Oxidation of Carbon Sources through the Tricarboxylic Acid Cycle in *Mycobacterium leprae* Grown in Armadillo Liver

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All the enzymes of the tricarboxylic acid cycle have now been demonstrated in extracts of *Mycobacterium leprae* grown in armadillo liver. Many were also present in homogenates of host-tissue, but biochemical evidence is presented which indicates that all enzymes detected in extracts from *M. leprae* were authentic bacterial enzymes. Further evidence for a complete tricarboxylic acid cycle in *M. leprae* was obtained by first establishing that citrate could be taken up and catabolized by whole *M. leprae* organisms, then showing that oxidation of radioisotopically labelled pyruvate to CO₂ by suspensions of *M. leprae* was stimulated by adding unlabelled citrate. Control of tricarboxylic acid cycle activity in *M. leprae* by the inactivation of fumarase by a protease is speculated upon.

INTRODUCTION

Knowledge of the catabolic pathways for possible carbon sources in *Mycobacterium leprae* is now becoming available. Glucose can be catabolized through glycolysis and the hexose monophosphate pathway, and glycerol is dissimilated through an intermediate, dihydroxyacetone phosphate, which is in the glycolysis pathway (Wheeler, 1982, 1983). Nevertheless, progress in elucidating metabolism in *M. leprae* is slow, as only 50–500 mg dry weight bacteria are obtained from one heavily infected armadillo, the only really practicable source.

Information on oxidative metabolism in *M. leprae* is rather fragmentary. Cytochrome *o*, a bacterial cytochrome, has been reported in extracts of *M. leprae* (Ishaque *et al.*, 1977); other cytochromes found in extracts may have been derived from host tissue. It is known that *M. leprae* can oxidize succinate (Wheeler, 1982) and malate (Wheeler & Bharadwaj, 1983), suggesting the presence of tricarboxylic acid cycle activity.

It has been suggested that *M. lepraemurium* lacks oxoglutarate dehydrogenase and therefore its tricarboxylic acid cycle has an anaplerotic function (Mori *et al.*, 1971). Recent work suggests that *M. lepraemurium* (a slow-growing, intracellular organism like *M. leprae*) is not a good model for metabolism in *M. leprae* (Wheeler & Gregory, 1980; Wheeler, 1982, 1983). However, other micro-organisms with specialized modes of growth have deletions of enzyme(s) in the Krebs cycle. Therefore the purpose of this study was to show whether or not the complete cycle operates in *M. leprae*. This involved looking for all the enzymes in extracts and testing whether rates of oxidation of substrates by intact *M. leprae* were stimulated by cycle intermediates. Before making comparisons of the operation of the Krebs cycle in *M. leprae* and *M. lepraemurium*, a further attempt to detect oxoglutarate dehydrogenase in *M. lepraemurium* was made.

METHODS

Mycobacteria. *Mycobacterium leprae* and *Mycobacterium lepraemurium* were harvested from armadillo liver and mouse spleen, respectively (Wheeler & Gregory, 1980). Purified suspensions (Report, 1980) were prepared by tissue homogenization followed by treatment with DNAase and Percoll (Pharmacia) density-gradient

Abbreviations: INT, *p*-iodonitrotetrazolium violet; NBT, nitroblue tetrazolium; PMSF, phenylmethylsulphonyl fluoride; TPP, thiamin pyrophosphate.

centrifugation (only for *M. leprae*). When cell-free extracts were required, suspensions were further purified on an aqueous two-phase system [7 g Dextran T500 (Pharmacia), 4.9 g polyethylene glycol 6000, 0.1 g polyethylene glycol monopalmitate, 0.01 M-NaCl, 0.01 M-potassium phosphate (pH 6.9); bacteria suspended in buffered Tween (see below); and distilled water to 100 g in total (Report, 1980)]. *Mycobacterium phlei* NCTC 10266 was grown as described by Wheeler & Gregory (1980). Cell-free extracts of mycobacteria were prepared by ultrasonication and centrifugation (Wheeler & Gregory, 1980). Since thiols are required to preserve some enzyme activities, some suspensions of *M. leprae* organisms were disrupted in sonication buffer (Wheeler & Gregory, 1980) containing 0.1 mM-dithiothreitol (DTT). Enzymes were assayed in cell-free extracts prepared in the presence or absence of this thiol.

