TRANSACTIONS, ILL. STATE ACAD. SCI.
Vol. 69, No. 3 (1976)

FATTY ACIDS OF *MYCOBACTERIUM SMEGMATIS* LIPIDS

Hugh J. O'Neill and Leon L. Gershbein
Biochemical Research Laboratories of
Northwest Institute for Medical Research
and Illinois Institute of Technology,
Chicago, Illinois 60634

ABSTRACT - Thin-layer chromatography was applied to the resolution of *Mycobacterium smegmatis* lipids and the fatty acids of the glycerides, the waxes and esterified components and the free fatty acids were analyzed. By gas chromatography-mass spectrometry, 10-methyloctadecanoic acid was identified in the fractions as well as in the total saponifiable portion of the lipids and was especially prominent in the free fatty acids, diglycerides and in the phospholipids. Cyclopropane fatty acids could not be detected in any of the lipids.

The lipids of *Mycobacterium tuberculosis* have been widely studied and several of the approaches extended to other virulent forms and to saprophytic mycobacteria. Thin-layer chromatography (TLC) of components as well as the differentiating characteristics of the fatty acids have been applied as aids to the classification of mycobacteria. Acetate incorporation into the fatty acids has been shown to increase markedly in the presence of other acids in various mycobacteria (Bekhtereva et al., 1971; Kanemasa and Goldman, 1965; McCarthy, 1971; Weir et al., 1972), the latter workers reporting a rapid incorporation of tracer levels of oleic acid into the triglycerides of *M. smegmatis*. The positional distribution of fatty acids in the triglycerides and phospholipids of mycobacteria has been determined (Okuyama et al., 1967; Walker et al., 1970). The fatty acid composition was found to be a function of the age of the mycobacterial cells in two virulent strains and two saprophytes screened, one of the latter being *M. smegmatis* (Chandramouli and Venkitasubramanian, 1973). The unsaturated and branched fatty acids noted with these saprophytes followed the distribution in *M. tuberculosis* (Bennet and Asselineau, 1970), *M. album* (Gerasimova et al., 1970), *M. phlei* (Lennarz et al., 1962; Ciarlini et al., 1964; Campbell and Naworal, 1969). 10-Methyloctadecanoic acid (19Br) has been found in the fatty acids from 90 strains of mycobacteria, of which twenty-five were atypical (Lucchesi et al., 1967).

The present study was undertaken to discern fatty acid profiles of lipid components of *M. smegmatis* separated by TLC, emphasis being directed to the waxes, glycerides, free fatty acids and the phospholipids and to the distribution of 19Br. A search for cyclopropane fatty acids was also instituted. Previously, the fatty acids of *M. smegmatis* tri-

glycerides and phospholipids were analyzed and several unsaturated fatty acids identified (Walker et al., 1970; Hung et al., 1970). The phospholipid content of this organism has been followed by use of TLC and tracer techniques (LaBelle and Walker, 1972). M. smegmatis has also been investigated in relation to mannophospholipids (Takayama et al., 1973), transacylases (Berset and Étémadi, 1973; Josse et al., 1973) and the mycolic acids (Asselineau and Bennet, 1964; Étémadi and Lederer, 1965; Étémadi and Pinte, 1966; Goren, 1972).

