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METABOLISM OF 2-HYDROXY-3-DEOXYESTRADIOL BY RAT LIVER MICROSOMES*

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Abstract—The biotransformation of [6,7-^{3}H]2-hydroxy-3-deoxyestradiol, which is a positional isomer of the naturally occurring estrogen, has been investigated with rat liver microsomes. 2-Hydroxyestradiol separated from the lipophilic fraction of the incubation mixture was unequivocally characterized by means of thin-layer chromatography, gas chromatography–mass spectrometry, and the reverse isotope dilution method. The formation of glutathione 1- and 4-thioethers of 2-hydroxyestradiol as water-soluble metabolites was also confirmed by chromatographic properties and coloration tests, followed by reverse isotope dilution analysis of the aglycone produced by desulfurization with Raney nickel. Incubation of [3-^{2}H]2-hydroxy-3-deoxyestradiol with rat liver microsomes yielded 2-hydroxyestradiol without any retention of the label, indicating that C-3 hydroxylation proceeded through a quinoid intermediate. When a 1 to 1 mixture of 3-deuterated and nonlabeled 2-hydroxy-3-deoxyestradiols was incubated, no substantial change was seen in the deuterium content with the recovered substrate, which implied the absence of the isotope effect in C-3 hydroxylation.

In the preceding papers of this series, the biotransformation of 3-deoxyestrone,† which is used as a lipid-shifting drug [1], has been studied in rabbits [2] and rats [3, 4]. Hydroxylation occurs at C-2 and C-3 in the aromatic ring, resulting in the formation of 2-hydroxy-3-deoxyestrone and estrone as the main metabolites. 2-Hydroxy-3-deoxyestradiol (1) (Fig. 1), which is a positional isomer of the endogenous female hormone, does not exhibit any significant estrogenic activity [5]. 2-Hydroxyestradiol, a major metabolite of female hormone in humans and rats [6–13], is further transformed into the glutathione conjugate and protein-bound metabolites in the rat [12–17]. It appears to be of interest to learn whether the close similarity in the structural feature of this modified steroid to estrogen will be associated with their metabolic fates. The present paper deals with the biotransformation *in vitro* of 2-hydroxy-3-deoxyestradiol by rat liver.

MATERIALS AND METHODS

Animals. Male Wistar rats (10- to 14-weeks-old) weighing 200–250 g were used.

Materials. NADPH and glutathione were purchased from Sigma Chemical Co. (St. Louis, Mo.), Amberlite XAD-2 resin from Rohm & Haas (Philadelphia, Pa.), Soluene 100 from Packard Instrument Co. (Downers Grove, IL), silica gel H and G from E. Merck AG (Darmstadt, West Germany), cellulose powder from Asahi Kasei Kogyo Co. (Tokyo, Japan), and [6,7-^{3}H]estradiol (48 Ci/m-mole) from the Radiochemical Centre (Amersham, England) respectively. [6,7-^{3}H]2-hydroxy-3-deoxyestradiol (10.8 μCi/m-mole) was synthesized chemically from [6,7-^{3}H]estradiol by the methods developed in these laboratories (total yield 28 per cent) [18, 19], and the radiochemical purity was checked by thin-layer chromatography (t.l.c.) prior to use. 2-Hydroxyestradiol was prepared in the manner described by Gelbke *et al.* [20]. 2-Hydroxyestradiol 1-SG and 4-SG were synthesized according to the procedure of Kuss [12, 13].

Synthesis of [3-^{2}H]2-hydroxy-3-deoxyestradiol. To a solution of 2-hydroxy-3-deoxyestradiol (500 mg) in glacial acetic acid (20 ml) a solution of $Hg(OAc)_2$ (375 mg) in glacial acetic acid (20 ml) saturated with I_2 (580 mg) was added dropwise over a period of 5 min and stirred at 45–55° for 2 hr. After removal of the precipitate by filtration the filtrate was poured

