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Dopa Metabolism by *Mycobacterium leprae:* Its Implications in Culture of the Bacillus and Chemotherapy of Leprosy*

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An integrated review is made of the investigations on a new type of tyrosinase (*o*-diphenoloxidase) detected in *Mycobacterium leprae*. The enzyme was different in several respects from tyrosinase obtained from plant or mammalian sources. Inhibitors of *o*-diphenoloxidase suppressed multiplication of leprosy bacteria in mouse footpads, indicating that the enzyme might have a key metabolic rôle in the growth of *Myco. leprae*. Suspensions of the organisms exposed *in vitro* to one of these inhibitors (diethyldithiocarbamate) completely lost their viability. The host tissues preferentially invaded by the bacilli (i.e. the skin and the peripheral nerves) are of ectodermal origin where metabolism of DOPA or its derivatives is important. Our results show that DOPA and other phenolic substrates are rapidly utilized *in vitro* by *Myco. leprae*. These observations suggest that, besides other factors, small amounts of DOPA continually generated by living cells may be essential for the survival and proliferation of the leprosy bacteria.

Introduction

Ever since the discovery of *Mycobacterium leprae* was reported in 1874, innumerable attempts have been made to cultivate the organism in bacteriological media; however, no established procedure is available as yet for culture of the bacillus. Similarly, little success had been achieved until recently to transmit the systemic (lepromatous) form of the disease to normal (immunologically intact) experimental animals. Now it has been found that some 9-banded armadillos (*Dasypus novemcinctus* L.) develop the systemic type of the infection on inoculation with *Myco. leprae* (Kirchheimer and Storrs, 1971). Previously it had been shown that a limited multiplication of the bacilli takes place in the footpads of mice (Shepard, 1960).

The sulphone drugs which are generally used at present in leprosy have to be administered for prolonged periods and many patients develop adverse reactions in the course of the treatment. Moreover the emergence of sulphone-resistant *Myco. leprae* is becoming increasingly evident (Browne, 1969; Jacobson and Trautman, 1971). The antibiotic, Rifampin (rifampicin), recently introduced in the treatment of leprosy has shown a profound bactericidal effect on the organisms (Rees, Pearson and Waters, 1970). However, it is known that resistance to Rifampin occurs not uncommonly in several other species of bacteria (Riva and Silvestri, 1972).

An understanding of the metabolic properties of the leprosy bacillus might be of use in developing more effective chemotherapeutic procedures and in attempts

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at cultivation of the organism. When we started our studies, almost nothing was known about the metabolism of *Myco. leprae.* In human leprosy the causative organism preferentially invades the skin and the peripheral nerve tissues. This may indicate the requirement of a common metabolite present in both these tissues for the survival and proliferation of *Myco. leprae.* The multiplication of brucellae in specific tissues of certain animals has been shown to be due to the presence of erythritol at these sites (Smith, 1968). There are reports that the body temperature in areas of the human body invaded by the leprosy bacilli is lower than normal (Binford, 1956; Brand, 1959; Hastings *et al.*, 1968). It may be pointed out, however, that the skin and the nerve tissues are of ectodermal origin and that the metabolism of 3,4-dihydroxyphenylalanine (DOPA) or its derivatives is important at these sites.

Source of Bacilli

Since no culture of *Myco. leprae* is available, the organisms used in our investigations are obtained from leprous human tissues or from the tissues of armadillos which developed massive infection after inoculation with the bacilli. The bacteria have to be prepared so as to be essentially free of host tissue material and in a metabolically active state. For this purpose solvents which might denature proteins and inactivate enzymes cannot be employed. We homogenize the infected tissues and separate the bacilli by differential and density gradient centrifugations in inert solutions such as those of sucrose and KCl. The preparations obtained by this method (on staining by the Ziehl-Neelsen procedure) proved to be concentrates of mycobacteria free of any visible tissue debris (Prabhakaran, Harris and Kirchheimer, 1969, 1971). The organisms are used in the intact state or are disrupted by ultrasonic oscillation. To remove any substances adsorbed from host tissue, the bacilli are treated with trypsin, acetone, and ether, or repeatedly washed with saline and water.

