Use of Carbon Sources for Lipid Biosynthesis in *Mycobacterium leprae*: a Comparison with Other Pathogenic Mycobacteria

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Carbon from glycerol and palmitate, but not significantly from five other carbon sources tested, was incorporated into lipids by suspensions of non-growing *Mycobacterium leprae* organisms. However, of the five other substrates three – citrate, glucose and pyruvate – were taken up. Non-growing *Mycobacterium microti* and *Mycobacterium avium* incorporated carbon into lipids from most simple carbon sources tested unless they were obtained from growth media including palmitate or from experimentally infected animals, when incorporation of carbon into lipids from carbon sources except palmitate occurred up to 20 times more slowly. Thus, utilization of simple carbon appeared to be repressible while utilization of the one fatty acid tested, palmitate, appeared constitutive. In *M. leprae*, carbon from glycerol was incorporated into the glycerol moiety of acylglycerols but not into the fatty acid moieties or into free fatty acids. *M. microti* and *M. avium* incorporated carbon from simple carbon sources into fatty acids, even (though very slowly) when these organisms were obtained from host tissue. Isocitrate lyase, malate synthase and acetate kinase were detected in *M. leprae*. However acetyl-CoA synthetase was not detectable and phosphoacetylase was deficient; thus, *M. leprae* may be incapable of making acetyl-CoA from acetate. Phosphotransacetylase was readily detected in both host-grown *M. avium* and *M. microti*.

INTRODUCTION

The ability to acquire carbon sources and use them to synthesize lipids is crucial for *Mycobacterium leprae* as, like most other mycobacteria, about 40% of its dry weight is lipid material (Ratledge, 1982). Moreover, like a number of pathogenic mycobacteria, *M. leprae* excretes large quantities of lipid into its environment. Tissue infected with the leprosy bacillus, *M. leprae*, contains up to 166 µg of a characteristic phenolic glycolipid for each mg dry wt of *M. leprae* (Cho *et al.*, 1986).

Although the identity of many lipids of *M. leprae* is known (Brennan, 1983; Draper, 1984), the substrates and metabolic pathways for lipid biosynthesis have not yet been shown in isolated leprosy bacilli. However, it is known that *M. leprae* takes up a number of carbon sources and can oxidize them to CO₂ (Wheeler, 1983, 1984*a*). Therefore, in this work the incorporation of various ¹⁴C-labelled substrates into lipid in *M. leprae* was ascertained. Previously, enzymes of glycolysis, the hexose monophosphate pathway (Wheeler, 1983) and tricarboxylic acid cycle (Wheeler, 1984*a*) were demonstrated in *M. leprae*. In this work, enzymes that might be involved in channelling carbon sources into lipid synthesis or involved in the metabolism of carbon derived from lipids were sought – i.e. enzymes for converting acetate and pyruvate to acetyl-CoA and the enzymes of the glyoxylate cycle.

Lipid biosynthesis and the related metabolic activities mentioned above are known to be regulated in mycobacteria (Bloch, 1977; Ratledge, 1982). Such activities may well be repressed or inhibited when mycobacteria are growing inside host tissue where lipids might be available as

Abbreviations: HPTLC, high performance thin layer chromatography; DTNB, dithio-bis(2-nitrobenzoic acid).

a carbon source. Therefore, all activities sought in *M. leprae* have been compared to those in *M. microti* and *M. avium* grown *in vivo* and *in vitro* in an attempt to determine whether the findings with *M. leprae* indicated possible metabolic incompetences in areas of lipid biosynthesis or whether *M. leprae* uses carbon sources for lipid biosynthesis in a way similar to other pathogenic mycobacteria when they are growing inside a host.

Additionally, *M. avium* and *M. microti* were grown in media with a single modification – such as the addition of a fatty acid – in an attempt to find out the effects of some exogenous carbon sources in lipid metabolism. Any deficiencies in metabolism in *M. leprae* that might be deduced from these studies might help in understanding why it cannot, as yet, be grown in axenic culture.

METHODS

Mycobacteria from experimentally infected animals. *M. leprae* was harvested from armadillo liver and spleen, *M. avium* CR1/69 (from AFRC Institute for Research in Animal Diseases, Compton, nr Newbury, Berkshire, UK) from spleen and liver of C57 Black mice, and *M. microti* OV 254 (from National Institute for Medical Research, London, UK) from lungs of CBA mice. Experimental infections and harvesting methods (World Health Organization, 1980) have both been described previously (Wheeler, 1987). Briefly, the bacteria were separated from tissue by a method including density gradient centrifugation using Percoll (Pharmacia). For suspensions which were sonicated to provide cell-free extracts for enzyme work, further purification was achieved in 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 mycobacteria.

Mycobacteria grown in cell-free media. *M. avium* and *M. microti* were grown in Modified Dubos medium alone, or with the following additions: (i) 1% (w/v) glycerol; (ii) 100 µg sodium palmitate ml⁻¹. These are referred to as Dubos medium, and Dubos medium with added glycerol or palmitate, in the Results and Discussion. Modified Dubos medium (see Chadwick, 1982) includes citrate, asparagine and Casamino acids as carbon sources as well as Tween 80. For some experiments, mycobacteria were grown in Modified Dubos medium with Tween 80 omitted. *M. microti* was also grown in Youmans and Karlson medium (see Chadwick, 1982) – which includes asparagine, glycerol and citrate only as carbon sources – plus 0.4% bovine serum albumin. Little or no growth was obtained when palmitate was added to Youmans and Karlson medium so it was not used in the principal experiments reported in this paper. All mycobacteria used in experiments were grown for 14 d at 37 °C in 100 ml medium in shaking (200 r.p.m.) 250 ml flasks stoppered with polyurethane bungs. The inoculum was a suspension (1 ml) grown previously in the same medium. After growth, bacteria were washed aseptically three times in buffered Tween 80 (0.1%, w/v, Tween 80 in 1.5 mM-MES, pH 7.0) or, when grown in Modified Dubos medium without Tween 80, washed twice in 10 mM-MES/KOH, pH 7.0.

