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Studies on Steroid Conjugates. XIV. Participation of Glucuronidation in Selective O-Methylation of Catechol Estrogen in the Rat[1]

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The studies on the mechanisms involved in the selective O-methylation of the catechol estrogen in the rat have been undertaken. Both *in vitro* and *in vivo* experiments revealed that the catechol 2-glucuronide and 3-sulfate underwent O-methylation preferentially at the unconjugated phenolic group yielding the 3- and 2-monomethyl ethers, respectively. It was also demonstrated that the catechol monoglucuronide was directly transformed into the corresponding methyl ether with retention of the glycoside linkage. These results are indicative of the active participation of conjugation in the metabolic transformation of steroid hormones in the living animals.

It is now well established that 2-methoxyestrogen, the principal metabolite of female hormone in man, is formed by two separate steps involving C-2 hydroxylation and subsequent O-methylation.[3,4] This selective O-methylation for the C-2 position is of particular interest, since the chemical properties of the two phenolic groups are virtually indistinguishable. In contrast the estrogen catechol 3-methyl ether is also produced besides the isomeric 2-methyl ether in the rat, though its relative amount is not significant.[5–7] Both *in vitro* and *in vivo* experiments with the rat have previously demonstrated that the prior sulfate formation participates in the selective O-methylation of catechol estrogen.[8,9] However, any plausible explanation for the distinct species difference in O-methylation between the rat and man has not yet been available. The present paper deals with the metabolic significance of conjugation, in particular glucuronidation, in the selective O-methylation at the unconjugated phenolic group of catechol estrogen in the rat.

Experimental

Material——Estrone-6,7-^{3}H (5.95 Ci/mmole) and estrone-6,7-^{3}H sulfate (40 Ci/mmole) were purchased from Daiichi Chemical Co. (Tokyo) and purified by thin-layer chromatography (TLC) prior to use. [^{3}H-Methyl]-S-adenosylmethionine (530 mCi/mmole), [^{14}C-methyl]-S-adenosylmethionine (44.5 mCi/mmole), and [^{14}C-methyl]-5-methyltetrahydrofolic acid (60 mCi/mmole) were also obtained from Daiichi Chemical Co. and

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2) Location: *Aobayama, Sendai.*

3) S. Kraychy and T.F. Gallagher, *J. Am. Chem. Soc.*, **79**, 754 (1957); *idem*, *J. Biol. Chem.*, **229**, 519 (1957).

4) L.L. Engel, B. Bagger, and P. Carter, *Endocrinol.*, **61**, 113 (1957).

5) A. Bartke, R.E. Steele, J.G. Williams, and K.I.H. Williams, *Steroids*, **18**, 303 (1971).

6) T. Nambara, S. Honma, M. Asama, S. Akiyama, and M. Nokubo, *Chem. Pharm. Bull.* (Tokyo), **21**, 914 (1973); S. Honma and T. Nambara, *ibid.*, **22**, 687 (1974); T. Nambara, J. Ishiguro, Y. Kawarada, and H. Tajima, *ibid.*, **22**, 889 (1974).

7) H. Watanabe and J.A. Menzies, *Steroids*, **21**, 123 (1973).

8) *a*) M. Miyazaki, I. Yoshizawa, and J. Fishman, *Biochemistry*, **8**, 1669 (1969); *b*) J. Fishman, I. Yoshizawa, and L. Hellman, *Steroids*, **22**, 401 (1973).

9) T. Nambara, S. Honma, and K. Kanayama, *Chem. Pharm. Bull.* (Tokyo), **20**, 2235 (1972).

used without further purification. 2-Hydroxyestrone,[10] 2-methoxyestrone,[11] 2-hydroxyestrone 3-methyl ether,[10] estradiol 3-glucuronide,[12] 2-hydroxyestrone 2-glucuronide,[13] 2-hydroxyestradiol 3-sulfate,[14] and 2-hydroxyestradiol 3-glucuronide[15] were prepared in these laboratories. 2-Hydroxyestrone-6,7-^{3}H 2-glucuronide was separated from rat bile after administration of estrone-6,7-^{3}H and purified repeatedly up to constant specific activity (2.18 mCi/mmole).

