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Catechol O-Methyltransferase

IV. Factors Affecting *m*- and *p*-Methylation of Substituted Catechols

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SUMMARY

The methylation of substituted catechols catalyzed by catechol *O*-methyltransferase (EC 2.1.1.6) with *S*-adenosylmethionine as methyl donor results in the formation of a mixture of *m*- and *p*-*O*-methyl derivatives. A variety of evidence indicates that a single enzyme catalyzes the formation of both *O*-methylated products. The ratio of *O*-methylated products and values for the apparent K_m and V_{max} obtained with a wide variety of substituted mono-, bi-, and polycyclic catechols, including catecholamines, amino acids, acids, esters, amides, and ketones, are presented. The results provide a guide for the prediction of the ratio of products to be expected with other catechols. The magnitude of the ratio of the *O*-methylated products is dependent upon the concentration of divalent cation, the pH of the medium, and the nature and position of the substituents on the catechol ring. It is proposed that the magnitude of this ratio results from the orientation in which the substrate binds to the enzyme. Polar groups, either anionic or cationic in nature, militate against binding of the catechol ring in the orientation that results in *p*-*O*-methylation. The ratio of *O*-methylated isomers obtained with various catecholamines and amino acids is discussed with respect to the probable preferred conformations of the side chains in these substrates.

INTRODUCTION

The enzyme catechol *O*-methyltransferase catalyzes the transfer of the methyl group of *S*-adenosylmethionine to one of the phenolic groups of catechol or substituted catechols. Formally, this reaction can be considered as the nucleophilic reaction of one of the phenolic groups of a catechol with the electron-deficient carbon of *S*-adenosylmethionine. The ratio of isomeric *O*-methylated products obtained from various substituted catechols should in this case reflect the relative nucleophilicities of the two phenolic groups. The ratio of *m*- and *p*-*O*-methylated catecholamines was, indeed, first rationalized on this basis (1). Subsequently it has been shown that the ratio of *m*- and *p*-*O*-methylated products depends strongly on the relative polarities of the substituent groups; i.e., with ionized groupings *m*-*O*-methylation predominates, while with un-ionized groupings the proportions of *m*- and *p*-*O*-methylated isomers are more nearly equal (2). The present paper reports the results of studies on over 50 substituted catechols and provides a guide for the prediction of the relative proportions of methylated products to be expected from both endogenous and xenobiotic catechols.

MATERIALS AND METHODS

S-Adenosyl-L-[*methyl*-^{14}C]methionine (55 mCi/mmole) was obtained from New Eng-

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land Nuclear Corporation. Catechol *O*-methyltransferase was purified from the livers of male Sprague-Dawley rats according to the method of Nikodejevic *et al.* (3). The authors gratefully acknowledge the donation of the following compounds: *N*-isobutyl-, *N*-propyl-, *N*-acetylbutyl-, and 6-methylnorepinephrine HCl, and (±)-*threo*-α-methylnorepinephrine HCl from Professor K. Zeile, C. H. Boehringer und Sohne; protokylol from Lakeside Pharmaceutical Corporation; (±)-*threo*-α-methylnorepinephrine HCl, (−)-*erythro*-α-methylnorepinephrine, (+)-*erythro*-α-methylnorepinephrine 2-furoate salt, (+)-isoproterenol (+)-bitartrate, (−)-isoproterenol HCl, α-ethyl-, α-ethyl-*N*-isopropyl-, and *N*-ethylnorepinephrine HCl, and 3-*O*-methyldopa from Drs. S. Archer and M. Levitt of Sterling-Winthrop Res. Inst,; 4-*O*-methyldopa from Dr. N. Kaubisch of Thomae-Bieberach; (+)-*erythro*-α-methylnorepinephrine HCl from Professor P. N. Patil of Ohio State University; and 6,7-dihydroxy- and 6,7-dihydroxy-*N*-methyl-1,2,3,4-tetrahydroisoquinoline from Burroughs-Wellcome and Company. The remaining compounds were obtained from commercial sources or synthesized by routine methods.

Meta:*para* ratios were determined by separation and measurement of the *O*-methylated products formed from various catechols after incubation in a reaction mixture containing the following components (final concentrations): catechol (2 mM), magnesium chloride (1.2 mM), *S*-adenosyl-L-methionine iodide (0.1 mM), *S*-adenosyl-L-[*methyl*-^{14}C]-methionine (0.1–0.5 μCi), dithiothreitol (2 mM), sodium phosphate buffer, pH 8.0 (100 mM), and 0.4–0.8 mg of purified catechol *O*-methyltransferase (3). The final volume was 0.5 ml. The reaction was started by the addition of enzyme, incubated at 37° for 30 min, and terminated by the addition of 5 N NH_4OH (0.5 ml) when the substrate was a basic catechol or 1 N HCl (0.1 ml) when the substrate was an acidic or neutral catechol. The appropriate authentic *m*- and *p*-*O*-methyl derivatives (0.1–0.4 μmole) were added as carriers for the radiolabeled products.

Periodate oxidation. *O*-Methylated products from *N*-alkyl and α-alkyl derivatives of norepinephrine and from 3,4-dihydroxyphenylserines were subjected to periodate oxidation in 2.5 N NH_4OH (4), and the *meta*:*para* ratio was determined by separation of the resultant aldehydes, vanillin and isovanillin. The ratio of the aldehydes formed remained constant when the time of periodate oxidation was varied from 4 to 20 min at 25°. The *meta*:*para* ratios obtained when periodate was used were identical with those obtained by direct chromatographic separation of the unmodified *O*-methyl isomers of compounds 7, 9, and 15 (Table 1).

