Reserpine Binding to Chromaffin Granules Suggests the Existence of Two Conformations of the Monoamine Transporter†

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ABSTRACT: The binding of [^{3}H]reserpine ([^{3}H]RES) to purified bovine chromaffin granule membranes has been studied at low membrane concentration. Saturation isotherms indicated a dissociation equilibrium constant K_D of 30 pM and a density of binding sites of 8 pmol/mg of protein at 30 °C. The association rate constant was 4.0×10^5 M^{-1} s^{-1}, and the calculated dissociation rate constant was 1.2×10^{-5} s^{-1}, corresponding to a half-lifetime of about 16 h. Although this dissociation was too low to be measured directly, [^{3}H]RES binding was indeed reversible since it was lost after addition of the detergent Triton X-100. Dihydrotetrabenazine (TBZOH) inhibited [^{3}H]RES binding in a time-dependent manner, EC_{50} varying from 37 nM after a 1-h incubation to 600 nM after 16 h. On the contrary, [^{3}H]RES binding inhibition by the substrate noradrenaline was time independent. It is proposed that the transporter exists in two different conformations which bind exclusively either tetrabenazine (TBZ) or RES and which are in equilibrium. The effects of detergents were consistent with this two-conformation model. The transporter solubilized by cholate bound [^{3}H]TBZOH, but not [^{3}H]RES. On the other hand, addition of cholate to membrane-bound [^{3}H]RES solubilized the membrane without releasing the ligand from its binding site. It is proposed that the TBZ-binding conformation is obtained by solubilization with cholate and that RES stabilizes the RES-binding conformation, allowing its solubilization by this detergent.

Reserpine (RES)[1] is the most important of the many alkaloids found in the extracts of *Rauwolfia serpentina*, a climbing shrub of India. It is a powerful sedative, which induces a marked and long-lasting depleting effect on the level of brain monoamines such as dopamine, noradrenaline, and serotonin [for a review, see Carlsson (1965)]. This effect is accounted for by an inhibition of the monoamine transporter present on the corresponding synaptic vesicles (Carlsson, 1965). RES has also a peripheral effect (Von Euler, 1972), which has been characterized at the molecular level on the monoamine uptake system of adrenal medulla chromaffin granules (Kirshner, 1962; Scherman & Henry, 1980; Zallakian et al., 1982). In this system as in synaptic vesicles, a specific transporter catalyzes a proton–monoamine antiport which is coupled to the proton electrochemical gradient (inside acidic and positively charged) generated by an ATP-dependent proton pump [for a review, see Johnson (1987)].

[^{3}H]RES binds to the monoamine transporter of purified bovine chromaffin granule membranes (Deupree & Weaver, 1984; Scherman & Henry, 1984) and of synaptic vesicles (Near & Mahler, 1983). A high-affinity binding has been characterized, which differs from that of the other inhibitors, tetrabenazine (TBZ) (Scherman et al., 1983) and ketanserin (Darchen et al., 1988), by the fact that kinetics of [^{3}H]RES binding are accelerated in the presence of the ATP-induced electrochemical gradient. This difference suggests the existence of two different binding sites on the monoamine transporter, a concept which is supported by pharmacological and biochemical data (Henry et al., 1987). However, two points remain obscure. First, bound [^{3}H]RES dissociates only very slowly, either in vivo or in vitro. In vivo experiments indicate a significant binding to rat chromaffin granules several days after administration (Alpers & Shore, 1969). Such an effect is consistent with the long-lasting depleting effect of RES observed in the brain (Carlsson, 1965). The persistence of these in vivo effects cannot be attributed to a covalent linkage of RES since in vitro experiments indicate that inhibition of monoamine uptake by RES (Kanner et al., 1979) and [^{3}H]-RES binding to chromaffin granule membranes (Scherman & Henry, 1984) are reversible. On the other hand, the persistence of this in vivo effect seems inconsistent with the published equilibrium dissociation constants: K_D = 9 nM (Deupree & Weaver, 1984), 0.3 nM (Scherman & Henry, 1984), and 1.25 nM (Near & Mahler, 1983), which in the reasonable hypothesis of an association rate constant of 10^6 M^{-1} s^{-1} would suggest a half-lifetime of less than 1 h. Second, the hypothesis that TBZ and RES bind to two distinct classes of sites raises another difficulty since these two drugs seem to compete in vivo for a common site. For instance, pretreatment of rats with TBZ just before RES administration induces a depletion of central monoamines which has the lifetime of a TBZ treatment (several hours) and not that of a RES one (several days), thus indicating that TBZ protects its target against the effect of RES (Carlsson & Lindqvist, 1966; Stitzel, 1977).

