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Interaction of Several Mono- and Dihydroxybenzene Derivatives of Various Depigmenting Potencies with L-3,4-Dihydroxyphenylalanine–Melanin[1,2]

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Certain mono- and dihydroxybenzene derivatives cause depigmentation of skin and hair, and appear to be selectively cytotoxic for melanized pigment cells. As direct physical and/or chemical interaction between depigmenter (DP) and pigment melanin may play a role in depigmentation, we have carried out preliminary studies in model systems where such interactions may easily be separated from effects due to tyrosinase, melanosomal proteins, and other components. We have used synthetic L-3,4-hydroxyphenylalanine (L-DOPA)-melanin as a protein-free model pigment and potassium ferricyanide as a model electron acceptor. Compounds studied were catechol, 4-t-butylcatechol, 4-methylcatechol, 3,4-dihydroxyphenylalanine (DOPA), 3,4-dihydroxyphenylacetic acid, hydroquinone, 4-methoxyphenol, 4-t-butylphenol, and 2,6, di-t-butyl-4-methylphenol (BHT) in 0.1 M phosphate buffer, pH 7.4. These compounds vary widely in their ability to depigment hair and skin. Ferricyanide reduction by DP in the presence and absence of melanin was monitored spectrophotometrically. The sparingly soluble BHT and 4-t-butylphenol did not reduce ferricyanide in the absence or presence of melanin. For the other compounds, kinetic analysis demonstrated direct interaction between each DP and melanin. Except for dihydroxyphenylacetic acid, reduction kinetics were consistent with a mechanism involving noninteractive binding of both DP and ferricyanide to melanin prior to coupled electron transfer through the melanin backbone. Kinetic analysis afforded K_B, a thermodynamic constant (M^{-1}) for DP-melanin binding, and k', a rate parameter ($M\ s^{-1}$) for electron transfer. A dimensionless enhancement factor (EF) was defined as $k'K_B/k_s$, with k_s a pseudo-first-order constant (s^{-1}) for ferricyanide reduction in the absence of melanin. Depending on the reductant, melanin either retards ($EF < 1$) or accelerates ($EF > 1$) the rate of ferricyanide reduction. There appears to be a direct relationship between EF and depigmenting potency. There is no relationship between depigmenting power and the ability per se of the DP to bind to melanin or to reduce ferricyanide.

Epidermal melanin is formed in subcellular organelles within the melanocyte known as *melanosomes*, which are secreted into upward-migrating kerotinocytes. *In vivo* melanoprotein consists of a protein moiety conjugated to a melanin pigment formed in the melanocyte by tyrosinase-mediated oxidative polymerization of tyrosine (1).

Melanin has excellent electron acceptor properties (2, 3), and can bind a large variety of molecules (2). The polyquinoid nature of the melanin polymer enables it to oxidize some compounds, to reduce others, and to couple oxidation of electron donors with reduction of electron acceptors (2, 4-

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6). The presence of the protein moiety can modify the observed redox properties of the melanoprotein (7).

Certain mono- and dihydroxybenzene derivatives (DPs)[4] can cause depigmentation of skin and hair, and appear to be selectively cytotoxic for melanized pigment cells (8-21). The cytotoxicity may depend on the ability of tyrosinase to mediate the formation of toxic DP oxidation products or intermediates (16, 17, 22). However, direct physical and/or chemical interaction between depigmenter and pigment melanin is also possible. In one case (6), we showed that melanin can couple oxidation of *p*-t-butylcatechol (tBC) to reduction of potassium ferricyanide, affording thermodynamic and kinetic parameters characteristic of such depigmenter-melanin interactions.

This work is concerned specifically with DP-melanin interactions, and their possible relationship to clinically observed depigmentation. A first approach to this problem is to use a simple model system, where direct DP-melanin interactions can easily be separated from modifications due to melanosomal protein, membranes, and tyrosinase. We have therefore chosen protein-free synthetic L-DOPA-melanin as a model compound for this preliminary work, and have studied its interaction with nine mono- and dihydroxybenzene derivatives having depigmenting capabilities ranging from "none" to "very potent." We utilized the model electron acceptor potassium ferricyanide to oxidize each derivative in the presence and absence of synthetic L-DOPA-melanin; the kinetics of ferricyanide reduction could easily be monitored by kinetic spectrometry. The ability of melanin to bind each derivative and to couple the redox reaction between each DP and ferricyanide was gauged, and where possible, thermodynamic and kinetic parameters were evaluated. Detailed knowledge of such parameters can provide quantitative information on DP-melanin interactions which may be specific enough to differentiate among the various DPs and/or types of melanin. Additional studies with extracted melanoprotein could, in turn, provide information as to the degree to which the protein moiety can modify such interactions.

