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Hydrophobicity of the Tetrabenazine-Binding Site of the Chromaffin Granule Monoamine Transporter

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SUMMARY

The catecholamine uptake inhibitor tetrabenazine (TBZ) binds to a high affinity site on the chromaffin granule membrane, presumably on the monoamine transporter. The hydrophobicity of the TBZ-binding site was investigated by comparing the potency of drugs to displace [^{3}H]dihydrotetrabenazine (TBZOH), a ligand of the TBZ-binding site, with the lipophilicity of these drugs reflected by their octanol/buffer apparent partition coefficient ($P_{o/b}$). Drugs tested were five substrates of the transporter, seven TBZ derivatives, and the inhibitors reserpine, haloperidol, and chlorpromazine. The validity of apparent $P_{o/b}$ as an index of lipophilicity was shown by measuring drug partitioning between buffer and chromaffin granule membranes. For most of the inhibitors tested, octanol/buffer and membrane/buffer apparent partition coefficients were correlated. For substrates of uptake and TBZ derivatives, the potency of a compound to displace [^{3}H]TBZOH from its binding site was correlated to its apparent $P_{o/b}$. This relationship was valid over a range of 5 orders of magnitude. These data are interpreted as indicating that the TBZ-binding site is hydrophobic and is in equilibrium with the ligand present in the membrane phase, and that substrates and TBZ derivatives are characterized by an equal intrinsic affinity for this site of about 1 μM. The 3-fold difference in affinity observed between α- and β-diastereoisomers of TBZOH was accounted for by a similar difference in apparent $P_{o/b}$. Reserpine, haloperidol, and chlorpromazine have much lower intrinsic affinity for the TBZ-binding site.

Chromaffin granules, the catecholamine storage organelles of adrenal medulla, accumulate monoamines (catecholamines and indoleamines) against their concentration gradient (1, 2). This active transport is mediated by a specific transporter driven by the proton electrochemical gradient generated by an ATP-dependent proton pump (3–8). Two specific inhibitors of this transport are known, reserpine and TBZ, which inhibit the monoamine transporter without affecting the electrochemical H^+-gradient (9, 10). Using [^{3}H]reserpine and a tritiated analog of TBZ, [^{3}H]TBZOH, inhibition of uptake has been shown to result from the binding of these drugs to high affinity sites on the monoamine transporter (11–13).

The TBZ-binding site (site T) has a low affinity for the substrates of uptake, catecholamines and indoleamines, as measured by displacement of bound [^{3}H]TBZOH. For noradrenaline, the dissociation constant derived from these measurements is in the mM concentration range and is 2 orders of magnitude larger than its K_M for ATP-dependent noradrenaline uptake (11). This difference is surprising, in view of the structural analogy existing between TBZ and catecholamines (Fig. 1). We thus investigated the possibility that the observed differences in the binding constants for site T of TBZ and of substrates might originate in differences in their physicochemical properties. In the present communication, this hypothesis was tested by comparing the potency of substrates and inhibitors of the transporter to displace [^{3}H]TBZOH with their hydrophobicity, determined from partition experiments with the octanol/buffer model system.

Materials and Methods

Chemicals. TBZ (Fluka) and reserpine (Sigma) stock solutions (1 mM) were prepared in 2 mM HCl and 200 mM acetic acid, respectively, and were kept at 4° in the dark. MIBG and [^{131}I]MIBG were gifts from Office des Rayonnements Ionisants, Commissariat à l'Energie Atomique (Gif sur Yvette, France). α- and β-[^{3}H]TBZOH (15 Ci/mmol) were synthetized either in the laboratory or by the Commissariat à l'Energie Atomique. Drugs were generally solubilized in 2 mM HCl, at 1 mM final concentration.

Synthesis of TBZ derivatives. [^{3}H]TBZOH, [2-^{3}H] 2-hydroxy-3-isobutyl-9,10-dimethoxy -1,2,3,4,6,7-hexahydro-11b(H)-benzo[*a*])quino-

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ABBREVIATIONS: TBZ, tetrabenazine; TBZOH, dihydrotetrabenazine (2-hydroxy-3-isobutyl-9,10-dimethoxy-1,2,3,4,6,7-hexahydro-11b(H)benzo[a] quinolizine; [^{3}H]TBZOH, [2-^{3}H]dihydrotetrabenazine; $TBZNH_2$, 2-aminotetrabenazine; TBA, arylazido tetrabenazine (*N*-(3-isobutyl-9,10-dimethoxy-1,2,3,4,6,7-hexahydro-11b(H)-benzo[*a*]quinolizine-2-yl)-4-[(4-azido-2-nitrophenyl)amino]butanamide); 5-HT, 5-hydroxytyrptamine (serotonin); MIBG, *meta*-iodobenzylguanidine; Hepes, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; apparent $P_{o/b}$, octanol/buffer apparent partition coefficient; apparent $P_{m/b}$, membrane/buffer apparent partition coefficient.

