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EFFECTS OF INTRAVENTRICULAR INJECTIONS OF 6-HYDROXYDOPAMINE ON AMINE METABOLITES IN RAT BRAIN AND URINE

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Abstract—The effects in rats of intraventricular injections of 6-hydroxydopamine (6-OHDA) on the urinary excretion 1–3 weeks later of 3-methoxy-4-hydroxyphenethylene glycol (MHPG), 3,4-dihydroxyphenethanol (DHPE), 3-methoxy-4-hydroxyphenethanol (MHPE), *p*-hydroxyphenylglycol (pHPG), homovanillic acid (HVA) and 3,4-dihydroxyphenylacetic acid (DOPAC) were examined. The excretion of MHPG was decreased to 63 and 71% of control on days 7 and 14, respectively, but had returned to control levels by day 23, even though the brain levels were decreased by 87%. Free and total HVA excretion was reduced on both days 7 and 23, but free and total DOPAC was reduced only on day 7. Based on these data, it can be estimated that about 39% of the free and 46% of the total HVA in urine originates in the CNS. The excretion of conjugated HVA was decreased by 70–80%, but this decrease does not support the notion that the conjugated form of HVA is derived principally from the brain and thus serves as a better marker of brain dopamine metabolism, since the level of this metabolite in the brain was not correspondingly decreased but was instead increased. Urinary DOPAC levels were generally more variable and derived to a greater extent from the periphery; therefore, DOPAC appears to be less suitable than HVA as a marker of brain dopamine. The results also indicate that as much as 35% of the urinary MHPG may originate in the CNS, although compensatory changes in catecholamine metabolism in either the brain or in the periphery may have somewhat influenced this estimate. The results also suggest that at least as much pHPG as MHPG in urine derives from the CNS. The data are consistent with the idea that the neutral dopamine metabolites largely derive from the brain, but the relatively small depletion in their brain levels produced by 6-OHDA prevented the exact proportion being determined accurately.

Brain amines are thought to have an important role in the regulation of mood and behavior as well as in the etiology and pharmacotherapy of certain psychiatric and neurological disorders (e.g. schizophrenia [1], mania and depression [2], and Parkinson's disease [3]). To reliably assess brain amine function in man, one frequently used approach involves measuring various metabolites in accessible body fluids, such as urine or blood, as indices of the turnover of the corresponding amines in brain [4]. Since the validity of this strategy is based partly on the assumption that a substantial proportion of the metabolites in peripheral body fluids derives from the brain, several laboratories have sought to test this for the catecholamines by injecting 6-hydroxydopamine (6-OHDA) into the brain of experimental animals to destroy catecholaminergic neurons and then determining to what extent the concentrations of catecholamine metabolites in urine or blood are reduced. Most studies to date have focused on the norepinephrine metabolite, 3-methoxy-4-hydroxyphenethylene glycol (MHPG) [5–10]. All except one of the studies in rats have found no effect on urinary MHPG [5–8] (in the one report MHPG was lowered by 29% [9]), and another study found plasma MHPG to be unaffected [10].

Although less attention has been paid to dopamine metabolites, there is some evidence to suggest that a significant proportion of certain urinary dopamine metabolites originates in the brain and may therefore be useful as markers of CNS dopamine function. For example, Hoeldtke *et al.* [6] reported that an injection of 6-OHDA that depleted brain dopamine by 80% decreased the excretion of homovanillic acid (HVA) by 27% but did not affect the excretion of 3,4-dihydroxyphenylacetic acid (DOPAC). From their data they inferred that approximately 34% of urinary HVA but very little of the DOPAC is derived from the CNS. Using rats with bilateral electrothermic lesions of the nigrostriatal pathway, the contribution of the striata to plasma levels of HVA and DOPAC has been estimated to be 40 and 25% respectively [11]. On the other hand, based on the effects of a unilateral injection of 6-OHDA in the substantia nigra, Peyrin *et al.* [12] estimated that both striata contribute about 88% of the conjugated HVA and 50% of the conjugated DOPAC in the urine, suggesting that levels of these conjugated metabolites are superior to the free metabolites as markers of central dopamine function.

In the present studies, we have examined simultaneously the effects of the central administration of 6-OHDA on the excretion of acidic and neutral metabolites of both norepinephrine and

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dopamine as well as of the trace amine octopamine as measured by gas chromatography-mass spectrometry (GC/MS).

MATERIALS AND METHODS

Animals. Male Sprague–Dawley rats were supplied by Zivic–Miller Laboratories (Allison Park, PA) 1 week before the beginning of each experiment. They were housed singly in a room with a 12 hr light:dark cycle (lights on 6:00 a.m. to 6:00 p.m.) and fed *ad lib.* a casein diet (No. 902487, ICN Nutritional Biochemicals, Cleveland, OH) and water.

