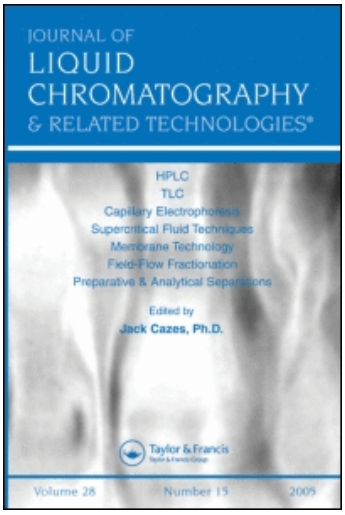

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CHROMATOGRAPHIC ANALYSES OF THE EFFECTS OF GLUTATHIONE, CYSTEINE AND ASCORBIC ACID ON THE MONOPHENOL AND DIPHENOL OXIDASE ACTIVITIES OF TYROSINASE

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ABSTRACT

The effects of glutathione, cysteine, and ascorbic acid on the monophenol and diphenol oxidase functions of tyrosinase were assessed by high performance liquid chromatography with electrochemical detection (HPLC-ED) at both oxidative and reductive potentials. The enzyme-catalyzed hydroxylations of tyrosine to dopa and tyramine to dopamine were inhibited completely by glutathione and cysteine, but not by ascorbic acid. However, the rates of oxidation of dopa and dopamine were enhanced approximately 5% by cysteine and 75% by glutathione. There was no chromatographic evidence to indicate that either thiol reduced o-quinones back to their respective o-diphenols, a reaction that was documented for ascorbic acid. Glutathione and cysteine each formed sulfhydryl conjugates with the oxidation products of both dopa and dopamine. The thiol-mediated alterations in tyrosinase activity are likely due to the direct interactions of these sulfhydryl compounds with the enzyme, suggesting that the

availability and relative quantities of glutathione and cysteine at the sites of o-quinone formation may have a profound effect on quinone cytotoxicity. Under certain conditions the nucleophilic addition of glutathione and cysteine to o-quinones may represent a mechanism for regulating quinone cytotoxicity. However, glutathione-enhanced diphenol oxidase activity can potentiate cytotoxic damage by generating oxyradicals, depleting cells of o-diphenols, and lowering the level of glutathione available for antioxidant activity.

INTRODUCTION

Tyrosinase (EC 1.14.18.1) plays a critical role in the biosynthesis of melanins by catalyzing two rate-limiting reactions, the initial hydroxylation of tyrosine to dopa (monophenol oxidase), and the ensuing oxidation of dopa to o-dopaquinone (diphenol oxidase). O-quinones derived from o-diphenols such as dopa and dopamine are extremely reactive substances that either undergo nucleophilic addition with sulphydryls forming thiol conjugates and precursors of pheomelanin, or become oxidized to indolequinones that form eumelanin (1). Alternation between eumelanogenesis and pheomelanogenesis is controlled by several factors including the availability of sulfhydryl compounds, the level of activity of tyrosinase and ancillary enzymes (dopachrome tautomerase and peroxidase), and the availability of certain metal ions, such as copper and iron, which accumulate in pigmented cells (1-3).

There is considerable interest in knowing how sulfhydryl compounds, such as glutathione and cysteine, modify melanogenic processes in both malignant melanocytes (4-11), and melanotic catecholaminergic neurons that are believed to be selectively destroyed in patients with Parkinson's disease (12-19). Conflicting reports are found in the literature concerning the actual mechanism(s) of interaction of these thiols with tyrosinase. While some studies show glutathione and/or cysteine to have no inhibitory effect on tyrosinase activity (20), other reports indicate that these compounds inhibit the enzyme (8, 21-26), suppress catechol oxidation (27), and interrupt melanogenesis (7, 26, 28). Jara *et*

al. (2) reported that both tyrosine hydroxylase and dopa oxidase functions of melanoma tyrosinase were inhibited by cysteine and increased by glutathione. With mushroom tyrosinase they reported increased monophenol oxidase activity with both thiol compounds, but their effects on the diphenol oxidase function of the enzyme were not investigated.

In this investigation high pressure liquid chromatography with electrochemical detection (HPLC-ED) was used to elucidate the differential effects of glutathione, cysteine, and ascorbic acid on both the monophenol and diphenol oxidase activities of mushroom tyrosinase. By employing extremely sensitive electrochemical methods enzyme incubation times are reduced. This essentially eliminates both the effects of reaction products derived from autoxidation, and the need for diphenol cofactors that otherwise compete with the monophenol substrate for the active site of the enzyme. In addition, the HPLC-ED methods provided a mechanism for continuous monitoring of the exact content of substrate, thiols, and products. The data obtained by these methods provide convincing evidence that at physiological concentrations, glutathione and cysteine are incapable of suppressing tyrosinase-mediated oxidations of dopa and dopamine, and in the case of glutathione, this function of the enzyme is enhanced significantly.

