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METABOLISM OF AMPHETAMINE BY RAT BRAIN TISSUE*

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Abstract—The metabolism of amphetamine by rat brain tissue was investigated *in vivo* and *in vitro* with the use of [^{3}H]amphetamine. After intracisternal administration of 0.53 nmole or 890 nmoles amphetamine, brain amphetamine content declined biphasically, with a $T_{\frac{1}{2}}$ of 6 min for the fast component and 60 min for the slow component. *p*-Hydroxyamphetamine (POHA), norephedrine (NOR) and *p*-hydroxynorephedrine (POHNOR) were isolated from brain tissue after either dose of amphetamine. Brain content of POHA and NOR declined quickly, while brain POHNOR content rose gradually to a maximum 1 hr after amphetamine administration and decreased only slightly between 1 and 4 hr. Metabolites could not be detected in brains of animals injected intraperitoneally with either dose, indicating that the metabolites found after intracisternal administration had not been formed peripherally. POHA and POHNOR but not NOR formation were decreased by pretreatment of rats with iprindole, an inhibitor of hepatic *p*-hydroxylation of amphetamine. Pretreatment of animals with U 14-624, an inhibitor of the enzyme dopmaine-β-hydroxylase, or with 6-hydroxydopamine, which destroys catecholamine nerve terminals, caused a reduction of POHNOR and NOR formation but did not affect brain AMPH content. All three hydroxylated metabolites were isolated from brain slices which had been incubated for 30 min in the presence of amphetamine. These findings indicate that amphetamine can be metabolized by brain tissue to the hydroxylated metabolites, POHA, NOR and POHNOR, although brain content of these metabolites represents a small fraction of the dose of amphetamine. The data also suggest that POHA is formed outside of catecholamine nerve terminals by a process which is inhibited by iprindole, while POHNOR and NOR are formed in noradrenergic nerve terminals by the enzyme dopamine-β-hydroxylase.

The sympathomimetic amine amphetamine is metabolized to some extent by *p*-hydroxylation in the liver in many species [1]. The quantitative contribution of this route of metabolism to the overall elimination of amphetamine may be critical in determining this drug's physiological and behavioral actions, since it results in the formation of two metabolites which in themselves disrupt catecholamine mechanisms. *p*-Hydroxyamphetamine (POHA) has actions on central and peripheral catecholamine neurons similar to those of amphetamine [2–4]. In addition, POHA is accumulated in noradrenergic nerve terminals, where it is metabolized by the enzyme dopamine-β-hydroxylase (DBH) to *p*-hydroxynorephedrine (POHNOR), a noradrenergic pseudotransmitter which displaces norepinephrine from its storage sites [5–7]. Finally, AMPH can be β-hydroxylated by DBH to form the sympathomimetic norephedrine (NOR), although amphetamine is a poor substrate for this enzyme and little NOR is found in tissues after intraperitoneal administration of amphetamine [8, 9].

The contribution of these metabolites to the actions of amphetamine remains controversial although their sympathomimetic activity has been demonstrated repeatedly. There is considerable evidence which suggests that accumulation of POHNOR contributes to the development of tolerance to some effects of amphetamine on the peripheral sympathetic nervous system in rats. However, it is assumed generally that POHA and POHNOR do not contribute to the actions of amphetamine in species that metabolize amphetamine mainly by deamination or to its effects in the central nervous system. In the former case, the blood–brain barrier limits the entry of hepatically formed POHA, and in the latter, the low rate of hepatic hydroxylation decreases the availability of POHA for uptake into tissues [1, 10, 11]. In these situations, the concentration of amphetamine in tissues greatly exceeds that of hydroxylated metabolites, and the effects of POHA and POHNOR on catecholamine mechanisms relative to those of amphetamine are assumed to be negligible [7, 12, 13]. Similarly, the rate of NOR formation is so low that little accumulates in tissues after AMPH administration [10].

Recent evidence suggests that the contribution of POHA and POHNOR to the actions of amphetamine should be reconsidered. There are several reports which indicate that the high AMPH:metabolite ratio found in tissues after AMPH administration is not an accurate indication of the relative concentrations of AMPH and its hydroxylated metabolites at their sites of action. POHA and POHNOR are accumulated actively in noradrenergic nerve terminals, and these intraneuronal stores comprise the major part of the tissue content of these compounds after administration of precursors or the compounds themselves [9, 10, 14–18, 19–21]. In contrast, amphetamine is not sequestered in noradrenergic nerve terminals or any other subcellular compartment, but appears to be distributed uniformly throughout "total cell water" [22, 23]. Therefore, the ratio of metabolites:AMPH

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within noradrenergic nerve terminals after AMPH administration might be considerably higher than is suggested by the overall tissue content of these compounds.

There is other evidence which supports the hypothesis that the hydroxylated metabolites of AMPH may contribute to its behavioral actions. Recent findings in the field of drug metabolism suggest that amphetamine could be metabolized to POHA in tissues other than liver. Several drug-metabolizing systems are found extrahepatically, including the cytochrome P-450 mixed-function oxidase, which mediates hydroxylation of AMPH [24–26]. Extrahepatic metabolism of amphetamine would allow accumulation of POHA and POHNOR in tissues like brain which are not exposed to high levels of hepatically formed POHA. Furthermore, hydroxylated metabolites formed extrahepatically could contribute significantly to the disruption of catecholamine mechanisms produced by AMPH in tissues that contain a high density of nerve terminals which accumulate POHA and POHNOR.

