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CHARACTERIZATION OF THE MONOAMINE UPTAKE SYSTEM IN CATECHOLAMINE STORAGE VESICLES ISOLATED FROM A PHEOCHROMOCYTOMA TAKEN FROM A CHILD

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Abstract—The catecholamine storage vesicles of a pheochromocytoma taken from a child have been isolated and characterized. The tumor contained almost exclusively noradrenaline and a large proportion of this amine was vesicle-bound. The noradrenaline-containing vesicles showed great resemblance to bovine chromaffin granules. Their catecholamine and dopamine β-hydroxylase contents were that of chromaffin granules; their morphology and density were similar to those of the subpopulation of these granules that contain noradrenaline. The pheochromocytoma vesicles contained in their membranes an abundant polypeptide of mol. wt 110,000, which was not apparent in bovine adrenal medulla vesicle membranes. Monoamine uptake by pheochromocytoma noradrenaline vesicles did not differ significantly from that observed in bovine chromaffin granules. The time-course, plateau level and K_M for noradrenaline were similar for both types of organelles. Both had an oligomycin-resistant ATPase with similar properties. Investigations using the tetrabenazine derivative [2-^{3}H]dihydrotetrabenazine (2-hydroxy-3-isobutyl-9,10-dimethoxy-1,2,3,4,6,7-hexahydro-11b-H-benzo[*a*]quinolizine), which binds specially to the bovine chromaffin granule monoamine carrier indicated that granule membranes from the tumor have a 10-fold increased number of [2-^{3}H]dihydrotetrabenazine binding sites, with no change in dissociation constant. As in the case of bovine chromaffin granules, [2-^{3}H]dihydrotetrabenazine can be totally displaced by noradrenaline and serotonin. To account for the discrepancy observed between the uptake data (which indicated no difference with bovine chromaffin granules) and the [2-^{3}H] dihydrotetrabenazine binding studies (which showed a large excess of binding sites in the tumor membranes), we propose that granules in the investigated tumor contained a large amount of inactive monoamine carrier.

Two types of pheochromocytomas commonly occur: (a) small tumors with low contents of catecholamines and rapid catecholamine turnover rates, which release mainly unmetabolized catecholamines into the circulation, and (b) relatively large tumors with high contents of catecholamines and slow turnover rates which release mainly catecholamine metabolites [1, 2]. Tumors harboured by children are almost always of the former type [1]. The mechanism by which these non-innervated tumors release their catecholamines is not understood. Winkler and Smith have proposed that release is not exocytotic and occurs by diffusion through the cytoplasmic membrane [3]. They hypothesized that the chromaffin storage granules are filled to capacity and are unable to accomodate the rapidly synthesized catecholamines. Chemical analysis of purified granules and measurement of the spontaneous release of catecholamines and ATP from isolated granules were taken as evidence for a normal system of hormone storage in these tumors [4]. A possible explanation for difference between tumors is altered properties of vesicular monoamine uptake. These have not been investigated. Progress has recently been made in understanding ATP-dependent monoamine uptake by bovine chromaffin granules. An ATP-dependent H^+-translocase (inwardly directed H^+-pump), located on the vesicle membrane, generates a proton electrochemical gradient $\Delta\mu H^+$, composed of a pH-gradient (inside acidic) and a transmembrane potential (interior positive) [5–7]. The $\Delta\mu H^+$ is in turn used by a specific reserpine and tetrabenazine-sensitive carrier to accumulate monoamines [8–10]. It has also recently become possible to quantitate catecholamine carrier molecules, using a tritiated derivative of tetrabenazine (TBZ)‡, [2-^{3}H]dihydrotetrabenazine (2-[2-^{3}H] hydroxy-3-isobutyl-9,10 dimetoxy-1,2,3,4,6,7-hexahydro-11b-H-benzo[a]quinolizine, [^{3}H]TBZOH) which binds to the carrier with high specificity [11].

In this communication, we report on the monoamine storage system of a pheochromocytoma removed from a child. We have characterized the tumor biochemically, purified its catecholamine storage granules and have investigated monoamine

‡ Abbreviations: TBZ, tetrabenazine; [^{3}H]TBZOH, [2-^{3}H]dihydrotetrabenazine [2-[2-^{3}H]hydroxy-3-isobutyl-9,10-dimethoxy-1,2,3,4,6,7-hexahydro-11b-H-benzo[*a*] quinolizine]; Hepes, *N*-2 hydroxyethylpiperazin-*N'*-2 ethane sulfonic acid; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; OX-V, bis [3-phenyl-5-oxoisoxazol-4 yl] pentamethine oxonol; DBH, dopamine β-hydroxylase; DCCD, *N,N'*-dicyclohexyl-carbodiimide; SDS, sodium dodecylsulfate.