Alkaline hydrolysis. *O*-Methyl products from *N*-carbobenzoxy, *N*-benzoyl, and *N*-acetyl derivatives of norepinephrine and dopamine were hydrolyzed under oxygen-free conditions in 2.5 N NaOH at 100° for 2–3 hr to give rise to the free amines.

Catalytic reduction. *O*-Methyl products of *N*-carbobenzoxynorepinephrine and *N*-carbobenzoxydopamine were reduced with hydrogen gas and 10% palladium-charcoal catalyst in ethanol for 24 hr to give rise to the corresponding amines (5).

Lithium aluminum hydride reduction. *O*-Methyl products from 3,4-dihydroxyphenylacetic and propionic acids were reduced to the corresponding alcohols by refluxing for 24–48 hr in dry ether with $LiAlH_4$ (6).

Thin-layer chromatography. The *O*-methylated or chemically modified products were extracted at an appropriate pH into ethyl acetate, the extract was concentrated under nitrogen, and the residue was streaked on a silica gel G plate (Analtech Inc., 5 × 20 cm) and developed in one of the solvent systems listed below. The carrier compounds were visualized with ultraviolet light (254 nm and 350 nm), and radioactivity was determined with a gas-flow scanner. When necessary, the plates were air-dried and rechromatographed until satisfactory separation was achieved. Sections (3 mm) of silica gel were removed from the plate and suspended in a xylene-based phosphor solution for determination of radioactivity by liquid scintillation spectrometry.

Separation of 3- and 4-O-methyldopa. Maximum formation of the *O*-methyl derivatives of dopa (1–2 μmoles) was achieved by increasing the *S*-adenosyl-L-methionine con-

centration to 2 mM in the above reaction mixture. The reaction was terminated, with no addition of carriers, by heating at 100° in 75% ethanol for 3 min. After centrifugation, the supernatant fluid was concentrated under vaccum, and the residue was chromatographed on Whatman No. 3 paper by the descending method in 1-butanol–50% acetic acid (2:1). The mixture of 3- and 4-*O*-methyldopa (R_F 0.3–0.5) was eluted from the paper, applied to a PA-28 resin column (50 cm, Beckman amino acid analyzer), and eluted with sodium citrate buffer, pH 4.28, at 33°. Elution times of 3.5 and 4.0 hr were obtained with 3- and 4-*O*-methyldopa, respectively. Elution times of authentic standards were identical with those obtained with the enzymatic products. Quantification was achieved by measuring the area under the analyzer curves obtained with the enzymatic products, either alone or after addition of authentic standards (50 and 100 μg).

The *O*-methylated products obtained from 4-methyl- and 4-ethylcatechol were separated by gas chromatography on a column of 10% Bentone 34–tricresylphosphate on 100-mesh Gas-Chrom P at 175°. Retention times for the *meta* and *para* isomers obtained from 4-methylcatechol were 2.2 and 1.2 min, respectively, and from 4-ethylcatechol, 1.8 and 1.0 min, respectively. In both cases the enzymatic products co-chromatographed with authentic standards. Quantitation was achieved by measuring the area under the curves.

The following notation is used to describe the chromatographic separations. Substrates are indicated by Arabic numerals (see tables); solvent systems are indicated by Roman numerals, followed by a hyphen and and the number of times the plate was rechromatographed. The R_F value and color reaction with the Gibbs reagent (7) (y = yellow and b = blue) for the *meta* and *para* isomers follow, in that order. Variations from this format are noted.

Solvent system and support phase: I, chloroform–triethylamine, 5:1; silica gel G. II, benzene–acetic acid–dioxane, 90:1:1; silica gel G. III, benzene–acetic acid–dioxane, 90:1:6; silica gel G. IV, benzene–acetic acid–dioxane, 90:25:4; silica gel G. V, 1-butanol–NH_4OH (concentrated), 4:1; Whatman No. 1 paper, descending. VI, 2-propanol–5 N NH_4OH–H_2O, 80:1:1.9; silica gel G. VII, 1-butanol–ethyl acetate–benzene–cyclohexane, 1:1:2:2; silica gel G. VIII, 1-butanol–ethyl acetate–benzene–methanol, 6:6:5:1; silica gel G. IX, 1-butanol–NH_4OH (concentrated)–ethyl acetate, 3:1:1; silica gel G. X, benzene–chloroform–ethyl acetate, 1:1:1; silica gel G. XI, toluene–piperdine, 5:2; silica gel G (activated at 100° for 60 min).

