Increased Synthesis of Catecholamines and Their Metabolites following the Administration of Phenoxybenzamine

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SUMMARY

The over-all synthesis rate of catecholamines was determined in control and phenoxybenzamine-treated rats both by measuring the incorporation of ^{14}C-L-tyrosine into norepinephrine and by assaying all the known major urinary metabolites of epinephrine and norepinephrine. The phenoxybenzamine-stimulated increase in synthesis of these hormones is firmly established. Consideration of the results of analysis of urinary metabolites reveals that (a) there is a large turnover of epinephrine and norepinephrine that must take place in sites not usually studied, and (b) studies of metabolism based on the intravenous administration of labeled norepinephrine must be interpreted with reserve.

INTRODUCTION

Factors which alter catecholamine synthesis and turnover have been the subject of intensive investigation in many laboratories over the past several years. There are several methods for estimating rates of synthesis *in vitro* and *in vivo*. In the earliest experiments, the labeled precursors ^{14}C-tyrosine or ^{3}H-3,4-dihydroxyphenylalanine were injected and the rate of decline of specific activity of the endogenously labeled catecholamines in various organs was followed (1–3). More recently the accumulation of label into endogenous catecholamines following ^{14}C-tyrosine administration has been used to estimate synthesis rates *in vivo* (4).

After it was demonstrated that infused norepinephrine was taken up by tissues (5), reports appeared describing the half-life and metabolic fate of the intravenously administered labeled amine (6, 7). This technique has been applied to the brain, the blood-brain barrier being circumvented by administering labeled norepinephrine into the lateral ventricle (8). When the rate-limiting step in catecholamine synthesis was elucidated (9) and an effective inhibitor of over-all synthesis was found (10), tissue turnover could be estimated by observing the rate of catecholamine depletion in organs after inhibition of synthesis (11). The limitations of each of these methods have been reviewed recently by Costa and Neff (12).

All of the above methods indicate that concomitant with increased sympathetic nerve activity there is an increased rate of synthesis of norepinephrine. Such studies have yielded information on turnover rates in individual organs such as heart, brain, and adrenal gland. Although they imply a net increase in synthesis of the transmitter by the whole organism, they do not establish it unequivocally. A prolonged elevation in the urinary excretion of catecholamines suggests an increased synthesis of the neurotransmitter. Although such findings have been reported (13–15), they are not

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convincing, since unchanged urinary norepinephrine and epinephrine represent an insignificant proportion of the total hormone secreted and metabolized by the rat. To ensure that urinary excretion represents synthesis rather than alterations in metabolism or kidney function, it is necessary to measure the excretion of all the urinary degradation products of norepinephrine as a function of sympathetic nerve activity. Only recently have suitable methods been developed for measuring catecholamines and their major metabolites in rat urine.[2] The present report describes the application of such methods to demonstrate that factors which increase sympathetic nerve activity, as measured by increased turnover of the catecholamines in individual organs, do result in a marked increase in the excretion of norepinephrine and all its metabolites. In this study sympathetic nerve activity was increased by administering the *alpha*-blocking agent phenoxybenzamine (16, 17).

METHODS AND MATERIALS

Four female Sprague-Dawley rats weighing 160 g were individually housed in metabolism cages over a container holding heptane cooled with Dry Ice, as reported by Denckla (18). The rats were allowed to eat ad libitum from a dish in the cage containing their usual food suspended as a mush in water. After being given 1 day to adapt to its new environment, each animal received 0.5 ml of 0.9% NaCl intraperitoneally. Urine was collected for the following 24 hr. At the end of this control period, each animal received phenoxybenzamine, 25 mg/kg intraperitoneally in 0.5 ml of 0.9% NaCl, and the urine was collected for the following 24 hr.

Analysis of urine. The urine was thawed, an aliquot was taken for creatinine determination, and the remainder was divided into two 10-ml portions. One portion was hydrolyzed for 18 hr at pH 5.0 with 1 ml of Glusulase (Sigma type H-2 contains 100,000 Fishman units of β-glucuronidase and 10,000 units of sulfatase per milliliter). Protein was then precipitated by making the urine 0.4 N with respect to perchloric acid, and catecholamines were removed by passage over alumina (19). Metanephrine and normetanephrine were isolated from the filtrate by passage over Amberlite CG-50 and were estimated according to the procedure of Bigelow and Weil-Malherbe (20). Both free and conjugated 3-methoxy-4-hydroxyphenylglycol (glycol metabolite), the major urinary metabolite, was extracted from the neutral filtrate of the Amberlite column into ethyl acetate and estimated as vanillin by a modification[2] of the method of Ruthven and Sandler (21). The second portion of the original urine specimens was hydrolyzed in acid, and the hydrolysate was analyzed for epinephrine and norepinephrine by the two-column procedure of Weil-Malherbe (19) and Weil-Malherbe and Bigelow (22).

