Isolation, Characterization, and Function of 6-Mycolyl-6′-acetyltrehalose in the H37Ra Strain of *Mycobacterium tuberculosis*†

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ABSTRACT: The major mycolic acid containing extractable lipid of the H37Ra strain of *Mycobacterium tuberculosis* was established to be 6-mycolyl-6′-acetyltrehalose (MAT). This new glycolipid was extracted from harvested cells with chloroform–methanol (2:1, v/v) and initially precipitated out in acetone. A series of column (DEAE-cellulose, silicic acid, and Sephadex LH-20) and preparative thin-layer chromatography steps yielded a homogeneous preparation. A single sugar was released by saponification and it was identified to be trehalose by paper chromatography and gas–liquid chromatography of the trimethylsilyl derivative. The lipid moiety was determined to be exclusively mycolic acids.The major mycolic acid component of this glycolipid was isolated, purified as the methyl ester, and characterized to be methyl α-mycolate by nuclear magnetic resonance spectroscopy and mass spectrometry. The molar ratio of trehalose to mycolic acids was determined to be 1:1. The other acyl group in MAT was established to be acetate by gas–liquid chromatography. Methylation analysis showed the mycolate and acetate ester linkages to the 6 and 6′ positions of trehalose. The time course of incorporation of ^{14}C-labeled acetate into the mycolates of both MAT and total cellular fatty acids was followed. These results showed that the synthesis of MAT is rapid and linear for the initial 20 min of incubation whereas the curve for the total cellular mycolates minus MAT (an estimate of the cell wall mycolates) had a 25–30-min lag period. When the label in the lipids was chased out with an excess of unlabeled acetate, relatively rapid decline in the labeled MAT resulted with a corresponding rise in the level of radioactivity in the mycolates of the nonextractable cellular fraction (assumed to be the cell wall fraction). Thus mycolic acids are rapidly transferred from MAT to the cell wall of *M. tuberculosis*.

Mycolic acids are complex β-hydroxy acids substituted at the α position with a long aliphatic chain. These acids are found as prominent parts of the cell wall structure of all mycobacteria, *Nocardia* and *Corynebacteriae* (Kanetsuna, 1968; Goren, 1972). Each species contains several characteristic types of mycolic acids. We recently became interested in how mycolic acids are synthesized in *Mycobacterium tuberculosis* H37Ra and how these fatty acids are utilized by the microorganism. This interest stems from the observation that isoniazid (isonicotinic acid hydrazide)[1] is a potent inhibitor of cellular mycolic acid synthesis (Wang and Takayama, 1972). It is reasonable to assume that the synthesis and utilization of mycolic acids probably involve some mycolate-containing free lipids (or mycolate precursors). The mycolate-containing lipids that were previously isolated from *Mycobacterium tuberculosis* are trehalose 6,6′-dimycolate (Bloch, 1950), wax D (Lederer, 1971), and trehalose 6-mycolate (Kato and Maeda, 1974). We have recently isolated but not identified a few of the mycolate-containing free lipids from *M. tuberculosis* H37Ra (Takayama, 1974).

In this report we shall describe the isolation and characterization of a new glycolipid, 6-mycolyl-6′-acetyltrehalose (MAT), which was found to be a major mycolate-containing free lipid of the H37Ra strain of *M. tuberculosis*. We shall show that this glycolipid is involved in the transfer of newly synthesized mycolic acids into the cell wall.

Materials and Methods

Materials. [1-^{14}C]Acetate was purchased from New England Nuclear, Boston, Mass. Water soluble fatty acid mixture containing 0.1% each of acetate, propionic, isobutyric, butyric, isovaleric, and valeric acids was obtained from Supelco, Inc., Bellefonte, Pa. Trehalose dimycolate isolated from the Aoyama B strain of *Mycobacterium tuberculosis* was a generous gift of Dr. Edgar Ribi. All other chemicals and reagents were obtained commercially and were reagent grade.

Bacterial Strain and Growth. M. tuberculosis H37Ra was grown in either glycerol-alanine-salts medium (medium A) at 37°C in a New Brunswick 28-l. capacity fermentor (New Brunswick Microferm) or in the Middlebrook 7H9 medium (Difco) (medium B) with Tween-80 and albumin-dextrose-catalase (ADC) enrichment on a rotary shaker at 37°C (Takayama et al., 1975).

Analytical Procedures. Sugar was determined by the phenol–sulfuric acid method (Dubois et al., 1956). The reducing sugar was determined by the Park and Johnson method (1949). Radioactivity was determined by adding 12 ml of toluene containing 4 g of 2,5-diphenyloxazole and 100 mg of 2,5-bis[2-(5-*tert*-butylbenzoxazolyl)]thiophene per liter and counting in a Packard TriCarb scintillation spectrometer. An internal standard was utilized to obtain disintegrations per minute values and the counting efficiency was established to be 74%. The following solvents were used

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[1] Abbreviations used are: isoniazid, isonicotinic acid hydrazide; MAT, 6-mycolyl-6′-acetyltrehalose; ADC, albumin-dextrose-catalase;