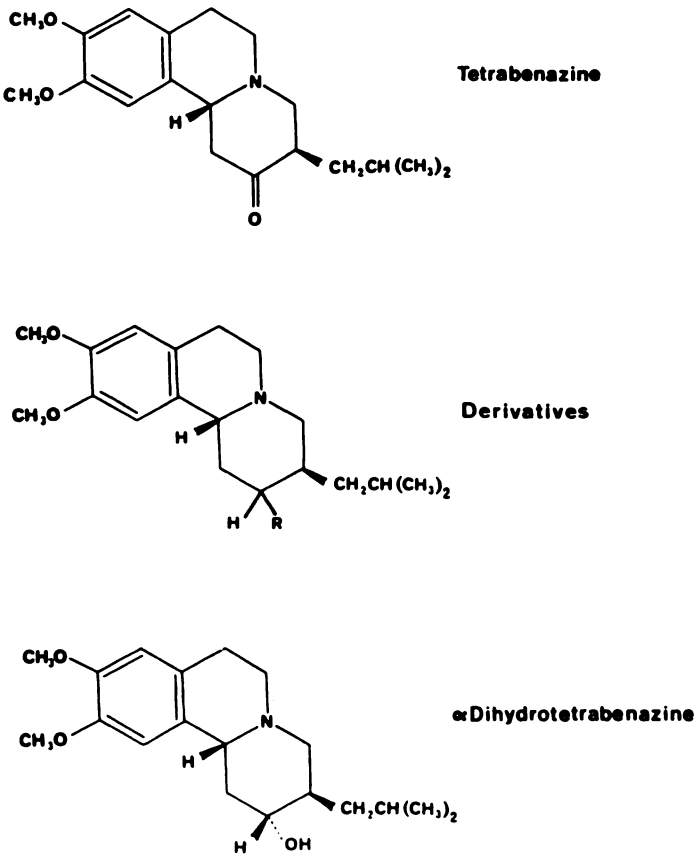

Fig. 1. Structural formulas of TBZ and TBZ derivatives. β-TBZOH is the diastereoisomer of α-TBZOH, which differs by an inverse configuration of the chiral carbon bearing the OH group. For the studied TBZ derivatives: acetyl TBZOH, R = $OCOCH_3$; succinyl TBZOH, R = $OCO(CH_2)_2COOH$; $TBZNH_2$, R = NH_2; TBA, R = $NHCO(CH_2)_3NHC_6H_3(NO_2)N_3$.

lizine (dihydrotetrabenazine), was prepared by reduction of TBZ with KB^3H_4 (11). α and β OH isomers were purified and separated by high performance liquid chromatography on a C_{18} μBondapak column with methanol/10 mM $(NH_4)CO_3H$ (70:30) as solvent. Unless indicated, [^{3}H]TBZOH indicates the more abundant α isomer. The purity of this compound was periodically checked and, if necessary, it was repurified by high performance liquid chromatography. $TBZNH_2$ (2-amino TBZ), TBA (*N*-(3-isobutyl-9,10-dimethoxy-1,2,3,4,6,7-hexahydro-11b(H)-benzo[*a*]quinolizine-2-yl)-4-[(4-azido-2-nitrophenyl)amino]butanamide), an arylazido derivative of $TBZNH_2$, and [^{3}H]TBA were prepared as described (14). Succinyl and acetyl TBZOH were prepared by reaction of unfractionated TBZOH isomers with succinic and acetic anhydrides, respectively. Acetic or succinic anhydride (30 mmol) was added to TBZOH (1 mmol) in 2 ml of dry pyridine. After one night at room temperature, the solvent was removed. The product was dissolved in water (10 ml) and sodium carbonate was added to neutrality. The aqueous phase was extracted three times with CH_2Cl_2 (10 ml) and the combined organic phases were evaporated. Succinyl or acetyl TBZOH esters were crystallized from ether.