[4] Abbreviations used: DP, depigmenter; tBC, *p*-t-butylcatechol; DOPA, dihydroxyphenylalanine; CAT, catechol; MCAT, 4-methylcatechol; DHPA, 3,4-dihydroxyphenylacetic acid; HQ, hydroquinone; MMEH, 4-methoxyphenol; BHT, 2,6-di-t-butyl-4-methylphenol.

MATERIALS AND METHODS

A. Reagents

Compounds used (DPs), their sources, and their abbreviations are given in Fig. 1 and Table I. Hydroquinone and *p-t*-butylcatechol were purified as previously reported (6). 3,4-Dihydroxyphenylacetic acid was recrystallized from cyclohexane-ethanol; all other compounds were used as received. Synthetic L-DOPA-melanin, obtained from Sigma Chemical Company, was treated as previously described (6). Reagent-grade potassium ferricyanide (Sigma Chemical Co.), was used without further purification.

Prior to each set of experiments, fresh DP solutions (ca 3.0 mM) and melanin suspensions (ca 330 μg/10 ml) were made up in subdued light in 0.1 M phosphate buffer, pH 7.4, and kept in the dark until measured. Control experiments in which light of wavelengths less than ca. 580 nm was rigorously excluded showed that the small amount of ambient light to which the melanin suspensions were exposed during their preparation had no noticeable effect on the results. Stock solutions containing 6.4 mM ferricyanide in buffer were stored in low-actinic glass volumetric flasks in the dark at 4°C (6).

B. Apparatus

Kinetic measurements were performed by monitoring ferricyanide reduction on an Aminco DW-2A kinetic spectrophotometer at room temperature (23-25°C). The probe wavelength was 420 nm (maximum of ferricyanide absorption), and except for DOPA, the reference wavelength was set at 530 nm (where no ferricyanide absorption occurred or absorption changed during the reaction). In the latter case, the

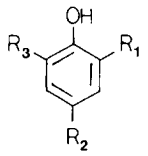

FIG. 1. Structural formula of all depigmenters (DPs) used in this work. See Table I for R_1, R_2, and R_3.

TABLE I

DPs USED IN THIS STUDY

Compound	Source[a]	Abbreviation	R_1[b]	R_2[b]	R_3[b]
Catechol	a	CAT	OH	H	H
4-t-Butylcatechol	a	tBC	OH	tBu	H
4-Methylcatechol	a	MCAT	OH	CH_3	H
3,4-Dihydroxyphenylalanine	b	DOPA	OH	$CH_2(CHNH_3^+)COO^-$	H
3,4-Dihydroxyphenylacetic acid	a	DHPA	OH	CH_2COOH	H
Hydroquinone	c	HQ	H	OH	H
4-Methoxyphenol	a	MMEH	H	OCH_3	H
4-t-Butylphenol	a	tBP	H	tBu	H
2,6-Di-t-butyl-4-methylphenol	a	BHT	tBu	CH_3	tBu

[a] (a) Aldrich Chemical, (b) Schwarz-Mann, (c) Sigma Chemical.
[b] See Fig. 1.

reference wavelength was 600 nm, and the formation of dopachrome was also monitored at 480 nm. Under these experimental conditions, the initial rate of dopachrome formation was equal to the initial rate of ferricyanide reduction.

The reaction was carried out in the dark in a 1.0-cm cuvette fitted with a small glass stirrer on a Cox 701 motor powered by a Tech-11 power-pak (with rheostat). The reaction was started by rapidly pipetting 0.3 ml ferricyanide (via an Eppendorf pipet) into the stirred cuvette, which contained 2.7 ml of the DP solution with or without 100 μg melanin. Total [DP] ranged from 200 to 1000 μM, and the initial ferricyanide concentration was always 640 μM. The "dead time" (approx 0.5 s) and absorbance of the ferricyanide at $t = 0$ were determined from runs in which DP had been omitted. Smooth kinetic curves were obtained, from which the initial velocity of ferricyanide reduction, V_0, was easily determined. The precision of the V_0 measurement (usually within ca. ± 15%) was determined by the mixing process. Each measurement was determined at least three times; error bars represent standard deviations.

C. Kinetic Analysis

Two simple cases are considered.

Case I: Absence of melanin. At constant initial ferricyanide (640 μM), the initial velocity of ferricyanide reduction, V_0, in the absence of melanin can be written in the form

$$V_0 = k_s [DP]_0 \qquad [1]$$

where $[DP]_0$ denotes initial concentration, and $k_s = k_2 [FE(CN)_6^{3-}]_0$ is a pseudo-first-order rate constant, with k_2 a second-order constant. Plots of V_0 vs $[DP]_0$ yield a straight line through the origin with slope equal to k_s.

