Isolation and Biochemical Activities of Trehalose-6-Monomycolate of *Mycobacterium tuberculosis*

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A monoester of trehalose linked at the 6-position with mycolic acids (trehalose-6-monomycolate) was isolated from the wax D fraction of virulent human *Mycobacterium tuberculosis*, and its biochemical action on host-cell mitochondria was studied. Trehalose-6-monomycolate showed a delayed toxicity for mice. The 50% lethal dose at 2 weeks was 452 μg. It induced in vitro a swelling of mouse liver mitochondria and uncoupled respiration and phosphorylation in the nicotinamide adenine dinucleotide pathway of the electron transport chain. The site of functional damage was located specifically at coupling site II. Mitochondrial adenosine triphosphatase was slightly stimulated by trehalose-6-monomycolate. These findings indicate that trehalose-6-monomycolate affects mitochondrial oxidative phosphorylation in a similar manner to, but to a lesser extent than, trehalose-6,6'-dimycolate (cord factor) of *M. tuberculosis*.

This paper deals with the isolation and characterization and the biological and biochemical activities of TMM of virulent human *M. tuberculosis*.

**MATERIALS AND METHODS**

**Isolation of glycolipid.** Virulent human *M. tuberculosis* H37Rv was grown as surface cultures on Sauton synthetic liquid medium for 4 weeks. After being autoclaved, the bacilli were repeatedly washed with acetone and ether-methanol (1:2). Wax D was prepared from the purified wax fraction according to the method of Noll (14). The chromatographic partitioning of wax D was done as described by Noll and Bloch (16) for the purification of TDM from the wax C fraction. The separation of the components was examined by melting point, acidity determination, and thin-layer chromatography on silica gel H (Merck) plates with chloroform-methanol (85:15) as developing solvent. Lipids were detected by spraying with either sulfuric acid-dichromate or modified anthrone reagent (18) and charring at 150 C. Preparative thin-layer chromatography was performed on plates (20 by 20 cm) covered by a layer of silica gel H (1-mm thickness).

**Analytical method.** The nonreducing glycoside obtained on alkaline hydrolysis of glycolipid was examined by either silica gel thin-layer chromatography (solvent A: n-butanol-acetic acid-water, 4:1:5) or paper chromatography (solvents A and B: isooamyl-alcohol-pyridine-water, 2:1:1). Trehalose was determined by a modified anthrone method (19).

Reducing sugars prepared by acid hydrolysis of glycoside were analyzed after deionization with Amberlite IR4B (OH−) by either paper chromatography (solvent B) or gas-liquid chromatography after methylation and trimethylsilylation with a Gas Chromatograph model 800 (Packard Instrument Co., Inc., La...
Grange, Ill.) equipped with an argon ionization detector. The column was packed with 3% Silicone SE 30 (methylsilicone) on Chromosorb W. Sugar was converted to the trimethylsilyl derivative with SIL PREP (Applied Science Laboratories, Inc., State College, Pa.).

For determining and identifying the fatty acid fraction contained in the glycolipid, the ether solution of the alkaline hydrolysis product was evaporated to dryness. After weighing, the lipid was esterified in methanol-hydrochloride and developed on silica gel thin-layer chromatography (solvent C: petroleum ether-ether, 80:20) with the methylated mycolic acid prepared from H37Rv. The product of pyrolysis at 250°C for 20 min of the esterified lipid was analyzed by gas-liquid chromatography.

Toxicity test. Male albino mice of random-bred ddO stock (10), 4 weeks of age, were used for the toxicity test. The glycolipid was dissolved in Bayol F (Serva Feinbiochimica GMBH & Co., Heidelberg, Germany) and sterilized by heating at 100°C for 15 min. Various amounts of glycolipid ranging from 100 to 500 μg in 0.1 ml of Bayol F were injected intraperitoneally into mice (10 in each group), and the number of deaths was recorded for 2 to 3 weeks. The 50% lethal dose (LDso) was calculated by the method of Reed and Muench (17).

Preparation of aqueous emulsion of glycolipid. The glycolipid was emulsified in 0.25 M sucrose containing 0.01 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride at pH 7.4 (sucrose-Tris) by a previously reported method (8).

Mitochondrial swelling. Mitochondria were prepared from mouse liver by a light modification (7) of Hogeboom's procedure (4). Protein was determined by the biuret method (3) modified by Jacobs et al. (5). The swelling of mitochondria was measured as previously described (6).

Assay methods. Phosphorylation coupled to the aerobic oxidation of either succinate (plus rotenone) or pyruvate plus malate was assayed manometrically by a slight modification (8) of a previously reported method (7). Phosphorylation at coupling sites II and III was measured as previously described (7). Mitochondrial respiration in the absence of a phosphorylation acceptor system was measured by substituting 2.5 mM adenosine triphosphate (ATP) for adenosine diphosphate and 0.15 M KCl for hexokinase and glucose by the method of Lardy and Wellman (11).

