Catabolic Pathways for Glucose, Glycerol and 6-Phosphogluconate in *Mycobacterium leprae* grown in Armadillo Tissues

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With radioisotopes, it was shown that suspensions of *Mycobacterium leprae* oxidized glycerol, 6-phosphogluconate, glucose, glucose 6-phosphate, and, at a low rate, gluconate, to CO₂. The incubation period in these experiments was usually 20 h, but after 140 h up to five times more glucose and gluconate had been converted to CO₂. Studies with differentially labelled glucose indicated that glycolysis and the hexose monophosphate pathway were used for glucose dissimilation.

Key enzymes of glycolysis, the hexose monophosphate pathway and glycerol catabolism were detected in cell-free extracts from purified *M. leprae*, but phosphoketolase, Entner–Doudoroff pathway activity and gluconate kinase were absent. All these enzymes were present also in host-tissue, but biochemical evidence is presented which indicates that all enzymes detected in extracts from *M. leprae* were authentic bacterial enzymes. Additionally, they could all be detected in extracts of *M. leprae* prepared by treatment with NaOH in which host enzymes adsorbed to *M. leprae* are inactivated.

INTRODUCTION

When the literature on the intermediary metabolism of mycobacteria was reviewed (Ramakrishnan *et al.*, 1972; Ratledge, 1976), nothing was known about the carbon metabolism of *Mycobacterium leprae*. Glutamate decarboxylase (Prabhakaran & Braganca, 1962) and *o*-diphenoloxidase (Prabhakaran, 1973) had been identified as enzymes of *M. leprae*, but their metabolic significance was (and is) not known.

Mycobacterium leprae has not been cultured, but the bacteria may be obtained from experimentally infected nine-banded armadillos (*Dasypus novemcinctus* Linn.). The discovery that armadillos develop a systemic infection (Kirchheimer & Storrs, 1971) has made available two to three orders of magnitude more leprosy bacilli for experimental work than were available from human biopsies, the previous best source. With *M. leprae* from armadillos, some glycosidases (Matsuo & Skinsnes, 1974; Wheeler *et al.*, 1982), acid phosphatase (Wheeler *et al.*, 1982), γ -glutamyl transpeptidase (Shetty *et al.*, 1981) and enzymes which catabolize the breakdown of oxygen-free radicals (Kusunose *et al.*, 1980; Wheeler & Gregory, 1980) have been identified as enzymes of *M. leprae*.

It has been established that glucose (Khanolkar, 1982) and possibly glycerol (S. R. Khanolkar, personal communication) are taken up by *M. leprae*, and preliminary evidence indicates that glycolysis and the hexose monophosphate pathway (HMP) are involved in glucose metabolism (Wheeler, 1982). In this study, suspensions of *M. leprae* were incubated with carbohydrates to look for catabolism to CO₂, and further key enzymes were assayed in extracts of purified *M. leprae*. Properties such as the effect of inhibitors, affinity for substrates, and electrophoretic

Abbreviations: DHAP, Dihydroxyacetone phosphate; EDP, Entner–Doudoroff pathway; HMP, hexose monophosphate pathway; ITP, inosine triphosphate; NBT, nitro blue tetrazolium; PMS, methylphenazonium methosulphate; UM-P, 4-methylumbelliferyl phosphate.

mobility of enzymes were studied, mainly to compare *M. leprae* enzymes with those from host-tissue. It is essential to make such comparisons because enzymes detected in extracts of *M. leprae* could be contaminating activities derived from host-tissue (Wheeler *et al.*, 1982; Wheeler & Gregory, 1980). Differences in properties would provide evidence for authentic *M. leprae* enzymes.

Initially, the catabolism of glucose by glycolysis and 'alternative' pathways, e.g. HMP, Entner–Doudoroff Pathway (EDP), as well as glycerol catabolism was studied. The observation of remarkably high levels of 6-phosphogluconate dehydrogenase and transketolase (Wheeler, 1982) led to experiments with 6-phospho[^{14}C]gluconate and [^{14}C]gluconate as possible substrates for *M. leprae*. There are two problems in the experiments in which suspensions of *M. leprae* are incubated with sugar phosphates. Experiments were designed so as to overcome possible problems due to the presence of different uptake systems for sugars and sugar-phosphates and to the degradation of sugar-phosphates by acid phosphatase.

METHODS

Mycobacteria. *Mycobacterium leprae* and *Mycobacterium lepraemurium* were harvested from armadillo liver or lymph node, and mouse liver, respectively (Wheeler & Gregory, 1980). Highly purified suspensions (Report, 1979) were prepared by tissue homogenization followed by treatment with DNAase and Percoll (Pharmacia) density-centrifugation (only for *M. leprae*). When cell-free extracts were required, suspensions of *M. leprae* 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, 1979)]. *Mycobacterium phlei* NCTC 10266 was grown as described previously (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) and enzymes were assayed in cell-free extracts prepared in the presence of (as well as without) this thiol reagent.

NaOH-treatment of M. leprae. Suspensions of pure *M. leprae* were incubated at 25 °C in 1 M-NaOH for 1 h; after neutralization with 2 M-HEPES, a cell-free extract was prepared (Wheeler & Gregory, 1980). Alternatively, the bacterial suspensions were incubated at 25 °C in 0.5 M-NaOH for 30 min; after neutralization they were washed three times and suspended in buffered Tween 80 [0.1% (w/v) Tween 80 in 1.5 mM-MES, pH 7.0]. Such suspensions were used in some incubations with radioisotopes.

EDTA-treatment of M. leprae. Immediately after the DNAase step in purification, EDTA (potassium salt) was added to a final concentration of 4 mM. 1 mM-Mg $^{2+}$ was already present at this stage (Report, 1979). Suspensions were incubated for 20 min before removing the bacteria by centrifugation at 10000 g for 10 min and continuing the purification scheme. Extracts free of organisms were prepared as described above, from the purified bacteria.

Extracts of armadillo tissues. Liver and lymph node tissue from uninfected armadillos 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 $_2$ (Brock, 1969) for use in assays. Extracts of liver prepared as described previously (Wheeler & Gregory, 1980) referred to here as 'EDTA-liver extract'; extracts free of *M. leprae* from infected armadillo livers (homogenized in Tris; pH 8.7) prepared by the method of Wheeler & Gregory (1980) were used, only where described in Results.

Chromatography. Bio-Gel P2 was equilibrated with distilled water and distilled water was used as an eluate to separate mixtures of glucose and gluconate (Brown, 1970). In the past, anion-exchange chromatography had been used to separate mixtures of sugar-phosphates, but not including 6-phosphogluconate (Benson, 1957). In this study, columns of volume 1 ml for analytical work, and 10 ml for preparative work, were made with Dowex-2 (200–400 mesh, 8% cross-linked; from Sigma). They were washed with 2.5 column volumes (vol.) water, charged with 2.5 vol. 1 M-HCl, then washed with water until free of chloride. Using 2.5 vol. eluent, glucose was eluted with water, gluconate and glucose 6-phosphate with 0.15 M-NaCl, and 6-phosphogluconate with 0.05 M-HCl in 0.15 M-NaCl. In each case, no radioactivity remained in the eluate after 2.5 vol. had been eluted through the column. Paper-chromatography with propan-2-ol/distilled water (4:1, v/v) was also used to separate the sugars and phosphates (Menzies & Seakins, 1969).