MATERIALS AND METHODS

Redistilled AR grade solvents were employed and all glassware was rinsed copiously with ether and chloroform-methanol (2:1 by volume). Concentration of fractions was carried out under vacuum or nitrogen and the samples were stored at 4° under nitrogen. M. smegmatis (ATCC 10143) was cultured in a Dubos-type medium (30 g tripticase soy broth (BBL), 7 g Dubos broth base (Difco), 10 ml glycerol and 1 liter deionized water) and the initial inoculum prepared by inoculating 150 ml of medium with a loopful of bacterial cells from an agar slant. The flasks which were placed in a rotary shaker for 72 hr at 32° comprised the inoculum for the larger flasks. The large batches of cells were prepared in 2 liter Erlenmeyer flasks containing 500 ml of culture medium and each was inoculated with 0.2% by volume of the growth from the initial inoculum flask. The flasks were incubated for 3 days at 32° on the shaker, after which time, the cells were filtered by centrifugation and washed twice with water; the thick paste was dried at 25°. A total of six different runs was made involving 208 flasks and of these, seven were discarded due to contamination which could be detected by appearance and odor. The total weight of dried cells was 700 g. When 420 g of cells was extracted in a Soxhlet assembly with ethyl ether, then with 2:1 v/v chloroform-methanol, the residues weighed 14.48 and 0.48 g, respectively. Extraction of the combined product with warm n-hexane gave rise to 5.09 g (34.1%) of lipids.

Separation of lipids by TLC was effected by the procedure advanced by this laboratory (Singh et al., 1965). By use of 98% acetone + 2% petroleum ether (b range 30-60°), 4 fractions were obtained, the least mobile one (I) containing the phospholipids, (II), the free fatty acids and among other components, (III), the wax esters and the nonfree fatty acids (mainly fatty alcohol esters) and fraction IV, the mono-, di- and triglycerides. Further resolution of fraction IV was accomplished by TLC employing 1-butanol saturated with water and of fraction III, by two-dimensional TLC with 95% ethanol in the first direction and chloroform in the second. The presence of glycerides was confirmed by IR analysis. An aliquot of the bacterial lipids was saponified with 20% sodium hydroxide in 95% ethanol according to the procedures detailed earlier (Gershbein et al., 1965; Gershbein and O'Neill, 1966; O'Neill and Gershbein, 1976); the unsaponifiable portion comprised 62% of the total lipids. The fatty acids were processed and converted to methyl esters by refluxing with methanol saturated with hydrogen chloride. The latter treatment was also accorded the phospholipids, the least mobile fraction in the TLC separation, following hydrolysis with acid in sealed tubes (Dawson, 1960; Singh and Gershbein, 1967) and the glycerides, after transesterification (Luddy et al., 1960).

For GC, a Loenco model 70, Hi-Flex gas chromatograph with dual hydrogen flame detectors was used; the packing was 3 wt% SE-30 on 120-130 mesh Anakrom ABS. Temperature programming was conducted over a range of 150° to 325°, isothermal operation being applied at the conclusion of the run until elution of the desired range of components had occurred. Standard mixtures were used for identification of the acids and where the components did not correspond to the saturated straight chain esters, these were given in terms of the fractional equivalent chain length (ECL). For GC-MS, a Hitachi Perkin Elmer model RMU-6D focusing spectrometer was utilized in conjunction with a Perkin Elmer model 881 gas chromatograph with a dual hydrogen flame ionization detector. The packing was 3 wt% methyl silicone (OV-I) in this case. The latter was employed in relation to the branched acids and a Varian model A-60 spectrometer, in conjunction with the cyclopropane fatty acids, a characteristic peak at 0.65 ppm being screened.

RESULTS AND DISCUSSION

Hexane was employed in the extraction of the crude mixtures as it removed lipids of lower polarity, thereby circumventing several of the conjugated polar types. The hexane-extracted lipids (545 mg) on dilution with 40 ml of ethyl ether deposited insoluble material (71.5 mg) on storage at 0° for 12 hr. The resulting filtrate on TLC fractionation gave rise to the analytical data presented in Table 1; the overall recovery was 86%. The order of increasing occurrence of the glycerides was: tri- > di- > mono-; the phospholipid and fatty acid portions were relatively high, constituting 18.1 and 9.8%, respectively. The esters and waxes accounted for 40.2% of the total lipids.

