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THIN-LAYER CHROMATOGRAPHY OF METHANOLYSATES OF MYCOLIC ACID-CONTAINING BACTERIA

D. E. MINNIKIN, IWONA G. HUTCHINSON and A. B. CALDICOTT

Department of Organic Chemistry, The University, Newcastle upon Tyne NE1 7RU (Great Britain)

and

M. GOODFELLOW

Department of Microbiology, The University, Newcastle upon Tyne NE1 7RU (Great Britain)

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SUMMARY

Whole-organism methanolysates of representative bacteria containing mycolic acids (long-chain 3-hydroxy 2-branched acids) were prepared by treating dry organisms with methanol–toluene–sulphuric acid (30:15:1) at 75° overnight. The mycolic acid esters from mycobacteria were not resolved satisfactorily by single dimensional thin-layer chromatographic analysis. Three distinct patterns of mycobacterial mycolates were produced by two-dimensional thin-layer chromatography, good examples of each pattern being the mycolates from *Mycobacterium tuberculosis*, *Mycobacterium avium* and *Mycobacterium fortuitum*.

INTRODUCTION

Mycolic acids are high-molecular-weight long-chain 3-hydroxy acids with a long alkyl branch in 2-position found in representatives of *Mycobacterium*, *Nocardia*, *Rhodococcus*, *Corynebacterium* and related bacteria[1–5]. Mycobacterial mycolic acids contain components having oxygen functions (=C=O, –COOH, –CH–OCH_3) in addition to the 3-hydroxy acid system but acids from other sources have only homologous mixtures of saturated and unsaturated components. Thin-layer chromatography (TLC) of mycobacterial mycolic acid methyl esters would, therefore, be expected to give multispot patterns and on this basis a system for distinguishing certain mycobacteria from other mycolic acid-containing taxa by TLC of whole organism methanolysates was developed[6]. Analysis of methanolysates of mycobacteria representing additional taxospecies showed that the simple one-dimensional TLC system using light petroleum (b.p. 60–80°)–diethyl ether (85:15)[6] was inadequate and this report gives details of more informative single- and two-dimensional TLC systems.

MATERIALS AND METHODS

Bacteria

Freeze–dried heat-killed cells of *Mycobacterium tuberculosis* strains C, PN and DT, *Mycobacterium avium* D4, *Mycobacterium bovis*, *Mycobacterium intracellulare* and *Mycobacterium johnei* 361F were supplied by Mr. H. B. Lee, Central Veterinary Laboratory, Weybridge, Great Britain. Frozen cell paste of heat-killed *Corynebacterium diphtheriae* PW8 was kindly provided by Dr. A. B. F. Standfast, The Lister Institute of Preventive Medicine, Elstree, Great Britain and lyophilised. *Nocardia asteroides* N317 (ATCC 19247), *Mycobacterium fortuitum* M62 (ATCC 9820), *Mycobacterium phlei* M207 (ATCC 19249) and *Mycobacterium smegmatis* M101 (ATCC 14468) were grown in shake culture at 30° for 2 to 5 days in modified Sauton's medium[7], checked for purity at maximum growth, killed by shaking with formalin (1 %), harvested by centrifugation, washed with distilled water and freeze–dried.

Whole-organism methanolysis

In initial studies dry organisms were subjected to acid methanolysis at 50° (ref. 6) but this method was superseded by the following procedure. Dry organisms (up to 50 mg) were placed in a suitable glass reaction vessel closed with a screw cap incorporating an inert polytetrafluoroethylene seal. A mixture (3 ml) of dry methanol–toluene–sulphuric acid (30:15:1) was added and the closed vessel kept at 75° in an oven or heating block overnight (16–18 h). After cooling to room temperature, 2 ml light petroleum (b.p. 60–80°) was added, the mixture shaken and centrifuged for 10 min at low speed. A small column (*ca.* 1 cm) of ammonium hydrogen carbonate was prepared dry in a short-form cotton wool-plugged Pasteur pipette, prewashed with diethyl ether (2 ml), the upper light petroleum layer pipetted on and the eluent collected in a small vial (1 dram or similar) suitable for subsequent storage. A further portion (1 ml) of light petroleum was added to the remaining reaction mixture and after shaking and centrifugation the upper layer added to the column. The pipette used for the transfer was placed in the column and the combination washed with diethyl ether (1 ml). The combined eluents were evaporated in a stream of nitrogen at $<40°$.

A suitable inexpensive glass reaction vessel for whole organism methanolysis was difficult to obtain. An ideal but expensive container was a 5-ml Reacti-Vial (Pierce, Rockford, Ill., U.S.A.; Cat. No. 13223) (or equivalent) using Tuf-bond seals (Pierce; Cat. No. 12718). A less expensive alternative was to use the same Tuf-bond seals and caps (Pierce; Cat. No. 13218) with standard 7-ml Universal bottles. Stoppered test tubes (100 × 14 or 16 mm) (Sovirel, Levallois-Perret, France; Cat. No. 4.611-51 or 4.611-52) (or equivalent) were also convenient but relatively expensive. The cheapest vessels obtained to date were disposable screw cap culture tubes (100 × 13 mm) (Corning Glass; Corning, N.Y., U.S.A.; Cat. No. 99447) with the normal seals replaced by ones cut by use of a cork borer from polytetrafluoroethylene backed washers (Sovirel; Cat. No. 4.708-39). A choice of vessel would depend on the funds available, the number of samples analysed and compatability with laboratory centrifuges.