Chromaffin granule membrane preparation. Bovine chromaffin granule membranes were prepared by osmotic lysis of granules isolated by centrifugation on a 1.6 M sucrose layer (15, 16). The preparation was frozen in liquid nitrogen and was kept at −80°. Protein concentration was estimated by the Lowry procedure (17).

Drug concentration determination. Concentrations of free drug (neutral and protonated) were determined spectrophotometrically for concentrations higher than 10 μM and, for lower concentrations, fluorometrically or by radioactivity measurements. Spectrophotometry and fluorescence measurements were performed in 1 mM HCl because the fluorescence of the protonated form of TBZ derivatives is more intense than that of the neutral form and because protonation is complete under these conditions. Absorbance and fluorescence characteristics were: TBZ derivatives, ϵ_{284} = 3,600 $M^{-1} \times cm^{-1}$, fluorescence/excitation 285 nm/emission 315 nm; reserpine, ϵ_{267} = 17,000 $M^{-1} \times cm^{-1}$ and fluorescence/excitation 295 nm/emission 365 nm; haloperidol, ϵ_{247} = 11,900 $M^{-1} \times cm^{-1}$; chlorpromazine, ϵ_{254} = 40,000 $M^{-1} \times cm^{-1}$; noradrenaline, adrenaline, serotonin, and tyramine, fluorescence/excitation 280 nm/emission 320 nm and ϵ_{275} = 15,000 $M^{-1} \times cm^{-1}$ for 5-HT. For TBZ derivatives, a common molecular absorption coefficient was assumed, which was verified by weighing in the case of TBZOH and succinyl TBZOH. For noradrenaline and adrenaline, fluorescence was also measured after adrenochrome formation (18).

[^{3}H]TBZOH binding. Membranes (10 μg of protein/ml) in 0.3 M sucrose/50 mM Hepes (Na) buffer, pH 7.5, containing 2.5 mM ATP/1.3 mM $MgSO_4$, were incubated for 2 hr at 30° with 2 nM [^{3}H]TBZOH and the studied compound at various concentrations. Bound [^{3}H]TBZOH was determined by filtration through Millipore HAWP filters (11). Nonspecific binding, determined by addition of 1 μM TBZ to the incubation mixture, was subtracted. The drug concentration corresponding to half-inhibition of [^{3}H]TBZOH binding (EC_{50}) was derived from the inhibition curve.

Octanol/buffer apparent partition coefficient (apparent $P_{o/b}$). Apparent $P_{o/b}$ determinations were performed essentially as in Ref. 19. Briefly, compounds were added to 20 mM Hepes (Na) (pH 7.5) saturated with octanol. Water-saturated octanol was then added, the mixture was stirred for 5 min at 20° and centrifuged for 15 min at 7500 × *g* at the same temperature, and the two phases were separated. For lipophilic inhibitors, which have an apparent $P_{o/b} > 1$, their concentration in the aqueous phase was measured as described above, and the concentration in octanol was calculated as the difference between total and aqueous phase concentrations. For hydrophilic uptake substrates with apparent $P_{o/b} < 1$, the octanol phase content was determined by reextracting this phase with an equal volume of 1 mM HCl. The concentration in the initial aqueous phase was obtained by subtraction.

Drug concentrations and volumes of the aqueous buffer and octanol phases were adjusted to give final concentrations in the 1–10 μM range in the aqueous phase for hydrophobic compounds or in the organic phase for hydrophilic compounds. Usually, five different concentrations were used. At the total concentration used for hydrophobic compounds (10–5000 μM), nonspecific adsorption on plastic tube walls could be neglected; however, these compounds were more strongly adsorbed to the plastic cones used to pipette the aqueous phase at pH 7.5, and the cones were thus routinely washed with 1 mM HCl.

Chromaffin granule membrane/buffer apparent partition coefficient (apparent $P_{m/b}$). Nonspecific adsorption of drugs to chromaffin granule membranes was determined in 20 mM Hepes (Na) buffer (pH 7.5) at 20°. Drugs were added to 50–200 μl of membranes in a Beckman Ultraclear Airfuge tube. After stirring for 1 min, the mixture was centrifuged for 20 min at 180,000 × *g* and the free drug in the supernatant was assayed as described above. Drug adsorbed to the membranes was obtained as the difference between total and free drug. Each experiment was performed as rapidly as possible to minimize time-dependent adsorption of the ligand onto centrifuge tube walls.

