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Melanogenesis inhibition by tetrahydropterines

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ABSTRACT

There is controversy in the literature concerning the action of tetrahydropterines on the enzyme tyrosinase and on melanogenesis in general. In this study, we demonstrate that tetrahydropterines can inhibit melanogenesis in several ways: *i*) by non-enzymatic inhibition involving purely chemical reactions reducing *o*-dopaquinone to L-dopa, *ii*) by acting as substrates which compete with L-tyr and L-dopa, since they are substrates of tyrosinase; and *iii*) by irreversibly inhibiting the enzymatic forms *met*-tyrosinase and *deoxy*-tyrosinase in anaerobic conditions. Three tetrahydropterines have been kinetically characterised as tyrosinase substrates: 6-*R*-L-erythro-5,6,7,8-tetrahydrobiopterin, 6-methyl-5,6,7,8-tetrahydropterine and 6,7-(*R*,*S*)-dimethyl-5,6,7,8-tetrahydropterine. A kinetic reaction mechanism is proposed to explain the oxidation of these compounds by tyrosinase.

1. Introduction

In the presence of molecular oxygen, 6-*R*-L-erythro-5,6,7,8-tetrahydrobiopterin ($6BH_4$) is the immediate electron donor for the hydroxylation of the aromatic aminoacids L-phenylalanine, L-tyr and L-tryptophan [1]. Hence, $6BH_4$ functions as an important regulator in the synthesis of the neurotransmitter serotonin [2] and catecholamines [3]. Keratinocytes and melanocytes need L-tyr to initiate catecholamine and melanin synthesis in these cells. It has been shown that in physiological conditions these cells are capable of the *de novo* synthesis/recycling/regulation of $6BH_4$ [4,5].

It has been described how $6BH_4$ can regulate tyrosinase (TYR) by uncompetitive inhibition [6]. It has been proposed that TYR has a binding domain for this cofactor at the site of Cu^A, while Ser^{128}, Lys^{130} and Glu^{131} participate through hydrogen bonds to the pyrimidine ring of the pterin [7].

More recently 6,7-(*R*,*S*)-dimethyl-tetrahydropterine ($DMBH_4$) and 6-(*R*,*S*)-tetrahydromonapterine have been studied as possible TYR inhibitors and it has again been suggested that these compounds, like $6BH_4$, act through an uncompetitive allosteric mechanism [8]. It has also been suggested that $6BH_4$ leads to the uncoupling of TYR, producing H_2O_2, followed by activation or inhibition of the enzyme, depending on the concentration of H_2O_2. The same authors propose that at a $6BH_4$ concentration of 10^{-6}M, the H_2O_2 produced by TYR oxidises the $6BH_4$ to 7,8-dihydrobiopterin even in the absence of the substrate L-tyr [8].

Other authors have suggested that the inhibition exercised by $6BH_4$ is apparent since it acts as a reductant of *o*-dopaquinone and, once exhausted, the reaction rate is the same as obtained in the absence of $6BH_4$ [9].

It is interesting that the proposed inhibition of TYR by $6BH_4$, or by its analogues, $DMBH_4$ and MBH_4, according to [8], only affects the monophenolase activity of the enzyme and not the dopa-oxidase. Furthermore, it has been described how TYR is inhibited by an excess of L-tyr in the absence of $6BH_4$ [8].

In a series of studies on the kinetic of TYR, we demonstrated how the enzyme is not inhibited by an excess of L-tyr, and emphasised the need for a rigorous measurement of the initial rates of the action of TYR on L-tyr [10–12]. The system needs to reach the steady-state if the measurements of V_{ss} (initial steady-state rate) in the action of the enzyme on monophenol (L-tyr) are to be correct. That is, the lag phase, τ, must be allowed to pass before the steady-state is reached. Since τ increases as the substrate concentration increases, the measurements must be made on a different time scales, *i.e.* longer times as the substrate concentration increases [10–12].

In a recent publication [13], we studied the effect of tetrahydropterines at μM concentrations on the monophenolase and diphenolase activities of TYR, and demonstrated that they are oxidised by *o*-dopaquinone, the systems reaching the steady-state when all the tetrahydropterines have been exhausted [13].

Pomerantz [14] demonstrated that $DMBH_4$ at a concentration of 2.4 mM eliminates the lag (τ) and stimulates the tyrosine hydroxylase activity of mammalian TYR, monitoring the activity by measuring the

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release of tritium from L-tyr-3,5-^{3}H into the solution in the form TOH [14].

Based on the experiments described in [13] and the results of [14], we decided to study the inhibition of melanogenesis by tetrahydropterines from several points of view. Our findings show that tetrahydropterines inhibit melanogenesis in several ways: *i*) by non-enzymatic inhibition, acting as reductants of *o*-dopaquinone; *ii*) inhibition through their acting as competitive substrates of TYR and *iii*) through the irreversible inhibition of *met*-TYR and *deoxy*-TYR. Different kinetic mechanisms are proposed to explain the results.

2. Materials and methods

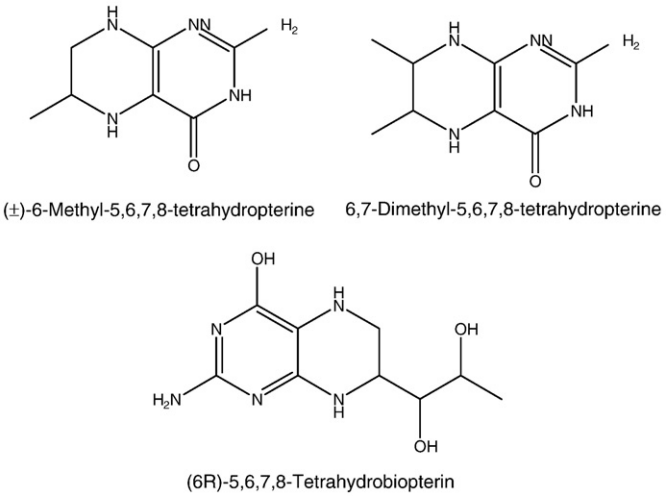
2.1. Reagents

6-*R*-L-erythro-5,6,7,8-tetrahydrobiopterin ($6BH_4$), 6-methyl-5,6,7,8-tetrahydropterine (MBH_4) and 6,7-*R*,*S*-dimethyl-5,6,7,8-tetrahydropterine ($DMBH_4$) (Scheme I) were purchased from Sigma (Madrid, Spain). L-tyrosine (L-tyr), L-dopa and 4-*tert*-butylcatechol (TBC) were obtained from Aldrich (Madrid, Spain). Stock solutions of the tetrahydropterines and the diphenolic and monophenolic compounds were prepared in 0.15 mM phosphoric acid and anaerobic conditions to prevent auto-oxidation.

Mushroom tyrosinase (TYR; EC 1.14.18.1, 3000 U/mg) was purchased from Sigma (Madrid, Spain) and purified according to [15]. Horseradish peroxidase (POD; EC 1.11.1.7, 297 U/mg), superoxide dismutase (SOD: EC 1.15.1.1, 4140 U/mg) and catalase (CAT; EC 1.11.1.6, 9300 U/mg) were obtained from Sigma (Madrid, Spain) The protein content was determined by Bradford's method using bovine serum albumin as standard [16].

