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Failure to detect *o*-diphenoloxidase in cultivable mycobacteria obtained from feral armadillos

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Summary We reported earlier that *Mycobacterium leprae* separated from lepromatous human as well as armadillo tissues contains an unusual form of *o*-diphenoloxidase which oxidized several diphenols, including D- and L-DOPA (3,4-dihydroxyphenylalanine) to quinones *in vitro*. It was not known whether any other species of mycobacterial separated from infected armadillo tissues would show *o*-diphenoloxidase activity. Recently, a few feral armadillos with mycobacterioses caused by cultivable bacilli became available. The data presented in this report demonstrate that cultivable mycobacteria obtained from the tissues of wild-caught armadillos did not contain the enzyme. Two species of nocardia tested converted DOPA to pigment, but this reaction was found to be non-enzymatic, being unaffected by heating. On the other hand, *o*-diphenoloxidase of the leprosy bacilli was sensitive to higher temperatures. Visual evidence on the occurrence of the enzyme in *M. leprae* is also presented.

Introduction

We have shown earlier that suspensions of *Mycobacterium leprae* purified from infected human or armadillo tissues contain the enzyme *o*-diphenoloxidase (EC 1.10.3.1), which actively oxidizes a variety of phenolic substrates including D- and L-DOPA (3,4-dihydroxyphenylalanine) (Prabhakaran & Kirchheimer, 1966;[1] Prabhakaran *et al.*, 1975).[2] In its wide substrate-specificity, resistance to inhibition by reducing agents and certain metal chelators, and in its kinetic properties, the bacterial enzyme was different from the *o*-diphenoloxidase occurring in vertebrate melanocytes (Prabhakaran, 1971;[3] Prahhakaran *et al.*, 1976).[4] At the time of our previous studies (Prabhakaran *et al.*, 1975),[2] we did not have any controls using other species of mycobacteria separated from armadillo tissues. Recently, a few wild-caught animals were found to have

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mycobacterioses caused by cultivable organisms. The mycobacteria separated from the infected tissues of feral armadillos and the organisms grown in culture did not contain *o*-diphenoloxidase. Data are also presented on the enzymatic nature of the oxidation of D-DOPA by *M. leprae* and the absence of the enzyme in two species of nocardia.

Materials and methods

ORGANISMS

Mycobacterium leprae was separated from the infected spleen or lymph nodes of armadillos, as described earlier (Prabhakaran *et al.*, 1976).[4] The organs were removed aseptically during autopsy and held at 0°C, before being homogenized in 0.2 M sucrose, using a Braun Model 853-202 homogenizer. The bacilli were separated by differential and density-gradient centrifugations in solutions of sucrose (0.2 M and 0.3 M) and KCl (1.5 M). The bacterial suspension was washed twice with cold saline and twice with deionized, glass-distilled water. All the operations were carried out at 0–4°C. The organisms were counted by the method of Hanks *et al.* (1964).[5] The same procedure was used for separating the cultivable mycobacteria from the spleen, lymph node or liver of feral armadillos. The bacilli were grown in Dubos' medium at 37°C for 3 weeks, harvested by centrifugation, and washed with saline and water. Four species of mycobacteria have been isolated from the tissues of the armadillos: *M. simiae, M. scrofulaceum, M. avium* and *M. terrae. Nocardia asteroides* (ATCC 19247) was grown for 2 weeks in Dubos' medium at 25°C, and *N. brasiliensis* (ATCC 19296) at 37°C. The cultured organisms remained viable and showed good respiratory activity.

CHEMICALS

DL-DOPA (G-^{3}H), 250 mCi/mmol was purchased from Amersham, Arlington Heights, Ill, USA; glass counting vials from Beckman, Fullerton, Calif, USA; the scintillation solution, Aquasol from New England Nuclear, Boston, Mass, USA, and D-DOPA from ICN, Cleveland, Ohio, USA. Other chemicals used were of the highest purity commercially available.

