

Mycolic Acids from "Noncultivable" Mycobacteria

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Chromatographic analysis, coupled to mass spectrometry with a high-resolution mass spectrometer, of materials isolated from skin lesions of patients with lepromatous leprosy allows the recognition of characteristic mycobacterial products, mycolic acids. This finding indicates that the "noncultivable" bacteria responsible for leprosy are mycobacteria.

Some years ago, one of us (A.H.E) applied the techniques of pyrolytic gas chromatography and mass spectrometry to the elucidation of mycolic acids structure (4-6, 9, 12-15). The combination of these and other techniques led to progress in our understanding of these characteristic mycobacterial products (for reviews see references 7 and 8). By 1966 it was possible to obtain precise structural information about submilligram samples of mycolic acids, and we decided to study these constituents of bacteria from leprosy lesions in humans. A brief mention of the results of these investigations was made in a review (6).

Since that time, an interesting idea has been suggested about the nature of the acid-fast organisms found in the lesions of leprosy. On the basis of observations on the staining properties of these organisms (2, 3) and other undoubted mycobacteria, Fisher and Barksdale (10, 11) have suggested that a diversity of organisms, as opposed to a single mycobacterial species (*Mycobacterium leprae*), may become acid-fast "leprosy bacilli" in contact with host tissues. It seemed important, therefore, to publish in more detail our findings about the mycolic acids of leprosy bacilli in the hope that they will contribute to a better understanding of the pathogenesis of leprosy.

MATERIALS AND METHODS

Materials collected from skin lesions of patients (30 patients) with lepromatous leprosy were crushed in a mortar and treated with 0.25% trypsin for 5 min at 37 C. After centrifugation and three washings, the precipitate was recovered and suspended in 100 ml of acetone. After a month of contact, the acetone extract was separated by centrifugation, and the sediment was extracted twice with 100 ml of a 50:50 (vol/vol) mixture of ethyl alcohol and diethyl ether. The extract was separated by centrifugation, and the residue was then subjected to three extractions by chloroform. The residue remaining after solvent ex-

tractions was saponified by refluxing for 4 h in the presence of 10 ml of 5% methanolic potassium hydroxide; ether-soluble products were extracted, dried over Na_2SO_4 , and treated with diazomethane. Analytical thin-layer chromatography was performed on Silica Gel G plates (10 by 20 cm). Either a petroleum ether-diethyl ether mixture (80:20, vol/vol) or a petroleum ether-acetone mixture (90:10, vol/vol) was used as eluant. Components for which identification was presumed by analytical thin-layer chromatography, with known compounds as reference samples, were purified by preparative thin-layer chromatography on plates (25 by 35 cm). Purified substances were subjected to mass spectrometry by using a high-resolution MS9 mass spectrometer; the mass spectra were compared to those of known samples.

RESULTS AND DISCUSSION

Extractions. It was observed that most solvent-extractable material was removed by acetone and that a very small chloroform extract was obtainable. Thus, 446, 17, and 1 mg of material was extracted with acetone, ethyl alcohol-diethyl ether, and chloroform, respectively; the residue left was 517 mg.

Preliminary treatments of solvent extracts. The mixture of acetone and alcohol-ether extracts was fractionated into hydro- and ether-soluble parts in a separatory funnel. The ether-soluble part was 243 mg. Preliminary studies were undertaken on this fraction. The complexity of the mixture was noted by thin-layer chromatography; however, its thorough examination was not pursued since it was contaminated with a compound of host origin, cholesterol. Before discontinuing experiments on this fraction, we ascertained, by saponification of a sample, that it did not contain an appreciable amount of mycolic acids. The scarcity of the chloroform extract prevented its further study.

Treatment of the residue of solvent extractions. Pioneer work of Anderson et al. (1)

has shown that mycobacterial residues, after solvent extractions, still contain lipid materials, "firmly bound lipids," from which mycolic acids can be isolated by saponification. We previously observed the richness of these residues in mycolic acids (7), and so we subjected the residue of solvent extractions to saponification. A 500-mg amount of this residue was treated with methanolic potassium hydroxide; the ether-soluble material, separated and treated by diazomethane, was subjected to thin-layer chromatography. By using known compounds as reference samples, we observed that characteristic mycobacterial products, α -mycolic acids, β -mycolic acids, pre- α -mycolic acids, and α -meromycolic acids, are present.

