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EDITORIALS

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Metabolism in *Mycobacterium leprae*: Its Relation to Other Research on *M. leprae* and to Aspects of Metabolism in Other Mycobacteria and Intracellular Parasites

The main purpose of this editorial is to present a detailed and critical review of the information on metabolism in *Mycobacterium leprae*. Comparisons are made with other mycobacteria (their metabolism has been reviewed[1, 2, 3]), especially *M. lepraemurium*, the etiologic agent of "rat leprosy," which has only recently been cultured[4, 5]. The suitability of *M. lepraemurium* (a more readily available bacterium) as a biochemical model for *M. leprae* will be considered. The bacteriology[6, 7], morphology[6, 8], and structural biochemistry[6, 9] of *M. leprae* have all been reviewed recently.

An understanding of the biochemistry and metabolism of *M. leprae* is important for fundamental reasons. *M. leprae* is a highly successful intracellular pathogen; up to 1% (dry weight) of the wet weight of some infected armadillo tissues (the best source of *M. leprae*) may be leprosy bacilli[10]. A figure, based on a comparison of wet weight of bac-

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[3] Ratledge, C. Nutrition growth and metabolism. In: *The Biology of the Mycobacteria.* Ratledge, C. and Stanford, J., eds. London: Academic Press, 1982, vol. 1, pp. 186–212.

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[5] Pattyn, S. R. and Portaels, F. *In vitro* cultivation and characterization of *M. lepraemurium*. Int. J. Lepr. **48** (1980) 7–14.

[6] Draper, P. The bacteriology of *Mycobacterium leprae*. Tubercle **64** (1982) 43–56.

[7] Stewart-Tull, D. E. S. *Mycobacterium leprae*: The bacteriologists' enigma. In: *The Biology of the Mycobacteria.* Ratledge, C. and Stanford, J., eds. London: Academic Press, 1982, vol. 1, pp. 213–308.

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[9] Minnikin, D. E. Lipids: Complex lipids, their chemistry, biosynthesis and roles. In: *The Biology of the Mycobacteria.* Ratledge, C. and Stanford, J., eds. London: Academic Press, 1982, vol. 1, pp. 95–185.

[10] World Health Organization. Report of the fifth meeting of the Scientific Working Group on the Immunology of Leprosy (IMMLEP). Annex 4. 1980. WHO document TDR/IMMLEP-SWG(5)/80.3.

teria in armadillo tissue (wet weight), of 4% has been published[11], although this probably represents a slightly lower load of bacteria. It would be useful to know how *M. leprae* survives *in vivo* and what nutrients it derives from the host. Knowledge of the metabolic capabilities of *M. leprae* may put attempts to culture *M. leprae* on a more rational basis. Finally, findings on specific inhibition of metabolic activities may help to provide clues for development of new drugs.

It is hoped that this review will stimulate ideas for culturing *M. leprae,* or at least stimulate further metabolic studies to that end. Especially interesting is the discovery of gaps in metabolic pathways since media might easily be devised to take advantage of such knowledge by making specific additions. However, finding gaps is necessarily tedious with *M. leprae* because it is impossible to do experiments analogous to screening for auxotrophs in cultivable bacteria. Individual activities must be looked for. More general knowledge of metabolic activities in *M. leprae* would allow the potential cultivator to know what the organism could use in media, and any unusual metabolic activities might suggest specific additions which could be tried in media. The use of aspects of metabolism of *M. leprae* for drug screening will also be discussed.

Attempts to culture *M. leprae*. The problem of cultivation was reviewed by Pattyn in 1973[12] and ten years later by Draper[6]. Between these reviews, many claims of cultivation have been made. A brief discussion of these claims is given here, since one of the paramount objectives in studying metabolism of *M. leprae* is to provide a rational basis for cultivation of the organism. The organisms isolated have generally been either related to the *M. avium-M. intracellulare-M. scrofulaceum* group of bacteria[13–15] or corynebacteria[16]. *M. leprae* is clearly distinct from both of these groups on the basis of taxonomic studies[17, 18] and detection of some characteristic lipids[19–22] which are clearly mycobacterial.

The most straight-forward explanation of the appearance of these bacteria in culture media is that the original inocula were contaminated and non-growing *M. leprae* are gradually swamped. One source, the skin, is notoriously difficult to sterilize, and armadillos are known to be both susceptible to[23, 24], and sometimes have natural infec-

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[11] Kirchheimer, W. F., Storrs, E. E. and Binford, C. H. Attempts to establish the armadillo (*Dasypus novemcinctus* Linn.) as a model for the study of leprosy. II. Histopathologic and bacteriologic post-mortem findings in lepromatoid leprosy in the armadillo. Int. J. Lepr. **40** (1972) 229–242.

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[15] Skinsnes, O. K., Kuba, B. A., Chang, P. M. C. and Kuwahara, T. *In vitro* cultivation of leprosy bacilli in hyaluronic acid-based medium. 2. Progress and developing concept of the role of hyaluronic acid suggested by culture and armadillo infection studies. Int. J. Lepr. **46** (1978) 394–413.

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[19] Draper, P., Dobson, G., Minnikin, D. E. and Minnikin, S. M. The mycolic acids of *Mycobacterium leprae* harvested from experimentally infected nine-banded armadillos. Ann. Microbiol. (Paris) **133B** (1982) 39–47.

[20] Draper, P., Payne, S. N., Dobson, G. and Minnikin, D. E. Isolation of a characteristic phthiocerol dimycocerosate from *Mycobacterium leprae.* J. Gen. Microbiol. **129** (1983) 859–863.

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[22] Payne, S. N., Draper, P. and Rees, R. J. W. Serological activity of purified glycolipid from *Mycobacterium leprae.* Int. J. Lepr. **50** (1982) 220–221.

[23] Binford, C. H., Meyers, W. M., Walsh, G. P., Storrs, E. E. and Brown, H. L. Naturally acquired leprosy-like disease occurring naturally in the nine-banded armadillo (*Dasypus novemcinctus,* Linn.): Histopathologic and microbiologic studies of tissues. J. Reticuloendothel. Soc. **22** (1977) 377–388.

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tions with[23, 25, 26], mycobacteria. A recent report confirms the presence of natural mycobacterial infections in armadillos in Texas. The organisms involved were *M. avium, M. intracellulare, M. scrofulaceum, M. gordonae,* and also *M. leprae* in 4.7% of wild armadillos[27]. An ingenious alternative explanation of the great differences between *M. leprae in vivo* and the cultivated organisms is based on the observation that many bacteria adapt readily to very different conditions of growth. Thus, the characteristics of *M. leprae* grown *in vitro* could not be predicted by looking at *M. leprae* grown *in vivo*[28]. Problems with this explanation are a) that some common characteristics can be found between *in vitro*- and *in vivo*-grown mycobacteria of the same species[1, 4, 5, 12] and b) the cultured organisms still need to be identified as *M. leprae*; they should revert to typical *M. leprae* on subculture or subsequent growth *in vivo*. Sometimes growth of these cultivated bacteria has been obtained in mice. It would have been interesting to see how many bacteria in the inoculum of cultivated bacteria could actually grow in mice. Serial dilutions could have be done before inoculation, and a most probable number—practicable for *M. leprae* suspensions[29]—could have been worked out. If the values had been only a few in an inoculum of, say, 10^4 acid-fast bacilli, one might wonder if these were surviving leprosy bacilli from the original inoculum which had not divided and been swamped by some other acid-fast organism.

At the time of writing, no cultivation method for *M. leprae* had been confirmed in another laboratory. When organisms grew in media they did not resemble *M. leprae*. Two recent attempts to confirm claims of growth in "M-Y medium"[30], and in a complex medium devised by Veeraraghavan (Kato, unpublished observations and Katoch and Desikan, unpublished observations) resulted in no growth of *M. leprae*.

There is now the possibility of characterizing organisms claimed to be cultivated *M. leprae*. About 10^{11} *M. leprae* can be obtained from a suspension inoculated into the nude mouse[31]. Since such numbers can be obtained after about two years from small inocula of *M. leprae*[31], the nude mouse could be used to obtain suspensions of *M. leprae* free of the contaminants, such as ADMs[25], which are often present in armadillo tissues. Such suspensions would be far more suitable for cultivation attempts than those obtained from the armadillo. The organisms grown in the nude mouse could be identified, since there are known characteristics which can be used for identifying *M. leprae* grown *in vivo*. Particularly promising are a number of lipids, typically mycobacterial, but characteristic of *M. leprae,* which have been identified recently[19–22].

There is, ultimately, the possibility of isolating deoxyribonucleic acid (DNA) from the cultured organisms and comparing it with DNA from *M. leprae* isolated from infected tissue. Homologies between DNA from *M. leprae* and other mycobacteria or corynebacteria have been worked out[31] but it is difficult to interpret them since DNA molecules of very different sizes are involved; very different degrees of homology were calculated when this was taken into account (Imaeda, personal communica-

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[25] Portaels, F., Francken, A. and Pattyn, S. R. Bacteriological studies of armadillo livers infected with *Mycobacterium leprae*. Ann. Soc. Belg. Med. Trop. **62** (1982) 233–245.

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[28] Kato, L. The Janus-face of *M. leprae*. Characteristics of *in vitro* given *M. leprae* are not predictable. Int. J. Lepr. **45** (1977) 175–182.

[29] Colston, M. J., Hilson, G. R. F. and Banerjee, D. K. The proportional bactericidal test. A method for assessing bactericidal activity of drugs against *Mycobacterium leprae* in mice. Lepr. Rev. **49** (1978) 7–15.

