FURTHER DEMONSTRATION OF BACTERIAL LIPIDS IN
MYCOBACTERIUM BOVIS HARVESTED FROM
INFECTED MOUSE LUNGS

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SUMMARY: Mycobacterium bovis grown in vivo were separated by dif-
ferential centrifugation from homogenate of the experimentally infected mouse
lungs and subjected to lipid fractionation by Anderson-Lederer method in
comparison with the same strain grown on Sauton synthetic liquid medium.
The per cent yield of chloroform-soluble wax was much smaller than that of
wax A in the lung bacilli. The esters of mycocerosic acid were the main
components of wax A of the in vivo bacilli, but some esters of mycolic acid
were not detected unlike the case of the Sauton bacilli. An arabinolipid,
probably arabinose mycolate, and tuberculostearic acid-containing cardiolipin
were separated from bound lipids and the phospholipid fraction, respectively, as
the lipids common in both kinds of bacilli.

INTRODUCTION

A question has long been raised as to whether or not mycobacteria grown in vivo
are similar to those grown in vitro in lipid composition. Studies to answer this
question have been able to afford some limited information: mycolic acids have been
identified from in vivo grown mycobacteria in three laboratories (Etemadi, 1967)
(Kanai, Wiegeshaus and Smith, 1970) (Goren, Brohl, Das and Lederer, 1970); phthiocerol
dimycocerosate was demonstrated by Kanai, Wiegeshaus and Smith
(1970). The present paper is concerned with some additional observations of
mycobacteria-specific lipids separated from the bacilli grown in the mouse lungs.

MATERIALS AND METHODS

Microorganisms: Mycobacterium bovis (Ravenel strain) was used as the test
microorganisms. Bacillary suspension was prepared from the pellicle growth on
Sauton synthetic liquid medium (Kanai and Kondo, 1971).

Infection of mice: Mice of the commercially available dd strain, male and
about 18 g in weight, were infected intravenously with 0.5 mg (semidried weight) of Ravenel strain.

Separation of "in vivo grown bacilli": The bacilli were separated by the method already described (Kai and Kondo, 1970) from the lungs of mice which were moribund or dead around 3 weeks after infection.

Separation of lipids from samples of "in vivo grown bacilli": The conventional Anderson-Lederer method (Asselineau, 1962) was employed to extract lipids from samples of the in vivo grown bacilli (the lung bacilli). Before extraction with organic solvents, the samples were digested with trypsin and washed repeatedly with distilled water.

Fractionation and identification of lipids: Separated lipids were further fractionated and identified by column chromatography on Florisil-Celite, thin layer chromatography on silica gel G, gas-liquid chromatography and infrared spectroscopy.

Column chromatography: Two parts by weight of magnesium silicate and one part of "Johns Manville" celite #545 were used as adsorbent in column chromatography. The mixture was washed and activated by the procedure of Bloch et al. (1953). The lipid fraction was subjected to chromatography on a column with an appropriate solvent system as described in each section of "Experimental".

Thin layer chromatography: This was carried out with silica gel G as adsorbent. The procedure was described in detail in our previous paper (Kondo et al., 1970).

Infrared spectroscopy: Infrared spectroscopy was conducted by the use of a Nihonbunko Type DS-301, to which lipids pressed with KBr were applied. The lipids of low-melting point were used as chloroform solution.

Gas-liquid chromatography: Fatty acid analysis was conducted according to the method described previously (Kondo et al., 1971). For analyses for sugar, the methods of Sweeley et al. (1963) and Yamakawa et al. (1964) were employed.

**EXPERIMENTAL**

**Yield of lipid fraction:** Table I shows the yield of the separated lipid fractions in per cent dry weight of the starting material. The amount of the starting material was 1 g for the lung bacilli and 5 g for the Sauton bacilli. The per cent yield of acetone-soluble fats from the lung bacilli was 30.9 %, being almost 3 times as much as the yield from the Sauton bacilli. The fat fraction of the former bacilli contained cholesterol in 36 % when determined by the method of Zak et al. (1954), and 64 % of the total cholesterol was in its esters. Neither cholesterol nor esters were detected in the Sauton bacilli.

