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Characterization of Serotonin Uptake in Cultured Pheochromocytoma Cells

Comparison with Norepinephrine Uptake

JOAN R. YOFFE[1] AND RONALD T. BORCHARDT

Department of Biochemistry, The University of Kansas, Lawrence, Kansas 66045

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SUMMARY

PC-12 cells, derived from a rat pheochromocytoma, were found to take up tritiated serotonin ([^{3}H]5-HT) from the external medium by means of a saturable mechanism which follows Michaelis-Menten kinetics. The apparent K_m of uptake was 0.39 μM and the V_{max} was 0.40 pmole/min/10^6 cells. The uptake was temperature-dependent, partially sodium-dependent, and inhibited by selected metabolic inhibitors (sodium azide, 2,4-dinitrophenol, and iodoacetamide). PC-12 cells also accumulated tritiated norepinephrine ([^{3}H]NE) by a saturable process, with an apparent K_m of 1.13 μM and a V_{max} of 1.72 pmole/min/10^6 cells. This NE uptake process was also temperature- and sodium-dependent and inhibited by metabolic inhibitors and ouabain. Desmethylimipramine (DMI, IC_{50} = 3.8 μM) was a better inhibitor of [^{3}H]NE uptake than fluoxetine (IC_{50} = 24.6 μM). The NE uptake process was structurally specific, since unlabeled NE was a better inhibitor of [^{3}H]NE uptake than 5-HT (IC_{50} = 19.6 and 171 μM, respectively). However, [^{3}H]5-HT uptake in PC-12 cells appeared to be a less structurally specific process, as it was equally inhibited by unlabeled 5-HT and NE (IC_{50} 4.9 μM and 4.3 μM, respectively). DMI was also a better inhibitor of [^{3}H]5-HT uptake than fluoxetine (IC_{50} = 85 and 411 μM, respectively). The neurotoxins 6-hydroxydopamine and 5,6-dihydroxytryptamine were cytotoxic to PC-12 cells, causing a time- and concentration-dependent inhibition of [^{3}H]thymidine incorporation into DNA. 5,7-Dihydroxytryptamine had little cytotoxic effect toward PC-12 cells in culture.

INTRODUCTION

A clonal cell line (PC-12) has recently been derived from an induced transplantable rat adrenal pheochromocytoma (1). These cells express the differentiated properties of adrenal chromaffin cells, such as high levels of enzymes capable of synthesizing catecholamines (2, 3) and high endogenous concentrations of catecholamines, which are stored in dense core granules and released by reserpine and depolarizing stimuli (4–6). In culture, PC-12 cells need ascorbic acid supplement to convert DA[2] to NE (7, 8). PC-12 cells are also capable of synthesizing and storing acetylcholine and γ-aminobutyric acid (9). Treatment with NGF or glial-conditioned medium (10) causes expression of neuronal properties, such as extension of neurites (11), cessation of cell division, (12) and acquisition of an electrically excitable membrane (13–15). The recent demonstration that human chromaffin cells respond to NGF in a similar way suggests that this response is normal and not associated with malignancy (16). The activity of the enzymes involved in transmitter synthesis increases with cell density and after treatment with dexamethasone (17–19). However, unlike neuroblastoma cells, in PC-12 cells the activity of tyrosine hydroxylase and the endogenous level of catecholamines decrease during differentiation (20). It has also been shown that differentiation does not alter the apparent K_m of NE uptake, although the V_{max} is considerably lower in untreated cells (21).

PC-12 cells have been used as model systems for adrenergic and cholinergic neurons. Since chromaffin granules also take up and store 5-HT (22), the aim of this work was to determine whether PC-12 cells possess an active uptake mechanism for 5-HT. If so, PC-12 cells might serve as a suitable model of serotonergic neurons

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[1] Present address, Department of Medicine, University Hospital of South Manchester, Manchester M20 8LR, United Kingdom.

[2] The abbreviations used are: DA, dopamine; NE, norepinephrine; NGF, nerve growth factor; 5-HT, 5-hydroxytryptamine; 5,6-DHT, 5,6-dihydroxytryptamine; 5,7-DHT, 5,7-dihydroxytryptamine; DMI, desmethylimipramine; 6-OHDA, 6-hydroxydopamine; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; TCA, trichloroacetic acid.