MATERIALS AND METHODS

5, 6-Dihydroxyindole (DHI) and 5,6-dihydroxyindole-2-carboxylic acid (DHICA) were obtained from Regis Chemical Company (Morton Grove, Illinois). Mushroom tyrosinase (EC 1.14.18.1, 3300 units/mg) and all other chemicals were obtained from Sigma Chemical Company (St. Louis, Missouri). The oxidations of dopa and dopamine were monitored spectrophotometrically (Δ OD 480 nm) by a Virion spectrophotometer interfaced with a recorder, and electrochemically by reversed-phase, ion-pairing high pressure liquid chromatography. The HPLC-ED system consisted of a Bioanalytical Systems (West Lafayette, IN) LC-4B electrochemical detector equipped with a glassy

carbon working electrode and a Ag/AgCl reference electrode. Oxidative potentials (+700 mV or +850 mV) were employed to monitor the oxidation of o-diphenols (dopa and dopamine) and to detect their immediate electrochemically oxidizable reaction products (leucodopachrome, leucodopaminechrome, and/or 5,6-dihydroxyindoles). A reductive potential (-100 mV) was employed to detect electrochemically reducible compounds, such as o-quinones, dopachrome and dopaminechrome, produced during the reactions. Separations were achieved at 40°C by a BAS Phase-II, 3-μm ODS reverse phase column (3.2 mm I.D. x 10 cm). A Gilson (Madison, WI) 712 HPLC System Controller was used to integrate peak areas. The standard mobile solvent system used throughout the study was comprised of 25 mM citrate buffer (pH 3.0) containing 2.5% acetonitrile, 0.5 mM sodium octylsulfate, and 0.7 mM Na_2EDTA. Two additional chromatographic conditions were used to identity electroactive reaction products. The second mobile phase was similar to the first except the concentration of citric acid was reduced to 15 mM. In the third solvent system 35 mM NaCl substituted for the acetonitrile. Electroactive components were identified by their co-elution with authentic standards under all three different chromatographic conditions, and amplification of their peak dimensions in proportion to the amount of standard co-injected with the reaction samples. The flow rate was maintained at 0.8 ml/min. Where appropriate, standard curves were prepared for each component in the reaction mixtures, and the data analyzed by linear regression. Calibration curves were calculated prior to and after each test using varying amounts (4 calibration points) of each component in the reaction mixtures. Known amounts of different catechols were occasionally incorporated in reaction mixtures to serve as internal standards. The correlation coefficient of the calibration curves established for the standards typically was greater than 0.98.

Enzyme activity was measured in incubation mixtures containing 20 μg enzyme protein and various concentrations (0-6.6 mM) of ascorbic acid, glutathione, or cysteine in a total volume of 300 μL in 0.16 M potassium

phosphate buffer (pH 7.2). Non-enzymatic oxidations of dopa and dopamine were initiated by incorporation of 0.3 mM $NaIO_4$ into the standard reaction mixture. For tyrosinase-mediated oxidations of the monophenols tyrosine and tyramine the concentration of substrate used was 1 mM, and for the diphenols dopa and dopamine the concentration was 0.3 mM. To clearly evaluate the different effects of ascorbic acid and thiol compounds on the monophenol and diphenol oxidase functions of tyrosinase, no diphenol cofactor was used in the monophenol assays. 5 mM stock solutions of each substrate as well as all other components used in the reaction mixtures were prepared daily, just prior to each assay, in ultrapure reagent-grade water obtained with a Milli-Q system (Millipore, Bedford, MA) and kept at 4°C protected from light. The incubations were performed at 22°C. All reactions were stopped by removing 40 μL aliquots from the incubation mixtures at specified times and placing each into 60 μL cold (4°C) 0.2M perchloric acid. Stopped reactions were stored at 4°C and analyzed immediately after each assay was performed. 5 μL of each stopped reaction mixture was injected for HPLC-ED analyses. All experiments were replicated at least 3 times. Quantitative electrochemical measurements of monophenol oxidase activity were made by monitoring the rates of conversion of tyrosine to dopa and tyramine to dopamine, and for diphenol oxidase activity the conversions of dopa and dopamine to their respective o-quinones were determined by measuring substrate depletion. Activity was expressed as pmols of either product or substrate change per min under the standard conditions established for the study.

The methods of Li and Christensen (29) were employed with slight modifications to verify the identity of those oxidized products derived from tyrosinase-catalyzed oxidations of dopa and dopamine that were detected by HPLC-ED in the reductive mode (-100 mV). Briefly, 1.3 mM ascorbic acid or $NaBH_4$ was incorporated into reaction mixtures at different times post-incubation with tyrosinase. 5μL of the treated samples were analyzed at an oxidative potential of +850 mV. Ascorbic acid reduces o-quinones back to their respective

o-diphenols without affecting aminochromes, whereas $NaBH_4$ reduces both o-quinones and the aminochromes, leucodopachrome and leucodopaminechrome.

Results are presented as the means $\pm$ SEM of the determinations specified. Differences between mean values were evaluated using the Student's *t*-test, and Duncan's new-range multiple comparisons test. Difference between 2 means was considered significant when $P < 0.05$.

RESULTS

Suitability of HPLC-ED Methods

The addition of tyrosinase into reaction mixtures containing either monophenols or o-diphenols but lacking antioxidants resulted in the solutions first turning pink, and then dark brown to black within 30 min. These color changes were not evident when antioxidants were incorporated into the reaction mixtures. The conventional spectrophotometric methods (ΔA 475 nm) used to monitor dopachrome formation provided some measure of the rate of formation of certain pigment precursors, but they were inadequate for assessing the roles of antioxidants in these reactions because thiols formed colorless reaction products (i.e. cysteinyl- and glutathionyl- conjugates with o-diphenols), and ascorbic acid reduced o-quinones back to o-diphenols. The HPLC-ED methods employed in this investigation effectively detected changes in the levels of monophenol and diphenol substrates and their principal electrochemically oxidizable and reducible products, and provided quantitative data with which to compare the effects of ascorbic acid, glutathione, and cysteine on both monophenol and diphenol oxidase functions of mushroom tyrosinase.