ENZYME ASSAY

Oxidation of D-DOPA was assayed spectrophotometrically by measuring the absorbance of the quinone formed in the reaction (Prabhakaran *et al.*, 1976).[4] The reaction system was as follows: Na_2HPO_4-KH_2PO_4 buffer (pH 6.8), 0.1 M (final concentration); D-DOPA, 0.002 M; bacilli, 5×10^9; volume, 3 ml;

temperature, 37°C; time, 60 minutes. The oxidation of ^{3}H-DOPA was determined as described before (Harris & Prabhakaran, 1975),[6] where the activity of tritiated water formed from labelled DOPA was measured in a liquid scintillation counter. The organisms were heated at 100°C for 15 minutes, and cooled in an ice bath. To study the heat-sensitivity of *o*-diphenoloxidase of *Mycobacterium leprae*, the bacterial suspensions were exposed to different temperatures in an oven; the maximum duration of the exposure was 15 minutes to ensure complete inactivation of the enzyme. Each experiment was done at least 3 times, except in the case of bacilli separated from the tissues of feral armadillos. With these organisms, the experiments were done once each, because only limited amounts of material were available. However, the assays were repeated when the mycobacteria were grown in culture. For each bacterial suspension, the results did not show variations exceeding 10%; the data presented are for representative experiments.

Results

CULTIVABLE MYCOBACTERIA FROM FERAL ARMADILLOS

The bacilli separated from the tissues of feral armadillos and the same organisms grown in culture failed to oxidize DOPA (Table 1). Sufficient bacteria for the assay were recovered from the tissues of 3 armadillos. However, the bacilli

Table 1. Oxidation of DOPA by mycobacteria from feral armadillos: absorbance 480 nm ($X10^{-3}$)

Bacilli	Unheated bacilli, + DOPA	Heated bacilli, + DOPA	DOPA
Separated directly from:			
Spleen	8	12	8
Liver	8	12	10
Lymph node	10	15	14
Grown in culture from			
Spleen (*M. simiae*)	20	30	10
Liver (*M. scrofulaceum*)	15	21	12
Lymph node (*M. terrae*)	20	22	10
Lymph node (*M. avium*)	8	12	10

were cultured from the tissues of many more animals. The slight activity observed with the bacteria was apparently not enzymatic, since heating the organisms failed to inactivate the reaction. This is in contrast to what was observed with *Mycobacterium leprae* where the activity was completely lost on heating. The data show that cultivable mycobacteria obtained from host tissues do not contain *o*-diphenoloxidase. The results corroborate our earlier finding

that *o*-diphenoloxidase of *M. leprae* is not due to tissue components adsorbed by the organisms, but is an intrinsic property of the bacilli.

Even with the highly sensitive radiometric method using labelled DOPA, little activity was observed in a culture of *M. scrofulaceum* isolated from the lymph node of an armadillo (Table 2). However, *M. leprae* readily oxidized the tritiated substrate.

Table 2. Oxidation of tritium-labelled DOPA by *Mycobacterium leprae* and *M. scrofulaceum*

Bacilli	p mol ^{3}H-DOPA oxidized*
M. scrofulaceum	0.35
M. leprae	59.70

*Values corrected for auto-oxidation of DOPA

NON-ENZYMATIC OXIDATION OF DOPA BY NOCARDIA

The oxidation of DOPA by *M. leprae* was compared to that observed with two species of nocardia. After centrifugation of the reaction mixtures, the spectra of the supernatant fractions were measured. The pigment formed from DOPA by nocardia gave a broad peak in the region 540–580 nm. The quinone formed from DOPA by *M. leprae* showed maximum absorbance at 475–480 nm (Table 3). The reaction observed with nocardia was not inactivated by heat, indicating that it is a non-enzymatic process, stimulated by inorganic ions. *o*-Diphenoloxidase which is heat-labile is the only exzyme that oxidizes DOPA to pigment. With *M. leprae*, heated bacilli showed no oxidation of DOPA.

Table 3. Comparison of the oxidation of DOPA by *Mycobacterium leprae* and *Nocardia* sp.: (absorbance $X10^{-3}$).

Organisms	Wavelength (nm)	Unheated organisms + DOPA	Heated organisms + DOPA	DOPA
N. asteroides	560	60	55	12
N. brasiliensis	560	60	64	10
M. leprae	480	73	10	12

VISUAL EVIDENCE OF *o*-DIPHENOLOXIDASE IN *M. leprae*

Unheated or heated *M. leprae* suspensions were incubated with or without DOPA. In the tube containing unheated bacilli and DOPA, the reaction mixture developed an intense dark colour, due to oxidation of the diphenol to pigment (Figure 1a). The tubes containing unheated bacilli or heated bacilli with or

without DOPA showed little colour change. Under the experimental conditions used, the substrate did not undergo auto-oxidation.

After incubation for 60 minutes, the reaction mixtures (except the one with DOPA, which remains in solution) were centrifuged at 25,000 *g* for 30 minutes. The precipitate in the tube containing unheated bacilli and DOPA was black; part of the pigment formed from DOPA sedimented with the bacilli (Figure 1b). The precipitate in the tubes containing unheated or heated bacilli alone or heated bacilli with DOPA showed no colour change. Evidently the *o*-diphenoloxidase of *M. leprae* is not catalysed by inorganic ions.