2.2. Spectrophotometric assays

Absorption spectra with a 60 nm/s scanning speed were recorded in an ultraviolet-visible Perkin-Elmer Lambda-35 spectrophotometer. Kinetic assays were also carried out with the above instruments by measuring the appearance of the products in the reaction medium. In the case of tetrahydropterines, the experimental conditions were: 30 mM sodium phosphate buffer (pH 7.0) at 25 °C, except when other conditions were indicated. Since both the substrate and product absorb at the measuring wavelength of 340 nm, it is necessary to know the extinction coefficient at 340 nm for the conversion of $6BH_4$ to $6BH_2$, (5040 $M^{-1}cm^{-1}$ [17]), of MBH_4 to MBH_2 and $DMBH_4$ to $DMBH_2$ (3800 and 3600 $M^{-1}cm^{-1}$, respectively [18]).



Scheme I. Chemical structures of the tetrahydropterines studied in this work.

2.2.1. Monophenolase and diphenolase activities of tyrosinase

Spectrophotometric measurements were made by measuring the accumulation of dopachrome at $\lambda = 475$ nm ($\varepsilon = 3600\ M^{-1}cm^{-1}$) during the oxidation of L-tyr or L-dopa to dopachrome.

2.3. Oxygen consumption

Oxygen consumption was measured using a Clark-type electrode coupled to a Hansatech oxygraph after calibrating with 4-*tert*-butylcatechol [19]. The reaction medium (2 ml) contained different concentrations of L-tyr, L-dopa, $6BH_4$, $DMBH_4$ and MBH_4 (see figures). The reaction was started by adding TYR in water.

2.4. Generation of E_{ox} and E_d

We set out to kinetically characterise the inactivation of the E_d form of TYR. E_d was generated from the native enzyme, adding micromolar concentrations (2 μM) of H_2O_2 so that the E_m form passed to E_{ox}. Then nitrogen was bubbled through the solution transforming all the E_{ox} to E_d ($E_{ox} \rightleftharpoons E_d + O_2$) [20,21].

2.5. Generation of E_m

The inactivation of E_m was characterised by first generating it from the native enzyme in two ways: (a) adding 2 μM catalase, so that $E_{ox} \rightleftharpoons E_m + H_2O_2$; the catalase acts on the H_2O_2 displacing the equilibrium, $2H_2O_2 + \text{catalase} \rightarrow O_2 + 2H_2O$, so that all E_{ox} is transformed into E_m at the end of the reaction. (b) Adding 2 μM of H_2O_2, so that all the enzyme passes to E_{ox} and then adding 2 μM catalase [20,21].

2.6. Evaluation of enzymatic species E_m, E_d and E_{ox} in an enzymatic preparation of TYR

It is known that in an enzymatic preparation of TYR from any source is found in three forms, E_m, E_d and E_{ox} [20]. Several authors have proposed spectrophotometric methods for evaluating these forms [20–23]. Here, we propose a kinetic method based on the fact that the inactivation of these forms by 2-mercaptoethanol [24] occurs over a wide time range: inactivation constants of 0.014 s^{-1}, $4\times10^{-5}s^{-1}$ and $1\times10^{-5}s^{-1}$ for E_{ox}, E_m and E_d, respectively. In aerobic conditions, at oxygen concentrations of 0.26 mM, practically the only forms existing are E_{ox} and E_m [25]. Note that the difference between k_i^{ox} and k_i^m is three orders of magnitude, a difference that can be used to evaluate these enzymatic forms. The experimental method was described in [25].

3. Results

To carry out the experiments with tetrahydropterines their propensity to auto-oxidation must be controlled before they can be studied in the presence of TYR and to study their effects on melanogenesis.

3.1. Auto-oxidation of tetrahydropterines

The auto-oxidation of tetrahydrobiopterin ($6BH_4$) was studied in [17]. The most controversial points of the process were the production of free $O_2^{\cdot -}$ radicals and the formation of H_2O_2. One molecule of $6BH_4$ reduces the O_2 to H_2O_2 and $6BH_2$. Subsequently, H_2O_2 slowly oxidises another molecule of $6BH_4$ and the following stoichiometry is fulfilled:

$$2\,6BH_4 + O_2 \rightarrow 2\,6BH_2 + 2H_2O. \quad (1)$$

As discussed below, this reaction can be carried out by TYR, optimising the experimental conditions.

3.1.1. Oximetric measurement of O_2 consumption in the auto-oxidation: evidence of the presence of superoxide anion and of H_2O_2 accumulation

The above process is outlined in Fig. 1, which shows the auto-oxidation of $6BH_4$ (Fig. 1, recording a), the effect of CAT (Fig. 1, recording b), the effect of SOD (Fig. 1, recording c) and the accumulation of H_2O_2, which is evaluated with CAT. The addition of SOD (Fig. 1, recording c) inhibits H_2O_2 formation, since, when the superoxide anion reacts with the substrate the stoichiometry is 1 $O_2^{\cdot-}$:1H_2O_2 and the reaction catalysed by SOD is ($2O_2^{\cdot-} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2$) with a stoichiometry of 2 $O_2^{\cdot-}$:1 H_2O_2. The addition of CAT, (Fig. 1, recording b), partly inhibits the oxidation of $6BH_4$ by H_2O_2 so that the auto-oxidation decreases and, since the H_2O_2 is consumed ($2H_2O_2 \xrightarrow{CAT} O_2 + 2H_2O$), O_2 consumption decreases. The presence of SOD and CAT, (Fig. 1 recording d) almost totally inhibits auto-oxidation, and, in this way, the optimal conditions for any assay involving tetrahydropterines are established (presence of SOD and CAT). The product of the oxidation of $6BH_4$ by O_2, the $6BH_2$, may also be oxidised by O_2, but the kinetics is much slower and does not interfere in the assays described below [26]. The concentration of H_2O_2 is shown in all the recordings (Fig. 1).

3.1.2. Spectrophotometric measurement of product formation in the auto-oxidation: evidence of the presence of superoxide anion and H_2O_2

Fig. 1 (Inset) depicts the auto-oxidation of tetrahydropterines, and the action of SOD and CAT. (Fig. 1 Inset, recording a) depicts the auto-oxidation that involves the generation of superoxide anion ($O_2^{\cdot-}$) and H_2O_2, as confirmed by Fig. 1 Inset, recording (b), in which the presence of CAT decreases the absorbance because it withdraws the H_2O_2. In Fig. 1 Inset, recording (c), the presence of SOD, which withdraws $O_2^{\cdot-}$ and inhibits the auto-catalytic reaction, further diminishes the absorbance. Lastly, in Fig. 1, recording (d), the simultaneous presence of both, the signal decreases, even more, although not totally. This set of assays indirectly reveals the presence of $O_2^{\cdot-}$ and H_2O_2.

