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Formation of a Stable Quinone Methide during Tyrosinase-Catalyzed Oxidation of α-Methyl Dopa Methyl Ester and Its Implication in Melanin Biosynthesis

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Mushroom tyrosinase-catalyzed oxidation of α-methyl dopa methyl ester resulted in the formation of "iminochrome" similar to the well-known dopa–dopachrome conversion. At pH 5, the iminochrome formed was found to be stable and accumulated in the reaction mixture. However, when the reaction was carried out under near neutral and slightly alkaline conditions, the iminochrome formed readily tautomerized into a quinone methide isomer, which accumulated in the reaction mixture. The structure of the quinone methide formed was established by ultraviolet and visible spectroscopy and cochromatography with a synthetic sample. These results demonstrate the facile generation of quinone methide from iminochrome under physiological conditions and strongly support the possible occurrence of a similar reaction during melanogenesis.

1. INTRODUCTION

A wide variety of catechol derivatives are found in nature and serve in diverse biological processes such as neurotransmission (*1*), hormonal action (*1*), oxidative browning of plant products (*2*), siderophore formation (*3*), melanization (*4, 5*), and sclerotization of arthropod cuticle (*6–9*). Central to the metabolism of catecholamines is their oxidation by ubiquitously present phenoloxidases (*10, 11*). Phenol oxidases [*o*-diphenol, oxygen oxidoreductase EC 1.10.3.1] oxidize a variety of catechols to their corresponding quinones. The fate of enzymatically generated quinones has not been determined in all cases. The quinones of dopa and dopamine either react internally followed by a series of transformations before yielding polymeric eumelanin products (*4, 5*) or react with external nucleophiles to account for pheomelanin products (*5*). Amino-protected (or modified) catecholamines in general do not exhibit the internal reactivity, but show usually external reactivity and play a key role in quinone tanning of insect cuticle (*6–9, 12, 13*) and oxidative browning of plant products (*2*). Other reactions of quinones have not been well understood.

As early as 1958, Witkop and his associates pointed out novel quinone methide formation from quinones derived from substituted catecholamine derivatives and

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suggested that such reactions might play a key role in the biosynthesis of norepinephrine derivatives (*14, 15*). Accordingly, in recent years we identified an enzymatic route for the generation of *N*-acylnorepinephrine in insects and characterized a novel quinone : quinone methide isomerase which converts 4-alkyl-*o*-benzoquinones to 2-hydroxy-*p*-quinone methides as an integral part of insect immunity and tanning machinery (*16–20*). In addition, we have demonstrated the presence of a new enzyme which converts enzymatically generated *N*-acetyldopamine quinone methide into 1,2-dehydro-*N*-acetyldopamine (*21–23*). We also reported the production of quinone methides during (a) enzyme-catalyzed oxidative decarboxylation of 3,4-dihydroxymandelic acid (*24*), (b) tyrosinase-catalyzed oxidation of 3,4-dihydroxyphenylacetic acid (*25*), (c) oxidative dimerization of 1,2-dehydro-*N*-acetyldopamine (*26, 27*), and (d) side chain desaturation of dihydrocaffeic acid derivatives (*28, 29*). Therefore, we felt that quinone methide formation should be more common to biology and began to investigate the detailed oxidation chemistry of a number of catecholamine derivatives. During our search, we came across an interesting finding by Musson *et al.* (*30*), who reported the production of a stable quinone methide by chemical oxidation of α-methyl dopa ethyl ester. In this communication, we present evidence that a similar quinone methide is also produced under physiological conditions during tyrosinase-catalyzed oxidation of α-methyl dopa methyl ester and propose that quinone methides may be involved as transient intermediates even during the biosynthesis of eumelanins originating from dopa and related compounds.