Table 1. Purification of noradrenaline vesicles and vesicle membranes

	Volume (ml)	Protein (mg)	Noradrenaline (μmole)	ATP (μmole)	DBH (u)	Noradrenaline/ ATP
Homogenate	5.5	82 ± 10	39 ± 7	4.3 ± 0.6	4.6 ± 0.8	9.1 ± 2.9
Low-speed supernatant	5.2	62 ± 6	31 ± 4	3.7 ± 0.5	3.8 ± 0.8	8.4 ± 2.3
27,000 *g* pellet (large granule fraction)	2.2	18 ± 1	19 ± 3	3.7 ± 0.7	2.8 ± 0.3	5.1 ± 1.8
19,000 *g* pellet (washed granules)	1.0	9 ± 2	14 ± 5	2.4 ± 0.3	1.9 ± 0.5	5.8 ± 2.9
Purified granules	1.0	6 ± 1	11 ± 3	1.5 ± 0.2	1.4 ± 0.1	7.3 ± 2.9
Membranes	0.3	1 ± 0.1	—	—	0.6 ± 0.2	—

The concentration of catecholamine in the tumor, as determined in the homogenate, was 39.4 μmole/g of tumor (6.7 mg catecholamine/g tumor), indicating a total content of 1970 μmole (335 mg) catecholamine for the whole tumor. Noradrenaline was the major amine, amounting to 99% of the monoamines of the homogenate. A large proportion of this noradrenaline was particle-bound since 63% of the low-speed supernatant amine was recovered in the large granule fraction. ATP and DBH activity which were abundant in the homogenate, were also mainly particulate (100 and 74% respectively). Data are mean (± S.D.) of 3–4 determinations and are expressed as calculated amounts derived from 1 g of tissue (wet weight). The purification steps are described in the experimental section.

uptake and [2-^{3}H]TBZOH binding by membrane vesicles derived from these granules. During completion of this work, a study with a similar aim has appeared in the literature [12].

MATERIALS AND METHODS

Case report. V.M. aged 13 years was referred to Necker-Enfants Malades Hospital, Paris (Pr. Royer) for severe hypertension (190/140 mmHg) and recent weight loss (−3 kg within 6 months). Her past history revealed a surgical intervention for bilateral club foot at 5 years of age and excision of lingual neuromas 5 years ago. Initial laboratory investigations showed highly abnormal urinary catecholamine excretion, according to our laboratory standards [13]: noradrenaline + adrenaline, 4805 nmole/24 hr (normal range: 0–145 nmole/24 hr); dopamine, 1729 nmole/24 hr (normal range: 150–750 nmole/24 hr); vanillylmandelic acid, 322 μmole/24 hr (normal range: 0–15 μmole/24 hr); homovanillic acid, 31.8 μmole/24 hr (normal range: 10–40 μmole/24 hr). Vasointestinal peptide was at normal level but thyrocalcitonin assay was slightly elevated, 480 pg/ml (normal range: 0–300 pg/ml). Preoperative diagnosis was left adrenal pheochromocytoma with multiple mucosal neuromas and possible carcinoma of thyroid gland, defining the well-known Görlin syndrome [14]. Surgical data were consistent with preoperative diagnosis and histology of the tumor revealed a typical pheochromocytoma. Post-operative urinary catecholamine levels remained within normal range: noradrenaline + adrenaline, 137 nmole/24 hr; dopamine, 683 nmole/24 hr; vanillylmandelic acid, 18 μmole/24 hr; homovanillic acid, 22.7 μmole/24 hr. Three months later thyrocalcitonin was at abnormal levels, leading to additional surgery on the thyroid gland.

