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# Oxidation of 3,4-Dihydroxyphenylalanine by Connective Tissue Constituents. Identification of *Mycobacterium leprae* not Related to Phenolase Activity[1]

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It has been shown that 3, 4-dihydroxyphenylalanine (DOPA) is oxidized by partially purified human leprosy bacilli harvested from human leprotic lesions, from the spleens of persons suffering with lepromatous leprosy ([9, 10]), and from armadillos infected with *M. leprae* ([2]). In view of these findings, *M. leprae* was prematurely considered unique among mycobacteria since none of the few tested cultivable mycobacteria as well as the noncultivated *M. lepraemurium* exhibited phenolase activity ([7]). Thus oxidation of DOPA has been widely accepted and erroneously used as an identification test of the human leprosy bacilli ([5, 11]).

Recently, Skinsnes *et al* ([13]) reported the successful cultivation of mycobacteria from human leprotic nodules. The cultures do not grow on Dubos, Lowenstein and Middlebrook media and based on immunofluorescence findings, the cultures are indistinguishable from human leprosy bacilli. The cultures oxidized DOPA. This was, however, due to the presence of hyaluronic acid in the liquid media ([13]). Prabhakaran was quick to deny that hyaluronic acid oxidizes DOPA ([6]).

The results of the Skinsnes group and the findings of Prabhakaran were verified in our laboratories using manometric and spectrophotometric technics. We already confirmed the findings of Skinsnes *et al* ([13]) and presented preliminary but unquestionable evidence that hyaluronic acid oxidizes DOPA and converted it to a pigmented product ([3]).[3] The obtained pigment was identical to that reported by Prabhakaran and considered to be a result of phenolase activity in *M. leprae*. In view of these conflicting reports, we investigated the oxidation of DOPA by polysaccharides present in the skin and related compounds. The present report provides evidence that DOPA, upon incubation with skin constituents, is oxidized into exactly the same pigmented products which has been mooted as a unique phenolase activity of *M. leprae* ([11]). The presented results will not only demystify the DOPA oxidation theory attributed to *M. leprae* but might advance further investigations into a new and hitherto unknown role of hyaluronic acid in the connective tissue.

## MATERIALS AND METHODS

**Chemicals.** All the phenolic compounds, hyaluronic acid (sodium salt, grade III-5 from human umbilical cord), hyaluronidase and mushroom tyrosinase were obtained from the Sigma Chemical Company, St. Louis, Missouri, U.S.A.

**Preparation of trypsinized umbilical cord, human skin, rat tissues, human lepromata and M. lepraemurium.** Human umbilical cords, obtained from a local hospital, were stored at –40°C. The frozen umbilical cords were thawed and washed with tap water to remove excess blood. Two grams of umbilical cord was cut into small pieces with a pair of scissors and homogenized in a Waring blender for two minutes in 100 ml 0.66 M sodium potassium phosphate buffer, pH 7.8. The homogenized tissue was then digested with 10 mg trypsin (trypsin 1:250) for one hour at 34°C. The resulting digested umbilical cord solution was quite viscous and was filtered through a nylon filter. The filtrate was adjusted with dilute HCl solution to pH 6.8, autoclaved for ten minutes at 15 lbs pressure and filtered again through Whatman filter paper. This filtrate was used to measure DOPA oxidation.

Subcutaneous rat lepromata were removed aseptically from Sprague-Dawley rats. Human lepromata were kindly provided by Dr. Languillon (Dakar). Purified cell suspension of *M. lepraemurium* was prepared as de-

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[3] See addendum.

scribed elsewhere (4). Rat lepromata, human lepromata and human skin, freshly obtained from surgery, were cut into small pieces and washed with cold distilled water to remove excess blood. They were all separately treated with trypsin the same way as umbilical cord.

To use umbilical cord, human skin, rat lepromata or human lepromata as controls, they were processed in the same manner except that trypsin was not added to the homogenate.

**Assay system.** Throughout these studies, 0.1M $Na_2HPO_4$-$KH_2PO_4$ buffer pH 6.8 was used to avoid auto-oxidation of DOPA in alkaline conditions. The reaction mixture in a final volume of 4 ml contained 360 $\mu$mole phosphate buffer and 4 mg hyaluronic acid in control flasks. Sample flasks in addition contained 0.002M phenolic compounds. The reaction mixture in Warburg flasks was incubated at 37°C.

The culture medium in which cultivation of *M. leprae* has been reported by Skinsnes *et al* (13) contains 0.1% hyaluronic acid. In the present study, the same concentration of hyaluronic acid, unless otherwise indicated, has been employed to measure the oxidation of DOPA or its derivatives.