Thin-layer chromatography

The chromatographic systems were developed using glass plates (20 × 20 cm) coated with layers (0.5 mm) of silica gel (Merck, Darmstadt, G.F.R., silica gel 60 HF_{254}, Cat. No 7739). More rapid analyses are currently obtained on small sheets (10 × 10 cm) cut from aluminium-backed sheets (20 × 20 cm) (Merck; silica gel 60 F_{254} TLC aluminium sheets, No. 5554). Large plates (20 × 20 cm) were developed in tanks made by cutting off the top of commercial clear glass bricks (24 × 24 × 8 cm) (Gerresheimer Glas, Düsseldorf, G.F.R.); for 10 × 10 cm sheets, the same bricks were cut in half.

For preliminary analyses of the more polar components occurring in methanolysates a two-step development system involving light petroleum (b.p. 60–80°)–acetone (70:30) to 8 cm followed, after drying, by light petroleum–diethyl ether (85:15) to 15 cm. The solvent systems used for single-dimensional development of methanolysates were light petroleum–acetone (95:5) (1 and 3 times), toluene–acetone (99:1) (1 and 3 times) and (97:3) (1 time) and light petroleum–ethyl acetate (94:6) (5 times). The first component in two-dimensional systems was a triple development with light petroleum–acetone (95:5) followed, in the second direction, by either toluene–acetone (97:3) (1 time) or (99:1) (3 times).

The positions of separated components were initially revealed by spraying with chromic acid followed by charring as described previously[6]. This method was superseded by spraying with a 10% ethanolic solution of molybdophosphoric acid followed by heating at 120° for 15 min[8]. Carbohydrate-containing components were detected by use of α-naphthol–sulphuric acid[9].

RESULTS AND DISCUSSION

Analyses of whole-organism methanolysates of mycolic acid-containing bacteria by thin-layer chromatography using light petroleum–diethyl mixtures as developing solvents[2,3,6,10] have given good indications of the general mycolic acid composition of these organisms. Systematic studies, currently in progress, of a wide range of methanolysates of mycobacteria have, however, shown a need for more sophisticated TLC systems which allow the whole range of mycolates and other long-chain components to be separated clearly.

Preliminary studies involved the use of more polar developing solvents in order to detect any long-chain components more polar in character than those revealed by, for example, light petroleum–diethyl ether (85:15) as developing solvent. Acid methanolysis of all mycolic acid-containing organisms at 50° (ref. 6) was found to produce extracts which contained substantial amounts of relatively polar components (Fig. 1). These compounds gave positive reactions with α-naphthol and periodate–Schiff reagents and were therefore judged to contain sugars. After isolation and repeated acid methanolysis of these polar components, mycolic esters were detected by TLC. It is probable, therefore, that these unknown compounds are carbohydrate esters of mycolic acids; mycolates of arabinose have been isolated previously from mycobacteria by mild acid hydrolysis[11]. Systematic studies were, therefore, directed towards improving the conditions of the acid methanolysis reaction in order to give the maximum yield of mycolic esters and other long-chain compounds. A good comprise was found to be an increased temperature of 75° (Fig. 1) and with the

Fig. 1. TLC of whole-organism methanolysates of A: *M. tuberculosis* C and B: *M. avium* D4 conducted at 75 and 50°. Developing system: light petroleum (b.p. 60–80°)–acetone (70:30) (to 8 cm) followed by light petroleum–diethyl ether (85:15) (to 15 cm). Abbreviations: F = fatty acid methyl esters; M = mycolic acid methyl esters; S = sugar derivatives.

proportion of methanol to toluene increased from 1:1 to 2:1. It is necessary to include a co-solvent such as toluene in order to dissolve the mycolic acid esters but if excess toluene is present two layers are sometimes not produced on addition of light petroleum. Removal of any traces of acid, by passing the light petroleum extract through a small column of ammonium hydrogen carbonate is a convenient way of producing a stable methanolysate for analysis. Acid methanolysis was preferred to alkaline hydrolysis or methanolysis in order to avoid the possibility of alkaline isomerisation of mycolates[12,13].

Many different combinations of common organic solvents were tried as developing systems for TLC; benzene and chlorinated solvents were avoided because of their elevated toxicity. The systems which emerged as being useful for routine analyses are shown in Figs. 2–5.

Preliminary analyses of whole-organism methanolysates were conveniently made by use of a single development with light petroleum–acetone (95:5) (Fig. 2a). This system was inferior in some respects to the popular light petroleum–diethyl ether systems used previously[2,3,6,10]. The three mycolic esters (Fig. 2a, A, B and C) from *M. tuberculosis*, for example, are better resolved by use of light petroleum–diethyl ether mixtures[6] and such systems separate the least polar mycolates of *M. smegmatis* (Fig. 2, A and A′) more efficiently[14]. Light petroleum–diethyl ether systems which give good resolution of the less polar mycolates did not, in our preliminary studies, succeed in moving the characteristic more polar components (Fig. 2, I, J) of *M. smegmatis* and *M. fortuitum* away from the origin.

