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THE TOXICITY OF MELANIN PRECURSORS

DOYLE G. GRAHAM, M.D., PH.D., SYLVIA M. TIFFANY, M.S., AND F. STEPHEN VOGEL, M.D.

Department of Pathology, Duke University Medical Center, Duke University, Durham, North Carolina, U.S.A.

The quinone intermediates resulting from tyrosinase-mediated oxidation of tyrosine were evaluated as sulfhydryl reagent inhibitors of purified calf thymus DNA polymerase α in order to determine which of these might be cytotoxic. Dopachrome and an oxidation product of 2,4,5-trihydroxyphenylalanine were relatively ineffective as inhibitors of DNA polymerase α. On the other hand, a dopaquinone analogue, 4-(2-*N*-acetylaminoethyl)-1,2-benzoquinone, synthesized from *N*-acetyl dopamine, was demonstrated to have marked affinity for this sulfhydryl enzyme. This property was shared by 1,2-benzoquinone. These studies point to dopaquinone as a significant toxic metabolite in melanin biosynthesis.

Since Hochstein and Cohen suggested that intermediates in melanin biosynthesis might be toxic to melanoma cells [1], a number of authors have documented the inherent autotoxicity of tyrosine metabolites in cells which synthesize melanin [2,3]. The well-known association of vitiligo with the increased MSH stimulation observed in adrenocortical insufficiency suggests that the melanocyte is increasingly vulnerable to injury and death under conditions of accelerated melanin synthesis [2,3]. Further, the existence of halo nevi and halo metastases lends support to the concept that cytotoxins or their precursors may be released from benign or malignant melanocytes during melanogenesis [4].

Several findings suggest that the metabolite released from nevus cells or melanoma cells is 3,4-dihydroxyphenylalanine (DOPA). This compound accumulates during normal melanogenesis [5], and its release from melanoma cells can be demonstrated by increased levels of DOPA in the urine of patients with metastatic melanoma [6]. Further, the observation that these patients also excrete 3-methoxytyrosine in the urine implies that DOPA release by melanoma cells is coupled with methylation in liver, heart, kidney, adrenal medulla or adrenergic neurons, or other sites of catechol-O-methyl transferase [7]. Likewise, the excretion of large amounts of 5-S-cysteinyl-DOPA might be expected in the urine of a red-haired person with metastatic melanoma, where it is a normal intermediate in pheomelanin synthesis [6,8]. However, the demonstration of this compound in the urine of people who synthesize eumelanin, whether or not they have melanoma [9], suggests that DOPA oxidation to dopaquinone also occurs outside melanosomes and perhaps in cells other than melanocytes, where the short-lived dopaquinone species can react with cysteine or reduced glutathione [10] as an alternative to cyclization to form leukodopachrome.

Given the likelihood that DOPA or its metabolites are cytotoxic, what are the possible mechanisms for this effect? The catechol, DOPA, may autoxidize to dopaquinone, generating superoxide and hydroxide radicals [11] which themselves may injure cells through membrane and DNA damage [11,12]. DOPA oxidation may be promoted by free superoxide radicals which can be generated through the action of cytoplasmic oxidases, such as xanthine oxidase, aldehyde oxidase, dihydroorotic dehydrogenase and NADPH-cytochrome c reductase [11,13]. The resulting dopaquinone or other quinone intermediates may then act as sulfhydryl reagents, inactivating steps in energy metabolism, e.g., pyruvate, α-ketoglutarate, and succinate dehydrogenases [14], or DNA synthesis, through inhibition of alpha class DNA polymerases [15].

In these studies the quinone intermediates expected to result from DOPA oxidation were evaluated as sulfhydryl reagents. These are given in Fig 1 where the Raper-Mason scheme for dopachrome synthesis is depicted along with an alternative pathway existant in fungi but as yet not documented in man [16]. These quinones included dopachrome, a dopaquinone analogue [4-(2-*N*-acetylaminoethyl)-1,2-benzoquinone] and p-topaquinone [5-(2-carboxy-2-aminoethyl)-2-hydroxy-1,4-benzoquinone], a stable hydroxyquinone intermediate in the oxidation of 2,4,5-trihydroxyphenylalanine (TOPA) to dopachrome [16]. 1,2-Benzoquinone was prepared from catechol. These quinones were compared with p-chloromercuribenzoate as inhibitors of purified calf thymus DNA polymerase α, a sensitive test enzyme for sulfhydryl reagents.