Monophenol Oxidase Activity

Tyrosinase-catalyzed hydroxylations of tyrosine and tyramine assessed at oxidative potentials documented both the depletion in the levels of these

monophenol substrates (detected at +850 mV), and the concurrent formation of their respective o-diphenols, dopa and dopamine (detected at either +700 mV or +850 mV) (Fig. 1). In control mixtures lacking antioxidants, tyrosine and tyramine were rapidly hydroxylated to dopa and dopamine, respectively. The o-diphenols in turn were oxidized further by tyrosinase to o-quinones and subsequently by non-enzymatic mechanisms to precursors of eumelanin, thereby diminishing the levels of dopa and dopamine in the analities.

There was no HPLC-ED evidence of monophenol oxidase activity in reaction mixtures containing glutathione or cysteine in concentrations ranging from 30 μM to 6.6 mM. In the presence of these thiols, substrate levels remained unaltered and no o-diphenols were produced during 30 min incubations. In reaction mixtures containing ascorbic acid the levels of dopa and dopamine produced in 3 min assays were 5 to 7 times greater than in the control incubations lacking ascorbic acid (Fig. 2). These elevated levels resulted from ascorbic acid reducing the o-quinones back to their respective o-diphenols, and not from any alteration in the activity of the enzyme. This was verified by HPLC-ED which in the reductive mode (-100 mV) showed no o-quinone formation, and in the oxidative mode (+850 mV) showed the rates of substrate depletion to be identical in both control and ascorbic acid incubation mixtures (not presented). Thus, monophenol oxidase activity was inhibited by glutathione and cysteine, but not by ascorbic acid.

Diphenol Oxidase Activity

Oxidative potentials also were used to study diphenol oxidase activity, with rates of reaction determined on the basis of the depletion of each o-diphenol substrate. A reductive potential (-100 mV) was used to monitor the formation of oxidized products derived from the o-diphenols. In control mixtures the rates of oxidation of dopa and dopamine averaged 298 pmol/min and 453 pmol/min, respectively (Table 1). Very little diphenol oxidase activity was detected in

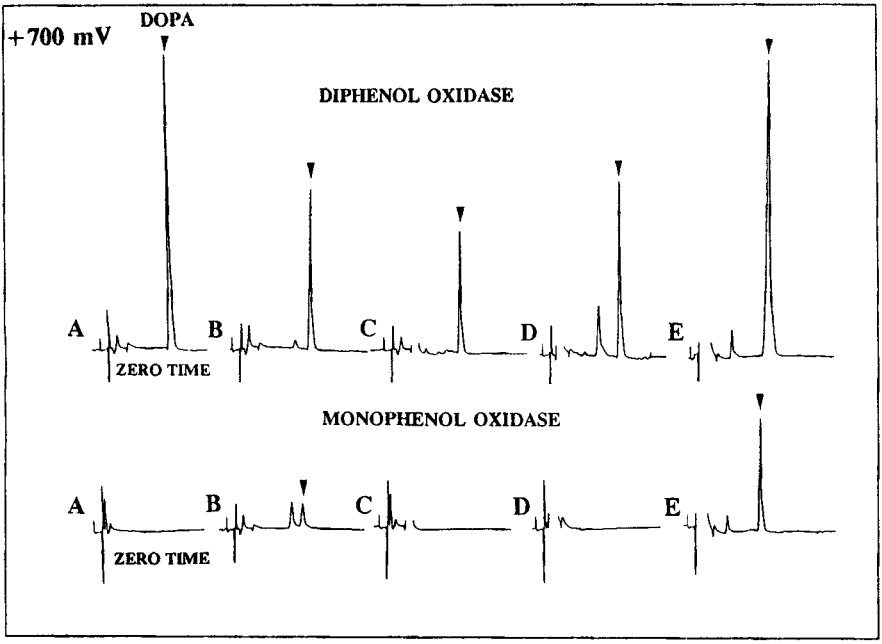

Figure 1. Representative HPLC-ED chromatograms obtained in the oxidative mode (+700 mV; 500 nA) illustrating the tyrosinase-catalyzed hydroxylation of tyrosine (monophenol oxidase function) and the oxidation of dopa (diphenol oxidase function). Control, buffer-mediated incubations are illustrated in (B) of both upper and lower series of chromatograms. Note the absence of dopa production in monophenol oxidase incubations containing glutathione (C) and cysteine (D), and the elevated level of dopa (arrow) with ascorbic acid (E). Diphenol oxidase activity, calculated by the rate of substrate delpletion, was enhanced by glutathione (C). Cysteine-mediated diphenol oxidase reactions (D) are similar to control reactions (B). Reaction mixtures contained 0.3 mM dopa or 1 mM tyrosine, 20 μg enzyme, and 1.3 mM antioxidant, and were incubated at 22°C for 1 min. The peaks produced by the antioxidants were removed to better accommodate the graphic representations.

