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SHORT COMMUNICATIONS

Catechol O-Methyltransferase

III. *m*- and *p*-O-Methylation of Catecholamines and Their Metabolites

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SUMMARY

In vitro, *O*-methylation of physiological substrates of catechol *O*-methyltransferase (EC 2.1.1.6) such as (nor)epinephrine, dopamine, 3,4-dihydroxyphenylacetic acid, 3,4-dihydroxymandelic acid, 3,4-dihydroxyphenylethanol, 3,4-dihydroxyphenylglycol, 3,4-dihydroxybenzoic acid, *N*-acetyldopamine, *N*-acetylnorepinephrine, and 3,4-dihydroxyphenylalanine affords mixtures of *m*- and *p*-*O*-methyl derivatives. The extent of *p*-methylation relative to *m*-methylation is low with substrates containing an ionized moiety in the ring substituent, i.e., amino acids, acids, and amines, and increases as the polar (hydrophilic) character of the ring substituent is decreased. With a nonpolar substituent, as in 4-ethylcatechol, the ratio of *meta* to *para* products is 1:1. The results suggest the presence of a nonpolar region in the catechol-binding site of catechol *O*-methyltransferase which militates against binding of polar substrates in the orientation necessary for *p*-methylation, while nonpolar substrates would appear to bind in a random fashion, resulting in the formation of nearly equal amounts of *m*- and *p*-*O*-methylated products. No change in the *meta*:*para* ratios was observed during more than 400-fold purification of catechol *O*-methyltransferase, suggesting that only one enzyme is involved in both *m*- and *p*-*O*-methylation.

The enzyme catechol *O*-methyltransferase (EC 2.1.1.6) plays an important role in the inactivation of catecholamines and in the detoxification of many xenobiotic catechols. *O*-Methylation of catecholamines and related physiological substrates *in vivo* results almost exclusively in the formation of *m*-*O*-methylated derivatives (1, 2). *In vitro*, however, COMT[1] catalyzes the formation of significant amounts of the *p*-*O*-methyl derivatives (2–5). Two possible explanations for this anomaly might be considered: (a) cellular disruption alters the nature of the active site of COMT so that *p*-methylation is now possible, or (b) the *p*-*O*-methyl metabolites are further metabolized *in vivo* by hepatic *O*-demethylation[2] or some other route. Administration of xenobiotic catechols such as arterenone (2), adrenalone (2), 3,4-dihydroxycinnamic acid (8), and 3,4-dihydroxybenzoic acid (9) does result in the formation and excretion of *p*-*O*-methylated metabolites. The present study focuses attention on the ratio of *O*-methylated isomers formed *in vitro* from the physiological substrates of COMT and delineates some of the factors which affect this ratio.

[1] The abbreviation used is: COMT, catechol *O*-methyltransferase.

[2] Enzymatic *p*-*O*-demethylation is usually favored over *m*-*O*-demethylation (3, 6, 7).

p-O-Methylation of a catecholamine was first reported in 1959, when it was shown that enzymatic *O*-methylation of dopamine resulted in the formation of a mixture of *m*- and *p-O*-methyl isomers in a ratio of 5.7–9.0 (2). This observation was confirmed with COMT from rat liver and brain, and a *meta*:*para* ratio of approximately 4 was obtained (5).[3] Norepinephrine and epinephrine were reported to yield mixtures of the *O*-methylated isomers with *meta*:*para* ratios of 7.5 and 10.0, respectively (3). *N*-Acetyldopamine formed a mixture of *O*-methylated isomers with *meta*:*para* ratios from 1.9 to 5.7, using a COMT preparation from brain (4). The *O*-methylation of arterenone, adrenalone, and 3,4-dihydroxyacetophenone catalyzed by rat liver COMT resulted in the formation of *O*-methylated isomers with a *meta*:*para* ratio of 1.5–1.7 (2, 3). The *meta*:*para* ratio obtained with 3,4-dihydroxyacetophenone was dependent upon the pH of the enzymatic reaction mixture and was shown to decrease with increasing pH. In this substrate, where the effect of the electron-withdrawing keto group increases the acidity of the *p*-phenolic group [pK_a approximately 7.5 (10)], ionization of the catechol moiety occurs at the *para* position. It was suggested that enzymatic methylation at higher pH values occurred preferentially at the ionized (more nucleophilic) *para* position. The preferential *m-O*-methylation observed at lower pH values was consistent, since in the *un-ionized* catechol the *m*-phenolic group is the better nucleophile, again because of the electron-withdrawing effect of the keto substituent. In this manner, the increase in *p-O*-methylation of 3,4-dihydroxyacetophenone with increasing pH was rationalized (2). Unfortunately, subsequent investigators have tended to apply this explanation even to substrates such as the catecholamines, where the pK_a values of the *m*- and *p*-phenolic groups and the relative nucleophilicities of the un-ionized phenolic groups are nearly identical. In addition, some investigators have concluded that hepatic COMT will preferentially form the *m-O*-methylation product with any catechol substrate (11).