2. MATERIALS AND METHODS

2.1 Materials

Mushroom tyrosinase [sp act 4000 U/mg] and α-methyl dopa were purchased from Sigma Chemical Co. (St. Louis, MO). α-Methyl dopa was esterified as follows: A mixture of α-methyl-L-dopa (5.27 g, 0.025 mol) in methanol (235 ml) was saturated with HCl gas and stirred at room temperature for 25 h. Removal of solvent on a rotary evaporator followed by chromatography of the product on a Bio-Gel P-2 column using 0.2 M acetic acid gave a white solid of methyl ester (2.2 g; yield, 39%). ^{1}H NMR: (DMSO-d_6) δ = 1.58 (singlet, 3H, CH_3), 3.05 (singlet, 2H, CH_2), 3.80 (singlet, 3H, $COOCH_3$), 6.35–6.92 (multiplet, 3H, ArH), 5.20–6.00 and 8.20–9.00 ppm (broad singlet, 4H, OH + NH_2—exchanged with D_2O).

The bicyclic quinone methide derived from oxidative cyclization of α-methyl dopa methyl ester was prepared as follows: To a mixture of $K_3Fe(CN)_6$ (376 mg) and $KHCO_3$ (125 mg) in water (10 ml)/benzene (5 ml) placed in an ice bath, a solution of α-methyl dopa methyl ester (100 mg) in 0.05 M sodium phosphate buffer, pH 7.0 (6 ml), was added slowly over a period of 10 min and the mixture was stirred for 1 h. The benzene layer was recovered at the end of this period and fresh benzene was added to extract the product. The process of extraction and separation was repeated five times. The recovered benzene layers were pooled, dried over anhydrous Na_2SO_4, and evaporated to dryness on a rotary evaporator.

Recrystallization of the product with benzene/hexane afforded 40 mg of yellow colored solid (yield 41%), mp 95–96°C; uv (methanol) λ_{max} = 422, 322(sh), 309 nm. ir (KBr) $\bar{\nu}$ = 3300, 3100, 1730, 1580, 1540, 1440, 1385, 1260, 1215, 1195, 1120, 940, 860 cm^{-1}. ^1H NMR (DMSO-d_6) δ = 1.58 (s, 3H, CH_3), 3.70 (s, 3H, $COOCH_3$), 5.45 (s, 1H, =CH), 6.22 (s, 1H, =CH), 6.90 (s, 1H, =CH), 9.15 ppm (broad s, 2H, NH and OH—exchanged with D_2O).

2.2 Methods

Spectral changes associated with the enzymatic oxidation of α-methyl dopa methyl ester were carried out using a Gilford Model 2600 spectrophotometer. The reaction mixture (1 ml) contained varying amounts of substrate and tyrosinase (5–50 μg) in 0.1 M phosphate buffer at different pH values.

HPLC studies were carried out using Altex Model 100A pumps, a Altex Model 210 50-μl loop injector, a variable wavelength detector set at 434 nm with a flow cell (Hitachi spectrophotometer, Model 100-30), and a recorder-integrator (Altex, Model C-R1A). Separations were achieved on an Ultrasphere ODS column (5 μm, 4.6 × 150 mm) using isocratic elution with 35% methanol–65% 50 mM acetic acid containing 0.2 mM sodium octyl sulfonate at a flow rate of 1 ml/min.

