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A kinetic study of the melanization pathway between L-tyrosine and dopachrome

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In the pathway of melanin biosynthesis originating from L-tyrosine, the dopachrome accumulation at physiological pH is produced with a pronounced lag period, during which the level of L-dopa increases, following a sigmoidal kinetics to reach a steady-state. A kinetic model has been proposed for the overall pathway of melanization from L-tyrosine to dopachrome; it explains the lag period present during the dopachrome accumulation as well as the influence of L-tyrosine and tyrosinase over this lag period. Use of this model is also valid to explain the kinetics of L-dopa accumulation in the reaction medium, as has been tested by simulation.

Introduction

The pathway of melanin biosynthesis from tyrosine described by Lerner and Fitzpatrick [1] in 1950 attached the tyrosinase pathway of plants and insects to the 'dopa-oxidase' pathway in mammals. The scheme presented in Fig. 1 is largely based upon studies by Raper [2], Mason [3], and Lerner et al. [4], the pathway being started by means of two enzymatic steps and continued by a series of chemical steps until the first stable product, dopachrome, has been obtained.

Both enzymatic steps are catalyzed by tyrosinase (EC 1.14.18.1), thus having two catalytic activities: (a) cresolase or monophenolase, producing hydroxylation of L-tyrosine into L-dopa; (b) catecholase or diphenolase activity, producing oxidation of L-dopa into *o*-dopaquinone.

Over the past decades, numerous reports on the mechanism of tyrosinase action have appeared [5–7]. On the other hand, in recent years, significant progress has been made in the elucidation of protein structure and the nature of the copper site [8,9], the mechanism for both monophenolase and diphenolase activities being shown in Scheme I. Although from the kinetic point of view enzyme studies have concentrated on diphenolase activity [10–12], only recently has a uni uni bi uni ping pong mechanism been considered [13], where the substrate L-dopa must bind twice to complete the catalytic cycle. However, most attempts to delineate the tyrosinase reaction course remain somewhat speculative [8], due to molecular heterogeneity and the complexity of the enzymatic reaction (monophenolase and diphenolase activities).

The chemical steps in the melanization pathway from *o*-dopaquinone-H^+ to dopachrome have re-

Abbreviations and trivial names: dopa, 3,4-dihydroxyphenylalanine; dopachrome, 2-carboxy-2,3-dihydroindole-5,6-quinone; dopaquinone, 4-(2-carboxy-2-aminoethyl-1,2-benzoquinone; leukodopachrome, 2,3-dihydro-5,6-dihydroxyindole-2-carboxylate.

