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Isolation and Identification of Mycolic Acids in *Mycobacterium leprae* and *Mycobacterium lepraemurium*[1]

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Mycolic acids, a series of high molecular weight β-hydroxy-fatty acids with a long alkyl branch at the α-position, are well known as one of the main components of cell walls of mycobacteria and related organisms. Although the physiological function of mycolic acids is not yet clear, their taxonomical usefulness has been pointed out ([13]). Detailed structures of mycolic acids in a number of strains of cultivable mycobacteria have been fully elucidated ([9,10,14]) due to recent progress in analytical methods. In this regard, Etemadi and Convit ([2]) have tried to isolate mycolic acid from skin lesions of leprosy patients. They isolated two kinds of mycolic acids, one of which was proposed to be an α (dicyclopropyl) mycolic acid. They concluded that the "noncultivable" bacteria responsible for leprosy are true mycobacteria. They needed, however, skin lesions from 30 patients and more than one month to isolate sufficient materials for the above experiment.

We developed an effective method using high-pressure-liquid-chromatography (HPLC) and mass-spectrometry to isolate and analyze mycolic acid from a very small amount of sample within one week. We have successfully utilized this method to isolate and analyze mycolic acids from a non-cultivable mycobacterium (*Mycobacterium leprae*) and a very slowly growing mycobacterium (*Mycobacterium lepraemurium*). An α-mycolic acid with a characteristic structure was obtained from *M. leprae* or *M. leprae*-containing tissues. These findings are described.

MATERIALS AND METHODS

Collection of mycobacteria grown *in vivo*. Frozen livers of nine-banded armadillos (*Dasypus novemcinctus*) with a) experimental leprosy and b) natural acquired leprosy-like disease were kindly provided by Dr. W. M. Meyers, Armed Forces Institute of Pathology, Washington, U.S.A. The mycobacteria were collected from these livers by the repeated centrifugal method as reported by Matsuki, *et al.* ([5]). From each 10 g wet weight of liver, about 0.5 g wet weight of mycobacterial cell-mass was obtained in each case. *In vivo* grown *M. lepraemurium* were collected by the same method. Spleens of C_3H mice, inoculated with *M. lepraemurium*, Hawaiian strain, 6 months before sacrifice, were kindly provided by Dr. H. Nakagawa, National Institute for Leprosy Research, Tokyo. From 10 g wet weight of the spleens, about 0.2 g wet weight of *M. lepraemurium* cell mass was obtained.

Isolation of mycolic acids from mycobacteria or directly from the host tissues. For isolating mycolic acids directly from animal sources, the tissues were chopped into small pieces with scissors. Saponification of these tissues or mycobacterial cells was then carried out by heating at 85°C for 4 hr in 5% KOH (w/v) in 50% ethanol (v/v). The suspension was then acidified (below pH 2) and extracted (3 times) with a mixture of n-hexane and diethyl ether (1:1 v/v). The extract was evaporated to dryness and the residue containing total fatty acids was esterified with p-bromo-phenacyl bromide ac-

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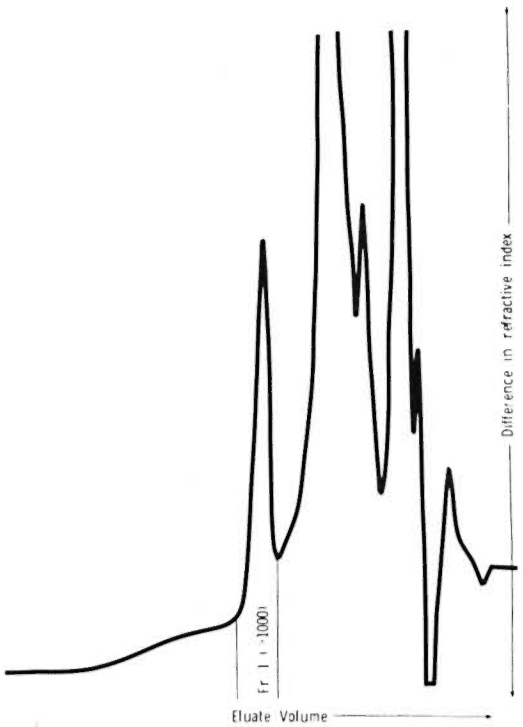


FIG. 1. Gel-permeation chromatography (GPC) of the total fatty acids in the footpad of a nude mouse with leprosy. The flow rate of the eluting solvent (THF) was fixed at 2 ml/min.

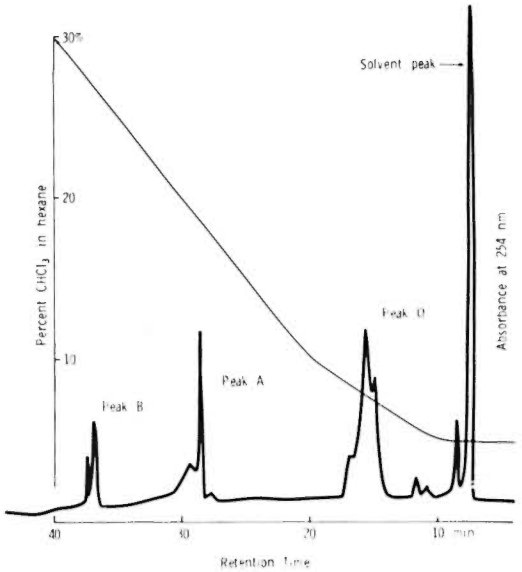


FIG. 2. Adsorption chromatography of fatty acids with more than 1000 molecular weight. Elution was performed with a linear gradient of $CHCl_3$ from 0–30% in n-hexane. The flow rate was fixed at 1 ml/min.

