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Binding Characteristics of Drugs to Synthetic Levodopa Melanin

K. SHIMADA, R. BAWEJA, T. SOKOLOSKI, and P. N. PATIL [x]

Abstract □ To define binding characteristics of drugs, levodopa melanin was prepared with the aid of mushroom tyrosinase. The binding of radiolabeled substances was studied with increasing concentrations of melanin in a fixed volume of potassium phosphate buffer (pH 7.4) at 37°. The affinity and capacity of the drug binding were calculated according to Langmuir's adsorption isotherm. The affinity constant of various sympathomimetic amines such as (−)-amphetamine, (+)-amphetamine, (−)-ephedrine, (±)-octopamine, and (+)-norepinephrine ranged from 1.1 to 2.8 × 10^5 M^{-1}. The binding capacity for the amines ranged from 1.4 to 3.2 × 10^{-9} mole/mg. Although the capacity of (±)-cocaine for binding was similar to that of the amines, the affinity was slightly higher, 8.9 × 10^5 M^{-1}. The binding of atropine to the synthetic melanin appeared to be a saturable process with the affinity and capacity values of 0.2 × 10^5 M^{-1} and 7.6 × 10^{-9} mole/mg, respectively. Although the binding lacks stereoselectivity, the drugs vary in their capacity and affinity to bind with melanin. The observed differential pharmacological and toxicological properties of drugs in the pigmented tissues may in part be related to their differential binding characteristics.

Keyphrases □ Melanin, synthetic—prepared by action of mushroom tyrosinase on levodopa, affinity and capacity of drug binding evaluated, various sympathomimetic amines □ Levodopa melanin—affinity and capacity of drug binding evaluated, various sympathomimetic amines □ Binding, drug-melanin—affinity and capacity evaluated, various sympathomimetic amines □ Sympathomimetic amines—binding to synthetic melanin, affinity and capacity evaluated □ Pigments—synthetic melanin, affinity and capacity of drug binding, various sympathomimetic amines

Several tissues such as iris, skin, hair, inner ear, and substantia nigra contain melanin. Many phenothiazines (1, 2), chloroquine (3), cocaine (4), antibiotics (5), and sympathomimetic drugs (6, 7) are markedly accumulated and retained for a long time by the pigmented tissue. The nonpigmented tissues from albino or nonalbino animals accumulate very little drug, and the accumulated substance is rapidly lost from the tissue (4, 6, 7).

In the pigmented tissue, the accumulation presumably occurs by the pigment cells and their constituent melanin granules. These granules consist of a lipoid membrane, a protein, and the melanin derived from levodopa (8). Since little is known about the binding characteristics of drugs by melanin, synthetic levodopa melanin was prepared and the affinity and capacity for drug binding were studied.

EXPERIMENTAL

Preparation of Synthetic Levodopa Melanin—Melanin was prepared by a modification of a reported method (2, 9). Ten grams of levodopa (L-dihydroxyphenylalanine)[1] and 66 mg of mushroom tyrosinase[2] (polyphenol oxidase) were placed in 3 liters of 0.1 *M* potassium phosphate buffer, pH 7.4, and stirred in a water bath at 37° for 4 hr. Buffers were prepared from reagent grade chemicals. Oxygen gas (95% oxygen–5% carbon dioxide) was bubbled through the preparation at room temperature for 90 min. The suspension was then stirred for an additional 4 hr. The mixture was then allowed to stand overnight (14 hr) with continual bubbling of oxygen.

The resultant precipitate of melanin was collected after centrifuging (19,000 rpm) at −2° and washed by resuspending in distilled water. The washings, followed by centrifugation, were performed until a clear supernatant solution was obtained. The washed precipitate thus obtained was freeze dried. The yield of melanin was approximately 1.41 g. The material exhibited a characteristic free radical signal in the electron spin resonance spectrometer[3] (like that seen in the natural melanins).

Methodology for Binding Studies—In all binding studies, the general procedure used was as follows. Aliquots of 1.0, 2.0, 4.0, 6.0, and 8.0 mg of synthetic melanin were placed in five 25-ml erlenmeyer flasks. Amounts of radiolabeled substances were added to each flask in the concentrations of 2 nCi/ml for ^{14}C-labeled materials and 6

[1] Merck Sharp & Dohme Research Laboratories, West Point, Pa.
[2] Sigma Chemical Co., St. Louis, Mo. (3690 units/mg).
[3] The spectra was supplied through the courtesy of Dr. L. Malspeis, College of Pharmacy, Ohio State University.