To measure the oxidation of DOPA by nondigested and trypsin digested human skin, umbilical cord, rat lepromata, human lepromata or cell suspensions of *M. lepraemurium,* the control flasks contained 1 ml of filtrate (cell suspension in case of *M. lepraemurium*) and 2 ml buffer, pH 6.8. The sample flasks in addition contained 0.002M D- or L-DOPA. The flasks were incubated at 37°C. In order to measure the oxidation of phenolic compounds by hyaluronic acid or oxidation of DOPA by trypsin digested materials at various time intervals, 2 ml samples were transferred in cuvettes of 1 cm light path and an increase in absorption was determined spectrophotometrically in Unicam SP 1800 Spectrophotometer or Cary Model 118C.

**Oxidation of phenolic compounds by mushroom tyrosinase.** The enzyme phenolase from *M. leprae* in the oxidation of DOPA to melanin has been shown to form an intermediate, namely indole-5, 6-quinone, which has a characteristic absorption maximum at 540 nm. Likewise, the same enzyme from mushroom in the oxidation of DOPA to melanin has been reported to form a quinone with an absorption maximum at 480 nm. During this investigation, we measured the increase in absorption both at 540 and 480 nm.

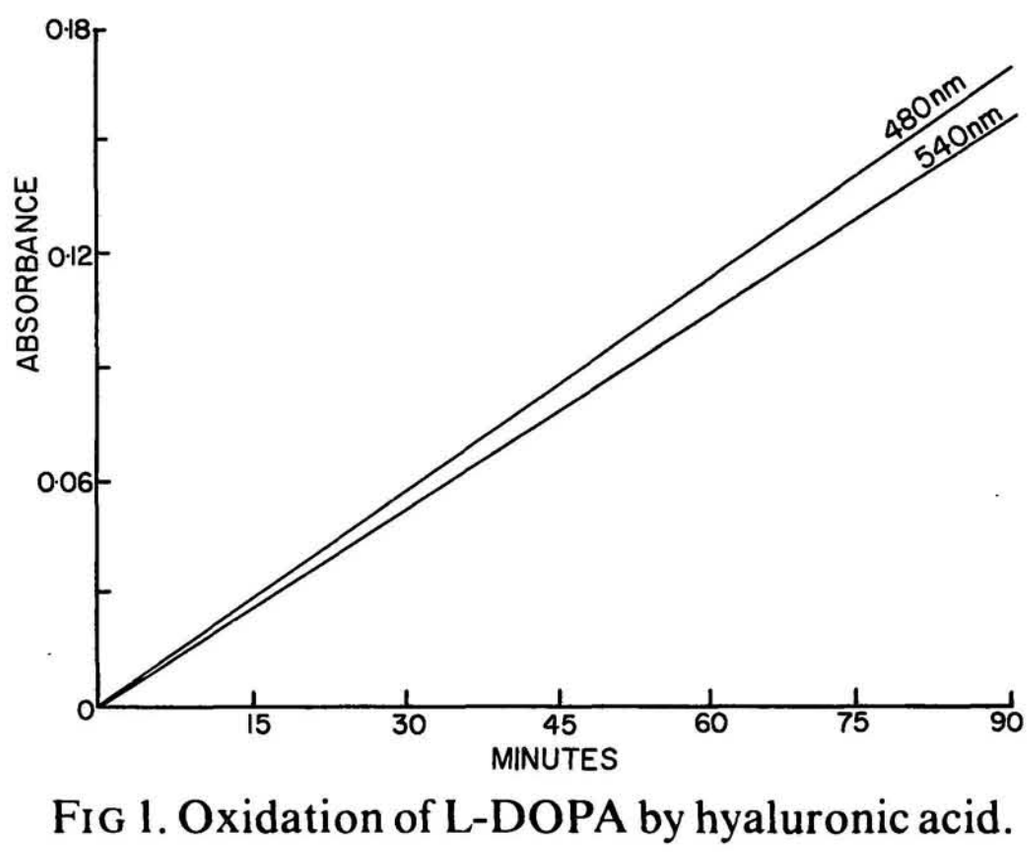


FIG 1. Oxidation of L-DOPA by hyaluronic acid.

The reference cuvette contained 0.002M phenolic compounds in a total volume of 2 ml phosphate buffer. In the sample cuvette containing 0.002M phenolic compounds and buffer, 10 $\mu$g mushroom tyrosinase was added by means of a microsyringe and increase in absorption at 480 nm was recorded in a Cary Model 118C. The temperature of incubation was kept at 25°C because of the rapid oxidation of DOPA by mushroom tyrosinase.

## RESULTS

The results in Figure 1 show that when L-DOPA was incubated with hyaluronic acid alone at 37°C, there was a development of color which gave increase in absorption at 540 nm as well as at 480 nm. Similar results were obtained upon incubation of D-DOPA with hyaluronic acid. When DOPA alone was incubated in buffer, under similar conditions, no appreciable change in color occurred in two to three hours.

Table 1 shows the effect of increasing concentrations of hyaluronic acid on D-DOPA oxidation. It is seen that the pigment formation due to DOPA oxidation, measured both at 540 and 480 nm, increased with the increase of hyaluronic acid concentration.