Extracts of host tissues. Liver tissue from uninfected armadillos or mice spleen was homogenized in 50 mM-phosphate ($\text{Na}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$, pH 7.5) buffer with 1 mM- MgCl_2 . Extracts of infected tissues were prepared from supernatants of the first homogenate (0.2 M-Tris; pH 8.7) after centrifugation at 16000 g for 10 min during the preparation of pure mycobacteria (Wheeler & Gregory, 1980).

*NaOH treatment of *M. leprae*.* NaOH treatment of leprosy bacilli (Wheeler & Gregory, 1980; Wheeler *et al.*, 1982) and tubercle bacilli (Kanai, 1967) abolished adsorbed host-derived activity. Such treatment does not affect *M. leprae* viability (A. C. R. E. Lowe, personal communication). Suspensions of *M. leprae* were incubated in 1 M-NaOH for 1 h at 25 °C, as described by Wheeler *et al.* (1982).

Desalting of extracts. Desalted extracts from *M. leprae* and armadillo liver were prepared with Amicon CF25 centrifuge ultrafiltration cones (700 g, 4 °C; molecular weight cut off 25000). After desalting, the salt concentration was 1% (w/v) of the level in extracts that were not desalted.

Radioisotopes. 2-Oxo[5- ^{14}C]glutaric acid, sodium salt (15.9 mCi mmol $^{-1}$; 0.58 GBq mmol $^{-1}$), [6- ^{14}C]glucose (52.7 mCi mmol $^{-1}$; 1.95 GBq mmol $^{-1}$), [2- ^{14}C]pyruvic acid (18.3 mCi mmol $^{-1}$; 0.68 GBq mmol $^{-1}$) and [1,5- ^{14}C]citric acid (111 mCi mmol $^{-1}$; 4.11 GBq mmol $^{-1}$) were obtained from Amersham.

*Incubations of *M. leprae* with radioisotopes.* Bacteria (0.5–1.4 mg dry wt) were incubated with ^{14}C -labelled substrates as follows: glucose – 1 μCi , 0.1 mM; pyruvic acid – 0.37 μCi , 0.1 mM; and citric acid – 1 μCi , 0.1 mM. The incubation mixtures (200 μl) also included HEPES (50 mM), MgSO_4 (5 mM), and $\text{Na}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$ (pH 7; 1 mM) adjusted to pH 7.0 with KOH. Additionally, penicillin (50 U ml $^{-1}$) was added to prevent growth of any chance contamination in the incubation mixtures, but this did not affect metabolic activities. Incubations were done in Warburg flasks at 34 °C for 20 h. The centre well contained 400 μl 1 M-NaOH to absorb CO_2 evolved from oxidation of the substrate. Reactions were stopped by addition of 0.1 ml 1.5 M- H_2SO_4 from a side-arm: 1 h later the NaOH was added to Ready-Solv EP (Packard)/distilled water (10:3, v/v) for scintillation counting. After incubations with citrate, bacteria were collected and washed by centrifugation and resuspension until the supernatant was free of radioactivity. The final pellet was incubated for 1 h in Soluene-350 (Packard) at 37 °C, then 10 ml Dimilume-30 (Packard) was added. The amount of radioactivity assimilated was estimated by scintillation counting of this pellet. Results are expressed as d.p.m. per mg dry wt mycobacteria; when pure *M. leprae* organisms are dried and weighed, 10 10 bacteria weigh approximately 1 mg (R. J. W. Rees, personal communication). Controls contained either no bacteria, or, for determination of assimilation, heat-killed (100 °C, 15 min) bacteria. In the results, d.p.m. in controls are always subtracted.

Enzyme assays and enzyme nomenclature. All enzymes were assayed in crude extracts at 25 °C; incubation volume was 500 μl unless otherwise stated. Carboxylic acids were added as sodium salts, or free acids were used and adjusted to assay pH with NaOH, unless otherwise stated.

Pyruvate dehydrogenase [EC 1.2.4.1; pyruvate:lipoamide oxidoreductase (decarboxylating and acceptor-acetylating)] was assayed by a modification of the methods used by Murthy *et al.* (1962). Extracts were incubated with 50 mM-HEPES (adjusted to pH 7.0 with KOH), 7 mM-pyruvate, 3.3 mM- MgSO_4 , 0.05 mM-CoA, 0.02 mM-thiamin pyrophosphate (TPP) and 0.1 mM-DTT, plus either 0.09 mM-NADP, in which case NADPH formation was followed by reading ΔA_{340} , or 0.17 mM-NAD, 0.6 mM-*p*-iodonitrotetrazolium violet (INT) and 0.05 units diaphorase (Sigma; from porcine heart), in which case reduction of INT was followed by reading ΔA_{500} ($\epsilon_{\text{mM}}^{1\text{cm}} = 12.6$).

