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# Studies on Steroid Conjugates. X. Significance of Conjugation involved in Biosynthesis of 2-Methoxyestrogen[1]

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The role of the conjugate in the biosynthesis of 2-methoxyestrogen in the living animal has been explored by the double-isotope technique. The isotope ratios of the isomeric catechol estrogen monomethyl ethers excreted in urine after simultaneous administration of estrone-4-$^{14}$C and estrone-6,7-$^{3}$H sulfate or estradiol-6,7-$^{3}$H 3-glucuronide were determined. Comparison of the $^{3}$H/$^{14}$C value with that of the administered steroid implies that the sulfate conjugation may possibly participate in the predominant formation of 2-methoxyestrogen, while the conjugation with glucuronic acid may not. The dimethyl ether of catechol estrogen was metabolized with random O-demethylation yielding two isomeric monomethyl ethers both in the rat and man. Therefore it appears that the selective O-demethylation may not be involved in the preferential formation of 2-methoxyestrogen.

2-Methoxyestrogen, the principal metabolite of female sex hormone in man, is formed by two separate steps, namely hydroxylation at C-2 and subsequent O-methylation.[3] This *in vivo* O-methylation is of particular interest in the high selectivity for the C-2 phenolic function. Similar property is observed with the catecholamine where *in vivo* O-methylation occurs exclusively at the *m*-hydroxylic group.[4] In contrast the incubation of the catechol estrogen with the human or rat liver in the presence of *S*-adenosylmethionine yields the mixture of the 2- and 3-monomethyl ethers in approximately equal amounts.[5] Transfer of the methyl group is catalyzed with catechol O-methyltransferase (COMT), which is present in the supernatant fraction of liver.[6] In order to explain the apparent discrepancy between the *in vivo* and *in vitro* results the extensive efforts have recently been made. Fishman, *et al.* presented the evidences on the basis of *in vitro* experiment that the sulfate formation would be an important factor in the selective O-methylation of the catechol estrogen.[7] On the other hand Breuer and his co-worker suggested that rapid demethylation of the 3-methyl ether may alter the ratio of the two isomeric monomethyl ethers and hence may favor the formation of 2-methoxyestrogen.[8]

In view of these attractive explanations the present study was undertaken to explore the possibilities that in the living animals the glucuronide or sulfate formation as a prior reaction stage, could be involved in the selective O-methylation and otherwise the preferential demethylation could participate in the biosynthesis of 2-methoxyestrogen.

1) This paper constitutes Part LIX of the series entitled "Analytical Chemical Studies on Steroids"; Part LVIII: T. Nambara and T. Kudo, *Chem. Pharm. Bull.* (Tokyo), **20**, 2156 (1972). This work has been presented at the 91st Annual Meeting of Pharmaceutical Society of Japan, April 1971, Fukuoka.
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7) J. Fishman, M. Miyazaki, and I. Yoshizawa, *J. Am. Chem. Soc.*, **89**, 7147 (1967); M. Miyazaki, I. Yoshizawa, and J. Fishman, *Biochemistry*, **8**, 1669 (1969).
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## Experimental

**Animal**——Male Wistar rats weighing, on the average, 250 g were housed in a cage that was designed to minimize the fecal contamination of the urine.

**Material**——Carrier 2-methoxyestradiol, 2-hydroxyestradiol 3-methyl ether and 2-hydroxyestradiol 2,3-dimethyl ether were prepared in these laboratories as previously reported[9,10] and their homogeneity was confirmed by thin-layer chromatography (TLC) and melting point determination. Estrone-4-$^{14}$C and estrone-6,7-$^{3}$H sulfate were purchased from New England Nuclear Corporation and purified by TLC prior to use. Estradiol-6,7-$^{3}$H 3-glucuronide and 2-hydroxyestradiol-6,7-$^{3}$H 2,3-dimethyl ether were prepared in these laboratories by the methods previously established.[9-11]

**Radioactive Counting**——Counting was carried out on a Kobe Kogyo Model EA-118 liquid scintillation counter. For toluene-soluble samples toluene containing 2,5-diphenyloxazole (4 g/liter) and 1,4-bis[2-(5-phenyloxazolyl)]benzene (100 mg/liter) was used as a scintillator. Aqueous samples were counted in a scintillator, composed of dioxane (1 liter), naphthalene (100 g), 2,5-diphenyloxazole (4 g) and 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene (100 mg). For quench corrections the channel ratio and external standard methods were employed.

