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Uptake of *meta*-Iodobenzylguanidine by Bovine Chromaffin Granule Membranes

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SUMMARY

meta-Iodobenzylguanidine, an adrenal imaging agent used for the scintigraphic detection of human pheochromocytoma, is a substrate for the monoamine uptake system of chromaffin granules. It is accumulated by bovine chromaffin granule membrane vesicles in the presence of ATP, and it can be released by an osmotic shock. The uptake is dependent upon the generation of an H^+-electrochemical gradient by an ATP-dependent H^+ pump since it is blocked by an H^+ ionophore and since *meta*-iodobenzylguanidine uptake can be driven by imposing an artificial pH gradient (inside acidic) on the membrane vesicles. The transport is saturable and its K_m value (2.0 μM at pH 8.0) is similar to that of noradrenaline (5.3 μM). Transport occurs through the monoamine transporter since it is blocked by the same inhibitors, tetrabenazine and reserpine, and also by the transporter substrates noradrenaline and serotonin. Noradrenaline inhibits *meta*-iodobenzylguanidine uptake competitively (K_i = 13 μM). In addition, *meta*-iodobenzylguanidine displaces dihydrotetrabenazine and reserpine from their binding sites on chromaffin granule membranes. It is thus likely that, after *in vivo* administration, [^{131}I] *meta*-iodobenzylguanidine is ultimately stored in chromaffin granules and that it is translocated by the monoamine transporter.

Radioiodinated MIBG has been developed as an adrenal imaging agent (1, 2). This drug is used for the detection of human pheochromocytoma and related tumors by scintigraphy (3). Its possible use for the treatment of this type of tumor is under investigation (4). These results indicate a specific accumulation of MIBG in adrenal medulla and related tissues. It has recently been shown (5) that MIBG enters the chromaffin cells of bovine adrenal medulla through nonspecific and specific routes via the noradrenaline uptake system, but the fate of this drug inside the cell is still unknown. The structures of MIBG and noradrenaline are shown in Fig. 1. In the chromaffin cell, catecholamines are mainly located in specific organelles, the chromaffin granules. The membrane of these organelles contains an ATP-dependent transport system responsible for the accumulation of catecholamines in the granule matrix (6, 7). Progress has been made in elucidating the molecular mechanism of this sytem of uptake, which is unrelated to the noradrenaline high affinity uptake system of the chromaffin cell membrane. Monoamine uptake by the chromaffin granule membrane involves a transporter which catalyzes a neutral amine/H^+ antiport and which is driven by the electrochemical proton gradient, $\Delta\mu H^+$ (inside positive and acidic) generated by an electrogenic ATP-dependent H^+ pump (8–12). Conversely, the noradrenaline transporter of the cell membrane catalyzes a catecholamine/Na^+ symport and is driven by the Na^+ gradient generated by Na^+, K^+ATPase (5, 13–15). The two transporters have different specificity since the granular one translocates 5-HT as well as catecholamines (16). In addition, they have different inhibitors, TBZ and reserpine (Fig. 1) for granule membrane (16) and desmethylimipramine for cell membrane (15). Binding sites for TBZ and reserpine have been characterized on the chromaffin granule membrane (17–19). We have examined the possibility that MIBG is a substrate for the monoamine uptake system of bovine chromaffin granules, a result that points to the granules as the ultimate sites of MIBG storage.

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Experimental Procedures

Materials. Nonradiolabeled MIBG was synthesized as described (1). [^{131}I]MIBG was obtained by an iodide exchange technique (20). The initial specific activity was 2.5 mCi/mg. *l*-[7,8-^{3}H]Noradrenaline

ABBREVIATIONS: MIBG, *meta*-iodobenzylguanidine; $\Delta\mu H^+$, electrochemical proton gradient; 5-HT, 5-hydroxytryptamine; TBZ, tetrabenazine; TBZOH, dihydrotetrabenazine; CCCP, carbonylcyanide *m*-chlorophenylhydrazone; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mes, 4-morpholineethanesulfonic acid.

