Competitive Binding between Cocaine and Various Drugs to Synthetic Levodopa Melanin

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Abstract □ The interaction of 15 drugs with synthetic levodopa melanin was studied by measuring their relative tendency to compete with ^{14}C-cocaine for sites on the polymer. The binding of cocaine to melanin followed a Type I Langmuir adsorption isotherm in the absence of added drugs. Cocaine in the presence of a ninefold greater concentration of (+)-norepinephrine, levarterenol, dextroamphetamine, levamfetamine, (−)-ephedrine, dopamine, cyclopentolate, tropicamide, and, perhaps, desipramine conformed to a Type I adsorption relationship modified to account for competitive binding. Reciprocal plots for the binding of cocaine in the presence of these drugs permit a comparison of their relative affinities to melanin. Chlorpromazine, promazine, fluphenazine, thioridazine, imipramine, and chloroquine in ratios of 9:1 (drug–cocaine) gave results that are not explainable by a model based on competitive inhibition of cocaine binding. However, studies at ratios of 1:1 for promazine and of 0.25:1 and 1:1 for chloroquine showed conformity with the competitive inhibition model, so affinities could be compared. The behavior of cocaine in the presence of thioridazine even at a 1:1 ratio cannot be judged as competitive inhibition. The overall results showed that the relative binding found was: some phenothiazines ≈ chloroquine > cyclopentolate > tropicamide > sympathomimetic amines.

Keyphrases □ Cocaine—competitive binding with various drugs to synthetic levodopa melanin □ Binding, competitive—cocaine and various drugs to synthetic levodopa melanin □ Levodopa melanin, synthetic—competitive binding with cocaine and various drugs □ Melanin, synthetic levodopa—competitive binding with cocaine and various drugs □ Narcotic anesthetics, topical—cocaine, competitive binding with various drugs to synthetic levodopa melanin □ Pigments—synthetic levodopa melanin, competitive binding with cocaine and various drugs

Many drugs are accumulated and retained by pigmented tissue (1–9). Since nonpigmented tissue accumulates only small amounts of drug, retention is assumed to be dependent on pigment cells and their constituents. The melanin of the pigment granules in these cells has been implicated as the intracellular material responsible for drug binding (1, 4–6).

An earlier study (10) attempted to quantify the drug interactions with synthetic melanin by assuming that the process involved was analogous to adsorption on a solid, permitting a calculation of affinity and capacity constants to characterize binding. It was observed (10) that (±)-cocaine hydrochloride had an affinity for melanin of a magnitude and reproducibility that suggested it might be used to study the binding of various drugs; the approach suggested was the measurement of the tendency of drugs to compete with labeled cocaine for melanin sites.

The present study reports the relative ability of several drugs to bind to melanin by measuring their competitive displacement of labeled cocaine. Cyclopentolate and tropicamide were selected because they are extensively used in ophthalmology (11) and, like cocaine, are less effective in pigmented iris (4). The phenothiazines, chlorpromazine, fluphenazine, promazine, and thioridazine, were studied to see if structural differences affected binding. Desipramine and imipramine were studied because of their structural similarity to phenothiazines. Chloroquine, like chlorpromazine, was used because it is reported to produce retinopathy and is known to bind to melanin (12). Dextroamphetamine [(+)-amphetamine] and levamfetamine [(−)-amphetamine], (+)-norepinephrine and levarterenol [(−)-norepinephrine], and dopamine were studied to determine if there was a stereospecific component to binding. The remaining sympathomimetic amine used, (−)-ephedrine, is known to be retained by the pigmented iris (5, 6).

EXPERIMENTAL

Preparation of Levodopa Melanin—The melanin was prepared as reported previously (10). The yield resulting was 4.38 g using exactly the same amounts and procedures. This yield is approximately three times more than was obtained earlier for unknown reasons. The resulting material, as well as that obtained earlier, showed that a free radical is present, as evidenced by its characteristic electron spin resonance signal[1] similar to that of melanin found in nature.

