Biochemical Pharmacology, Vol. 26, pp. 1367–1372. Pergamon Press, 1977. Printed in Great Britain.

THE ACCUMULATION OF *p*-HYDROXYAMPHETAMINE BY BRAIN HOMOGENATES AND ITS ROLE IN THE RELEASE OF CATECHOLAMINES

ARTHUR K. CHO, JOSEPH F. FISCHER and JAMES C. SCHAEFFER*

Department of Pharmacology, UCLA Center for the Health Sciences, Los Angeles, CA 90024 and *Department of Chemistry, University of Missouri, Kansas City, Kansas City, MO 64110, U.S.A.

(*Received* 21 *February* 1976; *accepted* 29 *October* 1976)

Abstract—*p*-Hydroxyamphetamine (*p*-OHA) is accumulated by synaptosome enriched preparations from both striatal and cortical tissues. In studies with P_2 preparations from rat cortex the accumulation of *p*-OHA-[^{3}H] was shown to be temperature dependent but different from the process involved in the accumulation of norepinephrine-[^{3}H](NE[^{3}H]) by this tissue. The uptake of *p*-OHA was less sensitive to DMI and cocaine and had a different time dependency. The accumulation of *p*-OHA was concentration dependent and did not show evidence for saturation at concentrations up to 10 μM. The relationship between *p*-OHA uptake and catecholamine release from the striatum and cortex was also examined. The results suggest that much of the observed cortical uptake of *p*-OHA is not due to the neuronal carrier for norepinephrine.

p-Hydroxyamphetamine (*p*-OHA) is an indirectly acting sympathomimetic amine which is of particular pharmacological significance because it is a metabolite of amphetamine in most species examined and a major metabolite in the rat [1]. The compound has been implicated in the actions of amphetamine by a number of investigators [2–5] and is found in the brain after systemic administration of amphetamine [2, 3]. More recent work examining different areas of the brain has revealed a relatively uniform distribution of *p*-OHA [6] after administration of amphetamine.

In studies with *in vitro* preparations *p*-OHA has been shown to inhibit uptake and cause release of norepinephrine (NE) from cortical tissues [7–9]. The releasing actions of *p*-OHA, like those of tyramine [10], would be expected to be cocaine sensitive reflecting the participation of the neuronal transport system in this indirect action. Consistent with this hypothesis, the accumulation of *p*-OHA by striatal preparations occurs by a process that is similar to that responsible for dopamine in terms of its sensitivity to inhibitors, as well as its sodium and temperature dependency [9]. This report describes the results of a study of the accumulation of *p*-OHA by rat cortex preparations. In contrast to the striatum, the cortex appears to accumulate *p*-OHA by a temperature sensitive process into a pharmacologically inert compartment. A preliminary account of this work has been presented [11].

MATERIALS AND METHODS

Uptake of p-*hydroxyamphetamine.* Male Sprague–Dawley rats weighing 180–250 g were killed by decapitation and the striata and cortices removed. Pooled tissues from at least six animals were homogenized in 9 vol. of 0.32 M sucrose and synaptosome enriched P_2 fractions [12] were prepared and washed as described previously [13]. The P_2 fractions were resuspended in 0.32 M sucrose to give final protein concentrations of 2–3.5 mg/ml and 10–13 mg/ml for striatal and cortical preparations respectively. A 0.1 ml aliquot of P_2 suspension was added to 20 ml glass vials containing 0.55 ml modified [13] Krebs–Ringer phosphate, pH 7.4, containing nailamide (12.5 μM) and glucose (8.4 mM). After a 5 min preincubation at 37°, *p*-OHA-[^{3}H] (0.24 μCi + carrier) was added to bring the final vol. to 0.7 ml and the mixture was incubated for the desired time period.