**TABLE I**

<table>
<thead>
<tr>
<th>Origin of bacilli</th>
<th>% yield (dry weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fats</td>
</tr>
<tr>
<td>Infected mouse lungs</td>
<td>30.9</td>
</tr>
<tr>
<td>Sauton culture</td>
<td>11.0</td>
</tr>
</tbody>
</table>
in the Sauton bacilli, indicating that they were of host origin.

The yields of phospholipids were 7.4 and 5.1%, respectively, also being higher in the lung bacilli. As described later, thin layer chromatography demonstrated that host-originating phospholipids were contained in the phospholipid fraction of the lung bacilli.

The yields of wax A were almost the same between the two kinds of bacilli, being 5.4 and 5.2%, respectively. The yield of alcohol-ether (1:1)-insoluble and Chloroform-soluble wax from the lung bacilli was 0.5%. This yield was so small as one-tenth that from the Sauton bacilli that further fractionation into wax, B, C and D was not possible. The yields of bound lipids extracted by the method of Azuma and Yamamura (1962) were 54.9 mg (5.5%) from 1 g of the lung bacilli and 344 mg (29.6%) from the same amount of the defatted Sauton bacilli. In this method, bound lipids were sequentially extracted with ether-alcohol and chloroform after acid hydrolyses with 0.1 N HCl and then 0.3 N HCl at increasing temperatures. These lipids were named Fractions A, B, C and D by Azuma and Yamamura (1962). The distribution of each subfraction in the total extractable bound lipids of each of the two kinds of bacilli is shown in Table II.

<table>
<thead>
<tr>
<th>Subfractions</th>
<th>Yield (mg) from In vivo bacilli</th>
<th>Yield (mg) from In vitro bacilli</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloroform-methanol (2:1) soluble</td>
<td>9.7*</td>
<td>55.7*</td>
</tr>
<tr>
<td>A</td>
<td>22.3*</td>
<td>80.3*</td>
</tr>
<tr>
<td>B</td>
<td>1.0*</td>
<td>57.2*</td>
</tr>
<tr>
<td>C</td>
<td>19.5</td>
<td>63.0</td>
</tr>
<tr>
<td>D</td>
<td>2.4</td>
<td>70.7</td>
</tr>
</tbody>
</table>

* These fractions of each kind of bacilli were combined and redissolved with chloroform. The soluble portion was subjected to chromatography as shown in Table IV and V.

In a separate experiment, bound lipids were obtained from the lung bacilli by the method of Kotani et al. (1959), in which the bacilli defatted with neutral organic solvents were refluxed with chloroform-methanol (2:1) containing 0.6 % HCl for 3 hr. The yield of bound lipids thus extracted was 5.8 % of dry weight of the starting material, in contrast to 14.7 % from the Sauton bacilli. It may be stated, therefore, that, despite of the different extraction methods, the yield of bound lipids from the lung bacilli was almost constant.

**Analysis of wax A**: Wax A was further fractionated by column chromatography and thin layer chromatography; some of the separated lipids were compared by infrared spectroscopy between the lung and Sauton bacilli. The method and results of column chromatography are shown in Table III; those of thin layer chromatography in Figs. 1 and 2. When a thin layer plate of silica gel G loaded with wax A of
Fig. 1. Thin-layer chromatography of wax A and its components (Table III) on silica gel G; the amount loaded was 250 µg each; developed with hexane-ethanol (99:1); the spots were detected with spray of the anthrone reagent.

The lung bacilli was developed with hexane-ethanol (99:1) and sprayed with the anthrone reagent, three orange spots were detected at Rf = 0.76, Rf = 0.96 and the front (Fig. 1). A portion of the loaded sample remained at the starting point. The figure also shows that the spots of Rf = 0.76 and Rf = 0.96 corresponded to the benzene-eluted fraction in the column chromatography and the spot in the front contained the fraction eluted with hexane-benzene (50:50) in the same column. Therefore, the lipids detected as three spots on the plate constituted the main component of wax A of the lung bacilli, amounting to 56% of the total. In wax A of the Sauton bacilli, however, the fraction eluted with hexane-benzene (50:50) from the column was so small in the yield (Table III). Besides, when analyzed on a thin layer plate, the benzene-eluted fraction which constituted 27% of the total wax A was found to be composed almost entirely of lipids of Rf = 0.96. A series of bands in infrared spectra of the hexane-benzene (50:50) and benzene fractions of the lung bacilli and the benzene fraction of the Sauton bacilli were at 2930, 2875, 1720, 1460, 1375, 1260, 1175, 1150, 1130, 1085, 970 and 922 (Fig. 3 a, b, c). This
Fig. 2. Thin-layer chromatography of wax A on silica gel G comparing the samples from the in vivo and in vitro bacilli; the loaded amount was 200 μg each, developed with benzene-ether (80:20) and the spots were detected by Anthrone reagent.