Table 2 shows that no detectable development of color was observed in two to three hours when DOPA was incubated with either skin extracts or umbilical cord extracts. However, DOPA was readily oxidized by both skin tissue and umbilical cord extracts when they were treated with trypsin. Likewise, there was no development of color when nontreated rat tissues and *M. leprae* extracts

TABLE 1. *Effect of different concentrations of hyaluronic acid on D-DOPA oxidation.*

| Hyaluronic acid concentration (mg) | Total volume (ml) | Time (min) | Increase in absorbance | |
|---|---|---|---|---|
| | | | 540 nm | 480 nm |
| 1 | 4 | 120 | 0.08 | 0.10 |
| 2 | 4 | 120 | 0.09 | 0.12 |
| 3 | 4 | 120 | 0.22 | 0.25 |
| 4 | 4 | 120 | 0.26 | 0.31 |

TABLE 2. *Oxidation of D-DOPA by normal and trypsin digested human skin and umbilical cord.*

| Material, extracts | Time (hrs) | Increase in absorbance | |
|---|---|---|---|
| | | 540 nm | 480 nm |
| Human skin (nontreated) | 3 | 0 | 0 |
| Human skin (trypsin treated) | 3 | 0.16 | 0.20 |
| Umbilical cord (nontreated) | 3 | 0 | 0 |
| Umbilical cord (trypsin treated) | 3 | 0.18 | 0.24 |

TABLE 3. *Oxidation of D-DOPA by normal and trypsin treated materials.*

| Material | Time (min) | Increase in absorbance | |
|---|---|---|---|
| | | 540 nm | 480 nm |
| Rat tissues[a] (nontreated) | 120 | 0.02 | 0.03 |
| Rat tissues[a] (trypsin treated) | 120 | 0.94 | 1.05 |
| *M. leprae*[b] (nontreated) | 120 | 0.02 | 0.03 |
| *M. leprae*[b] (trypsin treated) | 120 | 0.30 | 0.32 |
| *M. leprae*[b] (hyaluronidase treated) | 120 | 0.00 | 0.00 |
| *M. lepraemurium*[c] (trypsin treated) | 120 | 0.36 | 0.39 |

[a] Connective tissue of rat leprosy granuloma.
[b] From human subcutaneous leproma (LL, nontreated).
[c] Isolated from three month old subcutaneous rat leproma.

were incubated with D-DOPA (Table 3). It is interesting to note, however, that considerable pigment formation occurred upon incubation of DOPA with trypsin treated rat tissues, *M. leprae* and *M. lepraemurium.* When *M. leprae* suspension was treated with hyaluronidase, no oxidation of DOPA was observed. Our experimental data presented in Table 4 clearly show that in addition to L- or D-DOPA, phenolic compounds, namely epinephrine and norepinephrine, upon incubation with hyaluronic acid at 37°C for two hours developed a color which gave increase in absorption both at 540 and 480 nm. Mushroom tyrosinase is an extremely active enzyme and when a small amount of this enzyme (10 $\mu$g) was incubated at 25°C with L- or D-DOPA, pigment formation was very rapid as seen in Figure 2 and Table 4. Other