Materials. 6-Hydroxydopamine hydrobromide, harmaline hydrochloride and Type H-1 Helix pomatia sulfatase were purchased from the Sigma Chemical Co., St. Louis, MO. Compounds used as standards and internal standards include: 3-methoxy-4-hydroxyphenethyleneglycol (MHPG) piperazine salt (Calbiochem-Behring, La Jolla, CA); 3,4-dihydroxyphenethanol (DHPE) and 3-methoxy-4-hydroxyphenethanol (MHPE) (Regis Chemical Co., Morton Grove, IL); *p*-hydroxyphenylglycol (pHPG) (synthesized by Dr. B. L. Goodwin, London, U.K.); 3,4-dihydroxyphenylglycol (DHPG), 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), dopamine, norepinephrine, serotonin creatinine sulfate, 5-hydroxy-3-indole acetic acid (5-HIAA) and 3,4-dihydroxybenzylamine (DHBA) (Sigma); and 3,4-dihydroxyphenylpropionic acid (DOPPA) and 3-methoxy-4-hydroxyphenylpropionic acid (MHPPA) (ICN Pharmaceuticals, Inc., Plainview, NY). 3-Methoxy-4-hydroxyphenylpropanol (MHPP) was synthesized in our laboratory [13]. Ethyl acetate (Spectranalyzed, Fisher Chemical Co., Pittsburgh, PA) was redistilled over magnesium sulfate before use. Pentafluoropropionic anhydride (PFPA) and pentafluoropropanol (PFPOH) were obtained from the Pierce Chemical Co., Rockford, IL, and maintained under nitrogen. Octyl sodium sulfate was from the Eastman Kodak Co. (Rochester, NY) and methyl alcohol was from Burdick & Jackson (Muskegon, MI). All other reagents were purchased from Fisher and were the highest purity available.

Injection of 6-hydroxydopamine. The hydrobromide salt of 6-OHDA was dissolved in 0.9% saline containing 1 mg/ml ascorbic acid to give a concentration of the free base of 12.5 μg/μl. This solution was kept on ice, protected from light, and was used within 2 hr. Then 20 μl (containing 250 μg of 6-OHDA free base) was injected into the lateral ventricles, half into each side, of animals (146–178 g) lightly anesthetized with ether, using the method of Noble *et al.* [14]. The rate of injection was maintained at 1 μl/5 sec with the aid of a repeating dispenser fitted on a Hamilton microsyringe. The needle was removed from the lateral ventricles 30 sec after the completion of each injection. The rats were injected 24 hr later with an additional 250 μg of 6-OHDA following the same procedure. Where noted, a few animals were given only a single injection of 6-OHDA, 10 min after pretreatment with harmaline (30 mg/kg, i.p.), a reversible monoamine oxidase (MAO) inhibitor.

Collection of urine samples. Individual animals were placed in plastic metabolic cages (Maryland Plastics, Inc., Federalsburg, MD) at approximately 2:00 p.m., and urines were collected for 24-hr periods in receptacles containing 5 mg of sodium metabisulfite. After their volumes were measured, the urines were frozen (−20°) until assayed. The first urine collection served simply to acclimate the animals to the metabolic cages and, therefore, was not analyzed.

Preparation of brain and other tissue samples. Animals were killed by decapitation immediately following completion of the last 24-hr urine collection. The brains, hearts, spleens and adrenal glands were rapidly removed and blotted. In some cases, the brains were divided into left and right halves, so that one-half could be homogenized in 0.1 N perchloric acid for assay of catecholamines, serotonin, and 5-HIAA (see below). The tissues were then frozen on dry ice and stored at −45° until assayed, usually within 3 weeks.

Assay of neutral metabolites. Neutral metabolites were assayed in brain and urine samples as previously described [15, 16], with minor modifications. For assay of brain samples, they were thawed, weighed and homogenized with a Polytron for 15 sec in 6 ml (for whole brains) or 3 ml (for half-brains) of cold 40% ethanol containing 0.4 mg/ml ascorbic acid, 0.5 mg/ml EDTA, and 100 ng/ml of the internal standard, MHPP [13]. The homogenates were centrifuged at 26,000 *g* for 20 min in a Sorvall RC-2B refrigerated centrifuge. The supernatant fractions were removed by Pasteur pipette and stored overnight at 4°. Each supernatant fraction was evaporated to dryness using a rotary evaporator with a 36° water bath. The residues were redissolved in 1 or 2 ml of 2 mg/ml ascorbic acid.

Then 1 ml of each brain extract or 0.5 ml of each urine (to which 10 μg of MHPP had been added) was added to a 15-ml screw-cap tube. To each tube was added 0.5 ml of 1 M sodium acetate buffer (pH 6.0), containing 10 mg/ml of sulfatase (except for standards and for measuring unconjugated metabolites) and 0.25 ml of a solution of 2% EDTA and 0.4% ascorbic acid. The samples were incubated at 37° for 20 hr with gentle shaking and were then extracted with two 5-ml portions of ethyl acetate. The combined ethyl acetate layers were washed with 0.5 ml of 1 M $KHCO_3$ and evaporated to dryness using a rotary evaporator set at 36°. Each residue was redissolved in 2 ml of ethyl acetate, and either the entire amount (for brain samples) or 0.2 ml (for urine samples) was evaporated to dryness under a stream of nitrogen in 1 ml Reacti-Vials (Pierce). Throughout the extraction procedures, the samples were maintained under nitrogen. They were then redissolved in 10 μl (for brain samples) or 20 μl (for urine samples) of ethyl acetate and derivatized by adding 10 μl of PFPA. Aliquots of 1–4 μl were then analyzed with a Finnigan model 3200 quadrupole gas chromatograph-mass spectrometer which was operated in the chemical ionization mode with methane as the reagent gas (ion source pressure = 0.9 torr), as previously described [16, 17]. Generally, chromatographic separations were done using a 6 ft × 2 mm silanized column packed with 3% OV-1 on 80/100

mesh Supelcoport (Supelco, Inc., Bellefonte, PA) which was programmed at 6°/min from an initial temperature of 110°. Selected-ion monitoring was carried out by focusing on the ions having an *m/z* of 297 for MHPE, *m/z* 429 for DHPE and pHPG, *m/z* 459 for MHPG, and *m/z* 311 for the internal standard, MHPP. Quantitation for the samples was based on the ratios of the peak heights derived from each compound and the internal standard and comparing them with that of standard curves. The standard curves were derived by adding various amounts of each compound to pooled brain or urine samples of untreated rats and carrying them through the extraction procedure along with the unknown samples, except for the omission of the sulfatase. Blanks were run the same as the samples, except that water was used in place of urines and 2 mg/ml ascorbic acid in place of brain extracts. They were used to correct for the small amounts of metabolites present in some batches of sulfatase.

