STUDIES ON THE CHEMISTRY OF THE CORD FACTOR OF MYCOBACTERIUM TUBERCULOSIS

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A lipide component of the tubercle bacillus, which was termed “cord factor” (1–4), has been described in previous publications. This bacterial constituent is characterized by its exclusive occurrence in strains of Mycobacterium tuberculosis which have the ability to multiply in vivo, by a particular delayed toxicity for mice upon repeated injections of small amounts (2.5 to 5 γ of pure preparations), and by a definite rôle it plays in the virulence of the tubercle bacillus (5).

The present paper describes methods for obtaining highly purified preparations of cord factor, and it shows that cord factor is present in different virulent strains of M. tuberculosis of both the human and the bovine type. It presents a partial structural analysis of cord factor, showing that the compound is an ester of mycolic acid containing 1 molecule of glucose, the latter being attached through a glucoside linkage to an as yet unidentified aliphatic fragment. A detailed description of the biological properties of the cord factor will be reported elsewhere (6).

Anderson’s pioneer work on mycobacterial lipides has made use of solvent fractionation methods (7), and fractionation by chromatography has been profitably introduced in this field by Asselineau and Lederer (8). The present workers have benefited from both these methods and, in addition, made extensive use of infra-red spectroscopy1 as an aid in the identification of microquantities of lipides. The combined application of the three methods proved to be an excellent tool in the isolation and structural analysis of high molecular lipides of this class.

EXPERIMENTAL

Isolation and Purification of Cord Factor from Various Strains of Virulent Tubercle Bacilli

Bacteria—The bacilli were grown as surface cultures on a modified Lockemann medium (9) and harvested after about 3 weeks as described

1 The authors are greatly indebted to Dr. R. C. Gore, and Mr. N. B. Colthup, of the American Cyanamid Company, Stamford, Connecticut, for the courtesy of running a great number of infra-red spectra and for their invaluable help in interpreting the results.
previously (3). The following strains were used for the preparation of cord factor: (a) H37-Rv and (b) Brévannes, both virulent human strains; (c) PN, DT, C, a mixture of virulent human strains used in the production of tuberculin; (d) Vallée, a virulent bovine strain; and (e) a variant of H37-Rv, resistant to isonicotinic acid hydrazide, designated "Ciba."3

**Extraction and Isolation of Purified Wax**—All bacilli were extracted in the viable state, with the exception of bacilli (b) and (c), which were obtained as heat-killed cultures. Strain Brévannes was selected for the production of larger quantities of cord factor in most of the present studies.4 For practical reasons, steam-killed, 3 week-old cultures were used for all large scale extractions of the strain Brévannes.

An adaptation of Anderson's extraction scheme, as previously described (3), was employed with some minor modifications. In Anderson's procedure (10), the bacilli are defatted by preliminary extraction with ether-ethanol 1:1 (volume by volume) and subsequently extracted with chloroform. It was found that the cord factor-containing fraction, Wax C (11), of the chloroform extract was soluble in ether-methanol 1:1, but insoluble in a mixture of 1 part ether and 2 parts methanol. In order to prevent losses of cord factor-containing material, we used a mixture of 1 part ether and 2 parts methanol for the preliminary extraction.

Before the chloroform extraction, the bacilli were washed with acetone, dried, and pulverized. The purified wax obtained according to Anderson is usually contaminated with yellow pigments. These pigments were effectively removed by washing the purified wax with cold acetone on the filter.

The purified wax was separated into fractions of Waxes C and D by fractional precipitation with ether-methanol, as previously described (3). It is of the utmost importance to obtain a clean separation of Wax C from Wax D. Wax C (m.p. 40–50°) consists of low melting esters, including cord factor, while Wax D (m.p. 160–240°) is composed of high melting lipopolysaccharides (11). The presence of lipopolysaccharides in Wax C not only interferes with its chromatographic purification but also gives rise to misleading results in the chemical analyses of its components.

**Chromatographic Purification of Cord Factor**—For the chromatographic procedures, the following adsorbents were used: magnesium silicate-Celite

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2 The authors wish to thank Dr. H. D. Piersma of the Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York, for the generous supply of these bacterial cultures.

