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Importance of the Aromatic Ring in Adrenergic Amines

Nonaromatic Analogues of Phenylethanolamine as Substrates for Phenylethanolamine *N*-Methyltransferase

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SUMMARY

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Various nonaromatic analogues of phenylethanolamine were evaluated as substrates for phenylethanolamine *N*-methyltransferase (EC 2.1.1). In general the nonaromatic analogues were as good as, if not better than, phenylethanolamine itself as substrates for this enzyme. Evidence that these compounds were being *N*-methylated by phenylethanolamine *N*-methyltransferase was obtained by isolating and identifying the *N*-methylated products. Competitive experiments using phenylethanolamine also provided evidence that phenylethanolamine *N*-methyltransferase was the enzyme carrying out this methylation. The results of these studies suggest that the role of the aromatic ring of phenylethanolamine in binding to this enzyme would be due to its hydrophobic character, rather than its electron-rich nature. The data presented here are inconsistent with the possibility of a charge-transfer complex being formed during the binding of phenylethanolamine to phenylethanolamine *N*-methyltransferase, but instead suggest the existence of a hydrophobic binding site on the enzyme for this portion of the substrate molecule.

Phenylethanolamine *N*-methyltransferase (EC 2.1.1) is the enzyme which catalyzes the transfer of a methyl group from *S*-adenosyl-L-methionine to norepinephrine, resulting in the formation of the hormone epinephrine (1, 2). The enzyme has been isolated from the adrenal medulla, where it is highly localized in the soluble fraction (2–7). Because of the importance of epinephrine as a hormone in regulating cellular activity, considerable research interest has been directed toward the identifi-

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cation of inhibitors of this methyltransferase. A specific inhibitor of phenylethanolamine *N*-methyltransferase in the adrenal medulla would suppress the formation of epinephrine, without directly interfering with norepinephrine biosynthesis.

Several classes of inhibitors of this enzyme have been identified from studies *in vitro*, including (a) dead-end inhibitors such as phenylethylamines (8–10), benzylamines (11, 12), and aminobenzimidazoles (13, 14), and (b) product type inhibitors such as analogues of *S*-adenosylhomocysteine (15–18). The dead-end inhibitors appear to have an affinity for the substrate site on the enzyme, but are not themselves methylated.

The substrate specificity of phenylethanolamine *N*-methyltransferase has been extensively investigated (2, 19–22). The enzyme has an absolute requirement for a hydroxyl group at the β-position of the ethyl side chain. Therefore the enzyme has the ability to methylate a variety of physiological and nonphysiological phenylethanolamines, but not phenylethylamines and benzylamines. To date it has generally been assumed that the aromatic rings of both the substrates and dead-end inhibitors of the enzyme are crucial for maximum enzymatic binding. The apparent importance of the aromatic ring in the binding of such ligands has been investigated by evaluating numerous ring-substituted amphetamines (10, 23), benzylamines (11, 12), and phenylethanolamines (20, 22) as substrates or inhibitors of this enzyme. Our interest in the importance of the aromatic ring of adrenergic amines (24) in determining the degree and type of pharmacological activity suggested a study of a series of nonaromatic analogues of phenylethanolamine with varying degrees of π-electron character as substrates for phenylethanolamine *N*-methyltransferase. In this paper we show that the aromatic ring of phenylethanolamine is not an absolute requirement for binding to this enzyme and that, in fact, various nonaromatic analogues of phenylethanolamine are better substrates for phenylethanolamine *N*-methyltransferase than is the parent aromatic compound.

Phenylethanolamine *N*-methyltransferase was purified from bovine adrenal medulla (Pel-Freez Biologicals) according to Connett and Kirshner (3). The enzyme activity was assayed using *S*-adenosyl[*methyl*-^{14}C]methionine (New England Nuclear, 55.0 mCi/mmole) and phenylethanolamine (compound 1) according to a previously described radiochemical assay (3, 16–18). This enzyme preparation was found to be free of nonspecific *N*-methyltransferases, such as indolethylamine *N*-methyltransferase (25, 26), since no detectable methylation of phenylethylamine or *N*-methyltryptamine could be observed. The following compounds used in this study were commercially available from the indicated sources: 1-amino-2-propanol (compound 5) and 1-amino-2-pentanol (compound 4), Aldrich; phenylethanolamine (2-amino-1-phenylethanol, compound 1) and *S*-adenosyl-L-methionine, Sigma. Compounds 2, 3, and 6–10 were synthesized from the corresponding aldehydes by intermediate formation of the cyanohydrins, followed by reduction to the amines (27).

