EVIDENCE OF THE DIRECTIVE EFFECT OF 17 β -CONJUGATE GROUP ON THE ENZYMATIC O-METHYLATION OF CATECHOL ESTROGEN

ITSUO YOSHIKAWA, AKIKO NAKAGAWA, ERIKO KAMIYA, SHINJI ITOH, AND TAKETO OGISO
Hokkaido Institute of Pharmaceutical Sciences, 7-2, Katsuraoka-cho, Otaru, Hokkaido, 047-02, Japan

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Incubations of 2-hydroxyestradiol (I), 2-hydroxyestradiol 17 β -sulfate (II), and 2-hydroxyestradiol 17 β -glucuronide (III) with purified rat liver catechol O-methyltransferase were carried out at pH 7.2 in the presence of Mg²⁺ and (³H-Me)-S-adenosyl-L-methionine. The radioactive methylated products, 2-methoxyestradiol (IV) and 2-hydroxyestradiol-3-methyl ether (V), from each substrate were quantified by reverse isotope dilution method after their complete separation and acetylation. In the experiments of conjugated substrates, II and III, the analyses of the methylated products were done after their hydrolysis of 17 β -conjugate groups with acid or β -glucuronidase.

The product ratios (2-methoxy/3-methoxy) of substrates I, II, and III, were 1:1, 4:1, and 4.5:1, respectively. These results are suggesting that 17 β -conjugate groups of 2-hydroxyestradiol has directive effect on enzymatic O-methylation of estrogen catechols. Further, it is estimated that following process may be present in the estradiol metabolism in rat and/or humans: estradiol $\rightarrow$ estradiol 17 β -conjugates $\rightarrow$ 2-hydroxyestradiol 17 β -conjugates $\rightarrow$ 2-methoxyestradiol 17 β -conjugates.

Keywords—2-hydroxyestradiol; 2-hydroxyestradiol 17 β -sulfate; 2-hydroxyestradiol 17 β -glucuronide; catechol O-methyltransferase; transmethylation of catechol estrogens

Catechol estrogen, the principal metabolite of estrogenic hormone, estradiol, is converted *in vivo* to the 2-methyl ether without any evidence of methylation of the 3-methyl compound.¹⁾ This is particularly significant because the difference of the chemical reactivity between two phenolic groups is not so remarkable.²⁾ Methylation *in vivo* of the important catecholamines leads nearly exclusively to the *m*-methyl ether.³⁾

In sharp contrast with these results in living animals, *in vitro* methylation of catechols by rat liver catechol O-methyltransferase enzyme is relatively indiscriminate and yields mixtures of monomethyl derivatives, often in approximately equivalent amount.⁴⁾ The present study was undertaken to explore the possibility that 17 β -conjugation might be involved in the selectivity

of *in vivo* methylation.

The lack of selectivity for methylation of estrogen catechols *in vitro* was first confirmed by incubation of 600 μ g (2 μ mol) of 2-hydroxyestradiol (I) with purified catechol O-methyltransferase (41 units⁵⁾) from rat liver which was prepared by the method as described in a previous paper.⁵⁾ The incubation medium contained 1.5 ml of enzyme solution, 3.0 ml of 0.1 M phosphate buffer (KH₂PO₄/Na₂HPO₄) of pH 7.2, 1.0 mg of MgSO₄, and (³H-Me)-S-adenosyl-L-methionine (3.90 $\times 10^6$ dpm, specific activity: 4.40 $\times 10^8$ dpm/mmol). The mixture was shaken in nitrogen gas at 37° for 3 hr and then extracted with ether continuously for 24 hr. Carrier amount of 2-methoxyestradiol (IV) and 2-hydroxyestradiol-3-methyl ether (V) (about 30

mg each) were added to the ether extract and the product was separated carefully by preparative thin-layer chromatography (developed continuously with 2% acetone in benzene).⁶⁾ The materials separated were then acetylated with acetic anhydride and pyridine, and recrystallized from petroleum ether to constant specific activity. The product ratio found in this study was almost 1:1 as shown in Table I. The results are in good agreement with findings of Knuppen and Breuer⁷⁾ with non-radioactive compound and also our previous paper.⁸⁾