In the present study, the metabolism of AMPH to its active metabolites, POHA, POHNOR and NOR, has been investigated both *in vivo* and *in vitro* to evaluate the role of these compounds in the actions of AMPH.

METHODS

Male Sprague–Dawley rats weighing 200 ± 10 g were housed two/cage in a vivarium with a 12-hr light and dark cycle and ambient temperature of 22°. Two hr before the start of an experiment, animals were transported to the laboratory, where they were left undisturbed until the start of the experiment. All drug treatments were given between 8.00 and 11.00 a.m. to control for diurnal variations in drug-metabolizing activity.

Drug treatments. [^{3}H]amphetamine (0.53 nmole or 890 nmoles) or [^{3}H]tyramine (0.21 μg) were injected intracisternally (i.c.) in 10 μl of sterile saline by the technique described by Schanberg *et al.* [27]. In some studies, animals were injected with iprindole (100 μg, i.c., 15 min before [^{3}H]AMPH), U 14–624 (100 mg/kg, i.p., as a suspension in methyl cellulose 3 hr before [^{3}H]AMPH), FLA 63 (30 mg/kg, i.p., in Tween 80, 1 hr before [^{3}H]AMPH, 6-hydroxydopamine (200 μg, i.c., 3 weeks before [^{3}H]AMPH) or α-methyl-paratyrosine (200 mg/kg, i.p., 2 hr and 100 mg/kg 1 hr before [^{3}H]AMPH). Control animals were injected with vehicle alone. Animals were killed by cervical fracture at various times after [^{3}H]AMPH administration and brains were removed rapidly, weighed and analyzed for [^{3}H]AMPH, [^{3}H]POHA, [^{3}H]POHNOR and [^{3}H]NOR or [^{3}H]tyramine and [^{3}H]octopamine. Brain regions were dissected by a modification of the method of Glowinski and Iversen [28].

Brain slice preparation. Immediately after dissection, brains were placed in ice-cold Krebs–Henseleit buffer (122 mM NaCl, 3 mM NaCl, 3 mM KCl, 1.2 mM $MgSO_4$, 1.3 mM $CaCl_2$, 0.4 mM KH_2PO_4, 10 mM glucose and 25 mM $NaHCO_3$, buffered with 95% O_2, 5% CO_2) [29]. Slices were made freehand along the sagittal plane [30] with a Stadie Riggs blade, weighed immediately and placed in a 25-ml Erlenmeyer flask containing 4.9 ml of ice-cold buffer (approximately 250 mg/flask) which was kept on ice until all samples had been prepared.

Slices were pre-incubated for 10 min at 37°, then 0.1 ml buffer containing 2.5 μCi [^{3}H]AMPH diluted to the appropriate specific activity with nonradioactive amphetamine sulfate was added and the incubation continued for an additional 30 min. Incubations were carried out in a Dubnoff shaker. A mixture of 95% O_2 and 5% CO_2 was bubbled through a water trap and into the medium in each flask as recommended by Goldstein *et al.* [31]. At the end of the incubation period, slices were separated from the medium by filtration, rinsed with 10 ml of cold buffer and immediately assayed for amphetamine and metabolite content as described below.

Brain amphetamine and metabolite content. Brain content of [^{3}H]AMPH, [^{3}H]POHA, [^{3}H]POHNOR and [^{3}H]NOR was assayed after extraction from a brain homogenate as described by Belvedere *et al.* [32], dansylation and separation by thin-layer chromatography. Brains were homogenized in acetone formic acid, 85:15 (6 ml/g of tissue) and centrifuged at 10,000 *g* for 10 min. The pellet was re-homogenized in 4 ml of homogenization medium and re-centrifuged. A solution containing 30 nmoles of non radioactive, AMPH, POHA, NOR and POHNOR was added to the combined supernatants, followed by 0.1 ml formic acid (88 per cent) and 25 ml heptane chloroform (4:1). The mixture was shaken, centrifuged briefly and the organic layer discarded. The aqueous layer was re-extracted once with heptane chloroform and twice with 10 ml benzene. After the addition of 0.5 ml of 10 N NaOH and 1.5 g NaCl to the aqueous layer, it was extracted four times with 8 ml ethyl acetate. The ethyl acetate fraction was acidified with 3 ml of acidified methanol (9:1, methanol–1 N HCl) and then evaporated under a gentle stream of nitrogen. The dried extract was dissolved in 1 ml of 0.4 N perchloric acid, saturated with solid sodium carbonate, and 2 ml dansyl chloride (4 mg/ml in acetone) added. After shaking overnight, 0.1 ml proline (100 mg/ml in distilled water) was added to hydrolyze the excess dansyl chloride and then the samples were shaken in the dark for 2 hr more. After the excess acetone was evaporated under a stream of nitrogen, the dansylated amines were extracted twice into 3 ml benzene and the benzene layer was evaporated under a stream of nitrogen. The dried samples were re-dissolved in 40 μl benzene, immediately spotted on a Silica gel G plate (Brinkmann Instruments, Westbury, NY), and developed in Brinkmann Sandwich chambers. Plates were developed first with isopropyl ether–triethylamine continuously for 3 hr, followed by cyclohexane–ethyl acetate, 1:1, and then by chloroform–triethylamine, 5:1. Standards of AMPH, POHA, NOR, POHNOR and an aliquot of the [^{3}H]AMPH which had been injected were spotted on each plate. Plates were sprayed with a mixture of isopropanol–triethanolamine, 3:1, to facilitate elution of the compounds from the gel [33]. Spots were localized with a u.v. lamp, scraped into a glass vial and eluted with 10 ml benzene. Fluorescence intensity of each sample was measured in an Aminco–Bowman spectrophotofluorometer adapted to fit scintillation

