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Oxidation of 3,4-dihydroxyphenylalanine (DOPA) by *Mycobacterium leprae*[1]

K. Prabhakaran[2]

In spite of repeated attempts for nearly a century, no generally accepted method has been described for cultivating the presumed etiologic agent of leprosy, *Mycobacterium leprae, in vitro* or transmitting it to laboratory animals, except that of Shepard ([25]), who showed limited multiplication of the bacilli in the foot pads of CFW mice, leading to granulomatous lesions. Because of lack of adequate material, no extended biochemical investigations have been conducted on a proven mycobacterium of human leprosy, and its metabolism has therefore remained obscure. Any study of the metabolic activities of unquestioned *M. leprae* is entirely dependent on the use of lepromatous nodules obtained from infected persons.

The present paper describes dopa oxidase activity detected in *M. leprae* (separated from human leprosy nodules), and the significance of this finding is discussed in relation to the metabolism of the organisms and to the hypopigmented skin lesions characteristic of leprosy.

It has been established that the melanin pigment of the skin is formed through the enzymatic activity of melanocytes present in the basal layer of the epidermis ([17]). Embryologically, the melanocytes are derived from the neural crest; they may be considered as specialized nerve cells. The neural crest also gives rise to dorsal root ganglia of spinal nerves, the adrenal medulla, and Schwann cells of peripheral nerves ([23]). In mammals melanocytes are present in skin, eyes and leptomeninges; they occur also in certain surface membranes, such as the oral mucosa. The bulk of the melanin pigment is contained in the dendritic melanocytes located at the dermal-epidermal junction, and in the hair bulbs ([16]).

The enzyme complex that catalyzes the reactions leading to formation of melanin is variously called phenolase, polyphenol oxidase, phenolase complex and dopa oxidase or tyrosinase. It occurs in a variety of organisms, including vertebrates, invertebrates, higher plants, and fungi. The only report on the possible occurrence of the enzyme in bacteria relates to the oxidation of tyrosine to pigmented products by a small vibrio *Microspira tyrosinatica* ([15]).

In melanogenesis, tyrosine is first converted to 3,4-dihydroxyphenylalanine (dopa) which, through a series of reactions gives rise to melanin. The chemical steps involved in the reaction were studied by Raper in the insect *Tenebrio molitor* ([22]), and extended by Mason ([19]), using mushroom phenolase. The enzyme is assayed manometrically by measuring oxygen-uptake, or spectrophotometrically by measuring formation of dopachrome (absorption maximum 475 mμ) or indole-5,6-quinone with its absorption maximum at 540 mμ.

The presence of phenolase in mammalian melanocytes of skin as well as in melanomas is well established. In 1927, Bloch ([16]) showed that dopa, but not tyrosine, could be oxidized to melanin by frozen sections of normal human skin. Fitzpatrick *et al.* ([5]) reported the oxidation of tyrosine to melanin by sections of human skin from a person previously subjected to ultraviolet irradiation. Inhibition of tyrosine oxidation

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[2]K. Prabhakaran, M.Sc., Ph.D., Indian Cancer Research Centre, Parel, Bombay 12, India. *Present address:* U. S. Public Health Service Hospital, Carville, Louisiana 70721.

by extracts of human epidermis has been shown to be due to binding of Cu^{++} by sulfhydryl groups ([6]). Raper ([22]) and Lerner *et al.* ([18]) showed that dopa is the first product formed in the oxidation of tyrosine, and that both dopa and tyrosine are oxidized by the same enzyme preparations. Keilin and Mann ([13]) found that crude mushroom extracts oxidized both diphenols and monophenols; on purification, the enzyme became more and more specific for diphenols. It has also been observed that monophenolase activity is less stable than that of diphenolase 2 ([20]).

MATERIALS AND METHODS

Oxidation of dopa was studied by the Warburg manometric method by measuring O_2-uptake. The organisms were ground with sand in a chilled agate mortar, and the sand was removed by slow-speed centrifugation. The assay system consisted of 0.2 ml. of 0.5 M Na_2HPO_4-KH_2PO_4 buffer with a pH of 6.8, 0.3 ml. of a DL-dopa 10 mgm./ml. suspension, and 1 ml. of a bacterial suspension (protein concentration 40 mgm.). The final volume was made up with distilled water to 3.2 ml. The temperature was 37°C and the time 120 minutes.

