Site of inhibitory action of isoniazid in the synthesis of mycolic acids in *Mycobacterium tuberculosis*

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Abstract The cellular mycolate synthetase activity of *Mycobacterium tuberculosis* H37Ra was previously shown to be very sensitive to isoniazid (Wang, L., and K. Takayama. 1972. *Antimicrob. Agents Chemother.* **2**: 438-441). We have now examined the question of how isoniazid inhibits the synthesis of mycolic acids. The saponifiable ^{14}C-labeled lipids of control and isoniazid-treated cells (1.0 μg/ml, 60 min) were compared on a Sephadex LH-20 column, and it appeared that the synthesis of the intermediate-sized fatty acids was partially inhibited. These fatty acids were fractionated as their methyl esters by Sephadex LH-20 column chromatography and gas-liquid (6% Dexsil) chromatography. Mass spectral analysis of the fractionated lipids revealed several series of fatty acids: fraction II, $C_{39}-C_{56}$; fraction III, $C_{27}-C_{40}$. The long-chain fatty acids in three kinds of isoniazid-treated cells were examined: (*a*) long-term exposure (48 hr, 0.5 μg/ml), (*b*) short-term exposure (60 min, 1.0 μg/ml), and (*c*) variable exposure at low concentration (0-90 min, 0.2 μg/ml). Both long- and short-term exposure experiments showed that isoniazid inhibited the synthesis of saturated fatty acids greater than C_{26} and of unsaturated fatty acids greater than C_{24}. The variable-exposure experiment at low isoniazid concentration showed that the syntheses of mycolic acids and long-chain fatty acid fractions II and III were inhibited to the same extent. These fatty acids may thus be precursors of mycolic acids.

Supplementary key words long-chain fatty acids

INH (isonicotinic acid hydrazide) is an important drug for the treatment and prevention of tuberculosis. Since its discovery some two decades ago (1, 2), much work has been done in the attempt to elucidate its mechanism of action (3). Despite these efforts, little progress has been made. Recent work suggests that the drug's primary mode of action may be to inhibit the synthesis of mycolic acids (4-7). The α-mycolic acid (a major mycolate component of *Mycobacterium tuberculosis*) is one of a homologous series of $C_{74}-C_{84}$ fatty acids containing a long aliphatic chain at the α position, a hydroxyl group at the β-position, and two cy-

clopropane rings (8-10). These acids are present in the cell wall, wax D, and cord factor (trehalose 6,6'-dimycolate) (11-14). The cellular mycolate synthetase system of *M. tuberculosis* H37Ra has been shown to be very sensitive to INH (6).

We have now examined the question of how INH inhibits the synthesis of mycolic acids, and we report that it blocks the further elongation of the hexacosanoic acid. We shall show that this block leads to inhibition of the synthesis of a series of very long chain fatty acids ($C_{27}-C_{40}$ and $C_{39}-C_{56}$) that could be precursors of the mycolic acids.

MATERIALS AND METHODS

Growth of bacteria

The H37Ra strain of *M. tuberculosis* was grown at 37°C in either glycerol–alanine–salts medium (medium A) in a New Brunswick 28-l fermentor (New Brunswick MicroFerm) or Middlebrook 7H9 medium enriched with Tween 80 and ADC (Difco) (medium B) on a bench-top environmental shaker (model G-25, New Brunswick Scientific Co., New Brunswick, N.J.) operated at 150 rpm. Medium A contained the following in 1 l (pH adjusted to 6.6 with NaOH): glycerol, 20 ml; Bacto-Casitone (Difco), 0.3 g; ferric ammonium citrate, 0.05 g; K_2HPO_4, 4.0 g; citric acid, 2.0 g; L-alanine, 1.0 g; $MgCl_2 \cdot 6H_2O$, 1.2 g; K_2SO_4, 0.6 g; NH_4Cl, 2.0 g; Tween 80, 0.2 g; and Antifoam A (Dow Corning), 0.05 g. This medium was used for long-term drug exposure experiments where a high yield of cells was desired (a medium adaptable to the fermentor).

The cells in medium B were well dispersed and therefore one could follow the growth rate accurately and conveniently by measuring absorbance at 650 nm with a Coleman model 44 spectrophotometer. This medium was used for all short-term drug exposure experiments.

Analytical procedures

Radioactivity was determined by adding the sample to 12 ml of toluene containing 4 g of 2,5-diphenyloxazole (PPO) and 100 mg of 2,5-bis-[2-(5-*tert*-butylbenzoxazol-yl)]thiophene (BBOT) per liter and counting in a Packard Tri-Carb scintillation spectrometer. A counting efficiency of 74% was established by utilizing an external standard. Fatty acids were methylated with diazomethane. GLC of the methyl esters was carried out on a Barber-Colman model 5000 gas chromatograph as previously described (15) using a column (stainless steel, 0.43 cm ID by 1.83 m) of 6% Dexsil 300 on Gas-Chrom Q (Applied Science Laboratories, State College, Pa.). The temperature was programmed at 10°C/min up to 400°C. In radio-GLC analysis or preparative scale fractionation, the fractions were trapped in tubes containing glass wool, eluted with diethyl ether, dried, and either counted or subjected to analysis by mass spectrometry. Mass spectra were determined on an AEI model MS-902 mass spectrometer under the following operating conditions: ionization potential, 70 eV; electron current, 90 μA; source temperature, 125–150°C. Samples were introduced at the inlet with a probe.

The methyl esters of fatty acids were fractionated by reversed-phase thin-layer chromatography on a silicone oil-impregnated silica gel G thin-layer plate using the solvent system of methanol–water 95:5 (v/v) saturated with silicone oil; the plates were developed twice (16). Thin-layer chromatography of lipids was performed on silica gel G plates developed in petroleum ether–diethyl ether 8:2 (v/v). Lipid spots were visualized after exposure of the plates to iodine vapor. The radioactive bands on the plate were located with a Packard radiochromatogram scanner. Sephadex LH-20 columns of three different dimensions were used to fractionate the mycobacterial lipids: 2 × 66, 2 × 144, and 4 × 39 cm. The eluting solvent was chloroform–methanol 2:1 (v/v) containing 1 mM ammonium acetate.