Uptake was quenched by the addition of cold (0°) 0.9% NaCl that was 100 μM in *p*-OHA and the resulting mixture was filtered through a Milipore filter (0.8 μ). The filters were washed with an additional 5 ml of quenching solution, then dissoved in Bray's solution [14] for counting. Net uptake was calculated as the difference in radioactivity between uptake at 37° and 0°. A zero time blank analogous to that of Harris and Baldessarini [8] gives a slightly lower value (80% of 0° blank) but was not used. Each determination was carried out in quadruplicate. Amine accumulation was linear with respect to protein concentrations over the ranges used. The nature of radioactivity remaining in the baths after a 3 min incubation was examined by paper chromatographic procedures [15] to determine the extent of metabolism of the amines. The extent of metabolism of *p*-OHA, NE and DA were 12, 9 and 10 per cent, respectively, over this time period. The radioactivity in the tissue after a 3 min incubation with *p*-OHA was shown to be 90% *p*-OHA in both striatal and cortical preparations.

Efflux of catecholamines from P_2 *preparations.* Striatal and cortical tissues were coarsely minced with scissors and preincubated in 10 ml of the modified Krebs–Ringer phosphate at 37° for 5 min. The appropriate amine was then added to a final concentration of 0.5 μM and the mixture was incubated for 10 min at 37° to allow uptake. Since the presence of inorganic cations, particularly Ca^{2+} causes clumping it was

necessary to remove the inorganic ions before preparing the P_2 pellet. To remove the Krebs solution, the mixture was poured into a centrifuge tube, diluted with 20 ml of 0.32 M sucrose and centrifuged at 1000 *g* for 5 min. The pellet was resuspended and homogenized in 9 vol. of fresh 0.32 M sucrose and a P_2 suspension prepared as above. A P_2 suspension prepared in this way contained 3 mg/ml striatal, and 10 mg/ml cortical protein. The uptake capacity was equal to that prepared from fresh tissue within experimental variation.

A 0.1 ml aliquot of the resuspended P_2 pellet was added to 0.55 ml of the incubation media and after a 5 min preincubation at 37°, a 0.05 ml aliquot of phosphate buffer (0.1 M, pH 7.4) with or without the releasing amine was added. Release was terminated after 3 min as above by the addition of cold 0.9% NaCl and filtration. The radioactivity remaining on the filter was determined and the difference in activity between buffer and drug-incubated filters was used as the quantity released. Six to eight samples of each condition were run and the standard deviation of the means were about 5 per cent. Preloading the tissue with norepinephrine or dopamine in this manner did not alter the accumulation of *p*-hydroxyamphetamine.

The nature of the radioactivity released was examined by concentrating the Milipore filtrate at reduced pressure and examining the distribution of radioactivity by paper chromatography according to Levin [15]. The dopamine released by 1 μM *p*-OHA from striatal tissue was 42 per cent of the total radioactivity released and norepinephrine was 42 per cent of the total radioactivity released by *p*-OHA at 5 μM *p*-OHA from cortical preparations.

The concentration of *p*-OHA for half maximal release (ED_{50}) was estimated with regression procedures developed for a Wang 600 calculator. The concentration vs. release data over ranges of 0.1–3.0 μM for striatal tissue was fitted to the equation

$$\text{release} = \frac{R_{max} \cdot [p\text{-OHA}]}{ED_{50} + [p\text{-OHA}]}$$

by a nonlinear regression program [16].

Distribution of accumulated amines. Cortical and striatal P_2 pellets, containing *p*-OHA-[^{3}H], or NE-[^{3}H] prepared from minces of 3 brains as described above were resuspended either in 0.32 M sucrose or distilled water by gentle homogenization in a Teflon-glass tissue homogenizer. A 2 ml aliquot of the homogenate equivalent to 0.6 g brain was layered onto centrifuge tubes containing a discontinuous gradient of 4 ml aliquots of 0.4, 0.6, 0.8, 1.0, 1.2 and 1.4 M sucrose. The tubes were centrifuged at 50,000 *g* in a swinging bucket rotor for 2 hr. The contents of the tubes were removed by introducing 2.0 M sucrose through a needle inserted into the bottom at a rate of 1 ml/min. In this manner 1 ml fractions of the gradient were obtained, of which a 50 λ aliquot was removed for protein determination and the remainder placed in a scintillation vial for counting.

Chemical methods. Protein estimations utilized the procedure of Lowry *et al.* [17] with crystalline bovine serum albumin as the standard.

