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# KINETIC STUDY AND INTERMEDIATES IDENTIFICATION OF NORADRENALINE OXIDATION BY TYROSINASE

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**Abstract**—Characterization of intermediates formed in the noradrenaline oxidation by mushroom tyrosinase and sodium periodate has been performed by rapid scanning spectrophotometry and graphical analysis of obtained spectra. In a pH range from 5.0 to 6.0, it has been possible to detect *o*-noradrenalinequinone-$H^+$ as the first intermediate in these oxidations. The following steps for noradrenaline transformation into noradrenochrome would be: noradrenaline → *o*-noradrenalinequinone-$H^+$ → *o*-noradrenalinequinone → leukonoradrenochrome → noradrenochrome. It has been also verified that *o*-noradrenalinequinone-$H^+$ is transformed into noradrenochrome at a constant ratio. The stoichiometry for this converstion followed the equation: 2-noradrenalinequinone-$H^+$ → noradrenaline + noradrenochrome.

The pathway between noradrenaline and noradrenochrome has been studied as a system of various chemical reactions coupled to an enzymatic reaction. We have denominated this type of mechanism as an enzymatic–chemical–chemical mechanism, ($E_zCC$). Whole rate constants for the implicated chemical steps at different pH and temperature values have been evaluated from measurement of the lag period arising from the accumulation of noradrenochrome that takes place when noradrenaline was oxidized at pH 5–6. The lag period was independent on enzyme concentration, but was increased when pH and/or temperature were increased. Rate constants pH independent for the deprotonation of noradrenalinequinone-$H^+$ into noradrenalinequinone and for the internal cyclization of noradrenalinequinone into leukonoradrenochrome have been obtained. We conclude that this minor pathway of noradrenaline oxidation by tyrosinase follows a scheme similar to that established for L-dopa.

In recent years, several examples of adrenergic drugs oxidation metabolism have been described. Thus, there is strong evidence suggesting that dopamine, noradrenaline and also adrenaline can lead to melanin pigments similar to those obtained from dopa oxidation by tyrosinase [1, 2]. Harrison *et al.* [3] have shown in some *in vivo* experiments that L-α-metildopa and D,L-isoproterenol are incorporated into melanin and non-melanin components of the hair of albino and black pigmented guinea pigs; they have also suggested a minor pathway for these compounds that should account for 0.1% of the given dose. Evidence for the involvement of catecholamines in melanin formation by means of peroxidase oxidation has also been reported [4, 5]. Melanin accumulation in skin, brain, liver and lung of schizophrenic patients on chlorpromazine therapy has been shown [6, 7]; chlorpromazine, a commonly used antipsychotic agent, should increase the rate of catecholamine oxidation through a free radical mechanism [8]. All this experimental evidence seems to suggest that catecholamines could be involved, through minor pathways, in melanin formation.

In preceding papers, we have reported the kinetics of L-dopa oxidation to dopachrome, catalyzed by tyrosinase [9], and the spectrophotometric and voltammetric characterization of intermediates of this pathway [10]. On the other hand, Harrison [11] has published the detection of fluorescent products in the oxidation of noradrenaline by tyrosinase at reaction times greater than 15 min. This author did not study the reaction kinetics at short times and then failed to detect the *o*-quinone and the corresponding leukoaminochrome as possible intermediates originated in reaction mechanism.

The present paper deals with the characterization study of the intermediates of the enzymic oxidation of noradrenaline by tyrosinase, as well as with the kinetics of those reactions that proceed from the formation of noradrenaline *o*-quinone to that of aminochrome. We conclude that the oxidation pathway is parallel to that of dopa oxidation by tyrosinase, that we have previously described [9].

## MATERIALS AND METHODS

*Materials.* Mushroom tyrosinase (*o*-diphenol:$O_2$ oxido-reductase, EC 1.14.18.1, 2230 units/mg) and noradrenaline (L-arterenol) were from Sigma Chemical Co., Saint Louis, MO. Sodium periodate and all other chemicals were of analytical grade from the Merck Company.

*Intermediate identification.* Aminochrome accumulation was spectrophotometrically followed at 490 nm ($\varepsilon_{490} = 3.58 \times 10^3\ M^{-1}\ cm^{-1}$ at pH 5.9) [12]. At this wavelength neither noradrenaline nor any other possible intermediate present absorbance. The reaction medium was: 10 mM acetate buffer (pH as indicated in each case), 1.8 mM noradrenaline (i.e. in saturated conditions) and such an enzyme amount that once $t_{99}$ was reached ($t_{99}$ is the time necessary to attain the rate 0.99 $V_0/2$). In these conditions, absorbance increase was approx. 0.150. Determination of intermediate quinone with absorp-

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tion maximum at 390 nm ($\varepsilon_{390} = 1.23 \times 10^3\ M^{-1}\ cm^{-1}$ at pH 4.9), was made in the same conditions as those described above for aminochrome determination.

All spectrophotometric measurements were carried out with an Aminco DW-2 spectrophotometer equipped with a Hewlett–Packard recorder with kinetic response, which allows one to minimize the dead recording time and with scanning speed up to 20 nm $sec^{-1}$. Temperature was controlled by using a Gilson bath with a precision of ±0.1°.

*Oxygen determination.* Oxygen consumption of noradrenaline oxidation was followed by a Clark oxygen electrode connected to an oxygen monitor (Rank Brothers Co.).

*Protein determination.* Protein was evaluated by Hartree's procedure [13] using bovine serum albumin as standard.

## RESULTS

In an attempt to identify the intermediate compounds generated in the catecholamines oxidation through the minor catabolic pathway [3], their spectrophotometric detection was carried out upon oxidation of noradrenaline by tyrosinase enzyme and sodium periodate respectively. Both compounds have the property of oxidizing *o*-diphenols to respective *o*-quinones.