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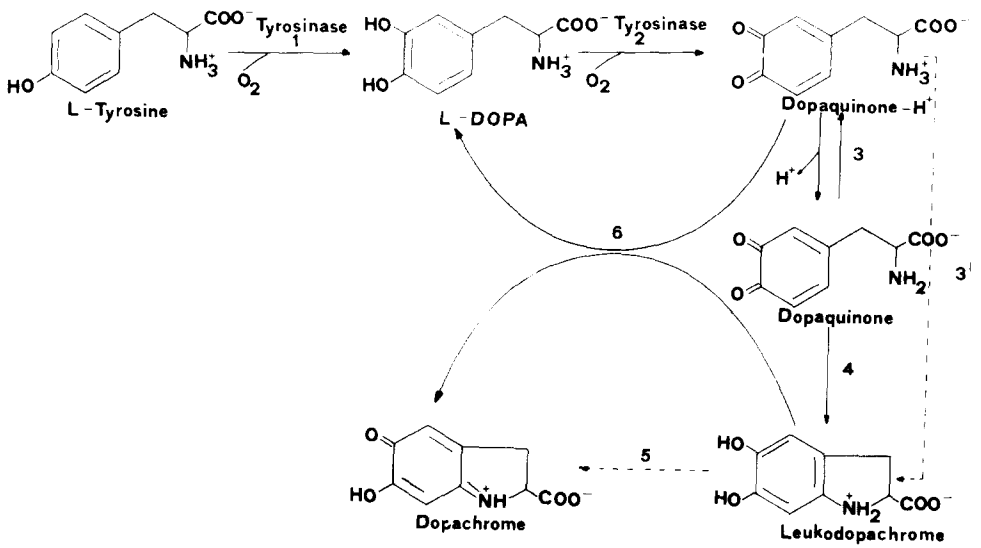
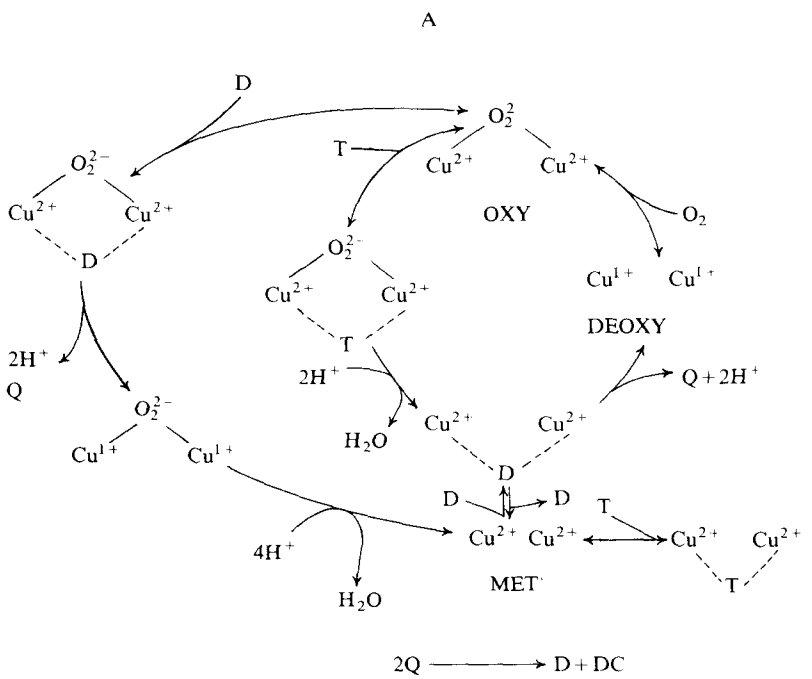
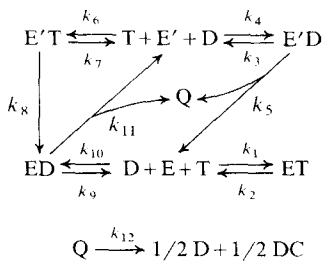


Fig. 1. The pathway from tyrosine to dopachrome proposed by Raper [2] in 1928, in which steps 1, 2, 3′ and 5 were suggested. Step 3 has been recently established to explain the role of pH in the synthesis of melanin from L-dopa [14,15] and step 6 to explain the stoichiometry of L-dopa transformation into dopachrome.





$k_1 = 25000\ M^{-1}\cdot s^{-1}$
$k_2 = 10\ s^{-1}$
$k_3 = 10\ s^{-1}$
$k_4 = 10^5\ M^{-1}\cdot s^{-1}$
$k_5 = 10^2\ s^{-1}$
$k_6 = 5000\ M^{-1}\cdot s^{-1}$
$k_7 = 10\ s^{-1}$
$k_8 = 10^2\ s^{-1}$
$k_9 = 5\ s^{-1}$
$k_{10} = 10^6\ M^{-1}\cdot s^{-1}$
$k_{11} = 10^2\ s^{-1}$
$k_{12} = 0.41\ s^{-1}$

Scheme I. (A) This scheme considers the different functional states established for the copper active site (met, oxy and deoxytyrosinase) and the binding of substrates. T = tyrosine; D = L-dopa. Q = *o*-dopaquinone and Dc = dopachrome are the obtained products. (B) The abbreviations in this scheme are: E: met-tyrosinase, E′ = oxytyrosinase; E′D and E′T are the complexes between oxytyrosinase and each substrate; ED and ET are the complexes between met-tyrosinase and each substrate. Q and Dc are as described in (A).

cently been re-studied, both from a kinetic point of view, using a theoretical design of an enzymatic-chemical-chemical mechanism [14], and considering pH dependence [15]. These studies allowed steps 3 and 6 to be introduced in the mechanism (Fig. 1), and step 5 to be eliminated, thereby explaining the stoichiometry and kinetics of the transformation of L-dopa into dopachrome by tyrosinase.