Membrane concentration was adjusted in such a way that 20–80% of the drug was adsorbed to the membrane. For each compound, the experiment was performed at several drug concentrations, and it was verified that the apparent partition coefficient was independent of membrane and drug concentrations. With the exception of TBA, the concentrations used were 3 orders of magnitude larger than the K_D for specific binding of the drug to the monoamine transporter. Specific binding could thus be neglected under these conditions. For [^{3}H]TBA at low concentrations, the monoamine transporter was saturated by 5 μM TBZ.

The membrane/buffer apparent partition coefficient, $P_{m/b}$, expressed in ml/g of membrane protein, was derived from the experimental data

according to the equation, $P_{m/b}$ = [Adsorbed drug]/[Free drug] × [Membrane concentration].

The apparent concentration ratio between the buffer phase and the hydrophobic phospholipid bilayer phase was tentatively deduced from $P_{m/b}$ using a value of 1.30 ml/g of membrane protein for the volume of the phospholipid bilayer in chromaffin granule membranes. This value was calculated using figures of 2.15 mmol/g of membrane protein for the phospholipid content of chromaffin granule membrane[1] (20), 2 nm for the mean length of a phospholipid acyl chain, and 2×10^6 phospholipid molecules/μm^2 for the phospholipid surface density in a bilayer.

Results

Effect of drugs on [^{3}H]TBZOH binding. We investigated the effects of 15 different compounds (see formulas in Fig. 1) on [^{3}H]TBZOH binding to chromaffin granule membranes. [^{3}H]TBZOH displacement curves are shown in Fig. 2. Compounds **1–4** are classical monoamine substrates of the transporter (5-HT, noradrenaline, adrenaline, and tyramine), which have a K_M in the μM concentration range; compound **6**, MIBG, is an adrenal medulla imaging agent, which has recently been shown to be a substrate of the monoamine transporter (K_M = 10 μM) and to have a higher affinity for site T than endogenous substrates (21). Compound **5** is reserpine, which inhibits ATP-dependent monoamine uptake in the sub-nM concentration range. Compounds **7–13** are various derivatives of TBZ. Compounds **14** and **15** are, respectively, haloperidol and chlorpromazine, which inhibit the transporter in the μM concentration range. A Hill plot of the data of Fig. 2 indicated Hill coefficients ranging from 0.82 to 1.19. It was thus assumed that EC_{50} values derived from this experiment and indicated in Table 1 reflected a simple competition process, following a Michaelis-Menten model. The results of Table 1 indicate a large range of affinity for site T, EC_{50} values differing by more than 5 orders of magnitude from TBZ to substrates.

It may be noted that site T has some sterospecificity since the two diastereoisomers obtained by reduction of the keto group of TBZ, α- and β-TBZOH, have a different affinity for the monoamine transporter (Fig. 3). α-TBZOH has a 3- to 4-fold higher affinity for site T than β-TBZOH (Table 1, Fig. 3).

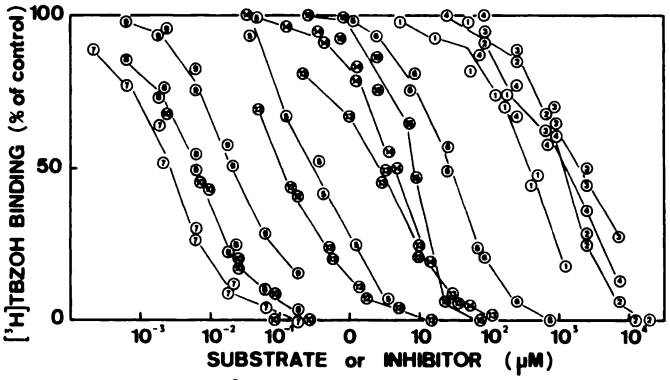

Fig. 2. Displacement of [^{3}H]TBZOH by various substrates and inhibitors of the monoamine transporter. The numbers refer to compounds listed in Table 1. The Hill coefficients and the linear regression coefficients derived from a Hill plot of the data were: **1**, −1.19 (r = 0.99, n = 6); **2**, −1.10 (r = 0.97, n = 8); **3**, −0.87(r = 0.97, n = 6); **4**, −0.82 (r = 0.96, n = 6); **5**, −1.03 (r = 0.99, n = 6); **6**, −1.07 (r = 0.99, n = 7); **7**, −0.94 (r = 0.99, n = 8); **8**, −0.90 (r = 0.99, n = 8); **9**, −1.00 (r = 0.99, n = 7); **10**, −0.93 (r = 0.99, n = 5); **12**, −0.92 (r = 0.99, n = 7); **13**, −0.87 (r = 0.99, n = 8); **14**, −1.12 (r = 0.99, n = 7); **15**, −1.19 (r = 0.91, n = 6).