Citrate synthase [EC 4.1.3.7; citrate oxaloacetate-lyase (*pro*-3S- $\text{CH}_2\text{COO}^- \rightarrow$ acetyl-CoA)] was assayed by a modification of the method of Weitzman (1969), with 100 mM-Tris/HCl (pH 8.0), 3.75 mM-oxaloacetate, 0.05 mM-acetyl CoA and 0.1 mM-5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB). The formation of the mercaptide ions of DTNB, stoichiometric with CoA formation, was followed by reading ΔA_{412} ($\epsilon_{\text{mM}}^{1\text{cm}} = 13.6$).

Aconitase [EC 4.2.1.3; citrate (isocitrate) hydro-lyase] was activated and assayed by the method of Fansler & Lowenstein (1969). Activation was in the presence of 5 mM-thiomalate/Tris (pH 7.8) and 2 mM-ferrous ammonium sulphate (the two solutions were gassed with nitrogen and kept separate until needed). Enzyme assay was with 20 mM-Tris/HCl (pH 7.4), 100 mM-NaCl and 0.2 mM-*cis*-aconitate. The fall in A_{240} due to disappearance of *cis*-aconitate ($\epsilon_{\text{mM}}^{1\text{cm}} = 4.88$) was followed.

Isocitrate dehydrogenase [EC 1.1.1.42; threo-D $_x$ -isocitrate:NADP oxidoreductase (decarboxylating)] was assayed by a modification of a method used for *M. phlei* (Dhariwal & Venkitasubramanian, 1975), with 40 mM-

Tris/HCl (pH 7.3), 5 mM-MgCl₂, 0.33 mM-NADP and 3 mM-threo-D₅-isocitrate. NADPH formation was followed by reading ΔA_{340} .

Oxoglutarate dehydrogenase [EC 1.2.4.2; 2-oxoglutarate:lipoamide oxidoreductase (decarboxylating and acceptor-succinylating)] was assayed by methods based on those used for *M. tuberculosis* (Murthy *et al.*, 1962). The reaction mixture was 100 mM-Na₂HPO₄/NaH₂PO₄ (pH 7.2), 0.05 mM-CoA, 10 mM-2-oxoglutarate, 0.33 mM-TPP and 1.5 mM-MgSO₄, plus either 0.33 mM-NADP, in which case ΔA_{340} was followed, or 0.33 mM-NAD, 0.6 mM-INT and 0.05 units diaphorase (Sigma; porcine heart), in which case ΔA_{500} was followed. Alternatively, the assay was done with 2-oxo[5-¹⁴C]glutarate (1 μ Ci; 2.5 mM), in which case the reaction mixture was altered in that 25 mM-phosphate buffer (pH 7.2) was used and the volume was 25 μ l. An alternative, radiochemical, assay included 2-oxo[5-¹⁴C]glutarate (1 μ Ci; 2.5 mM), 25 mM-phosphate buffer, 0.05 mM-CoA, 0.33 mM-TPP, 1.5 mM-MgSO₄, 0.33 mM-K₃Fe(CN)₆, and 3.3 mM-KCN. In all radiochemical assays, 5 mM-malonate, 0.5 mM-GDP and 0.05 units succinyl CoA synthetase (Sigma; from porcine heart) were included in the incubation mixture so that succinate was formed, but not oxidized. Succinate formed from 2-oxoglutarate was detected by stopping the reaction after 50 min with 4 μ l 6 M-HCl, removing the precipitate by centrifugation, applying 12 μ l samples to Kodak-Eastman 'Chromogram' cellulose thin-layer chromatography plates together with 60 nmol unlabelled carboxylic acids, developing with diethyl ether/acetic acid (glacial)/distilled water (13:3:1, by vol.), drying, spraying with acid-base indicator [0.04 g bromocresol green (3', 3'', 5', 5''-tetrabromo-*m*-cresolsulphone-phthalein) in 96% ethanol (100 ml); 0.1 M-NaOH added until a blue coloration just appeared], cutting out the spots corresponding to 2-oxoglutarate and succinate, and counting in 10 ml Ready-Solv EP (Packard). The amount of succinate formed was calculated from the proportion of total counts in the succinate spot; controls included distilled water in place of extract.