Case II: Presence of melanin. Melanin may "couple" ferricyanide reduction to DP oxidation in a manner analogous to that of a heterogeneous catalyst by binding both reactants on its surface prior to redox reduction (6). If the binding sites are noninteracting and at constant initial ferricyanide concentration, we have the relationship (6)

$$1/V_0 = 1/k'K_B [DP]_0 + 1/k' \qquad [2]$$

where K_B is the binding constant for DP to melanin (with dimensions of M^{-1}) and k' a rate parameter for ferricyanide reduction (in M s^{-1}). If such a "melanin-mediated" mechanism is operative, then $1/V_0$ vs $1/[DP]_0$ plots will be linear with slope $1/k'K_B$ and intercept $1/K'$.

From consideration of Eqs. [1] and [2], one may define a dimensionless enhancement factor, EF, as the ratio $k'K_B/k_s$. The quantity $k'K_B$ "looks like" a pseudo-first-order rate constant (in s^{-1}) which reflects the contributions of both DP-melanin complex formation and subsequent electron transfer to the overall rate of ferricyanide reduction.

RESULTS

Except for tBP and BHT (see below), all DP reduced ferricyanide in the presence or absence of melanin. In the latter cases, melanin significantly altered the reduction kinetics: the kinetic curves of the DP-melanin-ferricyanide systems were not equal to the sums of those for the respective DP-ferricyanide and melanin-ferricyanide components. In all cases, the extent of reaction and the product distribution was not altered by the presence of melanin (unpublished results).

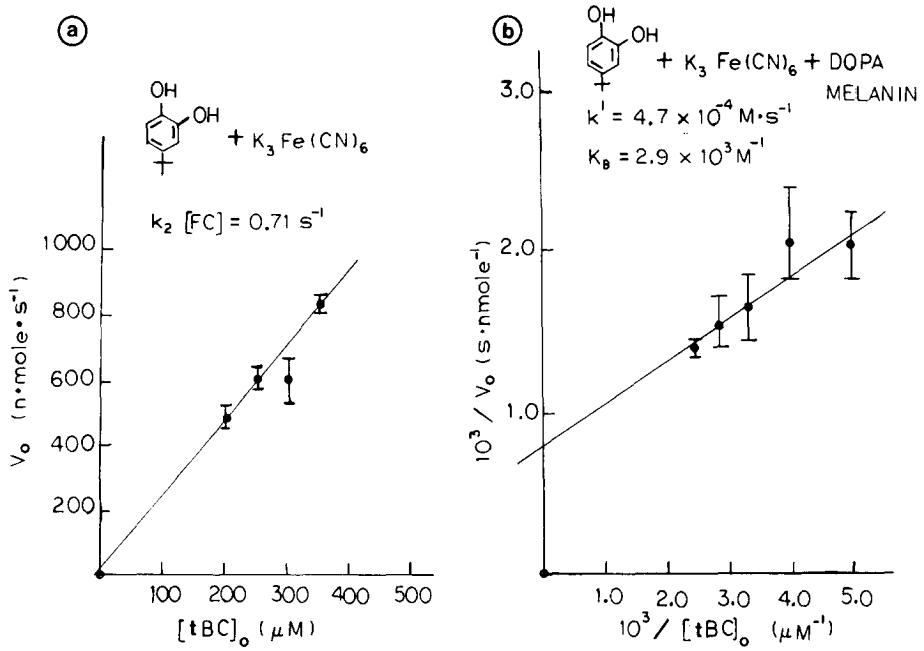

FIG. 2. (a) Plot of initial velocity of ferricyanide reduction, V_0, vs initial tBC concentration, $[tBC]_0$, in the absence of melanin. (b) Reciprocal velocity vs concentration plots in the presence of 100 μg L-DOPA melanin. Ferricyanide concentration was always 640 μM in 0.1 M phosphate buffer, pH 7.4.

Kinetic analysis of ferricyanide reduction for tBC, CAT, HQ, MCAT, DOPA, MMEH, and DHPA, are respectively shown in Figs. 2-8 in the absence (curves a) and presence (curves b) of melanin. With the exception of DHPA (see below), reciprocal

TABLE II

KINETIC AND THERMODYNAMIC PARAMETERS FOR REDUCTION OF POTASSIUM FERRICYANIDE BY MONO- AND DIHYDROXYBENZENE DERIVATIVES IN THE PRESENCE (a) AND ABSENCE (b) OF L-DOPA-MELANIN

Compound	$k' \times 10^4$[a] ($M\ s^{-1}$)	K_B[a] (M^{-1})	$k'K_B$[a] (s^{-1})	k_s[a] (s^{-1})	EF[b] (dimensionless)	Depigment potency[c]
tBC	4.7	2800	1.32	0.71	1.9	++
MCAT	3.4	2200	0.95	0.64	1.5	++
MMEH	1.5	850	0.13	0.028	4.7	++
HQ	13	420	0.55	0.27	2.0	± to +
CAT	22	225	0.50	0.58	0.9	± to +
tBP[d]	0	—	0	0	—	± to +
DHPA[e]	—	—	—	0.55	—	0
DOPA	13	400	0.53	0.69	0.77	0
BHT[d]	0	—	0	0	—	0

[a] See experimental section for details.