cording to the method of Drust, *et al.* ([1]). The resultant esters were dissolved in a small volume of tetrahydrofuran (THF) and applied to the first HPLC (Shimazu-LC 2) equipped with a gel-permeation column (HSG-15, Shimazu Co., 7.9 mm in diameter and 100 cm in length) using a differential refractometer detector (Shimazu RID-4). Various polystyrene standards with known molecular weights (Waters Co. Ltd.) were used to calibrate the column. Other conditions of the first HPLC are described in the legend to Fig. 1. The eluate corresponding to a peak of more than 1000 molecular weight as shown in Fig. 1 (Fr I), which was assumed to contain mycolic acids, was trapped and evaporated to dryness. The residue thus obtained was suspended in a small volume of n-hexane and applied to the second absorbing HPLC equipped with a porous silica gel column (μ Poracil, Waters Co., 4.0 mm in diameter and 20 cm in length) and with an ultraviolet photometer detector (λ: 254 nm, Shimazu UVD-4). Other conditions of the second HPLC are described in the legend to Fig. 2. Fr I of Fig. 1 was resolved into several peaks by this second HPLC as shown in Fig. 2. The eluates corresponding to the main peaks were trapped and evaporated to dryness. The residues obtained were suspended in small volumes of methanol and applied to the third HPLC equipped with a reverse-phased column (μBonda-C_{18}, Waters Co., 4.0 mm in diameter and 20 cm in length) and with the same UV detector as the second HPLC. Other conditions of the third HPLC are described in the legend to Fig. 3. Eluates corresponding to the main peaks shown in Fig. 3 were then trapped and evaporated to dryness. The residues thus obtained were then suspended in small volumes of chloroform and applied finally to an electron-impact (EI)-mass-spectrometric system connected to a data processing device (Hitachi Co. M-70 with 002B or M-80 with M003).

Bacterial strain and growth condition. *M. lepraemurium*, Hawaiian strain, were grown at 35° C on Ogawa's yolk medium supplemented with hemin as reported by Mori ([7]) and were harvested 6 weeks after inoculation.

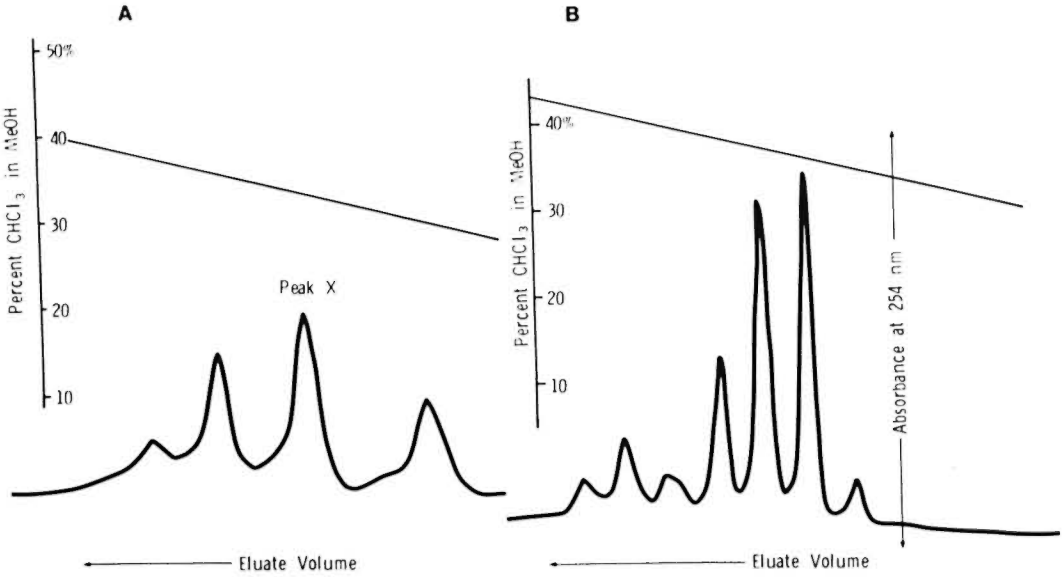


FIG. 3. Reverse-phased chromatography of eluates from the adsorption chromatography. A: eluate corresponding to peak A in Fig. 2. B: eluate corresponding to peak B in Fig. 2. Elution was performed with a linear gradient of $CHCl_3$ from 0–50% in methanol. The flow rate was fixed at 1 ml/min.

Chemicals. All solvents used for HPLC were HPLC-grade from commercial sources.

