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Purification of a [^{3}H]Dihydrotetrabenazine-Binding Protein from Bovine Adrenal Medulla

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SUMMARY

A high affinity binding site for [^{3}H]dihydrotetrabenazine is thought to be present on the monoamine transport protein from chromaffin granules. We describe a procedure for purification of this binding activity from frozen bovine adrenal tissue, and we partially characterize the purified preparation. Binding activity solubilized with sodium cholate and soybean lecithin was fractionated on wheat germ lectin-Sepharose, phenyl-Sepharose, Mono Q, and hydroxylapatite. Denaturing electrophoresis of the purified binding activity, followed by silver staining, revealed a single broad band centered at an apparent molecular weight of 85,000. This preparation bound [^{3}H]dihydrotetrabenazine with an apparent dissociation constant of 2.7 nM and had a site density of 10 nmol/mg. Treatment of the purified protein with neuraminidase reduced the apparent molecular weight by 9000, indicating the presence of terminal sialic acids on the oligosaccharide portion of this molecule.

Secretory organelles that contain catecholamines or serotonin possess an integral membrane transport system that is essential for packaging the organelles with these messenger substances (see Refs. 1–3, for reviews). In chromaffin granules, transport is driven by an outwardly oriented proton electrochemical gradient generated *in vivo* by a proton-translocating ATPase. The monoamine transporter mediates net exchange of one protonated amine for two protons (4, 5), but disagreement still exists concerning the exact species transported. Although most extensively studied in adrenal tissue, similar transporters exist in a variety of other monoaminergic tissues (1–3).

Several pharmacological agents potently inhibit the monoamine transporter. In the presence of a proton electrochemical gradient, the plant alkaloid reserpine will bind to a population of high affinity transporter binding sites (6). This binding is apparently irreversible, although denaturing agents will promote dissociation of unmodified reserpine (7). Reserpine may also bind to this high affinity site in the absence of a gradient, but the association rate is very slow (8). The density of the high affinity site in purified granule membranes is about 8 pmol/mg, and reported K_d values range from 30 pM (9) to 9 nM (10). Transport substrates inhibit reserpine binding to this site at concentrations on the same order as their K_m values for the transport process (8). Biphasic Scatchard plots of reserpine binding to chromaffin granule membranes (8) and the purified transporter (11) imply the presence of a more abundant, lower affinity site, in addition to the high affinity site discussed above.

Tetrabenazine is the prototype of a group of compounds that bind to a single class of high affinity sites on the transporter. [^{3}H]TBZOH, the radioligand commonly used to probe this site, binds with a dissociation constant of about 3 nM and a site density of 60 pmol/mg in purified membranes, approximately 7-fold greater than that for reserpine (8). Transport substrates inhibit only weakly at this site. An unrelated compound, ketanserin, also binds to this site but dissociates very rapidly at temperatures much greater than 4° (12). Although the high affinity reserpine and TBZOH binding sites appear distinct by their pharmacological characteristics, the drugs are mutually inhibitory under defined conditions (9, 13).

Several lines of investigation have suggested that transport substrates and reserpine bind to a protein distinct from the protein that binds [^{3}H]TBZOH. By radiation inactivation analysis, the functional molecular mass for reserpine binding activity was reported to be 37 kDa, in contrast to the value of 68 kDa obtained for [^{3}H]TBZOH-binding activity (14). Consistent with a smaller reserpine binding subunit was the purification of a serotonin-binding protein with a molecular mass of 45 kDa (15). Photoaffinity labeling of chromaffin granule membrane preparations with a derivative of tetrabenazine gave a molecular mass of 70 kDa (16). More recently, a ketanserin

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ABBREVIATIONS: TBZOH, dihydrotetrabenazine; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; FPLC, fast protein liquid chromatography; GlcNAc, *N*-acetylglucosamine; HA, hydroxylapatite; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; WGL, wheat germ lectin.

derivative has been used by two different groups to photolabel proteins migrating with apparent molecular masses of 65 (17) to 85 kDa (18), depending on the tissue source.

