Cytochrome-Linked Respiration in Host Grown *M. leprae* Isolated From an Armadillo (*Dasypus novemcinctus*, L.)[1]

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Mycobacterium leprae and *M. lepraemurium* are generally regarded as metabolically deficient, host dependent intracellular parasites. As a result of the failure of these mycobacteria to multiply in bacteriologic media, little is known of their physiologic requirements. Mori *et al* ([16]) and Kusaka *et al* ([13, 14]) claimed that *M. lepraemurium* were deficient in cytochrome pigments. This deficiency was cited as an explanation as to why these bacilli, when isolated, had no respiratory response to glucose or its intermediates ([3, 18]). Contrary to these reports, we have recently shown the presence of cytochromes of the $a + a_3$, b and c types ([6, 7]) in *M. lepraemurium*. Our more recent studies have shown that whole cell suspensions of *M. lepraemurium* catalyzed oxidation of succinate ([8]) and NADH was also found to be oxidized by cell-free preparations of this organism ([9]).

In the past, the only source of *M. leprae* has been human infected tissues and it was not possible to obtain pure cells in amounts sufficient for biochemical studies. As a result, little is known about the metabolic properties of this mycobacterium. However, the situation has greatly improved since Storrs ([20]) and Kirchheimer and Storrs ([12]) found that the nine-banded armadillo (*Dasypus novemcinctus*, L.) is susceptible to human leprosy. Armadillos infected with *M. leprae* yield tissues which contain enormous numbers of hitherto scarce leprosy bacilli. Thus, it has now been possible to isolate enough bacilli for metabolic studies. This paper describes investigations on the cytochrome systems, oxidation of succinate and NADH by *M. leprae* isolated from leprous tissues of an armadillo.

MATERIALS AND METHODS

Preparation of M. leprae cell suspensions. A nine-banded armadillo (AR-H6), infected subcutaneously and intraperitoneally 31 months previously, with a pooled suspension of *M. leprae* obtained from human lepromatous leprosy skin biopsies was sacrificed by cardiac exsanguination under sodium amytal anesthesia. Subcutaneous lepromas together with the spleen and the liver were removed aseptically and transported from Hawaii to Montreal in sterile containers on wet ice. The tissues were worked up within 48 hours. The pooled tissues (164 gm) were minced with scissors and washed twice with cold distilled water. They were then homogenized in 0.1 M potassium phosphate buffer, pH 7.2, in a Sorvall Omnimixer and the homogenate was filtered through a sterile nylon filter. The filtrate was centrifuged at 860 × g using JS-7.5 swing bucket rotor for eight minutes in a Beckman J-21B centrifuge. The supernatant was carefully decanted and the sediment was resuspended and similarly washed three times. The combined supernatants were centrifuged at 10,000 × g for ten minutes and the sediment washed twice with cold buffer. The fluffy superficial layer above the sedimented bacilli was poured off and the lipid sticking on the sides of the tubes was washed off. The sediment was resuspended in 0.1 M KPO_4 buffer, pH 7.2, and the final cell suspension, to be used for respiratory studies, was standardized turbidimetrically using a 540 mm filter, so that a 1:10 dilution gave a Klett unit reading of 450 which corresponded to about 12 mg of cells (dry weight) per milliliter. The cell suspensions were checked for purity and the stained preparations (Ziehl-Neelsen method) as well as the phase contrast microscopic examina-

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tions showed that the cell suspensions were practically free from such tissue contaminants as might interfere with the proposed studies ([10]).

Preparation of cell-free extracts. The washed bacilli were suspended (50% W/V) in a sonication medium containing 50 millimols tris-HCL (ph 7.2), 300 millimols sucrose, 5 millimols $MgCl_2$, 0.5 millimol EDTA-Na_2 and 0.5 millimol reduced glutathione. The cells were disrupted by passing three times through a chilled French press (Aminco, Silver Spring, Maryland, U.S.A.) at 1260 pSI. The unbroken cells were removed by centrifugation at 12,000 × g for 30 minutes and the cell-free supernatant was used as the enzyme source.

Measurement of succinate oxidation. The oxidation of succinate by intact cells of *M. leprae* was measured by the Warburg technic as described by Umbreit *et al* ([22]) under conditions similar to those described for *M. lepraemurium* ([8]).

