Fatty acid oxidation and the β -oxidation complex in *Mycobacterium leprae* and two axenically cultivable mycobacteria that are pathogens

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Intact, non-growing *Mycobacterium leprae*, *M. avium* and *M. microti* oxidized a wide range of 1- 14 C-labelled fatty acids (C_8 to C_{24}) to $^{14}CO_2$. Laurate (C_{12}) was oxidized most rapidly, and its oxidation by *M. leprae* was inhibited by the antileprosy agents Dapsone, clofazimine and rifampicin. Key enzymes of β -oxidation were detected in extracts from all three mycobacteria. All these activities (both in intact mycobacteria and the enzymes) were stimulated in *M. avium* grown in Dubos medium plus palmitate but activities in *M. microti* or *M. avium* grown either in Dubos medium with added liposomes or triolein, or *in vivo* were similar to those detected in the same strain grown in Dubos medium alone. *M. avium* could be grown in medium in which 95% of its fatty acyl elongase activity is acetyl-CoA dependent. In this medium growing *M. avium* organisms oxidized [1- 14 C]palmitate to $^{14}CO_2$ but simultaneously elongated palmitate to C_{24} acids and even longer. Acetyl-CoA-dependent elongase activity is similar but clearly not identical to reversed β -oxidation, but the exact point(s) of difference have not yet been identified.

Introduction

The envelope of *Mycobacterium leprae*, the leprosy bacillus is highly lipid-rich and the bacteria consist of up to 40% lipid. Amongst the lipids are structural lipids such as the mycolic acids, 80-carbon fatty acyl keto-esters, which are covalently bound to the cell wall. There are also unbound lipids, including the well-documented 'phenolic glycolipid-I', which appears in a capsule-like layer and may be involved in protection of the leprosy bacillus and even in pathogenesis. Although lipids are clearly important quantitatively and individually, very little is known about lipid metabolism in *M. leprae* (for a recent review of metabolism, see Barclay & Wheeler, 1989). Recent work has shown that palmitate is incorporated by intact *M. leprae* (Wheeler & Ratledge, 1988) and that the carbon from palmitate appears in 'phenolic glycolipid-I' – a lipid found only in *M. leprae* – on thin-layer chromatography (Franzblau *et al.*, 1987). Furthermore, palmitate is rapidly catabolized to CO_2 (Franzblau, 1988; Wheeler & Ratledge, 1988) and

stimulates oxygen uptake by suspensions of purified *M. leprae* (Ishaque, 1989).

The purpose of this study was to compare the utilization of a range of exogenous fatty acids through the β -oxidation pathway for fatty acid oxidation. In showing which fatty acids are most readily oxidized by *M. leprae*, this study may point to activities which may prove most useful to measure in devising radiometric tests for viability and drug susceptibility of *M. leprae*. Already, it is known that palmitate oxidation is inhibited by a wide range of antileprosy agents (Franzblau, 1988). The measurement of $^{14}CO_2$ release from ^{14}C -labelled fatty acids by *M. leprae* (Franzblau, 1988; Wheeler & Ratledge, 1988) reflects activity in non-dividing bacteria and is thus distinct from the BACTEC method in which ^{14}C -labelled fatty acid, included in the growth medium, allows rapid detection, by monitoring $^{14}CO_2$ release, of the actual multiplication of slow-growing organisms such as *M. tuberculosis* (see, for example, Camargo & Wagner, 1987; Heifets *et al.*, 1985).

Interest is added to the present study because the major activity for anabolism of fatty acids, which in *M. leprae*, *M. avium* (Wheeler *et al.*, 1990) and *M. lepraemurium* (Kusaka, 1977) is by fatty acid elongation, involves direct incorporation of acetyl-CoA into growing fatty acyl chains. In these mycobacteria, malonyl-CoA-dependent elongase activity, which is energetically a

Abbreviations: 8:0, octanoate; 10:0, decanoate; 12:0, dodecanoate (laurate); 14:0, tetradecanoate (myristate); 16:0, hexadecanoate (palmitate); 18:0, octadecanoate (stearate); 18:1 or 18:1 (c9), *cis*-octadec-9-enoate (oleate); 20:4, arachidonate; 24:0, lignocerate; DDS, 4,4-diaminodiphenyl sulphone (Dapsone); B663, clofazimine; DCPIP, dichlorophenol-indophenol, PMS, phenazine methosulphate.

Fig. 1. Scheme showing how the reactions studied in this work are involved in β -oxidation and acetyl-CoA-dependent fatty acyl chain elongation. The overall reactions are described in the boxes; β -oxidation/chain elongation *per se* is delineated by dashed lines. All the individual activities, except the essentially reversible reactions in parentheses, were studied in the work described in this paper. *A trace of acyl-CoA:NAD(P) oxidoreductase was detected; †assayed with *trans*-2-enoyl-CoAs only.

more favourable reaction is barely, if at all, detectable. This means either that β -oxidation enzymes (which release acetyl-CoA from fatty acyl-CoA esters) participate in these synthetic reactions by a reversal of activity (Nishimaki-Mogami *et al.*, 1987; Shimikata *et al.*, 1980) or that analogous but different enzymes must fulfil the means of elongating fatty acids by acetyl-CoA attachment (Fig. 1). Therefore, key enzyme activities for the reactions shown in Fig. 1 were sought for both catabolic and anabolic metabolism of fatty acids.