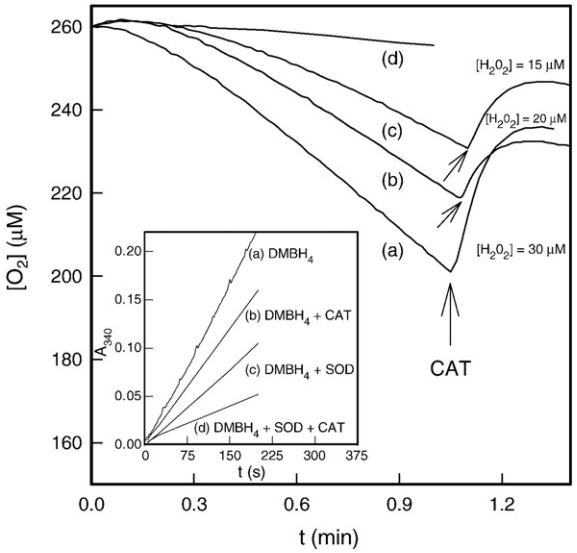


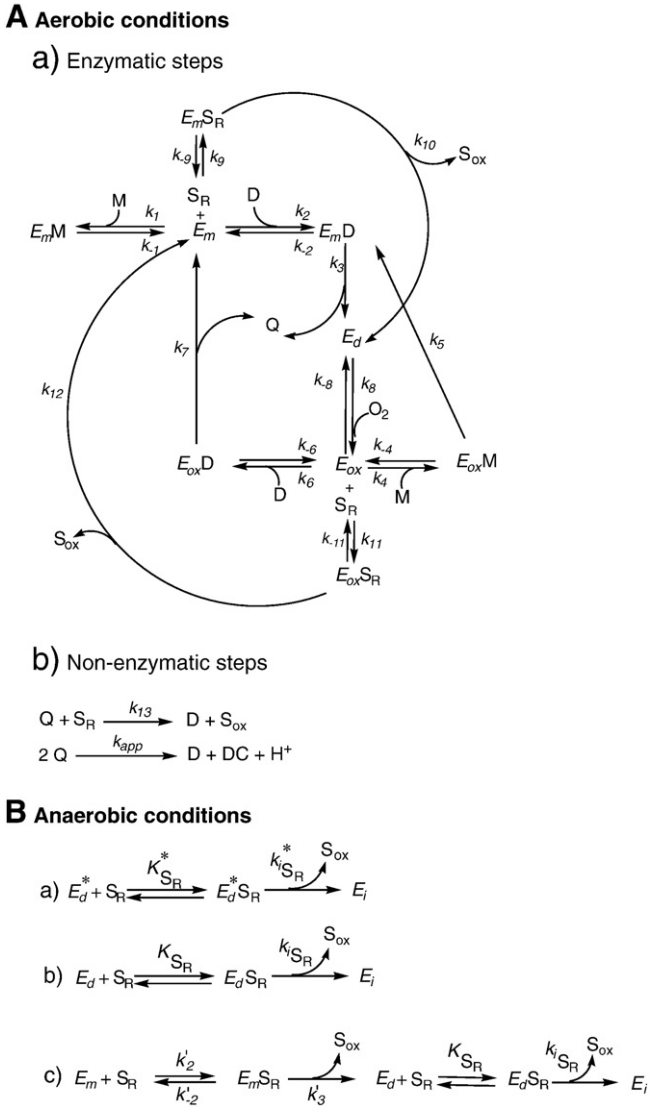
Fig. 1. Oxygraph recordings during the auto-oxidation of $DMBH_4$ and hydrogen peroxide generation. The experimental conditions were 30 mM sodium phosphate buffer (pH 7.0), 25 °C and $[DMBH_4]_0 = 4$ mM. Recording (a) is the control (substrate). The other recordings [(b)–(d)] are similar to (a) but including the indicated enzyme. (b) CAT 930 U/ml, (c) SOD 414 U/ml and (d) SOD 414 U/ml and CAT 930 U/ml. Arrows show when CAT were added. Inset. Spectrophotometric recordings of tetrahydropterine auto-oxidation. The experimental conditions were 30 mM sodium phosphate buffer (pH 7.0), 25 °C and $[DMBH_4]_0 = 0.2$ mM. Recording (a) is the control (substrate). The other recordings [(b)–(d)] are similar to (a) plus the indicated enzyme. (b) CAT 930 U/ml, (c) SOD 414 U/ml and (d) SOD 414 U/ml and CAT 930 U/ml.

3.2. Inhibition of melanogenesis by tetrahydropterines

Scheme II represents the different mechanisms proposed in this study to explain the inhibition of melanogenesis by tetrahydropterines. Scheme IIA describes the inhibition mechanisms for the enzyme acting in aerobic conditions. The most well known mechanism, the reduction of *o*-dopaquinone, is described in Scheme IIA (b). In Scheme IIA (a) the action of tetrahydropterines as competitive substrates with L-tyr and L-dopa is depicted. In anaerobic conditions, on the other hand (Scheme IIB), the enzymatic forms *deoxy*-tyrosinase and *met*-tyrosinase show the irreversible inhibition described in Scheme IIB (a) and IIB (b) for *deoxy*-tyrosinase and in Scheme IIB (c) for *met*-tyrosinase.

3.2.1. Inhibition of melanogenesis by the reduction of o-dopaquinone: effect of tetrahydropterines on the diphenolase and monophenolase activities of tyrosinase

Tetrahydropterines are easily oxidised, the product of the action of TYR on mono and *o*-diphenols (*i.e.* L-tyr and L-dopa), the *o*-dopaquinone



Scheme II. Kinetic mechanism showing different ways of melanogenesis inhibition by tetrahydropterines A) Aerobic conditions (a) Enzymatic steps of the action of TYR on L-tyr (M), L-dopa (D) and tetrahydropterine (S_R) (b) Non-enzymatic steps for the reduction of *o*-dopaquinone (Q) by tetrahydropterines (S_R) and the formation of dopachrome (DC) from *o*-dopaquinone (Q). B) Anaerobic Conditions (a) kinetic mechanism of the E_d^* inactivation in the presence of tetrahydropterines (b) kinetic mechanism of the E_d inactivation in the presence of tetrahydropterines (c) kinetic mechanism of the E_m inactivation in the presence of tetrahydropterines.

acts as oxidant, which is evident from a study of each of the activities of the enzyme in the presence of tetrahydropterines [Scheme IIA (b)][13].

3.2.1.1. Diphenolase activity. It should be remembered that tetrahydropterines reduce *o*-dopaquinone to L-dopa so that the effect on diphenolase activity depends on the concentration used and so two cases will be considered.

3.2.1.1.1. Low concentrations of 6BH$_4$. Fig. 2A shows the effect of low concentrations of 6BH$_4$ (μM) on diphenolase activity as measured by the consumption of oxygen and accumulation of dopachrome, Fig. 2A (Inset). As can be seen the rate of oxygen consumption does not vary (Fig. 2A, recordings a–d), and 6BH$_4$ only acts through