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for a study to determine the mechanism of action of dihydroxytryptamine neurotoxins (5, 6-DHT and 5,7-DHT). The results demonstrate that undifferentiated PC-12 cells can accumulate 5-HT by a process having the characteristics of 5-HT uptake in neuronal cells. It is concentration- and sodium-dependent and is inhibited by metabolic inhibitors, low temperatures, fluoxetine, and DMI. However, comparison with the NE uptake in these cells suggests that the 5-HT uptake system is less specific.

MATERIALS AND METHODS

Source of chemicals. [1,2-^{3}H]5-HT creatinine sulfate (32.1 Ci/mmole) and [7-^{3}H]NE (3.2 or 4.3 Ci/mmole) were purchased from New England Nuclear Corporation (Boston, Mass.) and diluted 1:10 in Buffer B or C. The following drugs were purchased from Sigma Chemical Company (St. Louis, Mo.): DMI·HCl, 5,6-DHT, and 5,7-DHT creatinine sulfates; 3,4-dinitrophenol, 5-HT creatinine sulfate, 6-OHDA·HBr, NE·HCl, and ouabain octahydrate. Other drugs used were fluoxetine·HCl (Lilly), iodoacetamide (Fischer Scientific), sodium azide (Aldrich Chemical), and [^{3}H]thymidine (60 Ci/mmole, Schwarz-Mann).

Cell culture. Starter cultures of pheochromocytoma cells clone PC-12 were kindly provided by Dr. X. O. Breakefield (Yale University School of Medicine). Cells were grown in monolayer culture and passaged by trituration. Cell doubling time was 60–70 hr. The medium used was DMEM [GIBCO], supplemented with 15% FCS (KC Biologicals or M. A. Bioproducts) and gentamycin sulfate, 70 mg/liter (Sigma Chemical Company). Cells were plated at 2×10^6 cell/100-mm dish, fed at 2- to 3-day intervals and used after 1 week.

Incubation conditions and procedures. Buffer A was an isotonic modified Dulbecco's phosphate-buffered saline, containing 129 mM NaCl, 2.5 mM KCl, 7.4 mM Na_2HPO_4, and 1.3 mM KH_2PO_4. Buffer B was prepared fresh daily by supplementing Buffer A with 0.63 mM $CaCl_2$, 0.74 mM $MgSO_4$, 5.3 mM glucose, and 0.1 mM ascorbic acid. Buffer C was prepared for sodium-free studies by substituting choline chloride for NaCl in Buffer A and supplementing with 0.63 mM $CaCl_2$, 0.74 mM $MgSO_4$, 5.3 mM glucose, and 0.1 mM ascorbic acid.

Confluent monolayer cultures were rinsed and taken up in Buffer B or C. Cell suspensions were prepared containing approximately 10^7 cells/ml. The uptake of [^{3}H]5-HT or [^{3}H]NE was terminated by placing the tubes in ice, diluting with 5 ml of cold Buffer A, and filtering immediately through GF/C glass-fiber filters in a Millipore manifold. Each tube was rinsed with 5 ml of cold Buffer A. The filters were rinsed with an additional 15 ml of cold Buffer A, placed in glass scintillation vials, dried in a warm oven for 15 min, and digested with 0.5 ml of NCS tissue solubilizer (Amersham). To each sample were added 10 ml of toluene-based preblended dry fluors 2a70 (Research Products International Corporation) and the samples were counted, after overnight storage in the dark, using a Beckman LS-7500 scintillation counter. The amount of radioactivity bound nonspecifically to the filters in the absence of cells was subtracted from total counts.

Uptake of [^{3}H]5-HT. To determine the time course of [^{3}H]5-HT uptake, aliquots (100 μl) of cell suspension were incubated for up to 20 min with 10 μl of [^{3}H]5-HT solution and sufficient Buffer B or C to give a final concentration of 0.1 μM. To determine the apparent K_m of uptake, 2.5–50 μl of [^{3}H]5-HT solution were added to 100 μl of cell suspension and the appropriate volume of Buffer B or C to give final concentrations of 0.025–0.5 μM. Incubation was carried out for 2 min at 37° or at 0–4°.

