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Reserpine Binding to Bovine Chromaffin Granule Membranes

Characterization and Comparison with Dihydrotetrabenazine Binding

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SUMMARY

[^{3}H]Reserpine bound reversibly *in vitro* to chromaffin granule membranes. Binding was temperature-dependent and slow, and had biphasic kinetics. The addition of ATP accelerated the kinetics, which became monophasic and comparable to those of [^{3}H] dihydrotetrabenazine, without affecting the binding equilibrium constants. The ATP effect was related to H^+-electrochemical gradient generation by the granule membrane H^+ pump. Binding of reserpine to chromaffin granule membranes occurred on two classes of sites: R_1, B_{max} = 7 pmoles/mg of protein and K_D = 0.7 nM, and R_2, B_{max} = 60 pmoles/mg of protein and K_D = 25 nM. Sites R_2 were considered to be equivalent to [^{3}H] dihydrotetrabenazine binding sites, as the densities of the R_2 and the [^{3}H]dihydrotetrabenazine binding sites were similar and because tetrabenazine displaced reserpine from R_2 sites. Sites R_1 were tetrabenazine-resistant; they were involved in monoamine uptake, since their K_D values were similar to the K_I values of reserpine for noradrenaline uptake. Sites R_1 were less abundant than sites R_2 on chromaffin granule membranes, but they were present at the same concentration in intact chromaffin granules. We propose that the monoamine carrier exists in two forms: (a) an active form bearing both high- and low-affinity sites for reserpine and (b) an inactive form with only the low-affinity R_2 sites.

INTRODUCTION

The chromaffin granules of adrenal medulla accumulate monoamines (catecholamines and serotonin) by an active ATP-dependent transport mechanism (1, 2). The transport involves a specific monoamine carrier (3), which is driven by the electrochemical H^+ gradient, $\Delta\mu H^+$ (4–8), generated by an ATP-dependent H^+ translocase (9–11). The carrier is inhibited by various drugs (1–3), among which the more efficient are reserpine, an indole derivative, and TBZ[1] a benzoquinolizine which may be considered a dopamine analogue. TBZ appears to be more specific than reserpine, which, at high concentrations induces various effects on chromaffin granule membranes (12, 13) and on other types of membranes (for a review, see ref. 14).

Therefore, to study the monoamine carrier, we recently synthetized a tritiated derivative of TBZ, [^{3}H]TBZOH, and we have investigated its binding to bovine chromaffin granule membranes (15). We have described high-affinity binding sites (K_D = 3.5 nM, B_{max} = 60 pmoles/mg of protein) which were identified as the monoamine carrier.

To confirm our results, we compared TBZOH and reserpine binding. In spite of abundant literature on the physiology and pharmacology of reserpine (14, 16), only few data are available on the *in vitro* binding of this drug (17). Using a newly available tritiated reserpine of high specific activity (35 Ci/mmole), we have performed this comparison on purified bovine chromaffin granule membranes. During completion of this work, Weaver and Dupree (18) made the interesting observation that [^{3}H] reserpine binds *in vitro* to chromaffin granules energized by the addition of ATP. We show in this report that binding also occurs in the absence of ATP and that ATP accelerates the rate of binding without affecting plateau values and equilibrium constants.

A preliminary account of some of these results has already been published (19).

MATERIALS AND METHODS

Chemicals. [*benzoyl*-G-^{3}H]Reserpine (34.1 Ci/mmole) was obtained from New England Nuclear Corporation (Boston, Mass.). Because of

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[1] The abbreviations used are: TBZ, tetrabenazine; TBZOH, dihydrotetrabenazine; HPLC, high-pressure liquid chromatography; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

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rapid radiolysis the product was periodically repurified by HPLC, using a C_{18} μBondapak column and methanol/10 mM (NH_4) CO_3H (70:30) as the solvent. Stock solutions (1 μM) were made in 10% EtOH/100 mM KCl/10 mM Hepes (pH 7.5) and were kept at −20°. [^{3}H]TBZOH was prepared as described (15). *l*-[7-^{3}H]Noradrenaline hydrochloride was obtained through the Radiochemical Centre (Amersham, United Kingdom). 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. TBZ and reserpine concentrations higher than 10 nM were determined by fluorescence (excitation 290, emission 360 nm for reserpine; excitation 288, emission 315 nm for TBZ). CCCP (Sigma) was dissolved in ethanol.

Chromaffin granules. Bovine chromaffin granules were prepared by differential centrifugation in 0.3 M sucrose/10 mM Hepes (pH 7.5) (12) and were used within 36 hr of slaughtering.

Chromaffin granule membranes. Chromaffin granule membranes were prepared by osmotic lysis of granules isolated by centrifugation on a 1.6 M sucrose layer; the preparation was frozen in liquid nitrogen and kept at −80° (15, 20).

[^{3}H]Reserpine and [^{3}H]TBZOH binding. Membranes (30–150 μg of protein per milliliter for reserpine binding or 10–30 μg of protein per milliliter for TBZOH binding) were incubated in a medium containing [^{3}H]reserpine or [^{3}H]TBZOH at maximal specific activity (29,500 and 9,800 cpm/pmole, respectively), drugs and ATP where indicated, and 40 mM Hepes (pH 7.5) in 100 mM KCl (KCl medium) or 0.3 M sucrose (sucrose medium). When the pH was varied, Tris-phosphate was substituted for Hepes buffer. When intact granules were used, the sucrose concentration of the medium was raised to 0.45 M to prevent lysis. Incubations were performed at 25° under constant stirring. This temperature was selected as a compromise between the rate of association of reserpine, which increases from 0° to 36°, and membrane degradation, which occurs during long incubations. At 25°, the rate of association was 60% of that observed at 36°. After 20 hr at this temperature, [^{3}H]TBZOH binding was unchanged and noradrenaline uptake was 50% of its initial value. After incubation, aliquots were withdrawn, diluted with 4 ml of wash buffer, and filtered under gentle vacuum over GF/C filters (Whatman) previously washed by the same medium. Filters were then washed twice with 4 ml of ice-cold wash buffer, and their radioactivity was measured by liquid scintillation spectrometry in Aqualuma (Lumac, Schaesberg, Holland). Glass-fiber filters were preferred to cellulose filters (Millipore) because [^{3}H]reserpine adsorption was 5 times lower on the former than on the latter. However, membrane retention was only 70% on GF/C filters. Unless indicated, this factor was not corrected for. The wash buffer was the incubation medium supplemented with 10 μM reserpine (for [^{3}H]reserpine binding) or 100 μM TBZ (for [^{3}H]TBZOH binding). The addition of the drugs to the wash buffer resulted in a 10-fold decrease (from 20% to 2% and from 10% to 1%, respectively) of the radioactivity nonspecifically bound to the filters. In some experiments (Fig. 4), the wash medium contained both 10 μM reserpine and 100 μM TBZ. Reserpine at low concentration bound readily and rapidly to glass or plastic tubes (for [^{3}H]reserpine, 70–80% and 20–30%, respectively, in the absence and in the presence of membranes). This factor was accounted for by measuring in aliquots the actual radioactivity in the assay at the end of the incubation. It was verified that concentrations of reserpine and TBZOH were identical at the beginning and at the end of the incubation period, in the presence or in the absence of membranes. In experiments with unlabeled reserpine, surface absorption was minimized by using large volumes (to decrease surface effects) and by increasing the drug concentration by 10%.