RESULTS

Isolation and identification of mycolic acids from a foot pad of a nude mouse infected with *M. leprae*. A foot pad of a nude mouse which had been inoculated with *M. leprae* 1 year earlier and which contained about 10^{10} bacilli was used as a source of *M. leprae*. The total fatty acids contained in the tissues were isolated and esterified with p-Br-phenacylbromide as described above. These esters were then applied to the first gel-permeating HPLC and the resulting chromatograph is shown in Fig. 1. The eluate corresponding to Fr I was then separated into three fractions (Peak A, B and O in Fig. 2) by the second absorbing HPLC. Eluates corresponding to Peak A and B in Fig. 2 were trapped and concentrated to apply to the third reverse-phased HPLC which gave the chromatographs shown in Fig. 3A and B respectively. Eluates corresponding to the major peaks in Fig. 3A were trapped and analyzed by mass-spectrometry. Fig. 4 shows the EI-mass-spectrum of the eluate corresponding to the largest peak (Peak X) in Fig. 3A. Fig. 5 shows a possible fragmentation pattern deduced from the spectrum of Fig. 4. As

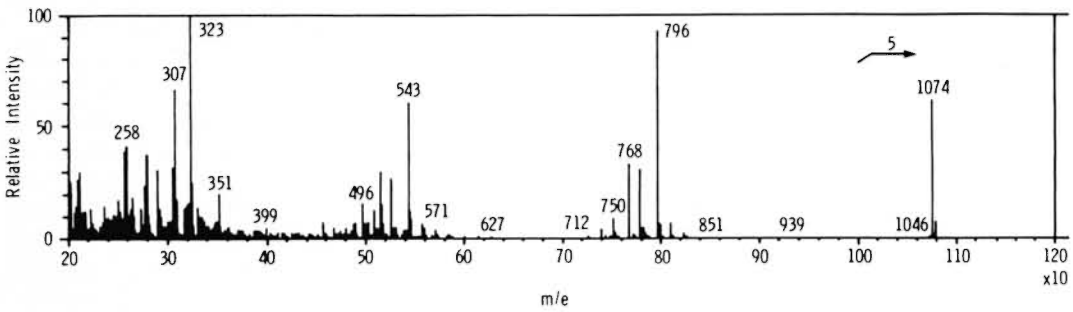


FIG. 4. Mass-spectrum of one of the main mycolic acids from the footpad of a nude mouse with leprosy. An Hitachi M-70 mass-spectrometer was used.

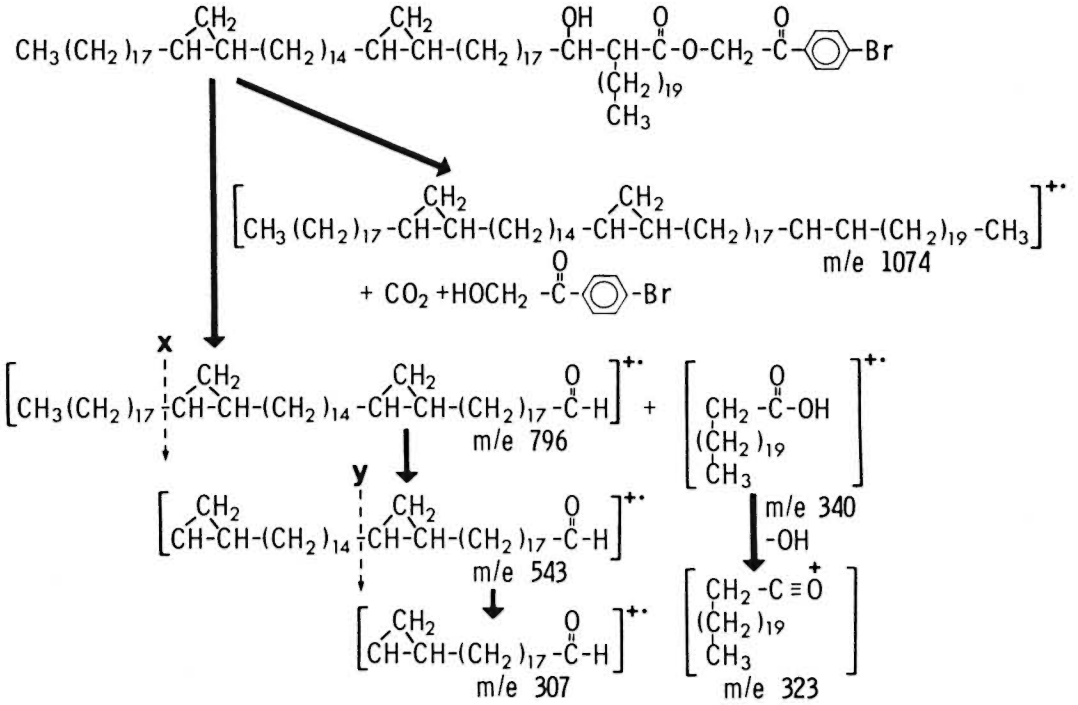


FIG. 5. Mass-fragmentation deduced from the spectrum of Fig. 4.

shown in Fig. 4 and Fig. 5, a single (M-258/260) fragment appeared at m/e 1074 and an intense meroaldehyde fragment appeared at m/e 796 with the corresponding (meroaldehyde-H_2O) fragment at m/e 778. Although the fragment of the α-branch acid (m/e 340) is not observable, an α-branch-acyl (acid-OH) fragment appeared at m/e 323 as the base (the most intense) peak. Cleavages at the dotted line x and y in Fig. 5 produced degraded aldehyde fragments appearing at m/e 543 and m/e 307, respectively. These fragments suggest that the compound in the eluate corresponding to Peak X in Fig. 3A is the p-Br-phenacyl ester of α (dicyclopropyl) mycolic acid whose structure is shown in Fig. 5.

EI-mass-spectrometry of eluates corresponding to the other peaks (other than Peak X) in Fig. 3A revealed that these eluates contained a series of α-mycolic acids. Among these α-mycolic acids some variation in the carbon chain length of the meroaldehyde part occurred but no change in the length of the α-branch (C_{20}) existed. The α-mycolic acid with the C_{20}-length α-branch seems to be a specific component of *M. leprae* as discussed later (The Table).