Step 6 substantially modifies the melanization scheme, since it allows the net accumulation of L-dopa by means of the oxidation-reduction reactions of *o*-dopaquinone-H^+.

Less is known regarding monophenolase activity kinetics, since the following particular characteristics can be found in the pathway from L-tyrosine to dopachrome:

(a) the monophenolase activity is always expressed together with the diphenolase activity acting on the monophenolase activity product (L-dopa);
(b) expression of the monophenolase activity has the same chemical steps as found in dopaoxidase activity from *o*-dopaquinone-H^+ to dopachrome;
(c) during expression of the monophenolase activity at pH 7.0 there is a characteristic lag period, either when consumption of oxygen or production of dopachrome is measured; elimination of this lag period by addition of small amounts of L-dopa has led some authors to postulate the existence of an activator site for L-dopa and evaluate a k_{act} (activation constant) of L-dopa on monophenolase activity [11,12,16].

In this paper, we have studied the monophenolase activity of frog epidermis tyrosinase on L-tyrosine. To allow us to obtain the accumulation curve of L-dopa during the catalytic process, a rapid, simple and quantitative method of measurement of L-dopa concentration in the reaction medium was set up.

A kinetic model for the melanization pathway from L-tyrosine to dopachrome was developed by numerical integration, considering this pathway as a model of an enzymatic-enzymatic-chemical mechanism (E_zE_zC) having particular characteristics imposed by the system, such as: (a) two enzymatic steps ,both catalyzed by the same enzyme; (b) a small percentage of the oxy form in the resting form of the enzyme; and (c) chemical steps necessary for the recycling of L-dopa from *o*-dopaquinone-H^+.

A high degree of correspondence was obtained between the theoretical results of the E_zE_zC model and the results of the experiments.

Materials and Methods

L-Dopa was purchased from Sigma Chemical Co. (Poole, Dorset, U.K.). All the other chemicals used were analytical grade.

Frogs (*Rana esculenta ridibunda*) were obtained from local suppliers from November to March. Epidermis was separated from dermis after incubation with a 2 M NaBr solution for 24 h at 0–4°C. After washing several times with bidistilled water, a negative bromide reaction took place and epidermis was lyophilized and kept at 0–4°C until used.

Enzyme preparation

Frog epidermis protyrosinase was extracted partly purified and activated by the procedure of Lozano et al. [17].

Enzyme activity determination

The dopachrome accumulation was determined spectrophotometrically at 475 nm ($\varepsilon = 3700\ M^{-1} \cdot cm^{-1}$). The reaction medium contained L-tyrosine as substrate (2 mM) in 10 mM sodium phosphate buffer (pH 7.0) at 25°C.

Catecholase activity was determined spectrophotometrically at 475 nm by the appearance of dopachrome in 0.01 M sodium phosphate buffer (pH 7.0) and 5.6 mM L-dopa. One unit of enzyme is taken as the amount of enzyme that produces 0.5 μmol of dopachrome per min, since this implies the production of 1 μmol of dopaquinone [14,15].

Protein concentration was determined by the method of Lowry et al. [18]. The enzyme concentration was calculated taking a value of $M_r = 62000$ [19].

The concentration of L-dopa accumulated in the reaction medium was deterined by measuring the absorbance at 475 nm produced at each reaction time after addition of 100 μl of 40 mM $NaIO_4$ as shown in Fig. 2.

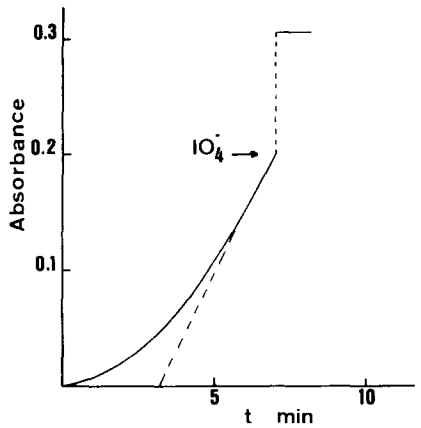


Fig. 2. Dopa determination during tyrosine oxidation by tyrosinase as described in Materials and Methods. [L-Tyrosine] = 1 mM, $[E] = 1 \cdot 10^{-7}$ M.