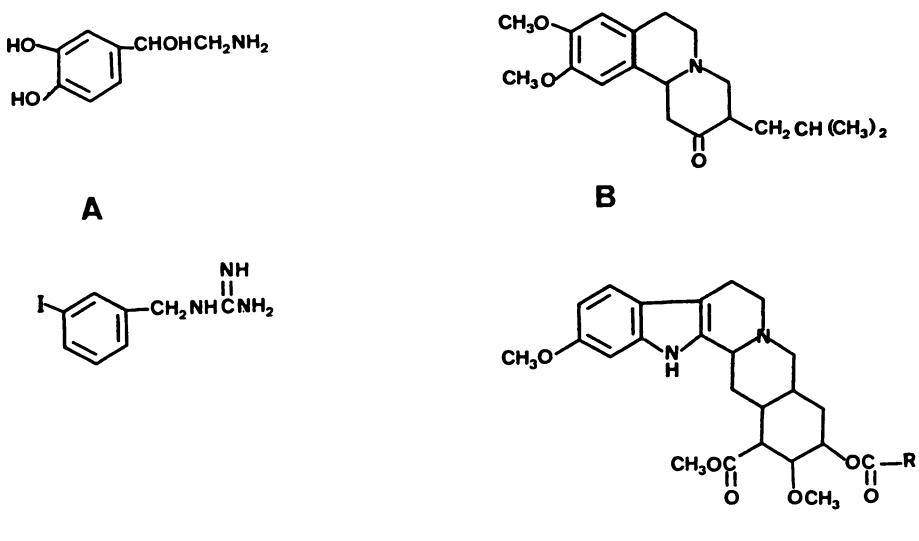

Fig. 1. Structures of noradrenaline (A), tetrabenazine (B), MIBG (C), and reserpine (D). R = 3,4,5-trimethoxyphenyl.

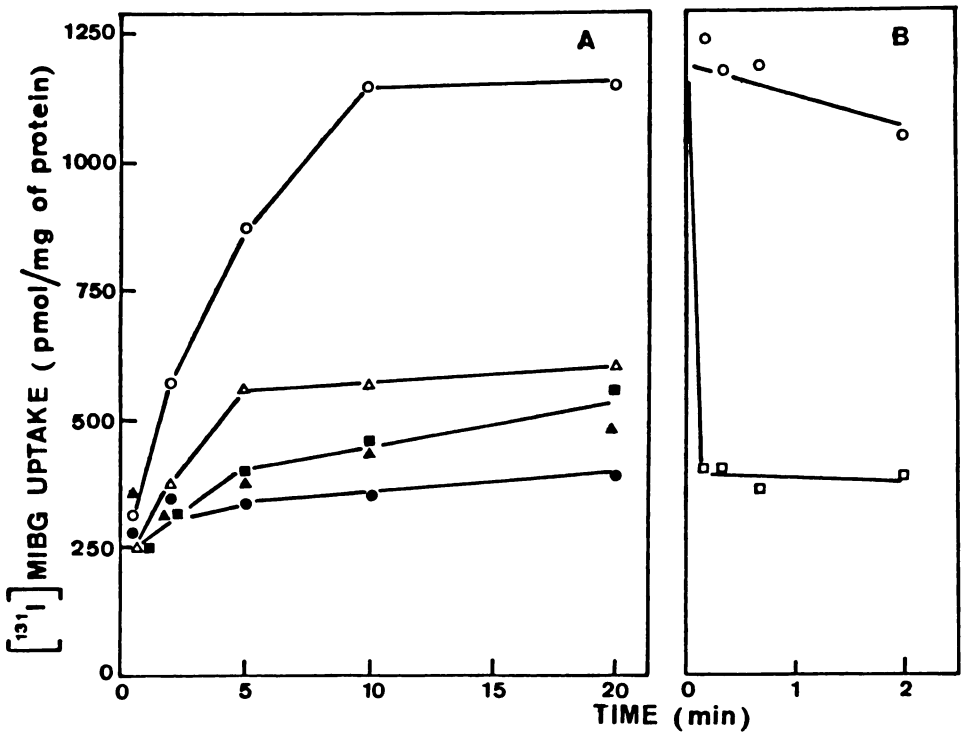

Fig. 2. ATP-dependent active transport of [^{131}I]MIBG. A. Chromaffin granule membranes (0.07 mg of protein/ml) were preincubated in 0.3 ml of 0.3 M sucrose/50 mM Hepes buffer (pH 7.5) without (△) or with 5 mM ATP, 2.5 mM $MgSO_4$ (○, ■, ▲, ●), and 7 μM CCCP (▲), 2 μM TBZ (■), or 2 μM reserpine (●). At zero time, [^{131}I]MIBG was added (final concentration 1 μM). Samples were incubated for the indicated periods of time and then filtered as described in Experimental Procedures. The experiment was repeated four times with similar results (MIBG accumulated after 20 min: 1145 ± 35 pmol/mg of protein, mean ± SD, n = 4). B. Membranes (0.07 mg of protein/ml) were preincubated for 20 min in 0.3 M sucrose buffer (pH 7.5) containing 5 mM ATP, 2.5 mM $MgSO_4$, and 1 μM [^{131}I] MIBG. Aliquots (300 μl) were withdrawn, diluted in 10 volumes of sucrose buffer (○) or in 5 mM Hepes buffer (pH 7.5) (□), and filtered after different periods of time.