Preparation of labeled methyl fatty acids from cells after short-term drug exposure

Cultures of *M. tuberculosis* H37Ra grown in 100 ml of medium B in 300-ml Nephlo-flasks for 7 days were used. INH was added to the culture to a final concentration of 1.0 μg/ml and the cells were incubated on the shaker at 37°C for 60 min. 200–250 μCi of [1-^{14}C]acetate was added to both control and INH-treated cells, and the cells were incubated for an additional 15 min. The cells were cooled in ice, harvested by filtering on a Millipore filter (0.45 μm), and washed with cold water. The harvested cells were
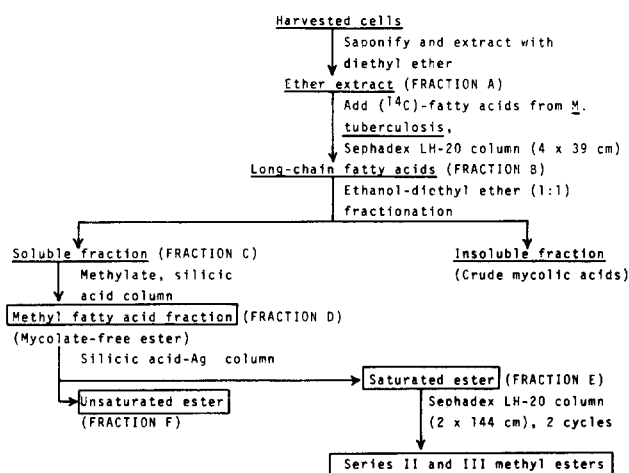

Fig. 1. Scheme for the isolation and partial purification of fraction series II and III long-chain fatty acids from *M. tuberculosis*.

refluxed in 30 ml of 5% KOH in 50% ethanol for 4 hr, cooled, acidified with 7.2 ml of 6 N HCl, and extracted three times with 30-ml portions of diethyl ether. The pooled ether extract was washed once with an equal volume of water, taken to dryness, and dissolved in 1.50 ml of chloroform–methanol 2:1 (v/v). The entire sample was applied to a 2 × 66 cm Sephadex LH-20 column in chloroform–methanol 2:1 (v/v) and eluted with the same solvent containing 1 mM ammonium acetate. 3-ml fractions were collected. In this way it was possible to obtain separation of the very large molecular weight fatty acids (mycolic acids) from the intermediate and lower molecular weight fatty acids. The latter two fatty acid fractions were recovered, pooled, methylated, and fractionated on silica gel G thin-layer plates developed in petroleum ether–diethyl ether 8:2 (v/v) in order to remove the last traces of mycolates. These samples were analyzed by radio-GLC and reversed-phase thin-layer chromatography.

Isolation and purification of fraction II and III long-chain fatty acids from *M. tuberculosis*

The procedure used for the isolation and purification of the long-chain fatty acids from *M. tuberculosis* H37Ra (fraction II, C_{39}–C_{56}; fraction III, C_{27}–C_{40}) is outlined in **Fig. 1**, and the results are shown in **Table 1**. Harvested cells grown in medium A in the fermentor were refluxed in 500 ml of ethanolic KOH solution, acidified, and extracted twice with equal volumes of diethyl ether. The pooled ether extract was evaporated, dissolved in 500 ml of ether, and washed with an equal volume of water (fraction A). ^{14}C-labeled fatty acids, obtained separately from *M. tuberculosis* by a procedure similar to that described in the previous section, were added to fraction A in 36 ml of chloroform–methanol 2:1 (v/v). The solution was divided into two 18-ml portions, and each portion was fractionated separately on a Sephadex LH-20 column (4 × 39 cm). A

TABLE 1. Partial purification of methyl esters of mycolate-free long-chain fatty acids obtained from control and INH-treated (long-term exposure) cells of *M. turberculosis*

| | Fatty Acid from | | | | | |
| | Control Cells[a] | | | INH-treated Cells[a] | | |
Fraction[b]	Radio-activity	Dry Wt	Specific Activity	Radio-activity	Dry Wt	Specific Activity
	$cpm \times 10^6$	g	$cpm \times 10^3/mg$	$cpm \times 10^6$	g	$cpm \times 10^3/mg$
A. Ether extract		7.3			3.1	
Added labeled fatty acid obtained from[c]						
Control cells	25.9		3.54			
INH-treated cells				24.4		7.87
B. Sephadex LH-20 column (4 × 39)	17.9	5.1	3.51			
C. Ethanol–ether-soluble	7.1	0.81	8.77			
D. Silicic acid column	3.0	0.102	29.4	16.5	0.330	50.0
Silicic acid–Ag column						
E. Saturated ester	2.2	0.074	29.7	14.7	0.300	49.0
F. Unsaturated ester	0.44	0.016	27.5	0.38	0.008	47.5
Sephadex LH-20 column (2 × 144 cm)						
fraction of saturated esters (two cycles)[d]						
Fraction II, C_{39}–C_{56}	0.045	0.002	23			
Fraction III, C_{27}–C_{40}	0.207	0.007	30			

[a] Harvested wet weights of cells were: control, 317 g; INH-treated (0.5 μg/ml, 48 hr), 167 g.
[b] Fractions A, B, and C are free acids.
[c] Labeled fatty acids were obtained separately from control and INH-treated (1.0 μg/ml, 60 min) cells exposed to [1-¹⁴C]acetate for 60 min.
[d] Saturated esters from INH-treated cells were not fractionated further.