Finally, intracellular mycobacteria inhabit a lipid-rich environment: the cells in which they reside are usually macrophages (Lowrie, 1983) or, for *M. leprae*, Schwann cells (Mukherjee & Antia, 1985). Therefore, the availability of host cell lipids for fatty acid oxidation in intact mycobacteria and their effects on activities of enzymes in the β -oxidation complex was also investigated in strains of *M. microti* and *M. avium* which are highly pathogenic for mice. These strains were grown in experimental animals as well as axenically with and without supplied lipids. In this way, results obtained with *M. leprae*, which can only be grown *in vivo*, can be interpreted in a more valid manner than by simply investigating fatty acid metabolism in *M. leprae* alone.

Methods

Mycobacteria from experimentally infected animals. *M. leprae*, *M. avium* CR1/69, and *M. microti* OV254 were obtained from heavily infected tissue as described previously (Wheeler, 1987). Briefly, bacteria were separated from tissue by a method (World Health Organization, 1980) involving differential then density-gradient centrifugation. For suspensions used to provide cell-free extracts for enzyme work, further purification was achieved on an aqueous two-phase system. Some suspensions were treated for 30 min with 0.5 M-NaOH at 25 °C (Wheeler *et al.*, 1982) to abolish host-derived activities adsorbed to the surface of the bacteria. Host-derived organisms were enumerated by direct counting. Compared with determination of dry weight, 10^{10} *M. leprae* $\cong$ 1 mg dry wt organisms.

Mycobacteria grown in cell-free media. Bacteria (the inoculum was 1 ml from a flask including the corresponding medium at the stage when harvesting was done; Wheeler, 1987) were grown in 100 ml Dubos medium (Chadwick, 1982) in 250 ml flasks, shaken at 200 r.p.m. at 37 °C, then harvested as described previously (Wheeler, 1987). *M. microti* OV254 was harvested after 15 d and *M. avium* CR1/69 after 20 d. Additions to the Dubos medium (see Table 2) were as follows. For liposomes, phosphatidylcholine, cholesterol and *sn*-1,2-dipalmitoyl-phosphatidic acid in the molar ratio 10:10:1, prepared as described previously (Kondo *et al.*, 1985; Wheeler *et al.*, 1990) and totalling 150 μ g lipid per ml of medium. Other additions were sodium palmitate, trioleoylglycerol (triolein) or methylaurate (Lancaster synthesis, Morecambe, Lancs, UK), all at 100 μ g lipid per ml of medium. These suspensions were prepared by first dispersing the lipid in water at 10 mg ml⁻¹ by sonication for about 10 s using a Dawe Soniprobe at low energy. Batches of *M. avium* were also grown in Sauton's medium (Chadwick, 1982) solely for determination of β -ketothiolase. Mycobacteria were enumerated by determination of their dry weight. Compared with direct counting, 1 mg dry wt *M.*

microti $\cong 10^{10}$ organisms and 1 mg dry wt *M. avium* $\cong 2 \times 10^{10}$ organisms.

Preparation of cell-free extracts of mycobacteria. Mycobacteria were ultrasonically disrupted and clarified as described previously (Wheeler *et al.*, 1990) to give extracts in 0.2 M-potassium phosphate, pH 7, with 5 mM-dithiothreitol, or 5 mM-MES/KOH, pH 7, with 1 mM-MgCl₂. Some extracts were dialysed using ultrafiltration cones with a cut-off of M_r 25000 as described previously (Wheeler, 1984). There was no marked difference in the specific activities of the enzymes studied in this paper between the extracts prepared in the different ways described above.

Incubations of mycobacteria with radioisotopically labelled substrates. Mycobacteria, as washed suspensions, were incubated in a buffer including 0.05% Tween 80 which did not support growth, and 1-¹⁴C-labelled fatty acid, as described for palmitic acid by Wheeler & Ratledge (1988) except that 5 nmol fatty acid was included in each 200 μ l incubation mixture. In order to achieve this, the following amounts of 1-¹⁴C-labelled fatty acid were added to each tube as solutions in toluene or toluene/ethanol (as supplied) and dried aseptically: octanoate, myristate, palmitate (all 2 TBq mol⁻¹), 10000 Bq; decanoate (1.92 Bq) 9620 Bq; laurate (2.15 TBq mol⁻¹), 10730 Bq; stearate, oleate (both at 2.07 TBq mol⁻¹), 10360 Bq; lignocerate (1.0 TBq mol⁻¹), 8510 TBq mol⁻¹; arachidonate (2.15 TBq mol⁻¹), 5500 Bq plus 2.4 nmol unlabelled arachidonic acid (Sigma). All radiochemicals were from Amersham except lignocerate which was from CEA, France. At the end of the incubation period at 34 °C – 1 h for *M. microti* and *M. avium*, 20 h for *M. leprae* – bacteria were collected on GFC filters (Whatman) and washed four times with buffered Tween 80. The ¹⁴CO₂ evolved during incubation was trapped in 400 μ l 1 M-NaOH. Assimilation was determined by measuring the radioactivity on the washed filters. Evolution of ¹⁴CO₂ was determined by measuring the radioactivity, as Na₂¹⁴CO₃, trapped in the NaOH. Incubations which were contaminated (checked by plating 5 μ l samples on to nutrient agar) were discarded. Controls contained heat-killed (100 °C, 15 min) bacteria, and values for radioactivity assimilated and for radioactivity evolved as ¹⁴CO₂ obtained in controls were subtracted from values for radioactivity in corresponding incubations with live bacteria before the results presented in the text and Tables 1 to 3 were calculated. Full details of all the components of incubation mixtures are presented in previous papers (Wheeler, 1984; Wheeler & Ratledge, 1988) and non-specific adsorption of fatty acids to intact mycobacteria as suggested by high values of radioactivity in controls was discussed previously (Wheeler & Ratledge, 1988). Some incubations included the antileprosy agents DDS (Dapsone) (Sigma), clofazimine (B663) (a gift from Dr S. O'Sullivan, Medical Research Council of Ireland, Dublin) or rifampicin (Sigma).