Radioisotopes. Radioisotopes (all ^{14}C) were obtained from Amersham. [^{14}C]Gluconate and 6-phospho[^{14}C]gluconate were prepared thus: 0.2 μmol D-[U- ^{14}C]glucose 6-phosphate (279 mCi mmol $^{-1}$; 10.3 GBq mmol $^{-1}$) was added to 0.4 μmol NADP and 4 μmol triethanolamine (adjusted to pH 7.6 with HCl) and incubated at 25 °C for 30 min with 0.25 U yeast glucose-6-phosphate dehydrogenase (Sigma). The volume at this stage was 500 μl . Yield of 6-phospho[^{14}C]gluconate (judged by A_{340}) was 80–85%. If [^{14}C]gluconate was required, 20 μl 1 M-Tris/HCl, pH 9.2 and 1 U *Escherichia coli* alkaline phosphatase (Sigma) were added and the reaction mixture (523 μl) was

incubated at 37 °C for 30 min. All available phosphate was removed from glucose 6-phosphate and 6-phosphogluconate as judged by the assay for inorganic phosphate (Ames, 1966). The [^{14}C]gluconate was separated from [^{14}C]glucose on a Bio-Gel P2 column (Brown, 1970). 6-Phospho[^{14}C]gluconate was separated from [^{14}C]glucose 6-phosphate on a Dowex-2 column, and judged to be pure by rechromatography or paper chromatography.

Incubations of M. leprae with radioisotopes. Mycobacteria (0.5 to 1.4 mg dry wt) were incubated with ^{14}C -labelled substrate (1 μCi ; 0.02 to 0.1 mM), HEPES (50 mM), MgSO_4 (1 mM), and K_2HPO_4 (5 mM), adjusted to pH 7.0 with KOH. Additionally, 50 U penicillin ml^{-1} could be added as it had no observable effects. This prevented growth of any chance contamination in the incubation mixtures. Mixtures (200 μl) were incubated in Warburg Flasks at 34 °C. 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 'Instagel' (Packard)/distilled water (10:3, v/v) for scintillation counting at 8 °C. 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. The term 'uptake' has been used for the total of radioactivity assimilated plus radioactivity in the CO_2 evolved. Results are expressed as d.p.m. (mg dry wt mycobacteria) $^{-1}$; when pure *M. leprae* organisms are dried and weighed, 10^{10} bacteria weigh 1 mg (R. J. W. Rees, personal communication). The same relationship between numbers and acid fast bacilli has been deduced for other mycobacteria in this report. 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 at 22 °C and a few were assayed also at 34 °C. The incubation volume for all assays was 500 μl except when NADP was included as a coenzyme (not for a coupling enzyme) for the enzyme being assayed; in this case the volume was either 500 μl , or 50 μl and, after the end of the incubation, NADPH formed was detected by a cycling assay (Lilius *et al.*, 1979). When the cycling method was not used, change of A_{340} was observed for incubations including NADP, and change of A_{340} was always observed for incubations including NADH or NADPH.

To minimize background readings, ATP and ADP were obtained from equine muscle and phosphoenolpyruvate as a tricyclohexammonium salt was used (all from Sigma): these details were essential. Other substrates with an acidic group were supplied (from Sigma) as sodium salts unless otherwise stated.

Hexokinase (EC 2.7.1.1; ATP:D-hexose 6-phosphotransferase) was assayed by incubating extracts with 0.64 mM-ATP, 20 mM-D-hexose, 8 mM- MgCl_2 and 40 mM-triethanolamine (adjusted to pH 7.6 with HCl) plus a coupling mixture consisting of 0.4 mM-NADH, 0.71 mM-phosphoenolpyruvate and lactate dehydrogenase/pyruvate kinase mixture (Sigma; 13 U ml^{-1} and 17 U ml^{-1} , respectively); this allowed for continuous detection of ADP production (Bergmeyer *et al.*, 1974). Glucose 6-phosphate (0.1 M), an inhibitor of hexokinase (Goldberg *et al.*, 1968), was added to controls. Alternatively, phosphorylation of glucose by hexokinase could be measured in extracts with triethanolamine (40 mM, adjusted to pH 7.6 with HCl), 20 mM-glucose, 8 mM- MgCl_2 , 0.64 mM-ATP, 0.91 mM-NADP and glucose-6-phosphate dehydrogenase from yeast (Sigma; 0.55 U ml^{-1}).

Phosphohexoisomerase (EC 5.3.1.9; D-glucose-6-phosphate ketol-isomerase) was assayed in extracts either with 1.4 mM-fructose 6-phosphate as substrate (Bergmeyer *et al.*, 1974) in 85 mM-triethanolamine (adjusted to pH 7.6 with HCl), 6.8 mM- MgCl_2 , 0.39 mM-NADP and glucose-6-phosphate dehydrogenase from yeast (Sigma; 0.46 U ml^{-1}), or with 1.4 mM-glucose 6-phosphate as substrate, in which case 6.8 mM- MgCl_2 and 85 mM-triethanolamine (adjusted to pH 7.6 with HCl) were included in the assay mixture, together with 2 U rabbit muscle phosphofructokinase (Sigma) ml^{-1} , 1.1 mM-ATP, 0.4 mM-NADH, 0.71 mM-phosphoenolpyruvate, and a lactate dehydrogenase/pyruvate kinase mixture from rabbit muscle (Sigma; 13 U ml^{-1} and 17 U ml^{-1} , respectively).

Phosphofructokinase (EC 2.7.1.11; ATP:D-fructose-6-phosphate 1-phosphotransferase) was assayed by a modification of the method of Ling *et al.* (1966). The incubation mixture was 80 mM-Tris (adjusted to pH 8.5 with HCl), 1 mM- MgCl_2 , 4 mM-KCl, 5 mM-fructose 6-phosphate, 0.92 mM-ATP, 1 mM-DTT, 0.23 mM-NADH, 60 U rabbit muscle aldolase (Sigma) ml^{-1} and α -glycerophosphate dehydrogenase/triose phosphate isomerase mixture (Sigma, 1.6 U ml^{-1} and 16 U ml^{-1} , respectively).