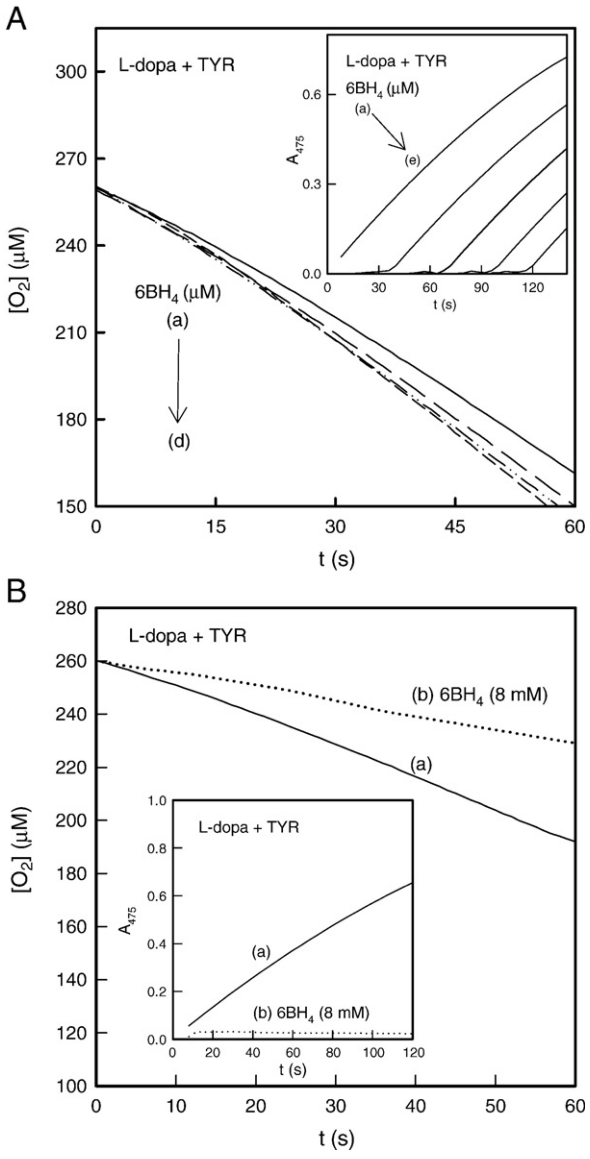


Fig. 2. Action of tetrahydropteridines on the diphenolase activity of tyrosinase. (A) At μM concentrations of 6BH$_4$. Oxygraph recording during the reaction of TYR (35 nM) on L-dopa (0.5 mM) in 30 mM sodium phosphate buffer (pH 7.0), at 25 °C, SOD 414 U/ml and CAT 930 U/ml, after the addition of 6BH$_4$ (μM) (a) 0; (b) 10; (c) 20 and (d) 40. Inset. Spectrophotometric recordings for dopachrome accumulation during the reaction of TYR on L-dopa using the same experimental conditions. 6BH$_4$ (μM): (a) 0, (b) 10, (c) 20, (d) 40 and (e) 50. (B) At mM concentrations of 6BH$_4$. Oxygraph recording during the reaction of TYR (30 nM) on L-dopa (0.5 mM) in 30 mM sodium phosphate buffer (pH 7.0), 25 °C, SOD 414 U/ml and CAT 930 U/ml. Addition of 6BH$_4$ (mM): (a) 0, (b) 8. Inset. Spectrophotometric recordings for dopachrome accumulation during the reaction of TYR on L-dopa using the same experimental conditions and 6BH$_4$ (mM): (a) 0; (b) 8.

oxidation/reduction reactions, that is, reducing Q to D. However, if dopachrome accumulation is measured [Fig. 2A, Inset, recordings (a–e)], there is a lag due to the reduction of *o*-dopaquinone. Once the 6BH$_4$ has been consumed, dopachrome is accumulated with a velocity (V_D^{DC}) equal to that of (Fig. 2A, Inset. recording a) in the absence of 6BH$_4$ [Scheme IIA (b) with [S_R] = 0].

3.2.1.1.2. High concentrations of 6BH$_4$. At high (mM) concentrations, besides the fact that 6BH$_4$ is consumed through oxidation/reduction reactions [Scheme IIA (b)], the oxygen consumption rate is slowed (Fig. 2B, recording b). If attempts are made to measure dopachrome accumulation, (Fig. 2B, Inset, recording b), the velocity is zero because Q is reduced to D by the 6BH$_4$. The inhibition of the oxygen consumption rate at high concentrations of 6BH$_4$ may be the result of the tetrahydropterine behaving as a competitive inhibitor or as alternative substrate to L-dopa [Scheme IIA (a) and (b)].

3.2.1.2. Monophenolase activity. As in the case of diphenolase activity, the effect of different tetrahydropterine concentrations was studied.

3.2.1.2.1. Low concentrations of 6BH$_4$. Experiments with L-tyr were carried out with low concentrations of 6BH$_4$ (μM), in which oxygen consumption was measured (Fig. 3A). Initially, no 6BH$_4$ has been added (Fig. 3A, recording a), while at increasing concentrations [Fig. 3A, recordings (b–d)], the lag period diminishes since, through the oxidation/reduction reactions between *o*-dopaquinone and 6BH$_4$, *o*-diphenol is accumulated in the medium and the system reaches the steady-state earlier. When dopachrome accumulation is measured, [Fig. 3A Inset, recordings (a–d)], the lag decreases at low concentrations since *o*-diphenol is accumulated first. However, at high concentrations the lag period is lengthened (Fig. 3A Inset, recording d), while the steady-state rates are the same [Scheme IIA (b) with [S_R] = 0].

3.2.1.2.2. High concentrations of 6BH$_4$. When oxygen consumption is measured at high concentrations of 6BH$_4$ (mM), Fig. 3B, the lag period disappears, (Fig. 3B, recording b), unlike in Fig. 3B, recording a, where no 6BH$_4$ has been added. In Fig.3B Inset, dopachrome accumulation is measured, and the zero rate measured in Fig. 3B Inset, recording (b), is due to the reduction of *o*-dopaquinone to L-dopa. From Fig. 3B, recording (b), it can be deduced that 6BH$_4$ reacts with the E_m form of the enzyme, so that the lag disappears and the enzyme reaches a pseudo steady-state with a slower rate than in the absence of 6BH$_4$ (Fig. 3B, recording a), indicating that tetrahydropterine can act as competitive substrate to L-tyr and therefore L-dopa [Scheme IIA (a)]. Note that in Fig. 3B, Inset, recording (b), no dopachrome is formed, demonstrating the inhibition of melanogenesis especially through the reduction of *o*-dopaquinone to L-dopa [Scheme IIA (b)].

3.2.2. Inhibition of melanogenesis by tetrahydropterines acting as competitive substrates: action of tyrosinase on L-tyrosine or L-dopa in the presence of tetrahydropterines

To explain this inhibition mechanism, we studied the action of tetrahydropterines as substrate of TYR.

3.2.2.1. Behaviour of tetrahydropterines as tyrosinase substrates: enzymatic catalysis. In the experiments described in Fig. 4 and Fig. 4 Inset A and Inset B, tetrahydropterines are assayed as TYR substrates [Scheme IIA (a), with M = 0, and D = 0].

3.2.2.1.1. Oxidation of tetrahydropterines by tyrosinase. Fig. 4 depicts the oxidation of 6BH$_4$ by TYR, where the appearance of isosbestic points indicates that the chemical species are linearly related. Insets A and B of this figure show the oxidation of MBH$_4$ and DMBH$_4$ by the enzyme respectively. TYR fulfils the stoichiometry described in Eq. (1) by means of a different auto-oxidation mechanism. The kinetic experiments on the oxidation of tetrahydropterines by TYR are shown in Fig. 5. As mentioned in section 3.1, auto-oxidation takes place, while this figure confirms the generation of superoxide and H_2O_2. In the control assay (Fig. 5, recording a), the reaction