MATERIALS AND METHODS

Preparation of DNA Polymerase

DNA polymerase α was isolated from calf thymus by the method of Yoneda and Bollum [17]. Calf thymus was obtained from Randolf Packing Co., Ashboro, N.C. and stored at −70°C. The partially thawed glands were run through a meat grinder into extracting buffer and unwanted proteins were removed by precipitation at pH 6.5. Then the enzyme activity was adsorbed with phosphocellulose, eluted, and passed through a DEAE cellulose column to remove nucleic acids. After a second phosphocellulose chromatography step the activity precipitating at 30-55% saturation in ammonium sulfate was then adsorbed onto hydroxyapatite and eluted with a linear 0.05 to 0.5 M potassium phosphate buffer, pH 7.5 containing 1 mM mercaptoethanol and 10% glycerol. The resulting fractions containing DNA polymerase α were dialyzed against 50% glycerol, 50 mM Tris HCl buffer, pH 7.2, with 1 mM dithioerythritol (DTE) and 1 mM ethylenediaminetetraacetate (EDTA) and stored at −20°C. Prior to exposure to sulfhydryl reagents the enzyme was dialyzed against two 1000-fold volumes of 50% glycerol buffer without DTE at −20°C.

Assay of Calf Thymus DNA Polymerase α

Calf thymus DNA polymerase α was assayed as described previously for the corresponding enzyme from L1210 leukemia [15] utilizing optimum assay conditions established in this laboratory. A final volume of 50 μl contained 50 mM Tris pH 7.5, 7 μM [^{3}H] TTP (12,600 dpm/pmol, New England Nuclear Corp., Boston, Mass.), 80 μM dATP, dGTP and dCTP (Sigma Chemical Co., St. Louis, Mo.), 4 mM $MgCl_2$, 2.5 μg of activated calf thymus DNA (Sigma, activated with pancreatic deoxyribonuclease as described earlier [15]), 5% glycerol, and sufficient enzyme to result in the incorporation of 2 to 3 pmoles of [^{3}H] TTP into DNA after 30 min of incubation at 37°C. The reactions were terminated by placing the tubes on ice and adding 0.1 ml of 0.1 M sodium pyro-

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Reprint requests to Dr. Doyle G. Graham, Department of Pathology, Duke University Medical Center, Duke University, Durham, North Carolina 27710.

Abbreviations:

BSA: bovine serum albumin
DTE: dithioerythritol
GHB: γ-L-glutaminyl-4-hydroxybenzene
NCS: tissue solubilizer obtained from Amersham Corp., Arlington Heights, Il.
PCMB: p-chloromercuribenzoate
TOPA: 2,4,5-trihydroxyphenylalanine

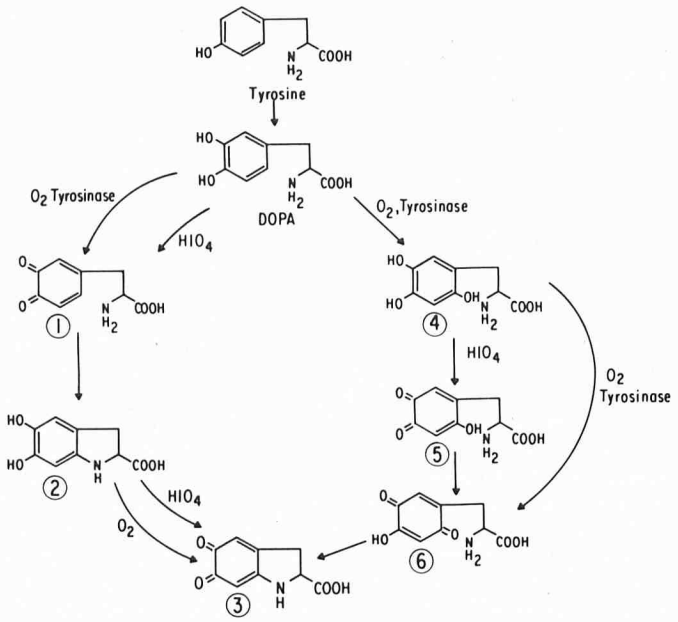

FIG 1. The Raper-Mason scheme for dopachrome synthesis is shown on the left and the TOPA pathway on the right. *1*, dopaquinone [4-(2-carboxy-2-aminoethyl)-1,2-benzoquinone]; *2*, leukodopachrome [2-carboxy-2,3-dihydro-5,6-dihydroxyindole]; *3*, dopachrome [2-carboxy-2,3-dihydroindole-5,6-quinone]; *4*, TOPA [2,4,5-trihydroxyphenylalanine]; *5*, o-topaquinone [5-(2-carboxy-2-aminoethyl)-4-hydroxy-1,2-benzoquinone]; *6*, p-topaquinone [5-(2-carboxy-2-aminoethyl)-2-hydroxy-1,4-benzoquinone].

