# Cytochemical Evidence for Aerobic Pathways in *Mycobacterium lepraemurium*[1]

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Although in the past numerous claims have been made for the *in vitro* cultivation of *Mycobacterium lepraemurium*, the increase in the bacterial mass obtained has been, at the most, very limited. So far, little information on the metabolism of the organism has been obtained and further studies are desirable.

The data presently available were derived from studies of infected animal tissue homogenates ([1, 6, 9, 10, 13, 14, 20]). The respiratory pathways of the organisms have been given much attention but conflicting data are on record.

Gray ([6]) found oxygen to be the terminal hydrogen acceptor whereas Kusaka *et al* ([11]) claimed that *M. lepraemurium* has no cytochrome system. Mori *et al* ([12, 13]) mention the presence of cytochromes of the b and a2 types, while Ishaque and Kato ([8]) and Kato *et al* ([10]) described the presence of the a + a3, b and c type cytochromes. Further evidence of aerobic metabolism in *M. lepraemurium* has been presented by Broman *et al* ([1]), Ito *et al* ([9]), and Tepper and Varma ([20]).

The enzymes of the tricarboxylic acid cycle were investigated by Mori *et al* ([14]) using enzymatic technics, and by Tepper and Varma ([20]) using radioisotopes.

It seemed to us that cytochemistry could be useful as an additional approach to these problems. Enzyme cytochemistry has been previously applied to *M. leprae* by Chatterjee ([2]). Using a light microscopic method he claimed to have observed cytochrome oxidase in the polar bodies of *M. leprae*.

In the present study, some key enzymes of aerobic pathways and the tricarboxylic acid cycle, e.g., cytochrome c oxidase, succinate dehydrogenase, peroxidase and catalase, were investigated in their ultrastructural localization in *M. lepraemurium* in infected mouse liver. The same technics were used on cultures of *M. fortuitum* selected as a control to evaluate the cytochemical methods used.

## MATERIALS AND METHODS

*M. fortuitum* strain M 1954 identified in our laboratory was used. The bacteria were cultured for 16-18 hours at 37°C in 5% bovine albumin, fraction V, in phosphate buffered saline (PBS). It was found that these conditions provided the greatest percentage of morphologically intact bacilli, particularly with respect to the plasma membrane.

Our *M. lepraemurium* strain ([15]) has been maintained in mice for more than ten years in our laboratory by I.V. passage every six to eight weeks of bacillary isolates from livers. For the study of *M. lepraemurium* 40 $\mu$ cryostat sections or small blocks of mouse liver were used. *M. fortuitum* suspensions were incubated and fixed in the culture tubes. After $OsO_4$ fixation, the suspension was coagulated in bovine albumin ([16]), allowing cutting the mass into small blocks which were handled subsequently in the same way as the tissue blocks.

Dehydration and embedding was as decribed by Spurr ([18]). Stained and unstained ultrathin sections were examined. Identical cytochemical methods were used for tissues and suspensions; only fixation and incubation times varied [times for suspensions are given in brackets].

**Cytochrome c oxidase.** After a two hour [one hour] fixation in pH 7.4 cacodylate buffered 3% glutaraldehyde, 40 $\mu$ cryosections were rinsed in buffered 7.5% saccharose solution. Incubation was performed at 37°C for one hour [one hour] in the following medium: 10 ml tris-HCl buffer pH 7.4; 10 mg DAB-HCl; 10 mg cytochrome-c (horse heart type III); 0.1 ml catalase (5 mg/25 ml); 0.75 gm saccharose. KCN 0.01 M was used as inhibitor. Postfixation was in 6% $OsO_4$ in veronal buffer pH 7.4.

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**Peroxidase and catalase.** For the discrimination of peroxidatic and catalitic activities, fixation and incubation conditions were as described by Herzog *et al* (7). For peroxidatic activity, fixation was in 6% cacodylate buffered glutaraldehyde; whereas for catalase this was 4% formaldehyde prepared from paraformaldehyde, both for one hour at 4°C. Composition of the media: 10 ml tris-HCl pH 10; 20 mg DAB-HCl; 0.5 gm saccharose; 0.1 ml 10% $H_2O_2$ (catalase) or 0.1 ml 0.5% $H_2O_2$ (peroxidase). Incubation was for two hours at room temperature. Controls were either by omitting addition of $H_2O_2$ or by 30 minute boiling of the samples.