The reaction mechanism assumed for catalysis of L-tyrosine to dopachrome by tyrosinase was the enzymic catalytic cycle proposed by Mason [5], together with the chemical steps involved between the *o*-quinone-H^+ and dopachrome previously proposed by us [14,15] (see Fig. 1). The reaction mechanism was simulated by the 4th order numerical integration method of Runge-kutta and by the predictor-corrector method of Hamming [20] introduced in a BASIC programme using an Olivetti M20 microcomputer.

Model of the melanization pathway

The model developed assumed that:

(1) the mechanism of the enzyme actuation was substantially that of Scheme I, which included the possibility of union of L-tyrosine at the met-tyrosinase, thereby producing an inhibition already postualted in some studies on the reciprocal inhibition of L-tyrosine on L-dopa [11,21];

2) there were subsequent chemical reactions on the initial product of enzymatic activity [2,3]. Recent kinetic studies of these reactions from L-dopa [14,15] have established the following balance:

$$2\ o\text{-dopaquinone-H}^+ \xrightarrow{k_{12}} \text{L-dopa} + \text{dopachrome}$$

thus representing a recycling of the diphenolase activity product into the substrate L-dopa in the proportion 2 : 1;

(3) in the native enzymes, there were variable amounts (2–30%) [10,22,23] of intrinsic oxytyrosinase. In our model a proportion of 10% of oxytyrosinase at zero-time was used.

The differential equations obtained for this model were:

$$[\dot{E}] = k_2[ET] + k_9[ED] + k_5[E'D] - (k_{10}[D] + k_1[T])[E]$$

$$[\dot{E}'] = k_7[E'T] + k_3[E'D] + k_{11}[ED] - (k_4[D] + k_6[T])[E']$$

$$[\dot{E}T] = k_1[T][E] - k_2[ET]$$

$$[\dot{E}D] = k_8[E'T] + k_{10}[E][D] - (k_9 + k_{11})[ED]$$

$$[\dot{E}'T] = k_6[E'][T] - (k_7 + k_8)[E'T]$$

$$[\dot{E}'D] = k_4[E'][D] - (k_3 + k_5)[E'D]$$

$$[\dot{Q}] = k_5[E'D] + k_{11}[ED] - k_{12}[Q]$$

$$[\dot{D}C] = \tfrac{1}{2}k_{12}[Q]$$

$$[\dot{D}] = k_3[E'D] + k_9[ED] - (k_4[E'] + k_{10}[E])[D] + \tfrac{1}{2}k_{12}[Q]$$

the initial conditions being $[E]_0 = [MET]_0 + [OXY]_0$, $T = T_0$, $D_0 = 0$ and $[MET]_0/[OXY]_0 = 90 : 10$.

Tyrosine concentration was considered constant through the whole experiment.

In Scheme I, values of the specific rate constants are given, k_{12} representing the overall apparent constant of chemical reactions subsequent to the enzymatic step, the value of which having been previously obtained [14]. Values for the unknown constants were chosen so that the model produced reasonable rates under the conditions of the simulation.