*Oxidation by tyrosinase.* When the oxidation of noradrenaline was achieved by mushroom tyrosinase for slightly acid pH values, it was possible to record an absorbance maximum at 390 nm (Fig. 1A). This maximum was ascribed to *o*-quinone of noradrenaline and was shifted with reaction time, towards another one of $\lambda_{max}$ at 490 nm, characteristic of aminochrome of noradrenaline [10]. At the beginning of the reaction no isosbectic point was defined,

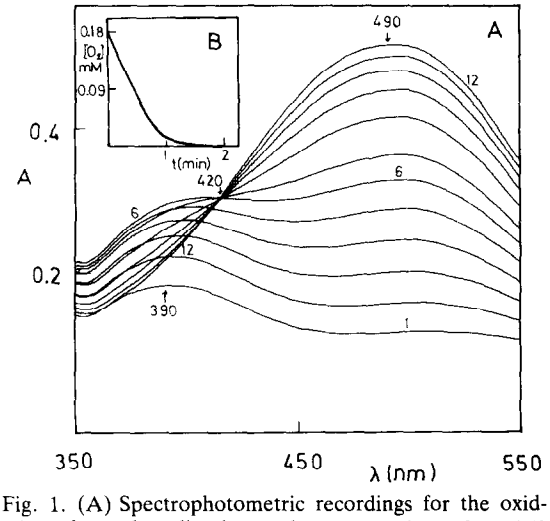



Fig. 1. (A) Spectrophotometric recordings for the oxidation of noradrenaline by mushroom tyrosinase from 350 to 550 nm at 12.5° in 0.01 M sodium acetate buffer, pH 4.9. Scan speed was up to 20 nm/sec and the first scan was started at 20 sec from the beginning of the reaction. The following spectra were recorded at 20 sec intervals. Noradrenaline at 1.8 mM was oxidized with tyrosinase (0.5 mg/ml). (B) Oxygen consumption corresponding to the reaction shown in Fig. 1A.

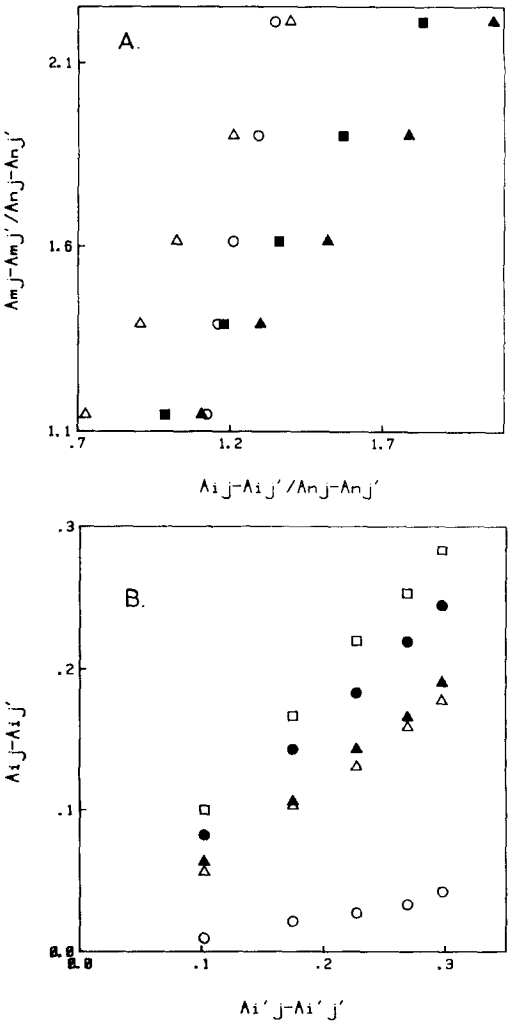



Fig. 2. Graphical analysis of spectra obtained in Fig. 1. (A) For the tracings 1–6, the matrix analysis for three species with restrictions was applied. In this analysis, *Aij* is the absorbance at wavelength *i* obtained during tracing *-j-*, being $m = 498$ nm, $n = 375$ nm and $j'$ = first, $i = 0$ (407 nm), $i$ = ▲ (466 nm), $i$ = ■ (530 nm), $i$ = △ (550 nm). (B) for the tracings 7 to 12, the test for two species with restrictions was applied, being $j'$ = tracing 7, $i' = 498$ nm, $i$ = ○ (420 nm), $i$ = △ (440 nm), $i$ = □ (466 nm), $i$ = ● (530 nm), $i$ = ▲ (550 nm).

as observed in Fig. 1A. Then the graphical analysis of the recordings in the visible spectrum by the matrix method of Coleman *et al.* [14] gave a good fit for three absorbing species in solution, as is shown in Fig. 2A. However, because the enzyme concentration used in the experiment of Fig. 1A was too high, the oxygen was depleted (Fig. 1B), and it was possible to determine an isosbectic point from trace six at $\lambda = 420$ nm. Matrix analysis of those spectra fitted for the test of two absorbing species kinetically related; however, as will be discussed below, there are three species in solution (Fig. 2B). This experiment also showed that neither enzyme activity nor oxygen took part in the conversion of quinones to their respective aminochromes, because oxygen was consumed from trace six of the spectra shown in Fig. 1A.

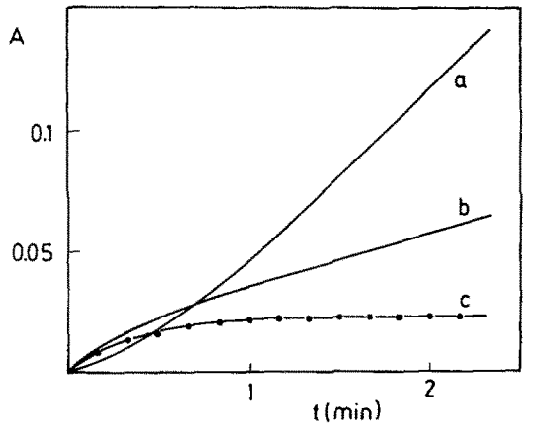



Fig. 3. Product formation with time for the noradrenaline oxidation with tyrosinase. (a) Representation of absorbance of noradrenchrome accumulation against time followed at $\lambda = 490$ nm during reaction of 50 $\mu$g/ml of tyrosinase with 1.8 mM noradrenaline at 15°, pH 4.9 (b) Representation of $\Delta$ A against time at $\lambda = 390$ nm for the same reaction as in (a). (c) *o*-Noradrenalinequinone-H$^+$ accumulation against time followed at $\lambda = 390$ nm for the same reaction as in (a). A correction for noradrenochrome absorbance at 390 nm was made using the relation:

$$A_{390}^{\text{aminochrome}} = \frac{\varepsilon_{390}}{\varepsilon_{490}} A_{490}^{\text{aminochrome}}; \frac{\varepsilon_{390}}{\varepsilon_{490}} = 0.26.$$

