Metabolism of *Mycobacterium leprae* Separated from Human Leprosy Nodules[1]

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This paper deals with studies on a number of oxidative enzymes in *Mycobacterium leprae* separated directly from human leprosy nodules. In an investigation such as this, certain limitations are inherent: (1) the microorganisms have to be separated from the infected tissue in pure form suitable for metabolic studies; (2) only those enzymes can be studied for which technics applicable to small quantities or material are available.

Study of *M. lepraemurium* has become a model system for investigations relating to human leprosy. Since these organisms have also not been cultivated in inert media, they are isolated directly from infected tissues of rats or mice. Gray ([9]), and Hanks ([10]), working with whole cells of *M. lepraemurium* separated from testicular lepromas of rats, could not obtain utilization of 50 different substrates, including various tissue extracts. Ito and Sonoda ([17]) demonstrated increase in oxygen-uptake of *M. lepraemurium* with heated extracts of rat testes and testicular lepromas. In cell-free extracts of *M. lepraemurium*, Kusaka ([18]) observed succinate oxidase and glucose-6-phosphate dehydrogenase, which were absent from the whole cells. Mori *et al.* ([19]) demonstrated diaphorase I and II and malate dehydrogenase in extracts prepared from lyophilized cells of *M. lepraemurium*.

METHODS AND MATERIALS

M. leprae was separated from human lepromatous nodules, as described elsewhere ([21]). Protein was estimated by a colorimetric method modified from that of Johnson ([16]).

Heated extract of lepromatous nodules was prepared by heating the crushed tissue in distilled water (about 1 gm. in 10 ml.) for 30 minutes at 100°C, and removing the particles by centrifugation.

Oxygen-uptake of the separated organisms with and without added substrates was studied by the conventional Warburg manometric method.

Succinate-cytochrome C reductase activity was estimated spectrophotometrically by measuring the increase in absorbance at 550 mμ in the Beckman spectrophotometer model DU.

Lactate dehydrogenase mediated by nicotinamide-adenine dinucleotide (NAD) was studied spectrophotometrically by measuring increase in absorbance at 340 mμ.

Pyruvate formed in the reaction medium was estimated by the carbonyl test ([7]), measuring absorbance of the hydrazone formed at 450 mμ.

Peroxidase activity was determined by measuring formation of purpurogallin from pyrogallol ([20]). Catalase activity was estimated by measuring liberation of oxygen from H_2O_2 ([3]).

Kedrowsky's bacillus and *M. phlei* were grown in glycerol broth for 21 days; *M. smegmatis* was grown in the same medium for 48 hours. *M. tuberculosis* H37Ra and BCG were cultured in Dubos medium for 21 days. The organisms were separated from the medium by centrifugation at 6,000 g for 10 minutes and were washed twice with 0.85 per cent NaCl and once with distilled water.

RESULTS

It is known that *M. leprae* isolated from

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lepromas shows a tendency to aggregation ([28]). In the present study various attempts to obtain a well-dispersed suspension of bacilli by use of Tween 80 and snake venom were not entirely successful. In preliminary experiments on oxygen-uptake of the intact organisms, a prolonged lag period was observed (Fig. 1). The possibility of a permeability barrier due to the lipopolysaccharide capsule of the bacilli was considered. To eliminate this, the organisms were ground with sand in a chilled agate mortar, and the sand was removed by slow speed centrifugation. This procedure resulted in a homogenous suspension, and apparently disrupted most of the bacilli, which were found to take up oxygen without a lag period. Since the method was found to be of advantage, it was generally followed throughout this study.

Results of respiration of *M. leprae* in the presence of heated extract of lepromatous tissue are given in Table 1. It is evident that the organisms showed *in vitro* respiration, which was enhanced by addition of the heated extract. The activity was rather low, possibly because of the small quantities of material used.