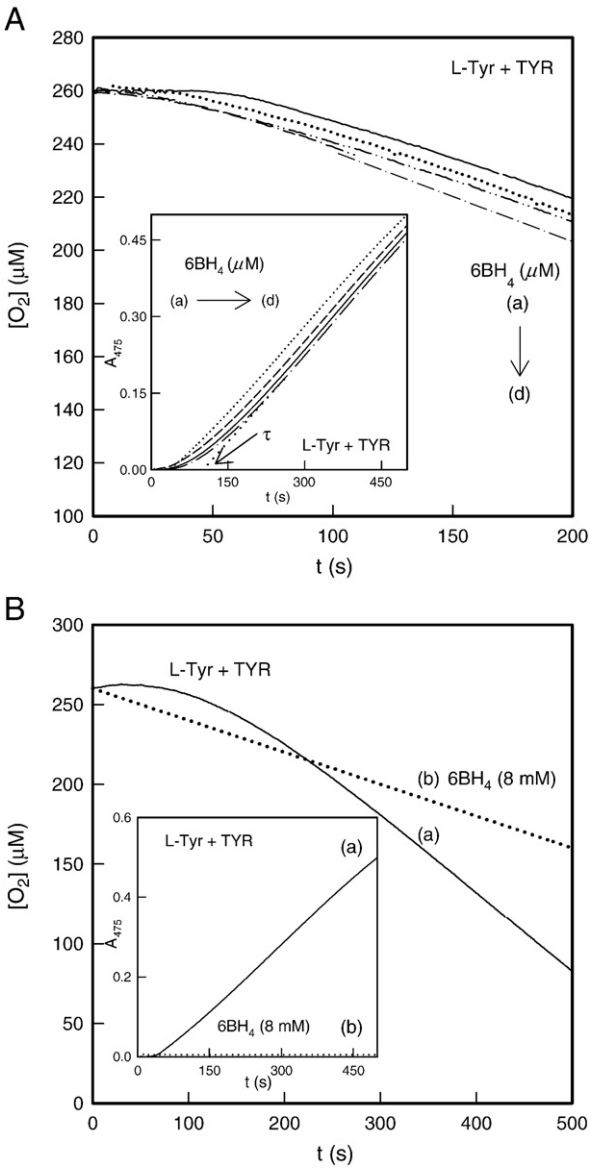


Fig. 3. Action of tetrahydropteridines on the monophenolase activity of TYR. (A) At μM concentrations of $6BH_4$. Oxygraph recording during the reaction of TYR (60 nM) on L-tyr (0.6 mM) in 30 mM sodium phosphate buffer (pH 7.0), 25 °C, SOD 414 U/ml and CAT 930 U/ml. Addition of $6BH_4$ (μM): (a) 0, (b) 10, (c) 20 and (d) 40. Inset. Spectrophotometric recordings for dopachrome accumulation during the reaction of TYR on L-tyr using the same experimental conditions. Addition of $6BH_4$ (μM): (a) 20, (b) 10, (c) 0 and (d) 40. (B) At mM concentrations of $6BH_4$. Oxygraph recording during the reaction of TYR (80 nM) on L-tyr (0.6 mM) in 30 mM sodium phosphate buffer (pH 7.0), 25 °C, SOD 414 U/ml and CAT 930 U/ml. Addition of $6BH_4$ (mM) (a) 0, (b) 8. Inset. Spectrophotometric recordings for dopachrome accumulation during the reaction of TYR on L-tyr using the same experimental conditions. Addition of $6BH_4$ (mM): (a) 0; (b) 8.

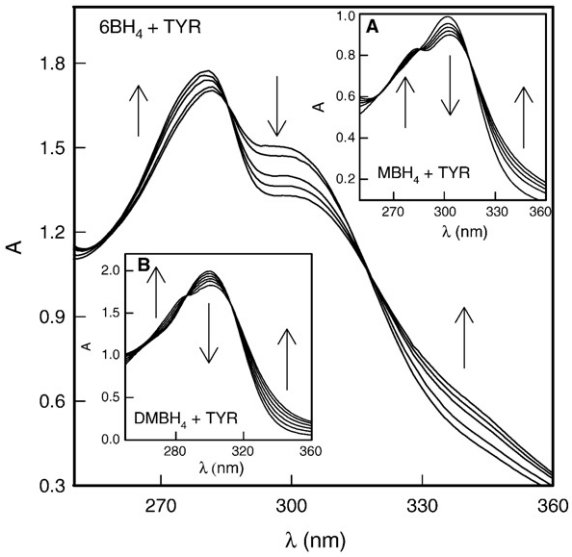


Fig. 4. Spectrophotometric scans of the oxidation of the different tetrahydropterines by TYR. The experimental conditions were 30 mM sodium phosphate buffer (pH 7.0), 25 °C, $[O_2]_0 = 0.26$ mM, SOD 414 U/ml and CAT 930 U/ml. The experimental recordings were made every 60 s. (A) $6BH_4$ oxidation by TYR, $[TYR]_0 = 0.4$ μM and $[6BH_4]_0 = 0.2$ mM. (B) MBH_4 oxidation by TYR, $[TYR]_0 = 0.4$ μM and $[MBH_4]_0 = 0.2$ mM. (C) $DMBH_4$ oxidation by TYR, $[TYR]_0 = 0.5$ μM and $[DMBH_4]_0 = 0.2$ mM.

involves $DMBH_4$ and TYR, while the addition of CAT gives the (Fig. 5, recording b), SOD captures the $O_2^{\cdot -}$ and provides (Fig. 5, recording c). The addition of both SOD and CAT practically eliminates the auto-oxidation and provides (Fig. 5, recording d), which really corresponds to the enzymatic activity if we take into account the small auto-oxidation of (Fig. 1 Inset). Fig. 5, recordings (e–h), obtained in the presence of POD show the formation of H_2O_2, especially (Fig. 5, recording e), although the presence of CAT, (Fig. 5, recording f), SOD, (Fig. 5, recording g) or of both enzymes, (Fig. 5, recording h), does not cancel the action of POD, so that the kinetic recordings must be made in the conditions of Fig. 5, recording (d), in the presence of both SOD and CAT. Fig. 5, Inset, shows the same effects but measuring the consumption of oxygen. (Fig. 5, Inset, recording a) corresponds to the auto-oxidation and action of TYR. The addition of SOD, (Fig. 5, Inset, recording b), decreases oxygen consumption, as happens with (Fig. 5, Inset, recording c) after the addition of CAT. (Fig. 5, Inset recording d), in the presence of SOD and CAT, corresponds to TYR activity.

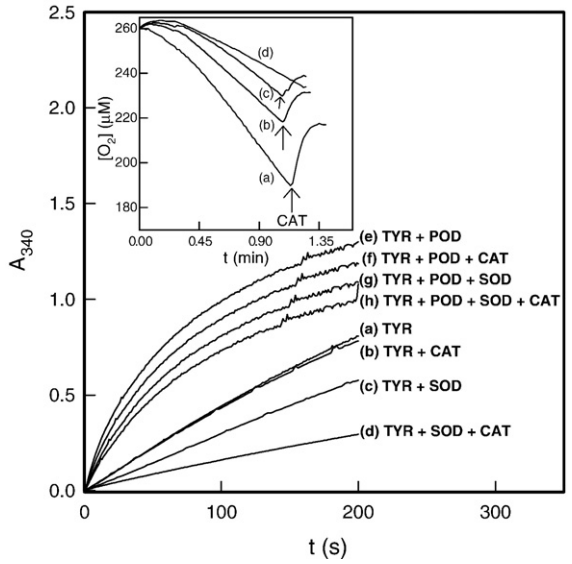


Fig. 5. Spectrophotometric recordings of the oxidation of $DMBH_4$ by TYR in different experimental conditions. The fixed experimental conditions in all cases were 30 mM sodium phosphate buffer (pH 7.0), 25 °C. Recording (a) is the control (TYR + substrate). The other recordings [(b)–(h)] are those of experiments with TYR plus the indicated enzyme. (a) Oxidation of $[DMBH_4]_0$ at 0.2 mM by $[TYR]_0$ (1 μM) recorded at λ = 340 nm. (b) $[CAT]_0 = 930$ U/ml. (c) $[SOD]_0 = 414$ U/ml. (d) $[SOD]_0 = 414$ U/ml and $[CAT]_0 = 930$ U/ml. (e) $[POD]_0 = 30$ U/ml. (f) $[POD]_0 = 30$ U/ml and $[CAT]_0 = 930$ U/ml. (g) $[POD]_0 = 30$ U/ml and $[SOD]_0 = 414$ U/ml. (h) $[POD]_0 = 30$ U/ml, $[CAT]_0 = 930$ U/ml and $[SOD]_0 = 414$ U/ml. Inset. Oxygraph recording in the presence of TYR in the same experimental conditions described above. (a) $[DMBH_4]_0 = 4$ mM and $[TYR]_0 = 0.17$ μM; (b) adding to (a) SOD 414 U/ml; (c) adding to (a) CAT 930 U/ml and (d) adding to (a) SOD 414 U/ml and CAT 930 U/ml.