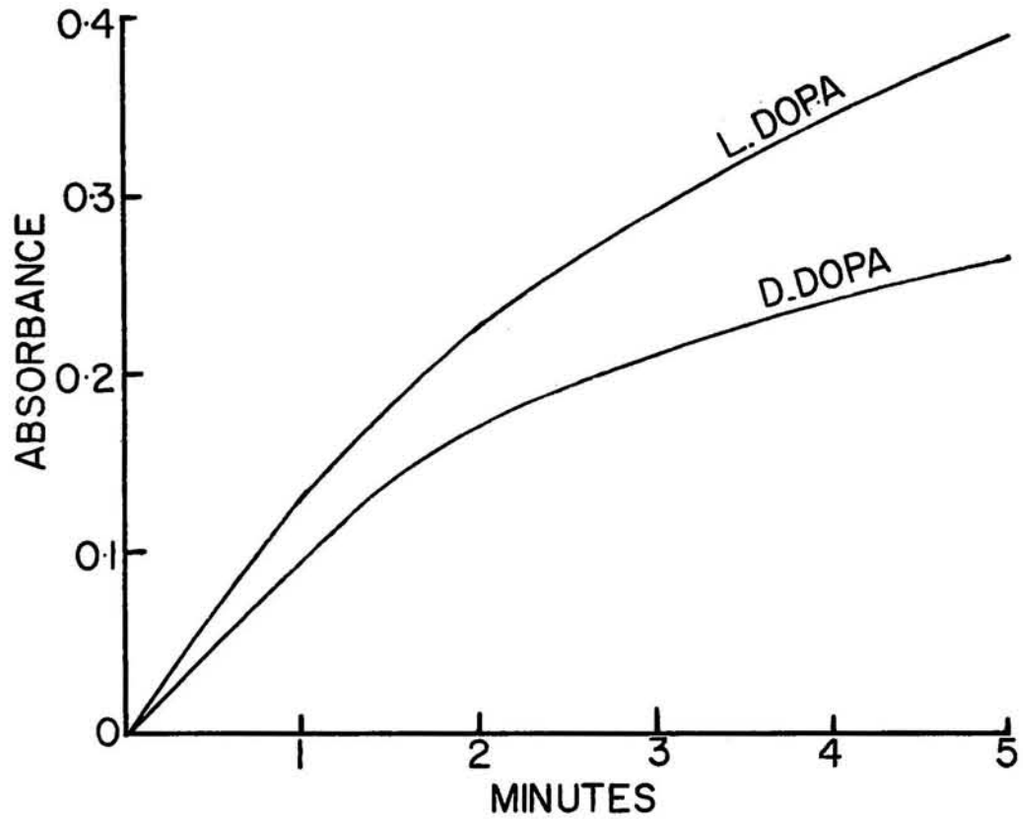


FIG. 2. Oxidation of L- and D-DOPA by mushroom tyrosinase.

Table 4. *Oxidation of phenolic compounds by hyaluronic acid and mushroom tyrosinase.*

| Compound | Increase in absorbance | | |
|---|---|---|---|
| | Hyaluronic acid[a] | | Mushroom tyrosinase[b] |
| | 540 nm | 480 nm | 480 nm |
| D-DOPA | 0.18 | 0.20 | 0.24 |
| L-DOPA | 0.26 | 0.31 | 0.38 |
| Epinephrine | 0.19 | 0.26 | 0.14 |
| Norepinephrine | 0.07 | 0.08 | 0.16 |

[a] Incubation time 120 minutes.
[b] Incubation time 5 minutes.

phenolic compounds such as isoproterenol, mimosine and trihydroxybenzoic acid, which have been shown to be oxidized by *M. leprae* ([12]), were also found to be oxidized by hyaluronic acid.

## DISCUSSION

Since the discovery of the leprosy bacillus more than 100 cultures have been registered as *in vitro* grown *M. leprae*. Some of them are mycobacteria with more or less acid-fastness. Others were mostly saprophytic contaminants or microorganisms present in the skin, e.g. diphtheroids. Lately, one of the so-called *M. leprae* cultures was identified as *Bacillus subtilis* ([1]). With careful investigations none of the presented cultures were recognized as *M. leprae.* Repeatedly, several technics and growth characteristics were proposed and even accepted to establish the identity of *M. leprae* grown on artificial media. Among other parameters, the DOPA oxidizing test was proposed and widely accepted as a technic for identifying *M. leprae* ([5]). It must be emphasized that the claimed presence of phenolase in *M. leprae* has been shown in bacilli isolated from host tissues which are extremely rich in acid mucopolysaccharides, e.g. hyaluronic acid. The tests were made with partially purified bacillary suspensions which are practically impossible to separate from the accompanying macromolecules. In the diseased host *M. leprae* multiplies in the histiocytes in the connective tissue matrix. The results of Skinsnes ([13]) and our present findings clearly indicate that hyaluronic acid or other skin mucopolysaccharides are responsible for DOPA oxidation rather than the leprosy bacilli in the experiments of Prabhakaran *et al.* These authors prepared the bacillary suspension of *M. leprae* from various human tissues such as skin nodules, testes and spleen as well as from armadillo tissues and mouse foot pads. All of these tissues are extremely rich in hyaluronic acid. Prabhakaran ([8]) included skin extract as control which did not oxidize DOPA. This latter result is confirmed by our present findings but is to be rejected as a control for DOPA oxidation by *M. leprae* isolated from the skin. In the connective tissue, hyaluronic acid is bound to protein and is not present as the free polysaccharide. When normal human skin was treated with trypsin, hyaluronic acid is liberated from its protein binding and becomes available as a functional molecule. Consequently, trypsinized skin extract oxidized DOPA with the same intensity as purified hyaluronic acid, thus leading to the same deep purplish color formation as obtained by Prabhakaran with partially purified *M. leprae* suspension from the skin.