Phenoloxidase

Measurement of oxygen uptake by the Warburg manometric technique showed that *Myco. leprae* rapidly oxidizes DOPA (Prabhakaran, 1967*b*). But this method requires large amounts of material which are not readily available, and therefore more sensitive techniques had to be adopted to assay the DOPA oxidase activity of the bacilli. The enzyme involved in the conversion of tyrosine or DOPA to pigmented products is referred to as tyrosinase, phenolase, or *o*-diphenoloxidase. Tyrosinase is not restricted to vertebrate melanocytes; it occurs in invertebrates and is widely distributed in the plant kingdom. Fungi are rich sources of the enzyme; lyophilized mushroom tyrosinase is commercially available. Extracts of mouse melanomas are used in our studies as sources of mammalian *o*-diphenoloxidase. Several species of mycobacteria tested, including *Myco. lepraemurium, Myco. tuberculosis* and some so-called *Myco. leprae* cultures showed no phenolase activity. Oxidation of DOPA has been proposed as an identification test of the leprosy bacilli (Prabhakaran and Kirchheimer, 1966, Kirchheimer and Prabhakaran, 1968). *Myco. leprae* did not oxidize tyrosine to quinone.

Two quinone intermediates in the oxidation of tyrosine or DOPA to melanin are chromophoric and have been identified by their characteristic absorption spectra (Mason, 1955). *o*-Diphenoloxidase from mammalian or plant sources gives

rise to the quinone dopachrome, which has an absorption maximum around 280 nm. Indoles-5,6-quinone, which is believed to polymerize to melanin shows a peak at 540 nm in the spectrum. The spectrophotometric method, being more sensitive than the manometric technique, was used in most of our experiments.

When the spectrum of the reaction intermediates was measured it was found that *Myco. leprae* obtained from leprous human tissues gives rise to indole-5,6-quinone from DOPA, whereas mammalian and plant tyrosinases produce dopachrome. The formation of indole-5,6-quinone involves a decarboxylation step. The results suggest that an active decarboxylase is associated with the *o*-diphenoloxidase of the leprosy bacilli separated from infected human tissues (Prabhakaran, 1968).

At present we are using the liquid scintillation-counting procedure, in which oxidation of tritiated DOPA by the bacilli is measured. By the radio-isotope tracer technique, oxidation of labelled DOPA has been demonstrated in organisms recovered from leprous human tissues as well as from tissues of experimentally infected armadillos (Prabhakaran, Harris and Kirchheimer, 1973).

It is known that the substrate specificity of *o*-diphenoloxidase becomes restricted with a rise in the phylogenetic scale (Mason, 1955). The enzyme from plants and microorganisms oxidizes several phenolic substrates, whereas tyrosinase from vertebrate melanocytes is relatively specific for L-tyrosine and L-DOPA. Because *Myco. leprae* is a micro-organism obtained from human tissues, it was of interest to study the substrate specificity of the enzyme in the bacilli. Our studies showed that *Myco. leprae* oxidizes D-DOPA at the same rate as L-DOPA and oxidizes also a variety of phenolic compounds *in vitro*, indicating that the enzyme has a wide substrate specificity. Mammalian tyrosinase obtained from melanomas rapidly converted L-DOPA to dopachrome; it showed very little activity towards D-DOPA and was completely inactive towards the other phenolic substrates (Prabhakaran, Harris and Kirchheimer, 1972).

Certain enzymes like acetylcholinesterase are inhibited by increasing substrate concentration (Davies and Green, 1959). In the oxidation of L-DOPA by mammalian tyrosinase a distinct inhibitory effect was observed when the substrate concentration was increased. In *Myco. leprae*, high substrate levels produced no inhibition of *o*-diphenoloxidase (Prabhakaran, Harris and Kirchheimer, 1972).

Besides *o*-diphenoloxidase, a few other enzymes and copper proteins oxidize phenolic substrates to pigmented products. Diphenols like DOPA are oxidized by peroxidase. It has been reported that along with tyrosinase, peroxidase is involved in the formation of melanin pigment in the human skin (Patel *et al.*, 1971). In our experiments peroxidase, as well as catalase oxidized DOPA in the presence of hydrogen peroxide. Without peroxide, these enzymes showed no activity towards DOPA. Phenolase of *Myco. leprae* and tyrosinase of plant or mammalian origin oxidized DOPA without added hydrogen peroxide and also did so when catalase was added to destroy any endogenous peroxide (Prabhakaran, Harris and Kirchheimer, 1972). Previous results showed that *Myco. leprae* contains both catalase and peroxidase (Prabhakaran, 1967*a*).

All the evidence led to the conclusion that the phenolase of the leprosy organisms is probably a true *o*-diphenoloxidase, and that other copper proteins and catalase or peroxidase are not involved in the enzyme activity observed (Prabhakaran, Harris and Kirchheimer, 1972). Presence of phenolase in *Myco leprae* has been corroborated by other workers (Beaman and Barksdale, 1970).