Radioactivity Counting——Counting was carried out on a Aloka Model LSC-651 liquid scintillation counter. A solution of 2,5-diphenyloxazole (4 g) and 1,4-bis[2-(5-phenyloxazolyl)]benzene (400 mg) dissolved in toluene to make the whole volume 1 liter was used as a scintillant.

Enzyme Preparation——Male Wistar rats weighing 250 g on the average, were decapitated and the liver was homogenized in ice-cold 0.25M sucrose solution. The homogenate was centrifuged at $1500 \times g$ for 20 min and the supernatant was used for the incubation study.

***In Vitro* Study**——The incubations were carried out in a medium consisting of estrogen substrate (200 μg), liver homogenate (5 ml), [^{3}H- or ^{14}C-methyl]-S-adenosylmethionine, $MgSO_4$ (3 mg), and sufficient 0.1M phosphate buffer (pH 7.5) to make the whole volume 8 ml. The mixture was incubated in air at 37° for 90 min and the reaction was terminated by cooling in an ice bath. The sulfate was solvolyzed in the usual manner at 37° for 18 hr.[16] The glucuronide was hydrolyzed with beef-liver β-glucuronidase (Tokyo Zōki-kagaku Co.) (20000 Fishman units) in 0.1M acetate buffer (pH 4.2) and the incubation mixture was deproteinized by treatment with EtOH at −20° for 4 hr and then centrifuged. The supernatant was separated and evaporated *in vacuo*. To a mixture of the free steroids thus obtained were added the two isomeric monomethyl ethers of 2-hydroxyestrone as a carrier and extracted with ether (70 ml×2). The organic layer was washed with H_2O, dried over anhydrous Na_2SO_4, and evaporated. The residue was dissolved in MeOH and reduced with $NaBH_4$ in the usual manner. After addition of AcOH to decompose the excess reagent the resulting solution was extracted with ether. The organic layer was washed with H_2O, dried over anhydrous Na_2SO_4, and evaporated. The residue was submitted to column chromatography on Al_2O_3 (E. Merck AG, Darmstadt) (2 g). Elution with 0.5% EtOH/benzene (50 ml) and 1% EtOH/benzene gave 2-methoxyestradiol and the isomeric 3-methyl ether, respectively. Each 5 ml fraction was divided into two portions and used for radioactivity counting and colorimetric determination by Kober reaction, respectively. When a larger amount of the carrier was employed, the two isomers were separated by preparative TLC on multiple runs using $CHCl_3$ as developing solvent.

***In Vivo* Study**——Male Wistar rats weighing 300—350 g were anesthesized with ether, cannulated to the bile duct with polyethylene tube (PE 50, Clay Adams, Parsippany, N.J.) by surgical operation, and housed in a Bollman cage for collection of bile. Estrone-6,7-^{3}H (2.4 μCi, 0.27 mg), estrone-6,7-^{3}H sulfate (3.8 μCi, 0.37 mg), 2-hydroxyestrone (5.0 mg), its 2-glucuronide-6,7-^{3}H (2 μCi, 0.466 mg) and 3-sulfate (3.0 mg) were intravenously administered, respectively. The pooled bile was passed through a column packed with Amberlite XAD-2 resin, washed with distilled water, and eluted with MeOH. The eluate was adjusted to pH 4.2 with 0.1M acetate buffer and incubated with β-glucuronidase (200000 Fishman units) at 37° for 40 hr. The hydrolyzate was then brough to 2N H_2SO_4 solution, saturated with NaCl, and extracted with AcOEt. The organic phase was allowed to stand at 37° for 48 hr and removal of the solvent provided the sulfate fraction. The glucuronide and sulfate fractions were combined, dissolved in MeOH, and treated with $NaBH_4$ in the manner as described in the incubation study. After usual work-up the reduction product obtained was chromatographed on Al_2O_3 and the isomeric monomethyl ethers of 2-hydroxyestradiol were separated. The estrogen value was colorimetrically determined and at the same time radioactivity was counted with an aliquot of each fraction.

