Solubilization of the Catecholamine Carrier of Chromaffin Granule Membranes in a Form That Binds Substrates and Inhibitors of Uptake†

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ABSTRACT: The component of bovine chromaffin granule membrane responsible for the binding of [2-^{3}H]dihydrotetrabenazine has been solubilized by treatment with detergents. The binding activity of the soluble material was measured either by equilibrium dialysis or by precipitation of the protein by poly(ethylene glycol) and filtration of the precipitate. The best yields and activities were obtained with sodium cholate, which solubilized more than 70% of the binding sites; deoxycholate had deleterious effects on the soluble activity, and among nonionic detergents, octyl β-glucoside was the most efficient. With cholate, [^{3}H]dihydrotetrabenazine binding occurred on soluble sites as judged by centrifugation of the bound material and by filtration on Sepharose 6B columns. Binding could be measured in the presence of cholate, but high detergent concentrations had a reversible inhibitory effect. The [^{3}H]dihydrotetrabenazine binding characteristics of the soluble material were similar to those of the granule membrane, with one class of binding sites (K_D = 23 nM, B_{max} = 90 pmol/mg of protein). The association and dissociation rate constants were 0.072×10^6 M^{-1} s^{-1} and 1.1×10^{-3} s^{-1}, respectively. The pharmacological properties of the binding sites were also similar to those of the membranes. Our results thus support the view that the catecholamine carrier has been solubilized in an active conformation that binds substrates and inhibitors of uptake.

Uptake of catecholamines by the chromaffin granules of adrenal medulla involves a specific carrier (Pletscher, 1976). This carrier is driven by the electrochemical proton gradient generated by an electrogenic ATP-dependent H^+-translocase (Johnson & Scarpa, 1979; Apps et al., 1980; Scherman & Henry, 1980a; Kanner et al., 1980; Knoth et al., 1980). Several attempts to solubilize the monoamine carrier with detergents have been reported (Maron et al., 1979; Isambert & Henry, 1981). In these studies, the carrier activity was assayed by reconstitution of phospholipid vesicles from the soluble extract and measurement of catecholamine uptake induced by an H^+ electrochemical gradient. To generate this gradient, either a pH gradient was artificially imposed on the reconstituted vesicles (Maron et al., 1979) or the ATP-dependent H^+-translocase was reconstituted together with the monoamine carrier (Isambert & Henry, 1981). The complexity of the reconstitution procedure and the difficulty in assaying vectorial monoamine transport have prevented purification of the carrier by this approach.

Among the inhibitors of ATP-driven catecholamine uptake, tetrabenazine has been shown to be highly specific for the monoamine carrier (Scherman & Henry, 1980b). We have recently shown (Scherman et al., 1983) that a derivative of tetrabenazine, [2-^{3}H]dihydrotetrabenazine (2-[^{3}H]hydroxy-3-isobutyl-9,10-dimethoxy-1,2,3,4,6,7-hexahydro-11b*H*-benzo[*a*]quinolizine, [^{3}H]TBZOH[1]), bound to saturable sites on chromaffin granule membranes (K_D = 3–6 nM, B_{max} = 60 pmol/mg of protein). The correlation between [^{3}H]TBZOH binding and monoamine uptake inhibition, and the displacement of [^{3}H]TBZOH from its binding sites by inhibitors and substrates of uptake have led us to propose that [^{3}H]TBZOH binds to the monoamine carrier. [^{3}H]TBZOH binding thus offers an alternate possibility to assay the carrier. In the present paper, this technique was used to follow the solubilization of the monoamine carrier. [^{3}H]TBZOH binding sites have been obtained in a soluble form retaining most of the properties of the membrane sites.

Experimental Procedures

Chemicals. TBZOH was obtained by reduction of tetrabenazine (Fluka) by $NaBH_4$ in methanol (Brossi et al., 1958). [^{3}H]TBZOH (12 Ci/mmol) was prepared as described (Scherman et al., 1981). Its purity was periodically checked by TLC (Scherman et al., 1981) or by HPLC on a C_{18} μBondapak HPLC column with methanol/10 mM NH_4HCO_3 (70:30) as the solvent. When necessary, the product was repurified by either technique.

Solubilization of Chromaffin Granule Membranes. Bovine chromaffin granule membranes were prepared by osmotic lysis of granules isolated by centrifugation on a 1.6 M sucrose layer (Smith & Winkler, 1967; Giraudat et al., 1980); they were frozen in liquid nitrogen and stored at −80 °C. Before use, they were rapidly thawed and then centrifuged at 120000*g* for 10 min with a Beckman Airfuge, and the pellet was resuspended in 10 mM Hepes buffer (pH 7.5) containing 100 mM KCl (buffer K).

Detergent (10%, w/v) solutions in buffer K were added dropwise to the membrane suspension containing 5.0 mg of protein/mL, with constant stirring. After 15–30 min at 25 °C, insoluble material was removed by centrifugation at 120000*g* for 50 min in the Airfuge. The clear yellow supernatant, containing the solubilized membrane proteins, was either used directly or rapidly frozen. The [^{3}H]TBZOH binding activity of the supernatant was stable after more than 3 weeks at −80 °C.