[b] $EF = k'K_B/k_s$.

[c] *In vivo* results; see Refs. (8, 9).

[d] Sparingly soluble in buffer; ferricyanide reduction not observed under solubilization conditions where CAT-to-tBC reduction is observed.

[e] System with melanin afforded nonlinear reciprocal plots according to Eq. [2]. Ferricyanide reduction strongly retarded in the presence of melanin, which implies EF < 1, if such a quantity were meaningful in this case.

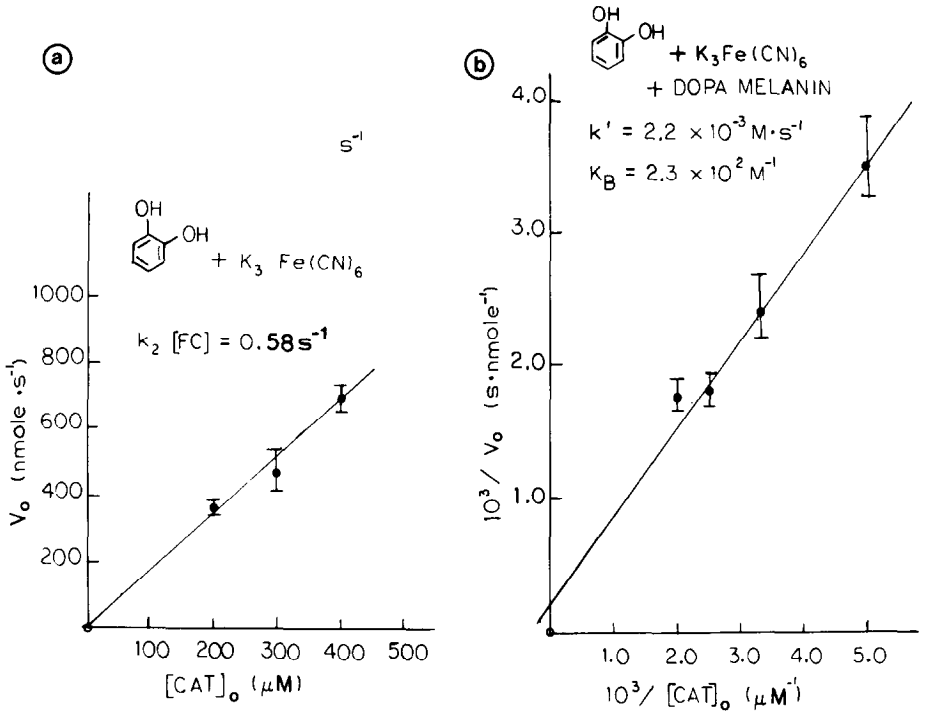

FIG. 3. (a) Plot of V_0 of ferricyanide reduction vs initial catechol concentration, $[CAT]_0$, in the absence of melanin. (b) Reciprocal velocity vs concentration plots in the presence of melanin. Other conditions are as in Fig. 2.

velocity vs concentration plots according to Eq. [2] are linear in the presence of melanin (Figs. 2b-7b), affording values of k' and K_B (Table II). In the absence of melanin, the kinetics of ferricyanide reduction are consistent with Eq. [1] (Figs. 2a-7a), with k_s ranging from 0.71 s^{-1} for tBC to zero for monohydroxy compounds BHT and tBP.

There appears to be an approximate reciprocal relationship between k' and K_B. Binding is strongest for tBC, whereas the value of k' is highest for CAT. Depending on the reductant, melanin either retards or accelerates the overall rate of ferricyanide reduction. Thus EF > 1 for tBC, MCAT, HQ, and MMEH, whereas EF < 1 for CAT and DOPA. In the presence of melanin, DHPA exhibits nonsimple reduction kinetics (Figs. 8a and b), which precludes evaluation of k' and K_B. Reduction is strongly retarded in the presence of melanin, which implies EF would be less than unity for DHPA if it could be evaluated.

In both the presence and the absence of melanin, dihydroxybenzene derivatives reduce ferricyanide much more rapidly than monohydroxy compounds. Reduction by the sparingly soluble tBP and BHT did not occur in either case, even under conditions of solubilization (20% aqueous ethanol) where tBC and CAT readily reduce ferricyanide.

