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Research Article

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Demonstration of Tuberculostearic Acid in Sputum from Patients with Pulmonary Tuberculosis by Selected Ion Monitoring

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ABSTRACT Selected ion monitoring was used to detect tuberculostearic acid (10-methyloctadecanoic acid) in sputum from patients with pulmonary tuberculosis. The specimens were autoclaved, lyophilized, extracted, and methanolysed before being subjected to thin-layer chromatography and injected into the gas chromatograph/mass spectrometer. Tuberculostearic acid could be detected in five of six tuberculous sputum specimens containing acid-fast rods detectable by light microscopy of Ziehl-Neelsen stained smears. After the sputum specimens had been cultured for five days on Löwenstein-Jensen medium, when still no colonies could be observed visually, the presence of tuberculostearic acid was demonstrated in all six cases of tuberculosis. In corresponding analyses of sputum from eight patients with non-tuberculous pneumonia, tuberculostearic acid was not found. This fatty acid, the presence of which was also demonstrated in cultures of various mycobacterial and nocardial species, is characteristic of organisms of the order *Actinomycetales*. The demonstration of tuberculostearic acid in sputum specimens may constitute a rapid and sensitive way of diagnosing pulmonary tuberculosis.

INTRODUCTION

Even though almost a century has passed since Robert Koch succeeded for the first time in isolating *Mycobacterium tuberculosis* in an artificial medium, it still takes many weeks to establish a diagnosis of tuberculosis by means of culture studies. Generally speaking, several weeks are needed for species identification of isolated mycobacteria. Obviously, there is a need for more rapid means with which to diagnose tuberculosis and other mycobacterial infections.

To speed up the identification of organisms isolated in the microbiological laboratory, gas chromatographic (GC)[1] analyses of culture media, head-space vapors and extracts of cellular compounds have been employed (1). GC has also been suggested for use in rapidly diagnosing certain infectious diseases, by demonstrating microbial metabolites in body fluids, *viz.*, in synovial and cerebrospinal fluids, in serum, urine, and pleural effusion. Chromatographic patterns, each characteristic of a given infectious disease, have thereby been demonstrated (2–4). The finding in body fluids of microbial metabolites specific to a particular organism represents a far less common application of GC. This latter type of analysis has involved relatively complicated derivatization techniques, employing electron capture detection of the products so obtained (5).

Mass spectrometry (MS) provides, particularly when combined with GC, a means of identifying a variety of compounds, including those of microorganisms. However, analytical difficulties frequently arise because of the chemically complex character of the specimens or the presence of only minute traces of the compound under study. Selected ion monitoring (SIM) allows the mass spectrometer to be scanned exclusively at preselected ions, maximizing the duty cycle of the instrument at the ions of interest. The sensitivity of the spectrometer is thereby increased by at least one order of magnitude, the increase being dependent on the relative intensity of the ion studied. By this technique it is frequently possible to detect a certain compound at a level of only a few picograms.

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[1] *Abbreviations used in this paper*: GC, gas chromatography, -chromatographic; MS, mass spectrometry; SIM, selected ion monitoring.

The technique also has the advantage of being selective; a specific microbial metabolite present in a complex mixture can be readily detected.[2]

Tuberculostearic acid (10-methyloctadecanoic acid) is a liquid saturated fatty acid, which was first isolated by Anderson and Chargaff (6) from *M. tuberculosis*. The acid has been demonstrated in many mycobacterial species and also in a limited number of other organisms of the order *Actinomycetales* (7–12). In *M. tuberculosis* the acid amounts to ≅10% (wt/wt) of the total fatty acid content. The natural acid is optically active (13, 14).

Mycocerosic acids constitute another group of optically active acids of high molecular weight (≅30 carbon atoms) (15–17). They are found exclusively in the lipids of *M. tuberculosis* and *M. bovis*, principally in the form of diesters of phthiocerol (18, 19).

SIM has previously been employed to detect mycobacteria and nocardiae by focusing the mass spectrometer on fragments characteristic of methylated tuberculostearic acid.[2] In the present investigation, we assessed whether the technique could be used to detect the specific acids mentioned in sputum samples from patients with active pulmonary tuberculosis. Synthesis of 10-methyloctadecanoic acid is described and the application of SIM for rapid diagnosis of mycobacterial infections is discussed.