phosphate, 1 drop of heat-denatured salmon sperm DNA (Sigma; 30 A_{260} units/ml), and 5 ml of cold 5% trichloroacetic acid. The precipitate was collected on Whatman GF/C glass fiber discs, washed with 15 ml of 1% trichloroacetic acid and 3 ml of 95% ethanol, dried, digested with 0.5 ml of Nuclear Chicago solubilizer, Amersham Corp., Arlington Heights, Ill (NCS), and counted in 15 ml of toluene with PPO (4 gm/liter) and POPOP (0.1 gm/liter) by the procedure outlined by Schrier and Wilson [18]. Counting efficiency for tritium, using a Beckman LS 150 scintillation spectrometer, averaged 48%.

Synthesis of Quinones

Dopachrome was prepared by incubating DOPA (Calbiochem, La Jolla, Calif.) at 0.5 mg/ml with mushroom tyrosinase (Worthington Biochemicals, Freehold, N.J.), 0.25 mg/ml in 0.025 M potassium phosphate buffer, pH 6.8, for 30 min at 25°C. One ml volumes were loaded onto 1 × 25 cm columns of Sephadex (Pharmacia Fine Chemicals, Piscataway, N.J.) G25 fine equilibrated and eluted with water. Similarly 2,4,5-trihydroxyphenylalanine (TOPA, Sigma) was oxidized with tyrosinase to yield p-topaquinone, which was then isolated by Sephadex chromatography.

Catechols, like 1,2-glycols, appear to form cyclic periodate esters which are cleaved in aqueous solutions to 1,2-benzoquinones [16]. Thus, quantitative oxidation of N-acetyl dopamine (Sigma) to 4-(2-*N*-acetylaminoethyl)-1,2-benzoquinone and of catechol to 1,2-benzoquinone was achieved by adding equimolar amounts of $NaIO_4$ in water at 25°C as outlined previously [16]. The quinones were then isolated by Sephadex chromatography as described above.

All quinones were evaluated as sulfhydryl reagents immediately after isolation. The instability of the quinones was minimized by conducting the chromatography in the dark and by the use of de-aired, demineralized water.

The ionic sulfhydryl reagent p-chloromercuribenzoate was dissolved in IN NH_4OH (1.5 ml/mg) with heating, then neutralized to pH 8 with IN acetic acid, diluted with water to a final concentration of 100 μM, and stored frozen at −20°C.

Molar extinction coefficients were determined in 0.015 M potassium phosphate buffer pH 6.7 by incubating with $NaIO_4$, 1 equivalent each with TOPA, catechol, and N-acetyl dopamine, for 120, 10, and 10 min at 25°C, and 2 equivalents of $NaIO_4$ with DOPA for 30 min. The reactions were followed by repetitive scanning of ultraviolet and visible spectra until maximum absorbance at the visible λ max was achieved and the spectrum matched that of the quinone isolated by Sephadex chromatography.

RESULTS

Dopachrome was identified by its characteristic chromophore in aqueous solutions with λ max at 302 nm (ε = 9300, pH 6.7) and 473 nm (ε = 3520). Absorption maxima for p-topaquinone were at 271 nm (ε = 5100) and 485 nm (ε = 1160). The dopaquinone analogue, 4-(2-*N*-acetylaminoethyl)-1,2-benzoquinone, demonstrated an absorption maximum at 392 nm (ε = 1300). The catechol oxidation product 1,2-benzoquinone had λ max at 385 nm (ε = 860) (Fig 2).

The inhibition of calf thymus DNA polymerase α by p-chloromercuribenzoate (PCMB) is consistent with the inhibition of alpha-class DNA polymerases from other sources by this agent [15]. Up to 94% inhibition was achieved with a 30 μM concentration of PCMB. A comparison of the concentration of quinones yielding 50% inhibition of calf thymus DNA polymerase α with the concentration of PCMB inhibiting to this extent is presented in Table I. Note that the N-acetyl dopamine product is the most potent sulfhydryl reagent. Somewhat less inhibition was observed with unsubstituted 1,2-benzoquinone. By contrast dopachrome was found to be a weak sulfhydryl