When the enzymatic oxidation was performed, but the absorbance increase with time was recorded at a fixed wavelength, the following results were obtained. When the recording was made at $\lambda = 490$ nm, a wavelength where the aminochrome absorbance is maximal, a lag time appeared (Fig. 3a). However, when the recording was made at $\lambda = 390$ nm, corresponding to the maximum of noradrenalinequinone-H$^+$, an apparent burst was detected (Fig. 3b). The *o*-quinone-H$^+$ accumulation is expressed in Fig. 3c, as difference between the total absorbance at 390 nm and the absorbance of aminochrome accumulation at the same wavelength. Note that *o*-noradrenalinequinone-H$^+$, the direct product of the enzymatic reaction, was accumulated following an apparent burst and then reaching a level of steady state (Fig. 3c). From the results presented in Fig. 3 it is possible to calculate that the rate of aminochrome accumulation in the steady state (20 $\mu$M/min, calculated from the slope of the linear zone of curve 3a) is half of the initial velocity of *o*-quinone-H$^+$ formation by the enzyme (40 $\mu$M/min, calculated from the tangent to the curve 3c at $t = 0$). These results suggest that a 2:1 stoichiometry must exist in the pathway with respect to aminochrome formation.

*Oxidation by periodate.* Noradrenaline oxidation was achieved with sodium periodate for several different concentration ratios. It is known that periodate oxidizes *o*-diphenols to give the corresponding *o*-quinone [15]; thus, this chemical model is similar to the reaction catalyzed by tyrosinase with respect to the reaction product, namely the corresponding *o*-quinone. When the concentration of *o*-diphenol is greater than that of periodate, [$NaIO_4$]/[noradrenaline] = 0.3, a yellow compound appears, with $\lambda_{max}$ at

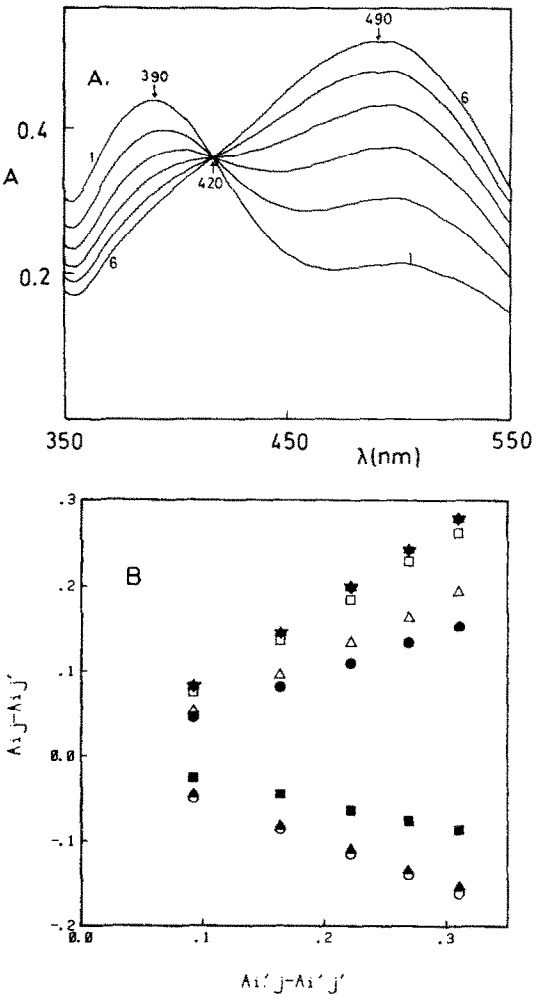



Fig. 4. (A) Spectrophotometric recordings for the oxidation of noradrenaline in defect of sodium periodate from 350 to 550 nm at 12.5° in 0.01 M sodium acetate buffer, pH 4.9. Scan speed was carried out as Fig. 1A, spectra were recorded at 20 sec intervals. Noradrenaline 1.8 mM was oxidized with 0.5 mM $NaIO_4$.
(B) Graphical analysis of recordings of Fig. 4A. The test for two absorbing species was applied, being $i' = 495$ nm, $j'$ = first, $i = \bigcirc$ (375 nm), $i = \blacktriangle$ (388 nm), $i = \blacksquare$ (406 nm), $i = \bullet$ (439 nm), $i = \bigstar$ (464 nm), $i = \square$ (526 nm) and $i = \triangle$ (547 nm).

390 nm, which was assigned to the *o*-quinone [16]; this absorption peak, just as in the case of oxidation by tyrosinase, shifts to another at $\lambda_{max} = 490$ nm, characteristic of the aminochromes. The plots are shown in Fig. 4A, where an isosbectic point at 420 nm can be seen, its appearance suggests, as a first approximation, the occurrence of two kinetically related species. The graphical analysis of these plots in the visible spectrum, by means of the matricial method of Coleman *et al.* [14] fits conveniently with the test for two absorbing kinetically related species in solution, as is shown in Fig. 4B. This case is similar to the conditions with oxygen depletion in the presence of tyrosinase (Fig. 1A).

From oxidation experiments carried out in these conditions, two points can be inferred. Firstly, all the $IO_{-4}$ is depleted in the reation with the *o*-

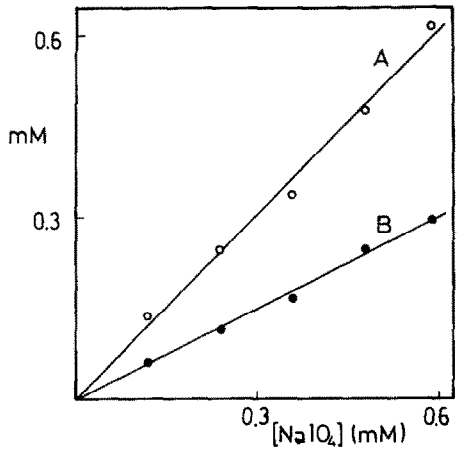



Fig. 5. Representation of the maximal *o*-noradrenalinequinone-$H^-$ (A) and noradrenachrome (B) concentrations at 390 and 490 nm, respectively; versus concentration of $NaIO_4$ for the different oxidations of noradrenaline with sodium periodate at 15° in 0.01 M acetic–acetate buffer, pH 4.9. In these assays, noradrenaline was always 2.2 mM and $NaIO_4$ (10 mM) was varied as indicated in the figure.