(13.7 Ci/mmol) and 5-hydroxy [G-^{3}H]tryptamine creatinine sulfate (12.3 Ci/mmol) were purchased from Amersham Corp. (Arlington Heights, IL); benzoyl-^{3}H(G)-reserpine (24.4 Ci/mmol) was from New England Nuclear (Boston, MA). [2-^{3}H]Dihydrotetrabenazine was prepared as described (17).

Chromaffin granule membrane preparation. Bovine chromaffin granule membranes were prepared by osmotic lysis of granules isolated by centrifugation on a 1.6 M sucrose layer (21). Membranes were frozen in liquid nitrogen and were stored at −80°.

Uptake experiments. Uptake was initiated by addition of the substrates to the membranes preincubated at 30° as indicated in the legends to the figures. Aliquots were withdrawn at intervals, diluted 10 times in ice-cold 0.3 M sucrose containing buffer at the pH of the incubation medium, and filtered through Whatman GF/C filters. Filters were washed twice with the same volume of dilution medium. Their radioactivity was measured either directly with a gamma scintillation counter for [^{131}I]MIBG or by liquid scintillation inAqualuma (Lumac, The Netherlands) for tritiated monoamines. Glass-fiber filters were preferred to cellulose filters (Millipore), because [^{131}I]MIBG adsorption was 3.5 times lower on the former than on the latter. However, membrane retention was only 70% on GF/C filters (18); figures were corrected for this factor.

[^{3}H]TBZOH and [^{3}H]reserpine binding. [^{3}H]TBZOH binding was measured as described (17). For [^{3}H]reserpine binding, the bound ligand was separated from the free ligand by centrifugation of 0.2-ml aliquots of the incubation medium on 1.0-ml SP-Sephadex columns previously dehydrated (22). The filtrate radioactivity was measured by liquid scintillation in Aqualuma.

Analysis of [^{131}I]MIBG after uptake experiments. [^{131}I]MIBG (4 μM) was incubated with chromaffin granule membranes (1 mg of protein/ml) and 5 mM ATP, 2.5 mM $MgSO_4$ in 0.3 M sucrose/50 mM Hepes buffer (pH 8.0) in the presence or absence of 10 μM TBZ for 20 min at 30°. After centrifugation at 140,000 × g for 10 min, the pellets were resuspended in 200 mM HCl, and the supernatants were acidified

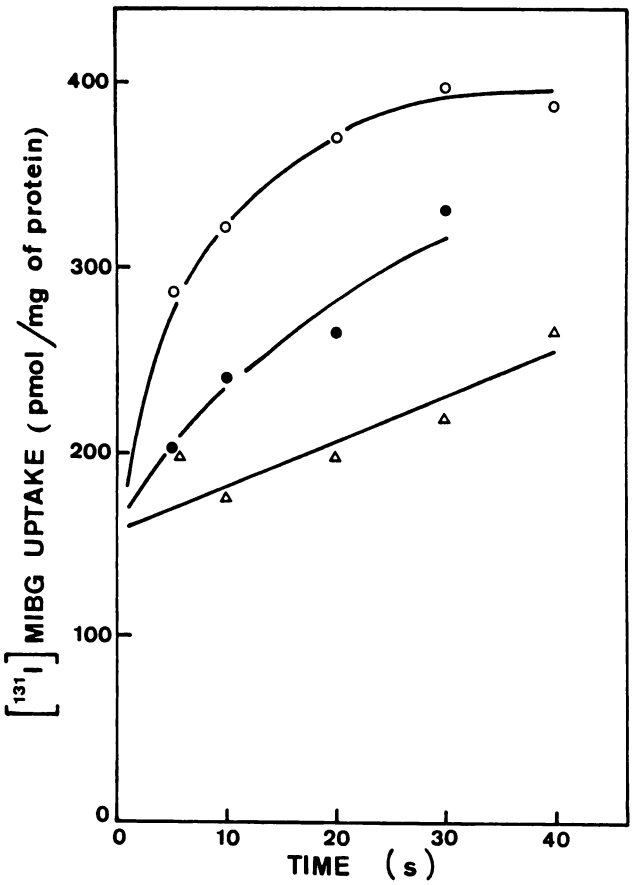

Fig. 3. ΔpH-driven [^{131}I]MIBG uptake. Membranes (0.43 mg of protein/ml) in 0.3 ml of 0.3 M sucrose/10 mM Mes buffer (pH 6.0) without (○) or with (●) 2 μM TBZ were diluted 6-fold in 0.3 M sucrose/40 mM Hepes (pH 8.5) containing 0.5 μM [^{131}I]MIBG without (○) or with (●) 1 μM TBZ. The control (△) experiment was performed by addition of the membranes to 0.3 M sucrose/40 mM Mes (pH 6.0). Similar results were obtained in three independent experiments.

