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Oxidation of Phenolic Compounds by *Mycobacterium leprae* and Inhibition of Phenolase by Substrate Analogues and Copper Chelators

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Experiments were conducted on the substrate specificity of phenoloxidase in *Mycobacterium leprae*, by using various phenolic compounds. Comparative studies were carried out with the enzyme from mammalian and plant sources. The phenolase of *M. leprae* was found to be similar to the enzyme of plant origin in oxidizing a variety of substrates; it was different from the mammalian enzyme, which has a limited substrate specificity. The findings confirmed that phenoloxidase is a specific property of *M. leprae* and is not a result of adsorption of host-tissue enzymes. The method used in separation of bacilli from infected tissues was evaluated for its effect on the viability of the organisms. This was tested by using *M. lepraemurium* as a model. The preparative procedure was found to have no adverse effect on the ability of the organisms to multiply in the mouse foot-pad. Several inhibitors of phenoloxidase have been tested—both substrate analogues and compounds which bind copper in the enzyme. Substances binding copper were found to be more effective. Since phenolase has been found to be a characteristic metabolic activity in *M. leprae*, nontoxic inhibitors of the enzyme offer possibilities of developing a rational chemotherapy of leprosy.

Because no proven culture of *Mycobacterium leprae* is as yet available, little progress has been made in understanding the metabolic properties of this microorganism. Recent investigations using concentrates of bacilli directly separated from infected tissues showed that *M. leprae* actively oxidizes 3,4-dihydroxyphenylalanine (dopa) to pigmented products (6). Further studies revealed that the organism oxidizes D-dopa at the same rate as L-dopa and also utilizes other phenolic compounds such as catechol, epinephrine, and norephinephrine (5, 7). It was therefore of interest to study more extensively this aspect of metabolism of *M. leprae* and to compare it with phenolase of mammalian and plant origin. The enzyme is widely distributed in nature and has been variously described as phenolase, phenoloxidase, phenolase complex, and tyrosinase. (E.C. 1.10.3.1, o-Diphenol: oxygen oxidoreductase).

M. leprae employed in the present investigation was separated from infected tissues. It would be useful to verify if the organisms retain their viability through the preparative procedure. This was tested by using *M. lepraemurium* as a model. It has also not been cultivated in bacteriological media; viability of the bacilli can be directly ascertained by inoculating in susceptible animals.

Dopa or its analogues are available in tissues of the human body (e.g., skin and peripheral nerve) which are the preferred sites of multiplication of the leprosy bacilli. Since phenoloxidase appeared to be a characteristic property of *M. leprae*, a study of inhibitors of this activity is important in developing a rational chemotherapy of leprosy.

MATERIALS AND METHODS

M. leprae was obtained at autopsy from the spleen of a patient who had died of lepromatous leprosy. The method employed and the purity of the preparation obtained were described earlier (5, 6). *M. lepraemurium* was separated from infected mouse spleen and liver, by employing the same procedure as for *M. leprae*. The *M. lepraemurium* preparation was suspended in Hanks' balanced salt solution. A known number of the bacilli was inoculated into foot-pads of C_{57} mice. The number of bacilli in the foot-pads was enumerated after 63 days (in the case of organisms obtained from spleen) and after 83 days (in the

case of bacilli from liver) by the method of Hanks et al. (2; modified by Kirchheimer).

Melanocytes of skin contain phenolase; the enzyme is present in a highly concentrated form in mammalian melanomas. A 10% homogenate of Harding-Passey mouse melanoma was centrifuged at 200 × *g* for 20 min, and the supernatant fluid was used as a source of mammalian phenolase. Mushroom tyrosinase was purchased from the Sigma Chemical Co., St. Louis, Mo. The melanoma extract and tyrosinase solution gradually became inactivated with time. Activity of the enzyme was assayed in a Beckman model DU spectrophotometer, with a 1.0-cm cuvette. Turbid reaction mixtures were centrifuged at 15,000 × *g* for 45 min before determining the spectrum. This method was adopted because oxidation products of dopa are well characterized by their absorption spectra (4), and it is suited to the small amounts of material available. Commercially obtained chemicals of the highest purity were used.

RESULTS AND DISCUSSION

Oxidation of phenolic compounds. Oxidation of L-dopa, D-dopa, catechol, and catecholamines by the leprosy bacilli has been reported earlier (5, 7). Additional substrates were tested with *M. leprae* to assess its substrate specificity. Comparative studies were also carried out with mammalian and plant enzymes. Oxidation of these compounds resulted in pigmented products and the activity could be visually assessed. This was further confirmed by measuring the spectrum of the reaction mixture and noting increase in absorbance (ultraviolet maximum) in each case. Each substance was tested at least three times. The values given are for typical experiments and have been corrected for auto-oxidation of the substrate and absorbance for the enzyme. The quinones formed by oxidation of these compounds gave different absorption peaks in the spectra (Table 1).

The phenolase of *M. leprae* is rather nonspecific, like the enzyme from plant sources, and oxidizes a variety of phenolic substrates. Like mushroom tyrosinase, *M. leprae* oxidizes D-dopa at the same rate as L-dopa and also utilizes catechol and catecholamines (5, 7). This is in contrast to what is obtained with the mammalian enzyme, where the substrate specificity is highly restricted. It oxidizes L-dopa; however, utilization of D-dopa or other phenolic compounds is rather limited (1). Our results indicate that phenolase of *M. leprae* is distinguishable from the mammalian enzyme. This finding confirms the earlier observation (7) that the activity of the bacilli could not be caused by adsorption of host tissue enzymes.