crude long-chain fatty acid fraction (fraction B), which was eluted at effluent volumes of 160–292 ml, was recovered and the solvent was evaporated. This sample was dissolved in 60 ml of diethyl ether, and 60 ml of ethanol was added with stirring. The mixture was centrifuged at room temperature to yield ethanol–diethyl ether-soluble and -insoluble fractions. The insoluble fraction was extracted twice as described above. The pooled ethanol–ether-soluble fraction (fraction C) was methylated and fractionated on a 36-g Bio-Sil BH (100–200 mesh, Bio-Rad Laboratories, Richmond, Calif.) column using 0.8% diethyl ether in petroleum ether. This fractionation removed the remaining traces
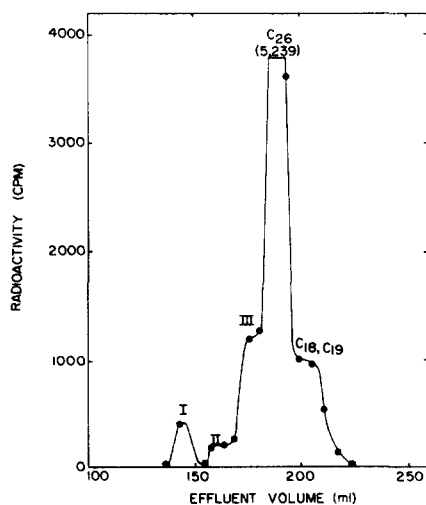

Fig. 2. Initial Sephadex LH-20 column chromatography of saturated long-chain fatty acid ester fraction E from control cells. Sample size was 74 mg (2.2 × 10⁶ cpm). A 2 × 144 cm column was used with chloroform–methanol 2:1.

of methyl mycolates. The first 160 ml of the effluent was recovered (fraction D). This fraction was further fractionated on a 50-g (3 × 18 cm) Hi-Flosil–Ag column (Applied Science). Saturated methyl fatty acids (fraction E) were eluted with 200 ml of 5% diethyl ether in petroleum ether, whereas the unsaturated fatty acids (fraction F) were eluted with 50% diethyl ether in petroleum ether. Further purification of the saturated long-chain methyl fatty acids was achieved on a Sephadex LH-20 column (2 × 144 cm) (see Fig. 2). The sample was dissolved in 1.0 ml of chloroform–methanol 2:1 (v/v), applied to the column, and eluted with chloroform–methanol 2:1 (v/v) containing 1 mM ammonium acetate. Fractions of 3.1 ml were collected after allowing the initial 100 ml to elute from the column. A pooled fraction with effluent volumes of 157–170 ml represented the C_{39}–C_{56} methyl fatty acids (fraction II), whereas 171–180 ml represented the C_{27}–C_{40} methyl fatty acids (fraction III). Each of these fractions was refractionated on the same column. The dry weights of these lipid fractions shown in Table 1 were determined after performing a water wash in a two-phase system of chloroform–methanol–water 10:5:6 (v/v). The specific radioactivity became relatively constant after the silicic acid column fractionation step.

Long-term INH-treated cells of *M. tuberculosis* grown in medium A in the fermentor (previously exposed to 0.5 μg/ml of the drug for 48 hr) were similarly processed to obtain the labeled fatty acids. The labeled fatty acids obtained from *M. tuberculosis* after a short-term INH treatment (0.5 μg/ml, 60 min) were added to the diethyl ether extract (fraction A) to serve as a labeled marker. We subsequently realized that the labeled fatty acids from the con-

trol cells would be a better marker. The initial Sephadex LH-20 column fractionation was omitted. Because the labeled mycolic and long-chain fatty acids were virtually absent, it would be difficult to determine how to pool the long-chain fatty acids. The ethanol–ether solvent fractionation was also omitted because of the low mycolic acid content.

Time course of inhibition of mycolic and long-chain fatty acids

INH (0.2 μg/ml) was added to cells grown in medium B and the cultures were placed on a shaker. 10-ml samples of the culture were removed at various time intervals and assayed for cellular mycolate synthetase activity as described previously (5), with two modifications. 50 μCi of [1-^{14}C]acetate (56 μCi/μmole) was used for each assay, and the synthesis of the mycolate-free fatty acids was measured after thin-layer chromatography of the methyl esters. The entire mycolate-free fatty acid fractions were recovered from the thin-layer plate and subjected to Sephadex LH-20 column chromatography (2 × 144 cm) to examine the inhibition of the synthesis of the long-chain fatty acids (C_{39}–C_{56} and C_{27}–C_{40}). The basis for the cellular mycolate synthetase assay is that [^{14}C]acetate is rapidly taken up by actively growing *M. tuberculosis* and incorporated into the fatty acids. Except for the initial moderate lag period of a few minutes, the rate of incorporation of the label into the mycolic acids is linear for at least 40 min.

Chemicals

[1-^{14}C]Acetic acid was obtained from New England Nuclear Corp., Boston, Mass. INH was purchased from Calbiochem, La Jolla, Calif. Standard normal (C_{14}, C_{16}, C_{18}, C_{20}, C_{22}, C_{24}, C_{26}, C_{28}, and C_{30}) and monoenoic (C_{16} and C_{18}) fatty acid methyl esters were obtained from Analabs, Inc., North Haven, Conn. All other chemicals used were reagent grade.