The distribution of fatty acids in the glycerides, wax esters, phospholipids and in the free and total acid mixtures is shown in Table 2. Cyclopropane fatty acids could not be detected in any of the products. A GC tracing is exemplified by the free fatty acid mixture in Figure 1. The latter displays unlabeled peaks of retention times shorter than C_{14} and represnet lower carbon chain fatty acids. These were not quantitated due to possible losses on volatilization during isolation. The patterns revealed the presence of a unique component exhibiting an ECL of 18.54. By MS, the acid displayed a small peak at m/e 171 (with rearrangement peaks at m/e 172 and 173) and strong peaks at m/e 199 and m/e 167. The latter results from removal of methanol (m/e 32) from the m/e 199 peak. The fragment distribution pattern was identical with the spectrum reported by Ryhage and Stenhagen (1960) for 19Br. The latter occurred to the extent of 7.5, 9.8 and 21.4% in the diglycerides, phospholipids and free fatty acids, respectively and comprised less than 1-2% of the other TLC fractions. It made up 16.3% of the total lipid saponifiable portion where it ranged second only to hexadecanoic acid (31.0%). The 18:0 acid was prominent in most lipid classes, especially in the esterified or wax components. Both the 16:1 and 18:1 acids generally ranged low but were elevated in the phospholipids and waxes. The acid, 24:0, was prominent in the di- and triglycerides and the phospholipids. The high level of the 19Br or of the acids in general in the free fatty acid portion may stem from an elevated phospholipase activity of the organism although data of this type were not obtained in the present study.

Table 1. Partial Analysis of M. smegmatis Lipids by TLC[a]

Fraction	Identification	Recovery mg	Recovery %
I	Phospholipids	73.3	18.1
II	Free fatty acids	39.8	9.8
V	Triglycerides	34.0	8.4
VI	Diglycerides	24.0	5.9
VII	Monoglycerides	9.8	2.4
X	Wax esters	21.2	5.2
XI	Nonfree fatty acids	142.0	35.0

[a] The recoveries are based on the respective weights of fractions isolated from the TLC plates.

A few discrepancies exist in the levels of the C_{16} and C_{18} acids among the phospholipids and triglycerides as compared to the findings of Walker et al. (1970) with the same strain of M. smegmatis but in the stationary phase of growth. However, different culture media were employed and variations may arise in the age of the cells harvested coupled with the differences in the experimental technique. Furthermore, the lower levels of some of the homologs may reside in the rapid uptake or utilization of the acids by the organism. The current data with the cells in the mid-log phase of growth, also show some similarity to the total acids from the organism employed by Chandramouli and Venkitasubramanian (1973), M. smegmatis (NCTC London). The latter workers observed that 19Br was the major acid at the third day of growth and a decrease in 18:1 resulted with a concomitant elevation in 19Br with culture age. The higher 16:1 content differentiated the organism from virulent strains.

The significant occurrence of the 19Br in the diglyceride and phospholipid fractions identifies the precursor relationship of the two classes and supports the findings of Zalkin et al. (1963) that in cyclopropane fatty acid formation, the phospholipid is the preferred substrate. The methyl group of the 19Br arises from methionine and the enzymatic synthesis of 19Br and the cyclopropane fatty acid has been studied by Akamatsu and Law (1968).

ACKNOWLEDGMENTS

The authors are indebted to Dr. W. E. Grundy of Abbott Laboratories, North Chicago, Illinois, for his untiring aid in the culturing of the bacterial cells and to Dr. R. G. Scholz for assistance in the mass spectrometry.