[30] Nakamura, M., Matsuo, Y., Mori, T., Okada, S., Nakayama, T., Saito, H., Ito, T., Koseki, Y., Ogawa, T., Ishihara, S., Kohsaka, K., Nakamura, K. and Ishidate, M. Failure to validate the growth of *Mycobacterium leprae* on M-Y 14b agar medium. Int. J. Lepr. **50** (1982) 480–487.

[31] Lancaster, R. D., Hilson, G. R. F., McDougall, A. C. and Colston, M. J. *Mycobacterium leprae* infection in nude mice: Bacteriological and histological responses to primary infection and large inocula. Infect. Immun. **39** (1983) 865–872.

tion). Nevertheless, the genome has been successfully isolated from *M. leprae*[32].

Problems in studying *M. leprae* metabolism. There are two major difficulties. Firstly, the amount of material is very limited; this is particularly acute since, by dry weight of *M. leprae*, most metabolic activities measured have proved to be low relative to the same activities in other mycobacteria. These low activities may be partly a reflection of the low viability of suspensions of *M. leprae* with accompanying denaturation of enzymes and loss of metabolic activity. Despite the great improvements in the availability of leprosy bacilli for research, the total amount of bacteria is rather limited. From 50 g of infected tissue, up to 250 mg dry weight of bacteria may be obtained. In practice, this has restricted the work to experiments with whole organisms or crude extracts.

Secondly, *M. leprae* organisms must be purified from the infected host tissue before they can be used for metabolic studies. This is tedious rather than problematical, since a method has been devised for preparing suspensions almost entirely free of host debris[10]. An easy way to examine suspensions of purified *M. leprae* is to prepare a smear and use soluble (aniline) blue which is useful as a counterstain. Suspensions appearing to be pure by examination with conventional counterstains (in the Ziehl-Neelsen procedure), such as methylene blue or malachite green, may prove to be heavily contaminated with tissue debris if soluble blue is used instead[10, 33].

Related to the problem of purity of suspensions is the possibility of detecting host-derived activities in suspensions and extracts of *M. leprae*. Since more vigorously purified suspensions of bacteria are more likely to have host-derived activities removed, methods of purification used by research workers are summarized (Table 1). But extensive purification is not good enough; even in highly purified suspensions of mycobacteria, some host-derived activities remain[34–36]. NaOH treatment is one way of abolishing host-derived activities from mycobacteria. Originally, NaOH treatment was used for decontamination of clinical smears which were to be investigated for *M. tuberculosis*. While other bacteria are killed by this treatment, mycobacteria survive well. It is possible that some damage is done to mycobacteria, possibly some surface-located enzymes might be affected by such treatment, but some *M. tuberculosis* enzymes[37] and many *M. leprae* enzymes can still be detected after incubation of purified bacteria in 1 N NaOH at 25°C for 1 hr[36, 38, 39]. NaOH treatment successfully removed host activities which were adsorbed to *M. tuberculosis*[34] and *M. leprae*[35, 36], and a large proportion of the leprosy bacilli remained viable after treatment (A. C. R. E. Lowe, personal communication).

While detection of a metabolic activity after NaOH treatment or possibly other surface treatments (a method using diazotization has been used with *M. leprae*[35]) is evidence for an authentic mycobacterial activity, it is necessary to have a number of criteria for distinguishing host activity from mycobacterial activity. The following techniques have been used to differentiate enzyme activities in host tissue and in extracts of *M. leprae*:

a) Substrate specificity. Enzymes of *M.*

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[32] Imaeda, T., Kirchheimer, W. F. and Barksdale, L. DNA isolated from *M. leprae*: Genome size, base ratio and homology with other related bacteria as determined by optical DNA-DNA reassociation. J. Bacteriol. **150** (1982) 414–417.

[33] Wheeler, P. R. and Draper, P. Soluble blue as a counterstain in the Ziehl-Neelsen procedure—a rapid communication. Int. J. Lepr. **48** (1980) 15–17.

[34] Kanai, K. Detection of host-originated acid phosphate on the surface of *in vivo* grown tubercule bacilli. Jpn. J. Med. Sci. Biol. **20** (1967) 73–90.

[35] Wheeler, P. R., Bharadwaj, V. P. and Gregory, D. N-acetyl-β-glucosaminidase, β-glucuronidase and acid phosphatase in *Mycobacterium leprae*. J. Gen. Microbiol. **128** (1982) 1063–1071.

[36] Wheeler, P. R. and Gregory, D. Superoxide dismutase, peroxidatic activity and catalase in *Mycobacterium leprae* purified from armadillo liver. J. Gen. Microbiol. **121** (1980) 457–464.

[37] Kanai, K. Resistance to NaOH treatment of *in vivo* grown tubercle bacilli. Jpn. J. Med. Sci. Biol. **20** (1967) 91–96.

[38] Wheeler, P. R. Metabolism of carbon sources by *Mycobacterium leprae*. A preliminary report. Ann. Microbiol. (Paris) **133B** (1982) 141–146.

[39] Wheeler, P. R. Catabolic pathways for glucose, glycerol and 6-phosphogluconate in *Mycobacterium leprae* grown in armadillo tissues. J. Gen. Microbiol. **129** (1983) 1481–1495.

TABLE 1. *Outlines of methods for obtaining suspensions of mycobacteria from infected tissues*[a,b] *for metabolic studies.*

Principal research worker(s)[c]	Organisms	Aspects of metabolism[d]	Method of purification[e]	Footnote no.
Ambrose, Antia and Khanolkar	ML	DOPA[f] Nucleic acid[f]	Homogenization only; activity detected by autoradiography.	119
Ambrose, Khanolkar and Chulawala	ML	DOPA[f] Nucleic acid[f]	Chopping then incubation in distilled water → trypsin treatment (twice, pH 7.2).	120
Dhople, Hanks and Storrs	ML, MLM[b]	ATP levels	Homogenization → low speed centrifugation to remove tissue debris; supernatant = suspension. Other purification methods compared with above[101,102].	99–102
Drutz and Cline	ML	Nucleic acid	None; cells infected with *M. leprae* were harvested and used intact.	131
Imaeda, Kirchheimer and Barksdale	ML, MLM	*M. leprae* genome[g]	Homogenization → pronase + SDS treatment → differential centrifugation → DNAase treatment → pronase treatment (all stages at pH 7.6).	32
Jayaraman, Mahadevan and Mester	ML	DOPA	Chopping then incubation in distilled water → trypsin treatment (twice, pH 7.2).	123
Kato	ML	Chemoautotrophism	Tissues shipped; storage at ambient temperature at pH 3 in HCl/NaCl → rinsing in distilled water → homogenization (pH 7.2) → differential centrifugation.	89
Kato, Ishaque and Adapoe	ML, MLM	Pyruvate and acetate Electron transport	Rinsing in distilled water → mincing (if skin) → homogenization (pH 6.8–7.4) → filtration through nylon filter → differential centrifugation.	84–86, 90, 95, 96
Katoch, Wayne and Diaz	ML	Catalase	Homogenization in Tris → DNAase treatment (pH 7.2) → Percoll-density gradient centrifugation (pH 7.2) → separation on aqueous two phase system (pH 6.9).	51
Khanolkar	ML	Glucose[g] Amino acids[g]	Homogenization then incubation in distilled water → trypsin treatment (pH 7.2) → Percoll-density gradient centrifugation (pH 7.2).	64, 121
Kulkarni and Seydel	ML	Folic acid synthesis	Tissues irradiated; homogenization in Tris → DNAase treatment (pH 7.2) → Percoll-density gradient centrifugation (pH 7.2) → separation on aqueous two phase system (pH 6.9).	139
Kusaka, Sato and Shoji	MLM	Electron transport	Chopping then homogenization in isotonic alkaline KCl → repeated differential centrifugation at 4°C, with protease.	93
Kusunose, Ichihara and Izumi	ML	Superoxide dismutase	Homogenization in Tris → DNAase treatment (pH 7.2) → Percoll-density gradient centrifugation (pH 7.2)[f] → separation on aqueous two phase system (pH 6.9).	56, 57
Mori and Khosaka	MLM	Pyruvate and acetate Electron transport	Mincing then homogenization in distilled water → differential centrifugation → alternate suspension and then centrifugation of bacteria in NaOH and distilled water.	91, 94

TABLE 1. *Continued.*

Principal research worker(s)[c]	Organisms	Aspects of metabolism[d]	Method of purification[e]	Footnote no.
Nath, Prasad and Sathish	ML	Nucleic acid	Homogenization only; activity detected in bacteria phagocytosed by macrophages.	130, 132–134
Prabhakaran and Braganca	ML	Dissimilation of carbohydrates Electron transport Amino acid	Washing → mincing → homogenization in sucrose → differential centrifugation in sucrose.	52, 82, 83, 105, 106
Prabhakaran, Kirchheimer and Harris	ML	Hydrolytic enzymes DOPA Glutamate	Homogenization in distilled water or sucrose → differential centrifugation in sucrose and KCl solution; repeated in KCl solution until no methylene blue staining material was observed in smears.	42, 104, 109, 110, 114–116, 125
Shetty, Antia and Krishnaswamy	ML	Glutamate	Rinsing in saline with penicillin → chopping → homogenization in distilled water → trypsin treatment.	40
Tepper and Varma	MLM	Dissimilation of carbohydrates	Homogenization in distilled water → differential centrifugation in distilled water → NaOH treatment.	70
Wheeler, Hall, Khanolkar and Gregory	ML	Hydrolytic enzymes Catabolism of oxygen-free radicals Dissimilation of carbohydrates[f] Iron uptake Purines and pyrimidines	Homogenization in Tris → DNAase treatment (pH 7.2) → Percoll-density gradient centrifugation (pH 7.2)[f] → separation on aqueous two phase system (pH 6.9) when cell free extracts were required.	35, 36, 38, 39, 61, 81, 88, 135, 145

[a] *M. leprae* from armadillo liver, lymph nodes, or spleen, or human lepromata; *M. lepraemurium* from subcutaneous lepromata in mice or rats; source of tissue is generally specified in the text.