ATP-dependent noradrenaline uptake. Noradrenaline uptake by granules or ghosts was determined by filtration on GF/C filters (Whatman) of aliquots diluted with 2 ml of ice-cold washing medium identical with the incubation medium (KCl or sucrose medium). The filters were washed twice with 2 ml of washing medium, and their radioactivity was measured in Aqualuma scintillation fluid.

Analytical techniques. Protein concentration was measured according to the method of Lowry *et al.* (21). Dopamine β-hydroxylase and cytochrome *c* oxidase activities were assayed as in ref. 15, and acid phosphatase and β-glucoronidase as in ref. 22.

RESULTS

Reserpine Binds to Chromaffin Granule Membranes in Vitro

[^{3}H]Reserpine was found to be associated with chromaffin granule membranes after incubation at 25° (Table 1). Under the experimental conditions (3-hr incubation), the addition of ATP-$MgSO_4$ increased the bound radioactivity by a factor of about 2. Excess reserpine (10 μM) decreased this radioactivity to background level, thus indicating the presence of saturable (specific) binding or transport sites. The radioactivity associated with the vesicles after incubation in the presence or in the absence of ATP could be extracted with chloroform and was identified as unmodified reserpine by HPLC.

Lysis in water of vesicles incubated in sucrose medium with [^{3}H]reserpine released only 14 ± 6% (n = 18) of the specific (saturable) radioactivity. This experiment indicates that [^{3}H]reserpine is bound to the membrane, since it behaves as [^{3}H]TBZOH (2 ± 4% release, n = 2), a bound inhibitor, and not as [^{3}H]noradrenaline (81 ± 3% release, n = 4), an accumulated substrate. Moreover, calculation of the internal concentration of [^{3}H]reserpine, assuming an internal volume of 4.7 μl/mg of protein (23), would indicate a considerable concentration gradient assuming an uptake mechanism (160 in the absence of ATP or in the presence of ATP and CCCP in the experiment of Fig. 1), which cannot be explained in the absence of any source of energy.

Kinetics of [^{3}H]Reserpine Binding

Association reaction. The binding of [^{3}H]reserpine to the chromaffin granule membrane is a very slow reaction in the absence of ATP (Fig. 1A). At 8 nM drug and at 25°, more than 24 hr were required to reach equilibrium,

TABLE 1

Binding of [^{3}H]reserpine

Membranes (0.4 mg of protein per milliliter were incubated in KCl medium for 3 hr at 25° with 20 nM freshly repurified [^{3}H]reserpine. Binding measurements were performed in duplicate on 20-μl aliquots. The nature of the bound radioactivity was analyzed by HPLC after $CHCl_3$ extraction (90% yield). In the three experiments, the elution profiles were similar, with more than 80% of the radioactivity in the reserpine peak.

Expt.	ATP-$MgSO_4$ (5 mM–2.5 mM)	Reserpine (10 μM)	[^{3}H]Reserpine bound		
			cpm ± SD	pmoles/mg protein	% of total
1	−	−	2300 ± 50	9.7	19.5
2	+	−	5000 ± 120	21.2	42.3
3	−	+	380 ± 15	1.6	3.2

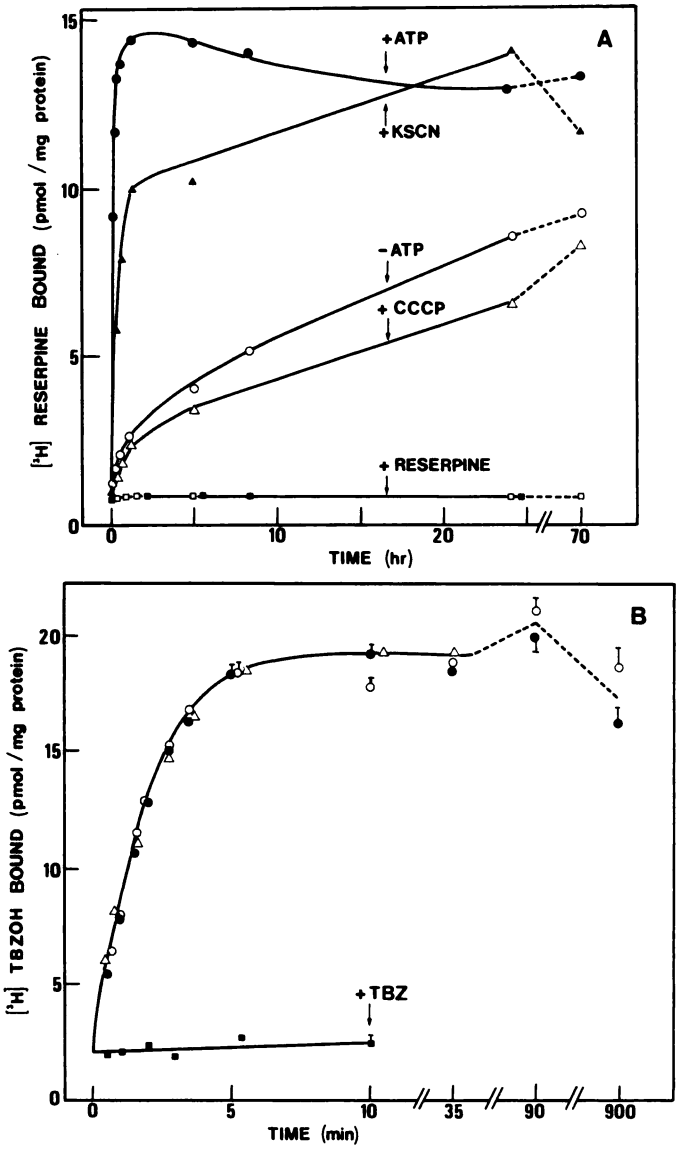

FIG. 1. *Kinetics of [³H]reserpine and [³H]TBZOH binding*

Membranes (150 and 90 μg of protein per milliliter in A and B, respectively) were incubated in sucrose buffer alone (○), or in sucrose buffer containing 2.5 mM ATP, 1.25 mM $MgSO_4$ (●, ▲, △, ■), 20 μM CCCP (△), 10 mM KSCN (▲), 2 μM reserpine (□, ■ in A) or 2 μM tetrabenazine (■ in B) in final volume of 500 μl. Aliquots (20 μl) were withdrawn at intervals and filtered. A, Binding of 8 nM [³H]reserpine; B, binding of 8 nM [³H]TBZOH.

and the reaction had biphasic kinetics (data not shown). This behavior contrasted with that of [³H]TBZOH, which at the same concentration was characterized by rapid ($t_{1/2}$ = 105 sec) monophasic kinetics (Fig. 1B). At 0°, both reactions were blocked, thus differing from [³H] imipramine, which binds readily at this temperature (24).