Uptake of [^{3}H]NE. To determine the time course of [^{3}H]NE uptake, aliquots (100 μl) of cell suspension were incubated for up to 60 min with 20 μl of [^{3}H]NE solution and sufficient Buffer B to give a final concentration of 1 μM. To determine the apparent K_m of uptake, 2–80 μl of [^{3}H]NE solution were added to 100 μl of cell suspension and the appropriate volume of Buffer B or C to give a final concentration of 0.1–4 μM and incubated for 5 min at 37° or at 0–4°.

Inhibition of [^{3}H]5-HT and [^{3}H]NE uptake. Aliquots (100 μl) of cell suspension were incubated for 15 min with 1 ml of the following drug solutions (final concentrations): iodoacetamide (5 mM), sodium azide (10 mM), 2,4-dinitrophenol (1 mM), ouabain (1 mM), fluoxetine (0.1–50 μM), and DMI (0.1–50 μM). Cells were separated by centrifugation and resuspended in 100 μl of Buffer B. The suspension was then incubated at 37° with [^{3}H]5-HT (0.1 μM) for 2 min or with [^{3}H]NE (1 μM) for 5 min.

Specificity of [^{3}H]5-HT and [^{3}H]NE uptake. Solutions of unlabeled 5-HT, NE, 6-OHDA, 5,6-DHT, and 5,7-DHT were prepared immediately before use. Aliquots of drug solutions giving final concentrations of 0.1–50 μM were added to 100 μl of cell suspension at the same time as sufficient tracer to give an over-all concentration of 0.1 μM [^{3}H]5-HT or 1 μM [^{3}H]NE. Cells were incubated at 37° for 2 or 5 min, respectively.

Cytotoxicity of neurotoxic analogues. Cytotoxicities of 6-OHDA, 5,6-DHT, and 5,7-DHT were determined by measuring the inhibition of incorporation of [^{3}H]thymidine into DNA. After incubation in drug solution for 30 or 90 min the cells were separated by centrifugation and washed once with Buffer A. The cell pellets were resuspended in 500 μl of DMEM, 1 μl of [^{3}H]thymidine was added, and the cells were incubated for a further 90 min. Incorporation was terminated by placing the tubes on ice and adding 5 ml of cold 5% TCA. After 10 min the precipated ^{3}H-labeled DNA was collected on GF/C glass-fiber filters previously wetted with 20% TCA, and the tubes were rinsed with 5 ml of cold 5% TCA. The filters were rinsed with 15 ml of 1% TCA and 3 ml of 95% EtOH, then dried in a warm oven, solubilized in 0.5 ml of NCS, and counted in 10 ml of toluene-based scintillation fluid as described above.

Expression and analysis of results. Results were standardized by expression as uptake per 10^6 cells. Each experiment was performed three times in duplicate and the mean values ± standard error of the mean are given. Counts per minute were converted to absolute values using external standards. The active uptake of [^{3}H]5-HT or [^{3}H]NE was calculated by subtracting the accumulation due to passive diffusion (0–4°) from the total accumulation at 37°. Kinetic analysis and calculation of IC_{50} values were carried out as described previously (23).

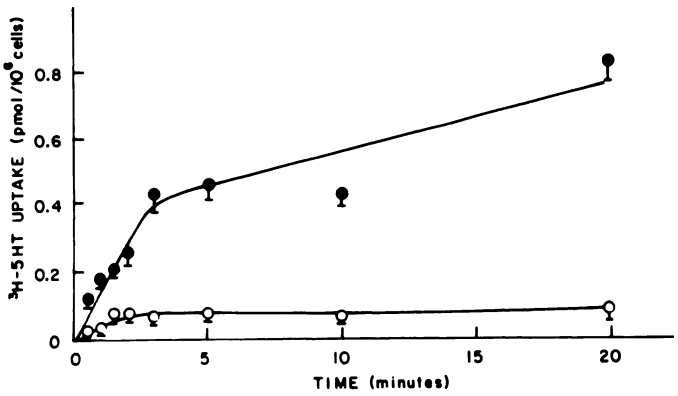

FIG. 1. *Apparent uptake of [^{3}H]5-HT as a function of time*
PC-12 cells in suspension (10^6 cells/100 μl) were incubated in 0.1 μM [^{3}H]5-HT at 37° (●) or at 0–4° (○) as described under Materials and Methods. [^{3}H]5-HT uptake was terminated after a 30-sec to 20-min incubation by dilution and filtration through glass-fiber filters, as described under Materials and Methods.