[b] ML = *M. leprae*; MLM = *M. lepraemurium.*

[c] If one paper, up to three names, or the first author. If more than one paper, all first authors and all co-authors of more than one paper—other co-authors have been omitted from this table. Arranged in alphabetical order by authors most cited within each group of papers or first authors if one paper.

[d] Refers to titles of sections in this review. Evidence for the metabolic activities being authentic *M. leprae* activities is discussed there.

[e] All start with tissue and finish with bacterial suspensions. Washings are done (by centrifugation and resuspension of bacterial pellets) between treatments and on the final suspension.

[f] Long incubation periods with whole *M. leprae* organisms, so contamination checks were made on the final suspension.

[g] Not strictly a study of metabolism.

leprae may have different specificities from similar enzymes from host tissue. A recent example is that *M. leprae* has a γ-glutamyl transpeptidase which is not stereospecific[40]. The enzyme from the host tissues is specific for L-amino acids.

b) Enzyme kinetics. These have rather limited use until enzymes can be purified from cell-free extracts of *M. leprae* but, with caution, simple experiments can be done with crude extracts. For example, the Km values for dihydroxyacetone phosphate of glycerol-3-phosphate dehydrogenase were, from armadillo liver, 0.1 mM and from *M. leprae,* 0.5 mM[38, 39].

c) Inhibition of metabolic activities. This has been particularly useful in identifying enzymes in extracts of *M. leprae* as either contaminating activities from the host or authentic bacterial activities. For example, transketolase in extracts of *M. leprae* is far more sensitive to inhibition by sulfate than

[40] Shetty, K. T., Antia, N. H. and Krishnaswamy, P. R. Occurrence of γ-glutamyl transpeptidase activity in several mycobacteria including *Mycobacterium leprae.* Int. J. Lepr. **49** (1981) 49–56.

transketolase in extracts of host tissues[38, 39]. Conversely, catalase detected in *M. leprae* extracts is identical to the host catalase with respect to inhibition by 3-amino-1,2,4-triazole[36].

d) Electrophoretic mobility. It is likely, since the substitution of one charged amino acid in a protein can affect this property, that similar enzymes from *M. leprae* and its host will have different electrophoretic mobilities. When isoenzymes are present, this method has an advantage over methods which rely on assaying crude extracts, when properties of individual isoenzymes cannot be separated, and there is a small risk that observed differences may be due to differences in the proportions of host isoenzymes in host-tissue and *M. leprae* extracts. Disc electrophoresis has been done successfully with, for example, the two dehydrogenases of the hexose monophosphate pathway (HMP) which were shown to be distinct from the host enzymes[38, 39] and with catalase which co-electrophoresed with host catalase[36]. In both cases crude extracts were used and gels were stained specifically for the enzymes in question.

Care must be taken not to overload electrophoretic gels—a problem when specific activities of enzymes are low, often the case in extracts of *M. leprae*—but if there is a risk of overloading, it is possible to mix small amounts of host-tissue extract (which will usually have a relatively high specific activity) and *M. leprae* extracts to see if bacterial and host activities can be separated on the same gel.

e) Serological tests. These can be done by purifying the host enzyme or mycobacterial enzyme from species other than *M. leprae* (sufficient material for purifying enzymes of *M. leprae* is not available) and raising antisera to the purified enzymes. Crossreaction with antiserum raised to host enzyme would indicate that the host enzyme is present in the extract, while any crossreaction with antisera to mycobacterial enzymes would depend upon the relatedness of the enzyme from *M. leprae* to the individual mycobacterial enzymes used to raise individual antisera.

It is desirable to use several criteria to differentiate between metabolic activities of *M. leprae* and host-derived activities. Thus, in the case of catalase in extracts of *M. leprae,* evidence that it was a contaminating activity from the host tissue was obtained from serological work, electrophoresis and use of an inhibitor, and supported by its inactivation by NaOH treatment. Finally, there is the possibility that the activity being studied may be absent in host tissue or abolished by the methods used for harvesting *M. leprae* from tissue. For example, no conversion of [^{14}C]-labelled substrates to CO_2 could be detected in uninfected armadillo tissue homogenized in the same way as infected tissue[38]. This metabolic activity depends on the functioning of whole pathways—and presumably the high degree of organization of the cell which is disrupted during the homogenization.

Hydrolytic enzymes

The association of two hydrolytic enzymes, N-acetyl-β-glucosaminidase (GlcNacase) and β-glucuronidase with *M. leprae,* was one of the reasons why Skinsnes and coworkers persevered with hyaluronic acid as a possible culture medium for *M. leprae.* They demonstrated these two glycosidases using cytochemical methods[41]. Prabhakaran detected the β-glucuronidase in cell-free extracts of *M. leprae* but claimed it was a host-derived enzyme[42]. However, it was shown that this conclusion was erroneous[35] because it was based on experiments using the inhibitor saccharo-1:4-lactone at levels too high to distinguish mammalian and bacterial β-glucuronidase[35, 43]. Using concentrations of lactones below those giving complete inhibition of both host and bacterial

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[41] Matsuo, E. and Skinsnes, O. K. Acid mucopolysaccharide metabolism in leprosy. 3. Subcellular localization of hyaluronic acid and β-glucuronidase in leprous infiltrates suggestive of a host-*M. leprae* metabolic relationships. Int. J. Lepr. **42** (1974) 399–411.

[42] Prabhakaran, K., Harris, E. B. and Kirchheimer, W. F. Absence of β-glucuronidase in *Mycobacterium leprae* and elevation of the enzyme in infected tissues. Lepr. Rev. **49** (1978) 203–213.

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enzymes, it was shown that β-glucuronidase and GlcNacase in extracts of *M. leprae* were authentic *M. leprae* enzymes[35]. Surface treatments (NaOH or diazotization) of *M. leprae* inactivated both these glycosidases, but identical treatments in other mycobacteria showed that such inactivation was typical for β-glucuronidase and GlcNacase located on the surface of mycobacteria[35].

Although β-glucuronidase and GlcNacase are present in *M. leprae* there are biochemical problems in proposing a pathway for hyaluronic acid utilization. Hyaluronidase, necessary to break down hyaluronic acid into the oligosaccharide substrates for β-glucuronidase, has never been detected in *M. leprae*[35, 41]. Key enzymes for the catabolism of glucuronate—one for each of two pathways—by glucuronate oxidase, or by the Entner-Doudoroff Pathway (detected previously only in glucose-grown *M. smegmatis* among the mycobacteria[44]) were not detected in *M. leprae*)[39]. Thus, pathways for catabolizing hyaluronic acid seem to be absent from *M. leprae*. β-Glucuronidase and GlcNacase are not even unusual activities of *M. leprae*; they are very widely distributed in the mycobacteria[45].

Acid phosphatase, which is also widely distributed in the mycobacteria, was detected in extracts of *M. leprae*[35]. Most of the activity was from host tissue but if the bacteria were incubated in 1 N NaOH before an extract was prepared, host enzymes were inactivated. *M. leprae*-acid phosphate was then revealed; the acid phosphatase from NaOH-treated *M. leprae* differed in its relationship of activity to pH, and its inhibition by vanadate ions, from the acid phosphatase from armadillo liver (i.e., host tissue)[35].

Catabolism of oxygen-free radicals

Catalase[46] and peroxidase[46] are present in most mycobacteria, a notable exception being isoniazid-resistant strains of *M. tuberculosis* which have neither activity and are susceptible to peroxide[47]. These strains have low virulence, partly due to their susceptibility to peroxide[47] which is produced by their host in its defense against the invading bacteria[48, 49]. Strains of *M. kansasii* isolated from water are generally high-catalase strains[50] which, unlike low-catalase strains of *M. kansasii*, are pathogenic[51].

Catalase and peroxidase were first detected in human-derived *M. leprae* in 1967, but there was no evidence to suggest that they were *M. leprae* (rather than host-derived) enzymes[52]. More recently, studies with the inhibitor 3-amino-1,2,4-triazole[36], and polyacrylamide gel electrophoresis[36] indicate that the catalase activity in armadillo-derived *M. leprae* extracts is host derived. Serological evidence broadly supports this view[53], but Katoch, *et al.* point out that since 80% of catalase activity in extracts of *M. leprae* is removed by immunoprecipitation with anti-armadillo liver antibody, it remains to be seen whether the remaining 20% is of host or mycobacterial origin[53]. If the 20% proves to be all bacterial, it would be surprising that it was not detected in the electrophoretic studies[36].