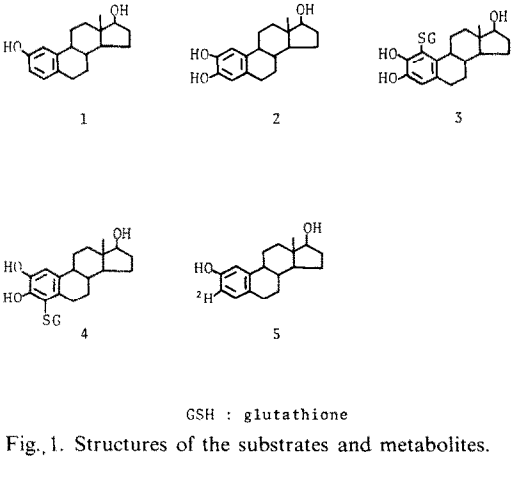

Fig. 1. Structures of the substrates and metabolites.

* Part CXVIII of "Studies on Steroids" by T. Nambara.

† The following trivial names are used in the text: 3-deoxyestrone, estra-1,3,5(10)-trien-17-one; estrone, 3-hydroxyestra-1,3,5(10)-trien-17-one; estradiol, estra-1,3,5(10)-triene-3,17β-diol; 2-hydroxy-3-deoxyestrone, 2-hydroxyestra-1,3,5(10)-trien-17-one; 2-hydroxy-3-deoxyestradiol, estra-1,3,5(10)-triene-2,17β-diol; 2-hydroxyestradiol, estra-1,3,5(10)-triene-2,3,17β-triol; 2-hydroxyestradiol 1-SG, S-(2,3,17β-trihydroxyestra-1,3,5(10)-trien-1-yl)-glutathione; 2-hydroxyestradiol 4-SG, S-(2,3,17β-trihydroxyestra-1,3,5(10)-trien-4-yl)-glutathione.

into 10% $Na_2S_2O_3$ solution. The precipitate was collected by filtration, washed with H_2O, and dried. The crude product obtained was dissolved in hexane and chromatographed on silica gel. Elution with hexane ethyl acetate and recrystallization of the eluate from ethyl acetate gave 2-hydroxy-3-iodo-3-deoxyestradiol (131 mg) as colorless prisms (m.p. 177–82°), $[\alpha]_D^{13}$ + 71.8° (c = 0.11 in methanol). Anal. Calcd. for $C_{18}H_{23}O_2I$: C, 54.28; H, 5.82. Found: C, 54.51, 54.28; H, 5.73, 5.77. NMR (0.5 per cent solution in $CDCl_3$) δ: 0.78 (3H, s, 18-CH_3), 3.70 (1H, t, J = 8 Hz, 17α-H), 6.88 (1H, s, 1-H), 7.30 (1H, s, 4-H).

To a solution of 2-hydroxy-3-iodo-3-deoxyestradiol (30 mg) in CH_3OD (2 ml) was added $PdCl_2$ (30 mg) and stirred at 0° under a stream of nitrogen gas. To this solution $NaBD_4$ (12 mg) was added in portions and stirred for 30 min. The reaction mixture was poured into 1% HCl and extracted with ether. The organic layer was washed with H_2O, dried over anhydrous Na_2SO_4, and evaporated. Recrystallization of the crude product from methanol gave [3-^{2}H]2-hydroxy-3-deoxyestradiol (15 mg) as colorless plates (m.p. 220–23°). Mixed melting point on admixture with the nonlabeled authentic sample showed no depression. Mass spectrum *m/e*: 273 (M^+) (84.2% d_1).

Thin-layer chromatography. Thin-layer chromatography was carried out on a plate coated with a layer (0.25 mm thick) of Silica gel H impregnated with ascorbic acid [21], silica gel G, and cellulose employing the following systems: TL-I hexane–ethyl acetate (1:1, v/v); TL-II benzene ether (1:1, v/v); TL-III 0.2 N acetic acid 0.1 N NH_4OH (14.7:20, v/v); TL-IV *n*-butanol–acetic acid–water (4:1:1, v/v), and the R_f value was given. The distribution of radioactivity on the chromatogram was determined by scraping off 0.5-cm-wide zones and counting after suspension of the silica gel in scintillator liquid.