Additionally, an experiment was done (in duplicate) involving incubating growing *M. avium* with [1-¹⁴C]palmitic acid. A 14-d-old culture, growing arithmetically (Wheeler, 1987) in Dubos medium with 100 μ g sodium palmitate ml⁻¹, was used. A 25 ml sample of the culture was poured aseptically into a sterile 100 ml flask containing 74 MBq solid [1-¹⁴C]palmitic acid. The flask was swirled for 1 min to disperse the [1-¹⁴C]palmitic acid, placed in a sealed container which also included a vial with 1 ml 0.5 M-NaOH, and shaken at 37 °C for 4 h; the trapped ¹⁴CO₂ was collected and determined as described above. Bacteria were collected on GFC filters (i) to determine assimilation and (ii) to extract unbound lipids. For the latter the bacteria, collected on the filters, were washed four times with buffered Tween 80, then each filter (with the bacteria) was refluxed for 1 h in 4 ml chloroform/methanol (2:1, v/v). Each filter was then removed, the solvent washed once with 2 ml distilled water, dried, and the fatty acyl moieties in the extracted lipids methylated under acid conditions (Minnikin *et al.*, 1980). Fatty acid methyl esters were separated from other lipids by TLC (Minnikin *et al.*, 1980) and analysed for their degree of saturation, and by gas

chromatography followed by collection of fatty acid esters and determination of their radioactivity. For the former, HPTLC plates (Merck; HPTLC silica gel 60 with concentrating zone) were dipped into a saturated solution of AgNO_3 in acetone, dried with a flow of cool air, and used immediately; a sample of fatty acid methyl esters was applied, with standards (16:0, 18:1, 18:2 and 18:3 methyl esters), dried, and plates were developed once in dichloromethane. The position of lipids was detected by spraying with 2,7-dichlorofluorescein. Relative positions of lipids were the same as when separated by argentation (AgNO_3)-TLC using standard TLC plates, with 16:0 methyl ester running nearest to the solvent front and 18:2 and 18:3 esters remaining near the origin and not always separating well. Radioactivity in the resolved lipids was detected by scraping and counting (Wheeler & Ratledge, 1988). Separation of labelled fatty acid esters by gas chromatography was done using a Pye Unicam chromatograph, injecting samples (up to 5 μl in chloroform) of fatty acid methyl esters into a column (2 m $\times$ 4 mm) of 10% diethylene glycol succinate (PhaseSep, Queensferry, Clwyd, UK). The column was maintained at 195 °C with N_2 as the carrier gas. Lipids were detected by flame ionization and collected in capillaries kept at -20 °C, the sample being split so that 1 part went through the detector to 19 parts into the capillary. Material was eluted with chloroform from each capillary into a scintillation vial, dried, and radioactivity determined by scintillation counting in Bray's fluid (4 g 2,5-diphenyloxazole, 60 g naphthalene, 100 ml methanol and 20 ml ethanediol, made up to 1 litre with dioxan). Duplicate samples of mycobacterial lipids were run, one with standards (methyl esters of saturated fatty acids of 16, 18, 19, 20, 21, 22 and 24 carbon atoms), and one without standards.

Enzyme assays. All were done at 25 °C; the reaction volume was 500 μl . Optimal assay conditions were established in previous work done with *M. smegmatis* (Kikuchi & Kusaka, 1984; Shimikata *et al.*, 1980).

Acyl-CoA dehydrogenase [EC 1.3.99.3; acyl-CoA:(acceptor) oxidoreductase – see Fig. 2] was assayed by incubating extracts with 50 mM-potassium phosphate (pH 7.6), 0.3 mM- Na_2EDTA , 30 μM -2,6-dichlorophenol-indophenol (DCPIP), 1.4 mM-phenazine methosulphate (PMS) and either 30 μM -decanoyl-CoA or palmitoyl-CoA. ΔA_{600} was followed against a control containing no acyl-CoA. In order to determine whether NAD^+ - or NADP^+ oxidoreductase (EC 1.3.1.8 – see Fig. 1) was present two procedures, with variants, were used: (i) EDTA and PMS were replaced either by 1 mM- NADP^+ and ΔA_{340} was followed, or by NAD^+ together with diaphorase (0.1 unit, porcine; Sigma) and 600 μM -*p*-iodonitrotetrazolium and ΔA_{500} followed; alternatively, (ii) 1 mM- NAD^+ and diaphorase (0.1 unit) or 1 mM- NADP^+ and ferridoxin: NADP^+ reductase (from spinach, with NADPH diaphorase activity; Sigma) replaced PMS, and controls were done without NAD^+ or diaphorase, and ΔA_{600} (for DCPIP) was followed.

Enoyl-CoA reductase [EC 1.3.1.8; acyl-CoA: NAD(P)^+ oxidoreductase – see Fig. 2] activity was assayed by incubating extracts with 0.1 M-potassium phosphate (pH 5.5), 100 μM -NADH, and either 100 μM -crotonoyl-CoA or 100 μM -*trans*-dec-2-enoyl-CoA. ΔA_{340} was followed against a control containing no enoyl-CoA. *trans*-Dec-2-enoic acid, purchased from Lancaster Synthesis, and CoA (lithium salt), from Sigma, were used for preparation of *trans*-dec-2-enoyl-CoA by the mixed anhydride method (Schulz, 1974).