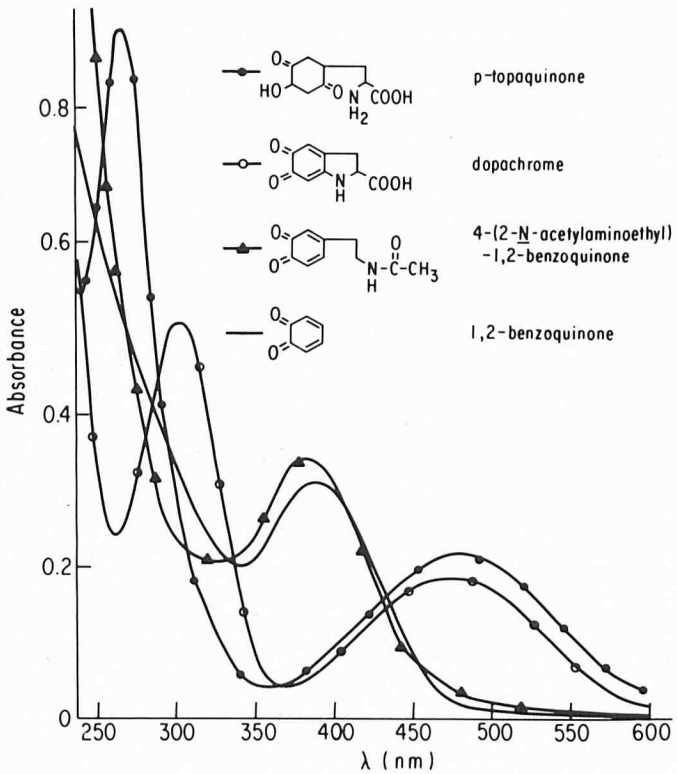

FIG 2. Absorption spectra recorded with a Beckman Acta III spectrophotometer after isolation by Sephadex G25 chromatography. The concentrations for each are 0.191 mM for p-topaquinone, 0.054 mM for dopachrome, 0.263 mM for 4-(2-*N*-acetylaminoethyl)-1,2-benzoquinone, and 0.360 mM for 1,2-benzoquinone.

TABLE I. *Inhibition of calf thymus DNA polymerase*

Inhibitor	Concentration Yielding 50% Inhibition
	μM
p-chloromercuribenzoate	4
4-(2-*N*-acetylaminoethyl)-1,2-benzoquinone	13
1,2-benzoquinone	24
dopachrome	155
p-topaquinone	>500

TABLE II. *Effect of free sulfhydryl groups on inhibition of calf thymus DNA polymerase α by sulfhydryl reagents*

Inhibitor	Concentration (μM)	% Inhibition	
		No DTE[a]	1 mM DTE
p-chloromercuribenzoate	2	35	0
	6	71	0
	10	84	0
	14	87	0
	20	87	0
4-(2-*N*-acetylaminoethyl)-1,2-benzoquinone	9	33	7
	18	66	40
	128	93	60
1,2-benzoquinone	59	75	68
	61	76	51
	118	88	59
dopachrome	27	8	0
	46	11	0
	91	36	0
p-topaquinone	134	2	0
	267	16	1

[a] DTE = dithioerythritol

reagent. Even less reactive was p-topaquinone which inhibited less than 30% with concentrations in excess of 500 μM.

Another measure of affinity of the quinones for the cysteine residues on calf thymus DNA polymerase α was achieved by comparing the amount of inhibition observed with quinones in the presence and absence of an excess of free sulfhydryl groups. Dithioerythritol at a final concentration of 1 mM was utilized for this purpose. In Table II one can see that the quinones gave inhibition of DNA polymerase in accord with the data in Table I, with the greatest degrees of inhibition exhibited by 1,2-benzoquinone and the dopaquinone analogue. Similarly, while PCMB was completely neutralized as a sulfhydryl reagent by excess DTE, substantial inhibition was still seen with the two 1,2-benzoquinones in spite of the excess of DTE. This finding suggests that 1,2-benzoquinones have a greater affinity for cysteinyl residues of DNA polymerase α than for the free sulfhydryl groups of DTE.