3-Methoxy-4-hydroxymandelic acid was not routinely estimated, since the rat excretes so much *p*-hydroxymandelic acid that methods based on the conversion of 3-methoxy-4-hydroxymandelic acid to vanillin often may not yield meaningful results, even when optical densities are taken at 360 mμ as recommended by Pisano *et al.* (23). Thin layer chromatography was used to show that the urinary 3-methoxy-4-hydroxymandelic acid in these studies did not exceed 5% of the urinary glycol metabolite and that this maximal estimate was unaffected by the treatment with phenoxybenzamine.

With the above methods of hydrolysis and assay, recoveries were of the order of 70% and 85% for epinephrine and norepinephrine, respectively, 90–95% for metanephrine and normetanephrine, and 80% for the glycol. The values shown in the tables are not corrected for recovery, but are adjusted in all cases to their molar equivalent of norepinephrine.

The phenoxybenzamine-treated animals were lethargic. They consumed little food or water, and their urine output was approximately one-third of the control period volume. The 24-hr excretion of creatinine was 10.6 ± 1.1 mg ($\pm$SEM) for the control collection period and 7.8 ± 0.5 mg for the phenoxybenzamine period (not signifi-

[2] L. B. Bigelow, manuscript in preparation.

TABLE 1

Effect of phenoxybenzamine on incorporation of radioactivity from ^{14}C-tyrosine into catecholamines in the rat

Rats were given phenoxybenzamine (25 mg/kg) intraperitoneally. Three (experiment 1), 16 (experiment 2), or 22 (experiment 3) hr later they received 25 μCi of ^{14}C-L-tyrosine intravenously. One hour following the administration of radioactive tyrosine, the rats were killed and the tissues were examined for catecholamines. The different experiments refer to studies carried out on different days.

Expt.	Time	Tissue	Catecholamine assayed	Newly formed catecholamine[a,b]	
				Control	Phenoxybenzamine[c]
	hr			*cpm/g tissue ± SEM*	
1	3	Heart	Norepinephrine	692 ± 142 (4)	1601 ± 87 (4)
	3	Brain	Norepinephrine	337 ± 15.5 (4)	556 ± 41.5 (4)
	3	Adrenals	Epinephrine	1290 ± 171 (4)	2398 ± 218 (4)
2	16	Heart	Norepinephrine	249 ± 22.4 (3)	1214 ± 106 (3)
	16	Brain stem	Norepinephrine	447 ± 19 (3)	768 ± 20 (3)
3	22	Adrenals	Epinephrine	1153 ± 49 (3)	2832 ± 250 (3)

[a] Adrenal data are expressed as counts per minute per adrenal pair.

[b] Numbers in parentheses refer to number of animals used.

[c] All phenoxybenzamine animals were significantly different from controls ($p < 0.01$).

cantly different by the two-tailed test). The reported values for catecholamines and metabolites are corrected to 10 mg of creatinine to compensate for any alteration in kidney function and possible errors in collection.

Estimation of rates of catecholamine synthesis by the isotopic method. A second set of rats was housed under conditions similar to those described above. On the second day, half of them were treated with phenoxybenzamine (25 mg/kg) intraperitoneally, and the other half with diluent. At varying time intervals after the phenoxybenzamine, each of the rats was given 25 μCi of ^{14}C-L-tyrosine intravenously (uniformly labeled; 376 μCi/μmole; New England Nuclear Corporation). Exactly 1 hr after the ^{14}C-L-tyrosine injection, the animals were killed and the heart, brain, and adrenals were removed and frozen. Incorporation of isotope into catecholamines was measured as described previously (4). Dopamine, norepinephrine, and epinephrine were isolated from tissues by methods published elsewhere (24). Norepinephrine and epinephrine were assayed by a modification of the trihydroxyindole procedure (25), and 3,4-dihydroxyphenylethylamine (dopamine) by the method of Drujan *et al.* (26). Tyrosine was isolated by a modification of the method of Lewander and Jonsson (27) and determined according to Waalkes and Udenfriend (28). For radioassay, samples were dissolved in Bray's solution (29) and measured in a scintillation spectrometer. The resultant counts were adjusted to an efficiency of 75%. Student's *t*-test was used to determine the significance of difference between the various means.