Isolation of catecholamine-containing particles. The procedures of Smith and Winkler [15] and Morris and Schovanka [16] were followed. The fraction (14.5 g) of the tumor used for biochemical studies was chopped, homogenized with a Teflon–glass homogenizer in 80 ml of 10 mM Hepes buffer (pH 7.0) containing 0.3 M sucrose and filtered through cheese cloth (Table 1, step 1). The homogenate was centrifuged at 1500 *g* for 10 min and the pellet was discarded (step 2). The resulting low speed supernatant was centrifuged at 27,000 *g* for 20 min and the pellet resuspended in the homogenization buffer (large granule fraction, step 3). The suspension was centrifuged at 19,000 *g* for 20 min to yield the washed granule fraction (step 4). Part of this fraction was layered onto 2.5 × its own volume of 1.6 M sucrose in 10 mM Hepes pH 7.0 and centrifuged for 90 min at 102,000 g_{av} in a type 65 Beckman rotor [15]. The pellets contained the purified granules which were resuspended in the homogenization buffer (step 5). Another part of the washed granule fraction was purified by isopycnic centrifugation in isotonic sucrose–metrizamide solutions [16]. Aliquots (2 ml) were layered on linear gradients prepared from 5 ml of 34.77% (w/v) metrizamide in 10 mM Mes (pH 6.0) ($d = 1.182$) and 5 ml of a mixture of 0.3 M sucrose in the same buffer and 34.77% metrizamide (68:32, v/v; $d = 1.09$) and were centrifuged for 90 min at 110,000 g_{av} in an SW 41 rotor. Fractions (0.38 ml) were collected by puncturing the bottom of the tubes.

Bovine chromaffin granules were prepared as described by Smith and Winkler [15].

Isolation of particle ghosts. Ghosts (Table 1, step 6) were derived from particles isolated on 1.6 M sucrose as described for chromaffin granules [17]. Preparation were frozen in liquid nitrogen and were kept at −80°.

ATP-dependent monoamine uptake. Membranes (0.02–0.2 mg of protein/ml final concentration) were added to a medium (1 ml final volume) containing 5 mM ATP, 2.5 mM $MgSO_4$, 0.3 M sucrose, 20 mM

Table 2. Comparison of pheochromocytoma storage vesicles and bovine chromaffin granules

Purification	Pheochromocytoma storage vesicles		Bovine chromaffin granules
	1.6 M sucrose	Metrizamide	
Density	—	1.14	1.123
Total catecholamines (μmole/mg protein)	1.8 ± 0.8	1.9 ± 0.2	2.5 ± 0.4
Noradrenaline (μmole/mg protein)	1.8 ± 0.8	1.9 ± 0.2	0.7 ± 0.1
Adrenaline (μmole/mg protein)	0.02 ± 0.01	0.03 ± 0.01	1.8 ± 0.3
ATP (μmole/mg protein)	0.25 ± 0.08	0.39 ± 0.05	0.56 ± 0.03
Catecholamines/ATP	7.3 ± 2.9	4.9 ± 1.1	4.5 ± 0.9
DBH (u/mg protein)	0.24 ± 0.06	0.29 ± 0.07	0.27 ± 0.03

For pheochromocytoma vesicles, data are derived from Table 1 (1.6 M sucrose) and from the peak fraction of granules purified on iso-osmotic sucrose/metrizamide gradient (metrizamide). For bovine chromaffin granules, data represent mean ± S.D. of 5 measurements performed as described in Methods.

Tris–succinate buffer pH 8.15 and, where indicated, a drug. After a 5-min incubation at 37°, the reaction was initiated by addition of [^{3}H]serotonin or [^{3}H] noradrenaline. Controls were performed by adding to the incubation medium either 5 μM CCCP or 10 μM tetrabenazine. Samples were processed according to [18]. The K_M for noradrenaline was measured at pH 8.15, as described in [18].

[^{3}H]*TBZOH binding.* [^{3}H]TBZOH binding was measured essentially as described in [11]. Briefly, membranes (2–20 μg of protein/ml) were incubated at 25° with various concentrations of [^{3}H]TBZOH at maximal specific activity (12 Ci/mmole) in 0.3 M sucrose, 20 mM Tris–succinate buffer pH 8.15 (0.5 ml final volume). Bound ligand was measured by filtration on Millipore HAWP filters. Aliquots of the incubation mixture were diluted in 4 ml of ice-cold incubation buffer containing 125 μM tetrabenazine and were immediately filtered. The filters were then washed twice with 4 ml of the same buffer and their radioactivity measured by liquid scintillation counting in Aqualuma (Lumac, The Netherlands).

OX-V spectral changes. The OX-V fluorescence decrease associated with the transmembrane potential induced by addition of ATP-Mg^{2+} was measured as described by Scherman and Henry [19].

Analytical techniques. Catecholamine assay: catecholamines were assayed essentially as described by Von Euler and Lishajko [20]. When a differential assay of adrenaline and noradrenaline was required, the assay was modified according to [21]. ATP assay: ATP was measured by the firefly luciferin–luciferase reaction [22]. DBH activity: the activity was assayed at pH 6.5 in 0.1 M phosphate buffer, with tyramine (10 mM) as a substrate. One activity unit corresponds to 1 μmol octopamine formed/min [23, 24]. ATPase activity assay: ATPase activity was measured as described [17]. Protein assay: Proteins were estimated by the Lowry procedure with bovine serum albumin as a standard, following precipitation in 5% trichloroacetic acid and redissolution in 2% deoxycholate–3% NaOH.