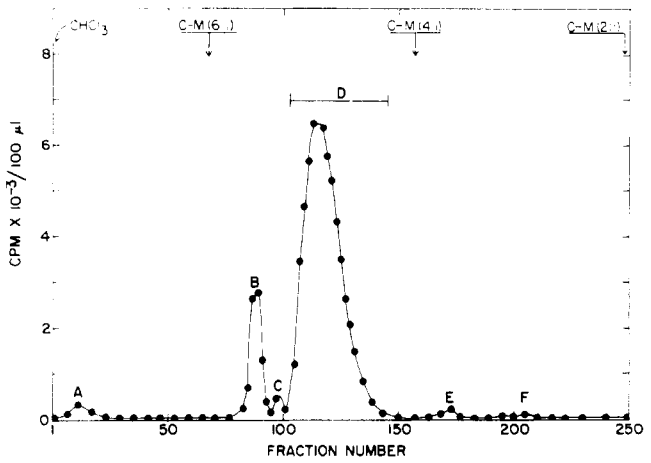


FIGURE 1: Silicic acid column fractionation of labeled acetone-insoluble, neutral lipids of *M. tuberculosis* H37Ra. The sample was dissolved in chloroform and applied to a silicic acid column packed in chloroform. The following solvents were used in a stepwise gradient elution: 350 ml of chloroform and 500 ml each of chloroform–methanol mixtures 6:1, 4:1, and 2:1 by volume. Only a trace of radioactivity was found beyond fraction number 250. The fraction size collected was 5 ml and 100-µl aliquots were analyzed for radioactivity.

in the chromatography of sugars on Whatman 3MM paper: A, ethyl acetate–pyridine–water (12:5:4, v/v); B, 1-butanol–ethyl acetate–acetic acid–water (40:30:25:40, v/v); C, 2-propanol–1-butanol–water (140:20:40, v/v); D, 1-butanol–acetic acid–water (12:3:5, v/v). Sugars were detected on paper by using the ammoniacal silver nitrate dip reagent (Aronoff, 1956). Thin-layer chromatography was performed on silica gel G (0.25 mm) plates with the following solvent systems: E, chloroform–methanol–water (80:20:2, v/v); F, petroleum ether–diethyl ether (8:2, v/v). Visualization was accomplished with 1% methanolic iodine and the dichromate–sulfuric acid sprays (Rouser and Fleischer, 1967). The radioactive bands on the plate were located with a Packard radiochromatogram scanner. The Barber Colman Model 5000 gas chromatograph was used with a column (stainless steel, 0.43 cm i.d. × 1.83 cm) of 15% SE-30 in anakron AB (Analabs, Inc., North Haven, Conn.) for the analysis of the trimethylsilyl derivative of sugars. The temperature was programmed at 10°C/min. Analysis of the volatile fatty acids was performed on a Becker Model 407 gas chromatograph by the procedure of Carlsson (1973). A sample of the water-washed GL-1A (7.82 mg) was saponified by refluxing in 10.0 ml of 5% KOH in 50% ethanol for 4 hr. A 1-ml aliquot was removed and passed through a 0.5 × 5 cm Dowex 50 (H^+) column; the effluent was made just basic (pH 8) with 0.1 *N* NaOH and evaporated to dryness. The dried sample was taken up in 50 µl of 0.1 *N* HCl and a sample volume of 0.5 µl was injected into glass column (0.20 cm i.d. × 1.83 cm) of Chromosorb 101, 80–100 mesh (Supelco, Inc., Bellefonte, Pa.), at the column temperatures of 170 and 190°C. Fourier transform nuclear magnetic resonance (NMR) spectra were determined with a 90-MHz Brucker model. Mass spectra were determined with a Hitachi-Perkin-Elmer RMU-6D mass spectrometer under the operating conditions of ionization potential, 70 eV, and source temperature, 210°C. Samples were introduced at the inlet with a probe. For the analysis of the permethylated sugar alditol acetate, a Dupont Model 491B gas chromatograph/mass spectrometer with glass transfer lines and glass jet separator was used. The column (glass, 0.21 cm i.d. × 1.83 cm) was 3% OV-1 in gas-chrom Q (Applied Science Lab., Inc., State College, Pa.). The injector temperature was 260°C and the source temperature was 250°C. The column was programmed at 10°C/min from 80 to 280°C.

Extraction and Solvent Fractionation of Lipids from Cells of M. tuberculosis. Harvested cells of H37Ra (667 g) grown in medium A were extracted with 4 l. of chloroform–methanol (2:1, v/v) by stirring overnight at room temperature. The suspension was filtered and the residue was extracted with the same volume of solvent. The two extractions were pooled and evaporated to dryness on a rotary evaporator. The pooled extract was dissolved in 80 ml of chloroform–methanol (2:1, v/v) and added slowly with stirring to 3.7 l. of acetone, stirred 15 min, and filtered. The yields of the acetone-soluble and -insoluble fractions were 11.556 and 7.968 g, respectively. The acetone-insoluble fraction contained the MAT.