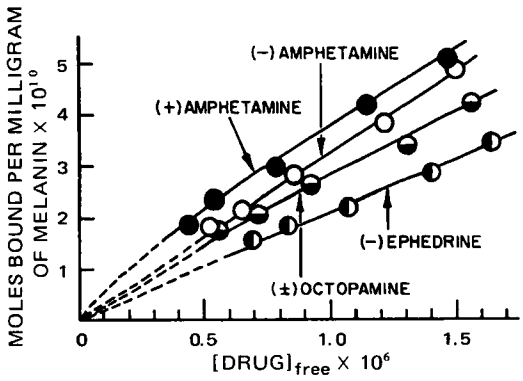

Figure 1—*Relationship between moles of drug bound per milligram of melanin for several different compounds and the molar concentration of free drug in equilibrium with amount bound.*

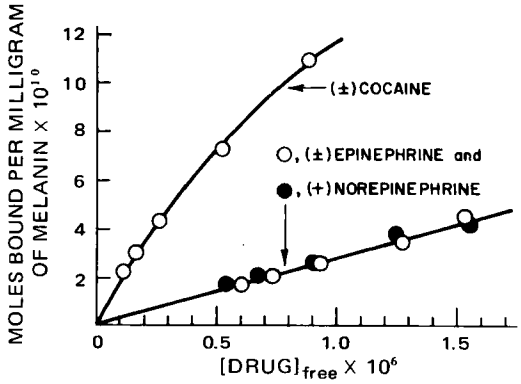

Figure 2—*Relationship between moles of cocaine, epinephrine, and norepinephrine bound per milligram of melanin and the molar concentration of free drug in equilibrium with amount bound.*

nCi/ml for ^{3}H-labeled compounds (2×10^{-6} *M*). The solutions were made with 0.1 *M* potassium phosphate buffer (pH 7.4); those containing epinephrine, norepinephrine, and octopamine contained sodium metabisulfite (0.1–1.9%) and edetic acid (0.001%) to minimize oxidation. In one study with (+)-amphetamine, 0.1 *M* sodium phosphate buffer (pH 7.4) was used.

The prepared suspensions were shaken in a water bath at 37° for 1 hr at 135 rpm. Then the mixture was centrifuged at 19,000 rpm for 30 min at −2°. One milliliter of the supernate was placed in a counting vial, and 1.0 ml of distilled water and 12.0 ml of liquid scintillation cocktail[4] were added. All samples were then counted[5] for 10 min. The counting efficiency was 86–87% for carbon-14 and 23–25% for tritium.

In studies with atropine, the same general procedure was followed. In one study, however, the concentration of atropine was three times that used for the other drugs. In another study using a constant amount of melanin (1 mg), cold atropine was added to the labeled compound in ratios of 10:1, 50:1, 100:1, 200:1, and 300:1 to determine the nature of binding at higher concentrations. All experiments were repeated five times.

The following drugs were used: (+)-[1-^{14}C-propyl]amphetamine sulfate[6], specific activity 1.99 mCi/mmole; (−)-^{14}C-amphetamine sulfate[7], specific activity 12.4 mCi/mmole; (±)-7-^{3}H(N)-epinephrine 1-bitartrate[8], specific activity 9.45 Ci/mmole; (−)-^{14}C-ephedrine hydrochloride[9], specific activity 2.8 mCi/mmole; (±)-2-^{3}H(N)-octopamine[8], specific activity 2.89 Ci/mmole; (±)-norepinephrine(methylene-^{14}C) *d*-bitartrate, specific activity 21.2 mCi/mmole; ^{3}H(G)-atropine[10], specific activity 407 mCi/mmole; and (±)-cocaine(methoxy-^{14}C)[8], specific activity 3.12 mCi/mmole. All radiolabeled substances were checked for purity. Only a single peak was observed by TLC in appropriate solvent systems.

All unlabeled drugs were used as obtained without further purification: (+)-amphetamine sulfate[11], (−)-amphetamine sulfate[12], (±)-epinephrine bitartrate[12], (−)-ephedrine hydrochloride[6], (±)-octopamine[13], (+)-norepinephrine bitartrate[14], atropine sulfate[1], and (−)-cocaine hydrochloride[1].