Polyacrylamide gel electrophoresis. Electrophoresis in presence of SDS was performed according to Laemmli [25], but on linear gradients of 8–18% acrylamide, overlaid with stacking gels of 5% acrylamide. Samples were solubilized by heating for 5 min at 100° in a mixture containing 6.4% SDS, 50 mM Tris–HCl (pH 6.8), 12% sucrose, 6 mM EDTA and 5% dithiothreitol.

Instrumentation. Fluorescence was measured with a JY 3C Jobin Yvon double monochromator fluorimeter.

Materials. [^{3}H]TBZOH was prepared as described [11]. 5-Hydroxy [G-^{3}H]tryptamine creatine sulfate (18 Ci/mmole) and l-[7, 8]-[^{3}H]noradrenaline (12 Ci/mmole) were purchased from Amersham. OX-V was a gift of Dr B. S. Cooperman. CCCP, oligomycin, DCCD and tributyltin were dissolved in ethanol; tetrabenazine (Fluka) was solubilized in 0.1 N HCl and reserpine (Sigma) was prepared according to Phillips [26].

RESULTS

Biochemical characterization of the catecholamine storage organelles of the tumor

Crude noradrenaline vesicles were purified by centrifugation through a 1.6 M sucrose layer (Table 1) or by isopycnic centrifugation on an iso-osmotic linear metrizamide–sucrose gradient (data not shown). Electron microscopy of the fraction purified by the first technique showed a homogenous population of vesicles in the 150–300 nm diameter range. Distribution of protein, noradrenaline, ATP and DBH activity on the linear gradient also indicated a homogenous population, characterized by a density of 1.14. The catecholamine, ATP and DBH content (per mg of protein) of the material obtained by the two techniques are given in Table 2. These figures

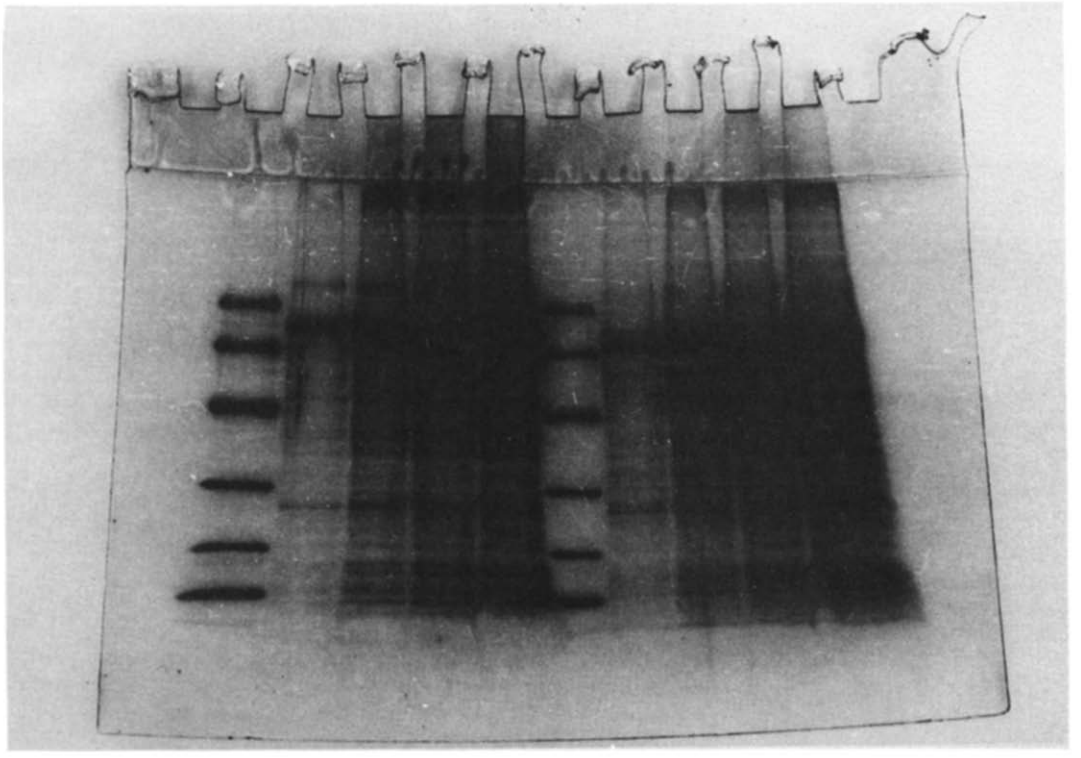

Fig. 1. Proteins of pheochromocytoma and adrenal medulla granule membranes separated by SDS polyacrylamide gel electrophoresis. Lanes: 1 and 6, standard polypeptides; 2–5, pheochromocytoma granule membranes (15, 30, 50, 75 μg protein); 7–10, adrenal medulla granule membranes at the same protein concentrations as pheochromocytoma ones. Standard polypeptides were phosphorylase b (94,000), albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), trypsin inhibitor (20,100), α.lactalbumin (14,400).