3 Obtained through the courtesy of Dr. R. L. Mayer, Ciba Pharmaceutical Products, Inc., Summit, New Jersey.

4 We are indebted to Dr. R. L. Mayer, Ciba Pharmaceutical Products, Inc., Summit, New Jersey, for providing us with large amounts of bacterial cultures.
545 (12)\(^6\) and silica gel "Davison." The separation of the components was followed by melting point and acidity determinations as well as infrared spectroscopy. The particulars of the techniques have been described (3).

Separation of Cord Factor and Mycolic Acid on Silica Gel—Pure cord factor is difficult to obtain from Wax C of old bacterial cultures because of

| Table I |

*Chromatogram I. Chromatography of Ether-Methanol Fractions Obtained after Chromatography of Wax C from Strains PN, DT, C on Magnesium Silicate-Celite*

310 mg. of material were adsorbed on 30 gm. of silica gel.

<table>
<thead>
<tr>
<th>Fraction No.</th>
<th>Eluted with 30 ml. of</th>
<th>M.p.</th>
<th>C.mm. 0.1 N MeONa per mg.</th>
<th>log(_a^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Petroleum ether</td>
<td>30.4</td>
<td>36-37</td>
<td>0.5</td>
</tr>
<tr>
<td>1</td>
<td>&quot;</td>
<td>17.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>&quot;</td>
<td>15.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>&quot;</td>
<td>10.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>&quot;</td>
<td>0.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>&quot; ether-benzene 1:1</td>
<td>6.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6-11</td>
<td>Benzene-ether 1:1</td>
<td>116.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>&quot; 1:1</td>
<td>32.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>&quot; 1:1</td>
<td>14.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>&quot; 1:1</td>
<td>7.7</td>
<td>52-54</td>
<td>5.9</td>
</tr>
<tr>
<td>15</td>
<td>&quot; 1:1</td>
<td>7.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>&quot; 1:1</td>
<td>6.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>&quot; 1:1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>Ether containing 5% MeOH</td>
<td>5.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>&quot; 5% &quot;</td>
<td>6.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>&quot; 5% &quot;</td>
<td>6.1</td>
<td>44-46</td>
<td>2.8</td>
</tr>
<tr>
<td>21</td>
<td>&quot; 5% &quot;</td>
<td>2.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>&quot; 10% &quot;</td>
<td>13.0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Recovered.............................. 298.4 = 96%

the latter's high content of free mycolic acid which is difficult to separate from cord factor. On magnesium silicate-Celite, repeated chromatographies are needed and the separation is still incomplete (3). Subsequent chromatography on silica gel permitted a more effective separation of the two components, as seen from a typical example in Table I. The toxic component, in contrast to its strong adsorption on magnesium silicate, is only slightly adsorbed on silica gel and eluted in the very beginning with petroleum ether.\(^6\)

\(^{6}\) We are indebted to Johns-Manville, New York, for a gift of Celite 545.

\(^{6}\) The mycolic acid fraction eluted with benzene-ether seems to be contaminated with toxic material, since its acidity (5.9 c.mm. of 0.1 N MeONa per mg.) is lower
The toxic fraction eluted from the silica gel column with petroleum ether still contained traces of acidity (Table I) which could not be completely removed by repeated chromatographies. Esterification with diazomethane yielded a completely neutral material which, after chromatography, was slightly acid again, suggesting that small amounts of mycolic acid were liberated on the column.

The sample of cord factor obtained by chromatography on silica gel had the physicochemical properties listed in Table III.

Infra-Red Spectrum (Fig. 1, Curve 1)—The spectrum shows a series of well defined bands all associated with the toxic fractions (3). The C—O stretching region, 1200 to 800 cm.\(^{-1}\), which is commonly used for the identification of closely related compounds, is highly characteristic for purified cord factor samples. The exact positions of the absorption maxima are 3350, 2900, 2840, 1715, 1470, 1380, 1275, 1170, 1150, 1100, 1075, 1050, 1020, 995, 915, 805, and 720 cm.\(^{-1}\). Upon repeated injections in mice, this sample displayed characteristic toxicity (5). The data mentioned above were found to be reproducible and consistent for various preparations obtained in different ways in this laboratory, as well as in the laboratory of Dr. E. Lederer in Paris.