Recently, however, a functional monoamine transporter was purified from chromaffin granule membranes (11). The purified transporter bound reserpine with high affinity, and substrate transport was potently inhibited by tetrabenazine, indicating that the transport activity, the high affinity reserpine binding site, and the tetrabenazine binding site were all present in this preparation. On SDS-PAGE the purified material migrated as a broad band centered at 80 kDa. These authors utilized [^{3}H] reserpine, tightly bound to a fraction of the transporter sites, as a probe to follow the purification of the transporter. In this report, we describe a procedure for purification of a functional [^{3}H]TBZOH-binding protein, taking advantage of the ability of this site to retain activity while solubilized and the higher density of this site relative to the density of the reserpine binding site.

Experimental Procedures

Materials. Frozen bovine adrenals were obtained from PelFreeze (Rogers, AK). Crude sodium cholate, GlcNAc, neuraminidase (type X), WGL-Sepharose 6MB, phenyl-Sepharose CL-4B, and a mixture of lyophilized molecular weight standards were obtained from Sigma (St. Louis, MO). An FPLC system and HR 10/10, HR 5/10, and Mono Q HR 5/5 columns were obtained from Pharmacia (Piscataway, NJ). Buffers for FPLC were made using Milli Q-filtered water (Millipore, Bedford, MA). Ultrafiltration/concentration devices were obtained from Amicon (Danvers, MA). Soybean lecithin (>95% phosphatidylcholine) was obtained from Avanti Polar Lipids (Pelham, AL). HA, Bio-Gel HTP, was obtained from Bio-Rad (Richmond, CA). Bio-Safe II was obtained from Research Products International (Mount Prospect, IL). Cholic acid (Malinckrodt, Paris, KY) was treated with activated charcoal and then recrystalized three times from 70% ethanol (19). After neutralization with NaOH, a 5% (w/v) solution of sodium cholate in 10 mM HEPES, pH 7.6 (buffer H), was further purified by ultrafiltration, using a Centriprep 10 or Centriprep 30 apparatus. The concentration of cholate was estimated from the absorbance at 221 nm, and a stock solution containing lecithin, 1:5 (w/w) with respect to sodium cholate, was prepared and used in all steps beyond the WGL column. Purified cholate was used because of discoloration of the Mono Q column top and possible proteinaceous contaminants. [^{3}H]TBZOH was prepared as described previously (13). Saturation binding experiments were performed using freshly repurified ligand. For all other experiments, the radiochemical purity was at least 98%. No differences were observed between fractionation experiments performed using freshly purified ligand and those that used ligand stored for periods up to 6 months. All other chemicals were obtained from standard commercial sources.

Preparation of soluble protein. Membranes were purified from frozen adrenal glands and solubilized as previously described (20), with minor modifications. Briefly, 3–5-g portions of medullary tissue were homogenized in 12.5 ml of 0.3 M sucrose in buffer H, diluted with 3 volumes of the same buffer, and filtered through gauze. After removal of nuclei and cellular debris, the crude membrane fraction derived from 18–30 g of medullary tissue was further purified by centrifugation through a discontinuous sucrose gradient, as described (20), in six tubes. The chromaffin granule membrane-enriched fraction was solubilized in a total of 100 ml of solubilization buffer, 1% (w/v) cholate, 0.2% lecithin, 150 mM KCl, 10 mM HEPES, pH 7.6 (buffer K).

Binding assay. Binding to membranes and solubilized protein was assayed in duplicate or triplicate, by incubating 100-μl samples with 200 μl of [^{3}H]TBZOH in 42 mM HEPES, 100 mM KCl, 10 mM NaCl, 5 mM $MgCl_2$, 1 mM EGTA, pH 7.6, for 1 hr at room temperature. Concentrated samples were diluted in buffer K before assay. The final [^{3}H]TBZOH concentration was 15 nM, except in saturation studies; for nonspecific binding determinations, 20 μM unlabeled tetrabenazine was included. The mixture was filtered over polyethyleneimine-treated filters (21) and the filters were dissolved in Bio-Safe II at least 8 hr before to liquid scintillation counting. Analysis of saturation binding experiments was performed as described by McPherson (22).

Purification of [^{3}H]TBZOH-binding activity. A WGL-Sepharose 6MB column (6.0 cm × 1.6 cm) at 4° was prewashed with 1 volume of buffer K. Soluble protein (100 ml) was loaded onto the column at 10 ml/hr. The column was washed with 20 ml of buffer K and then attached to an FPLC system at room temperature and eluted with a 50-ml linear gradient (0–250 mM) of GlcNAc in buffer K, at the same flow rate; 5-ml fractions were collected and the four fractions with the greatest binding activity were pooled. The column was regenerated by washing with 1 volume of 500 mM GlcNAc in buffer K, followed by 10 volumes each of water; 500 mM NaCl, 100 mM Tris, pH 8.5; 500 mM NaCl, 100 mM sodium acetate, pH 4.5; and water. This wash procedure was performed at least twice.