In this paper, these two points have been analyzed. The characteristics of [^{3}H]RES binding to purified chromaffin granule membranes and the effect of TBZ on this binding have been reinvestigated. In addition, the effects of detergents on the binding to the transporter of [^{3}H]RES and of the TBZ derivative [^{3}H]dihydrotetrabenazine ([^{3}H]TBZOH) have been

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[1] Abbreviations: RES, reserpine; TBZ, tetrabenazine (2-oxo-3-isobutyl-9,10-dimethoxy-1,2,3,4,6,7-hexahydro-11b*H*-benzo[*a*]quinolizine); 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; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

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compared in order to gain insights on the differences between the binding of these two drugs.

Experimental Procedures

Chemicals. [^{3}H]RES (13 Ci/mmol) was purchased from New England Nuclear (Boston, MA). Because of rapid radiolysis, [^{3}H]RES was periodically repurified by HPLC using a Nova Pak C_{18} column (Waters, Milford, MA) and methanol/10 mM NH_4HCO_3 (70:30) as solvent and following the elution by the optical absorption at 267 nm and by measurement of the radioactivity. The radiochemical purity of the material used was 91%. Stock solutions (1 μM) were made in 10% ethanol containing 100 mM HCl and were kept at −20 °C. [^{3}H]TBZOH (15 Ci/mmol) was from CEA (Saclay, France). RES and TBZ were obtained from Fluka (Buchs, Switzerland). Stock solutions were obtained by dissolution in 1 M acetic acid for RES (10 mM final concentration) and in 4 mM HCl for TBZ (2 mM final concentration). Cholate (sodium salt) and Triton X-100 were from Merck (Darmstadt, FRG) and Sigma (St. Louis, MO), respectively.

Chromaffin Granule Membrane Preparation. Bovine chromaffin granule membranes were prepared by osmotic lysis of granules isolated on a 1.6 M sucrose layer (Smith & Winkler, 1967; Giraudat et al., 1980). Membranes were frozen in liquid nitrogen and stored at −80 °C. Protein was measured according to Bradford (1976) with bovine serum albumin as a standard.

Solubilization of Chromaffin Granule Membranes. Membranes were diluted in 100 mM KCl/10 mM Hepes (K^+) buffer, pH 7.5, and centrifuged for 15 min at 30000*g*. The pellets were resuspended in the same buffer at a protein concentration of 5 mg/mL. A 10% sodium cholate solution was then slowly added under constant stirring to a final concentration of 0.75–1%. The mixture was kept at room temperature for 10 min and was then centrifuged for 40 min at 160000*g*. Supernatants were frozen in liquid nitrogen and stored at −80 °C.

[^{3}H]RES Binding to Chromaffin Granule Membranes. To measure [^{3}H]RES binding, chromaffin granule membranes (0.5–20 μg of protein/mL) were incubated at 30 °C in 0.3 M sucrose/40 mM Hepes (K^+) buffer, pH 8.0, containing 2.5 mM ATP/1.3 mM $MgSO_4$ and various [^{3}H]RES concentrations. The assay final volume was 4 mL when the membranes were in the 2.5–20 μg of protein/mL concentration range or 12 mL in the 0.5–1 μg of protein/mL concentration range. After incubation, the mixture was rapidly filtered through GF/C filters (Whatman, Clifton, NJ), preincubated in sucrose/Hepes buffer containing 10 μM unlabeled RES. The filters were washed twice with the same buffer, and their radioactivity was measured by liquid scintillation in Aqualuma (Lumac, Landgraaf, The Netherlands). Specific [^{3}H]RES binding was obtained by subtracting nonspecific binding determined in assays containing 50–100 nM RES.

[^{3}H]RES Binding in the Presence of Detergents. [^{3}H]RES was incubated either with detergent-solubilized membranes or with intact membranes, and in the latter case, detergents (cholate or Triton X-100) were added after completion of the binding. In the first type of experiments, soluble extracts (100 μg of protein/mL) were incubated with 2–4 nM [^{3}H]RES in 100 mM KCl/40 mM Hepes (K) buffer, pH 8.0, at 30 °C for 18 h. In the second case, membranes (10–40 μg of protein/mL) were incubated with 0.1–0.3 nM [^{3}H]RES in the indicated buffer containing 2.5 mM ATP/1.3 mM $MgSO_4$ at 30 °C for 2–4 h. Sodium cholate or Triton X-100 was then added as a concentrated solution, and the mixture was incubated at 30 °C for the indicated period of time. Binding was measured by filtration after precipitation of the protein. The incubation mixture was transferred at 0 °C, and 0.4 volume of 0.2% rabbit γ-globulins (Cohn fraction II) and 0.6 volume of 36% poly(ethylene glycol) 6000 were added (Cuatrecasas & Hollenberg, 1976). After 3–5 min, the suspension was diluted by addition of 100 mM KCl/20 mM Tris-HCl buffer, pH 8.0, containing 10 μM RES, and it was rapidly filtered through Whatman GF/C filters preincubated in the same buffer. The filters were washed twice with 2 mL of the same medium, and their radioactivity was measured by liquid scintillation in Aqualuma.