Could the host catalase be accreted by *M. leprae* to compensate for failure of the bacterium to make catalase? In *Plasmodium berghei*, it is suggested that the parasite spe-

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cifically adopts host cell superoxide dismutase (SOD) on the basis that the host SOD/hemoglobin ratio is much higher in the parasite than in the host (*P. berghei* lacks its own SOD)[54]. However, if the host catalase/host acid phosphatase ratio (both enzymes are lysosomal) is worked out from the literature on *M. leprae*[35, 36], the ratio is lower in extracts of *M. leprae* than in host tissue. Inactivation of catalase by NaOH lends support to the idea that the enzyme is probably adsorbed to *M. leprae* when lysosomes are ruptured during tissue homogenization.

In recent work, a peroxidase-like activity of *M. leprae* was detected on polyacrylamide gels, but this could have been a nonspecific peroxidatic activity, perhaps of a respiratory pigment; it had a different electrophoretic mobility from host (armadillo) peroxidase and catalase[36]. A crossreacting antigen between *M. leprae, M. smegmatis,* and *M. bovis* BCG was shown to be a peroxidase[55] and in *M. leprae* this may be the peroxidatic activity observed on polyacrylamide gels[36].

A manganese-dependent SOD in *M. leprae* was reported independently by two laboratories[36, 56]. It is serologically related to mycobacterial superoxide dismutases[57] and has a different mobility, on gel electrophoresis, to the superoxide dismutases in host tissues[36]. This enzyme is present in all other mycobacteria in which it has been looked for[58–60]. There is a discrepancy of nearly 100-fold in the level of SOD reported by the laboratories of Kusunose[57] and Wheeler[36]. This is hard to explain, since the two laboratories used the same method for isolation of *M. leprae* (Table 1) and agreed on the level of this enzyme in *M. phlei*[36, 57]. The availability of a more sensitive assay for SOD enabled Wheeler to assay a further 17 extracts of *M. leprae* for the enzyme. A revised value of 1.31 ± 0.21 (mean ± standard error) U (in cytochrome c assay) SOD/mg protein was obtained[61]. This was eight times higher than previously obtained[36], but the level of activity in those extracts used previously[36] was confirmed[61]. Some possible factors were looked at but the discrepancy[36, 57] was not explained. No host SOD was ever detected in *M. leprae*[36, 61] in contrast to another obligate parasite, *Treponema pallidum,* which is coated with the host enzyme[62].

Deficiencies in the enzymatic defense against oxygen-free radicals (no bacterial catalase detected and perhaps no true peroxidase) have been suggested as a factor in the slow growth of *M. leprae in vivo*[36], since the leprosy bacillus is found inside host phagocytes which produce superoxide and peroxide as a defense against bacteria[48, 49]. Peroxides form in many culture media, and it is possible that one of the reasons *M. leprae* is difficult to grow *in vitro* is that it may not be able to remove this peroxide effectively[36]. However, the discrepancy in the reported SOD level may be important in formulating ideas about the susceptibility of *M. leprae* to oxygen-free radicals; it has been suggested that very high SOD levels may compensate for the absence of catalase in some organisms, e.g., catalase negative strains of *Listeria monocytogenes*[63]. It would be useful to determine directly the susceptibility of *M. leprae* to superoxide and peroxide.

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TABLE 2. *Hexose monophosphate pathway in* M. leprae *and other mycobacteria.*

Bacterium	Carbon source	% Glucose metabolized by pentose cycle[b]	Footnote no.
M. leprae	NA[a]	9.3–10	38, 39
M. smegmatis	Glucose	9.2 (23)	44
	Glycerol	5.3 (14)	
	Pyruvate	0.4 (3)	
M. tuberculosis H37Rv:	Glycerol	2.7 (6)	67
Susceptible	Citrate + glucose	3.1	66
Streptomycin res.		4.4	
PAS res.		6.3	
Isoniazid res.		8.3	

[a] Grown in armadillo liver.

[b] Calculated from the data by the method of Wood, *et al.*[65]. Figures in parentheses were calculated by the author by the method of Chedelin[68]. The remainder of the glucose was metabolized by glycolysis.

Dissimilation of carbohydrates

Glucose. The observation that the uptake of glucose by *M. leprae* was partially inhibited by azide but that 2-deoxyglucose (which is not metabolized beyond phosphorylation) uptake was completely inhibited by azide indicated that *M. leprae* has an active transport system for glucose[64]. Given the slow rate of metabolism of glucose by suspensions of *M. leprae*[38, 39], it is likely that some glucose is made available for catabolism by a process of diffusion driven by metabolism[64].

Studies with [1-^{14}C]-glucose and [6-^{14}C]-glucose have been done on a number of strains of mycobacteria (Table 2). The proportion of glucose catabolized by the hexose monophosphate pathway (HMP) or pentose cycle (terminology of Wood[65]) was calculated from the proportions of differentially labelled glucose taken up that were converted to CO_2[65] by whole bacteria in the work of O'Barr and Rothlauf[66] and Wheeler[38, 39]. Bai, *et al.*[44, 67], on the other hand, used a method described by Chedelin[68] based on the proportions of differentially labelled glucose supplied that were converted to CO_2. Theoretical problems in quantifying the pathways for carbon metabolism are discussed in detail by Wood, *et al.*[65]. I have recalculated the involvement of the pentose cycle in mycobacteria by their method[65] and included the authors' values also (Table 2).

Methods based on evaluating the proportion of glucose supplied being converted to CO_2 are impracticable for *M. leprae*; in a 200 µl incubation, 1 mg (dry weight) leprosy bacilli in 20 hr only take up about 1% of the glucose (4–20 nmol) supplied[38, 39, 64].

A comparison of one overall metabolic activity, that of rates of conversion of glucose to CO_2 by mycobacteria, can be made from the literature. On the basis of the conversion of similar concentrations of [U-^{14}C]-glucose to CO_2 by a suspension of 1 mg/ml mycobacteria/20 hr, the rate was 0.05% for *M. leprae*[38, 39] and 4% for *M. phlei*[38]. With [6-^{14}C]-glucose, the rate was 0.03% for *M. leprae*[38, 39], 2% for *M. tuberculosis*[67], and 10–15% for *M. smegmatis*[44]. In these calculations it was assumed that scaling up or down of incubations would not affect the proportion of glucose metabolized, and that the rate of CO_2 production was proportional to the concentration of glucose supplied to the mycobacteria. While glucose is oxi-

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TABLE 3. *Comparison of levels of enzymes of glucose catabolism*[a] *in* M. leprae[38,39] *compared with other mycobacteria.*

Enzyme	Percentage activity[b] in extract compared with extracts from			
	M. smegmatis[44]	*M. tuberculosis* H37Rv[67,73]	*M. tuberculosis* H37Ra[148]	*M. lepraemurium*[39]
Hexokinase	5	16	ND[c]	ND
Phosphohexoisomerase	ND	ND	9	ND
Phosphofructokinase	7	0.7	1.5	ND
Aldolase	15	17	46	ND
Pyruvate kinase	0.11	0.40	ND	ND
Glucose-6-phosphate dehydrogenase	0.12–0.35	0.6	ND	24
6-Phosphogluconate dehydrogenase	13–17	143	ND	2100

[a] Also confirmed as *M. leprae* enzymes in glucose catabolism: glyceraldehyde-3-phosphate dehydrogenase, transketolase.

[b] (Sp. act. in extract of *M. leprae* ÷ sp. act. in extract of mycobacterium) × 100. Assay conditions (pH, temperature, use of crude extract) were similar for all extracts of mycobacteria in this table.

[c] Not done.

dized by most mycobacteria[69] including *M. leprae*[38, 39], it is an extremely poor substrate for oxidation by *M. lepraemurium*[38, 70]. Cell-free extracts prepared from mycobacteria and armadillo tissue from which *M. leprae* were harvested did not convert glucose, glycerol, and succinate to CO_2[38].

Key enzymes of the Embden-Meyerhoff Pathway (EMP) of glycolysis and the HMP were detected in crude extracts of *M. leprae,* and they have all been confirmed to be authentic *M. leprae* enzymes[38, 39]. A number of interesting properties of some of these *M. leprae* enzymes were noticed. Phosphohexoisomerase, phosphofructokinase, and glucose-6-phosphate dehydrogenase were inhibited by possible regulators of metabolic activity (6-phosphogluconate, phosphoenolpyruvate and stearoyl-CoA, respectively). Phosphofructokinase and pyruvate kinase have specific requirements for adenine nucleotides[39].

Observations on mycobacterial fructose-1,6-diphosphate aldolases are interesting. Aldolases can be divided into two types[71]: Class I aldolases, which form Schiff's bases with their substrates under reducing conditions and are insensitive to EDTA, are typically mammalian; whereas Class II aldolases, which do not form Schiff's bases, are inhibited by EDTA and require added thiols for full activity, are typically bacterial. *M. tuberculosis,* however, produces a Class I aldolase when fermenter grown[72] and a Class II aldolase when surface grown[73]. On the other hand, the aldolase isolated from surface-grown *M. smegmatis* is Class I[74]. The aldolase in extracts of *M. leprae* is Class II and, therefore, clearly distinguishable from the host (Class I) aldolase[39]. However, the above information on aldolase in other mycobacteria suggests that it may be unwise to attempt to infer very much about the metabolic state of *M. leprae* in the host solely on the basis of this enzyme.

Assay conditions and methods of preparation of crude extracts for a number of other mycobacteria were similar to those used for *M. leprae,* so it is possible to compare the levels of some of these enzymes in other mycobacteria with levels of similar enzymes in *M. leprae* (Table 3). Most of the work on other mycobacteria has been done with organisms grown *in vitro,* so caution is required in comparing the levels of their metabolic activities with *M. leprae* grown

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in vivo. However, the relatively low levels of most enzymes in *M. leprae* (Table 3) are consistent with the slow growth and low viability of suspensions of leprosy bacilli.