Racemic *p*-hydroxyamphetamine-[^{3}H] was obtained as a catalytic reduction product from 3,5,diiodo-4-

Table 1. Properties of amine accumulation processes

Amine	Temperature effects: Ratio of 37° to 0 uptake by P_2 fractions
Amphetamine (1.5 μM)	1.8
Antipyrine (3.2 μM)	1.1
p-Hydroxyamphetamine (1 μM)	14.6
l-Norepinephrine (1 μM)	20

Condition	*p*-Hydroxyamphetamine (0.1 μM) accumulation: Striatum	Cortex
Accumulation at 5 min	33 pmoles/mg protein	1.5 pmoles/mg protein
Percent released with* hypoosmotic conditions	75%	80%
Percent reduction with† low (23 mM) sodium ion concentration	60%	30%

The accumulation of the amines by brain P_2 fractions was measured with the tritium labelled amine and the Milipore filtration procedure described in Methods. The temperature effects were measured with P_2 fractions prepared from brains that had striata removed. Each value is the mean of at least 3 separate incubations.

* Hypoosmotic conditions utilized phosphate buffer (0.1 M) instead of isotonic NaCl as the quenching solution.

† The effect of reduced sodium ion concentration reflects a change from 123 meq/liter to 23 meq/liter.

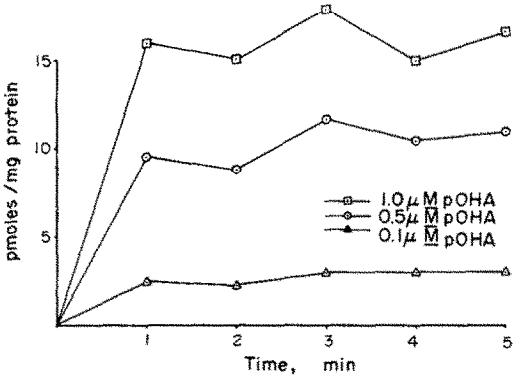

Fig. 1. Time course of *p*-OHA accumulation by cortical P_2 preparations. P_2 fractions from cortical tissue were incubated with *p*-OHA for varying time periods then worked up as indicated in Methods. Three different concentrations of *p*-OHA were used and are indicated in the figure.

Table 2. Accumulation of amines by P_2 fractions with time

Time (min)	Striatum DA (pmoles/mg)	Striatum *p*-OHA (pmoles/mg)	Cortex NE (pmoles/mg)	Cortex *p*-OHA (pmoles/mg)
1	25.8 ± 0.3	8.5 ± 0.3	0.9 ± 0.1	2.4 ± 0.6
2	34.4 ± 0.3	9.2 ± 0.2	1.7 ± 0.3	2.2 ± 0.6
3	37.6 ± 1.4	11.6 ± 0.1	4.1 ± 1.1	2.8 ± 0.6

The accumulation was measured at the indicated times at amine concentrations of 0.1 μM. Each value is the mean of ± S.D. of 6 determinations.

Table 3. Effect of reserpine pretreatment on accumulation

Time min	Striatum				Cortex			
	Control		Reserpine		Control		Reserpine	
	DA	*p*-OHA	DA	*p*-OHA	NE	*p*-OHA	NE	*p*-OHA
1	49.2 ± 6.0	39.6 ± 5.7	40.7 ± 0.4	15.7 ± 2.4	0.6 ± 0.02	1.8 ± 0.02	0.4 ± 0.05	2.1 ± 0.09
2	94.8 ± 7.5	46.8 ± 2.9	58.9 ± 2.9	20.8 ± 0.8	1.3 ± 0.10	1.7 ± 0.29	0.5 ± 0.05	2.1 ± 0.09
3	129.2 ± 11.2	63.9 ± 8.2	89.3 ± 5.3	28.1 ± 1.6	1.7 ± 0.15	2.2 ± 0.29	0.7 ± 0.10	2.1 ± 0.09

Animals were pretreated with reserpine (5 mg/kg i.p.) 20 hr before preparation of brain homogenates.
The accumulation of the amines by control and pretreated preparations were examined at amine concentrations of 0.2 μM for striata and 0.1 μM for cortex. The striatal accumulation experiments were conducted at 25° while cortical uptake experiments were carried out at 37°. Each value is the mean (pmole/mg protein ±S.D.) of 6 determinations.