**Succinate dehydrogenase.** The copper cyanide method as described by Geyer (5) was used.

## RESULTS AND DISCUSSION

Cytochrome c oxidase was demonstrated on the plasma membrane and on mesosomes in both *M. fortuitum* and *M. lepraemurium* (Figs. 1a, 1b). The deposits of electron-dense osmophilic material were in general rather slight and were not uniformly distributed over the entire membrane. In some experiments none were seen. This might be the result of differences in metabolic activity among bacteria in different experiments as noted by Tepper and Varma (20) and Tepper (19). Another reason might be a less efficient fixation to the added cytochrome c of mammalian origin by the bacterial oxidase. Two other key enzymes of aerobic metabolism that can be detected by electron microscopic cytochemistry are peroxidase and catalase.

Peroxidatic activity was demonstrable in both bacterial species, although at different sites. In *M. fortuitum* it was localized in granules lying in the nuclear mass (Fig. 2a). Even at high magnification (300,000 ×) a surrounding membrane was not detectable, but an electron lucent zone was clearly visible around the electron-dense enzymatically formed deposits. In *M. lepraemurium* this enzyme activity seemed to be associated with mesosomal membranes (Fig. 2b) and in some bacteria there was evidence of a large enzymatically active tubular system (Fig. 2c).

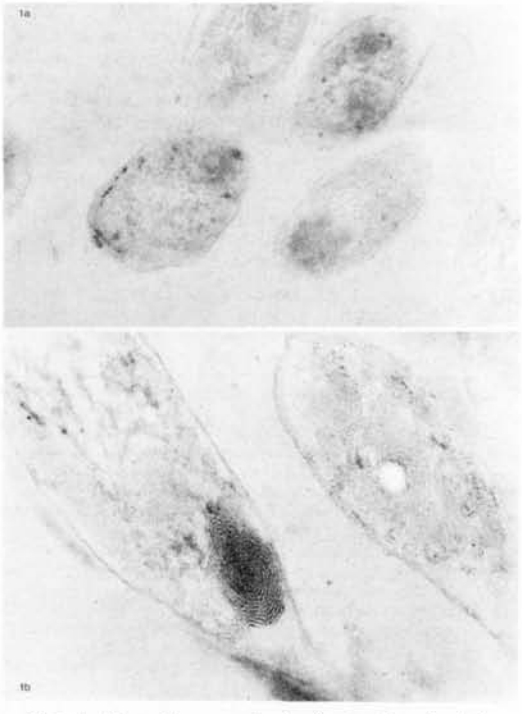
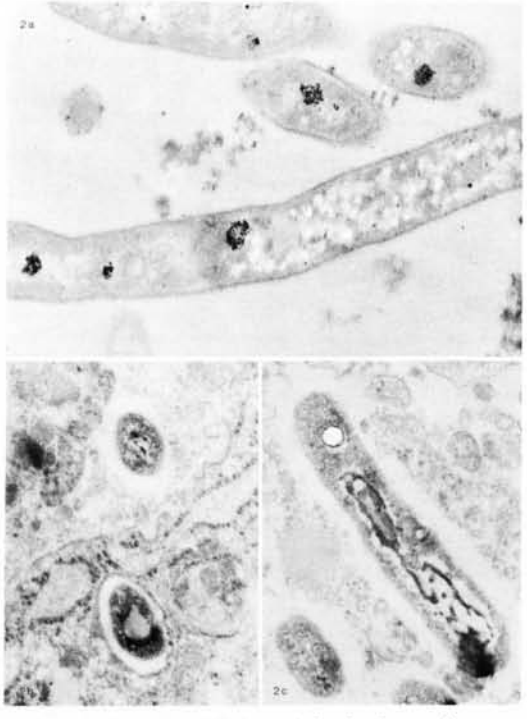


FIG. 1. Reaction product of cytochemical demonstration of cytochrome c oxidase in *M. lepraemurium* is located at the plasma membrane (Fig. 1a, magnification 140,000 ×) and at the mesosome (Fig. 1b, magnification 230,000 ×).