Assay of acidic metabolites. The acidic metabolites, HVA and DOPAC, were assayed by GC/MS, using the corresponding propionic acid analogs, MHPPA and DOPPA, as internal standards [13]. To each urine sample was added the two internal standards (10 μg of each), 0.5 ml of 1 M sodium acetate buffer (pH 6.0) containing 5 mg of sulfatase, and 0.25 ml of a solution of 2% EDTA and 0.4% ascorbic acid. To 50 μl of each brain homogenate was added 10 ng of each internal standard, 50 μl of 1 M sodium acetate buffer (pH 6.0) containing 0.5 mg of sulfatase, and 100 μl of a 2% EDTA–0.04% ascorbic acid solution. The sulfatase was omitted from the samples assayed for levels of free HVA and DOPAC and from the standards. The tubes were flushed with nitrogen and incubated for 16 hr at 37° with gentle shaking. Then, each sample was adjusted to pH 2 with concentrated HCl and was saturated with NaCl. The acidic metabolites were extracted with ethyl acetate, using two 1-ml portions for the brain samples or two 4-ml portions for the urine samples. For each urine sample, a 0.5-ml aliquot of the combined ethyl acetate layers was evaporated to dryness in a silanized Reacti-Vial (Pierce) under a stream of nitrogen. For each brain sample, the entire combined ethyl acetate layers were transferred to a Reacti-Vial and evaporated to dryness. The acidic compounds were then converted to the corresponding pentafluoropropionyl (PFP) derivatives by the two-step procedure of Wilk and coworkers [18]. First, they were reacted with 50 μl of a 4:1 mixture of PFPA:PFPOH for 15 min at 75°. After evaporating off the excess reagent under a stream of nitrogen, they were reacted with 30 μl of PFPA for 5 min at 75°. The excess reagent was again removed by evaporation, and the derivatives were dissolved in 30 μl of ethyl acetate containing 1% PFPA. Aliquots (1–2 μl) were analyzed by GC/MS as for the neutral metabolites, except that the instrument was run isothermally at 150° and was set to focus on ions at *m/z* 415 for DOPAC, *m/z* 283 for HVA, *m/z* 457 for DOPPA and *m/z* 325 for MHPPA.

Assay of catecholamines, DOPAC, serotonin and 5-HIAA by high-performance liquid chromatography (HPLC) with electrochemical detection. Tissue concentrations of catecholamines, DOPAC, serotonin and 5-HIAA were determined by an adaptation of the procedures of Reinhard and Roth [19]. For measuring the catecholamines and DOPAC, a 0.5-ml aliquot of each supernatant solution (obtained from samples homogenized in either 40% ethanol or 0.1 M $HClO_4$) was transferred to a 1.5-ml Beckman microfuge tube. After adding an appropriate amount (typically 60 ng) of the internal standard, DHBA, 200 μl of 1 M (pH 8.6) Tris buffer (containing 0.2 M EDTA and 3 mM sodium metabisulfite) and 50 mg of alumina were added, and the tubes were immediately mixed for 10 min to allow maximal adsorption of the catechols by the alumina. The alumina was washed with 1 ml of 0.1 M (pH 7.0) Tris buffer containing 10 mM EDTA and 1 mM sodium metabisulfite, and the catechols were eluted into 300 μl of 0.1 M $HClO_4$. Part of each eluate (50–100 μl) was injected into a model LC-153 Bio-Analytical Systems, Inc. (West Lafayette, IN) HPLC equipped with a 5 μm Biophase ODS reverse phase precolumn and column. The mobile phase, which consisted of 0.1 M KH_2PO_4, 0.1 mM EDTA, 1.5 mM sodium octylsulfate, and 14% methanol (pH 3.2), was pumped at a flow rate of 1.5 ml/min (3000 psi). Catecholic compounds were detected by a TL-5 glassy carbon electrode maintained by an LC-3A controller at an oxidation potential of 0.9 V vs an Ag/AgCl reference electrode.

Serotonin and 5-HIAA were analyzed by directly injecting aliquots of the supernatant fraction into the HPLC, using the same column but with a mobile phase of 0.1 N sodium acetate, 0.1 mM EDTA and 8% methanol (pH 4.2) and a flow rate of 1.7 ml/min. The detector was set as for the catechols.

RESULTS

In three separate experiments we found that intraventricular injections of 6-OHDA (2 × 250 μg) produced a depletion in the brain concentration of dopamine by 79–84% and of norepinephrine by 80–90% (Table 1). Brain DOPAC concentrations were decreased by 66–72%. The extent of catecholamine and DOPAC depletion appeared to be similar, whether the animals were killed about 1 or 3 weeks after 6-OHDA administration and whether they were fed a standard lab chow or a casein diet. The concentrations of serotonin and 5-HIAA were unchanged. Most of the results presented below refer to Experiment 3.