After incubation, the reaction mixture was centrifuged, and the spectrum of the supernatant was taken in a Beckman spectrophotometer, model DU for detection of indole-5,6-quinone, having an absorption maximum at 540 mμ.

Nitrogen estimation of the bacilli was made by the method of Johnson ([12]), and of the tissue extracts by the method of Folin and Wu, as described by Gradwohl ([7]).

Samples of normal human skin were obtained from amputated limbs of accident cases, and from cancer patients.

Kedrowsky's bacillus and *M. phlei* were grown in glycerol broth for 21 days and *M. smegmatis* in the same medium for 48 hours. *M. tuberculosis* H37Ra and BCG were cultured in Dubos medium for 21 days, and *M.sp. 607* was grown in the same medium for 7 days. The organisms were separated from the medium by centrifugation at 6,000 x g for 10 minutes and were washed twice with 0.85 per cent NaCl and once with distilled water.

RESULTS

Concentrates of mycobacteria were prepared from human leprosy nodules as described previously ([21]). The details of the procedure are given in Figure 1. It is to be noted that the method entailed some loss of material. However, the final fraction J was concentrated with respect to organisms and contained very little tissue debris, as far as could be detected by Ziehl-Neelsen staining technic (Figure 2a & b). The preparations were found to be metabolically active, for the organisms showed consistent oxygen-uptake. Normal human skin extract prepared under identical conditions was practically inert with respect to respiration.

Figure 3 shows oxygen-uptake of *M. leprae* in the presence of added dopa. It may be seen that there is considerable increase in O_2-uptake over the endogenous uptake, when the organisms are incubated with dopa.

Dopa is known to undergo auto-oxidation to melanin under certain conditions ([9]). It was therefore essential to determine if dopa is auto-oxidized in 2 hours at the pH of 6.8 employed in these studies. For this purpose, O_2-uptake of dopa due to auto-oxidation was measured at two different pH values. It may be seen in Figure 4 that at pH 8.3 there was considerable increase in O_2-uptake, whereas at pH 6.8 there was no significant increase.

Indole-5,6-quinone is one of the intermediates in the enzymatic conversion of dopa to melanin. Its presence is detected by its characteristic absorption maximum at 540 mμ. Curve 2 in Figure 5 shows the formation of indole-5,6-quinone in the oxidation of dopa by *M. leprae*. When the reaction mixture is kept for 18 hours, the absorption maximum due to indole-5,6-quinone disappears as a result of its conversion to melanin (curve 3). Dialysis of the bacterial preparation for 4 hours against distilled water at 4°C was found to have no effect on its dopa oxidase activity. Heating at 100°C for 15 minutes completely inactivated the enzyme.

The results of comparative studies with eight other mycobacteria on the oxidation

