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Melanosome Binding and Oxidation-Reduction Properties of Synthetic L-DOPA-Melanin as *In Vitro* Tests for Drug Toxicity

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SUMMARY

Fifteen drugs were assessed for their ability to interact with calf eye melanosomes and to inhibit synthetic L-DOPA-melanin-catalyzed oxidation-reduction reactions. All drugs were able to bind to calf eye melanosomes. The Scatchard plots of the saturation binding data were curvilinear. At a free drug concentration of 0.1 mM, binding ranged between 0.8 nmol/mg for pirenzepine and 71 nmol/mg for chloroquine, a compound which has been described as provoking toxic side-effects in melanin-containing tissues and adjacent structures. As a result of its electron transfer properties, synthetic L-DOPA melanin catalyzes the NADH oxidation/ferricyanide reduction reaction. Except for (−)-norepinephrine, which underwent rapid oxidation in the presence of ferricyanide, all of the investigated drugs were also able to inhibit this catalytic activity of L-DOPA-melanin. The degree of inhibition is dictated by the extent of binding rather than by the chemical nature of the drug itself. Chlorpromazine itself was able to catalyze the oxidation-reduction reaction and has been proposed to shunt normal electron transport sequences *in vivo*. The implications of melanin binding with respect to drug toxicity are discussed in the light of the present observations.

The melanin polymer is obtained by the oxidation of tyrosine in specialized cellular vesicles: the melanosomes (1). This pigment is present in various tissues of animals and humans, including the eye, the inner ear, skin, hair, hair follicles, and the brain. Its precise physiologic function is still not fully understood. Within the eye, melanin has the ability to reduce light scatter. In addition, melanin is a biological electron exchange polymer, which allows it to act as a free radical scavenger (2–4). It is therefore generally believed that one of the main functions of melanin is to protect tissues against photochemically induced free radicals.

Besides its protective function, this pigment has also been suspected of playing a pivotal role in the induction of toxic side-effects of several drugs. In this context, particular attention has already been paid to chloroquine, phenothiazine derivatives such as chlorpromazine, and the streptomycin group of antibiotics (5–9). Due to their high affinity for melanin, these drugs are retained by pigmented tissues such as the eye, skin, inner ear or pigmented nerve cells, and are accumulated in isolated melanosomes. These drugs are also able to provoke chronic lesions in the above-mentioned tissues. This marked coincidence has led to the suggestion that binding to melanin is implicated in the pathogenesis of lesions in pigmented tissues.

Drug-melanin binding has been studied by two main approaches. The first, a qualitative approach, is based on whole body autoradiography following *in vivo* injection of labeled drug (7, 10–13). The second, *in vitro* approach consists in the quantitative evaluation of drug binding to melanosomes prepared from bovine eyes (5, 7–9). Whereas many drugs appear to bind to melanin, only a few of them seem to cause toxicity. Therefore, drug toxicity might be related to alteration of the functional characteristics of melanin rather than to binding alone. In this context, the free radical/electron transfer properties can be determined *in vitro* by electron spin resonance spectroscopy (14, 15) or by investigating the ability of synthetic melanin to catalyze the oxidation of NADH and the reduction of ferricyanide (3, 16, 17).

In vitro binding studies have been used extensively to characterize drug-melanin interaction, whereas little is known about the potency of these drugs to impair the chemical properties and the biological function of melanin. In the present study, we have compared the ability of 15 aromatic drugs to bind to calf eye melanosomes with their capability of influencing redox catalysis by synthetic L-DOPA-melanin.

Materials and Methods

Chemicals. The following substances were obtained as generous gifts: (−)-alprenolol tartrate monohydrate (Hässle), dexetimide, and

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ABBREVIATIONS: L-DOPA, L-dihydrophenylalanine; (−)-QNB, (−)-quinulidinyl benzilate; RX 781094, idazoxan; SCH 23390, *R*-(+)-8-chloro-3-methyl-5-phenyl-7-ol-benzazepine.

spiperone (Janssen Pharmaceutical), prazosin hydrochloride (Pfizer Central Research), RX 781094 (Ricket and Colman Pharmaceutical Division), chloroquine sulfate (Rhone Poulenc), and SCH 23390 maleate (Schering Corp.). Chlorpromazine hydrochloride, (−)-norepinephrine bitartrate, (−)-scopolamine methyl bromide, clonidine hydrochloride, NADH, and potassium ferricyanide were purchased from Sigma; (−)-nicotine and L-DOPA were from Janssen Chimica; pirenzepine dihydrochloride monohydrate was from Dr. Karl Thomae GMBH; (−)-QNB was from Amersham International; and rauwolscine hydrochloride was from Carl Roth KG. [^{3}H]Chlorpromazine hydrochloride (22.4 Ci/mmol), [^{3}H]clonidine hydrochloride (41.5 Ci/mmol), (−)-[^{3}H]dihydroalprenolol (96.1 Ci/mmol), [^{3}H]pirenzepine (82.3 Ci/mmol), [^{3}H]prazosin (18 Ci/mmol), [^{3}H]rauwolscine (76.5 Ci/mmol), (−)-[*N-methyl*-^{3}H]scopolamine methyl chloride (85 Ci/mmol), and [^{3}H]spiperone (22.9 Ci/mmol) were obtained from New England Nuclear. (−)-[*N-methyl*-^{3}H]nicotine (60 Ci/mmol), (−)-[^{3}H]norepinephrine hydrochloride (16.3 Ci/mmol), (−)-^{3}H]QNB (35.2 Ci/mmol), [^{3}H]RX 781094 (60 Ci/mmol), and [*N-methyl*-^{3}H]-SCH 23390 (87 Ci/mmol) were obtained from Amersham, U.K. [^{3}H]dexetimide (14 Ci/mmol) was supplied by the Instiuut voor Radioelementen (Fleurus, Belgium). All other chemicals were of the highest grade commercially available.