The evidence supports the general statement that the EMP is the main pathway for glucose catabolism in mycobacteria (including *M. leprae*) and that the HMP (although quantitative assessments vary) has a minor but significant role.

6-Phosphogluconate and gluconate. One notable feature of the enzymes of *M. leprae* was that 6-phosphogluconate dehydrogenase (6PG-DH) activity was about 100 times higher[38, 39] than glucose-6-phosphate dehydrogenase (G6P-DH). This could not be accounted for by differential stability of the enzymes[38, 39] and is unique among the mycobacteria. The difference in levels of 6PG-DH and G6P-DH is reflected in the comparison in levels of these two enzymes with other mycobacteria (Table 3). Subsequently, it was shown that 6PG was oxidized, at a slightly higher rate than glucose, to CO_2[39]. An interesting but unexplained observation was the stimulation of 6PG oxidation by umbelliferyl phosphate (or possibly umbelliferone). This was clearly not a result of inhibiting phosphatases, since G6P oxidation and uptake were inhibited by the addition of umbelliferyl phosphate to incubations[39]. The utilization of 6PG suggested the metabolic role of scavenging 6PG for the disproportionately high level of 6PG-DH, analogous to the situation in plasmodia, which have no G6P-DH but may scavenge 6PG in erythrocytes[75]. A possible source of this substrate was suggested for *M. leprae in vivo*; it may be available as a byproduct of the metabolism of macrophages (particularly when activated)[48] in which leprosy bacilli reside.

Hydrolysis of the phosphate of 6PG would inhibit the rate of evolution of CO_2 since suspensions of *M. leprae* only oxidized gluconate very slowly[39] to CO_2. The enzymes for gluconate catabolism by *M. leprae* have not been detected. Unsuccessful attempts were made[39] to detect phosphotransferases for gluconate using ATP[76, 77] or polyphosphate[77] which are present in other mycobacteria. *Acetobacter*-like conversion of glucose to gluconate[78] could not be detected in *M. leprae*[39], but the metabolism of gluconate to 2-oxoglutarate[79] in the metabolic pathway for direct oxidation of glucose in *Acetobacter* has not yet been investigated in *M. leprae*.

The presently available evidence suggests that gluconate is of little or no importance as a substrate for *M. leprae*, but 6-phosphogluconate is readily metabolized by both whole cells and extracts of *M. leprae*.

Glycerol. Many mycobacteria oxidize glycerol. *M. leprae* oxidizes this substrate at 14% of the rate at which *M. lepraemurium*, another organism grown *in vivo*, oxidizes it[38]. Glycerol-3-phosphate dehydrogenase[38,39] and glycerol dehydrogenase[39] were detected and confirmed as mycobacterial enzymes in extracts of *M. leprae*. Thus glycerol can be oxidized by an NAD-dependent enzyme then phosphorylated, or vice versa, before catabolism of dihydroxyacetone phosphate by the EMP[39].

Fate of pyruvate and acetate. In skin biopsies from multibacillary leprosy patients, an additional lactate dehydrogenase isoenzyme was observed[80]. It has been suggested that this represents lactate dehydrogenase from *M. leprae*. Confirmation of this suggestion awaits electrophoresis of the enzyme from purified leprosy bacilli. Recently, a value of 0.6 mU lactate dehydrogenase/mg protein has been obtained in extracts of NaOH-treated *M. leprae* from armadillo

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tissue (Wheeler and Bharadwaj, unpublished results; lower activity in extracts of untreated *M. leprae*). At this level, 0.5 mg protein derived from about 2×10^{10} *M. leprae* would be required for a band to develop on a gel stained for dehydrogenase activity[39, 81]. On this basis, it appears that insufficient *M. leprae* lactate dehydrogenase would be present to account for the additional isoenzyme[80].

Previously, lactate oxidizing activity was demonstrated in partly purified suspensions (Table 1) of *M. leprae*[52, 82]. This could have been due to lactate dehydrogenase or lactate oxygenase, a characteristic mycobacterial enzyme[2]. The activity described in these reports[52, 82] could, however, have been host derived.

Lactate dehydrogenase and lactate oxygenase may represent a metabolic route for conversion of pyruvate to acetate in mycobacteria but the reaction (pyruvate → acetyl CoA) catalyzed by pyruvate dehydrogenase is more efficient in energy terms[2, 3]. In initial studies on the tricarboxylic acid cycle and related activities (including pyruvate dehydrogenase), it was shown that pyruvate[82, 83], succinate[52, 82, 84, 85], yeast extract[84], and a number of thiol-reagents[84] stimulated oxygen uptake by *M. leprae*. A similar, limited range of substrates stimulated oxygen uptake in *M. lepraemurium*[84, 86].

There may be a problem of permeability to possible substrates. In *M. smegmatis*, for instance, permeases to acetate and fumarate must be induced[87]. In incubations with some substrates, oxygen uptake was stimulated when extracts rather than whole *M. leprae* were used[82] and when suspensions of *M. lepraemurium* were frozen and thawed[84]. In this work, bacteria were not very rigorously purified (Table 1) and it is possible some of the oxygen uptake was an activity of remaining host contamination.

Subsequent work with more rigorously purified bacteria (Table 1) and radioisotopes (more sensitive than measuring changes in oxygen concentration) showed that, in a system where tissue or *M. leprae* extracts did not oxidize substrates to CO_2, succinate[38], citrate, [6-^{14}C]-glucose, and [2-^{14}C]-pyruvate[88] were converted to $^{14}CO_2$ by suspensions of *M. leprae*.

The labelling of glucose and pyruvate was such that evolution of $^{14}CO_2$ would occur only if they were metabolized through the Krebs cycle[88]. All of the enzymes of the Krebs cycle were identified in extracts of *M. leprae* and shown to be bacterial rather than host derived[81, 88]. Of particular interest were those involved in malate oxidation and fumarase. Malate dehydrogenase and malate-vitamin K reductase were present but malic enzyme (decarboxylating) was not detected in *M. leprae*[81]. This pattern is typical of slow growing mycobacteria[3]. It has been suggested that through malic enzyme, fast growers rapidly generate NADPH for biosynthetic purposes and slow growers may generate their less urgent requirements for NADPH through other reactions. In *M. leprae*, some of the tricarboxylic acid cycle enzymes can use NADP as well as (or in place of) NAD[88], and NADPH could also be generated through the HMP[39]. Fumarase activity fell rapidly in extracts of *M. leprae* kept at 4°C[88]. 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*. The inactivation of fumarase in *M. leprae* was strongly inhib-

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ited by phenylmethylsulfonyl fluoride, suggesting that the inactivation was due to a protease. It was suggested that this protease might control Krebs cycle activity, and therefore the rate of catabolism of substrates in *M. leprae,* if the protease could inactivate fumarase in the living organism as well as in extracts in experimental conditions[88].

Finally, it was important to demonstrate oxoglutarate dehydrogenase to confirm that the tricarboxylic acid cycle had an oxidative function in *M. leprae.* Many organisms in which the cycle has an anaplerotic function lack this enzyme, but it was recently demonstrated in *M. leprae* and shown to be an authentic bacterial enzyme[88]. Previously, Kato and coworkers failed to detect oxoglutarate dehydrogenase in *M. leprae* from human lepromata[89] but the storage conditions (pH 3, room temperature for eight days; see Table 1) were very unusual, and subsequently they had difficulty repeating some of the results obtained in that study. Oxoglutarate dehydrogenase has now been shown to be present in *M. lepraemurium*[88]. Although it was demonstrated previously in partly purified (Table 1) *M. lepraemurium*[90], evidence for its authentic bacterial nature was only obtained recently[88]. Initially, it was claimed that this enzyme was deleted in *M. lepraemurium*[91], but it appears likely that NaOH treatment used in that work (Table 1) abolished so much oxoglutarate dehydrogenase activity that the enzyme was not detectable[88]. Thus, while there are important differences in anaerobic pathways of carbon metabolism between *M. lepraemurium* and *M. leprae,* the operation of the tricarboxylic acid cycle in the two organisms is similar.

Chemoautotrophism in leprosy? The detection of ribulose 1,5 diphosphate carboxylase[89] together with the absence of NADH oxidase and oxoglutarate dehydrogenase[89] led Kato to suggest that *M. leprae* may be an autotroph. These results might have signalled a new approach in attempting to culture the leprosy bacillus but they need to be confirmed, especially since NADH oxidase activity in *M. leprae* was subsequently detected in Kato's laboratory[85].

A number of strains of mycobacteria—6Y, AU, and *M. gordonae* EO2—were shown to have inducible enzymes for autotrophic growth. Little or no ribulose 1,5 diphosphate carboxylase and no hydrogenase was detected unless these strains were grown autotrophically, when both enzymes were readily detected[92]. If these enzymes are also inducible in *M. leprae,* then it seems surprising that they were expressed in bacteria growing in host tissue.

Oxidative phosphorylation

Electron transport. The first observations which suggested an electron transport system in *M. leprae* were the reduction of cytochrome c in the presence of succinate and the uptake of oxygen in the presence of *p*-phenyldiamine by suspensions of the bacteria[52, 83]. These activities were observed after a long lag period which suggested that unbroken *M. leprae* presented a permeability barrier[52]. If that explanation is correct, then it is an indirect indication that the activity is bacterial, rather than host-derived contamination. However, no attempt was made to detect contamination by host material apart from staining smears[52]—it would have been easy to overlook contamination, since soluble blue was not used as the counterstain[33].