At 8 nM reserpine, the addition of ATP decreased the binding half-time from 500 to 5 min (Fig. 1A) and induced monophasic exponential kinetics (data not shown). The accelerating effect of ATP was dependent upon the generation of a $\Delta\mu H^+$ by the H^+ pump, since it was blocked by the proton ionophore CCCP. The experiment shown in Fig. 1A was conducted in sucrose medium, where the H^+ pump generates a considerable transmembrane potential, $\Delta\psi$, and a limited pH gradient, ΔpH (10, 25). However, the addition of 10 mM SCN^- to this medium, which collapsed the $\Delta\psi$ [as verified with the potential sensitive probe, oxonol-V (5)] and which allows the generation of a large ΔpH (5), has a small effect on the binding of [³H]reserpine in the presence of ATP (Fig. 1A). This observation was confirmed by the fact that the CCCP-sensitive accelerating effect of ATP was also observed when sucrose was replaced by 100 mM KCl, a medium with high permeant anion concentration (data not shown). It was therefore concluded that either component of the $\Delta\mu H^+$, the $\Delta\psi$ or the ΔpH, can accelerate reserpine binding.

The effect of ATP on [³H]reserpine binding was considerable at the beginning of the association reaction (15-fold increase after 10 min), but this effect decreased with time (1.6- to 2.0-fold increase after 24 hr). Thus, in spite of the difference still existing after 70 hr ([³H]reserpine binding to resting or uncoupled membranes is 70–75% of the figure obtained with energized membranes), this result was taken as indicating an effect of energization on the kinetics of association and not on the plateau levels. Similar results were obtained at high drug concentration: at 65 nM reserpine, the equilibrium was reached after 9 min in the presence of ATP whereas, in the absence of ATP, 65% of the plateau level obtained with energized membranes was already reached after 3 hr.

A completely different result was obtained with TBZOH, where energization of the membranes affected neither the kinetics nor the equilibrium of the binding (Fig. 1B and ref. 15).

Dissociation reaction. The association of [³H]reserpine with chromaffin granule membranes is reversible, but

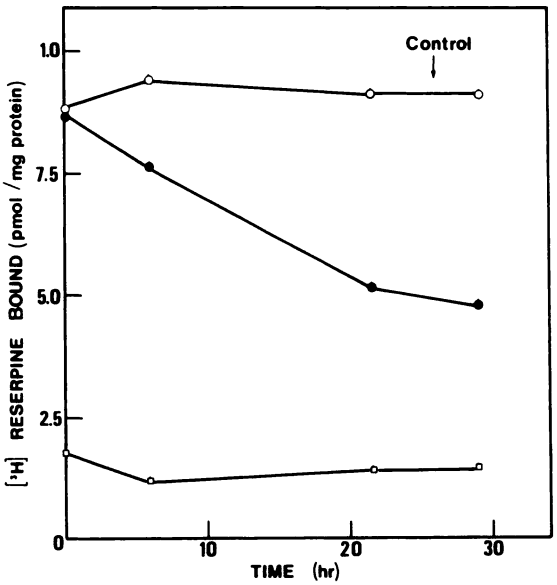

FIG. 2. *Reversibility of [³H]reserpine binding*

Membranes (70 μg of protein per milliliter) were preincubated for 28 hr in a KCl medium containing 25 nM [³H]reserpine alone (○, ●) or with 10 μM reserpine (□). The dissociation of the complex was followed by the addition of 10 μM reserpine to a sample (●), which was compared with an untreated sample (○). Aliquots (20 μl) were withdrawn at the indicated intervals. Points represent means of two measurements.

the dissociation reaction is very slow. In the absence of ATP, a rate constant, k_{-1}, of 8×10^{-6} sec^{-1} was measured (Fig. 2); however, for technical reasons, it was not possible to observe more than 50% of the dissociation. In the presence of ATP, dissociation was also very limited, only 38 ± 5% (n = 4) of the [^{3}H]reserpine-membrane complex being dissociated after 66 hr at 25° in the presence of an excess of reserpine. Determination of k_{-1} under these conditions was not attempted, since it was not known for how long the vesicles could be kept in an energized state.

Binding at Equilibrium

In the absence of ATP, [^{3}H]reserpine binding was considered to be at equilibrium after 24 hr at 25° (Fig. 1A). Under these conditions, binding was saturable with respect to ligand concentration (Fig. 3). Scatchard analysis of the specific binding gave a concave plot, indicating the existence of either two classes of sites or negative cooperativity (Fig. 4A). The same curvilinear plot was obtained in many experiments with different membrane preparations. The properties of the two classes of sites, derived from Scatchard plots, using an original iterative program (Fig. 4A, – – –), are summarized in Table 2. Sites R_1 have a higher affinity and a lower abundance than R_2 sites. Reserpine differed in this respect from TBZOH (15), which gave a perfectly linear Scatchard plot in the same concentration range (Fig. 4B; Table 2). The complexity of reserpine binding was also obvious in experiments in which a constant amount of tritiated ligand was incubated with increasing concentrations of cold ligand (Fig. 5). The slope at the midpoint of the curve describing reserpine binding was different from the theoretical value (– – –), thus differing from TBZOH binding.

Energization of the membranes by addition of ATP did not change [^{3}H]reserpine binding dramatically, which stayed biphasic (Table 2). In some experiments, the number of high-affinity R_1 sites was higher in presence of ATP, but this might reflect the fact that in absence of ATP equilibrium was not reached at the lowest concentrations of ligand, thus resulting in an underestimation of R_1 sites. In the experiments pre-

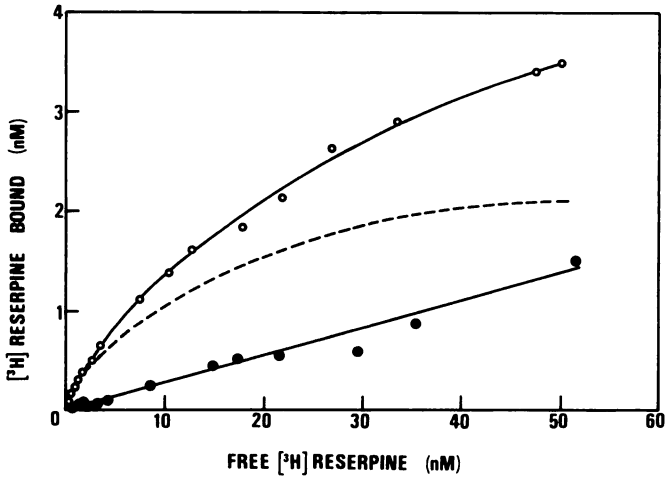

FIG. 3. *[^{3}H]Reserpine binding isotherm*

Membranes (60 μg of protein per milliliter) were incubated for 24 hr at 25° in 100 μl of KCl buffer with [^{3}H]reserpine in the 0.2–50 nM concentration range (○). Nonspecific binding was determined by the addition of 20 μM reserpine to the incubation medium (●). Dilutions were made from a stock solution of 1 μM [^{3}H]reserpine, the purity of which had been checked by HPLC. The ethanol concentration (1%) was adjusted in all samples. An aliquot (20 μl) of the incubation medium was used to determine the [^{3}H]reserpine total concentration, and the remaining part was filtered as described under Materials and Methods. Points represent means of two determinations. The coefficient of nonspecific binding was 0.022 (●).

