Journal of General Microbiology (1981), **127**, 385–389. *Printed in Great Britain*

Uptake of 3,4-Dihydroxy[^{3}H]phenylalanine by *Mycobacterium leprae* Isolated from Frozen (–80 °C) Armadillo Tissue

By SAROJ R. KHANOLKAR,[1]* E. J. AMBROSE[2]† AND P. R. MAHADEVAN[2]

[1] *National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, U.K.*

[2] *Foundation for Medical Research, 84A R. G. Thadani Marg, Sea Face Corner, Worli, Bombay 400 018, India*

(*Received 22 July 1980; revised 1 December 1980*)

Mycobacterium leprae separated from armadillo tissues stored at –80 °C is similar to that from human sources in its ability to take up ^{3}H-labelled 3,4-dihydroxyphenylalanine (DOPA). Several inhibitors were studied which showed complete or partial inhibition of [^{3}H]DOPA uptake. These findings suggest that *M. leprae* isolated from frozen tissue possesses an active uptake system for [^{3}H]DOPA.

INTRODUCTION

We have previously reported that it is possible to use radioactive markers to study the metabolic activity of *Mycobacterium leprae* obtained from human biopsies, by means of high-resolution autoradiography and scintillation counting techniques (Ambrose *et al.*, 1974; Khanolkar *et al.*, 1976). Recently, relatively large amounts of purified *M. leprae* have become available for study from tissues of infected armadillos (World Health Organization, 1976). Although the yield of bacilli from armadillo tissue is greater than from other sources, it is only possible to harvest at irregular intervals, as and when the animals become heavily infected. Therefore, methods for preserving the bacilli are required. Rees & Lowe (unpublished results) have shown by the mouse foot-pad technique that *M. leprae* suspensions stored at –80 °C retain some viability for up to 1·5 years. However, the effect of freezing on the metabolic activity of these bacilli has not yet been investigated.

Mycobacterium leprae isolated from lepromatous human tissue and from experimentally infected armadillo tissues contain an *o*-diphenol oxidase activity which is absent from other mycobacteria. The occurrence of this activity was first reported by Prabhakaran & Kirchheimer (1966) and confirmed in armadillo-derived bacteria by Prabhakaran *et al.* (1975).

We have used 3,4-dihydroxy[^{3}H]phenylalanine ([^{3}H]DOPA) oxidase activity as an indicator of metabolic activity and/or viability of *M. leprae* (Khanolkar *et al.*, 1976). Though [^{3}H]DOPA has been used to show uptake qualitatively, there is little information available about the kinetics of incorporation (Prabhakaran *et al.*, 1973, 1976; Ambrose *et al.*, 1974; Harris & Prabhakaran, 1975). The present studies were initiated to determine the optimal conditions, reproducibility and specificity of the uptake of [^{3}H]DOPA by *M. leprae* separated from infected armadillo tissues stored at –80 °C.

† Present address: The Mill House, Westfield, E. Sussex TN35 4RU, U.K.

0022-1287/81/0000-9453 $02.00

METHODS

Preparation of bacteria. Suspensions of *M. leprae* were prepared from spleens of experimentally infected nine-banded armadillos (*Dasypus novemcinctus* Linn.) by a modification (described below) of the method described previously (Ambrose *et al.*, 1978) for isolation of *M. leprae* from human biopsies. Spleen tissues from eight animals were processed; five were found to be free from microbial contamination, and 18 suspensions of *M. leprae* were made from fresh or stored samples of these tissues. The infected spleens were homogenized for 2 min at full speed in a Sorvall 'Omnimix' homogenizer and treated with distilled water at 4 °C for 30 min. The bacillary suspension was then digested with 0·25% (w/v) trypsin in phosphate-buffered saline, pH 7·2, followed by centrifugation at 27000 *g* for 1 h in density gradients formed from 30% (v/v) Percoll (Pharmacia). Under these conditions *M. leprae* bands at a density of about 1·09 (P. Draper, personal communication). The bacterial layer was suspended in 0·1 M-phosphate buffer, pH 6·8, plus 1% (w/v) glucose, and the number of bacilli per ml was determined by the method of Hart & Rees (1960). The extent to which the bacterial suspension was contaminated by armadillo tissue was observed by using 0·5% (w/v) soluble blue as a counter-stain (Wheeler & Draper, 1980). Each suspension was tested on nutrient agar for the presence of fast-growing contaminants; contaminated suspensions were discarded. The suspensions were stored at 4 °C.