Reducing Agents

In the formation of melanin pigment by skin melanocytes, substances like reduced glutathione are known to have a regulatory function. Decrease of reduced glutathione caused by ultraviolet rays results in greater pigment production. In our *in vitro* studies, using mammalian and plant phenolase, no quinone formation from DOPA was observed in the presence of reducing agents (ascorbic acid, reduced glutathione or cysteine). When these substances were added after the quinone was formed, it was rapidly decolorized, indicating that the quinone is reduced back to diphenol. However, in the case of *Myco. leprae*, reducing agents had no effect on the formation of quinone from DOPA. The reducing substances prevented further oxidation and polymerization of the quinone to melanin; but they did not reduce the quinone back to diphenol (Prabhakaran, 1971).

Detergent Treatment

The phenoloxidase of *Myco. leprae* was found to be firmly attached to particulate elements in the bacterial cell, unlike plant tyrosinase which is a soluble enzyme. Recently we have been able to release the phenolase of the bacilli from the particles by detergent-treatment. The anionic detergent, sodium dodecyl sulphate, liberated at least two-thirds of the enzyme activity of the disrupted bacteria into the soluble fraction. Deoxycholate was the most effective detergent for releasing mammalian tyrosinase from particles; this detergent, however, was ineffective in *Myco. leprae* (Prabhakaran, Harris and Kirchheimer, 1973).

Inhibitors of Phenolase

If phenoloxidase has a biological rôle in the leprosy bacilli, inhibitors of the enzyme might be of value in developing a rational chemotherapy of leprosy. Several inhibitors were tested *in vitro* on *o*-diphenoloxidase from *Myco. leprae* and from mammalian and plant sources. Copper chelators proved to be more effective than substrate analogues. Diethyldithiocarbamate (DDC) was found to be the most potent inhibitor (Prabhakaran, Harris and Kirchheimer, 1969). This compound produced total inhibition of the phenolase of *Myco. leprae* even at low concentrations. The molecular model showed that DDC has 2 ethyl groups (lipid-soluble, non-polar masses), which shadow the 2 sulphurs (the polar region). As such DDC could easily pass through lipid-predominant pores or membranes.

Chemotherapeutic Implications

To ascertain whether phenolase activity is important in the survival and proliferation of *Myco. leprae*, the effect of several inhibitors of phenoloxidase was tested on the multiplication of this organism in the mouse footpad (Prabhakaran, Harris and Kirchheimer, 1972). After inoculation of the mice with *Myco. leprae*, 30 μg of the drug in 0.03 ml of saline was injected once daily into the footpads, on 5 days a week for 6 months. Untreated mice and mice treated with saline (after inoculation with *Myco. leprae*) served as controls. Enumeration of the bacilli in the footpads showed that no multiplication of *Myco. leprae* occurred in

mice treated with phenolase inhibitors. Inoculation with normal saline had no effect. Penicillamine and mimosine are inhibitors of tyrosinase from mammalian and plant sources, but they do not inhibit the enzyme in *Myco. leprae*, either intact or disrupted. These compounds also did not suppress multiplication of the bacilli in the mice (Prabhakaran, Harris and Kirchheimer, 1972).

The above results do not indicate whether inhibitors or phenolase will have any effect on established infections of the bacilli in mouse footpads. Further studies, in which treatment was started 3 months after inoculation of the mice with *Myco. leprae*, showed that diethyldithiocarbamate (DDC) was the most effective phenolase inhibitor which prevented proliferation of *Myco leprae*. DDC penetrated intact bacilli and produced complete inhibition of phenoloxidase, whereas the other compounds partially inhibited the enzyme in disrupted bacilli. One of these inhibitors, thioadenine sulphate, was tested in the mice and it did not suppress multiplication of *Myco. leprae*, once the infection had established itself in the footpads (as revealed by histological examination of the footpads in untreated mice) (Prabhakaran, Harris and Kirchheimer, 1972).

In another series of experiments, the mice were treated with DDC, sulphoxone sodium or Rifampin 6 months after inoculation of the footpads with *Myco. leprae*. Before treatment, bacterial counts in control mice showed that the bacilli had multiplied. After 6 months' treatment, no significant reduction in the number of organisms was found in the footpads. The bacilli recovered from the control and the treated mice were reinoculated into new groups of mice. Six months later the organisms from untreated mice had multiplied normally, but only a slight increase in number was noted in the case of organisms obtained from mice treated with sulphoxone sodium. On the other hand, bacilli from animals treated with DDC or Rifampin showed no multiplication at all (Prabhakaran, Harris and Kirchheimer, 1972). These results indicate that the drugs probably had a bactericidal effect on *Myco. leprae*.