hydroxyamphetamine, which was prepared by iodination of *p*-hydroxyamphetamine in NH_4OH[18]. The product was recrystallized and a sample sent to the New England Nuclear Corp. for reduction. The crude *p*-hydroxyamphetamine-[^{3}H] obtained as the reaction product was chromatographed on paper with a butanol–ethanol–water (4:1:1) solvent. The desired product has an R_f of 0.54 under these conditions and a 3 cm section of the appropriate R_f was cut out and extracted for 2 hr in refluxing methanol. The methanol was evaporated and the residue dissolved in 0.01 N acetic acid. The concentration was determined by absorption at 275 nm and the sp. act. obtained was 17.7 Ci/m-mole. The purity of the material was reexamined by thin layer chromatography on cellulose with the same solvent and found to be 95 per cent. All experiments were conducted with racemic *p*-hydroxyamphetamine·HBr.

RESULTS

The accumulation of *p*-OHA described in these experiments is a temperature sensitive uptake with an accumulation at 37° of about 15 times that at 0° (Table 1). For comparison, the ratio of 37° to 0° uptake for the NE is 20 while the ratios for the freely diffusable antipyrine [19] and amphetamine are 1.1 and 1.8, respectively. As accumulation is measured by trapping particles in the incubation mixture on Milipore filters (0.8 μm), it could also include radioactivity bound to the outer surface of the particles. In an attempt to correct for this possibility, the uptake process was quenched by 10-fold dilution in cold isotonic NaCl that was 100 μM in carrier substrate so that surface bound radioactivity would be removed by exchange. When the incubate was quenched in the same vol. of 100 μM carrier in hypotonic buffer, a reduction of 75–80 per cent resulted indicating that the accumulation occurred in an osmotically sensitive compartment. Reduction of sodium ion concentration reduced accumulation with the striatal process being more sensitive.

The time course (Table 2, Fig. 1) of the *p*-OHA accumulation was different in the two P_2 fractions. Uptake into striatal P_2, while not linear with time, continuously increased from 0 to 5 min in a manner similar to that described by Snyder and Coyle [20]. Uptake into cortical P_2 was initially rapid, reaching a plateau at less than 1 min. The accumulation at 1 min exceeded that of norepinephrine which continued to increase with time and at 5 min exceeded *p*-OHA levels. This time pattern of *p*-OHA uptake was seen over a 10-fold concentration range (Fig. 1). Another difference in accumulation was seen in tissue from animals pretreated with reserpine (Table 3). In striatal preparations reserpine pretreatment reduced uptake of *p*-OHA by 60 per cent, while cortical uptake was not affected. The uptake of catecholamines was reduced in both preparations as others have reported [8].

Striatal uptake of *p*-OHA was similar to that of the dominant catecholamine of this area in terms of the time course of uptake and sensitivity to inhibitors [9] while *p*-OHA uptake into cortical preparations was considerably less sensitive to both metabolic and specific inhibitors of neuronal uptake (Table 4). Normetanephrine was included in the inhibitors studied because it is a potent inhibitor of uptake$_2$ [21], a non-neuronal uptake process in peripheral tissue.

The distribution of radioactive *p*-OHA and 1-NE accumulated by cortical P_2 fractions was examined on discontinuous sucrose density gradients. The results, shown in Fig. 2, indicate similar distribution patterns with peaks in radioactivity occurring at the 1.0 and 1.2 M sucrose layer interface, the synaptosome enriched fraction [22]. The percent of the total radioactivity present in the synaptosomal layer in cortical preparations was 22 per cent for *p*-hydroxyamphetamine and 48 per cent for norepinephrine. The corresponding values for striatal preparations were 34 per cent for DA and 28 per cent for *p*-OHA.

Table 4. Effects of inhibitors on cortical uptake of NE and *p*-OHA

Inhibitor (conc.)	Substrate NE (% control)	Substrate p-OHA (% control)
2,4 Dinitrophenol (100 μM)	59 ± 3	65 ± 2
Ouabain (100 μM)	28 ± 5	62 ± 2
Cocaine (10 μM)	10 ± 1	73 ± 2
DMI (10 μM)	7 ± 1	65 ± 4
Amphetamine (1 μM)	30 ± 4	77 ± 9
Normethanephrine (10 μM)	95 ± 2	100 ± 3

All values are the mean ± S.E. of 4 experiments. The accumulation was measured after 5 min incubation at substrate concentrations of 0.1 μM. Under these conditions NE accumulation was 20.5 pmoles/mg and *p*-OHA was 14.0 pmoles/mg protein.