From the presented results, it became evident that hyaluronic acid as it is present in the normal connective tissue does not oxidize DOPA. Following trypsin digestion, hyaluronic acid is released from its protein binding and consequently the trypsin treated skin homogenate oxidizes DOPA. The human leprotic nodule consists of thickly vascularized connective tissue, rich in histiocytes, which are loaded with leprosy bacilli. Consequently, "*M. leprae* in the human host are associated with the presence of the acid mucopolysaccharides of the host," as shown by Skinsnes and his co-workers ([12]). These authors also showed the presence of β-glucuronidase in *M. leprae,* an enzyme which is effective in the cleavage of hyaluronic acid. Phagocytic cells (histiocytes included) have a strong proteolytic activity similar to trypsin. In the lepromata, therefore, hyaluronic acid is not only attacked by

β-glucuronidase of *M. leprae* but also by proteolytic enzymes of the histiocytes. In the leprotic nodule, hyaluronic acid, therefore, must be present in a free and not protein-bound state and is consequently able to oxidize DOPA to a pigment. When partially purified human leprosy bacilli were submitted to hyaluronidase treatment DOPA oxidation did not occur. These results clearly show that, in the experiments of Prabhakaran, hyaluronic acid and not *M. leprae* is responsible for DOPA oxidation. Therefore, phenolase activity has not been shown to be associated with the metabolism of *M. leprae* and DOPA oxidation is not, "a unique characteristic of the human leprosy bacillus."

According to Prabhakaran ([7]), *M. lepraemurium* does not oxidize DOPA. When subcutaneous leprotic nodules of the rat were homogenized and digested for one hour with trypsin, the supernatant of these homogenates oxidized DOPA. Similary, when rat leprosy bacilli were separated from the leprotic nodules and a purified *M. lepraemurium* suspension was prepared, this bacillary suspension had an extremely strong DOPA oxidizing effect but only after digestion with trypsin. One might, therefore, argue that the trypsinized leprosy bacilli oxidized DOPA to a pigment. If this is true, then DOPA oxidation again is not a unique property of the human leprosy bacillus since trypsinized *M. lepraemurium* had the same effect on DOPA. Since hyaluronic acid alone or trypsin digested rat skin also oxidized DOPA, there is every reason to believe that skin constituents, probably hyaluronic acid, and not the bacilli are responsible for the pigment formation. The reaction of DOPA oxidation therefore must be rejected as a test for the identification of *M. leprae*.

The DOPA oxidizing enzyme or enzymes are still unidentified and poorly understood enzymes which act on certain phenolic compounds but their action leads to several intermediate pigments between DOPA and melanin. Under physiologic conditions, the skin pigments are formed in the connective tissue matrix in specific cells. These cells again float in the connective tissue matrix which is rich in polysaccharides. The present investigations and results did not clarify the chemical or physico-chemical processes by which these polysaccharides oxidize DOPA and transform the amino acids into pigmented products. The observations of Skinsnes and his co-workers ([13]) and our results might bring us closer to a better understanding of a pigment formation in the human skin in which enzymatic processes and the nuclear magnetic energy of light together with some still unknown role of skin mucopolysaccharides are jointly involved in the oxidation of DOPA, thus leading to the skin pigmentation which is so characteristic of human races.

## SUMMARY

The oxidation of 3, 4-dihydroxyphenylalanine (DOPA) was studied by spectrophotometric methods at pH 6.8. In the presence of L- or D-DOPA, a color development occurred in the presence of the following substances as measured by increase in absorption both at 540 nm and 480 nm: hyaluronic acid, trypsinized human skin and umbilical cord extract, trypsin treated rat tissue from subcutaneous rat leproma, trypsin treated *M. lepraemurium* isolated from rat lepromata, and trypsinized *M. leprae* isolated from non-treated lepromatous leprosy cases. Normal human skin and connective tissue extract and nontrypsinized connective tissue of rat leprosy granuloma did not oxidize DOPA. While the trypsin-treated partially purified *M. leprae* suspension oxidized DOPA at both wave-lengths, the hyaluronidase-treated same suspension of *M. leprae* failed to oxidize these phenolic compounds. Mushroom tyrosinase oxidized D-DOPA, L-DOPA, epinephrine and norepinephrine at 480 nm. Hyaluronic acid also oxidized epinephrine and norepinephrine at both wave-lengths. Since it is known that *M. leprae* in the human host is closely associated with the presence of the acid mucopolysaccharides of the skin, and since acid mucopolysaccharides and skin constituents strongly oxidized DOPA, and since the hyaluronidase treated *M. leprae* failed to oxidize DOPA, it became evident that hyaluronic acid and not *M. leprae* is responsible for DOPA oxidation, and phenolase activity is not associated with the metabolism of *M. leprae*. Evidence is presented that DOPA is not a unique characteristic of the human leprosy bacillus. For instance, trypsin-treated murine leprosy bacilli from the rat strongly oxidized DOPA. The reaction of DOPA oxidation, therefore, must be rejected as a test for the identification of *M. leprae*. The obtained results confirmed the pertinent findings of Skinsnes and his co-workers.