3.2.2.1.2. Effect of enzyme and substrate concentrations: effect of pH. When the substrate concentration is maintained constant, variations in enzyme concentration showed a linear dependence between the product formation rate and $[TYR]_0$ (results not shown). The enzyme concentration was then kept constant while the concentration of substrate was varied. Spectrophotometric recordings were made at 340 nm, where the greatest difference in absorption between the tetrahydropterines and their products, dihydropterins, was evident. Non-linear fitting of the values of V_0 *vs.* $[\text{tetrahydropterine}]_0$ (results not shown) provided the V_{max} and K_m for each of the substrates (Table 1). Knowing the variation in molar absorptivity for each of them (see Materials and methods section), the k_{cat} values can be obtained (see Table 1). The results (high K_m and low k_{cat}) suggest that tetrahydropterines are poor substrates for TYR, as was to be expected since their chemical structure differs greatly from that of physiological substrates of the enzyme (*i.e*: L-tyr and L-dopa) [10] (see Table 1).

When the effect of pH on the tetrahydropterines oxidation kinetics was studied, behaviour similar to that observed for diphenolase activity was obtained, with a kinetically significant pK_a (result not shown) [27].

Based on the results described above, tetrahydropterines are substrates of TYR and compete with monophenols and *o*-diphenols, according to the mechanism described in [Scheme IIA (a) and (b)]. From this mechanism, a kinetic analysis can be carried out. With M = 0, the system reaches the steady-state and the expression of the velocity can be deduced (see Supplementary material). With this mechanism, in the presence of monophenol M, the system does not reach the steady-state proper and so an analytical expression of the steady-state rate cannot be deduced.

3.2.2.2. Kinetic analysis of mechanism shown in Scheme II (A) with M = 0. The mechanism described in Scheme IIA with M = 0 is trisubstrate: *o*-diphenol (D), oxygen (O_2) and tetrahydropterine (reduced substrates, S_R). The equation corresponding to the oxygen consumption rate, $V_0^{O_2}$, is shown in the Supplementary material and is of the type 2:2 as regards *o*-diphenol (D) and tetrahydropterine (S_R) concentrations. Therefore, experiments carried out at a fixed concentration of reducing substrate (S_R), in the order of the Michaelis constant, and varying the concentration of *o*-diphenol (D), the representation of $V_0^{O_2}$ *vs* $[D]_0$ should not be hyperbolic. This is confirmed by Fig. 6, curve b.

Furthermore, if the concentration of reducing substrate, (S_R), or of *o*-diphenol (D) is zero, $V_0^{O_2}$ *vs.* $[D]_0$ or $V_0^{O_2}$ *vs.* $[S_R]_0$ should show hyperbolic behaviour, as happens in (Fig. 6 curves a and c), respectively, (see Supplementary material).

These results confirm that melanogenesis is inhibited by tetrahydropterines acting as alternative competitive substrates.

3.2.3. Irreversible inactivation of met-tyrosinase and deoxy-tyrosinase by tetrahydropterines

The inactivation of the forms E_d and E_m were studied in anaerobic conditions to maintain these enzymatic forms; this is why there are no turnovers and the inhibition is irreversible [Scheme IIB (a)–(c)].

Table 1
Kinetic constants of tetrahydropterin oxidase activities and comparison with monophenolase and diphenolase activities of TYR.

Actividad	K_m^S (mM)	k_{cat} (s^{-1})	$K_m^{O_2}$ (μM)
$6BH_4$oxidase	4.13 ± 0.87	6.61 ± 0.71	0.28 ± 0.06[a]
MBH_4oxidase	4.41 ± 0.91	4.52 ± 0.55	0.19 ± 0.04[a]
$DMBH_4$oxidase	5.01 ± 0.88	3.69 ± 0.60	0.16 ± 0.03[a]
Monophenolase (L-tyr)[b]	0.25 ± 0.03	7.91 ± 0.12	0.52 ± 0.02
Diphenolase (L-dopa)[b]	0.28 ± 0.01	107.31 ± 1.45	4.67 ± 0.06

[a] Calculated from $K_m^{O_2} = k_{cat} / k_8$ [15].
[b] Data obtained from reference [12].

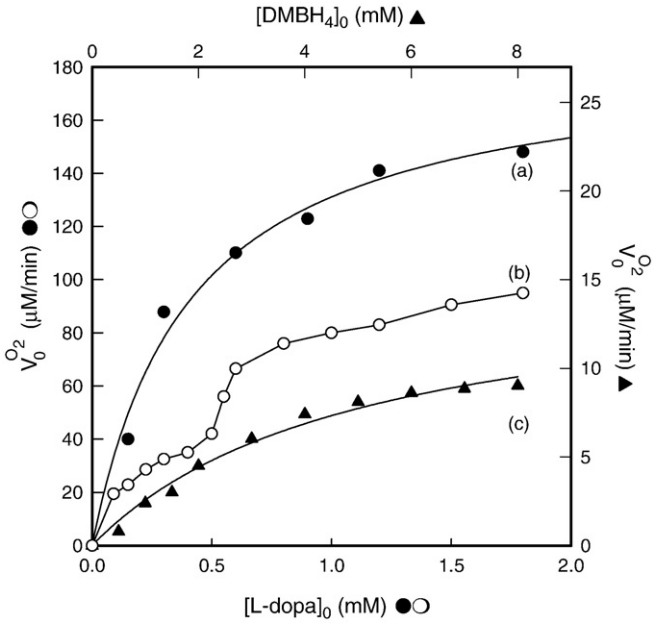


Fig. 6. Graphical representation of the initial rate of oxygen consumption versus substrate concentrations. The experimental conditions were 30 mM sodium phosphate buffer (pH 7.0), 25 °C, SOD 414 U/ml and CAT 930 U/ml. (a) Representation of $V_0^{O_2}$ *vs.* $[\text{L-dopa}]_0$. TYR concentration was 30 nM. (b) As (a) but in the presence of $[DMBH_4]_0 = 8$ mM. (c) Representation of $V_0^{O_2}$ *vs.* $[DMBH_4]_0$, using a TYR concentration of 0.4 μM. Data was normalized with respect to the enzyme concentration.

3.2.3.1. Evaluation of enzymatic species, E_m and E_{ox} in an enzymatic preparation of tyrosinase. *Oxy*-hemocyanin and *oxy*-tyrosinase are in equilibrium with both the E_m and E_d forms [20,28], as illustrated in Scheme III.

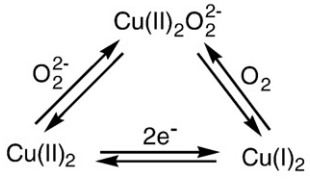
In the presence of oxygen (0.26 mM) and absence of H_2O_2, the principal forms are E_m ($Cu(II)_2$) and E_{ox} ($Cu(II)_2O_2^{2-}$). The concentration of each of these three forms was determined by measuring the inactivation of the native enzyme (1 μM) by irreversible inactivation with 2-mercaptoethanol (10 μM) as described in [25].

3.2.3.2. Inactivation of deoxy-tyrosinase by tetrahydropterines. The enzymatic specie E_d was generated as described in Materials and methods section [25]. In anaerobic conditions, E_d evolves slowly towards another enzymatic form that we denominate E_d^*, as described in Scheme IV.