Measurement of NADH oxidation. The oxidation of NADH by cell-free extracts of *M. leprae* was measured spectrophotometrically at 25°C and by following the decrease in absorbancy at 340 nm in a Cary model 118C spectrophotometer. The reaction mixture in a total volume of 2 ml contained 180 μmol of 0.1 M potassium phosphate buffer pH 7.0 and cell-free extracts containing 1.3 mg protein. The reaction was initiated by the addition of 0.05 μmol of NADH in the sample cuvette.

To study the effect of inhibitors on succinate or NADH oxidations, thenoyltrifluoroacetone, rotenone, antimycin A and HQNO (2-n-heptyl-4-hydroxyquinoline-N-oxide) were dissolved in 95% ethanol. Where indicated, the inhibitors were pre-incubated with the reaction mixture for three minutes before the reaction was begun.

Absorption spectra. The difference absorption spectra of cell-free extracts were obtained at room temperature in a Cary model 118C spectrophotometer using cuvettes having a 1 cm light path. Both cuvettes contained 2 ml of cell-free extract containing protein as indicated in each figure and the contents of the sample cuvette were treated with various reagents after a satisfactory baseline was obtained. In order to detect carbon monoxide-binding pigments, the contents of both cuvettes were first reduced with dithionite and then carbon monoxide was gently bubbled in the sample cuvette for three minutes in the dark.

Protein determination. Protein was determined by the method of Lowry *et al* ([15]) using bovine serum albumin (Sigma Chemical Co.) as a standard.

RESULTS

Difference spectra of M. leprae cell-free extract. The reduced minus the oxidized difference spectra of cell-free extracts (12,000 × g) of *M. leprae* are shown in Figure 1. Cell-free extracts treated with dithionite revealed a distinct absorption band at 605-607 nm and a shoulder at 443-445 nm which indicate, respectively, the alpha and gamma peaks of cytochromes of the a + a_3, type. The absorption peaks at 562, 530 and 429 nm represent, respectively, the alpha, beta and gamma peaks of cytochromes of the b type. The trough in the 465 nm region is attributable to flavoprotein (Trace A). The peaks characteristic of the c type cytochromes were not detectable in the dithionite reduced minus oxidized difference spectrum. Cell-free extracts of *M. leprae* were found to oxidize NADH and cytochromes can also be reduced

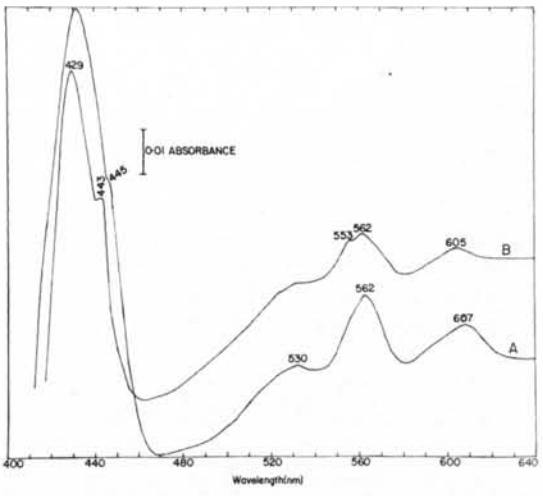

FIG. 1. Steady-state reduced minus oxidized difference spectra of cell-free extracts of *M. leprae* isolated from an armadillo (*Dasypus novemcinctus*, L.). The reaction mixture in a total volume of 2.0 ml contained cell-free extracts (2.6 mg protein) in 50 millimols sonication medium described in Materials and Methods. Trace A represents dithionite-reduced minus oxidized difference spectrum. Trace B shows NADH-reduced minus oxidized difference spectrum.

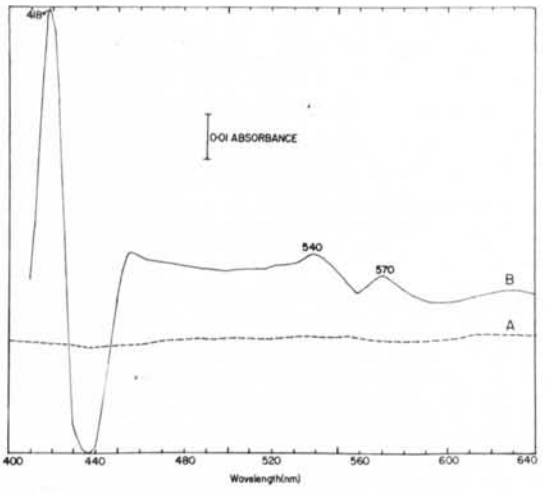

FIG 2. The carbon-monoxide difference spectrum of cell-free extracts of *M. leprae* isolated from an armadillo (*Dasypus novemcinctus*, L.). The reaction mixture in a total volume of 2.0 ml contained cell-free extracts (4 mg protein) in 50 millimols sonication medium. Trace A, represents baseline when the contents of both the sample and the reference cuvettes were treated with dithionite. Trace B shows dithionite-reduced plus CO minus dithionite-reduced spectrum.