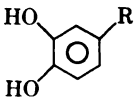
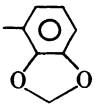
1. I, 0.37b, 0.20b. 2. IX, 0.58y, 0.50b. 2. I-2, 0.27y, 0.13b. 3. IX, 0.60y, 0.53b. 4. I-2, 0.72y, 0.53b. 5. I, 0.42y, 0.65b. 6. I-2, 0.45y, 0.25b. 7. IX, 0.64y, 0.55b. 9. IX, 0.74y, 0.64b. 15. IX, 0.84y, 0.74b. 7–26. Periodate oxidation; see 57. 27–28. Amino acid analyzer; see above. 29. Periodate oxidation; see 57. 30. I-3, 0.45b, *ortho*, 0.34b. 31. I-4, 0.50y, 0.26b. 31. VI, 0.62y, 0.72b. 32. I, 0.51b, 0.38b. 33–35. Periodate oxidation; see 57. 36. I, 0.45y, 0.29b. 36. VII, 0.83y, 0.72b. 37. I-2, 0.58y, 0.42b. 38. III, 0.14b, 0.39b. 39. Periodate oxidation; see 57. 40. III-3, 0.63y, 0.55b. 40. IV, 0.60y, 0.55b. 41. Reduction to alcohol; see 36. 42. III-6, 0.55y, 0.43b. 43. Reduction to alcohol; see 37. 44. VIII, 0.29y, 0.37b. 45. Alkaline hydrolysis; see 40. 46. Alkaline hydrolysis, reduction to alcohol; see 36. 47. IX, 0.80 brown, 0.66b. 48. XI, 0.59y, 0.72b. 49. VI, 0.52y, 0.89b. 50. VI-2, 0.85b, 0.38b. 50. II, 0.49b, 0.25b. 51. II, 0.40y, 0.33b. 52. I, 0.45b, 0.30b. 53. I-3, 0.60b, 0.42b. 54–56. vapor phase chromatography, see above. 57. II-3, 0.43y, 0.35b. 58. V, 0.59y, 0.84b. 59. V, 0.53y, 0.75b. 60. IX, 0.50y, 0.73b. 61. IX, 0.19y, 0.48b. 62. X, 0.80, 0.63 (visible yellow). 63. IX, 0.84y, 0.67b. 64. I-4, 0.69y, 0.56b. 65 II-6, 0.46b, 0.31b. 66. IX, 0.30, 0.39 (visible yellow). 68. II, 0.57 purple, *ortho*, 0.33b. 69. I-3, 6-*O*-Me, 0.25y, 7-*O*-Me, 0.40b. 70. I, 6-*O*-Me, 0.18y, 7-*O*-Me, 0.28b. 71. According to Knuppen and Breuer (8) and Ball *et al.* (9). 72. I-3, 7-*O*-Me, 0.89y, 6-*O*-Me, 0.23b. 73. I-2, 7-*O*-Me, 0.26b, 8-*O*-Me, 0.59 pink.

RESULTS AND DISCUSSION

The present findings extend and amplify our earlier conclusions (2) that the presence of a nonpolar region in the catechol-binding site of catechol *O*-methyltransferase militates against binding of polar substrates in the orientation necessary for *p*-methylation, while nonpolar substrates appear to bind in a more random fashion, resulting in the formation of nearly equal amounts of *m*- and *p*-*O*-methylated products. The *meta*:*para* ratios obtained with various amines (Table

TABLE 1

Meta:para ratios obtained with catecholamines and amino acids

No.[a]			*Meta:para* ratio[b]	K_m[c]	V_{max}[d]
				mM	
1		$-CH_2NH_3^+$	3.8		
2		$-CH_2CH_2NH_3^+$	6.9	0.78	0.90
3		$-CH_2CH_2NH_2^+CH_3$	4.4		
4		$-CH_2CH_2NH^+(CH_3)_2$	3.8		
5		$-CH_2CH_2NH_2^+CH_2C_6H_5$	2.0	0.87	1.49
6	DL	$-CH_2CH(CH_3)NH_3^+$ (α-methyldopamine)	18.1	0.67	0.69
7	D(−)	$-CH(OH)CH_2NH_3^+$ (norepinephrine)	5.3	0.26	0.66
8	L(+)	$-CH(OH)CH_2NH_3^+$ (norepinephrine)	7.4	1.62	0.30
9	D(−)	$-CH(OH)CH_2NH_2^+CH_3$ (epinephrine)	5.0	0.28	0.40
10	L(+)	$-CH(OH)CH_2NH_2^+CH_3$ (epinephrine)	5.7	0.63	0.99
11	DL	$-CH(OH)CH_2NH^+(CH_3)_2$	2.6		
12		$-CH(OH)CH_2NH_2^+CH_2CH_3$	1.8	0.79	2.24
13		$-CH(OH)CH_2NH_2^+CH_2CH_2CH_3$	1.5	0.36	2.19
14		$-CH(OH)CH_2NH_2^+CH_2CH_2CH_2CH_3$	2.7	0.61	1.60
15	D(−)	$-CH(OH)CH_2NH_2^+CH(CH_3)_2$ (isoproterenol)	2.3	0.04	0.12
16	L(+)	$-CH(OH)CH_2NH_2^+CH(CH_3)_2$	5.3	1.94	0.80
17	DL	$-CH(OH)CH_2NH^+CH(CH_3)CH_2CH_3$	0.9		
18		$-CH(OH)CH_2NH_2^+CH(CH_3)CH_2-$ 	0.7		
19	DL-*threo*	$-CH(OH)CH(CH_3)NH_3^+$ (α-methylnorepinephrine)	6.4		
20	DL-*erythro*	$-CH(OH)CH(CH_3)NH_3^+$ (α-methylnorepinephrine)	8.4		
21	D(−)-*erythro*	$-CH(OH)CH(CH_3)NH_3^+$ (α-methylnorepinephrine)	8.4	0.73	0.77
22	L(+)-*erythro*	$-CH(OH)CH(CH_3)NH_3^+$ (α-methylnorepinephrine)	13.5	0.55	0.46
23	DL	$-CH(OH)CH(CH_2CH_3)NH_2^+$ (α-ethylnorepinephrine)	9.6		
24	DL	$-CH(OH)CH(CH_2CH_3)NH_2^+CH(CH_3)_2$	3.1		
25	DL-*threo*	$-CH(OH)CH(NH_3^+)COO-$ (3,4-dihydroxyphenylserine)	13.9		
26	DL-*erythro*	$-CH(OH)CH(NH_3^+)COO-$ (3,4-dihydroxyphenylserine)	8.8		
27	L	$-CH_2CH(NH_3^+)COO-$ (3,4-dihydroxyphenylalanine)	19.8		
28	D	$-CH_2CH(NH_3^+)COO-$ (3,4-dihydroxyphenylalanine)	3.4		
29		6-Methylnorepinephrine[e]	13.3		
30		2,3-Dihydroxy-β-phenethylamine	1.0[f]	0.2	0.38

[a] The Arabic numeral for the substrates, used in this and the following tables, refers to the techniques used to separate the *O*-methyl isomers as listed under MATERIALS AND METHODS.