Figure 1 shows oxygen-uptake of *M. leprae* in the presence of p-phenylenediamine. While the endogenous respiration of the bacilli decreased after 60 minutes, in the experiment oxygen-uptake continued in a linear manner for over 210 minutes. When intact organisms were used, there was an initial lag period of 60 minutes, probably due to impermeability of the capsular coating present in *M. leprae*. The results suggest the operation of cytochrome C oxidase in the organisms, since p-phenylenediamine reduces cytochrome C, and reduced cytochrome C is oxidized by cytochrome oxidase.

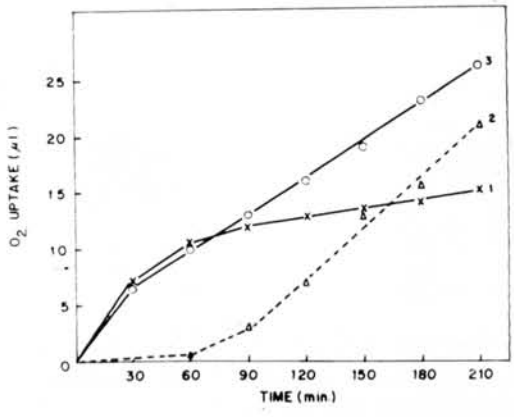

FIG. 1. Cytochrome C oxidase of *M. leprae*. The incubation system consisted of: Krebs-Ringer phosphate buffer, 0.2 ml.; PPD, 0.02 M; bacterial suspension, 1 ml. (protein concentration, 25 mgm.); volume 1.5 ml.; temperature 37°C. 1. Endogenous. 2. Intact bacilli plus PPD. 3. Crushed bacilli plus PPD.

Oxidation of succinate by *M. leprae* is shown in Figure 2. In the presence of succinate and cytochrome C, there was stimulation of respiration by the bacilli, which was inhibited by potassium cyanide (KCN). The overall activity was rather low. This was necessarily due to the small concentration of enzyme employed—a restriction imposed by the limited amounts of human material available.

Succinate-cytochrome C reductase activity was studied spectrophotometrically by measuring increase in absorbance at 550 mµ. Results presented in Table 2 show that there was considerable increase in absorbance at 550 mµ, due to reduced cytochrome C, when KCN was present in the system. This was abolished when cyanide was omitted, a fact providing further evidence for the presence of cytochrome oxidase in *M. leprae*.

TABLE 1. *Respiration of* M. leprae *in presence of heated extract of lepromatous tissue.*

Expt. No.	Time (min.)	Oxygen uptake (µl.)	
		Heated extract	
		Without	With
1	160	15.0	18.6
2	200	15.0	22.3

The incubation system consisted of: 0.5 M $Na_2HPO_4KH_2PO_2$ buffer pH 7.4, 0.2 ml.; bacterial suspension one ml. (protein concentration 18 mgm.); heated extract, 0.3 ml.; volume 1.5 ml.; temperature 37°C.

TABLE 2. *Succinate-cytochrome C reductase activity of* M. leprae.

Expt. No.	Time (min.)	E550 mμ		
		Enzyme with succinate-cytochrome C		
		KCN		
		Without	With	Increase
1	90	90	164	74
2	30	95	220	125

Incubation system same as in Figure 2. Protein concentration: (1) 15 mgm. (2) 26 mgm.; volume 4 ml.

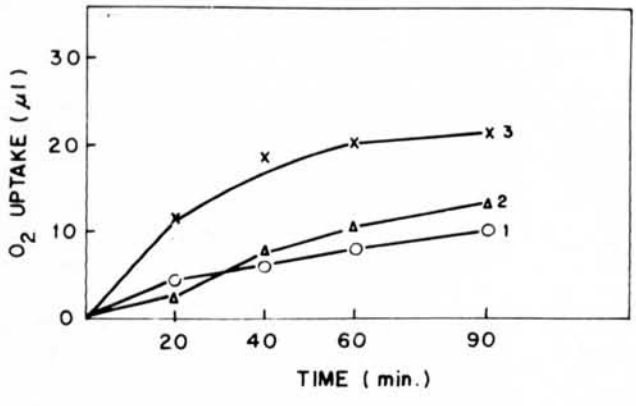

FIG. 2. Succinate oxidase of *M. leprae*. The incubation system consisted of: 0.5 M Na_2HPO_4-KH_2PO_4 buffer pH 7.4, 0.2 ml.; sodium succinate, 0.02 M; bacterial suspension, 0.5 ml. (protein concentration, 15 mgm.); KCN, 0.05 M; cytochrome C, 1 mgm.; volume 1.5 ml.; temperature 37°C. 1. Endogenous. 2. Enzyme plus sodium succinate plus cytochrome C plus KCN. 3. Enzyme plus sodium succinate plus cytochrome C.