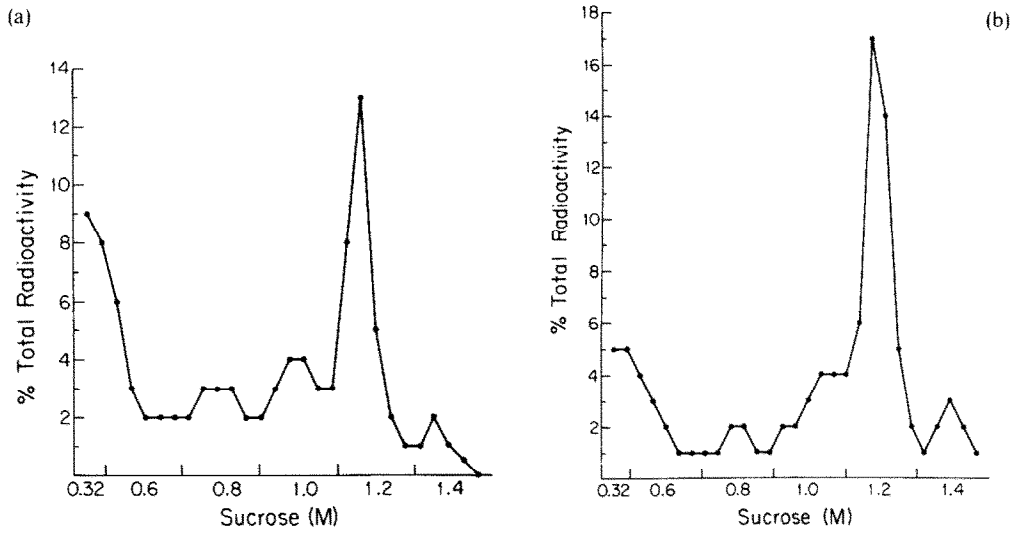

Fig. 2. Distribution of accumulated radioactivity in P_2 pellet. Distribution of tritium on sucrose density gradient after accumulation of [^{3}H]*p*-hydroxyamphetamine (2a) and [^{3}H]1-norepinephrine (2b) by cortical P_2 preparations under conditions described in Methods.

The relationship between accumulation and effect on neurotransmitter efflux was next examined. The increased efflux of amine or "release" caused by *p*-OHA is a characteristic action of indirectly acting sympathomimetic agents [23] and a dose dependent efflux of catecholamine from preloaded tissue (see Methods) was demonstrated for striatal and cortical tissue (Table 5, Fig. 3). Efflux was much greater from striatal tissue with maximal release occurring at a *p*-OHA concentration range of 1.5–2.5 μM. The accompanying accumulation of *p*-OHA was found to level off over this concentration range so that both uptake and release follow a similar pattern (Fig. 3). In contrast, cortical *p*-OHA uptake increased linearly