by NADH. Figure 1, Trace B, shows that when NADH was added in the sample cuvette under anaerobic conditions, in addition to cytochromes of the $a + a_3$ and b, it caused the appearance of c type cytochromes (550-553 and 523 nm). The gamma peak of cytochrome c was still masked by a larger peak of b type cytochrome at 429 nm. When cytochrome pigments were first reduced with NADH and then dithionite was added to the sample cuvette, the peak of b type cytochrome was obscured by a large peak at 560 nm. The enzyme preparations also contained cytochromes of o type (Fig. 2). When the cell-free extracts in both cuvettes were first treated with dithionite and then carbon monoxide gas was bubbled through the contents of the sample cuvette, the difference spectrum showed absorption maxima in the region of 570-572, 540 and 418 nm representing, respectively, the alpha, beta and gamma peaks due to cytochrome o-CO complex.

Oxidation of succinate by whole cells of M. leprae and the effect of inhibitors on succinate oxidation. When succinate was used as a substrate, whole cell suspensions of *M. leprae* actively oxidized succinate in the Warburg apparatus. While 31 μl of endogenous oxygen uptake occurred in two hours, in the presence of succinate as a substrate, 124 μl of O_2 uptake was observed giving an exogenous uptake value of 93 μl (Table 1). The oxidation of succinate by *M. lepraemurium* was markedly inhibited by the inhibitors of the respiratory chain ([8]). Therefore, the effect of the inhibitors rotenone, antimycin A, cyanide and thenoyltrifluoroacetone on succinate oxidation by *M. leprae* was investigated. Table 1 shows that succinate oxidation was not affected by 0.2 millimol rotenone. However, the process was strongly inhibited by 0.1 millimol thenoyltrifluoroacetone, 0.1 millimol antimycin A and 1 millimol cyanide.

Oxidation of NADH by cell-free extracts of M. leprae and the effect of inhibitors on NADH oxidation. The oxidation of NADH by cell-free extracts, as the decrease in absorbance at 340 nm, is shown in Figure 3. It is seen that NADH was readily oxidized by cell-free extracts of *M. leprae* (Trace A). While succinate oxidation was not affected by rotenone, it was a potent inhibitor of NADH oxidation. This process was inhibited

TABLE 1. *Effect of inhibitors on succinate oxidation by cell-free extract of* M. leprae.

Inhibitor	Concentration	μl O_2 uptake in two hours	% Inhibition[a]
—	—	93	0
Thenoyltrifluoroacetone	0.1 millimol	0	100
Rotenone	0.2 millimol	95	0
Antimycin A	0.2 millimol	13	86
Cyanide	1 millimol	0	100

[a] Actual O_2 uptake values for succinate without any inhibitor were taken as 100 and considered as 0% inhibition. Percent inhibition represents percent decrease in the amount of O_2 consumption in the presence of inhibitors indicated as compared to succinate.