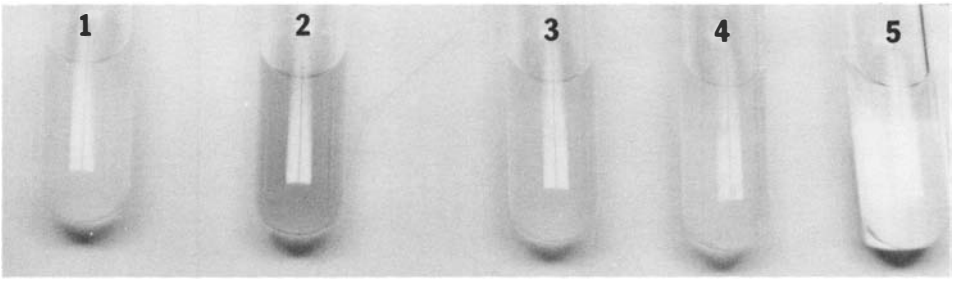

Figure 1a. *o*-Diphenoloxidase in *Mycobacterium leprae*: before centrifugation of the reaction mixtures. From left to right: 1, unheated bacilli; 2, unheated bacilli + DOPA; 3, heated bacilli + DOPA; 4, heated bacilli; 5, DOPA.

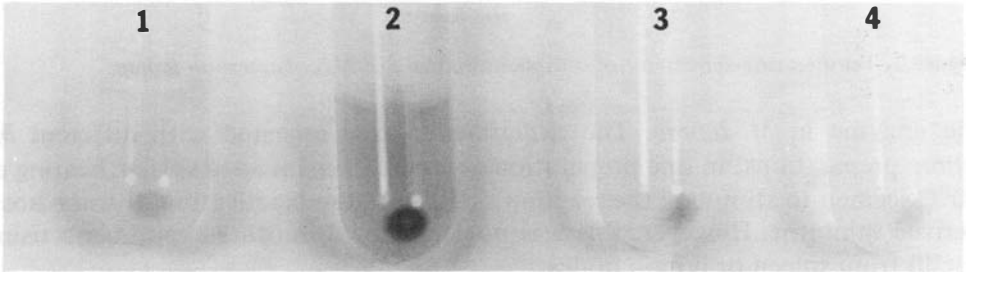

Figure 1b. *o*-Diphenoloxidase in *Mycobacterium leprae*: after centrifugation of the reaction mixtures. From left to right: 1–4 same as Figure 1a; sample 5 being a solution, was not centrifuged.

HEAT-SENSITIVITY OF *o*-DIPHENOLOXIDASE IN *M. Leprae*

The bacterial suspensions were exposed to different temperatures and tested for oxidation of D-DOPA. There was a gradual loss of activity from 37°C to 80°C, a sharp drop at 90°C, and at 100°C the enzyme was inactivated

completely. The decrease in activity was 21.6% at 60°C, 39.2% at 80°C, and 72.5% at 90°C (Figure 2). The bacilli were heated for varying periods at 100°C. There was complete loss of *o*-diphenoloxidase even when the exposure time was as low as 2 or 3 minutes, indicating the extreme temperature-sensitivity of

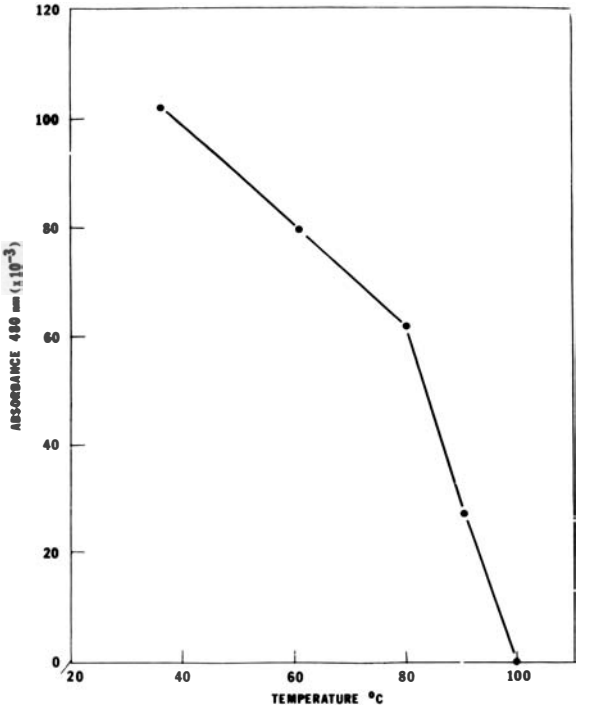

Figure 2. Temperature-sensitivity of *o*-diphenoloxidase of *Mycobacterium leprae.*

the enzyme in *M. leprae*. The experiments were repeated with different *M. leprae* preparations. In one preparation obtained from infected spleen, heating at 60°C seemed to stimulate the reaction, indicating the inactivation of some host-derived inhibitor. However, this was not confirmed in other experiments using bacilli from spleen or lymph nodes.

