Separation of Fatty Acids from Tubercle Bacillus 
by Gas Chromatography: Identification 
of Oleic Acid*

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Since gas phase chromatography has proved to be so much more effective than the best methods of fractional distillation for separation of complex mixtures, this technique is being applied to further investigation of fractions of acids from the lipides of tubercle bacillus, which have been separated previously by distillation and other techniques. The present report is concerned with preliminary separation of fractions previously termed the $C_{14}, C_{15}$ components and with identification of the unsaturated 18-carbon acid present in these fractions.

In each of the lots of tubercle bacillus which has been investigated in these laboratories, the fatty acids from the lipides have been found to present a similar gross composition. There were always present a large percentage of palmitic acid, a somewhat higher molecular weight fraction termed the $C_{16}, C_{17}$ fraction, and a third fraction consisting of a complex mixture of acids with more than 20 carbon atoms. The only component of the latter fraction which has been separated in what is believed to be a homogeneous condition is the physiologically active, intensively investigated $C_{14}$, phthiocanoic acid (1). The $C_{14}, C_{15}$ fraction from one lot of bacteria has been investigated with some thoroughness (2) and reported to consist of approximately 49 per cent tuberculostearic acid, 19 per cent stearic acid, and 32 per cent of a normal octadecenoic acid. This same fraction of acids (Lot I, Table I) has now been subjected to gas chromatography on silicone grease as partitioning agent. This agent separates largely on the basis of vapor pressure, hence the saturated and unsaturated 18-carbon components appear in the same band. The finding of 42.5 per cent of tuberculostearic acid and 49 per cent of $C_{14}$ components is in rather good agreement with the previously reported (2) results; however, the gas chromatography reveals the presence of small amounts of three additional acids. Of these, one is palmitic acid present in definitely detectable amount in spite of the fact that its ester boils about 20 degrees below the boiling point range of the fraction under investigation. More than half of the minor constituents consists of an ester whose retention time is the same as that of the n-heptadecanoate. Thus, the presence of a minor component between the palmitic and n-heptadecanoic esters is likely to be the ester of a branched 17-carbon acid, although a very highly branched acid with 18 carbons is possible.

Also included in Table I are the results of chromatography of the $C_{14}, C_{15}$ fractions from three additional lots of bacteria. It will be noted that in Lots II and III the pattern of acids is surprisingly similar to that of Lot I, with the heptadecanoate always present in much larger amounts than the branched isomers. The meaning of “trace” may be judged from the illustrative recorder tracings in Fig. 1. Chromatography of the two fractions from Lot III illustrates the rapid shift of composition with rise in boiling point. The fraction from Lot IV, which was chromatographed, represents a 1-degree cut with the boiling point of methyl stearate from a particularly careful fractional distillation. This did indeed eliminate the acids with less than 18 carbons; however, more than one-fourth of the material consists of fractions below or above stearate. It may be noted, further, that the concentration accomplished by the narrow cut in fractional distillation has raised to several per cent the content of ester which is probably that of a branched 18-carbon acid.

In the earlier investigation (2) of the acids from Lot I of bacterial cells, it was demonstrated that the unsaturated 18-carbon acid has the normal chain (isolation of stearic acid after hydro- 
genation); however, the position of the unsaturation was not established in this work or in earlier work (3, 4). Determination of this position becomes of interest since the careful investigations of Hofmann et al. (5–8) have shown that several microorganisms produce vaccenic acid rather than the oleic acid so common in higher plants and animals. These microorganisms included Lactobacillus arabinosus (5), Lactobacillus casei (6), a Streptococcus species (7), and Agrobacterium tumefaciens (8). Surprisingly enough, the unsaturated acid in all four lots of tubercle bacillus proved to be oleic acid, and there could be detected no other unsaturated acid in the $C_{14}$ fraction.*

The position of unsaturation in the octadecenoic acid was determined by ozonolysis of the $C_{14}$ fraction separated by gas

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** It should be emphasized that the fractions examined in this paper had been distilled in a 4-foot column designed especially for distillation of small amounts at low pressure and high temperature.
These times are reproducible to about 1 minute in 25 minutes, but a larger band gives a slightly longer retention time. Retention time becomes shorter as the column ages from long use at high temperature; however, correction for column aging may be accomplished by reference to known esters and adjustment of temperature or gas flow. Since the molecular weight range in these fractions is small, per cent of total area under a given band, as recorded in the table, is close to the weight per cent of that component in the mixture. Figures in parentheses refer to per cent of total area.