do not differ significantly from those obtained with bovine chromaffin granules [27]. The molar ratio of catecholamines to ATP in purified granules was 7.4 and 4.8 after centrifugation on sucrose and metrizamide, respectively. This ratio has also been calculated at various steps of the purification on sucrose gradient (Table 1).

Membranes of the noradrenaline vesicles

Resuspension of the granules purified on 1.6 M sucrose in hypotonic buffer induced an efficient lysis. Noradrenaline and ATP were reduced to an undetectable level (Table 1). This treatment released about 50% of the DBH activity, a figure similar to that observed for bovine chromaffin granules [27], but different from noradrenaline containing vesicles of bovine splenic nerves [28]. Washed membranes were resealed by suspension in isotonic sucrose [26] and appeared as closed vesicles ('ghosts') on electron micrographs (data not shown). These vesicles had a broad size distribution, a phenomenon which had been attributed to vesiculation of membrane fragments [26]. Some vesicles still contained an electron dense material.

When analysed by polyacrylamide gel electrophoresis in the presence of SDS, membranes of noradrenaline vesicles gave a characteristic pattern of proteins resembling that of bovine chromaffin granules (Fig. 1). The major component of the tumor membranes has an apparent mol. wt of 82,000 and

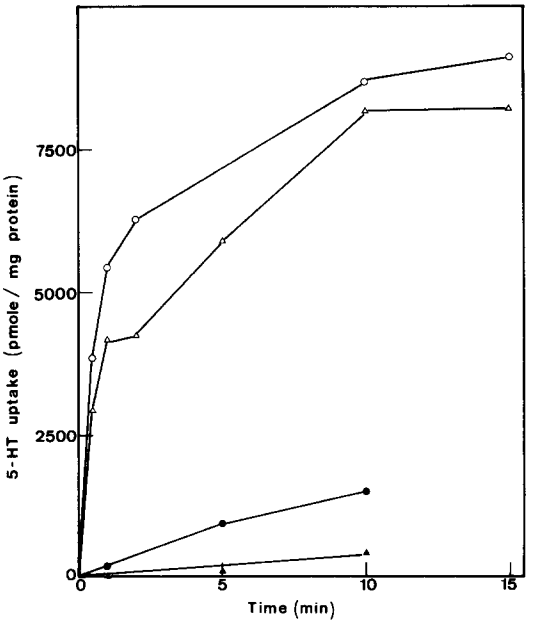

Fig. 2. Time-course of serotonin uptake by pheochromocytoma and adrenal medulla ghosts. Pheochromocytoma (○,●,0.18 mg protein/ml) and adrenal medulla (△,▲,0.17 mg protein/ml) particle ghosts were incubated with [^{3}H] serotonin, in presence (▲,●) or in absence (△,○) of 10 μM tetrabenazine. Points are mean of two determinations.

Table 3. Characterization of the ATPase of pheochromocytoma vesicles

	ATPase activity	
	nmol Pi/min/mg protein	%
Control	250 ± 8 (N = 6)	100
Oligomycin (10 μg/ml)*	239	96
DCCD (100 μM)*	97	39
Tributyltin (100 μM)*	85	34
CCCP (2 μM)*	287	114
CCCP (10 μM)*	359	143

* Mean of two determinations.

is likely to be DBH (mol. wt = 75,000 in chromaffin granules). One striking difference between the two membranes is the presence of an intensely stained band of high molecular weight (mol. wt = 110,000) in the pheochromocytoma membranes.

Monoamine uptake by noradrenaline granule ghosts

In the presence of ATP, ghosts derived from noradrenaline-containing granules accumulated serotonin (Fig. 2). The time-courses of uptake were similar for comparable concentrations of tumor ghosts and bovine adrenal ghosts. Plateau levels were also similar, indicating that similar serotonin concentration gradients were achieved by the two preparations. Investigation of the kinetics of noradrenaline uptake at pH 8.15 showed that the uptake of noradrenaline followed Michaëlis kinetics. A Lineweaver–Burk plot of the data showed the uptake to have a K_M for noradrenaline of 9.8 μM and a V_{max} of 625 pmole/min/mg protein. We have previously reported a K_M of 15.5 μM and a V_{max} of 1170 pmole/min/mg protein for the uptake of noradrenaline by chromaffin granule ghosts in the same conditions of pH [18].