Gas liquid chromatography (g.l.c.). The apparatus used was a Shimadzu model GC-3BF gas chromatograph equipped with a hydrogen flame ionization detector. A siliconized glass tube (3 mm i.d.) packed with 3% SE-30 (2 m) or 1.5% OV-1 (3 m) on Chromosorb W (60–80 mesh) was employed. The temperature of the column and detector was 230°, while the injection chamber was kept at 260°. Nitrogen gas was used as a carrier at a flow rate of 60 ml/min. The test samples were treated with trimethylchlorosilane and hexamethyldisilazane according to the method of Sweeley *et al.* [22] and the resulting trimethylsilyl derivatives were injected into the gas chromatograph.

Gas chromatography–mass spectrometry (GC–MS). Mass spectra were recorded on a Shimadzu model LKB-9000S gas chromatograph mass spectrometer. The operating conditions were as follows: injection port temperature 290°, column temperature 270°, separator temperature 260°, ion source temperature 200°, carrier gas (He) flow rate 40 ml/min, ionization energy 70 eV, and ionization current 75 μA.

Radioactivity counting. Counting was carried out on a Packard Tri-Carb model 3380 liquid scintillation spectrometer employing the Bray's scintillator [23]. The quenching was corrected by the external standard method.

Preparation of microsomes. The rats were sacrificed by decapitation, and liver was immediately removed and chilled on ice. All the subsequent procedures were carried out at 0–4°. The tissue was weighed, finely minced with scissors, homogenized with a 4-fold volume of an ice-cold 1.15% KCl solution by a Teflon glass Potter Elvehjem homogenizer, and centrifuged at 10,000 *g* for 30 min. The supernatant was carefully transferred and centrifuged at 105,000 *g* for 60 min by a Hitachi model 40P ultracentrifuge. The microsomal pellet separated was washed twice with 1.15% KCl for removal of endogenous glutathione and other soluble sulfhydryl compounds and then gently resuspended in 1.15% KCl in such a way that 1 ml was equivalent to 400 mg wet weight of liver. Microsomal protein was determined by the method of Lowry *et al.* [24] using bovine serum albumin as a reference. One g liver was found to be equivalent to 10–12 mg protein.

Enzyme assay. The incubation studies were undertaken with the following two systems: (1) a microsomal preparation (0.5 ml), NADPH (4 μmoles), glutathione (0.4 μmole), [6,7-^{3}H]2-hydroxy-3-deoxyestradiol or [6,7-^{3}H]estradiol (0.2 μmole) dissolved in 50% aqueous methanol (0.1 ml), and sufficient 0.05 M Tris–HCl buffer (pH 7.4) to make the total volume 1.3 ml; and (2) a microsomal preparation (5 ml), NADPH (40 μmoles), [3-^{2}H]2-hydroxy-3-deoxyestradiol or nonlabeled 2-hydroxy-3-deoxyestradiol (2 μmoles) dissolved in ethanol (0.18 ml), and sufficient 0.05 M Tris HCl buffer (pH 7.4) to make the total volume 10.2 ml. Incubations were carried out for 40 min at 37° under aerobic conditions. After addition of 1 N HCl (2 ml) and ascorbic acid (2 mg) the incubation mixture was brought to 10% trichloroacetic acid solution and centrifuged at 3000 *g* for 20 min to remove the protein-bound metabolites. The sediment obtained was washed with 10% trichloroacetic acid, ethanol and ethyl acetate, successively. The precipitate was dissolved in Soluene 100 and submitted to the radioactivity counting. The supernatant was then extracted with ethyl acetate (5 ml × 3). The organic layer was combined and evaporated to dryness *in vacuo*, and the residue obtained was dissolved in methanol (lipophilic fraction). In the case of the experiment using the ^{3}H-labeled substrate the remaining aqueous phase was adjusted to pH 5.0 with 2 N NH_4OH, gently poured onto a column packed with Amberlite XAD-2 resin (30 ml) and percolated. The column was washed with distilled water (100 ml) and then eluted with methanol (50 ml). The methanol fraction was evaporated *in vacuo* below 50° and the residue was dissolved in 0.5 N acetic acid (water-soluble fraction).