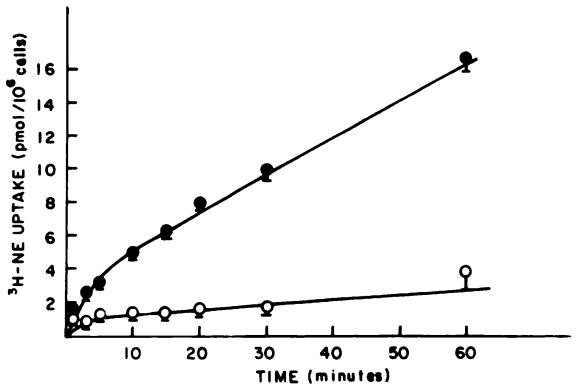

FIG. 3. *Apparent uptake of [^{3}H]NE as a function of time*
PC-12 cells in suspension (10^6 cells/100 μl) were incubated in 1 μM [^{3}H]NE at 37° (●) or 0–4° (○) for 1–60 min as described under Materials and Methods.

RESULTS

Characterization of [^{3}H]5-HT uptake. The rate of accumulation of [^{3}H]5-HT at 37° was constant for up to 3 min of incubation and thereafter occurred at a much slower rate (Fig. 1). At 0–4° the uptake exhibited saturation but was reduced to 10–30% of the values observed at 37°.

The velocity of uptake was determined over the concentration range 0.025–0.5 μM (Fig. 2). The uptake at 37° was concentration-dependent and exhibited Michaelis-Menten type kinetics. The uptake at 0–4° was also concentration-dependent but represented only 7–20% of the uptake at 37°. The values obtained at 0–4° were considered to be the nonsaturable linear component due to diffusion, and these values were subtracted from the total uptake to give the active uptake. In the absence of external sodium the active uptake was reduced by 43–53%.

Characterization of [^{3}H]NE uptake. The rate of accumulation of [^{3}H]NE at 37° was rapid for at least the first 5 min, and thereafter occurred at a much slower rate (Fig. 3). The uptake at 0–4° was reduced to 17–40% (n = 3). The velocity of uptake was determined over the concentration range 0.1–4 μM (Fig. 4). The uptake at 37° was concentration-dependent and exhibited saturation kinetics. At 0–4° or in the absence of sodium, the uptake of [^{3}H]NE was almost completely inhibited.

Lineweaver-Burk analysis. Lineweaver-Burk plots were drawn of the active uptake of [^{3}H]5-HT and [^{3}H]NE (Fig. 5). Analysis of these plots gave an apparent K_m of 0.39 ± 0.1 μM and V_{max} of 0.40 pmole/min/10^6 cells for the [^{3}H]5-HT uptake and an apparent K_m of 1.13 ± 0.7 μM and V_{max} of 1.72 pmoles/min/10^6 cells for the [^{3}H]NE uptake.

Effect of metabolic inhibitors. The ability of iodoacetamide (5 mM), sodium azide (10 mM), and 2,4-dinitrophenol (1 mM) to block the accumulation of [^{3}H]5-HT and

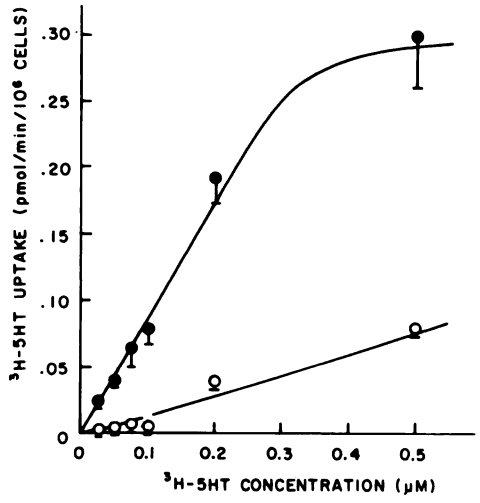

FIG. 2. *Apparent uptake of [^{3}H]5-HT as a function of concentration*
PC-12 cells in suspension (10^6 cells/100 μl) were incubated for 2 min with increasing concentrations of [^{3}H]5-HT at 37° (●), 0–4° (○) or in sodium-free buffer at 37° (▲), as described under Materials and Methods.