[b] Ratio of *meta* to *para* isomers formed enzymatically under the conditions described in the text.

[c] Values of the apparent Michaelis-Menten coefficient (K_m) were determined with substrate concentrations from 0.01 to 2 mM graphically and by the method of least squares. Correlation coefficient >0.995.

[d] Maximal velocity (V_{max}) is reported as the ratio of the value for a given substrate to that obtained with 3,4-dihydroxybenzoic acid with the same enzyme preparation. Four preparations were used, with V_{max} for this standard ranging from 10 to 55 nmoles/mg/min.

[e] 3,4-Dihydroxy-6-methyl-β-phenethanolamine.

[f] *Ortho:meta ratio.*

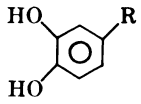
1) are ascribed to the presence of hydrophobic residues, which militate against binding in a manner leading to *p*-*O*-methylation. Clearly, the high *meta*:*para* ratios observed with these substrates is due to the presence of the ionized ammonium function in the side chain; i.e., the ratio decreases markedly as the pH is increased toward the pK_a of the amine (Table 5) and also when the amine function is converted to a neutral structure (Table 2). The variation in *meta*:*para* ratios as a function of side chain structure may also be rationalized in terms of probable preferred conformations of the various dopamine and norepinephrine derivatives and their interactions with hydrophobic centers in catechol *O*-methyltransferase. Preferred conformations of dopamine and norepinephrine have been calculated (10, 11). With dopamine, the lowest energy state consists of the *gauche* configuration (Formula Ia and b). If it is assumed that this conformer binds to the transferase, the *meta*:*para* ratio of 6.9 must reflect strong repulsive interaction of the cationic nitrogen with enzyme hydrophobic residues when the nitrogen is in either the Ia or Ib conformation. The decrease of the *meta*:*para* ratio to 3.8 in *N*,*N*-dimethyldopamine and to 2.0 in *N*-benzyldopamine might reflect the increasing stability of a completely staggered conformer (Formula II), in which interactions of the hydrophobic enzyme residues with the cationic moiety are reduced. Recent studies (12, 13) have suggested that the staggered conformer of dopamine analogues is the preferred conformation for catechol *O*-methyltransferase. A slight increase in the proportion of this conformer might therefore greatly affect the observed *meta*:*para* ratio. The conformations of amphetamine and *N*-substituted amphetamines have recently been measured by nuclear magnetic resonance spectroscopy (14); indeed, the proportion of the staggered isomer does increase with alkyl substitution on nitrogen. The decrease in *meta*:*para* ratio in the more highly substituted amines may also reflect an increase in the hydrophobic nature of the substituted nitrogen. The relative importance of this effect and of conformational changes cannot be assigned from the present data.

In the case of α-methyldopamine, an extremely high ratio of 18.1 is obtained. α-Methyldopamine, even more than dopamine, should be constrained to the *gauche* conformation (Formula IIIa and b), resulting in the strong repulsive interactions of substrate with enzyme, mentioned above, which militate against *p*-*O*-methylation.

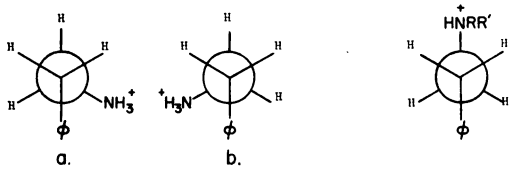
The results in the phenethanolamines series may also be rationalized in terms of staggered and *gauche* conformers. The additional factor of stereoisomerism in this series may provide further information on the geometry of the active site of catechol *O*-methyltransferase. Thus, in all pairs of

TABLE 2

Meta:*para* ratios obtained with neutral analogues of catecholamines

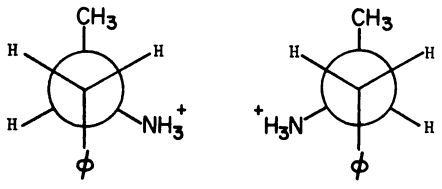
No.			*Meta*:*para* ratio	K_m	V_{max}
				mM	
31		$-CH_2CH_2NHCOCH_3$	1.7	0.52	0.70
32		$-CH_2CH_2NHCOOCH_2C_6H_5$	1.6		
33	DL	$-CH(OH)CH_2NHCOCH_3$	2.1	0.26	1.11
34	DL	$-CH(OH)CH_2NHCOC_6H_5$	2.0		
35	DL	$-CH(OH)CH_2NHCOOCH_2(C_6H_5)$	2.2		
36		$-CH_2OH$	2.0		
37		$-CH_2CH_2OH$	2.2	0.27	1.58
38		$-CH(OH)CH_3$	1.7	0.13	0.45
39		$-CH(OH)CH_2OH$	1.8	0.40	2.16