Table 1. Reverse isotope dilution analysis of 2-hydroxyestradiol formed from 2-hydroxy-3-deoxyestradiol by rat liver microsomes

No.	Crystallization* from	Sp. act.† (dis/min/mg)
1	Aqueous ethanol	23,200
2	Methanol	22,800
3	Methanol	22,700
4	Aqueous methanol	22,900

* Nonradioactive 2-hydroxyestradiol (25 mg) was added as a carrier.

† Calculated: 23,800 dis/min/mg.

Table 2. Reverse isotope dilution analysis of 2-hydroxyestradiol formed from water-soluble metabolites by desulfurization with Raney nickel

No.	Crystallization* from	Sp. act.† (dis/min/mg)
1	Methanol	1450
2	Aqueous methanol	1400
3	Aqueous methanol	1380

* Nonradioactive 2-hydroxyestradiol (20 mg) was added as a carrier.
† Calculated: 1650 dis/min/mg.

Desulfurization of glutathione conjugates with Raney nickel. To a solution of the glutathione conjugate (1.5×10^5 dis/min) in 0.5 N acetic acid, 2-hydroxyestradiol (2 mg) and Raney nickel W-6 [25] (100 mg) were added and the mixture was stirred at 4° for 3 days under a stream of nitrogen gas. The resulting solution was saturated with NaCl and then extracted with ethyl acetate (5 ml × 3). The organic layer was washed with water, dried over anhydrous Na_2SO_4, and evaporated to dryness under a stream of nitrogen gas. A portion of the residue was chromatographed on a thin-layer plate of silica gel H together with 2-hydroxyestradiol as a carrier.

RESULTS

[6,7-^{3}H]2-hydroxy-3-deoxyestradiol was incubated in the air with rat liver microsomes, and the ethyl acetate-soluble fraction (lipophilic fraction) was separated. The results of t.l.c. indicated the formation of the more polar metabolite, whose chromatographic behaviors were identical with those of 2-hydroxyestradiol (2) (TL-I 0.30; TL-II 0.42). The remaining portion of this fraction was applied to preparative t.l.c., and the area corresponding to 2-hydroxyestradiol was scraped off and eluted with ethyl acetate. To the eluate was added 2-hydroxyestradiol as a carrier and it was crystallized repeatedly to constant specific activity (Table 1). These results indicated that 2-hydroxy-3-deoxyestradiol underwent hydroxylation at C-3 to form catechol estrogen (ca. 13 per cent of the total radioactivity). For the definite structure elucidation the nonlabeled substrate was incubated and the more polar metabolite was isolated in the manner described above. The trimethylsilylated derivative was analyzed by g.l.c. on two stationary phases, 3% SE-30 and 1.5% OV-1, as well as by GC–MS. On g.l.c., the silyl ether exhibited retention values identical to those of 2-hydroxyestradiol (t_R 9.8 min on 3% SE-30; 12.3 min on 1.5% OV-1). In the mass spectra, the metabolite and authentic sample exhibited an identical fragmentation pattern with a molecular ion peak at *m/e* 502.

A portion of the water-soluble fraction which was equivalent to ca. 25 per cent of the total radioactivity was submitted to t.l.c. on silica gel G. The metabolites exhibited a single spot (TL-IV 0.65) of radioactivity, purple coloration with ninhydrin, and a positive test with potassium dichromate–silver nitrate reagent [26]. The R_f value was found to be identical to that of the synthetic 2-hydroxyestradiol 1- or 4-SG conjugate (3, 4). Being applied to t.l.c. on cellulose powder using solvent system TL-III, the metabolites were divided into two radioactive peaks of roughly equal height corresponding to 2-hydroxyestradiol 1- and 4-SG conjugates (R_f values of 0.62 and 0.45). The steroid aglycone formed from these metabolites by desulfurization with Raney nickel seemed to be 2-hydroxyestradiol as judged from the chromatographic behaviors on the thin-layer plate (TL-I 0.30; TL-II 0.42). In actuality, the structure of the steroidal moiety was unequivocally characterized by reverse isotope dilution analysis as listed in Table 2. Any detectable amounts of the water-soluble metabolites were not produced, unless glutathione was added prior to incubation. These results lend support to the argument assigning the structures 2-hydroxyestradiol 1- and 4-SG to the water-soluble metabolites.