than the theoretical value of 7.7 c.mm. of 0.1 N MeONa per mg. and its optical rotation ([\(\alpha\)]\(_D\) +9°) is higher than the value reported for mycolic acid ([\(\alpha\)]\(_D\) +1.8°) (7). For unknown reasons, a small amount of toxic material was always eluted in the tail fractions with ether methanol.
Isolation of Cord Factor from Different Strains—As has been pointed out previously (3), old cultures of tubercle bacilli are a poor source of cord factor. Thus, Wax C from the 12 week-old bacilli (e) yielded only 1 to 3 per cent, while 10 per cent or more was obtained from 2 to 3 week-old bacilli. The separation of cord factor from Wax C of the H37-Rv strain

**TABLE II**

Chromatogram II. Chromatography of 790 Mg. of Wax C from Strain H37-Rv Dissolved in Petroleum Ether Adsorbed on 60 Gm. of Magnesium Silicate-Celite

<table>
<thead>
<tr>
<th>Fraction No.</th>
<th>Eluted with 50 ml. of</th>
<th>M.p.</th>
<th>C.mm. of 0.1 N MeONa per mg.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Petroleum ether</td>
<td>237.8</td>
<td>29—31</td>
</tr>
<tr>
<td>2</td>
<td>&quot; &quot;</td>
<td>142.6</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>&quot; &quot;</td>
<td>46.7</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>&quot; &quot;</td>
<td>21.0</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>&quot; &quot;</td>
<td>25.8</td>
<td>32—33</td>
</tr>
<tr>
<td>6</td>
<td>ether-benzene 1:1</td>
<td>84.2</td>
<td>40—45</td>
</tr>
<tr>
<td>7</td>
<td>&quot; &quot;</td>
<td>20.8</td>
<td>48—55</td>
</tr>
<tr>
<td>8</td>
<td>&quot; &quot;</td>
<td>8.0</td>
<td>35—45</td>
</tr>
<tr>
<td>9</td>
<td>&quot; &quot;</td>
<td>6.5</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>&quot; &quot;</td>
<td>5.8</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>&quot; &quot;</td>
<td>5.2</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>Benzene-ether 1:1</td>
<td>9.8</td>
<td>54—60</td>
</tr>
<tr>
<td>13</td>
<td>&quot; 1:1</td>
<td>6.4</td>
<td>5.0</td>
</tr>
<tr>
<td>14 16</td>
<td>&quot; 1:1</td>
<td>4.1</td>
<td></td>
</tr>
<tr>
<td>17*</td>
<td>Ether containing 10% MeOH</td>
<td>67.7</td>
<td>41—42</td>
</tr>
<tr>
<td>18</td>
<td>&quot; &quot; 10% &quot;</td>
<td>25.4</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>&quot; &quot; 10% &quot;</td>
<td>6.7</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>&quot; &quot; 10% &quot;</td>
<td>4.0</td>
<td></td>
</tr>
<tr>
<td>21—32</td>
<td>&quot; &quot; 10% &quot;</td>
<td>12.0</td>
<td></td>
</tr>
</tbody>
</table>

Recovered ..................................... 740.8 = 94%

* The fractions representing cord factor are given in bold-faced type.

is shown in Chromatogram II (Table II). The ether-methanol eluate yielded cord factor of high purity after only one chromatography on magnesium silicate-Celite, since the Wax C of these bacilli contained little or no free mycolic acid. Similar results were obtained in the chromatography of Wax C from Brévannes, Vallée, and "Ciba" strains. Fig. 1 shows that the infra-red spectra of the toxic components obtained from five different bacterial strains are identical. Other data pertaining to these fractions are summarized in Table III. 7

7 Analyses by the Schwarzkopf Microanalytical Laboratory, Middle Village, New York.
Thus the same toxic fraction could be isolated from a variety of virulent strains of tubercle bacilli. The yields range from 5 to 20 per cent of Wax C. This is of particular significance in view of the biological rôle played by cord factor in tuberculous infection (6). In contrast, the nature of the non-toxic components of Wax C varies markedly with the different sources.

**Chemical Degradation of Cord Factor**

**Alkaline Hydrolysis of Cord Factor**—190 mg. of cord factor (Brévannes) were suspended in a solution of 5 cc. of isopropanol, 0.1 cc. of water, and 100 mg. of KOH. The solution was refluxed for 1 hour under a stream of nitrogen which was passed through a trap containing 5 cc. of a 0.1 N HCl solution. After about 10 minutes of heating, a clear solution of yellowish color was obtained. At the end of the heating period, the solution was cooled and neutralized with 2 cc. of 1 N HCl.