Enoyl-CoA hydratase (EC 4.2.1.17; L-3-hydroxyacyl-CoA hydrolyase) was assayed in crude extracts of the mycobacteria by a coupled assay system. Extracts were incubated with 50 mM-potassium phosphate (pH 7.0), 200 μM - NAD^+ , 600 μM -*p*-iodonitrotetrazolium violet, β -hydroxyacyl CoA dehydrogenase (1 unit, porcine heart; Sigma), diaphorase (0.1 unit, porcine; Sigma) and either 100 μM -crotonoyl-CoA or 100 μM -*trans*-dec-2-enoyl-CoA; ΔA_{500} was followed against a control containing no enoyl-CoA.

β -Hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35; L-3-hydroxy-

acyl-CoA: NAD^+ oxidoreductase) was assayed by incubating extracts with 100 mM-potassium phosphate (pH 7.0), 0.2 mg bovine serum albumin (Sigma) ml^{-1} , 0.1 mM-NADH and 30 μM -acetoacetyl-CoA; ΔA_{340} was followed against a control containing no acetoacetyl-CoA.

Acetyl-CoA acyltransferase (β -ketothiolase) (EC 2.3.1.16; acyl-CoA:acetyl-CoA C-acyltransferase) was assayed in extracts made by sonicating bacteria as described previously (Wheeler *et al.*, 1990) but in 0.5 M-Tris/HCl, pH 8 containing 25 mM- MgCl_2 , 5% (w/v) glycerol and 2 mM-2-mercaptoethanol. Extract (400 μl), or extract diluted with the above sonication mixture to 400 μl was mixed with substrates (final concentrations in the 500 μl mixtures shown) to give reaction volumes of 500 μl , as follows. For the biosynthetic direction, 30 μM -acetyl-CoA and either decanoyl-CoA or palmitoyl-CoA at either 25 μM or 75 μM were included, together with, as a coupled enzyme, β -hydroxyacyl-CoA dehydrogenase (1 unit, porcine heart; Sigma) and 100 μM -NADH. ΔA_{340} was followed against a control containing acetyl-CoA but no decanoyl-CoA or palmitoyl-CoA. Subsequently, it was found that omitting β -hydroxyacyl-CoA dehydrogenase did not affect the reaction rate, presumably because the activity of mycobacterial β -hydroxyacyl-CoA dehydrogenase was sufficient for the coupled reaction to take place. For the catabolic direction, 30 μM -acetoacetyl-CoA was included and ΔA_{303} was followed against a control containing no acetoacetyl-CoA (Binstock & Schulz, 1981). For acetoacetyl-CoA, $\epsilon_{\text{cm}}^{\text{nm}} = 16.9$.

Results

Oxidation of fatty acids by intact, non-growing mycobacteria

Suspensions of intact *M. leprae*, *M. microti* and *M. avium* all oxidized a similar range of fatty acids through to CO_2 . The complete range of fatty acids tested is shown for *M. leprae* (Table 1). For both *M. microti* and *M. avium*, the pattern of fatty acid oxidation was similar, i.e. lauric acid was oxidized most rapidly (rates shown in Table 2), followed by octanoate, myristate and decanoate at similar rates, while the long-chain lignocerate was oxidized relatively slowly. The rate of oxidation was not related to the rate of assimilation (the appearance of label in intact, living organisms, with background label in killed organisms subtracted) in *M. leprae* (Table 1), or in *M. microti* or *M. avium*, where ^{14}C was assimilated most rapidly in incubations with myristate or palmitate (usually over 50% of the label supplied per 10^{10} organisms in 1 h) followed by arachidonate.

The results in Tables 1 and 2 were obtained in incubations at pH 7.0. When additional, duplicate incubations with both laurate and palmitate at changed pH were done with an isolate of *M. leprae*, activity was similar to that shown in Table 1 at pH 5.5 (buffer 50 mM-MES/KOH) but about half the values shown in Table 1 when 50 mM-HEPES/KOH at pH 8.0 was used.

Growth of mycobacteria in media containing lipids did not have any clearly discernible effect on the rate at which they subsequently oxidized fatty acids, except that the oxidative activity was possibly higher in *M. avium*

Table 1. Incorporation and oxidation of $1\text{-}^{14}\text{C}$ -labelled fatty acids by suspensions of *M. leprae*

Intact *M. leprae* organisms (2 to 10×10^9) were incubated with 5 nmol $1\text{-}^{14}\text{C}$ -labelled fatty acid in $200\ \mu\text{l}$. Three isolates of *M. leprae* organisms, as suspensions, were prepared: two isolates from one armadillo and a third isolate from another armadillo. Each determination of assimilation and $^{14}\text{CO}_2$ evolved was done in duplicate for each isolate. From one infected armadillo, suspensions of NaOH-treated *M. leprae* and, on a separate occasion, untreated *M. leprae* organisms were obtained. There were no marked differences between their activities so results from the NaOH-treated as well as untreated *M. leprae* were used to calculate the mean values below. The values are the percentage of radioactivity supplied that was assimilated or evolved as $^{14}\text{CO}_2$ per 10^{10} organisms in 20 h, $\pm$ SEM.

