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SYNTHESIS OF TREHALOSE DIMYCOLATE (CORD FACTOR) BY A CELL-FREE SYSTEM OF MYCOBACTERIUM SMEGMATIS

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A 105,000 x g residue fraction of Mycobacterium smegmatis contains an enzyme (acyl transferase) that transfers endogenous mycolic acid to trehalose monomycolate to yield trehalose dimycolate. This enzyme activity is stable to repeated freezing and thawing and is unaffected by the antimycobacterial drug, ethambutol. These results show that trehalose monomycolate is a direct precursor of trehalose dimycolate and suggest the presence of activated mycolic acids (acyl donor) in the cell-free system.

INTRODUCTION

Mycobacteria, nocardia, and corynebacteria are unique in that they are able to synthesize mycolic acids (α-branched, β-hydroxy fatty acids) (1-3). Mycolic acids are found only in certain components of the cells, specifically the cell wall (4), TM (5), TD (6), and glycerol mycolate (7). Very little is known as to how these microorganisms synthesize the fatty acids. The synthesis of C_{24}-fatty acids by a purified cell-free system of Mycobacterium smegmatis which might represent the early pathway to the synthesis of mycolic acid was studied by Bloch and associates (8). Qureshi et al. (9) suggested that the very long-chain fatty acids ($>C_{30}$) which they characterized from Mycobacterium tuberculosis strain $H_{37}R_a$ are precursors of mycolic acid. The final steps in the assembly of mycolic acids and their transfer to either trehalose or cell wall are not known.

Abbreviations used: TM, trehalose monomycolate; TD, trehalose dimycolate; HPLC, high performance liquid chromatography; TLC, thin-layer chromatography; solvent A, chloroform-methanol-water (90:10:1 v/v/v).

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There are two important biologic properties of TD (also called cord factor). TD from mycobacteria is a toxic glycolipid. Kato (10,11) showed that this lipid affects the function of the mitochondrial membrane by inducing a decrease in respiration and a loss of oxidative phosphorylation. This action might be partly responsible for the pathogenicity of M. tuberculosis. Oil-water emulsions of TD and mycobacterial cell wall skeleton or TD and endotoxin are effective in reducing established line 10 tumors in syngeneic guinea pigs (12,13).

We have previously shown that exposure of M. smegmatis to the antituberculosis drug, ethambutol, causes the cellular accumulation and subsequent leakage of TM and TD (14). This suggested that the biosynthesis of both glycolipids is affected by this drug. We also showed that TM had a very rapid rate of turnover. In this communication, we show that a cell-free extract of M. smegmatis can synthesize TD from TM, which establishes a precursor role for TM.

MATERIALS AND METHODS

Materials. Distilled-in-glass isopropanol and hexane were purchased from Burdick and Jackson Laboratory. HPLC-grade water was from Alltech Associates. Sodium [1-^{14}C] acetate (58.7 μCi/μmol) was obtained from Amersham Corporation.

Preparation of cell-free extract. M. smegmatis was grown in glycerol-alanine-salts medium (15) in a 28-l New Brunswick Fermentor at 37°C for 24 hr. The cells were harvested by centrifugation to yield 42 g (drained weight) from 22 l of culture. The cells were then suspended in 0.10M potassium phosphate buffer (pH 7.0) at a concentration of 1-g harvested weight of cells per ml. The suspension was sonicated at full power using a Branson sonifier for 2.5 min at 4-10°C. The unbroken cells and cell debris were removed by centrifuging at 10,000 x g for 30 min. The supernatant was then centrifuged at 105,000 x g for 60 min to yield a pellet and a clear supernatant. The pellet was washed four times with 0.10M potassium phosphate buffer (pH 7.0) and finally suspended in the same buffer to a concentration of 6.08 mg protein/ml. Protein was determined by the method of Lowry et al. (16).

Preparation of unlabeled TD. M. smegmatis (200 g, wet weight) was suspended in 2 l of chloroform-methanol (2:1, v/v), stirred overnight, and then filtered. The residue was extracted again with the same solvents, and the pooled extract was dried, yielding 8.65 g of residue. The preparation was then passed through a 3.2- x 24-cm DEAE-cellulose column (acetate form) in chloroform-methanol (4:1, v/v) to yield 6.53 g of neutral lipids. The sample was applied to a silicic acid column (390 g of Bio Sil HA, 325 mesh), and the TD was eluted with chloroform-methanol (18:1, v/v). The yield was 522 mg of TD. Further purification of TD was achieved by preparative TLC, using silica gel G (1,000 μ) and solvent A.