The single development light petroleum–acetone (95:5) system (Fig. 2a) was

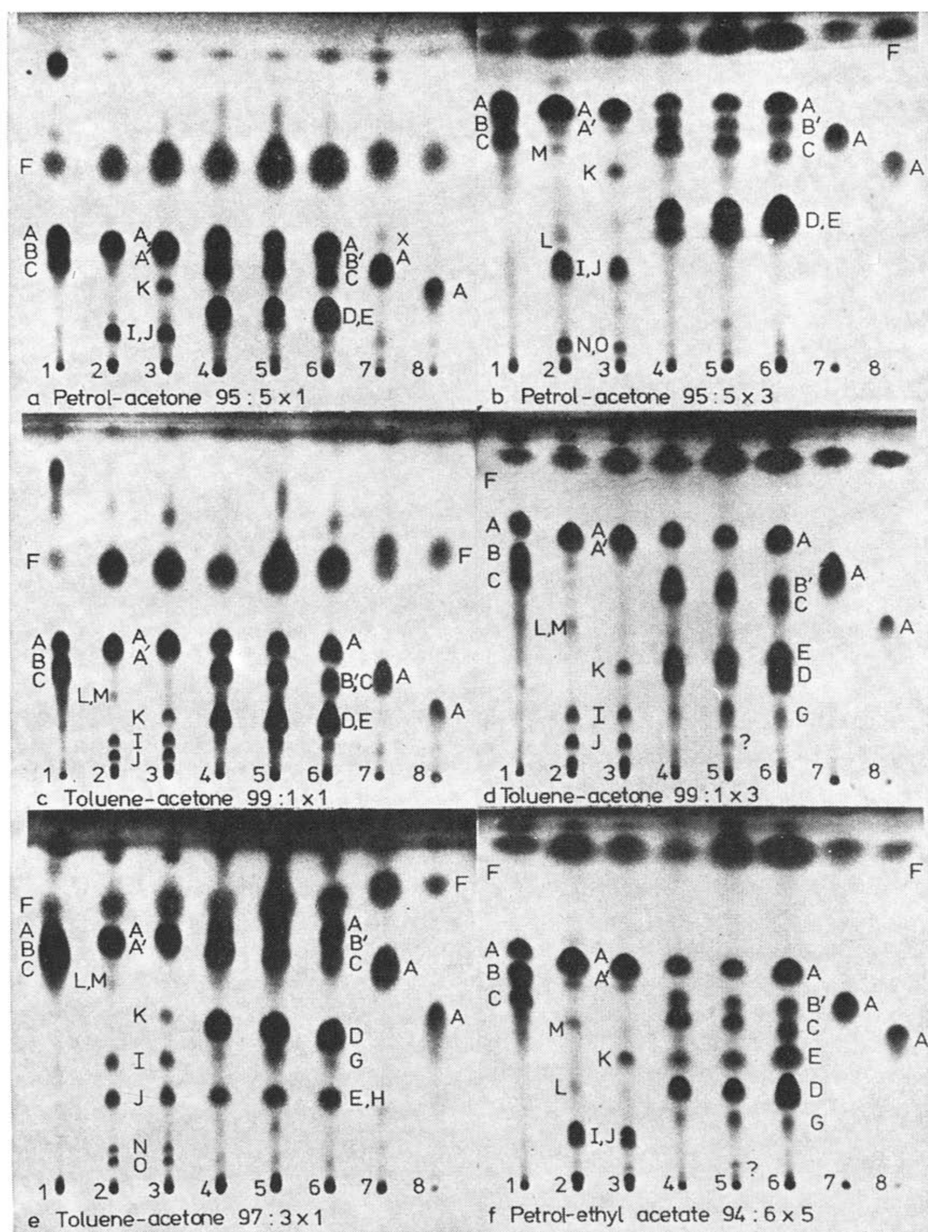

Fig. 2. Solvent systems developed for single dimensional TLC of whole-organism methanolysates of: 1, *M. tuberculosis* C; 2, *M. smegmatis* M101; 3, *M. fortuitum* M62; 4, *M. avium* D4; 5, *M. johnei*; 6, *M. phlei* M207; 7, *N. asteroides* N317; and 8, *C. diphtheriae* PW8. Abbreviations: A, A′ = methyl mycolates devoid of oxygen functions other than the 3-hydroxy ester system; B = methoxymycolate; B′ = unknown component from 4, 5, 6; C = ketomycolate; D = ω-carboxy mycolate methyl ester; E = 2-eicosanol and homologues; F = non-hydroxylated fatty acid methyl esters; G = unknown from 4, 5, 6; H = unknown from 6 (*M. phlei*, see Fig. 4d); I, J = unknowns from 2 and 3, K = unknown from 3; L, M = unknowns from 2; N, O = unknowns from 2 and 3; Petrol = light petroleum.