RESULTS

Effect of INH on synthesis of saponifiable lipids in *M. tuberculosis*

The incorporation of labeled acetate into the saponifiable lipid of control and INH-treated cells of *M. tuberculosis* (1.0 μg/ml, 60 min) was compared after separation on a Sephadex LH-20 column, as shown in **Fig. 3**. The samples used were derived from two identical 96-ml cultures grown in medium B with absorbances of 0.191. The cells were labeled with 250 μCi of [1-^{14}C]acetate (58 μCi/μmole). The yields of labeled fatty acids after saponification were 14.7 × 10^6 cpm for the control cells and 9.57 × 10^6 cpm for the INH-treated cells. All of both samples was fractionated.
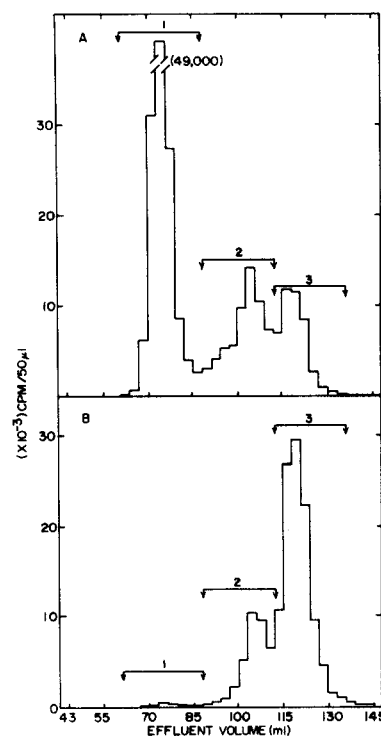

Fig. 3. Sephadex LH-20 column chromatography of saponifiable ^{14}C-labeled lipids obtained from (*A*) control and (*B*) INH-treated cells of *M. tuberculosis* (short-term exposure). Column size was 2 × 66 cm. The following methyl ester fractions were defined: *1*, very long-chain fatty acids (mycolic acids); *2*, intermediate-sized fatty acids; and *3*, small molecular weight fatty acids.

The results showed that the synthesis of the very long chain fatty acids (fraction 1, predominately mycolic acids) was completely inhibited, that of the intermediate-sized fatty acids (fraction 2) was partially inhibited, and that of the short-chain fatty acids (fraction 3, containing C_{16}–C_{19} fatty acids) was greater in the INH-treated cells.

Isolation and characterization of intermediate-sized saponifiable lipids of *M. tuberculosis*

From 317 g (wet weight) of cells of *M. tuberculosis*, 102 mg of mycolate-free methyl esters of long-chain fatty acids (fraction D) was isolated, as shown in Table 1, by a procedure outlined in Fig. 1. GLC of fraction D on a Dexsil 300 column showed it to be a complex mixture of fatty acids (**Fig. 4**). The methyl esters of fatty acids from C_{16} to C_{26} were identified by comparing their retention times with those of standards. Column fractions 26–40 were recovered and analyzed by mass spectrometry (**Table 2**). The pattern of elution of fraction D showed two series of prominent intermediate-sized fatty acids appearing at C_{24}–C_{28} and C_{34}–C_{38} (Fig. 4). Two gas chromatographic fractions, 27 and 28, were shown to contain fatty acids varying in carbon chain length from C_{25} to C_{28} and from complete saturation to the possible presence of three double bonds. A C_{32} fatty acid was detected in fractions 28 and 29; it was tenta-
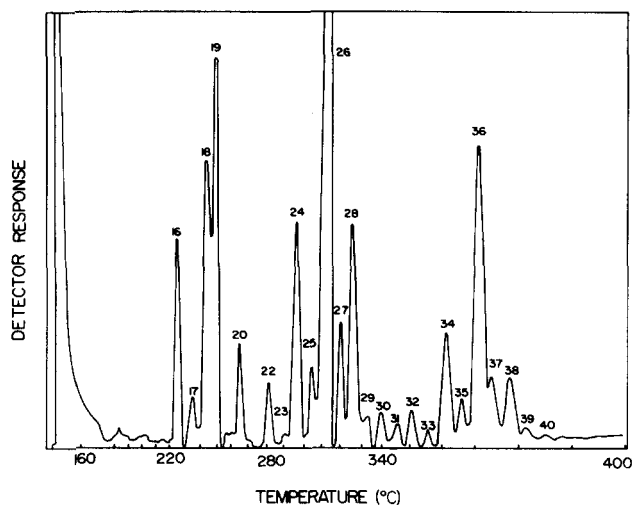
Fig. 4. Preparative GLC of the methyl ester of long-chain fatty acids from control cells of *M. tuberculosis* (fraction D; see Table 1). Sample size was 520 µg. A 6% Dexsil 300 column was used, and the temperature was programmed to 400°C. The numbers indicate the approximate chain lengths of the various fractions.

TABLE 2. Mass spectral analysis of methyl esters of long-chain fatty acids (fraction D) fractionated by GLC

GLC Fraction	Molecular Ion (m/e)	Intensity	Tentative Identity[a]
40	602		C_{40}, 2Δ
39	588		C_{39}, 2Δ
38	574		C_{38}, 2Δ
37	560		C_{37}, 2Δ
36	546		C_{36}, 2Δ
35	532	High	C_{35}, 2Δ
	516	Low	C_{34}, 3Δ
34	518	Equal	C_{34}, 2Δ
	488		C_{32}, 3Δ
33	474		C_{31}, 3Δ
32	490	Low	C_{32}, 2Δ
	460	High	C_{30}, 3Δ
31	476	Low	C_{31}, 2Δ
	446	High	C_{29}, 3Δ
30	466	Equal	C_{30}, saturated
	432		C_{28}, 2Δ
29	494	Low	C_{32}, mycocerosic acid
	418	High	C_{27}, 3Δ
28	494	Low	C_{32}, mycocerosic acid
	438	High	C_{28}, saturated
	436	Low	C_{28}, 1Δ
	434	Very low	C_{28}, 2Δ
	410	Low	C_{26}, saturated
	404	Very low	C_{26}, 3Δ
27	424	Very low	C_{27}, saturated
	422	Very low	C_{27}, 1Δ
	410	Low	C_{26}, saturated
	390	High	C_{25}, 3Δ
26	410	High	C_{26}, saturated
	376	Very low	C_{24}, 3Δ

[a] The mass spectral indication of unsaturation could alternatively be an indication of a cyclopropane ring. Δ, double bond.

tively identified as the methyl-branched mycocerosic acid (17). Fraction 27 contained as the major component a C_{25} fatty acid with three double bonds. Fatty acids containing three double bonds were major components in fractions 29 (C_{27}) through 34 (C_{32}) and a minor component in fraction 35 (C_{34}). The fatty acid containing two double bonds was a minor component in fractions 31 (C_{31}) and 32 (C_{32}) and either a major or a singular component in all other fractions up to fraction 40 (C_{40}).