Preparation of melanosomes. Melanosomes were prepared according to the method of Menon and Haberman (18) with modifications. Calf eyes were obtained from a local slaughterhouse. All subsequent steps were performed at 0–4°. The pigmented tissues (chorioid, iris, ciliary body) were dissected and homogenized in 50 mM sodium, potassium phosphate buffer, pH 7.0 (10 ml/g of tissue) with a Polytron mixer and subsequently with a Potter-Elvehjem homogenizer (three strokes at maximum speed). The homogenate was centrifuged at 120 × *g* for 8 min. The supernatant was collected and the pellet was rehomogenized in fresh buffer and recentrifuged. The combined supernatants were centrifuged at 7,700 × *g* for 10 min. The pellet was resuspended in buffer. Aliquots of 3 ml were placed over 20 ml of 1.6 M sucrose and centrifuged at 39,000 × *g* for 60 min. The pellets were resuspended in buffer and aliquots of 1 ml were centrifuged for 2 min at 9,000 × *g* in an Eppendorf centrifuge. The melanosome pellets were weighed (wet weight) and stored at 4° before use. No alterations of binding properties were observed within 2 weeks.

Preparation of L-DOPA-melanin. Synthetic L-DOPA-melanin was prepared by the autooxidation of L-DOPA according to the method of Froncisz *et al.* (19). One of g L-DOPA was dissolved in 400 ml of sodium, potassium phosphate buffer (50 mM, pH 8.0). The solution was stirred and bubbled with an air stream, previously passed through 2 M NaOH, for 72 hr at room temperature. The resulting black melanin was precipitated by addition of 1 ml of concentrated HCl and centrifugation at 3000 × *g* for 10 min. The pellet was resuspended in distilled water and precipitated by addition of HCl and centrifugation. This washing step was repeated twice. The final pellet was resuspended in 50 ml of distilled water and stored at 4° before use. Aliquots were lyophilized to determine the dry weight of melanin.

Saturation binding experiments. Calf eye melanosomes (2 mg wet weight/ml) were incubated at room temperature in Eppendorf tubes with increasing concentrations of tritium-labeled drug (10–200 μM) for 2 hr in 50 mM sodium, potassium phosphate buffer (pH 7.0) in a total volume of 1.0 ml. The tubes were placed in a rotating rack and shaken vigorously every 30 min. After incubation, the melanosomes were precipitated by centrifugation at 9000 × *g* for 3 min in an Eppendorf centrifuge. A 0.5-ml aliquot was removed from the supernatant and placed in a polyethylene scintillation vial with 8 ml of scintillation fluid (Picofluor 15 from Packard); then, the radioactivity content was counted in a Packard liquid scintillation spectrometer. This value, corresponding with the concentration of free drug, was subtracted from the total amount of radioactivity added to yield the concentration of drug bound to the melanosomes as well as to the tube wall. Binding to the wall was evaluated as a function of the free drug concentration by separate experiments, performed in the absence of melanosomes, and subtracted from total binding to evaluate the amount of melanin-bound drug. Binding of unlabeled chloroquine was determined as described above, except that the concentration of free drug was determined by measurement of its absorbance maximum at 342 nm with a Zeiss DM4 spectrophotometer. Each experiment comprised duplicate determinations and similar experiments were always carried out on different melanosome preparations.

Redox catalysis. Synthetic L-DOPA melanin catalyzes the redox reaction involving the oxidation of NADH and the reduction of potassium ferricyanide (3) according to the following global reaction scheme:

$$\text{Mel} + \text{NADH} + \text{H}^+ \rightleftharpoons \text{Mel}\cdot 2\text{H} + \text{NAD}^+ \quad (1)$$

$$\text{Mel}\cdot 2\text{H} + 2\ \text{Fe}^{3+} \rightleftharpoons \text{Mel} + 2\ \text{H}^+ + 2\ \text{Fe}^{2+} \quad (2)$$

L-DOPA-melanin (20 μg/ml) was incubated at room temperature with 0.1 mM NADH and 0.25 mM potassium ferricyanide [$K_3Fe(CN)_6$] either in the absence or in the presence of increasing concentrations of drug (0.1–100 μM) in 50 mM sodium, potassium phosphate buffer, pH 7.0. The reaction was started by addition of NADH and the mixture was immediately transferred to a 1-ml quartz cuvette. The concentration of NADH was determined as a function of time (0–5 min) by measurement of its absorbance maximum at 340 nm. The control cuvette contained the same mixture devoid of NADH and, except for mixtures containing (−)-norepinephrine, displayed a constant absorbance at 340 nm for the period of time investigated. The timewise decrease in NADH concentration was exponential in all instances. The rate of the reaction was calculated by linear regression analysis of the semilogarithmic absorbance versus time plot. A slow redox reaction was also observed when NADH was co-incubated with potassium ferricyanide only. This basal rate was not affected by any concentration of added drug except for chlorpromazine. Each experiment comprised duplicate determinations.