METHODS

Organisms. One sample of each *M. africanum, M. avium, M. fortuitum, M. kansasii, M. smegmatis, M. tuberculosis, Nocardia asteroides, N. brasiliensis,* and *N. rubra*, were studied.

Sputum samples. Sputum samples (2–4 ml) were collected from six patients with pulmonary tuberculosis, the diagnosis of which was confirmed by culture studies and guinea-pig pathogenicity tests. Sputa from all six patients contained acid-fast rods detectable by light microscopic examination of Ziehl-Neelsen-stained smears. In addition, sputa from eight patients with nontuberculous pneumonia were studied. The latter patients were all treated in an intensive care unit for infectious diseases, half of them in a respirator, when sampling was made. Each sample was divided into four parts: two parts were used for culture studies, one for microscopic investigation, and one was analyzed directly by SIM.

Culture technique. The mycobacterial strains studied were cultured in Proskauer-Beck medium (Difco Laboratories, Detroit, Mich.) and harvested as described elsewhere (20). Each *Nocardia* species was cultured in 5 ml Trypticase soy broth (Oxoid Ltd., England) at 37°C for 3 d before being washed with 10 ml of distilled water.

The sputa to be cultured for mycobacteria were treated with 5 ml each of an aqueous solution of sodium laurylsulphate (3%, wt/wt) and sodium hydroxide (1%, wt/wt) at room temperature for 20 min with shaking. After centrifugation at 3,000 rpm for 30 min, the pH of the pellets was adjusted to 7.0 with sulphuric acid (0.45%, wt/wt in water). The specimens were inoculated onto slants of Löwenstein-Jensen medium. After 5 d of incubation at 37°C, one of the duplicate cultures of each sputum sample was washed off its slant with 0.9% (wt/wt) aqueous NaCl.

Preparation of specimens. Both the sputa and the specimens from the cultures were autoclaved (121°C, 60 min, 1.1 kp/cm²) and lyophilized. The samples were then transferred to 2-ml glass ampules and extracted overnight with ≅1.5 ml of chloroform:methanol (2:1 vol/vol) at room temperature. After centrifugation for 20 min at 4,000 rpm, the supernates were transferred to new ampules and concentrated to dryness under a stream of nitrogen. 1 ml of 3% methanolic HCl was added, and the ampules were sealed by melting. The methanolysis was allowed to proceed at 80°C for 20 h, after which the ampules were again centrifuged (4,000 rpm, 20 min). The supernates were then evaporated to dryness and 100 μl of *n*-hexane was added. The mixtures were treated in an ultrasonic bath for 1 min at an out effect of 125 W, after which the solutions were subjected to thin-layer chromatography.

Thin-layer chromatography. The methanolysates were spotted as repeated applications, allowing drying in between. Unidimensional runs were made with a 0.25-mm layer of silica gel (Silica gel 60 F_{254}, Merck A. G., Darmstadt, Germany). A mixture of *n*-hexane and diethyl ether (9/1, vol/vol) was used as solvent. After 30–40 min, the bands corresponding to methyl esters were scraped off and the gel extracted with ethyl acetate. Before injection (0.1–0.2 μl) onto the GC column, the samples were evaporated to dryness and diluted with *n*-hexane.

Standards and reagents. 3% methanolic hydrogen chloride used for methanolysis was prepared by adding 5 ml of acetyl chloride to 100 ml of methanol. All solvents were of reagent grade and used without further purification.

17-methyloctadecanoic and *n*-nonadecanoic acids, the purities of which were >99% (GC) were supplied by Larodan Lipids AB (Limhamnsv Malmö, Sweden).

2-methyloctadecanoic acid (racemic) was prepared according to the method of Ställberg (21). The specimen of natural C32 mycocerosic acid was a gift from the late Professor Einar Stenhagen (Gothenburg, Sweden).