The uptake was ATP-dependent and omission of ATP decreased the rate of uptake to 20% of the control (data not shown). The transport observed in the absence of ATP is likely to originate in contaminating nucleotides since addition of the H^+-ionophore CCCP, which prevents ATP-dependent H^+-pumping (see below) resulted in a complete inhibition of the uptake. The ATPase activity of the noradrenaline vesicle membranes was the same as that of bovine chromaffin granule membranes and was oligomycin-resistant and DCCD and tributyltin-sensitive (Table 3). Its activity was increased by addition of CCCP, suggesting that it was associated with an ATP-dependent H^+-translocase. Fluorescence experiments with the probe OX-V, which monitors transmembrane potential changes [19], supported this hypothesis: addition of ATP polarized positively pheochromocytoma membrane vesicles (31% OX-V fluorescence decrease) as it did with chromaffin granule ghosts (24% fluorescence decrease). In both cases, the fluorescence changes were reversed by 5 μM CCCP, indicating that polarization was associated with electrogenic H^+-translocation (data not shown).

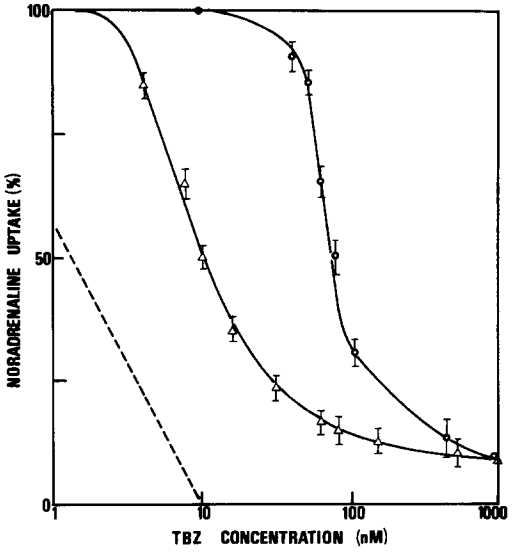

Fig. 3. Tetrabenazine inhibition of noradrenaline uptake. Pheochromocytoma (○) or adrenal medulla (△) particle ghosts were preincubated at 0.02 mg protein/ml for 30 min with TBZ–HCl at the indicated concentration. Noradrenaline (1 μM, 4 μCi/ml) was then added. Uptakes were measured on aliquots withdrawn at 1, 5 and 10 min on duplicate samples; uptakes were linear for more than 10 min. Blanks performed in presence of 10 μM TBZ were subtracted from all measurements. Controls in absence of TBZ were 20 and 32 pmole/min/mg protein for pheochromocytoma and adrenal medulla vesicles, respectively. The dotted line indicates the theoretical slope at midpoint assuming a Hill number of 1.0.

Pharmacological study

The uptake of both noradrenaline and serotonin by pheochromocytoma vesicles was inhibited by reserpine and tetrabenazine which interact specifically with the monoamine carrier [29]. Nevertheless, we observed a difference between reserpine which blocked monoamine uptake by pheochromocytoma and bovine chromaffin vesicles with the same efficiency (data not shown) and tetrabenazine (TBZ), which was apparently less efficient on tumor vesicles (Fig. 3). With 1 μM noradrenaline as substrate and at 0.02 mg membrane protein/ml, the IC_{50} for TBZ was 10 and 75 nM, with bovine chromaffin granule and human tumor vesicles, respectively.