[^{3}H]TBZOH Binding Assay. All experiments were performed at 25 °C. Binding was measured by equilibrium dialysis or by filtration. For equilibrium dialysis, Teflon cells (Dianorm, Diachema, Switzerland) with two 250-μL com-

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[1] Abbreviations: [^{3}H]TBZOH, 2-[^{3}H]hydroxy-3-isobutyl-9,10-dimethoxy-1,2,3,4,6,7-hexahydro-11b*H*-benzo[*a*]quinolizine; TLC, thin-layer chromatography; HPLC, high-pressure liquid chromatography; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IgG, immunoglobulin G.

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partments separated by a Visking membrane were used. Supernatants or membranes, cholate, and, where indicated, drugs were introduced in one compartment (200-μL final volume), and simultaneously, [^{3}H]TBZOH in the same volume of buffer K containing the same concentration of cholate was introduced in the other. Dialysis equilibrium was reached after 100 min at 25 °C with stirring. Aliquots (150 μL) were withdrawn and assayed for radioactivity by liquid scintillation spectrometry. Bound [^{3}H]TBZOH was determined as the difference between the compartments. It should be noted that with all types of dialysis membranes tested, 10–15% of total [^{3}H]TBZOH bound to the membrane. Preliminary experiments performed at 1 nM [^{3}H]TBZOH with intact chromaffin granule membranes (50–150 μg of protein/mL) indicated that under these conditions binding was easy to detect (more than 30% relative difference between the compartments) and was proportional to protein concentration.

Alternatively, binding was measured by filtration after precipitation of proteins by poly(ethylene glycol) 6000 (Cuatrecasas & Hollenberg, 1976). The soluble fraction to be assayed was incubated at 25 °C in buffer K (0.2–0.4 mL) with [^{3}H]TBZOH and, where indicated, drugs and detergent for 2–4 h. The incubation mixture was then transferred at 0 °C, and 0.4 volume of 0.2% rabbit γ-globulins in 0.2 M sodium phosphate, pH 7.4 (Cohn fraction II, Sigma), and 0.6 volume of 36% (w/v) poly(ethylene glycol) 6000 were added. During the 3–5 min required for precipitation of the proteins, it has been verified that the ligand–carrier complex did not dissociate. The suspension was diluted by addition of 4 mL of ice-cold buffer K containing 8% poly(ethylene glycol) and 100 μM tetrabenazine. It was immediately filtered on HAWP Millipore filters. The filters were washed twice with 4 mL of the same medium, and their radioactivity was measured by liquid scintillation in Aqualuma (Lumac) or Pico-Fluor (Packard). The use of a washing medium containing 100 μM tetrabenazine decreased the adsorption of [^{3}H]TBZOH on the filters from 5 to 0.5% of the total radioactivity, without displacing the label from the carrier because of the slow dissociation rate of the ligand–carrier complex (see Results). Controls performed by centrifugation of the suspension have indicated a complete retention on the filters of the poly(ethylene glycol) precipitate.

Analytical Techniques. Proteins were estimated by the Lowry procedure with bovine serum albumin as a standard. Dopamine β-hydroxylase was assayed at pH 6.5 in 0.1 M phosphate buffer with 10 mM tyramine as substrate (Wallace et al., 1973).

Results

Determination of Optimal Conditions for Solubilization. All solubilizations and [^{3}H]TBZOH binding assays were performed in 100 mM KCl/10 mM Hepes buffer, pH 7.5 (buffer K). The pH of this medium has been shown to be nearly optimal for [^{3}H]TBZOH binding (Scherman & Henry, 1983) and its high ionic strength, classically used for solubilization studies (Helenius & Simons, 1975), did not alter the [^{3}H]TBZOH binding parameters previously determined in 0.3 M sucrose/10 mM Hepes, pH 7.5 (Scherman et al., 1983).

Various detergents were tested for their ability to solubilize [^{3}H]TBZOH binding sites (Table I). In these experiments, solubilized fractions were assayed for [^{3}H]TBZOH binding after dilution of the detergent, and the results were expressed as percentage of the binding to untreated membranes. Since binding measurements were performed only at one drug concentration, low figures indicated a limited solubilization,

Table I: Solubilization of [^{3}H]TBZOH Binding Activity[a]

detergent	concn (%)	binding in supernatant[b] (%)	protein solubilized (%)
cholate	0.6	26.1	41.6
	1.0	38.2	50.4
deoxycholate	0.6	30.9	36.9
	1.0	47.2	46.1
octyl β-glucoside	1.0	1.5	15.1
	2.0	17.0	26.6
Lubrol PX	0.6	1.5	30.1
	1.0	4.5	32.2
	2.0	7.8	41.4
Nonidet P-40	1.0	0	28.5
	2.0	4.0	33.8