Characterization of 2-Hydroxyestrone Methyl Ether Glucuronide——The incubations were carried out in air at 37° for 90 min in a medium consisting of 2-hydroxyestrone monoglucuronide (1.8 mg), liver homogenate (20 ml), [^{3}H-methyl]-S-adenosylmethionine (15 μCi), $MgCl_2$ (6 mg), and sufficient 0.1M phosphate buffer (pH 7.5) to make the whole volume 24 ml. To this incubated mixture was added 6-fold volume of EtOH, allowed to stand at −20° overnight, and centrifuged. The supernatant was evaporated *in vacuo* and the residue obtained was chromatographed repeatedly on Sephadex LH-20 using 60% MeOH/0.2M AcOH as solvent. The eluate showed a radioactive spot on TLC ($CHCl_3$/iso-PrOH/HCOOH (12: 7: 0.7)) exhibiting a positive test with naphthoresorcinol and *Rf* value identical with that of the authentic sample. Three-fourth portion of the eluate dissolved in MeOH (5 ml) was treated with CH_2N_2 for 20 min and then with Ac_2O (0.5 ml)–pyridine (0.5 ml) overnight at room temperature. After addition of the carrier the acetate-methyl ester was crystallized repeatedly to constant specific activity. The remaining portion of the conjugate was dis-

10) J. Fishman, M. Tomasz, and R. Lehman, *J. Org. Chem.*, **25**, 585 (1960).
11) T. Nambara, S. Honma, and S. Akiyama, *Chem. Pharm. Bull.* (Tokyo), **18**, 474 (1970).
12) J.S. Elce, J.G.D. Carpenter, and A.E. Kellie, *J. Chem. Soc.* (*C*), **1967**, 542.
13) T. Nambara and S. Honma, to be published.
14) M. Miyazaki and J. Fishman, *J. Org. Chem.*, **33**, 682 (1968).
15) T. Nambara and S. Honma, *Chem. Pharm. Bull.* (Tokyo), **18**, 1191 (1970).
16) S. Burstein and S. Lieberman, *J. Biol. Chem.*, **233**, 331 (1958).

solved in H_2O (10 ml)–0.1 M acetate buffer (pH 4.2) (1 ml) and incubated with β-glucuronidase at 37° for 48 hr. After addition of the carrier to this incubated mixture 2-hydroxyestrone monomethyl ethers were extracted, separated, and crystallized repeatedly to constant specific activity.

Results

First, O-methylation of catechol estrogen with the rat liver homogenate in the presence of S-adenosylmethionine was examined on a variety of substrates. The 17-keto and 17β-hydroxylic steroids were indiscriminately employed as substrates, since it seems likely that the extent of catechol O-methylation is not dependent upon the oxygen function at C-17. The amounts of catechol estrogen 2- and 3-monomethyl ethers yielded were determined by hydrolytic cleavage of the conjugate, borohydride reduction of the 17-ketone of the liberated estrogen, chromatographic separation, and then radioactivity counting.

The results on 2-hydroxyestradiol, its 3-sulfate, and estrone sulfate were listed in Table I. As it has already been reported[17] the free catechol was randomly methylated yielding two isomeric monomethyl ethers in nearly equal quantities. In contrast the catechol 3-sulfate underwent O-methylation selectively at the unesterified phenolic group providing the 2- and 3-monomethyl ethers in a ratio of *ca.* 7 to 1. Estrone sulfate gave the methylated products in a similar proportion indicating the sequential bioconversion involving C-2 hydroxylation followed by O-methylation.

Table I. *In Vitro* Formation of Catechol Monomethyl Ethers from Estrogen Sulfates[a]

Substrate	2-Methoxy (dpm)	3-Methoxy (dpm)	Ratio 2-methoxy/3-methoxy
2-Hydroxyestradiol	10000	14300	0.7
2-Hydroxyestradiol 3-sulfate	15800	2200	7.2
Estrone sulfate	8300	1200	6.9

a) All incubations were carried out using substrate (200 μg) and [^{14}C-methyl]-S-adenosylmethionine (4.4×10^5 dpm).