3. RESULTS AND DISCUSSION

Mushroom tyrosinase has been shown to exhibit wide substrate specificity (*2, 11, 13, 24, 26*). Accordingly, it oxidized a number of *o*-diphenolic compounds such as catechol, 4-methylcatechol, 3,4-dihydroxybenzoic acid, 3,4-dihydroxybenzylamine, 3,4-dihydroxyphenylacetic acid, 3,4-dihydroxymandelic acid, 3,4-dihydroxyphenylpropionic acid, caffeic acid, dopa, dopamine, norepinephrine, epinephrine, *N*-acetyldopamine, *N*-β-alanyldopamine, *N*-acetylnorepinephrine, 1,2-dehydro-*N*-acetyldopamine, and α-methyl dopa methyl ester. In most cases, enzymatic oxidation resulted in the formation and accumulation of the corresponding *o*-benzoquinones in the reaction mixture. However, some compounds produced other products. For example, during the oxidation of 3,4-dihydroxymandelic acid, 3,4-dihydroxybenzaldehyde was generated as the final product (*24*). The oxidation of 3,4-dihydroxyphenylacetic acid yielded a mixture of products which included 3,4-dihydroxymandelic acid, 3,4-dihydroxybenzaldehyde, and 2,5,6-trihydroxybenzofuran (*25*). 1,2-Dehydro-*N*-acetyldopamine was converted into the benzodioxan dimer (*26, 27*). Dopa, dopamine, epinephrine, and norepinephrine were converted to the corresponding "iminochromes." Similarly, α-methyl dopa methyl ester was converted to the iminochrome which exhibited a visible absorbance maximum at about 475 nm (Fig. 1A). As shown in Fig. 1A, the iminochrome formed from α-methyl dopa methyl ester was reasonably stable at pH 5.0 and accumulated in the reaction mixture. However, when the pH was raised to 8.0, the iminochrome rapidly transformed to another product which exhibited a visible absorbance maximum at around 420 nm (Fig. 1D). In between, the transformation was found to be slower (Figs. 1B and 1C).

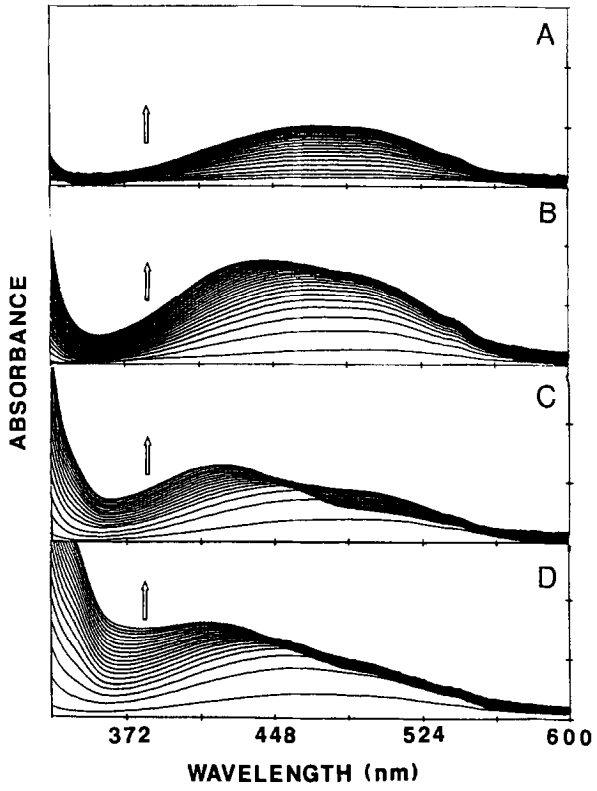

FIG. 1. Visible spectral changes associated with the oxidation of α-methyl dopa methyl ester. A reaction mixture containing 80 μg of α-methyl dopa methyl ester, specified amounts of mushroom tyrosinase in 1 ml of 0.1 M phosphate buffer was incubated at room temperature and the visible spectral changes associated with the oxidation were monitored at 2-min intervals. (A) With 25 μg enzyme in citrate phosphate buffer, pH 5.0, (B) with 5 μg enzyme in sodium phosphate buffer, pH 6.0; (C) with 5 μg enzyme in sodium phosphate buffer, pH 7.0; (D) with 5 μg enzyme in sodium phosphate buffer, pH 8.0.

Such a change in spectral properties was indeed due to the transformation of iminochrome to another product as confirmed by two different experiments. First, when the iminochrome was generated rapidly with excess tyrosinase and then the spectral changes were monitored, the formation of the compound absorbing at 420 nm coincided with the loss of iminochrome in the reaction mixture (Fig. 2). Second, HPLC studies of the reaction mixture attested to the rapid transformation of iminochrome to the new compound. Figures 3 and 4 show the HPLC analysis of the enzymatic reaction at two different pH values. At pH 5.0, the only product detectable by absorbance at the visible region (monitored at 434 nm) was the iminochrome (Fig. 3, 2.58-min peak). As is clear from the figure, the iminochrome formed accumulates in the reaction mixture. However, at pH 8.0, although iminochrome accumulated during the initial period, it was found to be rapidly transformed to additional products as evidenced by the appearance of new peaks (Fig.