Alternatively, binding was measured by filtration without precipitation, using filters preincubated for 1 h in 0.3% poly(ethylenimine) (Bruns et al., 1983). In this case, filters were washed twice with 0.2 M sucrose/100 mM KCl/20 mM Hepes buffer, pH 8.0, containing 10 μM RES. Nonspecific binding was determined in assays containing 50–100 nM RES.

[^{3}H]TBZOH Binding in the Presence of Detergents. The protocol was similar to that described for [^{3}H]RES binding. Solubilized extracts (100 μg of protein/mL) or membranes (10 μg of protein/mL) were incubated with 1.6 nM [^{3}H]-TBZOH in 0.2 M sucrose/100 mM KCl/40 mM Hepes (K^+) buffer, pH 8.0, for 4 h at 30 °C. The incubation mixture was precipitated with rabbit γ-globulins and poly(ethylene glycol) and filtered as described above, with the exception that 100 μM TBZ was substituted for 10 μM RES in the wash buffer. Nonspecific binding was determined in assays containing 1 μM TBZ.

Results

[^{3}H]RES Binding at Equilibrium. Correct measurement of an equilibrium dissociation constant requires that the concentrations of ligand used are of the order of magnitude of the dissociation constant and that the concentration of ligand binding sites does not exceed the value of the dissociation constant (Cuatrecasas & Hollenberg, 1976). Nonobservance of the latter condition leads to erroneous measurements of the free ligand concentration, especially at low concentration, and hence to an erroneous determination of the equilibrium constant. This point was investigated by measuring [^{3}H]RES bound as a function of membrane concentration, at low [^{3}H]RES concentration. At 4 pM [^{3}H]RES, bound ligand increased with membrane concentration up to 2 μg/mL at which point 20% of the ligand was specifically bound (data not shown). This membrane concentration is at least 1 order of magnitude lower than those previously used (Scherman & Henry, 1984).

Saturation isotherms were determined at such low membrane concentrations (Figure 1A). Scatchard plots of the data indicated one class of binding sites (Figure 1B). From the results of seven similar experiments performed at low membrane concentration, a K_D value of 30 ± 16 pM and a B_{max} of 8 ± 3 pmol/mg of protein were derived. It may be noted that the lowest K_D values were obtained for the lowest membrane concentration, suggesting that this K_D value was somehow overestimated (Figure 1B, inset).

Kinetics of [^{3}H]RES Binding. Under conditions where free and total ligand concentrations did not differ significantly, binding was characterized by pseudo-first-order kinetics; $\ln (B_{eq} - B/B_{eq})$ decreased linearly with time, with a slope proportional to $(k_{+1}[RES] + k_{-1})$, where B_{eq}, B, [RES], k_{+1}, and k_{-1} are respectively [^{3}H]RES bound at equilibrium and at time t, total [^{3}H]RES concentration, and association and dissociation rate constants. The experiment was repeated at different values of total ligand concentration [RES], and the parameter k_{+1} was derived from a secondary plot of k^* =