Results

Lag period in the accumulation of dopachrome

When the melanization pathway was started by the action of tyrosinase on L-tyrosine, a marked lag period was observed simultaneously with the appearance of the first stable product, dopachrome (Fig. 3). The system reached a steady state (linear rate) after the lag period [2], estimated by extrapolation of the linear portion of the curve to the abscissa [24]. This lag depended on both enzyme and substrate concentrations present in the

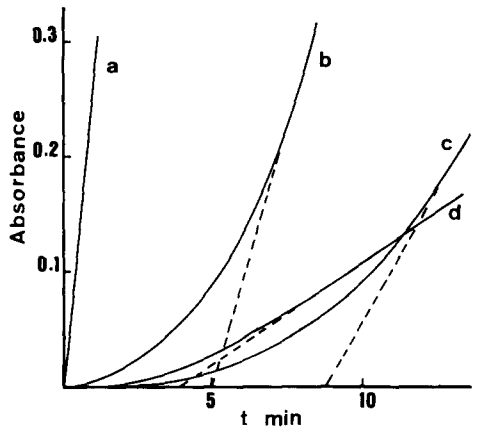


Fig. 3. Dopachrome accumulation measured by increased absorbance at 475 nm for different reaction media. (a) Dopachrome from L-dopa [dopa] = 6 mM and [E] = $6 \cdot 10^{-8}$ M. (b) Dopachrome from L-tyrosine [L-tyrosine] = 2 mM and [E] = $6 \cdot 10^{-8}$ M. (c) Dopachrome from L-tyrosine [L-tyrosine] = 2 mM and [E] = $3 \cdot 10^{-8}$ M. (d) Dopachrome from L-tyrosine [L-tyrosine] = 0.25 mM and [E] = $6 \cdot 10^{-8}$ M.

reaction medium (Fig. 3) (curves b, c, d).

When the melanization pathway was started using L-dopa at pH 7.0 no lag period was observed in the time scale used (Fig. 3a), this agreeing with previous publications regarding pH effect on the chemical steps between *o*-dopaquinone and dopachrome [14,15]. At this pH, there is an overall first-order apparent constant of 0.41 s^{-1}.

Lag period dependence on enzyme concentration

The lag period in the melanization pathway during accumulation of dopachrome from L-tyrosine at physiological pH was a parameter being controlled heavily by the enzyme concentration in the reaction medium. This influence is shown in Fig. 4A.

The results obtained by simulation of the model produced the same qualitative dependences as the ones obtained experimentally (Fig. 4B).

Lag period control by substrate concentration

As Fig. 3 shows, the lag period also depended on the level of L-tyrosine present in the reaction medium, this behaviour being shown in Fig. 4A. As can be observed, the substrate-dependent lag is a parameter tending towards a maximum value. The simulated results are shown in Fig. 4B; a similarity with the experimental results can be observed.

Accumulation of L-dopa during the melanization pathway

The accumulation of L-dopa when tyrosinase acts on L-tyrosine has been widely discussed

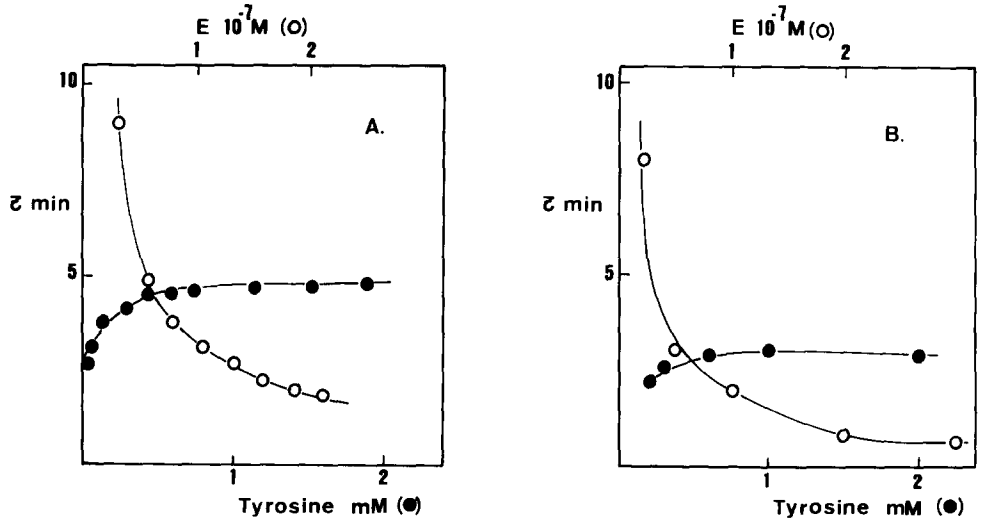


Fig. 4. (A) Experimental results of variation of lag period (τ) on L-tyrosine hydroxylation by frog tyrosinase: (●) with L-tyrosine concentration, [E] = $6 \cdot 10^{-8}$ M; (○) with enzyme concentration, [tyrosine] = 2 mM. (B) Variation of lag period obtained by simulation for variations in: (●) L-tyrosine concentration, [E] = $5 \cdot 10^{-7}$ M; (○) enzyme concentration, [L-tyrosine] = 1 mM. Results were obtained using the same constants as in Scheme I.

