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SHORT COMMUNICATION

Evidence That Dopamine Is Not a Substrate for Adrenal Phenylethanolamine *N*-Methyltransferase

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SUMMARY

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We found that dopamine is not a substrate for adrenal phenylethanolamine *N*-methyltransferase *in vitro* at concentrations from 1 μM to 10 mM, using both the bovine and the rabbit adrenal enzyme. In the adrenal glands of intact rats, [^{3}H]dopamine was rapidly converted to [^{3}H]norepinephrine and subsequently to [^{3}H]epinephrine. No significant levels of [^{3}H]epinine were detected.

The classical concept for catecholamine biosynthesis in the adrenal gland was first proposed by Blaschko (1) and Holtz (2) and involves the conversion of tyrosine to dopa and then to dopamine, followed by side-chain hydroxylation to form norepinephrine and *N*-methylation to yield epinephrine. The existence of each segment in the scheme has since been substantiated experimentally, and it has also been shown that all the enzymes for the pathway are located in the cytoplasm, with the exception of dopamine β-hydroxylase, which is localized in the catecholamine-containing storage granules (3).

Recently, however, Laduron (4) has reported that dopamine, a phenthylamine, is a substrate for bovine phenylethanolamine *N*-methyltransferase, with epinine as the methylated product. On the basis of these data he postulated that dopamine, and not norepinephrine, may be the preferred physiological substrate for phenylethanolamine *N*-methyltransferase and that, as a consequence, epinephrine is formed in the adrenal gland as shown in Scheme 1.

$$\text{Dopamine} \xrightarrow[\text{(cytoplasm)}]{\text{phenylethanolamine } N\text{-methyltransferase}} \text{epinine}$$

$$\text{epinine} \xrightarrow[\text{(granule)}]{\text{dopamine } \beta\text{-hydroxylase}} \text{epinephrine}$$

SCHEME 1

This scheme is attractive, since it would not be necessary for dopamine to enter the storage granule, be converted to norepinephrine, and then return to the cytoplasm to be methylated by phenylethanolamine *N*-methyltransferase, as is the case in the classical scheme for epinephrine biosynthesis. Because of the importance of this concept in understanding epinephrine biosynthesis, we have done studies, both *in vivo* and *in vitro*, to attempt to confirm that dopamine is a substrate for this enzyme.

A partially purified, lyophilized phenyl-

ethanolamine *N*-methyltransferase preparation, derived from either beef or rabbit adrenals, was obtained from Gallard-Schlesinger Company (Biozyme Laboratories), where it was generally prepared according to Saelens *et al.* (5). This method, which appears to differ in no essential way from that used by Laduron (14), involves homogenization in 0.1 M phosphate buffer (pH 7.0), centrifugation for 60 min at 23,000 × *g*, and double precipitation of the resulting supernatant with ammonium sulfate. The 25–60% ammonium sulfate precipitate is taken up into 3 mM phosphate buffer, after which it is dialyzed for 16–20 hr against the same buffer. The dialyzed material is lyophilized prior to use.

For experimental use, the enzyme was solubilized in phosphate buffer and the reaction was carried out in 300 μl constituted as follows: enzyme, 280 μg; phosphate buffer (pH 7.4), 50 μmoles; *l*-norepinephrine (Sigma), 9 nmoles, or dopamine at varying concentrations; and *S*-adenosyl[^{14}C]methionine (10,000 dpm, New England Nuclear), 9 nmoles. The label was localized on the reactive methyl group of the *S*-adenosylmethionine molecule. The reaction was conducted for 30 min at 37°, after which it was terminated with 1 N HCl (200 μl). Approximately 1 g of solid NaCl was added, and the solution was extracted with 6 ml of acid-washed, NaCl-saturated butanol. Then 1 ml of the butanol layer, containing labeled amine, was added to 10 ml of an aqueous 2,5-bis-[2′-(5′-*tert*-butylbenzoxazolyl)]thiophene phosphor, counted in a Packard Tri-Carb liquid scintillation spectrophotometer for 10 min, and quantitated in terms of nanomoles of methylated product. Dopamine was obtained from Sigma Chemical Company and was assayed as 99% pure. Each assay was performed in quadruplicate. A more complete description of this assay system, including the chromatographic identification of epinephrine as the methylated product when norepinephrine was the substrate, has recently been published (6).