Octanol/buffer partitioning. The hydrophobicity of the drugs was determined by measuring their distribution coefficients in an *n*-octanol/phosphate buffer system. Tritium-labeled drug (0.1 mM) was added to an Eppendorf tube containing equal amounts (500 μl) of saturated *n*-octanol and satured 50 mM sodium, potassium phosphate buffer (pH 7.0). The tubes were shaken vigorously for 30 sec and centrifuged for 1 min at 9000 × *g* in an Eppendorf centrifuge. One hundred-μl samples were taken from both of the phases and transferred into a scintillation vial containing 8 ml of scintillation liquid. The radioactivity content was determined by liquid scintillation counting. The concentration of chlorpromazine in the buffer phase was determined spectrophotometrically. This value was subtracted from the initial chlorpromazine concentration to yield its concentration in the octanol phase. The distribution coefficient, D was calculated by $D = [O]/[B]$ where $[O]$ represents the amount of drug in the octanol phase and $[B]$ the drug concentration in the buffer phase.

Results

Saturation binding. All 15 ligands tested were able to bind calf eye melanosomes in a dose-dependent fashion. As a typical example, the saturation binding data for [^{3}H]chlorpromazine, [^{3}H]spiperone, [^{3}H]RX 781094, and (−)-[^{3}H]nicotine are depicted in Fig. 1. Control experiments indicate that binding is not affected when the melanosome concentration is raised from 2 to 4 mg/ml or when the incubation time is raised from 2 to 24 hr (data not shown), suggesting that the saturation curves reflect equilibrium binding. The Scatchard plots (Fig. 1B) are curvilinear, indicating that the melanosomes contain multiple independent binding sites for these ligands. In Table 1 the extent of binding corresponding to a free drug concentration of 0.1 mM is represented. Binding values ranged between 0.8 nmol/mg for pirenzepine and 71 nmol/mg for chloroquine.

The octanol/buffer distribution coefficients of the investigated drugs are listed in Table 1. D values ranged between 0.02 for (−)-norepinephrine and 56.5 for spiperone. When deter-

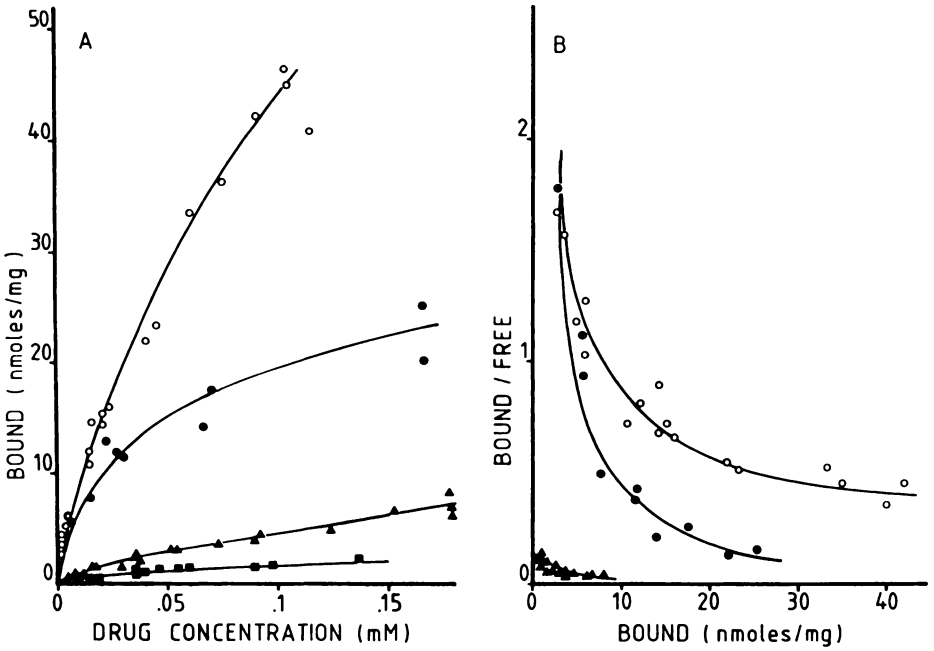

Fig. 1. A. Saturation binding of [^{3}H]chlorpromazine (○), [^{3}H]spiperone (●), [^{3}H]RX 78104 (▲), and (−)-[^{3}H]nicotine (■) to calf eye melanosomes. Melanosomes (2 mg/ml) were incubated with increasing concentrations of drug (10–200 μM) for 2 hr in 50 mM phosphate buffer, pH 7.0. Binding was determined as described under Materials and Methods. Data points represent single determinations. B. Scatchard plot of the saturation binding data shown in A.