Table II. *In Vitro* Formation of Catechol Monomethyl Ethers from Estrogen Glucuronides[a]

Substrate	2-Methoxy (dpm)	3-Methoxy (dpm)	Ratio 2-methoxy/3-methoxy
2-Hydroxyestrone 2-glucuronide	4900	3200	1.5
2-Hydroxyestradiol 3-glucuronide	60000	19000	3.2
Estrone	212000	30000	7.1
Estradiol 3-glucuronide	95000	2700	3.5

a) All incubations were carried out using substrate (200 μg) and [^{3}H-methyl]-S-adenosylmethionine (2.2×10^6 dpm).

The amounts of catechol estrogen monomethyl ethers formed from the glucuronides were collected in Table II. The less incorporation of the labeled methyl group into estradiol 3-glucuronide than into estrone would be ascribable to the necessity of deconjugation prior to hydroxylation at C-2. O-Methylation did take place with the catechol glucuronide, though

17) R. Knuppen, H. Breuer, and G. Pangels, *Z. Physiol. Chem.*, 324, 108 (1961); R. Knuppen and H. Breuer, *ibid.*, 346, 114 (1966).

its extent was less than that of the free estrogen. It was hereby suggested that the catechol estrogen glucuronide would undergo O-methylation without loss of the sugar moiety.

In order to clarify the possible occurrence of directive O-methylation the characterization of the methylated product was then undertaken. The structure of the biotransformation product of 2-hydroxyestrone 2-glucuronide was unequivocally characterized by leading to the acetate-methyl ester and subsequent reverse isotope dilution analysis (see Table III). In addition hydrolysis with β-glucuronidase provided 2-hydroxyestrone 3-methyl ether, which was unambiguously identified by the isotope dilution method (see Table IV).

Table III. Identification of 2-Hydroxyestrone 2-Glucuronide 3-Methyl Ether formed from 2-Hydroxyestrone 2-Glucuronide by Reverse Isotope Dilution Method[a)]

Crystallization No.	From	Specific activity (dpm/mg)
1	MeOH	141800
2	MeOH	163300
3	MeOH	164100
4	MeOH	160800

a) Methyl (3-methoxy-17-oxoestra-1,3,5(10)-trien-2-yl-2,3,4-tri-O-acetyl-β-D-glucopyranosid) uronate (14.4 mg) was used as a carrier.

Table IV. Identification of 2-Hydroxyestrone 3-Methyl Ether derived from Its 2-Glucuronide by Reverse Isotope Dilution Method[a)]

Crystallization No.	From	Specific activity (dpm/mg)
1	benzene–hexane	95100
2	benzene–hexane	102000
3	benzene–hexane	98800
4	MeOH	100500

a) 2-Hydroxyestradiol 3-methyl ether (15.2 mg) was used as a carrier.

Table V. Identification of 2-Methoxyestrone derived from Its 3-Glucuronide by Reverse Isotope Dilution Method[a)]

Crystallization No.	From	Specific activity (dpm/mg)
1	MeOH	84000
2	MeOH	81000
3	MeOH	81000

a) 2-Methoxyestrone (10.5 mg) was used as a carrier.

In a similar fashion the definite evidences for directive O-methylation have been obtained with the isomeric catechol 3-glucuronide by enzymatic hydrolysis and reverse isotope dilution analysis of the hydrolyzate (see Table V). Retention of the glucuronoside linkage in the methylated product was also demonstrated by leading to the acetate-methyl ester followed by the reverse dilution method as listed in Table VI.

A recent finding on O-methylation of biogenic amines[18)] prompted us to examine a possible role of 5-methyltetrahydrofolic acid as a methyl donor in the formation of methoxyestrogens. Incubation of 2-hydroxyestrone with the rat liver homogenate in the presence of the methyl

18) S.P. Banerjee and S.H. Snyder, *Science*, **182**, 74 (1973).