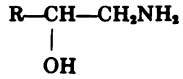
To elucidate the role of the aromatic ring in substrate binding to phenylethanolamine *N*-methyltransferase, various nonaromatic derivatives of ethanolamine were evaluated as substrates (Table 1). The nonaromatic analogues evaluated included those in which the phenyl ring of phenylethanolamine (compound 1) was replaced by a cyclohex-3-enyl group (compound 2), a cyclohexyl group (compound 3), a propyl group (compound 4), or a methyl group (compound 5). In addition, we also evaluated a series of nonaromatic analogues which possess varying degrees of π-electron character. These included analogues in which the phenyl ring was replaced by a cyclooctatetraenyl group (compound 6), a cycloocta-1,5-dienyl group (compound 7), a cycloocta-1,3-dien-2-yl group (compound 8), a cyclooctenyl group (compound 9), or a cyclooctyl group (compound 10).

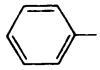
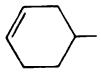
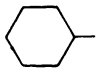
Shown in Fig. 1, as examples, are the reciprocal velocity vs. reciprocal substrate plots for compounds 2, 4, 6, and 7. In the concentration range of 20–300 μM linear plots of the type shown in Fig. 1 were obtained for each of the analogues tested. At substrate concentrations above 500 μM all the analogues showed strong substrate

TABLE 1

Michaelis-Menten constants for nonaromatic analogues of phenylethanolamine as substrates for phenylethanolamine N-methyltransferase

Phenylethanolamine *N*-methyltransferase was purified from bovine adrenal medulla (Pel-Freez Biologicals) according to Connett and Kirshner (3). The purification was carried through the isoelectric precipitation and dialysis steps, which resulted in 6-fold purification of the enzyme relative to the crude supernatant. The nonaromatic analogues of phenylethanolamine (compounds 2–10) were tested as the hydrochloride salts of the DL mixtures (27). The enzyme was assayed using a previously described radiochemical assay (3, 16–18) and a normal incubation mixture: water to a final volume of 0.25 ml; acceptor substrate, variable amounts; *S*-adenosyl-L-methionine, 0.25 μmole; *S*-adenosyl-L-[*methyl*-^{14}C]methionine, 0.05 μCi; phosphate buffer, pH 8.0, 25 μmoles; and enzyme preparation. Incubations were carried out for 30 min at 37°, the reaction was stopped with 0.25 ml of 0.5 M borate buffer (pH 10), and a mixture of toluene–isoamyl alcohol (7:3) was used to extract the product (16–18). Reciprocal velocities were plotted graphically against reciprocals of substrate concentrations as shown in Fig. 1. The data in the linear regions of these plots were used to calculate K_m and V_{max} values, using a previously described least-squares method (28).



Compound	R	Kinetic constants	
		K_m	V_{max}[a]
		μM ± *SEM*	±*SEM*
1		136 ± 12	2.1 ± 0.07
2		57 ± 6.8	1.37 ± 0.08
3		40 ± 1	0.99 ± 0.11
4	$CH_3CH_2CH_2$—	465 ± 73	4.04 ± 0.29
5	CH_3—	1085 ± 68	0.151 ± 0.66
6		430 ± 45	1.73 ± 0.1
7		155 ± 20	2.75 ± 0.25
8		128 ± 14	2.61 ± 0.19
9		110 ± 8	1.92 ± 0.09
10		26 ± 4	0.96 ± 0.08

[a] Nanomoles of product formed per milligram of protein per minute.

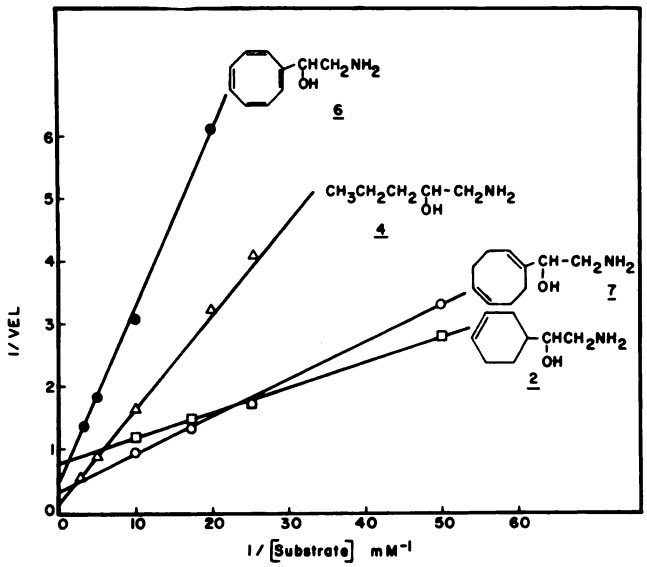


FIG. 1. *Reciprocal velocity vs. reciprocal substrate plots for nonaromatic analogues of phenylethanolamine* The enzyme assay conditions are described in Table 1. Vel = nanomoles of product formed per milligram of protein per minute. See Table 1 for the calculated K_m and V_{max} values.