This regimen of 6-OHDA caused an initial loss in body weight of about 44 g (compared to a gain of 3 g in animals injected with saline), but the 6-OHDA-treated animals rapidly recovered and, although they never caught up with the control animals, they gained weight in parallel with them between days 6 and 22. By day 22, the 6-OHDA-treated animals weighed 20% less than the control animals (288 ± 9.3 vs 358 ± 10.3 g, $P < 0.001$).

As shown in Table 2, the central administration of 6-OHDA had no effects on the concentration of catecholamines in any of the peripheral organs examined (i.e. heart, spleen and adrenal glands). However, the weights of the hearts were significantly lower (by 22%) in the 6-OHDA-treated rats ($P < 0.01$); by comparison, the weights of the spleens and adrenal glands were not statistically different

Table 1. Effects of intraventricular injections of 6-OHDA (2 × 250 μg) on brain concentrations of catecholamines, serotonin and their metabolites*

Expt.	Day	% Control				
		Norepinephrine	Dopamine	Free DOPAC	Serotonin	5-HIAA
1	7	13 (15)	16 (11)	34		
2	22	10	21	32		
3	23	20	20	28	95	93

* All data were obtained by HPLC, except for those in parentheses, which were obtained by gas chromatography with electron-capture detection [20]. Animals in Experiments 1 and 2 were fed Purina chow and animals in Experiment 3 were fed a casein diet. Day refers to the number of days after the second injection of 6-OHDA.

from the controls. The 22% decrease in heart weight is about equal to the 20% lower total body weight observed for the 6-OHDA-treated rats by the end of the experiment.

In one experiment (Expt. 2, Table 1), a few rats were pretreated with harmaline (30 mg/kg) 10 min before a single injection of 6-OHDA. This pretreatment was found to cause a depletion of brain dopamine of 89% and of brain norepinephrine of 77%, even though only one injection of 250 μg of 6-OHDA was administered. For comparison, Uretsky and Iversen [21] found that the same dose of 6-OHDA, given without any pretreatment, depleted brain dopamine by only 54% and norepinephrine by 66%. Thus, it appears that pretreatment with harmaline, like pargyline [22], may be a useful tool for selectively enhancing the destruction of dopamine neurons by 6-OHDA. Unlike pargyline, harmaline is a reversible MAO inhibitor, and therefore could be used in experiments, such as those described here, where the long-lasting inhibition of MAO caused by an irreversible inhibitor would interfere with the formation of deaminated metabolites. However, excessive dopamine depletion causes severe behavioral deficits, such as aphagia and adipsia [23], and since a reasonable dopamine depletion was achieved without pretreatment with MAO inhibitors, we did not use harmaline in further studies.

The effects of 6-OHDA on brain and urine levels of free and total HVA and DOPAC are shown in Fig. 1. Brain levels of free DOPAC were depleted by 64% ($P < 0.001$), total DOPAC by 72% ($P < 0.001$), free HVA by 49% ($P < 0.001$) and total HVA by 26% (NS, $P < 0.1$). By comparison, both norepinephrine and dopamine were depleted by 80% (Expt. 3, Table 1).

The excretion of free and total HVA was decreased significantly on both days 7 and 23: total HVA by 44% ($P < 0.005$) and 37% ($P < 0.001$) of control and free HVA by 37% ($P < 0.05$) and 31% ($P < 0.02$) of control on days 7 and 23 respectively. The most striking effect was the large decreases in conjugated HVA of about 70–80%. Although the precision of the measurement of conjugated HVA was limited due to its being determined indirectly as the difference between the free and total, the decrease in the excretion of conjugated HVA nevertheless appears to be similar to the degree of the dopamine depletion caused by the 6-OHDA. In contrast, conjugated HVA was *increased* in the brain of 6-OHDA-treated rats. In control rats, conjugated HVA was 31 ng/g, accounting for 16% of the total, but in 6-OHDA-treated rats it was 64 ng/g, accounting for 44% of the total.

The pattern for the effects of central 6-OHDA administration on urinary DOPAC levels differed markedly from that of HVA. Whereas on day 7 total urinary DOPAC levels were decreased by 64% ($P < 0.05$), on day 23 the DOPAC levels were not statistically different from controls. Free DOPAC levels tended to show a parallel change, although the

Table 2. Effects of intraventricular injections of 6-OHDA (2 × 250 μg) on catecholamine concentrations in the heart, spleen and adrenal glands*

Tissue	Weight (mg)	Norepinephrine	Dopamine	Epinephrine
Heart (ng/g)				
Control (4)	1203 ± 50	739 ± 46	ND†	58 ± 7
6-OHDA (4)	942 ± 37‡	831 ± 85	ND	48 ± 5
Spleen (ng/g)				
Control (4)	868 ± 153	814 ± 213	ND	ND
6-OHDA (4)	827 ± 144	708 ± 93	ND	ND
Adrenal gland (μg/g)				
Control (9)	58.4 ± 4.1	90.0 ± 9.6	3.0 ± 0.3	243 ± 19
6-OHDA (9)	55.8 ± 2.6	86.3 ± 6.9	3.9 ± 0.5	275 ± 15

* Data were obtained from Experiment 3 in Table 1. Values are means ± S.E. Number of determinations are in parentheses.

† Not determined.