The mitochondrial adenosine triphosphatase activity was measured as previously described (7).

Chemicals. Cytochrome c (type VI from horse heart), adenosine diphosphate (grade 1 from equine muscle), ATP, hexokinase (type III from yeast), and crystalline bovine serum albumin (BSA) were products of Sigma Chemical Co., St. Louis, Mo.; rotenone was obtained from K & K Laboratories, Plainview, N.Y.; N,N,N',N'-tetramethyl-p-phenylenediamine was purchased from Tokyo Kasei, Tokyo, Japan.

RESULTS

Purification of trehalose-6-monomycolate from the wax D fraction. Wax D was dissolved in petroleum ether and charged on a column of magnesium silicate-Celite (2:1). The column was washed successively with petroleum ether, petroleum ether-benzene (1:1), benzene, benzene-ether (1:1), and ether. After bulk separation of a glycolipid-rich fraction by eluting the column with ether-methanol (75:25) (15), the free mycolic acid contaminant was removed by a subsequent chromatography of the fraction on silica gel (16). The toxic glycolipid fraction eluted from the silica gel column with petroleum ether was again chromatographed on magnesium silicate-Celite (2:1), and tail fractions eluted with ether-methanol (99:1 and 80:20) were collected. The thin-layer chromatography pattern of these fractions is shown in Fig. 1. The fractions eluted with ether-methanol

![Fig. 1. Thin-layer chromatogram of glycolipids eluted from a magnesium silicate-Celite (2:1) column with the mixture of ether (E) and methanol (M).](http://iai.asm.org/Downloaded_from)
(99:1 and 95:5) contained one anthrone-positive spot of $R_t$ 0.70 which corresponded with the $R_t$ value of TDM purified from wax C. The fraction eluted with ether-methanol (90:10) contained two glycolipids, TDM and a more polar glycolipid with less mobility ($R_t$ 0.27). Fractions eluted with ether-methanol (85:15 and 80:20) contained only this polar glycolipid. Preparative thin-layer chromatography on silica gel H with chloroform-methanol (85:15) allowed the isolation of this polar glycolipid in the yield of 0.02% of dry bacterial weight. The purified polar glycolipid gave one single spot on silica gel thin-layer chromatography in several solvent systems. The $R_t$ values were: 0.65 in chloroform-methanol-water (65:25:4); 0.13 in chloroform-methanol-acetic acid (85:15:1); and 0.05 in diisobutylketone-acetic acid-water (85:25:4). The mobilities in all of these solvents were identical to those of TMM from the firmly bound lipids and much less than those of TDM (12).

**Chemical properties.** Table 1 compares the chemical properties of TMM with those of TDM. TMM showed much higher values of both melting point and optical rotation than TDM. The infrared spectra of TMM obtained from both wax D and the firmly bound lipids were typical of long-chain fatty acid esters (Fig. 2). The spectra showed a hydroxy group peak at 3,500 cm$^{-1}$ and an ester carbonyl peak at 1,720 cm$^{-1}$, together with a small peak at 808 cm$^{-1}$ which is associated with trehalose (1).

The nonreducing glycoside obtained by alkaline hydrolysis of TMM gave one single spot on silica gel thin-layer and paper chromatography as detected with anthrone reagent (18) in several solvent systems. The mobilities were identical to those of authentic $\alpha$, $\alpha'$-$\beta$-trehalose. The sugar content was, on the average, 19% of glycolipid (theoretical value for trehalose monomycolate = 21%). After acid hydrolysis of the nonreducing glycoside, descending paper chromatography yielded a single spot which corresponded to D-glucose in solvents A and B. Gas-liquid chromatography (SE 30, column 170 C, detector 210 C, argon 20 ml/min) of the trimethylsilyl derivative of the reducing sugar proved it to be identical with authentic D-glucose.

The fatty acid fraction obtained by alkaline hydrolysis of TMM had a melting point of 55 to 57 C and $[\alpha]_D^{20} +2.8$. Its acidity was 7.5 $\mu$liters of 0.1 N methanolic KOH per mg (theoretical value for mycolic acid = 7.7 $\mu$liters of 0.1 N methanolic KOH per mg). Its infrared spectrum was identical with that of a sample of mycolic acid of H37Rv. The methyl ester of the fatty acid gave one spot ($R_t$ 0.48) on silica gel thin-layer chromatography in solvent C corresponding with the $R_t$ value of methyl mycolate. Gas-liquid chromatography of the pyrolysis product of the fatty acid showed one peak which was identified as hexacosanoate by its retention time.

The glycolipid contained mycolic acid and trehalose in equal molar amounts (Table 2).