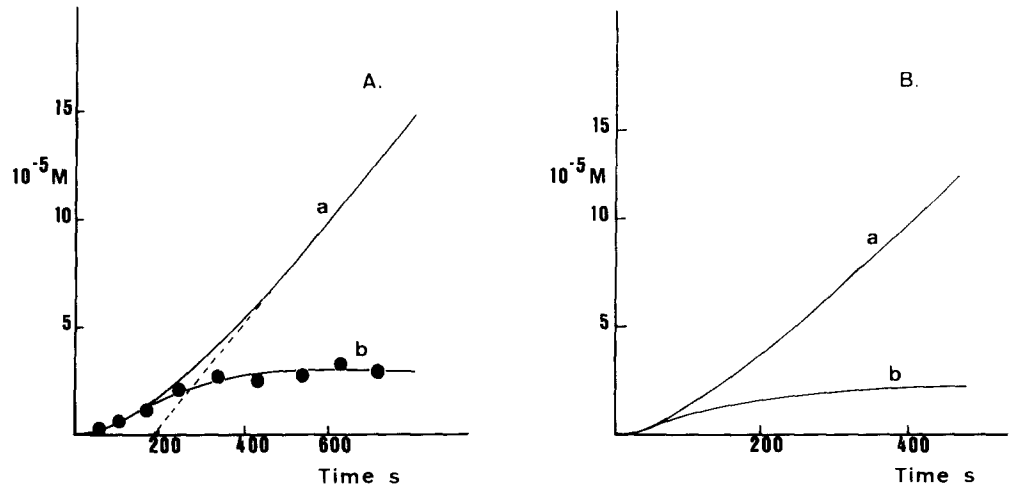


Fig. 5. Time course of (a) dopachrome and (b) L-dopa accumulation in the reaction medium. (A) Experimental results, [L-tyrosine] = 1 mM and [E] = $1 \cdot 10^{-7}$ M. (B) Simulated results, [L-tyrosine] = 1 mM and [E] = $1 \cdot 10^{-7}$ M.

[4,25,26] since Raper's original observations in 1928 [2]. Conflicting opinions could be due to the different methods used to evaluate the L-dopa accumulated, such as the measurement of L-[^{14}C]dopa from L-[^{14}C]tyrosine [25] by means of chromatographic separation, or by the more recent HPLC technique [26]. In both these and similar methods, a stopping of the reaction and a subsequent handling of the reaction mixture for at least several minutes took place. Such circumstances could influence the final evaluation of the L-dopa concentration.

In this paper, the concentration of L-dopa in the reaction medium has been evaluated by the specific and rapid oxidation of L-dopa by $NaIO_4$ [27], according to the procedure described in