TABLE 1

Binding to calf eye melanosomes, bound/free values, inhibition of redox catalysis of synthetic L-DOPA melanin, and the octanol/buffer distribution coefficients of the investigated drugs

Binding to melanosomes (in nmol/mg of melanosome, wet weight) was extrapolated for a free drug concentration of 0.1 mM from saturation binding curves and Scatchard plots as shown in Fig. 1, A and B, and bound/free values were recorded for 0.1 mM drug (total concentration). The percentage inhibition of the redox catalytic action of L-DOPA melanin for an 0.1 mM concentration of each drug is defined as described in the legend to Fig. 6. The octanol/buffer distribution coefficients (*D* values) were recorded for 0.1 mM drug (total concentration) as described under Materials and Methods.

Compound	Melanosome binding	Bound/Free	% Redox inhibition	*D*
	nmol/mg			
0. Chloroquine	71.2	2.31		1.54
1. Chlorpromazine	46.0	0.532	94.0	17.1
2. (−)-Quinuclidinyl benzilate	20.0	0.284	66.5	20.1
3. Spiperone	19.5	0.341	75.7	56.5
4. Prazosin	17.5	0.250	66.1	11.0
5. SCH 23390	6.5	0.164	54.1	21.6
6. (−)-Dihydroalprenolol	5.5	0.053	49.0	5.15
7. Clonidine	5.3	0.052	45.5	0.95
8. Rauwolscine	5.1	0.061	69.8	11.6
9. RX 781094	4.7	0.047	40.1	0.60
10. Dexetimide	3.8	0.039	58.1	5.37
11. (−)-Norepinephrine	3.6	0.036		0.02
12. (−)-Scopolamine	2.1	0.019	31.8	0.04
13. (−)-Nicotine	1.7	0.015	45.7	0.69
14. Pirenzepine	0.8	0.006	39.1	0.10

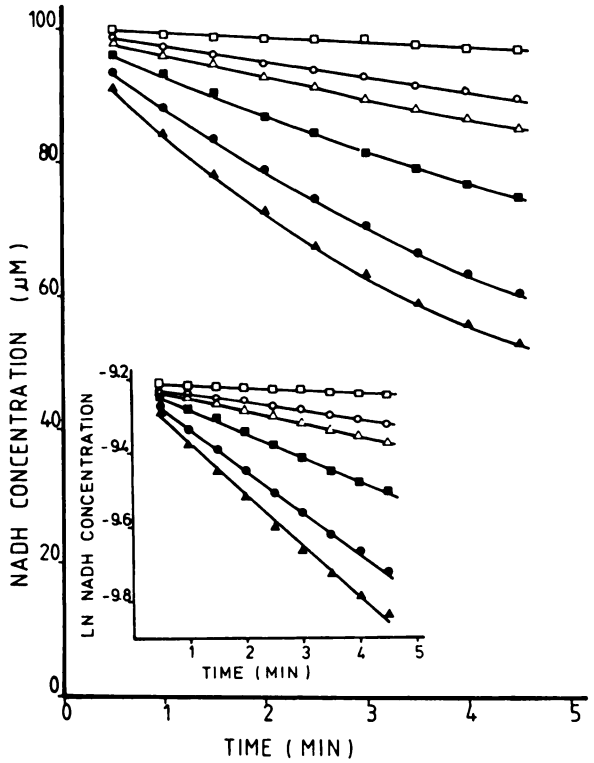

Fig. 2. NADH oxidation/potassium ferricyanide reduction: effect of increasing concentrations of synthetic L-DOPA-melanin. NADH (0.1 mM) and potassium ferricyanide (0.25 mM) were incubated in 50 mM phosphate buffer, pH 7.0, either alone (□) or in the presence of increasing concentrations of synthetic L-DOPA melanin: 3 μg/ml (○), 5 μg/ml (△), 10 μg/ml (■), 20 μg/ml (●), or 30 μg/ml (▲). The time dependence of the NADH concentration was recorded spectrophotometrically at 340 nm. Data points represent means of three experiments. *Inset*: Semilogarithmic representation of the same data. All plots are linear.

mined under the same experimental conditions (total drug concentration = 0.1 mM, pH = 7.0), there was no significant correlation between the distribution coefficients and the bound/free values of these drugs for melanosome binding (r = 0.02, $p > 0.1$).

Redox catalysis. The oxidation of NADH was monitored spectrophotometrically at 340 nm. Whereas a solution of 0.1 mM NADH in 50 mM sodium, potassium phosphate buffer at pH 7.0 was stable for 5 min, the addition of 0.25 mM potassium ferricyanide gave rise to a slow, timewise decrease in the NADH concentration (Fig. 2). When the NADH concentration is plotted on a logarithmic scale (Fig. 2, *inset*), the decrease is linear with the incubation time. The apparent first order rate constant (k_{ob}) of this spontaneous redox reaction is 0.012 min^{-1}.