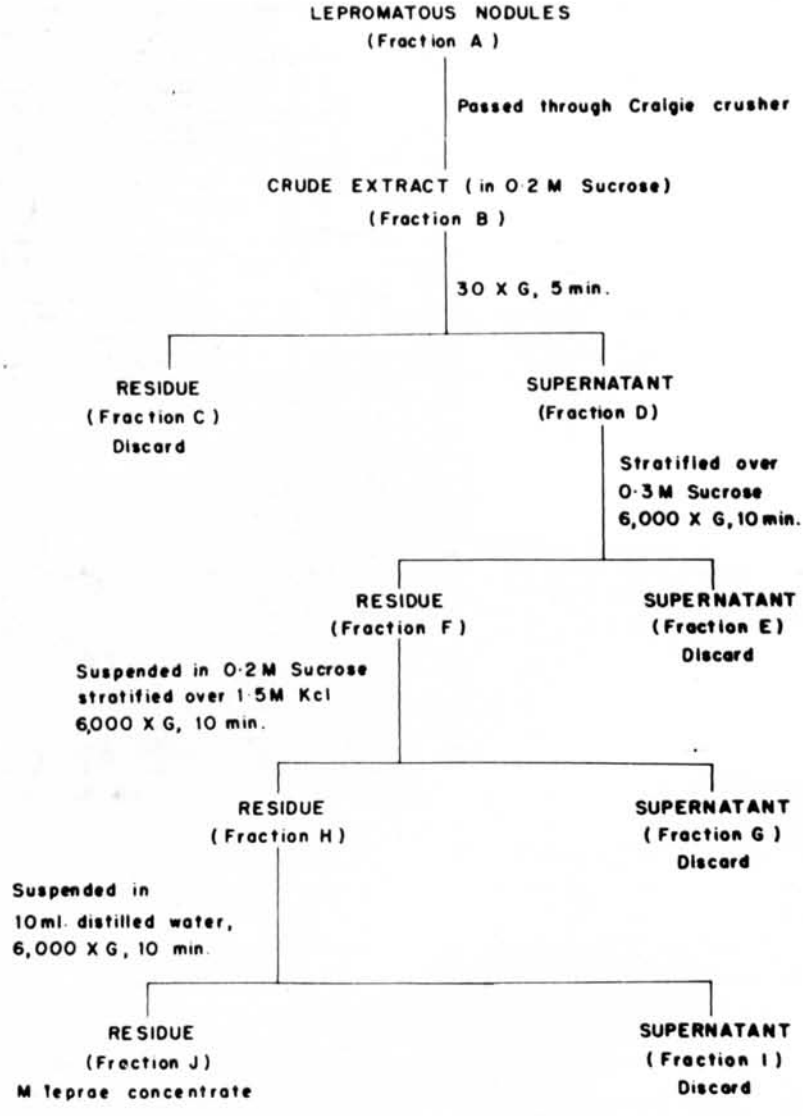

FIG. 1. Fractionation scheme for preparing *M. leprae* from human leprosy nodules.

of dopa are presented in Table 1. It can be seen that none of these mycobacteria, including *M. lepraemurium* and *M. tuberculosis* H37Rv could metabolize dopa. Thus, among nine organisms tested, only *M. leprae* was able to convert dopa to melanin with formation of indole-5,6-quinone.

Curve 2 of Figure 6 shows the formation of indole-5,6-quinone on incubation of human skin extract (fraction D; Fig. 1) with dopa. In view of the metabolism of dopa observed with the skin extract, and since *M. leprae* is separated from skin nodules, it was necessary to examine critically to determine if apparent oxidation of dopa by the bacilli was actually due to skin particles present in *M. leprae* preparations. For this purpose, the following experiments were carried out:

1. An attempt was made to prepare a particulate fraction from human skin, corresponding to the *M. leprae* fraction from leprosy nodules, and test its dopa oxidase activity. It was found that no packed sediment could be obtained from the skin extract when it was centrifuged at 6,000 x g, the force used for sedimenting *M. leprae*

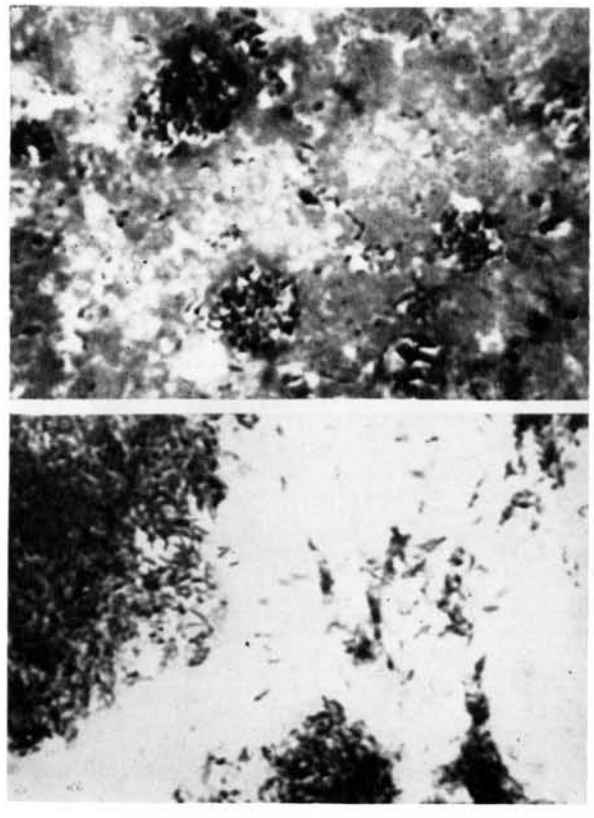

FIG. 2. (a) Fraction C (see Fig. 1). Ziehl-Neelsen stain. (b) Fraction J (see Fig. 1). Ziehl-Neelsen stain.

from nodule extract (Fig. 1). The skin preparation was therefore centrifuged at 18,000 x g and the various fractions were collected. Dopa oxidase activity of these fractions was compared to that of corresponding fractions from leprosy nodules. It may be noted from the results illustrated in Table 2 that the final residue from the skin (fraction J, comparable to *M. leprae* fraction from nodules) had no appreciable dopa oxidase activity.