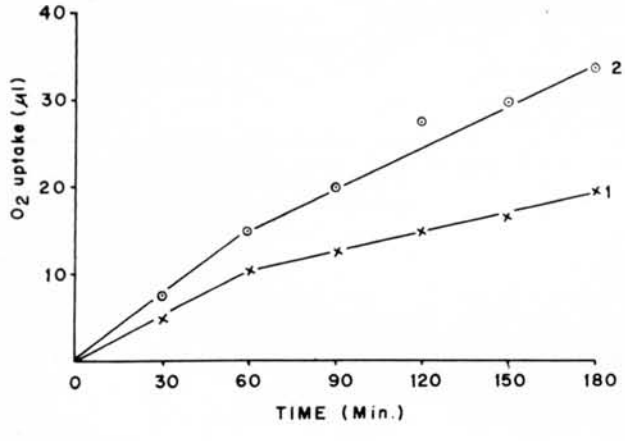

FIG. 3. Lactate dehydrogenase of *M. leprae*. The incubation system consisted of: 0.5 M Na_2HPO_4-KH_2PO_4 buffer pH 7.4, 0.2 ml.; L-sodium lactate, 0.02 M; NAD, 10^{-3} M; bacterial suspension, 1 ml. (protein concentration 70 mgm.); volume 1.5 ml.; temperature 37°C. 1. Endogenous. 2 *M. leprae* plus L-lactate plus NAD.

TABLE 3. *Lactate dehydrogenase activity of* M. leprae.

Expt. No.	Time (min.)	E340 mμ			
		Enzyme with			
		Lactate	Lactate & NAD	Lactate, NAD, KCN	Increase
1	60	34	—	102	68
2	60	—	85	295	210

Incubation system same as in Figure 3. Bacterial suspension, 1 ml. Protein concentration, (1) 45 mgm. (2) 80 mgm.; KCN, 0.05 M; volume 4 ml.

TABLE 4. *Lactate oxidation of* M. leprae *in relation to formation of pyruvate.*

Expt. No.	Treatment	Time (min.)	0_2 uptake (μl.)	E450 mμ (measure of pyruvate formed)
1	Dialyzed	120	20	820
2	Dialyzed	120	26	740
3	Undialyzed	180	34	206

Incubation system same as in Figure 3. Protein concentration: (1) and (2) 56 mgm. (3) 70 mgm.

Results given in Figure 3 show that the organisms actively oxidize lactate; there was 75 per cent increase in oxygen-uptake over the endogenous.

An attempt was made to see if reduction of NAD could be detected in the oxidation of lactate by *M. leprae*. NAD reduction was measured by estimating increase in absorbance at 340 mμ in the presence of KCN. Results given in Table 3 show that there was significant increase in absorbance at 340 mμ, a fact suggesting that lactate dehydrogenase of *M. leprae* is NAD-dependent.

Experiments in which products of lactate oxidation were analyzed showed that pyruvate was formed in the system. This could be demonstrated by the carbonyl test; the hydrazone formed had an absorption maximum at 450 mμ. Results given in Table 4 demonstrate that oxygen-uptake of *M. leprae* in the presence of lactate was inversely related to pyruvate accumulation in the reaction medium. This suggested that the pyruvate produced is further oxidized by the bacilli. However, attempts to detect increase in oxygen-uptake with added pyruvate were unsuccessful. Dialysis of the bacterial preparation against distilled water for four hours at 4°C apparently did not affect oxidation of lactate, whereas the pyruvate formed was not further oxidized and accumulated in the system (Table 4).