The abilities of the various compounds to produce depigmentation *in vivo* (see Refs. (8, 9)) are compared with the interaction parameters in Table II. The most potent depigmenters, tBC, MCAT, and MMEH, have relatively high values of K_B with moderate values of k', and EF > 1. DOPA and DHPA bind to melanin, and reduce ferricyanide, but are nonetheless nondepigmenters. Ferricyanide reduction by both compounds is significantly retarded in the presence of melanin (EF = 0.77 for DOPA). tBP shows moderate depigmenting activity, but does not reduce ferricyanide. It appears that compounds which clinically show high depigmenting

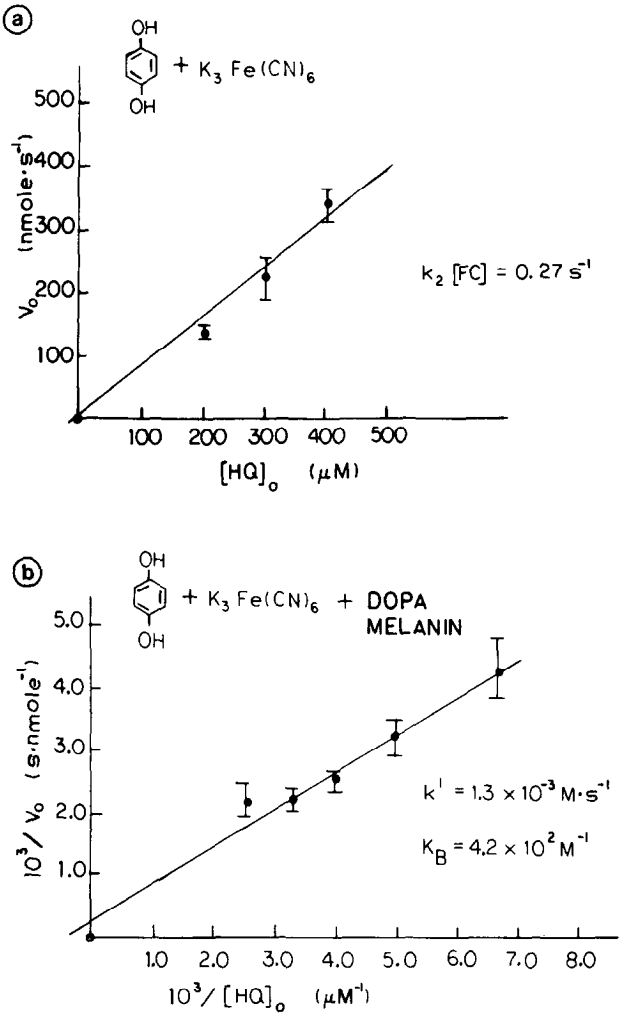

FIG. 4. (a) Plot of V_0 of ferricyanide reduction vs initial hydroquinone concentration, $[HQ]_0$, in the absence of melanin. (b) Reciprocal velocity vs concentration plots in the presence of melanin. Other conditions are as in Fig. 2.

potencies have higher values of EF than those which are poor depigmenters or non-depigmenters. There is no relationship between depigmenting power and the ability per se of a compound to bind to melanin or to reduce ferricyanide.

DISCUSSION

This work has been concerned with DP-melanin interactions and their possible relationship to clinically observable depigmentation potencies of the various DPs. As a first approach, we have used a simple model system which has the advantage of providing direct thermodynamic and kinetic data on such interactions. Our system is necessarily a step removed from more complicated, though more biologically relevant melanoproteins or melanosomes, but it yields straightforward results which provide a basis for interpreting future studies involving the latter systems.

Previous studies have indicated that, at least in some cases, melanocytotoxicity from DP is tied to its oxidation to more toxic quinone-type intermediates (15–17, 22, 23). In melanocytes, such oxidation may be mediated by tyrosinase. However, tyrosinase per se seems unlikely to explain