D- and L-isomers, the L-isomer affords a higher *meta*:*para* ratio. For norepinephrine, the respective ratios for the D(−) and L(+) forms are 5.3 and 7.4. For epinephrine, the ratios of 5.0 and 5.7 reflect the same trend, but the values are not statistically different. The most probable major conformers of epinephrine and norepinephrine are IV for the D-isomer and V for the L-isomer (11). The *meta*:*para* ratio for *both* isomers drops markedly for isoproterenol, perhaps indicating a greater preference of isoproterenol for the staggered conformations, VI and VII. However, a similar difference in *meta*:*para* ratios for the D and L forms of isoproterenol still pertains; i.e., 2.2 and 5.3, respectively. If this difference is due to the fact that greater hydrophobic interactions with the L-isomer prevent *p*-methylation, the difference in orientation of the hydroxyl group in the pair IV and V and the pair VI and VII should be important. Similar effects were noted in D- and L-*erythro*-α-methylnorepinephrine, for which ratios of 8.4 and 13.5 were observed. The most stable conformers would be VIII and IX. In the case



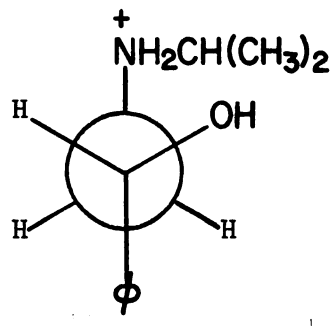
I

II

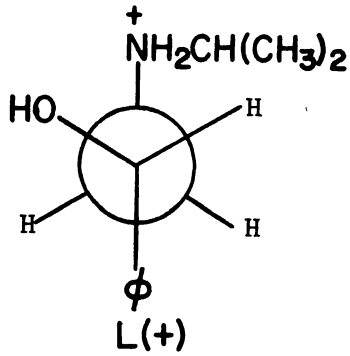


III

IV V

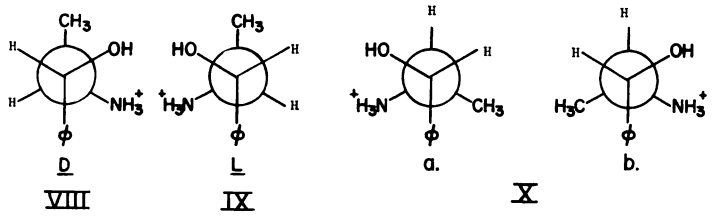


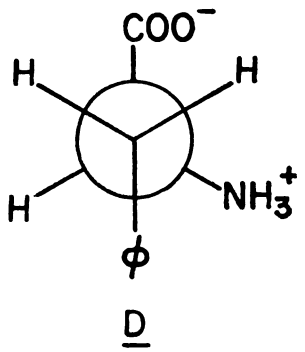
VI

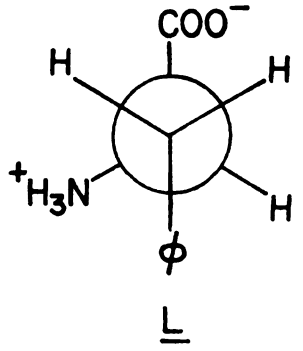


VII

of (±)-*threo*-α-methylnorepinephrine a ratio of 6.4 obtains. The conformers Xa and b are probably the most stable, although the decrease in ratio from 8.4 for the *erythro* to 6.4 in the *threo* isomer might reflect a partial shift to the staggered conformer. In the series of phenethanolamines with substituents larger than methyl on the nitrogen, a marked drop in the *meta*:*para* ratio, to values of 1.5–2.7, occurs. This may be rationalized in terms of increasing stability and hence a contribution of the staggered conformer in which hydrophobic interactions of enzyme with the cationic nitrogen are decreased. In the case of derivatives with large alkyl substituents on the nitrogen, the ratio becomes essentially 1.0; i.e., *m*- and *p*-methylation are equally favored. Recent studies with norepinephrine analogues have led to the suggestion (12, 15)







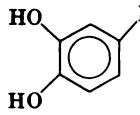
that the *gauche* conformer is the preferred conformation for interaction with catechol *O*-methyltransferase. As in the case of *N*-substituted dopamines, the increasingly hydrophobic nature of the substituted nitrogen may also favor a lower *meta*:*para* ratio.

The importance of the stereochemistry and conformation of the charged substituent is also apparent in the amino acids dopa and 3,4-dihydroxyphenylserine. In the case of dopa, the D-isomer (XI) gives a *meta*:*para* ratio of 3.4 while the L-isomer (XII) gives almost exclusively the *m*-methylation product, with a ratio of 19.8. The conformation of L-dopa in the crystal has been reported (16). It appears that clockwise placement of the polar groupings with respect to the phenyl ring, as in XII, prevents *p*-methylation to a greater extent than in the isomers such as XI, in which the polar groups are placed counterclockwise with respect to the phenyl ring. This was also the case with amines (VIII, IX, IV, V, VI, and VII).

p-Methylation is not favored by the presence of either a cationic or anionic substituent. In the case of the anionic carboxylates, ratios of 2.8 or higher are observed (Table 1). The highest ratios are found with the benzoates and phenyl acetates. Extending the chain to 3 carbon atoms results in a significant relative increase in the proportion of *p*-methylation.