With the partitioning agent used, methyl oleate has a slightly shorter retention time than does methyl stearate; however, a known ester of an acid with one less carbon atom, cause an aldehyde has the same retention time in gas chromatography as does the ester of an acid with one less carbon atom. This method of oxidation, followed by detection of products by gas chromatography, was adopted after extensive examination of various methods of oxidative degradation. This type of degradation has recently been examined by James and Webb (9), who concluded that the best procedure is oxidation with permanganate in acetic acid. These authors noted that the products of this type of oxidation include numerous lower homologues of the acids resulting directly from cleavage of the primary oxidation products; however, ozonolysis has been found to give a clean cut oxidation to the primary products contaminated with negligible amounts of lower homologues. In early experiments, ozonolysis appeared to yield large amounts of the next lower homologues to the primary oxidation products; however, it was subsequently established that this impression was received because an aldehyde has the same retention time in gas chromatography as does the ester of an acid with one less carbon atom. It has proved difficult if at all possible to completely oxidize the aldehydes obtained on ozonolysis, to the corresponding acids, when silver oxide is used as the oxidizing agent. Thus, chromatography of the esterified material gives double peaks for each degradation product, one for the ester and one for the aldehyde.

A representative tracing from ozonolysis of methyl oleate, folowed by silver oxide oxidation and esterification, is illustrated in Fig. 2. Also included in this figure is a representative tracing from degradation of the C₁₈ fraction from one lot of tubercle bacillus. The tracings from degradation products of the C₁₈ fractions from the other lots of bacteria were equally free from bands indicating any location for the double bond besides the 9,10 position.

In the course of the investigation of ozonolysis, it was discovered that the simplest method of treating the ozonide is to decompose it with water, then directly chromatograph the resulting product. This procedure gives a high yield of aldehydes, with only traces of acids. It has also been reported in a recent patent (10) that decomposition of ozonides with water produces aldehydes in good yield unless the pH of the solution is high. One of the tracings in Fig. 2 illustrates the homogeneity of the neutral degradation products obtained in this way.

The elegant method recently reported (11) for cleavage of a double bond by permanganate-catalyzed periodate oxidation in aqueous medium has also been examined, and the products were subjected to the highly sensitive analysis by gas chromatography. As illustrated by one of the tracings in Fig. 2, the primary degradation products are obtained in a highly homogeneous condition; the content of secondary products is considerably less than 1 per cent. Unfortunately, good results could be obtained by this method only in aqueous solution, hence the salt of the acid must be used. When methyl oleate was used in aqueous pyridine as solvent, according to the method which has been described (12),

### Table I

<table>
<thead>
<tr>
<th>Lot of bacteria*</th>
<th>Boiling point</th>
<th>Retention times of observed bands† and per cent of total area</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>°C./ dl mm.</td>
<td>(14.5)</td>
</tr>
<tr>
<td>I</td>
<td>175-181</td>
<td>(2.0)</td>
</tr>
<tr>
<td>II</td>
<td>173-184</td>
<td>19.0</td>
</tr>
<tr>
<td>III</td>
<td>168-175</td>
<td>(23.5)</td>
</tr>
<tr>
<td>IV</td>
<td>175-181</td>
<td>19.0</td>
</tr>
<tr>
<td>Known esters</td>
<td>15.0</td>
<td>(21.0)</td>
</tr>
<tr>
<td>Palmitic</td>
<td>(16.0)</td>
<td>21.2</td>
</tr>
<tr>
<td>Branched</td>
<td>(18.0)</td>
<td>(5.5)</td>
</tr>
<tr>
<td>n-C₁₁</td>
<td>(17.0)</td>
<td>(4.0)</td>
</tr>
<tr>
<td>n-C₁₇</td>
<td>(18.0)</td>
<td>(2.0)</td>
</tr>
<tr>
<td>C₁₈</td>
<td>(19.0)</td>
<td>(2.0)</td>
</tr>
<tr>
<td>Known identity</td>
<td>Probable identity of unknowns</td>
<td>Palmitic</td>
</tr>
</tbody>
</table>

* Refer to "Experimental" for further description of the fractions of esters utilized and references to original isolations.

† Retention time is taken as time elapsing between injection of the sample and the maximum in the peak traced by the recorder. These times are reproducible to about 1 minute in 25 minutes, but a larger band gives a slightly longer retention time. Retention times become shorter as the column ages from long use at high temperature; however, correction for column aging may be accomplished by reference to known esters and adjustment of temperature or gas flow. Since the molecular weight range in these fractions is small, per cent of total area under a given band, as recorded in the table, is close to the weight per cent of that component in the mixture. Figures in parentheses refer to per cent of total area.

‡ About one-fourth the area recorded in this tracing was under a series of bands of very short retention times. This material, presumed to have distilled as an azoetropic mixture, was not further investigated, and the total area used for calculations was that of the bands of longer retention times, as recorded in the table.

§ With the partitioning agent used, methyl oleate has a slightly shorter retention time than does methyl stearate; however, a known mixture of the two esters was not separable in the column used.