## RESUMEN

Se estudió la oxidación de la 3,4-dihidroxifenilalanina (DOPA) por métodos espectrofotométricos, a pH 6.8. Incubando L- o D-DOPA en presencia de las siguientes substancias, se observó el desarrollo de color medido por el incremento en la absorción tanto a 540 como a 480 nm: ácido hialurónico, extractos de piel y de cordón umbilical humanos digeridos con tripsina, extracto de un leproma subcutáneo de rata tratado con tripsina, *M. lepraemurium* aislado de lepromas de rata y tratado con tripsina, y *M. leprae* aislado de pacientes con lepra sin tratamiento y digerido con tripsina. La DOPA no fue oxidada por los extractos de piel y de tejido conectivo de personas sanas ni por el tejido conectivo del leproma de la rata sin tripsinizar. Mientras que la suspensión de *M. leprae* parcialmente purificada y tratada con tripsina si oxidó a la DOPA tanto a 540 como a 480 nm, la misma suspensión tratada con hialuronidasa no oxidó a compuestos fenólicos de este tipo. La tirosinasa fúngica (mushroom thyrosinase) oxidó a la D-DOPA, a la L-DOPA, a la epinefrina y a la norepinefrina a 480 nm. El ácido hialurónico también oxidó a la epinefrina y a la norepinefrina a ambas longitudes de onda. Puesto que se sabe que en el huésped humano el *M. leprae* está intimamente asocíado con la presencia en la piel de mucopolisacáridos ácidos y puesto que éstos y los constituyentes de la piel oxidaron energicamente a la DOPA, además de que el *M. leprae* tratado con hialuronidasa no oxidó a la DOPA, resulta evidente que el ácido hialurónico y no el *M. leprae,* es el responsable de la oxidación de la DOPA, y que la actividad de fenolasa no está asociada con el metabolismo del *M. leprae.* Se presentan evidencias de que la oxidación de la DOPA no es una caracteristica única del bacilo de la lepra humana ya que el bacilo de la lepra de la rata tratado con tripsina también oxidó a la DOPA. Por lo tanto, la reacción de oxidación de la DOPA no debe aceptarse como una prueba para la identificación del *M. leprae.* Estos resultados confirman los hallazgos del grupo de Skinsnes.



## RÉSUMÉ

L'oxydation de 3,4-dihydroxyphenylalanine (DOPA) à été étudiée par spectrophotométrie à pH 6.8. En présence de la L- ou de la D-DOPA, une couleur apparaît en présence des substances suivantes mesurée par l'augmentation de l'absorption soit à 540nm, soit à 480nm: l'acide hyaluronique, la peau humaine trypsinisée, les extraits de cordon ombilical, le tissu traité à la trypsine extrait de léprome sous-cutané de rat, le *M. lepraemurium* traité à la trypsine isolé d'un léprome de rat et un *M. leprae* trypsinisé isolé de cas de lépreux lépromateux non-traités.

La peu humaine normale et les extraits de tissus conjonctifs, les tissus conjonctifs non-trypsinisés de granulome lépreux de rat n'oxydent pas la DOPA. Alors que la suspension partiellement purifiée traitée à la trypsine du *M. leprae* oxyde la DOPA aux deux longueurs d'onde, la même suspension de *M. leprae* traitée à la hyaluronidase ne peut oxyder ses composés phénoliques.

La tyrosinase du champignon oxyde la D-DOPA, la L-DOPA, l'épinéphrine et la norépinéphrine à 480nm. L'acide hyaluronique oxyde aussi l'épinéphrine et la norépinéphrine aux deux longueurs d'onde. Comme il est connu que le *M. leprae* chez l'humain est associé de très près à la présence d'acide mucopolysaccharidique de la peau, comme l'acide mucopolysaccharidique et les constituants de la peau oxydent de façon très forte la DOPA, et comme le *M. leprae* traité à l'hyaluronidase n'oxyde pas la DOPA, il devient évident que l'acide hyaluronique et non le *M. leprae* est responsable pour l'oxydation de la DOPA et que l'activité phénolase n'est pas associée avec le métabolisme de *M. leprae.*

Les faits rapportés démontrent que la DOPA n'est pas une caractéristique unique du bacille lépreux humain. Par exemple, le bacille lépreux murin du rat traité à la trypsine oxyde très fortement la DOPA.

En conclusion, l'oxydation de la DOPA doit donc être rejetée comme une épreuve pour l'identification de *M. leprae.* Les résultats obtenus confirment les observations de Skinsnes et ses collaborateurs.


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## ADDENDUM

### DOPA Oxidation by Hyaluronic Acid and Yeast Extract

Over a period of ten years, the oxidation of DOPA (3,4-dihydroxyphenylalanine) has been promulgated as a unique property of *Mycobacterium leprae;* a property sufficiently characteristic to be accepted and used for the identification of this pathogen. Recently, hyaluronic acid and yeast extract either separately or in combination have been reported to give Prabhakaran's spot test (Skinsnes *et al:* Int. J. Lepr. **43** [1976] 193). However, more recently, Prabhakaran denied these findings and reported that neither yeast extract nor hyaluronic acid oxidizes DOPA to pigmented products. We investigated the oxidation

of DOPA in the presence of hyaluronic acid alone or in combination with yeast extract and the results are presented below.