**Test for Volatile Bases**—Microtitration of an aliquot of the 0.1 N HCl trap solution showed no decrease in acidity. Lyophilization of the trap solution left only an insignificant trace of a hygroscopic residue.

**Isolation of Mycolic Acid As Lipide Moiety**—After addition of 5 cc. of water, the acidified solution obtained after alkaline hydrolysis was repeatedly extracted with an excess of petroleum ether. The petroleum ether extracts were taken to dryness, and the lipide residue was dissolved in a small volume of ether and precipitated with methanol. The precipitate was filtered, washed with methanol, and dried in vacuo at room temperature; 158 mg. of a white powder, m.p. 54-55°, were obtained. The neutral equivalent was 1275 ± 25. Found, C 81.49, H 13.30, OCH₃ 1.71.

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* Noll, H., paper to be published.

**Table III**

**Properties of Cord Factor Preparations Isolated from Various Strains of Virulent Tubercle Bacilli**

<table>
<thead>
<tr>
<th>Strain</th>
<th>M.p.</th>
<th>[α]°d</th>
<th>Infra-red spectrum No.</th>
<th>C</th>
<th>H</th>
<th>N</th>
<th>N—CH₃</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>°C.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PN, DT, C</td>
<td>36-37</td>
<td>+31</td>
<td>1</td>
<td>76.3</td>
<td>12.5</td>
<td>0.8</td>
<td>1.0</td>
</tr>
<tr>
<td>H37-Rv</td>
<td>41-42</td>
<td>+30</td>
<td>2</td>
<td>76.4</td>
<td>12.7</td>
<td>0.7</td>
<td>1.0</td>
</tr>
<tr>
<td>Brévannes</td>
<td>40-41</td>
<td>+31</td>
<td>3</td>
<td>76.9</td>
<td>12.3</td>
<td>0.7</td>
<td>1.4</td>
</tr>
<tr>
<td>Vallée</td>
<td>38-39</td>
<td>+29</td>
<td>4</td>
<td>77.1</td>
<td>12.3</td>
<td>0.7</td>
<td>1.4</td>
</tr>
<tr>
<td>&quot;Ciba&quot;</td>
<td>42</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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* The theoretical value corresponding to one methoxyl group per molecule is 2.4 per cent. As pointed out by Dr. Lederer (personal communications), the methoxyl determinations on mycolic acids are often erroneous. On the other hand Aebi et al. (13) have shown that preparations of α-mycolic acid obtained from strain Brévannes...
These values agree with those published by Aebi et al. for \( \alpha \)-mycolic acid Brévannes (13). The infra-red spectrum (Fig. 2, Curve 6) is identical with that of a sample of \( \alpha \)-mycolic acid Brévannes obtained from Dr. Lederer.

The fact that one of these acids contains one methoxyl group while the other has a hydroxyl in place of the methoxyl group explains the low methoxyl values obtained by analysis of the mixture. It seems conceivable, therefore, that both of these acids can take part in the synthesis of cord factor. Preparations of cord factor obtained from the strains PN, DT, C yielded \( \alpha \)-mycolic acid, which was devoid of methoxyl.

In their extensive studies on the chemistry of mycolic acid (25), Dr. Lederer and co-workers have shown that the mycolic acids elaborated by different strains of *Mycobacteria* vary to some extent. Dr. Asselineau and Dr. Lederer (personal communications) also found that cord factor preparations obtained from various strains of virulent tubercle bacilli yielded different mycolic acids upon alkaline hydrolysis.
Isolation of Water-Soluble Moiety—The acidified water solution obtained after alkaline hydrolysis was deionized by passing through a mixed anion-cation exchange resin (Amberlite MB-3) and concentrated in vacuo to a volume of 2 cc. This concentrated solution was divided into two portions of 1 cc. each, designated in the following as Solutions 1 and 2. Solution 1 was lyophilized, yielding a white fluffy residue and turning into a sirup at atmospheric pressure. About 10 mg. of this hygroscopic residue were recovered and 1 mg. dissolved in 100 c.mm. of water which formed a cloudy solution. Aliquote of 10 c.mm. each were withdrawn for the following tests: (1) the reduction test according to Nelson (14), in which no reducing action was detected; (2) the sulfuric acid test according to Mendel et al. (15), in which the solution obtained after heating with concentrated sulfuric acid had a pink color. The spectrum was measured with a Beckman model DU spectrophotometer in the ultraviolet region between 215 and 350 mμ and in the visible region between 400 and 600 mμ. A sample of 100 γ of glucose per cc. served as a control. The spectra of the alkaline hydrolysate and of the glucose solution were identical, the characteristic maxima being shown at 255, 312, and 520 mμ. The extinction values of the alkaline hydrolysate were equal to those of 100 γ of glucose. According to Mendel et al., the pink color with a maximum at 520 mμ is specific for glucose, fructose, and their polymers.