DDC is a potent copper chelator and phenolase is known to be a copper enzyme. DDC has been successfully used in a patient with Wilson's disease (hepatolenticular degeneration), and in toxicity tests on animals the drug was found to be 5 times less toxic than penicillamine, which is generally used in the treatment of Wilson's disease (Sunderman, Jr, *et al.*, 1963). Diethyldithiocarbamate most likely inhibits tyrosinase by binding the copper moiety of the enzyme. As such, this compound promises to have two definite advantages over other drugs, especially in cases of leprosy where drug-resistance is encountered. (1) Rifampin-resistance in bacteria is reported to be mediated by a single-step mutation involving one amino-acid in the RNA polymerase enzyme, which is normally bound by the drug (Wehrli and Staehelin, 1971). Because DDC forms a complex with the copper moiety of phenolase and not the enzyme protein itself, any mutation involving amino-acid substitutions in the enzyme is not likely to influence its effectiveness. (2) Many bacteria become resistant to certain drugs because the bacterial cell membranes develop a permeability barrier. The molecular structure of DDC is such that it can easily penetrate lipid-predominant pores of membranes. These considerations show that DDC might be useful in cases where the bacilli have developed resistance to sulphones and other drugs employed in the treatment of leprosy. However, several problems regarding use of DDC in patients remain to be worked out. It has to be mentioned that DDC is unstable under acid conditions and has to be buffered before administration; moreover, it might produce copper depletion.

Physiological Rôle of *o*-Diphenoloxidase

The results reviewed here show that *o*-diphenoloxidase activity of *Myco. leprae* is probably essential for its multiplication. However, the exact physiological rôle of this enzyme is not clear. In plants, the quinones formed from DOPA have been reported to serve as electron carriers, undergoing reversible oxidation-reduction (Robinson and Nelson, 1944). In the presence of tyrosinase, reducing substrates interact with quinone and are oxidized; the quinoné which is reduced back to diphenol in the process is regenerated by the enzyme. *o*-Diphenoloxidase thus might serve as an alternative respiratory pathway. This would be important in organisms in which the cytochrome and flavoprotein systems are not fully active, as is probably the case in the leprosy bacillus (Prabhakaran, 1967*a*).

As mentioned earlier, *Myco. leprae* has a predilection for the skin and the peripheral nerve tissues where DOPA metabolism is important. In advanced cases of leprosy, the eyes are very often involved, the ciliary body and the iris being frequently invaded by the bacilli. Surprisingly, free DOPA has been demonstrated at these sites in the mammalian eye (Pirie, 1968). The testis is known to be a site where the leprosy bacilli proliferate; it has been found that human spermatozoa possess a characteristic enzyme which oxidizes DOPA (Ackerman, 1970). *Myco. leprae* also multiplies in the Schwann cells, the dorsal root ganglia of spinal nerves, and in the adrenal medulla. It may be significant that all these tissues have a common origin in the neural crest during embryonic development (Rawles, 1948). The melanocytes originate from the neural crest and are distributed in areas such as the skin and the mucosal membranes which are invaded by the leprosy bacilli. In the armadillo, in which systemic infection with *Myco. leprae* has been reported (Kirchheimer and Storrs, 1971), besides the special features mentioned by the authors, it may be noted that the skin of this primitive mammal has only a sparse coating of hair. As such, free melanocytes (not associated with the hair bulb) might be distributed in the skin, so differing from rodents and other mammals covered with hair. In animals like the mouse, *Myco. leprae* multiplies preferentially in areas such as the footpads and the ear lobes, which are free of hair.

When viable suspensions of *Myco. leprae* (which subsequently multiplied in mouse footpads) were added to cultures of melanocyte cells, the formation of melanin pigment was suppressed; non-viable bacteria (heat-killed organisms or those obtained from autopsies) failed to do so. It is likely that the utilization of DOPA by the bacilli produces this effect (Prabhakaran, Harris and Kirchheimer, 1971). Our studies *in vitro* demonstrate that the leprosy bacilli actively metabolize DOPA and its derivatives. These observations, and the predilection of the organisms for those specific sites in the human body derived from the neural crest, indicate that DOPA might be an essential metabolite in the growth of *Myco. leprae*. However, it is an unstable compound, which will undergo auto-oxidation if added to culture media. Probably a continual supply of small amounts of DOPA generated *in vivo* by the host tissue favours the multiplication of the leprosy bacteria, and an experimental system in which such conditions could be replicated *in vitro* has to be devised for the culture of *Myco. leprae*.

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