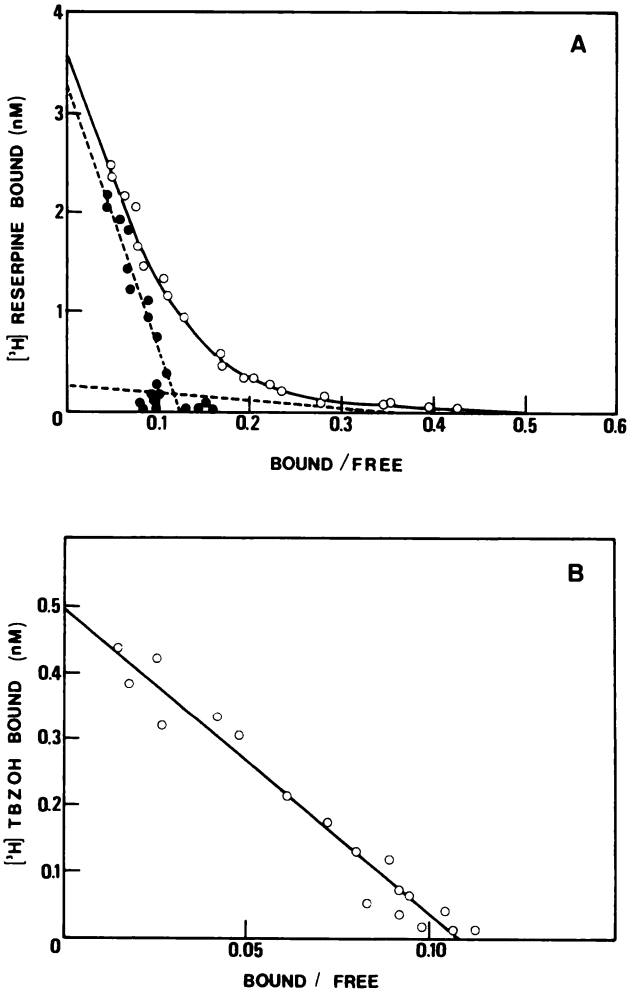

FIG. 4. *Scatchard plots of [^{3}H]reserpine and [^{3}H]TBZOH binding*

A. Reserpine binding. Experimental data (○) are from Fig. 3. The K_D of high affinity sites was derived from the slope of the straight line corresponding to the low-concentration samples (six points). The B_{max} of high-affinity sites and the B_{max} and K_D of low-affinity sites were calculated by an iterative program subtracting the contribution of high-affinity sites from total binding. Calculated points (●) and the corresponding linear regression are shown: R_1, B_{max} = 5.7 pmoles/mg of protein and K_D = 0.7 nM; R_2, B_{max} = 75.7 pmoles/mg of protein and K_D = 26.2 nM. B_{max} values are corrected for membrane retention on the filters.

B. TBZOH binding. Membranes (11 μg of protein per milliliter) were incubated for 20 hr at 25° in KCl buffer with [^{3}H]TBZOH in the 0.2–40 nM concentration range. Nonspecific binding was determined by the addition of 1 μM TBZ and was subtracted. Points represent means of two measurements. Parameters, derived by linear regression (r = 0.97), were as follows: B_{max}, 64.3 pmoles/mg of protein (corrected for filter retention); K_D = 4.6 nM.

TABLE 2

Characteristics of [^{3}H]reserpine and [^{3}H]TBZOH binding to membranes (A) and granules (B)

Saturation isotherms were generally determined from duplicate measurements at 20 different ligand concentrations in the 0.2–70 nM range. Protein concentrations varied from 0.025 to 0.065 mg of protein per milliliter. Results are means ± standard deviation.

Ligand	ATP-$MgSO_4$	n^a	Sites 1		Sites 2	
			$B_{max}{}^b$	K_D	$B_{max}{}^b$	K_D
			pmoles/mg protein	*nM*	*pmoles/mg protein*	*nM*
A. Membranes						
[^{3}H]Reserpine	−	8	7.1 ± 2	0.73 ± 0.28	58.7 ± 15	25.3 ± 7
[^{3}H]Reserpine	+	5	13.2 ± 4	0.30 ± 0.20	48.6 ± 8	18.5 ± 10
[^{3}H]TBZOH	−	5	—	—	57.9 ± 7	5.8 ± 1
B. Granules						
[^{3}H]Reserpine	+	2	9.8 ± 2	0.18 ± 0.01	9.4 ± 5	11.0 ± 6
[^{3}H]TBZOH	+	1	—	—	7.8	2.0

[a] Number of independent experiments.

[b] Experimental values multiplied by 1.4 to account for incomplete retention of membranes on GF/C filters (see Materials and Methods).

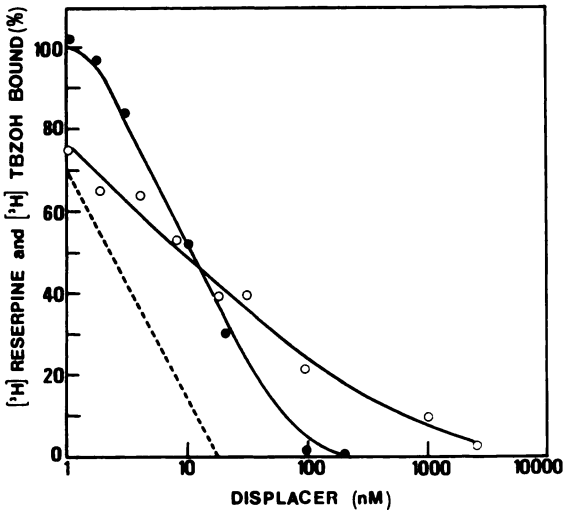

FIG. 5. *Competition with [^{3}H]TBZOH and [^{3}H]reserpine by the unlabeled molecules*

Membranes were incubated overnight at 25° in KCl buffer either at 50 μg of protein per milliliter in the presence of 1 nM [^{3}H]reserpine and various concentrations of reserpine (○) or at 25 μg of protein per milliliter in the presence of 2 nM [^{3}H]TBZOH and various concentrations of TBZOH (●). Total bound radioactivity and nonspecific binding, measured in the presence of 5 μM reserpine (○) or 2 μM TBZOH (●), were assayed in duplicate, and specific binding was expressed as percentage of controls (triplicate determinations) incubated without the competing agent (displacer). – – –, the slope, at the midpoint of a theoretical curve with a Hill coefficient of 1.0.

