Dynamic Aspects of Enzymatic O-Methylation and 
-Demethylation of Catechols in Vitro and in Vivo

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The importance of O-methylation of catecholamines and other 
catechols as a metabolic pathway has been amply shown (1-4). 
The isolation of "Substanz Y," a p-O-methylcatechol derivative 
from beef adrenal glands (5), and the occurrence of p-O-methyl-
catechol derivatives in urine (6) are suggestive of p-O-methyla-
tion in vivo. Previous studies (7) have shown that in vitro the 
enzyme O-methyltransferase effects both para and meta O-meth-
ylation. The following study demonstrates that (nor)epineph-
rine is converted to p-O-methyl-(nor)epinephrine in vitro and 
that 3,4-dihydroxyacetophenone, arterenone, and adrenalone 
undergo p-O-methylation in the intact rat. The para and meta 
O-methylated derivatives of 3,4-dihydroxyacetophenone (aceto-
ovanillone and acetovanillic acid) have been shown to undergo a 
novel interconversion in vivo.

MATERIALS AND METHODS

3,4-Dihydroxyacetophenone, acetovanillic acid, acetoisovanillic acid, 3,4-dihydroxyphenylmethylcarbinol, 3-hydroxy-4-methoxyphenylmethylcarbinol, and 4-hydroxy-3-methoxyphenylmethylcarbinol have been described in a previous paper (7). The isomeric monomethyl ethers of dopamine, epinephrine, and norepinephrine were synthesized at the Sterling-Winthrop Research Institute and made available to us through the courtesy of Dr. Sydney Archer. In analogy to (nor)metanephrine the name (nor)paranephrine is suggested for p-O-methyl-(nor)epinephrine.

Synthesis of p-O-Methyladrenalone—To a vigorously stirred mixture of 7 g of adrenalone and 4 g of sodium bicarbonate in 60 ml of water and 60 ml of ether at 0° a solution of 7.0 g of carbobenzyloxy chloride in 50 ml of ether was added dropwise. Stirring was continued for 24 hours at 0°. Filtration removed 3.0 g of unchanged adrenalone. The solution was then shaken three times with ethyl acetate. The extract was dried and the solvent was removed under reduced pressure. The residue was recrystallized from ethyl acetate-ether-petroleum ether to yield crude p-O-methyl-N-carbobenzyloxyadrenalone, m.p. 121-122°.

C_{1}H_{11}NO_{4}
Calculated: C 64.75, H 5.43, N 4.44
Found: C 64.77, H 5.48, N 4.51

A solution of 0.45 g of N-carbobenzyloxyadrenalone and 0.4 g of methyl iodide in 30 ml of methanol was refluxed with 1.6 ml of 1 n sodium hydroxide. After five hours the mixture was concentrated, the residue taken up in 150 ml of ethyl acetate, 
and the solution shaken four times with equal volumes of 0.05 n sodium hydroxide to remove any unchanged starting material. The product was then extracted into 1 n sodium hydroxide and precipitated by acidification. After recrystallization from ethyl acetate-ether-petroleum ether 110 mg of N-carbobenzyloxyadrenalone, m.p. 121-122°, were obtained.

C_{1}H_{11}NO_{4}
Calculated: C 51.84, H 6.09, N 6.05, Cl 15.31
Found: C 52.02, H 6.14, N 5.99, Cl 15.47

To 0.1 g of p-O-methyl-N-carbobenzyloxyadrenalone were added 3 ml of 32% hydrobromic acid in acetic acid. The solution was concentrated to dryness at room temperature under reduced pressure. The residue was recrystallized (Norit) from methanol-ethyl acetate to give crude p-O-methyladrenalone hydrobromide. The hydrobromide was dissolved in a small volume of water and the calculated amount of sodium carbonate was added. After standing overnight in the cold room the free base was collected and converted to the hydrochloride with a solution of hydrochloric acid in ethanol. Addition of ethyl acetate caused the precipitation of 60 mg of p-O-methyladrenalone-HCl, m.p. 245-246°.

C_{1}H_{11}NO_{4}
Calculated: C 51.84, H 6.09, N 6.05, Cl 15.31
Found: C 52.02, H 6.14, N 5.99, Cl 15.47

Synthesis of p-O-Methylarterenone—Carbobenzyloxylation of arterenone was carried out in the same way as described for adrenalone. From 10.2 g of arterenone HCl 4.8 g of N-carbobenzyloxyarterenone, m.p. 138-140°, were obtained; 2.2 g of unchanged arterenone were recovered.