Aldolase (EC 4.1.2.13; D-fructose-1,6-bisphosphate D-glyceraldehyde-3-phosphate lyase) was assayed by incubating extracts with 1 mM-fructose 1,6-diphosphate, 0.2 mM-NADH, 80 mM-triethanolamine (adjusted to pH 7.6 or pH 8.6 with HCl) and an α -glycerophosphate dehydrogenase/triose phosphate isomerase mixture from rabbit muscle (Sigma; 1.6 U ml^{-1} and 16 U ml^{-1} , respectively). Schiff's base formation with aldolase and dihydroxyacetone phosphate was carried out by a modification of the method of Grazi *et al.* (1962); to 3 mM-dihydroxyacetone phosphate in 0.1 M-potassium phosphate, pH 6.0, and 1.12 mg protein (ml cell-free extract) $^{-1}$ (armadillo liver or *M. leprae*), 2.8 M-sodium borohydride was added in four 5 μl amounts so that the final concentration was 0.14 M. The pH was maintained at 6.0 by addition of 2.5 M-acetic acid when required. After 30 min, the mixtures were desalted using an Amicon CF25 ultrafiltration cone (700 g, 4 °C). All stages were carried out at 0–4 °C.

Glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12; D-glyceraldehyde-3-phosphate : NAD⁺ oxidoreductase (phosphorylating), was assayed (Bergmeyer *et al.*, 1974) by incubating extracts with 82.5 mM-triethanolamine (adjusted to pH 7.6 with HCl), 6 mM-3-phosphoglycerate (cyclohexammonium salt), 0.9 mM-EDTA (sodium salt), 1.7 mM-MgSO₄, 0.2 mM-NADH, 1.1 mM-ATP, and 3-phosphoglycerate kinase from yeast (Sigma; grade free of glyceraldehyde-3-phosphate dehydrogenase; 15 U ml⁻¹).

Pyruvate kinase (EC 2.7.1.40; ATP : pyruvate 2-O-phosphotransferase) was assayed (Bergmeyer *et al.*, 1974) by incubating extracts in 80 mM-triethanolamine (adjusted to pH 7.6 with HCl), 0.54 mM-phosphoenolpyruvate, cyclohexammonium salt, 2.5 mM-MgSO₄, 10 mM-KCl, 4.7 mM-ADP (adjusted to neutrality with KOH), 0.2 mM-NADH and lactate dehydrogenase [LDH-1(H4) isoenzyme from beef heart], free of pyruvate kinase activity (Sigma; 9.2 U ml⁻¹).

Glucose-6-phosphate dehydrogenase (EC 1.1.1.49; D-glucose-6-phosphate : NADP⁺ 1-oxidoreductase) was assayed (Bergmeyer *et al.*, 1974) by incubating extracts in 80 mM-triethanolamine (adjusted to pH 7.6 with HCl), 7 mM-MgCl₂, 1 mM-glucose 6-phosphate and 0.39 mM-NADP.

6-Phosphogluconate dehydrogenase (EC 1.1.1.44; 6-phospho-D-gluconate : NADP⁺ 2-oxidoreductase (decarboxylating) was assayed (Bergmeyer *et al.*, 1974) by incubating extracts in 80 mM-triethanolamine (adjusted to pH 7.6 with HCl), 3.5 mM-MgCl₂, 2.7 mM-6-phosphogluconate and 3.4 mM-NADP. For this enzyme and glucose-6-phosphate dehydrogenase (above), NADPH formed was usually assayed by the cycling method (Lilium *et al.*, 1979).

Transketolase (EC 2.2.1.1; sedoheptulose-7-phosphate : D-glyceraldehyde-3-phosphate glycolaldehydetransferase) was assayed by the method of Horecker *et al.* (1956), modified in that glyceraldehyde 3-phosphate formed was monitored continuously by the addition of 0.1 mM-NADH and α -glycerophosphate dehydrogenase/triose phosphate isomerase mixture (Sigma; 0.8 U ml⁻¹ and 8 U ml⁻¹, respectively) to the assay mixture of 10 mM-glycylglycine buffer (adjusted to pH 7.6 with HCl), 6 mM-cysteine (adjusted to pH 7.6 with NaOH), 2 mM-MgCl₂, 0.1 mM-thiamine pyrophosphate, 0.8 mM-xylulose 5-phosphate, and either 0.8 mM-ribose 5-phosphate or 1.0 mM-erythrose 4-phosphate.

α -Glycerophosphate dehydrogenase (EC 1.1.1.8; sn-glycerol-3-phosphate : NAD⁺ 2-oxido-reductase) was assayed in extracts either by the method described by Bergmeyer *et al.* (1974) with 287 mM-triethanolamine (adjusted to pH 7.6 with HCl), dihydroxyacetone phosphate (0.007 mM to 2.10 mM; preliminary kinetic studies were done), and 0.2 mM-NADH, or by the method of Winder & Brennan (1966), in which the forward reaction was assayed by the inclusion of a tetrazolium salt, thiazolyl blue (MTT; Sigma) at 0.08 mM and 0.11 mM-methylphenazonium methosulphate (PMS) with extracts, 100 mM- α -glycerophosphate, 0.2 M-phosphate buffer (Na₂HPO₄/KH₂PO₄, pH 7), 0.167 mM-NAD or NADP, and 10 mM-KCN. Glycerol dehydrogenase was also assayed by substituting 100 mM-glycerol for α -glycerophosphate.

Phosphoketolase (EC 4.1.2.9; D-xylulose-5-phosphate : D-glyceraldehyde-3-phosphate-lyase (phosphate-acetylating) was assayed by the method of Goldberg *et al.* (1968), in which extracts were incubated with 40 mM-phosphate (Na₂HPO₄/KH₂PO₄, pH 6.0), 10 mM-glutathione (adjusted to pH 6 with NaOH), 10 mM-xylulose 5-phosphate, 1 mM-EDTA (Na salt), 3 mM-MgCl₂, 0.5 mM-thiamine pyrophosphate, glyceraldehyde 3-phosphate was monitored continuously by the inclusion of 0.1 mM-NADH and an α -glycerophosphate dehydrogenase/triose phosphate isomerase mixture (Sigma; 0.8 U ml⁻¹ and 8 U ml⁻¹, respectively).

Glucuronate reductase (EC 1.1.1.19; L-gulonate : NADP⁺ 1-oxidoreductase) was assayed by incubating extracts with D-glucuronate and NADPH in conditions similar to those used by York *et al.* (1961). Extracts were buffered with 60 mM-HEPES (adjusted to pH 7.2 with KOH) or 50 mM-Tris/glycine, pH 9.0, 10 mM-glucuronate and NADPH (0.12 mM or 0.46 mM) were included as substrates and assays were done with or without 1 mM-EDTA (sodium salt).

Gluconokinase (EC 2.7.1.12; ATP : D-gluconate 6-phosphotransferase) and polyphosphate : D-gluconate 6-phosphotransferase (EC 2.7.1.X) were assayed by first incubating extracts (at 22 °C or 34 °C) with 50 mM-Tris (adjusted to pH 8.6 with HCl), 5 mM-gluconate, 2.5 mM-MgSO₄ and either ATP or tetra-polyphosphate (Sigma) at 5 mM (total volume was 500 μ l). After this incubation, the mixture was heated at 60 °C for 3 min and 40 μ l 1 M-HEPES base, 30 μ l 80 mM-NADP and 0.1 U 6-phosphogluconate (yeast; Sigma) were added and incubated at 22 °C for 45 min (i.e. until no further NADPH was produced). NADPH was detected by reading directly A₃₄₀ (Szymona *et al.*, 1969), or by taking samples for the cycling assay (Lilium *et al.*, 1979).