The above results indicate that the glycolipid is a monoeaster of $\alpha$, $\alpha'$-$\beta$-trehalose with mycolic acid.

**Structural studies.** The glycolipid in anhydrous ether was permethylated with diazomethane with BF$_3$ catalysis according to Goren (2). The product was separated from incompletely

![FIG. 2. Infrared spectrum of trehalose-6-monomycolate isolated from (A) the firmly bound lipids and (B) the wax D fraction of M. tuberculosis H37Rv.](http://iai.asm.org/)

<table>
<thead>
<tr>
<th>Glycolipid</th>
<th>Elementary analysis</th>
<th>$\alpha_D^\circ$</th>
<th>$T_m$ (C)</th>
<th>Acidity*</th>
<th>Ninhydrin</th>
<th>Phosphorus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trehalose-6-monomycolate</td>
<td>73.05 11.85</td>
<td>$+47.1^\circ \pm 0.9^\circ$</td>
<td>163–167</td>
<td>0.10</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>Trehalose-6'-dimycolate</td>
<td>77.91 12.64</td>
<td>$+30^\circ \pm 1.0^\circ$</td>
<td>43–45</td>
<td>0.15</td>
<td>Negative</td>
<td>Negative</td>
</tr>
</tbody>
</table>

* Expressed as microliters of 0.1 N methanolic KOH per milligram.
methylated glycolipid by preparative thin-layer chromatography in benzene-ether (30:1). An infrared spectrum of the product showed only a minor absorption band of hydroxyl groups in the 3,500-cm\(^{-1}\) region. The permethylated glycolipid was solvolysed in ethanolic KOH for 48 h at 45 °C, and the product was partitioned between aqueous methanol and hexane.

The carbohydrate in the aqueous solution had a chromatographic mobility in thin-layer chromatography identical with that of authentic 2,3,4,6,2',3',4'-heptamethyl trehalose and different from a comparison sample of the 2,3,4,6,3',4',6'-isomer. In gas-liquid chromatography (SE 30, 1.8 m, 210 C), the unknown carbohydrate had a retention time identical with that of authentic 2,3,4,6,2',3',4'-heptamethyl trehalose and distinguished from the 2-hydroxy isomer.

The presumed 2,3,4,6,2',3',4'-heptamethyl trehalose was hydrolyzed for 18 h at elevated temperature in 1 N HCl, and the products were recovered by gentle evaporation. Gas-liquid chromatography of the trimethylsilyl derivatives (SE 30, 110 C) showed the components to be 2,3,4,6-tetra-O-methyl glucose and 2,3,4-tri-O-methyl glucose, by comparison with authentic samples. Both anomers of the two sugars were resolved; the trimethyl glucose was clearly distinguished from anomeric 2,3,6-, 2,4,6-, and 3,4,6-tri-O-methyl glucose. Therefore, the original glycolipid has the structure 6-mycoloyl-\(\alpha, \alpha'\)-trehalose.

**Biological and biochemical properties.**

TMM is toxic for mice, although it is much less toxic than TDM (Fig. 3). The LD\(_{50}\) of TMM at 2 weeks was 452 \(\mu\)g, whereas that of TDM was 50 \(\mu\)g (6). It appears that the toxicity of TMM is more delayed than that of TDM, killing mice between 2 and 3 weeks with apparent toxic manifestations. The LD\(_{50}\) value calculated at 3 weeks was 385 \(\mu\)g.

TMM induced in vitro swelling of mouse liver mitochondria in direct proportion to the amounts added to the test medium (Fig. 4).
This TMM-induced swelling was prevented by the presence of BSA in the incubation medium and was completely reversed by the addition of ATP plus magnesium ion or ATP plus magnesium ion plus BSA, whereas either ATP or BSA alone was without effect. These findings indicate that TMM induced only a slight structural damage in the mitochondrial membrane system.

Respiration and accompanying phosphorylation in the succinate pathway of the mitochondrial electron transport system was barely inhibited by TMM (Table 3). A slight uncoupling effect of TMM in this system was noted by the gradual decrease of respiratory control ratio with increasing concentration of TMM in the preincubation medium. By contrast, the reduced nicotinamide adenine dinucleotide (NADH) pathway of the mitochondrial electron transport chain was more sensitive to the inhibitory action of TMM than the succinate pathway. The rate of oxidation of pyruvate plus malate and the efficiency of associated phosphorylation were markedly affected, and respiratory control was lost. In both pathways, mitochondrial respiration in the absence of a phosphoryl acceptor system was not affected by TMM.

Table 4 compares the effects of TMM on phosphorylation at coupling sites II and III. The electron transfer process and respiratory control at coupling site II were affected by TMM, whereas the oxidation and phosphorylation at site III remained intact. Mitochondrial adenosine triphosphatase was slightly stimulated by TMM (Table 5). The effects of TMM and of 2,4-dinitrophenol to induce adenosine triphosphatase were additive.