In the protein-bound fraction, ca. 7 per cent of the total radioactivity remained even after thorough washing with ethanol and then with ethyl acetate. The result implied that the metabolites in this fraction would possibly be bound with protein through a covalent bond. The amounts of these three metabolites formed from 2-hydroxy-3-deoxyestradiol were much less than those from estradiol under identical conditions (Table 3).

In order to elucidate the mechanism of C-3 hydroxylation, [3-^{2}H]2-hydroxy-3-deoxyestradiol (5) was necessary as a substrate for the incubation study. The preparation of the desired compound was undertaken by reductive dehalogenation with the deuterated reagent. When 2-hydroxy-3-deoxyestradiol was treated with iodine in the presence of mercuric acetate as a catalyst, halogenation occurred preferentially at C-3, yielding 2-hydroxy-3-iodo-3-deoxyestradiol as a sole product. The orientation of an introduced halogen was confirmed by inspection of the aromatic ring proton signal in the nuclear magnetic resonance spectra. An initial attempt for dehalogenation with

Table 3. Conversion of [6,7-^{3}H]2-hydroxy-3-deoxyestradiol and [6,7-^{3}H]estradiol to 2-hydroxyestradiol, water-soluble and protein-bound metabolites by rat microsomes

Substrate	Metabolite* 2-Hydroxyestradiol	Water-soluble	Protein-bound
2-Hydroxy-3-deoxyestradiol	13 ± 1.8	25 ± 2.6	7 ± 1.7
Estradiol	24 ± 2.2	28 ± 3.0	14 ± 2.4

* Recovery of radioactivity of the combined organic, aqueous and protein phase was approximately 100 per cent. The figures express per cent of total radioactivity (mean ± S. E.).

lithium aluminum deuteride resulted in failure, since the 2-hydroxy-3-deoxyestradiol yielded showed no significant incorporation of deuterium. Recently, it was disclosed that a deuterium atom can be introduced into an aromatic system by treatment of the appropriate aryl halide with sodium borodeuteride in the presence of transition metal as a catalyst [27]. In actuality, 2-hydroxy-3-iodo-3-deoxyestradiol underwent reduction with sodium borodeuteride–palladium chloride, yielding [3-^{2}H]2-hydroxy-3-deoxyestradiol with satisfactory isotopic purity.

Incubation of [3-^{2}H]2-hydroxy-3-deoxyestradiol with liver microsomes yielded 2-hydroxyestradiol as a sole product. The content of labeled deuterium was determined by inspection of the molecular ion peak in the mass spectrum. Measurement by GC MS demonstrated that C-3 hydroxylation occurred without any retention of the heavy isotope.

In order to examine the isotope effect on C-3 hydroxylation, the 1 to 1 mixture of the 3-deuterio and nonlabeled compounds was used as a substrate for incubation under the same conditions. Determination of the isotope of the recovered substrate by GC–MS revealed that deuterium content was 43 per cent, and hence only 7 per cent of the label was lost during the incubation. Moreover, when the 1 to 1 mixture was incubated with boiled microsomes, the content of the isotope in the recovered substrate was 45 per cent. These results verified the lack of the isotope effect due to deuterium labeling at C-3.