Eluates corresponding to the main peaks in Fig. 3B were also trapped and analyzed by EI-mass-spectrometry. Typical fragmentation patterns corresponding to mycolic acid having an α-branch with the same length (C_{20}) as the α-mycolic acid described above but with a different structure in the meroaldehyde part were obtained. Further analysis is needed, however, to reveal the final structure of these mycolic acids. The eluate corresponding to Peak O in Fig. 2 was found mass-spectrometrically not to contain mycolic acid and was not analyzed further.

Isolation and identification of mycolic acids from mycobacteria collected from the liver of an armadillo experimentally infected with *M. leprae*. In order to compare the structures of mycolic acids isolated from *M. leprae* from nude mice with those of organisms from experimentally infected armadillo, mycobacteria were collected from the liver of an experimentally infected armadillo. The mycolic acids were isolated from these microorganisms and analyzed by the same procedures as described above. Fig. 6 shows the first gel-permeating HPLC of the total fatty acids from these

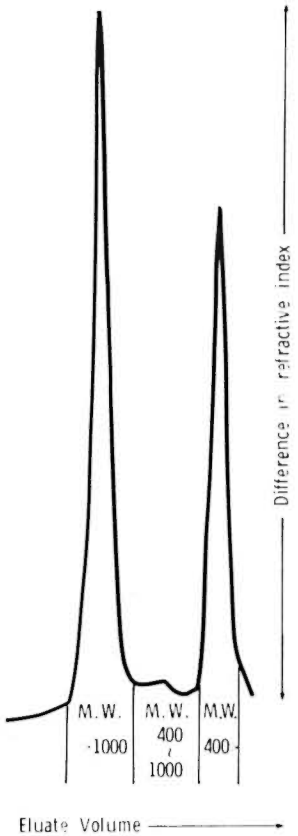


FIG. 6. Gel-permeation chromatography of the total fatty acids in mycobacteria collected from the liver of an armadillo with experimental leprosy. A refractometer was used as the detector. Other conditions were the same as Fig. 1.

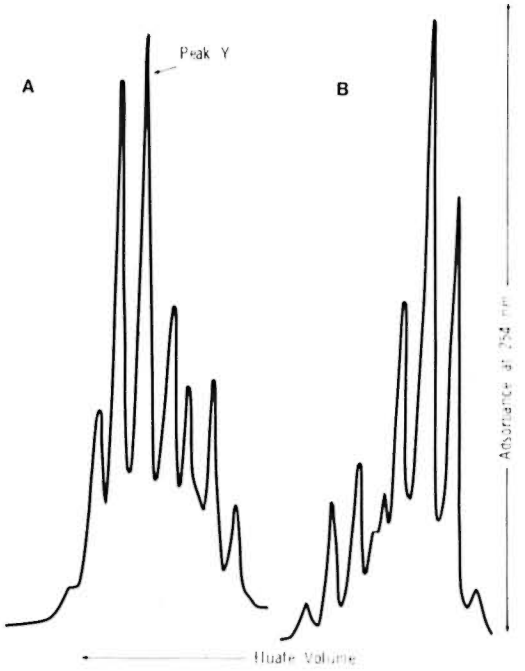


FIG. 7. Reverse-phased chromatography of mycolic acids of mycobacteria collected from the liver of an armadillo with experimental leprosy. The chromatographic conditions in Figs. 7A and 7B were the same as in Figs. 3A and 3B respectively.

mycobacteria. Fig. 7 shows the third reverse-phased HPLC of two series of mycolic acids from these mycobacteria. Eluates corresponding to the main peaks in Fig. 7A were trapped and analyzed by mass-spectrometry. The EI-mass-spectrum of the eluate corresponding to the largest peak in Fig. 7A (Peak Y) is shown in Fig. 8. In this spectrum, the peak at m/e 323 (α-branch acid-OH) is fairly intense but not the base peak as in the case of Fig. 4. However, the peak at m/e 340 corresponding to a fragment of the α-branch (C_{20}) acid, not observable in Fig. 5, seems intense enough to explain this discrepancy. Except for these differences, the other parts of the mass-spectrum in Fig. 8 are quite similar to those in Fig. 4. The two spectra (Fig. 4, Fig. 8) seem to provide sufficient evidence to conclude that the major mycolic acids isolated from *M. leprae* from experimentally infected armadillos are α-mycolic acids whose structures are the same as those from *M. leprae* from the foot pad of infected nude mice. Eluates corresponding to major peaks in Fig. 7B were also trapped and analyzed by EI-mass-spectrometry. This yielded about the same information about the structure of the mycolic acids as was the case of the eluates corresponding to major peaks in Fig. 3B.

Isolation and identification of mycolic acids from *M. lepraemurium* grown *in vitro* and *in vivo*. Mycolic acids were isolated from *M. lepraemurium*, a) grown *in vitro* and b) grown *in vivo*, by the methods described above. Fig. 9 shows the gel-permeating HPLC of the total fatty acids in *in vitro* grown *M. lepraemurium*. Fig. 10 shows the reverse-phased HPLC of two series of mycolic acids from this mycobacterium. Eluates corresponding to the main peaks in Fig. 10A were trapped and analyzed by mass-spectrometry. The EI-mass-

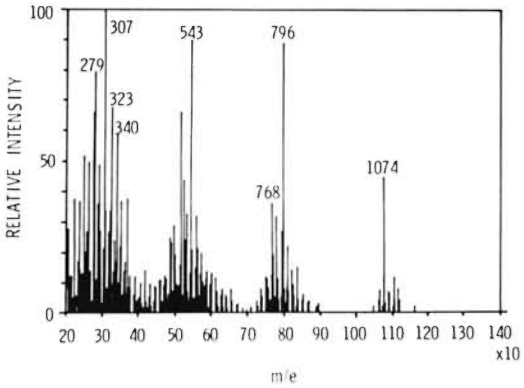


FIG. 8. Mass-spectrum of one of the main mycolic acids of mycobacteria collected from the liver of an armadillo with experimental leprosy. An Hitachi M-80 mass-spectrometer was used.