2. Since the experiment described above indicated that dopa oxidase may be a characteristic property of *M. leprae*, the experiment was repeated in terms of specific activity of the various fractions. The results (Table 3) show that in the skin the activity falls off from 1.76 in the crude extract to 0.54 in the final residue, whereas, in the nodule, the activity increases from 3.55 in the crude extract to 7.73 in the final fraction (J) consisting of bacilli. On comparison of fraction J from the control (skin) with that from the nodules, it is clear that the bacillus-containing fraction is 14 times as active as the skin preparation.

3. *M.607*, which did not oxidize dopa, was mixed with minced human skin, kept

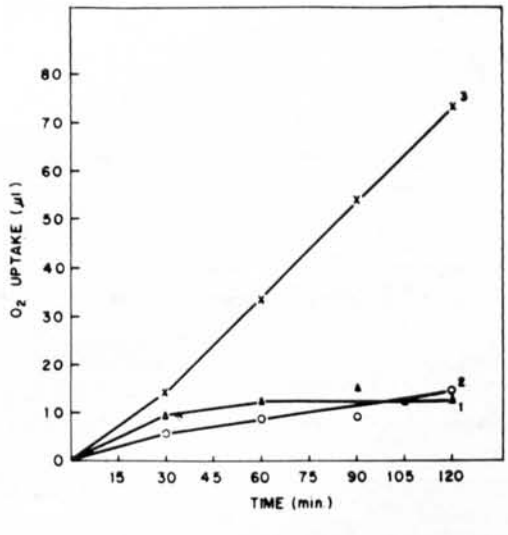

FIG. 3. Oxidation of dopa by *M. leprae*. For experimental conditions: see METHODS AND MATERIALS (1) Dopa. (2) Endogenous. (3) *M. leprae* + Dopa.

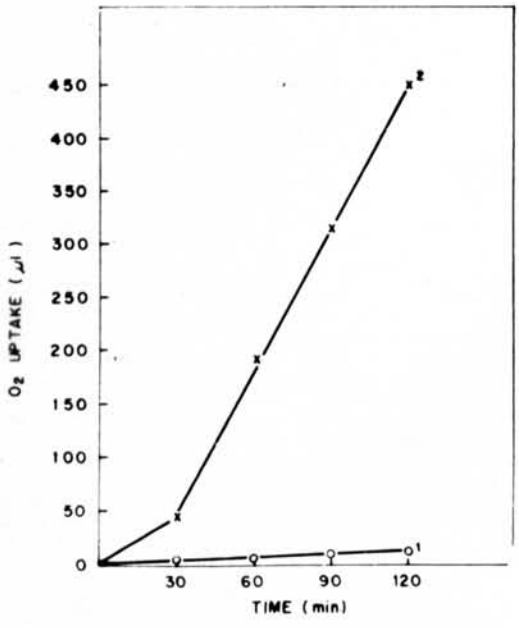

FIG. 4. Auto-oxidation of dopa at pH 6.8 and 8.3. The incubation system consisted of: 0.2 M Na_2HPO_4-KH_2PO_4 buffer, pH 6.8 or 8.3, 0.2 ml.; DL-dopa, 0.005 M; volume, 3 ml.; temperature, 37°C; time, 120 minutes. (1) pH 6.8; (2) pH 8.3.

frozen overnight, and separated from the tissue by the method used for *M. leprae*. The dopa oxidase of these organisms was tested to see if skin tissue enzymes were carried over in the final fraction of bacilli. The results were negative.

4. Attempts were made to determine if the dopa oxidase activity of *M. leprae* concentrates was due to enzymes firmly bound to the bacilli. The *M. leprae* fraction, as well as skin fraction F, was washed twice with distilled water and tested for dopa oxidase activity before and after washing. (Fraction F was chosen for comparison, since fraction J from skin had little activity.) Repeated washing of the *M. leprae* preparation produced no significant effect on its dopa oxidase activity, while the skin fraction lost over 50 per cent of its activity.