vials (excitation 350 nm, emission 490 nm) and radioactivity was assayed by liquid scintillation spectrometry in a toluene-based scintillation fluor (16 g 2,5-diphenyloxazole (PPO)/liter and 0.4 g 1,4-*bis*-[2-(4-methyl-5-phenyloxazole)]benzene (POPOP)/liter). Recovery was measured by comparing fluorescence intensity of standards added at dansylation to that of standards added at the beginning of the purification procedure (brains with no standards added served as blanks). Recovery averaged 90 per cent for AMPH, 85 per cent for POHA and 80 per cent for POHNOR and NOR. Data were corrected for recovery, loss of tritium during hydroxylation and the small amount of radioactivity (0.1 to 0.2 per cent of [^{3}H]AMPH) which traveled with metabolite spots in the chromatographic system used.

Brain content of tyramine and octopamine. The content of [^{3}H]tyramine and [^{3}H]octopamine in brain was analyzed as described by Breese *et al.* [34]. Brains were homogenized in ice-cold 0.4 N perchloric acid (10 ml/brain) and centrifuged at 10,000 *g* for 15 min. The supernatant was adjusted to pH 5 with 1 N KOH and adsorbed onto a 20 × 8 mm column of Biorex 70 (Biorad Labs, Whittier, CA). [^{3}H]neutral metabolites were assayed by adjusting a 2-ml aliquot of the column effluent to pH 7, extracting neutral metabolites with 10 ml ethyl acetate and assaying 8 ml of the ethyl acetate fraction for radioactivity. The same aliquot was adjusted to pH 1, and [^{3}H]acid metabolites were extracted and assayed in the same way. [^{3}H]octopamine was quantitated after its oxidation to *p*-hydroxybenzaldehyde, 2.5 ml of the NH_4OH eluate was mixed with 250 μl of sodium periodate (0.2% in distilled water), and 1 min later 250 μl sodium bisulfite (10 per cent in distilled water) was added. [^{3}H]*p*-hydroxybenzaldehyde was extracted into 10 ml toluene after addition of 0.8 ml of 12 N HCl to the reaction mixture, and 5 ml of the toluene layer was assayed for radioactivity. An aliquot of the original NH_4OH fraction also was assayed for radioactivity, and [^{3}H]tyramine values were calculated as the difference between the total radioactivity in this fraction and [^{3}H]*p*-hydroxybenzaldehyde. Recovery through the procedure was found to be greater than 90 per cent for all of the compounds involved, so data were not corrected for recovery.

Purification of [^{3}H]amphetamine. [^{3}H]AMPH had to be purified to remove contaminants: 1–2 per cent of the radioactivity co-chromatographed with POHA and 10–15% of the radioactivity was tritiated water. The 0.01 N HCl in which [^{3}H]AMPH was supplied was evaporated under a stream of nitrogen, and [^{3}H]AMPH was re-dissolved in distilled water and streaked on one-half of a Silica gel G plate. The plate was developed twice in benzene–glacial acetic acid–pyridine (30:10:1). After development, nonradioactive standards of AMPH, POHA, POHNOR and NOR which had been streaked on the other half of the plate were visualized with diazotized *p*-nitraniline [35], and zone corresponding to [^{3}H]AMPH was scraped and collected. [^{3}H]AMPH was extracted from the gel by filtration with 20 ml methanol on a Buchner funnel. The methanol was acidified (one to two drops glacial acetic acid) and then evaporated. After this procedure, only 0.1 to 0.2 per cent of the tritium co-chromatographed with amphetamine metabolites. This value did not change with repeated purification of [^{3}H]AMPH. In each experiment, an aliquot of the particular batch of repurified [^{3}H]AMPH which had been used was chromatographed with the nonradioactive standards, and the radioactivity in each metabolite spot was assumed to represent background. (The acid stability of [^{3}H]AMPH was confirmed by chemists at New England Nuclear Corp.). [^{3}H]AMPH was re-dissolved in 20% ethanol (1 mCi/ml) and stored in a refrigerator. The ethanol was evaporated before use and amphetamine re-dissolved in sterile saline for injection.

Statistics. Levels of significance were calculated by Student's *t*-test with two-tailed probability values reported [36].