Whereas synthetic L-DOPA-melanin (1–30 μg/ml) did not

provoke a perceptible decrease in the NADH concentration within 5 min (data not shown), the polymer increased the rate of the NADH oxidation/ferricyanide reduction reaction in a dose-dependent fashion (Fig. 2). The reaction scheme is depicted under Materials and Methods. The logarithm of the NADH concentration was always linear with the incubation time (Fig. 2, *inset*) and the increase of the k_{ob} values appeared to be linearly related to the L-DOPA-melanin concentration up to 20 μg/ml ($r = 0.99$, Fig. 3).

To investigate the influence of the drugs upon the redox-catalytic properties of L-DOPA melanin, we first verified their effect (at a concentration of 0.1 mM) on NADH or potassium ferricyanide either alone or in combination. Chloroquine was discarded due to its strong absorbance at 340 nm, the wavelength used to measure the NADH concentration. None of the drugs affected the NADH concentration, but (−)-norepinephrine underwent a reddish coloration (i.e., adrenochrome formation) in the presence of ferricyanide. Hence, (−)-norepinephrine was also discarded from the subsequent experiments. Except chlorpromazine, none of the drugs affected the rate of the NADH oxidation/ferricyanide reduction reaction. As shown in Fig. 4, this phenothiazine derivative was able to enhance the redox reaction. The k_{ob} values for the NADH disappearance increased significantly when the chlorpromazine concentration was 30 μM or above.

Interestingly, the redox catalytic actions of chlorpromazine and L-DOPA melanin were not additive. As shown in Fig. 4, the rate of NADH disappearance in the concomitant presence of 30 μM chlorpromazine and 20 μg/ml L-DOPA-melanin was intermediate between the effects produced by both compounds separately. The k_{ob} values were 0.100, 0.028, and 0.052 min^{-1} in the presence of L-DOPA-melanin, chlorpromazine, and their combination, respectively. This indicates that chlorpromazine is also able to provoke a decrease in the catalytic activity of L-DOPA-melanin. The concentration dependence of this chlorpromazine effect is illustrated in the *inset* of Fig. 4. The 12 remaining drugs were also able to inhibit the redox-catalytic action of L-DOPA-melanin. As a typical example, Fig. 5 illustrates the effect of different concentrations of spiperone. Fig. 6 compares the concentration dependency of chlorpromazine, spiperone, RX 781094, and (−)-nicotine to inhibit the L-DOPA-melanin-catalyzed redox reaction. For these and all the other drugs, the inhibition increased concentration-wise. Table 1 compares the inhibition produced by a 0.1 mM concentration of the different drugs tested. The degree of inhibition was drug dependent and ranged between 32% for (−)-scopolamine and 94% for chlorpromazine.

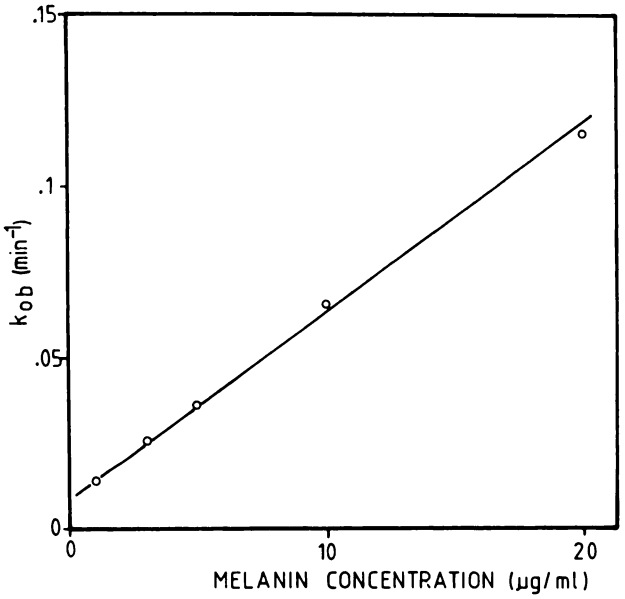

Fig. 3. Redox-catalytic action of L-DOPA-melanin: concentration dependency. The pseudo-first order rate constants (k_{ob}) for NADH disappearance were determined by linear regression analysis of the ln(NADH concentration) versus time plots shown in the *inset* of Fig. 2. The k_{ob} values are represented as a function of the concentration of added L-DOPA melanin. Co-incubation of NADH and potassium ferricyanide only (i.e., basal conditions) gave rise to a slow redox reaction with a k_{ob} of 0.012 min^{-1}.

