Biochemical Pharmacology, Vol. 39, No. 12, pp. 1959–1964, 1990.
Printed in Great Britain.

0006-2952/90 $3.00 + 0.00
Pergamon Press plc

EXTRAGRANULAR STORAGE OF THE NEURON BLOCKING AGENT META-IODOBENZYLGUANIDINE (MIBG) IN HUMAN NEUROBLASTOMA CELLS

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(*Received* 11 *July* 1989; *accepted* 25 *January* 1990)

Abstract—Human SK-N-SH neuroblastoma cells accumulate and store the adrenal imaging agent meta-iodobenzylguanidine (MIBG) with minor involvement of specialized cytoplasmic storage granules (Smets LA *et al.*, Active uptake and extravesicular storage of meta-iodo-benzylguanidine in human neuroblastoma SK-N-SH cells. *Cancer Res* **49**: 2941–2944, 1989). In the present study the mechanism of extravesicular MIBG retention was investigated and compared with granular storage of MIBG and norepinephrine (NE) in PC-12 pheochromocytoma cells. SK-N-SH cells concentrated both MIBG and NE by neuron-specific Uptake-1 but long-term retention was only observed with MIBG. Retention of accumulated NE was, however, promoted by inhibition of intracellular catecholamine degradation with pyrogallol. Drug release by controlled cell permeabilization and by KCl-induced exocytosis indicated that MIBG was mainly stored as freely diffusible, cytoplasmic molecules. SK-N-SH cells were depleted from stored MIBG by the Uptake-1 inhibitor imipramine but poorly so by the granule-depleting drug reserpine. Conversely, PC-12 cells were depleted by reserpine but insensitive to imipramine. The data suggest that extravesicular retention of MIBG in SK-N-SH cells is not based on intracellular sequestration but is solely due to efficient re-uptake of accumulated drug after leaking from the cells. The accumulation of MIBG in SK-N-SH cells, reflecting "pure" Uptake-1, appears to be a powerful system for exploring various cellular and molecular aspects of catecholamine uptake.

Active cellular uptake and subsequent granular translocation of catecholamines are widely studied archetypes of proton-driven transport systems. [^{131}I]MIBG, a radioiodinated functional analog of the neurotransmitter norepinephrine [1], is selectively accumulated by chromaffin tissues and by tumors of neural crest origin such as pheochromocytoma, neuroblastoma, medullary thyroidoma and various other tumors of the APUD series. The drug has gained considerable interest as a tumor-targeted radiopharmaceutical for the scintigraphic visualization of these tumors and their metastases [2, 3]. Local radiotherapy with high doses of [^{131}I]MIBG has been explored in pheochromocytoma [4] and neuroblastoma [5].

It has been well established that MIBG, like natural catecholamines, accumulates in competent cells by neuron-specific Uptake-1, a saturable sodium- and energy-dependent mechanism, sensitive to inhibition by tricyclic drugs such as cocaine and (des)imipramine [6–8]. Likewise, it is generally believed that MIBG shares with catecholamines a common storage mechanism [9, 10], namely the translocation into specialized storage granules by active, reserpine-sensitive transport and subsequent sequestration in complexes with ATP [11].

In SK-N-SH cells, a widely studied *in vitro* correlate of human neuroblastoma as well as in neuroblastoma tumors, storage granules are rare [10, 12, 13] compared with experimental, e.g. rat PC-12 [14], or clinical pheochromocytoma [10, 13]. In fact, we have demonstrated previously that the accumulated drug was not precipitable from cytosols of SK-N-SH cells by differential ultracentrifugation in a high-speed fraction and was also insensitive to the catecholamine-depleting effect of reserpine [12]. The mechanism by which SK-N-SH cells are then capable of long-term, extravesicular retention of a pharmacological amine is of both fundamental and clinical importance. In the present study retention of radiolabeled MIBG in SK-N-SH cells was investigated and compared with its granular storage in PC-12 cells. The two cell systems were investigated by pharmacological intervention and by competition with unlabeled substrates for uptake and storage. In addition, the effect of controlled cell permeabilization and membrane depolarization on drug retention was studied. The results have revealed a novel type of storage of a neuron-blocking agent.