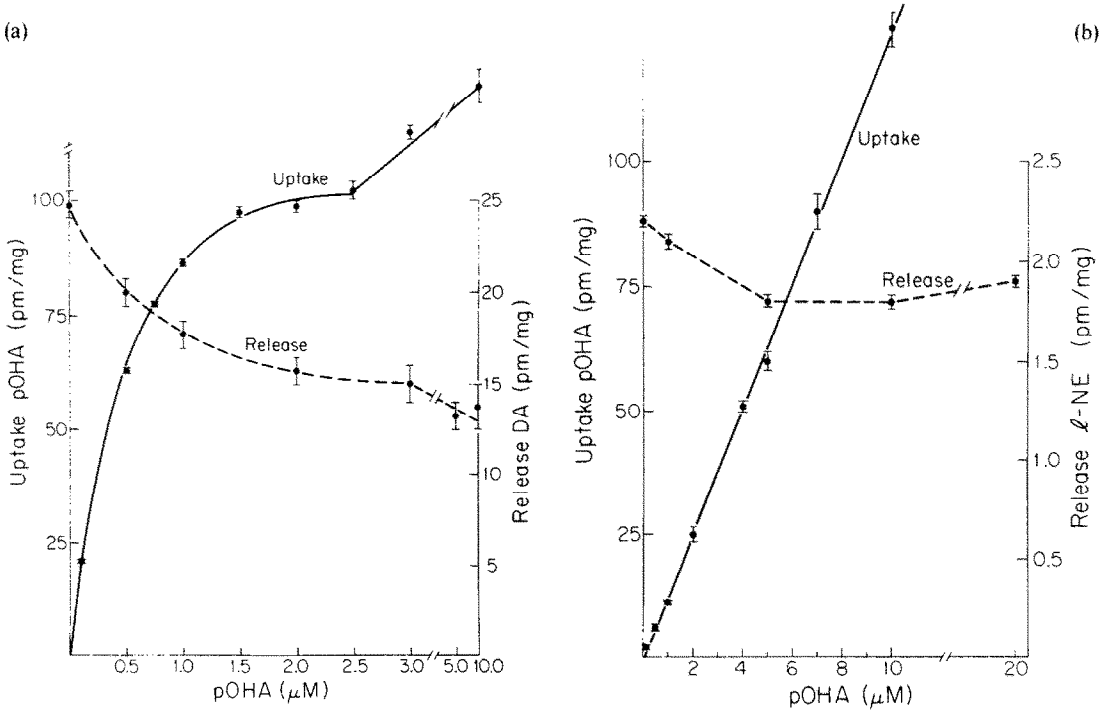

Fig. 3. Relationship between uptake of amine and transmitter efflux. P_2 fractions from striatum (3a) and cortex (3b) preloaded with [^{3}H]transmitter were incubated with *p*-hydroxyamphetamine at the indicated concentrations. The resulting tritium efflux was determined after 3 min by procedures described in Methods. In parallel experiments the accumulation of *p*-OHA was determined by incubation of [^{3}H]*p*-OHA at the indicated concentrations. The right and left ordinates refer to the amounts of radioactivity remaining in the tissue after 3 min. The ED_{50} for release from striatal tissue was 0.8 μm and for cortex between 2.5 and 3.0 μM. The maximal release induced by *p*-OHA was 4.2 pmoles/mg protein min^{-1} and 0.133 pmoles/mg protein min^{-1} for the striatum and cortex respectively.

Table 5. Uptake of p-OHA and efflux of catecholamines

Amine (μM)	Amount of p-OHA accumulated (pmole/mg protein) Striatum	Cortex	Amount of catecholamine released (pmole/mg protein) Striatum	Cortex
p-OHA (0.5)	62.5 ± 3.1	6.0 ± 0.3	4.5 ± 0.3	—
p-OHA (1.0)	82.5 ± 4.1	11.0 ± 0.6	6.5 ± 0.3	—
p-OHA (1.0) + cocaine (10)	36.3 ± 2.0	—	0	—
p-OHA (5.0)		60.0 ± 3.0	—	0.3 ± 0.05
p-OHA (6.5)		90.0 ± 3.6	—	0.4 ± 0.05
p-OHA (6.5) + cocaine (10)		73.8 ± 4.4	—	0.1 ± 0.04

P_2 preparations of [^{3}H]catecholamine-loaded tissues were incubated with the drug(s) at the indicated concentrations for 5 min at 37°. The radio-activity released during this time was determined as described in Methods. The accumulation of the amines was measured by incubation of P_2 preparations with [^{3}H]amine at the indicated concentration for 5 min at 37°. Accumulation and release are expressed as pmoles accumulated or released per miligram protein present in the incubation bath. The values are the mean ± S.D. of at least 8 replicates.

with concentration and did not display a plateau at maximal NE release. In addition, the quantities of catecholamine released from cortex and striatum differed both in terms of absolute amount released and in percent of total neurotransmitter present. Thus, p-OHA released maximally 40 per cent of the loaded DA-[^{3}H] and only 20 per cent of the loaded NE-[^{3}H]. p-OHA was thus more effective in releasing striatal DA than cortical NE with ED_{50} values of about 0.8 μM for release from striatal tissue and 2.5–3 μM for crtical tissue. The efflux of NE and DA caused by p-OHA is reversed with 10 μM cocaine (Table 5).