[1] D. Scherman and J. P. Henry, unpublished data.

TABLE 1

Effect of substrates and inhibitors on [^{3}H]TBZOH binding

Compound No.	Compound Name	Type[a]	[^{3}H]TBZOH binding, EC_{50}
			μM
1	5-HT	S	330
2	Noradrenaline	S	1000
3	Adrenaline	S	1000
4	Tyramine	S	1000
5	Reserpine	I	0.3
6	MIBG	S	26
7	TBZ	I	0.003
8	α-TBZOH	I	0.006
9	β-TBZOH	I	0.020
10	Acetyl TBZOH	I	0.006
11	TBA	I	0.047[b]
12	$TBZNH_2$	I	0.110
13	Succinyl TBZOH	I	2.5
14	Haloperidol	I	4.0
15	Chlorpromazine	I	8.0

[a] S, substrate; I, inhibitor.
[b] Taken from Ref. 14.

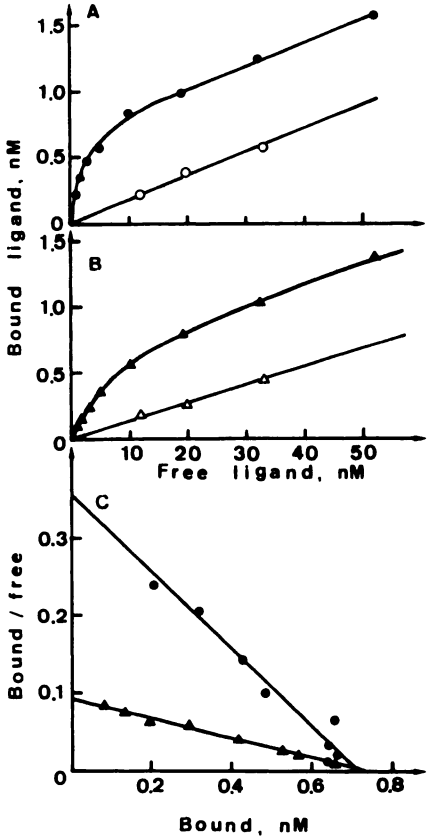

Fig. 3. α- and β-[^{3}H]TBZOH binding isotherms. Membranes (12 μg of protein/ml) were incubated for 2 hr at 25° in 0.3 M sucrose/10 mM Hepes (Na), pH 8.0, with various concentrations of α- and β-[^{3}H]TBZOH. A. α-[^{3}H]TBZOH binding in the absence (●) or presence (○) of 1 μM TBZ. B. β-[^{3}H]TBZOH binding in the absence (▲) or presence (△) of 1 μM TBZ. C. Scatchard plots of α-[^{3}H]TBZOH (●)- and β-[^{3}H]TBZOH (▲)-specific binding. Characteristics of the binding were: for α-TBZOH (●), K_D = 2.0 nM and B_{max} = 59.5 pmol/mg of protein (r = 0.967); for β-[^{3}H]TBZOH (▲), K_D = 7.9 nM and B_{max} = 60.5 pmol/mg of protein (r = 0.982).

Octanol/buffer and membrane/buffer apparent partition coefficients. The octanol/buffer partition coefficient of the investigated substrates and inhibitors of the monoamine transporter was determined. Since the partition coefficient of the protonated and the nonprotonated forms of these basic compounds are different (19), partition experiments were performed at pH 7.5, the pH value chosen for [^{3}H]TBZOH displacement experiments, and the results were expressed as an apparent partition coefficient at that pH value, $P_{o/b}$, describing the partition of the studied molecule, both protonated and unprotonated (Table 2). Uptake inhibitors concentrated in the organic phase, thus indicating a marked hydrophobicity. For these compounds, nonspecific adsorption to the membrane, observed under conditions where specific TBZ-binding sites on the transporter were saturated, was also measured. It was expressed as membrane/buffer apparent partition coefficient, $P_{m/b}$ (Table 2).