Preparation of cell-free extracts. These were prepared by ultrasonic disruption of bacteria as described previously (Wheeler & Gregory, 1980). Portions of some extracts were dialysed using ultrafiltration cones with a cut-off of *M*_w 25 000 as described previously (Wheeler, 1984a).

Extracts of armadillo tissue. An extract was prepared at pH 8.7 (the pH of the initial homogenate of infected tissue from which mycobacteria are harvested) as described previously (Wheeler & Gregory, 1980).

Radioisotopically labelled substrates. [U-¹⁴C]Palmitic acid (402 Ci mol⁻¹; 14.9 GBq mmol⁻¹), [2-¹⁴C]acetic acid, sodium salt (56 Ci mol⁻¹; 2.07 GBq mmol⁻¹), [U-¹⁴C]glycerol (170 Ci mol⁻¹; 6.3 GBq mmol⁻¹), [1-¹⁴C]pyruvic acid, sodium salt (26 Ci mol⁻¹; 0.96 GBq mmol⁻¹), [2-¹⁴C]malonic acid, sodium salt (20 Ci mol⁻¹; 0.74 GBq mmol⁻¹), [U-¹⁴C]glucose (270 Ci mol⁻¹; 10.0 GBq mmol⁻¹) and [1,5-¹⁴C]citric acid (170 Ci mol⁻¹; 6.3 GBq mmol⁻¹) were obtained from Amersham; [2-¹⁴C]pyruvic acid, sodium salt (12.5 Ci mol⁻¹; 0.46 GBq mmol⁻¹) was obtained from New England Nuclear.

Incubations of mycobacteria with radioisotopically labelled substrates. Bacteria (0.25 to 2.0 × 10¹⁰; 10¹⁰ *M. leprae* weigh approximately 1 mg dry wt) as washed suspensions in buffered Tween 80, were incubated with ¹⁴C-labelled substrates as follows: acetate, glucose, citric acid each at 0.5 µCi (18.5 kBq), 0.1 mM; glycerol (except where otherwise stated), pyruvic acid each at 0.25 µCi (9.25 kBq), 0.1 mM; malonic acid at 0.4 µCi (14.8 kBq), 0.1 mM and palmitic acid at 0.8 µCi (29.6 kBq) (2 nmol). Since palmitic acid was supplied as a solution in toluene, the 2 nmol portions were transferred into each incubation vial and the toluene was allowed to dry under aseptic conditions. All incubation mixtures were made up to a final volume of 200 µl which included one labelled substrate, bacteria, and the following adjusted to pH 7 with KOH: HEPES (50 mM), MgSO₄ (5 mM), Na₂HPO₄/KH₂PO₄ (1 mM) and penicillin G (50 U ml⁻¹). Incubations were at 34 °C; for *M. microti* and *M. avium* they were done for up to 2 h; for *M. leprae*, for 20 h. After 20 h incubations, a 5 µl sample was taken aseptically, and plated on to nutrient agar to check for any contamination – contaminated incubations were discarded. ¹⁴CO₂ evolved during incubation was trapped in 400 µl 1 M-NaOH. Assimilation of radioactivity into lipids was determined by collecting and washing the bacteria on filters as for determination of assimilation, then extracting

the bacteria (still on the filters) for 1 h with 4 ml hot chloroform/methanol/1 M-HCl (16:6:1, by vol.), removing and discarding the filters, washing the organic solvent phase once with 5 ml distilled water, removing any solid material in the solvent (bacteria, glass fibres from the filter) by filtration through Millipore Millex-HV 4 mm filters and evaporating the solvent in scintillation vials.

Radioactivity was determined – as $^{14}\text{CO}_2$ in NaOH, on filters, or in extracted lipids – by adding 10 ml Bray's scintillation fluid (4 g 2,5-diphenyloxazole, 60 g naphthalene, 100 ml methanol, 20 ml ethanediol made up to 1 l with dioxan), mixing thoroughly and scintillation counting at least 3 h after addition of scintillation fluid to the radioactive material. Controls contained: (i) heat-killed bacteria (100 °C, 15 min); (ii) formaldehyde-killed bacteria (18 h at 20 °C in 10%, v/v, formalin, followed by three washes, by centrifugation and resuspension in buffered Tween 80, of the killed bacteria); or (iii) live bacteria added to incubation media and collected immediately. In controls (i) and (ii) incubations were for the same period as parallel incubations with live bacteria. Controls (i), (ii) and (iii) had similar background radioactivity (except where shown in the results) and d.p.m. in controls is always subtracted from d.p.m. in incubations in the results.

Fractionation of neutral lipids. Labelled lipid material for fractionation was obtained by collecting and washing bacteria on filters as for determination of assimilation but then boiling in ethanol for 10 min, cooling, and extracting with 4 ml chloroform/methanol/1 M-HCl at 20 °C for 18 h. The organic solvent phase was washed with distilled water, filtered through Millex-HV 4 mm filters into glass tubes and evaporated. The material (at least 80% of the total) was applied to HPTLC plates (Merck; HPTLC silica gel 60 with concentrating zone) by adding 15 µl chloroform to each tube to dissolve it, applying to plates, and repeating the procedure twice. Unlabelled palmitate (R_F ~0.55), monopalmitoylglycerol (MPG; R_F ~0.05), dipalmitoylglycerol (DPG; R_F ~0.30) and tripalmitoylglycerol (TPG; R_F ~0.75) (20 µg each) were applied as carriers (fatty acyl chain length and saturation did not affect mobilities). The plates were developed once in light petroleum (b.p. 60–80 °C)/diethyl ether/glacial acetic acid (90:10:1, by vol.), and the position of lipids (R_F values given above) was detected non-destructively by spraying with 0.2% 2,7-dichlorofluorescin in ethanol/water (96:4, v/v). Spots corresponding to the above mentioned lipids, and areas of plate between spots, were scraped into scintillation vials, Bray's solution (see above) was added, and their radioactivity was determined by scintillation counting. The HPTLC method used in this work was based on a TLC method for separating neutral lipids of *M. smegmatis* (Dhariwal *et al.*, 1978).