The forms E_d and E_d^* have different K_m^{TBC} and so the transition can be followed from the measurement of the initial rate of the reaction rate of TBC, as shown in Fig. 7. Data analysis of Fig. 7 according to Eq. (2):

$$\frac{A}{A_0} = \frac{[E_a]}{[E_d]_0} = \alpha + \beta e^{-k_T t} \tag{2}$$

gives the apparent constant of the transition in the conditions described in Fig. 7, where $k_T = (6.5 \pm 0.5) \times 10^{-4} s^{-1}$ and A is the instantaneous enzymatic activity.



Scheme III. Equilibrium between E_m, E_{ox} and E_d forms of TYR.

$$E_d \xrightarrow{k_T} E_d^*$$

Scheme IV. Kinetic mechanism of the transition of E_d to E_d^*.

3.2.3.2.1. E_d^* inactivation by tetrahydropterines. By passing a stream of nitrogen through an enzymatic preparation in the form of E_d (Materials and methods section) for 90 min, the E_d^* formed becomes stable.

When different tetrahydrobiopterines are added to this enzyme form, irreversible inactivation occurs (Fig. 8), which can be described kinetically by Scheme IIB (a). The monoexponential behaviour observed in Fig. 8 can be fitted to the equation:

$$[E_d^*] = [E_d^*]_0 e^{-\lambda_{E_d^*}^{S_R} t} \quad (3)$$

with

$$\lambda_{E_d^*}^{S_R} = \frac{k_{i_{S_R}}^* [S_R]_0}{K_{S_R}^* + [S_R]_0} \quad (4)$$

where S_R, corresponds to $6BH_4$, MBH_4 or $DMBH_4$. The data analysis by non-linear regression shown in Fig. 8 Inset according to Eq. (4) gives the values of $k_{i_{S_R}}^*$ and $K_{S_R}^*$ shown in Table 2. From the data shown in Table 2 it can be seen that the order of $k_{i_{S_R}}^*$ is $DMBH_4 > MBH_4 > BH_4$, reflecting the donating effects of the methyl groups existing in $DMBH_4$ and MBH_4 with respect to BH_4. The values of $K_{S_R}^*$ are in the μM range. From these data, it is seen that in inactivation efficiency is in the order $DMBH_4 > MBH_4 > BH_4$.

3.2.3.2.2. E_d inactivation by tetrahydropterines. When the form E_d, generated from E_{ox} under the nitrogen stream, is made to react with the different tetrahydropterines, it gradually loses its activity. These results are shown in Fig. 9, while its kinetics is depicted in Scheme IIB (b). The monoexponential behaviour shown in Fig. 9, can be fitted to the Eq. (5) analogous to Eq. (3) but with the form E_d:

$$[E_d] = [E_d]_0 e^{-\lambda_{E_d}^{S_R} t} \quad (5)$$

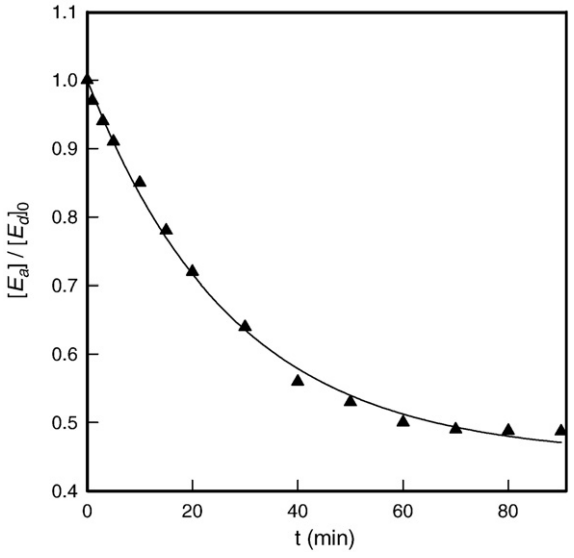


Fig. 7. Kinetic study of the transition of E_d to E_d^*. The enzyme form E_d was generated from E_{ox} by passing a stream of nitrogen over it, $[E]_0$ was 0.1 μM (see Materials and methods section) and $[H_2O_2]_0 = 2$ μM. Aliquots were taken at different times and enzymatic activity was detected with 4 mM TBC (λ = 410 nm). The values obtained were fitted by non-linear regression to Eq. (2).

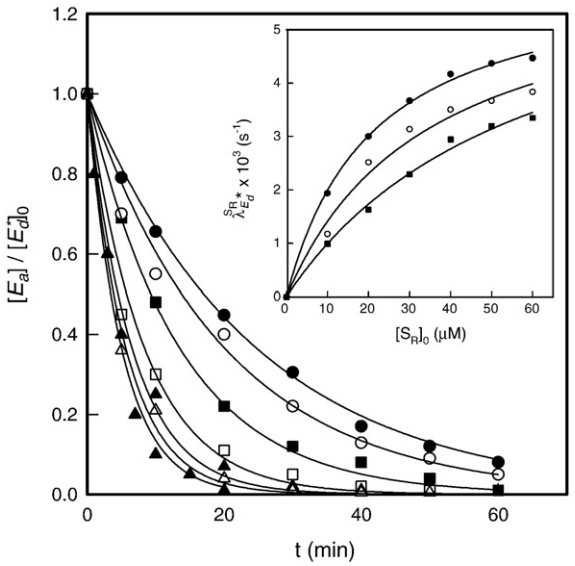


Fig. 8. Inactivation of the E_d^* form of tyrosinase by tetrahydropterines. The form E_d^* was obtained by allowing the form E_d obtained as described in Fig. 7 to evolve in anaerobic conditions for 90 min. At $t = 0$ each of the tetrahydropterines was added, and aliquots were taken at different times to measure the residual activity with 9 mM TBC (λ = 410 nm). The values obtained were fitted to Eq. (3) and the apparent inactivation constant ($\lambda_{E_d^*}^{S_R}$) was obtained at each substrate concentration. The experimental conditions were: 30 mM sodium phosphate buffer (pH 7.0), 25 °C, $[E]_0$ 0.1 μM (see Materials and methods section), $[H_2O_2]_0$ 2 μM and $DMBH_4$ (μM) of 10 ●, 15 ○, 20 ■, 30 □, 40 ▲, 50 △, and 60 ▼. Inset. Representation of $\lambda_{E_d^*}^{S_R}$ *vs.* $[S_R]_0$ for each compound used: $DMBH_4$ (●) MBH_4 (○) and $6BH_4$ (■). In the cases of $6BH_4$ and MBH_4, the concentrations were the same as for $DMBH_4$.

with

$$\lambda_{E_d}^{S_R} = \frac{k_{i_{S_R}} [S_R]_0}{K_{S_R} + [S_R]_0} \quad (6)$$

As above, S_R corresponds to $6BH_4$, MBH_4 or $DMBH_4$. Non-linear analysis of the data shown in Fig. 9 according to Eq. (5) provides the values of $\lambda_{E_d}^{S_R}$. In turn, the non-linear analysis of these values according to Eq (6) (Fig. 9, Inset A) provides the values of $k_{i_{S_R}}$ and K_{S_R} shown in Table 2. Note that the results indicated in Table 2 for E_d show that the order of $k_{i_{S_R}}$ is the same as that obtained for E_d^* but higher. These data can also be explained by the fact that the copper atoms of the active site in E_d^*, which evolves from E_d, are more separated than in E_d, as has been described for hemocyanin [29]. The values of K_{S_R} are in the same range as those of E_d^*. These data show that the inactivation efficiency is in this order $DMBH_4 > MBH_4 > BH_4$.