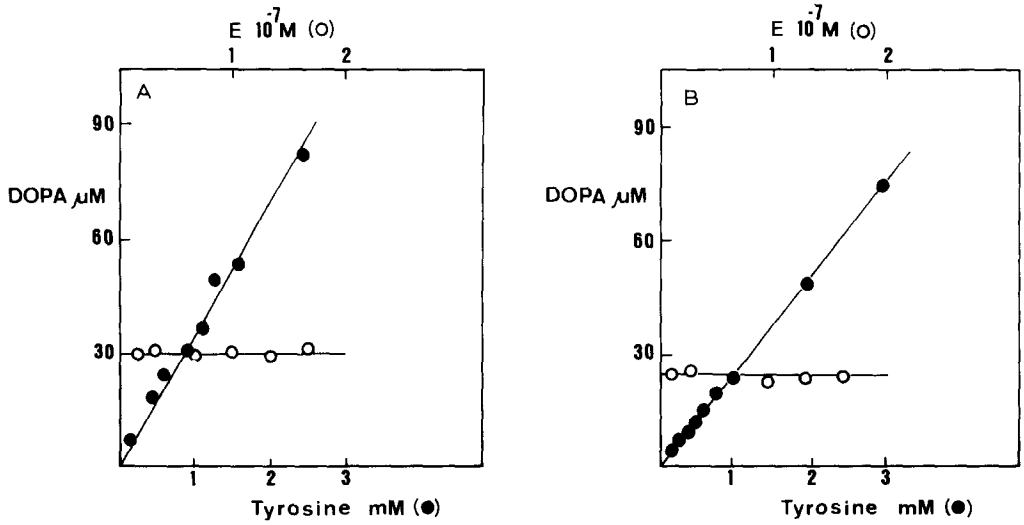


Fig. 6. (A) Experimental results of variation of $[D]_{ss}$ on L-tyrosine hydroxylation by frog tyrosinase: (●) with L-tyrosine concentration, [E] = $6 \cdot 10^{-8}$ M; (○) with enzyme concentration, [tyrosine] = 2 mM. (B) Variation of $[D]_{ss}$ obtained by simulation for variations in: (●) tyrosine concentration, [E] = $5 \cdot 10^{-7}$ M; (○) enzyme concentration, [tyrosine] = 1 mM. Results were obtained using the same constants as in Scheme I.

Materials and Methods. An experimental recording can be seen in Fig. 5A, showing the accumulation of dopachrome and L-dopa during the transition phase of the melanization pathway from L-tyrosine to dopachrome.

The parameter $[D]_{ss}$ was calculated as the L-dopa concentration when the system reached the steady state; attention should be drawn to the sigmoidal form of the L-dopa accumulation, as well as the coincidence between the L-dopa and dopachrome levels during the first part of the transition phase. Fig. 5B shows the results of the simulation of the model for this part of the melanization pathway.

Dependence of the steady-state level of L-dopa on enzyme concentration

The previously defined $[D]_{ss}$ is a steady-state parameter and, as such, able to be analyzed. The experimental results show that the L-dopa level in the steady state do not depend on enzyme concentration in the reaction medium (Fig. 6A). The same situation was found in the simulated experiments, as Fig. 6B shows.

Dependence of the steady-state level of L-dopa on L-tyrosine concentration

When the steady-state level of L-dopa was analyzed with regard to the tyrosine concentration, a linear dependence was obtained (Fig. 6A), obviously being the result of the competition taking place between L-tyrosine and L-dopa which are being bound to the same enzymatic forms in the catalytic 'turnover'. The simulated results plotted in Fig. 6B completely agree with the experimental results.

Discussion

The malanization pathway from L-tyrosine has the following characteristics:

(a) the two first steps of the pathway are catalyzed by only one enzyme, tyrosinase;
(b) *o*-dopaquinone-H^+, the product of the enzymatic reaction, is unstable at physiological pH, thus a series of chemical reactions leads to the stoichiometrical transformation of 2 *o*-dopaquinone-H^+ into L-dopa and dopachrome;
(c) the accumulation of dopachrome is accompanied by a marked lag period.

Although some approximations have been made, the kinetics of the melanization pathway has not been satisfactorily explained. Thus, Osaki [28] described the lag period of dopachrome accumulation as the result of two consecutive enzymatic reactions in different catalytic sites, L-tyrosine being a competitive inhibitor of diphenolase activity.

The mathematical solution of Osaki's model [28] was an exponential accumulation of L-dopa in the reaction medium; however, the experimental result of this accumulation (see Fig. 5A) did not correspond to the mathematical solution. On the other hand, Oskai's model did not take into account the detailed mechanism of enzymatic catalysis [5,8,9] where two activities take place at the same active site.