Effect of the preparative procedure on viability of the bacillus. Concentrates of *M. lepraemurium* were prepared from infected mouse spleen and liver by the procedure used for separating *M. leprae* (6, 5). A crude fraction (fraction I) before purification and the final fraction (fraction II) were employed in the study to see if the bacilli retained their viability through the preparative procedure. The purified fraction was free of visible tissue debris as ascertained by Ziehl-Neelsen staining method. Viability of the bacilli was assayed by their ability to multiply in the mouse foot-pad. The differential centrifugation, or the solutions used in the process, did not significantly affect the viability of the organisms (Table 2). It may be tentatively concluded that the technique employed is suited to preparation of pure (tissue free) and viable leprosy bacilli.

Effect of substrate analogues and copper chelators on oxidation of dopa. The property of oxidizing dopa is common to *M. leprae*, melanoma, and plant phenolase; moreover, the enzyme of the bacillus was found to be similar in substrate specificity to mushroom tyrosinase. Because leprosy organisms are obtained from infected human tissues available only in limited amounts, the inhibitors were initially tested on mushroom tyrosinase and melanoma extract. The effect of the added compound was estimated by its ability

TABLE 1. *Oxidation of phenolic compounds by Mycobacterium leprae, melanoma, and mushroom tyrosinase*[a]

Substrate	Increase in absorbance		
	M. leprae	Melanoma	Mushroom tyrosinase
3,4-Dihydroxyphenylalanine	180 (540)[b]	250 (480)	375 (480)
3,4-Dihydroxycinnamic acid	135 (600)	0	160 (480)
3,4-Dihydroxybenzoic acid	125 (560)	0	202 (350)
3,4,5-Trihydroxybenzoic acid	104 (560)	0	155 (380)
3-(3,4-Dihydroxycinnamoyl) quinic acid	143 (500)	0	110 (775)

[a] *M. leprae*, 2 mg of protein; melanoma, 15 mg; tyrosinase, 50 μg; substrate concentration, 0.002 M; *p*H, 6.8 for dopa and 6 for the other compounds; temperature, 37 C for *M. leprae* and melanoma and 25 C for tyrosinase; time, 60 min for *M. leprae* and melanoma and 30 min for tyrosinase; volume, 3 ml.

[b] Values in parentheses indicate ultraviolet maxima in mμ.

TABLE 2. *Effect of the preparative procedure on viability of Mycobacterium lepraemurium*

Source of inoculum	No. of bacilli inoculated	No. harvested	Generation time (days)
Bacilli from spleen			
Fraction I	$2.7 \pm 0.7 \times 10^4$	$1.8 \pm 0.2 \times 10^8$	3.9
Fraction II	$2.7 \pm 0.4 \times 10^4$	$4.5 \pm 0.6 \times 10^7$	4.5
Bacilli from liver			
Fraction I	$1.4 \pm 0.3 \times 10^4$	$8.0 \pm 1.0 \times 10^8$	5.3
Fraction II	$1.9 \pm 0.1 \times 10^4$	$5.8 \pm 0.6 \times 10^7$	7.1

TABLE 3. *Effect of inhibitors on phenoloxidase*[a]

Inhibitor	Absorbance (480 mμ)					
	Melanoma			Mushroom tyrosinase		
	Enzyme + dopa	Enzyme + dopa + inhibitor	Inhibition (%)	Enzyme + tyrosine	Enzyme + tyrosine + inhibitor	Inhibition (%)
Substances which bind copper						
Diethyl dithiocarbamate	460	25[b]	95	225	50[b]	78
1-Phenyl-2-thiourea	355	25[b]	93	285	115[b]	60
2-Thiouracil	315	50	84	305	130[b]	57
Thiourea	310	110	65	150	50	67
Substrate analogues						
DL-*p*-Fluorophenylalanine	250	215	14	215	130	40
O-fluorophenylalanine	150	125	17	270	235	13
DL-α Methylmetatyrosine	180	170	6	245	200	18
Dibromotyrosine	270	225[b]	17	150	115	23

[a] Melanoma, 30 mg protein; tyrosinase, 40 μg; dopa, 0.002 M; L-tyrosine, 0.001 M; inhibitors, 0.002 M; *p*H, 6.8; temperature, 37 C; time, 60 min for melanoma and 30 min for tyrosinase; volume, 3 ml.

[b] Indicates that there was no peak at 480 mμ, characteristic of dopachrome.

to inhibit formation of dopachrome in the reaction system. This intermediate in the conversion of dopa to melanin is characterized by its absorption maximum at 480 mμ. The results are given in Table 3.

Substances which combine with copper in the enzyme are good inhibitors; this is in agreement with reports in literature on melanoma extracts (3). Analogues of dopa or tryosine compete with the substrate for the active site of the enzyme. These were found to be less efficient than copper chelators. Preliminary studies on *M. leprae* have indicated that substances which bind copper also inhibit effectively the phenoloxidase activity of the bacillus.

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