DISCUSSION

The quinone intermediates in the proposed pathways for melanin biosynthesis include dopaquinone, dopachrome, 2-carboxyindole-5,6-quinone, indole-5,6-quinone, and the two intermediates in the TOPA pathway, o-topaquinone and p-topaquinone. In previous studies two quinones, dopaquinone and o-topaquinone, were observed only as transient intermediates in periodate oxidation of DOPA and TOPA [16]. Thus neither could be isolated for evaluation as sulfhydryl reagents. A dopaquinone analogue, however, could be synthesized from *N*-acetyl dopamine with periodate oxidation. *N*-acetylation renders the α-amino nitrogen non-nucleophilic, preventing cyclization of the nitrogen on the benzene ring. The resulting quinone was a potent sulfhydryl reagent as reflected in inhibition of calf thymus DNA polymerase α. The concentration required for 50% inhibition was less than that observed with 1,2-benzoquinone and other substituted 1,2-benzoquinones evaluated in this laboratory.*

Dopachrome is a much more stable quinone than dopaquinone in dilute aqueous solutions. Perhaps as a reflection of this stability its reactivity with DNA polymerase was notably less than that observed for the dopaquinone analogue. The TOPA oxidation product p-topaquinone is also stable in aqueous solutions, requiring 30 hr at 25°C for complete conversion to dopachrome [16]. This species was even weaker than dopachrome as a sulfhydryl reagent.

While we have not isolated indole quinones as products of DOPA or TOPA oxidation, an analogue of these quinones has been prepared with periodate oxidation of norepinephrine. This quinone is even less reactive with DNA polymerase α than either dopachrome or p-topaquinone†.

Thus, these studies point to dopaquinone as a major reactive species in the toxicity of melanin precursors. This conclusion is in accord with the production of 5-S-cysteinyldopa in patients with metastatic melanoma. The DOPA released from the melanosomes of melanoma cells would be oxidized in concept, in the cytosol of the melanoma cells or other cells, and the resulting dopaquinone would then react with free cysteine or the cysteinyl residues in glutathione or proteins. This hypothesis is supported by the observation that when DOPA and dopamine are oxidized with tyrosinase, xanthine oxidase, or NADPH-cytochrome c reductase in the presence of bovine serum albumin (BSA), the oxidized products are bound to the exposed cysteinyl residues of BSA [13]. When the cysteinyl residues so bound are at or near active sites of enzymes, profound inhibition results. Sulfhydryl reagent sensitive enzymes are particularly prevalent in DNA and energy synthesis, but are also found at many other steps in intermediary metabolism [14]. Thus, the intracellular generation of a potent sulfhydryl reagent would result in severe metabolic alterations in that cell.

The elucidation of dopaquinone as the likely toxic metabolite in melanogenesis gives direction to the design of possible chemotherapeutic agents for malignant melanoma. DOPA would have limited potential in the therapy of melanoma because of pressor effects, and indeed DOPA does not retard the growth of B16 melanoma [19]. Numerous catechols besides DOPA, however, have demonstrated utility as bleaching agents and are toxic to melanoma cells in vitro and in vivo [2,14,20]. One would suspect that even though catechols are readily oxidized to 1,2-benzoquinones by tyrosinase, these agents would not be highly specific for melanocytes, since alternative pathways including autoxidation exist for catechol oxidation, as outlined above. The observation that phenols are also toxic to melanocytes [2] implies that these cells can hydroxylate phenols to catechols, with subsequent oxidation to quinones.

While mushroom tyrosinase hydroxylates tyrosine to DOPA and will oxidize a variety of phenols to 1,2-benzoquinones*, controversy continues with regard to whether tyrosinase or peroxidase is the enzyme which converts tyrosine to DOPA in the melanocyte [3,21,22]. If mammalian tyrosinase is also capable of oxidizing phenols to quinones, then phenols would be expected to be highly selective for melanocytes. Evidence for such specificity is apparent with γ-L-glutaminyl-4-hydroxybenzene (GHB), a phenol originally isolated from the mushroom, *Agaricus bisporus*. GHB, given as a single parenteral injection at 400 mg/kg, results in transient depigmentation of hair of black mice, and, when given as multiple doses at 200 mg/kg/day prolongs the survival of mice bearing B16 melanoma without evidence of systemic toxicity [23]. The apparent specificity for normal and neoplastic melanocytes is postulated to be the result of GHB oxidation to a quinone with marked reactivity as a sulfhydryl reagent [15]. While there is as yet no direct evidence for hydroxylation of GHB by mammalian tyrosinase, the selective vulnerability of melanocytes to GHB and other phenols might still be a reflection of tyrosinase or "dopa oxidase" activity. This enzyme would be expected to oxidize catechols to quinones, whether hydroxylation were performed enzymatically as by peroxidases, or via a hydroxide radical-mediated reaction [24], thereby enhancing the rate of hydroxylation through mass action effect.

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* Tiffany SM, Graham DG, Jeffs PW, Cass MW, Vogel FS: Manuscript in preparation.

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