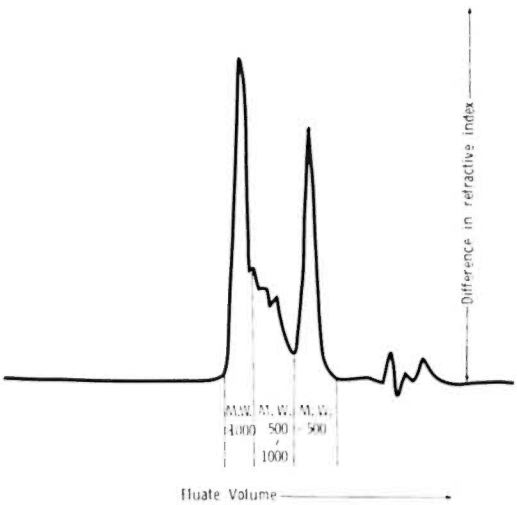


FIG. 9. Gel-permeation chromatography of the total fatty acids in *M. lepraemurium* grown *in vitro*. The chromatographic conditions were the same as in Fig. 1.

spectrum of the eluate corresponding to the largest peak in Fig. 10A (Peak Z) is shown in Fig. 11. Fig. 12 shows a possible fragmentation pattern deduced from the spectrum of Fig. 11. As shown in Fig. 11 and Fig. 12, a single (M-258/260) fragment appeared at m/e 1102 and the most intense meroaldehyde fragment appeared at m/e 796 (the base peak) with the corresponding (meroaldehyde-H_2O) fragment at m/e 778. Although the fragment of the α-branch acid (m/e 368) is not observable, an α-branch

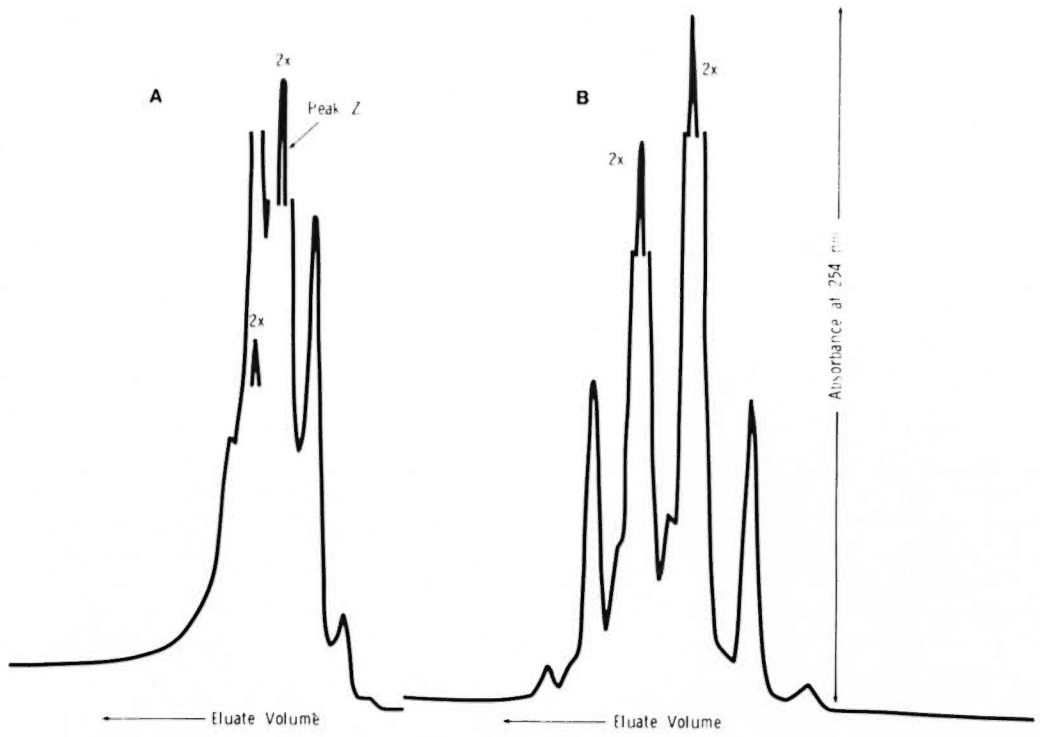


FIG. 10. Reverse-phased chromatography of mycolic acids of *M. lepraemurium* grown *in vitro*. The chromatographic conditions in Figs. 10A and 10B were the same as in Figs. 3A and 3B, respectively.