Fig. 4. Thin-layer chromatogram of fatty acids (mycolic acids and mycocerosic acid) separated from the lipid fractions (Table III) of wax A of the in vitro bacilli on silica gel G; the loaded amount was 200 μg each, developed with petroleum ether-ether (90:10), and the spots were detected by spray with a 0.01 % solution of rhodamine B dissolved in 0.25 M monosodium phosphate and then treated with 50% sulfuric acid and heat.

The absorption pattern was identical with that of the esters of phthiocereol with mycocerosic acid as reported by Noll and Bloch (1953) and Noll (1957). The above results of column chromatographic absorption, thin layer chromatography and infrared spectroscopy suggested that these lipids in wax A were the homologue of such esters.

With benzene-ether (80:20) as developing solvent, wax A was again fractionated on the same kind of thin layer plate (Fig. 2). Wax A of the Sauton bacilli produced four distinct spots including one in the front; however, almost all components of wax A of the lung bacilli went up to the front or remained at the starting point and other spots were extremely faint. In a separate experiment with chloroform-acetone (90:10) as developing solvent, the lipid of Rf=0.15 in the above plate was detected as a spot of Rf=0.67. It was considered to correspond to the fractions eluted with benzene-ether (50:50) and ether-methanol (99:1) from the column (Table III). This particular lipid constituted 27% of wax A of the Sauton bacilli. The characteristics of absorption in infrared specterum (Fig. 3 d, e) indicated a similarity to glyceryl monomycolate isolated from wax C of BCG by Tsumita (1956) and to a major constituent of wax C of the strains Brevannes and BCG obtained by
Fig. 3. Infrared spectra of various lipid fractions of wax A (Table III).
TABLE III

Column chromatography of wax A adsorbed on 6 g of Florisil-Celite for comparing the samples from the in vivo and in vitro bacilli

<table>
<thead>
<tr>
<th>Fraction No.</th>
<th>Eluted with</th>
<th>% yield of each eluted fraction from wax A of the bacilli grown</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>in vivo</td>
</tr>
<tr>
<td>1</td>
<td>Hexane</td>
<td>2.9</td>
</tr>
<tr>
<td>2</td>
<td>Hexane-benzene 85 : 15</td>
<td>3.6</td>
</tr>
<tr>
<td>3</td>
<td>Hexane-benzene 50 : 50</td>
<td>22.0</td>
</tr>
<tr>
<td>4</td>
<td>Benzene</td>
<td>34.3</td>
</tr>
<tr>
<td>5</td>
<td>Benzene-ether 95 : 5</td>
<td>5.8</td>
</tr>
<tr>
<td>6</td>
<td>Benzene-ether 80 : 20</td>
<td>trace</td>
</tr>
<tr>
<td>7</td>
<td>Benzene-ether 50 : 50</td>
<td>trace</td>
</tr>
<tr>
<td>8</td>
<td>Ether-methanol 99 : 1</td>
<td>6.5</td>
</tr>
<tr>
<td>9</td>
<td>Ether-methanol 95 : 5</td>
<td>5.8</td>
</tr>
<tr>
<td>10</td>
<td>Ether-methanol 80 : 20</td>
<td>9.7</td>
</tr>
<tr>
<td>11</td>
<td>Ether-acetic acid 98 : 2</td>
<td>9.4</td>
</tr>
</tbody>
</table>

Loaded amount (mg) 28 100

Fig. 5. Infrared spectra of mycolic acids separated from arabinose mycolate and from Fraction 7 of wax A of the in vitro bacilli (Table III).
Noll (1957). Therefore, separation and identification of mycolic acids from these lipids were attempted by Tsumita's method. The lipid (12.3 mg) was saponified in a mixture of 3 ml of butanol, 1.5 ml of ethanol, 0.2 ml of water and 100 mg of KOH. The mixture was refluxed for 1 hr. The reaction mixture, after cooled, was added with 5 ml of water and concentrated in vacuo to remove alcohol therefrom. The alkaline-water suspension was neutralized with N HCl and extracted with ether three times. The ether-soluble portion was dried over sodium sulfate and then evaporated to dryness. The resulting material was subjected to thin layer chromatography (Fig. 4) and infrared spectroscopy (Fig. 5), and identified as mycolic acid.