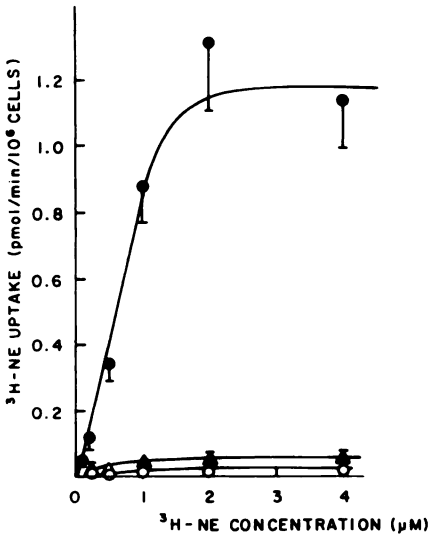

FIG. 4. *Apparent uptake of [^{3}H]NE as a function of concentration*
PC-12 cells in suspension (10^6 cells/100 μl) were incubated for 5 min with increasing concentrations of [^{3}H]NE at 37° (●), 0–4° (○), or in sodium-free buffer at 37° (▲) as described under Materials and Methods.

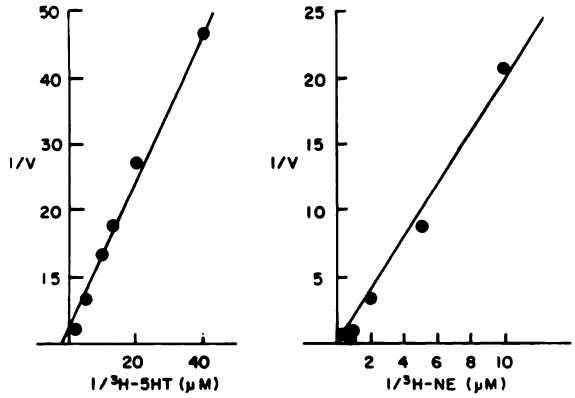

FIG. 5. *Lineweaver-Burk plots of the active uptake of [^{3}H]5-HT and [^{3}H]NE into PC-12 cells*

Active uptake was determined by subtracting the accumulation at 0–4° from the total accumulation at 37°. The reciprocals of the velocity (V) of active uptake (expressed as picomoles of [^{3}H]5-HT or [^{3}H]NE per 10^6 cells per minute) were plotted against the reciprocals of the external concentration of [^{3}H]5-HT or [^{3}H]NE.

Left. [^{3}H]5-HT (0.025–0.5 μM): K_m = 0.39 μM, V_{max} = 0.40 pmole/min/10^6 cells.

Right. [^{3}H]NE (0.1–4 μM): K_m = 1.13 μM, V_{max} = 1.72 pmoles/min/10^6 cells.

[^{3}H]NE uptake indicates that the uptake occurred by active transport processes (Table 1). Ouabain (1 mM) also inhibited [^{3}H]NE uptake but had no effect on [^{3}H]5-HT uptake.

Effect of antidepressants. Fluoxetine and DMI are known to inhibit both [^{3}H]5-HT and [^{3}H]NE uptake, but fluoxetine is usually more specific for [^{3}H]5-HT (24, 25) and DMI more specific for [^{3}H]NE. As expected, DMI was more effective at blocking [^{3}H]NE uptake than [^{3}H]5-HT uptake. However, it was also more effective than fluoxetine at inhibiting [^{3}H]5-HT uptake (Table 2).

Specificity of uptake. The uptake of [^{3}H]NE appeared to be more structurally specific as it was selectively inhibited by NE, whereas the uptake of [^{3}H]5-HT was equally inhibited by both NE and 5-HT (Table 2). However, 6-OHDA had no effect on [^{3}H]5-HT uptake whereas both 5,6-DHT and 5,7-DHT antagonized the uptake of [^{3}H]5HT, although less effectively than 5-HT.