The fact that the water-soluble moiety resulting from alkaline hydrolysis of cord factor gives no reducing action, but a positive glucose test with concentrated sulfuric acid, suggests that glucose is liberated under the strongly hydrolytic conditions of the test. It was indeed found that subsequent acid hydrolysis results in the liberation of a reducing sugar which was identified as glucose, as described in the following section.

Ultraviolet Spectrum of Water-Soluble Moiety—Except for the unspecific end-absorption at 215 mμ, no absorptions were detected between 220 and 400 mμ.

Infra Red Spectrum—The infra red spectrum of the hygroscopic residue obtained after lyophilization is pictured in Fig. 2, Curve 8. Taken from a smear, this spectrum shows the typical absorptions of sugars; i.e., a strong bonded —OH band at 3350, broad O—H bending absorptions between 1500 and 1300, and a system of well defined bands in the C—O stretching regions with maxima at 1150, 1105, 1080, 1035, 990, and 940 cm.⁻¹. The broad absorption between 1650 and 1600 cm.⁻¹ is probably due to water. The CH₂ band at 2940 cm.⁻¹ seems somewhat deeper than usual in sugars, indicating the presence of an aliphatic residue. Although the spectrum is typical for a carbohydrate, it is not identical with any of the spectra of the sugar and sugar derivatives published by Kuhn (16). This is not surprising, since the carbohydrate moiety obtained after alkaline hydrolysis of cord factor is a non-reducing derivative of glucose, and
the reducing sugar is liberated only after subsequent acid hydrolysis as described below.

**Acid Hydrolysis of Non-Reducing Carbohydrate Fragment Obtained after Alkaline Hydrolysis of Cord Factor**—To 1.0 cc. of Solution 2, 1.0 cc. of 3 N HCl was added, and the resulting solution heated in a sealed tube for 4 hours in a boiling water bath. An aliquot of 0.05 cc. of the hydrolyzed solution was withdrawn for a reduction test according to Nelson (14), which was strongly positive. Colorimetric determination gave a value corresponding to a total of 8.8 mg. of glucose in Solution 2. The acidic solution was divided into two equal portions, Solutions 3 and 4. Solution 3 was made alkaline with NaOH and extracted with ether. The ether extract was treated with dry HCl and a cloudy precipitate obtained which, after evaporation, left traces of a hygroscopic residue. This residue gave a reddish brown precipitate with Nessler's reagent.

**Identification of Sugar As Glucose**—After extraction with ether, the alkaline Solution 3 was passed through Amberlite MB-3 and concentrated in vacuo to a colorless sirup which was dissolved in 0.5 cc. of water. An aliquot of 0.1 cc. was removed and 0.005 cc. each was used for the following tests: (1) the sulfuric acid test (15), of which the intensity of the pink color formed corresponded to 35 γ of glucose; (2) the glucose oxidase test according to Keston (17), tests with this sensitive specific glucose reagent being strongly positive.

**Preparation of Potassium Gluconate**—The residual 0.4 cc. of the concentrated Solution 3, which contained 2.6 mg. of glucose as estimated by the sulfuric acid test, was taken to dryness and oxidized according to Moore and Link (18). This yielded 2.7 mg. of a crystalline powder, melting at 178–180° with decomposition which agrees with the melting point reported for potassium gluconate. A mixed melting point with a sample of potassium gluconate gave no depression. The infra-red spectrum of the derivative was found to be exactly identical with that of potassium gluconate.