to give the same HCl concentration. Aliquots of the two pellets and the two supernatants were analyzed by thin layer chromatography on SiO_2 plates using ethanol:ethylacetate (1:1) as the developing solvent. The radioactive spots were visualized by autoradiography. Only one major spot was detected with an R_f of 0.6, identical to that of [^{131}I]MIBG.

Results

Chromaffin granule membranes take up MIBG by an ATP-dependent active process. In the presence of 5 mM ATP/2.5 mM $MgSO_4$, chromaffin granule membranes accumulated [^{131}I]MIBG in a time-dependent manner (Fig. 2A). This accumulation was the result of the transport of [^{131}I]MIBG into the vesicle interior and not of the binding of this compound to the membrane, since the accumulated material was released by an osmotic shock (Fig. 2B). From the plateau level of Fig. 2A and the use of an internal specific volume of 4.5 μl/mg of protein (23), it was calculated that the observed accumulation results in a concentration gradient [MIBG] in/[MIBG] out of about 200. This value indicates an active transport. This hypothesis is substantiated by the fact that the accumulation is decreased in the absence of ATP and further inhibited in the presence of CCCP, an H^+ ionophore (Fig. 2A). The effect of CCCP, which is similar to that observed on the transport of catecholamines (24), indicates that the uptake of MIBG is dependent upon $\Delta\mu H^+$ generation by the ATP-dependent H^+ pump. MIBG accumulation is also blocked by 2 μM TBZ or reserpine (Fig. 2A), a result which suggests that MIBG uptake occurs through the monoamine transporter. Since MIBG is structurally unrelated to catecholamines, the possibility of a chemical modification of the molecule before its uptake was investigated. [^{131}I]MIBG was incubated with membranes under the conditions of the uptake experiment, and the radioactivity was then analyzed by thin layer chromatography (see Experimental Procedures). Neither within the ghosts nor in the medium was a chemically modified MIBG detected.$^{131}I^-$, a permeant anion (25), was present only as traces.

In the absence of ATP, MIBG was taken up by ghosts to which a pH gradient (inside acidic) was artificially imposed (Fig. 3), a result similar to that observed with catecholamines (26, 27). This uptake was partially inhibited by 2 μM TBZ, thus supporting the idea that the transporter-mediated uptake of MIBG is $\Delta\mu H^+$-dependent. The TBZ-resistant fraction of the uptake can be accounted for by nonspecific transport of MIBG, as observed with methylamine, the neutral form of which is rapidly translocated across biological membranes (28).

Characteristics of ATP-dependent MIBG uptake. The rate of [^{131}I]MIBG was saturable with respect to substrate concentration, and it followed Michaelis kinetics. At pH 7.5, a K_m of 4.8 μM and a V_{max} of 445 pmol/min/mg of protein were derived. The pH dependency of the reaction was then investigated (Fig. 4). At 0.5 μM MIBG, an optimum was observed at pH 8.0. At this pH value, K_m was 2.0 μM and V_{max} was 400 pmol/min/mg of protein. Under the same conditions, we obtained for the substrate noradrenaline a K_m of 5.3 μM and a V_{max} of 870 pmol/min/mg of protein. The increase of MIBG uptake rate with pH observed in the 7.0–8.0 pH range is similar to that reported for noradrenaline (23). This pH dependency originates in variations of the apparent K_m.

Inhibition of [^{131}I]MIBG uptake by substrates and inhibitors of the monoamine transporter. The kinetics of [^{131}I]MIBG uptake were analyzed at various noradrenaline concentrations (Fig. 5). Noradrenaline is a competitive inhibitor of MIBG uptake. This result supports the contention that MIBG and noradrenaline are translocated by the same transporter. A K_i of 13 μM for noradrenaline was derived, which is consistent with the K_m of noradrenaline determined in the same conditions (Table 1).