Mycobacterium intracellulare ATCC 13950 was maintained on Löwenstein-Jensen slopes; for experiments it was grown on slopes of Sauton medium, containing sodium succinate as a carbon source, at 32 °C. The bacteria were washed three times with phosphate buffer prior to experiments.

Assay of [^{3}H]DOPA uptake. L-[^{3}H]DOPA, ring-labelled in positions 2, 5 and 6 (specific activity 40 Ci mmol^{-1}; 1·5 TBq mmol^{-1}) was obtained from Amersham. Dimilume scintillation fluid, scintillation vials and Soluene-350 solubilizer were purchased from Packard. Other chemicals were purchased from Sigma.

The reaction mixture contained, in a final volume of 0·5 ml per tube, 0·1 M-Na_2HPO_4/KH_2PO_4 buffer, pH 6·8, 1 μCi [^{3}H]DOPA and 4–10 × 10^6 *M. leprae*. The temperature was maintained at 34 °C for 6 h. (Preliminary experiments had shown that bacteria kept at 32 °C or 37 °C were less active in taking up [^{3}H]DOPA.) The tubes were mounted on a roller tube unit, as used for tissue culture, rotating at about 1 rev. min^{-1}, to avoid aggregation of bacteria. Control tubes contained bacilli that were heat-inactivated (100 °C for 15 min). In two experiments with different suspensions, heat-killed bacteria were compared with γ-irradiated (2·5 Mrad from ^{60}Co) and formalin-fixed [10% (v/v) formalin for 24 h] bacteria for uptake of [^{3}H]DOPA. Substrate blanks were run simultaneously.

After incubation, the assay mixture was centrifuged at 4300 *g* for 15 min. The bacterial pellet was washed three times with cold isotonic saline; washing was completed within 1 h. The bacterial pellet was then dissolved in Soluene and transferred to scintillation vials containing Dimilume. Radioactivity was measured on a Packard automatic counter (model 2420).

Inhibitors were used in this assay at the following concentrations: diethyl dithiocarbamate (DDC), 4 mM; EDTA, 1 mM; 8-hydroxyquinoline, 1 mM; ascorbic acid, 4 mM; ouabain, 20 μM.

RESULTS

Incorporation of [^{3}H]DOPA by M. leprae

Mycobacterium leprae separated from armadillo spleen tissue stored at −80 °C showed appreciable uptake of [^{3}H]DOPA during the incubation period (Table 1). Heat-killed bacilli also exhibited some incorporation, but uptake by live *M. leprae* was two to three times that of heat-killed bacilli. The incorporation was similar in heat-killed, γ-irradiated and formalin-fixed bacteria (Fig. 1). The difference in uptake between live *M. leprae* and the controls was significant (paired *t*-test: $P = <0{\cdot}03$). A growing culture of *M. intracellulare* was tested for uptake of [^{3}H]DOPA by this method. It showed no detectable uptake.

Linearity of the relationship between numbers of bacilli per tube and radioactive counts

It was desirable to establish the relationship between the numbers of organisms and uptake of [^{3}H]DOPA. A progressive increase in radioactive counts with increasing numbers of bacilli was previously observed for *M. leprae* isolated from fresh human biopsy, but the relationship was not linear (Khanolkar, 1976). This may have been partly due to bacillary aggregation and the presence of tissue debris. The Percoll gradient method gave a purer and more uniform suspension than the earlier method, and incubating tubes on the roller drum reduced bacillary aggregation and sedimentation. Such homogeneous suspensions showed an approximately linear relationship between the number of bacilli and the extent of labelling (Table 2).