**Test for Volatile Bases**—Solution 4, resulting from acid hydrolysis, was made alkaline with 3 cc. of 1 N NaOH and refluxed for 4 hours. A stream of nitrogen which was kept bubbling through the solution was passed through a trap containing 5 cc. of 0.01 N HCl. At the end of the heating period, an aliquot of the trap solution showed no decrease in acidity. After lyophilization of the trap solution, a trace of a hygroscopic residue was left which was insoluble in ether. Addition of a drop of water and Nessler's reagent produced a reddish brown precipitate.

10 The authors are greatly indebted to Dr. A. S. Keston for a gift of his glucose oxidase reagent.

11 The derivative was prepared on a micro scale with only approximately 0.001 of the quantities described by Moore and Link. As little as 1 mg. of glucose can be converted into crystalline potassium gluconate with this microprocedure.
Reductive Cleavage with LiAlH₄—To an ether solution of 36 mg. of cord factor (PN, DT, C), a solution of LiAlH₄ in ether was slowly added. After refluxing for 1 hour, water was added and the ether layer separated. A waxy residue was obtained after evaporation of the ether which was purified by precipitation from ether with methanol, yielding 26 mg. of a white powder, m.p. 46-48°. Found, C 81.87, H 13.51; calculated for α-mycoly alcohol (C₈₅H₁₇₅O₃), C 82.29, H 13.97. The infra-red spectrum (Fig. 2, Curve 7) was identical with that of a sample of α-mycoly alcohol obtained after reduction of mycolic acid with LiAlH₄ (13).

The water solution was lyophilized after the insoluble hydroxides had been filtered off. The dry residue was then acetylated with acetic anhydride and sodium acetate. The acetylated carbohydrate fragment was isolated in the usual manner by extraction with chloroform, and a few mg. of a sirupy residue were obtained. It did not crystallize. Its infra-red spectrum (Fig. 2, Curve 9) has the characteristic bands of sugar acetates with strong acetyl absorptions at 1750, 1375, 1230, and 1040 cm⁻¹. The lack of amide C=O absorptions around 1650 cm⁻¹ indicates the absence of N-acetyl groups.

Derivatives of Cord Factor; Acetylation—It has been reported (19) that acetylation of cord factor with acetic anhydride in the presence of sodium acetate led to a partially acetylated product which was about 10 times less toxic. Asselineau found that a completely acetylated derivative could be obtained by treatment of cord factor with acetic anhydride in pyridine solution.12 According to his directions, 60 mg. of cord factor were dissolved in 1 cc. of benzene to which 3 cc. of pyridine and 1.5 cc. of acetic anhydride were added. After the solution was allowed to stand for 3 days at room temperature, it was concentrated on a boiling water bath, and the reaction product precipitated with methanol. Purification by precipitation from ether with methanol yielded 40 mg. of a white powder, m.p. 34°. Its infra-red spectrum (Fig. 3, Curve 10) shows strong acetyl bands. At the same time, the strong —OH band at 3350 cm⁻¹ has disappeared, indicating that acetylation was complete. The lack of absorptions in the vicinity of 1650 and 1550 cm⁻¹ indicates that no nitrogen is available for acetylation. Found, C 75.09, H 11.82, N 0.56. Analysis of the parent cord factor preparation gave the following: C 77.24, H 12.16, N 0.65. Calculation of the number of acetyl groups from the difference in the C and H values between the parent compound and the acetyl derivative, a computation which is subject to considerable uncertainty in the case of such a large molecule, gives rise to the estimate of two to five acetyl groups per molecule.

A more accurate method is the direct acetyl determination (20). Sa-

12 Asselineau, J., personal communication.
purification of 4.553 mg. of acetyl derivative in 5 cc. of isopropanol containing 100 mg. of KOH for 1 hour gave a volatile acid equivalent of 1.13 cc. of 0.013 N NaOH, or 13.6 per cent COCH₂. This corresponds to five to six acetyl groups per molecule, assuming a molecular weight of 1500 to 1600 for cord factor.