Materials—^{14}C-Methoxy-labeled-(±)-cocaine hydrochloride[2] (specific activity of 3.12 mCi/mmole) was found to be pure using TLC. The unlabeled drugs used without further purification were: fluphenazine[3], promazine hydrochloride[4], chloroquine diphosphate[5], imipramine hydrochloride[6], thioridazine hydrochloride[7], desipramine hydrochloride[8], dextroamphetamine [(+)-amphetamine] sulfate[9], levamfetamine [(−)-amphetamine] sulfate[9], (+)-norepinephrine (+)-bitartrate[5], levarterenol[10] [(−)-norepinephrine], dopamine hydrochloride[11], (−)-ephedrine hydrochloride[12], cyclopentolate hydrochloride[13], tropicamide[13], and (±)-cocaine hydrochloride[14]. All other chemicals were reagent grade.

Methodology for Binding Studies—Portions of 2, 4, 6, 8, and 10 mg of synthetic melanin were placed in separate 25-ml flasks. Nonlabeled drug was added to 5 ml of 0.1 *M* phosphate buffer, pH 7.4, to give a concentration normally nine times (5.24×10^{-5} *M*) that of labeled cocaine. The suspension was shaken at 37° for 1 hr in a thermostated water bath. Then labeled (±)-cocaine hydrochloride (0.15 ml of a stock solution) was added, resulting in a concentration of 5.83×10^{-6} *M* (5.8 nCi/ml), and shaking was continued for another hour.

The suspension was then centrifuged[15] at 19,000 rpm for 30 min at −2°. The supernate, 1 ml, was placed in a counting vial, and 1 ml of water and 12 ml of a liquid scintillation cocktail[16] were added. All samples were then counted[17] for 10 min. The counting efficiency for the instrument was 86–87%. In a few cases where results showed that the 9:1 ratio (unlabeled–labeled) was too high, a 1:1 or 0.25:1 study was performed.

In another study, saturation of melanin by cocaine was carried out using a constant amount of melanin (2 mg). Unlabeled cocaine was added to the labeled compound whose concentration was fixed at 5.83×10^{-6} *M* (5.8 nCi/ml). Ratios (unlabeled–labeled) of between 5:1 and 750:1 were used, and the systems were analyzed for free and bound drug as outlined previously (10).

All experiments were repeated five times. The concentration of cocaine bound (as free base) was calculated by taking the difference between the amount initially added and the amount remaining in the supernate after

[1] The spectrum was obtained through the courtesy of Dr. Louis Malspeis, College of Pharmacy, Ohio State University.
[2] Mallinckrodt Nuclear, St. Louis, Mo.
[3] Schering Corp., Bloomfield, N.J.
[4] Wyeth Laboratories, Philadelphia, Pa.
[5] Sterling-Winthrop Research Institute, Rensselaer, N.Y.
[6] Ciba Pharmaceutical Co., Summit, N.J.
[7] Sandoz Pharmaceuticals, Hanover, N.J.
[8] USV Pharmaceuticals Corp., Tuckahoe, N.Y.
[9] Smith Kline & French Laboratories, Philadelphia, Pa.
[10] Regis Chemicals, Chicago, Ill.
[11] General Biochemicals, Chagrin Falls, Ohio.
[12] Mallinckrodt, St. Louis, Mo.
[13] Alcon Laboratories, Fort Worth, Tex.
[14] Merck Sharp & Dohme Research Laboratories, West Point, Pa.
[15] Sorvall RC-2B centrifuge.
[16] Aquasol, New England Nuclear, Boston, Mass.
[17] Packard liquid scintillation spectrometer.