Materials. Rats were provided by Zivic-Miller (Allison Park, PA). [G-^{3}H]-*d*-amphetamine sulfate (10 Ci/m-mole) and [G-^{3}H]tyramine hydrochloride (10 Ci/m-mole) were obtained from New England Nuclear Corp. (Boston, MA). *d*-Amphetamine sulfate and *p*-hydroxyamphetamine hydrobromide were donated by Smith, Kline & French Laboratories (Philadelphia, PA); U 14-624 (2-thiazolyl-2-thiourea) and *p*-hydroxynorephedrine hydrochloride were supplied by Aldrich Chemical Co. (Milwaukee, WI). Norephedrine hydrochloride, tyramine hydrochloride, octopamine hydrochloride and L-proline were supplied by Sigma Chemical Co. (St. Louis, MO). Iprindole hydrochloride was donated by Wyeth Laboratories (Philadelphia, PA). FLA 63 was the gift of Dr. E. Ellinwood. Dansyl chloride was supplied by Pierce Chemical Co. (Rockford, IL). All organic solvents were MCB spectroquality (Matheson, Coleman & Bell, East Rutherford, NJ) except the triethylamine and isopropyl ether, which were supplied by Eastman Co. (Rochester, NY).

RESULTS

Metabolism of amphetamine after intracisternal administration. Brain AMPH content declined rapidly during hr 1 after i.c. injection of 890 nmoles, then declined more slowly (Fig. 1). The fall-off could be resolved into two exponential components with half-lives of 6 min (a pool containing 70 per cent of the dose) and 46 min (containing 5 per cent of the dose). The time course of amphetamine disappearance from brain after administration of a much smaller dose (0.53 nmole) was the same. Brain metabolite content at various times after i.c. administration of these doses of amphetamine is shown in Table 1. POHA could be detected within 5 min of injection, in most experiments, and by 10 min always (data not shown), and POHNOR and NOR within 15 min. Brain content of POHA and NOR declined rapidly and by 240 min had dropped below the limit of sensitivity of this method. In contrast, brain POHNOR content declined very little from 1 to 4 hr after amphetamine administration. When metabolite levels were highest, POHA and NOR metabolite represented 0.1 per cent and POHNOR represented 0.03 per cent of the injected amphetamine.

To establish that the metabolites found in brain had not been formed peripherally, brain AMPH and metabolite levels after i.c. injection of 890 nmoles AMPH were compared to those found after i.p. injec-

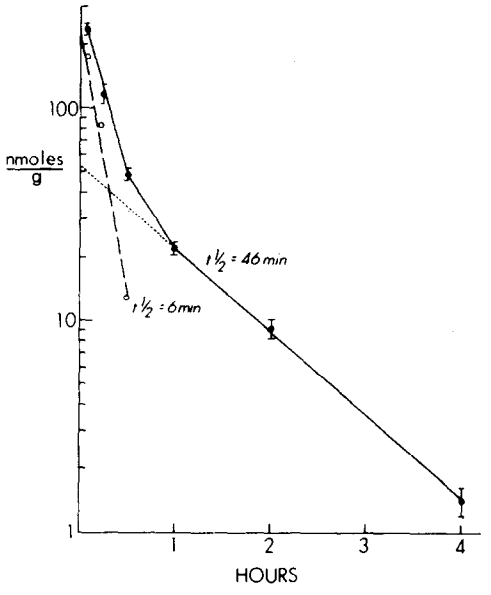

Fig. 1. Brain amphetamine content at various times after intracisternal administration of 890 nmoles. Values represent mean ± S. E. M. of six rats. Lines calculated by the method of least squares analysis. Key: - - - line = difference between experimental points and extrapolated slope of slow pool = slope of fast pool.

tion of 890 nmoles or after i.c. (890 nmoles) plus i.p. (890 nmoles) administration (Table 2). Brain AMPH levels were maximal within 15 min of drug administration by either route, but 5 and 15 min after administration, brain AMPH content of animals injected i.c. or i.c. plus i.p. was more than ten times that of animals injected i.p. alone. At 1 hr, AMPH content in brains of i.c. or i.c. plus i.p. animals was still three times that of animals injected i.p. alone. From 5 min to 1 hr, AMPH content declined rapidly in brains of animals injected i.c. or i.c. plus i.p., while brain AMPH content declined more slowly in animals injected i.p. From 1 to 4 hr, brain AMPH content declined more slowly in all animals regardless of route of administration. Brain region distribution of amphetamine differed considerably with route of administration. These data are shown in Table 3. After intracisternal administration, AMPH content of regions near the injection site (pons medulla, cerebellum and hypothalamus) was significantly higher than that of more rostral brain regions. In contrast, AMPH concentration was almost the same in all brain regions after intraperitoneal administration of an equal dose. The higher levels in brain regions of i.c.-injected animals reflect the earlier time of sacrifice. Since efflux of amphetamine from all regions is the same after either route of administration (data not shown), brain region distribution after the two routes of administration can be compared despite the different injection–sacrifice intervals used.