TABLE 3

Meta:*para* ratios obtained with catechol acids, esters, and amides

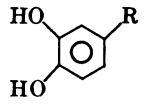
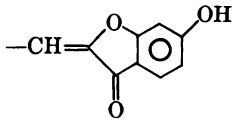
No.		*Meta*:*para* ratio	K_m	V_{max}
			mM	
40	—COO⁻	5.5	0.25	1.04
41	—CH_2COO^-	7.0	0.31	1.16
42	—$CH(OH)COO^-$	3.6	0.94	1.16
43	—$CH_2CH_2COO^-$	3.7	0.43	4.5
44	—$CH{=}CHCOO^-$	2.8	0.08	1.13
45	—$COOCH_2CH_3$	0.85	0.41	6.4
46	—CH_2COOCH_3	1.0		
47	—CH_2CONH_2	1.2		

In a variety of nonionized derivatives and isosteres of the cationic amines and anionic carboxylates, the *meta*:*para* ratio drops to near 2 or less (Tables 2 and 3).

The effects of a number of neutral 4-substituents of differing electronic properties

TABLE 4

Effect of un-ionized substituents on meta:para ratio

No.		*Meta:para* ratio	K_m	V_{max}
			mM	
48	—CH=CH(C_6H_5)	0.40		
49	—F	0.35	0.14	1.00
50	—CF_3	0.36	0.02	0.31
51	—C≡N	0.46		
52	—Br	0.55	0.70	1.53
53	—Cl	0.62	0.23	1.02
54	—CH_3	0.85	0.76	1.44
55	—CH_2CH_3	0.95	0.10	1.35
56	—$C(CH_3)_3$		0.35	4.17
57	—CHO	2.1	1.00	0.9
58	—$COCH_3$	1.2	0.02	0.70
59	—$COCH_2CH_3$	1.2	0.16	0.95
60	—$COCH_2CH_2CH_3$	1.5		
61	—CO(C_6H_5)	3.1	0.12	0.96
62	—NO_2	2.5	0.17	1.85
63	—$NHCOCH_3$	4.5		
64	—NHCO(C_6H_5)	2.6	0.06	1.2
65	—$NHSO_2$(C_6H_5)	1.3		
66		1.9		
67	—H		0.30	2.3
68	3-Methylcatechol	3.1[a]		

[a] *Ortho:meta ratio.*

on the *meta:para* ratios were also determined (Table 4). With methyl and ethyl substituents, the *meta:para* ratios are near unity, as might be expected from the electronic effects of alkyl substituents on the pK_a values and nucleophilicity of *m*- or *p*-phenols. The interpretation of the effects of other substituents is more difficult. One obvious problem is the fact that the *meta:para* ratios vary with pH (Table 5) and probably will be different when the catechol or the catecholate monoanion is the substrate. The relative pK_a values of the catechols of Table 4 vary considerably, so that some will be ionized and some will not be ionized at pH 8.0. In view of this, it is perhaps not surprising that structure correlations are not clearly evident. On the basis of pK_a values of *meta*- and *para*-substituted phenols (17), the fluoro, bromo, and chloro substituents should increase the nucleophilicity of the *p*-hydroxyl group in the catechol and thereby increase the proportion of *p*-methylated product, as evidenced by *meta:para* ratios of 0.35, 0.55, and 0.62, respectively. In the case of nitro-, cyano-, trifluoromethyl-, and carbonyl-substituted catechols, the *m*-hydroxyl group is the stronger nucleophile in the un-ionized molecule, which should result in *meta:para* ratios greater than 1.0. All these compounds possess lower pK_a values, however, so that the catecholate monoanion represents a significant species at pH 8.0. Ionization will have occurred at the *para* position, converting this hydroxyl group to the stronger nucleophile and favoring *meta:para* ratios less than 1.0. The observed result in these com-

TABLE 5

Effect of pH on meta:para ratio

Substrate	*Meta:para* ratio		
	pH 7.0	pH 8.1	pH 9.1
Dopamine	10.2	6.9	3.5
D(−)-Norepinephrine	9.8	5.3	3.0
D(−)-Epinephrine	8.5	5.0	4.1
DL-*N*-Butylnorepinephrine		2.7	1.5
3,4-Dihydroxybenzoic acid	6.1	5.5	5.3
3,4-Dihydroxycinnamic acid		2.8	2.7
N-Acetyldopamine	1.6	1.7	1.7
3,4-Dihydroxyphenylglycol	1.8	1.8	1.7
3,4-Dihydroxyacetophenone	1.25	1.27	1.55
4-Chlorocatechol	0.58	0.62	0.76
4-Bromocatechol	0.43	0.55	0.76
4-Nitrocatechol	2.2	2.5	3.5
3,4-Dihydroxyacetonitrile	0.43	0.46	0.45

pounds will be a composite of relative proportions of ionized and un-ionized species, their enzymatic reactivity, and relative nucleophilicities in each species.

Finally, the acyl- and sulfonylanilines appear to represent a unique case, since they will be un-ionized at pH 8.0 and the *p*-hydroxyl group should be the stronger nucleophile. However, *meta:para* ratios greater than 1.0, rather than the predicted <1.0, are observed. It may well be that the polar nature of the amide substituent is unfavorable for binding in the proper orientation for *p*-methylation.

In Table 6 some of the isomer ratios and kinetic parameters for a variety of other types of catechols are given. The marked influence of the nature and position of substituents on product formation is readily apparent in these compounds, as it was in the 4-substituted series of catechols.