The benzene-ether (95:5) fraction was obtained in the yield of 5.8% from the lung bacilli and 11.5% from the Sauton bacilli (Table III). From the experience of Demarteau-Ginsburg and Lederer (1963), this fraction was considered to contain mycoside B. The presence of mycoside B in *M. bovis* (Ravenel strain) grown in vitro was confirmed by Kanai, Wiegeshaus and Smith (1970). Regarding the samples here obtained, however, further identification was not made because of the limited amount of materials.

**Purification and identification of a glycolipid separated from bound lipids:** A glycolipid was isolated from bound lipids extracted from both kinds of bacilli by the

**TABLE IV**

<table>
<thead>
<tr>
<th>Eluted with</th>
<th>Yield (mg) of eluted fraction from the loaded sample of</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>In vivo bacilli</td>
</tr>
<tr>
<td>Chloroform</td>
<td>2.7</td>
</tr>
<tr>
<td>Chloroform-methanol (99 : 1)</td>
<td>2.1*</td>
</tr>
<tr>
<td>Chloroform-methanol (95 : 5)</td>
<td>4.0*</td>
</tr>
<tr>
<td>Chloroform-methanol (90 : 10)</td>
<td>2.7</td>
</tr>
<tr>
<td>Chloroform-methanol (80 : 20)</td>
<td>2.5</td>
</tr>
<tr>
<td>Chloroform-methanol (70 : 30)</td>
<td>1.5</td>
</tr>
<tr>
<td>Chloroform-methanol (60 : 40)</td>
<td>1.0</td>
</tr>
<tr>
<td>Chloroform-methanol (50 : 50)</td>
<td>2.1</td>
</tr>
<tr>
<td>Chloroform-methanol (40 : 60)</td>
<td>4.4</td>
</tr>
<tr>
<td>Methanol</td>
<td>4.2</td>
</tr>
</tbody>
</table>

* Rechromatographed and the results were as shown below

| Petroleum ether  | trace | 1.7 |
| Petroleum ether-benzene (50 : 50) | trace | 1.2 |
| Benzene-ether (50 : 50) | trace | 2.1 |
| Ether-methanol (95 : 5) | 7.4* | 47.7*** |
| Ether-methanol (90 : 10) | trace | 2.0 |

** m. p. 32-35 C, *** m. p. 38-39 C
Fig. 6. Infrared spectra of a glycolipid separated from bound lipids extracted by Kotani or Azuma and Yamamura method from the in vivo and in vitro bacilli.
method of Kotani et al. (1959) or of Azuma and Yamamura (1962). First, bound lipids of 58.4 mg were obtained by the method of Kotani et al. (1959) from 1 g of the lung bacilli. The chloroform-soluble portion of the bound lipids was subjected to chromatography on a Florisil-Celite column. The fractions eluted with chloroform-methanol (99:1 and 95:5) were combined and subjected to rechromatography with a different solvent system. The major fraction was eluted with ether-methanol (95:5). All the results are summarized in Table IV.

In the second trial, 54.9 mg of bound lipids was obtained from 1 g of the lung bacilli by the method of Azuma and Yamamura (1962). This bound lipid fraction was divided into 4 subfractions (Fr. A, B, C and D) by their original procedure. Fr. A and B were combined, which amounted to 32.3 mg. A chloroform-soluble portion (22.4 mg) of this was subjected to the same column chromatography as above. The yield of the fraction eluted with chloroform-methanol (95:5) was 7.3 mg (Table V).