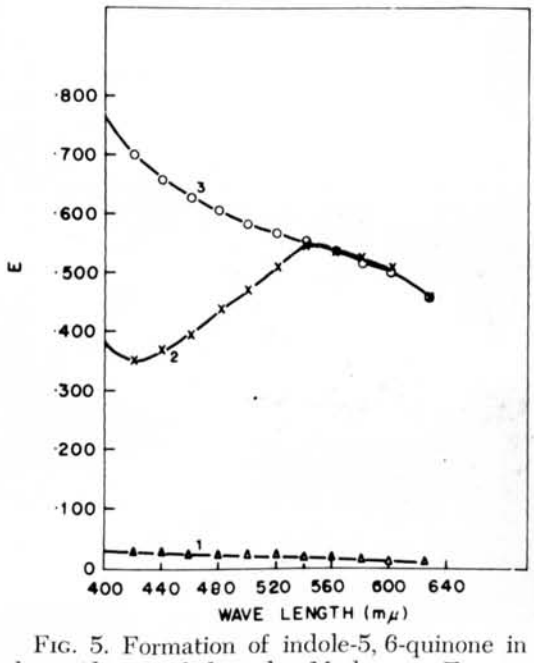

FIG. 5. Formation of indole-5, 6-quinone in the oxidation of dopa by *M. leprae*. For experimental conditions: see Table 2. (1) Dopa. (2) *M. leprae* + dopa, 2 hrs. (3) *M. leprae* + dopa, 18 hrs. (Dilution 3x).

TABLE 1. *Oxidation of dopa by various mycobacteria.*

Bacterial preparation	Oxygen uptake (μl) − Dopa	+ Dopa	% increase	Formation of indole-5, 6-quinone (Δ E540 mμ)
M. leprae	15	73	320	457
M. lepraemurium[a]	Not tested			
M. tuberculosis, H37Ra	14	16	—	—
M. tuberculosis, H37Rv[a]	Not tested			
M. tuberculosis, BCG	19	22	—	—
M. 607	Not tested			
M. smegmatis	18	16	—	—
M. phlei	45	45	—	—
Kedrowsky's bacilli	30	29	—	—

[a]Tested by Dr. R. J. W. Rees at the National Institute for Medical Research, London.
Auto-oxidation of dopa—10 μl.
For experimental conditions: see MATERIALS AND METHODS.
Protein concentration: *M. leprae*, 40 mgm.; other mycobacteria, 15-18 mgm.

TABLE 2. *Dopa oxidase activity of various fractions prepared from leprosy nodules and human skin (compared on wet weight basis).*

Fraction	E540 mμ	
	Skin	Nodule
D	340	1,250
E	125	760
G	55	140
I	15	25
J	60	550

The incubation system consisted of: 0.5 M Na_2HPO_4 –KH_2PO_4 buffer, pH 6.8, 0.4 ml.; DL-dopa 0.005 M; skin and nodule extract from 0.5 gm. tissue; volume, 6 ml.; temperature, 37°C; time 120 minutes.

TABLE 3. *Dopa oxidase activity of various fractions prepared from human skin and leprosy nodules (specific activity).*

Fraction	E540 mμ/mgm. protein	
	Skin	Nodule
B (crude extract)	1.76	3.55
D	1.14	4.00
E	0.36	2.25
J	0.54	7,73

The incubation system consisted of: 0.5 M Na_2HPO_4 –KH_2PO_4 buffer, pH 6.8, 0.3 ml.; skin extract, from 0.4 gm. wet weight; nodule extract from 0.2 gm. wet weight; DL-dopa, 0.0025 M; volume, 6 ml.; temperature, 37°C; time, 120 minutes.

The washed *M. leprae* fraction and the skin preparation were subjected to sonic oscillation in a 9 Kc Raytheon magnetostrictive oscillator for 10 minutes. Sonic vibration (which resulted in turbid homogeneous suspensions of fine particles) produced partial inactivation of both preparations. However, the decrease in activity was greater in the skin (24%) than in the bacilli (8%). The sonicates were centrifuged at 110,000 x g for 60 minutes in a Spinco model L ultracentrifuge. The particulate and supernatant fractions obtained were tested for dopa oxidase. In *M. leprae* 88 per cent of the activity (of the sonicate) was located in the particulate fraction; in the skin the activity was distributed more or less equally between the residue and the supernatant.