Preparation of [^{14}C] TM. A 1.5-l culture of M. smegmatis was grown at 37°C to an absorbance of 650 nm of 0.5. Sodium [1-^{14}C] acetate (2.0 mCi)

was then added; and the culture incubated for 60 min. The cells were harvested by centrifugation and extracted three times (24 hr/extraction) with 150 ml of chloroform-methanol (2:1, v/v) by stirring at 22°C. The pooled extract was dried, applied on a prescored 20- x 20-cm silica gel G (250 μ) TLC plate as an 18-cm streak, and developed with a solvent system of chloroform-methanol-water (80:20:2, v/v). The plate was broken into 5- x 20-cm sections, scanned with a Packard radiochromatogram scanner to locate the [^{14}C] TM band, and the crude [^{14}C] TM containing 1.85 x 10^8 dpm was recovered. This sample was then fractionated on a 2- x 144-cm Sephadex LH-20 column with chloroform-methanol (4:1, v/v), and the [^{14}C] TM peak was recovered (1:10 mg, 8.49 x 10^7 dpm/μmole based on an average m. wt. of 1,446)[1]. This preparation was dissolved in isopropanol to a concentration of 7.1 x 10^6 dpm/ml (warming was required) and used as the substrate for the TD synthetase assay.

TD synthetase assay. A reaction mixture containing from 0-18 μg of 105,000 x g residue fraction from M. smegmatis in 150-600 μl of 0.10M potassium phosphate buffer (pH 7.0) was warmed to 37°C. A boiled residue fraction was included in all assays. Then 840 pmoles (71,400 dpm) of [^{14}C] TM in 10 μl of isopropanol was added to start the reaction. After incubating at 37°C for various times, the reaction was stopped by adding 5.0 ml of chloroform-methanol (2:1, v/v) and mixing. Water (2.0 ml) was then added, mixed, and centrifuged at 5,000 x g for 5 min. The lower organic layer was filtered and dried. Carrier TD (50 μg) was added, and the extract was applied to a silica gel G (250 μ) plate as a 5-7-mm streak and developed with solvent A. The carrier TD was located by exposing the plate to I_2 vapor, and the TD area was scraped into vials containing 5.0 ml scintillation fluid (Aquasol, New England Nuclear) and counted in a liquid scintillation spectrometer.

HPLC fractionation. HPLC of TM and TD was performed with a previously described setup (9). The radial compression separation system with an 8-mm x 10-cm radialpak C_{18} cartridge (Waters Associates, Inc.) was used. A linear gradient of 0-60% hexane-isopropanol (2:1, v/v) in isopropanol-water (9:1, v/v) over a 60-min period was used at a flow rate of 2.0 ml/min. The separation was followed by measuring absorbance at 210 nm and by collecting 30-sec fractions for radioactivity determination.

RESULTS AND DISCUSSION

When a washed 105,000 x g membrane fraction from M. smegmatis was incubated with [^{14}C] TM at 37°C, the synthesis of [^{14}C] TD was observed as shown by TLC of the reaction mixture (Fig. 1). A clear separation of the [^{14}C] TM substrate (Rf, 0.10) was achieved from the [^{14}C] TD product (Rf, 0.59) on a silica gel G plate using solvent A. The product was further identified by HPLC as shown (Fig. 2). Using a reverse-phase mode of separation on a C_{18}-bonded silica cartridge, complete separation of TM and TD was achieved. This figure shows that the [^{14}C] TD product and carrier TD eluted at 40-54 min, whereas carrier TM eluted at 12-30 min. Separation according to the sizes of the mycolic acid residues in TM and TD was clearly indicated.

[1] We are using the average m. wt. of 1,122 for a diunsaturated C_{77} mycolic acid from M. smegmatis (17).