RESULTS

Effect of phenoxybenzamine on incorporation of ^{14}C-tyrosine into catecholamines.

TABLE 2

Effect of phenoxybenzamine on the specific activity of ^{14}C-tyrosine in rat tissues

Rats were given phenoxybenzamine (25 mg/kg) intraperitoneally. Sixteen hours later, they received 8 μCi of ^{14}C-L-tyrosine intravenously. One hour following the administration of tyrosine, the rats were killed and the tissues were assayed for tyrosine.

Tissue	Specific activity[a]	
	Control	Phenoxybenzamine
	cpm/μg tyrosine ± SEM	
Heart	181 ± 8.4	174 ± 8.8
Brain	246 ± 14.4	220 ± 10.7

[a] Four animals were used for each group.

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TABLE 3

Twenty-four-hour excretion of catecholamines and metabolites from rats treated with phenoxybenzamine

Four rats were given 0.9% NaCl intraperitoneally on the first day and phenoxybenzamine, 25 mg/kg intraperitoneally, on the second day. Urine was collected as described under METHODS AND MATERIALS.

Treatment	24-hr excretion[a]					
	Epinephrine	Norepinephrine	Metanephrine	Normetanephrine	Glycol[b]	Total
	μg/10 mg creatinine ± SEM					
0.9% NaCl	0.26 ± 0.08	3.36 ± 0.28	1.05 ± 0.16	9.75 ± 0.91	119 ± 7.0	133 ± 8.0
Phenoxybenzamine	2.30 ± 0.34[c]	23.0 ± 1.0[c]	6.01 ± 0.90[c]	45.4 ± 4.9[c]	178 ± 8.0[c]	252 ± 13.0[c]

[a] Values are corrected to molar equivalents of norepinephrine.

[b] Glycol, 3-methoxy-4-hydroxyphenylglycol.

[c] Significantly different from NaCl-treated controls ($p < 0.001$, one-tailed test).

TABLE 4

Catecholamine content of tissues from rats treated with phenoxybenzamine

Experimental conditions were identical with those of Table 1. Rats were killed 23 hr following phenoxybenzamine administration.

Tissue	Catecholamine assayed	Catecholamine content[a]	
		Control	Phenoxybenzamine
		μg/g ± SEM	
Heart	Norepinephrine	0 72 ± 0.01	0.36 ± 0 03[b]
Spleen	Norepinephrine	0.36 ± 0.07	0.23 ± 0.04
Brain	Norepinephrine	0.27 ± 0.02	0.29 ± 0.02
	Dopamine	0.54 ± 0.02	0.50 ± 0.01
Adrenal	Epinephrine	15.3 ± 1.17	14.3 ± 1.01

[a] Four animals were used for each group. Adrenal data are expressed as micrograms per adrenal pair.

[b] Significantly different from control ($p < 0.05$).

Dairman *et al.* (30) have previously shown that phenoxybenzamine increases catecholamine synthesis. Experiments were carried out with a set of animals to determine whether increased synthesis persisted during the entire experimental period. Table 1 shows that the incorporation of radioactivity from ^{14}C-L-tyrosine into norepinephrine and epinephrine was markedly stimulated 3, 16, and 22 hr after the administration of phenoxybenzamine. Table 2 shows that the specific activity of ^{14}C-L-tyrosine in tissues was not affected by the phenoxybenzamine. The above data show that throughout the entire 23-hr phenoxybenzamine treatment period catecholamine synthesis was stimulated.

Effect of phenoxybenzamine on urinary excretion of norepinephrine, epinephrine, and their metabolites. A second set of animals was used to follow the urinary excretion of catecholamines and their metabolites. Table 3 summarizes the 24-hr excretion of each of the metabolites measured and the total of all metabolites. Following phenoxybenzamine there were large increases in the excretion of epinephrine, norepinephrine, and both metanephrines (5–9-fold). The glycol level was also elevated, but to a lesser extent. It can be seen that the excretion of catecholamines and metabolites nearly doubled in the phenoxybenzamine-treated rats.

Effect of phenoxybenzamine on endogenous catecholamine content of the organs of the rat. The tissue levels of catecholamines shown in Table 4 were measured in rats 23 hr after they received phenoxybenzamine. Of the tissues examined, only the hearts of the drug-treated animals had a significantly lower catecholamine content.