The present study provides strong evidence that interactions between substrate and COMT which involve polar (charged) substituents on the catechol ring are far more important than electronic changes in the nucleophilicity of the catechol moiety in determining the extent of *m*- and *p-O*-methylation. Thus, as shown in Table 1, dopamine, norepinephrine, and epinephrine, which are almost completely in the cationic form at pH 8 [pK_a = 10.5, 9.7, and 9.9, respectively (14)], give rise predominantly to the *meta* isomer, with *meta*:*para* ratios of more than 6 (Table 1). The dipolar-ionic amino acid L-dopa also undergoes predominantly *m-O*-methylation, giving a *meta*:*para* ratio of 19.8. In the less polar acyl derivatives *N*-acetyldopamine and *N*-acetylnorepinephrine, the *meta*:*para* ratio is much lower, with a value of approximately 2. In view of these results, the *meta*:*para* ratios of 1.5–1.7 previously obtained with arterenone and adrenalone may not be due exclusively to electronic effects of the keto group on the nucleophilicity of the phenolic groups, as originally suggested (3), but may also reflect the fact that these α-ketoamines are much weaker bases ($pK_a < 8$)[4] and therefore exist in the un-ionized form to a significant extent at pH 7.9.

Catechols with substituents containing negatively charged moieties (acids, Table 1) also afford mainly *m-O*-methylated products. 3,4-Dihydroxybenzoic acid and 3,4-dihydroxyphenylacetic acid give *meta*:*para* ratios of 5.5 and 7.0, respectively, at pH 8, where they exist exclusively in the anionic form. The *meta*:*para* ratio with 3,4-dihydroxymandelic acid is 3.6, compared to 7.0 in the analogous 3,4-dihydroxyphenylacetic acid. In mandelic acid, internal hydrogen bonding might to some extent decrease the polar nature of the ionized carboxyl group. The great effect of an ionized substituent on the extent of

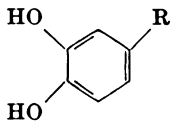
[3] The *p-O*-methylated isomer was erroneously reported in this reference as the major product owing to a typographical error (F. A. Kuehl, Jr., personal communication). Reference 7 contains a citation to this incorrect ratio of *p*- to *m-O*-methylated dopamine.

[4] Unpublished observations.

TABLE 1

Meta:para ratios of physiological substrates of COMT

Meta:para ratios were obtained by measuring the products formed in 30 min at 37° in a reaction mixture containing the following components: substrate, 1 μmole; $MgCl_2$, 0.6 μmole; *S*-adenosylmethionine-methyl-^{14}C, 0.1 μmole (0.108 μCi); sodium phosphate buffer, pH 8.0, 50 μmoles; and 60 units of COMT in a final volume of 0.5 ml. Unlabeled *O*-methylated products (0.4 μmole) were added, and the mixture was acidified with 0.1 ml of 1 N HCl or made basic with 0.5 ml of 5 N NH_4OH for acidic or basic substrates, respectively, and extracted with ethyl acetate (10 ml). The extract was concentrated with a stream of nitrogen, and the products were separated by thin-layer chromatography on a silica gel G plate in the following solvent systems: benzene–acetic acid–dioxane (9:1:1), lines 1 and 3; chloroform–triethylamine (5:1), lines 5, 7, and (iso)-vanillin; and 1-butanol–ammonium hydroxide–ethyl acetate (3:1:1), lines 8 and 9. Products in lines 6, 10, and 11 were first cleaved with periodate (12) to (iso)-vanillin; product 4 was first hydrolyzed with 1 N NaOH at 70° to the acids; product 2 was first reduced in dry refluxing ether with $LiAlH_4$ for 24 hr to the alcohols. Radioactivity was determined by scraping and counting 3-mm sections of silica gel by liquid scintillation spectroscopy. Products in line 12 were first isolated by descending paper chromatography (Whatman No. 1 paper; 1-butanol–acetic acid–water, 4:1:1) of the reaction mixture and then resolved on a Beckman amino acid analyzer (50-cm PA-28 resin column at pH 4.28 and 33°). Full details will be published elsewhere. The Michaelis constant (K_m) was determined with the reaction mixture described above, except that the concentration of *S*-adenosylmethionine was 10^{-3} M, the substrate concentration was varied from 2×10^{-4} to 10^{-5} M, and the reaction time was 10 min. The ^{14}C-methylated products were extracted as described previously (13). Values were obtained by the method of least squares.