Glucose oxidase (EC 1.1.3.4; β -D-glucose : oxygen 1-oxidoreductase) and glucose dehydrogenase (EC 1.1.1.47; β -D-glucose : NAD(P)⁺ 1-oxidoreductase) were assayed by methods used by Galante *et al.* (1963). 80 mM-Triethanolamine/HCl (pH 8.6) or 50 mM-MES (pH 6.6) were used as buffers and the substrate was 16 mM-D-glucose. To detect dehydrogenases, 1 mM-NADP or NAD was included; to detect glucose oxidase, 0.5 mM-KCN and 0.113 mM-2,6-dichlorophenolindophenol (sodium salt, Sigma) were included in place of coenzyme.

Enzymes of the EDP were assayed by a method (Kovachevich & Wood, 1955) for 6-phosphogluconate dehydratase (EC 4.2.1.12; 6-phospho-D-gluconate hydrolyase) modified in that keto-deoxygluconate was replaced by 6-phosphogluconate (50 mM) and that the pyruvate formed was monitored continuously by the

inclusion of 0.5 U rabbit muscle lactate dehydrogenase (Sigma) ml⁻¹ and 0.4 mM-NADH: extract, 50 mM-Tris (adjusted to pH 8.0 with HCl), 25 mM-glutathione (adjusted to pH 8.0 with NaOH), lactate dehydrogenase and NADH were mixed and then MnCl₂ was added to 0.1 mM, then 10 min later the 6-phosphogluconate was added and fall in A₃₄₀ was read.

Enzyme activities are expressed as units (U) where one unit catalyses the conversion of 1 µmol substrate min⁻¹. Inhibitors (all Sigma) were added to extracts in assays before coupling enzymes and 5 min before substrates.

PAGE. Extracts were applied to 7% (w/v) polyacrylamide gels at pH 9.0 (Davis, 1964) to detect glucose-6-phosphate dehydrogenase and at pH 6.5 (Chang *et al.*, 1979) to detect 6-phosphogluconate dehydrogenase. After electrophoresis, glucose-6-phosphate dehydrogenase was detected by the method of Mori *et al.* (1967). For detection of 6-phosphogluconate dehydrogenase, 10 mM-6-phosphogluconate was used instead of glucose 6-phosphate. Essentially, the methods involved adding nitro blue tetrazolium (NBT), PMS and KCN to the enzyme assay mixtures. Results were expressed as electrophoretic mobility relative to the mobility (1.00) of bromophenol blue.

Statistical analysis. A non-parametric test of significance (Nimmo & Atkins, 1979), the Wilcoxon test (Colquhoun, 1971) was used for experiments with radioisotopes. Null hypotheses tested were: (i) CO₂ evolution, and assimilation were equal in incubations with live bacteria and controls, (ii) proportions of [1-¹⁴C] and [6-¹⁴C]glucose converted to CO₂ by *M. leprae* were equal, (iii) CO₂ evolution and assimilation were not affected by treatments of *M. leprae*, or the addition of umbelliferyl phosphate or Casamino acids to incubations. Standard error of enzyme activity was calculated when four or more extracts of *M. leprae* were assayed.

RESULTS

Catabolism of substrates by unbroken M. leprae

[U-¹⁴C]Glucose was taken up and catabolized to CO₂ by suspensions of purified *M. leprae*. The addition of 10 mM-4-methylumbelliferyl phosphate (UM-P) to incubations, or the use of NaOH-treated suspensions did not ($P > 0.10$) affect either assimilation or catabolism (Table 1). In incubations with [1-¹⁴C]glucose, 3180 d.p.m. CO₂ mg⁻¹ per 20 h were evolved and 36% of the substrate taken up was catabolized to CO₂. With [6-¹⁴C]glucose, 2600 d.p.m. CO₂ mg⁻¹ per 20 h were evolved and 17% of the substrate taken up was catabolized to CO₂. The proportion of glucose converted to CO₂ was significantly higher ($P < 0.05$) for [1-¹⁴C]glucose than [6-¹⁴C]glucose, providing evidence for presence of the HMP. With the formula of Wood *et al.* (1963), it was calculated that 9.3% of glucose 6-phosphate formed from glucose was metabolized via the HMP (pentose cycle in the terminology of Wood *et al.*, 1963). [U-¹⁴C]glycerol was also catabolized to CO₂ by *M. leprae* (Table 1), but very high (>40000 d.p.m.) counts in the heat-killed bacteria made it impossible to detect significant uptake. The rate of CO₂ production (mg *M. leprae*)⁻¹ was also determined as a percentage of the rate in *M. lepraemurium* and *M. phlei*. For [U-¹⁴C]glucose it was 260% and 1.2%, respectively; for glycerol it was 20% compared with *M. lepraemurium*.

6-Phosphogluconate was oxidized to CO₂ at a similar rate to glucose by *M. leprae*. However, the proportion of 6-phosphogluconate catabolized to CO₂ was far greater than the proportion of glucose catabolized to CO₂ (Table 1). When the concentration of 6-phosphogluconate in incubation mixtures was 0.02 mM, 10% was hydrolysed (mg *M. leprae*)⁻¹ per 20 h. When 10 mM-UM-P was added to incubations, the rate of oxidation of 6-phosphogluconate doubled (Table 1) and hydrolysis of 6-phosphogluconate was completely inhibited. While this represented a significant ($P < 0.01$) increase for the rate of oxidation, the rate of assimilation of 6-phosphogluconate was not significantly different ($P > 0.10$) in incubations with UM-P. NaOH-treated *M. leprae* oxidized to CO₂ the same proportion of the 6-phosphogluconate taken up, but both assimilation ($P < 0.05$) and oxidation to CO₂ ($P < 0.01$) were significantly lower than with untreated *M. leprae*.

The proportion of glucose 6-phosphate taken up which was catabolized to CO₂ by *M. leprae* was similar to the proportion for glucose, although the rate of assimilation of glucose 6-phosphate was significantly lower ($P < 0.05$) than for glucose. NaOH-treatment of *M. leprae* did not affect assimilation or oxidation of glucose 6-phosphate, but incubation with 10 mM-UM-P (Table 1) inhibited oxidation significantly ($P < 0.05$), but affected assimilation less ($P < 0.10$). Limited hydrolysis (6% mg⁻¹ per 20 h) of 0.02 mM-glucose 6-phosphate by *M. leprae*

Table 1. *Metabolism of U-¹⁴C-labelled carbon sources by M. leprae*

Results with either NaOH-treated *M. leprae*, or 10 mM-UM-P or Casamino acids added to the incubation mixture, are presented separately only if either CO₂ evolution or assimilation were significantly ($P < 0.05$) different in incubations with untreated bacteria without UM-P or Casamino acids. Substrate concentration was 0.02 mM except for incubations with glucose, glycerol and 6-phosphogluconate, which were also tested at 0.1 mM. At the concentrations tested, substrate concentration did not affect CO₂ evolution or assimilation. At least four controls and five tests were done for each incubation mixture. Where *M. leprae* were harvested from different tissues, the tests described above were done for each (up to three) tissue. No significant differences were observed between metabolic activities in *M. leprae* from different tissues.