Enzyme assays. All were done at 25 °C; the reaction volume was 500 µl unless otherwise stated. Assay conditions were optimized in previous studies (see below and Reddy & Venkatasubramanian, 1975) and similar pH, as well as concentrations of substrates and metal ions were used in the present work. However, some assays were modified to improve their sensitivity. Pyruvate dehydrogenase [EC 1.2.4.1; pyruvate oxidoreductase (decarboxylating and acceptor acetylating)] and isocitrate dehydrogenase [EC 1.1.1.42; *threo*-D₅-isocitrate:NADP oxidoreductase (decarboxylating)] were assayed as described previously (Wheeler, 1984a).

Acetate kinase (EC 2.7.2.1; ATP:acetate phosphotransferase) was assayed by incubating extracts with 100 mM-sodium acetate (adjusted to pH 7.6), 1 mM-MgCl₂, 1 mM-MnCl₂, 5 mM-ATP, 1 mM-phosphoenolpyruvate and 100 mM-triethanolamine/HCl (pH 7.6) plus the coupling mixture 0.2 mM-NADH and a lactate dehydrogenase/pyruvate dehydrogenase from rabbit muscle (Sigma; 200 U ml⁻¹ and 32 U ml⁻¹ respectively). The decrease in A₃₄₀ in incubations with and without acetate was measured, the difference being proportional to acetate kinase activity. When background oxidation of NADH (i.e. without acetate) was very high, it was inhibited by adding 0.4 mM quinacrine dihydrochloride (Sigma; adjusted to pH 7.6 with Tris base). Quinacrine did not inhibit acetate kinase activity. Optimum assay conditions were established previously with the enzyme from *M. phlei* and *M. bovis* BCG (Andrejew *et al.*, 1975).

Phosphotransacetylase (EC 2.3.1.8; acetyl-CoA: orthophosphate acetyltransferase) was assayed by incubating extracts with 1 mM-acetyl-CoA, 19 mM-(NH₄)₂SO₄, 0.1 mM-5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB; added as a 10 mM-stock solution in dimethylsulphoxide), 20 mM-sodium phosphate, pH 7.5, and 86 mM-Tris/HCl, pH 7.5. Increase in A₄₁₂ was followed in incubations with or without phosphate, the difference being proportional to phosphotransacetylase activity. An alternative assay, using acetyl phosphate (at 7 mM) and acetyl-CoA, and following acetyl-CoA formation (increase in A₂₃₃), gave specific activities about three times higher in extracts of mycobacteria but was relatively insensitive because of high background A₂₃₃ values and high levels of noise in spectrophotometric readings at this short UV wavelength. Optimum assay conditions were established previously with enzyme from *M. phlei* and *M. bovis* BCG (Andrejew *et al.*, 1976).

A radiochemical assay for formation of acetyl-CoA and acetyl phosphate from acetate was also used. Extracts (44–74 µg protein) were incubated at 25 °C with 7 mM-sodium [2-¹⁴C]acetate (40 µCi ml⁻¹; 1.48 MBq ml⁻¹), 30 mM-Tris/HCl (pH 7.6), 2.5 mM-MgCl₂, 5 mM-ATP, 2 mM-CoA-SH (lithium salt) and 2 mM-dithiothreitol in a total volume of 25 µl. Samples (5 µl) were taken immediately, and after 10, 25 and 100 min, mixed with 1 µl of a mixture of unlabelled acetyl-CoA and acetyl phosphate (both at 50 mg ml⁻¹), and applied to PEI-cellulose TLC plates (Merck; PEI-cellulose F pre-coated on plastic sheets). The plates were developed in 0.2 M-LiCl (solvent front: 180 mm), allowed to dry in air, and sprayed with 0.04% bromocresol green in ethanol/water (96:4, v/v) to identify spots. Areas were cut out as follows: for acetyl-CoA, from origin to 15 mm; for acetyl phosphate, from 45 to

75 mm; for acetate, from 120 to 150 mm. Radioactivity in the areas of the TLC plate was determined by scintillation counting in Bray's fluid.

Isocitrate lyase (EC 4.1.3.1; *threo*-D₅-isocitrate glyoxylate lyase) was assayed in extracts by a modification of the method of Dixon & Kornberg (1959) using 1.6 mM-*threo*-L₅-isocitrate (Sigma; potassium salt), 5 mM-MgCl₂, 2 mM-cysteine.HCl, 3.3 mM-phenylhydrazine.HCl and 67 mM-HEPES/KOH, pH 6.85 (final pH of mixture 6.4). Increase in A_{324} was followed in incubations with or without isocitrate, the difference being proportional to isocitrate lyase activity. An alternative assay, coupled to glyoxylate reductase, gave specific activity values about three times higher in extracts of mycobacteria but was not very reproducible.

Malate synthase [EC 4.1.3.2; L-malate glyoxylate-lyase (CoA acetylating)] was assayed by incubating extracts with 0.5 mM-acetyl-CoA, 0.15 mM-glyoxylic acid, 10 mM-MgCl₂ and 50 mM-Tris/HCl, pH 7.5 (reaction volume 250 µl; controls were done by omitting glyoxylate). After 5 min, 250 µl 0.2 mM-DTNB in 10% (v/v) ethanol (from a stock solution of 2 mM-DTNB in ethanol) was added to each reaction mixture: enzyme activity was proportional to (A_{412} in mixtures including glyoxylate) minus (A_{412} in corresponding controls). An alternative, continuous assay in which malate formation was coupled to dye reduction by adding malate dehydrogenase, diaphorase, NAD, and *p*-iodonitrotetrazolium violet formation allowed formation of malate, linear with time, to be followed for 20 min. However, the continuous assay gave specific values about four times lower in extracts of mycobacteria.