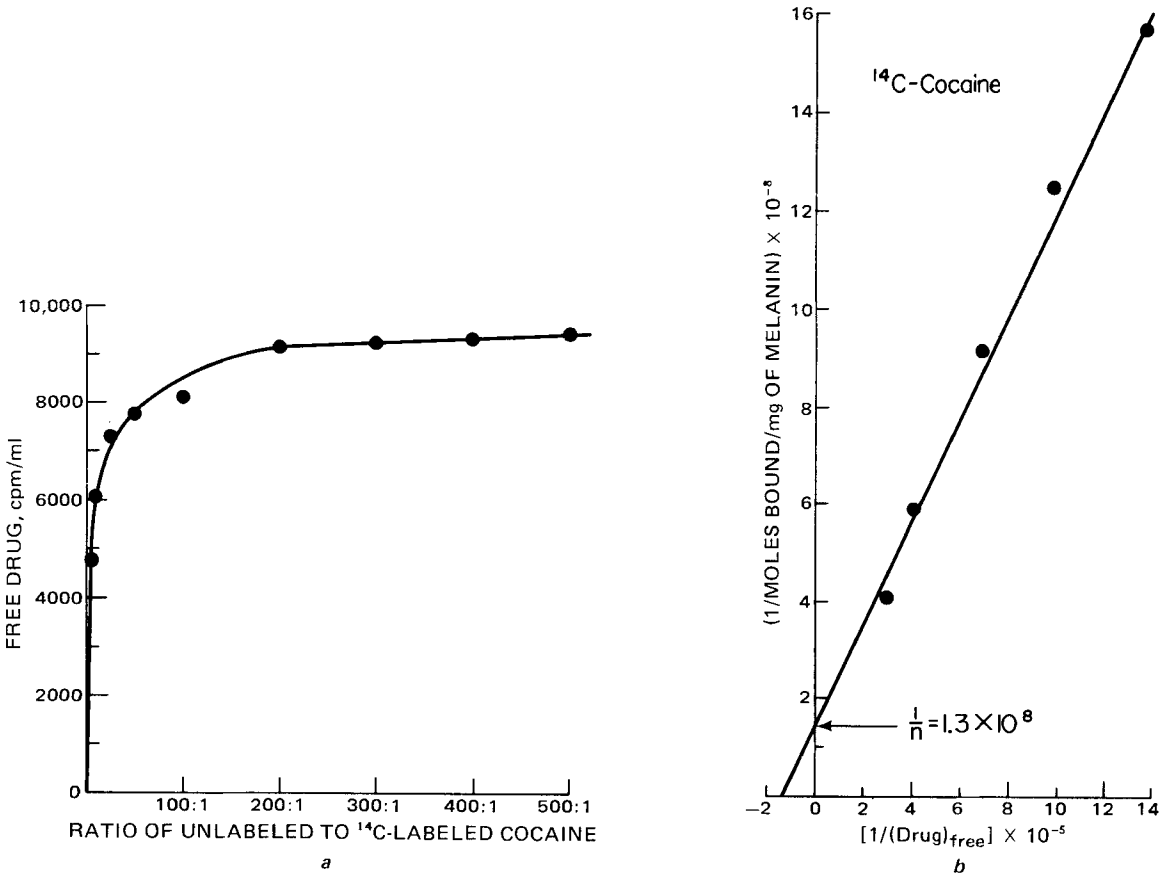

Figure 1—(a) *Free drug concentration as measured by counts per minute per milliliter for cocaine as a function of the cocaine concentrations where various proportions of unlabeled to labeled cocaine were used.* (b) *Reciprocal of the moles of cocaine bound per milligram of melanin as a function of the reciprocal of the free cocaine concentration. The affinity constant* K = 1.203×10^5 M^{-1}, *and the capacity* n = 7.69×10^{-9} *mole/mg.*

centrifugation. The average of the five determinations (standard error of mean between 1 and 5%) was used for data analysis.

Treatment of Binding Data—Cocaine binding to melanin was assumed to follow a Type I Langmuir adsorption isotherm according to:

$$r = \frac{nK[c]_{free}}{1 + K[c]_{free}} \quad \text{(Eq. 1)}$$

where r is the number of moles of cocaine bound per milligram of melanin, $[c]_{free}$ is the concentration of free cocaine, n is the maximum number of moles of cocaine that can be bound per milligram of melanin, and K is proportional to the affinity constant for cocaine binding under the experimental conditions. Equation 1 is modified when cocaine binding is studied in the presence of a drug that competes for cocaine binding sites. In the case of reversible inhibition by the added drug, Eq. 1 becomes:

$$r = \frac{nK[c]_{free}}{1 + K[c]_{free} + K'[D]_{free}} \quad \text{(Eq. 2)}$$

where K' is the constant related to the affinity of the drug and $[D]_{free}$ is the free inhibitor concentration. Defining the term K_{app} as:

$$K_{app} = \frac{K}{1 + K'[D]_{free}} \quad \text{(Eq. 3)}$$

and substituting into Eq. 2 gives the following equation upon rearrangement:

$$\frac{1}{r} = \frac{1}{n} + \frac{1}{nK_{app}} \frac{1}{[c]_{free}} \quad \text{(Eq. 4)}$$