Preparation of Labeled Lipids from the H37Ra Strain. To a 100-ml culture of *M. tuberculosis* H37Ra grown in medium B to an absorbance at 650 nm of 0.229, 100 µCi of [1-^{14}C]acetate (57.0 µCi/µmol) was added, incubated for 60 min, cooled in an ice bucket, and harvested by filtration through a Millipore filter (0.45 µm). Unlabeled cells of H37Ra strain (25 g, wet weight) grown in medium A were added to the labeled cells and extracted twice (overnight extractions) with 300-ml portions of chloroform–methanol (2:1, v/v). The pooled extract was dried and solvent fractionation was performed using acetone. The yield of the acetone-insoluble fraction was 13.0 × 10^6 dpm.

Isolation of the Neutral Lipids. The unlabeled and the labeled acetone-insoluble lipids were combined and applied to a 4 × 26 cm DEAE-cellulose column (acetate) in chloroform–methanol (2:1, v/v). The column was eluted with chloroform–methanol (9:1, v/v); the effluent was recovered and dried on the rotary evaporator. The yield of the neutral, acetone-insoluble lipid was 1.34 g (17%) and 10.0 × 10^6 dpm (76%).

Silicic Acid Column Fractionation. One-half of the acetone-insoluble, neutral lipid (0.670 g, 5.0 × 10^6 dpm) was dissolved in 9 ml of chloroform and fractionated on a 3.5 × 28 cm Bio-Sil HA column (−325 mesh, Bio-Rad Lab., Richmond, Calif.) using a stepwise gradient of chloroform–methanol. The elution profile of radioactivity is shown in Figure 1. Fraction D was found to contain MAT. After fractionating the entire neutral, acetone-insoluble lipid, the yield of fraction D was 123.0 mg (1.5%) and 5.92 × 10^6 dpm (46%).

Sephadex LH-20 Column Fractionation. Fraction D from the silicic acid column was dissolved in 2.0 ml of chloroform–methanol (2:1, v/v) and 1.0-ml portions were applied to a 2 × 144 cm Sephadex LH-20 column. The column was eluted with chloroform–methanol (2:1, v/v) containing 1 m*M* ammonium acetate and 3.0-ml fractions were analyzed for radioactivity. The result of such a fractionation is shown in Figure 2. Two pooled fractions were recovered: GL-1, effluent volume of 139–155 ml, and GL-2, effluent volume of 156–177 ml. Thin-layer chromatography of GL-1 in solvent E showed that this preparation was contaminated with a nonradioactive material that migrated ahead of the principal lipid fraction. Thus preparative thin-layer chromatography was performed with solvent E using 20 × 20 plates (0.5 mm) of silica gel G. The load was 500 µg/cm in a 0.7-cm wide streak. The desired lipid was located by spraying lightly with I_2 in methanol and recovered. This fraction was refractionated on the Sephadex LH-20

column to yield 91.2 mg (1.1%) and 4.24×10^6 dpm (33%) of purified GL-1A.

Assay for the Synthesis of Mycolic Acids and MAT. To a culture of *M. tuberculosis* H37Ra grown in medium B with an absorbance at 650 nm of 0.20, [1-^{14}C]acetate was added and incubated on the shaker. At specified time intervals, aliquots of 5.0 or 10.0 ml were transferred into tubes containing 0.100 ml of 1 *M* KCN to stop the incorporation of the label into the mycolic acids and cooled in the ice bucket. Unlabeled cells (0.64 g wet weight) were added to each tube, mixed, and centrifuged on a clinical centrifuge at full speed and the clear supernatant was carefully removed. The recovery of the cells was quantitative. Acetone (5.0 ml) was added to the pellet, mixed well, and centrifuged and the supernatant was discarded. These dehydrated cells could be saponified directly as previously described (Takayama et al., 1972) to determine the total mycolate content or extracted six times with chloroform–methanol (2:1, v/v) to obtain the extractable mycolic acid containing lipids. This extraction involved adding 5.0 ml of chloroform–methanol, vortexing for 1 min, followed by warming at 50°C for 10 min and centrifugation. A 400-μl aliquot of the pooled chloroform–methanol extract (in 25 ml) was chromatographed on a silica gel G plate using solvent E and scanned to locate the position of the labeled MAT. The sample was scraped from the thin-layer plate into counting vials containing 2,5-bis[2-(5-*tert*-butylbenzoxazolyl)]thiophene–toluene scintillation fluid and counted in a scintillation spectrometer. Authentic MAT was chromatographed along with the sample. The residue from the chloroform–methanol extraction was saponified and analyzed for the nonextractable mycolate content as previously described (Takayama et al., 1972).