DISCUSSION

The data in this report show that a drug which increases sympathetic nerve activity not only brings about increased turnover of norepinephrine in a few organs but leads to a large increase in urinary excretion of norepinephrine and its metabolites. Since the increased urinary excretion is not accompanied by significantly diminished tissue levels of catecholamine, with the exception of the heart, it appears that the additional excretion is not due to release of catecholamines from these tissues. In preliminary studies we have determined the norepinephrine and epinephrine content of rat carcasses from which the brain, heart, adrenals, and spleen were removed. Values on the order of 10 μg, consisting mainly of norepinephrine, were obtained for 200-g rats. This represents a fraction of the total urinary excretion. One can now say with assurance that the increased sympathetic nerve activity (16, 17) resulting from administration of phenoxybenzamine leads to increased synthesis of neurotransmitter. Most probably the increased sympathetic nerve activity due to any stimulus (cold, exercise, etc.) would also bring

about increases in urinary excretion of catecholamines and their metabolites.

Several important points must be noted concerning the quantitative aspects of the urinary excretion of norepinephrine, epinephrine, and their metabolites. Under control conditions glycol is by far the major metabolite, accounting for about 90% of all the metabolites in the urine. By contrast, following phenoxybenzamine administration the excretion of glycol is only 70% of the total. This indicates that over 50% of the additional norepinephrine and epinephrine secreted as a result of phenoxybenzamine was not deaminated. This finding is consistent with the observations that phenoxybenzamine, in addition to causing increased sympathetic nerve activity (16, 17), inhibits neuronal uptake of both exogenous (31) and endogenously released norepinephrine (32). A changed pattern of metabolism could be brought about in several other ways: (a) a general limitation of the capacity for oxidative deamination, (b) a major contribution during stimulation from tissues which deaminate to a lesser extent, or (c) preferential release of newly synthesized catecholamines. The last has been suggested by Kopin *et al.* (33). It should be noted that the distribution pattern of urinary catecholamines and metabolites in this report differs from the distribution pattern of radioactive urinary products obtained following the intravenous injection of labeled norepinephrine (6, 7). This finding will be the subject of a future report.

There is another striking feature of the urinary data. The control rats excreted appoximately 130 μg of catecholamines and metabolites (as norepinephrine) during the 24-hr period. However, the contributions or norepinephrine and epinephrine from heart, brain, and adrenal gland, as determined from turnover studies in the rat (11, 24, 34, 35), can only account for about 10 μg/day (Table 5). Even if all the brain dopamine were converted to norepinephrine (an unlikely assumption), one could only account for another 16 μg/day (11) at most. Thus, the bulk of the urinary catecholamines is unaccounted for by the tissues which have received the most intensive study and which store large amounts of catecholamines.

If one accepts the existing data for turnover of norepinephrine in the tissues, the only plausible explanation for the magnitude of the urinary excretion of catecholamine metabolites is the existence of other major sites of norepinephrine synthesis. To date, the tissues studied with respect to turnover have been those which store large amounts of catecholamines. Other tissues may exist which have extremely rapid turnover rates, but do not store as much catecholamines.

A likely candidate for such a site may

TABLE 5
Estimated rates of catecholamine synthesis in the rat

Tissue	Catecholamine	Tissue weight[a]	Estimated rates of synthesis		Reference
		g	*μg/g/hr*	*μg/tissue/24 hr*[b]	
Brain	Norepinephrine	1.6	0.094–0.165	3.62–6.35	(34)
Heart	Norepinephrine	0.7	0.060–0.106	1.0–1.79	
Submaxillary gland	Norepinephrine	0.05	0.120–0.234	0.14–0.28	
Brain	Norepinephrine	1.6	0.13	5.0	(35)
Heart	Norepinephrine	0.7	0.095	1.6	
Brain	Norepinephrine	1.6	0.071	2.72	(11)
Brain	Dopamine	1.6	0.432	16.50	
Heart	Norepinephrine	0.7	0.049	0.83	
Adrenal	Epinephrine	0.075	1.0	1.80	(24)

[a] Tissue weight estimated for a 200-g rat.
[b] Calculated on the basis of a 200-g body weight.

be the sympathetic innervation of the blood vessels. Hartman and Udenfriend (36), using an antibody fluorescent technique, have demonstrated the presence of dopamine β-hydroxylase in the blood vessels of the kidney and adrenal gland. Spector[3] has recently found that some arteries from guinea pigs and rats turn over norepinephrine with a half-time of approximately 2 hr and contain 1 to 3 μg of norepinephrine per gram. If the entire vascular bed has turnover times and endogenous levels of norepinephrine similar to this, one could account for as much as 100 μg of norepinephrine synthesis per 24 hr in a 200-g animal.

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