FIGS. 2a. Peroxydatic activity is shown in granules lying in the nuclear mass in *M. fortuitum* (50,000 ×).

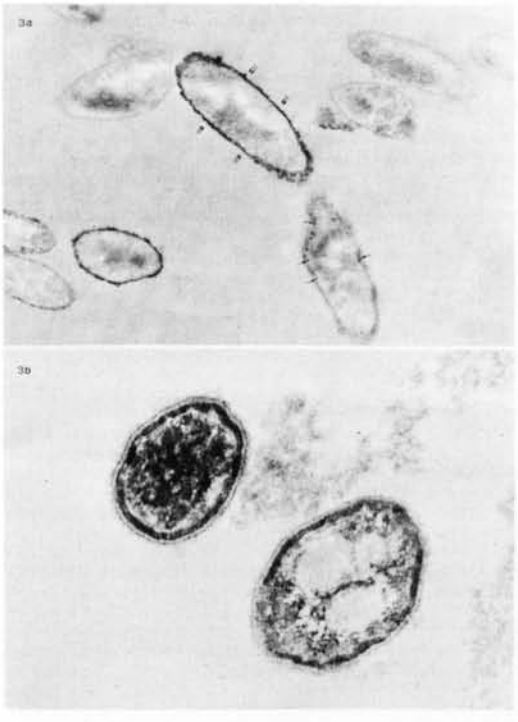
2b and 2c. This activity is associated with mesosomal membranes in *M. lepraemurium* (60,000 ×).

The three dimensional localization is now under investigation in a one million volt electron microscope. By this method the large granular localization of peroxidatic activity in *M. fortuitum* has already been confirmed.

The enzymes catalase and succinate dehydrogenase were only detected in *M. fortuitum*. For the catalase reaction two different localizations of electron-dense deposits have been observed: one on the plasma membrane and a second on the capsule (Fig. 3a). The former could be eliminated by boiling the preparation before incubation, whereas the latter was not affected by this treatment. In some bacteria the electron transparent zone between plasma membrane and cell wall is completely filled with a very fine deposit (Fig. 3b). Since this could also be removed by boiling, it is clearly a diffusion artifact. The deposits outside the cell wall must be interpreted as a nonenzymatic chemical reaction between diaminobenzidine and bacterial capsular constituents.

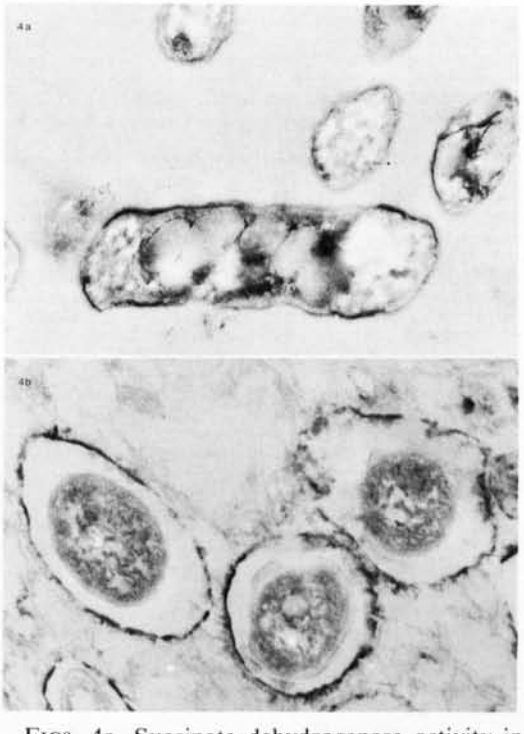
Succinate dehydrogenase in *M. fortuitum* was very active, resulting in a strong marking of the plasma membrane (Fig. 4a).