Succinyl-CoA synthetase (EC 6.2.1.4; succinate:CoA ligase (GDP-forming)) was assayed by a modification of a method described by Cha (1969), with 50 mM-Tris/succinate (22.7 mM with respect to succinate; pH 7.4), 10 mM-MgCl₂, 100 mM-KCl, 0.1 mM-GTP, 0.1 mM-CoA, 1.5 mM-phosphoenolpyruvate (tricyclohexammonium salt), 0.2 mM-NADH, and pyruvate kinase/lactate dehydrogenase mixture (Sigma, from rabbit muscle; 10 U ml⁻¹ and 12.5 U ml⁻¹ respectively). Oxidation of NADH, stoichiometric with succinyl-CoA and GDP formation, was followed by reading A_{340} .

Succinate dehydrogenase (EC 1.3.99.1; succinate:acceptor oxidoreductase) was assayed with 25 mM-Tris/succinate (11.4 mM with respect to succinate; pH 7.4), 1 mM-KCN, 10 μ M-phenazine methosulphate and 0.6 mM-INT. Reduction of INT was followed by reading A_{500} .

Fumarase (EC 4.2.1.2; fumarate hydro-lyase) was assayed by modifications of the method of Hill & Bradshaw (1969), with 20 mM-L-malate, K₂HPO₄ (50 mM), adjusted to pH 7.3 with NaOH. Alternatively, 1 mM-fumarate and 50 mM-K₂HPO₄/NaH₂PO₄ buffer was used. In each case, appearance or disappearance of the double bond in fumarate was followed by reading A_{240} ($\epsilon_{1\text{ cm}}^{1\%}$ for fumarate = 2.44).

Rate of reaction in the absence of substrate (i.e. in control incubations) was subtracted from rate of reaction in complete incubation mixtures. Enzyme activities were calculated from the initial velocity of the reaction, and expressed as units (U) where one unit catalyses the conversion of 1 μ mol substrate min⁻¹. Inhibitors (all from Sigma) were added to extracts in assays before the coupling enzymes and 5 min before substrates.

PAGE. Extracts (150–300 μ g protein) were applied to 7% (w/v) polyacrylamide gels and electrophoresed at about pH 6.5 (Chang *et al.*, 1979). After electrophoresis the gels were washed in the buffer of the incubation medium for 15 min, then incubated in the following. For detection of isocitrate dehydrogenase: 88 mM-Tris/HCl (pH 7.3), 10 mM-MnCl₂, 0.1 mM-NADP or NAD, 0.11 mM-phenazine methosulphate, 0.78 mM-3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and 6 mM-DL-isocitrate, for 3–4 h. For detection of succinate dehydrogenase: 50 mM-Tris/succinate (pH 7.4) (Tris/HCl was used for controls with no substrate); 1 mM-KCN, 0.07 mM-phenazine methosulphate or FAD and 0.3 mM-nitroblue tetrazolium (NBT).

Statistical analysis. The Wilcoxon (rank) test (Colquhoun, 1971) was used to test the following null hypotheses: (i) in experiments with enzyme inhibitors/activators, their effect on the activity being studied in both extracts of *M. leprae* and armadillo liver was identical (their effect was expressed as the percentage of the activity in a control incubation with no inhibitor/activator present); (ii) CO₂ evolution, in experiments with radioisotopes, was not affected by the addition of unlabelled tricarboxylic acid cycle intermediates; (iii) CO₂ evolution, in experiments with radioisotopes, was the same in controls and with live *M. leprae*.

RESULTS

Pyruvate dehydrogenase and tricarboxylic acid cycle enzymes

Pyruvate dehydrogenase. This enzyme was present at low specific activity in crude extracts of *M. leprae*; consequently it was not possible to detect it on polyacrylamide gels, nor to carry out studies with inhibitors. When TPP or CoA were omitted from incubations, no activity was

Table 1. *Activities of enzymes of the tricarboxylic acid cycle and pyruvate dehydrogenase*

Values for specific activities in untreated *M. leprae* include assays done with extracts of *M. leprae* sonicated with 0.1 mM-DTT; in these extracts, specific activity was not markedly different from that in extracts without DTT. Values for specific activities in NaOH-treated *M. leprae* represent assays done with one or two extracts of NaOH-treated *M. leprae*. Values for specific activities in armadillo liver were determined by assaying extracts from liver homogenized at pH 7.5 if activity could be detected in armadillo liver from infected tissue, which was homogenized at pH 8.7. Each figure represents an assay done at least in triplicate.