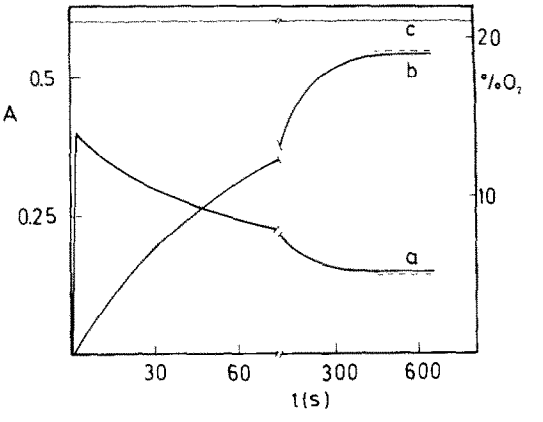



Fig. 6. Reaction progress for the oxidation of 3.7 mM noradrenaline with 0.3 mM $NaIO_4$, at pH 4.9 and 15°. (a) $A_{390}$ variation. (b) $A_{490}$ variations. (c) Oxygen concentration.

diphenol, with a 1:1 stoichiometry. This can be concluded from the data shown in Fig. 5A, where the concentration of *o*-quinone-$H^+$ at the beginning of the reaction (calculated from curve a of Fig. 6) was plotted against periodate concentrations, and a straight line of slope equal to 1 was obtained ($\varepsilon_{390} = 1.22 \times 10^3\ M^{-1}\ cm^{-1}$).

The isosbectic point suggest, in this case, that an amount of *o*-diphenol equivalent to that of periodate has been transformed into *o*-noradrenalinequinone, and, afterwards, the *o*-quinone has evolved to the corresponding aminochrome. In these experimental conditions, it is possible to study the involvement of oxygen in the oxidation of leukoaminochrome to aminochrome. Since the concentration of periodate was fully consumed, this oxidation could be due to oxygen or to the *o*-quinone. However, oxygen consumption could not be detected in the course of the reaction (curve c of Fig. 6). The oxidation of leukoaminochrome by its quinone can be explained by the redox potential difference between the pairs *o*-quinone/diphenol and aminochrome/leukoaminochrome [17]. Thus, by measuring the concentration of aminochrome when it reaches a constant value (0.15 mM in curve b of Fig. 6), it was found that these concentrations were equal to half of those of periodate, and, consequently, half those of the *o*-quinone. These results suggest that *o*-quinone oxidizes the leukocompound, being reduced in turn to noradrenaline. The slope of the straight line resulting from the plot of aminochrome concentration ($\varepsilon_{490} = 3.58 \times 10^3\ M^{-1}\ cm^{-1}$ (12)) versus periodate concentration was found to be equal to 0.5 (Fig. 5B).

When periodate concentration was greater than that of *o*-diphenol ([$NaIO_4$]/[noradrenaline] = 12.5), a set of plots showing two isosbectic points at 369 and 407 nm respectively were obtained, as can be seen in Fig. 7A. The graphical analysis of the spectra gave a good fit for two kinetically related absorbing species in solution (Fig. 7B). Finally, when oxidation

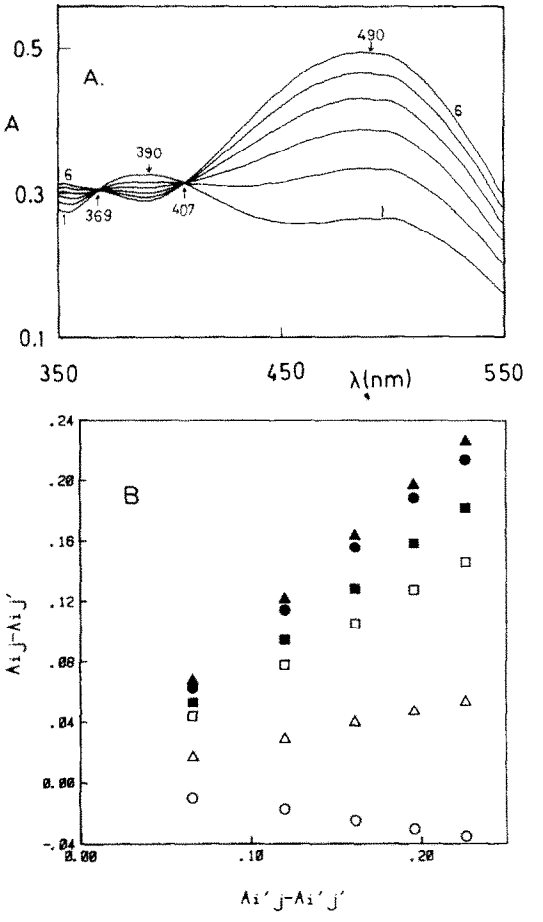



Fig. 7. (A) Spectrophotometric recordings for the oxidation of noradrenaline in excess of sodium periodate from 350 to 550 nm at 12.5° in 0.01 M sodium acetate buffer, pH 4.9. Scan speed was up to 20 nm and the first scan is started at 40 sec from the beginning of the reaction, spectra were recorded at 20 sec intervals. Noradrenaline 0.4 mM was oxidized with 5 mM $NaIO_4$.
(b) Graphical analysis of recordings of Fig. 7A. The test for two absorbing species was applied, being $i'$ = 498 nm, $j'$ = first, $i$ = ○ (388 nm), $i$ = △ (419 nm), $i$ = □ (440 nm), $i$ = ● (446 nm), $i$ = △ (475 nm) and $i$ = ■ (530 nm).