RESULTS

Binding of Drugs to Melanin—Centrifugation of the suspensions containing drug and melanin was a convenient means of separating free drug from drug bound to melanin. From the number of counts in the supernatant solution of the drug–melanin suspension, it was possible to determine the concentration of free drug. By knowing the amount of free drug and the total amount initially added, it was possible to determine by simple difference the amount of drug bound. The assumption is made that potential binding of drug to glass is not significant.

The number of moles of bound drug calculated as free base, for the five separate trials determined at each amount of melanin added, was averaged and the standard error of the mean was calculated. Treatment of the data obtained in this way showed that the standard error of the mean varied between only 1 and 5% of the amount of bound drug, indicating excellent reproducibility.

The average numbers of moles of the free base form of drug interacting per milligram of melanin in potassium phosphate buffer as a function of the free drug concentration in moles per liter are presented in Figs. 1–3. As the concentration of free drug increased, the amount of drug bound per milligram of melanin increased. For cocaine (Fig. 2) and atropine at high concentrations (Fig. 3), saturation of melanin possibly was approached.

When studies were made with (+)-amphetamine using a sodium phosphate buffer instead of a potassium phosphate buffer, slightly more drug was bound at approximately the same free drug concentration but the same almost linear relation as that presented in Fig. 1 was found. This observation emphasizes the need to make comparisons only in identical systems.

Treatment of Binding Data—The results obtained with cocaine (Fig. 2) and atropine (Fig. 3) suggested that the binding of the various drugs to melanin could be analyzed by assuming that the binding is analogous to the adsorption of a drug on a solid and follows a Type I Langmuir isotherm (10). Specifically, the amount of drug bound per milligram of melanin, r, should be related to the concentration of free drug, $[D]_{free}$, by:

$$r = \frac{r_{max} K [D]_{free}}{1 + K [D]_{free}} \qquad \text{(Eq. 1)}$$

where r_{max} is the maximum moles bound per milligram of melanin, that is, the amount needed to form a monolayer, and K is a constant related to the affinity or strength of the interaction. Rearrangement of Eq. 1 to:

$$\frac{1}{r} = \frac{1}{r_{max}} + \frac{1}{r_{max} K} \frac{1}{[D]_{free}} \qquad \text{(Eq. 2)}$$

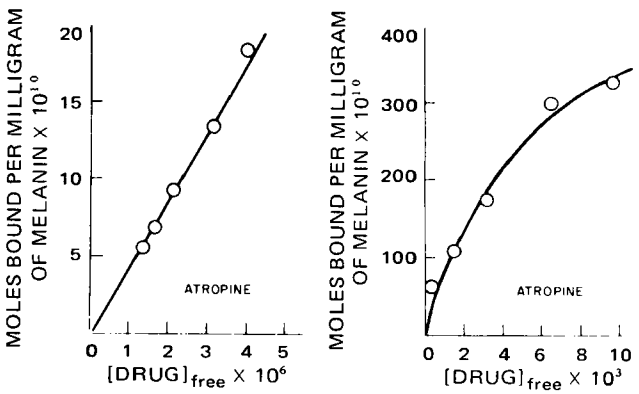

Figure 3—*Relationship between moles of atropine bound and the molar concentration of free atropine in equilibrium with the amount bound. Data show different concentration ranges.*

[4] Aquasol, New England Nuclear, Boston, Mass.
[5] Packard liquid scintillation spectrometer.
[6] Mallinckrodt (Nuclear), St. Louis, Mo.
[7] Schwarz/Mann, Orangeburg, N.Y.
[8] New England Nuclear, Boston, Mass.
[9] Supplied by Dr. Dennis Feller and Dr. L. Malspeis, College of Pharmacy, Ohio State University.
[10] Amersham/Searle, Arlington Heights, Ill.
[11] Smith Kline and French, Philadelphia, Pa.
[12] K & K Labs.
[13] Regis Chemicals, Chicago, Ill.
[14] Sterling-Winthrop Research Institute, Rensselaer, N.Y.