Statistical method. Student's *t*-test was used throughout to test significance.

RESULTS

Incorporation of carbon from exogenous substrates into lipids of M. leprae

M. leprae isolated from armadillo tissue readily incorporated carbon (traced using ¹⁴C) only from palmitate and glycerol (amongst carbon sources used in this study) into lipid material (Table 1). Carbon from glucose was detected in lipids at a trace level, 125 to 190 d.p.m. in 10¹⁰ cells of *M. leprae* (see Table 1) in all incubations using *M. leprae* from three separate isolates. In contrast, the trace incorporation of carbon into lipids from pyruvate (Table 1) was not detected in *M. leprae* from all isolates. Exogenous palmitate and glycerol were assimilated by *M. leprae* more rapidly than other carbon sources, but failure to assimilate carbon sources could not explain entirely the limited number of carbon sources used in lipid synthesis. While malonate and acetate were not assimilated by *M. leprae* in the incubation conditions used, glucose and citrate were assimilated, yet little or none of their carbon appeared in lipids. If incubations were done at pH 5 (substituting MES for HEPES in the incubation buffer), acetate was assimilated (1.1% of that supplied exogenously), but still none of the carbon appeared in the lipids. No radioactivity from any source was incorporated into isolated lipid material if incubations were done with just an extract of armadillo liver (1 mg protein) in place of mycobacteria.

Table 1. *Incorporation of carbon from substrates into lipids in M. leprae*

Incubations were at pH 7 and 34 °C for 20 h and included 8×10^9 to 2×10^{10} organisms, and 20 nmol ¹⁴C-labelled carbon source (except palmitate: 2 nmol) in 200 µl. Each value represents four to eight incubations from three to five isolates, except in the case of incubations with citrate and malonate, when values represent two or three incubations from two isolates. Values are expressed as the percentage of radioactivity supplied that was (i) assimilated into intact bacteria or (ii) incorporated into lipids (per 10¹⁰ organisms in 20 h $\pm$ SEM). In no incubation was > 16% of the substrate supplied utilized, including substrate converted to ¹⁴CO₂.

¹⁴ C-labelled substrate in incubation medium	Radioactivity in <i>M. leprae</i>	
	Assimilated	Incorporated into lipids
[U- ¹⁴ C]Palmitate	2.03 $\pm$ 0.58	1.65 $\pm$ 0.52
[2- ¹⁴ C]Acetate	0 $\pm$ 0.03	0 $\pm$ 0.03
[2- ¹⁴ C]Pyruvate	0.25 $\pm$ 0.07	0.07 $\pm$ 0.03*
[2- ¹⁴ C]Malonate	0 $\pm$ 0.01	0 $\pm$ 0.01
[U- ¹⁴ C]Glycerol	1.85 $\pm$ 0.24	0.67 $\pm$ 0.17
[U- ¹⁴ C]Glucose	0.50 $\pm$ 0.06	0.02 $\pm$ 0.002
[1,5- ¹⁴ C]Citrate	0.11 $\pm$ 0.04	0 $\pm$ 0.002

* Doubtfully significantly (0.05 < *P* < 0.10) above zero. Except for zeros themselves, all other values are significantly (*P* < 0.05) above zero.

Table 2. *Incorporation of carbon from substrates into lipids in M. microti*

Incubations were at 34 °C, for 1 h, and included 2.5×10^9 to 1.2×10^{10} organisms, and 20 nmol ^{14}C -labelled carbon source (except palmitate: 2 nmol) in 200 μl . Each value represents three to nine incubations from at least three cultures or isolates, except when bacteria were grown in Dubos + glycerol when values represent two incubations from a single, representative culture. Value are expressed as the percentage of radioactivity supplied that was (i) assimilated into intact bacteria or (ii) incorporated into lipids (per 10^{10} organisms per hour $\pm$ SEM). In no incubation was $>25\%$ of the substrate supplied utilized, including substrate converted to $^{14}\text{CO}_2$.

^{14}C -labelled substrate in incubation medium	Radioactivity in <i>M. microti</i>	Growth medium			
		Dubos	Dubos + 1% glycerol	Dubos + 100 μg sodium palmitate ml^{-1}	CBA mice
[U- ^{14}C]Palmitate	Assimilated	7.4 ± 1.6	13.3	6.6 ± 0.46	12.8 ± 3.3
	In lipids	6.7 ± 1.3	11.5	6.0 ± 0.98	9.6 ± 3.2
[2- ^{14}C]Acetate	Assimilated	13.6 ± 1.5	6.2	1.2 ± 0.39	1.2 ± 0.36
	In lipids	8.2 ± 1.4	3.8	0.27 ± 0.09	$0.43 \pm 0.15^*$
[2- ^{14}C]Pyruvate	Assimilated	1.1 ± 0.32	1.5	0.69 ± 0.22	0.80 ± 0.22
	In lipids	0.68 ± 0.08	0.74	$0.09 \pm 0.04^*$	0.25 ± 0.09
[U- ^{14}C]Glycerol	Assimilated	0.98 ± 0.17	0.42	0.10 ± 0.03	$0.20 \pm 0.11^*$
	In lipids	0.62 ± 0.20	0.27	0 ± 0.04	$0.02 \pm 0.01^*$
[2- ^{14}C]Malonate	Assimilated	2.02 ± 0.58	2.00	0.70 ± 0.12	1.73 ± 0.50
	In lipids	1.44 ± 0.34	0.78	0.38 ± 0.11	0.29 ± 0.12

* Doubtfully significantly ($0.05 < P < 0.10$) above zero.

There was some problem in evaluating assimilation and incorporation of carbon into lipids from palmitate in *M. leprae*. In a typical experiment, radioactivity in the lipids of samples (all containing 0.4×10^{10} cells of *M. leprae*) from 20 h incubations with [U- ^{14}C]palmitate was as follows: live *M. leprae*, 23900 d.p.m.; heat-killed (100 °C, 15 min) *M. leprae* 11700 d.p.m.; formaldehyde-killed *M. leprae*, 2300 d.p.m. When *M. leprae* was added to incubations and collected immediately (i.e. at zero time), radioactivity in the lipids was 13500 d.p.m.