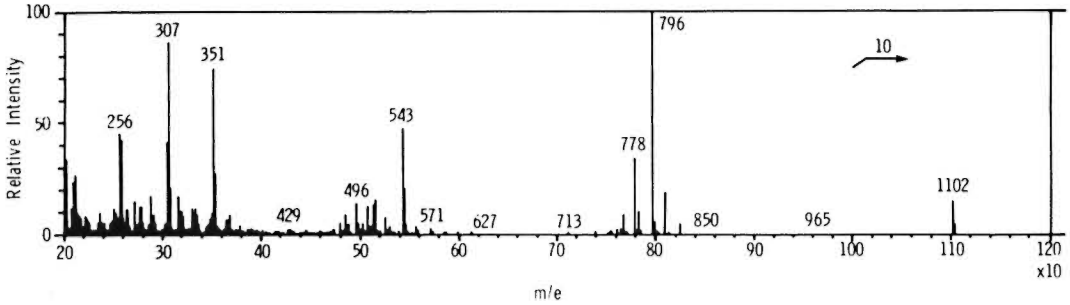


FIG. 11. Mass-spectrum of one of the main mycolic acids of *M. lepraemurium* grown *in vitro*. An Hitachi M-70 mass-spectrometer was used.

acyl (acid-OH) fragment appeared intensely at m/e 351. Cleavages at the dotted lines x and y in Fig. 12 produced degraded aldehyde fragments appearing at m/e 543 and m/e 307 respectively. These fragments suggest that the compound in the eluate corresponding to Peak Z in Fig. 10A was the p-Br-phenacyl ester of α (dicyclopropyl) mycolic acid whose structure is shown in Fig. 12.

EI-mass-spectrometry of eluates corresponding to the other peaks (other than Peak Z) in Fig. 10A revealed these eluates to contain a series of α-mycolic acids. Among these α-mycolic acids, some variation in the carbon chain length of the meroaldehyde part occurred but there were no changes in the length of α-branch (C_{22}). Thus the α-branch of the α-mycolic acid of *M. lepraemurium* is longer than that of the α-mycolic acid of *M. leprae* by two carbons.

Eluates corresponding to the major peaks in Fig. 10B were also trapped and analyzed by EI-mass-spectrometry. Typical fragmentation patterns corresponding to a my-

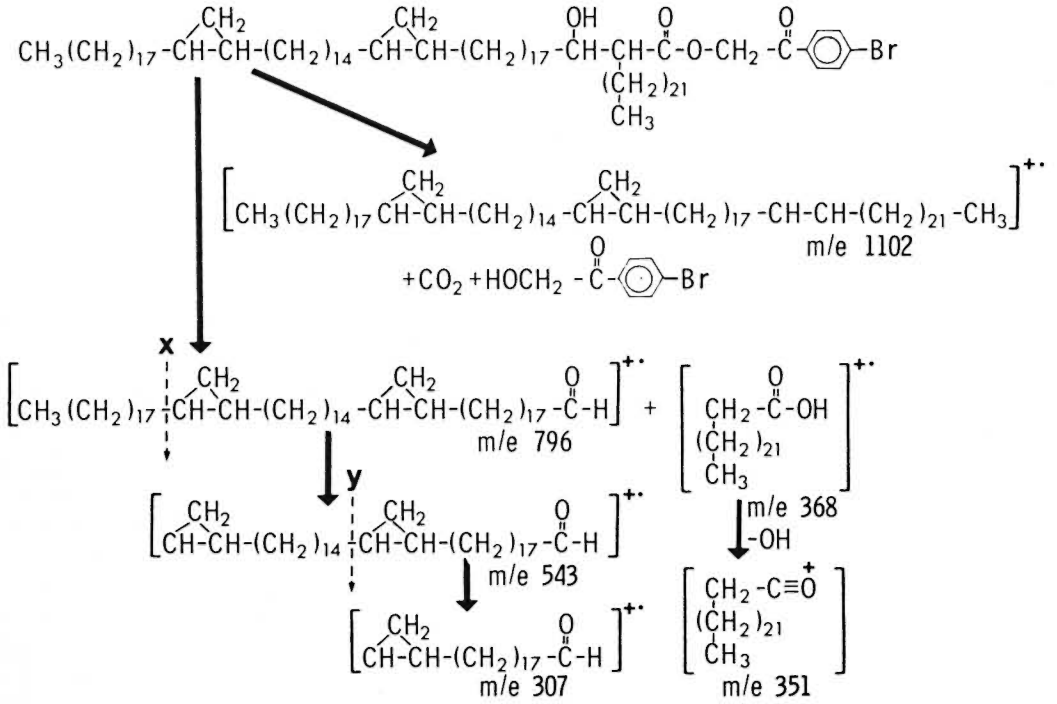


FIG. 12. Mass-fragmentation deduced from the spectrum of Fig. 11.

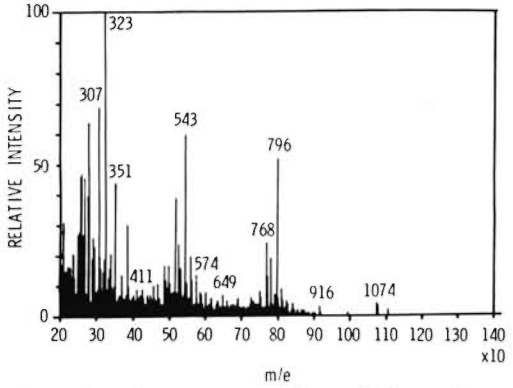


FIG. 13. Mass-spectrum of one of the main mycolic acids of mycobacteria collected from the liver of an armadillo with naturally acquired leprosy-like disease. An Hitachi M-80 mass-spectrometer was used.

colic acid having an α-branch with the same length (C_{22}) as the α-mycolic acid of *M. lepraemurium* but with a different structure in the meroaldehyde moiety were obtained. Further analysis is needed, however, to determine the structure of these mycolic acids.