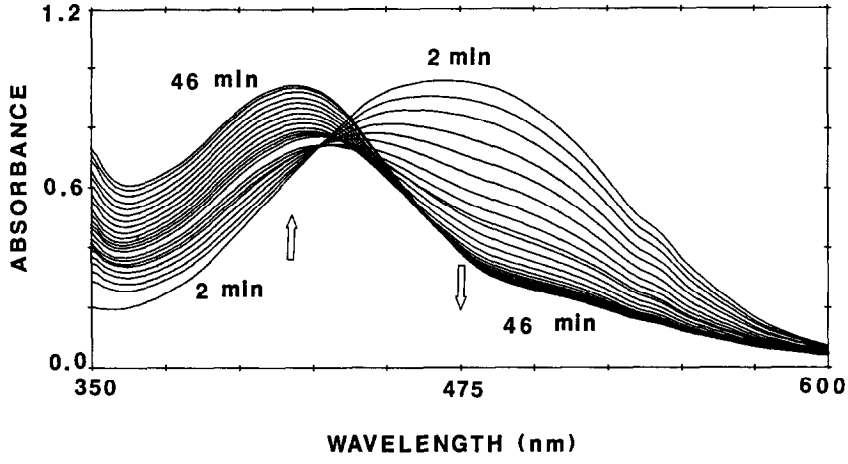

FIG. 2. Transformation of iminochrome into quinone methide. A reaction mixture containing 2 mg of α-methyl dopa methyl ester, 50 μg of mushroom tyrosinase in 1 ml of 0.1 M sodium phosphate buffer, pH 7.0, was incubated at room temperature and the visible spectral changes associated with the enzymatic oxidation were monitored at 2-min intervals. Note the rapid appearance of a 475-nm peak due to iminochrome and its transformation to quinone methide (420-nm peak).

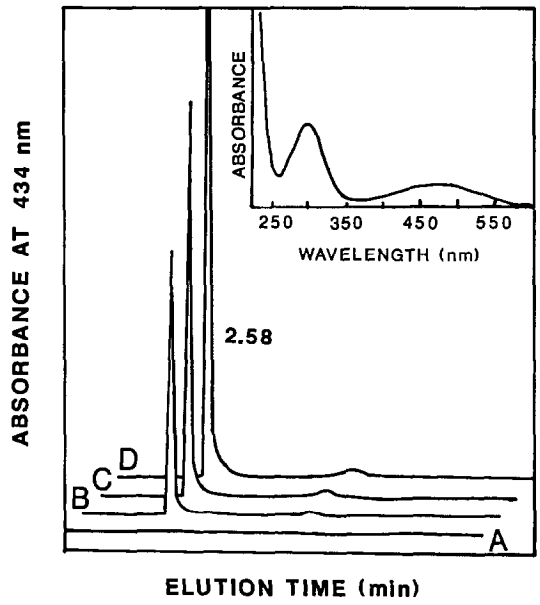

FIG. 3. HPLC analysis of the reaction mixture. A reaction mixture containing 200 μg of α-methyl dopa methyl ester, 5 μg of mushroom tyrosinase in 1 ml of 0.1 M citrate phosphate buffer, pH 5.0, was incubated at room temperature and a 20-μl aliquot was subjected to HPLC analysis as outlined under Materials and Methods at specified time intervals. (A) Zero time, (B) 12 min (C) 24 min; and (D) 72 min. Inset: Ultraviolet and visible spectrum of a 2.58-min peak. It corresponded well with that of iminochrome formed in the reaction mixture.