Radioactivity in the lipids was similar at zero time and 20 h for *M. leprae* killed by heating or with formaldehyde, contrasting with live *M. leprae*. Thus the radioactivity in lipids at zero time from live *M. leprae* probably represents radioactivity that is merely adsorbed, and therefore the percentage values (in Table 1) for utilization of exogenous palmitate by *M. leprae* are

$$\frac{(\text{d.p.m. in } 10^{10} \text{ } M. \text{ leprae after 20 h}) - (\text{d.p.m. in } 10^{10} \text{ } M. \text{ leprae at zero time}) \times 10^2}{\text{d.p.m. supplied } (1.76 \times 10^6)}$$

These values will be underestimates if the 'true' control value is that obtained with formaldehyde-killed *M. leprae*.

Incorporation of carbon from exogenous substrates into lipids of M. avium and M. microti

Both *M. microti* (Table 2) and *M. avium* (Table 3) that had been grown in Dubos medium incorporated a wide range of carbon sources into lipids in the incubations – using washed, non-growing organisms – done in this work. Glucose and citrate were both assimilated, but ^{14}C from these two substrates was incorporated very slowly, if at all, into lipids.

M. microti and *M. avium* grown in mice incorporated carbon into lipids from some substrates very slowly. In particular, carbon from the C-2 atom of acetate was incorporated into lipids 20 times more slowly in mycobacteria grown *in vivo* than in the same mycobacteria grown in Dubos medium (Tables 2 and 3). Growth in this medium appeared to reflect the areas of lipid metabolism under investigation more accurately in *M. microti* than in *M. avium*, notably the incorporation of carbon into lipids from glycerol could only be detected in mycobacteria grown in Dubos medium with palmitate in the case of *M. avium* (Table 3). Growth of *M. microti* was inhibited by palmitate: only 20 mg dry wt organisms per flask (100 ml medium) was obtained in 14 d growth as opposed to 35 mg dry wt organisms per flask in Dubos medium with no palmitate.

Table 3. *Incorporation of carbon from substrates into lipids in M. avium*

Incubations were at 34 °C and included 3×10^9 to 10^{10} organisms, and 20 nmol ^{14}C -labelled carbon source (except palmitate: 2 nmol) in 200 μl . Each value represents three to five incubations from two or three cultures or isolates except when bacteria were grown on Dubos + glycerol when values represent two incubations from a single, representative culture. Values are expressed as the percentage of radioactivity supplied that was (i) assimilated into intact bacteria or (ii) incorporated into lipids (per 10^{10} organisms per hour $\pm$ SEM). Incubations were for up to 2.5 h, during which period rates of assimilation and incorporation were linear. In no incubation was $>20\%$ of the substrate supplied utilized, including substrate converted to $^{14}\text{CO}_2$.

^{14}C -labelled substrate in incubation medium	Radioactivity in <i>M. avium</i>	Growth medium			
		Dubos	Dubos + 1% glycerol	Dubos + 100 μg sodium palmitate ml^{-1}	C57 Black mice
[U- ^{14}C]Palmitate	Assimilated	7.9 ± 0.33	8.8	7.9 ± 1.6	5.9 ± 0.44
	In lipids	5.9 ± 0.63	6.7	6.8 ± 1.8	4.3 ± 0.15
[2- ^{14}C]Acetate	Assimilated	10.0 ± 0.18	7.2	1.2 ± 0.08	0.25 ± 0.01
	In lipids	2.6 ± 0.64	2.7	0.25 ± 0.06	0.14 ± 0.01
[2- ^{14}C]Pyruvate	Assimilated	0.60 ± 0.18	0.60	$0.23 \pm 0.09^*$	$0.10 \pm 0.05^*$
	In lipids	0.18 ± 0.01	0.31	$0.02 \pm 0.01^*$	0.01 ± 0.001
[U- ^{14}C]Glycerol	Assimilated	8.8 ± 1.4	1.02	3.25 ± 0.05	0.26 ± 0.07
	In lipids	0.83 ± 0.11	0.33	0.26 ± 0.01	0.06 ± 0.01
[2- ^{14}C]Malonate	Assimilated	0.42	0.31	0.22	0.02
	In lipids	0.07	0.05	0.03	0.01

* Doubtfully significantly ($0.05 < P < 0.10$) above zero.

Addition of palmitate to Dubos medium had no effect on growth of *M. avium*. Nevertheless, the fact that palmitate is assimilated and clearly influences metabolism indicates that it is utilized by both these bacteria.

In general, growth rate and viability may affect apparent rates of metabolic activity. However, in these incubations, assimilation of [^{14}C]palmitate into both *M. microti* and *M. avium* varied little and could be used as a 'constitutive' activity with which to compare assimilation of, and incorporation of carbon into lipids from other ^{14}C -labelled sources.

Tween 80, a detergent containing an oleate moiety in its molecular structure, was routinely used at low concentrations (0.05 to 0.1%) for growth of mycobacteria *in vitro*. However, its omission from media made no discernable difference to the utilization of ^{14}C -labelled carbon sources by *M. microti* and *M. avium* (results not shown). Its omission did make experiments very difficult to do as the mycobacteria used in this study clumped very easily in its absence.

Addition of glycerol to Dubos medium had little effect on the utilization of ^{14}C -sources by *M. microti* (Table 2) and *M. avium* (Table 3) grown in its presence. The slightly depressed incorporation of carbon from glycerol into lipids in bacteria grown in the presence of glycerol may be a result of dilution of [U- ^{14}C]glycerol in elevated metabolic pools of glycerol and glycerol intermediates in the bacteria. However, growth on glycerol did affect some enzyme activities involved in utilization of carbon sources in lipid metabolism (see section below) so its effect on whole organisms is included in Tables 2 and 3. When *M. microti* was grown on Youmans and Karlson medium, which contains glycerol, the patterns of both utilization of exogenous carbon sources and enzyme activities (see below) were similar to those in *M. microti* grown in Dubos medium with added glycerol. Since it proved difficult to grow *M. microti* when Youmans and Karlson medium was modified (see Methods), these results are not shown.