Table 5 shows the catalase and peroxidase activities of *M. leprae* as compared to those of other mycobacteria. It is evident that *M. leprae* has strong peroxidase activity, while the saprophytic mycobacteria showed none. On the other hand, catalase activity in *M. leprae* was very low as compared with that in the other mycobacteria.

DISCUSSION

Human leprosy organisms separated from lepromatous nodules have thus been shown to oxidize several substrates. It may reasonably be assumed that the microorganisms universally occurring in lesions of leprosy are the causative agents of the disease and that the acid-fast bacilli isolated from lepromatous nodules are in fact con-

TABLE 5. *Catalase and peroxidase activities of* M. leprae *and other mycobacteria.*

Bacteria	Peroxidase (E420 mμ) (Klett units)	Catalase (μl. 0_2)	Concentration of enzyme (mgm. protein)	
			Peroxidase	Catalase
M. leprae	150	27.0	12.50	12.50
M. tuberculosis H37Ra	—	72.5	11.25	6.25
M. tuberculosis BCG	—	99.0	11.25	6.25
Kedrowsky's bacillus	—	75.0	10.00	3.75
M. phlei	—	79.0	12.50	3.75

Peroxidase: The incubation system consisted of 0.2 M acetate buffer, pH 4.0, 5 ml.; H_2O_2, 25 mgm.; pyrogallol, 5 mgm.; volume 7 ml.; temperature 30°C; time 10 minutes.

Catalase: The incubation system consisted of: 0.5 M, Na_2HPO_4-KH_2PO_4 buffer pH 7.0, 0.2 ml.; H_2O_2, 0.02 M; volume 3 ml.; temperature 30°C; time *M. leprae* 15 minutes, other bacilli 5 minutes.

centrates of *M. leprae*. The preparations employed in this study were practically free from contamination by tissue debris, as could be judged from stained slides. In analogous studies of mycobacteria isolated directly from animal tissues (*M. lepraemurium* from infected rat testes), Gray [9] suggested that enzymes of the host tissue do not interfere with the activity of the bacterial enzymes if the preparations are more than 85 per cent pure with respect to bacilli. In our experiments, when the organisms were ground with sand, the permeability barrier could be overcome, the lag period observed in the respiration of intact organisms being eliminated by this procedure.

As the substrate requirements of *M. leprae* were entirely unknown, a heated extract of the milieu in which it proliferates, viz., the lepromatous nodules, was employed as substrate-source. It was found that the oxygen-uptake of the organisms was stimulated by addition of the heated extract, a fact suggesting that the bacterial preparations were metabolically active. However, the oxygen-uptake was rather low. This was probably due to the small quantities of the organisms used. Electronmicroscopic studies have suggested that a significant proportion of bacilli present in human leprosy nodules are dead [22]. Such a condition might be partly responsible for the low metabolic response of the *M. leprae* preparations. However, the fact that increase in oxygen-uptake was found consistently in all the experiments suggests that the phenomenon is real.

It has been demonstrated that the ability of mycobacteria to oxidize several substrates decreases sharply as pathogenicity increases [2, 6]. Hanks and Gray [12] showed that as one passes from the saprophytes toward the more parasitic species, a step-wise limitation of oxidative capacity is observed. Segal and Bloch [23], who studied the metabolism of different strains of *M. tuberculosis*, found that the avirulent H37Ra had the maximum activity and the virulent H37Rv the least, while the attenuated strain BCG was intermediate. Under the experimental conditions of the studies here reported the metabolic activity of *M. leprae* was observed to be rather low. Hanks [11] and Hanks and Gray [13] observed that *M. lepraemurium* subjected to prolonged refrigeration showed increased hydrogen transfer capacity and infectivity. It has been suggested that inhibitors derived from the tissue are destroyed during refrigeration. In the present study *M. leprae* separated from the nodules was generally kept in the frozen condition before use, a procedure that might inactivate any inhibitors present.