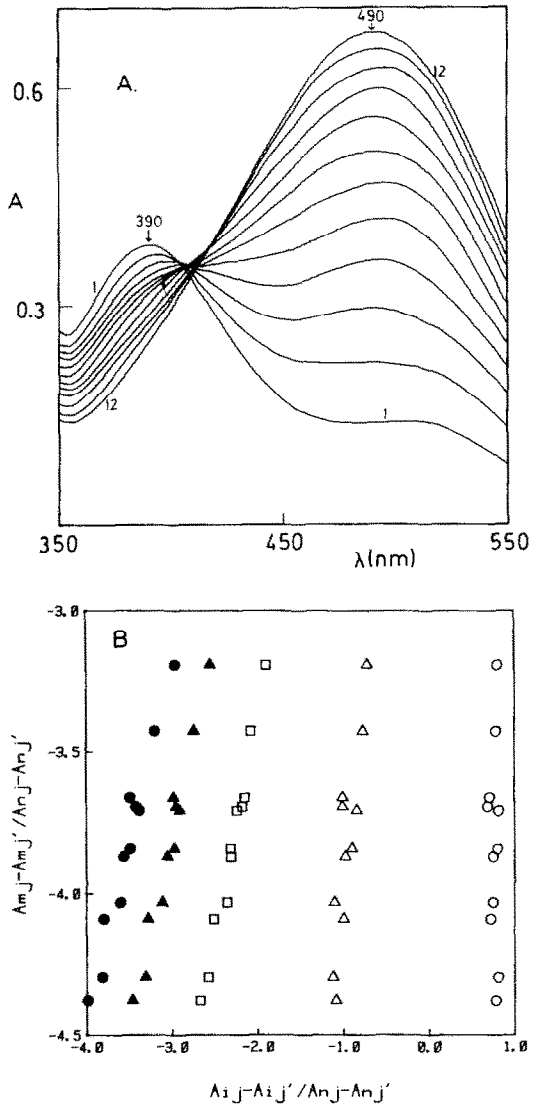



Fig. 8 (A) Spectrophotometric recordings for the oxidation of noradrenaline by sodium periodate in equimolar conditions. Scan speed was carried out as Fig. 1A. Spectra were recorded at 20 sec intervals. Noradrenaline at 0.4 mM was oxidized with 0.4 mM $NaIO_4$.

(B) Graphical analysis of recordings of Fig. 8A. The test for three absorbing species was applied being $m$ = 498 nm, $n$ = 376 nm, $j'$ = first, $i$ = ○ (390 nm), $i$ = □ (440 nm), $i$ = ● (466 nm), $i$ = ▲ (531 nm), $i$ = ■ (550 nm).

was carried out in equimolecular conditions ([$NaIO_4$]/[noradrenaline] = 1) no isosbectic point appeared, as is shown in Fig. 8A. The graphical analysis of the spectra showed the presence of three kinetically related absorbing species (Fig. 8B). This assay was similar to enzymatic oxidation in conditions where oxygen was not depleted.

*Kinetic approach.* In a previous paper [9], we have studied the kinetics of the melanization pathway between L-dopa and dopachrome. The identification of *o*-quinones as intermediates in dopa oxidation by tyrosinase, and the consideration of the potential difference between the diphenol/*o*-quinone and leukoaminochrome/aminochrome pairs [17] enables us to postulate a similar mechanism for the pathway

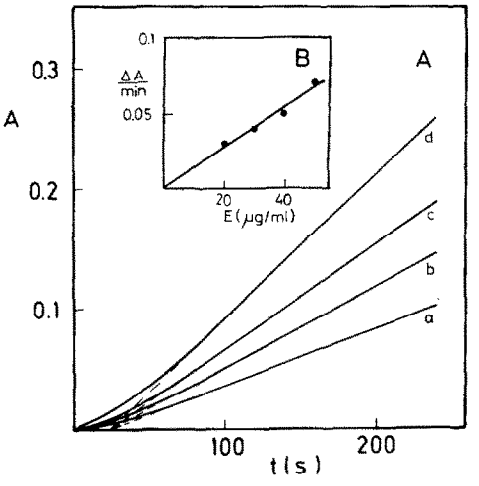



Fig. 9. (A) Noradrenochrome accumulation with time at pH 4.9 and 15° followed by absorbance increase at 490 nm for different enzyme concentrations (μg/ml): (a) 50; (b) 40; (c) 30 and (d) 20.

(B) Plot of noradrenochrome accumulation rate in steady-state conditions against enzyme concentration.

of these diphenols, that we have designed as Enzymatic–Chemical–Chemical ($E_zCC$) mechanism, with the same characteristics than those previously described [9]. The sequence of reactions is depicted in Scheme 1.

The kinetic studies were performed with mushroom tyrosinase and, although it is well known that the mammalian enzyme has a different specificity with respect to its substrates, it is, however, completely accepted that the oxidation of a diphenol to its corresponding quinone can be achieved by all the enzymes in the phylogenetic scale [16]. Since the reactions following the enzymatic step are merely chemical ones, mushroom tyrosinase seems an adequate system to study this minority pathway of oxidation of these compounds.

The derivation of the kinetic equations for such a mechanism is given in the Appendix. The experimental measurements consisted in the study of the

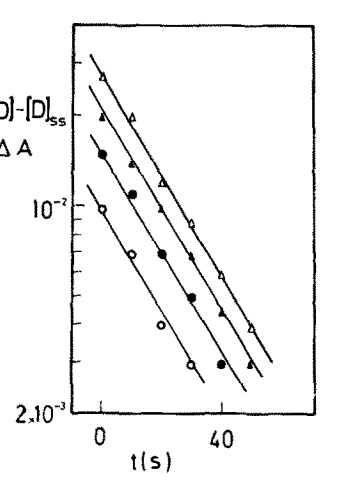
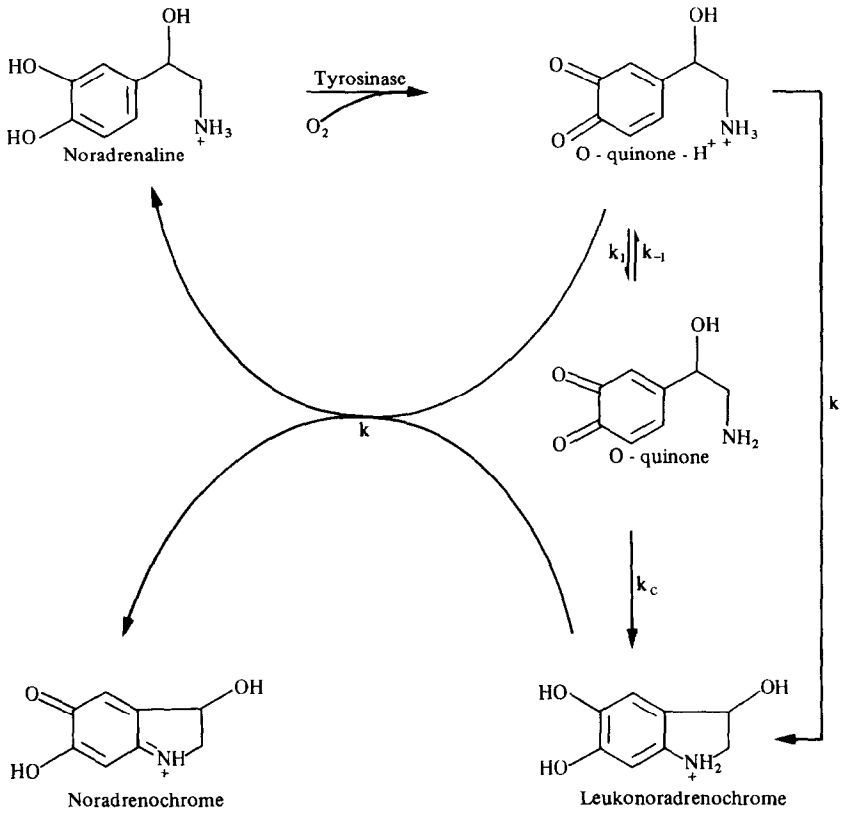