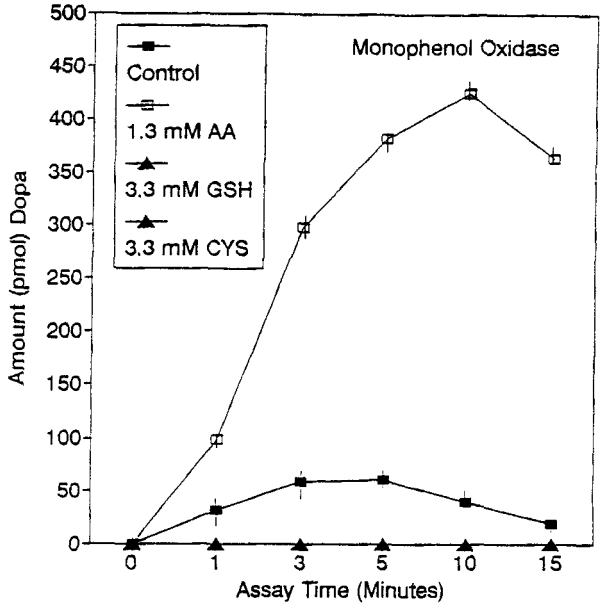

Figure 2. The effects of ascorbic acid (AA), glutathione (GSH) and cysteine (CYS) on the tyrosinase-catalyzed hydroxylation of tyrosine. Elevated levels of dopa with ascorbic acid result from the reduction by the antioxidant of dopaquinone back to the o-diphenol. Diminished levels of dopa in control incubations lacking antioxidant result from the enzyme-mediated oxidation of dopa to dopaquinone. Both thiol compounds used, glutathione and cysteine, inhibited the monophenol oxidase function of the enzyme (see chromatograms Figure 1C, D). Reaction mixtures (total volume 300 μL) were comprised of 1 mM tyrosine and 20 μg enzyme protein in 0.16 M phosphate buffer (pH 7.2). 5 μL of stopped reactions were injected for HPLC-ED analysis at +850 mV and 100 nA. Data points represent the means of 4 tests.

reaction mixtures containing ascorbic acid due to the regeneration of dopa and dopamine by ascorbic acid which reduced their o-quinones (Fig. 3). Unlike their role in inhibiting the monophenol oxidase function of tyrosine, neither glutathione nor cysteine could block the enzyme-catalyzed oxidations of dopa or dopamine. Instead, both thiols increased the diphenol oxidase function of the enzyme. With cysteine the rates of oxidation of dopa and dopamine were increased slightly

TABLE 1. The Effects of Glutathione, Cysteine, and Ascorbic Acid on Tyrosinase-catalyzed Oxidations of Dopamine and Dopa.

	Dopamine	Oxidation		Dopa	Oxidation	
	pmol/min	± (SE)	%	pmol/min	± (SE)	%
Controls	453	(5.6)	100	298	(1.1)	100
GSH	793	(7.2)	175	515	(3.9)	173
CYS	466	(3.2)	103	313	(2.3)	105
AA	12	(2.2)	3	5	(0.5)	2
GSH + AA	738	(3.7)	163	453	(4.0)	152

Reaction mixtures containing 0.33 mM substrate and 3.3 mM of components listed were incubated for 1 min at 22°C. 5μL aliquots were analyzed by HPLC-ED at +850 mV and 100nA.

(approximately 5%), but they were not significantly higher than reaction rates in control incubations lacking antioxidants. However, with glutathione the diphenol oxidase function of tyrosinase was enhanced significantly ($p < 0.05$), causing an increase of approximately 75% in the rates of oxidation of dopa and dopamine (Table 1).

Since diphenol oxidase activity was enhanced by glutathione, we next investigated how ascorbic acid would interact with the thiol in an environment where o-diphenols were oxidized to o-quinones. In reaction mixtures containing 3.3 mM ascorbic acid and glutathione, dopa oxidase and dopamine oxidase activities were virtually similar to those in reaction mixtures containing only glutathione (Table 1). These data clearly establish that the glutathione-mediated enhancement of the diphenol oxidase function of tyrosinase was not diminished