Is palmitate assimilated into M. leprae being metabolized?

When palmitate is assimilated into organisms it is inevitably extracted into lipid material even if it is not metabolized. However, preliminary evidence for its metabolism in *M. leprae* was obtained. When neutral lipids from 20 h incubations of live *M. leprae* with [U- ^{14}C]palmitate

were fractionated ($\sim 80\,000$ d.p.m. applied to HPTLC plate), 91% of the radioactivity appeared in the free fatty acid fraction and 3% in the monoacylglycerol fraction. In contrast, in the control incubations, neutral lipids from incubations either with no bacteria but with an extract of armadillo liver (1 mg protein), or with [U- ^{14}C]palmitate alone, 94 to 95% of the radioactivity appeared in the free fatty acid fraction and 0.2% in the monoacylglycerol fraction. Otherwise, the radioactivity in individual fractions was similar within $\pm 0.2\%$ to that in incubations with live *M. leprae* and control incubations. Results presented earlier in the paper suggest that $\sim 60\%$ of the radioactivity applied to the HPTLC plate may represent adsorbed [U- ^{14}C]palmitate; thus probably nearer to 7 to 8% of the [U- ^{14}C]palmitate truly assimilated by *M. leprae* is being incorporated into monoacylglycerols.

Remarkably rapid evolution of $^{14}\text{CO}_2$ was observed in incubations of *M. leprae* with [U- ^{14}C]palmitate: $16.3 \pm 4.9\%$ (SEM) of palmitate supplied was converted to $^{14}\text{CO}_2$ in 20 h by 10^{10} organisms (see Table 1 for details of incubations and the assimilation and incorporation into lipids). With all other ^{14}C -labelled sources, the radioactivity evolved as $^{14}\text{CO}_2$ was between 10% and 60% of the radioactivity assimilated for either *M. avium*, *M. microti* (both grown either *in vivo* or *in vitro*) or *M. leprae*.

Anabolic fate of glycerol

Apart from palmitate, comparative studies using *M. microti*, *M. avium* and *M. leprae* could only be done on the metabolism of glycerol, since insufficient carbon was assimilated into lipids when *M. leprae* was incubated with other carbon sources. An interesting difference in the anabolic fate of glycerol in *M. avium* and *M. microti* on the one hand, and *M. leprae* on the other, was observed. After incubations (1 h for *M. microti*, 2 h for *M. avium*) with [U- ^{14}C]glycerol, radioactivity (30 to 42% of the neutral lipids separated by HPTLC) was observed in the fatty acid fractions, regardless of the conditions under which the organisms had been grown. Further radioactivity was observed in acylglycerol fractions (results not shown). However, when neutral lipids from 20 h incubations of live *M. leprae* with $0.5\ \mu\text{Ci}$ [U- ^{14}C]glycerol were fractionated (~ 7000 d.p.m. applied to HPTLC plate), 92% of the radioactivity appeared in acylglycerol fractions but none at all in the free fatty acid fraction. Thus it appeared that the [U- ^{14}C]glycerol assimilated by *M. leprae* was only being esterified with endogenous fatty acyl moieties while in *M. avium* and *M. microti* some carbon from [U- ^{14}C]glycerol was being used (presumably metabolized via acetyl-CoA) for fatty acid biosynthesis or elongation.

In order to confirm that the carbon from glycerol was not being assimilated into fatty acyl moieties of acylglycerols in *M. leprae*, neutral lipids (two samples containing ~ 7000 d.p.m. extracted from a replicate incubation of *M. leprae* with $0.5\ \mu\text{Ci}$ [U- ^{14}C]glycerol) were each digested for 30 min at 37°C with lipase (*Candida cylindrica*; from Sigma; $0.2\ \text{mg}$ dry wt dissolved in $50\ \mu\text{l}$ $30\ \text{mM}$ -Tris/HCl, pH 7.6, and mixed with the lipid sample). One digested sample was dried and then dissolved in $25\ \mu\text{l}$ CHCl_3 and applied to an HPTLC plate. When neutral lipids were fractionated only 15% of the radioactivity appeared in acylglycerol fractions and again, none at all in the free fatty acid fraction. The second digested sample was acidified with $1\ \text{ml}$ $0.1\ \text{M}$ -HCl and extracted three times with $3\ \text{ml}$ light petroleum (b.p. $30\text{--}40^\circ\text{C}$). The combined light petroleum extracts were backwashed twice with $2\ \text{ml}$ distilled water; 97% of the radioactivity remained in the aqueous phase. Together these observations show that the carbon incorporated from glycerol into acylglycerols in *M. leprae* was all incorporated into the glycerol moiety.

Enzymes involved in utilization of carbon sources in lipid metabolism

The enzymes for conversion of acetate to acetyl-CoA, pyruvate to acetyl-CoA, of the glyoxylate bypass and the competing isocitrate dehydrogenase were assayed in *M. leprae*, and *M. avium* and *M. microti* grown in mice and the growth media described in Methods and above. Activities of all these enzymes in mycobacteria grown *in vivo* are presented in Table 4. Observations on their variation in the same *Mycobacterium* grown in different media are presented below. Some assays for each enzyme were done using dialysed extracts in case inhibitors were present in undialysed extracts. However, no marked difference of any activity was found between dialysed and undialysed extracts.

Table 4. *Enzymes involved in C-2 metabolism in mycobacteria from host tissues*

Enzyme assays were done at 25 °C as indicated in Methods. Values, $\pm$ SEM, represent specific activities determined by assaying in triplicate extracts prepared from bacteria from two or three isolates (except pyruvate dehydrogenase – one isolate only used, and phosphotransacetylase in *M. leprae*, five isolates used).