**Estrone Sulfate Study**——A solution of estrone-6,7-$^{3}$H sulfate ($9.0\times10^{7}$ dpm) and estrone-4-$^{14}$C ($1.56\times10^{7}$ dpm) ($^{3}$H/$^{14}$C=5.7) dissolved in propylene glycol (0.3 ml)-saline (3 ml) was injected intravenously to three rats and urine was collected in a bottle containing a few drops of toluene as a preservative for 4 days after administration. The pooled urine was adjusted to pH 4.2 with 0.1M acetate buffer (30 ml) and incubated with beef-liver $\beta$-glucuronidase (Tokyo Zōkikagaku Co.) (300 Fishman units/ml) and penicillin G (3000 units/ml) at 37° for 4 days. To the resulting solution were added 2-methoxyestradiol (33.3 mg) and 2-hydroxyestradiol 3-methyl ether (37.2 mg) as a carrier and extracted with ether, washed with $H_2O$ and dried over anhydrous $Na_2SO_4$. Evaporation of the solvent *in vacuo* gave the glucuronide fraction. The remaining aqueous layer was brought to 2N $H_2SO_4$ solution, saturated with NaCl and extracted with AcOEt. The organic phase was combined and allowed to stand at 37° for 2 days. The extract was washed with 5% $NaHCO_3$, $H_2O$, and dried over anhydrous $Na_2SO_4$. Removal of the solvent provided the sulfate fraction. The glucuronide and sulfate fractions were combined, dissolved in MeOH and treated with $NaBH_4$ (50 mg) at room temperature for 3 hr. After decomposition of the excess reagent with AcOH the resulting solution was diluted with ether, washed with 5% $NaHCO_3$ and dried over anhydrous $Na_2SO_4$. After usual work-up the reduction product obtained was submitted to the preparative TLC using benzene–MeOH (9:1) as developing solvent. The adsorbent of the area in *Rf* 0.60 corresponding to the two isomeric 2-hydroxyestradiol monomethyl ethers was eluted with acetone. The eluate was then chromatographed on neutral $Al_2O_3$ (E. Merck AG) (10 g) and each 5 ml fraction was collected. The estrogen value was colorimetrically determined by Kober reaction[12] and at the same time radioactivity was measured with a 0.7 ml aliquot of each fraction. As was illustrated in Fig. 1, elution with 0.1% EtOH/benzene and 0.5% EtOH/benzene gave 2-methoxyestradiol and 2-hydroxyestradiol 3-methyl ether, respectively. Treatment of these isomeric monomethyl ethers with pyridine and $Ac_2O$ in the usual manner gave the diacetates, which in turn were crystallized repeatedly to the constant specific activity.

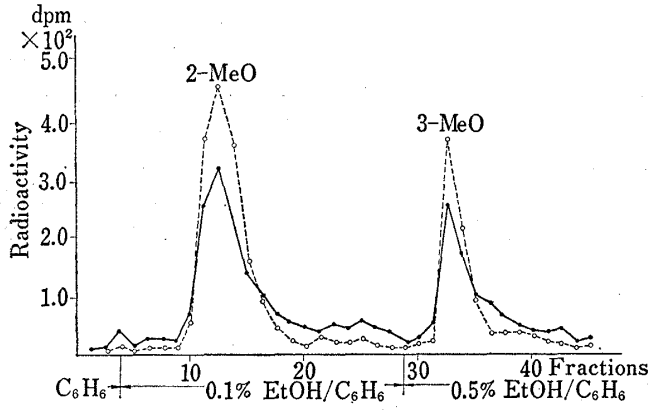


Fig. 1. Separation of 2-Hydroxyestradiol Monomethyl Ethers by Column Chromatography on Alumina

—●—: $^{3}$H ···○···: $^{14}$C

**Estradiol 3-Glucuronide Study**——A suspension of estradiol-6,7-$^{3}$H 3-glucuronide ($7.5\times10^{6}$ dpm, 51 mg) and estrone-4-$^{14}$C ($2.2\times10^{6}$ dpm, 30 mg) ($^{3}$H/$^{14}$C=3.4) with Tween 80 in saline (1.5 ml) was injected intravenously to three rats and urine was collected for 4 days after administration. Separation and estimation of the metabolites were similarly carried out without the addition of the inert material.