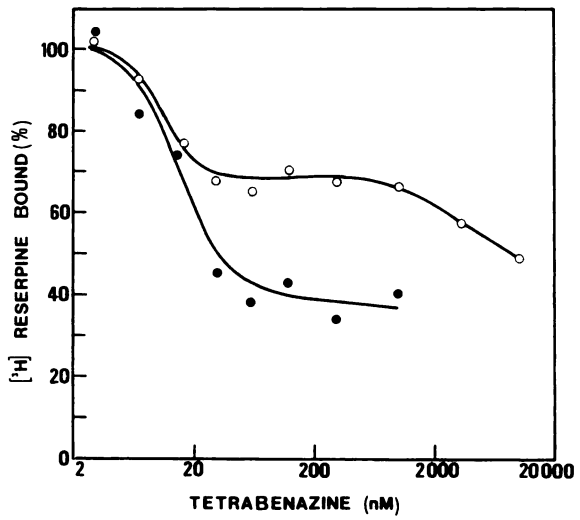

FIG. 6. *Competition with [^{3}H]reserpine at various concentrations by TBZ*

Conditions were as in Fig. 5. [^{3}H]Reserpine was 0.4 nM (○) or 50 nM (●). The experiment was also performed at intermediate concentrations demonstrating intermediate TBZ resistance: 4 nM, 70%; 8 nM, 60%; 60 nM, 42%; 160 nM, 35%.

sented above, frozen chromaffin granule membranes were used. With this type of preparation, R_2 and TBZOH binding sites had similar densities and R_1 was less abundant (Table 2A). In contrast, when freshly prepared intact granules were used, R_1 sites were found to be equivalent to TBZOH and R_2 sites (Table 2B).

Competition with [^{3}H]Reserpine by TBZ

TBZ competed with [^{3}H]reserpine for its binding sites (Fig. 6). The competition curves were biphasic, with first a decrease of the bound radioactivity in the 1–30 nM TBZ concentration range [where TBZ binds to the monoamine carrier (15)] and a second phase at much higher concentrations, corresponding to nonspecific effects. The percentage of [^{3}H]reserpine competed for specifically by TBZ (first part of the curve) increased with the concentration of reserpine used, thus suggesting that TBZ acted only on the low-affinity R_2 sites. To test this hypothesis, [^{3}H]reserpine saturation isotherms were studied in the absence or in the presence of 100 nM TBZ, a concentration without any unspecific effect (Fig. 7). This experiment allowed determination of the properties of the TBZ-resistant class of sites and, by difference, of the TBZ-sensitive class. The former had characteristics similar to those of R_1 whereas the latter were of the R_2 type (see legend to Fig. 7). In the hitherto described experiments, a constant concentration of unlabeled reserpine was used to measure nonspecific [^{3}H]reserpine binding. The effect of the concentration of [^{3}H]reserpine on the resistance to TBZ (Fig. 7) might then be explained by an incomplete saturation by unlabeled reserpine of the reserpine-specific sites. In a different type of experiment, nonspecific binding was determined by adding a constant 500-fold excess of reserpine to [^{3}H]reserpine. Under these conditions, there was again a significant difference

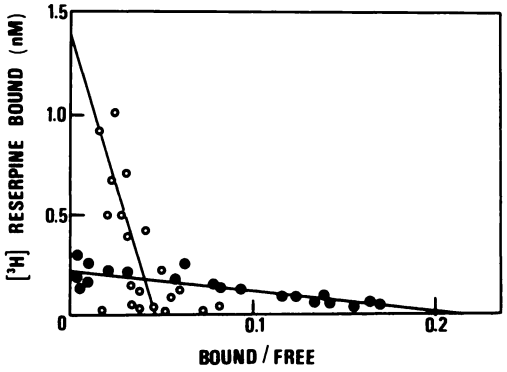

FIG. 7. *Scatchard plots of TBZ-resistant and difference between total and TBZ-resistant [^{3}H]reserpine binding*

Total and TBZ-resistant binding (measured in the presence of 100 nM TBZ) were determined as in Fig. 3, nonspecific binding being measured in the presence of 20 μM reserpine. The membrane concentration was 46 μg protein per milliliter. Total specific binding, when analyzed as described in Fig. 4, had the following characteristics: R_1, B_{max} = 5.6 pmoles/mg of protein and K_D = 0.40 nM; R_2, B_{max} = 42.6 pmoles/mg of protein and K_D = 23.2 nM. The TBZ-resistant binding (●) characteristics, determined by linear regression analysis, were as follows: B_{max} = 6.6 pmoles/mg of protein, K_D = 0.98 nM; those of TBZ-sensitive binding (○) (obtained as the difference between total and reserpine-resistant binding) were B_{max} = 43.6 pmoles/mg of protein, K_D = 29.5 nM. B_{max} values were corrected for filter retention.

in the level of TBZ-resistant [^{3}H]reserpine binding observed at low (0.6 nM) and at high (20 nM) [^{3}H]reserpine concentrations (68 ± 4% and 28 ± 5%, respectively). The specificity of TBZ for the low-affinity reserpine binding sites was observed in the presence as well as the absence of ATP (data not shown). TBZOH had the same specificity as TBZ (data not shown).

Localization of [^{3}H]Reserpine Binding Sites

[^{3}H]Reserpine binding was performed on membranes centrifuged on 0.45–1.45 M linear sucrose gradients (26). Both tetrabenazine-resistant (R_1) and tetrabenazine-sensitive (R_2) sites were found to be associated with [^{3}H] TBZOH binding activity and dopamine β-hydroxylase, a chromaffin granule membrane marker. Reserpine binding did not occur on either mitochondria or on lysosomes, as judged by the distribution of specific markers (Fig. 8).

Correlation of Biological Activity with [^{3}H]Reserpine Binding

Several technical difficulties were encountered in the determination of the K_I for the inhibition of noradrenaline uptake. These resulted from the low rate of association of reserpine, the low concentration of membranes (which was imposed by the high density of reserpine binding sites), and the presence of reserpine binding sites on walls of polystyrene tubes. When membranes (25 μg of protein per milliliter) were preincubated for 3 hr with reserpine in the presence of ATP, an IC_{50} for noradrenaline uptake of 0.5 nM was repeatedly observed (Fig. 9). A low figure was also obtained when membranes were preincubated for long periods of time with reserpine in the absence of ATP. An IC_{50} of 2 nM was measured after 18 hr at 25°. The effect of the concentration of substrate on IC_{50} values was investigated. Membranes were preincubated for 1 hr at 25° with reserpine, ATP-$MgSO_4$, and noradrenaline at 3, 15, and 50 μM. The rate of uptake was measured after the addition of [^{3}H]noradrenaline. A Dixon plot of the results indicated that the inhibition was of the competitive type and was characterized by a K_I of 0.5 nM (data not shown).

The similar values of K_D for the high-affinity binding sites R_1 and K_I suggested that these sites were involved in the monoamine carrier activity. That the TBZ-sensitive sites R_2 were not involved in the biological effects of reserpine was shown by the fact that the EC_{50} for [^{3}H] TBZOH displacement by reserpine differed by about 2 orders of magnitude from the IC_{50} of reserpine for noradrenaline uptake, the two constants being measured in the same experiment (Fig. 9).

To investigate the difference between reserpine and TBZ inhibition, the active sites of the carrier were indirectly titrated by analyzing uptake inhibition at high membrane concentration (1 mg of protein per milliliter), corresponding to a concentration of sites larger than the K_D (Fig. 10A). The IC_{50} values for reserpine and TBZ were 6.5 and 60 nM, respectively, corresponding to densities of sites of 13 and 120 pmoles/mg of protein. These figures are similar to B_{max} for R_1 and TBZOH sites given in Table 2A. As previously observed for binding at equilibrium (Table 2B), when the experiment was repeated with intact granules, the relative number of reserpine inhibitory sites increased and became equal to that of TBZ sites, 26 pmoles/mg of protein (Fig. 10B).