It may be recalled that Ito and Sonoda [15] demonstrated stimulation of respiration in *M. lepraemurium* with added heated extract prepared from lepromas of infected rats. The concentration of organisms employed was about 160 mgm. wet weight. Hanks [10] showed hydrogen transfer capacity of *M. lepraemurium* after prolonged anaerobic incubation with tetrazolium dyes. Gray [9] studied the respiratory metabolism of *M. lepraemurium*, but could not obtain enhancement of oxygen-uptake in the presence of various substrates, including chick embryo extract, heated extract of rat testes, and boiled suspensions of *M. phlei* and *M. lepraemurium*. The organisms employed consisted of intact cells, and the concentration was only 0.36 mgm. nitrogen per flask.

Cytochrome oxidase. Oxidation of p-phenylenediamine indicated that the organisms possess cytochrome oxidase activity. By a cytochemical method, Chatterjee *et al.* [4] demonstrated the presence of cytochrome oxidase in *M. leprae*. It has been pointed out by Hartree [14] that oxidation of p-phenylenediamine is not strictly specific, and should be considered in conjunction with other data for evidence of cytochrome oxidase activity. The fact that the increased absorbance at 550 mμ in the presence of succinate and cytochrome C was abolished when cyanide was omitted from the system, provides confirmatory evidence for the presence of the enzyme in *M. leprae*.

Succinate oxidase. Reduction of cytochrome C in the system shows that the succinate oxidation is linked with the cytochrome system. Gray [9] and Hanks [10] and Ito and Sonoda [15] could not demonstrate oxidation of succinate by intact cells of *M. lepraemurium*. When rat liver extract (which by itself had no effect) was

added to the system, however, Ito and Sonoda ([15]) found that succinate was oxidized by the bacilli, a fact suggesting the requirement of some cofactor for the activity. Kusaka ([17]) showed that the factor was derived from heat-stable components in liver mitochondria. In cell-free extracts of *M. lepraemurium*, he demonstrated succinoxidase activity, which was absent in whole cells ([18]). In the present study, the organisms employed have been ground with sand and cytochrome C has been added.

Several authors have reported oxidation of succinate by different mycobacteria ([6, 8]). Whole cells of *M. tuberculosis* H37Rv had no succinoxidase activity, but cell-free extracts prepared by sonic vibration of the organisms for 20 minutes showed succinate dehydrogenase with ferricyanide as electron acceptor ([27]).

Lactate dehydrogenase. Oxidation of lactate by *M. leprae* was found to be NAD-dependent, and pyruvate has been detected as the reaction product. It is well known that lactate dehydrogenase of mammalian tissues is mediated by NAD ([24]). In yeast, lactate dehydrogenase is identical with cytochrome b_2. The enzyme brings about oxidation of lactate by cytochrome C, and pyruvate is the reaction product ([1]). The lactate oxidase of *M. phlei* catalyzes the oxidative decarboxylation of lactate to acetate ([26]). Yamamura *et al.* ([30]) isolated two lactic oxidases from *M. avium*. Lactic oxidase I converted lactate to acetate, and reacted directly with oxygen. Lactic oxidase II catalyzed the oxidation of lactate to pyruvate; it required the NAD-cytochrome C-cytochrome oxidase system or methylene blue for activity. Suryanarayanamurthy *et al.* ([25]) observed that lactate dehydrogenase of sonic extracts of *M. tuberculosis* H37Rv is NADP-dependent. It is evident that a variety of lactate oxidases linked to different carriers are present in mycobacteria. The lactate dehydrogenase detected in *M. leprae* corresponds to the lactic oxidase II of Yamamura *et al.* ([30]), in converting lactate to pyruvate.