Enzyme	Specific activity [mU (mg protein ⁻¹)] in cell-free extracts of :		
	<i>M. leprae</i>	<i>M. microti</i> OV 254	<i>M. avium</i> CR1/69
Acetate kinase	0.17 $\pm$ 0.03	0.35 $\pm$ 0.04	0.96 $\pm$ 0.11
Phosphotransacetylase	0.02 $\pm$ 0.01*	27 $\pm$ 7.4	6.8 $\pm$ 1.2
Pyruvate dehydrogenase	0.09	0.39	0.29
Isocitrate dehydrogenase†	37 $\pm$ 1.0	13 $\pm$ 2.3	7.8 $\pm$ 0.3
Isocitrate lyase	0.35 $\pm$ 0.02	1.1 $\pm$ 0.13	1.6 $\pm$ 0.44
Malate synthase	18 $\pm$ 3.1	11.4 $\pm$ 0.5	8.5 $\pm$ 3.4

* Mean of activity in the five extracts; no activity detected in two of them; not significantly ($P > 0.10$) above zero.

† Activity five to eight times lower in extracts made from bacteria treated with NaOH. All other enzymes were present at similar activities in NaOH-treated and untreated bacteria.

Acetate kinase and phosphotransacetylase. Acetate kinase was present in all extracts of *M. leprae* in which it was assayed for. However, phosphotransacetylase was only possibly detected, at very low activity, in three extracts out of five. This finding might be due to the activity (see Table 4) being little above the minimum detectable [~ 0.01 mU (mg protein)⁻¹ in the assay using acetyl-CoA and phosphate as substrates] but as activity is not significantly ($P > 0.10$) above zero the existence of phosphotransacetylase in *M. leprae* remains in doubt. Mycobacteria convert acetate to acetyl-CoA using these two enzymes (Ratledge, 1982) but it might be that *M. leprae* has an acetyl-CoA synthetase instead. The synthetase catalyses the direct conversion of acetate to acetyl-CoA, at the same time converting ATP to ADP. Therefore, the assay for acetate kinase, dependent upon detecting ADP formation, could indicate acetyl-CoA synthetase activity also. However, this acetate-dependent conversion of ATP to ADP was not stimulated by CoA at 0.1, 0.25 or 0.5 mM. Furthermore, 0.1 mM-DTNB, which would bind any CoA in extracts of *M. leprae*, failed to inhibit completely acetate-dependent conversion of ATP to ADP, indicating that CoA was not necessary and the *M. leprae* possesses a true acetate kinase.

In a further attempt to detect acetyl-CoA synthetase, extracts of *M. leprae* were assayed for the enzyme using the radiochemical assay described in Methods. Acetyl-CoA was formed at the rate of 61–84 pmol min⁻¹ (mg protein)⁻¹ (range; three extracts, one of which was made from NaOH-treated *M. leprae*). However, this could be a result of conversion of acetate first to acetyl phosphate, then to acetyl-CoA; since radioactivity appeared in acetyl phosphate during this assay equivalent to 980–1860 pmol min⁻¹ (mg protein)⁻¹ and phosphotransacetylase, the enzyme for the second step was detected spectrophotometrically in two of the three extracts incubated with [¹⁴C]acetate in these experiments, at just above the limit for its detection [~ 0.05 mU (mg protein)⁻¹ – i.e. 50 pmol acetyl-CoA formed min⁻¹ (mg protein)⁻¹ from acetyl phosphate].

Acetate kinase and phosphotransacetylase activities varied considerably in extracts prepared from both *M. microti* and *M. avium* grown in the different media used in this study. Acetate kinase activity in extracts of mycobacteria grown *in vivo* (Table 4) was relatively low. However, phosphotransacetylase activity was relatively high in extracts of *M. microti* but relatively low in extracts of *M. avium* (though still easily detected), both grown *in vivo* (Table 4). When *M. microti* was grown in Dubos medium or Dubos with added palmitate both activities were relatively high: acetate kinase was detected at 4.6–6.2 mU (mg protein)⁻¹ and phosphotransacetylase was detected at 19–34 mU (mg protein)⁻¹. Only when they were grown in Dubos with glycerol added, or in Youmans and Karlson medium (which contains glycerol) were activities comparable with

activities found in mycobacteria grown *in vivo*: when they were in Dubos with glycerol added, acetate kinase and phosphotransacetylase were detected at 0.20 and 2.7 mU (mg protein)⁻¹ respectively in *M. microti* and 0.69 and 2.7 to 3.5 mU (mg protein)⁻¹ respectively in *M. avium*. The levels of acetate kinase and phosphotransacetylase activity do not correlate with the ability of intact bacteria to incorporate carbon from acetate into lipids (see Tables 2 and 3) but some activity is essential for conversion of acetate to acetyl-CoA in mycobacteria.

Isocitrate lyase and malate synthase. Both these enzymes were detected in extracts from *M. leprae* (Table 4). Their activities in both *M. microti* and *M. avium* grown *in vivo* are also shown in Table 4. Compared with activity in mycobacteria grown *in vivo* isocitrate lyase activity in bacteria grown on Dubos with glycerol added was about half, and in bacteria grown on Dubos, or Dubos with added palmitate, about double. A similar pattern of variation between activities in extracts from mycobacteria grown in the different media above and in mice was observed for malate synthase, but activity varied fivefold in *M. microti*, and sevenfold in *M. avium*.

Isocitrate dehydrogenase and pyruvate dehydrogenase. The presence of both these enzymes in *M. leprae* was confirmed (Table 4). Pyruvate dehydrogenase appeared constitutive, activity varying from 0.25 to 0.29 mU (mg protein)⁻¹ in extracts from *M. avium* and 0.33 to 0.48 mU (mg protein)⁻¹ in extracts from *M. microti* whether the organisms were grown in Dubos, Dubos with glycerol or palmitate added, or in mice (Table 4).