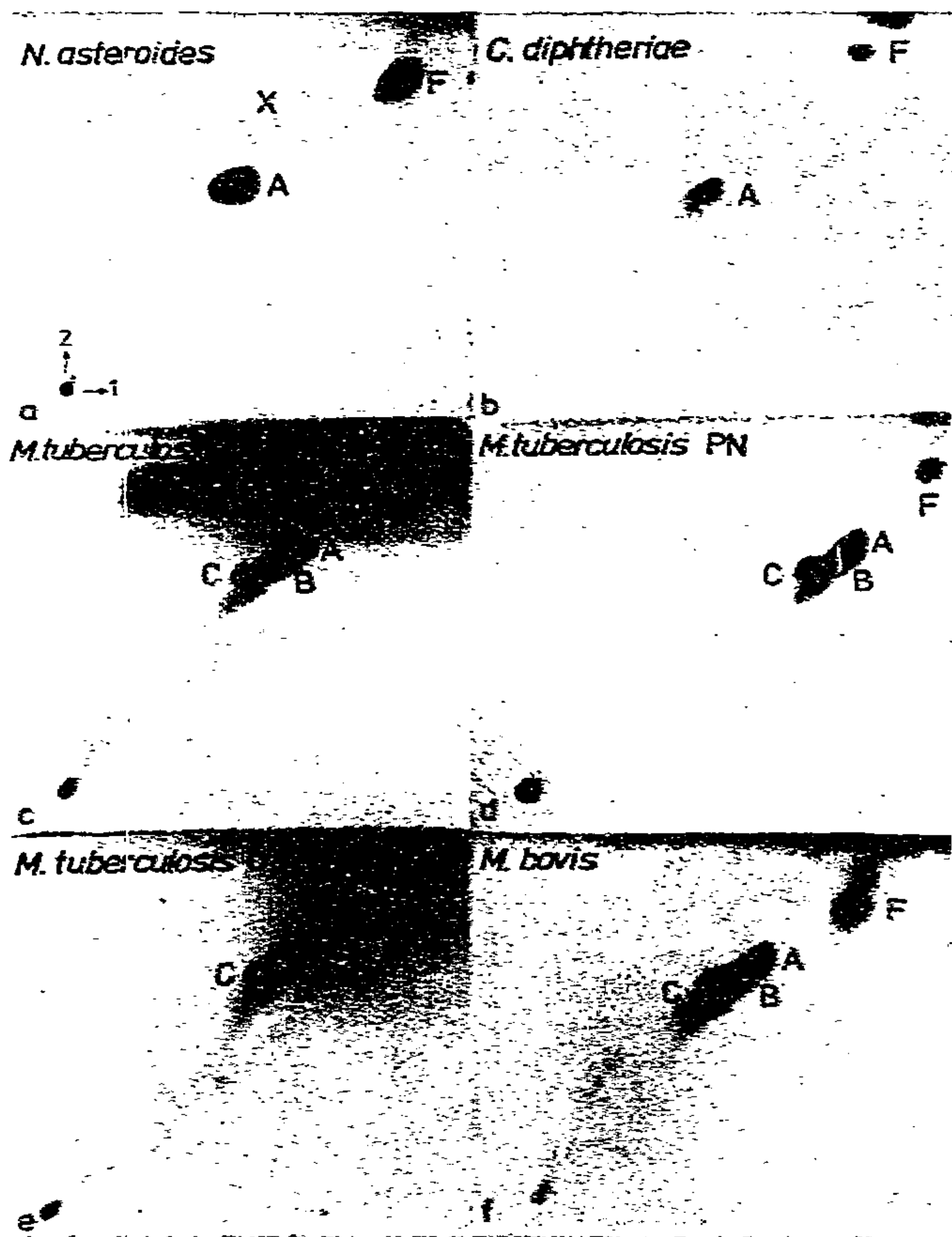

Fig. 3. Two-dimensional TLC of whole-organism methanolysates of (a) *N. asteroides* N317; (b) *C. diphtheriae* PW8; (c) *M. tuberculosis* C; (d) *M. tuberculosis* PN; (e) *M. tuberculosis* DT; and (f) *M. bovis*. 1st direction: light petroleum (b.p. 60–80°)–acetone (95:5) (3 times) 2nd direction: toluene–acetone (97:3) (1 time). See Fig. 2 for abbreviations.