Enzyme	Specific activity [mU (mg protein) ⁻¹] in extracts of:			
	Untreated <i>M. leprae</i> *	NaOH-treated <i>M. leprae</i>	Armadillo liver	<i>M. phlei</i>
Pyruvate dehydrogenase	0.093	0.083	nil†	0.80
Citrate synthase	0.321	0.183	12.8	5.1
Aconitase	13.1	9.3	4.5	52.0
Isocitrate dehydrogenase	36.4	6.1	26.7	ND
Oxoglutarate dehydrogenase	0.17	0.14	nil†	2.0
Succinyl-CoA synthetase	4.7	5.3	21.4	5.1
Succinate dehydrogenase	5.4	6.2	0.69	2.9
Fumarase	40.7	38.3	35.9	17.5

ND, Not done.

* Contains some contaminating host enzymes (Wheeler *et al.*, 1982).

† Limit of detection is 0.010 mU (mg protein)⁻¹.

Table 2. *Differences between enzymes in extracts of M. leprae and armadillo liver*

Extracts were assayed with inhibitors/activators of enzyme activity, or NADP in place of NAD, and the results are expressed as the percentage of the activity in incubations without inhibitor/activator, or with NAD, respectively. Each figure represents an assay done at least in triplicate. For isocitrate dehydrogenase and succinate dehydrogenase, differences between enzymes from extracts of *M. leprae* and armadillo liver were demonstrated by PAGE, followed by staining specifically for these enzymes (Fig. 1).

Enzyme	Property studied	Value obtained*	
		<i>M. leprae</i>	Armadillo liver
Pyruvate dehydrogenase†	Activity with NADP	nil	ND†
Citrate synthase	Activity with 5 mM-ATP	35%	45%
Aconitase	Activity with 0.1 mM-fluorocitrate	40%	60%
Oxoglutarate dehydrogenase†	Activity with NADP	105%	ND†
Succinyl-CoA synthetase	Activity with 10 mM-NH ₂ OH	40%	125%
Fumarase	Inactivation by a protease from <i>M. leprae</i> (6 h)	95%	<15%

ND, Not done.

* Differences in properties were all significant ($P < 0.05$) except with ATP inhibition of citrate synthase ($P > 0.10$).

† Enzyme not detected in armadillo liver extract. Activity of mammalian enzyme with NADP would be nil.

detected. In the absence of added DTT, activity was only 40% of that in the presence of DTT, except when extracts were made with DTT in the sonication buffer. The presence of this enzyme in extracts from NaOH-treated *M. leprae* is evidence for its authenticity as a bacterial enzyme (Table 1). The chance of contamination with host enzyme was very low in any case, since the enzyme was not detected in infected armadillo liver tissue (Table 1).

Citrate synthase. ATP may be a more potent inhibitor of the *M. leprae* enzyme than the host enzyme, but this difference was not statistically significant ($P > 0.10$) (Table 2). However, citrate synthase activity was detected in extracts of NaOH-treated *M. leprae* (Table 1).

Aconitase. *cis*-Aconitate was included in incubations at a low concentration (Fansler & Lowenstein, 1969) since a 1 cm light path and crude extracts with a high absorbance at 240 nm were used. Aconitase in extracts of *M. leprae* was readily detected by this method and was distinguished from the host enzyme (Table 2) on the basis of its greater sensitivity ($P < 0.05$) to

Fig. 1. PAGE of extracts on gels at pH 6.5. (a) Stained for isocitrate dehydrogenase (tracks 2-6) and compared with control (no substrates: track 1). NADP was the coenzyme in tracks 1-5, and NAD in track 6. Extracts (some gels with two applied together) were from: *M. leprae* (tracks 3, 4, 6); NaOH-treated *M. leprae* (tracks 1, 2); uninfected armadillo liver (tracks 1, 4, 5, 6). (b) Stained for succinate dehydrogenase (tracks 8, 9, 10, 12, 13) and compared with controls (tracks 7, 11). Phenazine methosulphate was omitted in track 12. Extracts were from: *M. leprae* (tracks 11-13); uninfected armadillo liver (tracks 7-9) - this liver extract was ultrasonicated (11 min at 100 W) by the method described for mycobacteria (Wheeler & Gregory, 1980) in tracks 8 and 9. Controls (above) were all without enzyme substrate. m, denotes enzyme from *M. leprae*; a, denotes enzyme from armadillo liver; b, denotes bromophenol blue (marker; only visualized after staining when NBT was used).