Table I—Linear Regression Parameters for the Langmuir Treatment of the Binding of Drugs to Melanin

Drug	Slope × 10^{-3} (Lower/Upper Limit)[a]	Intercept × 10^{-8} (Lower/Upper Limit)[a]	Maximum Moles Bound per Milligram of Melanin[b]	Affinity[c], M^{-1}
(−)-Amphetamine	2.7 (2.5/2.9)	3.1 (−0.4/6.6)	3.2×10^{-9}	1.1×10^{5}
(+)-Amphetamine	2.1 (1.7/2.4)	5.7 (1.0/10)	1.8×10^{-9}	2.8×10^{5}
(±)-Epinephrine	3.5 (3.1/3.9)	0.25 (−4.1/4.6)	39.7×10^{-9}	0.072×10^{5}
(−)-Ephedrine	4.0 (3.3/4.8)	5.6 (−0.6/11.7)	1.8×10^{-9}	1.4×10^{5}
(±)-Octopamine	2.8 (2.2/3.3)	7.2 (0.8/14)	1.4×10^{-9}	2.6×10^{5}
(+)-Norepinephrine	2.7 (2.4/3.1)	5.8 (1.4/10)	1.7×10^{-9}	2.1×10^{5}
Atropine	2.3 (2.1/2.4)	0.57 (0.07/1.1)	17.6×10^{-9}	0.25×10^{5}
(±)-Cocaine	0.48 (0.45/0.51)	4.2 (2.8/5.7)	2.4×10^{-9}	8.9×10^{5}

[a] 95% confidence intervals calculated at $p = 0.05$. [b] Calculated as the reciprocal of the y-intercept. [c] Calculated from intercept and slope.

shows that a linear relation should be obtained when $1/r$ is plotted as a function of $1/[D]_{free}$. The extrapolated y-axis intercept is $1/r_{max}$, and the slope is $1/r_{max}K$. Thus, such a treatment of the binding data would permit a calculation of r_{max} and K for each drug and provide a basis for comparisons of the binding of drugs to melanin.

When the data of Figs. 1–3 were plotted according to the treatment, linear relationships were obtained, thus providing some justification for the use of this method. If binding to glass complicated the treatment of data, such a linear relationship would not be expected. Typical results are presented in Figs. 4 and 5. Linear regression analyses of data were made for all drugs (Figs. 4 and 5), and the regression parameters thus generated are given in Table I together with the 95% confidence limits calculated at $p = 0.05$. Values for r_{max} and K, calculated from the slopes and intercepts, are also listed in this table.

DISCUSSION

The drug binding with melanin is of considerable interest in pharmacology and toxicology. Retinopathy, skin coloration, bleaching of hair, ototoxicity, and an extrapyramidal syndrome caused by some drugs can be correlated to drug accumulation by the pigment (2, 4, 11). The longer duration of atropine mydriasis in the pigmented rabbit is related to drug binding by melanin (12).

The data from the present study indicate that synthetic levodopa melanin does have a varying capacity and affinity for binding to the synthetic drugs. Among the substances examined, cocaine has the highest affinity for binding to synthetic levodopa melanin. Irrespective of the differences in the chemical structure of the sympathomimetic amines studied, the net binding was similar. The affinity of (±)-epinephrine appears to be low, but the capacity is high. The product of these two numbers, capacity and affinity, determines the net binding and is the same for all sympathomimetic amines. The binding of the amines to synthetic melanin does not parallel the binding by the pigmented iris.

The nonphenolic amines such as ephedrine and β-phenethylamine are accumulated more by the pigmented iris than are the phenolic amines such as tyramine, phenylephrine, epinephrine, and norepinephrine (6, 7). The high liposolubility of the nonphenolic amines may be responsible for their greater accumulation by the pigmented iris. In other words, these substances could easily be transported across the lipoid membrane of the melanin granules. Binding of drugs to natural melanin presumably occurs after the transport. If the drugs pass the lipoid membrane with difficulty, the low binding of nonphenolic amines to the pigmented tissue is understandable. Hence, a strict parallelism between the drug binding to synthetic melanin and that of the melanin granules or the pigmented tissue may not be observed.