With the cytochemical technic used for the latter enzyme we were unable to detect any activity in *M. lepraemurium* in rat liver. Marked deposits were formed in mitochondria, and surprisingly in the bile canaliculi and on the membranes of the phagosomes (Fig. 4b). This activity could be blocked by malonate, suggesting genuine succinate dehydrogenase activity. However, it seems more likely that the electron-dense deposits are the reflections of an oxidative reaction that catalyzes hydrogen peroxide formation in the phagosomes with the resulting bactericidal effect ([3]). If this is so, the absence of catalase activity in the bacterium is not easy to understand. Perhaps the electron transparent zone ([4]) surrounding the bacteria effectively protects them not only against hydrolytic enzymes but also against hydrogen peroxide produced by the host. In this connection it may be mentioned that Tepper ([19]) detected very high activity of superoxide



FIGS. 3a. Catalase activity in *M. fortuitum* on the cytoplasmic membrane (small arrow) and as a nonenzymatic chemical reaction between DAB and bacterial capsular constituents (double arrow) (48,000 ×).

3b. Fine granular deposit in the space between plasma membrane and cell wall (128,000 ×).



FIGS. 4a. Succinate dehydrogenase activity in *M. fortuitum* at the plasma membrane (85,000 ×.)

4b. Succinate dehydrogenase activity on the walls of phagosomes containing *M. lepraemurium* (150,000 ×).

dismutase in *M. lepraemurium*.

The protective layer around the bacilli could also explain the inability of cytochemical methods to detect both a catalase and a succinate dehydrogenase.

Mori *et al* [13], in extracts from ground suspensions of *M. lepraemurium*, detected succinate dehydrogenase but no α-ketoglutarate dehydrogenase and produced evidence for the persistence of a glyoxalate pathway. Tepper and Varma [20], by radioisotopic tracer technics, found evidence for a functional tricarboxylic acid cycle.

The results presented in this paper lead to the conclusion that succinate dehydrogenase is either absent from *M. lepraemurium* or present in very small amounts, undetectable by ultramicroscopic histochemistry. The first eventually would imply the presence of a glyoxalate cycle in this organism; the second would signify that *M. lepraemurium* is an obligate autotrophic organism [17].

In view of the fact that our present results may be affected by the intraphagosomal localization of the bacteria studied, similar observations should be made on purified suspensions of *M. lepraemurium*.

## SUMMARY

Three enzymes of aerobic pathways (cytochrome c oxidase, peroxidase and catalase) and one key enzyme of the tricarboxylic acid cycle (succinate dehydrogenase) were investigated for their ultrastructural localization in *M. lepraemurium* in infected mouse liver and in cultures of *M. fortuitum* as a control. All four enzymes were localized in *M. fortuitum*.

To *M. lepraemurium* only cytochrome c oxidase and peroxidatic activity were detected. The localization of the latter enzyme activity was different compared with *M. fortuitum*. Succinate dehydrogenase was not detected in *M. lepraemurium* but rather surprisingly was found in the membrane of the phagosomes containing the bacteria.

It is concluded that *M. lepraemurium* can function aerobically and has either a glyoxalate pathway or is an obligate autotroph.

## RESUMEN

Se investigó la localización ultraestructural de 3 enzimas del metabolismo aeróbico (oxidasa del citocromo C, peroxidasa y catalasa) y de la deshidrogenasa succínica, una enzima "clave" en el ciclo de los ácidos tricarboxílicos, en el *M. lepraemurium* localizado en el hígado de ratones infectados y, como control, en cultivos del *M. fortuitum*.

Mientras que en el *M. fortuitum* se encontraron las cuatro enzimas, en el *M. lepraemurium* sólo se encontraron la oxidasa del citocromo C y la peroxidasa, aunque esta última tuvo una diferente localización en el *M. fortuitum*. Sorpresivamente, la deshidrogenasa succínica no se encontró en el *M. lepraemurium* en sí, sino en la membrana de los fagosomas que contenían a la bacteria. Se concluyó que el *M. lepraemurium* puede funcionar aeróbicamente y que utiliza el ciclo del glioxilato o que es un autótrofo obligado.