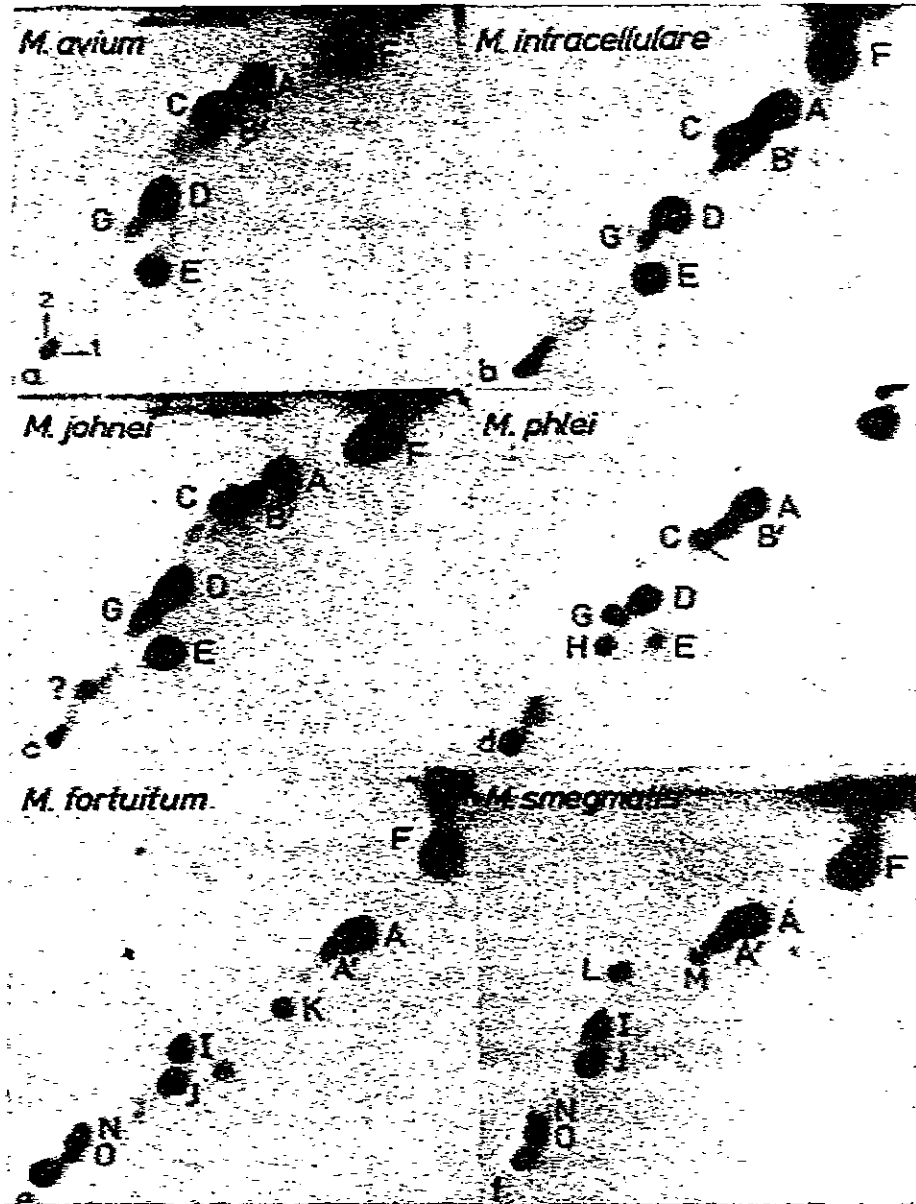

Fig. 4. Two-dimensional TLC of whole-organism methanolysates of (a) *M. avium* D4; (b) *M. intracellulare*; (c) *M. johnei*; (d) *M. phlei* M207; (e) *M. fortuitum* M62; and (f) *M. smegmatis* M101. Same developing system as Fig. 3. See Fig. 2 for abbreviations.

devised with the possibility of extending its value by means of multiple development[15] to resolve the mycolic esters and related components. Three successive developments are a convenient number to perform and the results are shown in Fig. 2b. Advantages of this system are the clear revelation of unknown components (B') in extracts of