MATERIALS AND METHODS

Tissue culture and incorporation studies. Human neuroblastoma SK-N-SH cell and rat PC-12 pheochromocytoma cells were cultured as described [12]. For experiments on uptake and storage, SK-N-SH cells were plated in Costar 6-well cluster dishes at 100,000 cells in 3 mL of medium and grown for 3–4 days to a density of approximately 500,000 cells/well. The cultures were then incubated in 2 mL of medium containing [^{125}I]MIBG or [^{3}H]NE at fixed radioactive concentration of 0.01 and 0.1 μCi/mL, respectively. Unlabeled drug was added to obtain the desired molar concentrations. At the end of the incubations, the cultures were washed three times with cold phosphate-buffered saline (PBS) and extracted twice with 0.25 mL of 0.5 N perchloric acid (PCA). The combined extracts were counted by

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liquid scintillation counting and the cell residue was dissolved in alkali for protein determination. Corresponding experiments with PC-12 cells were performed on a single cell suspension obtained from stock cultures by forced aspiration. Aliquots of approximately 500,000 cells in 2 mL were incubated as described above.

Cell permeabilization. To investigate the effect of controlled cell permeabilization [15], cultures of SK-N-SN were loaded with [^{125}I]MIBG for 2 hr and washed twice with Ca^{2+}- and Mg^{2+}-free PBS. The cultures were subsequently incubated for 5 min in cold Earle's Balanced Salt Solution without Ca^{2+} and Mg^{2+} (EBSS$^-$) containing 10^{-3} M EDTA. In case of PC-12 cells, single cell suspensions were prepared from prelabeled stock cultures by aspiration and the suspension was distributed over Eppendorf vials at 200,000 cells/vial. After centrifugation (3 min, 1000 *g*) the cells were resuspended in 1 mL of cold EBSS$^-$. Controls for membrane damage consisted of cultures prelabeled with [^{3}H]deoxyglucose (2 hr; 0.1 μCi/mL) as low molecular weight marker. Permeabilization with digitonin was as described by Wilson and Kirsner [16]. Digitoxin was added to final concentrations ranging from 5 to 30 μg/mL to EBSS containing 1% bovine serum albumin with or without 0.9 mM Ca^{2+}. Cells were incubated for the indicated time at 30° and samples of the supernatant medium and PCA extracts of the pelleted or adherent cells were counted to calculate the percentage of released label. In addition to the [^{3}H]deoxyglucose marker, permeabilization was also controlled by trypan blue staining and by determination of the cytoplasmic marker lactate dehydrogenase (LDH) in the supernatant medium, using DuPont acd^{tm} chemical analyser (courtesy of Dr W. Nooijen, Dept. Clinical Chemistry). Membrane depolarization was achieved by replacing the loading medium for isotonic growth medium containing 56 mM KCl.

Chemicals. MIBG ($I.C_6H_4CH_2NH(=NH)CNH_2$) was synthesized from the monoamine precursor meta-iodobenzylamine (MIBA: $I.C_6H_4CH_2NH_2$; Janssen Pharmaceutics, Beerse, Belgium) as described [12, 17]. Imipramine, pargyline, pyrogallol, digitonin and norepinephrine (artenorol: $(HO)_2C_6H_3CH(CH_2NH_2)OH$) were purchased from the Sigma Chemical Co. (Poole, U.K.) and formulated reserpine (Serpacill) was kindly provided by Ciba Geigy (Basel, Switzerland). [^{3}H]NE from Amersham was diluted according to the instructions of the manufacturer with addition of carrier NE to a final specific activity of 1 Ci/mmol. [^{3}H]Deoxyglucose (2-deoxy-D-[1-^{3}H]glucose; sp. act 17 Ci/mmol) was from Amersham. [^{125}I] MIBG (sp. act. 40 mCi/mg) was a kind gift of Amersham Braunschweig GmbH, G.F.R. Acid washed alumina to concentrate catecholamines as described by Kaplan [18] was kindly provided by Dr A. van Gennip, University of Amsterdam, Academic Hospital.