For reserpine and most of the TBZ derivatives, apparent $P_{o/b}$ and $P_{m/b}$ were correlated ($p \leq 0.01$; $r = 0.974$, $n = 5$). The slope of the regression line (±SE) is 0.59 ± 0.08 (Fig. 4). However, when data for haloperidol, chlorpromazine, and TBA were included, no correlation was observed ($p > 0.25$); for these three compounds, the observed apparent $P_{m/b}$ is larger than 0.6 times $P_{o/b}$. This point will be discussed below.

Relationship between the apparent affinity of the ligands for site T and their octanol/buffer apparent partition coefficient. For TBZ derivatives and substrates of uptake, there was an excellent correlation between the EC_{50} for [^{3}H]TBZOH displacement and the apparent $P_{o/b}$. On a log-log plot (Fig. 5), the regression line corresponding to these compounds (Fig. 5, *solid circles*) had a slope (±SE) of −1.069 ± 0.083 ($r = 0.971$; $p \leq 0.0001$). This plot indicated a linear reciprocal relationship between EC_{50} and apparent $P_{o/b}$. From the y axis intercept (0.675 ± 0.169, SE), the following relation was deduced:

$$EC_{50} \times \text{apparent } P_{o/b} = 4.7\ \mu M \qquad (1)$$

TABLE 2

Octanol/Buffer and membrane/buffer apparent partition coefficients

Results are the mean of 5–10 determinations.

Compound No.	Compound Name	Octanol/buffer apparent partition coefficient ($P_{o/b}$)	Membrane/buffer apparent partition coefficient ($P_{m/b}$)
			ml/g of protein
1	5-HT	0.0135	
2	Noradrenaline	0.0050	
3	Adrenaline	0.0028	
4	Tyramine	0.048	
5	Reserpine	5,200	3,270
6	MIBG	1.5	
7	TBZ	480	403
–	TBZOH[a]	105	130
8	α-TBZOH	139	
9	β-TBZOH	62	
10	Acetyl TBZOH	1,900	612
11	TBA	103	6,120
12	$TBZNH_2$	34	217
13	Succinyl TBZOH	0.6	
14	Haloperidol	534	2,300
15	Chlorpromazine	1,315	10,950

[a] –, unfractionated mixture of α and β isomers (70:30).

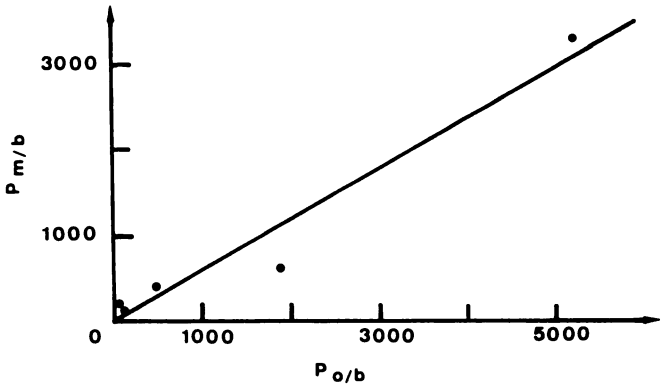

Fig. 4. Correlation between octanol/buffer ($P_{o/b}$) and membrane/buffer ($P_{m/b}$) apparent partition coefficients. Data for TBZ, TBZOH, acetyl TBZOH, $TBZNH_2$, and reserpine obtained from Table 2 are plotted. The line was fitted to the points by a least squares analysis ($r = 0.974$, $p \leq 0.01$).