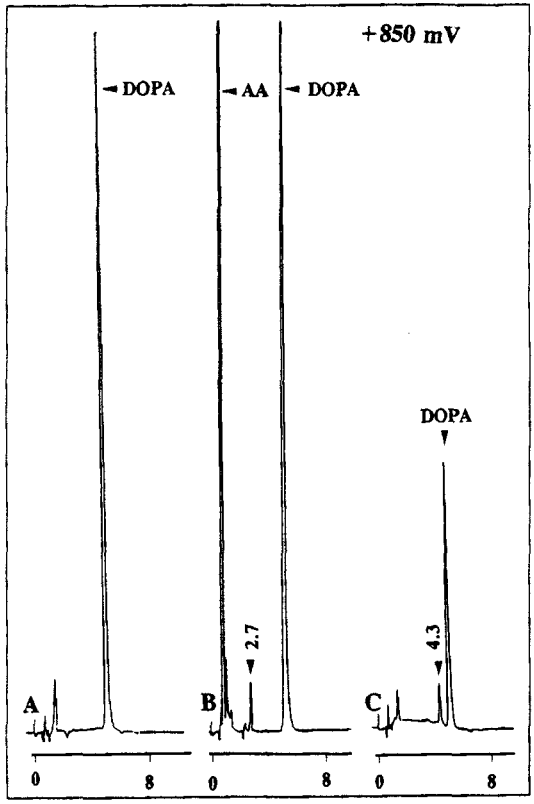

Figure 3. Representative HPLC-ED chromatograms obtained in the oxidative mode (+850 mV; 500 nA) depicting the extent of dopa oxidation in reaction mixtures containing 1.3 mM ascorbic acid (AA) and in those lacking the antioxidant. Dopa level at time zero (A) represents 0.6 nmol substrate. Less than 5% of the o-diphenol is oxidized in 1 min when ascorbic acid is present in the incubation mixture (B), whereas approximately 50% of the substrate is oxidized in control incubations lacking the antioxidant (C; and Fig. 4). A single oxidation product with a retention time of 2.7 min was detected in ascorbic acid reaction mixtures, whereas a product with a retention time of 4.3 min was formed in mixtures lacking antioxidant.

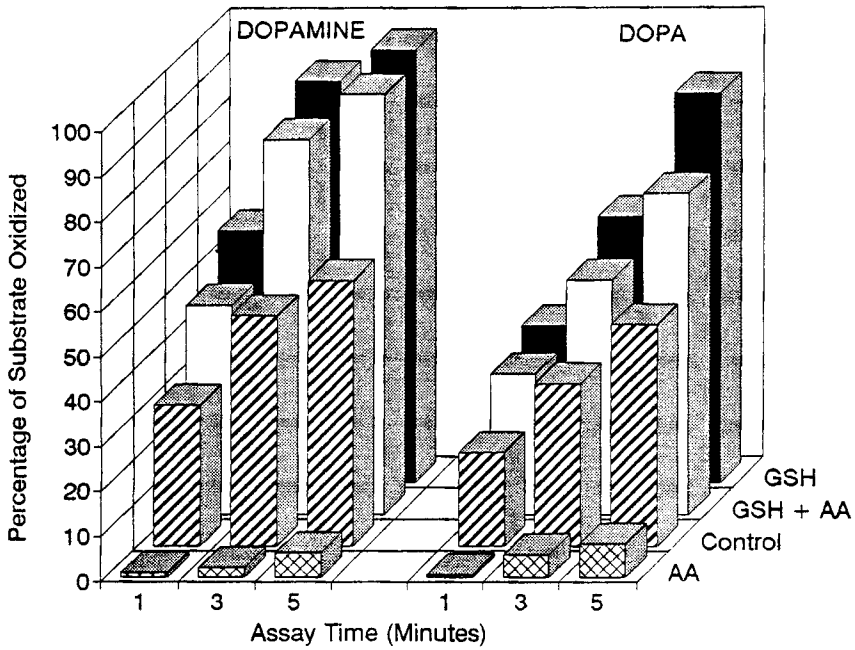

Figure 4. Histograms illustrating the separate and combined effects of ascorbic acid (AA) and glutathione (GSH) on the diphenol oxidase function of tyrosinase. Reaction mixtures contained 0.3 mM substrate (dopa or dopamine), 3.3 mM glutathione, and 6.6 mM ascorbic acid. Each bar represents the mean of 4 tests. Each experimental group is statistically different ($p < 0.05$) compared to controls, but comparative data involving GSH and GSH + AA are not significantly different. Data were obtained by HPLC-ED in the oxidative mode (+850 mV; 100 nA).

significantly by ascorbic acid. Identical results were obtained when the concentration of ascorbic acid was doubled (6.6 mM), and the time of incubation extended to 5 min (Fig. 4).

The effects of different concentrations of glutathione and cysteine on the diphenol oxidase activity of tyrosinase were investigated using dopa as substrate and incubating the reaction mixtures for 1 min (Table 2). With cysteine incorporated into reaction mixtures in concentrations ranging from 30 μM to 6.6

TABLE 2. The Effects of Varying Concentrations of GSH and Cysteine on the Tyrosinase-catalyzed Oxidations of Dopa.

	Dopa Oxidation pmol/min ± (SE)	
Controls	**309 (3.3)**	
Concentration (mM)	**GSH**	**Cysteine**
6.6	**510 (5.4)**	**299 (5.3)**
3.3	**508 (6.2)**	**301 (4.4)**
1.0	**514 (2.2)**	**307 (3.3)**
0.3	**450 (4.2)**	**302 (2.9)**
0.06	**320 (3.6)**	**300 (2.0)**
0.03	**314 (3.7)**	**301 (3.3)**

Reaction mixtures contained 0.3 mM DOPA and were incubated for 1 min at 22°C. 5μL aliquots of the stopped reactions were analyzed by HPLC-ED at +850 mV and 500 nA.

mM there was no statistically significant alteration in dopa oxidase activity. With glutathione, statistically significant ($p < 0.05$) increases in dopa oxidase activity were noted when the concentration of this thiol in reaction mixtures was 0.3 mM and higher.