**2-Hydroxyestradiol 2,3-Dimethyl Ether Study**——A suspension of 2-hydroxyestradiol-6,7-$^{3}$H 2,3-dimethyl ether ($5.4\times10^{6}$ dpm, 30 mg) with Tween 80 in saline (3 ml) was injected intravenously to eight rats

9) T. Nambara, S. Honma, and S. Akiyama, *Chem. Pharm. Bull.* (Tokyo), **18**, 474 (1970).
10) J. Fishman, M. Tomasz, and R. Lehman, *J. Org. Chem.*, **25**, 585 (1960).
11) J.S. Elce, J.G.D. Carpenter and A.E. Kellie, *J. Chem. Soc.* (C), **1967**, 542.
12) J.B. Brown and H.A.F. Blair, *J. Endocrinol.*, **20**, 331 (1960).

and urine was collected for the following 4 days. In a separate run 2-hydroxyestradiol-6,7-$^{3}$H 2,3-dimethyl ether ($2.0\times10^{7}$ dpm, 9 mg) together with lactose (30 mg) as a vehicle were orally given to a normal woman (42 years). The urine was collected for 3 days after administration and processed in the manner as described above.

## Result

The excreted amounts of the radioactive metabolites derived from estrone-6,7-$^{3}$H sulfate and estrone-4-$^{14}$C for 4 days after administration were found to be both *ca.* 4%. Each monomethyl ether of 2-hydroxyestradiol was diluted with the carrier steroid, acetylated and crystallized to the constant specific activity. As was shown in Table I the bioconversion rates from estrone-6,7-$^{3}$H sulfate and estrone-4-$^{14}$C into 2-methoxyestradiol were 1.37 and 0.24%, while those into 2-hydroxyestradiol 3-methyl ether were 1.26 and 0.56%, respectively. In addition the $^{3}$H/$^{14}$C values of 2-methoxyestradiol and the isomeric 3-methyl ether were found to be 20.0 and 7.7. Comparison of the isotope ratio of the former with that of the administered steroid implies that estrone sulfate was converted into 2-methoxyestrogen to the much more extent than estrone itself.

The total urinary excretions of radioactivity for 4 days after administration of estradiol-6,7-$^{3}$H 3-glucuronide and estrone-4-$^{14}$C were 4.9 and 5.4%. Two isomeric monomethyl ethers were obtained by borohydride reduction and subsequent chromatographic separation in the manner as described with the sulfate. The $^{3}$H/$^{14}$C values of the two isomeric 2- and 3-methoxy compounds were determined to be 0.84 and 1.12, respectively (see Table II). The isotope ratio of both metabolites was somewhat smaller than that of the administered steroids.

Table I. *In Vivo* Formation of Catechol Estrogen Monomethyl Ethers from Estrone-6,7-$^{3}$H Sulfate and Estrone-4-$^{14}$C in the Rat[a,b]

| | Amount added (mg) | Specific activity (dpm/mg) | | |
|---|---|---|---|---|
| | | $^{3}$H | $^{14}$C | $^{3}$H/$^{14}$C |
| 2-Methoxy | 33.3 | 308 | 15.4 | 20.0 |
| 3-Methoxy | 37.2 | 254 | 33.0 | 7.7 |

*a*) Estrone-6,7-$^{3}$H sulfate ($9.0\times10^{7}$ dpm) and estrone-4-$^{14}$C ($1.56\times10^{7}$ dpm) ($^{3}$H/$^{14}$C=5.7) were administered.
*b*) Radioactivity of total urinary metabolites was as follows; $^{3}$H, $7.49\times10^{5}$ dpm; $^{14}$C, $2.18\times10^{5}$dpm.