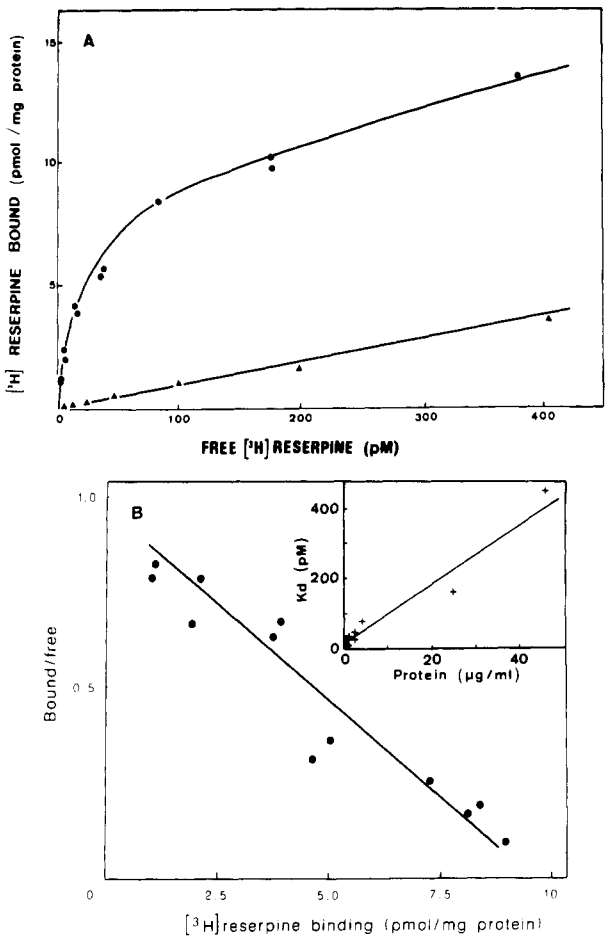

FIGURE 1: Binding of [^{3}H]RES to chromaffin granule membranes. (A) Saturation isotherms. Membranes (2.5 μg of protein/mL) were incubated with [^{3}H]RES (6–400 pM) at 30 °C for 4 h (●). Each point is the mean of two determinations. Nonspecific binding (▲) was proportional to free [^{3}H]RES concentration with a proportionality coefficient of 0.0087. (B) Scatchard plot of specific binding. K_D and B_{max} values derived by linear regression were 26.3 ± 2.7 pM and 9.9 ± 0.5 pmol/mg of protein (r = 0.93), respectively. (Inset) Relation between K_D value and the concentration of membrane used. This graph suggests an extrapolated K_D value of 15–20 pM.

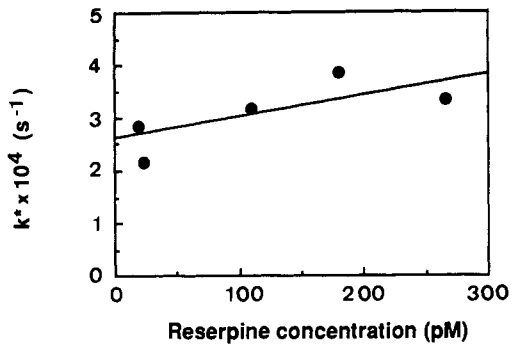

FIGURE 2: Effect of [^{3}H]RES concentration on the association rate constant. The time course of association between membranes (2.5 μg of protein/mL) and [^{3}H]RES was studied over a ligand concentration range of 19–265 pM. Initial time course (from 0 to 90 min) was linearized according to the equation of pseudo-first-order reaction. The figure is a secondary plot of the slope of the straight lines thus obtained as a function of [^{3}H]RES concentration. The rate constant k_{+1} value derived from the slope of the line was $4 \times 10^5 \pm 2 \times 10^5$ M^{-1} s^{-1}.

k_{+1}[RES] + k_{-1} as a function of the concentration of RES (Figure 2). A figure of $4.0 \times 10^5 \pm 2.0 \times 10^5$ M^{-1} s^{-1} was obtained for k_{+1} at 30 °C.

Attempts were also made to measure directly the dissociation rate constant. However, dissociation was too slow to be measured accurately and the half-lifetime of dissociation was estimated to be larger than 12 h (data not shown). Such a value is consistent with that predicted by the equation $k_{-1} = k_{+1}K_D$, from which a half-lifetime of 16 h may be calculated. These values are given in Table I, where the corresponding values for the binding of [^{3}H]TBZOH have also been indicated. It may be noted that the reversibility of [^{3}H]RES binding was demonstrated by the fact that addition of Triton X-100 to a concentration of 1% results in the rapid dissociation of bound [^{3}H]RES (see Figure 5B).

Table I: Characteristics of [^{3}H]RES and [^{3}H]TBZOH Binding

	[^{3}H]RES	[^{3}H]-TBZOH[a]
equilibrium dissociation constant K_D (nM)	0.030	3.0
association rate constant k_{+1} (s^{-1} M^{-1})	4.0×10^5	2.2×10^5
dissociation rate constant k_{-1} (s^{-1})	1.2×10^{-5} [b]	1.8×10^{-3} [c]
$t_{1/2}$ (h)	16	0.1

[a] Values taken from Scherman et al. (1983). [b] Calculated as $k_{-1} = K_D k_{+1}$. [c] Measured directly.