Detection of O-Diphenol Products by Reductive Electrochemistry

Under reductive HPLC-ED conditions (-100 mV; 500 nA) the tyrosinase-catalyzed oxidation of dopa produced a single compound with a retention time of 1.1 min. With dopamine as substrate a single product also was detected but with

a retention time of 2.5 min (Fig. 5). Both o-diphenol derivatives also were generated non-enzymatically by $NaIO_4$-mediated oxidations of the o-diphenols. No electrochemically reducible compound was produced when reactions were initiated with ascorbic acid, glutathione or cysteine present in incubation mixtures in concentrations ranging from 30 μM to 6.6 mM. Once the compounds were produced in control mixtures, subsequent attempts to reduce them by adding ascorbic acid, glutathione or cysteine to the reaction mixtures as early as 1 min post-incubation were unsuccessful. However, both compounds were completely reduced upon the addition of 1.3 mM $NaBH_4$ to the reaction mixtures. Analyses of the $NaBH_4$-treated reaction mixtures at oxidative HPLC-ED conditions (+850 mV) showed that the o-diphenol substrates were rejuvenated to nearly their initial levels (Fig. 5). The stability of the two compounds after treatment with ascorbic acid eliminates the $NaBH_4$-reducible substances as o-quinones, since the latter are reduced by ascorbic acid. Since $NaBH_4$ has the ability to regenerate o-diphenols by reducing aminochromes (Li and Christensen, 1993), the two reducible compounds produced by the enzyme-mediated oxidations of dopa and dopamine are dopachrome and dopaminechrome, respectively.

Detection of O-Diphenol Products by Oxidative Electrochemistry

HPLC-ED analyses of tyrosinase-catalyzed oxidations of dopa at an oxidative potential (+850 mV; 500 nA) showed a single product with a retention time of 4.3 min (Fig. 3). This electrochemically oxidizable compound was identified as 5,6-dihydroxyindole (DHI) in co-elution experiments using the authentic standard and three different solvent systems (Fig. 6). A single electrochemically oxidizable product also was derived from the oxidation of dopamine, but this substance could not be identified. The incorporation of thiols into reaction mixtures generated o-diphenol-sulfhydryl conjugates that were detected at oxidative potentials (Figs. 7, 8). With dopamine as substrate, a single electrochemically oxidizable compound was formed with each thiol. The retention time for the cysteinyldopamine conjugate was 23.1 min (Fig. 7A), and

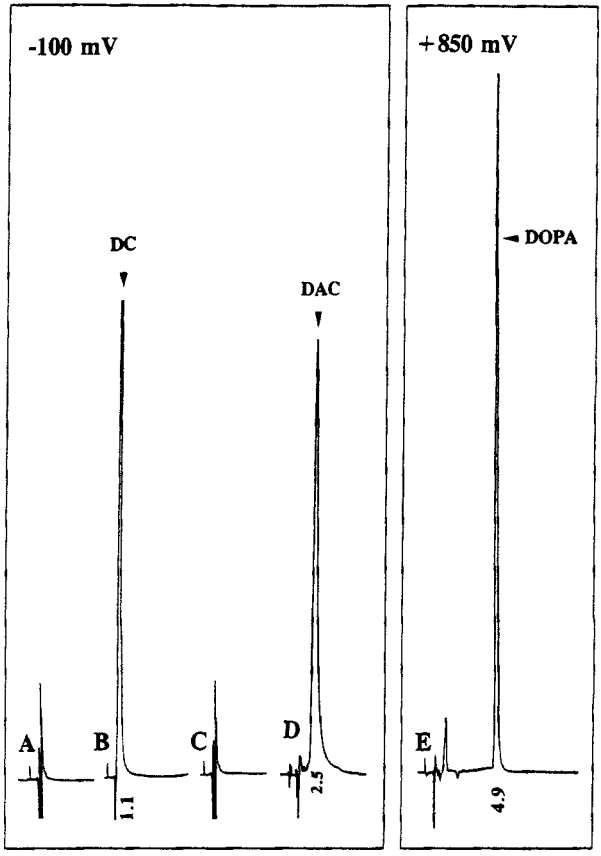

Figure 5. Chromatograms A - D obtained in a reductive mode (-100 mV; 500 nA) showing glutathione suppression of electrochemically reducible compound in tyrosinase-catalyzed reactions of dopa (A) and dopamine (C). Dopachrome (DC) and dopaminechrome (DAC) were produced in 1 min assays when the antioxidant was not incorporated into the reaction mixtures (B and D). The aminochromes were identified by $NaBH_4$ reduction to o-diphenols, a reaction detected at +850 mV. $NaBH_4$ reduction of DC formed a compound (4.9 min) identified as dopa (E). Under identical conditions $NaBH_4$ reduction of dopaminechrome (D) produced dopamine (not presented).

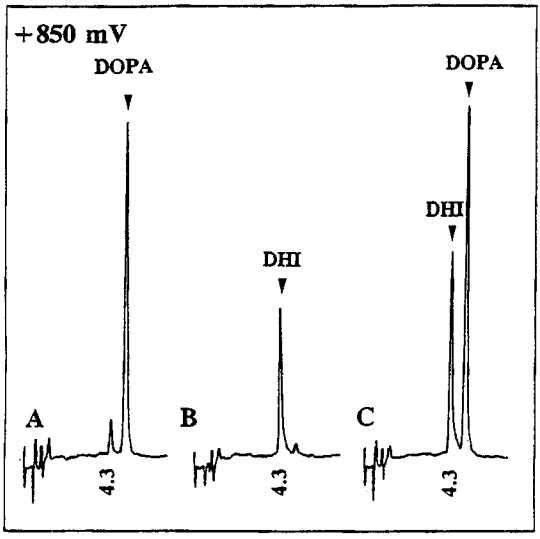

Figure 6. (A) Chromatograph showing the presence of a single oxidation product with a retention time of 4.3 min obtained during the tyrosinase-catalyzed oxidation of dopa (1 min incubation) in phosphate buffer (7.2 pH). (B) 0.2 nmol of authentic standard DHI. (C) Chromatogram showing co-elution at 4.3 min of the oxidation product with 0.2 nmol of DHI standard incorporated into 5 μL sample that was injected. Co-elution was demonstrated with three solvent systems (see Materials and Methods).