Fig. 10. Semilogarithmic representation of ([D] -$[D]_{ss}$) against time, according to Eqn 17. The values of [D] correspond to those of Fig. 9A.




Scheme 1. Pathway postulated for the noradrenaline oxidation by tyrosinase.

accumulation of aminochrome with time, that followed equation 10 of the Appendix. From this equation it was possible to define the steady-state accumulation of noradrenochrome (Eqn 11). In accordance with this equation, when the appearance of aminochrome is measured, half the enzymic velocity is detected. On the other hand, this expression also justifies the appearance of a lag period which is dependent on the chemical steps. This lag period (L) can be defined as the intercept on the abscissa axis of the straight zone prolongation of noradrenochrome accumulation curve. So, when the rise in absorbance at 490 nm, a wavelength where only aminochromes absorb, was followed by the aminochrome of noradrenaline, the reaction progress showed a marked lag period (Fig. 3a), which strongly depended on pH and temperature, but was independent of the enzyme concentration. Figure 9A shows the aminochrome formation for different enzyme concentrations at 15° and pH 4.9. The transient phase is defined by the lag period (L) whose value is $1/2k$, according to Eqn 11 and therefore is independent of the enzyme concentration. However, the slope of the linear zone of the curve depended directly on the enzyme concentration, as shown by Fig. 9B, and its value is $V_0/2$ in accordance with Eqn 11.

An analysis of the experimental curves of Fig. 9A, according to Eqn 17, is shown in Fig. 10. The rate constant value calculated from the slope of the straight lines was the same as estimated from the lag period; therefore, $k$ values will always be estimated from the lag period.

If the reaction progress was followed at 390 nm ($\lambda_{max}$ for *o*-quinone-$H^+$), a phenomenon of apparent burst was observed (Fig. 3b). The *o*-noradrenalinequinone-$H^+$ concentration (Fig. 3c) reached a maximum value when noradrenochrome accumulation took place with a constant velocity, *viz.* $V_0/2$. Then, this apparent burst represents the accumulation of noradrenoquinone-$H^+$ until the steady state is reached as established by Eqn 7.

Table 1 summarizes the values of $1/k = 2L$ for the different pH and temperature values studied. The lag period increased when pH and/or temperature were increased. Application of Eqn 16 to the observed rate data from Table 1, gives nicely linear plots of $1/k$ against $[H^+]$, expressed in Fig. 11. From these linear regression fitted plots, $k_1$ can be evaluated from the intercepts on the ordinate axis and $k_c$ from the slopes of lines. the first order rate constants pH-independent for deprotonation of *o*-quinone-$H^+$ to *o*-quinone and for the cyclization of *o*-quinone to leukoaminochrome at different temperatures were

Table 1. Reciprocals of the first order rate constants for the conversion of noradrenalinequinone-$H^+$ in noradrenochrome, at several pH and temperature values

| | $1/k$(sec) | | | | |
|---|---|---|---|---|---|
| pH/$t$(°) | 5 | 10 | 12.5 | 15 | 20 |
| 4.9 | 190 | 103 | 92 | 48 | 28 |
| 5.2 | 80 | 52 | 50 | 24 | 10 |
| 5.5 | 56 | 30 | 26 | 13 | 6.8 |
| 5.8 | 31 | 14 | 11.2 | 6.4 | 3.4 |

Table 2. First order rate constants ($k_1$) for deprotonation of noradrenalinequinone-H$^+$ to noradrenalinequinone and for the cyclization of noradrenalinequinone to leukonoradrenochrome at various temperatures

| $t$ (°) | $k_1$ (sec$^{-1}$) | $k_c$ (sec$^{-1}$)* |
|---|---|---|
| 5 | 0.2 | 423 |
| 10 | 0.37 | 757 |
| 12.5 | 0.5 | 832 |
| 15 | 1.52 | 1606 |
| 20 | 3.13 | 3105 |

* Derived from Eqn 16 by using a value of $k_a = 1.66 \times 10^{-10}$ M [19].

expressed in Table 2. Therefore, two [H$^+$]-depending *o*-quinone forms can be postulated, and, in agreement with the variation shown by the apparent first order constant with respect to pH, mainly the protonated form has to exist, since the half-lives at 25°, estimated from the deprotonation and cyclization rate constants were approximatly 0.1 sec and 0.2 msec for *o*-quinone-H$^+$ and unprotonated *o*-quinone formes of noradrenaline, respectively.

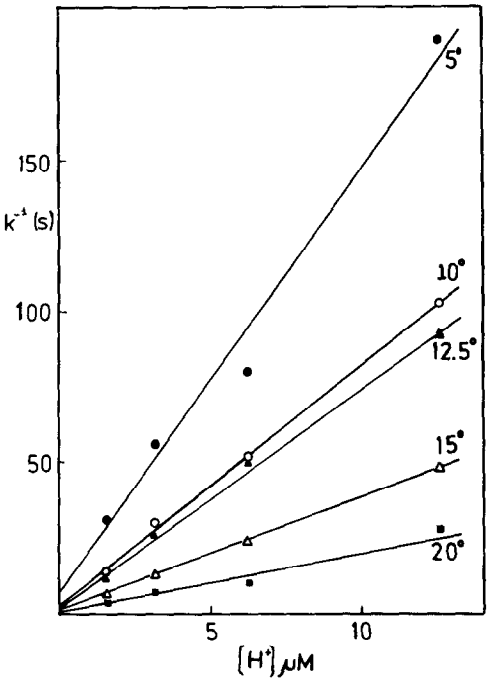



Fig. 11. Plot of the reciprocals of observed rate constants against proton concentrations at different temperatures, in the process of conversion of noradrenalinequinone-H$^+$ to noradrenochrome (see Eqn 15).