Thus, a plot of the reciprocal of the amount of cocaine bound per milligram of melanin as a function of the reciprocal of the free cocaine concentration should be linear in the presence of a constant concentration of inhibitor. In competitive inhibition of cocaine binding, the y-axis intercept should be the same as that found in the absence of inhibitor but the slope should be greater since K_{app} must be less than K. The greater the slope at a constant inhibitor concentration, the greater is the affinity, K', of the inhibitor for melanin. If the added drug is an irreversible inhibitor of cocaine binding, both the slope and y-axis intercept should increase. However, the value for K, the x-axis intercept, remains the same in the presence and absence of drug inhibitor.

RESULTS

When cocaine binding to melanin was studied using a constant weight of melanin (2 mg) and increasing amounts of cocaine (ratios of unlabeled to labeled drug increased from 5:1 to 750:1), the relation between the counts per minute per milliliter and the increasing ratio reached a plateau above ratios of about 200:1 (Fig. 1*a*). Since an increase in counts per minute per milliliter reflected displacement of bound labeled cocaine, saturation of 2 mg of melanin in 5.15 ml of buffer occurred at concentrations of about 1.17×10^{-3} M. This result showed that the subsequent studies involving cocaine binding to melanin in the presence and absence of inhibitors were conducted at levels well below cocaine saturation.

In studies using a constant concentration of labeled cocaine (5.83×10^{-6} M) with varying amounts of melanin added (2–10 mg/5.15 ml), a plot of the reciprocal of the experimentally measured amount bound per milligram of melanin as a function of the reciprocal of the free cocaine concentration was linear (Fig. 1*b*). The result conforms with that predicted by Eq. 1, where the n and K values found (7.7×10^{-9} mole/mg and 1.20×10^5 M^{-1}, respectively) are of the same order of magnitude as those reported earlier (7) where $n = 2.4 \times 10^{-9}$ mole/mg and $K = 8.9 \times 10^5$ M^{-1}. The values are not expected to be exactly the same because of the error involved in getting the constants (Table I) and because different batches of synthetic melanin were used where the physical characteristics (external surface area) affecting binding may have been quite different. Thus, in spite of different melanins used, the values for cocaine can be considered approximately equal within the limits of experimental error. The relationship found for cocaine alone (Fig. 1*b*) was used as the reference for all subsequent drug comparison studies.

Competition for binding sites between cocaine and drugs of several pharmacological classes was characterized by plotting the reciprocal of the amount of cocaine bound as a function of the reciprocal of the free cocaine concentration. Linear relationships were always obtained where slopes in plots for cocaine in the presence of another drug were always greater than the slope found for cocaine alone (Table I). The amine salts, (+)-norepinephrine, levarterenol, dextroamphetamine, levamfetamine, (−)-ephedrine, and dopamine, studied at a ratio of 9:1 (amine–cocaine) gave good linear double-reciprocal plots as typified by those for (+)-

Table I—Linear Regression Parameters for the Langmuir Treatment of the Binding of ^{14}C-Cocaine to Melanin in the Presence of Several Drugs

Drug	Ratio, Drug–Cocaine	Slope, mg/liter × 10^{-3} (Lower/Upper Limit)[a]	Intercept, mg/mole × 10^{-8} (Lower/Upper Limit)[a]
(+)-Norepinephrine	9:1	1.9 (1.4/2.3)	4.1 (1.1/7.1)
Levarterenol	9:1	1.7 (1.3/2.0)	2.9 (1.7/4.2)
Dextroamphetamine	9:1	2.2 (1.7/2.7)	2.0 (0.5/3.5)
Levamfetamine	9:1	2.3 (1.8/2.8)	2.1 (0.5/3.8)
(−)-Ephedrine	9:1	2.1 (1.5/2.6)	3.1 (0.9/5.4)
Dopamine	9:1	2.3 (1.4/3.1)	3.3 (0.6/5.9)
Cyclopentolate	9:1	3.1 (2.6/3.6)	2.2 (−0.4/4.8)
Tropicamide	9:1	2.5 (2.2/2.8)	0.8 (−0.7/2.3)
Chlorpromazine	9:1	2.0 (1.2/2.8)	10.4 (5.9/14.8)
Fluphenazine	9:1	2.2 (1.7/2.6)	9.4 (5.5/13.2)
Promazine	9:1	2.0 (1.5/2.6)	14.4 (11.7/17.1)
	1:1	2.4 (1.8/3.0)	1.7 (−2.4/5.8)
Thioridazine	1:1	1.6 (1.4/1.9)	8.1 (6.0/10.2)
Imipramine	9:1	2.2 (1.8/2.8)	6.1 (3.4/8.9)
Desipramine	9:1	2.2 (1.9/2.6)	4.0 (2.0/6.0)
Chloroquine	9:1	1.9 (1.4/2.4)	17.4 (13.4/21.4)
	1:1	2.0 (1.3/2.7)	7.6 (0.6/14.7)
	0.25:1	2.1 (1.4/2.9)	5.8 (−1.8/13.4)
(±)-^{14}C-Cocaine	Control	1.1 (0.9/1.3)	1.3 (−0.2/2.8)