Methylation Analysis of the Glycolipid. The permethylated alditol derivative of the labeled GL-1A was prepared according to Noll et al. (1956) with modifications. GL-1A (30.2 mg) was refluxed in CH_3I and Ag_2O for 9 days and the resulting permethylated GL-1A was fractionated on a 2 × 18 cm Bio-Sil HA column (−325 mesh, Bio-Rad Lab., Richmond, Calif.) using the solvent system of petroleum ether–diethyl ether (1:4, v/v). Two major fractions (peak 1, 6.5 mg; and peak 2, 14.7 mg) were obtained and analyzed by infrared spectroscopy. Peak 1 was found to be completely devoid of free hydroxyl groups as determined by the absence of an absorption band at 3500 cm^{-1}. Thus this fraction was saponified in 1.0 ml of 5% KOH in 50% ethanol at 45°C for 48 hr and the free mycolic acids were then extracted out with 2.0 ml of hexane. This mycolic acid preparation was methylated with diazomethane and chromatographed on a thin-layer plate using solvent F. The lipid migrated considerably ahead of authentic methyl mycolates which suggested that the β-hydroxy group in the mycolic acids of the glycolipid was originally free and later methylated during the permethylation step. The aqueous layer remaining after the extraction was neutralized with HCl and the permethylated trehalose was recovered by acetone extraction. This sample was then hydrolyzed in HCl to yield the permethylated monosaccharide. The hydrolyzed sample was reduced by incubating in 150 μl of 0.17 *M* $NaBH_4$ at room temperature for 48 hr. The reduced sample was passed through a 0.5 × 6 cm Dowex 50 (H^+) column and the borate was removed by repeated additions and evaporations of methanol.

The dried, permethylated alditol was acetylated by adding an equal volume (5 ml each) of pyridine and acetic anhydride and incubating at 25°C for 18 hr. Water was added to the sample to destroy the excess acetic anhydride, the sample was extracted three times with 25-ml portions of petroleum ether, and the pooled extract was dried over anhydrous Na_2SO_4. The solvent of the extract was evaporated to dryness and the residue was dissolved in $CHCl_3$. This sample represented the partially methylated monosaccharide derivative of GL-1A in the form of the alditol acetate and it was subjected to gas chromatography/mass spectrometry.

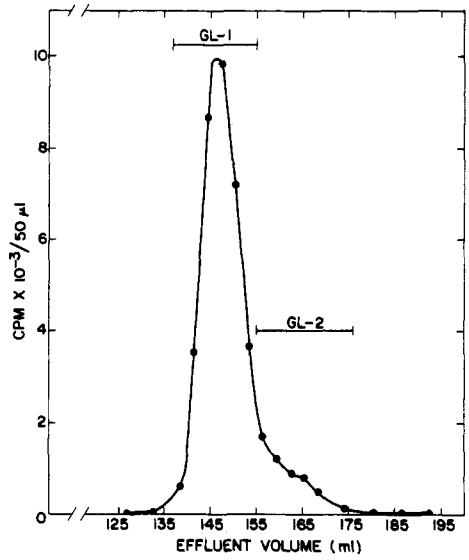


FIGURE 2: Sephadex LH-20 column chromatography of fraction D obtained from the silicic acid column. Fraction D (61.5 mg, 2.96×10^6 dpm) dissolved in chloroform–methanol (2:1, v/v) was applied to a Sephadex LH-20 column and eluted with the same solvent containing 1 m*M* ammonium acetate. The fraction size collected was 3.0 ml and 50-μl aliquots were analyzed for radioactivity.

Results

Major Mycolic Acid Containing Free Lipid from M. tuberculosis H37Ra. Previous work showed that there are three different mycolic acid containing free lipids in the H37Ra strain of *M. tuberculosis* (Takayama, 1974). We have now investigated the nature of one of these lipids. ^{14}C-labeled whole cells of the H37Ra strain were extracted with chloroform–methanol, the extract was passed through a DEAE-Sephadex column (acetate), and the neutral effluent was analyzed by thin-layer chromatography. The result of such an analysis revealed the presence of a major mycolic acid containing free lipid that did not correspond to the trehalose dimycolate (cord factor) which is known to be a prominent component of the virulent strain of *M. tuberculosis* (Bloch, 1950).

Isolation and Purification of a Mycolic Acid Containing Lipid. Over 90 mg of the major mycolic acid containing free lipid was isolated and purified from *M. tuberculosis* H37Ra. The purity of the final preparation (designated GL-1A) was checked by silica gel G thin-layer chromatography and it revealed a single char-positive spot that was radioactive. The yield of this sugar-containing lipid represented about 0.07% of the dry weight of cells. This compares with a yield of 0.02% obtained by Kato and Maeda (1974) for the trehalose 6-mycolate isolated from *M. tuberculosis* H37Rv. Fraction B obtained from the silicic acid column fractionation (Figure 1) contained trehalose dimycolate and other unidentified lipids. The GL-2 fraction obtained from Sephadex LH-20 column (Figure 2) was refractionated on the same column and analysis showed the presence of acyl-