The effect of MIBG on ATP-dependent [^{3}H]noradrenaline and [^{3}H]5-HT uptake was also investigated. In both cases, MIBG had an inhibitory effect (Table 1). Using the fluorescent probe oxonal V, which monitors transmembrane potentials (29), we verified that this effect was not due to a collapse of the H^+ electrochemical gradient (data not shown). Analysis of kinetic data showed that MIBG inhibited competitively [^{3}H]noradrenaline uptake (Fig. 6) and that the K_i value was similar to that of the K_m for [^{131}I]MIBG uptake (Table).

The effect of inhibitors of the monoamine transporter on ATP-dependent [^{131}I]MIBG uptake was then tested. At a membrane concentration of 0.07 mg of protein/ml, reserpine inhibited the uptake of 1 μM MIBG with an IC_{50} of 1 nM (Table 1), a value similar to that reported for the inhibition of noradrenaline uptake (18). The slope of the inhibition curve indicated that the effect of reserpine resulted from its binding on one class of sites. TBZ also inhibited [^{131}I]MIBG uptake, and,

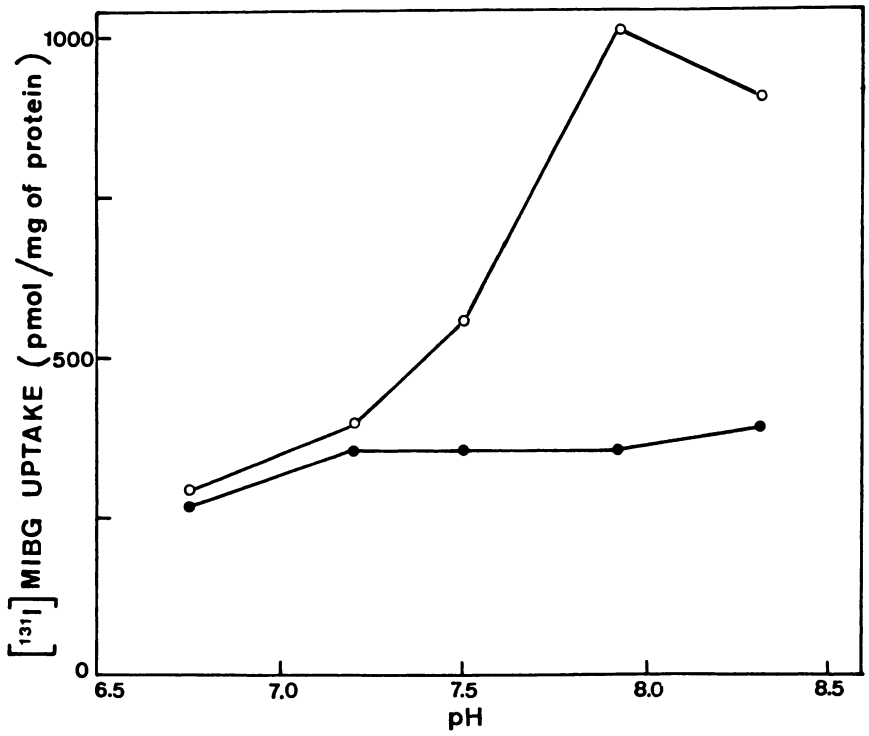

Fig. 4. pH dependency of ATP-driven [^{131}I]MIBG uptake. Membranes (0.08 mg of protein/ml) preincubated in 0.3 M sucrose containing 50 mM Hepes at the indicated pH, 2.5 mM ATP, 1.25 mM $MgSO_4$, with (●) or without (○) 1 μM reserpine, were incubated for 3 min with 1 μM [^{131}I]MIBG and filtered. The same pH profile was obtained when uptake was measured at 5 and 7 min (not shown).