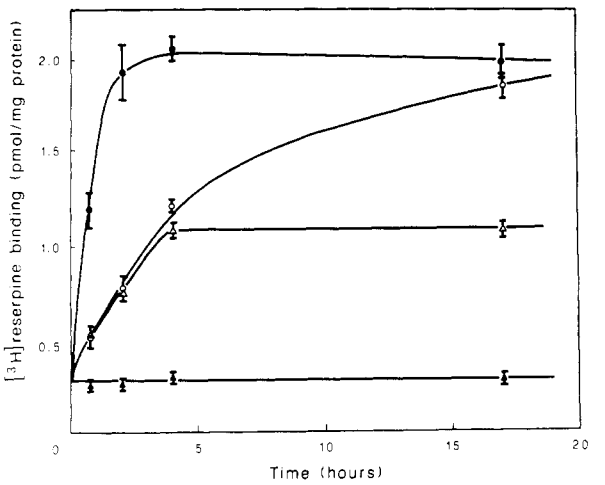

FIGURE 3: Kinetics of [^{3}H]RES binding to chromaffin granule membranes. Membranes (2.5 μg of protein/mL) were incubated with 19 pM [^{3}H]RES for the indicated period of time in 0.3 M sucrose/40 mM Hepes buffer (pH 8.0) containing 2.5 mM ATP/1.3 mM $MgSO_4$ in the absence (●) or in the presence of inhibitors: (○) 50 nM TBZOH; (△) 50 μM noradrenaline. Nonspecific binding (▲) was determined in the presence of 250 nM RES. Each point is the mean of three determinations. Similar results were obtained in four other independent experiments. Addition of protease inhibitors (leupeptine, 3 μg/mL; aprotinine, 5 μg/mL; EDTA, 1 mM) to the incubation bath and use of membranes pretreated with 1 mM PMSF had no significant effect.

Relationship between RES and TBZOH Binding. In order to look for a possible interaction between TBZOH and RES binding, we followed a protocol similar to that used by physiologists to demonstrate a competition between the two drugs (Carlsson & Lindqvist, 1965). Membranes were first preincubated with TBZOH and ATP. [^{3}H]RES was then added at zero time, and bound [^{3}H]RES was followed as a function of time (Figure 3). Initially, the preincubation with TBZOH decreased [^{3}H]RES binding, thus indicating a competition between the two drugs. However, this effect decreased after larger incubation periods, and after 18 h, TBZOH no longer affected [^{3}H]RES binding. This behavior was analyzed by measuring the apparent EC_{50} of TBZOH for the displacement of [^{3}H]RES as a function of time (Table II). EC_{50} values increased by a factor of more than 20 during the 16-h incubation.

It may be noted that the effect of TBZOH contrasted with that of noradrenaline which, in the same type of experiment,

Table II: Inhibition of [^{3}H]RES Binding by TBZOH and Noradrenaline[a]

	EC_{50} (μM)	
incubation time (h)	TBZOH	noradrenaline
0.5	0.025 ± 0.005	20 ± 8
1.0	0.037 ± 0.008	18
1.5	0.040 ± 0.014	
4.0	0.180 ± 0.040	25
16.0	0.610 ± 0.050	25

[a] Hill numbers were not significantly different from 1.0.