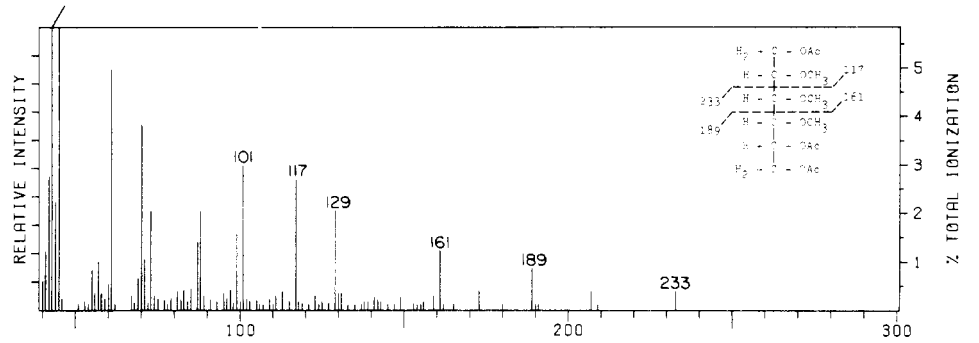
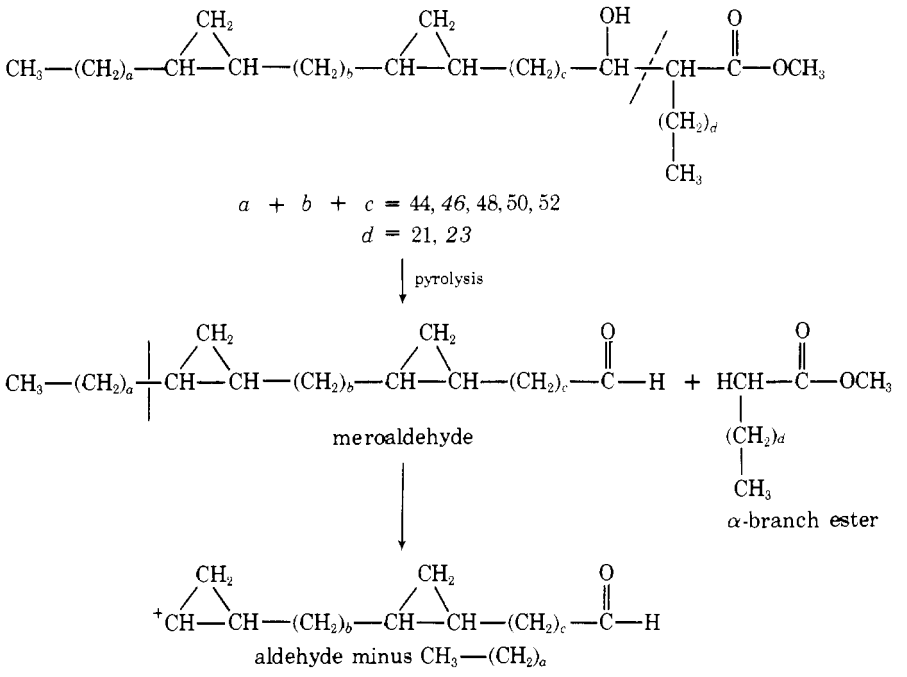


FIGURE 3: Mass spectrum of the partially methylated alditol acetate product derived from GL-1A and fractionated by gas–liquid chromatography. The peak at *m/e* 207 is column background.

Scheme I



ated trehalose in which the acyl groups were tetracosanoic and hexacosanoic acids.

Nature of the Sugar Moiety. GL-1A was found to contain trehalose as the water-soluble moiety after saponification. This identification was established by paper chromatography in solvents A, B, and C by gas–liquid chromatography of the trimethylsilyl derivative and by the observation that the liberated sugar was devoid of reducing group. After acid hydrolysis, the reducing sugar was identified to be glucose by paper chromatography in solvents C and D.

Nature of the Lipid Moiety. The lipid moiety of GL-1A was isolated as the free acid after saponification, methylated with diazomethane, and purified by a combination of silica gel G thin-layer and Sephadex LH-20 column chromatography as previously described (Takayama et al., 1972). The nonvolatile fatty acids were determined to be exclusively mycolic acids. A methyl α-mycolate fraction was obtained and analyzed by nuclear magnetic resonance spectroscopy and mass spectrometry. The chemical shifts obtained from nuclear magnetic resonance analysis revealed the presence of protons for the following functional groups: *cis*-cyclopropane ring (τ 9.40 and 10.30), terminal and branch methyl (τ 9.12), internal methyl (τ 8.74), and methyl ester (τ 6.29). Mass spectral fragmentation of methyl α-mycolate is known to cause a pyrolytic cleavage as shown in Scheme I (Malani and Polgar, 1963; Etemadi, 1964). The following assignments were made: the meroaldehyde series fragments at *m/e* 740, 768, 796, 824, and 852; the aldehyde minus CH_3-$(CH_2)_a$ fragments at *m/e* 459, 487, 515, 543, and 571; and the α-branch ester fragments at *m/e* 382 and 410. We could not detect the molecular ions.[2] Thus the major lipid moiety of GL-1A was identified to be α-mycolic acid which is present in a homologous series. These results compare favorably with those of Minnikin and Polgar (1967) and Etémadi and Lederer (1965) for the methyl α-mycolate isolated from the human strain of tubercle bacilli. From such an analysis, the molecular weight and the molecular formula of the most abundant methyl α-mycolate was determined to be 1178 and $C_{81}H_{158}O_3$, respectively. The methyl α-mycolate obtained from the whole cells was identical with that obtained from the isolated glycolipid.

Ester Linkages of the Acyl Groups to the Trehalose. Gas–liquid chromatography/mass spectrometry was performed on the permethylated monosaccharide of the alditol acetate of GL-1A. Gas–liquid chromatography revealed a single peak that gave mass spectral fragmentation ions at *m/e* 117, 161, 189, and 233 (Figure 3). Björndal et al.