[a] Membranes (6 mg of protein/mL) were incubated for 30 min with the indicated detergent as described under Experimental Procedures. Triton X-100 (at 0.6, 1.0, or 1.5%), digitonin (at 0.6, 1.0, or 2.0%), Lubrol WX (1.0 or 2.0%), and Tween 20 and 80 (1.0 or 2.0%) have also been tested by the same procedure and gave negative results. The first three detergents solubilized from 20 to 44% of membrane protein whereas the two other ones were less efficient (6–15% of the proteins). [b] Supernatants were diluted 60-fold in 400 μL of buffer K and were assayed for binding with 10 nM [^{3}H]TBZOH by the filtration technique. Binding and protein concentration are expressed as percent of controls obtained with membranes prior to solubilization (2.2 nM bound ligand corresponding to 20 pmol of [^{3}H]TBZOH/mg of protein). In all experiments, nonspecific binding, measured in the presence of 2 μM TBZOH, was subtracted. Values are the mean of triplicate determinations.

a low affinity of the solubilized material, or a combination of both possibilities. Nonionic detergents solubilized poorly the [^{3}H]TBZOH binding sites. Tween 20 and 80 were inefficient in solubilizing any membrane protein whereas Triton X-100, digitonin, Lubrol WX and PX, and Nonidet P-40 solubilized some protein but gave fractions with a low [^{3}H]TBZOH binding activity. Among nonionic detergents, the best results were obtained with octyl β-glucoside, which, at 2% concentration, solubilized 17% of the binding activity with a good specific activity. However, bile salts proved to be more efficient than octyl β-glucoside, the yield of activity solubilized being about 40% at 1% detergent concentration. In the experiments of Table I, deoxycholate gave slightly better results than cholate. Nevertheless, when membranes solubilized by 0.6% deoxycholate or cholate were assayed in the presence of the same concentration of detergent, 80% of the deoxycholate-solubilized activity was lost whereas the same cholate concentration was without any effect (see below). This experiment indicated that deoxycholate had more deleterious effects than cholate at the concentration required for solubilization, and for this reason, cholate was prefered throughout this work.

The effect of cholate concentration was investigated next (Figure 1). Increasing the detergent concentration increased the solubilized activity with a concomitant decrease of the pellet activity up to 1.5% cholate. The specific activity of the supernatant, calculated from protein and binding activity solubilization (Figure 1), increased with cholate concentration up to 1%, thus showing that a high detergent concentration was required to solubilize the hydrophobic amine carrier. It should be noted that, in the experiment of Figure 1, [^{3}H]-TBZOH binding was measured by equilibrium dialysis and that similar data were obtained by poly(ethylene glycol) precipitation of the protein and filtration of the precipitate (Cuatrecasas & Hollenberg, 1976). The latter technique was used in all subsequent experiments.

Effect of Cholate Concentration on [^{3}H]TBZOH Binding. As previously noted, [^{3}H]TBZOH binding to cholate-treated

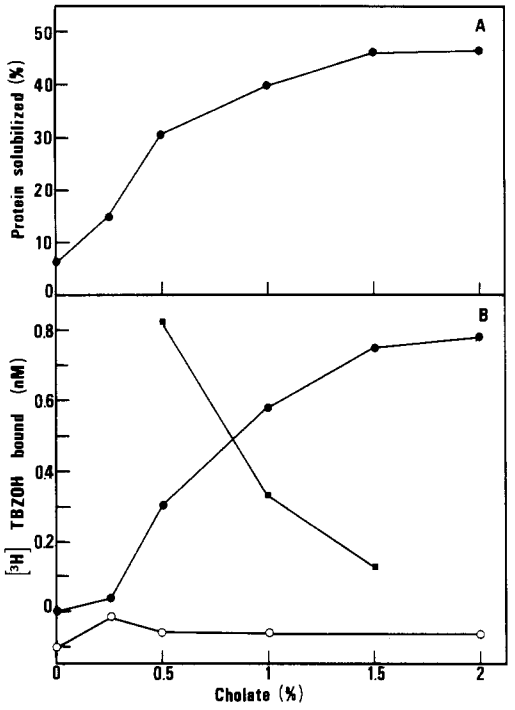

FIGURE 1: Effect of cholate concentration on solubilization of binding sites. Membranes were treated with various concentrations of cholate, as described under Experimental Procedures. (A) Solubilization of membrane proteins; (B) binding of 2 nM [^{3}H]TBZOH by the solubilized fraction (●, ○) and the insoluble residue (■), with (○) or without (●, ■) 2 μM tetrabenazine. Binding was measured by equilibrium dialysis. Cholate final concentration in the assay was 0.05%. The subzero values for the controls in the presence of 2 μM tetrabenazine (○) presumably reflect the fact that [^{3}H]TBZOH equilibration was not complete in this experiment.