40.6 min for the glutathionyldopamine conjugate (Fig. 8A). With dopa as substrate, three electrochemically oxidizable compounds were generated with glutathione, and two with cysteine. One compound with a retention time of 3.7 min was common to glutathione- and cysteine-mediated oxidations of dopa (Figs. 7B and 8B). The oxidation of dopa in the presence of ascorbic acid also produced a single unidentified compound with a retention time of 2.7 min (Fig. 3).

DISCUSSION

Eumelanin and pheomelanin originate from a common metabolic pathway involving two sequential tyrosinase-catalyzed reactions, the conversion of tyrosine

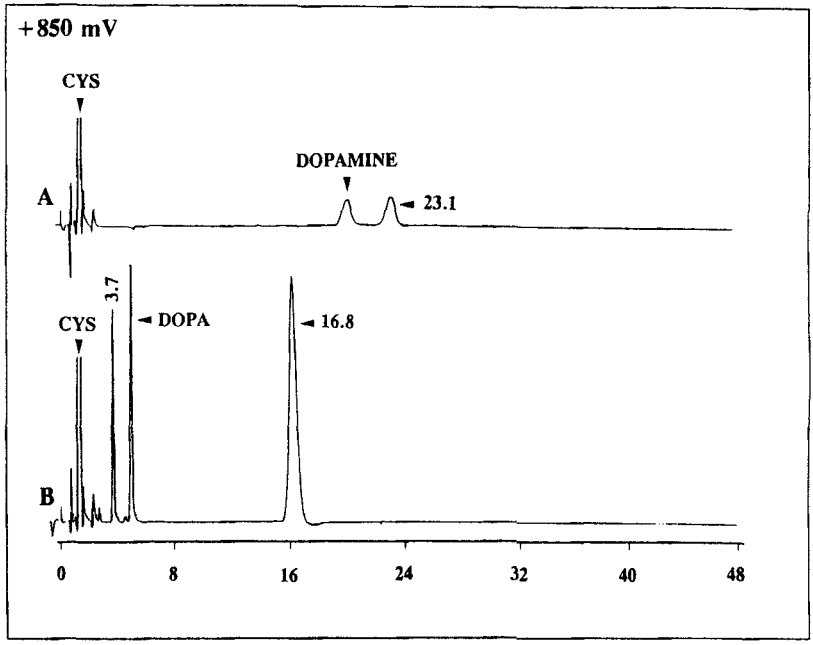

Figure 7. Chromatograms of tyrosinase-catalyzed oxidations showing the formation of a single cysteinyldopamine conjugate at 23.1 min (A), and two cysteinyldopa conjugates at 3.7 and 16.8 min (B). The compound with a renetion time of 3.7 min was also formed in glutathione-mediated oxidations (see Figure 8B).

to dopa, and the oxidation of dopa to dopaquinone. Ensuing spontaneous reactions involving cyclization and oxidative polymerization convert o-quinones into precursors of eumelanin. An alternate pathway is provided by thiols which react with o-quinones to produce colorless addition compounds and precursors of pheomelanin. The capacity of cells to transiently modify their biosynthetic activity to alternate between the synthesis of eumelanin and pheomelan may be important either in their defense against cytotoxic quinone-generated intermediates derived from oxidant stress, or in generating such molecules for protection against pathogens. Several enzymes play pivotal roles in regulating melanin biosynthesis

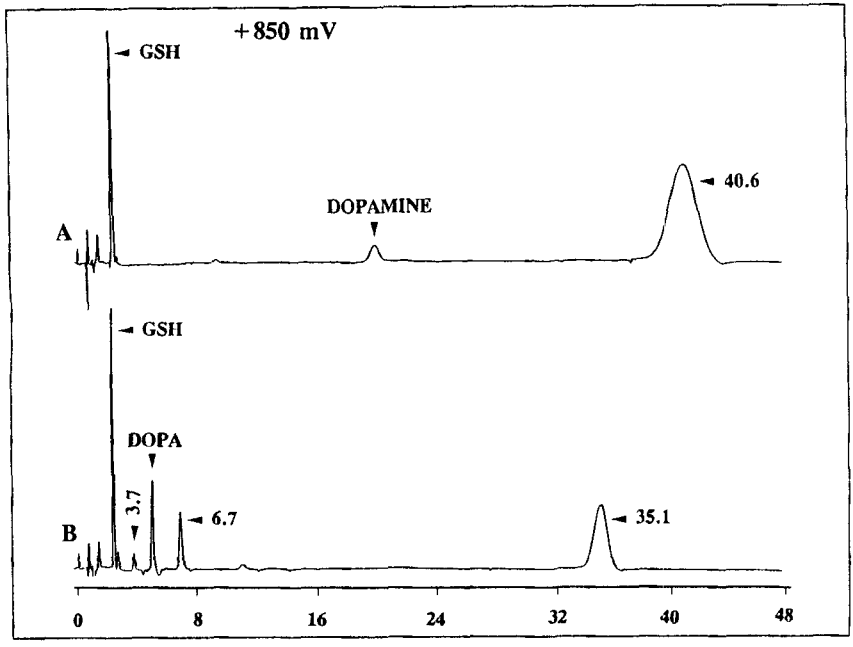

Figure 8. Chromatograms of tyrosinase-catalyzed oxidations showing the formation of a single glutathionyldopamine conjugate at 40.6 min (A), and three glutathionyldopa conjugates at 3.7, 6.7, and 35.1 min (B).