All of these effects of TMM on the mitochondrial enzymatic processes mimic those of TDM, although the former was much less active than the latter (8).

**DISCUSSION**

Data presented in this paper demonstrate that a glycolipid which contains α,α'-trehalose and mycolic acids in equal molar ratio (TMM) is present as a native constituent of virulent human tubercle bacilli. Mycolic acids are esterified at the 6-position of trehalose. The yield of TMM from the wax D fraction of M. tuberculosis H37Rv was 0.02% of the dry bacterial weight. It has been reported that the firmly bound lipids of the same bacterial strain contained 0.04% of TMM of the bacterial dry weight (12). Thus, the total yield (approxi-
mately 0.06%) of TMM is about one-half of the yield of trehalose-6,6′-dimycolate (cord factor, TDM) which has been known as a sole toxic lipid component of virulent tubercle bacilli (13). The big difference in polarity between TMM and TDM allowed a clear separation of both glycolipids in silica gel thin-layer chromatography with a mixture of chloroform and methanol (85:15) as developing solvent.

Chemical and physicochemical properties sharply differentiated TMM from TDM. TMM, which lacks one mycolic acid residue of TDM, was much more polar than the latter and had a markedly higher melting point and rotatory power.

It is of interest that TMM is less toxic than not only TDM but also a number of semisynthetic TDM analogues containing different sugars, methyl 6-mycoloyl-α-D-glucopyranoside, and 6,6′-dimycocolyl sucrose (9). This seems to support our previous view (7) that the proportion of hydrophobic mycolic acid chain with hydrophilic hydroxyl groups of trehalose plays, in part at least, an important role in the toxicity of TDM. In fact, the incorporation of a nonsubstituted glucose residue to methyl 6-mycoloyl-α-D-glucoside (LD₅₀ = 187 μg [9]) to give trehalose-6-mycolate greatly reduced the toxicity of the molecule. This decrease in toxicity of TMM compared with methyl 6-mycoloyl-α-D-glucoside seems to be the result of increased polarity corresponding to replacement of the glycosidic methyl group with the larger and more polar hydrophilic glucose of the trehalose moiety of TMM.

In accord with its lowered toxicity for mice, the action of TMM on the structure and function of mouse liver mitochondria was less marked than that of TDM (7) and of toxic semisynthetic analogues (9). TDM induced in vitro a swelling of mouse liver mitochondria which was not prevented by BSA and was partially reversed by ATP, magnesium ion, and BSA, indicating that it gave an irreversible change to the mitochondrial membrane structure (6). However, the mitochondrial swelling induced by TMM was prevented by BSA and was completely reversed by ATP, magnesium ion, and BSA. It appears from these findings that TMM affects very slightly the structural integrity of the mitochondrial membrane.

TDM (7), as well as its toxic semisynthetic analogues (9), inhibited phosphorylation accompanying the oxidation of both succinate and a number of NADH-linked substrates and induced a loss of respiratory control in mouse liver mitochondria. Phosphorylation at coupling site II was damaged by preincubation of the mitochondrial suspension with TDM. In contrast to this, TMM inhibited the mitochondrial oxidative phosphorylation only in the NADH pathway and caused uncoupling of respiration and phosphorylation at site II, which was detectable only by the decline of the respiratory control ratio.

However, the effects of TMM and TDM are common in that both compounds affected more markedly the NADH pathway than the succinate pathway of the mitochondrial electron transport chain, since III was entirely unaffected by either TDM or TMM, the mitochondrial adenosine triphosphatase was stimulated by both glycolipids, and their induction of adenosine triphosphatase and 2,4-dinitrophenol was additive.

These findings indicate that TMM acts on mitochondria in an identical mechanism with TDM, but that the former induces a less marked structural and functional damage in host-cell mitochondria than the latter. The metabolic correlation between TMM and TDM in the biosynthetic processes in mycobacteria and a possible synergistic action of both glycolipids in the toxic phenomena in mice are under investigation.

**ACKNOWLEDGMENTS**

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**LITERATURE CITED**


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**TABLE 5. Effect of trehalose-6-monomycolate on mitochondrial adenosine triphosphatase**

<table>
<thead>
<tr>
<th>TMM concn (μg/mg of protein)*</th>
<th>Adenosine triphosphatase*</th>
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<tbody>
<tr>
<td></td>
<td>- DNP*</td>
</tr>
<tr>
<td>0</td>
<td>12</td>
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<td>25</td>
<td>18</td>
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<tr>
<td>50</td>
<td>25</td>
</tr>
<tr>
<td>100</td>
<td>38</td>
</tr>
</tbody>
</table>

* See footnote to Table 3.
* Expressed as nanomoles of inorganic phosphate liberated per minute per milligram of protein.

*2,4-Dinitrophenol.