Brain metabolite patterns observed after i.c. or i.c. plus i.p. AMPH administration also differed considerably from those found in animals injected i.p. alone. POHA, POHNOR and NOR could not be detected 60 min after injection in brains of animals injected i.p. alone, while these metabolites could be isolated consistently from brains of animals injected i.c.

The effect of several drugs on the metabolism of amphetamine after i.c. administration was tested in order to characterize the amphetamine-metabolizing system more completely. These data are shown in Table 4. Pretreatment with iprindole, an inhibitor of hepatic *p*-hydroxylation of AMPH [37], resulted in a significant decrease in brain POHA and POHNOR content 1 hr after amphetamine administration, although neither AMPH nor NOR levels were affected by this treatment. Animals pretreated with U 14-624, an inhibitor of DBH [38], had significantly lower brain POHNOR and NOR levels 1 hr after AMPH than did animals pretreated with vehicle alone, but brain AMPH content was the same in both groups. Similar results were obtained when animals were pretreated with another DBH inhibitor, FLA 63 [39], and then sacrificed 3 hr after amphetamine administration. POHNOR and NOR levels were significantly lower in drug-pretreated animals. POHA and NOR could not be detected at this time point in either group. In animals pretreated with 6-hydroxydopamine 3 weeks before amphetamine, the amphetamine metabolite pattern was similar to that after inhibition of DBH. Brain NOR and POHNOR content was significantly lower in 6-hydroxydopamine animals than in controls, but brain POHA and AMPH content was unaffected by this treatment.

Pretreatment of rats with α-methyl-para-tyrosine, an inhibitor of tyrosine hydroxylase, did not affect AMPH or POHA levels 1 hr after i.c. administration of AMPH, although NOR levels were elevated significantly.

To establish that 6-hydroxydopamine and U 14-624 effectively inhibited DBH, rats treated with identical doses of these compounds were injected i.c. with [^{3}H]tyramine, and [^{3}H]tyramine and [^{3}H]octopamine (the β-hydroxylated derivative) were isolated from brain 1 hr after injection (Table 5). Brain octop-

Table 1. Metabolite content of whole brain at various times after intracisternal administration of amphetamine*

	Amphetamine (890 nmoles)			Amphetamine (0.53 nmole)		
Time (min)	POHA	NOR	POHNOR	POHA	NOR	POHNOR
5	630 ± 170	ND†	ND	0.36 ± 0.08	ND	ND
15	590 ± 140	760 ± 190	230 ± 80	0.42 ± 0.07	0.34 ± 0.06	0.15 ± 0.03
60	220 ± 40	210 ± 40	170 ± 10	0.09 ± 0.01	0.11 ± 0.03	0.17 ± 0.03
240	ND	ND	200 ± 20	ND	ND	0.17 ± 0.02

* Results are expressed as nmoles/g. Values represent mean ± S. E. M. of at least six animals.
† Not detectable.

Table 2. Brain amphetamine and metabolite content after administration of amphetamine (890 nmoles) by various routes*

Time (min)	Route of administration	AMPH (nmoles/g)	POHA (pmoles/g)	NOR (pmoles/g)	POHNOR (pmoles/g)
5	i.c.	140 ± 30	ND†	ND	ND
	i.p.	5 ± 1			
	i.c. + i.p.	120 ± 10			
15	i.c.	72 ± 7	420 ± 70	280 ± 40	70 ± 15
	i.p.	7 ± 1	ND	ND	ND
	i.c. + i.p.	77 ± 4	315 ± 80	330 ± 30	60 ± 10
60	i.c.	13 ± 1	250 ± 40	160 ± 40	100 ± 10
	i.p.	4 ± 1	ND	ND	ND
	i.c. + i.p.	15 ± 1	160 ± 50	100 ± 20	100 ± 10
240	i.c.	0.8 ± 0.1	ND	ND	140 ± 20
	i.p.	0.7 ± 0.1	ND	ND	ND
	i.c. + i.p.	0.6 ± 0.1	ND	ND	160 ± 10

* Results are expressed as nmoles/g. Values represent mean ± S. E. M. of at least six animals.
† Not detectable.

amine content 1 hr after tyramine was 14 per cent that of control in U 14-624-treated animals, indicating significant inhibition of DBH. Similarly, pretreatment of animals with 6-hydroxydopamine resulted in a significant decrease in octopamine formation. Neither 6-hydroxydopamine nor U 14-624 pretreatment resulted in a significant change in brain content of [^{3}H]acid or [^{3}H]neutral metabolites of tyramine (data not shown) or in brain content of [^{3}H]tyramine.

In summary, iprindole inhibited *p*-hydroxylation of amphetamine and the subsequent formation of POHNOR, while DBH inhibition or destruction of catecholamine nerve terminals decreased the formation of NOR and POHNOR and elevated brain content of POHA. Brain AMPH content after intracisternal administration was not affected by pretreatment with any of these drugs.