10-methyloctadecanoic acid (tuberculostearic acid, racemic), which is not commercially available, was synthesized by the same principal route as that of Linstead et al. (22). 14.09 g (0.088 mol) methyl hydrogen 3-methyl-glutarate (racemic) and 18.70 g (0.130 mol) *n*-octanoic acid were subjected to mixed anodic coupling (Kolbe electrosynthesis) in 250 ml methanol to which 0.59 g (0.011 mol) of sodium methoxide was added. The current was 4 A at a voltage of 50 V. After 3 h, when the pH had reached 7.0, the reaction mixture was evaporated to dryness, the residue rubbed with light petroleum (boiling point, 60–80°C), and the suspension subsequently filtered. The filtrate was shaken with a 5% (wt/wt) aqueous solution of sodium carbonate, washed with water, and again evaporated. The residue was hydrolyzed by refluxing for 5 h in the presence of 20 g KOH, 50 ml water, and 200 ml ethanol. Water was added to the reaction mixture, which was subsequently extracted with light petroleum. The aqueous phase was then acidified to pH 2 with dilute hydrochloric acid and the organic acid taken up in diethyl ether. After work up, the residue was distilled. 3-methylundecanoic acid boiled at 125–129°C at 0.8 mm Hg. The yield was 5.5 g and the purity >98% (wt/wt), as indicated by GC of the corresponding methyl ester. The identity of the reaction product was confirmed by MS.

[2] Larsson, L., P-A. Mårdh, and G. Odham. 1979. Detection of tuberculosteric acid in mycobacteria and nocardiae by gas chromatography and mass spectrometry using selected ion monitoring. *J. Chromatogr. Biomed. Appl.* In press.

The 3-methylundecanoic acid obtained was chain-lengthened via a second anodic coupling. 5.50 g (0.028 mol) of the acid and 3.70 g (0.019 mol) methyl hydrogen azelate was electrolysed in methanol (50 ml) containing 0.12 g (0.002 mol) of sodium methoxide at 1.6 A and 70 V for 1.5 h (pH 7.0). The reaction mixture was worked up as described above. The residue (6 g) was chromatographed on 100 g silicic acid (silicar CC-4, Mallinckrodt, Inc., St. Louis, Mo.) with diethyl ether:light petroleum (1:50; vol/vol; boiling point, 40–60°C). The combined fractions (2.3 g) of methyl 10-methyloctadecanoate had a purity of >98% (wt/wt), as indicated by GC peak heights. The methyl ester was hydrolysed as described above. The yield of 10-methyloctadecanoic acid was 1.9 g (6.8% calculated on the first half ester). The identity of the product was confirmed by MS (Fig. 1).

GC and MS. A mass spectrometer (Varian Associates, Instruments Div., Palo Alto, Calif., MAT, model 112) coupled to a gas chromatograph (Varian Associates, model 1400) was used. The chromatographic conditions were as follows. Two glass columns (i.d., 2 mm) were employed: (*a*) 3-m column packed with 3.7% OV-17 on Chromosorb W, HP 80/100 mesh and (*b*) 2.3-m column packed with 1.8% OV-101 and 1.8% OV-17 on Chromosorb W, HP 80/100 mesh (Varian Associates, Palo Alto, Calif.). The injector port temperature was 270°C and the oven temperature 230°C (detection of methyl tuberculostearate) or 270°C (detection of methyl C 32 mycocerosate). The carrier gas was He with flow rate 20 ml/min.

The mass spectrometer was operated under the following conditions. The separator temperature was 250°C, the ion source temperature 230°C or 270°C, and the electron energy 70 eV. Measurements were performed by single ion detection monitoring on the ions at the mass:charge ratio (*m/e*) 312 (molecular peak) and *m/e* 167 for methyl 2-methyloctadecanoate, methyl 10-methyloctadecanoate (methyl tuberculostearate), methyl 17-methyloctadecanoate, and methyl nonadecanoate. Methyl C32-mycocerosate was monitored at *m/e* 494 (molecular peak).

RESULTS

MS. In Fig. 1, the mass spectrum of the methyl ester of natural tuberculostearic acid has been reproduced. It indicates the expected molecular weight of 312 (M, 4%) for the C19 methyl ester. In addition, abundant fragments at *m/e* 171 and *m/e* 199, characteristic of the methyl side chain at position 10, are found. The latter fragment readily loses a molecule of methanol, giving rise to ions of *m/e* 167, virtually absent in mass spectra of other C19 methyl ester positional isomers, including the straight chain ester.

Selected ion monitoring of cell extracts and the reference esters. A mass chromatogram is shown, obtained when monitoring the methyl esters of an extract of *M. tuberculosis* at *m/e* 312 (Fig. 2A). The single peak obtained indicates that the cellular material separating on the column does not interfere with the determination of tuberculostearic acid. The experiment was repeated, focusing at *m/e* 167, and using the same amount of extract (Fig. 2B). This chromatogram shows a significantly smaller tuberculostearate peak.