samples was measured after dilution of the detergent. In order to examine if that dilution resulted in reconstitution of a particulate activity, [^{3}H]TBZOH binding to extracts solubilized at different cholate concentrations was determined in the presence of various concentrations of detergent (Figure 2). For extracts solubilized at cholate concentrations up to 0.75%, [^{3}H]TBZOH binding could be measured without dilution of the detergent, thus showing that the drug bound to a soluble material. Higher cholate concentrations in the binding assay had deleterious effects on the activity. It was striking, however, that the concentration of detergent affecting [^{3}H]TBZOH binding was dependent upon the solubilization conditions: increasing the detergent concentration used to solubilize the membranes (up to 0.75% cholate) raised the tolerance of the binding reaction. It should also be noted that the effect of cholate was reversible, since solubilization at 1.2% detergent gave an appreciable binding when assayed at 0.75% cholate. When soluble fractions were assayed under optimal conditions (left part of the sigmoid curves), [^{3}H]TBZOH binding was linear with the extract concentration, up to the point where 30–40% of the total radioactivity was bound (data not shown).

Properties of the 0.75% Cholate Extract. Solubilization of the membranes by 0.75% cholate followed by binding measurement in the presence of 0.6% detergent was selected as optimal to study [^{3}H]TBZOH binding sites in a soluble state. Two different types of experiments confirmed the soluble nature of the binding sites thus obtained. In the first one, a 0.75% cholate extract was incubated with 4 nM [^{3}H]TBZOH in the presence of 0.6% cholate. After 1 h at 25 °C, the mixture was centrifuged for 50 min at 100000*g*. More than 92% of the binding was found in the supernatant. In the second type of experiment, the [^{3}H]TBZOH binding activity

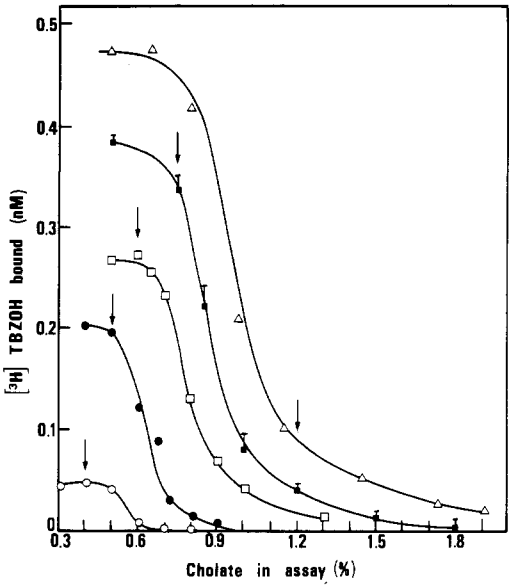

FIGURE 2: Effect of cholate concentration in the assay on [^{3}H]TBZOH binding. Aliquots (10 μL) of supernatants obtained by treating membranes with various concentrations of cholate [(○) 0.4%, (●) 0.5%, (□) 0.6%, (■) 0.75%, (△) 1.2%] were incubated for 1 h at the indicated final detergent concentration in the presence of 4 nM [^{3}H]TBZOH (400-μL final volume). Arrows indicate cholate concentration during the solubilization step. [^{3}H]TBZOH binding was measured by the filtration technique, and nonspecific binding determined in the presence of 2 μM tetrabenazine was subtracted. Each point is the mean of two measurements, standard error bars being indicated in only one instance. Protein concentration of the supernatants was, in mg/mL, (○) 1.3, (●) 1.5, (□) 2.5, (■) 2.5, and (△) 2.7.

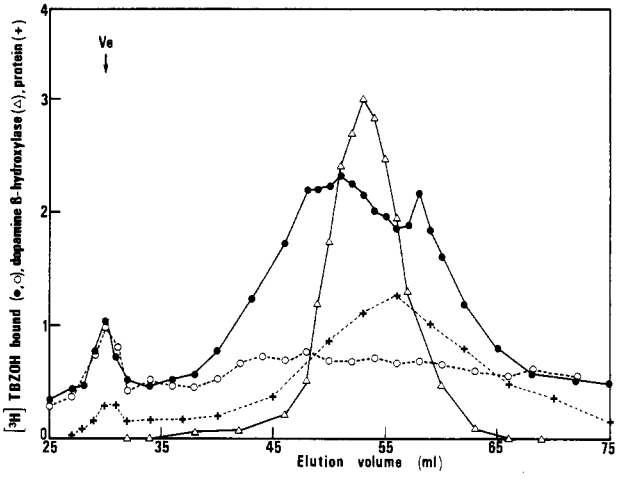

FIGURE 3: Gel filtration of solubilized [^{3}H]TBZOH binding activity. The soluble extract (1 mL) obtained by treating membranes with 0.75% cholate was layered onto a Sepharose 6B column (45 × 1.6 cm) equilibrated in buffer K containing 0.6% cholate and was eluted at 4 °C by the same buffer. [^{3}H]TBZOH binding was determined in duplicate by the filtration technique on 0.1-mL aliquots. The incubation mixture (0.2-mL final volume) contained 10 nM ligand, 0.6% cholate, and the fraction to be assayed, in the absence (●) or in the presence (○) of 2 μM tetrabenazine. Dopamine β-hydroxylase (△) and protein concentration (+) were also measured. The arrow indicates the elution volume of Dextran blue (V_e) determined in the same buffer. Total specific [^{3}H]TBZOH binding, dopamine β-hydroxylase activity, and protein concentration have been normalized.

of a 0.75% cholate extract was analyzed by gel filtration in the presence of 0.6% cholate. On Sephacryl S-200, the activity was found in the void volume whereas, on Sepharose 6B (Figure 3), [^{3}H]TBZOH saturable sites were retarded. Under the conditions used, the activity was eluted as a broad peak at about the same position as dopamine β-hydroxylase.