Then Ishaque, *et al.* showed that extracts of *M. leprae* isolated from armadillo tissues had NADH oxidase activity[85], in contradiction to an earlier report by some of the same workers[89]. They showed that the NADH oxidase activity was completely inhibited by 0.1 mM rotenone but that oxygen uptake stimulated by succinate was not inhibited by rotenone, suggesting that different flavoproteins were involved in succinate and NADH oxidation[85]. No attempt was made to distinguish true bacterial from host activities, although the use of respiratory

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TABLE 4. *Cytochromes in* M. leprae *and other mycobacteria grown* in vivo.

Cytochrome(s)	Source[a]	Reduced λ max			Footnote no.
		α	β	γ	
A class or a_2	*M. lepraemurium*, BCG[b,c]	—[d]	—	—	93
a + a_3	*M. lepraemurium*[c]	605–607	—	445	95, 96
a + a_3	*M. leprae*[e]	605–607	—	443–445	85
a_2	*M. lepraemurium*	630	—	440	94
a_3-CO	*M. lepraemurium*	590	—	432	96
B class	*M. lepraemurium*, BCG	—	—	—	93
b	*M. lepraemurium*	560[f]	530	430	95, 96
b	*M. leprae*	562	530	429	85
b_1	*M. lepraemurium*	561	NF[g]	433	94
c	*M. lepraemurium*, BCG	—	—	—	93
c	*M. lepraemurium*	552	523	427?	95
c	*M. leprae*	553	523?	NF[g]	85
o-CO	*M. lepraemurium*	572	540	417	96
o-CO	*M. leprae*	570–572	540	418	85

[a] Extracts or particulate fractions were used except when whole bacteria grown *in vivo* were used as well as extracts[93], and when only whole bacteria were used[96]. Concentrations of bacteria, or estimates of what the concentrations would have been had no breaking/fractionation been done, were: 5–8 mg/ml[85], 20 mg/ml[95,96], 15–20 mg/ml[93], and 12–30 mg/ml[94].

[b] *M. bovis* BCG.

[c] Mouse grown.

[d] Component or reaction absent.

[e] Armadillo grown.

[f] λ max (α-band) was 562 nm when NADH was used instead of dithionite to reduce cytochromes.

[g] NF = None found or not detected. There should be a value for λ max, but it was either swamped by λ max for another component or insufficient material was used.

inhibitors[85] may provide a useful lead. NADH oxidation recently has been observed as background activity in other enzyme assays, even with extracts from NaOH-treated *M. leprae*[38], supporting evidence for the presence of NADH oxidase in *M. leprae.*

In early work on cytochromes of mycobacteria, Kusaka, *et al.* failed to demonstrate cytochromes in *M. lepraemurium* or *M. bovis* BCG grown *in vivo,* but they detected cytochromes in a number of mycobacteria grown *in vitro*[93]. Subsequently, using similar quantities of bacteria, Mori, *et al.*[94] and then Ishaque, *et al.*[95, 96] detected cytochromes in *M. lepraemurium* grown *in vivo* (Table 4). Mori, *et al.* treated their bacteria with NaOH; this may have removed some bacterial cytochromes but the two which they detected would be mycobacterial. On the other hand, Ishaque, *et al.* did not use NaOH treatment, so it is necessary to distinguish any cytochromes detected in their work from host cytochromes. Cytochrome c of *M. lepraemurium* was clearly bacterial[95]. In the only work on the cytochromes of *M. leprae,* cytochromes a + a_3, b, c and o (Table 4) were detected[85]. Some of these may be derived from host tissue, but since cytochrome o is not found in mammalian tissue it may be considered to be an authentic cytochrome of *M. leprae.* Comparisons of the spectral characteristics of cytochromes from host tissue and host-derived mycobacteria need to be done in order to evaluate the remaining cytochromes detected in *M. leprae* and *M. lepraemurium.* While there is now evidence for the presence of cytochromes in *in vivo*-grown mycobacteria, including *M. leprae,* none of the very distinct cytochrome b species ob-

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[96] Ishaque, M. and Kato, L. The cytochrome system in *Mycobacterium lepraemurium.* Can. J. Microbiol. **20** (1974) 943–947.

served in *M. phlei* (α-band at 574 nm[97]) or multiple forms[98] have been seen in *M. leprae* or *M. lepraemurium*. The cytochrome a_2 of *M. lepraemurium*[94] may be similar to the "cytochrome a + a_3" (α-band at 623 nm[97]) of *M. phlei*.

ATP levels and leakiness. Coupled electron transport would result in ATP formation. The level of ATP measured in *M. leprae* was 12.4 pg/mg (dry weight) bacteria, about half that detected in *M. lepraemurium*[99]. A very simple purification procedure was used (Table 1), but it was shown that no ATP from the host would have been measured in the suspensions of bacteria[99, 100]. When mycobacteria are more thoroughly purified (including NaOH and other treatments), ATP levels remain constant in all mycobacteria (including *M. leprae*) studied[101], except *M. lepraemurium*[101, 102] in which ATP levels fall. It has been suggested that ATP levels can be correlated with the viability of suspensions of mycobacteria and, indeed, the ATP content of *M. lepraemurium* has been shown to be related to the ability of the bacterium to grow *in vitro*[100]. However, *M. lepraemurium* purified very extensively (differential centrifugation followed by separation on a sucrose gradient) were still fully infectious for mice (R. J. W. Rees, personal communication), although the ATP levels of the bacteria were very low[102].

The fall in ATP levels in *M. lepraemurium* on purification suggested that the bacteria may be "leaky"[102]. In the mycobacteria, only *M. lepraemurium* appears to be "leaky." An alternative possibility to leakiness of *M. lepraemurium* is that the organism has a transport system for ATP like that found in mitochondria and the rickettsiae[103].

Amino acid metabolism

General. Some of the earliest observations on the metabolism of *M. leprae* were concerned with amino acid metabolism. In human tissue from leprosy cases, the level of tyrosine is low compared with uninfected tissue and γ-aminobutyrate is present outside nerve tissue[82]. *o*-Diphenoloxidase[104, 105] and glutamate decarboxylase[106] were subsequently demonstrated in the leprosy bacilli. Both enzymes were demonstrated in leprosy bacilli from human and armadillo tissues.

Suspensions of *M. leprae* take up amino acid mixtures ([^{14}C]-protein hydrolysate). Uptake was enhanced in the presence of glucose, included as an energy source, and inhibited by azide[64], an inhibitor of active processes. Some of the radioactivity was incorporated into trichloroacetic acid-insoluble material. This incorporation was inhibited strongly by puromycin and chloramphenicol, suggesting that the bacteria were synthesizing protein[64]. The time for maximal uptake of amino acids by *M. leprae* was 72 hr; by *M. tuberculosis* H37Rv (grown *in vitro*), it was 0.25 hr[107]. Incorporation of amino acids in a cell-free protein

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synthesizing system has been demonstrated for *M. tuberculosis*[108] but not for *M. leprae*.

3,4-Dihydroxyphenylalanine. *o*-Diphenoloxidase activity can be detected in extracts[109] or suspensions[105] of *M. leprae*. A wide range of 3,4-dihydroxyphenols are oxidized, and even mimosine, in which a keto group replaces a hydroxyl group, is oxidized[110]. Unlike mammalian *o*-diphenoloxidase, the *M. leprae* activity oxidizes both D- and L-isomers[110] of DOPA (3,4-dihydroxyphenylalanine). DOPA oxidation in extracts of *M. leprae* is inhibited by a number of structural analogues, and more strongly by the copper chelator, diethyldithiocarbamate[110]. Even after sonication, the activity is still membrane bound[109].

The enzymatic nature of this diphenoloxidase activity has been questioned, on the basis that contaminants of suspensions of *M. leprae* such as hyaluronic acid oxidize DOPA, which is in any case very easily autooxidized[111]. Artifacts giving false positives in the similar DOPA reaction for mammalian cells occur; these may be related to processes of importance to the cell but are not related to tyrosinase[112]. The only evidence for the activity of *M. leprae* being enzymatic is the instability of the activity to heat[113], while stimulation of DOPA oxidation by some cuproproteins[110] and hyaluronic acid[113] is not heat sensitive. Reaction products of the activity are mainly indole-5,6-quinone[105,109] and a melanin-like polymer[114], both possible products of autooxidation. Some extracts of *M. leprae* appear not to have diphenoloxidase activity. This has been attributed to an as yet unindentified inhibitor from armadillo tissue[115]. Since DOPA is extremely unstable, especially in the presence of copper which is associated with the DOPA oxidase activity, further evidence for the enzymatic nature of *M. leprae* diphenoloxidase activity is essential.

Because diphenoloxidase activity is not present in any other mycobacteria[116], it has been suggested as a diagnostic test for *M. leprae*[114]. Criticism of some claims of growth have been made on the grounds that the alleged cultivable *M. leprae* do not possess DOPA oxidase[117]. Furthermore, Reza, *et al.* claimed that phenoloxidase activity can be detected in the serum of untreated lepromatous leprosy patients, but not tuberculoid leprosy patients or uninfected patients[118]. I was unable to repeat their observations, using serum from human lepromatous patients or from armadillos with a systemic infection (unpublished results). Debate on this matter must remain of academic rather than clinical interest, since the problems of auto-oxidation and, sometimes, unexplained inhibition make DOPA oxidation too unreliable and awkward a test for routine use.