RESULTS

Uptake and storage in SH-N-SH cells

SK-N-SH cells were incubated in increasing concentrations of [^{125}I]MIBG with or without the Uptake-1 inhibitor imipramine (1.25×10^{-6} M) to discriminate between active Uptake-1 and nonspecific uptake [6, 12]. After reaching steady state of uptake in 2 hr, part of the cultures were washed and kept in drug-free medium for a further 6 hr to assess the plateau levels of storage. MIBG was concentrated up to 20-fold over nonspecific uptake as evident from the ratio of incorporation by control over imipramine-inhibited cultures (Fig. 1). Moreover, a large fraction (about 70%) of accumulated drug was retained after removal of the labeling medium. This fraction was constant at all extracellular concentrations tested and stable for at least 24 hr (not shown). In contrast, the cells failed to retain [^{3}H]NE. After removal of the labeling medium the intracellular levels rapidly returned to that of nonspecific uptake (Fig. 2). Kinetic analysis revealed comparable uptake capacities, namely V_{max} values of 2.5 and 2.0 nmol/mg protein for [^{125}I]MIBG and [^{3}H]NE, respectively. However, the affinity constant K_m was 0.31×10^{-6} M for [^{125}I]MIBG uptake versus 1.80×10^{-6} M for [^{3}H]NE, suggesting that MIBG had a higher apparent affinity for Uptake-1 than the natural substrate. This was confirmed by investigating the uptake of radiolabeled MIBG and NE in the presence of graded concentrations of unlabeled MIBG, NE or the mono-amine precursor MIBA. Radiolabeled MIBG and NE were used at 1 and 2×10^{-6} M, respectively, i.e. the concentrations which nearly saturated their uptake at steady state conditions (compare with Figs 1 and 2). [^{3}H]NE uptake was very sensitive to competition by unlabeled MIBG, being almost completely inhibited by an equimolar concentration of competing analog but only by 42% by cold NE (Fig. 3). MIBA appeared a weak inhibitor with about 100-fold lower ID_{50} compared to MIBG. In the reciprocal experiments, uptake of [^{125}I]MIBG at 1×10^{-6} M was inhibited

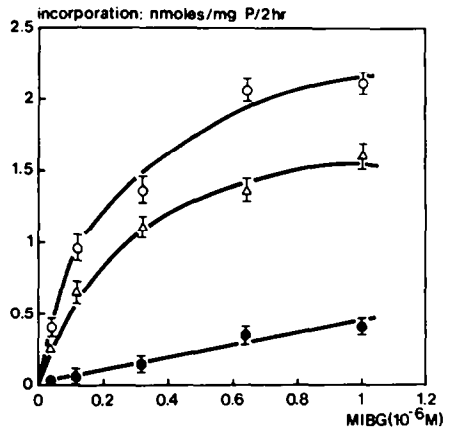

Fig. 1. Uptake and retention of [^{125}I]MIBG in SK-N-SH neuroblastoma cells as a function of extracellular concentration of the radiopharmaceutical. Cells were incubated in the indicated concentration of [^{125}I]MIBG for 2 hr without (0; total uptake) or with the Uptake-1 inhibitor imipramine at 1.25×10^{-6} M (●; nonspecific uptake) and the cell-associated radioactivity was determined. For kinetic analysis, specific uptake was calculated from the difference total − nonspecific uptake. Drug retention (△) was measured as the residual radioactivity at 6 hr after removal of the labeling medium. Values are averages (±SE) of three experiments.

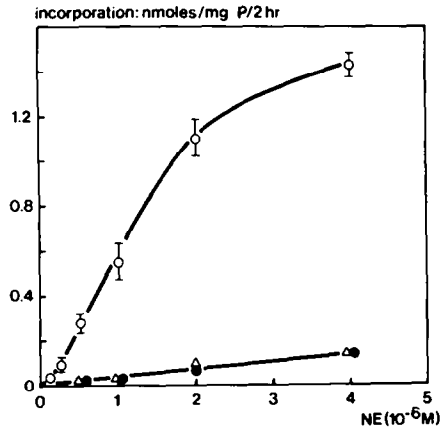

Fig. 2. The active uptake and deficient retention of [^{3}H]NE as a function of extracellular drug concentration in SK-N-SH cells. Cells were incubated in the indicated concentrations of [^{3}H]NE for 2 hr without or with imipramine or incubated for a further 6 hr in label-free medium for the determination of total uptake (○), non-specific uptake (●) and retained radioactivity (△) as described in the legend to Fig. 1.