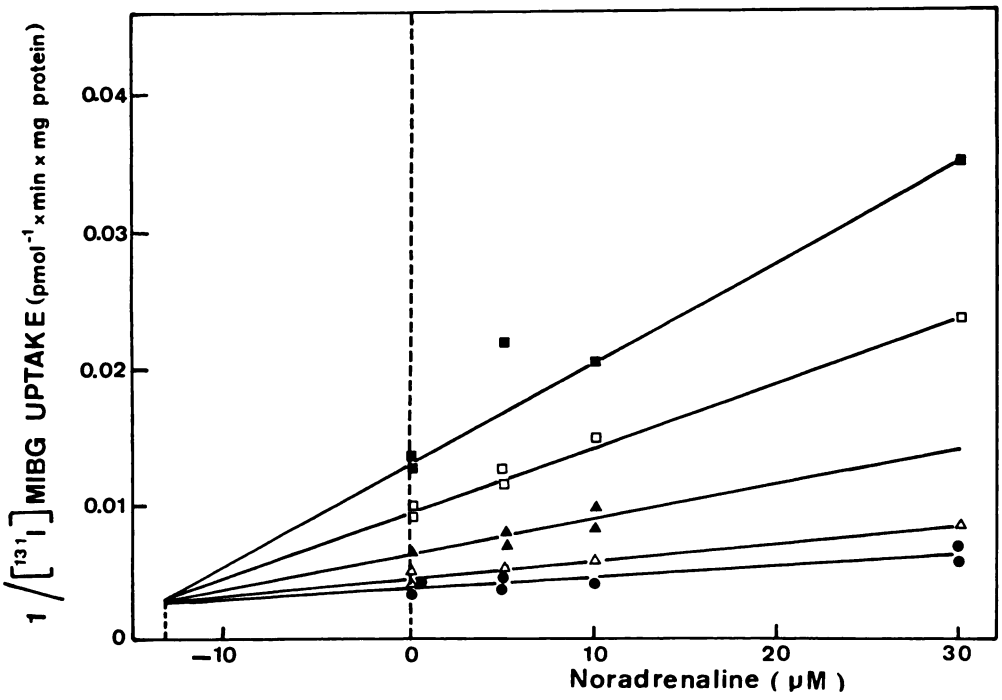

Fig. 5. Effect of noradrenaline on [^{131}I] MIBG uptake. Membranes (0.09 mg of protein/ml) were preincubated for 10 min at 30° with 2.5 mM ATP, 1.25 mM $MgSO_4$, and the indicated concentration of noradrenaline in 0.3 M sucrose, 50 mM Hepes buffer (pH 7.5). Uptakes were measured 4 min after addition of [^{131}I]MIBG: 0.5 μM (■), 0.75 μM (□), 1.5 μM (▲), 2.5 μM (△), and 5 μM (●). Controls performed in the presence of 1.5 μM reserpine were subtracted from all measurements. In the absence of noradrenaline, K_m and V_{max} values for MIBG were 2.0 μM and 400 pmol/min/mg of protein, respectively.

under the same experimental conditions, this drug inhibited [^{3}H]5-HT and [^{131}I]MIBG uptake with the same efficiency.

Inhibition of TBZ and reserpine binding to chromaffin granule membranes by MIBG. Noradrenaline has been reported to displace efficiently [^{3}H]reserpine from its binding sites (18), but to displace poorly [2-^{3}H]TBZOH, a ligand of TBZ binding sites (17). The efficiency of MIBG to displace [^{3}H]TBZOH and [^{3}H]reserpine was investigated and compared to that of noradrenaline (Fig. 7). MIBG efficiently displaced [^{3}H]TBZOH: the EC_{50} was in the same concentration range as the K_m for MIBG uptake and 2 orders of magnitude lower than the EC_{50} for the displacement of [^{3}H]TBZOH by noradrenaline (Fig. 7A). Conversely, MIBG was inefficient in displacing [^{3}H] reserpine since the EC_{50} was 300 μM, two orders of magnitude higher than the K_m for MIBG uptake (Fig. 7B).

Discussion

In the presence of ATP, chromaffin granule membranes accumulate MIBG since 1) trapped MIBG is released by osmotic shock and 2) there is an important concentration gradient between internal and external compartments. This uptake reaction has many features in common with that of monoamines. The uptake is dependent upon the generation of a $\Delta\mu H^+$ by an

TABLE 1
Inhibition of ATP-dependent amine uptake

Substrate	Inhibitor	K_m, K_i, or IC_{50}
		μM
[^{131}I]MIBG		2.0[a]
[^{3}H]Noradrenaline	MIBG	8.4[b]
[^{3}H]5-HT	MIBG	10[c]
[^{131}I]MIBG	noradrenaline	13[b]
[^{3}H]Noradrenaline		5.3[a]
[^{131}I]MIBG	reserpine	1×10^{-3}[c]
[^{3}H]Noradrenaline	reserpine	0.5×10^{-3}[d]

[a] K_m values were derived from linear regression ($r \geq 0.98$) of a Lineweaver-Burk plot of the data.
[b] K_i values were derived from the experiments of Figs. 5 and 6.
[c] IC_{50} values were measured at a substrate concentration of 1 μM.
[d] Taken from Ref. 15.