Fraction D was separated into saturated (fraction E) and unsaturated (fraction F) esters as shown in Fig. 1. When fraction E was further separated on a 2 × 144 cm Sephadex LH-20 column, three fractions of interest were obtained, I, II, and III (Fig. 2). Gas–liquid chromatographic analysis of fraction III showed it to contain the C_{27}–C_{40} fatty acids. Fraction II could not be fractionated by GLC. The results of mass spectral analysis of fraction II (after a second fractionation on the same column) are shown in **Table 3**. Fraction II was a complex mixture of fatty acids with the chain length varying from C_{39} to C_{56}. From this complex mixture, a list of four series of long-chain esters was compiled according to the degree of unsaturation as indicated by mass spectral analysis and whether the straight-chain lengths were assumed to be even- or odd-numbered. From the series with two double bonds, the range of the molecular ions for the even-numbered esters was 658–826 and the range for the odd-numbered esters was 644–812. In the series with three double bonds, the molecular ion range was 586–810 for the even-numbered esters and 656–824 for the odd-numbered esters. Catalytic reduction of fraction II followed by mass spectral analysis showed all of the completely saturated esters (range of molecular ions, 592–830) that corresponded to the original

components. Fraction II was unaffected by ozonolysis, which confirmed the absence of unsaturation. The results thus suggest the presence of two or three cyclopropane rings. As the α-mycolate contains only two cyclopropane rings (other forms of mycolates contain a single ring), it is presently difficult to explain the presence and significance of two series of long-chain esters containing three cyclopropane rings.

Gas–liquid chromatographic analysis of long-chain fatty acids from control and INH-treated cells of *M. tuberculosis* (long-term drug exposure)

Methyl esters of long-chain fatty acids were obtained from control and INH-treated cells of *M. tuberculosis* (0.5 µg/ml, 48 hr) and fractionated as shown in Fig. 1. The results of these fractionations (Table 1) showed that, although the recoveries of the lipids from the two sources were different, the specific radioactivity of the partially purified methyl esters became relatively constant after the silicic acid column fractionation step. Fraction D obtained from the drug-treated cells had the full complement of the original non-mycolate fatty acids whereas a similar fraction from the control cells had about 50% of the total non-myco-

TABLE 3. Mass spectral analysis of purified fraction II obtained from
Sephadex LH-20 column chromatography[a]

Number of Carbons	Molecular Ion (m/e) of Catalytically Reduced Fraction II	Molecular Ion (m/e) of Fraction II[b]			
		2Δ (Even No.)	2Δ (Odd No.)	3Δ (Even No.)	3Δ (Odd No.)
56	830	826			824
55	816		812	810	
54	802	798			796
53	788		784	782	
52	774	*770*			768
51	760		756	754	
50	746	*742*			740
49	732		728	726	
48	718	*714*			*712*
47	704		700	698	
46	690	*686*			*684*
45	676		672	670	
44	662	658			656
43	648		644	*642*	
42					
41	620			614	
40					
39	592			586	

[a] Fraction E was fractionated on a Sephadex LH-20 column (2 X 144 cm) twice to yield purified fraction II.

[b] This table was prepared by assuming that the unsaturation indicated by mass spectral analysis was due to the presence of a cyclopropane ring. The even- and odd-number designations refer to the number of carbon atoms assumed to be present in the straight-chain portions of the fatty acids. The odd-numbered series could alternatively be methyl-branched fatty acids. The most abundant molecular ions in each ester series are in italic type.

late fatty acids (in the form of short-chain fatty acids) removed by the initial Sephadex LH-20 column fractionation. Thus, the sample sizes for GLC analyses were correspondingly adjusted. We also adjusted the two sample sizes by normalizing the C_{24} fatty acid content. GLC analysis of fraction D (**Fig. 5**) showed that the lipids of the control cells contained two series of prominent long-chain fatty acid fractions, 24–28 and 34–38. When the lipids of the INH-treated cells were similarly examined, only fractions 24 and 26 were observed. All other long-chain fatty acids were absent. In order to obtain a GLC detector response for the C_{34}–C_{38} methyl esters in fraction D of the INH-treated cells that was similar to the corresponding esters of the control cells as shown in Fig. 5, *A*, an amount greater than 1 mg had to be injected. These results showed that further elongation of fatty acids beyond fraction 26 (corresponding to a C_{26} fatty acid) was inhibited by INH.

Both samples were then further fractionated on a silicic acid–AgNO₃ column to separate the saturated from the unsaturated fatty acids (see Table 1 for results). The two preparations were again compared by GLC analysis. **Fig. 6, A,** shows that the lipids from the control cells contained a series of prominent fatty acid fractions, 24–28, but the series of fractions 34–38 (present in Fig. 4) were absent. This provided evidence that these latter fatty acids were saturated and contained two cyclopropane rings. The methyl fatty acid peaks corresponding to fractions 30, 32, and 34 were detected in small amounts. Fig. 6, *B*, shows that the lipids from the INH-treated cells had smaller amounts of fractions 25, 27, and 28 and the methyl fatty acid peaks corresponding to fractions 30, 32, and 34 were absent. These results show that the elongation of the unsaturated fatty acids beyond fraction 26 (probably a C_{24} fatty acid) was inhibited by INH. The unsaturated long-chain fatty acids obtained from the INH-treated cells represented minor components before the fractionation (about 4% of the total counts, see Table 1). Consequently, peaks 25, 27, and 28, which were not detectable in the GLC analysis of the total long-chain fatty acids (Fig. 5, *B*), appeared upon GLC analysis of the unsaturated long-chain esters (Fig. 6, *B*).