Substrate	*Meta:para* ratio	K_m	V_{max}[a]
		mM	
Acidic			
1. R = —COOH	5.5 ± 0.4	0.25	1.21
2. —CH_2COOH	7.0	0.31	1.16
3. —CH(OH)COOH	3.6	0.94	1.16
Neutral			
4. R = —$COOCH_2CH_3$	0.9	0.15	1.87
5. —CH_2CH_2OH	2.2	0.26	3.17
6. —$CH(OH)CH_2OH$	1.8	0.40	0.63
7. —$CH_2CH_2NH(COCH_3)$	1.7	0.52	4.94
8. —$CH(OH)CH_2NH(COCH_3)$	2.3	0.26	4.43
Basic			
9. R = —$CH_2CH_2NH_2$	6.9 ± 0.7	0.16	0.9
10. —$CH(OH)CH_2NH_2$	7.03 ± 1.2	0.26	0.66
11. —$CH(OH)CH_2NHCH_3$	7.2	0.38	0.49
Amphoteric			
12. R = —$CH_2CH_2NH_2(COOH)$	19.8		

[a] Maximal velocity is expressed as mμmoles of product formed per unit of COMT per minute (1 unit catalyzes the *O*-methylation of 1 mμmole of 3,4-dihydroxybenzoic acid per minute; see Table 2).

p-O-methylation is demonstrated on comparison of the *meta:para* ratio of 5.5 obtained for 3,4-dihydroxybenzoic acid with the ratio of 0.85 obtained for its un-ionized ethyl ester. In this pair of substrates, the electronic effect of the substituent on the nucleophilicity of the phenolic hydroxyl groups is similar, while the *meta:para* ratios obtained on *O*-methylation are strikingly different.

Other metabolites of catecholamines such as 3,4-dihydroxyphenylethanol and 3,4-

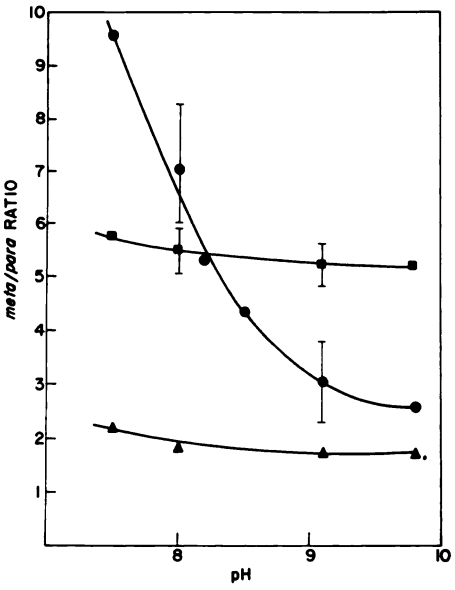


FIG. 1. *Effect on pH on meta:para ratios obtained with norepinephrine (●), 3,4-dihydroxyphenylglycol (▲), and 3,4-dihydroxybenzoic acid (■)*

Meta:para ratios were obtained as described in Table 1.

dihydroxyphenylglycol do not contain an ionized substituent moiety, and the *meta:para* ratio is close to 2. These compounds do, however, have polar alcohol groups. With 4-ethylcatechol, no polar group is present in the side chain and the *meta:para* ratio is 1.0. Thus, with this compound and with ethyl 3,4-dihydroxybenzoate, there is no preference for *m*- or *p*-*O*-methylation. In the series of substrates 4-ethylcatechol, 3,4-dihydroxyphenylethanol, 4-dihydroxyphenylacetic acid, and dopamine, the electronic effect of the side chain on the catechol portion of the molecule is nearly identical; yet the *meta:para* ratio varies from 1 to 7, depending on the polarity of a substituent isolated from the catechol ring by one or two intervening methylene groups. Based on these studies, *O*-methylation of relatively nonpolar metabolites such as 3,4-dihydroxyphenylacetaldehyde and 3,4-dihydroxymandelaldehyde would be expected to result in *meta:para* ratios close to unity. Likewise, *O*-methylation of many xenobiotic catechols with nonpolar subsubstituents affords *meta:para* ratios near unity.[4]

The effect of pH on the *meta:para* ratios obtained with three types of substrates was ascertained (Fig. 1). With norepinephrine, the *meta:para* ratio decreased as the pH was increased from 7.5 to 9.8. At the higher pH, a significant portion (more than 50%) of the less polar, nonprotonated amine would be available for binding and *O*-methylation. With a neutral substrate, 3,4-dihydroxyphenylglycol, and with an acidic substrate, 3,4-dihydroxybenzoic acid,