inhibition by fluorocitrate (barium salt). Comparisons of enzyme activities in different extracts were made with non-activated extracts (Tables 1 and 2). After activation (25 °C, 30 min), 38 mU aconitase (mg protein)⁻¹ was detected in the extract from NaOH-treated *M. leprae*.

Isocitrate dehydrogenase. The NADP-dependent enzyme was detected in extracts of both *M. leprae* and armadillo liver. On gels, it was shown that the enzymes from these two sources had different electrophoretic mobilities (Fig. 1, tracks 2-5), and that there was no activity in either extract when NAD was substituted for NADP (Fig. 1, track 6).

Oxoglutarate dehydrogenase. The values for specific activity in Table 1 were derived from results with both the spectrophotometric and the radioisotopic method, with NAD in the incubation mixture. The values obtained were similar with the two methods, although the concentration of substrate was 10 mM and 2.5 mM respectively. The reaction rate was linear up to 100 min in the spectrophotometric assay. The comparison between activity with NADP and NAD (Table 2) was made with the radioisotopic method only. When ferricyanide was used in place of the pyridine nucleotides, the rate of appearance of succinate, in incubations including *M. leprae* extracts, was enhanced by 50%.

This enzyme was also detected in extracts of *M. lepraemurium*. The rate was noticeably more rapid in the first 4 min (spectrophotometric method), and specific activities in extracts calculated after 4 min (six incubations) were: from untreated bacteria, 0.63 mU (mg protein)⁻¹; from NaOH-treated bacteria, 0.22 mU (mg protein)⁻¹. Lower values (50% of total above) were calculated from the radioisotopic method, by which it could be shown that both bacterial extracts used NADP as coenzyme at 80% the rate of NAD. No activity was detected in an extract of mouse spleen.

The low activity in extracts, and complex requirement for cofactors, made it impossible to detect the enzyme from *M. leprae* or *M. lepraemurium* on gels.

Succinyl-CoA synthetase. This enzyme was present at a relatively high specific activity in extracts of *M. leprae* (Table 1). It was inhibited by 10 mM-hydroxylamine (NH₂OH), in contrast to the activity in extracts from armadillo liver, which was slightly increased (Table 2).

Succinate dehydrogenase. Activity at the level shown in Table 1 could be measured in extracts of *M. leprae* even if phenazine methosulphate was omitted from the reaction mixture. However, a band of activity corresponding to *M. leprae* succinate dehydrogenase was only detected on gels (after electrophoresis) when phenazine methosulphate was included in the incubation mixture (Fig. 1). Succinate dehydrogenase in armadillo liver extracts did not migrate into gels unless the extracts were sonicated (11 min, 100 W, as for *M. leprae* organisms) before electrophoresis: the activity observed after sonication had a different mobility from the activity in extracts of *M. leprae* (Fig. 1, tracks 9 and 13). No bands were detected when FAD was substituted for phenazine methosulphate.

Fumarase. In these studies, fumarase in extracts of *M. leprae* was the only enzyme activity to decrease appreciably after storage at 4 °C (activity was retained, as for the other enzymes, when extracts were stored at -80 °C). This inactivation was 95% of the original activity in extracts from NaOH-treated *M. leprae* in 6 h (Table 2) but it could be inhibited by addition of 0.2 mM-phenylmethylsulphonyl fluoride (PMSF: added from a 5 mM stock solution in dimethyl sulphoxide immediately on thawing extracts) – inactivation was 30% in 6 h. In contrast only 20% loss of activity of fumarase in armadillo liver extract, not preventable by addition of 0.2 mM-PMSF, occurred over 6 h at 4 °C. When an extract of NaOH-treated *M. leprae* and an extract of armadillo liver (both freshly thawed) were mixed in the ratios (by weight, protein) 1:4 or 2:1, the inactivation of the fumarase from armadillo liver was 35% in both mixtures over 6 h at 4 °C. The result with the latter mixture was from triplicate incubations. When 0.2 mM-PMSF was added to the second mixture, inactivation was 28%. Differences between (i) liver extract and (ii) liver extract/*M. leprae* extract mixture $\pm$ PMSF can be used to calculate inactivation over 6 h of the liver fumarase due to mixing with *M. leprae* extract; the maximum value was 15% inactivation. In a mixture (1:1 by weight, protein) of *M. phlei* extract and *M. leprae* extract (stored at 4 °C for 2 h), fumarase activity from *M. phlei* was lost at the same rate as fumarase activity in an *M. phlei* extract only. The loss of activity in both the mixture or the *M. phlei* extract alone was 40% of the original *M. phlei* activity in 6 h, and was not inhibited by PMSF.