To establish the separation efficiency of the OV-17 stationary phase for other C19 isomers, a mixture of methyl 2-methyl- (*a*), 10-methyl- (*t*), and 17-methyl- (*c*) octadecanoate and methyl nonadecanoate (*d*), was analyzed at *m/e* 312 using the SIM technique (Fig. 2C). These compounds are known to occur occasionally in biological material. As is evident from the figure, the phase facilitated separation of the isomers. A less satisfactory result was obtained when using the mixture OV-17 and OV-101 as stationary phase.

Monitoring of the reference mixture at *m/e* 167 (the mass number characteristic of the 10-methyl-substituted ester) resulted as expected in one single peak (Fig. 2D).

Employing ion selection of C32 methyl mycocerosate (17) at the molecular peak (*m/e* 494) proved less successful. Although giving rise to the expected single peak, the signal:noise ratio was only about 1/10 of that obtained when analyzed tuberculostearate at *m/e* 312. Consequently, we omitted analyses of C32 mycocerosic acid in our later studies.

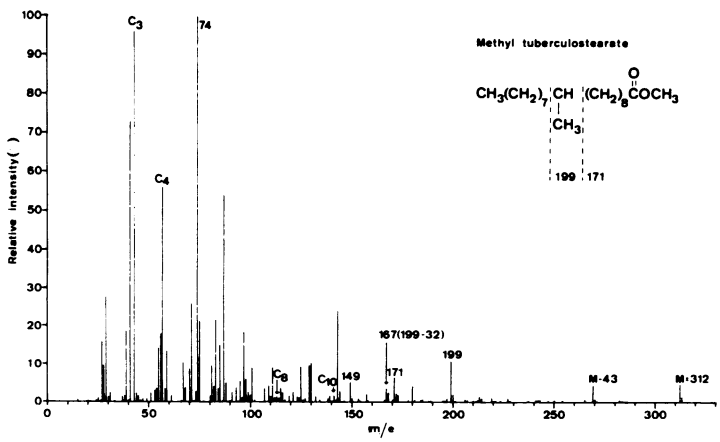

FIGURE 1 Mass spectrum of methyl tuberculostearate isolated from *M. tuberculosis*.

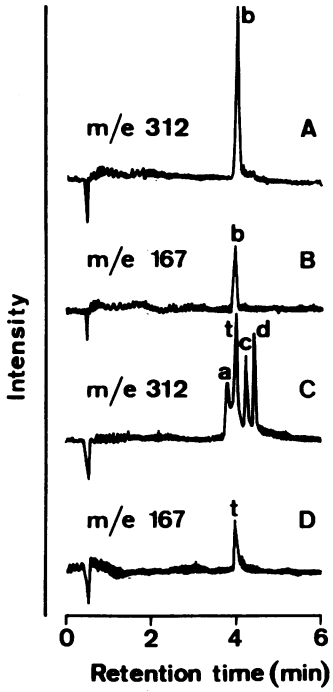

FIGURE 2 Mass chromatograms of a methanolysate of *M. tuberculosis* at *m/e* 312 (A) and *m/e* 167 (B), and of a reference mixture containing ≅200 pg of each methyl ester of 2-methyl-octadecanoic (*a*), 10-methyloctadecanoic (*t*), 17-methyloctadecanoic (*c*), and nonadecanoic acid (*d*) at *m/e* 312 (C) and *m/e* 167 (D). OV-17 was used as stationary phase.

Quantitative determination of bacterial tuberculostearic and C32 mycocerosic acids. The sizes of the peaks representing methyl tuberculostearate, obtained when analyzing lyophilized cells of one strain each of the *Mycobacterium* and *Nocardia* species studied at *m/e* 312, were compared with those produced by known amounts of synthesized methyl 10-methyloctadecanoate. The mycobacterial strains studied were found to contain 0.3–0.8% (wt/wt) of tuberculostearic acid. *N. asteroides*, *N. brasiliensis*, and *N. rubra* contained 0.5, 0.7, and 0.1% (wt/wt) of tuberculostearic acid.