The results of our study using bovine phenylethanolamine *N*-methyltransferase in our standard assay are shown in Fig. 1

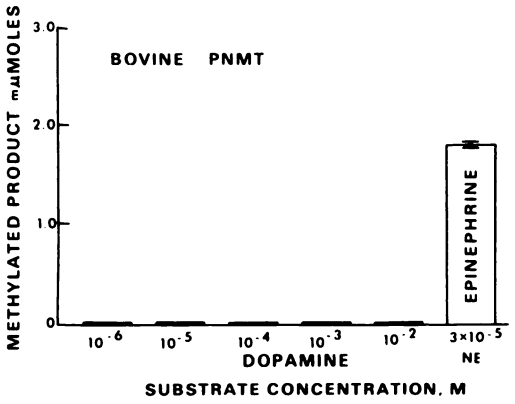

FIG. 1. *Effect of dopamine as substrate for bovine phenylethanolamine N-methyltransferase (PNMT)*

Norepinephrine (NE) was placed in the assay at 30 μM for comparative purposes, and each reaction was run for 30 min. All points are the means of quadruplicate measurements. Bars indicate standard error of the mean. Each dopamine mean value was slightly below the blank value (see the text).

and indicate that dopamine showed no substrate activity at medium concentrations ranging from 1 μM to 10 mM. In addition, the compound was tested at the K_m reported by Laduron (4) (2 mM) with exactly the same results. In fact, in each set of tubes, the average number of counts in the butanol phase (per milliliter) was slightly (2–14 dpm) less than in the blank set, which contained no phenethylamine substrate. In contrast, the tubes containing norepinephrine (30 μM) had 275 ± 5 dpm (mean ± standard error) in the butanol phase per milliliter, exclusive of the blank (72 ± 2 dpm). We observed no color change in the reaction tubes during the study, and subsequently found that epinine, added to the acidified reaction mixture, was substantially (92%) extracted into the butanol phase.

An identical study, except that the specific activity of *S*-adenosyl[^{14}C]methionine was doubled (20,000 dpm/9 nmoles), was performed, using rabbit phenylethanolamine *N*-methyltransferase at each of the dopamine concentrations used in the above study. The results likewise showed no evidence of substrate activity for dopamine at concentrations from 1 μM to 10 mM. Increasing the *S*-adenosyl[^{14}C]methionine

specific activity with the bovine enzyme also was without detectable effect.

Laduron has recently reported that low concentrations of norepinephrine (0.3 μM) can completely inhibit dopamine *N*-methylation (7) by the adrenal enzyme. We analyzed our rabbit phenylethanolamine *N*-methyltransferase preparation for norepinephrine (8) and can rule out a final concentration in our reaction vessels of more than 10 nM. Nonetheless, this may be a possible explanation for the fact that we could not demonstrate dopamine methylation *in vitro*.

We also assayed our rabbit enzyme preparation under our standard test conditions for nonspecific *N*-methyltransferase activity (7) by using 5-methyltetrahydrofolate as the potential methyl donor at a final concentration of 30 μM. No significant methylation of either norepinephrine (30 μM) or dopamine (2 mM) was detected under these circumstances.