The only drug binding property common to both synthetic melanin and the pigmented tissue is the lack of stereoselectivity. Pigmented guinea pig iris binds both (−)- and (+)-^{14}C-ephedrine to the same extent (7). In the present study, there was a lack of selectivity in the

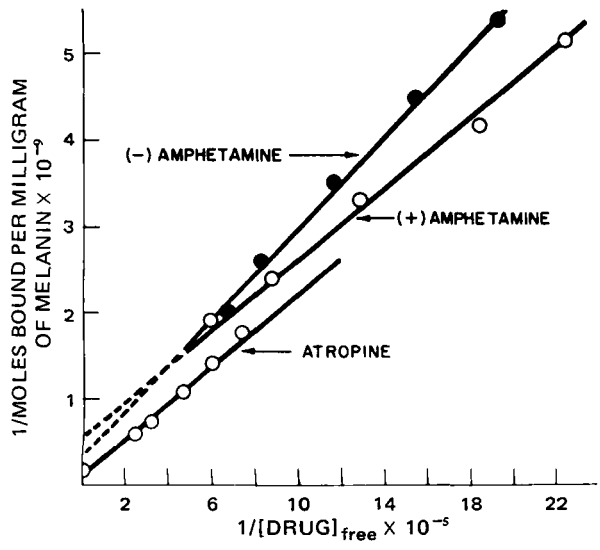

Figure 4—*Langmuir plots: reciprocal of amount of drug bound* versus *reciprocal of molar free drug concentration for amphetamines and atropine.*

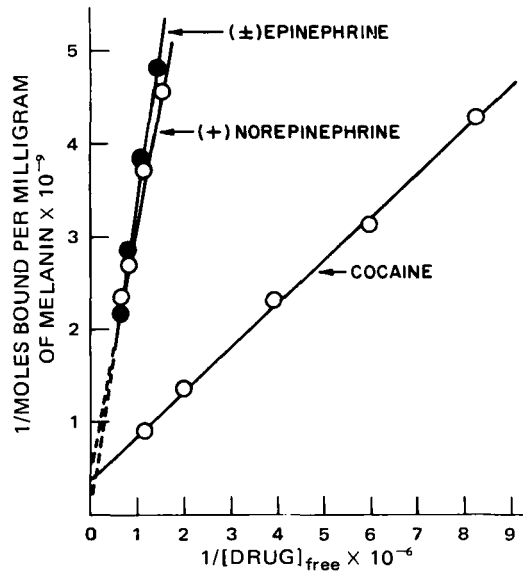

Figure 5—*Langmuir plots: reciprocal of amount of drug bound* versus *reciprocal of molar free drug concentration for epinephrine, norepinephrine, and cocaine.*

binding of (−)- or (+)-amphetamine by synthetic melanin. Data indicate that the α-methyl group is not involved in the binding of the amines, as evidenced by a comparison of the results found for amphetamine and ephedrine with those of norepinephrine and epinephrine (Table I).

Among several biopolymers examined, sepiomelanin showed outstanding affinity for binding certain drugs (13). The details of the molecular mechanism of binding of drugs to natural melanin have been the subject of much speculation (2, 14, 15). Under certain conditions, melanin can be considered as a free radical which can attract and bind some drugs with unshared electrons (2, 15, 16). As yet, there is no unanimity regarding the orientation of indole units of synthetic or natural melanin (17). Hence, the binding site or sites with which a drug interacts will be purely speculative.

A question emerges as to how drug binding to the synthetic melanin compares with the drug binding to the plasma proteins. In terms of affinity, the constants observed for the binding of drugs to melanin, 10^5–$10^6\ M^{-1}$, are comparable to the affinity constants for the binding of drugs to serum albumin. For example, thyroxine showing strong binding to albumins has a primary affinity constant of $1.6 \times 10^6\ M^{-1}$ for bovine serum albumin (18).

Since the molecular weight of melanin is unknown, comparisons of capacity between melanin and albumins should be based on the net binding of drug per unit mass of material. Drugs showing a capacity of about 2×10^{-9} mole/mg of melanin, as found for some drugs in this study, would be equivalent to approximately 0.1 mole bound/mg of serum albumin where a molecular weight of 70,000 is assumed for the albumin. This calculated equivalent value for serum albumin capacity is lower than that usually found for drugs, but surface area plays a significant role in determining capacity in the case of melanin. The surface area per unit mass of the synthetic melanin used in these studies may be considerably less than that found within naturally occurring melanosome, so the actual capacity found *in vivo* may be considerably greater.

The accumulation of atropine appears to be a saturable process, and presumably similar saturation occurs with other drugs. As stated earlier, the toxicity of several drugs could be correlated to their local accumulation by the pigment. However, drug binding to melanin may not necessarily mean toxicity; since drugs vary in their affinity for the binding, theoretically it should be possible to displace a toxic drug by a nontoxic drug. Such a study should be valuable in reducing the toxic effects of drugs related to the binding.

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