[a] Parentheses contain 95% confidence intervals calculated at $p = 0.05$.

norepinephrine and levarterenol (Fig. 2). The slopes for all six compounds were greater than the slope for cocaine alone, and their intercepts do not appear to be statistically different from the intercept of cocaine (Table I).

Cyclopentolate and tropicamide gave slopes larger than those found for amine salts (Table I). In comparing the two drugs, cyclopentolate gave a slightly larger slope (Fig. 3).

Studies at ratios of 9:1 (drug–cocaine) using the tranquilizers promazine, chlorpromazine, fluphenazine, thioridazine, imipramine, and desipramine and the retinotoxic drug chloroquine gave reciprocal plots with slopes greater than those for cocaine alone (Table I). Except for desipramine, the y-axis intercepts were greater and statistically different from those for cocaine alone, as represented in Fig. 4*a* for chloroquine. Desipramine showed a y-axis intercept (Fig. 4*c*) with its lower 95% confidence limit falling within the range for cocaine (Table I). Table I shows that the intercept limits for fluphenazine, imipramine, chlorpromazine, promazine, and chloroquine were outside the error limit for cocaine. For

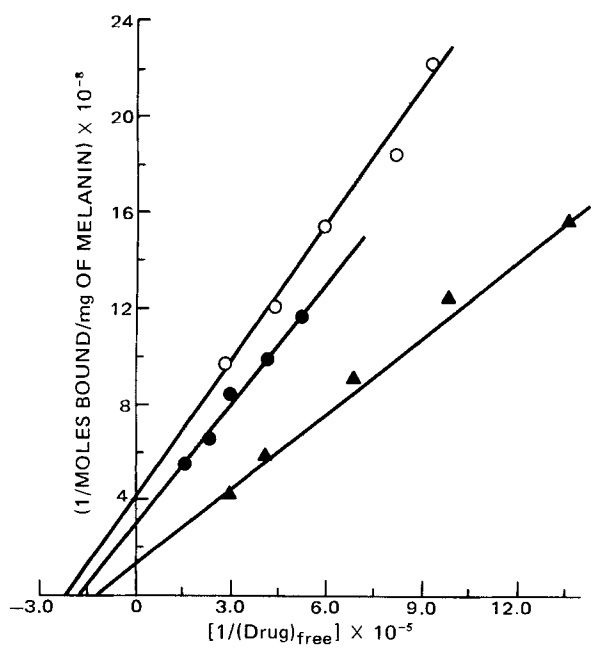

Figure 2—*Reciprocal of the moles of cocaine bound per milligram of melanin as a function of the reciprocal of the free cocaine concentration. Key: ▲, cocaine alone; ●, cocaine in the presence of levarterenol (1:9); and ○, cocaine in the presence of (+)-norepinephrine (1:9).*