Fatty acid	Percentage of radioactivity supplied that was:	
	Assimilated	Evolved as $^{14}\text{CO}_2$
Octanoate	9.3 ± 4.5	6.2 ± 1.5
Decanoate	10.4 ± 2.1	4.5 ± 1.2
Laurate	7.4 ± 2.3	11.9 ± 1.2
Myristate	10.4 ± 2.1	4.0 ± 0.5
Palmitate	8.3 ± 1.1	3.4 ± 1.0
Stearate	9.9 ± 1.6	0.5 ± 0.1
Lignocerate	$7.8 \pm 9.3^*$	0.10 ± 0.03
Oleate	6.2 ± 1.7	0.25 ± 0.11
Arachidonate	10.0 ± 3.3	0.7 ± 0.3

* For each separate isolate, radioactivity in live bacteria was always higher than in controls: the considerable variation was between isolates. Also, radioactivity associated with live bacteria was only 1.2 to 2 times the radioactivity associated with heat-killed controls.

grown in the presence of a fatty acid or fatty acid methyl ester (Table 2). More striking, however, was the great variation between isolates from tissues or between individual batches of bacteria grown axenically in the same medium (Table 2), even though bacteria were always harvested during a phase of slow, arithmetic growth before the stationary phase (see Wheeler, 1987, for growth curves).

Despite the variation between isolates, oxidation of fatty acid by replicate incubations of mycobacteria from the same isolate was consistent (see legend to Table 2). This finding was exploited in previous work to test the effects of inhibitors and antileprosy agents on oxidation of $[1\text{-}^{14}\text{C}]$ palmitate to $^{14}\text{CO}_2$ (Franzblau, 1988). In the present work, oxidation of $[1\text{-}^{14}\text{C}]$ laurate by *M. leprae* was shown to be inhibited by DDS and B663 at all concentrations used and rifampicin at $3\ \mu\text{g ml}^{-1}$ and above (Table 3). The solvents used to dissolve the agents (dimethylsulphoxide at up to 0.1% in incubations for B663 and rifampicin; HCl at up to 0.1 mM for DDS) did not effect oxidation of lauric acid.

Table 2. Oxidation of laurate and palmitate by individual isolates of *M. microti* and *M. avium*

Intact mycobacteria (4 to 12×10^9) were incubated at 34°C with all the $1\text{-}^{14}\text{C}$ -labelled fatty acids shown in Table 1; only results with laurate and palmitate are shown here (see text for results of other incubations). Individual isolates or batches of bacteria are identified below by the medium from which the bacteria were harvested. For each isolate, determinations of $^{14}\text{CO}_2$ evolved from each $1\text{-}^{14}\text{C}$ -labelled fatty acid were done in duplicate, and were always within 15% of the mean values shown below. Values are the percentage of radioactivity supplied evolved as $^{14}\text{CO}_2$ per 10^{10} organisms in 1 h.

Isolate/batch	Percentage of radioactivity evolved as $^{14}\text{CO}_2$			
	<i>M. microti</i> plus:		<i>M. avium</i> plus:	
	Laurate	Palmitate	Laurate	Palmitate
Dubos (1)	10.7	1.1	9.3	0.5
Dubos (2)	5.6	0.7	10.7	0.7
Dubos + liposomes* (1)	1.5	0.2	6.5	2.2
Dubos + liposomes* (2)	9.3	1.0	1.9	0.2
Dubos + palmitate*	ND	ND	23.1	1.8
Dubos + methyl laurate*	ND	ND	17.6	2.7
Dubos + triolein*	ND	ND	8.0	0.5
Mice (1)	5.0	0.6	1.5	0.1
Mice (2)	ND	ND	5.0	1.0

ND, Not done, usually because of severe inhibition of axenic growth of *M. microti* by palmitate or triolein.

* Total lipid content 100 to $150\ \mu\text{g ml}^{-1}$: for details of how supplied, and content of liposomes, see Methods.

Table 3. Effect of antileprosy agents on laurate oxidation by *M. leprae*

Intact *M. leprae* was incubated with $[1\text{-}^{14}\text{C}]$ lauric acid as described in Table 1. The mean rate of $^{14}\text{CO}_2$ evolution in six control incubations including live *M. leprae* was $934\ \text{Bq per } 10^{10}\ M. leprae$ in 20 h. This rate is assigned a relative activity of 100 – of the six incubations, the lowest individual incubation had a relative activity of 72. Incubations with antileprosy agents were done at the same time as the controls, using the same isolate. For each agent at each concentration, incubations were done in triplicate.

Antileprosy agent	Concn	Relative activity	P*
DDS	$10\ \text{ng ml}^{-1}$	38	<0.05
	$30\ \text{ng ml}^{-1}$	30	<0.05
	$100\ \text{ng ml}^{-1}$	24	<0.05
B663	$200\ \text{ng ml}^{-1}$	36	<0.05
	$20\ \mu\text{g ml}^{-1}$	27	<0.05
Rifampicin	$1\ \mu\text{g ml}^{-1}$	70	>0.10
	$3\ \mu\text{g ml}^{-1}$	26	<0.05
	$10\ \mu\text{g ml}^{-1}$	15	<0.05

* Determined using the Wilcoxon rank-sum test (see Colquhoun, 1971); significant inhibition at $P < 0.05$.

Enzyme activities involved in β -oxidation and acetyl-CoA-dependent chain elongation

Except for acetyl-CoA acyltransferase, all activities of the β -oxidation pathway assayed were detected in extracts of mycobacteria, including mycobacteria harvested from tissues of experimentally infected animals (Table 4). It was shown that acetyl-CoA acyltransferase was present in the mycobacteria only by growing them to as high a yield as possible: *M. avium* in Sauton's medium, *M. microti* in Dubos medium and *M. leprae* (a single isolate) harvested from armadillo spleen. Not only were the specific activities of this enzyme low, it was also unstable, with 20 to 40% of the activity being lost on storage at either 4 °C or (with freezing and thawing) at -80 °C in 24 h.