[2] This observation is not unusual (D. E. Minnikin, personal communication).

Table I: Determination of Trehalose to Mycolate Molar Ratio in GL-1A.

Labeled Lipid	Amount (mg)	Specific Radioactivity (× 10^4 dpm/mg) Obsd	Calcd[a]
Preparation 1			
GL-1A	50.0	5.08	
Methyl α-mycolate from GL-1A	28.5	6.82	6.43
Methyl α-mycolate from trehalose dimycolate[b]			5.78
Preparation 2			
GL-1A	19.6	19.9	
Methyl α-mycolate from GL-1A	11.6	25.8	25.5
Methyl α-mycolate from trehalose dimycolate[b]			22.5

[a] The following average molecular weights were used in the calculations: GL-1A, 1530; trehalose dimycolate, 2634; methyl α-mycolate, 1178. [b] Calculated with the assumption that GL-1A is trehalose dimycolate.

(1970) have shown that such a pattern corresponds to the structure of 2,3,4-tri-*O*-methyl-1,5,6-tri-*O*-acetyl-D-glucose. The 2,3,4,6-tetra-*O*-methyl-1,5-di-*O*-acetyl-D-glucose peak was clearly absent on gas–liquid chromatography. Thus the acyl linkages in the GL-1A must be to both 6 and 6′ positions of trehalose.

Trehalose to Mycolate Molar Ratio. The trehalose to mycolate molar ratio in GL-1A was determined by measuring the specific radioactivities of the purified lipid sample and the corresponding methyl ester of the α-mycolic acid (Table I). One preparation of GL-1A had a specific radioactivity of 5.08 × 10^4 dpm/mg. If the purified glycolipid was a trehalose monomycolate, the methyl mycolate obtained from the glycolipid should have a specific radioactivity of 6.43 × 10^4 dpm/mg. The radioactivity in the mycolic acids was 98% of the GL-1A. The observed value of the methyl α-mycolate from GL-1A was 6.82 × 10^4 dpm/mg. Similar results were obtained from another preparation of GL-1A. Thus the trehalose to mycolate molar ratio in GL-1A is 1:1 and the glycolipid is a trehalose monomycolate.

Nature of the Other Acyl Group. Since both 6 and 6′ positions of trehalose in GL-1A are occupied by acyl groups and the molar ratio of trehalose to mycolate is 1:1, the nature of the other acyl group at the 6′ position of trehalose was investigated. The nonvolatile fatty acids were shown to be exclusively mycolic acids and saponification revealed only a single nonvolatile, water-soluble compound (trehalose). The acyl group at the 6′ position of GL-1A could thus be a volatile, short-chain fatty acid. The saponified and desalted sample of GL-1A was analyzed by gas–liquid chromatography for volatile fatty acids. The volatile acyl group present in GL-1A was thus shown to be acetate (Figure 4). Quantitation showed 0.50 μmol of acetic acid/mg of sample which corresponded to an acetic acid to MAT molar ratio of 0.77:1.00.

Analytical Data on GL-1A. The carbon–hydrogen elemental analysis was performed on GL-1A and methyl α-mycolate. For GL-1A the observed values were C, 74.81; H, 12.32 which compares with the calculated values of C, 74.73; H, 11.63. For methyl α-mycolate the observed values were C, 82.88; H, 13.42 which compares with the calculated values of C, 82.51; H, 13.41. The optical rotation for

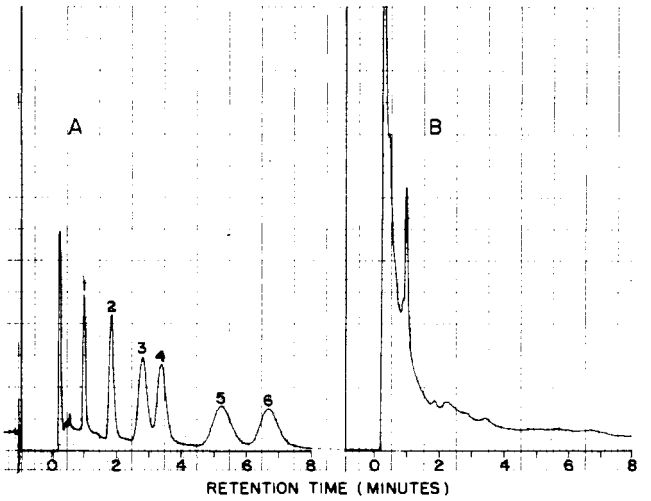


FIGURE 4: Gas–liquid chromatography of volatile fatty acids in (A) standard mixture and (B) GL-1A. Fatty acids in the standard mixture: (1) acetic acid; (2) propionic acid; (3) isobutyric acid; (4) butyric acid; (5) isovaleric acid; (6) valeric acid. The volatile fatty acid in the saponified and desalted sample from 7.82 μg of GL-1A was analyzed at a column temperature of 170°C.