M. tuberculosis contained 0.1% (wt/wt) of C32 mycocerosic acid.

Linearity of instrument response and detection limit. In Fig. 3 the peak areas of methyl 10-methyloctadecanoate, monitored at *m/e* 312, have been plotted against known amounts of the compound injected onto the column. The curve indicates that quantitative estimations can be made by direct comparison of peak areas. The detection limit was found to be ≅20 pg.

A similar experiment was performed employing ion selection at *m/e* 167 of the ester. Under these conditions, the sensitivity was reduced by a factor of approximately five compared with that obtained when using *m/e* 312.

Analysis of sputum samples. From four of the patients, the sputum samples yielded confluent growth of *M. tuberculosis* when incubated on slants of Löwenstein-Jensen medium, whereas from another patient the growth was less abundant. From a sixth patient, heavy growth of *M. avium* was demonstrated. From all six patients, microscopy of Ziehl-Neelsen-stained sputum specimens revealed acid-fast rods, the morphology of which suggested them to be mycobacteria. From the remaining eight patients, cultures and microscopy of sputum specimens did not reveal mycobacteria.

In Fig. 4, representative chromatograms (A) of a sputum sample from a patient infected with *M. tuberculosis*, obtained by ion monitoring at *m/e* 312 and *m/e* 167, are shown. Chromatograms (B) from the same sample to which synthetic 10-methyloctadecanoate (*t*) had been added, are also shown. Four prominent peaks are clearly visible in each chromatogram at *m/e* 312. The component (*b*) represents the methyl ester of tuberculostearic acid. The other components were identified as the 2-methyl-substituted C18 isomer (*a*), the iso- (*c*), and straight-chained (*d*) C19 acid methyl esters by comparison with GC data from studies of the reference substances. When monitoring at *m/e* 167, the chromatograms obtained were less easy to interpret. However, a peak corresponding to tuberculostearate (*b*) could be identified.

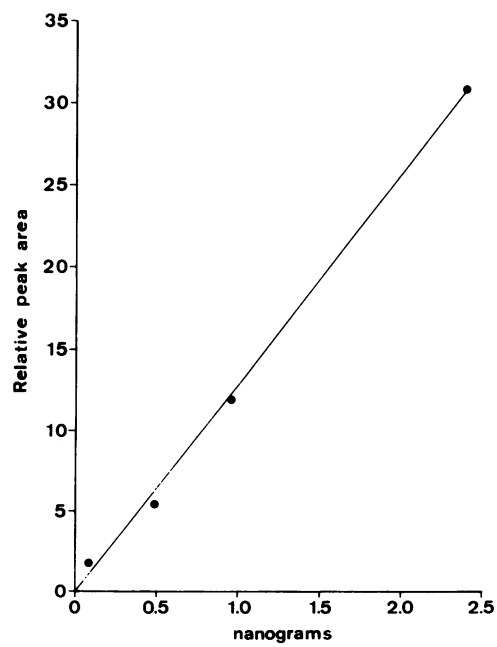

FIGURE 3 Integrated ion intensity vs. sample size of methyl 10-methyloctadecanoate.

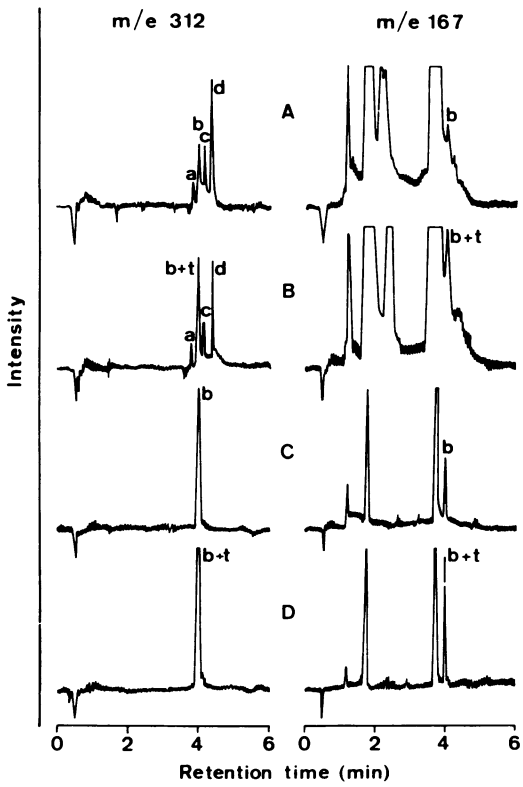

FIGURE 4 Representative mass chromatograms at *m/e* 312 and *m/e* 167 of a sputum specimen from a patient with pulmonary tuberculosis, analyzed directly (A) and after 5 d of incubation at 37°C on Löwenstein-Jensen medium (C). Of a final sample volume of 400 μl, 0.2 μl was injected. The chromatograms obtained after addition of synthesized 10-methyloctadecanoic acid methyl ester (*t*) to the same preparations are also shown (B and D). For explanation of letters *a*–*d*, see legend to Fig. 2. OV-17 was used as stationary phase.