DISCUSSION

The neuronal uptake process is thought to be responsible for the removal of released neurotransmitter from the synaptic cleft and the mechanism by which some indirectly acting sympathomimetics gain access to the neuronal cytoplasm where they cause an efflux of transmitter [23]. Thus, the effects of tyramine can be reversed by cocaine which inhibits the uptake process although it potentiates the actions of the directly acting sympathomimetic amines [24]. For this reason, the accumulation of p-OHA by striatal tissue by a mechanism similar to that for DA uptake would be anticipated and has been documented [9] together with similar data for other phenolic phenethylamine derivatives [25–27].

A similar phenomenon would be expected for cortical tissue since it has been demonstrated to accumulate NE which can be released by compounds such as amphetamine [28]. However, there are substantial differences in the accumulation of p-OHA by striatum and cortex. The uptake of cortical p-OHA is a rapid process which attains equilibrium sooner than that for NE which suggests a diffusion process might be occurring. But the accumulation of p-OHA by cortical tissue is temperature sensitive, displaying a greater than 10-fold increase with an increase in temperature of 37°, while under identical conditions antipyrine, a freely diffusable compound [19], exhibits no temperature dependency. An alternative explanation for temperature sensitivity could be an effect on base strength. The pK_a values of organic bases showed significant temperature coefficients, becoming weaker bases at higher temperature [29]. A base weakening effect would increase the concentration of the neutral species at the same pH so that the concentration of diffusable form of the drug would increase with temperature. This does not appear to be likely since the uptake of amphetamine, a base of approximately the same pK_a as p-OHA, is not temperature sensitive.

The accumulation by cortical tissue is not saturable over a concentration range of 0.1–10 μM and does not show the same pattern of inhibition as NE. p-OHA uptake was only slightly reduced by either cocaine or desmethylimipramine (DMI) under conditions that inhibited NE uptake by 90 per cent. The effect of a potent inhibitor of uptake$_2$, normethanephrine [21], was also examined and at 10 μM it had no effect on p-OHA uptake in the preparations used here. Thus, the process by which cortical tissue accumulated p-OHA here has neither the characteristics of neuronal uptake of catecholamine nor those of uptake$_2$, a non-neuronal process described in the peripheral system. One additional possibility if β hydroxylation of p-OHA to the false transmitter, p-hydroxyephedrine [30] which is retained by certain tissues. However, the dopamine β hydroxylase inhibitor, fusaric acid, had no effect on accumulation [11] and 90 per cent of the accumulated radioactivity is identifiable as p-OHA.

The cortical uptake could reflect a temperature dependent surface binding but that does not appear likely since quenching in the presence of a 100-fold excess of p-OHA should result in a rapid dilution of surface bound radioactivity. In addition, the loss of 80 per cent of the accumulated amine with hypoosmotic shock indicates most of the compound is contained in an intracellular compartment. This compartment has bouyant density characteristics similar to synaptosomes but does not appear to involve neuronal vesicles since uptake is not reduced in tissue from reserpine pretreated animals. Striatal p-OHA uptake is reduced by 60 per cent in such preparations.

The present experiments show that while *p*-OHA can increase efflux or release catecholamines from both striatal and cortical preparations, there are quantitative differences in its interactions with these two brain areas. *p*-OHA is a more effective releaser of striatal catecholamines and is accumulated by the tissue in proportion to the catecholamine released. In the cortex preparations, the maximal release is lower and the accumulation appears to involve a compartment unrelated to the loss of catecholamine. While the precise mechanism by which this increase in efflux occurs is not established, the greater efficacy of DA release by *p*-OHA may be a characteristic of this class of indirect amine since similar differences have been noted for amphetamine. In studies with brain slices Heikkila *et al.* [31] found 60 per cent of striatal DA-[^{3}H] and about 10 per cent of cortical NE-[^{3}H] was released by *d*-amphetamine while tyramine released with comparable efficacy. Ferris *et al.* also observed differences in the amphetamine-induced catecholamine release from these two areas [28].

Acknowledgements—This work was supported by USPHS Grant number MH-23839. The *dl-p*-hydroxyamphetamine HBr was a generous gift of the Smith, Kline and French Laboratories, Philadelphia, PA 19101.

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