‡ $P < 0.01$ (two-tailed *t*-test).

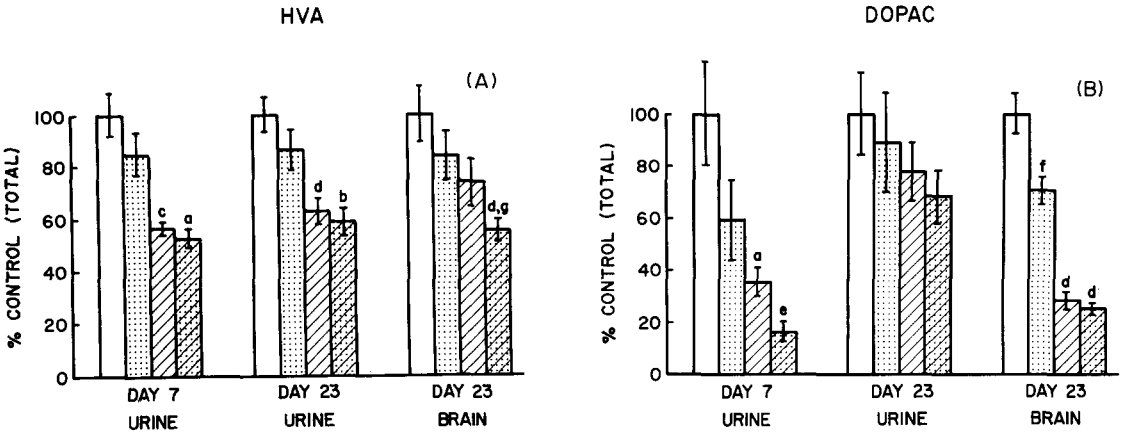

Fig. 1. Effects of intraventricular injections of 6-OHDA (2 × 250 μg) on urinary and brain concentrations of free and total HVA (panel A) and DOPAC (panel B). Control groups are represented by bars without diagonal lines: (□) total; and (▨) free. 6-OHDA-treated groups are represented by bars with diagonal lines: (▨) total; and (▩) free. The height of each bar is given as the mean percentage of the total (free + conjugated) metabolite level in the corresponding control group. (Standard errors of the means are indicated by the vertical lines.) The absolute values of these control groups were as follows: total HVA in urine on day 7 = 10.6 μg/24 hr, on day 23 = 28.9 μg/24 hr, and in brain on day 23 = 196 ng/g; total DOPAC in urine on day 7 = 17.2 μg/24 hr, on day 23 = 73.4 μg/24 hr, and in brain on day 23 = 218 ng/g. Key: $^{a-d}$Statistically significant difference (two-tailed Student's *t*-test) between the 6-OHDA group and the corresponding control group: $^{a}P < 0.05$; $^{b}P < 0.02$; $^{c}P < 0.005$; and $^{d}P < 0.001$. $^{e-g}$Statistically significant difference (two-tailed Student's *t*-test) between the free and total metabolite levels of the same group: $^{e}P < 0.05$; $^{f}P < 0.02$; and $^{g}P < 0.01$.

decrease in free DOPAC in the experimental group on day 7 did not achieve statistical significance ($P < 0.1$).

Next, the effects of 6-OHDA on brain and urine levels of neutral metabolites were examined (Fig. 2). Total MHPG levels in brain were depleted by 87% ($P < 0.001$) at the time of sacrifice, 23 days after 6-OHDA was administered. Total MHPE was depleted by 60% ($P < 0.05$). In this particular experiment, total DHPE was not altered significantly; and even though pHPG was decreased by 48%, this difference did not reach statistical significance.

Measurements of free metabolite levels in these brain samples (data not shown) did, however, reveal a small decline in free DHPE of 25% in 6-OHDA-treated rats ($P < 0.05$). On the other hand, free MHPG was lowered by 86% ($P < 0.001$), which was virtually identical to the reduction in total MHPG. Free MHPE and pHPG were undetectable.

Because of the uncertainties in the effects of 6-OHDA on brain DHPE and pHPG concentrations, the experiment was repeated, and the following results were obtained. Total MHPG was depleted by 85% ($P < 0.001$), in close agreement with that found above. Total DHPG, which was not determined in the experiments above, was found to be depleted by an even greater amount (93%, $P < 0.001$). In contrast to the previous experiment, both total DHPE and pHPG were reduced significantly in 6-OHDA-treated rats: by 53% ($P < 0.005$) and 70% ($P < 0.005$) respectively.

Analyses of urinary neutral metabolites from Experiment 3 (Table 1) indicated that the urinary