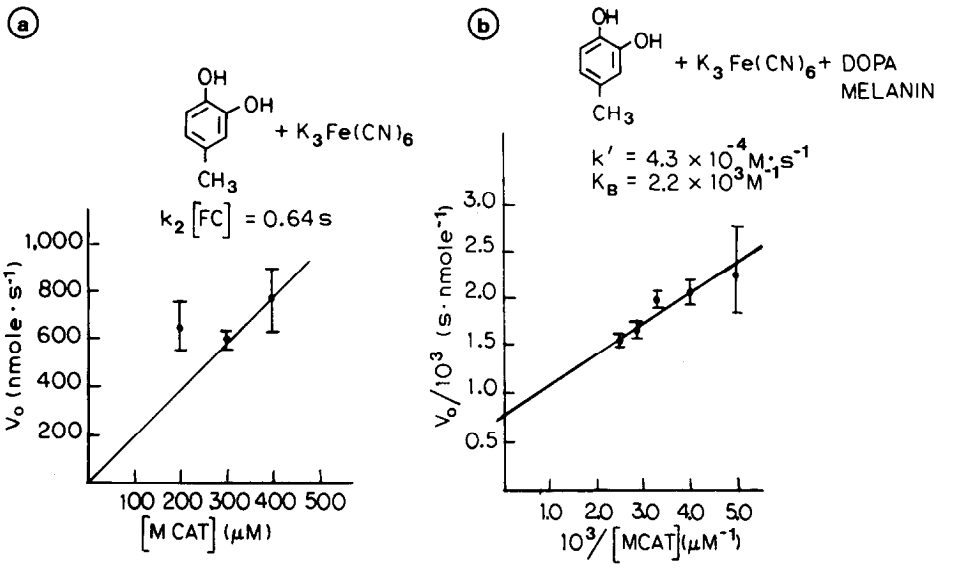

FIG. 5. (a) Plots of V_0 of ferricyanide reduction vs initial 4-methylcatechol concentration, $[MCAT]_0$, in the absence of melanin. (b) Reciprocal velocity vs concentration plots in the presence of melanin. Other conditions as in Fig. 2.

depigmentation (23). In this work, we have demonstrated that the DOPA-melanin pigment can bind to, and accept electrons from, mono- and dihydroxybenzene derivatives, so that one might envision an alternate route for DP oxidation. However,

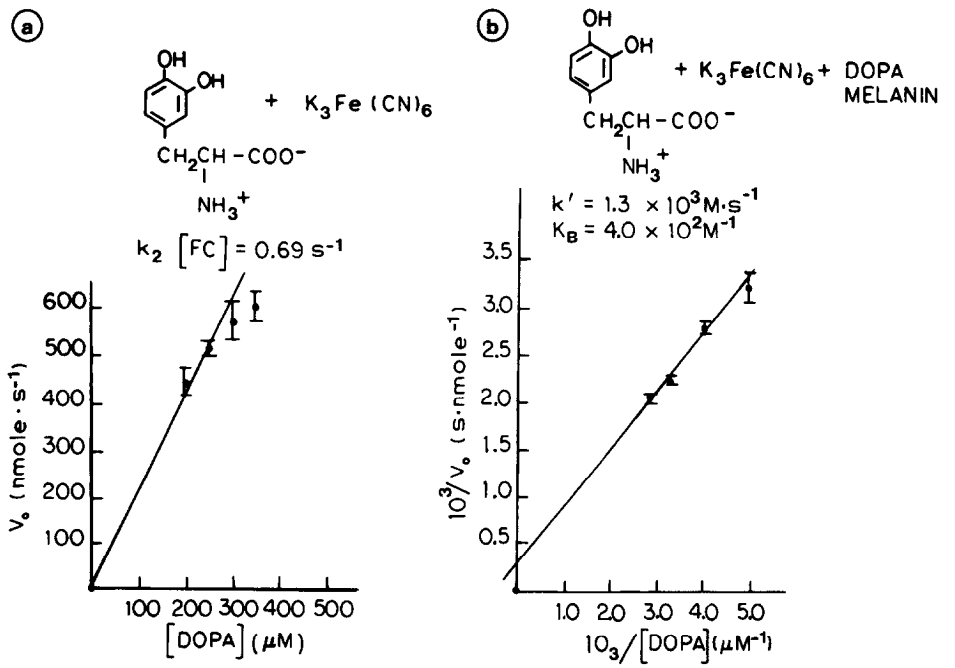

FIG. 6. (a) Plot of V_0 of ferricyanide reduction vs initial DOPA concentration, $[DOPA]_0$, in the absence of melanin. (b) Reciprocal velocity vs concentration plots in the presence of melanin. Other conditions are as in Fig. 2.