Table 1. *Uptake of [^{3}H]DOPA by two different suspensions of M. leprae*

The incubation mixtures contained, in a final volume of 0·5 ml per tube, 0·1 M-Na_2HPO_4/KH_2PO_4 buffer, pH 6·8, 5 × 10^6 *M. leprae* bacilli isolated from frozen tissue and 1 μCi [^{3}H]DOPA. After incubation at 34 °C the bacteria were washed three times with saline and their radioactivity was counted. Further details are given in Methods. The counts obtained for duplicate determinations are shown for each incubation (means in parentheses).

	Radioactivity (c.p.m.)	
Incubation	Viable *M. leprae*	Heat-killed *M. leprae*
Suspension 1		
30 min	430, 750 (590)	188, 162 (175)
2 h	3013, 3092 (3052)	1097, 1666 (1382)
6 h	8009, 5304 (6657)	2860, 2550 (2705)
Suspension 2		
30 min	823, 711 (767)	454, 369 (411)
2 h	1781, 2001 (1891)	1019, 1005 (1012)
6 h	6332, 6333 (6332)	3226, 3016 (3121)

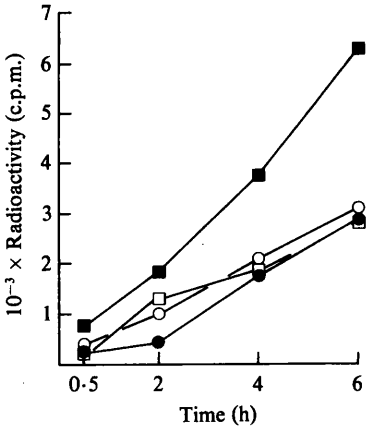

Fig. 1

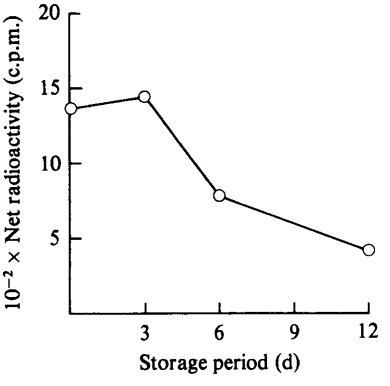

Fig. 2

Fig. 1. Uptake of [^{3}H]DOPA by viable and inactivated *M. leprae*. Incubation conditions were as in Table 1: ○, heat-killed bacteria; ●, γ-irradiated (2·5 Mrad) bacteria; □, formalin-fixed (10% formalin for 24 h) bacteria; ■, viable bacteria.

Fig. 2. Effect of storage at 4 °C on uptake of [^{3}H]DOPA by *M. leprae*. Bacterial suspensions were stored at 4 °C and after various periods the uptake of [^{3}H]DOPA was measured as described in Methods. Net radioactivity represents the radioactivity of viable bacteria minus that of heat-killed bacteria. Each point represents the mean of two determinations.

Effect of storage at 4 °C on metabolic activity of M. leprae

The uptake of [^{3}H]DOPA was used to assay the metabolic activity of bacilli isolated from stored armadillo tissues and then kept as purified suspensions for different periods at 4 °C (Fig. 2). Good incorporation was observed after storage for up to 6 d; after longer periods of storage incorporation progressively decreased. This loss of activity occurred at a similar rate to the loss of viability measured in mouse foot-pad studies (R. J. W. Rees, personal communication).

Effect of inhibitors on incorporation of [^{3}H]DOPA by M. leprae

The effects of various inhibitors on uptake of [^{3}H]DOPA by *M. leprae* are shown in Table 3. Ascorbic acid (a reducing agent) completely inhibited the uptake of [^{3}H]DOPA by *M.*

Table 2. *Relationship between amount of labelling with [^{3}H]DOPA and number of M. leprae per tube*

Incubation conditions were as described in Methods. The three suspensions were obtained from different armadillos. Duplicate tubes were incubated for 6 h. Net radioactivity represents the radioactivity of viable bacteria minus that of heat-killed bacteria.