Metabolism of amphetamine by brain slices. To further test the hypothesis that brain tissue can metabolize amphetamine, brain slices were incubated in Krebs–Henseleit buffer containing [^{3}H]AMPH diluted with various amounts of nonradioactive *d*-amphetamine. The content of amphetamine in brain slices at the end of the incubation approximated the range of amphetamine concentrations found in brain after administration of doses ranging from those that minimally stimulate locomotor activity to those which approximate an LD_{50}. Table 6 shows temperature-dependent uptake of amphetamine into brain slices after a 30-min incubation (at this time diffusion equilibrium had been attained). The ratio of accumulation at 37° to that at 0° increased as the concentration of amphetamine in the medium decreased. All three hydroxylated metabolites of amphetamine could be detected in brain slices after a 30-min incubation with [^{3}H]amphetamine (Table 7). At all three concentrations of amphetamine used, approximately equal amounts of all three metabolites were isolated from tissue slices. Each metabolite represented from 0.3 to 0.5 per cent of slice tritium content at the end of incubation. Metabolite content of slices incubated at 0° was not above background.

Table 3. Brain region distribution of [^{3}H]amphetamine after intraperitoneal or intracisternal administration*

	Intraperitoneal administration	Intracisternal administration
Cerebellum	190 ± 13	1109 ± 125
Pons medulla	174 ± 15	792 ± 74
Hippothalamus	206 ± 17	412 ± 67
Midbrain	217 ± 16	275 ± 36
Corpus striatum	215 ± 20	58 ± 4
Cortex	215 ± 19	52 ± 5
Hippocampus	208 ± 4	116 ± 11

* Animals were injected intraperitoneally with 5 mg/kg (30 μCi) [^{3}H]amphetamine 30 min before sacrifice or intracisternally with 0.53 nmole (2 μCi) [^{3}H]amphetamine 10 min before sacrifice. Results are expressed are cpm × 10^{-3}. Values represent mean ± S. E. M. of six to eight animals.

DISCUSSION

Tissue content of the sympathomimetic amphetamine metabolites, POHA and POHNOR, is thought to be too low to contribute significantly to the actions of amphetamine because little accumulation of these compounds occurs in tissues that have a permeability barrier to peripherally formed POHA (e.g. brain) or in tissues of the many species that metabolize amphetamine mainly by deamination. However, the studies reported here demonstrate that accumulation of POHA and POHNOR can occur in at least one situation in which availability of hepatically formed POHA is limited. Our findings suggest that amphetamine is metabolized in brain to three compounds with sympathomimetic activity: *p*-hydroxyamphetamine, *p*-hydroxynorephedrine and norephedrine.

All three compounds were isolated from brain after intracisternal administration of amphetamine and from brain slices which had been incubated with amphetamine. The reported isolation of POHA and POHNOR from brain tissue after intraventricular administration of amphetamine to rats is in accord with these findings [40].

Two results indicate that the hydroxylated amphet-

Table 4. Effect of drug pretreatments on metabolism of intracisternally administered amphetamine*

	AMPH (nmoles/g)		POHA (pmoles/g)		NOR (pmoles/g)		POHNOR (pmoles/g)	
	Control	Expt.	Control	Expt.	Control	Expt.	Control	Expt.
Iprindole	8.7 ± 0.5	9.6 ± 0.5	120 ± 20	70 ± 41	117 ± 41	97 ± 12	190 ± 20	70 ± 8
Per cent of control		103		57		83		37
P value		NS†		$P < 0.025$		NS		$P < 0.001$
U 14-624	4.3 ± 0.4	3.3 ± 0.2	90 ± 18	130 ± 10	130 ± 19	19 ± 2	170 ± 3	80 ± 9
Per cent of control		79		140		15		47
P value		NS		$P < 0.01$		$P < 0.001$		$P < 0.025$
FLA 63	1.2 ± 0.3	2.0 ± 0.2					120 ± 10	30 ± 5
Per cent of control								25
P value								$P < 0.025$
6-Hydroxydopamine	10.5 ± 0.3	12.0 ± 1.5	75 ± 20	60 ± 10	160 ± 30	50 ± 15	180 ± 50	50 ± 15
Per cent of control		114		80		37		28
P value		NS		NS		$P < 0.025$		$P < 0.05$
α-Methyl tyrosine	6.6 ± 0.9	5.6 ± 0.4	60 ± 10	50 ± 10	120 ± 40	250 ± 60	140 ± 30	92 ± 11
Per cent of control		87		81		200		71
P value		NS		NS		$P < 0.001$		NS

* Animals were injected with iprindole (100 μg, i.c., 15 min before amphetamine), U 14-624 (100 mg/kg, i.p., as suspension in methyl cellulose 3 hr before amphetamine), FLA 63 (30 mg/kg, i.p., in Tween 80 1 hr before amphetamine), 6-hydroxydopamine (200 μg, i.c., 3 weeks before amphetamine) or α-methyl-para-tyrosine (200 mg/kg, i.p., 2 hr before and 100 mg/kg 1 hr before amphetamine as suspension in distilled water). Controls were injected with vehicle only. Amphetamine (0.53 nmole) was injected i.c. and animals were sacrificed 1 hr after injection, except for FLA 63 experiment, in which animals were injected 3 hr after injection. Results are expressed as nmoles/g of tissue or pmoles/g of tissue. Each value represents the mean ± S. E. M. of at least six animals.

† Not significantly different from control.