Mycolic acids of *M. lepraemurium* grown *in vivo* were also analyzed and α-mycolic acid with the same structure as that of *M. lepraemurium* grown *in vitro* was obtained (data not shown). Analysis of the series of mycolic acids other than the α-mycolic acids in this microorganism are not yet completed.

Isolation and identification of mycolic acids from mycobacteria collected from the liver of an armadillo with naturally acquired leprosy-like disease. A comparison was made between the structure of mycolic acids in mycobacteria harvested from the liver of an armadillo with naturally acquired leprosy-like disease and those of organisms from experimentally infected armadillos. Mycobacteria were collected and mycolic acids were isolated using the same methods as described above. All the data obtained from HPLC in isolating the mycolic acids from these mycobacteria were identical to those obtained with experimental armadillo leprosy (data not shown). Fig. 13 shows the EI-mass-spectrum of an eluate corresponding to the largest peak obtained by the reverse-phased HPLC of the p-Br-phenacyl esters of the mycolic acids. The main fragments in this spectrum, indicating M-258/260 (m/e 1074), meroaldehyde (m/e 796), α-branch acid-OH (m/e 323), cleaved meroaldehyde (m/e 543 and 307) are quite similar to those in Fig. 4. Accordingly, α (dicyclopropyl) mycolic acid, having an α-branch with a 20 carbon chain length could be postulated as one of the main mycolic acids in these mycobacteria from an armadillo with naturally acquired leprosy-like disease.

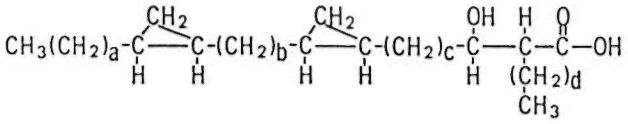
DISCUSSION

Qureshi and Takayama have studied EI-mass-spectra of mycolic acids in *Mycobacterium tuberculosis* $H_{37}Ra$ ([9]). The patterns of EI-mass-fragments shown in Figs. 4, 8 and 11 are quite similar to those of α (dicyclopropyl) mycolic acids in *M. tuberculosis*. Accordingly, our proposed fragmentation schema shown in Figs. 5 and 12 seem reasonable.

As mentioned, Etemadi and Convit ([2]) isolated α mycolic acids from skin lesions of leprosy patients in 1974. At that time, they cautiously used the term "non-cultivable mycobacteria" to refer to the origin of these mycolic acids rather than using "*M. leprae*." The structure of the α-mycolic acid proposed by them is the same as that of the α-mycolic acids isolated by us from mycobacteria from the liver of an armadillo with experimental leprosy. Moreover, mycolic acids isolated directly from the foot pad of a nude mouse inoculated with *M. leprae* had the same structure. Thus, there is now good evidence to conclude that the α-mycolic acids isolated by Etemadi and Convit and by us originated from *M. leprae*.

Minnikin, *et al.* ([6]) used a two dimensional thin-layer-chromatographic technique and were able to distinguish three patterns of mycobacterial mycolic acids, good examples of each pattern being the mycolic acids from *M. tuberculosis*, *M. avium*, and *M. fortuitum*. These patterns were produced by differences in the structure of the meroaldehyde part of the main mycolic acid in each species of mycobacteria. However, this means of classifying mycolic acids may not be absolutely consistent. It has been reported that changing the growth temperature of *Mycobacterium phlei* induces some changes in the carbon chain

THE TABLE. *Comparative structural data of α-mycolic acids from some strains of mycobacteria.*



Strain	Carbon chain length				Reference
	a	b	c	d	
M. tuberculosis $H_{37}Ra$	17, 19	10	17, 19	23	Qureshi & Takayama (1978)[9]
M. tuberculosis Brevannes	17, 19	14	11, 13	23	Qureshi & Takayama (1978)[9]
M. kansasii	17	14	17	21	Etemadi, *et al.* (1964)[3]
M. avium	17	14	17	21	Walczak & Etemadi (1965)[12]
M. lepraemurium	17, 19	12, 14	17	21	Present study
M. leprae	17, 19	14	17	19	Etemadi & Convit (1974)[2] and present study

length of the meroaldehyde part but no change in the α-branch of mycolic acids from this organism [11].

The structure of the α-branch in mycolic acids seems, therefore, to be another important taxonomical criterion. The Table compares the structures of α (dicyclopropyl) mycolic acids of some mycobacteria. The carbon chain length of the α-branch is expressed by "d" in the Table. At least in regard to the mycobacteria listed in the Table, α-mycolic acids having a C_{20} α-branch ("d" is 19) seem unique for *M. leprae*. (It has been reported [14] that the main mycolic acids of *M. parafortuitum* or *M. vaccae* also have a C_{20} α-branch, but their meroaldehyde parts are monoenoic or monocyclopropylic). Accordingly, it seems reasonable that detection of an α (dicyclopropyl) mycolic acid with a C_{20} α-branch from animal sources is a useful criterion for identifying *M. leprae* therein. In our experience, samples containing about 10^9–10^{10} mycobacterial cells are sufficient to detect such mycolic acid structures by our method. We have been able to detect an α-mycolic acid of *M. leprae*, using our method, in skin leproma from a patient with leprosy. The tissue had a wet weight of about 800 mg and was kindly provided by Prof. N. Nakamura, Kurume University (data not shown).