Suspension	No. of *M. leprae* per tube	Net radioactivity (c.p.m.)
Stored tissue	4×10^6	2092
	8×10^6	5100
Stored tissue	4×10^6	3025
	8×10^6	7356
Fresh tissue	5×10^6	634
	1×10^7	1113

Table 3. *Effect of chelating agents and a reducing agent on uptake of [^{3}H]DOPA by M. leprae*

Incubation conditions were as described in Methods. Where indicated, inhibitor was added to the mixture with the [^{3}H]DOPA.

Incubation	Radioactivity (c.p.m.) Incubation period: 30 min	2 h	4 h	6 h
Buffer blank	132	387	351	484
M. leprae	518	1094	4057	6482
Heat-killed *M. leprae*	334	722	1842	3764
M. leprae + ascorbic acid (4 mM)	268	645	173	177
M. leprae + DDC (4 mM)	316	346	519	513
M. leprae + EDTA (1 mM)	236	468	452	220
M. leprae + 8-hydroxyquinoline (1 mM)	349	527	509	572
M. leprae + ouabain (20 μM)	761	893	1112	954

leprae and also inhibited its auto-oxidation in the absence of bacteria (giving very low substrate blanks). It did not have such a pronounced effect on the incorporation of [^{3}H]DOPA by fresh bacilli derived from human biopsy material (Ambrose *et al.*, 1978). The delayed effect of ascorbic acid supports the view that its penetration into the bacilli is slow. In a separate experiment (results not shown) ascorbic acid even penetrated slowly into bacilli which had been frozen and thawed. The copper-chelating agent DDC and the more general bivalent cation chelator EDTA showed total metabolic inhibition, and 8-hydroxyquinoline, which chelates Mg^{2+} and Zn^{2+}, showed considerable inhibition. Ouabain, which inhibits ATPase in the Na^+/K^+ pump, showed a marked inhibitory effect after 6 h.

DISCUSSION

The results presented above show that *M. leprae* isolated from armadillo tissues behaves towards DOPA in the same way as isolates of the organism from human sources. They also show that these bacilli are as active when prepared from frozen tissues as when fresh, at least in short-term experiments.

The uptake of [^{3}H]DOPA by live organisms was confirmed, as was the fact that heat-killed bacilli and other inactivated controls also showed some incorporation of radioactive label (about one-half to one-third that of live bacteria). These results suggest that non-specific binding of [^{3}H]DOPA occurs. γ-irradiated bacteria would be expected to have surface and other macromolecular structures similar to or only slightly changed from those in live

bacteria. The similarity of the behaviour of the heat-inactivated bacteria to that of the γ-irradiated and formalin-fixed controls provides further evidence that the difference between the incorporation by fresh and killed organisms represents a vital function of the live bacilli. These results agree well with those obtained using bacteria from fresh human tissues (Khanolkar *et al.*, 1978).

The presence of *o*-diphenol oxidase activity in *M. leprae* has been considered to be a specific marker for this organism. Previous experiments using autoradiography (Ambrose *et al.*, 1974) showed that although 'solid'-staining *M. leprae* were clearly labelled with [^{3}H]DOPA, *M. phlei, M. smegmatis* and *M. tuberculosis* failed to show any silver grains. This specificity is further confirmed by the present studies, which failed to show uptake by *M. intracellulare*, using scintillation counting.

Mouse foot-pad tests have been recognized as the only biological method for assessing the viability of *M. leprae*. These tests revealed a progressive decrease in viability on storing the fresh bacilli (R. J. W. Rees, personal communication). Tests of the effect of storage on [^{3}H]DOPA uptake showed a reduction in the incorporation of label after 6 d storage at 4 °C (Fig. 2). This further supports the view that the amount of labelling is related to the metabolic activity of *M. leprae*.