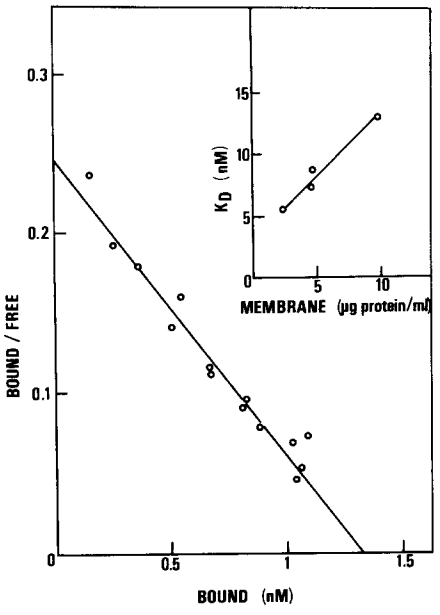

Fig. 4. [^{3}H]TBZOH binding to pheochromocytoma vesicle membranes. Total [^{3}H]TBZOH binding was determined at 2.3 µg membrane protein/ml on 0.5 ml incubation mixtures. Non-specific binding was measured in the presence of 1 µM TBZ and was subtracted from total binding to give specific binding (more than 92% of total binding). Data are presented as a Scatchard plot of [^{3}H]TBZOH specific binding (points are mean of two determinations), from which a K_D of 5.5 nM and a B_{max} of 590 pmol/mg protein were derived. Inset: K_D measurements as a function of membrane concentration.

With 10 µM serotonin and at 0.15 mg membrane protein/ml, the same difference was observed, the IC_{50} being 50 nM for the former preparation and 360 nM for the latter (data not shown).

The origin of this difference was investigated by comparing the binding of [^{3}H]TBZOH to the two types of membranes. Scatchard plots indicated that pheochromocytoma vesicles have one class of binding sites (Fig. 4), as has been shown for chromaffin granule membranes [11]. But the two membrane preparations differed significantly in the number of [^{3}H]TBZOH binding sites. A saturation experiment performed at 0.010 mg protein/ml gave a B_{max} of 585 pmole/mg protein and a K_D of 12.8 nM for the tumor vesicles, whereas at the same concentration under the same conditions, figures of 73 pmole/mg protein and 4.1 nM were obtained for chromaffin granules. In view of the high B_{max} obtained on the tumor preparation and since K_D can be determined correctly at site concentrations lower than K_D, the experiment was repeated at lower membrane concentrations. B_{max} values of 625 and 590 pmole/mg protein and K_D values of 8.0 and 5.5 nM were obtained at 0.005 and 0.002 mg of membrane protein/ml, respectively (Fig. 4, inset). The measurement performed at the lowest membrane concentrations indicates similar K_D values for tumor and bovine adrenal vesicles (5.5 and 4.4 nM, respectively).

To characterize the tumor [^{3}H]TBZOH binding sites further, displacement experiments were done at low membrane concentration (Fig. 5). [^{3}H]TBZOH was fully displaced from its binding sites by TBZ as well as by the uptake substrates noradrenaline and serotonin. The K_D for noradrenaline (0.7 mM) derived from the EC_{50} value did not differ significantly from that measured at the same pH on chromaffin granule membranes (0.5 mM) [30].

DISCUSSION

The studied pheochromocytoma was noradrenergic and it contained a larger amount of catecholamine than human adrenal medulla (6.7 vs 1.6 mg/g tissue) [31]. Most of amines were vesicular. Noradrenaline storage granules could be purified from this pool by procedures used for bovine adrenal granules [15, 16]. On linear sucrose–metrizamide gradient which separates noradrenaline and adrenaline chromaffin granules [32], distributions of protein, DBH activity, ATP and catecholamines were unimodal. The buoyant density of the granules was 1.14, a value similar to that reported for bovine adrenal medulla noradrenergic granules (1.15) and different from that of isolated adrenergic granules (1.10–1.12) [32]. The molar ratio of catecholamine to ATP was somewhat higher in tumor granules purified on 1.6 M sucrose than on metrizamide. The former value was confirmed by measurements at the various purification steps (Table 1). The ratio found for both preparations of tumor vesicles was in any case much lower than has been reported earlier (for a review, see [33]), but was similar to that reported recently for an adrenergic tumor [12]. This ratio is thus not correlated with the noradrenaline content of the tumor. A similar conclusion has been proposed for bovine chromaffin granules [34].

The general aspect of the proteins of the vesicle membrane visualized after electrophoresis on polyacrylamide gels confirmed the resemblance that tumor granules bear to adrenal ones. The major difference between the two membranes is the presence in the pheochromocytoma of a strongly stained band of 110,000 mol. wt. Polypeptides of high mol.