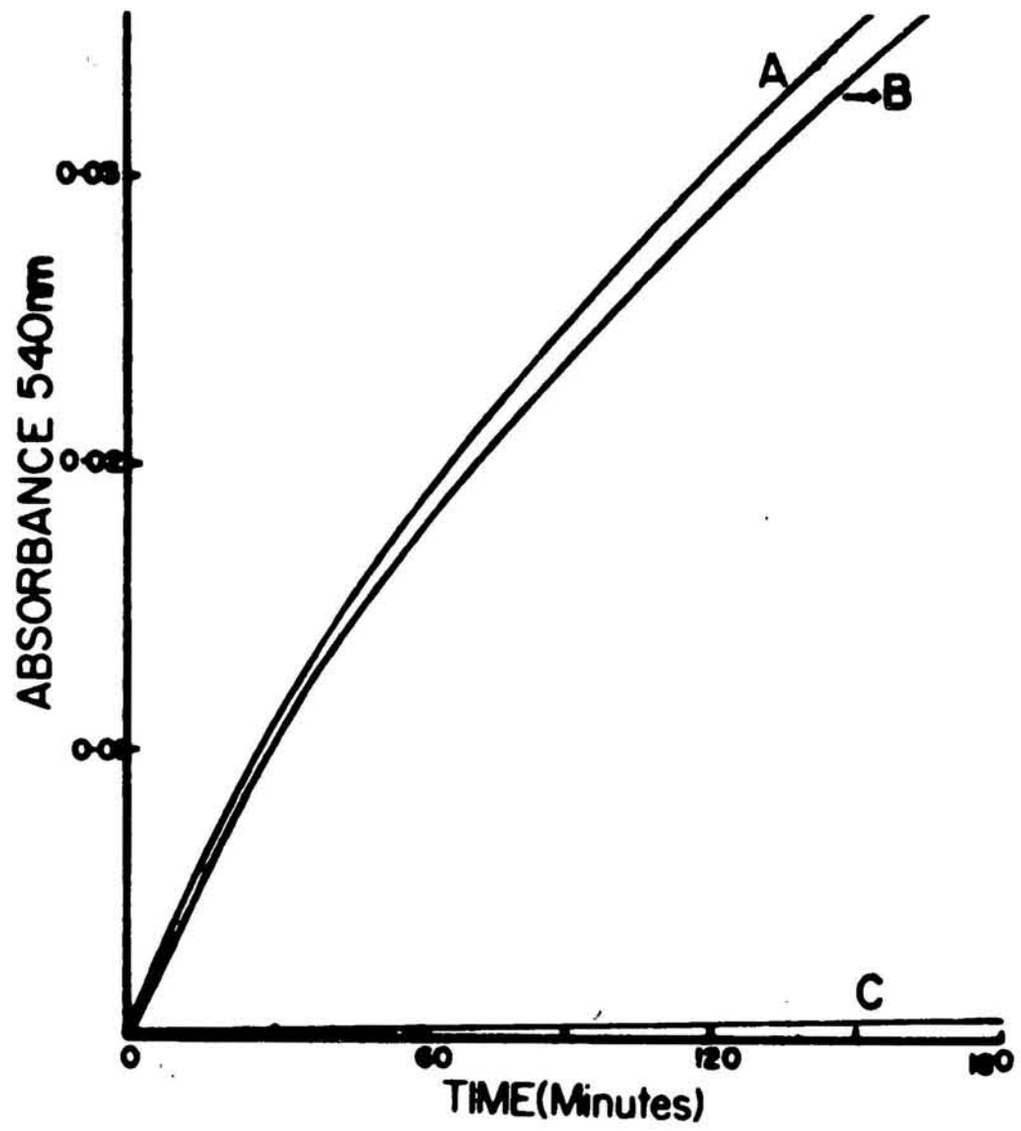


FIG. 1. Oxidation of L-DOPA by hyaluronic acid and yeast extract measured at 540 nm; Curve A, L-DOPA + hyaluronic acid. Curve B, L-DOPA + hyaluronic acid + yeast extract. Curve C, L-DOPA + buffer.