inhibition, which appears to be characteristic of most substrates for this methyltransferase (3, 20, 22). Table 1 summarizes the kinetic constants for these substrates. Of general interest is the observation that each of these nonaromatic derivatives is a substrate for phenylethanolamine *N*-methyltransferase. In fact, the cyclohex-3-enyl derivative (compound 2) and the cyclohexyl derivative (compound 3) had substantially lower K_m values (2–3-fold) for this enzyme than phenylethanolamine itself. However, phenylethanolamine appeared to be methylated at twice the rate of the more highly saturated analogues 2 and 3. Of particular interest was the observation that the simple propyl derivative 4 was a fairly good substrate for this enzyme. A comparison of the cyclohexyl derivative 3 ($K_m = 40 \pm 1$ μM) and the propyl derivative 4 ($K_m = 465 \pm 73$ μM) indicates that a decrease in the size of the hydrocarbon side chain results in a substantial increase in the magnitude of K_m. This conclusion is further substantiated by the very high K_m value (1085 ± 68 μM) observed for the methyl derivative 5. Furthermore, ethanolamine itself does not appear to be methylated by this enzyme preparation.

Analogues 6–10, which possess varying degrees of π-electron character, exhibited interesting substrate properties. The cyclooctatetraenyl derivative 6, which is nonaromatic but π-electron-rich, was a good substrate for phenylethanolamine *N*-methyltransferase. It appeared to be methylated at essentially the same rate as phenylethanolamine, but it had a higher K_m for the enzyme than the parent molecule. Of particular interest is the observation that the less π-electron character present in the analogues of this series, the lower the K_m for the enzyme. For example, the K_m values for this series of analogues follow the order $6 > 7 \cong 8 > 9 > 10$, which correlates with a decrease in π-electron character. While the apparent affinities (K_m) of this enzyme for the completely unsaturated analogue 6 and the completely saturated analogue 10 differ by a factor of about 17, the rates of methylation only differ by a factor of 2. Therefore it appears that such structural changes have little effect on the rates of enzymatic methylation, but markedly affect the K_m values of these substrates.

In order to obtain evidence that we were indeed observing the enzymatic formation of the *N*-methyl-^{14}C derivatives of these nonaromatic analogues of phenylethanolamine (compounds 2–10), the products from enzyme incubation mixtures were separated and identified by paper chromatogra-

phy. Such studies were carried out using the methyl derivative 5 and the cyclooctyl derivative 10. In both cases the radioactive peaks formed by enzymatic methylation corresponded in R_F values to authentic samples of the corresponding *N*-methyl derivatives. To substantiate our earlier observation that ethanolamine itself is not a substrate for phenylethanolamine *N*-methyltransferase, we found that no radioactive product was formed which had an R_F value on paper chromatography corresponding to *N*-methylethanolamine when ethanolamine was tested as a substrate.

To rule out the possibility that a nonspecific *N*-methyltransferase rather than phenylethanolamine *N*-methyltransferase was responsible for methylation of these nonaromatic analogues of phenylethanolamine (compounds 2–10), various competitive studies with phenylethanolamine (compound 1) as a substrate were carried out. Advantage was taken of the fact that each of the analogues 2–10 showed strong substrate inhibition. For example, concentrations of analogue 10 above 500 μM produced strong substrate inhibition. Table 2 shows the effects of an inhibitory concentration of the nonaromatic analogue 10 on the methylation of phenylethanolamine. When the nonaromatic analogue 10 was present at a concentration of 1.0 mM, the methylation of phenylethanolamine was inhibited by about 90%. This finding would suggest that the nonaromatic analogue was competing for the same enzyme as phenylethanolamine. The data shown in Table 2 and similar data for the other nonaromatic analogues 2–9 provide evidence that the enzyme catalyzing the methylation of these nonaromatic substrates is phenylethanolamine *N*-methyltransferase, the same enzyme which methylates phenylethanolamine.

TABLE 2

Effect of a nonaromatic analogue of phenylethanolamine on methylation of phenylethanolamine

Phenylethanolamine *N*-methyltransferase was assayed as described in Table 1.

Reaction mixture	Additions		Enzyme activity[a]
	Phenylethanolamine	Compound 10	
	mM	mM	
1	1.0	0	1.32
2	0	1.0	0.137
3	1.0	1.0	0.155

[a] Nanomoles of product formed per milligram of protein per minute.

In conclusion, we have shown in these studies that the aromatic ring of phenylethanolamine is not crucial for binding to the enzyme phenylethanolamine *N*-methyltransferase. Various nonaromatic analogues of phenylethanolamine are as good as, if not better than, phenylethanolamine itself as substrates for this enzyme. The role of the aromatic ring of phenylethanolamine in binding to this enzyme appears to be due to its hydrophobic character, rather than its electron-rich nature. The data presented here are inconsistent with the possibility of a charge-transfer complex being formed during the binding of phenylethanolamine to phenylethanolamine *N*-methyltransferase, but instead suggest the existence of a hydrophobic binding site on the enzyme for this portion of the substrate molecule. We believe the observations made in this study will provide a new approach to the design of inhibitors of phenylethanolamine *N*-methyltransferase.

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