The results are presented in Table 4. It will be seen that dopa oxidase of *M. leprae*

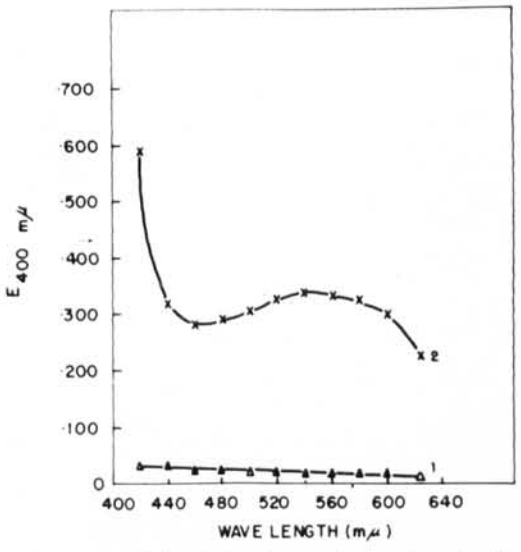

FIG. 6. Indole-5, 6-quinone formation in the oxidation of dopa by human skin extract (Fraction D). For experimental conditions: see Table 2. (1) Dopa. (2) Skin extract + dopa.

is not easily dissociated from the particulate elements, whereas in the skin the activity is more easily removed from the particles. The results suggest that the oxidation of dopa by *M. leprae* is due to enzymes firmly bound to the cell structure, and all evidence points to the conclusion that the activity is not an artifact due to skin particles.

DISCUSSION

It has thus been demonstrated that *M. leprae* (separated from human leprosy nodules) actively oxidizes 3,4-dihydoxyphenylalanine (dopa), giving rise to pigmented products. Indole-5,6-quinone has been identified as an intermediate of the reaction. This is in agreement with detection of this compound in the enzymatic oxidation of dopa by mushroom phenolase ([19]). Dopa was not metabolized by *M. lepraemurium*, *M. tuberculosis* (H37Rv and H37Ra), BCG, *M.607*, *M. phlei*, *M. smegmatis*, and Kedrowsky's bacilli. Among these mycobacteria, *M. lepraemurium* and *M. tuberculosis* H37Rv are pathogenic organisms, and *M. lepraemurium*, like *M. leprae*, is directly isolated from infected

TABLE 4. *Effect of washing and sonic vibration on dopa oxidase activity of* M. leprae *(J) and skin fraction (F).*

Treatment	E540 mμ		% activity	
	Fraction J (*M. leprae*)	Fraction F (skin)	Fraction J (*M. leprae*)	Fraction F (skin)
Untreated	260	265	100	100
Washed 2 x	240	125	92.3	47.1
After sonic vibration of washed fractions	220	95	84.6	35.9
110,000 x g. supernatant	40	45	16.9	17.0
110,000 x g. precipitate	193	60	74.2	22.6

The incubation system consisted of: 0.5 N Na_2HPO_4 –KH_2PO_4 buffer, pH 6.8, 0.2 ml.; DL-dopa, 0.0025 M; *M. leprae* (J) from 0.3 gm. wet weight of tissue; skin fraction (F) from 0.5 gm. wet weight of tissue; volume, 4 ml.; temperature, 37°C; time, 120 minutes.

tissues. Experiments on auto-oxidation of dopa at different pH values have shown that at pH 6.8, the H ion concentration employed for studies with *M. leprae*, dopa auto-oxidation could be excluded. The effects of dialysis and heat on the dopa oxidase of *M. leprae* revealed, further, that the activity is not catalyzed by inorganic ions present as contaminants; heat-instability indicates that the reaction is probably enzymatic in character.

Since *M. leprae* preparations were obtained from leprosy skin nodules, and since it is known that melanocytes in skin can convert dopa to melanin ([17]), control experiments were carried out with the use of normal human skin. After subjecting the skin to the procedure employed in the separation of *M. leprae* from the nodules, dopa oxidase activity was determined in all fractions obtained from the skin and the nodules. In the skin the most active fraction was the starting material; the final fraction (corresponding to *M. leprae* concentrates) showed little dopa oxidase. In the case of the leprosy nodules, on the other hand, the specific activity increased with concentration of organisms by the procedure used in the preparation. It was apparent from these observations that the dopa oxidase detected in the bacilli could not be an artifact caused by skin enzymes.