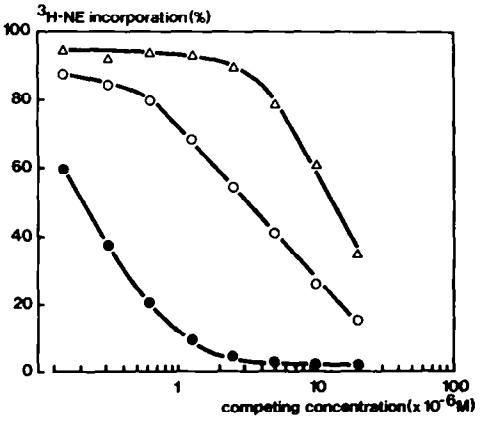

Fig. 3. The inhibition in SK-N-SH cells of the uptake of [^{3}H]NE by graded concentrations of unlabeled MIBG (●), norepinephrine (○) and meta-iodobenzylamine (△). Cells were incubated for 2 hr in medium containing [^{3}H]NE in the uptake-saturating concentration of 2×10^{-6} M (cf. Fig. 2) to which the competing unlabeled drugs had been added in the indicated concentrations. Mean values from duplicates of three experiments.

by 55%, 15% and 0% by equimolar concentrations of unlabeled MIBG, NE and MIBA, respectively.

Intracellular degradation of NE

To control for intracellular degradation as the cause of failing [^{3}H]NE retention, preloaded SK-N-SH cells were incubated with inhibitors of catecholamine-degrading enzymes. Pargyline, the inhibitor for monoamine-oxidase (MAO) was without effect whereas pyrogallol, the inhibitor of catechol-*O*-methyltransferase (COMT), significantly promoted the retention of [^{3}H]NE (Fig. 4). Moreover, pyrogallol (but not pargyline) included in the labeling medium stimulated total uptake up to fourfold (not shown). Attempts to assess [^{3}H]NE levels

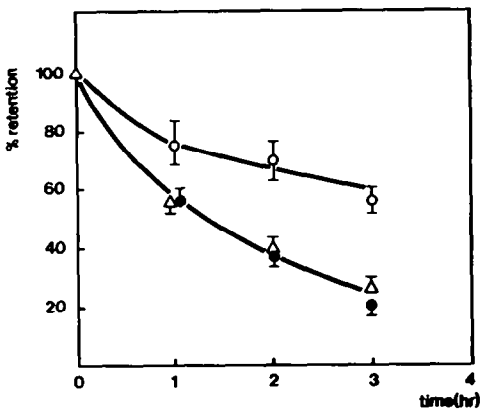

Fig. 4. The effect of inhibitors of catecholamine degradation on the retention of [^{3}H]norepinephrine by SK-N-SH cells. Cells were loaded with [^{3}H]NE at 10^{-7} M for 2 hr, washed and incubated in drug-free medium (● = control) or medium containing the MAO inhibitor pargyline (△; 5 μg/mL) or the COMT inhibitor pyrogallol (○; 2 μg/mL). Average values ± SE of four experiments.

by HPLC in lysates of preloaded SK-N-SH cells or in the post-labeling medium were unsuccessful due to poor adsorption of [^{3}H]-radioactivity to alumina. In contrast, [^{3}H]NE dissolved in fresh culture medium or extracted from prelabeled PC-12 cells was recovered by >80% using this method. Apparently, in SK-N-SH cells extragranular [^{3}H]NE is subject to rapid and complete breakdown by COMT.