Table II: Characteristics of Membrane-Bound and Solubilized Sites

expt	cholate in solubilization step (%)	cholate in assay (%)	K_D[a] (nM)	B_{max}[a] (pmol/mg of protein)	% of initial binding sites
1[b]			8.2	65	100
2	0.6	0.6	25.6	80	63
3	0.75	0.6	22.7 ± 2.3 (n = 4)	91 ± 10 (n = 4)	73
4	1.1	0.6	19.1	109	90
5	0.75	0.9	54.6	81	65
6	0.75	1.0	48.0	30	24

[a] K_D and B_{max} were derived from Scatchard analysis as described in Figure 5. Plots were linear, indicating the existence of only one class of binding sites. Protein concentrations ranged from 10 to 40 μg/mL, with the exception of experiments 5 and 6, performed at 220 μg of protein/mL. [b] Membranes in buffer K.

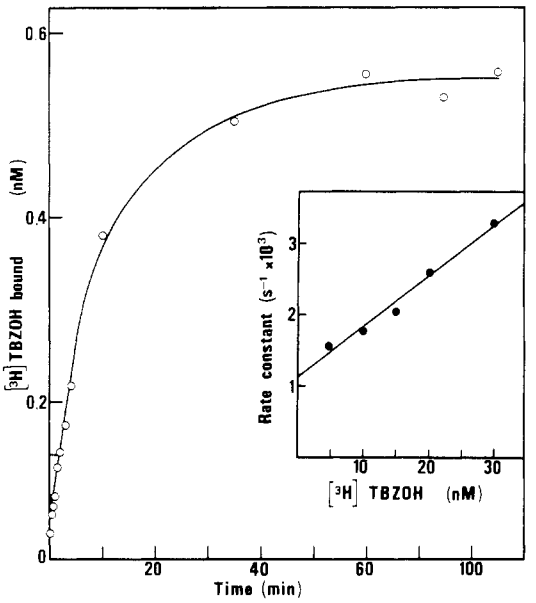

FIGURE 4: Kinetics of [^{3}H]TBZOH association. Time course of the reaction. An extract solubilized by 0.75% cholate was incubated in buffer K containing 0.6% cholate with 5 nM [^{3}H]TBZOH. Aliquots (0.25 mL) were withdrawn at intervals, to which 10 μM tetrabenazine was added to stop the reaction. They were filtered after a 3-min incubation in the presence of poly(ethylene glycol) and IgG. The plot of ln ([B_{eq}] − [B]) as a function of time (where B_{eq} and B are bound [^{3}H]TBZOH at equilibrium and at a given time, respectively) was linear for 5 min, characterized by a time constant of 1.57 × 10^{-3} s^{-1}. (Inset) Variation with [^{3}H]TBZOH concentration of the rate constant. B_{eq} values were determined after a 90-min incubation. With the preparation used, B_{max} and K_D measured at equilibrium were 97 pmol/mg of protein and 24.9 nM, respectively.