Table 2. Relative Weight Percentages of Fatty Acids of Various Lipid Classes from TLC Fractionation of *M. smegmatis*

(SE-30) ECL[a]	Total acids[b]	Mono-glycerides	Di-glycerides	Tri-glycerides	Phospho-lipids	Free fatty acids	Nonfree fatty acids	Wax esters
13.84	0.36	1.1	2.5	1.3	1.8	0.07	2.5	3.9
14.00	3.9	6.8	5.0	3.9	2.9	3.7	16.7	2.6
.25	-	-	-	-	-	0.48	-	-
.54	0.39	1.0	0.70	-	0.22	0.20	-	0.34
.67	-	1.2	0.61	3.3	-	-	-	0.91
.74	0.24	-	-	-	0.52	0.29	0.30	-
.90	-	1.8	-	-	0.59	-	0.20	-
15.00	2.4	3.5	1.8	1.7	0.52	0.25	0.83	1.5
.08	-	-	-	7.2	-	1.5	-	-
.25	-	-	1.5	-	0.82	-	2.8	-
.33	-	-	-	0.61	-	0.13	-	-
.54	-	1.2	0.70	2.8	-	0.40	-	-
.74	2.2	-	2.8	2.0	5.4	-	7.0	2.7
16.00	31.0	28.3	31.8	32.4	21.4	29.2	22.3	20.5
.33	-	0.29	-	-	-	-	-	-
.54	2.6	0.21	1.2	4.6	1.8	1.5	0.49	0.32
.74	-	-	-	-	0.89	0.20	0.83	4.4
17.00	1.2	4.7	1.7	2.6	1.3	0.75	0.99	7.1
.25	-	0.62	-	-	-	0.22	-	1.1
.56	0.56	0.95	0.47	2.4	-	2.1	-	-
.74	9.5	2.7	1.4	0.52	24.2	-	11.1	7.8
18.00	7.6	8.3	10.7	5.4	7.0	4.9	17.1	10.2
.54	16.3	0.54	7.5	1.5	9.8	21.4	0.81	0.84
19.00	2.6	2.3	2.3	1.3	1.0	1.4	1.1	2.8
.54	0.78	0.95	1.4	1.5	-	14.6	-	0.25
.74	-	-	-	-	1.0	-	0.94	1.1
.85	0.89	-	-	-	0.30	-	3.8	1.1
20.00	0.78	2.5	3.9	2.0	0.48	0.60	0.50	6.9
.54	0.53	4.1	0.25	0.92	0.12	0.88	-	0.30
.74	0.63	0.21	-	-	-	-	-	0.25

Table 2. Concluded

(SE-30) ECL[a]	Total acids[b]	Mono-glycerides	Di-glycerides	Tri-glycerides	Phospho-lipids	Free Fatty acids	Nonfree fatty acids	Wax esters
21.00	-	3.1	0.94	0.92	0.55	2.6	1.0	2.2
.25	1.6	-	-	1.8	-	3.2	-	-
.64	-	1.9	2.6	0.92	0.52	-	0.80	-
22.00	1.4	5.8	4.5	2.4	1.9	1.8	3.1	11.8
.33	-	3.7	-	-	-	0.21	-	-
.54	-	-	0.17	0.22	-	-	-	-
.74	0.12	-	-	-	-	0.03	-	-
23.00	0.01	0.82	0.40	0.22	0.22	0.05	0.80	1.4
.33	0.06	-	-	4.2	-	1.1	-	-
.64	-	2.1	0.14	-	0.15	0.26	0.46	1.0
24.00	12.1	3.5	11.3	7.7	14.6	1.6	2.3	3.3
.26	-	0.31	-	0.39	-	-	-	-
.64	-	3.0	-	0.39	-	1.1	-	-
25.00	0.05	2.0	0.28	0.74	-	0.08	0.66	2.8
.54	-	-	-	1.7	-	-	-	-
26.00	0.07	0.50	1.2	0.74	-	3.2	0.33	0.59

[a] The integral part of the equivalent chain length, ECL, identifies the lower carbon number of the homologous series of fatty acid esters and the fractional portion refers to the relative retention time (position) between an ester and the next higher homolog.

[b] Mixture from saponification of the initial lipids with alcoholic alkali.