Cell permeabilization and exocytosis

Controlled cell permeabilization [15] was applied to investigate whether MIBG was sequestered in macromolecular complexes. Digitonin (5–30 μg/mL) evoked a time- and concentration-dependent but calcium-independent release of MIBG from SK-N-SH cells. This response was already maximal at a digiton concentration of 15 μg/mL which caused only 20% release of the cytoplasmic LDH marker (Fig. 5), suggesting that MIBG was present as molecules of low MW and not contained in calcium-excitable granules. Since Ca^{2+}-dependency of catecholamine release is often masked by damage of the permeabilizing agent to storage granules [15], control experiments were performed with [^{3}H]NE or [^{125}I]MIBG stored in PC-12 cells and with [^{3}H]deoxyglucose as a low MW cytoplasmic marker. In addition, exocytosis was induced in both cell systems by membrane depolarization with 56 mM KCl. The results (Table 1) revealed that the exocytotic response induced by 56 mM KCl was modest in SK-N-SH and about three fold lower than in PC-12 cells. In the latter cells, no significant differences between the release of [^{3}H]NE or [^{125}I]MIBG was observed. Digitonin alone (15 μg/mL) caused a large release of MIBG and deoxyglucose from SK-N-SH cells. This response which was clearly antagonized by Ca^{2+}, possibly by interference with the permeabilizing agent which was used at suboptimal concentration. In contrast, the release of [^{3}H]NE from permeabilized PC-12 cells was stimulated by Ca^{2+}. However, the net calcium effect of about 20% specific release, was relatively low compared to the

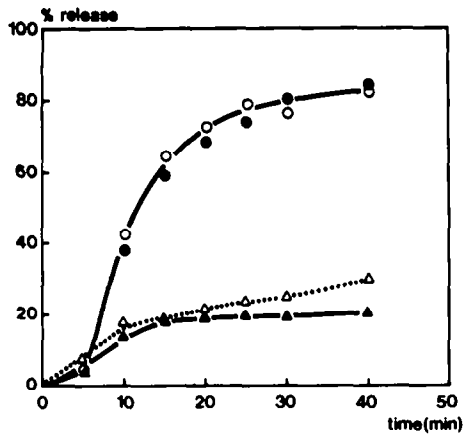

Fig. 5. The release of [^{125}I]MIBG from SK-N-SH cells permeabilized with digitonin. Cells were preloaded with [^{125}I]MIBG at 10^{-7} M for 2 hr (final level: 1.0 nmol/mg P), washed and incubated in a balanced salt solution containing 15 μg digitonin/mL with or without 0.9 mM Ca^{2+}. Samples of the supernatant medium were taken at the indicated times to determine the amount of released drug in the presence (●) or absence (○) of Ca^{2+}. Digitonin-induced released of the cytoplasmic LDH marker (▲) and the spontaneous release of [^{125}I]MIBG in the absence of digitonin (△) was assessed in parallel cultures. Mean values from duplicates in two experiments.

high level (59.5%) of nonspecific, calcium-independent release.

Pharmacological intervention

The hypothesis was tested that SK-N-SH cells retain MIBG by rapid re-uptake of the drug during leakage from the cells. Specific inhibition of re-uptake by imipramine during the postlabeling period caused rapid and near-complete depletion of preloaded SK-N-SH cells (Fig. 6A) which were minimally sensitive to reserpine. Conversely, reserpine rapidly depleted PC-12 cells whereas imipramine had little effect in these cells (Fig. 6B). The specific effect of imipramine in SK-N-SH cells strongly suggested that efficient re-uptake of [^{125}I]MIBG was indeed sufficient cause for its long-term retention. The apparent efficiency of the proposed mechanism of leakage/re-uptake suggest short-range interactions in or near the plasma membrane, minimizing the diffision of leaking drug into the surrounding medium. This notion was supported by the observation (data not shown) that MIBG retention in SK-N-SH was not affected by continuous agitation of the culture medium in cultures mounted on a gyratory shaker (60 strokes per min) nor by replacing the culture medium for a fresh one at hourly intervals.