Solid KCl was added to the WGL peak fractions to 750 mM. Phenyl-Sepharose CL-4B in an HR 10/10 column was prewashed with 10 ml of 1% sodium cholate, 0.2% lecithin, 10 mM KCl, in buffer H (elution buffer), and then 25 ml of the same buffer with 750 mM KCl (input buffer). The WGL pool was injected, the column was washed with 15 ml of input buffer, and binding activity was eluted with a 20-ml linear gradient (750–10 mM KCl), followed by 20 ml of elution buffer. The flow rate was 30 ml/hr, and 5-ml fractions were collected. The column was regenerated as previously described (23).

The three phenyl-Sepharose peak fractions were pooled, diluted with 1 volume of 10 mM KCl in buffer H, and then injected onto a Mono Q HR 5/5 column that had been prewashed with 2 volumes of water. The column was washed briefly with 0.5% sodium cholate, 0.1% lecithin, 10 mM KCl, in buffer H (input buffer), and eluted with a 20-ml linear gradient of KCl (10–750 mM) in the same buffer. The flow rate was 30 ml/hr, and 2-ml fractions were collected. The column was regenerated by washing twice with 4 volumes of 750 mM KCl in buffer H, followed by 4 volumes of water. The second peak fraction was diluted with 4.3 volumes of 0.5% sodium cholate, 0.1% lecithin, in buffer H (to give a KCl concentration of 100 mM), and was injected again onto the column. After washing with input buffer until 10 ml of cholate-containing buffer had been loaded, binding activity was eluted with the same gradient used for the first Mono Q column. Flow rate and fraction size were as described above. The column was regenerated as described above and then cleansed according to the manufacturer's instructions.

HA in a HR 5/10 column was washed with several volumes of water before injection of the Mono Q peak fraction. After injection, the column was washed with 1% sodium cholate, 0.2% lecithin, in buffer H (input buffer), until the absorbance at 280 nm returned to baseline. The column was washed with 2 ml of 1.1 M $MgCl_2$ in input buffer, by injection of the solution and washing with input buffer until the peak cleared. In the same fashion, the column was washed with 2 ml of 1.1 M KCl in input buffer. After washing with 7 ml of 10 mM K_2HPO_4 in input buffer, binding activity was eluted with a 20-ml linear gradient to 300 mM K_2HPO_4 in the same buffer. The flow rate was 15 ml/hr, and 2-ml fractions were collected. Four peak fractions were pooled for the experiment summarizing the purification, and three were pooled for the saturation analysis and neuraminidase digestion.

Neuraminidase treatment. Peak fractions from the HA column were concentrated and exchanged into 1% sodium cholate in buffer H, using a Centricon 10 apparatus, according to the manufacturer's instructions. Neuraminidase (0.1 unit) in 75 μl of 50 mM sodium acetate, pH 4.8, was added to 25 μl (1.1 pmol) of the HA concentrate and incubated for 4 hr at 37°. Identical buffer mixtures lacking the neuraminidase or the purified binding activity were incubated simultaneously as controls. The reaction was terminated by precipitation, as for SDS-PAGE samples.

SDS-PAGE. Samples of >100 μl were first precipitated with 1 volume of −20° acetone. After 5 min on ice, the precipitate was pelleted

for 2 min in a microfuge, and the supernatant was discarded. For samples of <100 μl and these precipitates, the method of Wessel and Flügge (24) was used to prepare electrophoresis samples. Dried pellets were dissolved in 10 μl of SDS sample buffer (7 M urea, 5% 2-mecaptoethanol, 2% SDS, 10% glycerol, 62.5 mM Tris·HCl, pH 6.8, with bromophenol blue as a tracking dye). A discontinuous buffer system was used (25), with a 8- × 10-cm slab electrophoresis system; stacking gels contained 4% acrylamide and 1% bisacrylamide, whereas separating gels contained 9% acrylamide and 1% bisacrylamide. The acrylamide composition of the gel was chosen empirically to minimize staining in the stacking gel and the top of the separating gel. Constant current of 5 mA was applied for 3 hr, followed by silver staining (26). Molecular weight estimates were derived from comparison with standards of known molecular weights.