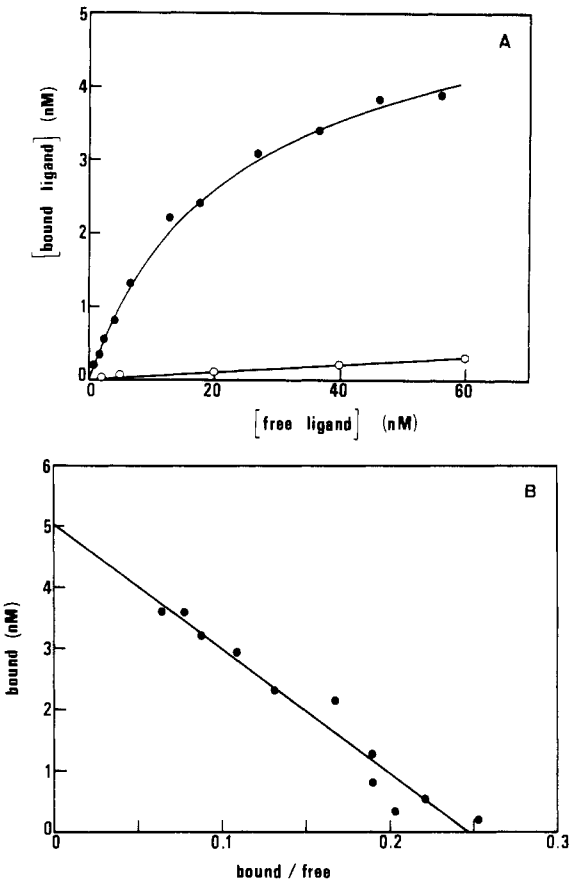

FIGURE 5: [^{3}H]TBZOH binding at equilibrium. (A) Saturation isotherm. The material solubilized at 0.75% cholate (53 μg of protein/mL) was incubated in buffer K containing 0.6% cholate with [^{3}H]TBZOH (1–60 nM), in the presence (○) or absence (●) of 2 μM tetrabenazine. Bound [^{3}H]TBZOH was determined in duplicate by the filtration technique; free ligand was obtained as the difference between total and bound [^{3}H]TBZOH. Nonspecific binding (measured in the presence of tetrabenazine) was 0.5% of the free ligand. The line is a theoretical curve calculated from the data of the Scatchard plot. (B) Scatchard plot. B_{max} = 95 pmol/mg of protein; K_D = 20.4 nM (r = 0.975). Hill coefficient is 1.0.

Binding Properties of the Solubilized [^{3}H]TBZOH Binding Sites. The association reaction was characterized by an exponential time-course (Figure 4). Since at the extract concentration used less than 15% of the ligand was bound, the free-ligand concentration can be considered as constant, and the reaction kinetics can be analyzed as a pseudo-first-order reaction. Plotting the rate constant as a function of [^{3}H]-TBZOH concentration gave a straight line (Figure 4, inset). The association k_1 and dissociation k_{-1} rate constants of [^{3}H]TBZOH were determined as, respectively, the slope and the intercept on the y axis of this line (Kitabgi et al., 1977). Values of 0.072 × 10^6 M^{-1} s^{-1} and 1.1 × 10^{-3} s^{-1} were obtained for k_1 and k_{-1}, from which an equilibrium dissociation constant of 15.2 nM was derived. The dissociation rate constant was also measured directly by addition of 10 μM tetrabenazine to an extract preincubated with 15 nM [^{3}H]TBZOH. Under constant stirring, the dissociation rate constant was first order for more than 100 min, characterized by a rate constant k_{-1} of 0.22 × 10^{-3} s^{-1}. The origin of the discrepancy between this value and that derived from analysis of the association reaction (Figure 4, inset) has not been investigated. It can be related to the fact that the system under study may not be a homogeneous reaction system since the lipophilic TBZOH is concentrated in the hydrophobic part of the solubilized proteolipids.

Binding at equilibrium was saturable (Figure 5A). Analysis of the specific binding by the method of Scatchard gave one class of binding sites (Figure 5B), characterized by a K_D of 23 nM (Table II, experiment 3). This value is consistent with that derived from kinetic experiments (K_D = 15 nM) but is significantly larger than those obtained with native membranes either in K^+ buffer (Table II, experiment 1) or in sucrose

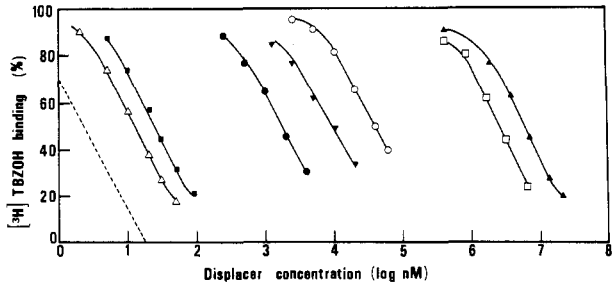

FIGURE 6: Displacement of [^{3}H]TBZOH. The extract solubilized by 0.75% cholate (45 μg of protein/mL, corresponding to 4.0 nM [^{3}H]TBZOH binding sites) was incubated for 1 h in buffer K containing 0.6% cholate, 5 nM [^{3}H]TBZOH, and various concentrations of tetrabenazine (△), TBZOH (■), reserpine (●), haloperidol (▼), chlorpromazine (○), serotonin (□), and noradrenaline (▲). Displacement by noradrenaline was not affected by addition of 2.5 mM ATP/1.25 mM $MgSO_4$. Binding was measured on duplicate samples, the relative difference being always less than 3%. Maximal binding was 0.75 ● 0.02 nM (n = 6). Since free ligand varied only slightly, nonspecific binding was nearly constant and was subtracted. The dotted line indicates the theoretical slope at midpoint, assuming a Hill number of 1.0.