Pharmacology of [^{3}H]Reserpine Binding

Substrates or inhibitors of the carrier were incubated at various concentrations with 15 nM [^{3}H]reserpine and membranes in the absence of ATP (Table 3). Under these conditions, [^{3}H]reserpine was mainly bound to R_2 sites. Chlorpromazine and haloperidol, which are inhibitors of noradrenaline uptake less efficient and less specific than reserpine and TBZ (3, 12), displaced [^{3}H] reserpine from up to 75% of its binding sites, with an EC_{50} in the micromolar range. The substrates serotonin and noradrenaline were less efficient, with an EC_{50} in the millimolar range. In the same table, the IC_{50} for noradrenaline uptake inhibition or the K_m of substrates and the EC_{50} for displacement of [^{3}H]TBZOH from its binding sites are also indicated. For the four compounds studied, the EC_{50} for reserpine displacement were reasonably well correlated with [^{3}H]TBZOH binding or noradrenaline uptake data.

In a different set of experiments designed to investigate R_1 sites, 0.4 nM [^{3}H]reserpine was incubated with ATP-$MgSO_4$, membranes, and various concentrations of substrate for 1 hr. Under these conditions, EC_{50} values of 4.8 and 20 μM were obsereved for serotonin and noradrenaline, respectively (data not shown). These values are of the order of magnitude of the apparent K_m determined at the same pH (23).

pH Dependency of [^{3}H]Reserpine Binding

At 1 nM [^{3}H]reserpine, binding increased with the pH up to pH 8.0 and stayed constant up to pH 9. Half-

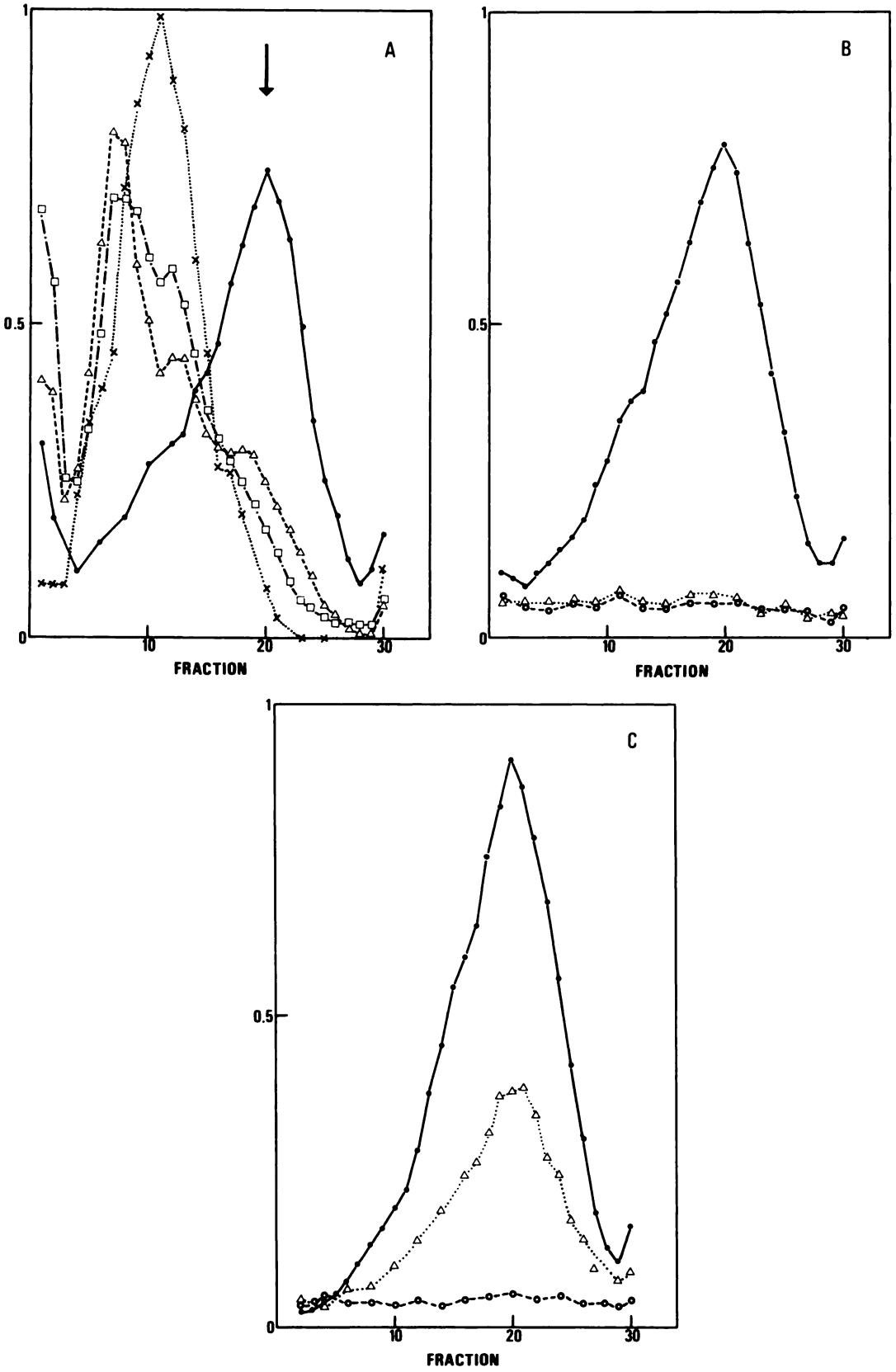

FIG. 8. *Distribution of [^{3}H]reserpine binding after isopycnic centrifugation on sucrose gradients*

A linear 0.45–1.45 M sucrose gradient was layered onto membranes (2.0 mg of protein) in 0.5 ml of 50% sucrose, and the tube was centrifuged for 3 hr at 270,000 × *g* in an SW 41 rotor. A. Dopamine β-hydroxylase (●——●), cytochrome *c* oxidase (×····×), β-glucuronidase (□—·—□), and acid phosphatase (Δ– – –Δ) were measured in 10-μl aliquots. B. [^{3}H]TBZOH binding was measured by incubating 7-μl aliquots in KCl medium for 2 hr at 25° with 2 nM [^{3}H]TBZOH in the absence (●) or in the presence of 100 nM (Δ) or 2 μM (○) TBZ (100-μl final volume). C. [^{3}H]Reserpine binding in the absence (●) or in the presence of 100 nM TBZ (Δ) or 2 μM reserpine (○) was measured with 2 nM [^{3}H]reserpine under the same conditions as [^{3}H]TBZOH binding, but 2.5 mM ATP and 1.25 mM $MgSO_4$ were added to the incubation medium. Total collected activities have been normalized to unity.

