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EFFECT OF SELECTIVE DESTRUCTION OF CENTRAL AND PERIPHERAL CATECHOLAMINE-CONTAINING NEURONES WITH 6-HYDROXYDOPAMINE ON CATECHOLAMINE EXCRETION IN THE RAT

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1 The contribution of various tissues to some of the pools of catecholamine metabolites in urine has been estimated by measuring the excretion of these compounds by rats given DOPA-free diets and intravenous, intraventricular, or intracisternal 6-hydroxydopamine.

2 Destruction of peripheral sympathetic neurones by repeated intravenous doses of 6-hydroxydopamine led to a 34% decrease in noradrenaline excretion, and a 38% decrease in 4-hydroxy-3-methoxyphenylglycol sulphate excretion. Depletion of brain noradrenaline (by 67%), after intracisternal 6-hydroxydopamine, was unassociated with changes in the excretion of noradrenaline or of 4-hydroxy-3-methoxyphenylglycol sulphate. This suggests that these compounds in rat urine are derived mainly from peripheral tissues.

3 Depletion of brain dopamine (by 80%) by intraventricular 6-hydroxydopamine was associated with a 27% decrease in the excretion of homovanillic acid. Destruction of peripheral sympathetic neurones with intravenous 6-hydroxydopamine led to a 25% decrease in homovanillic acid excretion. The data suggest that the homovanillic acid in rat urine derives partially from brain dopamine and partially from dopamine released from or metabolized within sympathetic neurones.

4 Neither depletion of brain dopamine, nor destruction of sympathetic neurones, caused alterations in the excretion of dopamine or dihydroxyphenylacetic acid.

Introduction

Catecholamines are present in, and released from, a variety of mammalian tissues, including adrenal medullary chromaffin cells, the brain, and all sympathetically innervated organs (Anton & Sayre, 1964). For this reason, it is difficult to relate changes in the excretion of catecholamines or their metabolites to alterations in catecholamine metabolism within any specific tissue. In the dog (Maas & Landis, 1968), rat, and guinea-pig (Breese, Chase & Kopin, 1969), 4-hydroxy-3-methoxyphenylglycol sulphate (HMPG) may be the principal metabolite of brain noradrenaline (NA). Thus, urinary HMPG has been proposed as an index of brain NA metabolism (Jones, Maas, Dekirmenjian & Fawcett, 1973). In rats, however, immunosympathectomy, which causes the degeneration of only peripheral sympathetic neurones, leads to a 50% decrease in urinary HMPG (Ceasar, Ruthven & Sandler, 1969). Correspondingly little information is available concerning the origins of dopamine metabolites in the urine. We believe that the examination of the tissue source of urinary dopamine and its metabolites has been complicated by an unsuspected nutritional artefact, i.e., the contribution of the metabolites of exogenous 3-4-dihydroxyphenylalanine (DOPA) which is present in rat food (Hoeldtke, Baliga, Issenberg & Wurtman, 1972) and is synthesized in the rat stomach by a cereal constituent, presumably a tyrosinase (Hoeldtke & Wurtman, 1972). Rats consuming a DOPA-free diet in which casein was the sole protein source excreted less than 20% as much free and conjugated dihydroxyphenylacetic acid (DOPAC) as animals consuming a standard cereal-based diet (Hoeldtke & Wurtman, 1974). In the present study, we attempted to avoid the

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influence of exogenous DOPA by feeding the animals a casein diet.

6-Hydroxydopamine (6-OHDA) can be used to selectively destroy either peripheral (Thoenen & Tranzer, 1968; Clark, Laverty & Phelan, 1972) or central (Uretsky & Iversen, 1969; Breese & Traylor, 1970) catecholamine-containing neurones. We have measured the concentrations of dopamine and noradrenaline, and some of their major metabolites in urines from such 6-OHDA-treated animals, in order to estimate the relative contributions of particular catecholamine-containing tissues to each urinary compound.