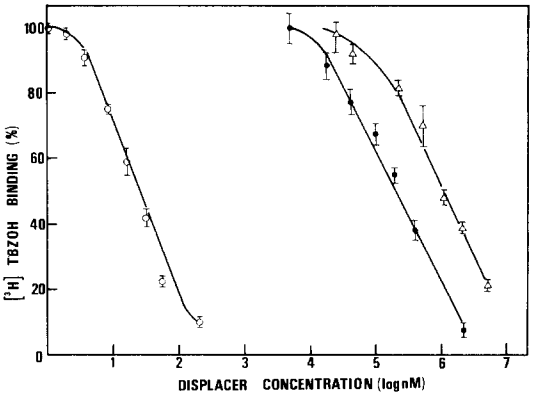

Fig. 5. [^{3}H]TBZOH displacement. Membranes (2.4 µg protein/ml) were incubated for 2 hr at 25° with 2 nM [^{3}H]TBZOH and various concentrations of TBZ (○), serotonin (●), or noradrenaline (△). Incubations with substrates also included 2.5 mM ATP and 1.25 mM $MgSO_4$. Points are mean ± S.D. (N = 3).

wt are present in gel patterns of reduced chromaffin granule membranes but they are only faintly stained. No identified protein has been localized to this region of the gel. It has not yet been determined if this membrane component is specific to human tissues or to human tumors.

Monoamine uptake was studied on ghosts derived from the storage vesicles since the high noradrenaline concentration of native granules might have interfered with transport experiments. Pheochromocytoma ghosts accumulated noradrenaline and serotonin and the characteristics of the transport (time-course, plateau level and K_M for noradrenaline) were indistinguishable from those of bovine adrenal ghosts. The experimental evidence presented support the idea that pheochromocytoma vesicles, like chromaffin granule ghosts, possess an ATP-dependent electrogenic proton pump and that, for both types of organelles, the H^+ electrochemical gradient generated by the pump drives monoamine uptake. However, studies with [^{3}H]TBZOH indicated an important difference between the investigated tumor and bovine adrenal medulla vesicles: the former contained ten times more binding sites than the latter, the binding constant being unchanged. This observation explains the relative inefficiency of TBZ inhibition of monoamine uptake by pheochromocytoma vesicles at the high [^{3}H]TBZOH binding site concentration used in this type of experiment. Under these conditions, the binding sites were in excess and the values obtained thus do not represent affinity constants but are titration curves. This is shown by the deviation of the slope from the theoretical value (Fig. 3), and by calculation of B_{max} values. That a similar effect is not observed with reserpine is somehow surprising. However, [^{3}H] reserpine binding to bovine chromaffin granule membranes is different from that of [^{3}H]TBZOH. Preliminary data (Scherman and Henry, unpublished) indicate that reserpine binding is a slow reaction accelerated by ATP and which occurs on two classes of binding sites. Our observation raises also a puzzling question: if [^{3}H]TBZOH binding sites are located on the monoamine carrier, why are the kinetics of the transport unchanged in a situation where the binding sites are significantly more abundant? A first possibility is that the carrier does not catalyse the rate-limiting step. But this possibility is unlikely in view of the high activity of the ATPase (200 nmole/min/mg protein) compared to the V_{max} of the carrier (0.6 nmole/min/mg protein). Proton pumping would be rate limiting only if the H^+ conductance of the membrane were high. Experiments with the fluorescent probe OX-V did not indicate a decreased ATP-dependent polarization of the tumor vesicles. A second possibility would imply different kinetic properties of the carrier of the two preparations. If $V_{max} = K_{cat} \times [C]$, where K_{cat} is the catalytic rate constant of the transport and $[C]$ the carrier concentration, a ten-fold increase of $[C]$ might be compensated for by a concomitant ten-fold decrease of K_{cat}. Although difficult to rule out, such a possibility is not very attractive. According to a third hypothesis, [^{3}H]TBZOH would bind not only to the monoamine carrier, but also to other undefined and more abundant sites. However, Scatchard plots indicated only one class of binding sites (Fig. 4), with a K_D very similar to that of chromaffin granules when determined under appropriate conditions (low membrane concentration). Moreover, [^{3}H]TBZOH was fully displaced from its binding sites by appropriate concentrations of noradrenaline and serotonin, two substrates of the monoamine carrier. The IC_{50} of TBZ for noradrenaline uptake (75 nM, Fig. 3), is apparently larger than the K_D of [^{3}H]TBZOH (5.5 nM, Fig. 4), a result which apparently indicates that monoamine uptake is unrelated to [^{3}H]TBZOH binding. But as pointed out above, the inhibition curve (Fig. 3) for pheochromocytoma vesicle membranes is a titration curve and the IC_{50} value derived from this experiment is different from the inhibition constant K_I. Noradrenaline uptake determination at lower protein concentrations (0.002 mg protein/ml) is technically difficult. We propose that the sites detected by [^{3}H]TBZOH binding might thus represent an inactive form of the carrier. One interesting possibility is that the carrier has an oligomeric structure, and the subunit carrying [^{3}H]TBZOH binding sites is in large excess in the tumor vesicle membrane. Work is now in progress to assess the generality of this observation in human pheochromocytomas.

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