Comparison of ¹⁴C-labeled fatty acid content in control and short-term INH-treated cells of *M. tuberculosis*

Partially purified methyl esters of mycolate-free ¹⁴C-labeled fatty acids were prepared from control and short-term INH-treated cells of *M. tuberculosis* (1.0 μg/ml, 60 min). Two identical cultures of *M. tuberculosis* (100 ml) with absorbances of 0.192 were used. The control and drug-treated cells were incubated with 200 μCi of [1-¹⁴C]acetate (56 μCi/μmole) for 60 min. Labeled fatty acids obtained from control cells (30.8 X 10⁶ cpm) and INH-
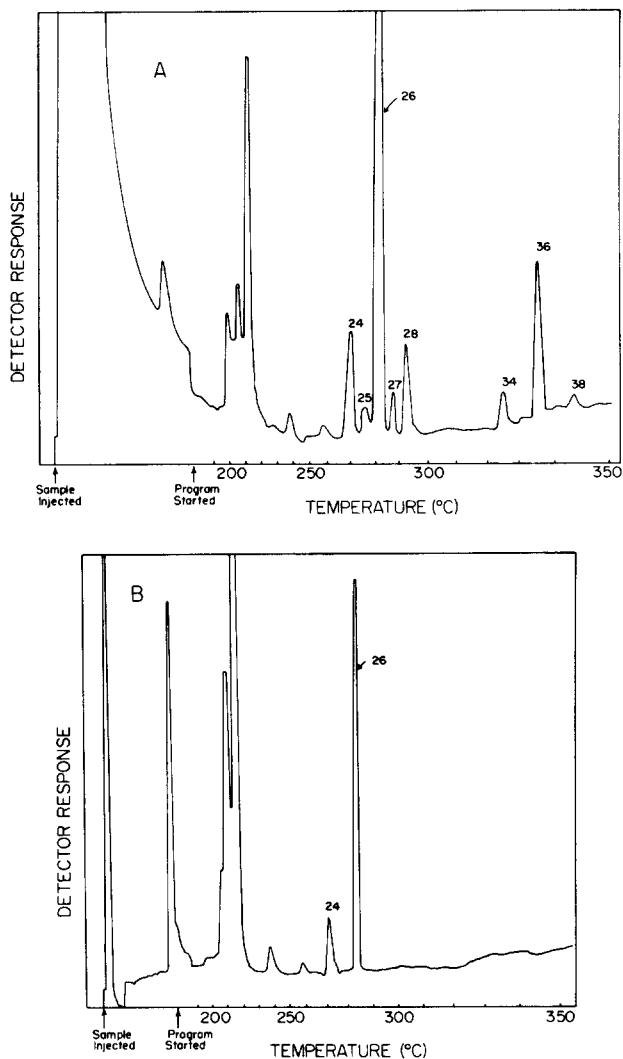
Fig. 5. GLC of methyl esters of long-chain fatty acids (fraction D) from (A) control and (B) INH-treated cells of *M. tuberculosis* (long-term exposure). Sample sizes were: *A*, 105 μg; *B*, 130 μg. A 6% Dexsil 300 column was used with temperature programming. The initial isothermal temperature was 180°C. Chain lengths of the various fractions are indicated.
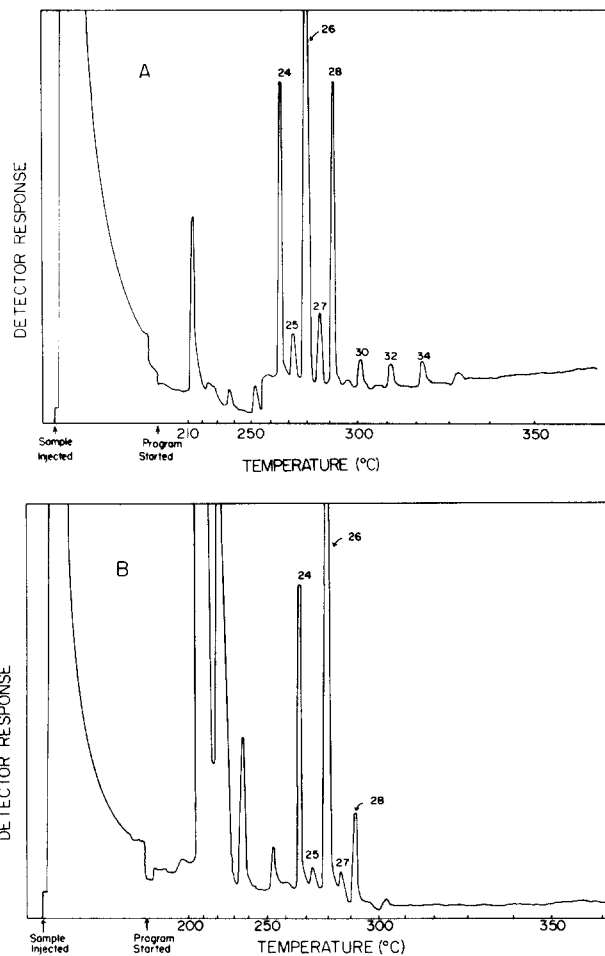

Fig. 6. GLC of methyl esters of long-chain unsaturated fatty acids (fraction F) from (A) control and (B) INH-treated cells of *M. tuberculosis* (long-term exposure). Sample sizes were: *A*, 110 μg; *B*, 210 μg. The initial isothermal temperature was 170°C.