3.2.3.3. E_m inactivation by tetrahydropterines. The form E_m is stable in anaerobic conditions but, in the presence of tetrahydropterines,

Table 2
Kinetic constants which characterise the inactivation of E_d, E_m and E_d^* by tetrahydropterines in anaerobic conditions.

Enzymatic form	Substrate	K_{S_R} (μM)	$k_{i_{S_R}}$ (s^{-1})	$k_{i_{S_R}} / K_{S_R} \times 10^2$ ($\mu M^{-1} s^{-1}$)
E_d^*	BH_4	58.72 ± 9.81	0.37 ± 0.04	0.51 ± 0.09
	MBH_4	34.51 ± 7.68	0.41 ± 0.04	1.19 ± 0.51
	$DMBH_4$	20.98 ± 1.68	0.47 ± 0.01	2.24 ± 0.59
E_d	BH_4	68.29 ± 9.97	0.55 ± 0.04	0.81 ± 0.04
	MBH_4	35.16 ± 8.81	0.56 ± 0.06	1.59 ± 0.61
	$DMBH_4$	22.53 ± 4.41	0.58 ± 0.05	2.57 ± 0.04
E_m	BH_4	68.29 ± 9.97	0.55 ± 0.04	0.81 ± 0.04
	MBH_4	35.16 ± 8.81	0.56 ± 0.06	1.59 ± 0.61
	$DMBH_4$	22.53 ± 4.41	0.58 ± 0.05	2.57 ± 0.04

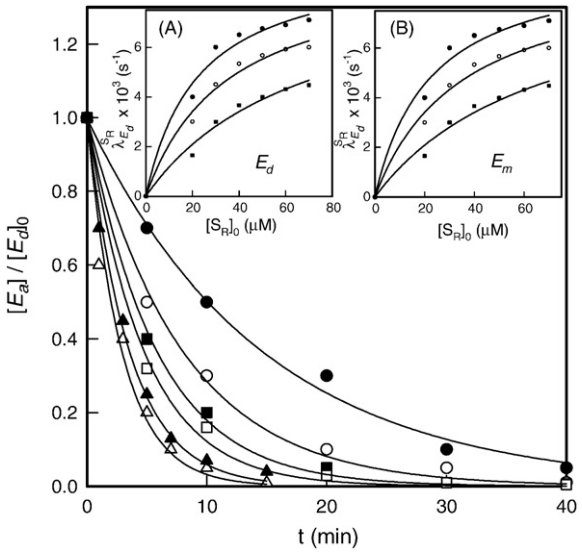


Fig. 9. Inactivation of the E_d form of TYR by tetrahydropterines. The form E_d was obtained (see Materials and methods) and immediately incubated with tetrahydropterines. Aliquots were taken at different times to measure the residual activity with TBC. The experimental conditions were: 30 mM sodium phosphate buffer (pH 7.0), 25 °C, $[TBC]_0$ 9 mM ($\lambda = 410$ nm), $[E]_0$ 0.1 μM (see Materials and methods section), $[H_2O_2]_0$ 2 μM and $DMBH_4$ (μM) of 20 ●, 30 ❍, 40 ■, 50 □, 60 ▲ and 70 △. The data were fitted to Eq. (5) and the apparent inactivation constants ($\lambda_{E_d}^{S_R}$) were obtained. Inset (A). Representation of $\lambda_{E_d}^{S_R}$ *vs.* $[S_R]_0$; $DMBH_4$ (●). MBH_4 (❍) and $6BH_4$ (■). In the cases of $6BH_4$ and MBH_4, the concentrations were the same as for $DMBH_4$. Inset (B). In the case of E_m the same procedure was followed as for E_d. Representation of $\lambda_{E_m}^{S_R}$ *vs.* $[S_R]_0$; $DMBH_4$ (●). MBH_4 (❍) and $6BH_4$ (■).

undergoes an inactivation which can be kinetically characterised in the following way. The form E_m, generated as described in Materials and methods section [25], is preincubated anaerobically with each of the tetrahydropterines. Aliquots are taken at different times to determine residual activity. To explain the irreversible inactivation, Scheme IIB (c), is proposed.

The residual activity with time shows analogous behaviour to the form E_d. The transition from E_m to E_d occurs rapidly, since it corresponds to stages of the catalytic cycle of TYR governed by k_2,k_{-2} and k_3. The form E_m of the enzyme is rapidly transformed into E_d, Scheme IIB (c). Fitting of the residual activity data by non-linear regression according to Eq. (5) gives the values to $\lambda_{E_m}^{S_R}$, which when analysed according to Eq. (6) provides the constants shown in Table 2, which are the same as those calculated for E_d.

3.2.3.4. Oxygen protects against the inactivation of tyrosinase. TYR catalyses a bisubstrate reaction with O_2 and a reductant, in this case, tetrahydropterines. The most instable form in the enzyme's turnover, Scheme IIA, is E_d. However, this form is practically non-existent in the turnover since it binds to the O_2 with a low constant [15]. The $K_m^{O_2}$ values are also small [30], and, at the oxygen concentration of the solutions (0.26 mM), the enzyme is saturated by O_2. The other substrate, tetrahydropterines, and, generally speaking, most of the *o*-diphenols and triphenols are true suicide substrates, this process occurring during electron transfer to the copper atoms.

4. Discussion

Recently, it has been suggested that the coenzyme $6BH_4$ and their analogues (MBH_4 and $DMBH_4$) might act as regulators in melanin biosynthesis through their action on TYR [8,31–33]. It has been observed that $6BH_4$ inhibits the monophenolase but not the diphenolase activity of TYR [6,33], which has led to the suggestion that it has two different active sites for each kind of activity [6,33].

These experimental results have been poorly interpreted. In the first place, the large body of experimental evidence demonstrating the existence of only one active site for both activities of TYR has not been considered [34,35]. Neither have the many non-enzymatic coupled reactions which originate the heteropolymer complexes denominated eumelanins and phaeomelanins from *o*-dopaquinone [36] been taken into account.

In the present article, we demonstrate that $6BH_4$ (and their analogues) reduce *o*-dopaquinone non enzymatically, in agreement with experimental observations made in the literature [9,13,14], both at low [13] and high [14] concentrations of $6BH_4$. This reducing property of *o*-dopaquinone would be the principal physiological cause of the inhibitory effect of $6BH_4$ on melanogenesis when $6BH_4$ exists inside the melanosomes. Melanogenesis would continue more rapidly when this $6BH_4$ inside the melanosomes was used up.

Furthermore, we demonstrate that $6BH_4$ can act as substrate of TYR, although with low affinity ($K_m = 4.13 \pm 0.87$ mM) with respect to L-Tyr ($K_m = 0.25 \pm 0.03$ mM) and L-dopa ($K_m = 0.28 \pm 0.01$ mM). Consequently, $6BH_4$ could act as a competitive substrate with L-Tyr and/or L-Dopa with respect to TYR, but only when low concentrations of L-Tyr and L-Dopa in the melanosomes were accompanied by high concentrations of $6BH_4$. The inhibition of melanogenesis, therefore, by $6BH_4$ through its action as competitive substrate of TYR, would only take place in very specific conditions.

Lastly, we have also demonstrated that $6BH_4$ may act as irreversible inhibitor of TYR in anaerobic conditions. In this way, $6BH_4$ would inhibit melanogenesis when it is present in high concentrations in the melanosomes, accompanied by low concentrations of L-Tyr and L-dopa and a very low concentration of oxygen.

Taking into consideration the three mechanisms of melanogeneis by $6BH_4$, it seems that its physiological significance decreases in the following order: non-enzymatic reductant through *o*-dopaquinone > competitive substrate of TYR > irreversible inhibitor of TYR.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbapap.2009.08.011.

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