Methods

Sprague-Dawley male rats (150-200 g) (Charles River Laboratories, Wilmington, Mass.) were allowed free access to food and water. For four days prior to urine collection, the animals were fed a casein diet (General Biochemical, Test Diet 71373, Chagrin Falls, Ohio). The rats readily consumed the diet (13.7 g/24 h) and gained weight normally.

Drugs and injections

1. The intravenous administration of 6-hydroxydopamine. Fifty mg/kg 6-OHDA hydrobromide (Regis Chemical, Chicago) was dissolved in 0.5 ml 0.001 N HCl, and injected into the tail vein. One week later, 100 mg/kg of the drug was similarly administered on each of two successive days. Eight days after the last injection, animals were placed in metabolic cages, and urine was collected and immediately frozen.

2. The intraventricular administration of 6-hydroxydopamine. Animals were anaesthetized with ether. 6-OHDA (250 μg) (303 μg 6-OHDA hydrochloride (AB BIOTEC, Stockholm, Sweden) dissolved in 25 μl 0.1% ascorbic acid in 0.9% w/v NaCl solution (saline)) was injected into the left lateral brain ventricle according to the technique of Noble, Wurtman & Axelrod (1967). Forty-eight hours later, the injection was repeated into the right brain ventricle. Control animals were injected with the vehicle. Ten days after the second injection, urine was collected.

3. The intracisternal administration of 6-hydroxydopamine. Animals were anaesthetized with ether. 6-OHDA (250 μg) (303 μg 6-OHDA hydrochloride dissolved in 25 μl 0.1% ascorbic acid in saline) was injected intracisternally, and again 48 h later. Control animals received 25 μl of the solvent. Urine was collected 18 days after the second injection.

Assay of tissue noradrenaline and dopamine

The animals were killed by cervical fracture after the last day of urine collections. The brains and hearts were removed and frozen immediately on dry ice. The tissues were weighed and then homogenized in 10 ml 0.4 N perchloric acid. Dopamine and NA were extracted from the perchloric acid supernatant by alumina column chromatography (Anton & Sayre, 1964). The dopamine in the acetic acid eluates was then assayed fluorimetrically by the technique of Carlsson & Waldeck (1958). NA was assayed by the method of von Euler & Lishajko (1961).

Assay of urinary catecholamines and metabolites

Proteins were precipitated from rat urine by the addition of one-tenth volume 4 N perchloric acid, and then centrifugation at 30,000 *g* for 15 minutes. Catecholamines were then extracted from the supernatant fluid by alumina chromatography, followed by Amberlite ion exchange resin chromatography (Weil-Malherbe, 1968). The NA concentrations in the eluates were assayed by the method of Weil-Malherbe & Bigelow (1968). Other portions of the eluates were adjusted to pH 5.9, and their dopamine concentrations assayed by the procedure of Carlsson & Waldeck (1958). The reported catecholamine concentrations were corrected for average recoveries of 50%. Catechol acids (i.e., DOPAC and dihydroxymandelic acid) were eluted from the alumina columns with 3 ml 1 N H_2SO_4 (Weil-Malherbe & Van Buren, 1969). DOPAC was then extracted from the sulphuric acid eluate into ether, separated from dihydroxymandelic acid, and assayed according to the procedure of Spano & Neff (1971). The reported DOPAC concentrations were corrected for average recoveries of 66%.

The alumina effluents were collected in beakers containing 1 ml 2 N HCl. A second deproteinization was then performed; 1 ml 40% sodium tungstate was added, and the pH was adjusted to less than 1.5 with 60% perchloric acid. The solutions were left in ice for 30 min, and were then centrifuged for 15 min at 30,000 *g*. HMPG, liberated from its sulphate conjugate by incubation with β-glucuronidase (Type H-2, Sigma Chemical, St. Louis, Missouri), was then extracted from the hydrolysate, oxidized to vanillin, and measured photometrically (Bigelow, Neal & Weil-Malherbe, 1971).