**TABLE V**

<table>
<thead>
<tr>
<th></th>
<th>Yield (mg) of eluted fraction of chloroform-methanol as above</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>In vivo bacilli</td>
</tr>
<tr>
<td>Chloroform</td>
<td>3.3</td>
</tr>
<tr>
<td>Chloroform-methanol (99 : 1)</td>
<td>3.8</td>
</tr>
<tr>
<td>Chloroform-methanol (95 : 5)</td>
<td>7.3</td>
</tr>
<tr>
<td>Chloroform-methanol (90 : 10)</td>
<td>1.1</td>
</tr>
<tr>
<td>Chloroform-methanol (80 : 20)</td>
<td>1.0</td>
</tr>
<tr>
<td>Chloroform-methanol (70 : 30)</td>
<td>1.1</td>
</tr>
<tr>
<td>Chloroform-methanol (60 : 40)</td>
<td>0.5</td>
</tr>
<tr>
<td>Chloroform-methanol (50 : 50)</td>
<td>/</td>
</tr>
<tr>
<td>Methanol</td>
<td>1.8</td>
</tr>
</tbody>
</table>

Defatted bacilli of 4.3 g were obtained from 5 g of the Sauton bacilli. Bound lipids (147 mg) were obtained by the method of Kotani et al. (1959). A chloroform-soluble portion (137 mg) of the bound lipids was loaded on a column; the fraction eluted with chloroform-methanol (95:5) was obtained in an amount of 53.8 mg. In rechromatography of this lipid, the major fraction (47.7 mg) was eluted with ether-methanol (95:5) as show in Table IV. When Azuma and Yamamura's method was applied to 1 g of the defatted Sauton bacilli, total bound lipids obtained were 344 mg, of which the chloroform-methanol (2:1) soluble fraction, Fr. A and Fr. B were 166 mg. A chloroform-soluble portion of this was subjected to column chromatography; the fraction eluted with chloroform-methanol (95:5) was 38.5 mg.

The melting point of the lipid eluted from the lung bacilli with chloroform-methanol (95:5) as above was 32 to 35 C and that from the Sauton bacilli 38 to
Fig. 7. Gas-liquid chromatography of sugar components separated from the glycolipids as shown in Fig. 6.
Analyses were carried out with Shimazu GC-4A PTF with a flame ionization detector. Column, 4 mm × 2 m, 5 % Ucon-coated Gaschrom. CLH; carrier gas, nitrogen; gas flow rate, 1.12 kg per cm²; column temperature 185 C.
The infrared spectrum was characteristic of typical glycolipid and the exact positions of the absorption maxima expressed in cm\(^{-1}\) were as follows: 3530, 2910, 2860, 1730, 1717, 1461, 1370, 1305, 1270, 1167, 1127, 1090, 1082, 1015, 955, 898, 714 (Fig. 6). These data suggest that the material had the characteristics similar to arabinose mycolate described by Azuma and Yamamura (1962).

To confirm this possibility, an attempt was made to demonstrate the presence of arabinose and mycolic acid in this lipid. Acid hydrolysis of the lipid (1.1 mg) from the lung bacilli was made with a sample dissolved in 1 ml of benzene added with 1 ml of 6% hydrochloric acid in absolute methanol. The mixture in a sealed tube was heated at 105°C for 3 hr. The reaction mixture, after cooled, was added with a small volume of water and extracted with petroleum ether to yield a fatty acid methyl ester fraction. The extraction was repeated 3 times. The methanol layer was concentrated to dryness after the Amberlite IR 4B treatment. The residue was suspended in 0.5 ml of anhydrous pyridine, to which 0.2 ml of hexamethyl disilazane and 0.1 ml of trimethylchlorosilane were added. After heated at 60°C for 2 hr and allowed to stand at room temperature for 30 min, the reaction mixture was extracted with 2 ml of chloroform and 4 ml of water. The chloroform layer was separated and washed with water two more times. It was concentrated to dryness and redissolved in a small amount of chloroform, an aliquot of which was injected onto the column of gas-liquid chromatography. Sugar derivatives to serve as references were obtained by methanolysis and trimethyl-silation of arabinose and glucose. The patterns obtained in chromatography are shown in Fig. 7. In the samples from the lung bacilli, the presence of arabinose and glucose in a proportion of 6.4 to 1.0 was demonstrated. However, in the glycolipid from the Sauton bacilli, arabinose alone was detected.

Fig. 8. Thin-layer chromatogram of methyl esters of mycolic acid obtained from a glycolipid of bound lipids extracted from the in vivo and in vitro bacilli; the conditions in chromatography were the same as those described in Fig. 4.
The petroleum ether layer in the above fractionation was dried over sodium sulfate and evaporated to dryness. The methyl esters of fatty acids here obtained were examined by thin layer chromatography. The thin layer plate of silica gel G loaded with the samples was developed with petroleum ether-ether (90:10) according to Lannele (1963). The spots of the samples from the lung and Sauton bacilli showed exactly the same Rf values (Fig. 8).