DISCUSSION

Although it is generally believed that the neuron-blocking drug MIBG, similar to biogenic amines, is retained in specialized storage granules, no evidence for a significant contribution of this storage mechanism was obtained in case of SK-N-SH neuroblastoma cells, containing few electron dense cytoplasmic granules [12]. Permeabilization with digitonin under conditions of limited, i.e. 20% release of the macromolecular 134 kDa LDH marker caused rapid leakage of stored MIBG in a calcium-independent manner which paralleled that of the low MW deoxyglucose marker (Fig. 5; Table 1). Apparently, MIBG is not sequestered intracellularly in macromolecular complexes but as freely diffusible molecules. In view of the large nonspecific release of [^{3}H]NE from digitonin-treated PC-12 cells, indicative of gross damage to storage granules, it is obvious that these experiments are barely conclusive with regard to the possibility of intracellular compartimentalization of MIBG. On the other hand, in SK-N-SH cells exocytosis of MIBG was limited and the cells were insensitive to reserpine in comparison with corresponding experiments in PC-12 cells. All this would exclude a major contribution of cytoplasmic organelles, functionally related to storage granules, to drug storage in the neuroblastoma cells.

Depletion of SK-N-SH cells by the Uptake-1 inhibitor imipramine (Fig. 6A) indicated that high intracellular levels of MIBG are maintained in a dynamic equilibrium, generated by rapid re-uptake of drug diffusing from the cells. In other words, in SK-N-SH cells uptake and storage of MIBG are essentially similar processes, resembling those involved in maintaining ATP-driven ionic gradients. A depleting effect of imipramine by an action on intracellular stores is unlikely since the drug actually inhibits catecholamine release in bovine adrenomedullary cells [19]. [^{3}H]NE was not retained by a similar mechanism of re-uptake (Figs 2 and 4), a phenomenon which was attributable to intracellular breakdown of the natural catecholamine by pyro-

Table 1. Effect of membrane depolarization and cell permeabilization on the release* of MIBG, norepinephrine (NE) and deoxyglucose (dG1) in SK-N-SH and PC-12 cells†

	SK-N-SH		PC-12	
Treatment	[^{3}H]dG1	[^{125}I]MIBG	[^{3}H]dG1	[^{3}H]NE
Controls	13.3 ± 0.9	12.4 ± 1.5	18.6 ± 2.0	11.7 ± 3.8
56 mM KCl	0.3 ± 1.1	19.3 ± 2.7	5.5 ± 2.2	66.3 ± 4.0
Digitonin − Ca^{2+}	79.7 ± 2.5	84.6 ± 2.5	73.1 ± 2.2	59.1 ± 3.5
Digitonin + Ca^{2+}	54.4 ± 4.0	73.9 ± 3.2	66.4 ± 4.1	70.4 ± 2.8

* Determined after 20 min as total release in controls or as specific release (total − control) in the treated groups. Values are expressed in per cent of total radioactivity and are average values ± SE of three experiments.

† Cells were preloaded for 2 hr in 10^{-7} M of radiolabeled MIBG or NE. Average control levels (100%) were 1.0 nmoles MIBG/mg P and 2.4 nmoles NE/mg P, respectively.