Products revealed after being sprayed with

rhodamine B were collected by scraping off the materials from the Silica Gel G plate and eluting with diethyl ether. Quantitatively, 2.8 mg of methyl α -mycolates, 1.2 mg of methyl β -mycolates, 1.65 mg of pre- α -mycolates, and 0.3 mg of α -meromycolates were collected in this way. Methyl mycolates were characterized further by mass spectrometry.

Mass spectrometric study of characteristic mycobacterial constituents in material from human lesions. We show here only the significant part of the mass spectrum of methyl esters of α -mycolic acids purified from bacteria contained in human lepromatous leprosy lesions (Fig. 1).

As previously reported (4-6, 9, 12-15), methyl esters of α -branched, β -hydroxylated fatty

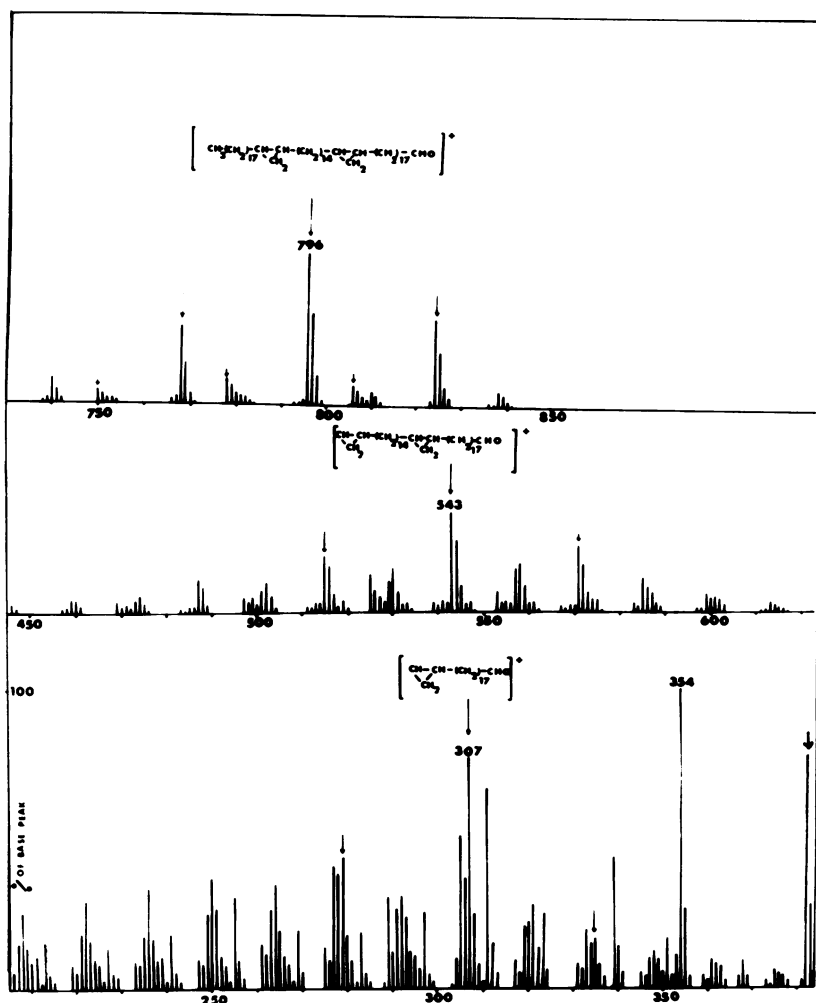
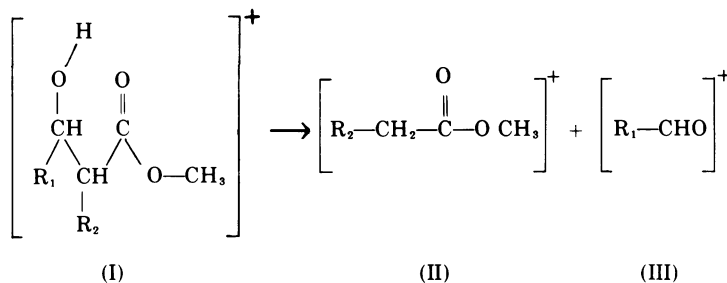


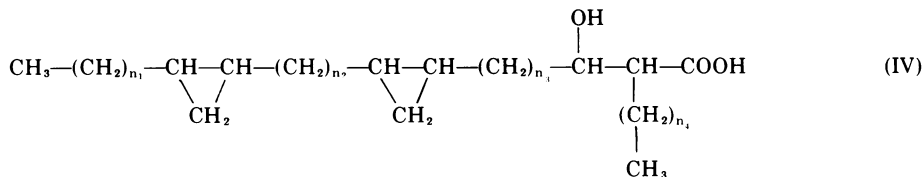
FIG. 1. Part of mass spectrum of methyl esters of α -mycolic acids isolated from bacteria in skin lesions of patients with lepromatous leprosy. The base peak is taken as the molecular peak of methyl behenate: m/e = 354.

acids (I) undergo fragmentation, giving ions of ester (II) and aldehyde (III). As the following scheme shows, the charge remains either with the ester or with the aldehyde.