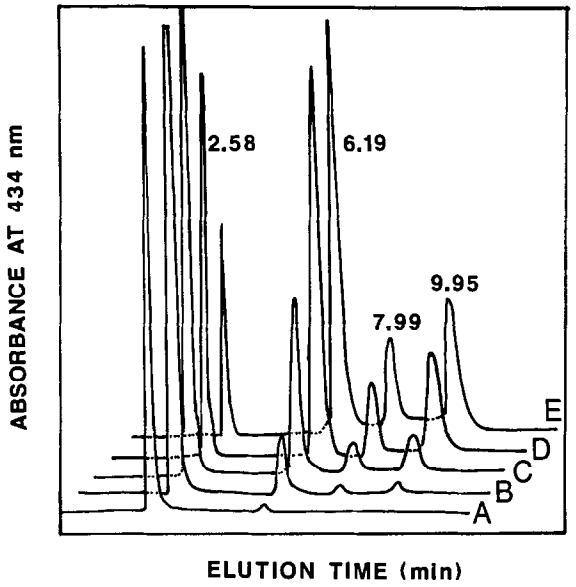

FIG. 4. HPLC analysis of the reaction mixture. A reaction mixture containing 200 μg of α-methyl dopa methyl ester, 5 μg of mushroom tyrosinase in 1 ml of 0.1 M sodium phosphate buffer, pH 8.0, was incubated at room temperature and a 20-μl aliquot was subjected to HPLC analysis as outlined under Materials and Methods at specified time intervals. (A) 30 s, (B) 12 min, (C) 24 min, (D) 60 min, and (E) 72 min. The peak at 2.58 min is due to iminochrome and the peak at 6.19 min is due to quinone methide. The minor peaks at 7.99 and 9.95 min arise from these compounds and were not identified due to their instability and low concentration.

4). Earlier, Musson *et al.* (*30*) have reported the isolation of a quinone methide as the stable product of oxidation of α-methyl dopa ethyl ester. Therefore, we chemically synthesized the quinone methode derived from α-methyl dopa methyl ester using the procedure developed by Musson *et al.* (*30*) and compared its chromatographic and spectral behavior with those of the enzymatic product. As shown in Fig. 5 (trace B), the synthetic quinone methide migrated with the same retention time as that of the enzymatic product. On cochromatography, both the 6.19-min peak and the synthetic sample migrated together under different HPLC conditions, indicating that they are one and the same compound. Finally, the ultraviolet and visible spectra of both synthetic and enzymatic products shown in Fig. 6 also attested to their identity. For comparison, the spectrum of iminochrome is also shown in the same figure.

The 7.99-min and 9.95-min peaks observed in Figs. 4 and 5 arose probably by the decomposition of iminochrome or the quinone methide in a pH-dependent reaction, as we witnessed the production of these two compounds more at the alkaline range than at the neutral pH. Due to the instability of these compounds and their low concentration, the structure of these compounds could not be determined.

Figure 7 accounts for the observed quinone methide formation from α-methyl

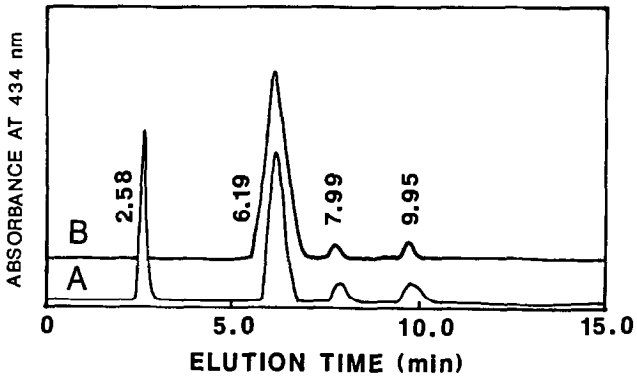

FIG. 5. HPLC analysis of the reaction mixture and synthetic quinone methide. (A) A reaction mixture containing 200 μg of α-methyl dopa methyl ester, 10 μg of mushroom tyrosinase in 1 ml of 0.1 M sodium phosphate buffer, pH 7.0, was incubated at room temperature for an hour and a 20-μl aliquot was subjected to HPLC analysis as outlined under Materials and Methods. (B) Synthetic quinone methide. For peak identification see legend to Fig. 4.