Isocitrate dehydrogenase activity did vary depending on how bacteria were grown, but not to any discernible pattern. For instance, in *M. microti*, activity was higher when bacteria were grown in Dubos with glycerol added [35 mU (mg protein)⁻¹] than when they were grown in Dubos with palmitate added [5.4 mU (mg protein)⁻¹]. In contrast, in *M. avium*, activity was higher when bacteria were grown in Dubos with palmitate added [8.8 to 22.5 mU (mg protein)⁻¹; three extracts] than when they were grown in Dubos with glycerol added [5.7 mU (mg protein)⁻¹]. Isocitrate dehydrogenase activity was labile when host-grown mycobacteria were treated with 0.5 M-NaOH (Table 4). The objective of the treatment was to abolish host-derived activities adsorbed to the surface of mycobacteria (Kanai, 1967; Wheeler, 1984*b*) but the activity in extracts of untreated host-grown mycobacteria is considered authentic since previous studies using PAGE showed that no tissue-derived isocitrate dehydrogenase could be found in such extracts (Wheeler, 1984*a*).

DISCUSSION

M. leprae, like all mycobacteria, invests a great deal of effort in synthesizing lipids (about 40% of its dry weight). Many lipids are complex and characteristic such as mycolic acids in the cell wall, glycolipids and some membrane lipids. All of these it must synthesize itself. However, it is far from certain that *M. leprae* can synthesize all the intermediates required for complex lipid synthesis. The results obtained here may indicate that *M. leprae* lacks the ability to synthesize some intermediates in lipid synthesis, in particular fatty acids, from simple carbon compounds. In that case, the intracellular *M. leprae* would depend to some extent on being able to scavenge fatty acids from its host.

Alternatively, the pathways for the synthesis of intermediates in lipid synthesis may be repressed or inhibited in mycobacteria when they are growing in the host, or more generally with an exogenous source of lipid. Fatty acid synthesis *de novo* in mycobacteria is known to be exquisitely sensitive to feedback inhibition by fatty acyl-CoA (Bloch, 1977), and repression or inhibition of fatty acid synthesis appears to occur in *M. convolutum* grown in the presence of alkenes (Ascenzi & Vestal, 1979) and *M. lepraemurium* grown in lipid-rich medium (Kusaka, 1977). Results obtained in this work also suggest that repression or inhibition does occur. Both intact *M. microti* and *M. avium* organisms incorporated carbon from acetate, pyruvate or glycerol considerably more slowly (in many cases over 20 times more slowly) when they were grown *in vivo*, or *in vitro* in the presence of palmitate (a fatty acid) than in Dubos medium alone (i.e. in the absence of added fatty acids) and it may be that the failure of *M. leprae* isolated from tissue to incorporate carbon from most carbon sources into lipid is also a result of metabolic control of activities.

Two substrates – acetate (at pH 7) and malonate – were not assimilated into *M. leprae*

organisms and therefore could not be utilized in lipid synthesis. Transport of substrates might itself be subject to metabolic control, certainly assimilation of acetate and glycerol into *M. avium* or *M. microti* grown *in vivo* takes place relatively slowly and this might limit the rate of lipid synthesis. However, some substrates (e.g. citrate) are clearly assimilated into mycobacteria but not used in lipid synthesis.

Lack of enzyme activity may explain the failure to use some carbon sources in lipid synthesis. Although isocitrate lyase, malate synthase and acetate kinase were fairly readily detected in *M. leprae*, phosphotransacetylase activity could not reliably be detected in *M. leprae*. Although phosphotransacetylase activity was susceptible to metabolic control in *M. microti* and *M. avium* its activity remained high when bacteria were grown *in vitro* in the presence of added palmitate and *in vivo* in the case of *M. microti*, so experiments with *M. microti* and *M. avium* cannot explain why phosphotransacetylase activity is absent or extremely low in *M. leprae*. Deficiency of phosphotransacetylase in *M. leprae* may explain its failure to utilize exogenous acetate, since the enzyme for conversion of acetate to acetyl phosphate (acetate kinase) was detected but no route for the rapid formation of acetyl-CoA from either acetate or acetyl phosphate was shown. Acetyl-CoA is essential for lipid synthesis, and it would appear that pyruvate dehydrogenase, which converts pyruvate to acetyl-CoA, is the key enzyme for acetyl-CoA production from carbohydrates in *M. leprae*. It seems that *M. leprae* is unable to metabolize efficiently any acetate which might be formed as a result of deacetylation reactions. Such an inability is not fatal, but restricts the number of carbon sources on which the microbe can grow, as evinced by mutant *E. coli* strains which cannot convert acetate to acetyl-CoA (Le Vine *et al.*, 1980).

Apart from the deficiency of phosphotransacetylase in *M. leprae*, most of the present findings on *M. leprae* might be explained as the metabolic activities of a Mycobacterium growing as *in vivo*. The other unusual finding in *M. leprae* was the failure to detect fatty acid synthesis or elongation (no radioactivity in fatty acids after incubations with [¹⁴C]glycerol). This is probably not a result of repression or inhibition of activity by lipids since some radioactivity was always traced in fatty acids after similar incubations with *M. avium* or *M. microti* grown *in vivo* or *in vitro* in the presence of palmitate, when fatty acid synthesis would be repressed. An explanation of this finding, and further elucidation of lipid biosynthesis in *M. leprae* would appear to await the next logical step – a study of the enzymes for fatty acid synthesis *de novo*, and for elongating existing fatty acids (such as palmitate which is so readily utilised) in intracellular mycobacteria including *M. leprae*.

Finally, formation of ¹⁴CO₂ from [¹⁴C]palmitate is the most easily detectable activity of *M. leprae* so far found, and this activity is inhibited by a number of antileprosy agents (Franzblau, 1988). Thus, it appears very promising for use in a simple, sensitive method to screen for activity of antileprosy agents.

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