Acyl-CoA dehydrogenase activity was slightly higher in all extracts with decanoyl-CoA as the substrate than with palmitoyl-CoA as the substrate (Table 4a). Palmitoyl-CoA dehydrogenase activity was observed using PMS as an electron acceptor, but without NAD⁺, suggesting a flavin-linked enzyme of type EC 1.3.99.3 (see Methods and Fig. 2). When diaphorase and NAD⁺ were included in assays instead of PMS, to show NAD⁺-linked activity, the activity was 8% of that observed with PMS; endogenous levels of diaphorase and NAD⁺ were negligible, as no activity was observed if either NAD⁺ or diaphorase or both were omitted in assays without PMS. No activity was observed if NADP⁺ was included in assays instead of PMS and DCPIP but if NADP⁺, 0.1 unit ferridoxin:NADP⁺ reductase (NADPH diaphorase) and DCPIP were included in assays instead of PMS, activity was 3% of the activity observed with PMS, indicating a trace of NADP⁺-linked activity. The above experiments, in which PMS was replaced with other acceptors, were done with extracts of *M. avium* and *M. microti* grown in Dubos medium and then confirmed with an extract of *M. avium* grown in mice. Activity, whether of type EC 1.3.1.8 or EC 1.3.99.3, was never detected if *p*-iodonitrotetrazolium violet was used as the terminal electron acceptor in assays in place of DCPIP. In the biosynthetic direction (see Table 4b), *trans*-2-enoyl-CoA was reduced in the presence of NADH or NADPH, showing activity of the type EC 1.3.1.8. (see Fig. 2). This suggests that acyl-CoA dehydrogenase (EC 1.3.99.3) acts in the catabolic, β -oxidation pathway, whereas enoyl-CoA reductase acts mainly in the anabolic direction. However, the trace of NAD(P)⁺-linked acyl-linked oxidoreductase activity shown – essentially the reverse reaction of enoyl-CoA reductase suggests that this enzyme activity may be able to function, perhaps as a minor activity, in the catabolic direction as well as the anabolic direction (Fig. 1).

Other key enzymes of β -oxidation in extracts of mycobacteria *in vivo* are shown in Table 4. When *M.*

Table 4. Enzyme activities in extracts from host-grown mycobacteria

All assays were done at 25 °C as indicated in Methods. Values are means of specific activities [mU (mg protein)⁻¹] $\pm$ SEM, obtained by performing two to four (usually four) assays with each of extracts prepared from two or three isolates of host-grown bacteria, except for acetyl-CoA acyltransferase when one isolate of *M. leprae* and *M. avium* (grown in Sauton's medium) and *M. microti* (grown in Dubos medium) was used for preparing each extract.

	Extract from:		
	<i>M. leprae</i>	<i>M. microti</i>	<i>M. avium</i>
(a) Assayed in the catabolic, oxidative direction			
Acyl-CoA dehydrogenase			
With decanoyl-CoA	2.5 $\pm$ 0.1	16 $\pm$ 3	31 $\pm$ 4
With palmitoyl-CoA	1.5 $\pm$ 0.3	14 $\pm$ 1	20 $\pm$ 2
Enoyl-CoA hydratase			
With crotonyl-CoA	2.1 $\pm$ 0.1	8.1 $\pm$ 2.0	5.5 $\pm$ 0.9
With dec-2-enoyl-CoA	2.2 $\pm$ 0.2	7.0 $\pm$ 0.5	4.9 $\pm$ 1.1
β -Hydroxyacyl-CoA dehydrogenase	Assayed in the anabolic direction only		
Acetyl-CoA acyltransferase, β -ketothiolase activity with acetoacetyl-CoA	0.10 $\pm$ 0.03	1.2 $\pm$ 0.3	1.6 $\pm$ 0.2
(b) Assayed in the anabolic, fatty-acyl-elongating direction			
Enoyl-CoA reductase with dec-2-enoyl-CoA*			
With NADH	4.3 $\pm$ 1.7	26 $\pm$ 4	17 $\pm$ 2
With NADPH	4.1 $\pm$ 1.9	5.1 $\pm$ 2.4	10 $\pm$ 2
Enoyl-CoA hydratase	Assayed in the catabolic direction only		
β -Hydroxyacyl-CoA dehydrogenase, with acetoacetyl-CoA	170 $\pm$ 33	99 $\pm$ 7	78 $\pm$ 11
Acetyl-CoA acyltransferase			
With 25 μ M-decanoyl-CoA	ND	0.8 $\pm$ 0.3	0.6 $\pm$ 0.1
With 25 μ M-palmitoyl-CoA	ND	0.8 $\pm$ 0.1	0.9 $\pm$ 0.1
With 75 μ M-decanoyl-CoA	ND	0.7 $\pm$ 0.2	0.3 $\pm$ 0.1
With 75 μ M-palmitoyl-CoA	ND	0.7 $\pm$ 0.2	0.4 $\pm$ 0.1

ND, Not done.