Pipsylation (21)—48 mg. of cord factor were dissolved in 10 cc. of chloroform containing 0.1 cc. of pyridine and 205 mg. of 1³¹-radioactive p-iodobenzene sulfonyl chloride. After the solution was allowed to stand at 25° for 30 minutes, it was concentrated to 5 cc. on a water bath and extracted successively with 1 N NaOH and water until radioactivity measurements indicated that pipsylic acid was no longer being extracted. The chloroform layer was dried over Na₂SO₄ and the solvent evaporated. The residue was dissolved in ether, precipitated with methanol, filtered, and washed with methanol. 35 mg. of a white powder, m.p. 36-38°, were recovered. The specific radioactivity of this product corresponded to about half a pipsyl group per cord factor molecule. The infra-red spectrum also suggested that the reaction product was a mixture containing unchanged cord factor. Chromatography of this mixture on magnesium silicate-Celite yielded two fractions: (1) eluted with benzene-ether 1:1 (sharp band), m.p. 33-34°, containing most of the radioactivity; (2) eluted with ether containing 5 per cent methanol, m.p. 38-40°, its weak radioactivity suggesting that it contained a trace of the pipsyl derivative as an impurity.

The infra-red spectrum of the radioactive fraction (1) (Fig. 3, Curve 11)

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The radioactive pipsyl reagent was obtained through the courtesy of Dr. A. S. Keston.
shows the characteristic absorptions of covalent sulfonate at 1370 and 1180 and the p-substituted phenyl bands at 1575, 815, and 730 cm\(^{-1}\). The intensity of the \(-\)OH band is somewhat less than in cord factor but still strong; at the same time its position has shifted from 3350 to 3450 cm\(^{-1}\), indicating less hydrogen bonding. The decrease in OH absorption, as well as the position of the \(S=O\) bands, suggests that the derivative is a sulfonate rather than a sulfonamide (in the latter, the \(S=O\) bands are usually lower). This is further evidence for the absence of a primary or secondary amine group, since the latter is more reactive than are hydroxyls.

The radioactivity was measured with a Geiger counter with thin end window tubes. The specific activity was determined by adding a volumetrically determined amount of the sample solution on to a filter paper disk placed in a dish. After evaporation of the solvent, the sample was counted. Before the determination of the specific activity of the pipsyl reagent standard, its purity was ascertained by determination of melting point and ultraviolet absorption. The following values were obtained: (a) specific activity of pipsyl derivative Fraction 1 = 3280 c.p.m. per mg. and (b) specific activity of the pipsyl residue = 22,600 c.p.m. per mg.

Assuming one pipsyl group per cord factor molecule, we obtain \(M = 1570\) for the molecular weight of cord factor (267 times 22,600, divided by \(M\) plus 267, equals 3280, 267 being the molecular weight of the pipsyl residue).

This value agrees well with estimations derived from the degradation experiments. The pipsyl derivative was not toxic for mice.

Fraction 2, m.p. 38–40°, which was eluted with ether-methanol, has the typical infra-red spectrum of cord factor and was toxic, thus proving that the unchanged part of the original reaction mixture had been removed during the chromatographic purification of the latter.

**DISCUSSION**

Although the final elucidation of the chemical structure of cord factor has not yet been accomplished, the identification of mycolic acid and glucose as structural units allows its characterization as a new type of glycolipide which has not been previously isolated from tubercle bacilli.

From the data collected in the experimental part, it is possible to outline broadly the structure of the cord factor molecule. It has been shown that a two-step hydrolysis splits the molecule into at least three fragments according to the following sequence:

\[
\begin{align*}
\text{KOH} & \quad \text{mycolic acid (83\%)} + \text{glucoside} \\
\text{HCl} & \quad \text{glucose (9\%)} + R
\end{align*}
\]

It is evident from this that the fragments are connected with each other by two different types of linkages, one broken by the action of alkali and
LiAlH₄ and the other by acid. The former is an ester linkage involving the carboxyl group of mycolic acid, since it is split by saponification as well as reduction, liberating mycolic acid or mycolic alcohol, respectively.

The acid-sensitive linkage must be of glucosidic nature because its cleavage gives rise to a reducing sugar identified as glucose. The fact that it is stable towards alkali is in accordance with this conclusion.