Since results *in vitro* may be misleading, we also attempted to demonstrate that dopamine was a substrate for phenylethanolamine *N*-methyltransferase or any other adrenal *N*-methylating enzyme *in vivo*. In this study a group of 40 male Charles River rats, weighing 240–265 g, were intravenously injected with 50 μCi of [7-^{3}H]dopamine (Amersham/Searle; specific activity, 9.8 Ci/mmole). Groups of five were killed at various time intervals (Fig. 2) by decapitation, and the adrenals were removed and homogenized in 5 ml of 0.4 N $HClO_4$. Then 4 ml of the extracts were placed on alumina columns at pH 8.4–8.6 and eluted with 5 ml of 0.2 N acetic acid; 2 ml of each eluate were taken for acetylation. One milligram each of unlabeled dopamine, epinine, norepinephrine, and epinephrine was added to each sample prior to the addition of 0.5 ml of acetic anhydride and sufficient sodium bicarbonate to attain a pH of 7.0. The acetylated samples were extracted into methylene chloride, evaporated to dryness under N_2, and redissolved in 0.25 ml of methyl alcohol. Then 50 μl of this sample were spotted on silica gel (Merck, F254) thin-layer plates and triply developed, using a chloroform–acetic acid–ethanol–benzene (95:5:5:10) solvent system. The

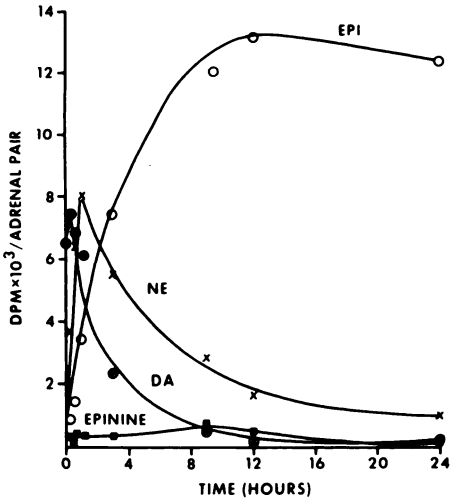

FIG. 2. *Time course for conversion of [^{3}H]dopamine (DA) to [^{3}H]norepinephrine (NE), [^{3}H]epinephrine (EPI), and [^{3}H]epinine*

[^{3}H]Dopamine was intravenously injected into each rat at zero time. Each value is the mean obtained from five animals. The *maximum* standard error was ±18%.

TABLE 1

R_F values for acetylated catecholamine derivatives

A mixture of the four amines below was acetylated as described in the text and carried through our standard extraction and concentration procedures prior to spotting on a silica gel thin-layer plate. The plate was triply developed, and the final migration of the solvent front was 11.5 cm from the origin.

Amine	R_F
Epinine	0.82
Dopamine	0.70
Epinephrine	0.61
Norepinephrine	0.39

spots for the four amines were clearly separable and were identified both under ultraviolet radiation and by I_2 vapor staining. The R_F value for each substance is listed in Table 1.

No significant counts (all samples were less than twice background) were found in the epinine spot at any time after the administration of dopamine. Furthermore, on the basis of the temporal relationships, it is apparent that the data are consistent with the classical reaction sequence: dopamine → norepinephrine → epinephrine.

The recoveries for the various amines from the alumina columns were as follows: epinine, 92%; norepinephrine, 90%; epinephrine, 88%; dopamine, 90%.

The final recoveries of tracer amounts of norepinephrine, epinephrine, and dopamine, which were added to a cold acetic acid eluate and ultimately scraped off the thin-layer plates, were 70%, 69%, and 72%, respectively. The final recovery of epinine could not be determined because of the nonavailability of labeled compound, but it presumably was similar to those of the close congeners described above, particularly since, in preliminary experimentation, only a single spot was observed on our thin-layer plates after epinine was subjected to the above acetylation and extraction procedures. The radioactive levels shown in Fig. 2 were not corrected for recoveries.

The mean endogenous epinephrine values in each of the above groups, which were corrected for column recovery, varied from 18.4 ± 0.7 to 21.6 ± 2.3 μg/adrenal pair. Corresponding norepinephrine values ranged from 5.3 ± 0.4 to 7.7 ± 0.7 μg/adrenal pair. The level of dopamine was less than 0.1 μg/adrenal pair in all samples. Each of these catecholamine levels was determined spectrofluorometrically by methods previously described (8). These data, both *in vivo* and *in vitro*, indicate an agreement with the observations of many other workers (9–12) that dopamine is not a substrate for phenylethanolamine *N*-methyltransferase.

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