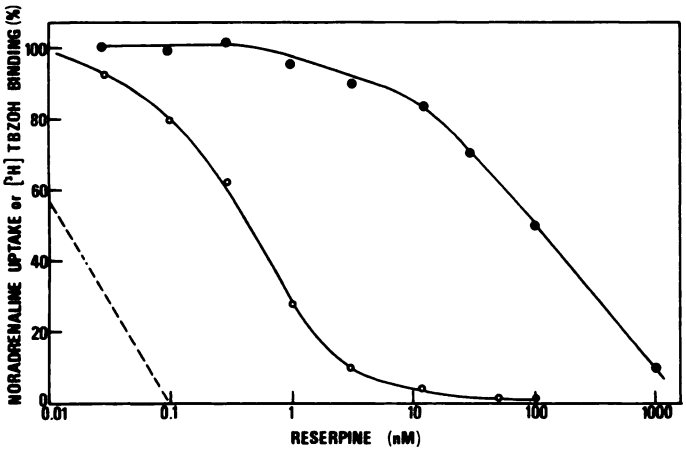

FIG. 9. *Inhibition by reserpine of [^{3}H]noradrenaline and [^{3}H]TBZOH binding at low membrane concentration*

Membranes (25 μg of protein per milliliter) were preincubated for 3 hr at 25° in KCl medium containing 2.5 mM ATP, 1.25 mM $MgSO_4$, and various concentrations of reserpine. Aliquots were withdrawn to assay for [^{3}H]noradrenaline uptake (○) or [^{3}H]TBZOH binding (●). In the first case, the aliquots were added to KCl medium containing ATP-$MgSO_4$ at the same concentration and 5 μM [^{3}H]noradrenaline (10^6 cpm). Monoamine uptake was measured at 20, 40, and 60 min. The maximal rate of uptake was 120 pmoles/min/mg of protein. The IC_{50} was 0.5 nM. [^{3}H]TBZOH binding was measured after incubation for 1 hr with 2 nM [^{3}H]TBZOH and ATP-$MgSO_4$. Maximal binding was 18 pmoles/mg of protein. The apparent K_D for reserpine, calculated as in ref. 27, was 40 nM.

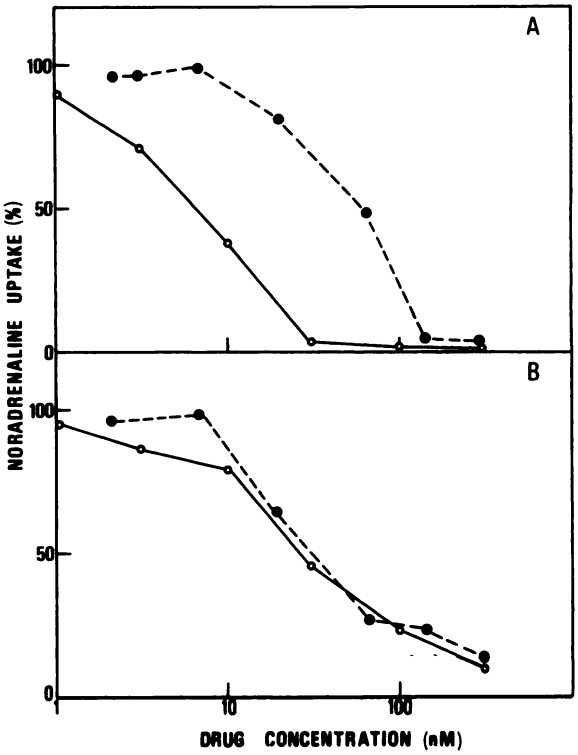

FIG. 10. *Inhibition of noradrenaline uptake by reserpine or TBZ at high membrane or granule concentration*

In A, membranes (1 mg of protein/ml) were preincubated for 3 hr at 25° in a KCl medium containing 5 mM ATP, 2.5 mM $MgSO_4$, and various reserpine (○) or TBZ (●) concentrations. In B, granules (2 mg of protein/ml) were preincubated for 1 hr at the same temperature in a 0.45 M sucrose medium containing ATP-$MgSO_4$ and drugs. At the end of the preincubation, 50 μM [^{3}H]noradrenaline was added and monamine uptake was measured. Maximal uptake values, after correction for nonspecific uptake occurring at 1 μM reserpine (○) or 5 μM TBZ (●), were 320 ad 30 pmoles/min/mg of protein for membranes and granules, respectively. The low value obtained with the granule preparation is likely to be due to a decrease in specific radioactivity caused by catecholamine leakage. The IC_{50} values for reserpine of membranes and granules measured at low protein concentrations were 0.5 and 0.85 nM, respectively, in this set of experiments.

maximal binding was observed at pH 6.45, a value close to the drug pK_a (6.6). Similar results were obtained at high (25 nM) reserpine concentration, with half-maximal binding at pH 6.25, and in presence of ATP (half-maximal binding at 6.5 and 6.15 at 0.3 and 40 nM [^{3}H]reserpine, respectively). This result is similar to that observed with [^{3}H]TBZOH and it can be taken as indicating that the carrier binds only the neutral form of reserpine, as it does for TBZ and noradrenaline (28).

DISCUSSION

Our results clearly show that reserpine binds *in vitro* in a (partially) reversible manner to saturable sites on chromaffin granule membranes. Although it is possible to detect the presence of contaminating organelles (mitochondria and lysosomes) in the purified membrane preparation used in this work, subcellular fractionation experiments (Fig. 8) indicate that reserpine binding sites are localized on the chromaffin granule membrane. Binding of reserpine to rat chromaffin granules has been reported previously; however, in these studies binding was observed only after administration of the drug *in vivo* (17). *In vitro*, binding was low and nonsaturable (17). Recently, an interpretation of these results was offered by Weaver et Dupree (18), who proposed that ATP-Mg was a requirement for [^{3}H]reserpine binding. We now show that [^{3}H]reserpine can indeed bind to chromaffin granule membrane in absence of ATP. It is likely that this reaction has remained undetected until now because of the use of short incubation times and low temperature (0°).

[^{3}H]Reserpine binding differs from [^{3}H]TBZOH binding in the following aspects: (a) in the absence of ATP, the former binds much more slowly than the latter; (b) ATP accelerates the rate of reserpine binding; (c) reserpine binds to two classes of sites, R_1 and R_2; (d) TBZ does not displace reserpine from R_1 sites; and (e) in the presence of ATP, the substrates, noradrenaline and serotonin, compete with reserpine much more effectively than TBZOH. These differences deserve some comment.

The mechanism of the ATP effect is obscure. It appears to occur through the H^+ pump, since the effect is $\Delta\mu H^+$-driven. As pointed out before, ATP does not induce [^{3}H]reserpine accumulation inside ghosts, but it accelerates its rate of binding to the membrane. This suggestion is further supported by the fact that the B_{max} for [^{3}H]reserpine (Table 2) is not increased in the presence of ATP. Moreover, the efficiency (EC_{50}) of reserpine in displacing [^{3}H]TBZOH (Table 3) is consistent with K_D values for [^{3}H]reserpine binding to sites R_2, and both are unaffected by ATP. Two hypotheses might account

for the ATP effect. According to the first hypothesis, reserpine binding would occur only on the inner face of the vesicle. It would be preceded by an inward translocation of the drug, which would be the rate-limiting step, accelerated by ATP. An asymmetrical binding of reserpic acid has been recently reported by Njus (29).