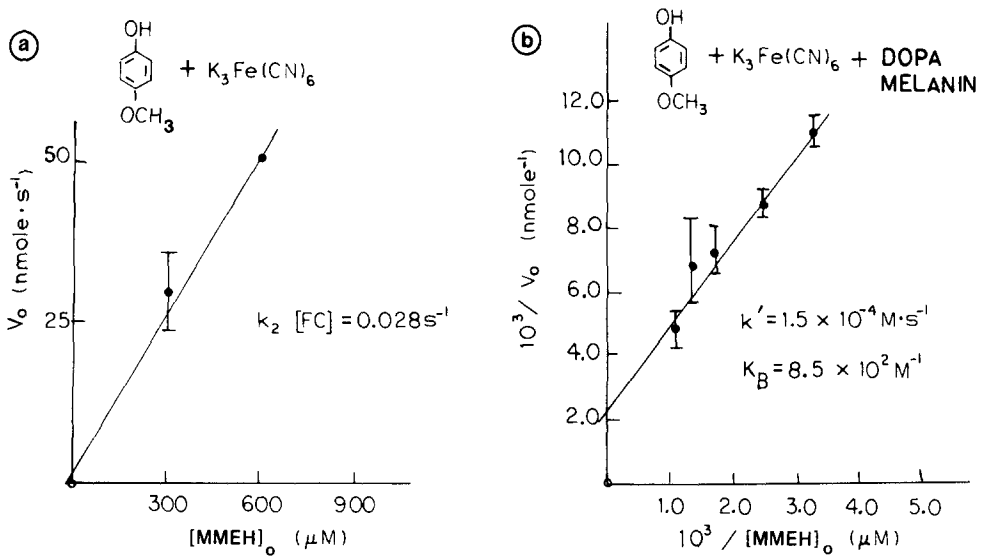

FIG. 7. (a) Plot of V_0 of ferricyanide reduction vs initial monomethyl ether of hydroquinone, $[MMEH]_0$, in the absence of melanin. (b) Reciprocal velocity vs concentration plots in the presence of melanin. Other conditions are as in Fig. 2.

in this case, the presence of a facile electron acceptor is required. Aerated DP solutions in the presence or absence of DOPA-melanin are inefficiently oxidized in the absence of tyrosinase or ferricyanide. The latter observation might explain why mel-

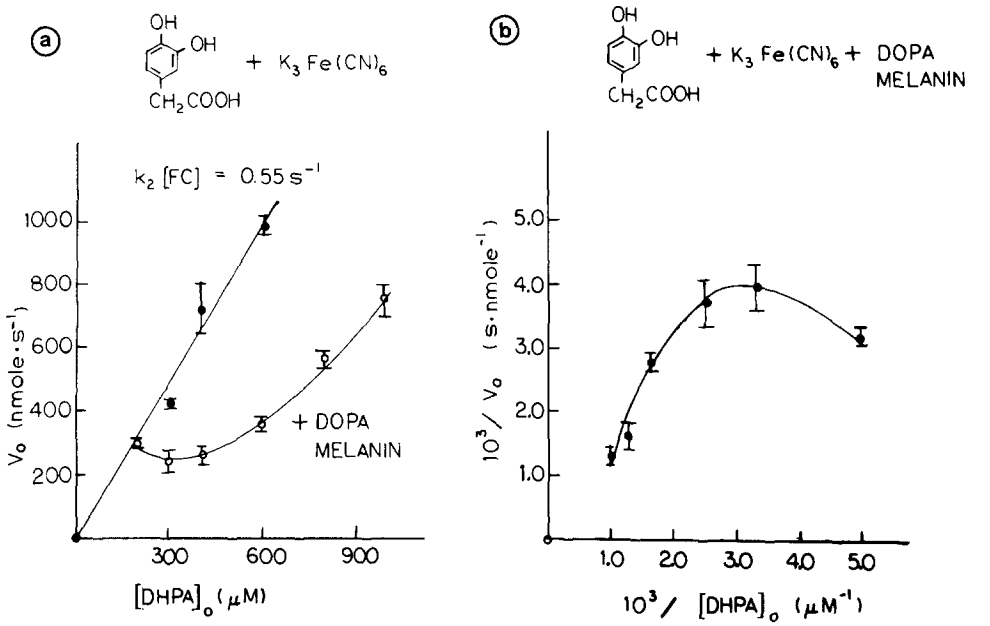

FIG. 8. (a) Plot of V_0 of ferricyanide reduction vs initial 3,4-dihydroxyphenylacetic acid concentration, $[DHPA]_0$, in the absence of melanin. (b) Reciprocal velocity vs concentration plots in the presence of melanin. Other conditions are the same as in Fig. 2. The lower curve in (a) is a replot of the data in (b) as velocity vs concentration.

anin-containing keratinocytes (with inactivated tyrosinase) (24) do not appear to be attacked by melanocytotoxic DPs. In this regard, it is of interest that Breathnach *et al.* (22) were able to demonstrate MMEH cytotoxicity to keratinocytes in tissue culture in the presence of added mushroom tyrosinase.

The demonstration that melanin can bind to, and accept, electrons from DP is of relevance to *in vivo* depigmentation in at least two other ways: (1) In melanized melanosomes, tyrosinase is in close proximity with pigment; DP-melanin binding can therefore physically facilitate DP-tyrosinase interaction. (2) Melanin can *modify* DP-tyrosinase-O_2 reaction; we observe a marked increase in the rate of tyrosinase-catalyzed oxygenation of MMEH and tBP in the presence of mushroom tyrosinase (unpublished results).