Further experiments by repeated washing and sonic vibration of the organisms also demonstrated that in *M. leprae*, dopa oxidase is firmly bound either to the cell membrane or to particulate elements in the cytoplasm. Electronmicroscopic studies by Brieger *et al.* ([3]) and Imaeda and Ogura ([11]) of ultrathin sections of *M. leprae*, have in fact revealed a system of intracytoplasmic membranes in the bacillus, often in continuity with the cell membranes. In the skin, the activity could be removed by washing, and partially solubilized. It is therefore reasonable to conclude that oxidation of dopa by *M. leprae* is an inherent property of the organisms. Comparative studies with eight other mycobacteria (including *M. lepraemurium* and *M. tuberculosis* H37Rv) showed that none of them could oxidize dopa, thus indicating a high degree of specificity for dopa oxidase in *M. leprae*.

The nature of the enzyme catalyzing the oxidation of dopa in *M. leprae* is not clear. The organisms failed to oxidize tyrosine to melanin. This failure is probably due to the presence of some inhibitor, as has been found in human skin ([5]). It is known also that monophenolase activity is less stable than that of diphenolase ([2, 20]). Kertesz and Zito ([14]) believe that hydroxylation of monophenols is probably a nonenzymatic process and that oxidation of diphenols is the only enzyme-catalyzed reaction in phenolase. If this is the case, dopa oxidase of *M. leprae* functions as a true phenolase (Enzyme Commission 1.10.3.1, o-diphenol:0_2 oxidoreductase).[3]

The observation that *M. leprae* could

[3]Identification numbers assigned by Enzyme Commission appointed by the International Union of Biochemists.

oxidize dopa, essential for formation of melanin pigment, gains added significance in light of the fact that hypopigmented skin patches are characteristic lesions of leprosy. In the human skin melanocytes located at the dermal-epidermal junction and in the hair bulbs produce melanin pigment. Embryologically, as noted above, melanocytes are derived from the neural crest, which gives rise also to dorsal root ganglia of spinal nerves, the adrenal medulla, and Schwann cells of the peripheral nerves ([23]). It is interesting that *M. leprae* invades tissues of the body where melanocytes and other cells derived from the neural crest occur. In view of the facts that melanocytes are of neural origin ([23]) and that the human leprosy bacilli have a particular affinity for nerve tissue ([4]), it appears possible that *M. leprae* is associated with melanocytes in skin. In this connection, the following observations are relevant: (1) Electronmicrographs of lesions produced in hamsters by inoculation of *M. leprae*, by Imaeda *et al.* ([10]), show the organisms to be in intimate association with cells containing pigment granules. (2) In the tissue-culture experiments by Hanks ([8]), where attempts were made to cultivate *M. leprae*, it was observed that the bacilli survived longest in pigment-containing cells. In view of the fact that tyrosine is converted to dopa in the melanocytes ([17]), it is conceivable that the organisms in close contact with these cells compete, through their dopa oxidase activity, for the substrate dopa, which normally would lead to pigment production in the skin. Such a deviation of dopa from normal melanin formation may be reflected finally in the hypopigmented skin patches typical of leprosy.

It should be pointed out that catecholamines are structural analogs of dopa, and may be oxidized to pigmented products. The presence of these compounds is a factor to be reckoned with in considering the invasion of peripheral nerves by *M. leprae*.

On the basis of the above reasoning, which attempts to explain the source of dopa available to *M. leprae* at the site of infection, the question arises as to the *in vivo* metabolism of this compound in the organisms. Boswell and Whiting ([1]) were the first to suggest that phenolase could function as a respiratory enzyme. The oxidation of ascorbic acid by mushroom phenolase has been demonstrated by Keilin and Mann ([13]). Robinson and Nelson ([24]) showed that, in presence of oxidizable substrates like ascorbic acid, dopa could function aş a true hydrogen carrier. Wosilait and Nason ([26]) have also reported that phenolase could oxidize $NADH_2$ or $NADPH_2$ through the quinones formed in the reaction:

$$\text{Quinone} + \text{NADH}_2 \text{ or } \text{NADPH}_2 \rightarrow \text{Diphenol} + \text{NAD or NADP}^4.$$