Incubation mixture	Incubation period (h)	Radioactivity [d.p.m. (mg <i>M. leprae</i>) ⁻¹]		CO ₂ evolution (% of total uptake)
		In CO ₂ evolved	Assimilated	
Glucose	20	4430	7340	37
Glucose	140	19500	50100	28
Glucose + Casamino acids	20	1186	2602	31
Glucose + Casamino acids	140	2363	10100	19
Glycerol	20	10700	ND	ND
Glucose-6-phosphate	20	2300	4120	36
Glucose-6-phosphate + UM-P	20	439	2390	16
6-Phosphogluconate	20	5450	2010	73
6-Phosphogluconate + UM-P	20	11300	2270	83
6-Phosphogluconate, NaOH-treated <i>M. leprae</i>	20	1360	499	68
Gluconate	20	804	3460	18
Gluconate	140	4120	29200	13
Gluconate + Casamino acids	140	2240	11500	16

ND, Not determined.

suspensions was completely inhibited by 10 mM-UM-P. In incubations for 20 h with either 0.02 mM-glucose 6-phosphate or 6-phosphogluconate and 1 mg *M. leprae*, 60% of the 10 mM-UM-P was hydrolysed to 4-methylumbelliferone.

Since 6-phosphogluconate was a good substrate for oxidation by *M. leprae*, gluconate oxidation was studied. Over 20 h very little gluconate was oxidized to CO₂ and the proportion taken up that was oxidized was also low (Table 1). When the incubation mixture was supplemented with 1% (w/v) Casamino acids (Difco), no significant difference in assimilation or CO₂ production from gluconate was observed.

Some 140 h incubations were carried out, with gluconate or glucose $\pm$ Casamino acids. Compared with 20 h incubations, gluconate oxidation to CO₂ was increased fivefold, and assimilation increased eightfold, but when Casamino acids were included in the incubation mixture (Table 1), the increase was only threefold for both oxidation and assimilation. The inhibition of assimilation of gluconate by Casamino acids was statistically significant ($P < 0.05$). Glucose oxidation to CO₂ and assimilation were both significantly depressed ($P < 0.05$ in all cases) by Casamino acids in 20 h and 140 h incubations. Similar increases in oxidation and assimilation in 140 h incubations compared with 20 h incubations were observed with glucose as with gluconate (Table 1).

Key enzymes of glucose metabolism by glycolysis

Hexokinase. Glucose actually inhibited oxidation of NADH when a coupled assay system for ADP was used to detect hexokinase in extracts, so glucose 6-phosphate was used to inhibit hexokinase (Hammes & Kochavi, 1962) in controls instead of omitting hexoses. Although hexokinase could be measured in extracts of *M. leprae* by detecting glucose 6-phosphate formed by coupling the reaction with glucose-6-phosphate dehydrogenase and measuring NADPH formation, measurement of the ADP formed was more useful because phosphorylation of different D-hexoses could be compared (measurements of activity with glucose by the two methods were in agreement). Relative rates of phosphorylation of glucose : deoxyglucose : gluco-

Table 2. *Enzymes of glycolysis*

Enzymes were assayed in extracts of armadillo liver and cell-free extracts of *M. leprae*. The bacteria were either untreated or NaOH-treated. Values for specific activities of phosphofructokinase, hexokinase, and glyceraldehyde-3-phosphate dehydrogenase include assays done in triplicate with extracts of *M. leprae* sonicated with 0.1 mM-DTT; in these extracts, specific activity did not differ markedly from that of extracts without DTT. When extracts were measured with inhibitors, the results are expressed as the percentage inhibition of the activity in incubations without inhibitor. Each figure represents an assay done at least in triplicate with each of two extracts.

Enzyme	Specific activity [mU (mg protein) ⁻¹] in extracts of:			Inhibitor (and concentration)	Percentage inhibition	
	<i>M. leprae</i>	NaOH-treated <i>M. leprae</i>	Armadillo liver		<i>M. leprae</i>	Armadillo liver
Hexokinase	0.54	0.49	5.6	None*	—	—
Phosphohexoisomerase	36	17	410	Deoxyglucose 6-phosphate (0.1 mM)	75	40
				6-Phosphogluconate (0.3 mM)	40	85
Phosphofructokinase	0.12	0.06	6.3	Phosphoenolpyruvate (5.0 mM)	90	0
Aldolase	10.7	8.1	2100	DHAP/NaBH ₄ †	0	100
Glyceraldehyde-3-phosphate dehydrogenase	0.064	0.043	120	Threose-2,4-diphosphate‡ (0.2 µM)	70	50
Pyruvate kinase	0.23	0.094	700	Threose-2,4-diphosphate (2 µM)	100	80
				None§	—	—

* Rates of phosphorylation of D-hexoses compared instead; see text.

† Irreversible inhibition; see text for details of Schiff's base formation with DHAP and aldolase.

‡ The concentration of D-threose-2,4-diphosphate was calculated on the basis of 1 mol from 8 mol α-glycerophosphate (Fluharty & Ballou, 1959).

§ Rates of pyruvate production with ADP and GDP compared instead; see text.

samine were 100:71:72 for *M. leprae* and 100:92:84 for armadillo liver extracts. Loss of activity (35% loss) from both bacterial and liver extracts was observed after freezing and thawing.

Phosphohexoisomerase. With fructose 6-phosphate as the substrate, no increase in A_{340} was observed if yeast 6-phosphogluconate dehydrogenase (Sigma 1 U ml⁻¹) was added to the incubation mixture, for extracts of either *M. leprae* or armadillo tissues (liver or lymph node). Therefore, specific activities were calculated on the basis of 2 mol NADP reduced (mol fructose 6-phosphate isomerized)⁻¹. Phosphohexoisomerase was not inhibited by phosphoenolpyruvate (up to 5 mM), but it was inhibited by 2-deoxy-D-glucose (Wick *et al.*, 1957). Differential inhibition of the enzyme in extracts of armadillo liver and *M. leprae* was observed with 2-deoxyglucose at 1.0 mM (Table 2), but not at 0.2 mM (only 25–30% inhibition).