Eight hundred μg ($2 \mu\text{mol}$) of 2-hydroxyestradiol 17 β -sulfate (II)⁹⁾ was incubated with the same enzyme under conditions identical with those used for free steroid. After being acidified with 10% H_2SO_4 solution, the incubation mixture was diluted with a couple of carrier steroid and then extracted with ether continuously. The methylated products were similarly separated by preparative thin-layer chromatography and acetylated. 2-Methyl ether (IV) was obtained in a yield of almost 4 times that of 3-methyl ether (V) as shown in Table I. Since sulfatase activity was

demonstrably absent in the purified enzyme, it is reasonable to suppose that methyl ethers were derived from sulfate.

Studies were made in a similar fashion by using 900 μg ($1.8 \mu\text{mol}$) of 2-hydroxyestradiol 17 β -glucuronide (III)⁹⁾ and catechol O-methyltransferase under the same incubation conditions used in the above experiments. After incubation, the mixture was passed through Amberlite XAD-2 resin¹⁰⁾ column (10 ml) and steroidal conjugate fraction eluted with methanol was evaporated under reduced pressure to give residues. The methylated glucuronide was then incubated with β -glucuronidase (calf liver) in 0.1 M acetate buffer of pH 4.9 at 37° for 48 hr. The enzymatic activity of the incubation mixture was 13000 Fishman units/ml. After dilution with authentic carrier steroids, the mixture was extracted with ether continuously. The separation, acetylation, and recrystallization of the products to constant specific activity was done by the same way as described before. The results obtained are shown in Table I. This substrate yielded about five times as much 2-methoxy

TABLE I. *Isotope Contents of Methylated Metabolites of 2-Hydroxyestradiol (I) and Its 17 β -Sulfate (II) and 17 β -Glucuronide (III) with Rat Liver Catechol O-Methyltransferase in the Presence of Mg^{2+} and ($^3\text{H-Me}$)-S-Adenosyl-L-methionine (3.90×10^6 dpm, specific activity: 4.40×10^8 dpm/mmol)^{a)}*

Substrates		Metabolites		Ratio
		2-Methoxy	3-Methoxy	2-Methoxy/3-Methoxy
2-Hydroxyestradiol (I)	exp. 1	355,800	352,700	1.01
	2	378,100	367,100	1.03
2-Hydroxyestradiol 17 β -sulfate (II)	exp. 1	644,700	176,500	3.65
	2	689,400	172,800	3.99
2-Hydroxyestradiol 17 β -glucuronide (III)	exp. 1	441,200	98,700	4.50
	2	390,200	81,300	4.80

a) Each value is expressed as dpm. About $2 \mu\text{mol}$ of substrates were incubated with enzyme at pH 7.2 under nitrogen gas at 37° for 3 hr. Experiments were conducted in duplicate.

compound as the isomeric 3-methoxy steroid. Since β -glucuronidase activity was absent in the purified catechol O-methyltransferase, the specific formation of IV implies that 17 β -glucuronyl group is also directing the O-methylation of catechol ring.

The present results suggest that 17 β -conjugate formation of catechol estrogen may be an important factor in the selectivity of methylation found in normal humans. Indeed, since sulfate participation in catechol estrogen 3-sulfate has been demonstrated,¹¹⁾ it is not too daring to speculate that following metabolic pathways may be present, estradiol $\rightarrow$ estradiol 17 β -sulfate (or glucuronide) $\rightarrow$ 2-hydroxyestradiol 17 β -sulfate (or glucuronide) $\rightarrow$ 2-methoxyestradiol 17 β -sulfate (or glucuronide). The implications of this conclusion are being explored further.

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