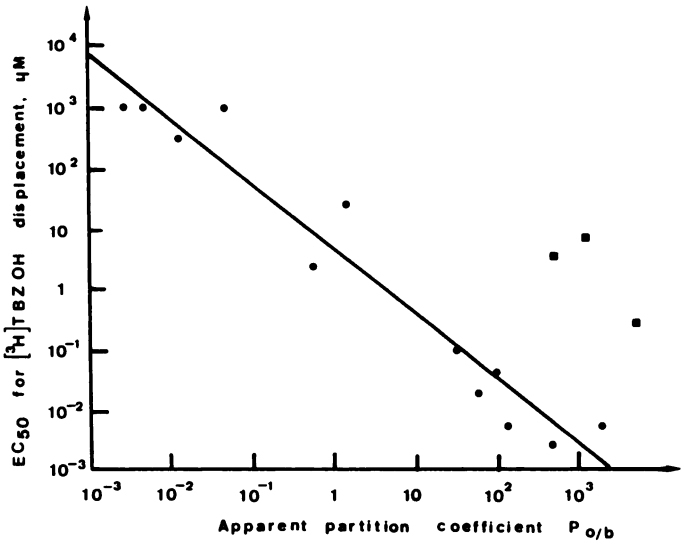

Fig. 5. Correlation between octanol/buffer ($P_{o/b}$) apparent partition coefficient and EC_{50} for [^{3}H]TBZOH displacement. Data were taken from Tables 1 and 2. ●, substrates of uptake and TBZ derivatives; ■, reserpine, haloperidol, and chlorpromazine. Characteristics of the regression line derived from data on substrates of uptake and TBZ derivatives (●) were: $r = 0.971$, $p \leq 0.0001$; slope (±SE): −1.069 ± 0.083; *y* axis intercept: 0.675 ± 0.169 (±SE).

This relationship is valid for the various TBZ derivatives tested, the apparent $P_{o/b}$ of which extends over a range of 3 orders of magnitude. Moreover, the relationship is also valid for the substrates of the transporter, which are less hydrophobic than TBZ derivatives, thus extending the validity domain of the correlation to 5 orders of magnitude in apparent $P_{o/b}$. Among the substrates, tyramine and MIBG are located above the line. For these two compounds, EC_{50} values are about 10 times larger than those calculated from relation 1 (Eq. 1).

The regression line thus obtained does not fit the points corresponding to reserpine, chlorpromazine, and haloperidol, which are indicated by the *solid squares* in Fig. 5. The affinity of these compounds for TBZ-binding sites cannot be described by Eq. 1, which would underestimate their EC_{50} by 3 orders of magnitude.

Discussion

Octanol/buffer and membrane/buffer apparent partition coefficients. The octanol/buffer system has been pro-

posed as a model to study the apparent partition of hydrophobic compounds between aqueous buffers and biological membranes (22). The apparent $P_{o/b}$ is generally proportional to the apparent $P_{m/b}$ derived from nonspecific adsorption to membranes (22). We have observed such a linear relationship for reserpine and for most of the TBZ derivatives (Fig. 4). For TBA, haloperidol, and chlorpromazine, apparent membrane partitioning is larger than predicted from apparent $P_{o/b}$. A similar behavior of chlorpromazine has been reported in the study of the erythrocyte membrane (22). This behavior of chlorpromazine might be attributed to ionic interactions between the drug and membrane proteins, since apparent $P_{m/b}$ decreases with increasing ionic strength (23). Such an interpretation might also be proposed for TBA, which bears three amine functions. Thus, apparent $P_{o/b}$ which, contrary to apparent $P_{m/b}$, is not affected by these ionic interactions, seems to reflect better the liposolubility of a compound and, hence, the concentration ratio between the aqueous buffer and the membrane phase.

It must be noted that, for chlorpromazine, the apparent $P_{m/b}$ in chromaffin granule membranes is similar to that reported in erythrocyte membranes, when measured under the same low ionic strength conditions and expressed in the same units. A value of 3200 ml/g dry weight has been reported for erythrocyte membranes (23) and a value of 4200 ml/g dry weight was calculated for chromaffin granule membranes, assuming for the latter a figure of 3.4 g of membrane dry weight/g of protein (20).

Another advantage of the octanol/buffer system is that it can be used to estimate the liposolubility of the hydrophilic monoamine substrates. To derive an apparent $P_{m/b}$ for these compounds from nonspecific adsorption to membranes would be difficult, since substrates are transported and trapped by closed structures. Adrenaline, noradrenaline, and 5-HT have a low lipophilicity (Table 2). Tyramine and MIBG are more lipophilic, thus accounting for the lower plateau value of uptake observed with these two compounds (10, 21): influx rates are comparable to that of catecholamines and 5-HT, but efflux rates are higher for the lipophilic compounds, due to diffusion through the lipid bilayer.