As in the case for NADH, melanin can couple oxidation of these compounds to ferricyanide reduction. Except for BHT and tBP, which do not reduce ferricyanide, the presence of melanin either increases or decreases the overall rate of reduction without altering the equilibrium of the reaction. There appears to be a direct relationship between the magnitude of EF and depigmenting potency (Table II); clinically effective depigmenters have higher EF values than nondepigmenters. DHPA would obey this trend if linear reciprocal plots were obtained. The EF is a measure of the overall rate of reduction, reflecting both DP-melanin complex formation and electron transfer to ferricyanide. It is almost as though melanin exerts more "control" over electron transfer reactions of nonpotent depigmenting agents than it does for those involving good-to-excellent depigmenters. This is interesting, since electron transfer may contribute to cytotoxic reactions *in situ.* tBP has moderate depigmenting activity even though it fails to reduce ferricyanide. It is possible that this reflects either complexities in depigmentation mechanisms and/or possible metabolism to tBC *in vivo* via tyrosinase (11).

Ferricyanide reduction kinetics for tBC, MCAT, HQ, MMEH, CAT, and DOPA in the presence of melanin take place according to Eq. [2]. This is consistent with a mechanism whereby melanin acts like a heterogeneous catalyst in coupling a redox reaction between adsorbed but noninteracting DP and ferricyanide. We confirmed DP-melanin binding in all cases by static uptake experiments (Menter, unpublished). Ferricyanide binding can be inferred, but the interpretation is complicated by chemical reduction of 15-20% of it (presumably by quinol moieties within the melanin polymer) in a rapid reaction.

The exact nature of DP-melanin binding is not known. Potts has proposed that charge-transfer interactions are important (25). Larsson and Tjalve (26) have implicated the presence of more than one class of binding site for the catonic chlorpromazine, chloroquine, paraquat, and Ni^{2+}, and have indicated the importance of electrostatic and van der Waals ("stacking") forces (26) in drug-melanin binding. In the present cases (except for DHPA, with its ionized carboxyl group), neutral phenols and catechols predominate at pH 7.4, so that one might expect the predominant interactions to involve charge-transfer, van der Waals, and conceivably hydrogen-bonding forces. Since K_B is a noncooperative Scatchard constant, it is evident that the bound molecules are separated enough to prevent steric interference with each other, and that binding apparently introduces no significant conformational changes in the polymer. The present type of binding apparently differs from enzyme-substrate binding in that the catalytic effect is much smaller than in typical enzyme reactions.

On the other hand, the failure of DHPA to afford linear plots according to Eq. [2] indicates a more complicated kinetic picture, in which interaction among sites may be significant. In this case, it is possible that the ionized DHPA carboxylate group may play a role, perhaps via "nonproductive" binding or repulsive interactions, possibly resulting in steric hindrance or conformational changes.

Comparison of k' and k_s values (Table II) illustrates the extent to which melanin can

modify the relative rates of electron transfer among the various DPs. From the approximate reciprocal relationship between k' and K_B (Table II), it appears that strong binding confers on the DP properties which tend to retard electron transfer. Tighter binding may retard the rate of turnover of reacted/unreacted DP on the melanin surface. Alternatively, change-transfer or hydrogen-bonding interactions might conceivably reduce the amount of DP active reducing species, probably the mono- and/or dianion (27, 28). Additional studies assessing the pH and ionic strength dependence of the redox reactions in the presence and absence of melanin should help resolve these questions.

No ferricyanide reduction was obtained with tBP and BHT, in either the presence or absence of melanin. Low solubility of these compounds in aqueous buffer is not per se responsible, since reduction also did not occur under solubilization in 20% aqueous ethanol, where CAT and tBC readily reduce ferricyanide. Saleem and Wilson (27) have shown that reduction of cytochrome *c*, another iron-containing one-electron system, does not occur for aromatic derivatives which lack two conjugated hydroxyl groups (e.g., phenols and resorcinol), presumably because conjugated hydroxyls are necessary for radical intermediates formed during electron transfer to dismute to yield quinone products. The lack of ferricyanide reduction in the presence of melanin indicates that neither tBP nor BHT readily donates electrons to melanin.

Although formally a phenol, MMEH can be oxidized to *p*-benzoquinone with periodate (29), and it can stabilize a phenoxy radical, formed on electron loss, by delocalization on the unpaired electron to the *p*-type lone pair on the methoxy oxygen (30). Thus MMEH reduces ferricyanide, although more slowly than the dihydroxy compounds. In the presence of melanin, MMEH reduction of ferricyanide is enhanced (EF = 4.7), possibly reflecting melanin's role as an oxidation-reduction coupler capable of facilitating electron transfer.

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