Protein determination. Protein was measured by the method of Peterson (27) or the method of Schaffner and Weissmann (28) for samples after the phenyl-Sepharose column.

Results and Discussion

In a previous report describing the interaction of solubilized [^{3}H]TBZOH-binding activity with WGL-Sepharose 6MB, we noted that a portion of this activity could be eluted with 10 mM GlcNAc and another portion of similar purity eluted with 200 mM GlcNAc (20). Using gradient elution, we show here that, although binding activity eluted over the entire gradient, the bulk of activity appeared as a single peak, rather than discrete isoforms with different affinities for the lectin. A typical chromatographic profile is shown in Fig. 1. The peak fractions from the WGL-Sepharose column, when pooled, had a specific activity increased 14.6-fold over that of the soluble protein (Table 1).

Most of the WGL eluate bound to phenyl-Sepharose in the presence of 750 mM KCl, as shown in Fig. 2. The peak of binding activity, which eluted several fractions after the bottom of the KCl gradient, had a specific activity increased 3.2-fold over that in the previous step (Table 1). A prominent component of this eluate migrated in a broad band centered at 52.5 kDa on SDS-PAGE (Fig. 3, *lane 3*). A chromaffin granule glycoprotein referred to as glycoprotein IV, a quantitatively minor WGL-binding protein, migrates with a similar molecular mass (29) and has yet to be purified and characterized. This

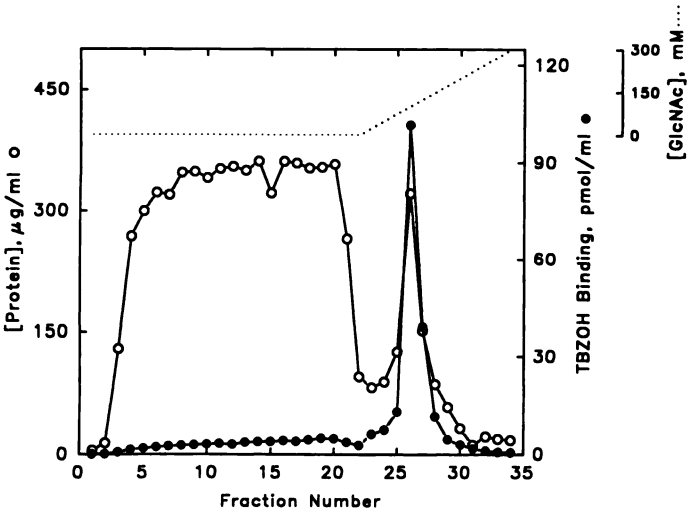

Fig. 1. Fractionation of [^{3}H]TBZOH-binding activity on WGL-Sepharose. Chromatography and analysis were as described in Experimental Procedures.

TABLE 1

Summary of purification of [^{3}H]TBZOH-binding activity

The purification of [^{3}H]TBZOH-binding activity and characterization of the individual steps were as described in Experimental Procedures.

	Binding activity	Protein	Specific activity	Yield
	pmol	*mg*	*pmol/mg*	%
Chromaffin granule membranes	1710	70.4	24.3	
Soluble protein	1410	46.3	30.4	100
WGL-Sepharose pool	843	1.90	443	60.0
Phenyl-Sepharose pool	365	0.260	1400	25.9
Mono Q step 1	76.6	0.0225	3400	5.45
Mono Q step 2	49.1	ND*	ND	3.49
HA pool	11.8	0.00340	3460	0.837

* ND, not determined.

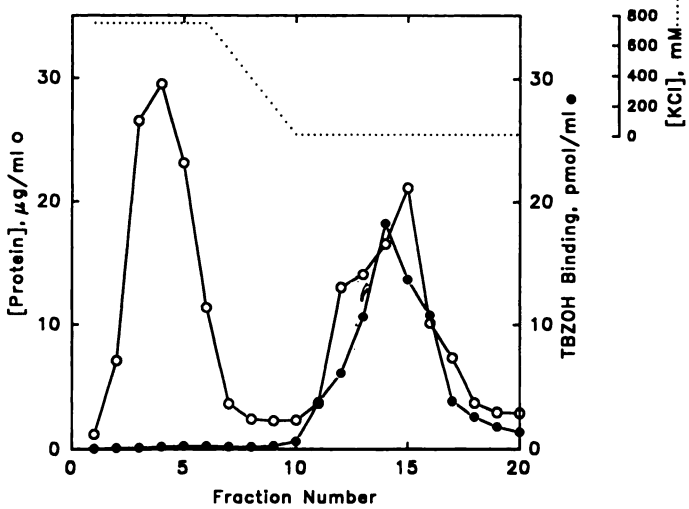

Fig. 2. Fractionation of [^{3}H]TBZOH-binding activity on phenyl-Sepharose. Chromatography and analysis were as described in Experimental Procedures.