* No activity (<0.2) detected with crotonyl-CoA.

avium was grown in Dubos medium or Dubos medium with either liposomes or triolein added, activities were similar (Table 5). However, when *M. avium* was grown in Dubos medium plus palmitate specific activities of all the enzymes assayed were elevated at least 4-fold (Table 5). It was important to use good quality triolein in the growth media: we used Sigma Grade, 99% pure, to obtain the above results; when we used practical grade (65% pure) we obtained similar results to those obtained when palmitate was added to growth medium and, indeed, found that about 10 to 20% of the lipid in practical grade triolein was free fatty acid. Activities were not elevated in *M. microti* grown in Dubos medium with added palmitate, but palmitate inhibited the yield of *M. microti* by about 70%: its low growth rate may therefore result in depressed enzyme activities.

EC 1.3.99.3

EC 1.3.99.3 –
proceeds without
addition of NAD(P)

EC 1.3.1.8

EC 1.3.1.8 – assayed with
diaphorase and
CoA oxidation or without
diaphorase and
CoA reduction

Fig. 2. Assay of acyl-CoA dehydrogenase (EC 1.3.99.3) and 2-enoyl-CoA reductase (EC 1.3.1.8) activities. Systematic enzyme names are given in Methods.

Table 5. Stimulation of enzymes for β -oxidation in *M. avium* grown in the presence of palmitate

All assays were done at 25 °C as indicated in Methods. Values are (i) ranges of specific activities [mU (mg protein)⁻¹] for *M. avium* grown in Dubos medium with either no addition, with the addition of either triolein or liposomes (see Methods for details) or grown in mice – the activities in individual assays overlapped considerably in extracts of *M. avium* grown in these different ways, and could not be distinguished from each other; (ii) values for specific activities of *M. avium* grown in Dubos medium + palmitate (sodium salt, 100 µg per ml of medium) are means $\pm$ SEM, determined by performing two or three assays with each of two extracts prepared from different batches of *M. avium*.

	Growth medium for <i>M. avium</i>	
	Without free palmitic acid	With sodium palmitate
<i>(a) In the oxidative direction</i>		
Acyl-CoA dehydrogenase		
With palmitoyl-CoA	16–30	87 $\pm$ 10
Enoyl-CoA hydratase		
With crotonyl-CoA	3.5–7.5	55 $\pm$ 13
With 2-decenoyl-CoA	2.8–6.5	32 $\pm$ 6
<i>(b) In the fatty acyl elongation direction</i>		
Enoyl-CoA reductase		
With NADH	14–18	57 $\pm$ 7
With NADPH	7–12	52 $\pm$ 12
β -Hydroxyacyl-CoA dehydrogenase		
With acetoacetyl CoA	55–98	402 $\pm$ 64

Simultaneous elongation and catabolism of supplied fatty acids

When 100 µg [1-¹⁴C]palmitate ml⁻¹ was included in Dubos growth medium, *M. avium* [initially 6 mg dry wt (1.2 $\times$ 10¹¹ bacteria in 25 ml) converted 1.4% (1038 Bq) to ¹⁴CO₂. During 4 h growth 20% (14670 Bq) of the radioactivity supplied appeared in lipids associated with the bacteria which could be extracted with chloroform/

methanol (see Methods). After methylation and fractionation by TLC, a fraction representing fatty acid methyl esters containing 4170 Bq, could be further fractionated by AgNO₃-TLC (see Methods) into saturated (86%), monounsaturated (12%) and diunsaturated (2%) fatty acid methyl esters. Only 4 Bq (0.1%) was recovered in the fraction containing esters with three or more double bonds. Radio-gas chromatography showed that 74% of the radioactivity was in esters of fatty acids of 16 or fewer carbon atoms, including 16:1 and 16:2 (which appear after 16:0 but before 18:0 in the method used). The remaining 26% of the radioactivity was associated with esters of fatty acids 18:0 or longer. Since virtually no radioactivity was detected in polyunsaturated fatty acid esters (which would be retarded during chromatography), this distribution of radioactivity shows that chain elongation was occurring. Fractionation of the longer-chain fatty acid methyl esters in a duplicate sample, in which 28% of the radioactivity was in 18:0 ester or longer chain esters, showed peaks of radioactivity associated with the following: 18:0 (8.5% of the radioactivity in all fatty acid methyl esters); 20:0 (2.0%), 21:0, an unidentified mycobacterial component (2.9%); 22:0, a further unidentified mycobacterial component (2.6%); 24:0 (1.4%); and a fraction including 24:1 and 26:0 (8.6%).

Discussion

The results in this paper show that *M. leprae*, *M. microti* and *M. avium* catabolize exogenous fatty acids to CO₂. This is done through the β -oxidation cycle for which the key enzymes have now been demonstrated (Table 4). The observation that laurate was most rapidly oxidized agrees with previous studies with other mycobacteria,

notably host-grown *M. lepraemurium* (Camargo *et al.*, 1979).

Uptake and utilization of decanoate and palmitate were compared in a previous paper (Wheeler *et al.*, 1990) so, where appropriate, β -oxidation enzyme activities were assayed here using decanoyl-CoA and palmitoyl-CoA. The activity of acyl-CoA dehydrogenase is higher for decanoyl-CoA than for palmitoyl-CoA, as shown in extracts of all mycobacteria studied (Table 4a), which may be one of the reasons why intact mycobacteria oxidize decanoate more rapidly than palmitate (see Table 1 and the section in the Results on oxidation of fatty acids by intact mycobacteria). However, acetyl-CoA-dependent elongation of fatty acyl-CoA to longer-chain fatty acyl-CoA esters (the complete reaction is given in Fig. 1) was previously shown to occur at a higher rate for decanoyl-CoA and palmitoyl-CoA in all mycobacteria studied (Wheeler & Ratledge, 1990), but the first individual enzyme activity – acetyl-CoA acyltransferase – involved in elongation (Fig. 1) had similar reaction velocities for decanoyl-CoA and palmitoyl-CoA (Table 4b). Therefore, the activity of this enzyme cannot account for the greater rate of elongation of decanoyl-CoA by the complete elongase (Wheeler *et al.*, 1990).