DOPA was the first amino acid shown to be taken up by *M. leprae*, either from human[119, 120] or armadillo tissue[121]. The op-

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[120] Ambrose, E. J., Khanolkar, S. R. and Chulawalla, R. G. A rapid test for bacillary resistance to dapsone. Lepr. India **50** (1978) 131–143.

timal temperature for DOPA uptake was 34°C[121]; this is the only metabolic activity for which a temperature optimum has been published. DOPA uptake was inhibited by the metal-ion chelators diethyldithiocarbamate, EDTA, and 8-hydroxyquinoline[121]. Some importance has been attached to the inhibitory effect of diethyldithiocarbamate on DOPA uptake[121] and metabolism[110] since it alleviates experimental leprosy in mice[110]. This copper-chelator could, however, be generally inhibiting amino acid uptake into *M. leprae*. Copper-mediated amino acid uptake was reported for other mycobacteria[122]. DOPA incorporation was also inhibited by deoxyfructose-serotonin[123], claimed to be a possible antileprosy drug, and dapsone[120], an established antileprosy drug. Caution is needed in interpreting these results. DOPA uptake may be reflecting the viability of the suspension of *M. leprae*[121] and its inhibition may not be a direct result of the drug on DOPA metabolism. Deoxyfructose-serotonin may have inhibited DOPA uptake as a structural analogue, but the significance of this is doubtful since the DOPA in these experiments[123] was swamped by at least 70-fold molar excess of deoxyfructose-serotonin. It is difficult to postulate a direct effect of any drug on DOPA metabolism since the function of DOPA uptake and oxidation is unknown, although the temptation to look for drugs against what appears to be an activity unique among mycobacteria to *M. leprae* is great. Perhaps workers on DOPA metabolism should start looking at its relation to the metabolism of aromatic compounds in general, in *M. leprae*.

Glutamate. Incubation of suspensions of human-derived *M. leprae* with L-glutamic acid and pyridoxal phosphate at 37°C and pH 5 resulted in the formation of γ-amino butyrate[106]. Since glutamate decarboxylase is not present in some of the tissues[124] from which *M. leprae* was harvested[106], this suggests that the activity observed is bacterial. Recently, glutamate decarboxylase was measured by the rate of $^{14}CO_2$ evolution from [1-^{14}C]-glutamate and some differential inhibition studies were done on whole bacilli[125]. These results are difficult to interpret since the presence of an intact plasma membrane must be accounted for and whole organisms may decarboxylate glutamate through the tricarboxylic acid cycle. Glutamate decarboxylase has been detected in some but not all mycobacteria[82]. I have detected glutamate decarboxylase in extracts of *M. leprae* from armadillo liver. The specific activity, judged by formation of both CO_2 and γ-amino butyrate from glutamate, was 0.68 mU/mg protein, 35 times higher than that detected in extracts of host tissue (unpublished results). This confirms the presence of an *M. leprae* glutamate decarboyxlase but more work needs to be done before the role of this enzyme in *M. leprae* can be judged. One possibility that could easily be tested, by looking for the key enzyme succinic semialdehyde dehydrogenase, is that it is a way of catabolizing glutamate into the tricarboxylic acid cycle. Such a pathway has been demonstrated in germinating spores of *Neurospora crassa*[126].

γ-Glutamyl transpeptidase has been detected in suspensions of *M. leprae* from human biopsy and other mycobacteria[40]. The enzyme activity from *M. leprae* could be distinguished from the host-tissue enzyme since it could use glycyl-D-amino acid dipeptides as acceptors for glutamate; the host enzyme is stereospecific for L-amino acid peptides. Peptidases are important in forming and breaking cross-peptide links in the cell walls of mycobacteria[40, 127], and both

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glycine and D-amino acids are present in the cell walls of *M. leprae*[128]. The information on possible transpeptidase substrates in *M. leprae* was severely limited by lack of material.

Nucleic acids

Pyrimidine metabolism. One attraction of being able to demonstrate nucleic acid synthesis in *M. leprae* is that it might be used to indicate the viability of leprosy bacilli[129, 130]. Methods relying on plating on nutrient agar or broth are clearly impossible for *M. leprae,* and growth in mice takes at least six months to assess. It is possible to investigate thymidine incorporation by *M. leprae* in macrophages[130, 131] since macrophages do not incorporate thymidine. Since thymidine is a precursor of DNA but not RNA, its incorporation represents a fundamental correlate (DNA synthesis) with viability of *M. leprae.*

The first demonstration of thymidine incorporation by *M. leprae* was by Drutz and Cline[131] who used infected monocytes from the blood of bacteremic patients. On culture, silver grains were seen above leprosy bacilli in macrophages incubated with [^{3}H]-thymidine. There were no cultivable mycobacteria present. Subsequently, macrophages from animals not infected with leprosy were cultured and then infected with *M. leprae.* Nath and coworkers showed that thymidine is incorporated by human-derived *M. leprae* in infected macrophages from mice[130] or humans[132].

Thymidine incorporation in the macrophage system is inhibited by a number of antileprosy drugs including dapsone (at 3 ng/ml) and can be used to assess dapsone (DDS) resistance of *M. leprae;* incorporation in macrophages at different levels of dapsone was related to growth in the mouse foot pad of isolates of *M. leprae* with different degrees of dapsone resistance[133]. Lymphokines from tuberculoid and borderline leprosy patients also inhibited thymidine incorporation of *M. leprae* in macrophages[134], showing that the macrophage system for thymidine incorporation could be used to assess the killing of *M. leprae* mediated by the immune system.

A problem was that not all isolates of *M. leprae* incorporated thymidine[130, 132]. The lack of activity was neither accounted for by the batch of macrophages used[132] nor correlated with the number of bacteria used, the concentration of radiolabel, nor the morphological index (MI) of the bacteria[130]. In the most recent work, 25 out of 26 strains incorporated thymidine[133, 134] so the problem of non-incorporation may be technical.

Thymidine is also incorporated into suspensions of *M. leprae* in a maintainance medium[120, 129] or in buffer[135]. It was possible to inhibit incorporation by dapsone at 10 ng/ml with susceptible *M. leprae,* but incorporation into *M. leprae* suspected of being dapsone resistant was not inhibited by dapsone[120]. Thus thymidine incorporation into *M. leprae* in macrophages or suspensions appears to be promising for checking the viability of leprosy bacilli and drug screening, although the incorporation into suspensions of *M. leprae* was not compared quantitatively with the established foot pad technique[120].

Although uracil may be incorporated into

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cobacterium smegmatis. Enzymatic properties and sensitivity to β-lactam antibiotics. Eur. J. Biochem. **86** (1978) 97–103.

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TABLE 5. *Purine scavenging*[a] *in* M. leprae. *Comparison with pyrimidine incorporation.*

	pmol/10^{10} *M. leprae* (or extract thereof)/24 hr		
	In whole *M. leprae*		In extracts
	Incorporation into CCl_3COOH-insoluble material supplied at:		Rate of conversion to nucleoside monophosphates[b]. Substrate at 80 μM except for phosphoribosyltransferase assays (60 μM).
Substrate	17 μM	80 μM	
Adenine	47	106	180[c]
Adenosine	120	465	2.6×10^{5}[d]
Hypoxanthine	74	280	2400[c]
Inosine	6	ND[f]	70[d]
Guanine	71	ND	2450[e]
Guanosine	11	38	290[d]
Orotic acid	10	ND	ND
Uracil	9	ND	ND
Thymidine	6	19	ND

[a] Purine synthesis *de novo* at an extremely low rate was also detected using a different method from that for scavenging activity.
[b] Precursors of nucleotides and thus nucleic acid synthesis.
[c] Conversion by phosphoribosyltransferase.
[d] Conversion by nucleoside kinase.
[e] Based on Khanolkar and Wheeler[135].
[f] ND = Not done.

RNA as well as DNA, it was incorporated at similar rates in *M. leprae* as thymidine[135]. However, orotic acid, a precursor of all pyrimidines, was incorporated slightly more rapidly (Table 5)[135].

Purine metabolism. In similar conditions, purine bases were generally incorporated into *M. leprae* at six to 20 times the rate of pyrimidines (Table 5)[130]. Like thymidine, incorporation of hypoxanthine was inhibited by dapsone and other antileprosy drugs[135]. Although the incorporation of radioisotopically labelled purines and pyrimidines reflects uptake, dilution in pools of precursors, and nucleic acid synthesis, it was suggested that the pool sizes were quite small since the ratio of incorporation at 80 μM: 17 μM was similar for labelled substrates with a 20-fold range of rates of incorporation at 17 μM[135]. Rates of incorporation of supplied purines into *M. leprae* could be accounted for by the levels of phosphoribosyltransferases in extracts of *M. leprae,* and it was suggested that these enzymes operated for purine scavenging. The alternative scavenging pathway (purine base → nucleoside → nucleotide) had one deletion, or enzyme at extremely low level, for each purine (Table 5)[135].

The concept of *M. leprae* as an organism scavenging purines was strengthened by observations that whole organisms could interconvert purines (e.g., supplied with ^{14}C-adenine, label could be found in guanine). Enzymes for such interconversions were detected in extracts. However, *M. leprae* organisms also synthesized purines *de novo* at a very slow rate; label from ^{14}C-serine was repeatedly detected in purines from hydrolyzed nucleic acids[135]. Thus while purines are scavenged efficiently by *M. leprae,* the bacterium appeared not to be entirely dependent upon a supply of preformed purines.