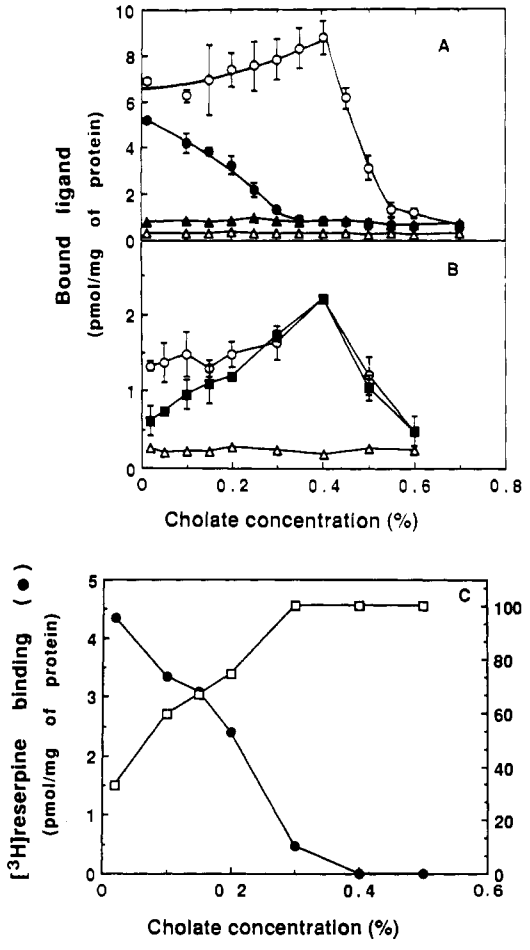

FIGURE 4: Effect of cholate on [^{3}H]RES and [^{3}H]TBZOH binding to soluble extracts of chromaffin granule membranes. (A) Soluble extracts prepared in 1% cholate (100 μg of protein/mL) were incubated with 3.8 nM [^{3}H]RES (●) or 6.4 nM [^{3}H]TBZOH (○) in 150 mM KCl/50 mM Hepes buffer (pH 8.0) containing the indicated concentration of cholate for 18 h at 30 °C. Nonspecific binding was determined in assays containing 2 μM RES (▲) or 2 μM TBZOH (△). Each point is the mean of two determinations. (B) Soluble extracts (100 μg of protein/mL) were incubated for 1 h with 6.4 nM [^{3}H]TBZOH as previously. Binding was measured by filtration of 100 μL of the mixture (○) or of the same volume of a supernatant obtained by centrifugation of the mixture for 1 h at 100000g (■). Nonspecific binding (△) was determined in assays containing 2 μM TBZOH. (C) Simultaneous plots of [^{3}H]RES binding (●, derived from panel A) and membrane solubilization (□, derived from the ratio of soluble to total [^{3}H]TBZOH binding).

inhibited [^{3}H]RES binding in a constant time-independent manner (Figure 3).

Lack of [^{3}H]RES Binding to Detergent-Solubilized Membranes. We have previously shown that the monoamine transporter could be solubilized in a form which binds [^{3}H]-TBZOH (Scherman & Henry, 1983). For instance, membranes solubilized by addition of 0.75% cholate bind [^{3}H]-TBZOH with an equilibrium dissociation constant of about

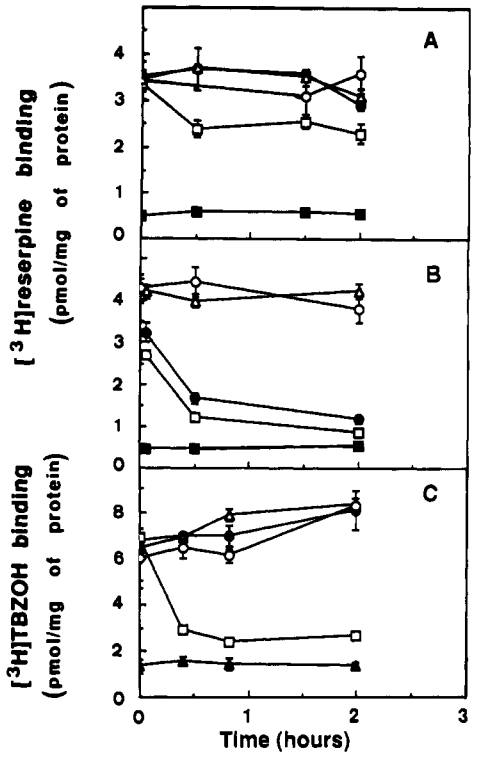

FIGURE 5: Effect of detergents on [^{3}H]RES and [^{3}H]TBZOH bound to chromaffin granule membranes. (A) Effect of cholate on bound [^{3}H]RES. Membranes (10 μg of protein/mL) were preincubated with 300 pM [^{3}H]RES in 3.0 mL of 0.15 M sucrose/0.1 M KCl/40 mM Hepes buffer (pH 8.0) containing 2.5 mM ATP/1.3 mM $MgSO_4$ for 4 h at 30 °C. Cholate was added, and the mixture was incubated at the same temperature. Aliquots (40 μL) were withdrawn at intervals and assayed for bound [^{3}H]RES. Cholate concentration was (○) 0, (△) 0.01%, (●) 0.1%, and (□) 1%. Nonspecific binding was determined in assays containing 50 nM RES (■). Each point is the mean of two or three determinations. (B) Effect of Triton X-100 on bound [^{3}H]RES. Same conditions as in (A). Triton X-100 concentration was (○) 0, (△) 0.01%, (●) 0.1%, and (□) 1%. (C) Effect of cholate on bound [^{3}H]TBZOH. Membranes were preincubated as in (A) with 1 nM [^{3}H]TBZOH. Cholate was then added as in the experiment of (A). Nonspecific binding (▲) was determined in the presence of 1 μM TBZ.

10 nM. However, such an extract did not bind [^{3}H]RES. [^{3}H]RES binding to intact membranes is greatly accelerated by the proton electrochemical gradient generated by the H^+ pump, but it can also be observed in the absence of such a gradient after long incubation periods (Scherman & Henry, 1984). Thus the lack of [^{3}H]RES binding to the solubilized extract does not originate merely in the suppression of the proton gradient.