Makino *et al.* (1974) have shown that the active site of mushroom tyrosinase contains a pair of antiferromagnetically coupled copper ions. Prabhakaran *et al.* (1975) reported that the *M. leprae* *o*-diphenol oxidase was inhibited by chelators of copper, but reducing agents had little effect on the enzyme. In the present study, DDC, an effective chelator of copper, reduced uptake to the host of heat-inactivated bacilli. A similar effect was observed in the presence of EDTA and ascorbic acid. The reduction in uptake in the presence of inhibitors was similar to that caused by heat-killing. However, the results observed may be due to slight damage to the permeability barrier of the bacterial cell membrane obtained from the frozen tissue. These results are consistent with, but do not prove, that the uptake of [^{3}H]DOPA is a two-stage process: uptake (diffusion) through a permeability barrier, followed by oxidation.

We thank Drs P. Draper and R. J. W. Rees for their advice and many fruitful discussions during this work. S. R. Khanolkar was in receipt of a Heiser Fellowship.

REFERENCES

AMBROSE, E. J., ANTIA, N. H. & KHANOLKAR, S. R. (1974). Uptake of radioactive DOPA by *M. leprae*. *Nature, London* **249**, 854–855.

AMBROSE, E. J., KHANOLKAR, S. R. & CHULAWALLA, R. G. (1978). A rapid test for bacillary resistance to dapsone. *Leprosy in India* **50**, 131–143.

HARRIS, E. B. & PRABHAKARAN, K. (1975). Uptake of radioactive DOPA by *Mycobacterium leprae*. *Microbios* **12**, 119–124.

HART, P. D'ARCY & REES, R. J. W. (1960). Effect of macrocyclon in acute and chronic pulmonary tuberculosis infection in mice as shown by viable and total bacterial counts. *British Journal of Experimental Pathology* **41**, 414–421.

KHANOLKAR, S. R. (1976). *Metabolic studies on Mycobacterium leprae in vitro using radioisotopes*. Ph.D. thesis, University of Bombay, India.

KHANOLKAR, S. R., ANTIA, N. H. & AMBROSE, E. J. (1976). Autoradiographic studies of *M. leprae*. *Leprosy Review* **47**, 267–273.

KHANOLKAR, S. R., AMBROSE, E. J., CHULAWALLA, R. G. & BAPAT, C. V. (1978). Autoradiographic and metabolic studies of *M. leprae*. *Leprosy Review* **49**, 187–198.

MAKINO, N. P., MCMAHILL, P. & MASON, H. S. (1974). The oxidation state of copper in resting tyrosinase. *Journal of Biological Chemistry* **249**, 6062–6066.

PRABHAKARAN, K. & KIRCHHEIMER, W. R. (1966). Use of 3,4-dihydroxyphenylalanine oxidation in the identification of *Mycobacterium leprae*. *Journal of Bacteriology* **92**, 1267–1268.

PRABHAKARAN, K., HARRIS, E. B. & KIRCHHEIMER, W. F. (1973). Particulate nature of *o*-diphenoloxidase in *M. leprae* and assay of the enzyme by the radioisotope technique. *Microbios* **8**, 151–157.

PRABHAKARAN, K., HARRIS, E. B. & KIRCHHEIMER, W. F. (1975). *o*-Diphenoloxidase of *Mycobacterium leprae* separated from infected armadillo tissues. *Infection and Immunity* **12**, 267–269.

PRABHAKARAN, K., HARRIS, E. B. & KIRCHHEIMER, W. F. (1976). Binding of ^{14}C-labelled DOPA by *M. leprae in vitro*. *International Journal of Leprosy* **44**, 58–64.

WHEELER, P. R. & DRAPER, P. (1980). Soluble blue as a counter stain in the Ziehl-Neelsen procedure. *International Journal of Leprosy* **48**, 15–17.

WORLD HEALTH ORGANIZATION (1976). *Report of the Second IMMLEP Task Force Meeting, 1-5 December 1975*. (Reprinted in *Leprosy Review* **47**, 313–332.)