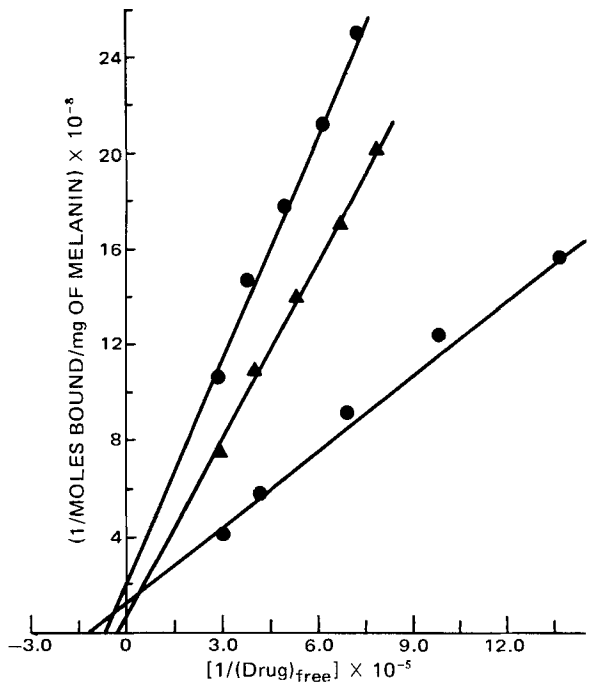

Figure 3—*Reciprocal of the moles of cocaine bound per milligram of melanin as a function of the reciprocal of the free cocaine concentration. Key: ●, cocaine alone; ▲, cocaine in the presence of tropicamide (1:9); and ○, cocaine in the presence of cyclopentolate (1:9).*

an unexplained reason, thioridazine at a 9:1 ratio gave results that were not analyzable.

With the assumption that the findings obtained were a result of high affinities of the several drugs (except desipramine) for melanin, which effectively displaced cocaine from binding sites with a concomitant introduction of large errors in the calculation of bound cocaine, studies were made using chloroquine at ratios of 1:1 and 0.25:1 (drug–cocaine) and promazine and thioridazine at ratios of 1:1. At the 1:1 ratio, analyzable results were obtained for thioridazine. The result for chloroquine at the 1:1 ratio is presented in Fig. 4*b*. The other compounds gave similar results. The slopes of the reciprocal plots obtained in these studies were greater than those of cocaine alone and the intercepts, except for thioridazine, were not statistically different from those found for cocaine alone (Table I).

DISCUSSION

The linear relationship obtained in the reciprocal plots for the six amine salts in the presence of cocaine at ratios of 9:1, for desipramine at the same ratio, for chloroquine at ratios of 1:1 and 0.25:1, and for promazine at a ratio of 1:1, where the y-axis intercepts were not significantly different from the intercept for cocaine studied alone, strongly suggests compliance with Eq. 3. The equation assumes that a drug reversibly competes with cocaine for binding sites on melanin where the concentration of free inhibitor is relatively constant. Compliance by the several drugs suggests that a comparison of the slopes obtained for cocaine in the presence of drugs with the slope obtained for cocaine alone can be used to compare the relative affinities of the several drugs for melanin. Thioridazine is the only drug that does not comply with Eq. 3 at a 1:1 ratio.

With the amine salts, an increase in the slope of the reciprocal plots occurred at ratios of 9:1; therefore, among the compounds studied, the amine salts showed the weakest binding to melanin. Furthermore, dextroamphetamine and levamfetamine had similar slopes, as did (+)-norepinephrine and levarterenol. This result indicates that competitive binding is nonstereoselective. The observations with the stereoisomers of amphetamine are consistent with the previous studies where the binding of each labeled isomer was investigated (10).

Cyclopentolate and tropicamide showed a greater slope and, hence, a greater affinity for melanin than the amine salts at similar ratios of drug to labeled cocaine. Cyclopentolate reflected a slightly greater inhibitory effect and presumably has a greater affinity for melanin than tropicamide, which may be a result of their differing chemical structures.