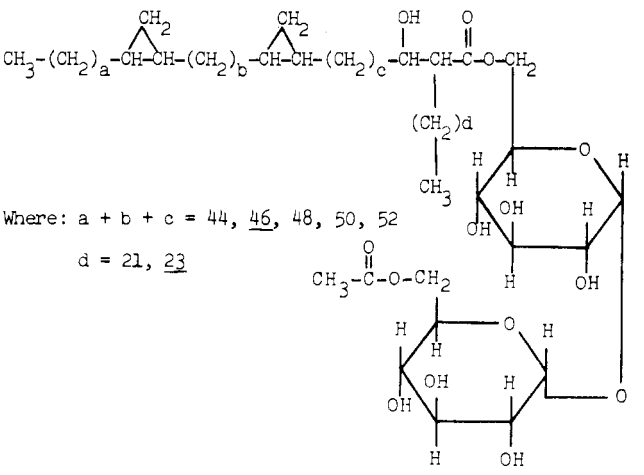


FIGURE 5: Structure of MAT.

GL-1A was determined to be +46.6°. The reported optical rotation values for trehalose mycolate and trehalose dimycolate of *M. tuberculosis* H37Rv are +47.1 and +30°, respectively (Kato and Maeda, 1974; Asselineau, 1966). The structure of MAT is shown in Figure 5. The molecular weight of the most abundant form of MAT is 1530 and the molecular formula is $C_{94}H_{178}O_{14}$.

Function of MAT in M. tuberculosis. The time course of synthesis of MAT and total mycolate and the difference (total mycolate minus MAT) in the H37Ra strain of *M. tuberculosis* incubated in the presence of ^{14}C-labeled acetate are shown in Figure 6. It showed a 3-min lag in the synthesis of total mycolic acids before it became linear. The rate of synthesis of MAT was initially rapid and linear up to the first 20 min after which there was a gradual decrease. A plot of the difference revealed a 25–30-min lag period before the rate of synthesis became linear. It would indirectly measure the incorporation of mycolic acids into the cell wall. These results suggested that MAT could function as the intermediate product in the transfer of mycolic acids into the cell wall.

^{14}C-labeled acetate was pulsed into cells of H37Ra for 20 min and the labeled mycolic acids were chased out into

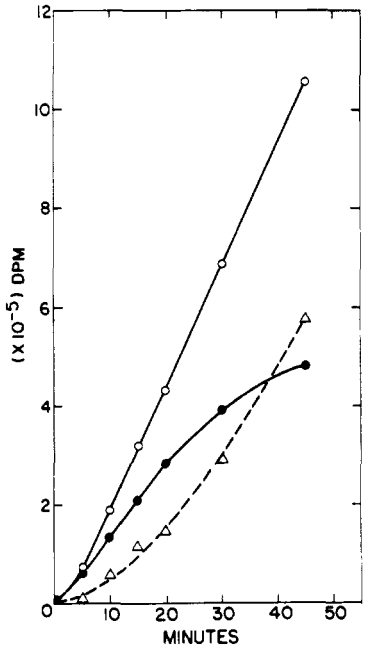


FIGURE 6: Time course of synthesis of total mycolic acids and MAT in cells of *M. tuberculosis* H37Ra. To a 90-ml culture, 450 μCi of [1-^{14}C]acetate (57.0 μCi/μmol) was added and incubated; 5.0-ml aliquots of labeled cells were analyzed at various time intervals for total mycolates (○) and MAT (●); (△) total mycolates minus MAT.

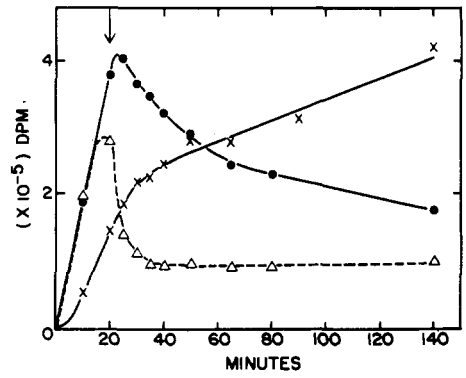


FIGURE 7: A pulse-chase experiment showing the synthesis of labeled MAT and the transfer of the labeled mycolates into the nonextractable cellular lipid fraction. To 220-ml culture of *M. tuberculosis* H37Ra, 1.0 mCi of [1-^{14}C]acetate (58.1 μCi/μmol) was added and incubated. The pulse phase in the presence of label was 20 min and the chase was initiated by adding a 58-fold excess of unlabeled acetate at the time indicated by the arrow; 10.0-ml aliquots of labeled cells were analyzed for MAT (●); nonmycolated fatty acids (△); and nonextractable mycolates (X).

other lipids by adding an excess of unlabeled acetate (Figure 7). The effectiveness of the chase is shown by the sharp drop in the non-mycolate fatty acid content. After initiating the chase, the MAT content showed a gradual decrease with time down to a level of about 46% of the maximum value after 140 min. There was a corresponding increase in the cellular nonextractable mycolic acid content after the chase. These results showed that the labeled mycolic acids were chased out of MAT into the cell wall fraction.