including tyrosinase, peroxidase, dopachrome tautomerase, sulphydryl-metabolizing enzymes (i.e., glutathione-reductase and gamma-glutamyl transpeptidase), and catechol-o-methyltransferase (3, 30, 31). Regulatory roles have been ascribed to antioxidants also, but the mechanism(s) by which these compounds interact with o-quinones has not been completely elucidated.

In this investigation quantitative HPLC-ED data establish that ascorbic acid maintains the level of o-diphenols in tyrosinase-catalyzed reactions by reducing the o-quinones that are produced, and not by modifying the activity of the enzyme. Glutathione and cysteine were found to inhibit the monophenol oxidase function of tyrosinase and increase the diphenol oxidase function. The diphenol oxidase function of tyrosinase was enhanced approximately 5% by cysteine and

75% by glutathione. Both thiols formed catechol-thiol conjugates during tyrosinase-catalyzed oxidations of dopa and dopamine. These reactions proceeded with no electrochemical evidence of thiol regeneration of o-diphenols, a function readily documented with ascorbic acid. Thus, nucleophilic addition of glutathione and cysteine may be the primary if not sole mechanism by which these thiols engage the oxidation products of dopa and dopamine.

The fact that the glutathione-mediated enhancement of diphenol oxidase activity was not altered significantly by ascorbic acid suggests that glutathione may be more effective than ascorbic acid in engaging o-quinones. However, when glutathione was added to reaction mixtures containing o-dopaquinone or o-dopaminequinone, there was no detectable evidence the thiol reduced these compounds to their respective o-diphenols. These findings indicate that the glutathione-mediated enhancement of diphenol oxidase activity results from a specific interaction of glutathione with tyrosinase, and that thioether formation occurs rapidly without the generation of free o-quinones. When monophenols are involved, glutathione or cysteine interact with the enzyme to inhibit tyrosine hydroxylation. It is not known how these varied activities are produced, but since sulfhydryl compounds form stable complexes with copper ions (2, 22, 24, 32), the thiols likely interact differently with the copper active site of tyrosinase during monophenol and diphenol oxidase activities. The enzymatic activities of tyrosinase are dependent on its capacity to coordinate phenolic substrates at the binuclear copper active site, a reaction sequence that requires a concomitant copper-mediated, four-electron reduction of dioxygen. Thiols may inhibit monophenol oxidase function by interfering with the incorporation of an oxygen atom into the aromatic ring, and enhance diphenol oxidase activity by a redox exchange involving the substrate and the binuclear copper ions at the active site of the enzyme.

Our results of the effects of thiols on the diphenol oxidase function of tyrosinase support in part the findings of Agrup *et al.* (20) who showed no inhibition of the dopa oxidation by cysteine. Our chromatographic data clearly

establishing thiol-suppression of monophenol oxidase activity and thiol-enhancement of diphenol oxidase activity of mushroom tyrosinase are in contrast to those of Jara *et al.* (2) who reported that both functions of melanoma tyrosinase were inhibited by cysteine but enhanced by glutathione. This disparity with respect to diphenol oxidase activity is attributed to their assessment of rates of reaction only after cysteine was exhausted from the reaction mixtures, at which time dopachrome appears. The extremely sensitive electrochemical methods employed in this investigation for diphenol oxidase activity provided a quantitative assessment of the actual changes in the amount of o-diphenol substrate consumed. Our assessment of thiol-mediated suppression of monophenol oxidase activity was based on the absence of detectable changes in substrate and the fact that no electrochemically active products were evident under either oxidative or reductive modes.

Regardless of the mechanism(s) involved, glutathione and cysteine can, under certain circumstances, serve as a detoxification mechanism by both inhibiting tyrosine hydroxylation and scavenging potentially cytotoxic o-quinones and other eumelanogenic quinoids to form pheomelanin (31). However, since cysteinyldopas have the ability to bind to proteins through their sulfhydryl groups (33), thiol-catechol conjugates may be as potentially damaging to cells as quinone oxidation products. Also, the availability of glutathione during the oxidation of dopa or dopamine will accelerate the formation of o-quinones, possibly depleting cells of these o-diphenols and potentiating quinone cytotoxicity by generating reactive oxygen species. In this regard it is of interest to note that correlations have been made by numerous investigators between neurodegenerative conditions in the substantia nigra of Parkinson's patients and the presence of neuromelanin in the catecholaminergic neurons targeted for destruction (34). Neuromelanin is a mixed-type melanin comprised of oxidation products of dopamine and cysteinyldopamine (35). Although the mechanism by which dopamine is oxidized in the substantia nigra is still unclear, the immediate oxidation product is o-dopaminequinone. Alterations in brain levels of glutathione or cysteine would

have a pronounced effect on the rate of oxidation of the o-diphenol and the pathophysiological manifestations of such a reaction.

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