Cytotoxicity of neurotoxic analogues. 6-OHDA was the most effective neurotoxic analogue, causing considerable inhibition of [^{3}H]thymidine incorporation after 30 and 90 min of incubation (Fig. 6). 5,6-DHT was less effective after 30 min but equally cytotoxic after 90 min of incubation. 5,7-DHT had no significant cytotoxic effects.

TABLE 1

Effect of metabolic inhibitors on the uptake of [^{3}H]5-HT and [^{3}H]NE

PC-12 cells in suspension (10^6 cells/100 μl) were incubated with the metabolic inhibitors for 15 min. The cells were washed, then incubated with 0.1 μM [^{3}H]5-HT for 2 min or with 1 μM [^{3}H]NE for 5 min. Results were expressed as percentage inhibition of active uptake.

Inhibitor	Concentration	% Inhibition of [^{3}H]5-HT uptake	% Inhibition of [^{3}H]NE uptake
	mM		
Iodoacetamide	5	51 ± 4	47 ± 3
Sodium azide	10	40 ± 7	46 ± 2
2,4-Dinitrophenol	1	53 ± 6	61 ± 1
Ouabain	1	5 ± 9	42 ± 3

TABLE 2

Effect of antidespressants and unlabeled analogues on the uptake of [^{3}H]5-HT and [^{3}H]NE

PC-12 cells in suspension (10^6 cells/100 μl) were either preincubated with fluoxetine (0.5–50 μM), DMI (0.5–50 μM), or unlabeled analogues added at the same time as [^{3}H]5-HT and [^{3}H]NE. Cells were incubated with 0.1 μM [^{3}H]5-HT for 2 min or with 1 μM [^{3}H]NE for 5 min. Results were expressed as concentrations causing 50% inhibition of active uptake (IC_{50}).

Drug	IC_{50}	
	[^{3}H]5-HT	[^{3}H]NE
	μM	*μM*
Fluoxetine	411	24.6
DMI	85	3.8
5-HT	4.9	171
NE	4.3	19.6
6-OHDA	No effect	
5,6-DHT	21.8	
5,7-DHT	47.6	

DISCUSSION

The results of this study indicate that PC-12 cells can accumulate 5-HT from the external medium by a nonsaturable diffusion process and by a high-affinity transport process. The high-affinity process is saturable, temperature-dependent, and followed apparent Michaelis-Menten kinetics. The apparent K_m of uptake was 0.39 μM, which was very similar to that reported for serotonergic neurons and platelets (26). This process was energy-dependent and partially sodium-dependent but not

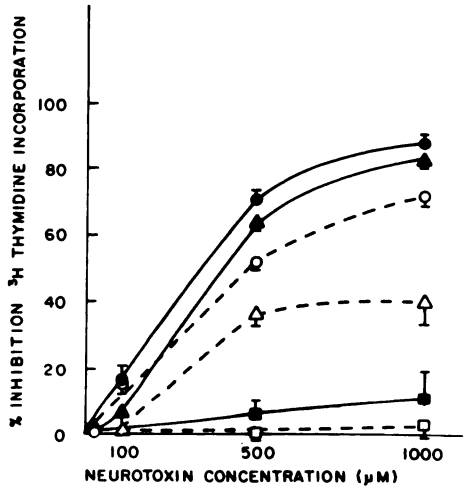

FIG. 6. *Cytotoxicity of 6-OHDA, 5,6-DHT, and 5,7-DHT*

PC-12 cells in suspension (10^6 cells/100 μl) were incubated for 30 min (*open symbols*) or for 90 min (*closed symbols*) with 6-OHDA (○, ●) 5,6-DHT (△, ▲), or 5,7-DHT (□, ■). The cells were washed once with Buffer A, then incubated for an additional 90 min with [^{3}H]thymidine (0.5 μCi/500 μl). [^{3}H]Thymidine incorporation was terminated by TCA precipitation and filtration through glass-fiber filters, as described under Materials and Methods. Cytotoxicity was expressed as percentage inhibition of [^{3}H]thymidine incorporation.