## RÉSUMÉ

Trois enzymes de la voie aérobie (cytochrome c oxidase, peroxidase et catalase) et un enzyme clef du cycle tricarboxylique (succinate dehydrogenase) ont été recherchés chez *M. lepraemurium* par la technique de la localisation ultramicroscopique, dans le foie infecté de la souris. Des cultures de *M. fortuitum* servirent de controles. Les quatre enzymes furent localisé chez *M. fortuitum*. Seules la cytochrome c oxidase et la peroxidase furent retrouvées chez *M. lepraemurium*. La localisation de la dernière était différente par rapport à celle dans *M. fortuitum*. La succinate dehydrogénase ne fut pas détectée dans *M. lepraemurium* mais était présente, d'une facon surprenante dans la membrane des phagosomes contenant les bacilles. On arrive à la conclusion que *M. lepraemurium* est un organisme aérobie, soit avec un cycle glyoxalate soit autotrophe obligatoire.

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## REFERENCES

1. Broman, R. L., Keele, B. B. and Tepper, B. S. Enzymatic evidence for an aerobic metabolism in *Mycobacterium lepraemurium*. Eighth Joint Leprosy Conference, San Francisco, 1973.
2. Chatterjee, K. R. Electron microscopy and cytochemistry of *Mycobacterium leprae* and leprous tissue. Lepr. India **30** (1958) 79-82.
3. De Chatelet, L. R. Oxidative bactericidal mechanisms of polymorphonuclear leucocytes. J. Infect. Dis. **131** (1975) 295-303.
4. Draper, P. and Rees, R. J. W. Electron transparent zone of mycobacteria may be a defense mechanism. Nature **228** (1970) 860-861.

5. GEYER, G. *Ultrahistochemie,* Stuttgart: Gustav Fischer Verlag, 1973, p 430.
6. GRAY, C. T. The respiratory metabolism of murine leprosy bacilli. J. Bacteriol. **64** (1952) 305-313.
7. HERZOG, V. and FATIMI, H. D. Colorimetric and cytochemical studies for determination of optimal conditions for demonstration of catalase. *In:* Electron Microscopy and Cytochemistry, E. Wisse, W. Th. Daems, I. Molenaar, and P. van Duijn, eds., Proc. 2nd Int. Symp. 1973, Amsterdam and London: Elsevier Publishing Company, 1974, pp 111-113.
8. ISAHQUE, M. and KATO, L. The cytochrome system in *Mycobacterium lepraemurium.* Can. J. Microbiol. **20** (1974) 943-947.
9. ITO, T., SONODA, R. and KOHSAKA, K. Biochemical studies on murine leprosy bacillus. Biken J. **1** (1958) 166-172.
10. KATO, L., ISHAQUE, M. and BONIN, M. C. Cytochrome c chez *Mycobacterium lepraemurium.* Ann. Microbiol. **125A** (1974) 399-402.
11. KUSAKA, T., YAMANOUCHI, T. and SATO, R. Lepro **33** (1964) 28-37.
12. MORI, T. Biochemical properties of cultivated *Mycobacterium lepraemurium.* Int. J. Lepr. **43** (1975) 210-217.
13. MORI, T., KOHSAKA, K. and DOHMAE, K. Terminal electron transport system of *Mycobacterium lepraemurium.* Int. J. Lepr. **39** (1971) 813-828.
14. MORI, T., KOHSAKA, K. and TANAKA, Y. Tricarboxylic acid cycle in *Mycobacterium lepraemurium.* Int. J. Lepr. **39** (1971) 796-812.
15. PATTYN, S. R. Comportement de diverses espèces de mycobactéries après infection dans la patte de la souris. Ann. Inst. Pasteur **109** (1965) 309-313.
16. SHANDS, J. W. Embedding free-floating cells and microscopic particles; serum albumin coagulum epoxy resin. Stain Technol. **43** (1968) 15-17.
17. SMITH, A. J., LONDON, J. and STANIER, R. Y. Biochemical basis of obligate autotrophy in blue-green algae and thiobacilli. J. Bacteriol. **94** (1967) 972-983.
18. SPURR, A. R. A low viscosity epoxy resin embedding medium for electron microscopy. J. Ultrastruct. Res. **26** (1969) 31-43.
19. TEPPER, B. S. *In vitro* metabolic activity of *Mycobacterium lepraemurium* isolated from different tissue lesions. Int. J. Lepr. **41** (1973) 533.
20. TEPPER, B. S. and VARMA, K. G. Metabolic activity of purified suspensions of *Mycobacterium lepraemurium.* J. Gen. Microbiol. **73** (1972) 143-152.