The identification of the mycobacteria found in armadillos with naturally acquired leprosy-like disease is of considerable importance. Certain data such as non-cultivability, positive *o*-diphenol oxidation [8], etc. indicated that this mycobacterium was indistinguishable from *M. leprae*. On the other hand, some discrepancies still seemed to exist. It was interesting, therefore, to compare the structures of the mycolic acids isolated from this microorganism with the structures of the mycolic acids isolated from nude mice and armadillos experimentally infected with *M. leprae*. α-Mycolic acids with the same structures were found from all three sources. Thus it seems very likely that the pathogenic mycobacteria of armadillos with naturally acquired leprosy-like disease is *M. leprae*, or at least very closely related to *M. leprae*.

Recently Young [15] proposed a convenient method for identifying *M. leprae*, using a thin-layer chromatographic classification of the mycolic acids extracted from 10^9 mycobacterial cells. He has contended that *M. leprae* contain only two types of mycolic acids (assumed to be α and β) while other, cultivable mycobacteria contain three types. However, some ambiguities seem to exist in his report. For example, there is no direct evidence proving that the two spots developed on the thin-layer-chromatograph for identifying *M. leprae* really correspond to α and β types of mycolic acids. In any case, his method seems useful especially for a preliminary identification of mycobacteria as *M. leprae*. On the other hand, we feel that other methods, probably including ours, are necessary for the definite identification of *M. leprae*.

Finally, we would like to point out that the present work is not only applicable for identifying *M. leprae* but also suggests a

special position of this microorganism in mycobacterial phylogeny. For example, from our previous ([4]) and present work, one can imagine that *M. leprae* has some remarkable deviations in its fatty acid synthesizing enzyme systems.

SUMMARY

Mycolic acids with a characteristic structure were isolated by high performance-liquid-chromatography (HPLC) and mass-spectrometry from a foot pad of a nude mouse inoculated with *Mycobacterium leprae*. Mycolic acids with the same structure were also obtained from mycobacteria collected from the liver of an armadillo with experimental leprosy.

Mycolic acids were isolated from *Mycobacterium lepraemurium* grown both *in vivo* and *in vitro* and these mycolic acids had different structures from those of *M. leprae*.

Mycolic acid structures have great taxonomical significance. The methods used for isolating and analyzing mycolic acids appear applicable for the rapid identification of *M. leprae* in samples containing at least 10^9–10^{10} mycobacterial cells.

Using our method, mycolic acids with the same structure were found in mycobacteria from armadillos experimentally infected with *M. leprae* and from armadillos with naturally acquired leprosy-like disease. It is likely, therefore, that the pathogenic mycobacteria of the naturally acquired disease are the same as, or at least closely related to, *M. leprae*.

The present work suggests that *M. leprae* has a special position in mycobacterial phylogeny.

RESUMEN

Se aislaron ácidos micólicos con una estructura caracteristica a partir de las almohadillas plantares de ratones desnudos inoculados con *Mycobacterium leprae* por cromatografía de líquidos de alta resolución y por espectrometría de masas. También se aislaron ácidos micólicos con la misma estructura a partir de micobacterias colectadas del hígado de un armadillo con lepra experimental.

Los ácidos micólicos aislados de *Mycobacterium lepraemurium* crecido tanto *in vivo* como *in vitro*, tuvieron estructuras diferentes de aquellos aislados del *M. leprae*.

Las estructuras de los ácidos micólicos tienen gran importancia taxonómica. Los métodos usados para aislar y analizar los ácidos micólicos, pueden ser aplicados a la identificación rápida del *M. leprae* en muestras conteniendo cuando menos 10^9–10^{10} micobacterias.

Usando nuestro método, se encontraron ácidos micólicos con la misma estructura en micobacterias aisladas de armadillos infectados experimentalmente con *M. leprae* y de armadillos con "lepra" adquirida naturalmente. Es probable, por lo tanto, que la micobacteria patogénica de la enfermedad adquirida naturalmente sea la misma que, o una muy relacionada con, *M. leprae*.

El presente trabajo sugiere que *M. leprae* tiene una posición especial en la filogenia de las micobacterias.

RÉSUMÉ

On a eu recours à la chromatographie liquide à haute performance (HPLC) et à la spectrométrie de masse, pour isoler des acides mycoliques avec strutures caractéristiques à partir des coussinets plantaires de souris glabres inoculées avec *Mycobacterium leprae*. On a obtenu des acides mycoliques présentant la même structure chez des mycobactéries recueillies à partir du foie d'un armadillo atteint de lèpre expérimentale.

Des acides mycoliques ont été isolés de *Mycobacterium lepraemurium* s'étant développé tant *in vivo* que *in vitro*; ces acides mycoliques présentaient des strutures différentes de celles des acides mycoliques.

Les structures de l'acide mycolique one une signification taxonomique importante. Les méthodes utilisées pour isoler et analyser les acides mycoliques se sont révélés applicables pour l'identification rapide de *M. leprae* dans des échantillons contenant au moins 10^9–10^{10} cellules mycobactériennes.

En utilisant cette méthode, on a identifié des acides mycoliques ayant la même structure dans des mycobactéries obtenues d'armadillos infectés expérimentalement par *M. leprae*, et d'armadillos présentant une maladie semblable à la lèpre acquise naturellement.

Il est dès lors vraisemblable que les mycobactéries pathogéniques de la maladie acquise naturellement sont les mêmes, ou tout au moins sont étroitement reliées, à *M. leprae*. Le travail accompli suggère que *M. leprae* bénéficie d'une position particulière dans la phylogénie mycobactérienne.

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