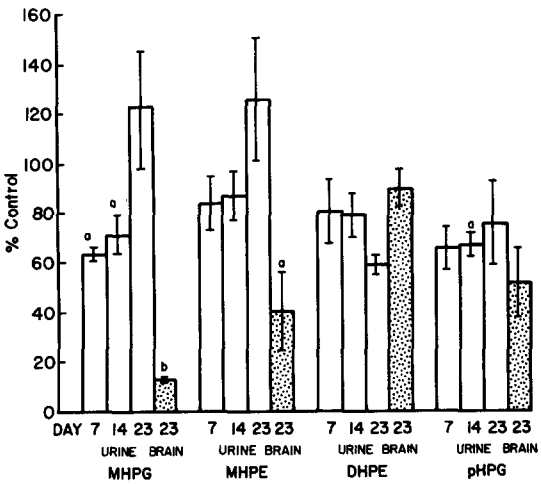

Fig. 2. Effects of intraventricular injections of 6-OHDA (2 × 250 μg) on urinary and brain levels of total neutral amine metabolites. Results are for the same experiment as in Fig. 1. Control values were: MHPG in urine on day 7 = 34.0 μg/24 hr, day 14 = 41.3 μg/24 hr, day 23 = 10.3 μg/24 hr, and in brain on day 23 = 83.9 ng/g; MHPE in urine on day 7 = 1.9 μg/24 hr, day 14 = 1.5 μg/24 hr, day 23 = 1.0 μg/24 hr, and in brain on day 23 = 0.55 ng/g; DHPE in urine on day 7 = 3.0 μg/24 hr, day 14 = 3.1 μg/24 hr, day 23 = 1.9 μg/24 hr, and in brain on day 23 = 11.1 ng/g; pHPG in urine on day 7 = 1.2 μg/24 hr, day 14 = 2.4 μg/24 hr, day 23 = 0.7 μg/24 hr, and in brain on day 23 = 2.7 ng/g. The N = 9 for all groups except N = 6 for day-7 samples. Key: ^{a,b}Statistically significant (two-tailed Student's *t*-test) compared with the control group: $^{a}P < 0.05$; $^{b}P < 0.001$.

excretion of MHPG was significantly lowered in 6-OHDA-treated rats on days 7 and 14 (by 37 and 29% of control, respectively, $P < 0.05$) but not on day 23 (123% of control) (Fig. 2). The excretion of each of the other neutral metabolites was lower in the 6-OHDA-treated rats than in controls at every time point (except for MHPE excretion of day 23), but none of these differences reached statistical significance, with the exception of pHPG excretion on day 14, which was decreased by 33% ($P < 0.05$).

DISCUSSION

The results presented in this paper provide new information on the contribution of the CNS to the excretion of both acidic and neutral metabolites of catecholamines and octopamine. While several previous studies on the effects of 6-OHDA on urinary metabolites have been reported [5–9, 12], their interpretation is somewhat limited since they have usually focused on a single metabolite (most commonly MHPG) and have differed in their methodologies. A number of experimental factors can have a profound influence on the results. For example, the nature of the diet fed to the animals is extremely important, since standard laboratory chow contains large amounts of DOPA [24] and markedly increases the excretion of both acidic and neutral catecholamine metabolites.* Moreover, as shown by the results of the present study (Fig. 1), the results may be dependent on the time interval between administering 6-OHDA and collecting urine.

Our results indicate that free and total HVA in urine were reduced by 31 and 37%, respectively, 23 days after 6-OHDA treatment. Since at this time brain dopamine levels were reduced by only 80%, by extrapolation we can estimate that, if brain dopamine was totally depleted, this would result in a decrease in free HVA excretion of 39% (31% × 100/80) and in total HVA excretion of 46% (37% × 100/80), meaning that these percentages of free and total HVA in urine had originated from the CNS. The estimated amount of free HVA that is derived from the CNS is in good agreement with the 34% reported by Hoeldtke *et al.* [6].

The estimates in both studies are based on the assumption that 6-OHDA did not cause any compensatory increase in dopamine synthesis in the brain. Such a compensatory change has been proposed to occur when there is partial destruction of catecholamine neurons [25] and would result in the above calculations being an underestimation of the proportion of urinary HVA derived from the brain. The smaller decreases in brain free and total HVA levels than in dopamine levels would appear to support the concept of a compensatory increase in dopamine turnover. However, the relatively smaller decrease in brain HVA levels more likely reflects an effect of 6-OHDA on its transport out of the brain (see below) rather than an increase in dopamine turnover. Although previous studies have shown that 6-OHDA produces an increase in the DOPAC/dopamine ratio in the striatum [26, 27], data in the present study indicate that in the whole brain the decrease in the free DOPAC levels (72%) was nearly the same as the 80% decrease in dopamine (Table 1). Thus, these data are consistent with the conclusion of Hoeldtke *et al.* [6] that destruction of dopamine neurons with 6-OHDA probably produces little or no compensatory increase in dopamine turnover in the surviving neurons of the whole brain. It would therefore seem unlikely that any compensatory changes in brain dopamine turnover would be sufficiently large as to have a major influence on our calculations.

Peyrin *et al.* [12] have suggested previously that conjugated HVA is a much more selective marker of brain dopamine metabolism than is the free metabolite. This conclusion was based on the 42% decline in the excretion of conjugated HVA in rats following the unilateral destruction of the substantia nigra with 6-OHDA, providing an estimate that 88% of the conjugated HVA in rat urine derived from the striatum. In agreement with those results, we have found that urine levels of conjugated HVA (as measured by the differences between the free and total levels) were profoundly decreased in animals injected intraventricularly with 6-OHDA. The decrements in conjugated HVA (80% on day 7 and 71% on day 23) were not only greater than those of free HVA but were nearly the same as the depletion in brain dopamine.