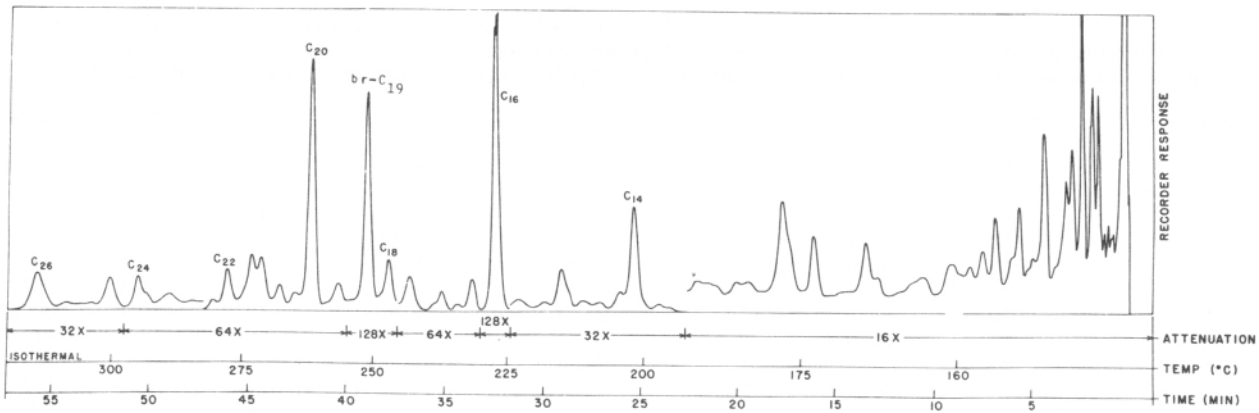

Figure 1. Gas chromatographic separation of methyl esters of M. smegmatis free fatty acids on SE-30.

LITERATURE CITED

Akamatsu, Y. and J. H. Law. 1968. Enzymatic synthesis of 10-methylene stearic acid and tuberculostearic acid. Biochem. Biophys. Res. Commun. 33:172-176.

Asselineau, J. and P. Bennet. 1964. In Metabolism and Physiological Significance of Lipids, R. M. C. Dawson and N. Rhodes, eds. John Wiley and Sons, New York, p. 116.

Bekhtereva, M. N., N. M. Gerasimova and A. T. Donetz. 1971. Lipids of the saprophyte mycobacteria grown on the media with fatty acids. Mikrobiologii 40:813-819.

Bennet, P. and J. Asselineau. 1970. Influence de l'âge sur la teneur en acides gras à chaîne ramifée du bacille tuberculeux. Ann. Inst. Pasteur 118:324-329.

Berset, C. and A. H. Étémadi. 1973. Acyl-CoA-ACP-transacylase of Mycobacterium smegmatis. An analytical study. Biochimie 55:1381-1394.

Campbell, I. M. and J. Naworal. 1969. Composition of the saturated and monounsaturated fatty acids of Mycobacterium phlei. J. Lipid Res. 10:593-598.

Chandramouli, V. and T. A. Venkitasubramanian. 1973. Effect of age on the fatty acids C_{14} to C_{19} of mycobacteria. Am. Rev. Respir. Dis. 108:387-390.

Ciarlini, E., F. Mossini and G. Serventi. 1964. Studio gas-cromatografico degli acidi grassi di Mycobacterium phlei. Nuovi Ann. Ig. Microbiol. 15:349-352.

Dawson, R. M. C. 1960. A hydrolytic procedure for the identification and estimation of individual phospholipids in biological samples. Biochem. J. 75:45-53.

Étémadi, A. H. and E. Lederer. 1965. Biosynthèse de l'acide alpha-smegma-mycolique. Biochim. Biophys. Acta 98:159-167.

Étémadi, A. H. and F. Pinte. 1966. Sur une nouvelle voie metabolique des acides palmitique et stéarique mise en évidence chez M. smegmatis. C. R. Acad. Sci. [D], Paris 262:1151-1154.