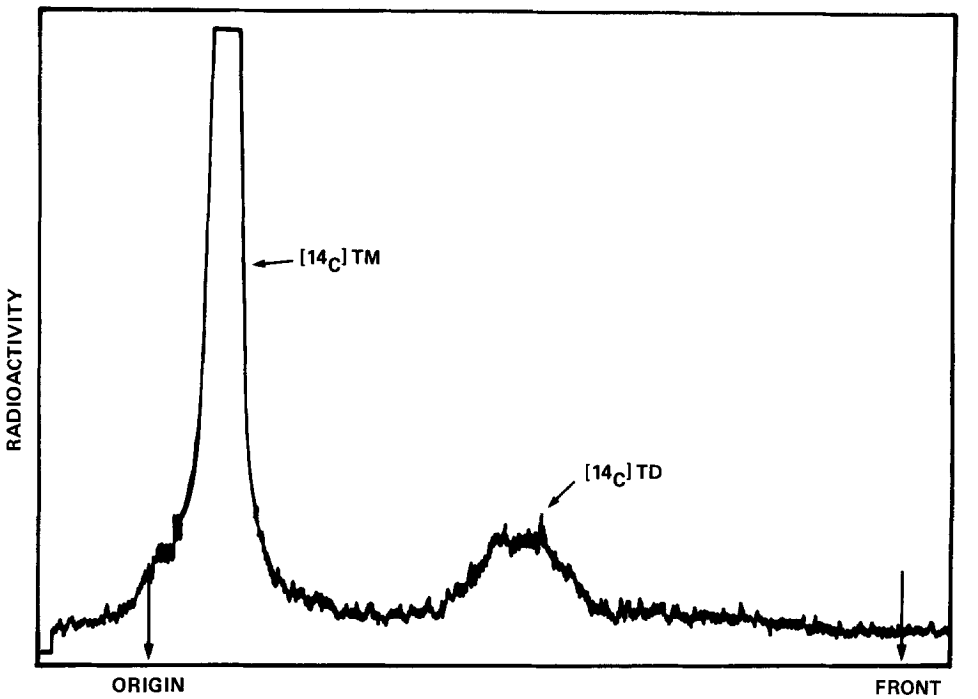

Fig. 1. A scan of TLC of [^{14}C] TD synthesized by a cell-free enzyme system of M. smegmatis. The reaction mixture containing 1.50 mg of 105,000 x g residue from M. smegmatis and 4.16 nmoles (357,000 dpm) of [^{14}C] TM in 3.0 ml of 0.10 M potassium phosphate buffer (pH 7.0) was incubated at 37°C for 15 min. The reaction mixture was extracted with chloroform-methanol (2:1, v/v) and TLC was performed on the extract using solvent A. Carrier TM and TD (50 µg each) were added to the sample. The plate was scanned, sprayed with dichromate-sulfuric acid reagent, and charred to locate the standards.

The TD synthetase activity was unaffected by the presence of the antimycobacterial drug, ethambutol, and [^{14}C] TM prepared from M. tuberculosis $H_{37}R_a$ was utilized as a substrate as efficiently as [^{14}C] TM prepared from M. smegmatis, indicating a lack of species specificity by the M. smegmatis enzyme (data not shown). Furthermore, this enzyme was found to be stable to repeated freezing and thawing. The 105,000 x g supernatant fraction showed a lower TD synthetase activity per µg of protein. However, other preparations have shown greater activity in the supernatant fraction. No explanation is offered for these results at this time.

As shown in Figure 3, the synthesis of TD was dependent on the incubation time. The linear portion of the curve was the first 15 min. The synthesis of TD was also dependent on the protein concentration (data not