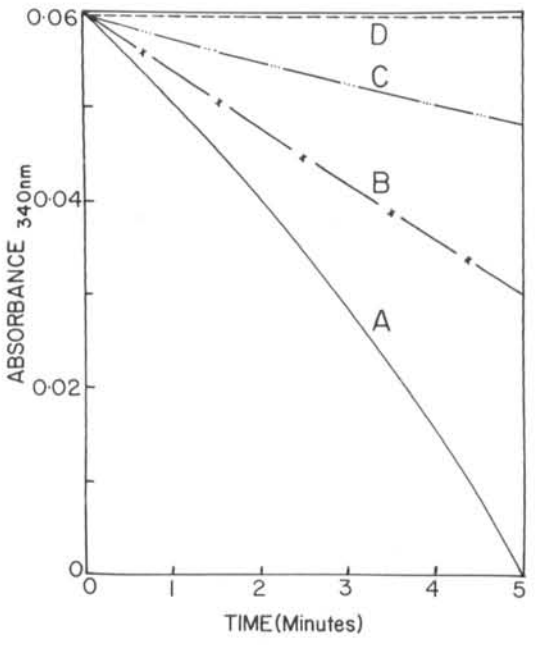

FIG. 3. Oxidation of NADH by cell-free extracts of *M. leprae* isolated from an armadillo (*Dasypus novemcinctus*, L.) and the effect of inhibitors on NADH oxidation. Trace A shows oxidation of NADH (decrease in absorption at 340 nm). Traces B, C and D represent NADH oxidation by cell-free extracts preincubated for five minutes with 5 millimols cyanide, 0.1 millimol antimycin A and 0.1 millimol rotenone, respectively, followed by the addition of 0.025 millimol NADH.

to 80% by 0.1 millimol antimycin A (Trace C) and only to 50% by 5 millimols cyanide (Trace B).

DISCUSSION

The results presented here indicate that a complete respiratory system is present in *M. leprae* isolated from leprous tissues of an armadillo. Thus, cell-free extracts reduced with dithionite exhibited cytochromes of the $a + a_3$ and b type while cytochrome of c type was not detected. However, presence of c type cytochrome became evident in the NADH minus oxidized difference spectrum (Fig. 1). Although cytochrome pigments were completely reduced by dithionite, b type cytochrome was not fully reduced by NADH even under anaerobic conditions. Based on the CO-difference spectrum, cytochrome o also appears to be present in *M. leprae* (Fig. 2). There may be quantitative differences but similar types of cytochromes were present in *M. lepraemurium* ([6, 7]) as well as in *M. leprae* isolated from subcutaneous lepromas of armadillos. Although spectral evidence for the presence of cytochromes in *M. leprae* has been obtained, the role of these cytochromes in energy conservation has not previously been investigated.

Extensive efforts have been made to cultivate *M. leprae in vitro* but no substrate has definitely been found to have a respiration stimulating effect on this mycobacterium. Recently, Skinsnes *et al* ([19]) have claimed successful cultivation of *M. leprae* in a hyaluronic acid-based artificial medium. Our studies show that one of these cultures (HI-75) did not utilize hyaluronic acid by oxidation. Whole cell suspensions prepared from host-grown *M. lepraemurium* were also unable to oxidize hyaluronic acid ([6]). Tepper and Varma ([21]) using radioactive tracer technics have shown that c-labeled acetate, glycerol, α-ketoglutarate, glutamine and succinate were oxidized and assimilated at extremely slow rates by *M. lepraemurium*. Our recent studies have shown that whole cell suspensions of *M. lepraemurium* when frozen for a very short period and thawed, catalyzed the oxidation of succinate which is mediated through the electron transport chain. However, intact cells of *M. leprae* catalyzed succinate oxidation without being frozen and this process appears to involve the enzymatic transfer of electrons from succinate to oxygen. This is supported by the fact that succinate oxidation is markedly sensitive to low concentrations of thenoyltrifluoroacetone, antimycin A and cyanide (Table 1). Inhibition of succinate oxidation, not by rotenone but by antimycin A, indicates that electrons from succinate oxidation couple with the electron transport chain at cytochrome b level. Marked inhibition of succinate oxidation by one millimol cyanide is indicative of involvement of terminal oxidase in the process. The concentration of cytochromes of $a + a_3$ type seems to be significantly higher in *M. leprae* and *M. lepraemurium* as compared to other mycobacteria and several bacterial systems and the terminal oxidase may play a major role in energy conservation in these host-grown mycobacteria.

Among the cultivable mycobacterial, reactions associated with the NAD-linked respiratory chain have been studied in detail

only in *M. phlei* ([1,2]) and *M. tuberculosis* ([5,11]). The oxidation of NADH by *M. lepraemurium*, cell-free extracts has recently been shown to be mediated by cytochromes of the $a + a_3$, b and c type ([9]). The cell-free preparations from *M. leprae* also catalyzed oxidation of NADH. Although succinate oxidation was not affected by rotenone, NADH oxidation was markedly inhibited indicating that two different flavoproteins are involved in succinate and NADH oxidations. The oxidation of NADH was sensitive to a lesser extent to antimycin A and relatively higher concentrations of cyanide (5 millimols) were required to achieve 50% inhibition (Fig. 3). These results suggest that the cytochrome of the o type is involved in the oxidation of NADH rather than the normal cytochrome oxidase ($a + a_3$). The occurrence of cytochromes of the o type has been shown in cell-free extracts of *M. leprae* (Fig. 2). Cytochrome o which is characterized by its ability in the reduced form to combine with carbon monoxide, has been found to be present in *M. phlei* ([17]), *M. lepraemurium* ([5]) and in many different strains of bacteria ([4]).