Gerasimova, N. M., A. T. Donetz, V. V. Kotelev and M. N. Bekhtereva. 1970. Changes in the fatty acid ratio in the lipid composition of Mycobacterium rubrum 874 and Mycobacterium album 88 during growth. Mikrobiologii 39:1005-1009.

Gershbein, L. L. and B. K. Krotoszynski. 1965. Fractionation and analysis of components of human hair and scalp lipids. J. Gas Chromatog. 3:378-380.

Gershbein, L. L. and H. J. O'Neill. 1966. Alcoholic components of human hair and scalp lipids. J. Invest. Dermatol. 47:16-21.

Goren, M. B. 1972. Mycobacterial lipids: selected topics. Bacteriol. Rev. 36:33-64.

Hung, J. G. C. and R. W. Walker. 1970. Unsaturated fatty acids of mycobacteria. Lipids 5:720-722.

Josse, M., C. Berset and A. H. Etemadi. 1973. Palmityl-CoA-ACP-transacylase et malonyl-CoA-ACP-transacylase de Mycobacterium smegmatis. Biochimie 55:497-505.

Kanemasa, Y. and D. S. Goldman. 1965. Direct incorporation of octanoate into long-chain fatty acids by soluble enzymes of Mycobacterium tuberculosis. Biochim. Biophys. Acta 98:476-485.

LaBelle, Y-S. and R. W. Walker. 1972. The phospholipids of Mycobacterium smegmatis. Am. Rev. Respir. Dis. 105:625-628.

Lennarz, W. J., G. Scheuerbrandt and K. Bloch. 1962. The biosynthesis of oleic and 1-methylstearic acids in Mycobacterium phlei. J. Biol. Chem. 237:664-671.

Lucchesi, M., C. Cattaneo and G. C. de Ritis. 1967. The chromatographic separation of fatty acids (C11-C20) from mycobacteria. Bull. Int. Union Tuberc. 39:65-70.

Luddy, F. E., R. A. Barford and R. W. Riemenschneider. 1960. Direct conversion of lipid components to their fatty acid methyl esters. J. Am. Oil Chem. Soc. 37:447-451.

McCarthy, C. 1971. Utilization of palmitic acid by Mycobacterium avium. J. Am. Soc. Microbiol., Infection and Immunity 4:199-204.

Okuyama, H., T. Kankura and S. Nojima. 1967. Positional distribution of fatty acids in phospholipids from Mycobacteria. J. Biochem. (Tokyo) 61:732-737.

O'Neill, H. J. and L. L. Gershbein. 1976. Analysis of fatty acid and alcoholic components of sebaceous lipid types. J. Chromat. Sci. 14:28-36.

Ryhage, R. and E. Stenhagen. 1960. Mass spectrometric studie. IV. Esters of monomethyl-substituted long chain carboxylic acids. Ark. Kemi 15:291-315.

Singh, E. J. and L. L. Gershbein. 1967. Phospholipids of human hair lipids. J. Chromatog. 31:20-27.

Singh, E. J., L. L. Gershbein and H. J. O'Neill. 1966. Isolation and characterization of glycerides in human hair lipids by thin-layer and gas chromatography. Lipids 1:274-278.

Takayama, K., H. R. Schnoes and E. J. Semmler. 1973. Characterization of the alkali-stable mannophospholipids of Mycobacterium smegmatis.

Walker, R. W., H. Barakat and J. G. C. Hung. 1970. The positional distribution of fatty acids in the phospholipids and triglycerides of Mycobacterium smegmatis and M. bovis BCG. Lipids 5:684-691.

Weir, M. P., W. H. R. Langridge III and R. W. Walker. 1972. Relationships between oleic acid uptake and lipid metabolism in Mycobacterium smegmatis. Am. Rev. Respir. Dis. 106:450-457.

Zalkin, H., J. H. Law and H. Goldfine. 1963. Enzymatic synthesis of cyclopropane fatty acids catalyzed by bacterial extracts. J. Biol. Chem. 238:1242-1248.