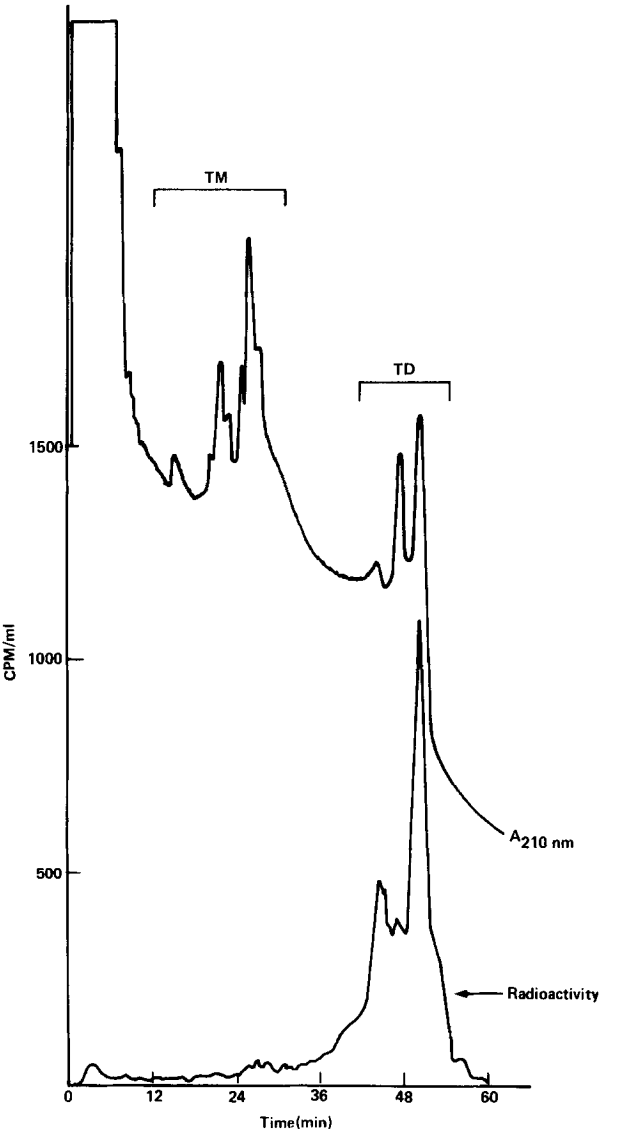

Fig. 2. HPLC of [^{14}C] TD synthesized by a cell-free enzyme system of M. smegmatis. The synthesized [^{14}C] TD (30,000 dpm) previously purified by TLC was combined with 1.0 mg of TM and 2.0 mg of TD (used as carriers) and fractionated on a C_{18}-bonded silica cartridge using a linear gradient of 0-60% hexane-isopropanol (2:1, v/v) in isopropanol-water (9:1, v/v) over a 60-min period at a flow rate of 2 ml/min. The carrier TM and TD were detected by following the absorbance at 210 nm. Fractions (30 sec) were collected and assayed for radioactivity.

shown). Figure 3 shows that the product formation becomes approximately maximal with 330 pmoles of TD formed after about 180 min. This result suggests that the 105,000 x g residue contains at least 166 pmoles of activated mycolic acid (possibly a protein-bound thioester) per μg of

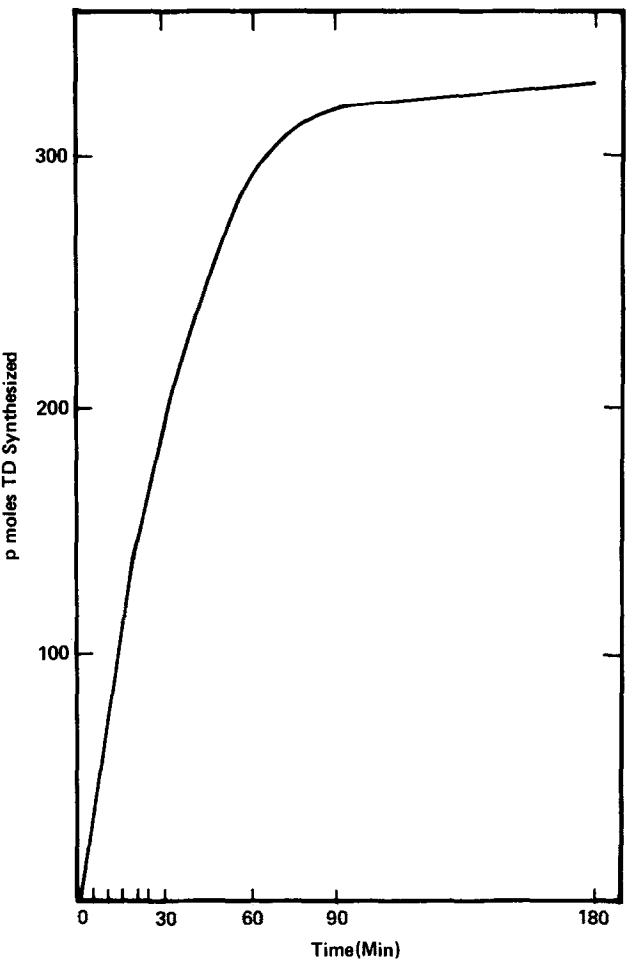

Fig. 3. Time course of TD synthesis. Each reaction mixture contained 2.0 µg of the 105,000 x g residue from M. smegmatis and 840 pmoles (71,400 dpm) of [^{14}C] TM in a final volume of 150 µl of 0.10 M potassium phosphate buffer (pH 7.0). The reaction mixtures were incubated at 37°C, and the synthesis of [^{14}C] TD was followed by TLC.