When extracts solubilized in 1% cholate were diluted in order to decrease the detergent concentration, two effects were observed (Figure 4). On the one hand, [^{3}H]RES binding as well as [^{3}H]TBZOH binding could then be detected (Figure 4A). On the other hand, some material was reconstituted, as shown by the fact that an increasing fraction of the bound [^{3}H]TBZOH could be sedimented (Figure 4B). Figure 4C shows that [^{3}H]RES binding is correlated to the amount of particulate material, thus indicating that this binding occurs only on reconstituted material. The characteristics of this binding have not been investigated in detail, but saturation isotherms indicate a K_D of 7 nM and a B_{max} of 25 pmol/mg of protein (data not shown).

Effect of Detergents on Membrane-Bound [^{3}H]RES. In a different type of experiment, we tested the effect of detergents on [^{3}H]RES already bound to membranes in the presence of ATP. Cholate had no effect at concentrations of 0.01

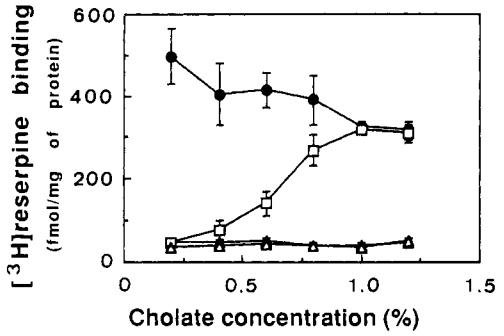

FIGURE 6: Solubilization by cholate of bound [^{3}H]RES. Membranes (40 μg of protein/mL) were preincubated with 100 pM [^{3}H]RES in 0.1 M KCl/0.25 M sucrose/40 mM Hepes buffer (pH 8.0) containing 2.5 mM ATP/1.3 mM $MgSO_4$ for 2 h at 37 °C. Cholate was then added at the indicated concentration and the mixtures were incubated for 2.5 h. A first set of 1.8-mL aliquots (●) were directly assayed for bound [^{3}H]RES by filtration through filters preincubated with poly(ethylenimine). A second set of aliquots of the same volume were centrifuged for 45 min at 100000g (□), and bound [^{3}H]RES was assayed on the supernatant by the same technique. Nonspecific binding was determined in assays containing 350 nM RES, with (△) or without (▲) the centrifugation step. Each point is the mean of four determinations.

or 0.1% and at 1% decreased the binding by about 30% (Figure 5A). It may be noted that the resistance of [^{3}H]RES binding to cholate addition is not the result of a covalent binding, since another detergent, Triton X-100, released [^{3}H]RES at 0.1% (Figure 5B). On the other hand, when the experiment was repeated on membranes preincubated with [^{3}H]TBZOH, bound ligand was rapidly and extensively released after addition of 1% cholate (Figure 5C).

These experiments suggested that soluble [^{3}H]RES binding sites could be obtained by first binding [^{3}H]RES to intact membranes and then solubilizing the membranes by 1% cholate. The results of such an experiment are shown in Figure 6. Incubation with 1% cholate released about 30% of the bound [^{3}H]RES. However, centrifugation of the material incubated in the presence of 1% cholate indicated that this material was in a soluble form. This experiment was repeated several times with the same result.

Discussion

The present data show that the affinity of RES for the monoamine transporter was previously underestimated (Deupree & Weaver, 1984; Scherman & Henry, 1984; Near & Mahler, 1983). The methodological problem in the determination of the equilibrium dissociation constant lies in an abundance of binding sites (due to the use of purified material) associated with a very low value of the dissociation constant. In spite of experimental difficulties, the present value of 30 pM, measured with a membrane concentration of about 1 μg of protein/mL, seems to be acceptable. In Table I, the dissociation equilibrium constant and rate constant of RES have been compared to those of TBZ. The dissociation of TBZ is about 2 orders of magnitude faster than that of RES, a result which is consistent with in vivo data on the transient amine depleting effect of TBZ in the brain (Pletscher et al., 1958). This result is also consistent with the clearance of [^{3}H]RES administered in vivo (Alpers & Shore, 1969). The very slow dissociation rate of [^{3}H]RES makes it a valuable ligand for in vivo labeling experiments (Richards et al., 1979). After administration, nonspecific binding is rapidly eliminated, and after one day, a good signal to noise ratio is observed.