DISCUSSION

The present study has demonstrated that 2-hydroxyestradiol and its glutathione 1- and 4-thioethers are formed when 2-hydroxy-3-deoxyestradiol is incubated with rat liver microsomes in the presence of NADPH and glutathione. Inspection of the properties, chromatographic behaviors, and reactivity with Raney nickel revealed that the water-soluble metabolites should be 2-hydroxyestradiol glutathione conjugates, where the thiol is bound to the steroid nucleus at C-1 and C-4 via a thioether linkage. It is of particular interest that estradiol and 2-hydroxy-3-deoxyestradiol similarly undergo ortho hydroxylation to form catechol estrogen and subsequent conjugation with glutathione at C-1 and C-4, yielding the water-soluble metabolites in the rat, although the latter is metabolized at a slower rate than the former. On the other hand there can be seen a somewhat different metabolic pattern between these two substrates. Being incubated with rat liver microsomes, estradiol undergoes hydroxylation at C-2 and C-16 [13, 28], whereas 2-hydroxy-3-deoxyestradiol yields solely the 3-hydroxylated metabolite. In a preceding paper of this series, we reported that pretreatment with 3-methylcholanthrene influences the 2- and 3-hydroxylase activities in a different way, indicating the enzyme systems involving these two aromatic hydroxylations to be different in nature [4]. It seems to be worthwhile to clarify whether or not 2- and 3-hydroxylases which are capable of forming catechol estrogen would be identical to the corresponding hydroxylases involving 3-deoxyestrone.

The formation of the glutathione conjugates from estrogen is explained in terms of the reaction sequence involving the initial hydroxylation at C-2, followed by oxidation of the resulting catechol into *o*-quinone or semiquinone [13–16]. However, we have previously suggested an alternative mechanism for the formation of the glutathione conjugate where conjugation does not require the preceding ortho hydroxylation and proceeds via the arene oxide as an intermediate [29]. As for the biotransformation of 2-hydroxy-3-deoxyestradiol, the similar mechanism may be also operative for the formation of the glutathione conjugates.

It is well known that catechol estrogen is further metabolized into the methyl ether by catechol-*O*-methyltransferase (COMT) [30–32], and enzymatic methylation of the phenolic group is an important biotransformation to inactive catecholamines [33]. Recently, it was demonstrated that both catechol substances underwent *O*-methylation by the same COMT [34], and in addition inactivation of neurotransmitters was strongly inhibited *in vivo* [35, 36] and *in vitro* [34, 37] by catechol estrogen. In the light of these findings, it is supposed that the highly elevated production rate of 2-hydroxylated estrogen [38] may be associated with the prolonged effect of neurotransmitters and the occasionally occurring hypertension during pregnancy [34, 39, 40].

The physiological significance of glutathione conjugation and protein binding in liver in connection with the action of the female hormone appears to be of interest, but still remains unclear. The blockage of a sulfhydryl group by a nonspecific binding reaction may result in alteration or inactivation of enzymes and disturbance of metabolic transformation in the liver cell. Therefore, it can be speculated that a certain mechanism for protecting the biologically important protein against damage may be operative in the intact liver [40]. The soluble thiol compounds such as glutathione may trap preferentially the active estrogen metabolites which may possibly react with protein.

The result of deuterium analysis demonstrated that the 3-hydroxylated metabolite lost completely the heavy isotope initially labeled at C-3; in other words the "NIH shift" did not occur during the formation of catechol estrogen. This result is not surprising, since the lack of retention of deuterium during ortho hydroxylation has already been reported with estradiol [41] and other instances of catechol formation [42]. The loss of the label can be explained in such a way that hydroxylation proceeds through a quinoid intermediate which would release readily ortho hydrogen.

There could be seen no substantial difference in the deuterium content of 2-hydroxy-3-deoxyestradiol before and after incubation with the enzyme preparation. The retention of deuterium in the recovered substrate implied that hydrogen removal from C-3 is not a reversible process and C-3 hydroxylation proceeds without the isotope effect. The present finding is in good accord with the results on C-2 hydroxylation of estradiol in humans [41].

2-Hydroxy-3-deoxyestradiol does not possess any estrogenic acitivity, but its mode of biotransformation is very similar to that of estradiol. Recently considerable attention has been directed to the physiological effects of catechol estrogen on the level of luteinizing hormone [43] as well as catecholamine. In these re-

spects, the biological activity of this modified steroid in the living animals is a fertile field for further investigation.

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