Table 5. Effect of pretreatment with 6-hydroxydopamine or U 14-624 on brain tyramine and octopamine content 1 hr after intracisternal administration of tyramine*

	Tyramine	Per cent of control	P value	Octopamine	Per cent of control	P value
6-Hydroxydopamine						
Control	0.7 ± 0.1			0.12 ± 0.01		
Experimental	0.6 ± 0.1	78	NS†	0.03 ± 0.01	25	<0.001
U 14-624						
Control	0.7 ± 0.1			0.15 ± 0.01		
Experimental	0.6 ± 0.1	82	NS	0.02 ± 0.01	14	<0.001

* Animals were injected with 210 ng tyramine and sacrificed 1 hr later. Results expressed as pmoles/g of tissue. Each value represents mean ± S. E. M. of six animals.
† Not significantly different.

amine metabolites detected in this study were formed in brain. Metabolites produced in brain slices obviously were formed in brain. In addition, metabolites were found in brain after i.c. but not after i.p. administration of a very small dose of amphetamine.

The failure of measurable amounts of peripherally formed POHA to enter brain during the time course of this study probably resulted from its relatively low lipid solubility compared to that of amphetamine. AMPH diffuses rapidly out of brain because its high lipid solubility enables it to move rapidly through the blood–brain barrier [41]. However, POHA is less lipid soluble [42] and penetrates the blood–brain barrier much more slowly: only 0.01 per cent of an intraperitoneal dose of POHA ever reaches the brain [10]. Even if 100 per cent of the intracisternally administered AMPH had been *p*-hydroxylated in the liver, the amount of POHA that would have re-entered the brain during the time course of this study could not be distinguished from background by the method used to quantitate amphetamine and its metabolites. Therefore, metabolites formed in the periphery could not account for brain content of POHA, POHNOR and NOR after i.c. administration of AMPH.

Table 6. Accumulation of amphetamine in brain slices*

Concn of amphetamine in medium (μM)	37°	0°	37°:0°
22.0	67.9 ± 6.8	24.1 ± 1.9	2.8
2.2	5.3 ± 0.8	1.8 ± 0.1	2.9
0.22	0.8 ± 0.1	0.1 ± 0.1	8.0

* Slices were prepared and incubated as described in Methods. Results expressed as nmoles/30 min/sample. Values = mean ± S. E. M. of six samples.

Table 7. Metabolite concentration in brain slices incubated in amphetamine*

Concn of amphetamine in medium (μM)	POHA (pmoles/g)	NOR (pmoles/g)	POHNOR (pmoles/g)
22	227 ± 35 0.3†	365 ± 65 0.5†	230 ± 63 0.3†
2.2	24 ± 5 0.5†	21 ± 2 0.4†	28 ± 5 0.5†
0.22	2.9 ± 0.6 0.4†	2.5 ± 0.5 0.3†	3.3 ± 0.7 0.4†

* Slices were prepared and incubated as described in Methods. Results are expressed as pmoles/g/30-min incubation. Metabolite values at 0° were not above background. Values represent mean ± S. E. M. of six samples.
† Per cent amphetamine content.

The exact mechanism of amphetamine *p*-hydroxylation is not clear. The inhibition of amphetamine *p*-hydroxylation by iprindole suggests that some component of the brain *p*-hydroxylating system may be similar to that which mediates amphetamine metabolism in the liver. Iprindole may act either by inhibiting amphetamine uptake into sites of metabolism (as it inhibits uptake into peripheral tissues) [43] or by competing with amphetamine for some component of the drug-metabolizing system, as several tricyclic antidepressants do in liver [37, 44, 45]. The ability of iprindole to decrease the formation of POHA suggests that the cytochrome P-450 mixed-function oxidase system may be involved in this process, since this is the site at which iprindole competes with amphetamine in the liver. Additional evidence for this hypothesis is provided by the reports of metabolism in brain of several other drugs which normally are metabolized by this system in liver [24, 46, 47]. However, cytochrome P-450 is barely detectable in brain, and it has been suggested that it is not involved in the metabolism of drugs in brain [24, 48].

An alternative mechanism for the formation of POHA in brain which can be postulated is hydroxylation by enzymatic mechanisms which normally utilize endogenous substrates. Two such mechanisms for this conversion are hydroxylation by the system which is thought to form tyramine from phenylethylamine [49–51] and hydroxylation by the process in mitochondria which contributes slightly to amphetamine metabolism in the liver [52]. The possibility that amphetamine is metabolized by some nonspecific chemical process also must be considered.

Two hypothetical sites of amphetamine *p*-hydroxylation were excluded by the results of this study. *p*-Hydroxylation probably does not occur within catecholamine nerve terminals, since 6-hydroxydopamine pretreatment did not decrease brain content of this compound. In addition the hypothesis of Costa and Groppetti [7] that tyrosine hydroxylase is responsible for the *p*-hydroxylation is not supported by

the fact that alpha-methyl-para-tyrosine pretreatment did not decrease POHA formation.