Table II. *In Vivo* Formation of Catechol Estrogen Monomethyl Ethers from Estradiol-6,7-$^{3}$H 3-Glucuronide and Estrone-4-$^{14}$C in the Rat[a]

| | Radioactivity (dpm) | | |
|---|---|---|---|
| | $^{3}$H | $^{14}$C | $^{3}$H/$^{14}$C |
| 2-Methoxy | 1334 | 1590 | 0.84 |
| 3-Methoxy | 936 | 836 | 1.12 |

*a*) Estradiol-6,7-$^{3}$H 3-glucuronide ($7.5\times10^{6}$ dpm, 51 mg) and estrone-4-$^{14}$C ($2.2\times10^{6}$ dpm, 30 mg) ($^{3}$H/$^{14}$C=3.4) were administered.

The total amount of the urinary metabolites in the rat administered with 2-hydroxyestradiol-6,7-$^{3}$H 2,3-dimethyl ether was found to be 21%. The metabolites were deconjugated, reduced with sodium borohydride and then separated by the preparative TLC followed by column chromatography on alumina. A typical chromatogram of two isomeric monomethyl ethers and 2,3-dimethyl ether of 2-hydroxyestradiol was illustrated in Fig. 2. It is evident from the data listed in Table III that the 2- and 3-monomethyl ethers were

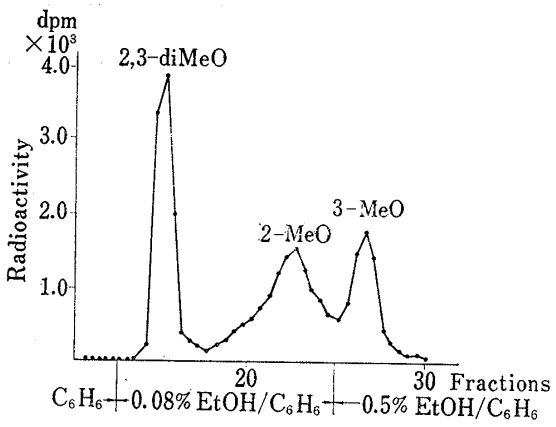


Fig. 2. Separation of *O*-Demethylated Metabolites Formed from 2-Hydroxyestrone Dimethyl Ether by Column Chromatography on Alumina

formed in a ratio of 2.5 to 1. The structures of the isomeric metabolites were characterized with a portion of these two by the reverse isotope dilution method (see Table IV).

The total radioactivity of the metabolites excreted in the human urine for 3 days after oral administration of the 2,3-dimethyl ether was 53%. The urinary metabolites were divided into the glucuronide and sulfate fractions. The radioactivity of the 2- and 3-monomethyl ethers in the glucuronide fraction was estimated to be $42.5\times10^4$ and $29.0\times10^4$ dpm, respectively. To the remaining sulfate fraction were added the 2-methyl ether (31.9 mg) and 3-methyl ether (31.5 mg) as the carrier and separation of these two isomers was performed according to the established procedure. The results on the reverse isotope dilution method are listed in Table V.

Table III. O-Demethylated Metabolites formed from 2-Hydroxyestradiol-6,7-$^3$H 2,3-Dimethyl Ether in the Rat

| | Radioactivity (dpm) | % |
|---|---|---|
| 2-Methoxy | 106050 | 43.1 |
| 3-Methoxy | 45980 | 18.7 |
| 2,3-Dimethoxy | 94260 | 38.3 |

Table IV. Identification of Metabolites formed from 2-Hydroxyestradiol-6,7-$^3$H 2,3-Dimethyl Ether by Reverse Isotope Dilution Method (Rat)[a]

| No. | Crystallized from | Specific activity (dpm/mg) | |
|---|---|---|---|
| | | 2-Methoxy | 3-Methoxy[b] |
| 1 | MeOH | 57 | 145 |
| 2 | MeOH | 48 | 119 |
| 3 | MeOH | 55 | 133 |

*a*) 2-Methoxyestradiol (28.0 mg) and 2-hydroxyestradiol 3-methyl ether (28.5 mg) were used as the carrier, respectively. *b*) Crystallization was carried out on the diacetate.