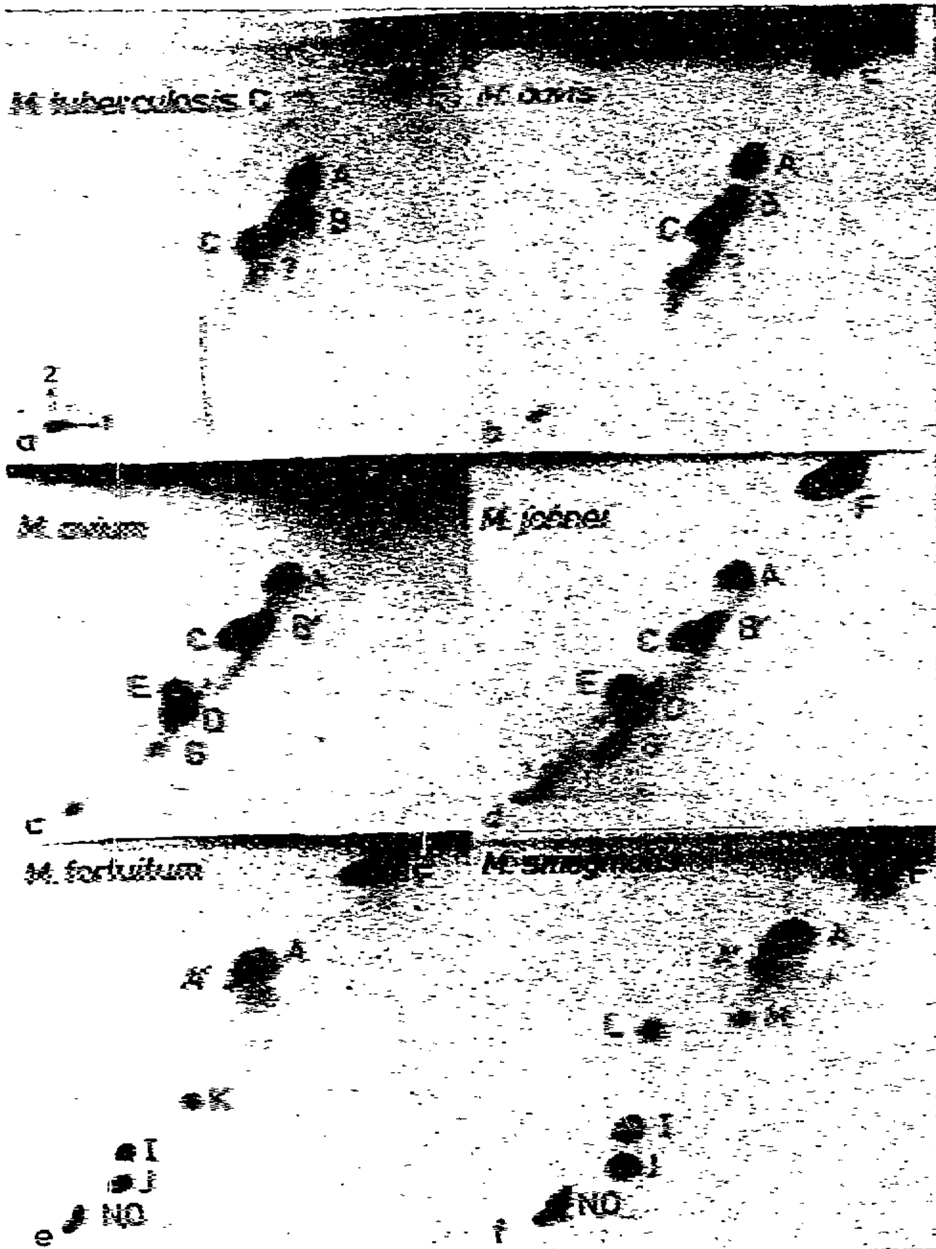

Fig. 5. Two-dimensional TLC of whole-organism methanolysates of (a) *M. tuberculosis* C; (b) *M. bovis*; (c) *M. avium* D4; (d) *M. johnei*; (e) *M. fortuitum* M62; and (f) *M. smegmatis* M101. 1st direction: light petroleum (b.p. 60–80°)–acetone (95:5) (3 times); 2nd direction: toluene–acetone (99:1) (3 times). See Fig. 2 for abbreviations.

M. avium, *M. johnei* and *M. phlei*; and very polar long-chain compounds (N and O) in *M. smegmatis* and *M. fortuitum*.

Single-dimensional systems, however, proved inadequate for a true understanding of the complexity of the mixtures of long-chain compounds in mycobacterial methanolysates and two-dimensional systems were investigated. The triple development with light petroleum–acetone (95:5) (Fig. 2b) was adequate for the first direction and attention was focussed on toluene–acetone systems for the second direction because such systems were found to be the only ones capable of clearly resolving components I, J, N and O (Fig. 2c–e) found in extracts of *M. smegmatis* and *M. fortuitum*. Initially toluene–acetone (99:1) (Fig. 2c) was explored and this is a useful alternative to light petroleum–acetone (95:5) (Fig. 2a) for analysis of all the components in the methanolysates since it resolves the polar components (I and J) from *M. smegmatis* and *M. fortuitum*. Better resolution of the mycolic ester and related compounds was obtained by triple developments with this system (Fig. 2d) and the two-dimensional combination with triple developments with light petroleum–acetone (95:5) gave useful patterns (Fig. 5). Toluene–acetone systems have the disadvantage, however, that they develop very slowly, taking two to three times longer than the light petroleum–acetone mixture. This is a serious problem using 20 × 20 cm plates which is, however, alleviated by use of 10 × 10 cm sheets. Other drawbacks are the poor resolution of 2-eicosanol (E) from the ω-carboxymycolic acid methyl esters (D) (Fig. 2c, d; Fig. 5) and the lack of mobility of components N and O (Fig. 5e, f). A satisfactory compromise was achieved by using a single development of toluene–acetone (97:3) (Fig. 2e) in the second direction of the two-dimensional system (Figs. 3, 4). This two-dimensional combination gave excellent identification of 2-eicosanol (Fig. 4a–d) and allowed components N and O (Fig. 4e, f) to be observed. Toluene–acetone (97:3) gave poor separations of the less polar mycolates (A–C) (Figs. 2e, 3, 4) but, since light petroleum–acetone (95:3) (3 times) resolved these, the two-dimensional system (Figs. 3 and 4) were satisfactory.