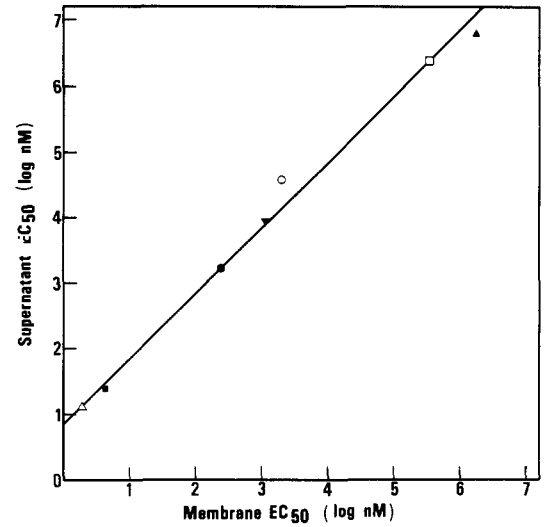

FIGURE 7: Correlation between [^{3}H]TBZOH displacement constants obtained on membranes and on soluble extract. Symbols are as in Figure 6. Soluble extract and membrane data are taken from Figure 6 and from Scherman et al. (1983), respectively. The slope of the line is 1.0.

medium (3.5 nM; Scherman et al., 1983). The effect of cholate has been systematically investigated. Increasing the cholate concentration used in the solubilization step increased the yield of solubilized binding sites without affecting the K_D measured at 0.6% detergent (Table II, experiments 2–4). In contrast, an increase of the cholate in the assay medium gave rise first to an increase of K_D at constant B_{max} (Table II, experiments 3 and 5) and, at a higher detergent concentration (experiment 6), to a decrease of the number of binding sites.

The pharmacology of the solubilized binding sites was then explored. [^{3}H]TBZOH was displaced from its binding sites by inhibitors and substrates of catecholamine uptake (Figure 6). As for membrane-bound sites (Scherman et al., 1983), displacement curves were sigmoids, characterized by a Hill number of 1.0, corresponding to competitive binding. The EC_{50}s measured on the extracts were consistent with those of intact membranes, the former being larger than the later by a constant factor of 7 (Figure 7). This factor is of the same order as the ratio between the K_D for [^{3}H]TBZOH of the solubilized extract (23 nM), measured in K^+ buffer, and that of native membranes (3–5 nM), measured in sucrose medium.

Discussion

Solubilization of Monoamine Carrier. In the reported attempts to solubilize the monoamine carrier of chromaffin granule membranes (Maron et al., 1979; Isambert & Henry, 1981), positive results have only been obtained with cholate. This detergent is also more efficient in solubilizing [^{3}H]-TBZOH binding sites. With the exception of octyl β-glucoside, neutral detergents affected binding as they did carrier activity. The superiority of octyl β-glucoside over Triton X-100 has been demonstrated in several systems (Stubbs & Litman, 1978; Gould et al., 1981). Octyl β-glucoside induced a limited solubilization of [^{3}H]TBZOH binding sites, suggesting that this detergent could be used in conjunction with cholate as reported for the solubilization of the H^+ pump of chromaffin granule membranes (Roisin & Henry, 1982). Deoxycholate was as effective as cholate for solubilization but had a more drastic effect on binding. It should be stressed that in the experiments of Table I, [^{3}H]TBZOH binding was measured after dilution of the detergents and that binding might reflect a high critical micellar concentration (cholate, deoxycholate, and octyl β-glucoside), allowing reconstitution of a particulate material (Helenius & Simons, 1975; Tanford & Reynolds, 1976). However, our results indicate that [^{3}H]TBZOH binds to soluble components of the cholate extract since (i) binding occurred without dilution of the detergent, (ii) bound [^{3}H]-TBZOH was not spun down after 50 min at 100000g, and (iii) the binding sites had a Stokes radius similar to that of dopamine β-hydroxylase on Sepharose 6B columns. In this respect, cholate was very efficient since, under standard conditions (Table II, experiment 3), 73% of the binding sites were solubilized with an increase of specific activity of 1.4.

High cholate concentration (1.2%) affected [^{3}H]TBZOH binding, but this effect was reversed by dilution of the detergent (Figure 2). [^{3}H]TBZOH binding thus appears to be more resistant than catecholamine transport activity, which could not be detected in reconstituted vesicles unless exogenous lipid (asolectin) was added to the solubilization mixture (Maron et al., 1979; Isambert & Henry, 1981). Nevertheless, the results of Figure 2 and Table II might indicate a dependence upon the phospholipid to cholate ratio similar to that observed for the reconstitution of uptake activity and that might be attributed to stabilization of the catecholamine carrier by a phospholipid environment. Consistent with this hypothesis, it may be noted (Figure 2) that (i) cholate at high concentration inhibits binding, presumably by removing more endogenous phospholipid from the carrier environment, and (ii) the amounts of protein and phospholipid solubilized increase with the concentration of detergent used. Thus, the increase of the inhibitory cholate concentration observed when the detergent used for solubilization was increased suggests that the detergent to solubilized protein or phospholipid ratio is a critical factor in the conservation of the carrier in an active conformation.

Properties of the Soluble Binding Sites. Equilibrium dialysis is an effective technique for measuring [^{3}H]TBZOH binding to soluble sites. This technique has been previously used to measure catecholamine uptake by chromaffin granule membranes (Pletscher, 1976). Nevertheless, it suffers from a lack of sensitivity since bound ligand is obtained as a difference between two compartments. The requirement for high site concentration is not convenient for determining low K_D values (Chang et al., 1975), and kinetic studies are not possible by this method. Precipitation of the soluble sites by poly(ethylene glycol) in the presence of carrier IgG, followed by filtration on Millipore filters, has been described for the assay

of solubilized insulin binding sites (Cuatrecases & Hollenberg, 1976). This technique does not have the drawbacks of equilibrium dialysis, and the convergence of the results obtained by the two techniques justifies its use.