Discussion

We are interested in the pathway to the synthesis of mycolic acids and how these complex fatty acids are transferred ultimately to the cell wall. This interest was brought about by the observation that the cellular mycolate synthetase activity in the H37Ra strain of *M. tuberculosis* is extremely sensitive to isoniazid (Wang and Takayama, 1972). We are assuming that there is an important mycolic acid containing free lipid(s) involved in this pathway. Such an assumption appears to be valid and is supported by recent work of Promé et al. (1974) who showed the synthesis of a single tetradecyl-2-keto-3-octadecanoate (a keto derivative of corynomycolic acid) of trehalose from 2 mol of palmitic acid by *Corynebacterium diphtheriae*. A similar lipid in the *M. tuberculosis* could be MAT.

A review of the literature shows that several mycolic acid containing free lipids have been isolated from *M. tuberculosis*. The first of these was trehalose 6,6′-dimycolate (a toxic glycolipid) by Block (1950) whose structure was determined by Noll et al. (1956). This glycolipid accumulates only in the virulent strain of *M. tuberculosis*. Purified wax D also contains mycolic acids but it is considered to be a product of autolysis and is thus of little interest here (Goren, 1972). Recently Kato and Maeda (1974) isolated trehalose 6-monomycolate from the crude wax D fraction of *M. tuberculosis* H37Rv. Their characterization, although extensive, did not include a thorough analysis of the lipid moiety. Their methylation analysis revealed that the 6′ position of trehalose was unsubstituted, thus the glycolipid they isolated is different from MAT. We have not examined the presence of trehalose 6-mycolate in *M. tuberculosis* H37Ra. They were interested in the delayed lethal toxicity of the glycolipid to mice by repeated interperitoneal injections. We have isolated and characterized a major mycolic acid containing free lipid of the H37Ra strain to be 6-mycolyl-6′-acetyl-α,α-D-trehalose. This characterization included a detailed analysis of the lipid moiety (mycolic acid) of the glycolipid.

The results of our work clearly establish MAT to be an important lipid in the metabolic pathway of mycolic acids. Time-course study shows that this glycolipid is rapidly synthesized with virtually no lag period, whereas the rate of incorporation of labeled acetate into the cell wall mycolates shows an initial lag period of 25–30 min. The pulse-chase experiments shows that mycolic acids are transferred from the MAT to the cell wall.

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Separation of Chromatin Containing Bromodeoxyuridine in One or Both Strands of the DNA†

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ABSTRACT: A method has been devised for the separation of chromatin containing 5-bromodeoxyuridine (BrUdRib) in one strand (HL) of the DNA from that with BrUdRib in both strands (HH). Ultraviolet light breaks chromatin containing HH DNA into smaller fragments than chromatin containing HL DNA and the two species can be partially resolved on neutral sucrose gradients. Unfiltered ultraviolet light is not suitable since it causes considerable alteration in the electrophoretic pattern of chromatin-associated proteins. Irradiation with 313-nm light causes much less damage to the associated proteins. The ability to separate, isolate, and examine chromatin containing HL and HH DNA makes studies on the distribution of chromatin-associated proteins possible.

The replication of eukaryotic chromatin involves the semiconservative synthesis of DNA (Taylor et al., 1957; Chun and Littlefield, 1961) and the association of chromosomal proteins with DNA in a specific manner to yield daughter molecules that are functionally identical with the parent. How the proteins present in parental chromatin are distributed to the daughter chromatin has for sometime been a matter of interest (Prescott and Bender, 1963: Weintraub, 1973; Tsanev and Russev, 1974). This communication details a method for separating chromatin containing DNA with BrUdRib[1] in one strand from chromatin containing DNA with both strands substituted. The technique should facilitate investigations of the distribution of chromatin-associated proteins during cell replication.

The technique is based on the observation that irradiation of DNA containing BrUdRib in both strands causes double-stranded breaks (Hutchinson, 1973). The primary lesion induced by the ultraviolet light is a single-strand nick at the site of a photoactivated BrUdRib molecule (Hutchinson, 1973). Double-stranded breaks occur where two single-strand nicks are sufficiently close together in opposite strands (Freifelder and Trumbo, 1969). Fewer double-strand breaks would be induced in DNA containing BrUdRib in one strand (HL DNA) than in DNA containing BrUdRib in both strands (HH DNA) because the unsubstituted (L) strand in the HL molecule breaks with much lower frequency than the substituted (H) strand (Hutchinson, 1973). Such differential sensitivity of HL and HH DNA suggests that ultraviolet light might also cause chromatin containing HH DNA to break into smaller fragments than chromatin containing HL DNA. The present communication describes experiments which demonstrate the validity of this prediction and detail a procedure for separating the two species of chromatin. In addition a method is given for extraction and gel electrophoresis of chromatin-associated proteins from the resultant products.

Methods

Cells and Culture Media. Chinese Hamster Ovary (CHO) cells (Puck et al., 1958) were cultured on a roller apparatus in F10 medium (Ham, 1963) containing 15% calf serum (Gibco), 7.5 m*M* Hepes buffer (Calbiochem), 100 units/ml of penicillin, and 100 μg/ml of streptomycin. The doubling time was 19 hr. "F10 minus Thy" medium is F10 medium lacking thymidine.

Incorporation of BrUdRib and Radioactive Tracers. To

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[1] Abbreviations used are: BrUdRib, 5-bromodeoxyuridine; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.