Chromatograms representing a sample of sputum that had been inoculated onto Löwenstein-Jensen medium and incubated for 5 d are shown in Fig. 4C. The chromatograms are simpler than those obtained by direct analysis of the same sputum sample. Notably, mycobacteria were not visible on the agar slants to the naked eye until 14–20 d after incubation of the duplicate culture. The identity of peak (*b*) was confirmed by the addition of authentic methyl 10-methyloctadecanoate (*t*) (Fig. 4D).

Tuberculostearic acid could be detected in five of the six sputa from the tuberculous patients at *m/e* 167 and *m/e* 312, although in two of the cases the peak was smaller than in Fig. 4A. In specimens collected after 5 d of incubation of the same sputum sample on Löwenstein-Jensen medium, this compound could be detected in all six cases studied. Analyses of sputa collected from the eight patients with nontuberculous pneumonia produced chromatograms like that exemplified in Fig. 5A. In no case was a peak with the same retention time as methyl tuberculostearate detected. The example shows (Fig. 5B) a chromatogram of a specimen with methyl 10-methyloctadecanoate added (*t*). Chromatograms representing a 5-day-old culture were completely blank (Fig. 5C).

DISCUSSION

The demonstration of acid-fast rods, i.e., mycobacteria, in sputum samples is a comparatively laborious procedure. Furthermore, to trust the test results, one requires the technician to have a great deal of experience. The diagnosis of tuberculosis by culture studies is time consuming, the culturing time required for isolation of *M. tuberculosis* being 3–6 wk (23).

For the differential diagnosis of mycobacteria, we have earlier performed GC analysis of trifluoroacetylated methanolysates of whole mycobacterial cells (20). GC has also been applied in the diagnosis of mycobacterial infections by direct study of serum and cerebrospinal fluid specimens. The GC elution profiles obtained appeared characteristic of tuberculosis when compared with chromatograms of corresponding body fluids of patients with other infectious diseases

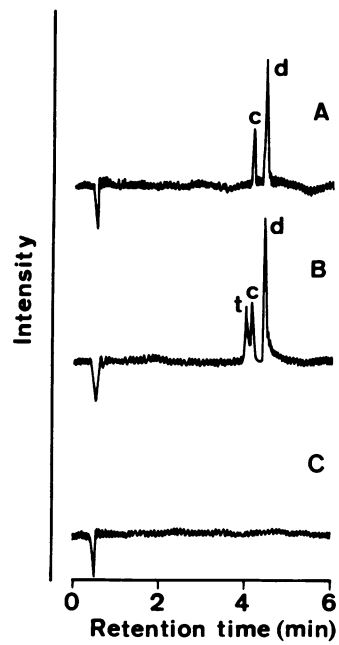

FIGURE 5 Representative mass chromatograms at *m/e* 312 of a sputum sample obtained from a patient with nontuberculous pneumonia, analyzed directly (A) and after 5 d of incubation at 37°C on Löwenstein-Jensen medium (C). Of a final sample volume of 400 μl, 0.2 μl was injected. A chromatogram obtained by addition of synthesized 10-methyloctadecanoic acid methyl ester (*t*) to the former sample is also shown (B). For explanation of letters *c* and *d*, see legend to Fig. 2. OV-17 was used as stationary phase.

(2, 4). No attempts were made to identify the compounds represented by the peaks in the chromatograms obtained. Thus, no unique constituents of mycobacteria could be demonstrated. Brooks et al. (5) identified 3-(2′-ketohexyl) indoline in cerebrospinal fluid samples from patients with tuberculous meningitis, whereas this compound was not detected in cases of cryptococcal and aseptic meningitis.