On the other hand, if conjugated HVA serves as a marker of brain dopamine metabolism, as suggested by the hypothesis of Peyrin *et al.* [12], one would predict that conjugated HVA would be depleted in brain to about the same extent as dopamine. However, our data (Fig. 1) show that 6-OHDA treatment was associated with an *increase* rather than a decrease in conjugated HVA. The cause of this increase is unclear, but one possibility is that 6-OHDA partially inactivated the probenecid-sensitive transport system that is responsible for the efflux of acidic metabolites from the brain. Although the lack of any effect of 6-OHDA on free 5-HIAA, which is also a substrate for this transport system [28], seems to argue against this idea, it is possible that a partial inactivation of the probenecid-sensitive system would have a selective effect on conjugated HVA. It is known, for example, that although this transport system is involved in the elimination of both free and conjugated HVA [29, 30], low doses of probenecid preferentially enhance the accumulation of the conjugated form [30]. It is conceivable that a similar inactivation of the acid transport system caused by 6-OHDA might selectively block conjugated HVA efflux from the brain without affecting that of 5-HIAA. An alternative explanation for our results is that 6-OHDA treatment by some unknown mechanism independently alters the degree of HVA conjugation in the CNS and the periphery, increasing it in the one case and decreasing it in the other. In view of the lack of a corresponding change of conjugated HVA levels in the brain and urine following 6-OHDA, it appears doubtful that measuring conjugated urinary HVA levels has any advantage over the free or total levels as markers of central dopamine metabolism.

In contrast to HVA, DOPAC excretion was

* M. L. Sedlock, J. Ravitch and D. J. Edwards, *Biochem. Med.*, in press.

unequally affected 7 and 23 days after 6-OHDA. The data obtained on day 23 indicated that the 6-OHDA treatment caused only a small, nonsignificant fall in the excretion of both free and total DOPAC. These results are similar to those previously reported by Hoeldtke *et al.* [6]. Combined with our finding that brain DOPAC was depleted by more than 70%, these data suggest that most of the DOPAC in urine was from the periphery. On the other hand, the large 64% decrease in total DOPAC excretion 7 days after 6-OHDA suggests that most of the urinary DOPAC derived from the CNS. The apparent discrepancy between the data obtained on days 7 and 23 might be explained by either of two possibilities. First, most of the DOPAC in urine might originate in the periphery (as indicated by the data for day 23), but destruction of central catecholamine neurons that interact with peripheral dopamine (or norepinephrine) neurons might lead to a transient decrease in peripheral dopamine metabolism, accounting for the reduction in DOPAC excretion on day 7. Since our treatment with 6-OHDA destroys both norepinephrine and dopamine, depletion of either or both of these neurotransmitters might be responsible for a reduction in peripheral dopamine metabolism. However, if this possibility is correct, it would mean that peripheral dopamine turnover adapts to a persisting interruption of central inputs and is restored to normal by day 23. A second possibility that would account for our finding is that a large proportion of urinary DOPAC does derive from the brain, as would be suggested by our data from day 7, but that the lesioning of central catecholamine neurons leads to an increase in peripheral dopamine turnover that develops between days 7 and 23. In this instance, our data would suggest that at least 80% of total DOPAC in the urine is of central origin. Further studies will be needed to distinguish between these two possibilities. Nevertheless, since in our experience the excretion of DOPAC is much more variable and more greatly influenced by the diet than is HVA, we believe that urinary HVA is a better marker of CNS dopamine function than is DOPAC.

The neutral metabolites are expected to be more selective markers for the CNS than are the corresponding acidic metabolites. This is due to the fact that mitochondria in brain, in contrast to those in liver and other organs, are relatively high in the activity of NADPH-linked aldehyde reductase [31] and low in the activity of aldehyde dehydrogenase [32]. Consequently, aldehydes formed from the corresponding amines by the action of brain mitochondrial monoamine oxidase would be preferentially reduced to neutral, alcoholic products, whereas in liver and other peripheral tissues (where aldehyde reductase is localized primarily in the cytoplasm), the aldehydes would be principally converted to acidic metabolites [33]. An example of experimental verification of this idea is provided by the finding that norepinephrine is primarily metabolized to MHPG in the CNS [34] and to vanillylmandelic acid (VMA) in the periphery [35]. Although this supports the validity of using levels of MHPG in peripheral body fluids as a marker of CNS norepinephrine turnover (for review, see ref. 36), determining the exact percentage of the metabolite that derives from the CNS has proven to be rather difficult and the results controversial. For example, based on the measured difference in venous-arterial levels, Maas *et al.* [37] estimated that the brain accounts for 63% of the total body production of MHPG in human subjects. The accuracy of this estimate has been challenged, however, by the finding that in man MHPG is rapidly converted to VMA [38, 39]. Based on the latter results, it has been suggested that perhaps only 20% of urinary MHPG in man is derived from the brain [38]. However, this lower estimate is also in doubt, since an extremely large, nonphysiological amount of deuterated MHPG was injected [40].

Several investigators have used experimental animals to determine the origin of urinary MHPG by measuring the effect of 6-OHDA administered directly into the brain. Studies in rhesus monkeys treated with an intraventricular injection of 6-OHDA have yielded discrepant results, with urinary MHPG excretion being lowered in two studies [41, 42] but not in another [9]. Based on the results of one study, it was calculated that up to 65% of the excreted MHPG originated in the CNS [41], but in another study the decrease in MHPG excretion was not accompanied by a depletion in brain catecholamines [42].