C_{1}H_{11}NO_{4}
Calculated: C 64.75, H 5.43
Found: C 64.86, H 5.43

The methylation of N-carbobenzyloxyarterenone was carried out as described above for adrenalone. From 0.6 g of ethyl acetate-ether-petroleum ether to yield 2.6 g of N-carbobenzyloxyadrenalone, m.p. 180-183°.

C_{1}H_{11}NO_{4}
Calculated: C 64.75, H 5.43, N 4.44
Found: C 64.77, H 5.48, N 4.51
The decarbobenzylosylation of p-O methyl N-carbobenzyloxyarterenone and the conversion of the resulting hydrobromide to the hydrochloride were carried out as described above. The hydrochloride of p-O-methylarterenone had m.p. 254-256°.

\[ C_{10}H_{18}NO_3 \cdot HCl \]

Calculated: C 49.65, H 5.56, N 6.44, Cl 16.29

Found: C 49.69, H 5.87, N 6.09, Cl 16.39

Synthesis of m-O-Methyladrenalone—Three grams of 4-acetoxy-3-methoxyacetophenone (8) in 50 ml of glacial acetic acid were cooled and stirred while 2.3 g of bromine was added in 2 ml of acetic acid. The reaction was stirred for 1 hour and the temperature then raised briefly to 70°. After concentration to dryness under reduced pressure the residue was triturated with water. The reaction mixture was then treated with acetone and stirred while 2.3 g of bromine was added in 2 ml of glacial acetic acid. The reaction was stirred for 1 hour and the temperature then raised briefly to 70°. After concentration to dryness under reduced pressure the residue was triturated with water and benzene. The benzene layer was removed and dried over sodium sulfate. On addition of petroleum ether, 3.1 g of 4-acetoxy-3-methoxy-o-bromoacetophenone were obtained, m.p. 77-79°.

\[ C_{10}H_{18}BrO_4 \]

Calculated: C 46.01, H 3.86, Br 27.84

Found: C 45.70, H 3.70, Br 27.89

A solution of 1 g of 4-acetoxy-3-methoxy-o-bromoacetophenone in 50 ml of ethyl acetate was added slowly to 50 ml of ethyl acetate through which was passed a stream of methylamine for 10 minutes. The reaction mixture was allowed to stand for 30 minutes and then concentrated to dryness under reduced pressure. The residue was dissolved in 20 ml of 3% hydrochloric acid, refluxed for 3 hours on the steam bath, and the suspension was shaken with isoamyl alcohol. The ammonia solutions were acidified after 5 minutes and extracted with ethyl acetate. The (dried) extracts were concentrated under reduced pressure. Frac- tional recrystallization from methanol-ethyl acetate afforded 100 mg of m-O-methyladrenalone hydrochloride, m.p. 250-254°.

\[ C_{10}H_{18}NO_3 \cdot HCl \]

Calculated: C 51.84, H 5.56, N 6.44, Cl 16.29

Found: C 51.67, H 6.12, N 6.10, Cl 15.68

Synthesis of m-O-Methylarterenone—The compound was prepared as described above for m-O-methyladrenalone with ammonia instead of methylamine. From 1 g of 4-acetoxy-3-methoxy-o-bromoacetophenone 0.2 g of m-O-methylarterenone hydrochloride was obtained, m.p. 249-251°.

\[ C_{10}H_{18}NO_3 \cdot HCl \]

Calculated: C 49.69, H 5.56, N 6.44, Cl 16.29

Found: C 49.47, H 5.80, N 6.38, Cl 16.50

Enzymatic O-Methylation of Catecholamines—Enzymatic O-methylation of epinephrine, norepinephrine, adrenaline, and ar- terenone was carried out by incubating the hydrochloride of the catecholamine (10 μmoles), dissolved in 1 ml of water, for 1.5 hours at 37° with 4 ml of the soluble supernatant fraction of rat liver, 2.5 ml of 0.5 M phosphate buffer, pH 7.9, 4 μmoles of S-adenosylmethionine, and 0.1 ml of 2.0 M magnesium chloride (9).

Enzymatic Demethylation of O-Methylated Catechols—The demethylation of O-methylcatechols was carried out as described by Axelrod (10). The formaldehyde formed in the oxidative demethylation was assayed with the Nash reagent (11). The results are presented in Table II.