With D-glucose 6-phosphate as substrate, rates of enzyme activity were similar to those observed with fructose 6-phosphate. 6-Phosphogluconate (Kahana *et al.*, 1960), at 0.3 mM, inhibited the enzyme from armadillo tissues more strongly than the enzyme from extracts of *M. leprae* (Table 2).

Phosphofructokinase. ITP (inosine triphosphate) was as effective a substrate for armadillo liver phosphofructokinase as ATP, but the enzyme in extracts of *M. leprae* used ITP at only 25% the rate of ATP. Inhibition of phosphofructokinase by 5 mM-phosphoenolpyruvate (Yoshida *et al.*, 1971) was far stronger in extracts of *M. leprae* than in extracts of armadillo liver (Table 2); at 1 mM it inhibited the activity in extracts of *M. leprae* by 60%.

If EDTA-treated liver extract was used, the specific activity was <0.5% of the value in Table 2. Cell-free extracts from EDTA-treated *M. leprae* contained 0.095 mU phosphofructokinase (mg protein)⁻¹.

The enzyme in *M. leprae* and liver extracts had, respectively, 2.2-fold and 2-fold greater activity at 34 °C than at 22 °C. In all extracts assayed, the rate of phosphorylation of D-fructose 6-phosphate increased with time up to 100 min for *M. leprae* extracts and 10 min for liver extracts. Specific activities (Table 2) were calculated after incubations of 60 min and 6 min, respectively.

Aldolase. The ratio of enzyme activity at pH 8.6:pH 7.6 was 0.64 to 0.72 in extracts of *M. leprae* (including NaOH-treated *M. leprae*). In extracts from armadillo tissues, the ratio was 3.72 for liver and 1.29 for lymph node.

Treating extracts with the substrate dihydroxyacetone phosphate (DHAP) in the presence of nascent hydrogen had a differential effect on aldolase in extracts of armadillo liver and in extracts of *M. leprae* (Table 2; the inhibitor is described as DHAP/NaBH₄). The ratio of activity at pH 8.6:7.6 was 0.61 in extracts of *M. leprae* treated with DHAP and NaBH₄.

Glyceraldehyde-3-phosphate dehydrogenase. Reduction of 1,3-diphospho-D-glycerate could be followed in *M. leprae* extracts for 60 min at 22 °C but only 20 min at 34 °C, after which time the rate of reaction had fallen considerably compared with the initial velocity. D-Threose 2,4-diphosphate (Fluharty & Ballou, 1959), prepared from glycolaldehyde phosphate (Loring *et al.*, 1956), inhibited the enzyme in extracts of *M. leprae* more strongly than the enzyme in extracts of armadillo liver (Table 2).

Pyruvate kinase. The activity of this enzyme with GDP was 85% of that obtained with ADP in extracts of armadillo liver. The enzyme could not be detected in *M. leprae* when GDP was substituted for ADP.

Key enzymes of 'alternative' metabolic pathways, including glycerol catabolism

Glucose-6-phosphate dehydrogenase. This enzyme was found in each of seven extracts of *M. leprae* and was present in one extract (Ex 146) of untreated *M. leprae* at about 10% the activity in all other extracts (Table 3). EDTA- and NaOH-treatment of *M. leprae* did not affect the enzyme activity. Enzyme activities in extracts of armadillo liver homogenized in phosphate/MgCl₂ (Table 3) and in extracts of infected liver, which was homogenized in Tris, were similar. The enzyme in *M. leprae* extracts could be incubated at pH 9.2 in triethanolamine/HCl for 1 h and retained full activity (the assay was performed after adjusting the pH to 7.6 with HCl), and was suitable for electrophoresis in the system of Davis (1964). The electrophoretic mobilities of the

Table 3. Enzymes of 'alternative' metabolic pathways

Enzymes of the HMP and the pathway for glycerol catabolism were detected in extracts of armadillo liver and cell-free extracts of *M. leprae*. See text for details of enzymes not detectable in extracts of *M. leprae*, and glycerol dehydrogenase (present in extracts of *M. leprae*). One extract in the case of *M. leprae* sonicated with DTT and a minimum of two extracts in other cases were assayed at least in triplicate for each value of specific activity. When properties of the enzymes were studied, results were obtained with at least two extracts assayed in triplicate. Unless stated in the text, only extracts of untreated *M. leprae* were used for these experiments.

Enzyme	Specific activity [mU (mg protein) ⁻¹] in extracts of:			Property studied	Value obtained	
	<i>M. leprae</i>	<i>M. leprae</i> sonicated with DTT	NaOH-treated <i>M. leprae</i>		<i>M. leprae</i>	Armadillo liver
Glucose-6-phosphate dehydrogenase	0.131* ± 0.021 (0.017 in Ex 146)†	0.091	0.22	7.6	See Fig. 1(a) 10%	See Fig. 1(a) 20%
6-phosphogluconate dehydrogenase	13.2* ± 2.0 (1.23 in Ex 146)†	40	19.8	20	See Fig. 1(b) 60	See Fig. 1(b) 10
Transketolase	3.5	7.1	4.2	0.74		
α-glycerophosphate dehydrogenase	0.57	ND	0.52	100 <i>K_m</i> for DHAP	0.52 mM	0.097 mM

ND, Not done.

* Lowest value for extract other than Ex 146 was 0.086 mU mg⁻¹ for glucose-6-phosphate dehydrogenase and 8.0 U mg⁻¹ for 6-phosphogluconate dehydrogenase; more than four extracts (other than Ex 146) were assayed and a value for S.E. is given.

† Specific activities of these enzymes in Ex 146 were remarkably low and are not included in the mean values for specific activity.

Fig. 1. PAGE of extracts on gels with different pH. (a) pH 9.0 gels were stained for glucose-6-phosphate dehydrogenase (tracks 2, 4, 6) and compared with controls (tracks 1, 3, 5). The stain was made up without glucose 6-phosphate. Extracts were from: *M. leprae* (tracks 1, 2); uninfected armadillo liver (tracks 3, 4); infected armadillo liver (tracks 5, 6). (b) pH 6.5 gels were stained for 6-phosphogluconate dehydrogenase (tracks 8, 9) and compared with a control (track 7). The stain was made up without 6-phosphogluconate. Extracts were from: *M. leprae* (track 9); uninfected armadillo liver (tracks 7, 8). No bands were detected in the control with *M. leprae* extracts. Specific activity of the enzyme in infected armadillo liver extract was too low for electrophoresis. M denotes enzyme from *M. leprae*; A denotes enzyme from armadillo liver; B denotes bromophenol blue (marker).

enzymes in extracts of infected liver were the same as in extracts of uninfected tissue (0.7 mU of each applied to each gel) and clearly distinct from the enzymes in extracts of *M. leprae* (Fig. 1a). The enzyme from both sources was unable to use NAD instead of NADP and was not strongly inhibited by up to 0.02 mM-stearoyl-CoA (Neufeldt *et al.*, 1965). For the incubations with stearoyl-CoA, extracts were preincubated with 0.2 mM-phenylmethylsulphonyl fluoride (from a 20 mM stock solution in acetone) to inactivate esterases (Fahrney & Gold, 1963) present (unpublished results). The ratio of activity at 34 °C to 22 °C was 3.5 in *M. leprae* extracts and 3.8 in armadillo liver extracts. The enzyme was also detected in extracts of *M. lepraemurium*, at 0.41 mU (mg protein)⁻¹.