Catabolism of substrates by unbroken M. leprae

[¹⁴C]-labelled citrate and succinate were both taken up and catabolized to CO₂ by suspensions of purified *M. leprae* (Table 3). Citrate and glutamate (known to be a substrate for *M. leprae*; Prabhakaran & Braganca, 1962) were then used to supply tricarboxylic acid cycle intermediates, and to determine whether their addition to suspensions of *M. leprae* stimulated glucose and pyruvate oxidation. ¹⁴CO₂ evolution with suspensions of *M. leprae* plus either [6-¹⁴C]glucose or [2-¹⁴C]pyruvate was measured (Table 3) with and without the two cycle intermediates (all at 0.1 mM). Only pyruvate oxidation was stimulated; mean d.p.m. in CO₂ evolved was similar when citrate or glutamate was added, representing ~50% stimulation. However, only the stimulation by citrate was statistically significant ($P < 0.05$).

DISCUSSION

All the enzymes of the tricarboxylic acid cycle have been demonstrated in extracts of *M. leprae*. They could all be detected at similar levels in extracts from untreated and NaOH-treated *M. leprae*, although isocitrate dehydrogenase was present in NaOH-treated *M. leprae* at 17% the level in untreated *M. leprae*. This degree of inactivation may reflect the location of isocitrate dehydrogenase in *M. leprae* at a (surface?) site where it is exposed to NaOH treatment since electrophoresis shows clearly that isocitrate dehydrogenase in untreated *M. leprae* is not host-derived (Fig. 1). Enzyme activities adsorbed to mycobacteria from host tissue are completely abolished by NaOH treatment (Kanai, 1967; Wheeler & Gregory, 1980; Wheeler *et al.*, 1982). Since two of the enzyme activities are higher in NaOH-treated than untreated *M. leprae* (Table 1) it is suggested that the differences between activities in treated and untreated *M. leprae* reflect

Table 3. *Effect of tricarboxylic acid cycle intermediates on oxidation of carbon sources*

Incubations were for 20 h, with untreated, intact *M. leprae*. No CO₂ was evolved when extract from *M. leprae* or armadillo liver was substituted for the whole bacteria. Both labelled and unlabelled substrates were at 0.1 mM; 1 μ Ci radiolabel was included except in the case of [2-¹⁴C]pyruvate (0.37 μ Ci). Values in the table were subtracted from values obtained with heat-killed controls or (CO₂ evolved only) controls with no bacteria. All were significantly above control values ($P < 0.01$ except when indicated). At least four controls and nine incubations (except with glucose, three and six respectively) with live *M. leprae* were done. No significant differences were observed between metabolic activities in *M. leprae* from different tissues.

Substrates		CO ₂ evolved (d.p.m. per mg <i>M. leprae</i>)
Labelled*	Unlabelled	
Glucose	None	2650
Glucose	Citrate	2460
Glucose	Glutamate	2770
Pyruvate	None	4150†
Pyruvate	Citrate	6170
Pyruvate	Glutamate	6230
Citrate	None	2930†‡

* Distribution of radioisotopic labelling was selected to avoid, as far as possible, ¹⁴CO₂ evolution without catabolism in the tricarboxylic acid cycle.

† Assimilation (d.p.m. per mg *M. leprae*) was 2600 for pyruvate and 3060 for citrate.

‡ $P < 0.05$.

variation in the preparation of different extracts and do not constitute evidence for the removal of residual Krebs cycle enzymes from the host. Activity varied widely from enzyme to enzyme, both in terms of units and relative to *M. phlei* (Table 1). However, no significance could be attached to this variation, which could be a result of assaying the enzymes in very different conditions to those in the living organism. For instance, enzymes of the tricarboxylic acid cycle are generally attached to membranes in bacteria (Doelle, 1975). In these experiments, very little activity is attached to membranes (as shown by successful electrophoresis of the enzymes) after the ultrasonication treatment used to disrupt the mycobacteria.