Gas chromatography of certain fractions of methyl esters of acids isolated from lipides of tubercle bacillus

Tracings were obtained from the injection of about 15 pl. of the ester mixture into a 3-m. X 15-mm. (outer diameter) Pyrex glass column. Partitioning agent was high vacuum silicone grease dispersed on 30-60 mesh Celite fire-brick (4 parts grease: 10 parts Celite.) Column temperature was 270°C; helium pressure, 17.7 cm. of mercury; flow rate, 145 ml. per minute. A thermal detection cell was used. Illustrative tracings (Lots I and II) are shown in Fig. 1.
gas chromatography revealed that about 77 per cent of the oleate was recovered. Also ineffective in our hands was the osmium tetroxide-catalyzed periodate oxidation, utilizing a two-phase mixture of ether and water as solvent (13). In this instance, recovery of oleate was about 95 per cent. Thus, ozonolysis followed by simple decomposition of the ozonide with water appears the most generally useful method for locating the position of unsaturation. In instances where an acid may be used in water solution, the periodate-permanganate oxidation is equally satisfactory.

EXPERIMENTAL

The ester fractions used for the presently described investigations had been obtained, in work previously described, by extraction of the bacterial cells with alcohol-ether, saponification of the extracted lipides, and finally fractional distillation of the methyl esters of the acids recovered from the saponification. There follows identification of the four lots of bacterial cells supplying esters for the separations recorded in Table I, and references to the earlier isolations.

Lot I: Strain H-37 grown on Long's medium; esters were Fractions 5 to 7, Fig. 2 in (2).

Lot II: Culture M-920 of a strain isolated in 1899 by Ravenel and Gilliland, grown on veal medium with 5 per cent glycerine; esters were Fraction 3, Table II, in (14).

Lot III: Cell residues from preparation of old tuberculin, with strain DT; esters were Fractions 3 and 4, respectively, Table I, in (14).

Lot IV: A human strain grown on Long's medium; esters were the first half of Fraction 10 (of b.p. indicated in Table I), Fig 1 in (2).

Procedure for Ozonation—In 14 ozonations of methyl oleate, the effectiveness of the degradation was found to be rather insensitive to temperature or solvent, and absorption of ozone was quite slow after about 1 equivalent had been absorbed. In most of the runs, the products obtained after decomposition of the ozonide with water were oxidized with silver oxide, then esterified. Gas chromatography of the resultant mixture of aldehydes and esters (Fig. 2, Curves I and II) fixed the position of the double bond with certainty. This procedure was used for ozonation of the C18 fractions separated by gas chromatography (cf. Table I). In some runs, the product obtained by decomposition of the ozonide with water was not oxidized. Separation of the small amount of acid and chromatography of the neutral material yielded a single band for each degradation product (cf. Fig.
2, Curve III). A typical procedure for ozonolysis of methyl oleate is described; it may be applied to samples of 20 mg. or less.

A solution of 92.8 mg. (0.31 mmole) of methyl oleate in 60 ml. of chloroform (dried over calcium chloride and distilled) was cooled to -60° in a Dry Ice-acetone bath. Ozone in oxygen was passed through the solution for 1 minute, 42 seconds at the rate of 0.185 mmole of ozone per minute. The total of 0.31 mmole of ozone was absorbed. After the solution had been kept at -60° for one-half hour it was allowed to come to room temperature and immediately subjected to oxidation with silver oxide in presence of water. Decomposition of the ozonide by heating with water before silver oxide oxidation did not alter the results.

For preparation of silver oxide, excess 0.5 N potassium hydroxide was added to a solution of 152 mg. (0.92 mmole) of silver nitrate in 10 ml. of water. The precipitate was washed three times with water by decantation, then suspended in 10 ml. of water. This suspension (pH 8 to 9) was added to the solution of ozonide, and the two-phase solution was stirred under reflux for 3 hours. Extending the period of stirring to 9 days reduced the amount of aldehyde remaining unoxidized but did not eliminate it. The cooled reaction mixture was acidified to Congo red with 6 N hydrochloric acid, then stirred for one-half hour. During this stirring period, the colloidal silver becomes adsorbed on the precipitated silver chloride. The chloroform layer was separated and the aqueous phase was extracted with two 60-ml. portions of chloroform. The combined extracts were washed with two portions of water and evaporated at reduced pressure and a bath temperature below 50°. The residue, which consisted of 119.5 mg. of colorless oil, was esterified by heating under reflux for 2 hours with 15 ml. of methanol containing 10 per cent by weight of sulfuric acid. The esterification mixture was diluted with 10 volumes of water and extracted with three 50-ml. portions of ether. After the extract had been washed and dried it was evaporated at reduced pressure with a bath temperature below 40° (to avoid excessive loss of monobasic ester and mononic aldehyde). The residue of 102 mg. of slightly yellow esters was dissolved in 1 ml. of benzene, and 0.06 ml. of this solution was injected into the 3 m. × 15 mm. (outer diameter) column for gas chromatography. At 255°, with helium flow of 170 ml. per minute, retention times were: palergonic aldehyde, 3:10 (minutes, seconds); palergonate, 3:40; aldehyde ester of azelaic acid, 5:50; azelate, 7:20. No other peaks were observed. Retention times of known samples of methyl palergonate and dimethyl azelate were found to check those observed for the degradation products. Known compounds were chromatographed each time that degradation products were run; the oxidation products shown in Fig. 2, Curve IV, were frequently used for reference. When chromatography was carried out at higher temperature, no significant band was observed at the retention time of methyl oleate.