Table VI. Identification of 2-Methoxyestrone 3-Glucuronide formed from 2-Hydroxyestrone 3-Glucuronide by Reverse Isotope Dilution Method[a)]

Crystallization No.	From	Specific activity (dpm/mg)
1	MeOH	179000
2	MeOH	162000
3	MeOH	133000
4	MeOH	137000
5	MeOH	136000

a) Methyl (2-methoxy-17-oxoestra-1,3,5(10)-trien-3-yl-2,3,4-tri-O-acetyl-β-D-glucopyranosid)uronate (10.0 mg) was used as a carrier.

donor was carried out and the yielded amounts of the two isomeric monomethyl ethers were determined. It is evident from the data in Table VII that any incorporation of the labeled methyl group was not attained with 5-methyltetrahydrofolic acid, while significant amounts of the methyl ethers were formed with S-adenosylmethionine.

The *in vivo* study on O-methylation of the catechol estrogen conjugate was then undertaken with the fistula rat. The excreted amounts of radioactivity in bile after administration of 2-hydroxyestrone-6,7-^{3}H 2-glucuronide, estrone-6,7-^{3}H, and estrone-6,7-^{3}H 3-sulfate were determined. As illustrated in Fig. 1 the biliary elimination constant of the glucuronide (k=0.029 min^{-1}) was found to be much higher than those of the sulfate (k=0.011 min^{-1}) and

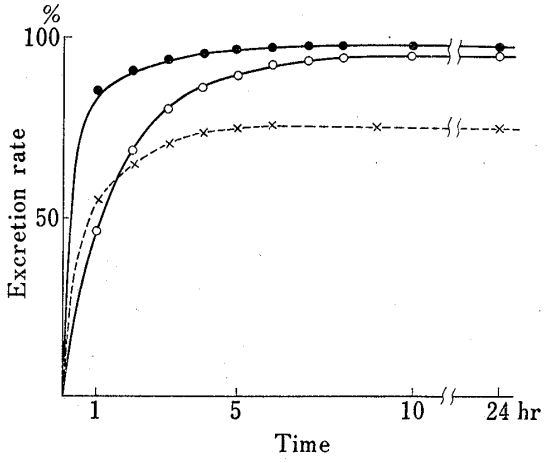

Fig. 1. Cumulative Biliary Excretion Rate of Radioactivity following Administration of Estrogens

2-OHE$_1$ 2-G, 2-hydroxyestrone 2-glucuronide; E$_1$, estrone; E$_1$ 3-S, estrone 3-sulfate
—●—: 2-OHE$_1$ 2-G —○—: E$_1$ --×--: E$_1$ 3-S

Table VII. *In Vitro* Formation of Catechol Estrogen Monomethyl Ethers with S-Adenosylmethionine and 5-Methyltetrahydrofolic Acid[a)]

Methyl donor	2-Methoxy		3-Methoxy	
	dpm	%	dpm	%
S-Adenosylmethionine	189×10^4	35	107×10^4	19
	237	45	106	19
5-Methyltetrahydrofolic acid	1.00	0.60	0.02	0.01
	0.06	0.03	0.10	0.06

a) [^{3}H-Methyl]-S-adenosylmethionine (5.05×10^{6}dpm) and [^{14}C-methyl]-5-methyltetrahydrofolic acid (2.20×10^5 dpm) were used.

free estrogen (k=0.014 min^{-1}). It is of interest that the conjugate form was distinctly reflected on the excretion rate. The quantities of catechol estrogen monomethyl ethers excreted in bile after administration of each substrate were then determined. The results hereby obtained are collected in Table VIII. The ratios of the isomeric monomethyl ethers formed from the various substrates were nearly close to those observed by the *in vitro* experiment except the case of 2-hydroxyestrone.