For the evaluation of the activation energies for the cyclization and deprotonation processes, Arrhenius plots were made using the $k_c$ and $k_1$ obtained values. The calculated activation parameters are summarized in Table 3 for the cyclization process as well as for the deprotonation one.

## DISCUSSION

The identification of intermediates in this minoritary pathway of melanin biosynthesis is difficult to establish, because of the great reactivity of these short half-life compounds. The occurrence of a maximum centered at 390 nm when tyrosinase acts on tyrosine led to the proposal that *o*-quinone-H$^+$ was the product of the enzymatic action. Nevertheless, identification of this compound as the direct product of the enzyme action on dopa has never been achieved [16]. It was possible to observe the appearance of *o*-dopaquinone-H$^+$ working with tyrosinase at acid pH values (from 3.5 to 6.0) and with a rapid scan spectrophotometer [10].

The oxidation of noradrenaline, because of its similarity to L-dopa, should proceed by the same mechanism, and the appearance of the quinone-H$^+$ form can be followed at acid pH (Fig. 1A). The formation of the aminochrome has been shown to proceed following scheme I. In accordance with this scheme, the cyclization of the quinonic form by an intramolecular 1,4 addition of Michael is required for the formation of the aminochrome from *o*-quinone-H$^+$. However, this cyclization is only feasible when the amino group is deprotonated, and thus, the reaction is pH-dependent. For pH values close to the physiologic, one can fail to detect the *o*-quinone-H$^+$ intermediate by means of graphical analysis because the cyclization is very rapid, so that intermediate concentrations remain low. For low pH values (less than 5.8), the reaction becomes slower, and thus the detection by means of graphical analysis becomes possible (Fig. 2A and 2B). When the system reaches the steady state, after a lag time aminochromes accumulate at a constant rate. From the experiments performed with tyrosinase and shown in Fig. 3, where it can be seen that the velocity of aminochrome accumulation is half that of *o*-quinone-H$^+$ appearance, as well as from the experiments of oxidation by periodate shown in Fig. 5 and 6, where no oxygen consumption was detected, we can deduce that, as in the case of dopa oxidation [10], the following stoichiometry should occur:

2-*o*-quinone-H$^+$ → leukoaminochrome +
*o*-quinone-H$^+$ → aminochrome + *o*-diphenol.

When the oxidation of these diphenols is carried out with mushroom tyrosinase at high concentra-

Table 3. Thermodynamic activation parameters for the deprotonation of noradrenalinequinone-H$^+$ into noradrenalinequinone and for the cyclization of noradrenalinequinone into leukonoradrenochrome

| | $E_a$ (kcal. mol$^{-1}$) | $\Delta H^*$ (kcal. mol$^{-1}$) | $\Delta G^*$ (kcal. mol$^{-1}$) | $\Delta S^*$ (EU) |
|---|---|---|---|---|
| Deprotonation | 30.2 | 29.6 | 16.4 | 44.3 |
| Cyclization | 21.1 | 20.5 | 12.4 | 27.2 |

tions, the graphical analysis shown in Fig. 2A fits, at short reaction times, with three species, namely noradrenaline, *o*-quinone-$H^+$ and aminochrome. However, for longer reaction times (from line 6 in Fig. 2B) the graphical analysis nicely fits with the case of two kinetically related species. In these conditions, the oxygen is depleted from the medium (Fig. 1B), and thus, formation of *o*-quinone-$H^+$ can no longer proceed. In these conditions, the graphical analysis detects the protonated *o*-quinone, and another species. This last species should be formed by noradrenaline + aminochrome, in a 1:1 ratio. Both noradrenaline and aminochrome should be formed at this constant ratio through the reaction time, so that they should be detected as a single species by graphical analysis. The same result is obtained when the oxidation is carried out with $NaIO_4$ in defect (Fig. 4A). In these conditions, an isosbectic point appears at 420 nm. Graphical analysis fits with two kinetically related species, but, as we have described above, three species are present, namely *o*-quinone-$H^+$, noradrenaline and aminochrome. The experiments carried out with $NaIO_4$ in excess showed the appareance of two isosbectic points different from the precedent, at $\lambda = 369$ and 407 nm. This can be explained by the transformation of *o*-quinone-$H^+$ into aminochrome, following a 1:1 stoichiometry. The excess of periodate prevents the accumulation of *o*-diphenol in the reaction medium, and can also oxidize the leukoaminochrome formed, thus preventing the action of an oxidizing agent of *o*-quinone-$H^+$. In this case, the graphical analysis (Fig. 7B), fits nicely for the test of two species kinetically related: *o*-quinone-$H^+$ and aminochrome.

When the oxidation is carried out with small amounts of enzyme, oxygen is not depleted in the medium and the reactions of noradrenaline oxidation and *o*-quinone-$H^+$ cyclization to the corresponding aminochrome proceed at the same time. The graphical analysis fits with the test of three species kinetically related: diphenol, *o*-quinone-$H^+$ and aminochrome.

This same situation is reached when the oxidation by periodate is achieved in stoichiometric amounts of diphenol and periodate, as is shown in Fig. 8A. The graphical analysis then fits with the test for three species. The third intermediate in the pathway of formation of the aminochrome, the leukoaminochrome, is not detected by graphical analysis, and therefore its concentration in reaction media should be very low, since it is rapidly oxidized by *o*-quinone-$H^+$.

Thus, we conclude that this minor pathway of oxidation of noradrenaline by tyrosinase follows a scheme similar to that established for L-dopa [9]. However, the specific rate constants for the processes of deprotonation and cyclization are higher. This fact should justify its small efficiency as cytostatic drugs against malignant melanoma [18] because of the great instability of *o*-noradrenalinequinone.