protein and an acyl transferase enzyme. In this reaction, we make a reasonable assumption that the endogenous donor mycolic acid is transferred to the [^{14}C] TM acceptor to form [^{14}C] TD.

The volume of the reaction mixture influenced the rate of synthesis of TD as shown in Figure 4. There was a linear decrease in the rate of synthesis with increases in volume from 150-625 µl. At this substrate and enzyme content, the maximum rate extrapolated to zero volume was 60.3 pmol/ 15 µg/10 min. At the lower range of 25-125 µl, the enzyme activity decreased rapidly with decreasing volume. This drop corresponded to a sharp rise in

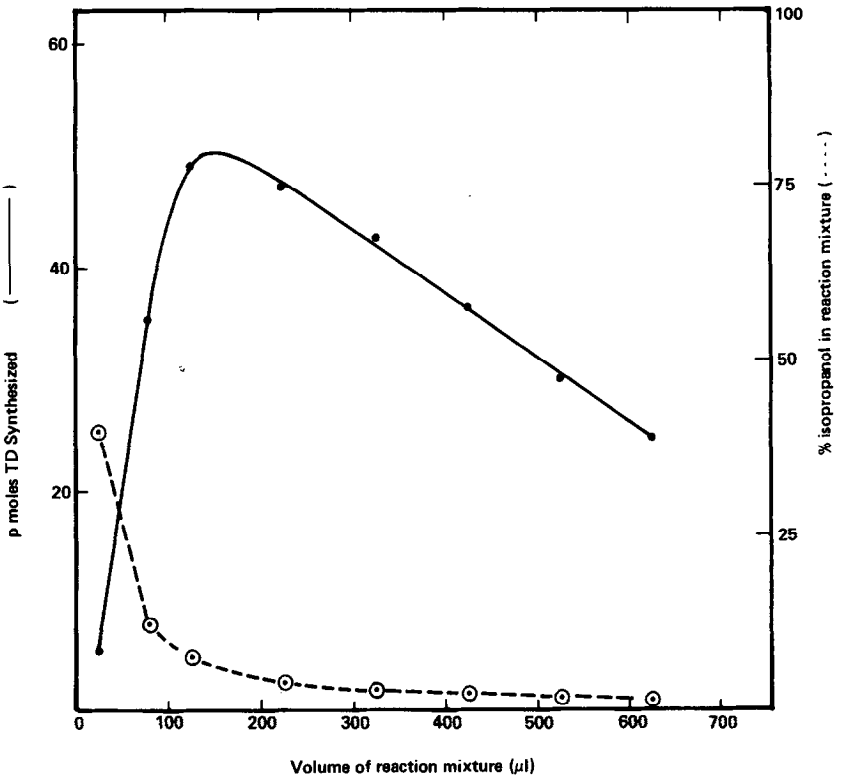

Fig. 4. Effect of reaction mixture volume on the synthesis of TD. The reaction mixtures contained 15 µg of 105,000 x g residue from M. smegmatis and 840 pmoles of [^{14}C] TM in 0.10 M potassium phosphate buffer (pH 7.0). The volumes of the reaction mixtures varied from 25-625 µl. Incubation was at 37°C for 10 min.

the isopropanol concentration. We conclude that an isopropanol concentration above 8% inhibits the enzyme activity. The volume-dependent property of the TD synthetase could be due to the low solubility of the substrate. The diffusion coefficient of TM in an aqueous medium might be so low that it becomes a rate-limiting factor in the TD synthetase activity. In this regard, we did not investigate the effects of detergent on the enzyme activity.

This study clearly establishes a precursor role for TM in the synthesis of TD. We are presently studying the nature of the acyl transferase and the endogenous activated mycolic acid.

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