The homovanillic acid (HVA) in 20 ml portions of the deproteinized salt-saturated alumina effluents was extracted into 20 ml ethyl acetate by mechanical shaking. This process was repeated and the ethyl acetate extracts pooled, after which 3 ml

sodium bicarbonate (10%) was added to the organic phase for back-extraction of the HVA into an aqueous phase (Armstrong, Shaw & Wall, 1956). The organic phase was discarded and the sodium bicarbonate phase was adjusted to pH 4.5, and 0.5 ml 0.1 M ammonium acetate buffer (pH 4.5) was added. The HVA was then adsorbed onto an anionic Dowex column (AG 1 x 4, 100-200 mesh, chloride form) and assayed as described by Sato (1965).

Results

The repeated administration of large intravenous doses of 6-OHDA caused a nearly total depletion of cardiac NA, without affecting brain NA (Table 1). Depletion of peripheral NA stores was associated with significant decreases in urinary NA (34%), HMPG sulphate (38%), and HVA (25%). Dopamine and DOPAC excretions were not significantly altered.

The bilateral intraventricular administration of 6-OHDA caused a large (>80%) decrease in brain dopamine. These animals excreted moderately decreased (27%) quantities of HVA, but unchanged amounts of NA, dopamine, and DOPAC (Table 2).

The intracisternal administration of 6-OHDA caused a marked (67%) depletion of brain NA but only a moderate (45%) fall in brain dopamine (Table 3). The depletion of brain NA was unassociated with alterations in urinary NA or HMPG sulphate.

Discussion

In agreement with previous studies (Thoenen & Tranzer, 1968), the repeated administration of large doses of intravenous 6-OHDA caused a nearly total depletion of cardiac NA (Table 1). Brain NA was unaffected, suggesting that the circulating 6-OHDA did not enter the brain tissue. In spite of

Table 1 Urinary catecholamines and their metabolites following depletion of peripheral noradrenaline stores by repeated doses of intravenous 6-hydroxydopamine (6-OHDA).

		Control *n = 9*	*6-OHDA-treated* *n = 10*	*Significance*
Tissue catecholamines (μg/organ)	Brain noradrenaline	0.696 ± 0.49	0.769 ± 0.93	N.S.
	Cardiac noradrenaline	0.684 ± 0.16	undetectable	$P < 0.001$
Urinary catecholamines (μg/24 h)	Noradrenaline	1.84 ± 0.26	1.20 ± 0.16	$P < 0.05$
	Dopamine	4.36 ± 0.48	3.56 ± 0.28	N.S.
	3,4-dihydroxyphenylacetic acid	8.13 ± 0.81	6.95 ± 0.57	N.S.
	Homovanillic acid	26.5 ± 3.3	19.4 ± 1.7	$P < 0.05$
	4-hydroxy-3-methoxyphenylglycol	41.6 ± 3.71	25.6 ± 2.8	$P < 0.01$

The groups were compared by the non-paired *t* test.

Table 2 Urinary catecholamines and their metabolites following depletion of brain dopamine by intraventricular administration of 6-hydroxydopamine (6-OHDA).

		Control *n = 16*	*6-OHDA-treated* *n = 18*	*Significance*
Brain dopamine (μg/brain)		1.17 ± 0.05	0.23 ± 0.05	$P < 0.001$
Urinary metabolites (μg/24 h)	Noradrenaline	1.10 ± 0.10	1.02 ± 0.12	N.S.
	Dopamine	3.34 ± 0.28	2.86 ± 0.22	N.S.
	3,4-dihydroxyphenylacetic acid	5.30 ± 0.555	4.98 ± 0.47	N.S.
	Homovanillic acid	18.6 ± 1.9	13.5 ± 1.5	$P < 0.05$

The groups were compared by the non-paired *t* test.