These compounds undergo further fragmentation characteristic of each of the two categories of substances (for more detailed discussion see references 4 and 6), and knowledge about these fragments leads to that about the original material. However, since mycolic acids are always mixtures of homologues (7, 8), they show peaks for more than one ester and one aldehyde.

In the particular case of α -mycolic acids of the causative agent of lepromatous leprosy, the structure established by comparison with known compounds is as shown in the following scheme:



where $n_1 = 15, 17, \text{ and } 19$; $n_2 = 12, 14, \text{ and } 16$; $n_3 = 15, 17, \text{ and } 19$; and $n_4 = 19 \text{ and } 21$. (In all cases, the number corresponding to the most abundant entity is italicized.)

Two esters are recognized on the mass spectrum of methyl α -mycolates: these present molecular peaks at 354 and 382. Other peaks of these esters (namely, methyl behenate and methyl tetracosanoate) are seen on the spectrum. This determines the values of n_4 : $n_4 = 19 \text{ and } 21$.

Peaks are observed on the spectrum showing m/e values of 768, 796, and 824; these are molecular peaks for meroaldehydes. Another series of peaks at $m/e = 750, 778, \text{ and } 806$ at M-18 (M being the molecular weight of the aldehydes) exists on the spectrum. From these two series

of peaks, the values $n_1 + n_2 + n_3 = 46, 48, \text{ and } 50$ are easily deduced. On the other hand, peaks originating from the fragmentation of aldehydes (scheme V) are observed on the spectrum (see

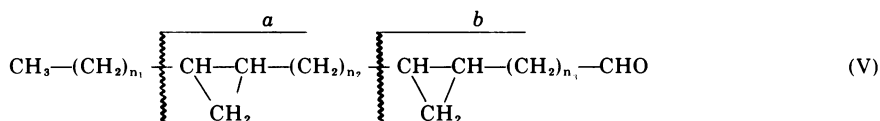
scheme V at bottom of page) where $n_1 = 15, 17, 19$; $n_2 = 12, 14, \text{ and } 16$; and $n_3 = 15, 17, \text{ and } 19$.

A series of peaks at $m/e = 515, 543, \text{ and } 571$, as well as a series of peaks at M-18, for these fragments exists on the spectrum. The former series of peaks is due to fragmentation following *a* as shown in scheme V; this allows the calculation of $n_2 + n_3 = 29, 31, \text{ and } 33$. Another series of peaks at $m/e = 279, 307, \text{ and } 335$, originating from fragmentation following *b*, is observed; this allows the calculation of $n_3 = 15, 17, \text{ and } 19$. From the reported data, the values $n_2 = 12, 14, \text{ and } 16$ and $n_1 = 15, 17, \text{ and } 19$ are deduced. The

structure of the main α -mycolic acid of the bacteria responsible for lepromatous leprosy is that of scheme IV: $n_1 = 17, n_2 = 14, n_3 = 17, \text{ and } n_4 = 19$.

The mass spectrum of methyl esters of β -mycolic acids from bacteria of human lepromatous leprosy is very similar to that of *Mycobacterium tuberculosis* strain Test (O. Torquebiau and A. H. Etemadi, unpublished data; see also references 7 and 8), the difference being that in the present case the methyl esters resulting from the cleavage of methyl mycolates are methyl behenate and methyl tetracosanoate, whereas methyl mycolates of strain Test give methyl hexacosanoate as the mass spectrometric cleavage product.

Our conclusion is that, beyond any doubt, the



material isolated from human leprosy lesions contains mycobacteria, since mycolic acids of the types reported are characteristic for these organisms and are found, in particular, in *Mycobacterium tuberculosis* and *Mycobacterium kansasii* (7, 8). Thus, ideas concerning the nonmycobacterial etiology of leprosy are not supported by this work.

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