Intrinsic affinity of substrates and inhibitors for TBZ-binding site. The major finding of this work is the large validity domain of the linear correlation existing between the reciprocal of apparent $P_{o/b}$ and EC_{50} for [^{3}H]TBZOH displacement (Fig. 5). This correlation is described by relation 1 (Eq. 1), which may be formulated in a different way. The compounds following the correlation (TBZ derivatives and substrates) are competitive inhibitors of [^{3}H]TBZOH binding as previously shown (11, 24) and as indicated by their Hill coefficients (Fig. 2). For these displacers, EC_{50} and K_D values are proportional, according the the Cheng and Prusoff formula (25). Under our experimental conditions, K_D is about 0.6 times the EC_{50}. The correlation between EC_{50} and apparent $P_{o/b}$ can now be formulated as:

$$K_D \times \text{apparent } P_{o/b} = 2.8\ \mu M \qquad (2)$$

If we assume, as discussed before, that the apparent $P_{o/b}$ reflects the liposolubility of the compounds, then relation 2 (Eq. 2) suggests that site T equilibrates with the ligand present in the membrane phase, and that this site has the same "intrinsic" affinity for all compounds located on the correlation line of Fig. 5. The corresponding equilibrium dissociation constant, $K_{D,intrinsic}$, is related to the apparent K_D by the relation:

$$K_{D,intrinsic} = K_D \times R_{m/b}$$

where $R_{m/b}$ is the concentration ratio between the buffer and the hydrophobic phospholipid bilayer phase. This ratio can be tentatively derived from $P_{m/b}$ values (see Materials and Methods), and we find for TBZ derivatives and for the natural substrates of uptake an approximate value of 1 μM for $K_{D,intrinsic}$.

Relation 2 (Eq. 2) thus suggests that all compounds located on the correlation line have the same "intrinsic" affinity for site T, about 1 μM, although their apparent affinities are very different. For these compounds (natural substrates and TBZ derivatives), true differences in affinity cannot be completely ruled out. However, a fortuitous correlation over 5 orders of magnitude is unlikely. The diversity of the apparent dissociation constants may thus be attributed to differences in the concentration ratio between the aqueous and membrane phases.

Activity-structure relationship. The experimental data suggest that 5-HT, adrenaline, noradrenaline, and TBZ derivatives have the same "intrinsic" affinity for site T. Tyramine, which lacks one phenol group, and MIBG, which is neither a catechol nor an amine, have an intrinsic affinity which is decreased by only a factor of 10. The structural differences between these compounds are thus not critical for their specific interaction with the binding site. Accordingly, site T has a broad specificity and recognizes only a few elements of the ligand molecules. An aromatic ring and a basic function precisely located with respect to the aromatic ring may be the only structural requirements. It is interesting to note that the apparent stereoselectivity observed with α and β isomers of TBZOH (Fig. 3) is accounted for by a difference in the apparent $P_{o/b}$ of these isomers (Fig. 5).

Reserpine, haloperidol, and chlorpromazine have very large values of the product $K_D \times$ apparent $P_{m/b}$. This difference with the other molecules tested (about 3 orders of magnitude) indicates a low intrinsic affinity for site T. For reserpine, this result confirms the existence of a low affinity binding of this compound to site T (12). It should be pointed out that high affinity reserpine binding requires membrane energization by ATP (12, 13), and that about 80% of that binding is lost with the type of preparation used (26). Thus, competition between TBZ and reserpine for binding on their respective high affinity site cannot be analyzed with the type of preparation used. The low "intrinsic" affinity of chlorpromazine and haloperidol may have a different origin. Inhibition of catecholamine uptake and of [^{3}H]TBZOH binding by these two drugs occurs in the μM range, where a detergent-like action of these compounds has been reported (10). Moreover, it has been suggested that chlorpromazine inhibits catecholamine uptake by affecting the membrane permeability rather than by competitively inhibiting the monamine transporter (27). Thus, a 3-order of magnitude lower "intrinsic" affinity of chlorpromazine and of haloperidol may simply reflect their nonspecific action.

The fact that site T is in equilibrium with the ligand in the membrane phase might also explain the pH dependence of TBZ and TBZOH apparent affinity (19, 24). The unprotonated form of these amines has a larger apparent $P_{o/b}$ value than the protonated form, thus resulting in an increase with pH of the ligand concentration in the membrane phase. This increase in

turn might account for the apparent increase of affinity of the binding site with the pH.

In conclusion, the apparent high affinity of site T for TBZ may be explained by the hydrophobic character of this site, rather than by structural differences between this drug and the substrates. Our results suggest a localization of the TBZ-binding site inside of the membrane.

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