dopa methyl ester. Tyrosinase-catalyzed oxidation of α-methyl dopa methyl ester (structure A, Fig. 7) generates the corresponding *o*-benzoquinone which undergoes a rapid intramolecular cyclization to produce the dienone (structure C, Fig. 7). Dienone phenol rearrangement of the latter yields the leucochrome which is oxidized to the iminochrome (structure E, Fig. 7). Iminochrome isomerizes to the quinone methide (structure F, Fig. 7) in a pH-dependent reaction. At present it is

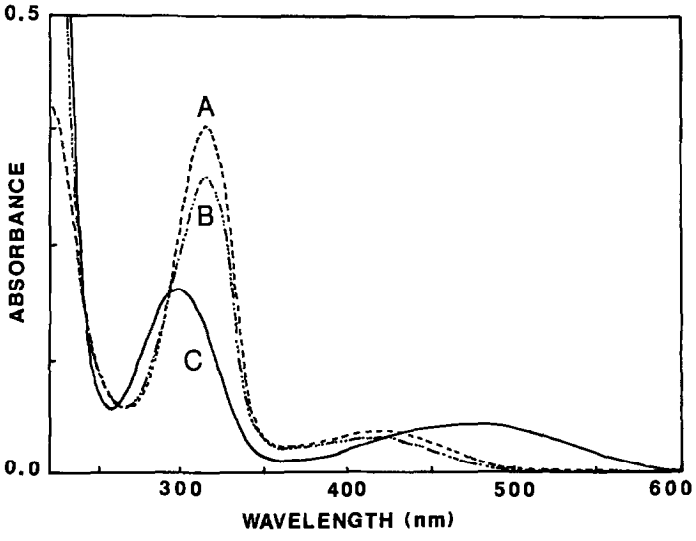

FIG. 6. Ultraviolet and visible spectrum of (A) the quinone methide isolated from enzymatic reaction, (B) the synthetic quinone methide, and (C) iminochrome derived from α-methyl dopa methyl ester.

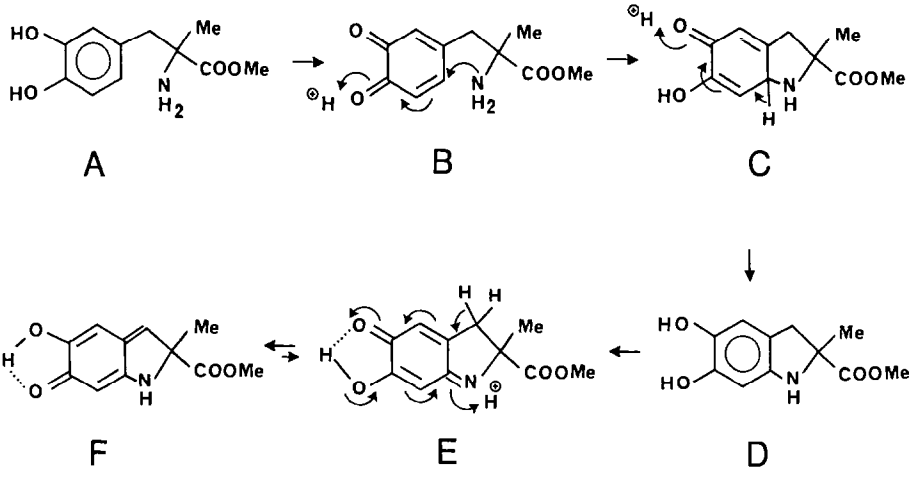

FIG. 7. Proposed mechanism for quinone methide formation. Tyrosinase-catalyzed oxidation of α-methyl dopa methyl ester (A) generates the corresponding quinone (B) which undergoes rapid intramolecular cyclization to form the leuchrome (D). Oxidation of leucochrome produces the iminochrome (E) which undergoes isomerization to the quinone methide (F).