Table V. Identification of Metabolites formed from 2-Hydroxyestrone-6,7-$^3$H Dimethyl Ether by Reverse Isotope Dilution Method (Man)[a]

| No. | Crystallized from | Specific activity (dpm/mg) | |
|---|---|---|---|
| | | 2-Methoxy | 3-Methoxy[b] |
| 1 | MeOH | 13400 | 15500 |
| 2 | MeOH | 13700 | 15700 |
| 3 | MeOH | 13000 | 15700 |

*a*) 2-Methoxyestradiol (31.9 mg) and 2-hydroxyestradiol 3-methyl ether (31.5 mg) were used as the carrier, respectively. *b*) Crystallization was carried out on the diacetate.

## Discussion

First, it is to be noted that the occurrence of the 3-methyl ether in the rat urine has been unambiguously confirmed by means of chromatography and reverse isotope dilution method. The isolation of catechol estrogen 3-methyl ether from the biological fluid was hitherto unsuccessful though the extensive efforts were made.[13] The amount of the 3-methyl ether derived from estrone was approximately three times as much as that from estradiol 3-glucuronide, whereas almost the equal amount of the 3-methyl ether was formed from estrone and its sulfate. The marked difference in the bioconversion rate between these two conjugates may be ascribable to the steps involving C-2 hydroxylation and/or deconjugation. The $^{3}H/^{14}C$ value of 2-methoxyestrogen derived from estrone-4-$^{14}C$ and estradiol-6,7-$^{3}H$ 3-glucuronide demonstrates that estrone was transformed into the 2-methoxy derivative with four-fold rate as compared with the 3-glucuronide. This result implies that hydroxylation at C-2 and subsequent O-methylation would proceed after hydrolysis at the initial stage. The direct C-2 hydroxylation, if present, may take place to much smaller extent with the 3-glucuronide than with estrone itself. Our separate experiment also supports this explanation demonstrating that *in vitro* methylation of 2-hydroxyestradiol 3-glucuronide catalyzed by catechol O-methyltransferase hardly proceeded with the rat liver.[15]

The isotope ratio of the 2-methoxyestrogen formed from estrone-6,7-$^{3}H$ sulfate and estrone-4-$^{14}C$ indicates that the 2-methoxyestrogen was produced from estrone sulfate with four-fold rate as compared with estrone. Many recent works revealed that the steroid sulfate is not always the end product and in certain case serves as the more suitable substrate for the biotransformation.[16] In the present case, however, whether C-2 hydroxylation occurs with or without retention of the sulfate bond is yet unclear. Fishman, *et al.* reported that the 3-monosulfate underwent *in vitro* O-methylation at C-2 more selectively resulting in formation of the 2-methoxyestrogen.[7] Recently this finding was also confirmed by the authors.[15] In addition 2- and 3-monosulfates of 2-hydroxyestrone were isolated from the urine after administration of 2-hydroxyestrone or estrone.

In order to examine the possibility that 2-methoxyestrone may be produced from the dimethyl ether of the catechol estrogen by the selective demethylation, the metabolic fate of 2-hydroxyestradiol-6,7-$^{3}H$ 2,3-dimethyl ether was explored. In the rat the formation rate of the 2-methoxy derivative was two and a half fold as compared with that of its isomer suggesting the more facile demethylation at C-3 than at C-2. In man two isomeric monomethyl ethers were formed in almost the equal amounts with the recovery of the unchanged 2,3-dimethyl ether in *ca.* 60%. Anyhow the dimethyl ether was metabolized with the random O-demethylation to yield both monomethyl ethers. These results indicate that the proposed mechanism involving preferential demethylation is not necessarily explicable for the predominant formation of the 2-methoxyestogen.

It is hoped that further work in progress in these laboratories will provide the data necessary for the more precise knowledge on the selective O-methylation in man.

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13) During the preparation of this manuscript the occurrence of 2-hydroxyestrone 3-methyl ether as a biliary glucuronide metabolite of radioactive estrone and estradiol in the rat has been reported.[14]
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15) T. Nambara, S. Honma, and K. Kanayama, in preparation.
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17) After completion of this work the authors learned a paper dealing with the metabolism of catechol estrogen ethers in man.[18]
18) I. Yoshizawa and J. Fishman, *J. Clin. Endocrinol. Metab.*, **31**, 324 (1970).