The idea for the present study emanated from the well-documented fact that tuberculostearic acid is unique for organisms belonging to *Actinomycetales* and that mycocerosic acids are specific for *M. bovis* and *M. tuberculosis*. Tuberculostearic and mycocerosic acids have attracted interest ever since 1934, when the structure of the former was elucidated by Spielman by oxidative degradation by means of chromic acid (24). The structure, which was that of 10-methyloctadecanoic acid, was confirmed by synthesis. Independently, Prout et al. (13) and Ställberg-Stenhagen (14) showed that the natural acid is weakly levorotatory.

The mass spectra of the racemic and optically active methyl ester are identical. As a consequence, the present work was carried out with the racemic reference substance.

We found earlier that SIM gives chromatograms containing only one peak of methyl tuberculostearate when analyzing whole-cell methanolysates of mycobacteria and nocardiae at *m/e* 312 and *m/e* 167.[2] The chromatograms obtained by corresponding analyses of a great number of other bacterial species encountered in pneumonia were totally blank.[2] Apart from its specificity, SIM proved to be ≅50 times as sensitive as a conventional GC flame ionization detector.

In the present study, we were able to demonstrate tuberculostearic acid in five patients with pulmonary infections caused by *M. tuberculosis* or *M. avium*. The failure to demonstrate this acid in one of the tuberculous sputum specimens, even when a concentrated sample was injected, might be a result of the analysis of a nonrepresentative aliquot of the sample. Mycobacteria can become unevenly distributed in such specimens, particularly when having a high viscosity.

The advantages of the SIM technique used became particularly evident in analyses of specimens from mycobacterial cultures that had been incubated for only a few days. A large peak of methyl tuberculostearate could be observed even when injecting <0.001 part of the extract prepared from such cultures in all six cases of pulmonary mycobacterial infection. Furthermore, these chromatograms were found simpler to interpret than those obtained from analyses of the uninoculated sputum samples, because irrelevant peaks were then either absent (monitoring at *m/e* 312) or much smaller (monitoring at *m/e* 167) (Fig. 4). The largest peaks in the latter chromatograms probably arise from natural carbon isotopes of methyl palmitate and stearate, respectively. Whether these acids are metabolized by the cultured mycobacteria, or merely adhere to the Löwenstein-Jensen slants when the mycobacteria are being washed off the slants, was not evaluated in the present investigation.

The presence of tuberculostearic acid in mycobacteria grown in vivo has been reported previously. Lungs of mice infected with *M. bovis* were collected and the mycobacteria (66 mg) harvested. After extraction of bacterial lipids, GC analysis of the methanolysed extracts was performed (25).

OV-17 was found to provide an acceptable separation of the reference substances studied. However, it was not evaluated how well it could separate the esters of C19 acids with methyl branching at other positions. Improved separation of such compounds, although not likely to occur in biological specimens, might be employed by the use of other stationary phases, or capillary columns. Improved results might also be obtainable by employing centrifugation procedures or selective extractions. Such studies would also make clear whether tuberculostearic acid exists either free or linked as an ester in sputum specimens, or whether it is mainly present in intact bacteria or cell fragments.

Because the sensitivity in detecting C32 mycocerosic acid was relatively poor compared with that of tuberculostearic acid, we have so far refrained from further analyses of the former acid. However, its detection may be used as a means to differentiate between nocardiae and anonymous mycobacteria, *viz.*, mycobacteria belonging to Runyon's groups I–IV, on the one hand, and *M. tuberculosis* and *M. bovis* on the other. Such a differentiation is not possible when solely studying tuberculostearic acid. But as infections with *Nocardia* species are extremely rare, at least in our hospital catchment region, this drawback does not seem to constitute a serious diagnostic dilemma. Whether or not the presence of apathogenic mycobacteria in sputum specimens may denote a diagnostic error in the diagnosis of tuberculosis by the method described requires more extensive studies. It also remains to be established whether other organisms of the order *Actinomycetales*, some species of which contain tuberculostearic acid (7–12), might imply such an error. Quantitative determination of tuberculostearic acid may be a way of overcoming these, at least potential, diagnostic errors.

A more extensive study, using SIM for analysis of pleural effusions and gastric lavage samples (apart from sputum samples), is in progress to assess the value of the technique for the diagnosis of mycobacterial infections on a routine basis.

Because microorganisms may produce compounds

unique for a given genus or species (26, 27), SIM would seem to constitute a means for the rapid diagnosis of a number of infectious diseases.

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