The effect of TBZ on [^{3}H]RES binding has been analyzed under correct equilibrium conditions, i.e., at picomolar ligand

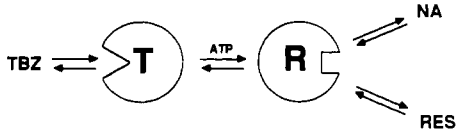

FIGURE 7: Kinetic model of the monoamine transporter.

concentrations and with appropriate low membrane concentrations. The experiment has been performed with a protocol similar to that used in vivo (Carlsson & Lindqvist, 1966), and a similar result was obtained: preincubation with a TBZ derivative prevents RES binding over short incubation periods. Such as result had not been observed in our previous work (Scherman & Henry, 1984) where long incubation periods were used, and we had previously defined the high-affinity RES binding site as TBZ resistant. The apparent paradox between in vivo and in vitro data appears to be due to the fact that the inhibition by TBZ derivatives of [^{3}H]RES binding is clearly time dependent (Figure 3).

To interpret these data, it may be noted that competition experiments do not indicate necessarily that RES and TBZ compete for the same site, but merely that binding of these two drugs are exclusive processes. Therefore, it may be proposed that the monoamine transporter exists in two different conformations, each conformation binding only one type of ligand, TBZ or RES (Figure 7). According to this interpretation, addition of TBZ would pull the conformational equilibrium toward the TBZ binding conformation (conformation T which is unable to bind [^{3}H]RES). However, the differences in the dissociation rate constants for TBZOH and RES ($t_{1/2}$ = 10 min and 16 h for TBZOH and RES, respectively) would explain the slow increase of the RES-binding conformation (conformation R, unable to bind TBZ and TBZOH), and thus explain the lack of inhibition of RES binding by TBZOH after long incubation times.

A similar conformational change has been proposed to account for the kinetic effect of the proton electrochemical gradient on [^{3}H]RES binding (Henry et al., 1987). The proton gradient would favor conformation R, and in its absence [^{3}H]RES binding would be limited by the rate of an ATP-independent transition from conformation T to conformation R.

This interpretation is also supported by the effect of noradrenaline on [^{3}H]RES binding. At variance with the results obtained with TBZOH, the inhibition by noradrenaline does not vary with time. This result might suggest that substrates such as noradrenaline bind only to conformation R, a hypothesis which is consistent with the fact (Scherman & Henry, 1984) that noradrenaline displaces efficiently [^{3}H]RES (with an EC_{50} in the micromolar concentration range) and inefficiently [^{3}H]TBZOH (with a millimolar EC_{50}).

Experiments with detergents indicate a clear difference between [^{3}H]TBZOH and [^{3}H]RES binding. It had previously been shown that cholate solubilized the transporter in a conformation which bound [^{3}H]TBZOH. We now show that such a preparation is unable to bind [^{3}H]RES. This result might be intrepreted by an inactivation of the [^{3}H]RES binding site in the solubilized material. Alternatively, it may be interpreted as indicating that cholate solubilizes the transporter in the T conformation. According to this latter interpretation, the fact that [^{3}H]RES cannot bind to the transporter might suggest either that conformation R is unstable in the presence of the detergent or that the T or R conformational change is unlikely under these conditions. However, since solubilization of bound [^{3}H]RES occurs without release of the ligand, it may be proposed that RES

stabilized conformation R, which now could be observed in the soluble state. This stabilization might be due to the low dissociation rate of RES.

In the hypothesis of a conformation change, these experiments suggest an effect of the environment on the conformational transition. Substitution of the natural phospholipidic environment by cholate did not allow the T to R transition, and substitution by Triton X-100 induced a denaturation of the R conformation, which is stable in the presence of cholate. Similar differential effects of detergents on the conformational equilibrium of the mitochondrial adenine nucleotide transporter have been described (Block & Vignais, 1986).

In conclusion, it is proposed that the monoamine transporter has two conformations: (i) a RES-binding conformation, favored in the presence of the H^+ electrochemical gradient and which has a high affinity for the substrates, and (ii) a TBZ-binding conformation, with a low affinity for the substrates. It is tempting to speculate that these two conformations alternate in the transporter catalytic cycle (Henry et al., 1987). In the presence of ATP, the transporter would take conformation R and would bind the monoamines present in the cytosol. Substrate binding would be followed by a conformation change to conformation T, with concomitant translocation of the substrate. Because of the low substrate affinity of conformation T, the monoamine would dissociate in the intragranular compartment. Substrate release would allow recycling of the transporter.

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Registry No. RES, 50-55-5; TBZ, 58-46-8; dihydro-TBZ, 3466-75-9; cholic acid, 81-25-4.

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