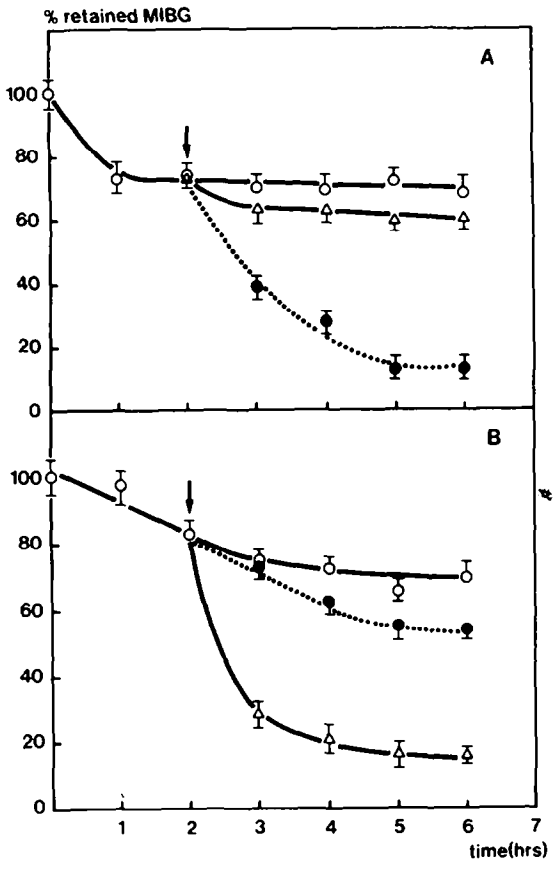

Fig. 6. The depleting effects of reserpine (2×10^{-6} M) and imipramine (1.25×10^{-6} M) on [^{125}I]MIBG stored in SK-N-SH and PC-12 cells. Cells were incubated with [^{125}I]MIBG at 10^{-7} M for 2 hr, washed and kept in regular medium for a further 2 hr (control levels: 1,1 and 0.8 nmoles MIBG/mg P for SK-N-SH and PC-12, respectively). Drugs were added from 100-fold concentrates (arrows) and the residual cell-associated radioactivity was determined at hourly interval in control cultures (○) and in cultures exposed to reserpine (△) or imipramine (●). (A) The depleting effect of imipramine on MIBG storage in SK-N-SH cells. (B) The depleting effect of reserpine on MIBG storage in PC-12 cells. Values are averages (±SE) of three experiments.

gallol-sensitive COMT. The metabolic stability of MIBG has been documented in animal experiments and in human patients [20]. In bovine adrenomedullary cells [6], SK-N-SH neuroblastoma cells [7] and explanted pheochromocytoma tumors [8] only minor differences have been observed in the kinetic parameters V_{max} and K_m of the rate of uptake of MIBG and NE. The apparent superior affinity of MIBG over NE for Uptake-I as demonstrated in the present experiments is most likely due to selective breakdown of NE and not necessarily in conflict with the published kinetic data. In fact, our data may be more representative of clinical administration of MIBG, usually in 4-hr infusions.

The question whether extragranular storage also contributes to MIBG retention in clinical neuroblastoma remains to be resolved. These tumors are efficiently portrayed by [^{131}I]MIBG scintigraphy in spite of low granule content (see the introduction). Tumors generated in nude mice by injection of SK-N-SH cells accumulate and retain [^{131}I]MIBG similarly as observed with human neuroblastoma [21]. Primary explants of such tumors respond to pharmacological intervention almost identical as described for the parent cells in Fig. 6, i.e. demonstrating extragranular accumulation up to 70% of total uptake (unpublished). Rapid depletion of extragranular MIBG *in vivo* by tumor blood flow could not be simulated in the *in vitro* model by agitation or frequent replacement of the culture medium. Accordingly, extragranular storage of MIBG *in vivo* may be a realistic possibility which is currently under investigation.

Apart from its possible relevance as a model of clinical neuroblastoma, the uptake and retention of MIBG by SK-N-SH cells appears an attractive tool to investigate neuron-specific Uptake-I. For instance, in granule-rich adrenomedullary and pheochromocytoma cells, catecholamine uptake kinetics reflect aggregated values of the tandem array of active Uptake-1 at the plasma membrane and active translocation into storage granules. As a result, damage to storage granules in PC-12 cells by reserpine [12] or by lipid peroxidation [22] strongly inhibits the overall uptake of catecholamines. Moreover, the mono-amine precursor MIBA, whose lipophylic iodinated benzyl moiety is identical to that of MIBG, was a weak competitor of MIBG and NE uptake (Fig. 3). This would suggest that the structural requirements for high-affinity interaction with the transport system reside in the hydrophylic diamine side chain of MIBG and not in the lipophylic ring moiety as was suggested by Jaques *et al.* [8]. Accumulation of metabolically stable MIBG in granule-poor SK-N-SH cells, reflecting "pure" Uptake-1, thus possesses interesting potential for exploring these and various other properties and structural requirements of catecholamine uptake in intact cells.

Acknowledgements—This study was supported by the Dutch Cancer Society under grant NKI-87-16. E.M. was a visiting scientist from Cairo University, supported by the Channel System between the Arabic Republic of Egypt and the German Federal Republic.

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