Table VIII. Excretion of Catechol Estrogen Monomethyl Ethers in Bile[a]

Substrate	2-Methoxy (%)	3-Methoxy (%)	Ratio 2-methoxy/2-methoxy
2-Hydroxyestrone	9.40	2.80	3.4
2-Hydroxyestradiol 3-sulfate	6.20	0.50	12.0
2-Hydroxyestrone 2-glucuronide	0.20	0.15	1.3
Estrone	4.70	0.70	6.7
Estrone sulfate	4.20	0.70	6.0

a) Expressed as per cent of dose administered intravenously to the fistula rat.

Discussion

In vivo O-methylation occurs selectively at the C-2 phenolic function in man, even though a very small amount of the 3-methyl ether is excreted in pregnancy urine.[19] In contrast *in vitro* O-methylation of 2-hydroxyestrone with human and rat liver is indiscriminate yielding a mixture of the two isomeric monomethyl ethers in approximately equal amounts.[17] These divergent results have recently been explained in terms of the active participation of the sulfate in the directive O-methylation.[8,9] In actuality it has been ascertained that the catechol 3-sulfate is methylated preferentially at the unesterified phenolic group. The selective O-methylation at the C-2 hydroxyl group was similarly observed with estrone sulfate as with the catechol 3-sulfate. This result has been further confirmed by the *in vivo* study in the rat. It is evident from these findings that in the living animals the 3-sulfate would play an important role in the formation of 2-methoxyestrogen, though the occurrence of the catechol 3-sulfate in blood still remains unclear. Recently, Fishman, *et al.* demonstrated by means of the double-isotope technique that in man the metabolic transformation proceeds *via* the sequence: estrone →estrone sulfate→2-hydroxyestrone 3-sulfate→2-methoxyestrone.[8b]

With regard to the formation of the isomeric 3-methyl ether there has been observed a distinct species difference between the rat and man. It has previously been reported that uridine diphosphate-glucuronyltransferase is highly specific for the C-3 phenolic function with the catechol substrate yielding solely the 3-glucuronide in man.[20] In contrast catechol estrogen 2-glucuronide is exclusively produced in the rat as well as in the hamster and guinea pig.[21] Therefore it seems unlikely that catechol estrogen 3-glucuronide would be involved in the selective O-methylation at C-2 in the rat. The structure of the methylated product derived from the catechol monoglucuronide was unequivocally characterized by leading to the acetate-methyl ester and hydrolysis with β-glucuronidase. The catechol estrogen glucuronide underwent evidently O-methylation at the unconjugated phenolic group to afford the monomethyl ether with retention of the conjugate. The ratio of two isomeric methyl ethers in bile formed from 2-hydroxyestrone 2-glucuronide was found to be 1.3, while those derived from 2-hydroxyestrone and estrone were 3.4 and 6.7, respectively. These data strongly support the assumption that one of the principal metabolites, 2-hydroxyestrone 2-glucuronide, would undergo *in vivo* O-methylation at C-3 with retention of the glycoside linkage and in another word the prior glucuronidation at C-2 would be associated with the formation of the 3-methyl ether in the rat. The result is of particular interest in suggesting the active participation of the glucuronide in metabolic transformation of steroids.

19) R. Kunppen, O. Haupt, and H. Breuer, *Biochem. J.*, **128**, 1369 (1972).
20) I. Yoshizawa and J. Fishman, *J. Clin. Endocrinol. Metab.*, **29**, 1123 (1969).
21) K.I.H. Williams, *Steroids*, **15**, 105 (1970); I. Yoshizawa, K. Fujimori, and M. Kimura, *Chem. Pharm. Bull.* (Tokyo), **19**, 2431 (1971).

It has recently been demonstrated that 5-methyltetrahydrofolic acid is capable of being a natural methyl donor in O- and N-methylation of biogenic amines.[18] In the formation of methoxyestrogen, however, there has been obtained no evidence for the participation of this methyl donor. Whether the enzyme system involved in transmethylation of S-adenosylmethionine toward the catechol monoglucuronide is identical with catechol O-methyltransferase (COMT) or not will be a subject to be explored in the future.

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