Thus phenolase could find a place in the pathway of the respiratory enzymes helping to carry out oxidation of a variety of substrates. On the basis of the above mechanism, it appears that dopa oxidase does not proceed to melanin-formation in the presence of other oxidizable substances, the quinones produced being reconverted to diphenol. Thus, in the *in vivo* state, where utilizable substrates are present, normal melanin-formation from dopa would not occur. It is most likely that in *M. leprae* dopa oxidase provides an alternative mechanism by which different substrates could be oxidized effectively by the organisms.

SUMMARY

Human leprosy bacilli separated from lepromatous nodules actively oxidized 3,4-dihydroxyphenylalanine (dopa) to pigmented products. Indole-5,6-quinone was detected as an intermediate of the reaction.

Dopa oxidase activity showed a high degree of specificity for *M. leprae*. Eight other mycobacteria tested, including *M. lepraemurium* and *M. tuberculosis* H37Rv, did not oxidize dopa.

Various control experiments with normal human skin revealed that dopa oxidase of *M. leprae* was a characteristic property of the bacilli, and not due to accompanying skin tissue enzymes. Heat-inactivation and

[4]NAD = Nicotinamide-adenine dinucleotide. NADP = Nicotinamide-adenine dinucleotide phosphate.

dialysis of the bacterial preparations demonstrated that the activity was not catalyzed by inorganic ions.

Sonic vibration of *M. leprae* suspension and ultracentrifugation of the product showed that dopa oxidase activity of the organisms was firmly bound to particulate elements in the cell structure.

It is suggested that dopa oxidase provides an alternative mechanism by which a variety of substrates could be oxidized by the bacilli.

RESUMEN

Bacilos leprosos humanos separados de nódulos lepromatosos activamente oxidaron 3,4-dihydroxyphenylalanine (dopa) a productos pigmentados. Indole-5,6-quinone fué revelado como efecto intermedio de la reacción.

La actividad de oxidación de dopa mostró un alto grado de especificidad para *M. leprae*. Otros ocho mycobacterias experimentados, incluyendo *M. lepraemurium* y *M. tuberculosis* H37Rv, no oxidaron dopa.

Varios experimentos de control con piel humana normal revelaron que dopa oxidasa de *M. leprae* era una característica propia de los bacilos, y no una consecuencia de las enzimas que acompañan los tejidos de la piel. Inactivación por el calor y la dialysis de las preparaciones de las bacterias demostró que la actividad no era catalizada por iones inorgánicos.

La detrucción sónica de una suspensión de *M. leprae* y la ultracentrifugación del sedimento demostró que la actividad dopa oxidase de los organismos estaba firmemente relacionada con elementos particulares en la estructura celular.

Se sugiere que dopa oxidasa constituye un mecanismo alterno por el cual una variedad de sustratos podrían ser oxidados por los bacilos.

RÉSUMÉ

Ajouté à des composés pigmentés, des bacilles de la lèpre humaine séparés de nodules lépromateux ont oxydé activement la 3,4-dihydroxyphenylalanine (dopa). L'indol-5,6-quinone a été decelée comme intermédiaire de la réaction.

L'activité de la dopa-oxydase a montré un haut degré de spécificité pour *M. leprae*. Il n'y a pas eu d'oxydation de la dopa chez huit autres mycobacteries qui ont été étudiées, parmi lesquelles *M. lepraemurium* et *M. tuberculosis* H37Rv.

Diverses expériences témoins menées avec de la peau humaine ont montré que la dopa-oxydase de *M. leprae* constituait une propriété caractéristique des bacilles et n'était pas due aux enzymes présents dans le tissu cutané. L'inactivation par la chaleur et la dialyse des préparations bactériennes a démontré que l'activité n'était pas catalysée par des ions inorganiques.

La dislocation par ultra-sons d'une suspension de *M. leprae* et l'ultracentrifugation du produit passé aux ultra-sons a montré que l'activité dopa-oxydasique des organismes était fermement liée aux élements particulés de la structure cellulaire.

On émet la suggestion que la dopa-oxydase fournit un mécanisme de remplacement grâce auquel nombre de substracts peuvent être oxydés par les bacilles.

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