Table 3 Urinary noradrenaline and 4-hydroxy-3-methoxyphenylglycol (HMPG) following depletion of brain noradrenaline by intracisternal 6-hydroxydopamine (6-OHDA).

	Control $n = 11$	*6-OHDA-treated* $n = 8$	*Significance*
Brain noradrenaline (μg/brain)	0.75 ± 0.07	0.25 ± 0.02	$P < 0.001$
Brain dopamine (μg/brain)	1.27 ± 0.08	0.72 ± 0.09	$P < 0.001$
Urinary noradrenaline (μg/24 h)	1.32 ± 0.22	1.00 ± 0.18	N.S.
Urinary HMPG (μg/24 h)	41.6 ± 4.9	39.5 ± 2.6	N.S.

The groups were compared by the non-paired *t* test.

the dramatic decrease in cardiac NA, the urinary excretion of NA and HMPG were only moderately decreased.

The relative resistance of urinary NA (and of its metabolite HMPG) to doses of 6-OHDA which deplete the heart of this catecholamine suggests that some sympathetic neurones are not destroyed by the 6-OHDA: the adrenal medulla contributes a negligible fraction of the NA excreted into the urine (Leduc, 1961) while the brain secretes little if any of the catecholamine without prior deamination (Maas and Landis, 1968). One possible locus of surviving sympathetic neurones is in vascular tissue, inasmuch as vascular stores of NA are less affected by 6-OHDA than NA elsewhere. Berkowitz, Spector & Tarver (1972) found that a dosage schedule of 6-OHDA similar to that employed in our study caused a marked depletion of cardiac and splenic NA, but only a mild reduction in vascular NA concentrations. Since intravenously administered [^{3}H]-NA is poorly taken up within sympathetic nerve terminals in blood vessels, it was suggested that these terminals also fail to concentrate the structurally related compound, 6-OHDA, and thus are spared its toxic effects (Berkowitz, Tarver & Spector, 1971). Immunosympathectomy also causes a more dramatic decrease in cardiac than in vascular NA (Hamberger, Levi-Montalcini, Norberg & Sjöquist, 1965). As might be expected, the excretion pattern for catecholamine metabolites observed in immunosympathectomized animals is similar to that found in our rats treated with intravenous 6-OHDA, i.e., immunosympathectomy caused a 50% decrease in free urinary NA (Brody, 1964; Carpi & Oliverio, 1964), and a comparable decrease in HMPG sulphate excretion (Ceasar, Ruthven & Sandler, 1969). Thus the data obtained from both the immunosympathectomized and 6-OHDA-treated rats suggest that a large fraction of the NA excreted into the urine derives from sympathetic nerve terminals present in blood vessels.

The relative resistance of urinary NA and HMPG concentrations to 6-OHDA treatment could also reflect a compensatory increase in the synthesis of NA and of adrenaline, also a precursor of urinary HMPG, within the adrenal medulla. 6-OHDA causes a fall in blood pressure which is associated with a reflex increase in the presynaptic input to the adrenal medulla, and an increase in tyrosine hydroxylase activity (Mueller, Thoenen & Axelrod, 1969). If the increase in tyrosine hydroxylase activity is associated with enhanced catecholamine secretion, the anticipated falls in urinary NA and HMPG after 6-OHDA treatment would be diminished. Mueller *et al.* (1969) found that the increase in adrenal tyrosine hydroxylase activity persisted for five days after 6-OHDA treatment; however, Brimijoin & Molinoff (1971) observed that the activity of the adrenal enzyme had returned nearly to normal after three days and was back to control values on day seven. This suggests that increased secretion of adrenomedullary catecholamine was probably not an important factor in our animals, since urine was collected eight days after the last dose of 6-OHDA.