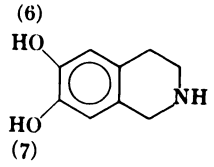
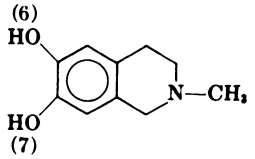
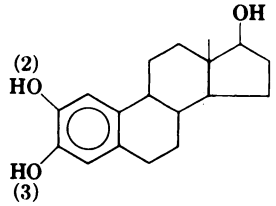
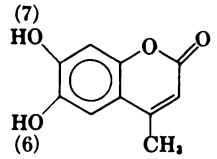
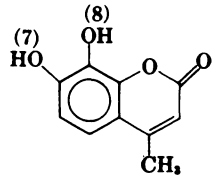
The concentration of metal ions also appears to have an effect on *meta:para* ratios (Table 7). The effect of metal ions on catechol *O*-methyltransferase activity with different substrates is extremely complicated (see ref. 18), and will be the subject of a subsequent publication.

The *meta:para* ratio does not change during the course of the enzymatic reaction from 1 to 120 min with L-norepinephrine or 3,4-dihydroxyacetophenone, nor can it be changed by partial inhibition of the reaction with competitive or noncompetive inhibitors (Table 8). Extensive purification of catechol *O*-methyltransferase did not affect the *meta:para* ratio with four different substrates (2). These observations tend to indicate that only one enzyme is involved in *m*- and *p*-*O*-methylations. *Meta:para* ratios for dopamine were similar with soluble and particulate enzyme preparations from the livers of a variety of rodents and a species of monkey (Table 9).

With dopamine, 3-*O*-methyldopamine, or 4-*O*-methyldopamine as substrate, permeth-

TABLE 6

Meta:para ratio obtained with bi- and polycyclic catechols

	Substrate	Positions O-methylated	Ratio
69		6 7	0.62
70		6 7	0.91
71		2 3	2.1
72		7 6	1.1
73		8 7	11.7

ylation to form 3,4-dimethoxyphenylamine was not detected with any of the preparations of catechol O-methyltransferase used. In addition, with purified enzyme, no interconversion of 3-O-methyl- and 4-O-methyldopamine could be demonstrated using purified, radioactive O-methylated substrates. The probable reason for apparent interconversion of 3- and 4-O-methylated dopamines previously reported (19) is that these substrates, as supplied commercially, contain small amounts of dopamine, which undergo O-methylation. Interconversion of an O-methylated catechol *in vivo* probably involves intermediate formation of the catechol (20).

The influence of substrate structure on the kinetic parameters with catechol O-methyltransferase is apparently quite complicated. The affinity of substrates for the enzyme, as reflected by the K_m value, varies from 0.02 to 2.0 mM, and the V_{max}, from 0.5 to 6.0. However, no obvious correlation with structure is apparent. Furthermore, in the case of the catecholamines, the apparent K_m and V_{max} vary over the range of substrate concentrations used, with different enzyme preparations, and according to the presence or absence of dithiothreitol. The nature of this variation is under investigation. For this reason, it is important to stress that the kinetic parameters given for the catecholamines in Table 1 represent those obtained with a single enzyme preparation over a range of amine concentration from 0.02 to 0.4 mM. An analysis of the structure-activity relationship with various semirigid and rigid analogues of dopamine and norepinephrine as substrates of catechol O-methyltransferase has recently been reported (12, 13, 15). It was concluded that for the norepinephrine analogues a *gauche* conformer was preferred, while for the dopamine analogues a staggered conformer was preferred for the enzyme. These results are in general agreement with kinetic parameters in Table 1 and with

TABLE 7

Effect of cation concentration on meta:para ratio

Substrate	*Meta:para* ratio		
	No Mg^{++}	0.06 mM Mg^{++}	1.2 mM Mg^{++}
D(−)-Norepinephrine	1.8	4.3	5.3
3,4-Dihydroxybenzoic acid	8.0	7.7	6.9
3,4-Dihydroxyacetophenone	1.9	1.6	1.3
4-Nitrocatechol	4.4		2.5

TABLE 8

Effect of enzyme inhibition on meta:para ratio

Inhibitor	Concentration	Inhibition	*Meta:para* ratio	
			3,4-Dihydroxyacetophenone	Dopamine
	mM	%		
None	0	0	1.36	6.9
3,5-Dihydroxy-4-methoxybenzoic acid[a]	1.0	87.5	1.59	
	3.0	92.5	1.20	
3,5-Dihydroxy-4-methoxyphenethylamine[a]	1.0	22.8	1.44	
	3.0	26.8	1.42	
5-Hydroxy-3,4-dimethoxyphenethylamine[a]	0.02	2.7	1.26	
	0.1	5.0	1.33	
	0.5	13.5	1.36	
	1.0	28.0	1.32	
4-Chlorocatechol[b]	0.40	75		6.8
	0.20	58		7.0
	0.05	20		6.9

[a] Noncompetitive inhibitor (3).

[b] Competitive inhibitor, which itself undergoes predominantly p-O-methylation.