Activities of enzymes in the β -oxidation cycle did not appear to be stimulated in host-grown mycobacteria except with *M. avium* grown in the presence of a free fatty acid, in which all the individual enzyme activities were stimulated, suggesting them to be co-induced as observed in other microbes (Finnerty, 1989) including *Escherichia coli* (Nunn, 1986). However, *M. avium* is possibly unique and certainly different from *M. microti* in that its growth is stimulated, though slightly, by adding palmitate to the culture medium (McCarthy, 1984), whereas with *M. microti* the growth rate is diminished (Wheeler *et al.*, 1990). (The effect of palmitate on *M. leprae* is, of course, unknown due to the inability to cultivate it axenically.)

Acetyl-CoA-dependent elongation of fatty acyl-CoA esters, which is the predominant route in mycobacteria (see also Wheeler *et al.*, 1990), apparently involves the same enzyme activities as are found in β -oxidation enzymes but working in the opposite direction. In *E. coli*, synthesis of the enzymes is under the same regulation as catabolic β -oxidation (Nishimaki-Mogami *et al.*, 1987). Results obtained in this study suggest that regulation of these pathways in mycobacteria is slightly different: in contrast to the activities observed for individual enzymes of β -oxidation, acetyl-CoA-dependent chain elongation is stimulated in *M. microti* and *M. avium* grown in host animals or axenically in the presence of liposomes (Wheeler *et al.*, 1990).

β -Oxidation and chain elongation rely on the same reactions but it is inconceivable that these processes can

occur simultaneously – which they do as has been shown here – and be catalysed by the same enzymes. Thus we suggest that there must be two separate enzyme complexes, functionally identical but spatially distinct. This would therefore involve the two sets of enzymes being structurally distinct as site-recognition must be different. In this way, two apparently similar enzyme complexes can simultaneously catalyse two sets of reactions but in opposite directions.

Consideration of the evidence presented in this paper suggests that there are differences between the two putative complexes. For the elongating system, the first enzyme of the sequence (see Fig. 1) is acetyl-CoA transferase. In *E. coli*, where the same processes also occur, there is no evidence for two distinct enzymes (Binstock & Schulz 1981; Nishimaki-Mogami *et al.*, 1987). Although Shimikata *et al.* (1980) reached a similar conclusion working with *M. smegmatis*, there could be more than one acetyl-CoA acyltransferase in the pathogenic mycobacteria studied here as there is clearly separate regulation of elongation and β -oxidation. Such regulation could be argued to occur at this first reaction as the equilibrium strongly favours the reverse (cleavage) reaction. Thus to achieve elongation, the condensation reaction must be highly integrated as part of a complex to ensure that the product of the reaction (β -ketoacyl-CoA) is rapidly removed.

For β -oxidation, the first individual enzyme that acyl-CoA must bind to is acyl-CoA dehydrogenase (Fig. 1). In *E. coli*, this enzyme is different from that involved in acetyl-CoA-dependent chain elongation, which is a specific *trans*-2-enoyl-CoA reductase (Mizugaki *et al.*, 1982). Likewise, the flavin-linked acyl-CoA dehydrogenase, demonstrated in this work, is possibly involved in catabolism of fatty acids while the NADP(H)-dependent enoyl-CoA oxidoreductase is part of the elongating complex.

Enoyl-CoA hydratase is another activity for which there could be separate enzymes involved in acetyl-CoA-dependent chain elongation and oxidation. In *E. coli*, polyclonal antibodies raised against the β -oxidation complex also inhibited acetyl-CoA-dependent chain elongation, providing further evidence for the involvement of similar enzymes in β -oxidation and elongation; however, the antibodies did not completely inhibit hydratase activity with dec-2-enoyl-CoA. For *M. smegmatis*, two distinct enoyl-CoA hydratases have been purified (Fujita *et al.*, 1980). One (type I) had activity for crotonoyl-CoA and dec-2-enoyl-CoA; the other (type II), which was the only one that participated in acetyl-CoA-dependent acyl-CoA elongation, had no activity with crotonoyl-CoA but was active with dec-2-enoyl-CoA. In the present study using crude extracts, activity with both crotonoyl-CoA and dec-2-enoyl-CoA was observed. As

the purified type I enzyme from *M. smegmatis* has about ten times greater activity with crotonyl-CoA than with dec-2-enoyl-CoA (Fujita *et al.*, 1980), the results in Table 4(a) suggest that type II hydratase must have been present along with type I hydratase.

Overall, the present work shows that all the enzyme activities for β -oxidation and acetyl-CoA-dependent chain elongation exist in pathogenic mycobacteria. Further progress will require the purification of enzymes so that similar enzymes can be distinguished and the stereospecificity of the substrates worked out.

Finally, as antileprosy agents inhibited the evolution of $^{14}\text{CO}_2$ from $[1-^{14}\text{C}]$ laurate and the rate of evolution of $^{14}\text{CO}_2$ from this substrate was considerably higher than with $[1-^{14}\text{C}]$ palmitate, the former substrate should replace $[1-^{14}\text{C}]$ palmitate in the potential drug-screening methods developed by Franzblau (1988), which depend on oxidation of ^{14}C -labelled fatty acids to $^{14}\text{CO}_2$. This would give a 3- to 4-fold increase in the sensitivity of such a test system.

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