Whether the third fragment, i.e. the still unknown residue $R$, is attached to $C_1$ of glucose through oxygen or nitrogen cannot be decided. The possibility of an $N$-glucoside is conceivable since the glucosidic fragment $R$ most probably contains the 1 atom of nitrogen found in the cord factor molecule. However, from what is known about $N$-glucosides, they are apparently rather unstable unless the nitrogen, being a member of an unsaturated ring system, is stabilized by resonance (22). The possibility of the presence of an unsaturated ring structure, however, seems unlikely because of the lack of the corresponding absorptions in the ultraviolet as well as in the infra-red region of the spectrum.

Also it is questionable whether the mycolic acid residue is attached to one of the hydroxyls of glucose or to the aglycon $R$: mycolic acid-glucose-$R$; mycolic acid-$R$-glucose. Attempts to decide this question by direct acid hydrolysis of cord factor failed because of incomplete reaction due to the great insolubility of the material in aqueous solvents. Since in these experiments both glucose and mycolic acid were liberated in small amounts, it is obvious that conditions permitting the selective hydrolysis of the glucoside linkage have not yet been found.

The fragment which is linked to $C_1$ of glucose has not yet been isolated. Information about its nature can be deduced only by inference. If the molecular weight of cord factor is known, the size of the unknown fragment can be estimated by difference. The molecular weight of cord factor was determined by three independent methods: (1) pipsylation, (2) calculation based on recovery (83 per cent) and titration equivalent (1300) of mycolic acid, and (3) calculation based on C, H distribution in mycolic acid and cord factor. All three values were in close agreement, corresponding to 1570 ± 25. Since the total recovery of mycolic acid and glucose was 92 per cent, the unknown fragment cannot constitute more than 8 per cent, corresponding to a molecular weight of 100 to 150. It is likely to contain the $N$—CH₃ group found in cord factor. The infra-red spectra of the acetyl derivatives fail to show any $N$-acetyl absorptions. Infra-red spectroscopy also indicated that the non-reducing glucosidic fragment contains no $C$—O function (acid, ester, keto, amide). The relative intensity of the $CH₂$ bands, on the other hand, is stronger than in glucose. All these facts together suggest that the unknown fragment is a non-volatile aliphatic trisubstituted amine, possibly containing alcoholic —OH.

Glucose has been obtained from tubercle bacilli only in the form of the
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non-reducing disaccharide trehalose. Anderson has shown that trehalose is the structural unit of a polysaccharide extracted from tubercle bacilli (23), but he also found it as a water-soluble component after alkaline hydrolysis of the acetone-soluble neutral fat (24). He pointed out the peculiarity that acid-fast bacteria grown on a glycerol-containing medium synthesize a disaccharide such as trehalose with which the fatty acids are combined to form neutral fat. Although cord factor appears to be another example of this type of structure, the difference between the two compounds is nevertheless striking. Instead of the relatively low molecular fatty acids of the acetone-soluble fat, cord factor contains high molecular mycolic acid as the lipide component.

The relationship between the lipopolysaccharide (Wax D (25)) or the similar "Pmko" of Choucrour (26) and cord factor has been discussed in a previous paper (3). Both cord factor and Wax D are carbohydrate- and nitrogen-containing esters of mycolic acid, and both are components of Anderson's purified wax fraction, but, according to Anderson and Lederer,14 the polysaccharide part of Wax D is composed of galactose, arabinose, and glucosamine, whereas the glucose content of cord factor makes this compound a chemically distinct constituent of the purified wax of the tubercle bacillus.

SUMMARY

The isolation of a toxic glycolipide "cord factor" from various strains of virulent tubercle bacilli is described. It was found to be a characteristic component of the Wax C fraction of the purified wax.

Chemical studies show that cord factor is an ester of mycolic acid having a molecular weight of about 1570. Alkaline hydrolysis splits the ester linkage, liberating 1 molecule of mycolic acid and 1 molecule of a non-reducing glucoside. Subsequent acid hydrolysis of the latter cleaves the glucosidic linkage, yielding glucose and a non-volatile aliphatic fragment which has not yet been identified. Chemical data suggest that the unknown aglycon contains 1 atom of nitrogen which carries a methyl group and cannot be acetylated.

BIBLIOGRAPHY


14 Dr. E. Lederer informs us that he and his collaborators have never isolated glucosamine from Wax D.
H. NOLL AND H. BLOCH

STUDIES ON THE CHEMISTRY OF THE CORD FACTOR OF MYCOBACTERIUM TUBERCULOSIS
Hans Noll and Hubert Bloch


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