In the run whose product was chromatographed as shown in Fig. 2, Curve III, the ozonide was decomposed by stirring the methylene chloride solution under reflux with water only. In a run in which were used 200.8 mg. of methyl oleate, there were recovered from the methylene chloride solution 203.8 mg. of product, which was separated by countercurrent extraction in a three-stage Kies apparatus (15) to yield 9.9 mg. of acidic material and 187.4 mg. of neutral material. Chromatography of the neutral material gave the recording reproduced in Fig. 2, Curve III. When this neutral material was dissolved in methylene chloride and oxidized with silver oxide as described above, chromatography showed bands for the two esters as well as the two aldehydes.

**Oxidation with Permanganate-Periodate.—(a) Water solvent:** In a procedure similar to that described by Lemieux and Rudloff (11), there was stirred for 24 hours at room temperature a solution, in 400 ml. of water, of 282 mg. (1 mmole) of oelic acid, 414 mg. (3 mmole) of potassium carbonate, 1.536 gm. (8 mmoles) of periodic acid, 320 mg. (8 mmole) of sodium hydroxide, and 22 mg. (0.155 mmole) of potassium permanganate. At the end of the reaction period, there was added excess sodium bisulfite and 10 ml. of 6 N sulfuric acid, then the resultant colorless solution was extracted continuously with benzene. The acids remaining after evaporation of the benzene were esterified to yield 301.5 mg. of esters which were chromatographed to yield the tracing shown in Fig. 2, Curve IV. When this mixture was chromatographed at 285°, with a helium flow rate of 145 ml. per minute, the band for recovered oleate was observed at 16:10 (known sample at 16:09), and the area under this band was only 5 per cent of the total area under the three bands; therefore, oxidation of oleate was nearly complete.

(b) Aqueous pyridine solvent: In a procedure slightly different from that described by Rudloff (12), a solution of 240 mg. (0.81 mmole) of methyl oleate in 25 ml. of pyridine was added to a solution of 1.48 gm. (6.4 mmoles) of periodic acid dihydrate and 18 mg. (0.114 mmole) of potassium permanganate in 20 ml. of water. Finally, there was added a solution of 870 mg. of potassium carbonate in 5 ml. of water, and the red solution was stirred at room temperature for 30 hours (some white solid precipitated after 30 minutes). At the end of the reaction period, 60 ml. of 6 N sulfuric acid were added with stirring and cooling, then the mixture was extracted with three 60-ml. portions of ether. After the extract had been washed and dried, solvent was removed to leave 212.4 mg. of yellow oil. This was esterified and chromatographed in the usual fashion. There were observed bands for both aldehydes and esters from degradation (as in Fig. 2, Curves I and II), but 77 per cent of the total area under all bands was in that corresponding to methyl oleate. Thus, about three-fourths of the oleate remained unattacked, and part of that oxidized yielded aldehydes (or possibly esters of the acids which are next lower homologues of the acids obtained as primary degradation products).

**SUMMARY**

The fraction of esters previously termed the C13, C14 fraction, from four lots of tubercle bacillus, has been examined by use of gas phase chromatography. In each, similar components were found, although in varying ratios. In addition to palmitic, stearic, and tuberculostearic acids, there were also present smaller amounts of three other acids whose esters had retention times between palmitic and stearic esters. These components are probably a branched C17 acid, the normal C17 acid, and a branched C15 acid.

Ozonolysis of the C16 fraction separated by chromatography...
from each lot showed that the unsaturated C_{18} acid is oleic acid in each instance, not vaccenic acid as found in other microorganisms.

Investigation of several methods for location of a double bond by oxidation and gas chromatography of the degradation products has indicated that ozonolysis is usually the most convenient and effective. Decomposition of the ozonide with water yields aldehydes as degradation products accompanied by less than 10 per cent of acids. If an unsaturated acid, which may be dissolved in aqueous alkali, is being oxidized, periodate-permanganate oxidation is also convenient and gives homogeneous degradation products.

REFERENCES

Separation of Fatty Acids from Tubercle Bacillus by Gas Chromatography: Identification of Oleic Acid
James Cason and Peter Tavs