Finally, to identify the fatty acids as mycolic acid by infrared spectroscopy, separation and purification of them from the glycolipid were attempted with 20 mg of the material from the Sauton bacilli. Twenty mg of the glycolipid was dissolved in 3 ml of alcoholic KOH (10 ml of iso-proponol, 0.1 ml of water and 100 mg of KOH). The mixture was refluxed for 1.5 hr, cooled and neutralized with N HCl. After addition of 5.0 ml of water, the reaction mixture was extracted with ether. The ether-soluble portion was separated, dried over sodium sulfate and then evaporated to dryness. The resulting material of 16.3 mg was dissolved in a small volume of ether and added with 10 volumes of methanol to produce precipitate. The precipitate was subjected to infrared spectroscopy. The pattern shown in Fig. 5 is the typical one of mycolic acid and also is exactly the same as that of mycolic acid obtained from the esters separated from wax A (Fig. 5). Though infrared spectroscopy of mycolic acid from the glycolipid of the lung bacilli was not possible to perform.

![Thin-layer chromatogram of phospholipid fractions separated from the in vivo and in vitro bacilli](image)

**Fig. 9.** Thin-layer chromatogram of phospholipid fractions separated from the in vivo and in vitro bacilli; each sample was loaded in the amount of 200 µg on a plate of silical gel G, developed with chloroform-methanol-water (65:25:4) and the spots were detected with Dittmer reagent.
because of the limited amount of the sample, all the above observations suggest that
the glycolipid of the lung bacilli consisted mainly of arabinose mycolate contaminated
with mycolic acid ester of glucose.

The per cent yields of this lipid in the two separate experiments according
to the methods of Kotani et al. (1959) and of Azuma and Yamamura (1962) were
respectively 0.74 and 0.73 in the lung bacilli and 4.12 and 3.31 in the Sauton
bacilli.

Analysis of phosphatides: Phosphatides from the in vivo and in vitro bacilli
were subjected to thin layer chromatography. The resulting spots showed the
presence of cardiolipin, phosphatidylethanolamine, lecithin, sphingomyeline and
lysolecithin in the phospholipids of the lung bacilli; cardiolipin, phosphatidylethanol-
amine and the other two spots in those of the Sauton bacilli (Fig. 9).

To examine the constituent fatty acids of cardiolipin, a larger amount of the
phospholipids was applied onto a thin layer plate and purified into single spots by
rechromatography. The spot was localized by spraying water and it was sucked off
by aspiration. The silica gel containing cardiolipin was extracted with chloroform-
methanol (2:1) into screw-cap vials. After the solvent had been evaporated, 3 ml
of 3% HCl in methanol was added to the vial. Methanolyis was made according
to the method already described (Kondo et al., 1971). As shown in Fig. 10,
tuberculostearic acid was detected in 4.57% in the lung bacilli and in 9.96% in the

![Fig. 10. Gas-liquid chromatography of the constituent fatty acids of cardiolipin separated
from the in vivo and in vitro bacilli.](image)

Analyses were carried out with Yanagimoto Type 8 with a flame ionization detector. Colu-

mn, 3 mm×2.25 m, 5% SE-30 coated chromosorb W; carrier gas, nitrogen; gas flow rate,
12.5 ml/min; column temperature 187°C.
Sauton bacilli. This result meant that cardiolipin of the lung bacilli was of bacillary origin, at least partly.

**DISCUSSION**

The physical and chemical conditions in the mouse lung may extremely be different from those on Sauton synthetic liquid medium; nutrients available for bacillary growth may not be the same in these two environments. Host factors interfering with the physiological and biochemical activities of bacilli may prevail in the mouse lungs, but not on Sauton meidum. Therefore, it seems natural to suspect that lipid composition of mycobacteria grown in vivo and in vitro differs from each other to a considerable extent. Fundamental differences, however, were not detected in this respect between the two kinds of bacilli, except some quantitative ones.