buffer system and the phenyl-Sepharose resin may be useful to this end. This protein was the major contaminant for the remainder of our purification.

When the phenyl-Sepharose eluate was diluted and applied to the Mono Q column, two peaks of binding activity eluted with increasing KCl (Fig. 4). Binding activity corresponding to the first peak could be eluted with input buffer alone, at a volume inversely related to the cholate and KCl concentrations of the input buffer, but the second peak remained bound even after that volume was reached. Binding activity from each peak retained its chromatographic behavior when the KCl concentration was lowered by dilution and the fractions were reapplied to the Mono Q column (data not shown). The data might be interpreted as supporting the existence of two discrete protein isoforms possessing similar binding activity. The chromatographic conditions, however, do not allow us to rule out the artifactual separation of a broad peak due to the buffer system. In any case, the more acidic [^{3}H]TBZOH-binding proteins elute with a greater specific activity and are, thus, more amenable to purification.

A second Mono Q chromatography step proved to be necessary to obtain a highly purified preparation, but protein concentrations were not determined for this step, due to limited quantities. A lower purity preparation with greater yield could

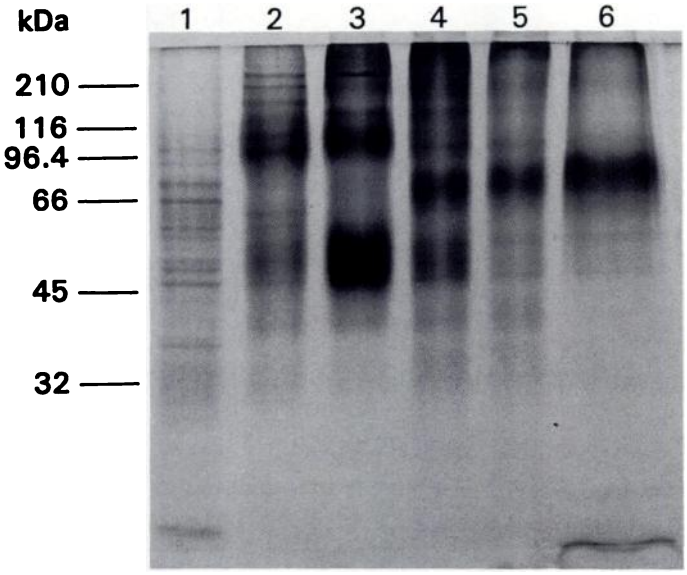

Fig. 3. SDS-PAGE of various fractions from the purification of [^{3}H]TBZOH-binding activity. *Lane 1*, soluble protein, 0.11 pmol; *lane 2*, WGL-Sepharose 6MB pool, 0.25 pmol; *lane 3*, phenyl-Sepharose pool, 0.80 pmol; *lane 4*, Mono Q column 1 peak fraction, 2.5 pmol; *lane 5*, Mono Q column 2 peak fraction, 2.5 pmol; *lane 6*, HA pool, 1.5 pmol. Samples were prepared and electrophoresed as described in Experimental Procedures.

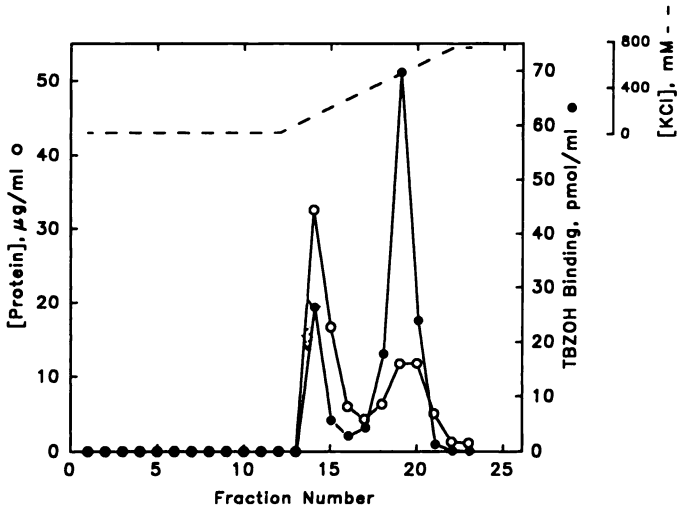

Fig. 4. Fractionation of [^{3}H]TBZOH-binding activity on Mono Q HR 5/5 column 1. Chromatography and analysis were as described in Experimental Procedures.