On the other hand, the transport of reserpine is likely to be a fast event because of the liphophilicity of the drug. In the second type of hypothesis, the $\Delta\mu H^+$ would unmask reserpine binding sites by affecting membrane lipids or proteins. The sites would mainly preexist in a nonbinding state and would be converted to a binding state slowly in absence of ATP or rapidly in its presence. It should be pointed out that reserpine binding sites would differ in this respect from TBZOH binding sites.

Our data indicate the existence of two classes of binding sites for reserpine (Fig. 4). Nonlinear Scatchard plots also arise from artifacts. In the case of reserpine, the main difficulty is the low rate of association, which might hamper measurements at equilibrium. Nevertheless, samples at the lowest ligand concentrations should be the farthest from equilibrium, thus resulting in convex and not concave plots. In addition, the same biphasic plots were also observed in the presence of ATP, where the association reaction was much faster. The existence of two different classes of sites is further confirmed by the slope of the curve describing the competition with [^{3}H]reserpine by unlabeled reserpine (Fig. 5) and by the differential effect of TBZ on [^{3}H]reserpine binding (Figs. 6 and 7). A study of the distribution of [^{3}H]reserpine after *in vivo* administration to rats has indicated the existence of two rate constants for the clearance of the drug in adrenergic tissues (30). The first and second phases might be associated with R_2 and R_1 sites, respectively.

A comparison of the properties of [^{3}H]TBZOH and [^{3}H]reserpine binding sites lends support to the view

TABLE 3

Pharmacological properties of [^{3}H] TBZOH and [^{3}H]reserpine sites

Experimental conditions were as described in Fig. 5, except where indicated.

Drug	15 nM [^{3}H]Reserpine[a] EC_{50}	1 nM [^{3}H] TBZOH EC_{50}	Noradrenaline uptake IC_{50} or K_m
	nM	*nM*	*nM*
TBZ	16[b]	4.4[c]–11	3.2[e]
Reserpine		19[c]–26[d]	0.5
Haloperidol	3×10^3	1×10^{3e}	0.55×10^{3e}
Chlorpromazine	9×10^3	1.9×10^{3e}	2.5×10^{3e}
Serotonin	200×10^3	345×10^{3e}	—
Noradrenaline	6000×10^{3f}	1720×10^{3e}	60×10^{3g}

[a] Membrane concentration was 150 μg of protein per milliliter.

[b] Derived from the TBZ-sensitive fraction of the sites as defined in Fig. 6.

[c] Preincubation of the membranes (8 μg of protein per milliliter) for 24 hr at 25° with reserpine or TBZ in KCl medium, followed by a 3-hr incubation with [^{3}H]TBZOH.

[d] As in *c*, but 2.5 mM ATP and 1.25 mM $MgSO_4$ were added at the beginning of the preincubation.

[e] Data from ref. 15.

[f] Incubation in the presence of superoxide dismutase (10 μg/ml) to decrease noradrenaline autoxidation.

[g] Data from ref. 23.

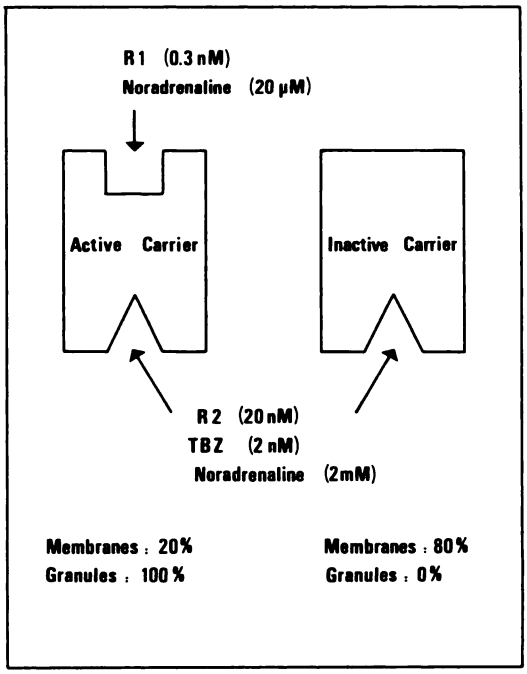

FIG. 11. *Model of drugs and substrate binding to the monoamine carrier*

that the former are identical with R_2 sites which are thus borne by the monoamine carrier: (a) Both have the same density, either in membrane or in granule preparations (Table 2). (b) R_2 sites are sensitive to TBZ and TBZOH and they have the same affinities for reserpine and TBZ as [^{3}H]TBZOH sites. Thus, the K_D of R_2 sites (Table 2) is similar to the EC_{50} for displacement of [^{3}H]TBZOH by reserpine (Table 3), and the EC_{50} values for displacement by TBZ of [^{3}H]reserpine from R_2 sites or of [^{3}H] TBZOH are similar (Table 3). (c) Displacement experiments with haloperidol, chlorpromazine, serotonin, and noradrenaline indicated similar EC_{50} values for TBZOH and R_2 sites.

R_1 sites are more difficult to interpret. The low value that we have obtained for the K_I of reserpine for noradrenaline uptake indicates that these sites are involved in catecholamine uptake. Our K_I value is more than 1 order of magnitude lower than those previously reported (13, 15, 31). This discrepancy is probably explained by the low temperature-dependent rate of association of reserpine in the absence of ATP. Maximal inhibition is seen only after preincubation of the drug with membranes in the presence of ATP at 25°; if the membranes are preincubated at low temperature or in the absence of ATP, the time-course of noradrenaline uptake is nonlinear, indicating that binding proceeds during uptake. If R_1 sites are involved in monoamine translocation, it should, however, be noted that their density on chromaffin granule membranes is 5–10 times lower than that of R_2 or [^{3}H]TBZOH binding sites (Table 2A; Fig. 10). This result can be interpreted in various ways. It might indicate the existence of regulatory R_1 sites, each controlling several transport sites R_2. Nevertheless, in intact granules, the density of R_1 sites is equal to that of R_2 or [^{3}H]TBZOH binding sites (Table 2B). Therefore, an alternate possibility would be that both R_1 and R_2 sites

are borne by the monoamine carrier but that the monoamine carrier of membrane preparations is heterogeneous: the carrier exists in an active form possessing both R_1 and R_2 sites and in an inactive form devoid of R_1 sites (Fig. 11). According to this hypothesis, the inactive form of the carrier would bind TBZ and, with low affinities, reserpine and noradrenaline. In the active form, a second class of sites would be present, which would bind reserpine and noradrenaline with higher affinities (0.5 nM and 20 μM, respectively). The appearance of the inactive form would be a result of the preparation of membranes (Fig. 10), either due to protein denaturation or to topological problems which would prevent generation of a correct $\Delta\mu H^+$. Reserpine would thus be a very useful tool in the study of the carrier, since it would probe only the active carrier molecules whereas [^{3}H] TBZOH would bind to the active and inactive ones.

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