6-Phosphogluconate dehydrogenase. Like glucose-6-phosphate dehydrogenase, this enzyme was present at a very low specific activity in Ex 146 relative to the specific activity in six other extracts of *M. leprae* (Table 3). Such very low enzyme activities in Ex 146 were confined to these two enzymes: phosphofructokinase and glyceraldehyde-3-phosphate dehydrogenase were present in Ex 146 at levels similar to the mean values in Table 2 (lack of material restricted further assays on Ex 146).

EDTA- and NaOH-treatment of *M. leprae* organisms did not affect 6-phosphogluconate dehydrogenase but, after incubation of extracts of *M. leprae* at pH 9.2 (as described in previous section), only 5% of the 6-phosphogluconate dehydrogenase activity remained. Activity of the enzyme from *M. leprae* or armadillo liver could not be detected on polyacrylamide gels run at pH 9.0 (Davis, 1964) so the method of Chang *et al.* (1979) was used. The mobility of the one band

of enzyme activity from host-liver (1 mU applied to each gel) was distinct from 6-phosphogluconate dehydrogenase from *M. leprae* (Fig. 1*b*).

In extracts of *M. leprae*, 6-phosphogluconate dehydrogenase was found at levels one hundred times greater than glucose-6-phosphate dehydrogenase (Table 3). This may be an underestimate, since an even higher level of activity (Table 3) was observed in extracts of *M. leprae* if 0.1 mM-DTT was added to the sonication buffer. The enzyme was present at 0.54 mU (mg protein)⁻¹ in extracts of *M. lepraemurium*.

Transketolase. The specific activity of transketolase in extracts was high relative to many other enzymes from *M. leprae* and was elevated when 0.1 mM-DTT was present in the sonication buffer for *M. leprae* (Table 3); enzyme activity in extracts of *M. leprae* was similar with erythrose 4-phosphate or ribose 5-phosphate as a substrate. The enzyme in *M. leprae* extracts was more strongly inhibited by Na₂SO₄ than the enzyme in extracts of armadillo liver (Table 3).

α-Glycerophosphate dehydrogenase. Experiments with inhibitors of this enzyme were impracticable; for instance, high levels of aldolase in extracts would interfere with fructose 1,6-diphosphate, a possible allosteric inhibitor (Beisenhertz *et al.*, 1953). However, a difference in affinity for DHAP was observed between the enzyme in extracts of *M. leprae* and armadillo liver. With 2.1 mM-DHAP, the enzyme activity at 34 °C in extracts of *M. leprae* was 50% greater than at 22 °C. This enzyme was also detected in both extracts with α-glycerophosphate as a substrate (Winder & Brennan, 1966).

Glycerol dehydrogenase. This enzyme was present at a specific activity of 0.11–0.14 mU (mg protein)⁻¹ in extracts of untreated and NaOH-treated *M. leprae*; a lower level, 0.04 mU (mg protein)⁻¹ was found in armadillo liver extracts. The tetrazolium salt in the assay could be reduced directly even in desalted (Amicon CF25 cone) extracts, but *in vivo* it is likely that NAD or NADP was reduced (Winder & Brennan, 1966). Specific activities were too low for gel electrophoresis, although the assay could have been used directly to detect the enzyme on gels.

Enzymes not detectable in extracts of M. leprae

Phosphoketolase, glucuronate reductase, gluconate phosphotransferases (with ATP or tetrapolyphosphate), enzymes for direct oxidation of glucose to gluconate (Chedelin, 1961; Galante *et al.*, 1963), and the EDP enzymes could not be detected in extracts of *M. leprae*, even when 0.1 mM-DTT was included in the sonication buffer. The lowest specific activities that could be detected were 5 μU (mg protein)⁻¹ for phosphoketolase, glucose oxidase, glucose dehydrogenase and the EDP enzymes and, using the cycling method for NADP, 0.3 μU (mg protein)⁻¹ for gluconate kinase and 0.9 μU (mg protein)⁻¹ for glucuronate reductase. Gluconate kinase was detected in extracts of *M. phlei*.

DISCUSSION

Mycobacterium leprae catabolizes glucose by glycolysis and the HMP. The proportion of glucose catabolized by the HMP is as high as previously observed for other mycobacteria (Bai *et al.*, 1975*b*, 1976; O'Barr & Rothlauf, 1970; Ramakrishnan *et al.*, 1972). The values for the proportion of glucose catabolized by the HMP in the work of Bai *et al.* (1975*b*, 1976) can be recalculated by the method of Wood *et al.* (1963). If this is done, the maximum value for catabolism through the pentose cycle (Wood *et al.*, 1963) observed in their work is 9.2%. In contrast to *M. leprae*, this study confirmed that *M. lepraemurium* does not use glucose very effectively as a substrate for oxidation (Tepper & Varma, 1972).

All enzymes detected in extracts of *M. leprae* had properties different from similar enzymes in liver extracts. Differences in substrate-specificity or affinity, the effects of inhibitors, or electrophoretic mobility were detected. From these results, all the enzymes discussed were authentic *M. leprae* enzymes. Additionally, after treatment of *M. leprae* with NaOH (Wheeler *et al.*, 1982) or EDTA (Brock, 1969) to inactivate any host-enzymes which may have been adsorbed to the surface, all of the enzymes could still be detected in extracts from the treated bacteria. Where electrophoretic analysis was possible it was possible to show that *M. leprae* glucose-6-

phosphate dehydrogenase was not present in extracts of infected tissues after the bacteria had been removed.

The properties of some enzymes were of particular interest. Some of the enzyme inhibitors, e.g. 6-phosphogluconate against phosphohexoisomerase (Kahana *et al.*, 1960), phosphoenolpyruvate against phosphofructokinase (Yoshida *et al.*, 1971), and stearyl-CoA against glucose-6-phosphate dehydrogenase (Neufeldt *et al.*, 1965), are known to regulate enzyme activity and may do so in *M. leprae*. Nucleotide requirements of pyruvate kinase and phosphofructokinase of *M. leprae* were more specific than those of the armadillo liver enzymes. Phosphorylation of hexoses was attributed to hexokinase but an ATP:glucose phosphotransferase (glucokinase) may be present: this possibility does not diminish the evidence for an authentic *M. leprae* hexokinase since the relative rates of phosphorylation of glucosamine and deoxyglucose are different in extracts from *M. leprae* and host tissues. Conversely, the glucokinase reported in crude extracts of some mycobacteria (Bai *et al.*, 1975*b*, 1976) could be hexokinase. *Mycobacterium leprae*, like *M. tuberculosis* (Bai *et al.*, 1975*a*) has a class II fructose-diphosphate aldolase. In contrast, host tissue has a class I aldolase. Generally, class I is mammalian and class II is bacterial (Rutter, 1964). Unlike the aldolase of *M. tuberculosis* H₃₇Rv (Bai *et al.*, 1975*a*), and the armadillo enzyme, *M. leprae* aldolase had higher activity at pH 7.6 than pH 8.6.