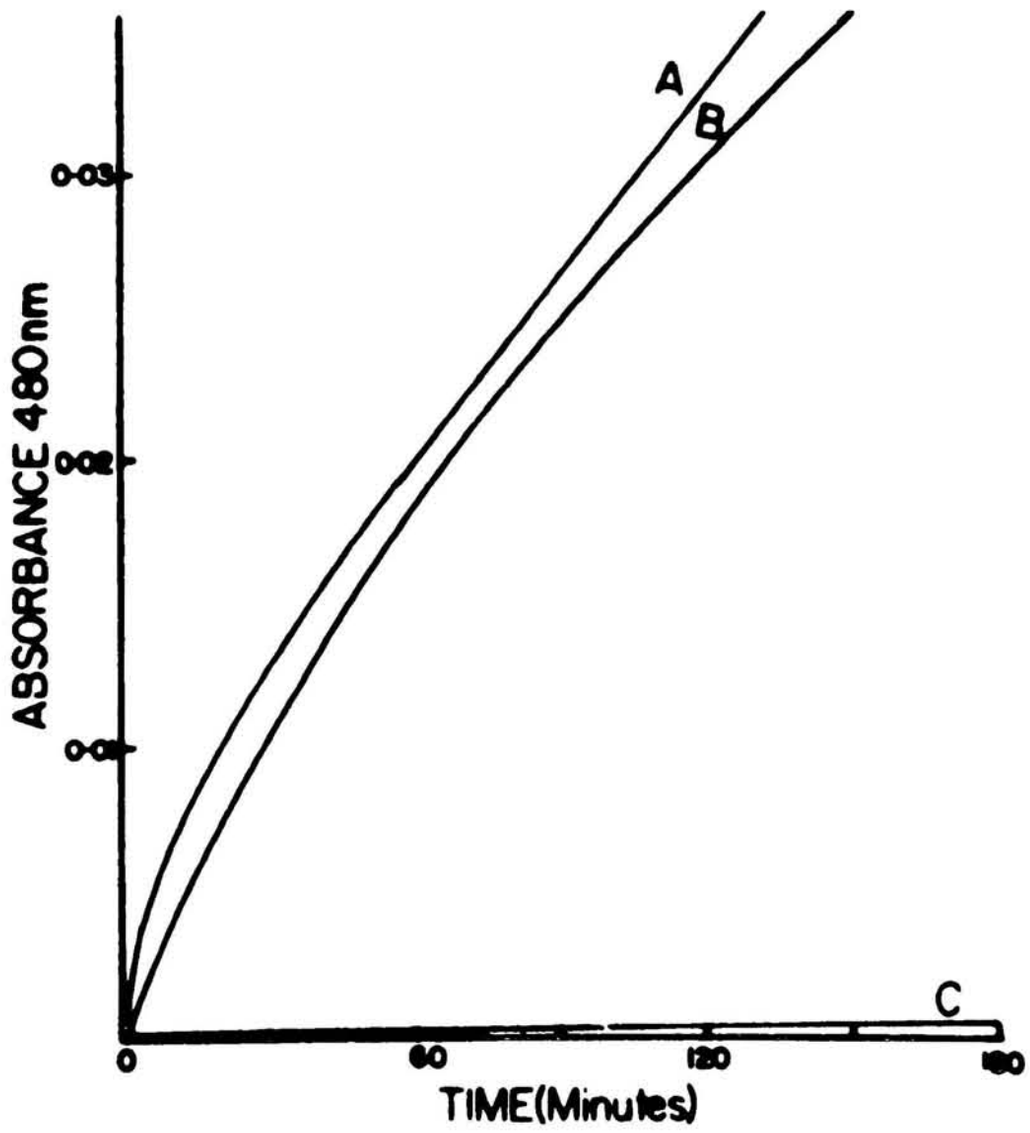


FIG. 2. Oxidation of L-DOPA by hyaluronic acid and yeast extract measured at 480 nm; Curve A, L-DOPA + hyaluronic acid. Curve B, L-DOPA + hyaluronic acid + yeast extract. Curve C, L-DOPA + buffer.

In several previous investigations, the oxidation of DOPA has been studied by its characteristic absorption at 540 nm in the presence of *M. leprae* and at 480 nm in the presence of mushroom tyrosinase and melanoma extract. We obtained DOPA and hyaluronic acid (sodium salt, grade III) from Sigma Chemical Company, and yeast extract (dry powder) from Difco. In these studies, the reaction mixture in a total volume of 4 ml contained 400 $\mu$moles of $Na_2HPO_4KH_2PO_4$ buffer, pH 6.8, and 800 $\mu$g of hyaluronic acid alone or hyaluronic acid 800 $\mu$g + yeast extract 2 mg in control flasks. Sample flasks in addition contained 2.5 mg of L-DOPA. The final volume was made up with distilled water. The reaction mixture (4 ml) was incubated in Warburg flasks at 37°C for 15 minutes and thereafter 2 ml was transferred to standard cuvettes (1 cm light path) and any increases in absorption, both at 540 and 480 nm, were measured at room temperature (22°C) in a Cary spectrophotometer, Model 118C equipped with a recorder.

The results in Figure 1 clearly show that when DOPA was incubated with hyaluronic acid alone (curve A) there was immediate pigment formation which gave rise to an increase in absorption at 540 nm. Similar results were obtained on incubation of DOPA with hyaluronic acid + yeast extract (curve B), again there was a development of color which increased in intensity with time and could easily be measured at 480 nm. The increase in the optical density was recorded at about 22-23°C, and it is noted that it might well have been much higher at 37°C. When DOPA alone in buffer was incubated, no change in color occurred (curve C).

Memorandum circulated
May 17, 1976—M. Ishaque
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