ATP-dependent H^+ pump since it is blocked by the H^+ ionophore CCCP (which collapses the $\Delta\mu H^+$) and since an externally imposed pH gradient can drive MIBG uptake. MIBG is translocated by a transporter since its uptake is saturable: saturation cannot originate from the dissipation of the $\Delta\mu H^+$ by incident entry of the MIBG free base since 1) the transmembrane potential generated by the H^+ pump is not collapsed by addition of MIBG; and 2) the internal pH is buffered by 50 mM Hepes, and it has been shown that the internal pH of such vesicles suspended in sucrose media is not affected by the H^+ pump, which generates mostly a membrane potential under these conditions (10). Based on the following evidence, the transporter which translocated MIBG is the same as that of monoamines: 1) noradrenaline is a competitive inhibitor of MIBG uptake and, inversely, MIBG is an inhibitor of noradrenaline and 5-HT uptake; 2) MIBG uptake is inhibited by reserpine and by TBZ, and IC_{50} values are similar to those observed for the inhibition of 5-HT and noradrenaline uptake. MIBG is thus a substrate for the system of catecholamine uptake not only in the plasma membrane (5), but also in that of chromaffin granules.

The fact that MIBG is a substrate for the monoamine transporter is surprising, in view of the structural differences between MIBG and catecholamines: MIBG is neither a catechol nor an amine. The pK_a values for MIBG ($pK_a = 12$) and noradrenaline ($pK_a = 9$) differ significantly as do the lipophilicity reflected in the partition coefficients at pH 7.5 in the octanol/H_2O system (0.005 and 1.4 for noradrenaline and MIBG, respectively). Both parameters are important for substrate binding and translocation (30). In fact, some differences are observed between noradrenaline and MIBG which might be explained by their different physicochemical properties: 1) the V_{max} value for MIBG uptake appears to be lower than that of noradrenaline, although both have similar K_m values; 2) MIBG is more efficient than noradrenaline at displacing [^{3}H]TBZOH from its binding sites, whereas the opposite is true for [^{3}H]reserpine binding sites (Fig. 7). The second observation is interesting since the transporter substrates described so far (noradrenaline and 5-HT) have a low affinity for [^{3}H]TBZOH binding sites, with K_D values much larger than their K_m values (17), a fact which might have suggested that TBZOH binding sites were not directly involved in amine translocation but were internal regulatory sites. The low affinity of MIBG for reserpine binding sites is a symmetrical situation which does not support the idea of regulatory TBZOH binding sites. We thus propose the existence of two sites involved in the transport mechanism: sites R_1, with a high affinity for reserpine and noradrenaline, and sites T, with a high affinity for TBZ and MIBG.

It is likely that MIBG, when administered *in vivo*, is ultimately stored in chromaffin granules. Its entry into the cytoplasm of chromaffin cells has been described (5), and our results show that it is a substrate for the monoamine uptake system of chromaffin granules. This hypothesis is supported by results of subcellular fractionation after *in vivo* administration (2). In