One of them was that the per cent yield of wax D and other chloroform-soluble waxes was much smaller in the lung bacilli. Because of its biological activity as an immunologic adjuvant, wax D has been the principal interest in lipid chemistry of mycobacteria. For this reason, the above observation may deserve discussion in some detail.

Since many years ago, various conditions have been found to reduce the amount of chloroform-soluble wax of mycobacteria growing in vitro. Pokoroný (1962) observed that the wax with a high melting point was reduced in amount when glycerol in the culture medium was replaced with glucose. This lead us to raise a question which is more easily available in vivo for the growth of mycobacteria, glucose or glycerol. Asselineau (1962) experienced that wax D was very sensitive to enzymatic hydrolysis and autolysis of cultures. This reminds us of a possibility that wax D might be destroyed in vivo by digestive enzymes of macrophages.

According to Migliore (1971), wax D increased in amount after the culture came to the stationary phase of growth. The lung bacilli we collected were evidently in the logarithmic phase of growth (Kondo et al., 1971).

Quite recently, Draper and Rees (1970) suggested that a peribacillary electron-transparent zone surrounding ingested mycobacteria within the phagocytic vacuole of macrophages is wax D, and that it is easily liberated from the bacilli even by a delicate mechanical manipulation into the supernatant of tissue homogenate. Then, a question came up to our mind whether or not our lung bacilli became devoid of wax D during the mechanical procedure to separate them by centrifugation from the mouse lung homogenate. An attempt is now under way to isolate wax D from the supernatant of such homogenate.

Wax A was obtained in a relatively high proportion from the lung bacilli; as will be reported in a separate paper, a fraction having a toxicity like hcord-factorh was demonstrated in this wax A. These observations suggest a possibility that some portion of chloroform-soluble waxes might have gone into the fraction of wax A in our experiments.

Wax A of the lung bacilli was not the same in chemical composition as that of the Sauton bacilli. The major components of the former were the esters of phthiocerol with mycocerosic acid. This kind of lipids in various strains of *M. tuberculosis* grown in vitro were studied by Philpot and Wells (1952), Asselineau (1962), Noll and Bloch (1953) and Noll (1957). Noll and Bloch (1953) reported that
a large amount of the esters in the form of phthiocerol dimyocceronate was present in wax C. Tsumita (1956) also separated the esters from wax C of BCG. Smith and his coworkers (1960) demonstrated the presence of phthiocerol dimyocceronate in 28 out of 30 strains of *M. tuberculosis* and all of 7 strains of *M. bovis* which they tested, and claimed that this ester was a type-specific lipid of these two species of mycobacteria. Finally, Kanai, Wiegeshaus and Smith (1970) separated and identified this lipid in *M. bovis* (Ravenel strain) grown in the mouse lungs.

In the present study, however, thin layer chromatography revealed at least 3 homologues of phthiocerol ester with mycocerosic acid in wax A of the lung bacilli. They may probably be different in their fatty acid compositions. On the other hand, wax A of the Sauton bacilli had substantially only one of them. The fact that these esters were isolated in a high yield from wax A of the lung bacilli may suggest that they are essential metabolites of mycobacteria produced irrespective of the difference in their growth environment.

The ester of mycolic acids, whose infrared spectrum looked like that of glycerclymonomycolate isolated by Tsumita (1956) from wax A and C of BCG grown on Sauton medium, was not detected in the lung bacilli, though it was present as the most abundant component in the Sauton bacilli. It is an open question whether glycerol in Sauton medium is directly available for mycobacteria to esterify mycolic acid.

The isolation of an arabinolipid, most probably arabinose mycolate, from bound lipids of the lung bacilli is of utmost interest in connection with the previous papers of Kotani et al. (1963), Azuma, Yamamura and Fukushi (1968), Kanetsuna (1968) and Acharya and Goldman (1970). These authors demonstrated the presence of wax D, bound lipids and arabinose mycolate as the main components of the mycobacterial cell wall synthesized in vitro. Our present finding appears, therefore, to provide the starting point for the future study on the chemical structure of mycobacterial cell wall synthesized in vivo. It is not difficult to suspect that the lipidic surface of mycobacteria grown in vivo may have a particular interaction with the membrane structure of host macrophages. From the research conducted by us along the line, the host-parasite relationship in tuberculous infection will be understood from a new angle.

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**LIPIDS OF MYCOBACTERIA GROWN IN VIVO**