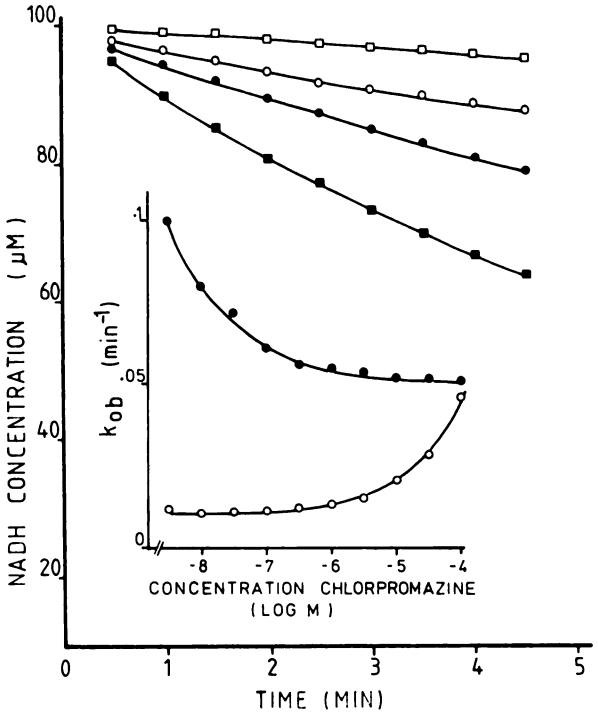

Fig. 4. NADH oxidation/potassium ferricyanide reduction: combined effect of synthetic L-DOPA melanin and chlorpromazine. NADH and potassium ferricyanide were incubated as described in Fig. 2, either alone (□) or in the presence of 20 μg/ml synthetic L-DOPA-melanin (■), 30 μM chlorpromazine (○), or L-DOPA-melanin plus chlorpromazine (●). The time dependence of the NADH concentration was recorded spectrophotometrically at 340 nm. Data points represent means of three experiments. *Inset*: The k_{ob} values for NADH disappearance are represented as a function of the concentration of chlorpromazine either in the absence (○) or presence of 20 μg/ml L-DOPA melanin (●).

Fig. 7 compares the percentage of redox inhibition (I) with the extent of melanosome binding (B) for each drug at a free concentration of 0.1 mM. There was a significant correlation between both drug parameters ($r = 0.78$, $p < 0.01$). Nevertheless, the relationship appeared to deviate from linearity, the I/B ratio being much higher for low B values. A similar relationship was observed when I and B values were compared for different free concentrations of each single drug. As a typical example, both values for chlorpromazine are represented in the *inset* of Fig. 7.

Discussion

In this study we have employed two convenient techniques for the *in vitro* characterization of drug-melanin interactions:

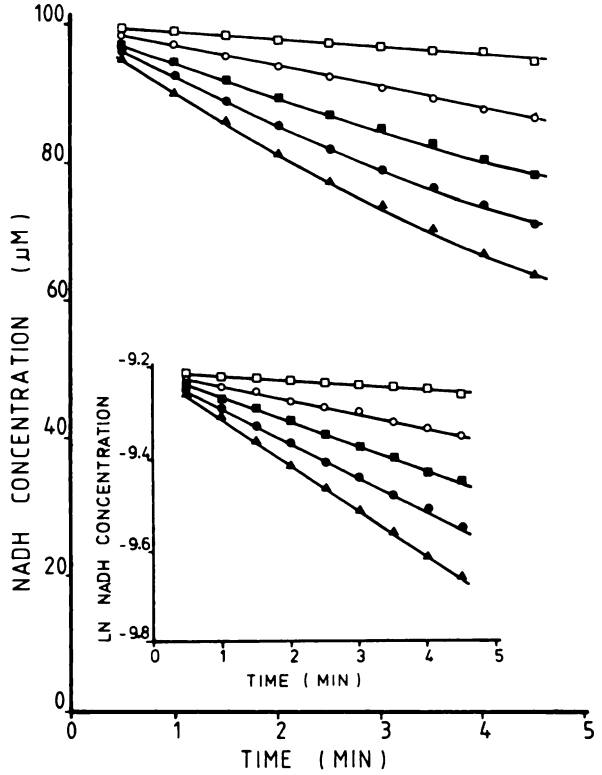

Fig. 5. Inhibition of the redox-catalytic action of L-DOPA-melanin by spiperone. NADH and potassium ferricyanide were incubated as described in Fig. 2 either alone, (□) or in the presence of 20 μg/ml synthetic L-DOPA-melanin (▲) or L-DOPA-melanin plus increasing concentrations of spiperone: 1 μM (●), 10 μM (■), or 100 μM (○). *Inset*: Semilogarithmic representation of the same data. All plots are linear.