The soluble [^{3}H]TBZOH binding sites do not differ very much from the membrane-bound ones (Scherman et al., 1983). Under the conditions used, only one class of sites was found (Figure 5). The dissociation constant K_D (Table II) and the EC_{50} for inhibitors and substrates (Figure 6) of the soluble sites differed by less than one order of magnitude from those of the membrane sites (Figure 7). Comparison of the kinetic parameters of the soluble sites ($k_1 = 0.07 \times 10^6$ M^{-1} s^{-1} and $k_{-1} = 1 \times 10^{-3}$ s^{-1}) and of the membrane-bound ones ($k_1 = 0.22 \times 10^6$ M^{-1} s^{-1} and $k_{-1} = 0.7 \times 10^{-3}$ s^{-1}) suggests that the difference originates mainly in the rate of association. Part of this difference is likely to be accounted for by the nature of the medium used for binding studies (sucrose or KCl). The large similarities that exist between the properties of the soluble and the membrane-bound sites strongly support the view that the monoamine carrier has been solubilized in an active conformation.

Acknowledgments

We thank Dr. A. M. Michelson for sustained encouragements and B. Gasnier for his participation in some experiments. We are indebted to M. Dupuis (Service Vêtérinaire des Abattoirs de Mantes) for collecting bovine adrenals.

Registry No. Dihydrotetrabenazine, 3466-75-9; octyl β-D-glucoside, 65309-84-4; deoxycholic acid, 83-44-3; cholic acid, 81-25-4; tetrabenazine, 58-46-8; reserpine, 50-55-5; haloperidol, 52-86-8; chlorpromazine, 50-53-3; serotonin, 50-67-9; noradrenaline, 51-41-2.

References

Apps, D. K., Pryde, J. G., & Phillips, J. H. (1980) *FEBS Lett. 111*, 386–390.

Brossi, A., Chopart dit Jean, L. H., & Schnider, O. (1958) *Helv. Chim. Acta 41*, 1793–1800.

Chang, K. J., Jacobs, S., & Cuatrecasas, P. (1975) *Biochim. Biophys. Acta 406*, 294–303.

Cuatrecasas, P., & Hollenberg, M. D. (1976) *Adv. Protein Chem. 30*, 251–451.

Giraudat, J., Roisin, M. P., & Henry, J. P. (1980) *Biochemistry 19*, 4499–4505.

Gould, R. J., Ginsberg, B. U., & Spector, A. A. (1981) *Biochemistry 20*, 6776–6781.

Helenius, A., & Simons, K. (1975) *Biochim. Biophys. Acta 415*, 29–79.

Isambert, M. F., & Henry, J. P. (1981) *Biochimie 63*, 211–219.

Johnson, R. G., & Scarpa, A. (1979) *J. Biol. Chem. 254*, 3750–3760.

Kanner, B. I., Sharon, I., Maron, R., & Schuldiner, S. (1980) *FEBS Lett. 111*, 83–86.

Kitabgi, P., Carraway, R., Van Rietschoten, J., Granier, C., Morgat, J. L., Menez, A., Leeman, S., & Freychet, P. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 1846–1850.

Knoth, J., Handloser, K., & Njus, D. (1980) *Biochemistry 19*, 2938–2942.

Maron, R., Fishkes, H., Kanner, B. I., & Schuldiner, S. (1979) *Biochemistry 18*, 4781–4785.

Pletscher, A. (1976) *Bull. Schweiz. Akad. Med. Wiss. 32*, 181–190.

Roisin, M. P., & Henry, J. P. (1982) *Biochim. Biophys. Acta 681*, 292–299.

Scherman, D., & Henry, J. P. (1980a) *Biochim. Biophys. Acta 601*, 664–677.

Scherman, D., & Henry, J. P. (1980b) *Biochem. Pharmacol. 29*, 1883–1890.

Scherman, D., & Henry, J. P. (1983) *Mol. Pharmacol.* (in press).

Scherman, D., Jaudon, P., & Henry, J. P. (1981) *C. R. Hebd. Seances Acad. Sci., Ser. C 293*, 221–224.

Scherman, D., Jaudon, P., & Henry, J. P. (1983) *Proc Natl. Acad. Sci. U.S.A. 80*, 584–588.

Smith, A. D., & Winkler, H. (1967) *Biochem. J. 103*, 480–482.

Stubbs, G. W., & Litman, B. J. (1978) *Biochemistry 17*, 215–219.

Tanford, C., & Reynolds, J. A. (1976) *Biochim. Biophys. Acta 457*, 133–170.

Wallace, E. F., Krantz, M. J., & Lovenberg, W. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 2253–2255.