TABLE 9

Invariance of meta:para ratio with liver catechol O-methyltransferases from various sources

Enzyme source	*Meta:para* ratio			Activity
	Dopamine	3,4-Dihydroxy-benzoic acid	3,4-Dihydroxy-propriophenone	
				nanomoles/mg N/min
Rat				
Sprague-Dawley, soluble	6.8	5.8	1.0	5.2 ± 0.4
Sprague-Dawley, microsomal	6.9	6.0	0.9	1.3 ± 0.04
Wistar, soluble	6.7	5.3	1.0	6.9 ± 0.6
Wistar, microsomal	6.1	5.7	0.8	0.9 ± 0.03
Aoki-SHR, soluble	6.2	5.3	1.1	22.4 ± 0.9
Aoki-SHR, microsomal	6.3	5.3	0.9	3.3 ± 1.0
Guinea pig				
Hartley, soluble	6.0	6.0	2.0	2.6
Hartley, microsomal	7.2	5.4	1.2	0.8
Rabbit				
New Zealand, soluble	6.2	5.1	1.0	0.3
New Zealand, microsomal	6.0	5.3	0.8	1.0
Mouse				
NIH regular, soluble	6.7	5.3		6.1 ± 0.5
NIH regular, microsomal	8.4	5.4		1.8 ± 0.1
Monkey				
Rhesus, soluble	8.0	3.6	1.4	10.6
Rhesus, microsomal	7.2	3.5	1.6	1.8

the probable preferred conformations of the various analogues.

CONCLUSIONS

The orientation of binding of catechol substrates to catechol *O*-methyltransferase, and hence the *meta:para* ratio of *O*-methylated products, appears to be strongly influenced by the polarity and orientation of substituents. Nucleophilicity of the phenolic groups of the catechol plays a lesser role in determining the *meta:para* ratio.

The relevance of *m*- and *p*-methylation to drug metabolism and to deactivation of endogenous catechols is readily apparent. Awareness of the occurrence of *p-O*-methylated metabolites has increased. There are now many reports of such metabolites. For example, homoisovanillic acid has been reported as a metabolite of L-dopa in the rat (21) and as a normal urinary constituent in man (22–24). However, the *meta:para* ratios of *O*-methylated catechols obtained *in vivo* (20, 25) are frequently much higher than those obtained *in vitro*. Indeed, with epinephrine, the *p-O*-methylated compound could not be detected *in vivo* (20). Whether the decrease in *p*-methylated products observed *in vivo* reflects the formation of a different *meta:para* ratio of methylated catechols by catechol *O*-methyltransferase in its microenvironment in the cell, or whether the decrease derives from the greater rate of demethylation of *p-O*-methylated catechol (20, 26), is at present an unresolved question.

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REFERENCES

1. S. Senoh, J. Daly, J. Axelrod and B. Witkop, *J. Amer. Chem. Soc.* **81**, 6240 (1959).
2. C. R Creveling, N. Dalgard, H. Shimizu and J. W. Daly, *Mol. Pharmacol.* **6**, 691 (1970).
3. B. Nikodejevic, S. Senoh, J. W. Daly and C. R. Creveling, *J. Pharmacol. Exp. Ther.* **174**, 83 (1970).
4. J. J. Pisano, C. R. Creveling and S. Udenfriend, *Biochim. Biophys. Acta* **43**, 566 (1960).

5. R. A. Boissonnas, *Advan. Org. Chem.* **3**, 159 (1963).
6. W. G. Brown, *in* "Organic Reactions" (R. Adams, ed.), Vol. 7, p 469. Wiley, New York, 1951.
7. H. G. Bray, W. V. Thorpe and K White, *Biochem. J.* **46**, 271 (1950).
8. R. Knuppen and H. Breuer, *Hoppe-Seyler's Z. Physiol. Chem.* **346**, 114 (1966).
9. P. Ball, R. Knuppen and H. Breuer, *Eur. J. Biochem.* **21**, 517 (1971).
10. L. B. Kier and E. B. Truitt, Jr., *J. Pharmacol. Exp. Ther.* **174**, 94 (1970).
11. L. Pedersen, R. E. Haskins and H. Cable, *J. Pharm. Pharmacol.* **23**, 216 (1971).
12. E. E. Smissman and R. T. Borchardt, *J. Med. Chem.* **14**, 702 (1971).
13. E. E. Smissman and R. T. Borchardt, *J. Med. Chem.* **14**, 383 (1971).
14. G. A. Neville, R. Deslauriers, B. J. Blackburn and I. C. P. Smith, *J. Med. Chem.* **14**, 717 (1971).
15. E. E. Smissman and R. T. Borchardt, *J. Med. Chem.* **14**, 377 (1971).
16. A. Mostad, T. Ottersen and C. Romming, *Acta Chem. Scand.* **24**, 1864 (1970).
17. A. I. Biggs and R. A. Robinson, *J. Chem. Soc.* 388 (1961).
18. S. Senoh, Y. Tokuyama and B. Witkop, *J. Amer. Chem. Soc.* **84**, 1719 (1962).
19. F. A. Kuehl, Jr., M. Hichens, R. E. Ormond, M. A. P. Meisinger, P. H. Gale, V. J. Cirillo and N. G. Brink, *Nature* **203**, 154 (1964).
20. J. W Daly, J. Axelrod and B. Witkop, *J. Biol. Chem.* **235**, 1155 (1960).
21. O. M. Bakke, *Acta Pharmacol. Toxicol.* **29**, 106 (1971).
22. L. P. O'Gorman, O. Borud, I. A. Khau and L. R. Gjessing, *Clin. Chim. Acta* **29**, 111 (1970).
23. P. Mathieu and L. Revol, *Bull. Soc. Chim. Biol.* **52**, 1039 (1970).
24. E. Comoy and C. Bohuon, *Clin. Chim. Acta* **35**, 369 (1971).
25. O. M. Bakke, *Acta Pharmacol. Toxicol.* **28**, 28 (1970).
26. J. Axelrod, *Biochem. J.* **63**, 634 (1956).