One further TLC system, light petroleum–ethyl acetate (94:6) (5 times), is recommended for use in difficult cases, since it gives very clear patterns (Fig. 2f) but on 20 × 20 cm plates it is very time-consuming. The system only works well with the proportions 94:6, and, given this fixed polarity, at least five developments are necessary.

The systems described above allow the general patterns of the mycolic esters of representative mycobacteria and related bacteria to be compared systematically. Their resolving power is considerably greater than the simple single-dimensional system using light petroleum-diethyl ether mixtures introduced by Lanéelle[10] and used in systematic chemotaxonomic investigations[6,16,17]. A good example is the apparent simplicity of the pattern obtained on analysis of methanolysates of *M. avium* using light petroleum–diethyl ether mixtures in which only three spots are seen[6,16,17]. The present systems in fact allow the recognition of twice the number of chromatographically distinct components (Figs. 2a–f, 4a, 5c). The separated components seen in Figs. 2–5 are still probably homologues mixtures unresolvable by adsorption chromatography; the application of argentation chromatography would no doubt resolve further those components differing in the number and stereochemistry of their double bonds[14,18–20]. Further studies will be necessary to compare the present systems with an alternative TLC system involving analysis of underivatised mycolic acids[21,22].

TABLE I

SOME MYCOBACTERIAL MYCOLIC ESTERS AND RELATED LONG-CHAIN COMPOUNDS

Compounds	*Formulae*
I	$CH_3\cdot(CH_2)_l\cdot\underset{cis}{\overset{CH_2}{CH{-}CH}}\cdot(CH_2)_m\cdot\underset{cis}{\overset{CH_2}{CH{-}CH}}\cdot(CH_2)_n\cdot\overset{OH}{CH}\cdot\overset{COOCH_3}{CH}\cdot(CH_2)_o\cdot CH_3$
	$l = 19, m = 14, n = 11, 13, o = 23$
II	$CH_3\cdot(CH_2)_l\cdot\overset{CH_3}{CH}\cdot\overset{OCH_3}{CH}\cdot(CH_2)_m \left[\begin{matrix} cis \\ -\overset{CH_2}{CH{-}CH}\cdot CH_2- \\ or \\ -\overset{CH_3}{CH}\cdot\overset{CH_2}{CH{-}CH}- \\ trans \end{matrix}\right] (CH_2)_n\cdot\overset{OH}{CH}\cdot\overset{COOCH_3}{CH}\cdot(CH_2)_o\cdot CH_3$
	$l = 17, m = 16, n = 16, o = 23$
III	$CH_3\cdot(CH_2)_l\cdot\overset{CH_3}{CH}\cdot\overset{O}{\overset{\Vert}{C}}\cdot(CH_2)_m \left[\begin{matrix} cis \\ -\overset{CH_2}{CH{-}CH}\cdot CH_2- \\ or \\ -\overset{CH_3}{CH}\cdot\overset{CH_2}{CH{-}CH}- \\ trans \end{matrix}\right] (CH_2)_n\cdot\overset{OH}{CH}\cdot\overset{COOCH_3}{CH}\cdot(CH_2)_o\cdot CH_3$
	$l = 17, m = 16, n = 18, o = 23$
IV	$CH_3\cdot OOC\cdot(C_n\cdot H_{2n-2})\cdot\overset{OH}{CH}\cdot\overset{COOCH_3}{CH}\cdot(CH_2)_o\cdot CH_3$
	$n = 32\text{–}39, o = 21$
V	$CH_3\cdot(CH_2)_{17}\cdot\overset{CH_3}{CH}\cdot OH$
VI	$CH_3\cdot(CH_2)_{17}\cdot\overset{CH_3}{CH}\cdot O\cdot CO\cdot(C_nH_{2n-2})\cdot\overset{OH}{CH}\cdot\overset{COOCH_3}{CH}\cdot(CH_2)_o\cdot CH_3$
	$n = 32\text{–}39, o = 21$
VII	$CH_3\cdot(CH_2)_l\cdot\underset{cis}{CH{=}CH}\cdot(CH_2)_m\cdot\underset{cis}{CH{=}CH}\cdot(CH_2)_n\cdot\overset{OH}{CH}\cdot\overset{COOCH_3}{CH}\cdot(CH_2)_o\cdot CH_3$
VIII	$CH_3\cdot(CH_2)_l\cdot\underset{cis}{CH{=}CH}\cdot(CH_2)_{m-1}\cdot\underset{trans}{CH{=}CH}\cdot\overset{CH_3}{CH}\cdot(CH_2)_n\cdot\overset{OH}{CH}\cdot\overset{COOCH_3}{CH}\cdot(CH_2)_o\cdot CH_3$
IX	$CH_3\cdot(CH_2)_l\cdot\underset{cis}{\overset{CH_2}{CH{-}CH}}\cdot(CH_2)_{m-1}\cdot\underset{trans}{CH{=}CH}\cdot\overset{CH_3}{CH}\cdot(CH_2)_n\cdot\overset{OH}{CH}\cdot\overset{COOCH_3}{CH}\cdot(CH_2)_o\cdot CH_3$
VII–IX:	$l = 15, 17, 19, m = 14, 16, n = 15, 17, o = 21$