Glycerol is a good substrate for oxidation by *M. leprae*, like other mycobacteria. The key enzyme of glycerol catabolism, α -glycerophosphate dehydrogenase (Hunter, 1953) was detected in extracts of *M. leprae*, but glycerol dehydrogenase was also detected. Thus, *M. leprae* can reduce glycerol either directly or after phosphorylation like *M. smegmatis* (Hunter, 1953) and old cultures of *M. tuberculosis* BCG (Winder & Brennan, 1966), but unlike other mycobacteria (including, otherwise, BCG). The metabolite DHAP could then be metabolized further by the Embden-Meyerhof Pathway.

The assay of enzymes in crude extracts has provided much information about mycobacterial metabolism (e.g. Bai *et al.*, 1975*b*, 1976). In this study, substrates for the enzymes were used at saturating concentrations. Any substrate removed by interfering enzymes in the extracts did not affect the rate of the reaction being studied. During the incubations of extracts (5–15 min for armadillo, 15–60 min for *M. leprae*), after a lag phase in some cases, the rate of reaction was generally linear. However, with *M. leprae* glyceraldehyde-3-phosphate dehydrogenase, the rate fell with time: this enzyme proved to be unstable, particularly at 34 °C. With some enzymes, the rate increased throughout the incubation period; this may have been due to the removal of substrate-inhibition or even stimulation by the product, as reported for phosphofructokinase (Ramaiah *et al.*, 1964). Studies with inhibitors were done only if they were not metabolized by interfering enzymes. Therefore, incubations with inhibitors were considered to distinguish reliably bacterial from host-tissue enzymes and the specific activities were a reliable guide to enzyme activities in *M. leprae*.

While activities of individual enzymes were very different in *M. leprae*, this was also seen in *M. smegmatis* (Bai *et al.*, 1976) and *M. tuberculosis* (Bai *et al.*, 1975*b*; Bastarrachea *et al.*, 1961). Activities of enzymes in extracts of *M. leprae* relative to *M. tuberculosis* H₃₇Rv (Bai *et al.*, 1975*b*) or H₃₇Ra (Bastarrachea *et al.*, 1961) were between 0.4 and 1.5% for phosphofructokinase, pyruvate kinase and glucose-6-phosphate dehydrogenase; between 9 and 17% for phosphohexoisomerase, hexokinase and aldolase, and the activity of α -glycerophosphate dehydrogenase was 23% of the activity in extracts of *M. tuberculosis* BCG (Winder & Brennan, 1966). The activity of 6-phosphogluconate dehydrogenase was 143% of the activity in extracts of *M. tuberculosis* H₃₇Rv (Bai *et al.*, 1975*b*). 6-Phosphogluconate dehydrogenase activity in *M. smegmatis* (Bai *et al.*, 1976), *M. tuberculosis* H₃₇Rv (Bai *et al.*, 1975*b*) and *M. lepraemurium* was almost the same as glucose-6-phosphate dehydrogenase activity, but in *M. leprae* it was 100 times higher. The results obtained with the two HMP enzymes in *M. leprae* suggest that the enzyme at low activity, glucose-6-phosphate dehydrogenase, was less labile to the manipulations in this work. So the difference in specific activities could not be explained by differential denaturation. The observation that *M. leprae* used 6-phosphogluconate most efficiently as a source for oxidation (measured by CO₂ production) suggested that there is a metabolic significance in the elevated activity of 6-phosphogluconate dehydrogenase.

It may be that *M. leprae* scavenges 6-phosphogluconate, which may be available as a by-product of the metabolism of activated macrophages (Karnovsky *et al.*, 1975), in which *M. leprae* resides. It is interesting that the obligate intracellular parasites, *Plasmodium spp.*, which have no glucose-6-phosphate dehydrogenase but do have 6-phosphogluconate dehydrogenase, may scavenge 6-phosphogluconate produced by host erythrocytes (Sherman, 1979).

Enhancement of 6-phosphogluconate oxidation by UM-P was far greater than expected by its effect of inhibiting the limited hydrolysis of the substrate by acid phosphatase. Indeed, UM-P had an opposite effect on uptake and oxidation of glucose 6-phosphate by *M. leprae*. These results may represent a difference in metabolic control, or uptake of the sugar-phosphates, mediated by UM-P (or umbelliferone). Neither sodium nor potassium ions affected assimilation or CO₂ evolution with either sugar-phosphate (unpublished results) suggesting that a sodium-dependent transport system for sugar-phosphates (Roseman, 1977) was not operating in *M. leprae*. The difference between glucose and glucose 6-phosphate uptake (in untreated *M. leprae*) may reflect differences in the uptake mechanisms; their metabolism, measured as proportions oxidized and assimilated, was very similar.

Since gluconate catabolism is inducible in some other mycobacteria (Szymona *et al.*, 1969), this might have explained why it was a poor substrate for oxidation for *M. leprae*. When Casamino acids were added to the incubation medium, and when the incubation period was extended, gluconate catabolism may have been induced. However, the amino acids inhibited oxidation of ¹⁴C-labelled substrates to CO₂ and may have been acting as carbon sources themselves. The absence of any gluconate-phosphorylating activity in *M. leprae* suggested that the role of the high level of 6-phosphogluconate dehydrogenase does not play a part in gluconate catabolism.

The low levels of HMP enzymes in Ex 146 and not in any other extracts might be explained by an 'environmental' effect, since some of the other extracts were made from *M. leprae* grown in other armadillos but from the same inoculum as Ex 146. Unfortunately, it is impossible to plan experiments on induction of metabolic activities by *M. leprae* at the moment since there is only one practicable (*in vivo*) source of the bacteria for biochemical studies.

Mycobacterium leprae, on the basis of these results, can use glucose and glycerol as energy sources, like many other mycobacteria, and also 6-phosphogluconate. Some negative results are of interest: the absence of both glucuronate reductase and EDP activity indicate that glucuronate cannot be used as an energy source by *M. leprae*. This is important because hyaluronic acid has been suggested as a growth substrate for *M. leprae* (Matsuo & Skinsnes, 1974) on the basis of the detection of glycosidases, which would be part of a pathway of hyaluronic acid dissimilation. However, hyaluronic acid is not oxidized by suspensions of *M. lepraemurium* (Kato *et al.*, 1976), and, although the presence of the glycosidases has been confirmed (Wheeler *et al.*, 1982), it is shown in this study that key enzymes for the breakdown of one of the products of the glycosidases are absent in *M. leprae*.

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