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## REFERENCES

1. F. Binns, J. A. G. King, S. N. Mishra, A. Percival, N. C. Robson, G. A. Swan and A. Waggott, *J. chem. Soc. (Drg)* **15**, 2063 (1970).
2. T. B. Fitzpatrick, W. C. Quevedo, G. Szabo and M. Seiji, in *Biology of Melanin Pigmentary System in Dermatology and General Medicine* (Eds T. B. Fitzpatrick, K. A. Arndt, W. H. Clark, A. Z. Eisen, E. J. Van Scott and J. H. Vaughan), p. 117, McGraw-Hill, New York (1971).
3. W. H. Harrison, R. M. Gray and L. M. Solomon, *Acta Dermotovener (Stockholm)* **54**, 249 (1974).
4. M. H. Van Woert, K. N. Prasad and D. C. Borg, *J. Neurochem.* **14**, 707 (1967).
5. M. H. Van Woert and L. M. Ambani, *Adv. Neurol.* **5**, 215 (1974).
6. A. C. Greiner and G. A. Nicolson, *Lancet* **ii**, 1165 (1965).
7. G. A. Nicolson, A. C. Greiner, W. J. G. McFarlane and R. A. Baker, *Lancet* **i**, 344 (1966).
8. R. A. Løvstad, *Gen. Pharmac.* **10**, 437 (1979).
9. F. García-Carmona, F. García-Cánovas, J. L. Iborra and J. A. Lozano, *Biochim. biophys. Acta* **717**, 124 (1982).
10. F. García-Cánovas, F. García-Carmona, J. Vera, J. L. Iborra and J. A. Lozano, *J. biol. Chem.* **257**, 8738 (1982).
11. W. H. Harrison, *Archs. Biochem. Biophys.* **101**, 116 (1963).
12. R. A. Løvstad, *Acta chem. Scand.* **25**, 3144 (1971).
13. E. Hartree, *Analyt. Biochem.* **48**, 422 (1972).
14. J. S. Coleman, L. P. Varga and S. H. Mastin, *Inorg. Chem.* **9**, 1015 (1970).
15. S. W. Weidman and E. T. Kaiser, *J. Am. chem. Soc.* **88**, 5820 (1960).
16. D. G. Graham and P. W. Jeffs, *J. biol. Chem.* **252**, 5729 (1977).
17. G. A. Swan, *Fortsch. Chem. Org. Naturst.* **31**, 521 (1974).
18. M. M. Wick, *J. Natl. Cancer Inst.* **63**, 1465 (1979).
19. G. P. Lewis, *Bri. J. Pharmac.* **9**, 488 (1954).

## APPENDIX

The mechanism postulated in scheme I propose the recycling of the product of the enzymic reaction into the substrate of this same step by a chemical reaction, according to the following equations:

$$AH \xrightarrow[V_0]{E} BH \quad (1)$$

$$BH \xrightarrow{k} C \quad (2)$$

$$C + BH \underset{K}{\rightleftharpoons} AH + D \quad (3)$$

where AH = noradrenaline, BH = noradrenaline-quinone-$H^+$, C = leukonoradrenochrome, and D = noradrenachrome.

Equation 1 assumes the enzymatic conversion of noradrenaline to noradrenoliquinone-$H^+$; Eqn 2 involves two chemical reactions of schem I, the deprotonation of noradrenalinequinone-$H^+$ and a ring closure process, whose whole rate constant is defined by $k$. Equation 3 assumes the recycling of the product of the enzymatic reaction into the substrate of this same step by a chemical reaction. Since the equilibrium constant $K$ for Eqn 3 should be very high in accordance with the electrochemical data

[16], it may be seen that the leukonoradrenochrome concentration in the reaction medium tends to zero throughout the whole reaction. On the assumption that noradrenaline concentration is saturating or its variation during reaction time is very small, we ought to consider that the enzymatic reaction rate remains constant ($V_0$).

Taking into account the former conditions, the following equations can be established:

$$\frac{d[BH]}{dt} = V_0 - 2k[BH] \quad (4)$$

$$d[D] = k[BH] \quad (5)$$

$$[C]_t = 0. \quad (6)$$

Integrating Eqn 4 and given that [BH] = 0 for $t$ = 0, we obtain:

$$[BH] = \frac{V_0}{2k}(1 - e^{-2kt}). \quad (7)$$

For $e^{-2kt} \ll 1$, BH in the steady state can be defined as,

$$[BH]_{ss} = V_0/2k. \quad (8)$$

From Eqn 7 and 8 the following can easily be deducted,

$$\ln([BH]_{ss} - [BH]) = \ln[BH]_{ss} - 2kt. \quad (9)$$

On the other hand, integrating Eqn 5 and taking Eqn 7 into account yields,

$$[D] = \frac{V_0}{2}\left(t + \frac{1}{2k}e^{-2kt} - \frac{1}{2k}\right). \quad (10)$$

For $e^{-2kt} \ll 1$, it is possible to define how D is accumulated when the system reaches its steady state:

$$[D]_{ss} = \frac{V_0}{2}\left(t - \frac{1}{2k}\right). \quad (11)$$

Since $k$ is the rate constant of the whole process of noradrenalinequinone-$H^+$ conversion into leukonoradrenochrome, it can be expressed as a function of the constants implied in each step, using a steady state approximation for the noradrenalinequinone intermediate (B). It can be established that:

$$\frac{d[B]}{dt} = k_1[BH] - k_{-1}[HB^+][B] - k_c[B]. \quad (12)$$

And from

$$\frac{d[B]}{dt} = 0, \frac{d[D]}{dt} = k[BH] \text{ and } \frac{d[D]}{dt} = k_c[B] \quad (13)$$

it is deduced that

$$k = \frac{k_1 k_c}{k_{-1}[H^+] + k_c} \quad (14)$$

where $k_c$ is the specific rate constant for the ring closure (intramolecular 1,4 addition of Michael), provided that this reactions is accepted to be of first order.

Equation 14 can be transformed into,

$$\frac{1}{k} = \frac{1}{K_a k_c}[H^+] + \frac{1}{k_1} \quad (15)$$

where $K_a$ is the ionization constant for amino group of noradrenalinequinone-$H^+$, and Eqn 15 can be also rewritten as a function of the lag period,

$$2L = \frac{1}{K_a k_c}[H^+] + \frac{1}{k_1}. \quad (16)$$

On the other hand, using Eqns 10 and 11, it can be established that,

$$\ln([D] - [D]_{ss}) = \ln\frac{V_0}{4k} - 2kt. \quad (17)$$

Hence a plot of $\ln([D]-[D]_{ss})$ against time gives straight lines whose slopes enable us to evaluate the rate constant $k$.