be obtained by omitting the second Mono Q column. The peak fraction from the second Mono Q column was applied directly to the HA column, which was washed and then eluted with a phosphate gradient. The yield of activity in the final step was 24%, but partial inactivation likely occurred, because little activity was present in other fractions (data not shown). A similar degree of inactivation was noted in the final step of the purification scheme of Stern-Bach *et al.* (11). An SDS-PAGE gel of samples from each step in the purification is shown in Fig. 3. If urea was not included in the sample buffer, dark staining at the top of the gel was accompanied by diminished staining in the 85-kDa band present in the final fractions (data not shown), most likely due to aggregation of the protein. Because glycoproteins often migrate anomalously on SDS-

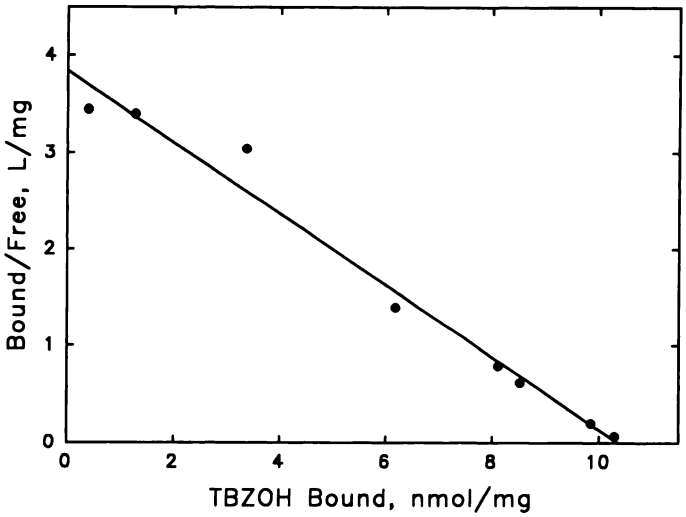

Fig. 5. Scatchard plot of [^{3}H]TBZOH binding to the purified preparation. A saturation binding study was performed in triplicate, as described in Experimental Procedures, using between 0.13 and 160 nM [^{3}H]TBZOH. The data, analyzed using the iterative program LIGAND (34), fit best to a single class of binding sites, with a K_d of 2.7 nM and a site density of 10 nmol/mg.

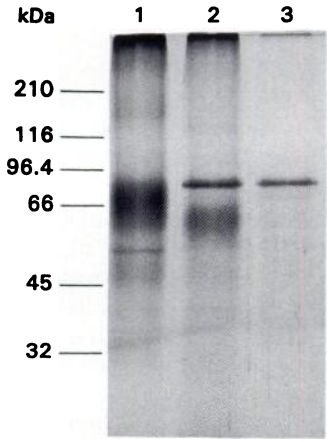

Fig. 6. SDS-PAGE of the purified [^{3}H]TBZOH-binding protein after neuraminidase treatment. *Lane 1*, binding site alone; *lane 2*, binding site and neuraminidase; *lane 3*, neuraminidase alone. Samples were treated, prepared for electrophoresis, and electrophoresed as described in Experimental Procedures.

PAGE (30), we consider the value of 85 kDa to be a rough estimate. A summary of the purification procedure is provided in Table 1. The specific activity of the final fraction varied somewhat between preparations, probably because of differential inactivation during the final purification step.

Analysis of a saturation binding study of the HA eluate from another preparation gave a K_d of 2.7 nM and B_{max} of 10 nmol/mg (Fig. 5). This K_d value agreed well with those reported for freshly prepared granules. Assuming a M_r of 85,000 and a single site on each protein, one would expect a specific activity of 12 nmol/mg. However, any conclusions concerning the stoichiometry of [^{3}H]TBZOH binding that might be derived from the B_{max} would be premature, for several reasons. Protein concentration was difficult to measure accurately in the pure fraction, due to small sample size, and an accurate molecular weight for the proteinaceous portion of the molecule is not yet available. The loss of binding activity in the final purification step and the variability in specific activity for the purified component

between preparations suggest that partial inactivation was probable.