treated cells (30.3 × 10^6 cpm) were initially fractionated on a Sephadex LH-20 column, and a fraction partially depleted of mycolic acids was recovered and esterified. These samples were further fractionated on a silica gel G thin-layer plate to yield the following mycolate-free labeled methyl esters: 12.2 × 10^6 cpm for the control sample and 15.7 × 10^6 cpm for the drug-treated sample. Such samples were examined by GLC and reversed-phase thin-layer chromatography. **Fig. 7** shows the GLC pattern of recovery of the labeled fractions from the two samples collected at 10°C intervals. Labeled methyl ester fatty acids greater than C_{26}, including C_{34}–C_{38} lipids, were present in the sample from the control cells (Fig. 7, *A*), but these large lipids were absent in the sample from the INH-treated cells (Fig. 7, *B*). Reversed-phase thin-layer chromatography of the same two samples also revealed the virtual ab-

sence of ^{14}C-labeled fatty acids greater than C_{26} in the lipid sample from INH-treated cells (see the scan from origin to C_{26} in **Fig. 8**).

Time course of inhibition of synthesis of mycolic and long-chain fatty acids by INH

Because INH inhibits the synthesis of mycolic acids (the largest of the fatty acids) and the C_{27}–C_{40} fatty acids, we examined its effect on the synthesis of all the long-chain fatty acids. An 88-ml culture of *M. tuberculosis* with an absorbance of 0.225 was exposed to 0.2 μg/ml of INH for various time intervals and analyzed for the synthesis of mycolic and mycolate-free long-chain fatty acids. This culture was previously stored overnight at 5°C and reincubated overnight on the shaker at 37°C before use. Such a procedure enhanced cellular uptake and incorporation of labeled acetate into the mycolic acids. **Fig. 9** shows that the inhibition of the synthesis of mycolic acids begins after 30 min of incubation and gradually declines with time until complete inhibition (after approximately 75 min of incubation).

The mycolate-free ^{14}C-labeled long-chain methyl esters obtained from the above-mentioned labeled lipids were further fractionated on a 2 × 144 cm Sephadex LH-20 column; the results are shown in **Fig. 10.** The amounts of labeled fatty acid esters recovered for column chromatography were as follows: no exposure, $3.6 × 10^5$ cpm; 50 min, $3.7 × 10^5$ cpm; 60 min, $5.0 × 10^5$ cpm; 90 min, $5.7 × 10^5$ cpm. The fatty acid fractions were identified: II, C_{39}–C_{56}; III, C_{27}–C_{40}; IV, C_{26}; and V, C_{16}–C_{19}. The identity of fraction I has not been established. INH inhibition of the synthesis of fractions I, II, and III did not occur until after 30 min of incubation. The results show that within the time intervals of 30 to 90 min of exposure to INH, there was a gradual decline in the synthesis of fractions I, II, and III, whereas the synthesis of fractions IV and V was enhanced. Thus, the pattern of inhibition in the synthesis of these three lipid fractions was similar to that of mycolic acids (Fig. 9).

DISCUSSION

We have isolated and partially characterized the long-chain fatty acids from *M. tuberculosis* H37Ra. (The complete characterization of these lipids is in progress.) They were fractionated into the following series according to chain length by utilizing Sephadex LH-20 column chromatography: fraction II, C_{39}–C_{56}; and fraction III, C_{27}–C_{40}. Mass spectral analysis showed that the major molecular ions of fraction III contained two equivalents of unsaturation (either double bond or cyclopropane ring) and fraction II contained two and three equivalents of unsaturation. The synthesis of these fatty acids was shown to be inhibited by very low concentrations of INH (0.2 μg/ml) to the same extent as the mycolic acids. Such an observation is consistent with the possibility that these long-chain fatty acids are precursors of mycolic acids. Walker, Prome, and Lacave (18) recently showed that the synthesis of corynomycolic acid in *Corynebacterium diphtheriae* involves the condensation of one molecule of palmitic acid to the α carbon of another molecule of palmitic acid. A similar mechanism involving the condensation of very long chain fatty acid to the α carbon of a hexacosanoic acid may be occurring in *M. tuberculosis* for the biosynthesis of mycolic acids.

The INH inhibition of the synthesis of mycolic acids could involve a block in the further elongation of saturated C_{26} or unsaturated C_{24} fatty acids. INH could be acting at one of the three possible points in the pathway to the synthesis of long-chain fatty acids in *M. tuberculosis*: (*a*) desaturation of fatty acids, (*b*) introduction of cyclopropane rings, or (*c*) elongation of fatty acids.

There are three separate enzyme systems in the pea plant (*Pisum sativum*) that are responsible for the synthe-
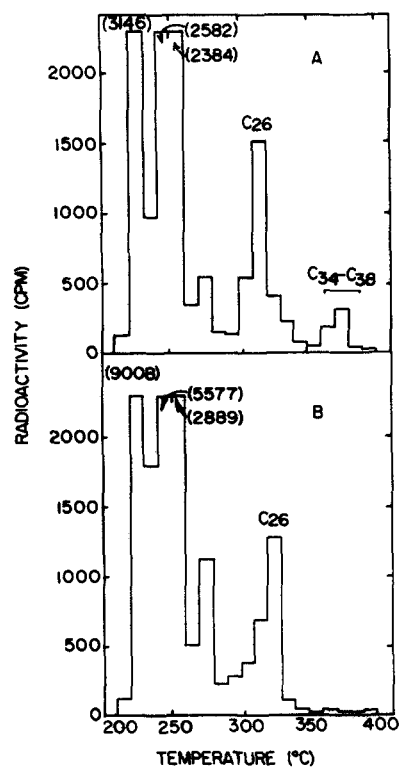