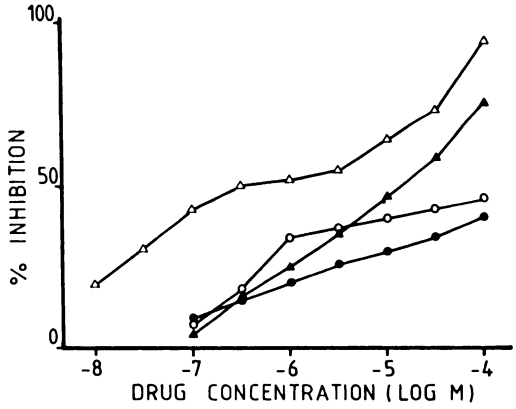

Fig. 6. Concentration-dependent inhibition of the catalytic action of L-DOPA-melanin by chlorpromazine, spiperone, RX 78104, and nicotine. NADH and potassium ferricyanide were incubated with 20 μg/ml synthetic L-DOPA-melanin in the presence of the indicated concentrations (*abscissa*) of chlorpromazine (△), spiperone (▲), RX 78104 (●), and (−)-nicotine (○). Data for 1–100 μM spiperone are shown in Fig. 5. Percentage inhibition of the redox-catalytic action of L-DOPA melanin is defined as: $100 \times (k_M + k_D - k_{MD} - K_O)/(k_M - k_O)$ for chlorpromazine and as: $100 \times (k_M - k_{MD})/(k_M - k_O)$, for the other drugs ($k_O$, k_M, k_D, and k_{MD} are k_{ob} values for NADH disappearance under basal conditions, in the presence of melanin, of drug, and of melanin plus drug, respectively).

binding to isolated melanosomes and inhibition of L-DOPA-melanin-catalyzed redox reactions. The 15 drugs investigated could be classified on the basis of their pharmacologic specificity as adrenergic agonists [clonidine, (−)-norepinephrine] and antagonists [prazosin, rauwolscine, RX 781094, (−)-alprenolol],

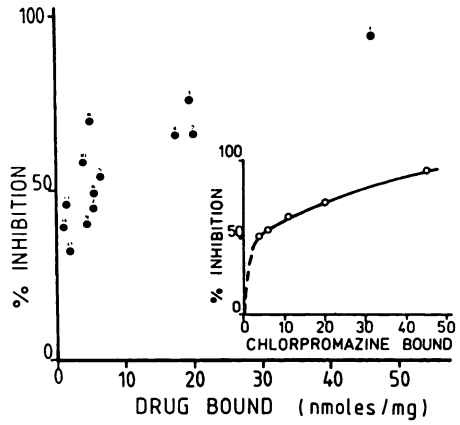

Fig. 7. Inhibition of redox catalysis as a function of the extent of melanosome binding. Points represent situations for the drugs listed in Table 1 [compounds **0–14**; except (−)-norepinephrine and chloroquine] at a free concentration of 0.1 mM. Percentage inhibition of the redox-catalytic action of L-DOPA-melanin (*I*) and the extent of melanosome binding (*B*) are given in Table 1. *Inset*: *I* versus *B* for increasing concentrations of chlorpromazine (1–100 μM, free concentration). *B* values were extrapolated from the saturation binding curve shown in Fig. 1A.

dopaminergic antagonists (SCH 23390, chlorpromazine, spiperone), cholinergic antagonists [(−)-nicotine, (−)-scopolamine, pirenzepine, (−)-QNB, dexetimide], and the antimalarial agent chloroquine. Besides their different structures, all drugs contain one or more aromatic nuclei and amino groups.

As expected, all drugs were found to bind to calf eye melanosomes. As for most compounds investigated so far, saturation binding experiments yielded curvilinear Scatchard plots (Fig. 1B). The upward concavity reflects the presence of at least two classes of binding sites with different affinity. Since the structural heterogeneity of melanin suggests a large number of different sites, a quantitative evaluation of the drug binding parameters according to a two-site or even a three-site analysis remains highly speculative. Factors such as accessibility, sterical hindrance, and the strength of binding forces are likely to dictate the binding parameters of each class of sites for a given drug. In this context, a semiquantitative evaluation reveals striking differences in the extent of drug-melanosome binding. At equal concentrations, chloroquine and chlorpromazine bound to a higher extent to calf eye melanosomes than the other drugs (Table 1). (−)-QNB, spiperone and prazosin bound to an appreciable extent as well. Whereas chloroquine and chlorpromazine have been described to provoke several toxic side-effects in melanin-containing tissues, such effects have not yet been reported for the latter drugs (e.g., for prazosin) (20). Binding of the other drugs was 3.5–89 times lower, compared to chloroquine. This marked difference cannot solely be attributed to the occurrence of hydrophobic interactions. Indeed, there is no significant correlation between the extent of melanosome binding and the octanol/water distribution coefficients of the drugs investigated. Van der Waals forces at the conjunction between the aromatic rings of the drugs and those of melanin (21) and ionic attractions between the positively charged amino groups of the drugs and the anionic carboxyl groups of melanin (8) probably also contribute to the extent of drug-melanin binding.

Our observations are in agreement with the general contention that, whereas many compounds are able to bind to melanosomes, only a few of them seem to be toxic in melanin-containing tissues. In this context, it has been put forward that

compounds which bind reversibly by electrostatic interaction cause no toxicity, whereas drugs which might undergo free radical interactions give rise to toxicity (1). Melanin possesses stable free radicals, which are assumed to play a crucial role in the protection of tissues against reducing and oxidative conditions and against irradiation (2–4). Since chlorpromazine is a good electron donor, it might participate in a charge transfer complex with melanin (8, 9, 22). This would result in a reduction of the free radical content of melanin (22), its electron transfer properties, and, hence, its ability to inactivate toxic free radicals. Alterations of the melanin electron spin resonance signals by chloroquine have been reported as well (14).