**Results**

Enzymatic p- and m-O-Methylation of Epinephrine and Nor- epinephrine—After incubation of the reaction mixture containing either norepinephrine or epinephrine the pH was adjusted to 10 and the suspension was shaken with isooamyl alcohol (9). The isoamyl alcohol extract was then extracted with 0.1 N hydrochloric acid and this acid solution was concentrated under reduced pressure to dryness. The residue was taken up in a small amount of 95% ethanol for chromatography on paper (Whatman No. 1). The Rp values in a variety of systems and the color reaction with dichloroquinonechlorimide (7) of the enzymatically formed O-methyl compounds were identical with those of the corresponding isomic catecholamine monomethyl ethers. Chromatographic resolution of the isomeric monomethyl ethers of metanephrine and paraneprine, and of normetanephrine and norparaneprine, however, was possible only by way of the azo- benzene sulfonate derivatives. With the use of the coupling method described by Senoh et al. (7), the azobenzenesulfonate derivatives of metanephrine, paraneprine, normetanephrine, norparaneprine, and the enzymatically formed O-methyl ethers of (nor)epinephrine were formed and subjected to chromatographic resolution. The results are given in Table I. They show that epinephrine and norepinephrine are enzymatically transformed to mixtures of the corresponding p- and m-O-methyl ethers. From the relative intensity of the two spots it was esti-mated that both with epinephrine and norepinephrine, the p-O- methyl ether represented only 10 to 15% of the methylation mixture. Confirmation of this was obtained in further experiments in which the final 0.1 N HCl extract (50 ml) was treated with 5 ml of concentrated ammonium hydroxide and 2 ml of a 2% solution of periodic acid, a reaction which quantitatively con- verts (nor)metanephrine to vanillin and (nor)paraneprine to isovanillin (12). The ammonia solutions were acidified after 5 minutes and extracted with ethyl acetate. The (dried) extracts were concentrated under reduced pressure. The residue was taken up in ethanol and subjected to paper chromatography. Two spots were detected whose Rp values in a variety of solvent systems were identical with those of vanillin and isovanillin. The color reactions with dichloroquinonechlorimide gave a light green color for vanillin and a blue color for isovanillin. Under ultraviolet light the light green vanillin spot fluoresced dark vio-
let, whereas the blue isovanillin spot fluoresced bright yellow.

The vanillin and isovanillin obtained by periodate oxidation of the enzmyatic mixture also showed these properties. In addition, when the ethanol extract was distributed along the starting line of Whatman No. 1 paper, developed with butanol-concentrated ammonia (4:1), and the areas corresponding to vanillin and isovanillin eluted with a measured volume of 0.1 N sodium hydroxide and the ultraviolet absorption curves determined, they were found to be identical with the ultraviolet absorption curve of authentic vanillin or isovanillin. The amounts of vanillin and isovanillin could be calculated from the ratio of extinctions of $\lambda_{\text{max}}$ 347 mp (vanillin in 0.1 N NaOH), and $\lambda_{\text{max}}$ 248 mp (isovanillin, 0.1 N NaOH). By this method, samples of enzymatically $O$-methylated epinephrine and norepinephrine were found to contain 9 and 12% of the pure isomers, respectively.

Enzymatic $p$- and $m$-$O$-Methylation of Arterenone and Adrenalone—After incubation the pH of the reaction mixture was adjusted to 9 and the suspension was shaken with isooamyl alcohol. The extracted products were transferred into 0.1 N HCl, and this solution was concentrated to dryness under reduced pressure. Under these conditions only small amounts of unreacted adrenalone or adrenone are extracted into the isooamyl alcohol, whereas the $p$- and $m$-$O$-methyl ethers are extracted almost quantitatively. The methylation products were taken up in a small volume of ethanol and subjected to paper chromatography. Synthetic samples of $p$- and $O$-methyladrenalone and -arterenone served as reference. The reaction of $O$-methyladrenalone with dichloroquinonechlorimide gave an initial blue-green color which turned yellow-brown; the $O$-methylarterenones gave a more greenish color which also turned brown. Under ultraviolet light the spot from the $m$-$O$-methyl ethers fluoresced violet, that from the $p$-$O$-methyl ethers fluoresced yellow. Mixtures of the isomers did not separate in a variety of solvent systems. However under ultraviolet light, after treatment with dichloroquinonechlorimide, the single spot was in some solvents (n-butanone-propionic acid-water, 15:5:6) seen to consist of a violet fluorescing portion preceding a yellow fluorescing portion. The enzymatically formed $O$-methylated compounds gave the same $R_p$ values and color reactions as described above for mixtures of the synthetic reference samples.