It was possible to demonstrate at least one difference between enzymes in extracts of *M. leprae* and armadillo liver, when activity could be detected in host tissue. Pyruvate and oxoglutarate dehydrogenases were not detectable in armadillo liver homogenized in Tris at pH 8.7 (Table 1) but a property of these dehydrogenases in some bacteria – the ability to use NADP as well as NAD – could be shown in *M. leprae*. Many of the tricarboxylic acid cycle enzymes were at low levels in the extract of host-tissue, and this may be a result of using homogenization methods unsuitable for the isolation of mitochondrial enzymes. In this context, the isocitrate dehydrogenase detected from host tissue was the NADP-dependent cytoplasmic enzyme. Mitochondrial, NAD-dependent isocitrate dehydrogenase was not detected (Fig. 1).

An 'incomplete Krebs cycle' in micro-organisms is generally a result of the deletion of oxoglutarate dehydrogenase (Doelle, 1975). This has been suggested for *M. lepraemurium* (Mori *et al.*, 1971) so it was of particular interest to demonstrate this enzyme in *M. leprae* (Table 1). However, the enzyme was also detected in extracts of *M. lepraemurium* during this work. Oxoglutarate dehydrogenase in extracts of both bacteria was demonstrated by NAD and NADP reduction in the presence of oxoglutarate, and formation of succinate in incubations with oxo[5-¹⁴C]glutarate. There is a risk that NaOH treatment may remove enzymes of *M. leprae* located in the surface as well as activities adsorbed from the host and it is possible that Mori and co-workers abolished the *M. leprae*-derived activity by their NaOH-treatment; in this work similar treatment of *M. lepraemurium* abolished 62% of the activity.

Further evidence for the complete nature of the tricarboxylic acid cycle in *M. leprae* was obtained by showing that pyruvate oxidation (to CO₂) could be stimulated by citrate (Table 3). This observation was made with pyruvate radioisotopically labelled in such a position (C-2) that ¹⁴CO₂ would be evolved in Krebs cycle rather than in the reaction catalysed by pyruvate dehydrogenase. It replaced the classical demonstration of Krebs cycle activity in which

endogenous or pyruvate-dependent O_2 uptake is catalytically stimulated by a cycle intermediate. The evolution of $^{14}CO_2$ from $[6-^{14}C]$ glucose is almost completely dependent upon tricarboxylic acid cycle activity – the alternative possibility for limited $^{14}CO_2$ release is extensive cycling through the hexose monophosphate pathway (Wood, 1955), which can be neglected in *M. leprae* where hexose monophosphate pathway activity is limited (Wheeler, 1983). Evolution of CO_2 was not stimulated by citrate or glutamate, but it is possible that some enzymes in the Embden–Meyerhoff pathway, through which most glucose is catabolized in *M. leprae* (Wheeler, 1983), are inhibited by cycle intermediates.

Previous research on anaerobic pathways of carbon metabolism (Wheeler, 1982, 1983) and oxidation of malate (Wheeler & Bharadwaj, 1983), and the present work, suggest that *M. leprae* is competent in its ability to catabolize different carbon sources such as glucose, glycerol and 6-phosphogluconate completely to CO_2 . Thus, failure to cultivate the leprosy bacillus appears not to be related to difficulty in finding suitable substrates for energy production. However, metabolic activity of suspensions of *M. leprae* is low and the growth of *M. leprae* is very slow. The low levels of enzymes in extracts of *M. leprae* relative to other mycobacteria may be related to these observations. An interesting observation is that there is a protease (evidence for the protease nature of the activity is restricted to its inhibition by PMSF) in extracts of *M. leprae* which has been shown to act only against the fumarase of *M. leprae*. Loss of activity of other tricarboxylic acid cycle enzymes in extracts of *M. leprae* was not noticeable, and fumarases in extracts of armadillo liver or *M. phlei* were not inactivated when those extracts were mixed with *M. leprae*. It may be possible for this protease to control Krebs cycle activity, and therefore the rate of catabolism of substrates in *M. leprae*, if the protease can inactivate fumarase in the living organism as well as in extracts in experimental conditions.

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