The mechanism of β-hydroxylation of AMPH and POHA was characterized more definitely than the mechanism of *p*-hydroxylation: the data indicate that NOR and POHNOR were formed in noradrenergic nerve terminals by the enzyme dopamine-β-hydroxylase. Intracisternal injection of 6-hydroxydopamine, which destroys noradrenergic nerve endings [53, 54], or with U 14-624 or FLA 63, which inhibit DBH [38, 39], resulted in a decrease in the tissue levels of POHNOR and NOR. This finding provides more support for the hypothesis that POHNOR is formed and stored in these terminals after i.c. AMPH administration. The effectiveness of these drug treatments in inhibiting DBH was demonstrated by inhibition of the conversion of tyramine to octopamine in brain.

The total proportion of amphetamine which was metabolized after intra-cisternal AMPH administration or incubation of brain slices with AMPH was very small (0.1 to 0.3 per cent of the original dose). The low rate of metabolism after i.c. administration might have resulted from several characteristics of the experimental design. The rapid efflux of amphetamine from brain after i.c. administration might have limited precursor availability at sites of metabolism, as could the uneven distribution of AMPH throughout different brain regions. AMPH distribution after i.c. administration was similar to that of many other compounds after administration into the ventricular system of the brain [55–58]. High concentrations of AMPH were found near the site of injection (pons medulla, cerebellum, and hypothalamus) but levels in brain regions farther from the injection site (cortex and corpus striatum) were much lower. Therefore, this particular route of administration limits access of AMPH and POHA to several regions with a high density of catecholamine nerve terminals which represent potential sites for uptake and storage of hydroxylated AMPH metabolites.

Similar methodologic limitations may have resulted in a low rate of *p*-hydroxylation in the brain slice preparation. The use of slices made freehand, which may have been thicker than the 0.5 mm maximum for diffusion of oxygen [29], efflux of POHA and NOR from slices as well as the inactivity of the amphetamine *p*-hydroxylating system *in vitro* [20, 44, 52, 59, 60] may have limited the rate of amphetamine metabolism in slices.

Alternatively, the low rate of amphetamine metabolism might reflect the low rate of amphetamine *p*-hydroxylation by brain instead of simple methodologic limitations on the rate of metabolism. The rate of metabolism of other drugs by brain tissue is similarly slow [46] and brain content of all elements of the cytochrome P-450 mixed-function oxidase system is very low [51, 61]. In most studies of extrahepatic drug metabolism, activity of drug-metabolizing systems in brain is very low or undetectable. Even if AMPH is metabolized by an enzyme system which normally utilizes natural substrates, the low rate of metabolism could reflect poor affinity of AMPH for the enzyme system rather than limitation of AMPH access to sites of metabolism by the particular mode of administration.

The low rate of norephedrine formation probably does reflect the actual rate of amphetamine β-hydroxylation. AMPH is a poorer substrate for the system involving neuronal and vesicular uptake and hydroxylation than POHA, so the rate of NOR formation is lower than that of POHNOR despite the greater availability of AMPH [62, 63]. These findings are consistent with their report that brain content of NOR is less than 10 per cent that of POHNOR after intraperitoneal administration of AMPH [9]. Furthermore, active phenylethylamine uptake into nerve terminals and the dopamine-β-hydroxylase activity have been demonstrated in brain slices [31, 64] and so the low rate of amphetamine-β-hydroxylation in slices cannot be attributed to an inactivated metabolizing system.

The contribution of these cerebrally formed metabolites to the biochemical and behavioral effects of amphetamine is difficult to evaluate. However, this study demonstrates that measurable metabolism of amphetamine can occur. Furthermore, the high rate of POHA conversion to POHNOR (the ratio of POHA–POHNOR at the time of peak brain content of both was roughly 1:2) suggests that local accumulation of POHNOR in noradrenergic nerve terminals can result from extrahepatic formation of POHA. This means that accumulation in brain of the pseudotransmitter POHNOR may occur in species in which little POHA is formed hepatically and so provide support for the involvement of POHNOR in the development of tolerance to amphetamine in species other than the rat. In addition, the amount of POHA produced in brain (roughly 0.2 per cent of the available amphetamine) might be high enough to contribute to brain content of this compound after intraperitoneal administration of amphetamine since brain POHA content is less than 1 per cent that of AMPH after intraperitoneal AMPH administration (Kuhn and Schanberg, manuscript in preparation) [12, 13]. Furthermore, the rat has more limited capacity for extrahepatic drug metabolism than many other mammalian species [25], and the amount of hydroxylated metabolites produced in brains of other species could be greater than that observed in this study. The isolation of POHNOR from urine and bile of many species after amphetamine administration [65] further suggests that hydroxylated metabolites may accumulate in noradrenergic nerve endings in other species, and indicates that the role of these compounds in the behavioral and biochemical effects of amphetamine merits further attention.

In summary, these studies have demonstrated that amphetamine is hydroxylated to form POHA, NOR and the pseudotransmitter POHNOR in brain after intracisternal administration of amphetamine or after incubation of brain slices with amphetamine. POHA is produced by an unknown mechanism which is inhibited by iprindole, while NOR and POHNOR are formed in noradrenergic nerve terminals by the enzyme dopamine-β-hydroxylase. The results of these studies re-emphasize the importance of considering the pharmacological actions of amphetamine metabolites when evaluating the actions of amphetamine after acute and chronic administration.

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