A common feature of membrane glycoproteins is the presence of terminal sialic acids on the oligosaccharide portion of the molecule. When the HA eluate was treated with a highly purified preparation of neuraminidase and then analyzed by SDS-PAGE, the molecular weight of the binding site was reduced by 9000 (Fig. 6). At least part of the acidic behavior of this molecule can, therefore, be attributed to its sialic acid content. The broadness of the staining pattern was not appreciably affected by neuraminidase treatment, suggesting that variable sialation cannot account for the apparent microheterogeneity observed.

Although we have yet to demonstrate transport activity in our purified preparations, several lines of evidence suggest that the polypeptide carrying the [^{3}H]TBZOH site is the transporter. First, tetrabenazine inhibits transport in a similar preparation at nanomolar concentrations. Second, both the functional transporter and the [^{3}H]TBZOH site bind to WGL, anion exchange, and HA resins. Third, both proteins migrate as broad bands at approximately 85 kDa on SDS-PAGE. And fourth, our observation of heterogeneous binding to the Mono Q column is consistent with the different charge isoforms observed by Stern-Bach *et al.* (11) by pI determination of bound [^{3}H] reserpine.

The greater density of [^{3}H]TBZOH binding sites relative to high affinity reserpine binding sites becomes more puzzling, now that it appears unlikely that two distinct subunits comprise the active transporter. To explain the excess of [^{3}H]TBZOH sites in resealed chromaffin granule ghosts, Gasnier *et al.* (31) proposed that these sites represent nonfunctional transporters, somehow inactivated in the lysis/resealing steps. It should be noted, however, that a similar excess of [^{3}H]TBZOH sites was observed in freshly prepared bovine striatal synaptic vesicles (32, 33). Another possibility is the existence of more than one [^{3}H]TBZOH site per polypeptide. As mentioned, we cannot eliminate the possibility of an underestimation of the specific activity in our purified preparation, because the site seems to be partially inactivated, at least in the final step. An alternative explanation is that an oligomeric conformation is required to form the high affinity reserpine binding site.

The relationship between binding sites is further clouded by the low affinity reserpine site, which had been proposed to correspond to the TBZOH site. A low affinity reserpine site has been observed in both energized (10) and unenergized (8) ghost preparations, but a more recent study by one of these groups failed to observe the low affinity site in energized ghosts (9). The latter result appears to be incompatible with the notion that the TBZOH and low affinity reserpine binding sites are equivalent. Reserpine bound to the purified preparation of Stern-Bach *et al.* (11) at both high and low affinity sites under unenergized conditions. The B_{max} of 310 pmol/mg obtained for the high affinity reserpine site by Scatchard analysis is clearly an underestimate of the true site density, because energized conditions increased [^{3}H]reserpine binding severalfold. It was not clear, however, that the increase in the high affinity binding came at the expense of the low affinity site, which was >10-fold more abundant under unenergized conditions. A saturation analysis with energized proteoliposomes was not undertaken. Indeed, the appropriateness of Scatchard analysis of equilibrium binding studies to assess the characteristics of the high affinity reserpine binding site remains questionable in our minds, because it has yet to be demonstrated that this binding process obeys simple reversible mass action laws.

Despite the differences in the probes used, a comparison can be made between our preparation and that of Stern-Bach *et al.* (11). The yield of [^{3}H]TBZOH sites is about 20% of their estimated yield of reserpine binding sites, with respect to the amount of protein in the initial membrane fraction (11). We do not yet know whether this preparation will be useful for transport studies. Further work is needed to assess the transport capacity of various fractions containing the [^{3}H]TBZOH binding site. If the ability to bind [^{3}H]TBZOH can be dissociated from the transport capacity, this binding activity will be useful in characterizing the molecular basis underlying both activities. In conclusion, we describe a procedure for purification of a [^{3}H]TBZOH-binding protein that is reproducible, simple to perform, not limited by a requirement for a fresh tissue source, and amenable to scale-up. Because of its stability in the detergent buffer system used, [^{3}H]TBZOH-binding activity is a useful marker for this molecule.

Acknowledgments

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