The electron transfer properties of melanin can be assessed *in vitro* by investigating its ability to catalyze oxidation-reduction reactions. Van Woert (22, 23) first reported the ability of synthetic L-DOPA-melanin to catalyze the oxidation of NADH under aerobic conditions. Interestingly, chlorpromazine was found to decrease the catalytic activity as well as the electron spin resonance signal of L-DOPA-melanin (22). This constituted good evidence for the ability of chlorpromazine to reduce the free radical content of melanin. Subsequently, Gan and colleagues (3, 16, 17) demonstrated that the rate of L-DOPA-melanin-catalyzed NADH oxidation could be appreciably enhanced when this reaction was coupled to the reduction of ferricyanide. Besides the short incubation time needed (less than 5 min), this procedure also offers the considerable advantage that the concentration of oxidant is under control, so that the experiments are highly reproducible. We have adopted this experimental procedure in the present study to compare the ability of the investigated drugs to affect the catalytic activity of L-DOPA-melanin. (−)-Norepinephrine was discarded since it underwent rapid oxidation in the presence of ferricyanide, and chloroquine was discarded due to its strong absorbance at 340 nm. This study gave rise to two rather unexpected results: 1) all of the 13 investigated drugs were, to a greater or lesser extent, able to inhibit the catalytic activity of L-DOPA-melanin (Table 1); and 2) chlorpromazine itself was able to catalyze the NADH oxidation/ferricyanide reduction reaction in a concentration-dependent manner (Fig. 4).

The decrease in the electron transfer properties of L-DOPA-melanin might result from an actual decrease in its free radical content but also from the ability of the drugs to shield or hide its catalytic sites. Several observations suggest that these alternative mechanisms might play an important, if not primordial, role.

First, the inhibition of the catalytic activity of L-DOPA-melanin (expressed as per cent redox inhibition) seems to be dictated by the extent of binding rather than by the chemical nature of the drug itself. Hence, charge transfer complex formation does not play a key role. Indeed, comparable curves are obtained when the per cent redox inhibition is plotted as a function of the extent of melanosome binding for (a) all the drugs at a free concentration of 0.1 mM and (b) the individual drugs at different free concentrations (Fig. 7). Further experimental work is needed to clarify whether or not the nonlinear relationship between these parameters reflects differences in the melanins employed in both assays.

Second, Gan *et al.* (24) have reported that the catalytic activity of melanin is reduced when the polymers are attached to proteins. This phenomenon was held responsible for the fact that NADH oxidation is appreciably slower in the presence of melanosomes than in the presence of free L-DOPA-melanin. These data also suggest that any increase in the compactness of melanin is reflected by a decrease in the accessibility of its catalytic sites. In this context, bound drugs might increase the compactness of L-DOPA melanin by intercalating their aromatic rings between those of melanin. The ability of high concentrations of chlorpromazine (>1 mM) to provoke aggregation of L-DOPA melanin pleads in favor of this assumption.

Being soluble, synthetic L-DOPA melanin has the considerable advantage of an easily accessible catalytic surface. However, our data indicate that the decrease in the catalytic activity of synthetic L-DOPA-melanin is merely related to the amount of bound drug so that the investigation of redox catalysis does not add substantially new information with respect to binding studies.

Protoporphyrin is known to produce symptoms of photosensitivity in patients with porphyrias and has been reported to catalyze the NADH oxidation/ferricyanide reduction reaction (25). Interestingly, chlorpromazine was also able to catalyze this redox reaction (Fig. 4). This catalytic action can be explained by the observation of Borg and Cotzias (26) that ferric ions can oxidize chlorpromazine into its semiquinone form. This form is labile and is able to react with electron donors and electron acceptors. These authors proposed that chlorpromazine could shunt normal electron transport sequences *in vivo* or alter the local availability of rate-limiting ionic cofactors in some essential biochemical processes. In the light of these assumptions, the NADH oxidation/ferricyanide reduction reaction itself might prove to be a very suitable model system for the determination of drug toxicity.

Although drugs such as chlorpromazine (26–28) and chloroquine (29) might be capable of provoking cell damage by a variety of mechanisms which are unrelated to melanin binding, we still have to deal with the fact that melanin-containing tissues are the most vulnerable ones. We do not rule out that chlorpromazine and chloroquine might be capable of interfering with the normal *in vivo* electron transfer properties of melanin. Rather, our data indicate that a decrease in the redox-catalytic properties of soluble L-DOPA-melanin does not constitute an adequate *in vitro* indication for the occurrence of such a mechanism. Alternatively, melanin might also be seen to act as an intracellular reservoir, slowly releasing its drug content. This phenomenon has been invoked to explain the prolonged action of certain drugs in the eye (30). The same property of melanin might also result in the prolonged presence of toxic compounds in pigmented tissues, whereas they are only transiently present in the other tissues. According to this mechanism, melanin does merely act as a reservoir, and drugs may be toxic by mechanism which are unrelated to melanin binding. Since melanin binding appears to be implicated in the etiology of serious side-effects of certain therapeutic drugs, a better understanding of the exact role of this pigment should be beneficial for the development of safer drugs.

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