Because of the unsatisfactory chromatographic separation of the isomers of $O$-methyladrenaline and -arterenone, a method was sought for the selective reduction of the keto group in $p$- and $m$-$O$-methylated arternones and adrenalone to the corresponding ethers of norepinephrine and epinephrine, so that the more sensitive and convenient oxidation with periodic acid to vanillin or isovanillin could be employed. The following method was found satisfactory: The aqueous 0.1 N HCl extract (25 ml) was treated with 25 ml of 2.5% sodium carbonate and 20 mg of sodium borohydride. After heating for 30 minutes at 60–70°C, an excess of acetone (~1 ml) to decompose any unreacted sodium borohydride, and then 5 ml of a 2% aqueous solution of periodic acid were added. After standing for 10 minutes, the solution was cooled, acidified, and shaken with ethyl acetate. The ethyl acetate layer was dried and the solvent removed under reduced pressure; the residue was dissolved in a small amount of ethanol and subjected to paper chromatography. Vanillin and isovanillin, identical in all respects ($R_p$ values, color reactions, ultraviolet absorption spectra) with authentic samples, were identified and assayed. This method showed the $O$-methylation mixture from adrenalone to contain 37% $p$-O methyl and 63% $m$-O methyl ether. For arterenone this ratio was 40% $p$- and 60% $m$-O-methyl ether.

$p$- and $m$-$O$-Methylation of Adrenalone in vivo—All compounds were administered intraperitoneally. Two adult male rats received 120 mg per kg of iproniazid, an inhibitor of monoamine oxidase, 6 hours and 1 hour before the experiment, and then 120 mg per kg of adrenalone hydrochloride in three divided doses over 6 hours. Urine was collected for 20 hours, pooled, adjusted to pH 6 with acetate buffer, incubated for 8 hours at 37°C with 10,000 units of bacterial $\beta$-glucoronidase (Sigma Chemical Company) and 2,000 units of snail sulfatase (gusulase, Endo Products).

An aliquot was adjusted to pH 9 and the $m$- and $p$-$O$-methyladrenalones were isolated as described above. Chromatography was carried out in a variety of solvent systems. The $R_p$ values and the color reaction with dichloroquinonechlorimide indicated the presence of a mixture of $O$-methyladrenalones. The material was treated as described above for the conversion of the $O$-methyl adrenalone or arterenones to vanillin and isovanillin. $R_p$ values, color reactions, and ultraviolet absorption spectra proved the presence of vanillin and isovanillin and indicated the formation of 16% $p$-O-methyl and 84% $m$-O-methyl ether from adrenalone. Direct treatment of urine without isolation of the amine fraction with sodium borohydride and periodic acid yielded a mixture of 9% isovanillin and 91% vanillin. The $O$-methylated derivatives represented about 20% of the administered adrenalone hydrochloride.

$p$- and $m$-$O$-Methylation of Arterenone in vivo—The methylation of arterenone in the rat and the subsequent assay was carried out as described above for adrenalone. Chromatography of the purified products indicated the presence of a mixture of $O$-methylated arterenones. Treatment with sodium borohydride and periodic acid yielded vanillin and isovanillin ($R_p$, color reaction, ultraviolet spectrum) in relative amounts of 18% of $p$-O-methyl and 82% of $m$-O-methyl isomer in the purified extract. The direct treatment of the urine with sodium borohydride and periodic acid yielded a mixture of 92% vanillin and 8% isovanillin. The recovery of $O$-methylated derivatives in the urine was 16% of the administered arterenone hydrochloride.

Methylation of Epinephrine in vivo—Similar studies as above with rats were conducted with 40 mg per kg (in four divided doses) of $L$-(+)-epinephrine (13, 14), the physiologically less active antipode of the natural hormone, with or without administration of iproniazid. No conversion to isovanillin could be demonstrated.

Administration of parancinephrine or metancinephrine intraperitoneally to rats with or without administration of iproniazid, followed by extraction of the amine fraction from the urine and vanillin-isovanillin assay, showed no detectable interconversion of the isomerics others, the recovery of which was essentially the same (20 to 25%) within the limits of error.