Discussion

For the first time, we show that cultivable acid-fast bacteria separated from the organs of feral armadillos with mycobacterioses, do not possess *o*-diphenoloxidase. As such, the oxidation of phenolic substrates by *Mycobacterium leprae* prepared from the organs of experimentally infected armadillos could not be due to host tissue elements adsorbed on the bacilli. The

oxidation of DOPA by *M. leprae* is catalysed by *o*-diphenoloxidase which is a temperature-sensitive enzyme. The non-enzymatic conversion of the diphenol to pigment by the cultured organisms is probably caused by inorganic ions adsorbed from the media; the activity was not abolished on heating.

Certain precautions have to be observed to demonstrate *o*-diphenoloxidase in *M. leprae* obtained from lepromatous tissues. The tissues have to be held at 0°C or below and the preparative procedures done in the cold to prevent enzyme denaturation. When the armadillo tissues are stored for over 2 months, the bacilli gradually lose the enzyme activity. If the bacterial preparation is contaminated with host tissue material, no enzyme activity would be detected (Prabhakaran *et al.*, 1979).[7] Some suspensions of *M. leprae* prepared from infected armadillo liver might have a dark-brown or greenish tinge, indicating the presence of bile pigments. Such preparations do not oxidize DOPA, while bacilli separated from the spleen or lymph nodes of the same animal would be active. Probably, this phenomenon depends on the degree of liver damage in the individual animal. Occasionally we have found that leprosy organisms separated from the subcutaneous nodules of armadillos are hard to purify and would show very little enzyme activity. We have not encountered such discrepancies in *M. leprae* derived from human tissues. Cultures of mycobacteria used for testing DOPA oxidation have to be grown in liquid media and washed thoroughly to exclude false positive results. Control experiments using heated bacilli should be done, to determine non-enzymic conversion of DOPA to pigment by metal ions. To detect enzymatic oxidation of dopa by *M. leprae*, prolonged incubation of the reaction mixtures at 37°C is unnecessary. The substrate would undergo little auto-oxidation if the incubation conditions are properly controlled. In our experience, quinone formation from DOPA can be observed within 15 minutes if the bacterial suspension is enzymatically active. Failure to observe these precautions would, in fact, lead to anomalous results (Binford *et al.*, 1977;[8] Kato *et al.*, 1976).[9]

In our previous experiments where *M. leprae* was derived from frozen tissues, the pigment produced from DOPA gave a peak at 540 nm, characteristic of indole-5,6-quinone (Prabhakaran *et al.*, 1976).[4] In recent experiments where bacilli separated from fresh tissues were used, the peak was near 480 nm, characteristic of dopachrome. The enzyme was heat-labile in both cases. A decarboxylation step is involved in the conversion of dopachrome to indole-5,6-quinone. Probably, a decarboxylase is associated with the bacilli from frozen tissues. The reason for this variation remains to be elucidated.

We have shown earlier that β-glucuronidase detected in *M. leprae* preparations is adsorbed from the host tissue (Prabhakaran *et al.*, 1978).[10] However, *o*-diphenoloxidase seems to be a constitutive enzyme of the bacillus. So far, we have tested *M. leprae* for *o*-diphenoloxidase after five passages in the armadillo, and the bacilli have retained the activity unimpaired. Our efforts to induce the enzyme in two species of cultivable mycobacteria did not succeed

(Prabhakaran *et al.*, 1969).[11] The species-specificity of DOPA metabolism for *M. leprae* (Kirchheimer & Prabhakaran, 1968)[12] has been corroborated by other workers and is being used as an identification test of the bacillus (Ambrose *et al.*, 1974;[13] Hall & Rightsel, 1978;[14] Kohsaka *et al.*, 1978).[15] Melanocytes do not lose *o*-diphenoloxidase in spite of repeated passages *in vitro* (Prabhakaran *et al.*, 1975);[2] it is likely that the leprosy bacteria would retain the activity, even if it were possible to grow the organisms in a bacteriological medium.

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