Fig. 7. Radio-GLC analysis of methyl esters of ^{14}C-labeled fatty acids from (*A*) control and (*B*) INH-treated cells of *M. tuberculosis* (short-term exposure). Samples of mycolate-free methyl esters of ^{14}C-labeled fatty acids obtained from the control (71,400 cpm) and drug-treated cells (80,000 cpm) were fractionated on a 6% Dexsil 300 column GLC. The temperature was programmed to 400°C, fractions were collected at 10°C intervals and transferred into counting vials, and the radioactivity was determined with a scintillation spectrometer. Chain lengths of some of the fractions are indicated. Recovery of the fraction collector system was established to be about 20% with methyl [^{14}C]palmitate.
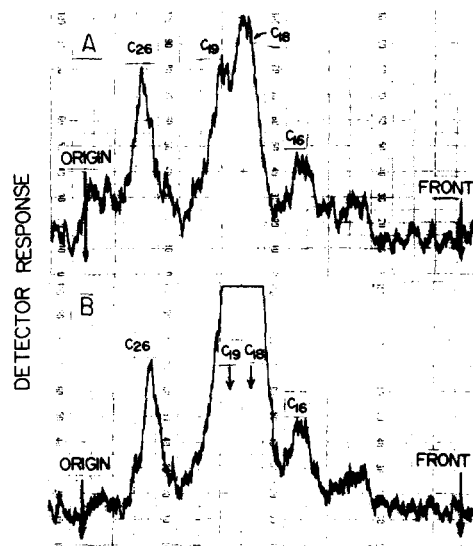

Fig. 8. Radioactivity scan of reversed-phase thin-layer chromatograms. The samples were mycolate-free methyl esters of ^{14}C-labeled fatty acids from (*A*) control (71,400 cpm) and (*B*) INH-treated cells (80,000 cpm) of *M. tuberculosis* (short-term exposure). Chain lengths of the various fractions are indicated.
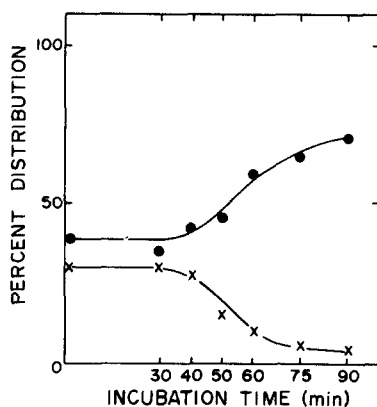
Fig. 9. Effect of exposure time to INH on the ability of *M. tuberculosis* cells to incorporate labeled acetate into mycolic and mycolate-free fatty acids. Cells were exposed to 0.2 $\mu g/ml$ INH for various periods of time, and the radioactivity incorporated into mycolic and mycolate-free fatty acid fractions from labeled acetate was determined by thin-layer chromatography. Symbols: ●, labeled mycolate-free fatty acids; ×, labeled mycolic acids.

sis of palmitate, the elongation of palmitate to stearate, and the elongation of stearate to long-chain fatty acids (19). Decenylsuccinic acid and the carbamate weed killers were shown to specifically reduce the formation of long-chain fatty acids by pea. Buckner and Kolattukudy (20) showed that the elongation of long-chain fatty acids in young pea leaves is inhibited by trichloroacetic acid. Similarly, there may be a separate system(s) in the tubercle bacillus that carries out the elongation of the long-chain fatty acids. INH may be acting on a specific enzyme system that blocks the further elongation of the C_{26} fatty acid.

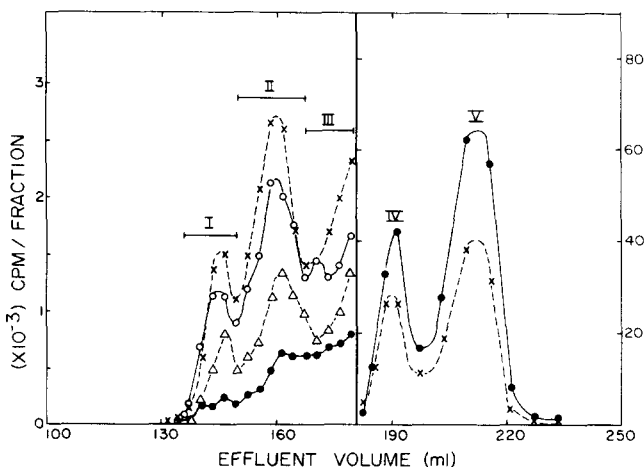Fig. 10. Sephadex LH-20 column chromatography of purified methyl esters of mycolate-free ^{14}C-labeled fatty acids obtained from *M. tuberculosis* cells that were exposed to INH (0.2 $\mu g/ml$) for various periods of time and then allowed to incorporate labeled acetate for 15 min. Column size was 2 × 144 cm. The sample was applied to the column in 1.0 ml of chloroform-methanol 2:1 (v/v), and 3.1-ml fractions were collected. The labeled methyl ester fatty acids were obtained from cells exposed to INH for the following periods: ×, no exposure; O, 50 min; △, 60 min; ●, 90 min. Identification of the methyl fatty acids: II, C_{39}-C_{56}; III, C_{27}-C_{40}; IV, C_{26}; V, C_{16}-C_{19}.

With the exception of mycolic acids, this is the first report documenting the existence of fatty acids with unusually long chains (C_{27}-C_{56}) in *M. tuberculosis*. The isolation of corrinic (C_{35}) and corynolic (C_{52}) acids from *C. diphtheriae* has been reported (17). Recently, Uchida (21) reported the presence of saturated and monounsaturated fatty acids with chain lengths up to C_{30} in the S-14 strain of *Lactobacillus heterohiochii*. Fatty acids with chain lengths up to C_{26} were isolated from *M. smegmatis, M. phlei,* and *M. bovis* BCG (22, 23). Such studies showed that the odd-numbered saturated fatty acids greater than C_{19} contained the methyl branch. This may explain the existence of such fatty acids in *M. tuberculosis*. ▮▮

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