Duckworth and Coleman [11] sustained a similar point of view, stating that the appearance of *o*-diphenol was the immediate product of monophenol oxidation and as a consequence the lag period was therefore suppressed by the addition of small amounts of L-dopa. However, this theory offers no explanation for the lag period, since for the turnover to be accomplished the enzyme needs all the *o*-diphenol molecules formed from L-tyrosine and, in this way, net accumulation of *o*-diphenol is unable to take place in the reaction medium.

On the other hand, Pomerantz, and Murthy [29] have suggested that the 3 h lag period of hamster melanoma tyrosinase could be in agreement with the proposition that the tyrosine hydroxylation reaction requires a cosubstrate to furnish electrons for the reduction of an atom of oxygen into water. When L-dopa was not added, non-enzymatic reactions slowly synthesized a small amount of L-dopa from L-tyrosine, thus leading to a full-scale reaction. With pure tyrosinases from *Vibrio tyrosinaticus,* however, a lag in hydroxylation of less than a minute was observed. In this case Pomerantz and Murthy [29] explained that the most probable reason was that L-dopa was even more tightly bound to bacterial tyrosinase than to melanoma tyrosinase.

This explanation could obviously be correct for melanoma tyrosinase, but not in general; for bacterial tyrosinase the explanation seems improb-

able, this binding not having, in fact, been detected.

Hearing and Ekel [30] have suggested the existence of an allosteric site, where L-dopa could act as a positive allosteric effector for tyrosine hydroxylation by tyrosinase. To date, this hypothetical site has not been demonstrated, either by kinetic or structural studies.

In this paper, a kinetic model has been presented which explains the dependences observed for the lag period of frog epidermis enzyme and for the accumulation of L-dopa in the reaction medium, according to the variation of enzyme and substrate. This model incorporated three basic well-known facts:

(1) an internal mechansim of enzymaitc catalysis, mainly based on structural studies of different forms of copper in the active site [31,22] and on kinetic studies of the disappearance of an oxy form for *o*-diphenols and monophenols, thereby demonstrating that this form is involved in the catalytic turnover of both activities as an intermediate;
(2) the presence of an oxy form (2–30%) in resting tyrosinase [8];
(3) the existence of chemical steps, subsequent to the enzymatic activity, which at pH 7.0 rapidly transform *o*-dopaquinone into dopachrome and L-dopa [14,15].

The lag period represents an expression of the complex kinetics established in the system, which means the production, in a short time, of low levels of *o*-dopaquinone and the obtaining of almost all the enzyme in the met-tyrosinase form. The latter is a dead-end complex, the enzyme recovering slowly back into the catalytic cycle by means of the L-dopa recycling in the subsequent chemical reactions.

The increment of the L-dopa level produced a greater transformation of enzyme into the catalytic active form and, subsequently, a higher transformation of L-tyrosine into *o*-dopaquinone. This process continued until the rate of transformation from L-dopa to *o*-dopaquinone by oxytyrosinase was the same as the rate of dopa regeneration by chemical steps. When these identical rates had been reached the steady state for the system was also obtained.

From this point of view, calculation of an activation constant for L-dopa [11,16] on the L-tyrosine hydroxylase activity cannot be used to indicate the meaning of a binding constant between the activator and the enzyme; it is, however, related to the value of L-dopa which allows the steady-state situation between the enzymatic and chemical systems to be reached. Bearing in mind that L-dopa and L-tyrosine compete for oxytyrosinase, the apparent activation constant for L-dopa will be dependent on the L-tyrosine concentration, thereby explaining the results of Duckworth and Coleman [11] who, applying the ideas of Pomerantz and Warner [16], found different values for the L-dopa k_{act} depending on the level of L-tyrosine used.

The model present in this paper is of general application for lag period characteristics observed in tyrosinase from varying sources: *Calliphora erythrocephala* [32], mushroom [11,28], melanoma [16], *Vibrio tyrosinaticus* [29]. Only the modification of the rate constants involved in the kinetic equations of the model are necessary for the respective qualitative and quantitative values to be obtained.

Furthermore, this model could explain the question raised in a recent review of tyrosinase [9] about the lag period that procedes hydroxylation of monophenols and which remained as an open question.

Acknowledgements

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