inhibited by ouabain. The 5-HT uptake in PC-12 cells was less susceptible to inhibition by DMI and fluoxetine than the uptake mechanism in neuroblastoma cells (23) and other cell types (24–26). DMI was 5 times more effective than fluoxetine in inhibiting 5-HT uptake in PC-12 cells. These findings with DMI and fluoxetine and the observation that unlabeled 5-HT was less effective in reducing [^{3}H]5-HT uptake in PC-12 than in neuroblastoma cells (23) and no more effective than unlabeled NE suggest that the serotonin carrier in PC-12 cells is quite nonspecific. However, [^{3}H]5-HT uptake was inhibited by both 5,6-DHT and 5,7-DHT, whereas 6-OHDA caused only 3–18% inhibition. The effect with 6-OHDA was not dose-dependent (results not shown).

The NE uptake system in PC-12 cells has been characterized by Greene and Rein (21). The results of this investigation confirm that undifferentiated PC-12 cells possess a mechanism for taking up NE from the external medium similar to that present in central and peripheral noradrenergic nerve endings. The uptake was saturable and followed apparent Michaelis-Menten kinetics. The apparent K_m of uptake was 1.13 μM, which is comparable to that reported in noradrenergic nerve endings (27) and dissociated cell cultures of rat sympathetic neurons (28) and slightly lower than that previously observed in PC-12 cells (21). The apparent V_{max} of uptake was 1.72 pmoles/min/10^6 cells. This is considerably lower than that previously reported, 14 pmoles/min/10^6 cells, and may be related to the fact that these experiments were conducted with cells in suspension rather than in monolayers. Differentiation by NGF caused a further increase in observed V_{max} to 35 pmoles/min/10^6 cells, and this is believed to be due to the greater surface area of process-bearing cells rather than a direct effect on the uptake carrier (21). Nonmalignant bovine adrenal chromaffin cells in culture possess a similar high-affinity NE-uptake system (29). The K_m was lower than that observed with PC-12 cells (0.35–0.48 μM), but the V_{max} was similar (1.1–1.7 pmoles/min/10^6 cells). Neurite extension was seen after increased time in culture, but unlike PC-12 cells this change was not accompanied by dramatic changes in the kinetic characteristics of the NE uptake system.

The current study showed that [^{3}H]NE uptake was substantially inhibited at 0–4° and in the absence of external sodium, indicating that there was negligible accumulation by passive diffusion. The uptake was inhibited by metabolic inhibitors and ouabain. It also showed structural specificity, as it was inhibited by DMI at concentrations 6 times lower than the concentration of fluoxetine and by unlabeled NE at concentrations 9 times lower than the concentration of 5-HT.

These results suggest that, in PC-12 cells, NE is taken up by a selective carrier, whereas 5-HT is taken up by a much less specific mechanism. However, the K_m of apparent uptake of [^{3}H]5-HT observed was much lower than that reported for its uptake by the NE carrier (30), which suggests that there are two separate carriers present. Thus, the accumulation of [^{3}H]5-HT observed in this study may be by a combination of high-affinity and low-affinity active mechanisms.

In PC-12 cells, as in neuroblastoma cells (23), 6-OHDA and 5,6-DHT were shown to be equally cytotoxic, although 6-OHDA was more rapidly effective. 5,7-DHT again had very little cytotoxic action, despite its affinity for the serotonin receptor as indicated by its ability to reduce [^{3}H]5-HT uptake. This supports the theory that, whereas 5,6-DHT produces small amounts of a very reactive product, 5,7-DHT produces larger amounts of a much less reactive product (31). 5,6-DHT and 5,7-DHT are the most selective serotonin neurotoxins available at present but they are not very specific and cause considerable damage to noradrenergic neurons *in vivo*. Their selectivity *in vivo* has been increased by pretreatment with DMI (32) and this may be a useful technique to try when studying these drugs in PC-12 cells.

The results of this investigation suggest that PC-12 cells are a useful model with which to study 6-OHDA and, with reservations, 5,6-DHT and 5,7-DHT to elucidate their mechanism of action.

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Send reprint requests to: Dr. Ronald T. Borchardt, Department of Biochemistry, 2095 Avenue A, West Campus, Smissman Research Laboratories, The University of Kansas, Lawrence, Kan. 66045.