In the present study three general patterns of mycobacterial mycolic esters and related long-chain compounds can be observed (Table I, I–IX). The simplest pattern is that shown by methanolysates of *M. tuberculosis* and *M. bovis* (Figs. 2; 3c–e; 5,a,b) containing components A, B and C corresponding to dicyclopropyl (I), methoxy (II) and keto (III) mycolates, respectively[2,3,23,24]. The closely related patterns (Figs. 2; 4a–d; 5c,d) found for *M. avium*, *M. intracellulare*, *M. johnei (M. paratuberculosis)* and *M. phlei* are, as noted above, more complex than those reported for *M. avium*[2,3], *M. johnei (M. paratuberculosis)*[24] and *M. phlei*[2,3,25,26]. Components A, C and D in these patterns correspond to the expected unsaturated, keto and ω-carboxy (IV) mycolic esters[2,3,25–27] and E co-chromatographs with authentic 2-eicosanol (V) which is expected to co-occur with the ω-carboxymycolate (IV) since both are formed by breakdown of a long-chain wax ester mycolate (VI)[2,3,25,27]. The remaining major (B′) and minor (G) components are unknown in structure.

The related patterns shown by the methanolysates of *M. fortuitum* and *M. smegmatis* (Figs. 2; 4e, f; 5e, f) have not been reported previously. Studies having been limited to the analysis of the least polar mycolates, corresponding to A and A′ of *M. smegmatis (Mycobacterium butyricum)*[2,3,14,28]. Mycolates corresponding to component A have been reported[2,3] to be a diunsaturated mixture (C_{76} to C_{82}) comprising *cis*-diolefinic (VII), *cis,trans*-diolefinic methyl branched (VIII) and *cis*-cyclopropyl *trans*-olefinic methyl branched (IX) components. The slightly more polar mycolates, corresponding to A′ were shown in *M. smegmatis*[2,3] to be lower molecular weight diunsaturated (C_{66} and C_{68}) and monounsaturated (C_{62}) mycolic esters. The characteristic more polar components I, J, N, O found in both *M. fortuitum* and *M. smegmatis* (Figs. 2; 4e, f; 5e, f) are of unknown structure, as are the minor components K (Figs. 2, 4e, 5e); L and M (Figs. 2, 4f, 5f). It has been reported that ketomycolates were present in *M. smegmatis*[2,3] but in the strain examined in this study no components co-chromatographing with ketomycolates (C) of *M. tuberculosis* (Fig. 2; 3c–e; 5a) were detected.

Methanolysates of *N. asteroides* and *C. diphtheriae* had the expected simple TLC patterns (Figs. 2; 3a,b) containing single spots corresponding to saturated and unsaturated mycolates having no oxygen functions in addition to the 3-hydroxy ester system[6,16]. The lower TLC mobility of these mycolates, in comparison with those from mycobacteria, is a reflection of the relatively lower size of the ester from *N. asteroides* (C_{48} to C_{58})[29] and *C. diphtheriae* (C_{26} to C_{34})[30]. Certain nocardiae, particularly *N. asteroides* ATCC 9969 (ref. 31), contain substantial proportions of long-chain alcohols (nocardols) structurally closely related to mycolic acids. The presence of nocardols may lead to misinterpretation of single-dimensional TLC analyses of methanolysates of strains of *Nocardia* and related bacteria since in addition to the single spot recognisable as a mycolic ester another component having similar TLC properties is observed (Fig. 2a, component X). The two-dimensional system shown in Fig. 3 is of assistance in recognising the presence of nocardols since in toluene–acetone they migrate with similar mobility to that of the non-hydroxylated fatty acid methyl esters.

For routine analyses of organisms suspected to contain mycolic acids, methanolysates should be screened using the one-dimensional light petroleum–acetone (95:5) system (Fig. 2a); increased resolution of the mycolic esters may be then obtained by a triple development using the same solvent mixture (Fig. 2a). These simple systems are sufficient if only a single component, corresponding to a mycolic ester, is observed

in addition to the non-hydroxylated fatty acid methyl esters. If a multispot pattern is obtained the two-dimensional system involving a triple development with light petroleum–acetone (95:5) combined with a single run of toluene–acetone (97:3) (Figs. 3 and 4) allows the type of pattern to be established. The second two-dimensional TLC system (Fig. 5), involving a triple development with toluene–acetone (99:1) in the second direction, may then be used to resolve the less polar mycolate types. In difficult cases developing five times in a single dimension with light petroleum–ethyl acetate (94:6) (Fig. 2f) gives useful alternative patterns. Single-dimensional toluene–acetone systems are useful for recognising the patterns of the *M. fortuitum* and *M. smegmatis* mycolates and are also useful in preparative TLC.

The TLC systems developed in the present study allow the true complexity of natural mycolic acid mixtures to be established for the first time and provide a firm foundation for systematic structural studies. Mycolic esters that are homogeneous by adsorption TLC may be isolated and fractionated further by argentation TLC[14,18–20], high-performance liquid chromatography[32,33] and gas chromatography[34,35] so that the structures of individual components may be determined.

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