Similar studies in rats all failed to show any effect of 6-OHDA on urinary MHPG [5–8], except for a 29% decrease observed by Breese *et al.* [9]. In our studies, we also found no difference in MHPG excretion between 6-OHDA and saline-treated animals when measured 23 days after injection. The fact that brain MHPG levels were depleted to the same extent as norepinephrine indicated that a compensatory increase in norepinephrine turnover in the neurons not lesioned by 6-OHDA could not explain our results. The lack of such a compensatory change is also supported by the data of Helmeste *et al.* [10] in which the levels of norepinephrine and MHPG in the forebrain of 6-OHDA-treated rats were reduced to 26 and 34% respectively. Moreover, Logue *et al.* [43] recently reported that, following treatment with DSP-4, a neurotoxin believed to damage central norepinephrine neurons, MHPG-SO_4 and norepinephrine levels were lowered to the same extent in all brain regions except the cortex and hippocampus.

In contrast, MHPG was decreased in the urines of 6-OHDA-treated rats collected either 7 or 14 days after injection. The differences in the results obtained 1–2 vs 3 weeks after 6-OHDA might explain previous discrepancies. For example, in the previously mentioned studies of monkeys the urines were collected 10–15 days after treatment in the one study in which urinary MHPG was depressed by 6-OHDA [41] but in the negative study they were collected 6 weeks after the last treatment [9]. However, as in the case with DOPAC (see above), it is impossible to say which of the results provides a more accurate indication of the percentage of urinary MHPG that is of central origin. Descending norepinephrine neurons from the locus coeruleus are known to influence the activity of the sympathetic nervous system [44]. This has been demonstrated, for instance, by the finding that electrical stimulation of the locus coeruleus produces an increase in plasma

MHPG that is partially blocked by the ganglionic blocker hexamethonium [45]. Thus, the decrease in activity of central norepinephrine neurons following sympathectomy might reduce MHPG production in the periphery. Such an effect, however, must be of short duration and reversed by day 23 to be consistent with our data. Another possibility is that a decrease in peripheral MHPG formation is an indirect effect of some of the transient behavioural deficits that are associated with depleting brain catecholamines with 6-OHDA [23]. On the other hand, it is possible that 6-OHDA decreases urinary MHPG due to a reduction in the amount of this metabolite coming from the brain, but that this is negated by a compensatory increase in peripheral MHPG production that is manifested by day 23. If so, we can calculate the percentage of urinary MHPG that derives from the CNS based on the data of days 7 and 14. Since the degree of norepinephrine depletion remains constant over the range of 2 to 75 days after 6-OHDA injections [21], we can assume that brain norepinephrine levels were decreased by 80% on days 7 and 14 as they were on day 23 when the brains were assayed. Based on the 29 and 37% decreases in urine MHPG levels on days 7 and 14, respectively, and by extrapolating the results to total norepinephrine depletion (as described above for HVA), we can estimate that 36% (29% × 100/80) to 46% (37% × 100/80) of the MHPG in urine originates in the CNS. This is in good agreement with our finding that treatment with the peripheral monoamine oxidase inhibitor, debrisoquin, decreases MHPG excretion by 68%, suggesting that 32% originates in the CNS.* This is also similar to the results of Karoum *et al.* [5], who used the same regimen of debrisoquin to perturb the metabolism of [^{3}H]norepinephrine and calculated that 30% of urinary MHPG in the rat is derived from the brain.

The excretion of the neutral dopamine metabolites, DHPE and MHPE, tended to be about 20% lower in the 6-OHDA-treated rats, although these differences were not statistically significant. A lack of decrease in MHPE excretion following 6-OHDA has also been reported by Karoum and Costa [46]. However, in their paper the combined amount of the sulfate and glucuronide conjugates of MHPE excreted by control animals was 13 μg/24 hr, which is considerably more than the amount of total (free + conjugated) MHPE (about 2 μg/24 hr) we have found in our studies [15]. This discrepancy may be due to the greater specificity of the GC/MS assay or to differences in the diets.

The fact that MHPE and DHPE excretion was not decreased significantly by 6-OHDA is not surprising in view of the fact that MHPE levels in the brain were lowered by only 60% and DHPE levels were not affected significantly. This may be a result of a compensatory increase in the rate of synthesis of these metabolites following 6-OHDA (reflecting a compensatory increase in dopamine synthesis or release) or it may reflect these metabolites being formed in a compartment outside of dopamine neurons that is resistant to 6-OHDA. Which of these possibilities is correct is not at present clear.

The excretion of pHPG in 6-OHDA-treated rats was lowered by only 24–34% on days 7, 14 and 23. However, because the decrease was statistically significant only on day 14 and because the values for brain pHPG levels were not statistically reduced by 6-OHDA due to the large variance, the proportion of urinary pHPG that derives from the CNS cannot be accurately calculated in this experiment. However, based on the results of a subsequent experiment using the same conditions of 6-OHDA treatment in which brain pHPG levels were reduced by 70% ($P < 0.001$), we can infer that approximately 1/3 to 1/2 of the pHPG in urine is of central origin. It therefore appears that (when dietary sources are eliminated) the contribution of the brain to the urinary levels of pHPG is similar or somewhat greater than that for MHPG. The possibility that it is greater is also suggested by our recent finding that debrisoquin has a smaller effect on pHPG excretion than on MHPG excretion.*

In conclusion, these results show that the central administration of 6-OHDA can be used for estimating simultaneously the proportions of several acidic and neutral urinary metabolites that originate in the CNS. Although interpretation of the data must consider possible compensatory changes in amine turnover in both the CNS and the periphery of 6-OHDA-treated animals, the data suggest that a substantial amount of MHPG, pHPG and HVA in rat urine derives from the brain. On the other hand, the data did not support the idea that conjugated HVA in urine is a better marker of brain dopamine metabolism than is the unconjugated metabolite.

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