Folic acid synthesis

Folic acid biosynthesis in *M. leprae* is of great interest because of the extreme sensitivity of the organism to an inhibitor of folate synthesis[136], DDS (dapsone; 4,4′-diaminodiphenylsulfone), the most widely used antileprosy drug. Most other mycobacteria are also sensitive to DDS[137], but

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only "*M. lufu*" is as sensitive as *M. leprae*[138]. Extracts of *M. leprae*, "*M. lufu*," *M. smegmatis* 607 and *Escherichia coli* synthesize folate at the relative rates 1:9:11:133 in identical conditions[139], and the I_{50} (concentration in inhibiting folate synthesis by 50%) of dapsone is 0.71 μM in "*M. lufu*"[139], 0.42 μM in *M. leprae*[139], and 35 μM in *E. coli*[139, 140], the latter sensitivity being similar to that of most mycobacteria[137, 140]. The I_{50} of DDS for growth of *M. leprae* (but not "*M. lufu*") is lower still. Perhaps levels of para-amino benzoic acid (PABA) are very low in *M. leprae*[139] or maybe DDS is accumulated; this happens in a strain of *M. kansasii*[141]. By antagonizing the action of DDS on folate synthesis by PABA, it can be shown that DDS has an extremely high affinity for the folate synthetase of "*M. lufu*" and *M. leprae* compared with other bacteria[139].

Crossresistance with sulfonamides occurs in *M. leprae*[142–144]. Sulfonamides, like DDS, form a pteroic acid analogue with DDS and 7,8-dihydro-6-OH-methylpterin pyrophosphate in *E. coli*[140]. However, no such analogue forms in "*M. lufu*" or *M. leprae*[139], so the great sensitivity of the leprosy bacilli to dapsone appears to be solely due to the extremely high affinity of DDS for their folate synthetases. The more readily available "*M. lufu*" (cultivable; *in vitro* generation time = 24 hr) appears to be an excellent model for folate synthesis and inhibition in *M. leprae*.

Iron uptake

The ability to take up iron is essential for the survival of bacteria. Most produce iron chelators and mycobacteria are no exception. Mycobacteria can scavenge iron from their environment by producing exochelins, which are powerful chelators, from which the organisms take up the iron[3]. Iron chelated to the exochelins from *M. neoaurum* was taken up by a suspension of *M. leprae* over 15 hr. No uptake occurred when the iron was chelated with exochelins from *M. bovis* BCG or *M. smegmatis* or to a single exochelin from *M. vaccae*[145]. The latter result was quite surprising, since *M. vaccae* and *M. neoaurum* are very closely related and the exochelins are very similar. Uptake into *M. leprae* appeared to be by facilitated diffusion since it was not inhibited by either $HgCl_2$, NaN_3, or 2,4-dinitrophenol. This was similar to the mode of uptake of ferriexochelin into *M. neoaurum* itself. These preliminary results[145] represent the first demonstration of iron uptake into *M. leprae*.

SUMMARY AND CONCLUSIONS

Recently, some knowledge of metabolic pathways, rather than individual enzyme activities of *M. leprae*, is becoming available. Ultimately this may be useful in devising culture media for *M. leprae*. Knowledge restricted to individual reactions may be misleading. For instance, the detection of GlcNacase and β-glucuronidase and the subcellular localization of hyaluronic acid led to attempts to cultivate *M. leprae* on hyaluronic-acid based medium. Subsequent investigations suggested that there was no pathway for the breakdown of hyaluronic acid in *M. leprae*.

The biochemical pathways for breaking down glucose and glycerol seem to be complete, and thus similar to many bacteria, but there is an unusually high level of one enzyme, 6-phosphogluconate dehydrogenase

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(6PGDH). Although 6-phosphogluconate is oxidized by *M. leprae,* and this is an unusual activity, reflecting very high levels of 6PGDH, glycerol may be a preferable energy source (on the basis of rates of oxidation by suspensions) for *M. leprae* in attempts to cultivate the bacterium. The utilization of 6-phosphogluconate might be important for other aspects of *M. leprae* metabolism not yet investigated (e.g., pentose metabolism) or it may be an adaption, not needed *in vitro,* to its existence in host macrophages. Alternatively, its oxidation may be a way of rapidly generating NADPH at critical times for the bacterium.

Other unusual activities which have been reported are the presence of an enzyme characteristic of chemoautotrophism[89], completely surprising in view of the biology of *M. leprae.* This report needs to be confirmed—some aspects, in fact, have failed to be confirmed. *o*-Diphenoloxidase activity is unique, among mycobacteria, to *M. leprae,* but there is still doubt over whether or not it is an enzymatic activity and its function is unknown. A transpeptidase which may be involved in cell wall synthesis, recently demonstrated in *M. leprae,* is a typical mycobacterial enzyme.

It is now known that iron could be supplied to *M. leprae* in potential media in the form of ferriexochelin from *M. neoaurum.*

Two "deletions" in the metabolic processes of *M. leprae* have been observed. Catalase appears to be absent in *M. leprae*; its addition to media stimulates the growth of some organisms[146] since peroxides form in the bacteriological media[147]. Purine synthesis *de novo* occurred at a very low rate compared with purine scavenging. Whether this is an adaption to growth *in vivo* is not known. The investigators are planning to compare these activities in other mycobacteria which can be grown *in vivo* and *in vitro* before deciding whether preformed purines might be a useful addition to potential culture media[135].

Generally, metabolic activities in *M. leprae* are low compared with other mycobacteria, including *in vivo*-grown *M. lepraemurium.* This seems to correlate with the low viability of suspensions of *M. leprae.* Some of the comparisons of levels of activities in this review are made with mycobacteria grown *in vitro* and some enzyme systems may be activated in bacteria grown in culture medium.

A great difficulty is assessing the efficacy of additions to potential media, since stimulation of growth cannot be assessed. The viability of suspensions of *M. leprae* in media can be checked by the mouse foot pad method, but results take too long to obtain if additions and alterations to media are to be made on the basis of their effect on the viability of suspensions of *M. leprae.* However, a suggested application of research on the metabolism of *M. leprae* is to use levels of metabolic activity to assess viability. It is important in this context that the activity is a function of living bacteria. While individual activities may persist for a while in dead bacteria and could be misleading, two promising potential correlates of viability—synthesis of nucleic acids from labelled thymidine or hypoxanthine and ATP levels—should require the complex organization of living organisms. A detailed study is still required to see if these activities correlate quantitatively with results obtained in the mouse foot pad.

The discovery of unusual or characteristic bacterial activities in *M. leprae* may lead to ideas for developing new drugs. So far, such development has been restricted to agents which inhibit DOPA uptake. These agents are diethylthiocarbamate and deoxyfructose-serotonin, both of which suppress leprosy in mice. A problem with designing drugs to antagonize DOPA metabolism is that since the metabolic significance of DOPA uptake and oxidation is unknown it is impossible to know if, in antagonizing the activity, the drug will affect the viability of *M. leprae.* Inhibition of a metabolic activity in whole *M. leprae* is not, per se, evidence for that activity being the target for the inhibitor, since the activity may be reflecting the viability of *M. leprae* and being inhibited

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as a result of killing of the bacteria rather than directly, for instance, inhibition of thymidine incorporation by DDS is known not to be a primary effect. In using the inhibition of uptake of DOPA and incorporation of thymidine into *M. leprae* to monitor drug sensitivity (i.e., as a potential drug screen), the results seem almost too good. When drugs were tested at their lowest concentrations (e.g., DDS, 3–10 ng/ml; rifampin, 1 μg/ml), they inhibited the activities very strongly within about one generation time (a fraction of the time which the drugs, in particular DDS, take to have an effect on growing *M. leprae*). The results are undoubtedly reproducible, and significant inhibition by a drug does not occur with strains of *M. leprae* resistant to that drug[120, 133] corresponding with testing for sensitivity to drugs by growing *M. leprae* in mouse foot pads[133]. An explanation is needed for the exquisite sensitivity of these metabolic activities of *M. leprae* organisms to antileprosy drugs and the rapid effect of the drugs on metabolic activities. Perhaps in the case of dapsone, folate is present at a very low level in *M. leprae* or it is rapidly depleted, so that inhibition of metabolic activities of *M. leprae* follows quickly after dapsone is added to suspensions of *M. leprae.*

I have shown that, despite the difficulties, and discounting work—particularly where enzyme and metabolic activities are not confirmed to be activities of *M. leprae*—which one must treat with skepticism, information on the biochemistry of *M. leprae* is becoming available. Can this process be accelerated by using more readily available mycobacteria as models for *M. leprae* metabolism? Such an approach needs care. Specific evidence should be sought to show that another mycobacterium is suitable as a model. For instance, observations on *M. leprae* and "*M. lufu*" suggested that "*M. lufu*" could be used as a model for folate synthesis and dapsone sensitivity in *M. leprae.* The point in this case is that some information on *M. leprae* was necessary in the first place before a model could be proposed. Studies on other mycobacteria can give leads to the biochemical study of *M. leprae* and may be used to look at general problems, such as the differences between *in vivo*- and *in vitro*-grown mycobacteria. However, *M. lepraemurium,* thought of as a suitable general model for *M. leprae,* now appears to be in many ways quite different from *M. leprae.* Already differences in the metabolism of glucose, in the levels of 6PGDH and catalase and in the amino acid composition of the cell wall peptidoglycan have been shown. Now that *M. leprae* is available in sufficient quantities for experimental work, it is essential that metabolic studies with leprosy bacilli should continue to be a very important part of research on *M. leprae.*

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