For most phenothiazines at a 9:1 ratio, it was not possible to charac-

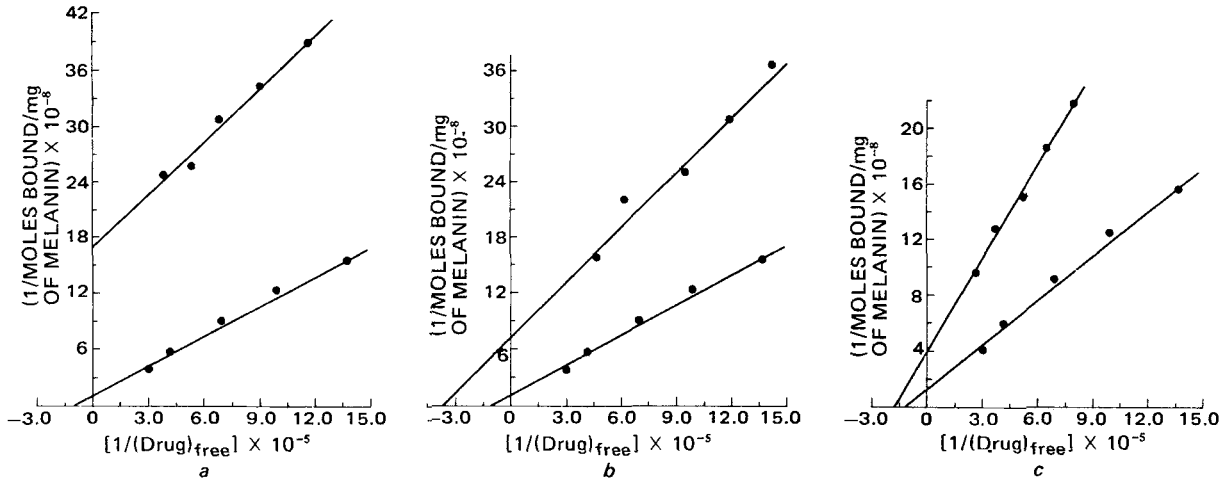

Figure 4—*Reciprocal of the moles of cocaine bound per milligram of melanin as a function of the reciprocal of the free cocaine concentration in the presence and absence of (upper curves) chloroquine (9:1) (a), chloroquine (1:1) (b), and desipramine (9:1) (c). The lower curves are ^{14}C-cocaine alone.*

terize the exact nature of the inhibitory effect since the isotherms obtained were not typical of the Type I adsorption. This result probably is a consequence of both the amount of phenothiazine present and the strength of the interaction. Desipramine is apparently a much weaker inhibitor than other phenothiazines studied since its behavior at a 9:1 ratio was similar to that of other phenothiazines studied at a 1:1 ratio. Chloroquine at a 9:1 ratio behaved similarly to most of the phenothiazines (not desipramine) at the same ratio. Just as with the phenothiazines, this behavior is probably related to its concentration and affinity for melanin.

The phenothiazines displaced cocaine at a 1:1 ratio, analogous to the amine salts at a 9:1 ratio, which indicates that phenothiazine affinity is greater than amine salt affinity. Chloroquine at ratios of 1:1 and 0.25:1 showed competitive effects similar to the amine salts at 9:1; it probably has a melanin affinity greater than the amine salts. Cyclopentolate and tropicamide, both at a 9:1 ratio, exhibited greater inhibitory effects than the amine salts at a similar ratio. When the anomalous behavior of phenothiazines at a 9:1 ratio is compared with the behavior of cyclopentolate and tropicamide at the same ratio, the phenothiazines show a greater affinity for melanin than these drugs.

The significance of these studies is that there is a difference among drugs in their affinity for melanin: some phenothiazines ≈ chloroquine > cyclopentolate > tropicamide > sympathomimetic amines. It has been suggested that binding occurs with melanin *via* interaction of unshared electrons on drugs with the free radical of melanin (13). Further study is necessary to clarify the mechanism of drug binding to melanins.

Because of the binding, regardless of its mechanism, it is possible that a drug may localize in the vicinity of melanin in melanin-containing tissue. It is easy to visualize such a phenomenon occurring, for example, in the eye where a localized high total concentration could remain over a long period (8, 9). Such a situation might foster toxicity. Chloroquine and chlorpromazine are known to produce ocular toxicity (12), and indeed they are among the strongest binders to melanin. Thus, it is tempting to suggest that there is a relationship between melanin affinity and toxicity.

The pharmacological and toxicological significance of the interaction of certain drugs with melanins is well recognized (1–6, 8, 9). Since many other drugs also interact and bind with melanins, the pharmacological and toxicological significance of these interactions is perhaps underestimated.

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ACKNOWLEDGMENTS AND ADDRESSES

Received October 26, 1976, from the *Divisions of Pharmaceutics and Pharmacology, College of Pharmacy, Ohio State University, Columbus, OH 43210.*

Accepted for publication January 17, 1977.

Supported in part by the National Eye Institute, U.S. Public Health Service Grant EY-01308, and in part by Alcon Laboratories, Fort Worth, Tex.

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