TABLE 2

Effect of enzyme purity on meta:para ratio

Enzyme fraction	Purification[a]	*Meta:para* ratio[b]			
		Dopamine	L-Norepinephrine	3,4-Dihydroxybenzoic acid	3,4-Dihydroxypropiophenone
	-fold				
Crude homogenate				5.0	1.0
100,000 × *g* supernatant	6	6.9	6.1	5.9	0.9
30–50% $(NH_4)_2SO_4$	54	6.5	6.5	5.8	1.1
$(NH_4)_2SO_4$ backwash	95	6.5	7.0	5.2	1.0
Calcium phosphate gel	121	6.9 ± 0.7	7.03 ± 1.2	5.5 ± 0.4	1.0 ± 0.13
Sephadex G-50	346	7.0	6.2	6.4	1.0
Acrylamide gel	429				0.9

[a] COMT was purified from the livers of male Sprague-Dawley rats as described earlier (13). The specific activity of the enzyme following gel filtration on Sephadex G-50 with 3,4-dihydroxybenzoic acid as substrate was 114.3 mμmoles/mg of protein per minute.

[b] *Meta:para* ratios were determined as described in Table 1. The *O*-methylated products formed from 3,4-dihydroxypropiophenone were separated by descending paper chromatography in 1-butanol–15% NH_4OH (4:1 by volume).

pH had relatively little effect on *meta*:*para* ratio over the range of 7.5–9.8. Within this pH range, no significant changes in ionization of the *catechol ring substituent* would pertain with these substrates. Ionization of the catechol moiety to the anionic form does occur within this pH range, with a pK_a of approximately 9 for norepinephrine (14) and 3,4-dihydroxyphenylglycol.

During extensive purification of COMT (13), the *meta*:*para* ratio with four different substrates did not vary significantly (Table 2). This provides strong evidence that only one enzyme is involved in both *m*- and *p*-*O*-methylation of catechols.

At least two explanations for the effect of polar substituents on *p*-methylation by COMT may be considered: (a) catechols containing substituents with a highly polar moiety are bound by means of this polar substituent in an orientation that favors transfer of the methyl group to the *meta* position, or (b) a hydrophobic region is present in the active site of COMT which prevents random binding of polar substrates by repulsive interactions, so that binding occurs primarily in a configuration favoring *m*-*O*-methylation. Since the apparent affinities of polar catechol substrates for COMT do not differ markedly (K_m values, Table 1) from those of nonpolar catechol substrates, and since both anionic and cationic polar substituents have the same effect on *p*-*O*-methylation, the latter explanation is favored. Figure 2 illustrates a manner in which such a hydrophobic region adjacent to the active site of COMT could influence the *meta*:*para* ratios which obtain with polar substrates. The possibility that interaction of polar substrates with COMT induces an allosteric change that alters the active site of COMT so as to favor *m*-*O*-methylation must also be considered.

The present results indicate that with highly purified COMT (13), the ionic or polar nature of the substituent greatly influences the formation of *m*- and *p*-*O*-methylated isomers. It is postulated that COMT is an enzyme which contains a catechol-binding site flanked by a hydrophobic region (Fig. 2). With catechols bearing relatively nonpolar substituents such as 4-ethylcatechol and ethyl 3,4-dihydroxybenzoate, the manner of binding at the active site of COMT is indiscriminate, and equal amounts of *p*- and *m*-*O*-methylated products are formed. With catechols containing a substituent ionized at the pH of the reaction medium (amino acids, acids, amines), binding at the catechol site occurs in such a way (Fig. 2A) that *m*-*O*-methylated products are preferentially formed. Catechols with an alcohol or amide moiety in the side chain represent a class of substrates of intermediate polarity, and intermediate *meta*:*para* ratios are obtained. These preliminary results suggest that further information on the topography of the active site of COMT can be obtained by a study of catechols in which the position of the ionized or polar substituent is rigidly fixed, and such studies are in progress.

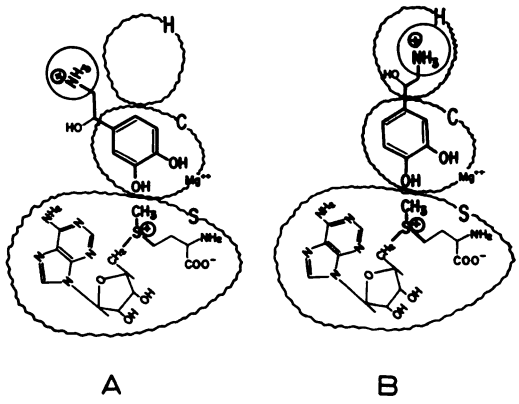


FIG. 2. *Alternative orientations of a catechol substrate (norepinephrine) at catechol-binding site (C) of COMT, resulting in m-O-methylation (A) and p-O-methylation (B).*

S represents binding site for *S*-adenosylmethionine. The proposed hydrophobic region (*H*) could influence binding of polar substrates so as to favor orientation A and *m*-*O*-methyl transfer.

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