not clear why this bicyclic quinone methide is stable while the corresponding simple quinone methides are known to be highly unstable and undergo rapid Michael 1,6-addition reactions (*6–9*). But the facile generation of quinone methide from iminichrome indicates that a similar reaction is likely to occur even with the parent compound, dopachrome. Dopachrome is a key intermediate formed during melanin biosynthesis from tyrosine and its hydroxylated derivative, dopa (*4, 5*). Recently it has been shown that dopachrome is converted either enzymatically by dopachrome oxidoreductase to 5,6-dihydroxyindole-2-carboxylic acid or nonenzymatically to both 5,6-dihydroxyindole and 5,6-dihydroxyindole-2-carboxylic acid (*31, 32*). The mechanism shown in Fig. 8 (B → C → D) has been proposed to account for the conversion of dopachrome to 5,6-dihydroxyindole-2-carboxylic acid. According to this mechanism, the dopachrome is converted into the indolidine derivative which rapidly tautomerizes to generate the indole. However, the conversion of iminochrome to an indolidine derivative, as shown by the electron rearrangement, attributes an electron donating property to the imino group. But in reality this group is electron withdrawing and is in fact responsible for the observed stability of iminochrome (since both the carbonyl group and the imino group withdraw electrons in the opposite directions, the quinonoid structure is comparatively stabilized). The alternate mechanism (Fig. 8, B → E → D) involving quinone methide intermediate overcomes this difficulty and is supported by the results presented in this study. The quinone methide formed by the rearrangement of iminochrome will rapidly tautomerize to more a stable indole derivative. We have proposed a similar tautomerization route for the conversion of quinone methide to α,β-unsaturated catecholamine derivatives, in particular for the biosynthesis of 1,2-dehydro-*N*-acetyldopamine from *N*-acetyldopamine quinone

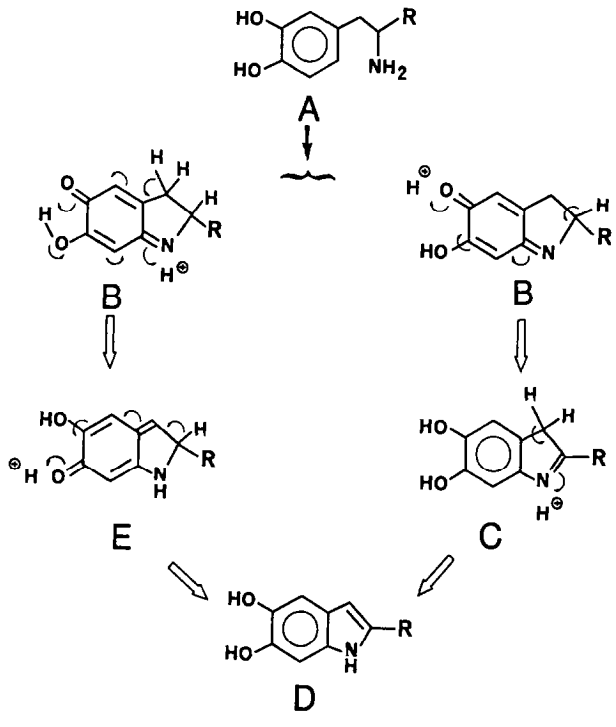

FIG. 8. Two possible mechanisms for the formation of 5,6-dihydroxyindoles. Dopachrome (B, R = COOH) derived from the oxidative coupling of dopa (A, R = COOH) is converted to an indolidine derivative (C, R = COOH) which tautomerizes to form 5,6-dihydroxyindole-2-carboxylic acid (D, R = COOH). A similar reaction of dopaminechrome (B, R = H) yields 5,6-dihydroxyindole (D, R = H). Alternatively, these compounds can also arise through the quinone methide (E) as indicated.

methide (*7–9*), and recently confirmed the existence of such a route by isolating and characterizing the enzyme involved in this process (*21–23*). Therefore, it is likely that such a route is operative even during the conversion of dopachrome to indole derivatives. However, further experiments are essential to throw more light on this aspect and we are currently extending our studies in this direction.

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