The nonoccurrence of $p$-$O$-methylation of $L$-(+)-epinephrine was also demonstrated in mice.

$p$- and $m$-$O$-Methylation of 3, 4-Dihydroxyacetonaphone in vivo—Two groups of adult male rats were used. One of these received 120 mg per kg of 3, 4-dihydroxyacetonaphone in divided doses over a period of 4 hours whereas the other group served as control throughout the experiment. The urine from each was collected for 20 hours, adjusted to pH 6 with acetate buffer, and
incubated overnight with 10,000 units of bacterial $\beta$-glucuronidase. The solutions were brought to an acid strength of 1 m in hydrochloric acid, heated for 90 minutes in a boiling water bath, cooled, and adjusted to $\sim$pH 2. Aliquots were extracted five times with benzene, a procedure that extracts acetovanillone and acetoisovanillone but not 3,4-dihydroxyacetophenone (7).

After concentration to dryness under reduced pressure, and so-

times with benzene, a procedure that extracts acetovanillone

3,4-dihydroxyphenylmethylcarbinol, and dopamine undergo

37% and the ratio of meta (acetovanillone) to para


crude enzyme preparation of 0-demethylase (10) indicated that

Table II

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Rat liver</th>
<th>Guinea pig liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-Hydroxy-3-methoxyphenyl-methylcarbinol</td>
<td>0.6</td>
<td>0.9</td>
</tr>
<tr>
<td>3-Hydroxy-4-methoxyphenyl-methylcarbinol</td>
<td>1.0</td>
<td>1.4</td>
</tr>
<tr>
<td>Acetovanillone</td>
<td>1.3</td>
<td>1.2</td>
</tr>
<tr>
<td>Acetoisovanillone</td>
<td>1.7</td>
<td>1.8</td>
</tr>
<tr>
<td>$m$-O-Methyldopamine-HCl</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>$p$-O-Methyldopamine-HCl</td>
<td>0.4</td>
<td>0.5</td>
</tr>
<tr>
<td>Metanephrine-HCl</td>
<td>0.3</td>
<td>0.6</td>
</tr>
<tr>
<td>Paraneprine-HCl</td>
<td>0.3</td>
<td>1.0</td>
</tr>
<tr>
<td>Norparaneprine-HCl</td>
<td>0.3</td>
<td>0.5</td>
</tr>
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<td>$m$-O-Methylarterenone-HCl</td>
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<tr>
<td>$p$-O-Methylarterenone-HCl</td>
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<td>$m$-O-Methyladrenalone-HCl</td>
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<td>$p$-O-Methyladrenalone-HCl</td>
<td>1.1</td>
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</table>
The interconversion of m- and p-O methyl catechol ethers in the intact rat may be the result of demethylation to the catechol by the TPNH-dependent microsomal enzyme (10) followed by remethylation. Since the catechol is excreted largely as the m-methoxy derivative when given in vivo, the relatively greater conversion of p- to m-O-methyl ether may express the relative rates of methylation \( k_d > k_a \) in the two positions. Further analysis of the factors determining the fates of these compounds requires determination of the number of enzymes capable of methylating and demethylating the catechol derivatives under study, the specificity and affinities of these enzymes, their distribution in the organism, the distribution of the substrates, and other factors.

p-O-Methylation of catechol derivatives in vivo introduces new metabolic aspects and will have to be studied further before any general conclusions may be reached.

**SUMMARY**

1. Methods for the synthesis of p-O-methyladrenalone, p-O-methylarterenone, m-O-methyladrenalone, and m-O-methylarterenone are described.
2. The formation of p-O-methylated compounds in vitro with O-methyl transferase has been demonstrated for epinephrine, nor-epinephrine, arterenone, and adrenalone.
3. The methylation in vivo of 3,4-dihydroxyacetophenone, arterenone, and adrenalone occurs at the para as well as meta positions.
4. An interconversion of the isomeric m- and p-methyl ethers of 3,4-dihydroxyacetophenone was observed in rats, the para isomer undergoing this interconversion to a greater extent.
5. Enzymatic demethylation studies in vitro showed that in most cases the para O-methyl ethers are demethylated more rapidly than the meta isomers.
6. No formation of paranephrine from epinephrine could be detected in vivo nor was any interconversion of paranephrine to metanephrine observed in vivo.

**REFERENCES**

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