The oxidation of pyruvate by *M. leprae* could only be inferred from the results obtained on its lactate dehydrogenase activity. When the product of lactate oxidation by *M. leprae* was studied, it was found that pyruvate was formed in the system. It was observed also that accumulation of pyruvate in the reaction medium was inversely proportional to oxygen-uptake in the presence of lactate, a fact indicating that the pyruvate produced was further oxidized by the bacilli. However, externally added pyruvate was not utilized. Sutton ([27]) has shown that in the oxidation of lactate by *M. phlei*, pyruvate-formation could be detected under anaerobic conditions. He suggested that the pyruvate produced is strongly bound to the enzyme, since carbonyl-trapping agents did not inhibit its oxidation under aerobic conditions. It is probable that the pyruvate formed in the oxidation of lactate by *M. leprae* is bound to the enzyme and readily oxidized. When the bacterial extracts were dialyzed, lactate oxidation remained unaffected, whereas pyruvate accumulated in the system, a fact suggesting that some factors essential for pyruvate oxidation are lost during dialysis.

Catalase and peroxidase. Ito and Sonoda ([15]) have demonstrated catalase and peroxidase in *M. lepraemurium*. Catalase activity is present in all aerobes and facultative aerobes; anaerobic and lactic acid bacteria are usually catalase-negative ([2]). *M. tuberculosis* H37Rv has been shown to possess peroxidase, and nonpathogenic mycobacteria such as *M. tuberculosis* H37Ra, BCG, *M. phlei* and *M. smegmatis* have been found to lack this activity ([29]). By analogy, the strong peroxidase of *M. leprae* is probably correlated with its pathogenicity. It is of interest to note that frozen human skin tested under identical conditions had no measurable peroxidase activity. This probably indicates that the metabolic property detected in *M. leprae* is specific for the bacillus and not due to tissue enzymes. Several saprophytic mycobacteria tested did not have this property. The catalase of *M. leprae* has been found to be very low as compared to that of several nonpathogenic strains studied. The intracellular life of these organisms under low oxygen tension might explain their reduced catalase activity. Whether peroxidase and catalase function physiologically as protective agents against peroxide toxic-

ity or as oxidative catalysts is still an open question ([5]).

The experiments reported indicate that *M. leprae* isolated from human leprosy nodules possesses enzymes associated with the citric acid cycle. However, the activity of the enzymes was rather low. The results suggest that these organisms have the biologic machinery to oxidize substrates aerobically, and might be able to obtain energy, at least in part, for growth and proliferation through oxidative mechanisms.

SUMMARY

Human leprosy bacilli (separated from lepromatous nodules) showed consistent *in vitro* respiration, which was enhanced by the addition of a heated extract prepared from lepromatous tissue.

M. leprae oxidized p-phenylenediamine, a fact indicating the presence of cytochrome C oxidase in the bacilli.

The organisms oxidized succinate, and reduction of added cytochrome C was observed in the system.

Lactate dehydrogenase mediated by NAD was demonstrated. Pyruvate was found to be the reaction product, and evidence obtained showed that pyruvate formed in the reaction medium was further oxidized by the bacilli.

RESUMEN

Bacilos leprosos humanos (separados de nódulos lepromatosos) mostraron consistente respiración *in vitro*, que fué aumentada añadiendo un extracto calentado preparado de tejido lepromatoso.

M. leprae oxidó p-phenylenediamine, un hecho que indica la presencia de cytochromo C oxidasa en los bacilos.

Los organismos oxidaron el succinato, y se observó una reducción del cytochromo C que fué agregado al sistema.

Lactato dehydrogenaso mediato por NAD fué demostrado. El pyruvate se encontró ser el producto de la reacción, y evidencia obtenida mostró que el pyruvate formado en el seno de la reacción fué mas adelante oxidado por los bacilos.

RÉSUMÉ

Les bacilles de la lèpre humaine (séparés des nodules lépromateux) ont témoigné régulièrement d'une respiration *in vitro*, qui était augmentée par l'addition d'un extrait chauffé préparé à partir de tissu lépromateux.

Le *M. leprae* a oxydé la p-phenylènediamine, phénomène qui a révélé la présence de cytochrome C oxydase dans les bacilles.

Les organismes ont oxydé le succinate et une réduction du cytochrome C ajouté au système a été observée.

On a mis en évidence la dehydrogénase lactique avec intervention de NAD. On a observé que le produit de réaction était du pyruvate, et les données obtenues ont montré que le pyruvate formé dans le milieu de réaction était plus tard oxydé par les bacilles.

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