The presented results clearly show that cytochrome linked respiration of *M. leprae* is as functional and metabolically competent as in any other cultivable mycobacteria thus far tested. With such a functional cytochrome linked respiratory mechanism, *M. leprae* should be able to grow in culture media, with appropriate substrates added. Succinate and NADH oxidation by *M. leprae* are similarly indicative that this microorganism is able to multiply independently from host cells and should not be considered as a metabolically deficient, host dependent, obligate intracellular parasite.

SUMMARY

The bacilli were isolated from an armadillo (*Dasypus novemcinctus*, L.) and cytochrome systems as well as oxidation of succinate and NADH by *M. leprae* were studied. Cell-free extracts of *M. leprae* contained cytochromes of the $a + a_3$, b, c and o type. Whole cell suspensions catalyzed the oxidation of succinate. The process was unaffected by rotenone but was markedly inhibited by thenoyltrifluoroacetone, antimycin A and cyanide. Cell-free preparations of *M. leprae* also oxidized NADH with oxygen as the terminal electron acceptor. Although NADH oxidation was completely inhibited by rotenone, the process was inhibited to only 50% by 5 millimols cyanide. The results indicated that complete respiratory system is present in *M. leprae* isolated from leprous tissues of an armadillo. The effect of inhibitors on succinate and NADH oxidations showed that the respiration in host-grown *M. leprae* is mediated through the cytochrome system with oxygen as the final electron acceptor.

RESUMEN

Se infectaron armadillos (*Dasypus novemcinctus*, L.) con *M. leprae*. Se estudió el sistema de citocromos en los bacilos recuperados de un armadillo así como su capacidad para oxidar al succinato y al NADH. Los extractos libres de células del *M. leprae* contuvieron citocromos de los tipos $a + a_3$, b, c, y o. Las suspensiones de células completas catalizaron la oxidación del succinato. Este proceso no fue afectado por la retenona pero se inhibió en forma muy marcada por la tenoiltrifluoroacetona, la antimicina A y el cianuro. Los extractos libres de células del *M. leprae* también oxidaron al NADH teniendo al oxígeno como aceptor final de electrones. Aunque la retenona inhibió completamente la oxidación del NADH, este proceso sólo se inhibió en un 50% en presencia de 5 milimoles de cianuro. Los resultados indicaron que el *M. leprae* aislado de los tejidos infectados de un armadillo posee un sistema respiratorio completo. El efecto de los inhibidores usados sobre las oxidaciones del succinato y del NADH, demostró que la respiración del *M. leprae* crecido en el armadillo está mediada por un sistema de citocromos donde el oxígeno es el aceptor final de electrones.

RÉSUMÉ

Las bacilles furent isolés d'un tatou (*Dasypus novemcinctus*, L.) et le système des cytochromes, de même que l'oxydation du succinate et du NADH par *M. leprae*, furent étudiés. Les extraits acellulaires de *M. leprae* contenaient les cytochromes du type $a + a_3$, b, c et o. Les suspensions de bacilles entiers catalysaient l'oxydation du succinate. Le processus n'était pas affecté par le rotènone, mais on notait une forte inhibition en présence de thenoyltrifluoroacétone, antimycin A et la cyanure. Des extraits acellulaires de *M. leprae* oxydaient le NADH avec l'oxygène comme accepteur final des électrons. Bien que le rotènone inhibait complètement l'oxydation du NADH, le processus n'était inhibé qu'à 50% par 5mM de cyanure. Les résultats indiquent qu'un système complet de respiration est présent chez *M. leprae* isolé de tissus lépreux de tatou. L'effet des inhibiteurs sur l'oxyda-

tion du succinate et du NADH montre que la respiration des cellules de *M. leprae* cultivées dans l'hôte se fait par la chaîne des cytochromes avec l'oxygène comme accepteur final des électrons.

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