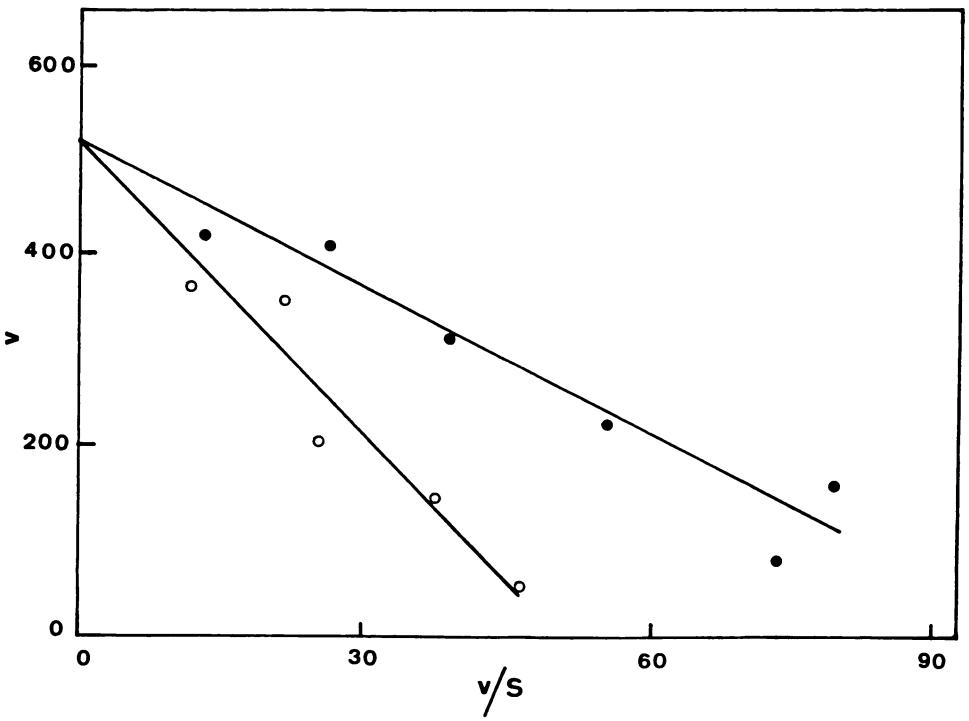

Fig. 6. Competitive inhibition of [^{3}H]noradrenaline uptake by MIBG. Membranes (0.15 mg of protein/ml) were preincubated for 5 min at 30° with 2.5 mM ATP, 1.25 mM $MgSO_4$, 0.3 M sucrose, 50 mM Hepes buffer (pH 8.0) in the absence (●) or presence (○) of 8.4 μM MIBG. Uptakes were measured 2 min after addition of [^{3}H]noradrenaline in the 1–32 μM concentration range. Controls performed in the presence 7 μM TBZ were subtracted from all measurements. Results were plotted according to the Wolff-Hoffstee plot (*v*, pmol of accumulated noradrenaline/min/mg of protein; *S*, μM noradrenaline concentration). The K_i value for MIBG was derived from a secondary plot of the data.

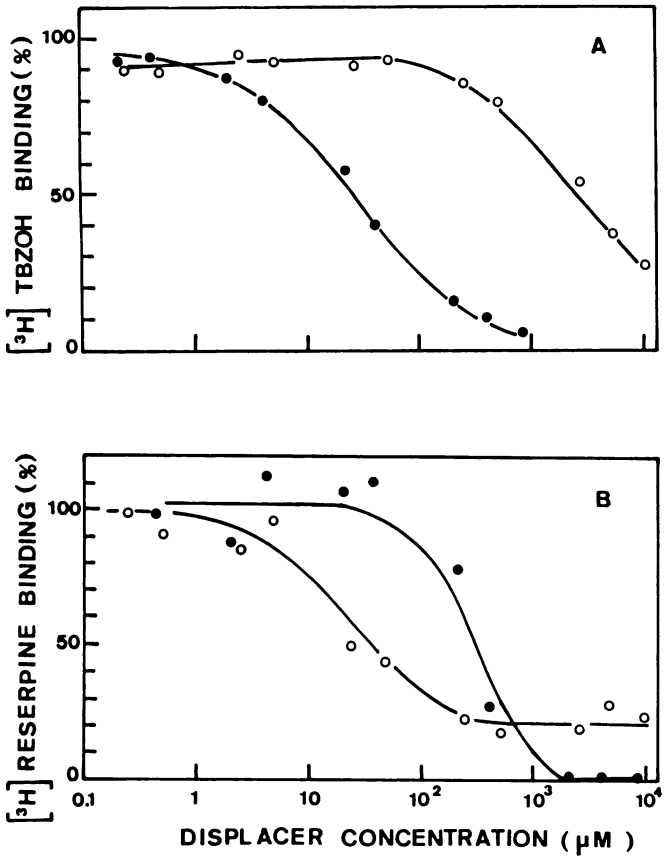

Fig. 7. Comparison of the effects of MIBG and noradrenaline on [^{3}H] reserpine and [^{3}H]TBZOH binding. Chromaffin granule membranes (0.075 mg of protein/ml were incubated for 30 min at 30° in 0.3 M sucrose/50 mM Hepes (pH 7.5) with 2.5 mM ATP, 1.25 mM $MgSO_4$, noradrenaline (○) or MIBG (●) at the indicated concentration and 1.8 nM [^{3}H]TBZOH (A) or 1.7 nM [^{3}H]reserpine (B). Nonspecific binding was determined by the addition of 1 μM TBZ (A) or 2 μM reserpine (B) and was subtracted. Binding at equilibrium in the absence of displacer was 19 ± 1 (n = 4) and 4.5 ± 0.3 (n = 3) pmol/mg of protein for TBZOH and reserpine, respectively. Similar results were obtained in three independent experiments.

addition, well contrasted scintigraphic images of animal adrenals or human pheochromocytoma are seen at least 24 hr after the injection of the drug, consistent with the low turnover of the adrenal granular pool of catecholamines. Finally, our data raise the possibility that granular accumulation of MIBG might be affected by the cytoplasmic catecholamine level, since we have shown that noradrenaline is a competitive inhibitor of MIBG uptake and that the K_i is in the estimated range of cytoplasmic catecholamines.

Acknowledgments

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