Intravenous 6-OHDA did not depress the excretions of dopamine or DOPAC, but caused a small but significant (25%) decrease in urinary HVA (Table 1). This alteration in HVA excretion suggests that some of the dopamine present in sympathetic neurones is either released as such or metabolized intraneuronally, and not β-hydroxylated. Collins & West (1968) incubated the rabbit ileum with [^{3}H]-DOPA or [^{3}H]-dopamine, and found that stimulation of sympathetic innervation of the ileum caused [^{3}H]-dopamine release. Similarly, [^{3}H]-dopamine has been found to be taken up by sympathetic neurones in the cat spleen, and

released by sympathetic nerve stimulation (Musacchio, Fischer & Kopin, 1966). In neither study was evidence presented for the release or intraneuronal metabolism of the small amount of endogenous DA in the sympathetic neurones.

There is considerable evidence that the introduction of 6-OHDA into the cerebrospinal fluid selectively affects brain neurones. Breese & Traylor (1970) have shown that intracisternally administered 6-OHDA does not affect cardiac catecholamines in adult rats. Similar results were obtained when the drug was administered intraventricularly to monkeys (Maas, Dekirmenjian, Garver, Redmond & Landis, 1972).

Rats treated bilaterally with intraventricular 6-OHDA had greatly decreased (80%) brain dopamine (Table 2). These animals excreted moderately decreased (27%) amounts of HVA but normal quantities of NA, dopamine, and DOPAC.

There is evidence that depletion of brain dopamine with 6-OHDA leads to parallel decrease in brain dopamine synthesis. Animals pretreated with the drug have a decreased capacity to synthesize [^{3}H]-dopamine from [^{3}H]-tyrosine when the isotope is administered intravenously or intracisternally (Breese & Traylor, 1970). Such animals also exhibited decreased tyrosine hydroxylase activity in the whole brain or caudate nucleus. The rate at which [^{3}H]-NA disappeared from the brain was also reduced in the 6-OHDA-treated animals, by 85% within the hypothalamus and 50% in the pons-medulla (Uretsky, Simmonds & Iversen, 1971). This suggests that there is probably little or no compensatory increase in brain catecholamine turnover with those catecholamine-containing neurones that escape destruction by 6-OHDA. The available evidence thus suggests that the decrease in brain dopamine concentrations in the 6-OHDA-treated animals is associated with parallel decreases in brain dopamine synthesis and turnover. The observed decrease in urinary HVA in the animals receiving intraventricular 6-OHDA provides further support for this concept.

Assuming that brain dopamine concentrations are indicative of its turnover in the brain, it becomes possible to estimate the contribution of brain dopamine to the urinary HVA pool. If an 80% depletion of brain dopamine leads to a 27% decrease in urinary HVA, then total depletion of brain dopamine might be expected to cause a 34% decrease. Thus, it appears that approximately one-third of urinary HVA in the rat is derived from brain. The data obtained from rats receiving 6-OHDA intravenously suggest that approximately one-fourth of the excreted compound is derived from sympathetic neurones sensitive to 6-OHDA (Table 1). The source of the remaining 40% of urinary HVA remains to be identified; it could be the adrenal medulla, or surviving sympathetic neurones.

Intracisternal 6-OHDA caused a 67% depletion of brain NA, but only a 43% decrease in brain dopamine. The relative resistance of brain dopamine to intracisternal 6-OHDA has previously been reported (Breese & Traylor, 1970). The NA depletion, however, was unassociated with changes in the excretion of HMPG (Table 3). More dramatic brain NA depletion (82%) has, however, been reported to cause a 29% decrease in HMPG excretion (Breese, Prange, Howard, Lipton, McKinney, Bowman & Bushnell, 1972). Similarly, in the monkey, a 72% depletion of brain NA has been found to cause a 33% decrease in urinary HMPG (Maas *et al.*, 1972). Thus, the lack of alteration in HMPG excretion in our study may have resulted from insufficient depletion of brain NA. Intravenous 6-OHDA treatment led to a larger decrease (38%) in HMPG excretion than reported in either of the above studies on brain. Thus it appears that sympathetic neurones are quantitatively the most important source of urinary HMPG.

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