[Chem. Pharm. Bull. 32(7)2745—2751(1984)]

Evidence for 4-Hydroxylation of Estradiol 17-Sulfate by Rat Liver Microsomes (Clinical Analysis on Steroids. XXIX[1])

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(Received October 17, 1983)

When estradiol 17-sulfate was incubated with rat liver microsomes in the presence of a reduced nicotinamide adenine dinucleotide phosphate (NADPH)-generating system, 4-hydroxyestradiol 17-sulfate was obtained as a minor product accompanying the major metabolite, 2-hydroxyestradiol 17-sulfate. This 4-hydroxylation was shown to occur without cleavage of the conjugate group.

At the substrate concentration of 200 μM, which is about twice the K_m value of estradiol 17-sulfate 2-hydroxylase, the amounts of 2- and 4-hydroxylated products formed by liver microsomes from male rats were 15.03 ± 1.43 and 0.23 ± 0.01%, respectively. Analogous results were obtained with microsomes from female rats under the same conditions: the yields of 2- and 4-hydroxylated metabolites were 8.75 ± 0.97 and 0.14 ± 0.02%, respectively. Thus, the relative ratios of 2- and 4-hydroxylation of estradiol 17-sulfate were approximately 60 : 1 in both sexes.

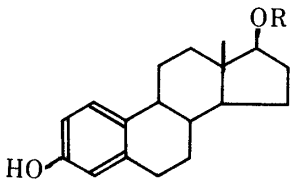
A synthesis of the authentic conjugate, 4-hydroxyestradiol 17-sulfate, and a method for its assay by high-performance liquid chromatography with an electrochemical detector are also described.

Keywords——catechol estrogen; 2-hydroxyestradiol; 4-hydroxyestradiol; estradiol 17-sulfate; 4-hydroxyestradiol 17-sulfate; rat liver microsome

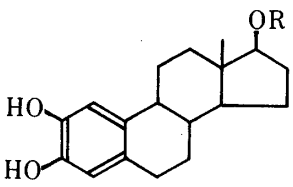
Catechol estrogen is a general term for the main metabolites of the female hormone estrogen in which the aromatic positions *ortho* to the phenolic hydroxyl group at C-3 are oxidized.[2] The principal catechol estrogens such as 2-hydroxyestrone and 2-hydroxyestradiol (IIa) are not merely catabolites of estradiol (Ia), but they themselves have important roles in endocrine organs. This is also the case for another type of catechol estrogens having a hydroxyl group at C-4, such as 4-hydroxyestrone and/or 4-hydroxyestradiol (IIIa).

The ratio of hydroxylation at C-2 or C-4 is dependent on the animal tissues employed.[3] For example, 2-hydroxylation of estradiol by liver microsomes from male rat exceeds 4-hydroxylation by about 50—60 times, whereas in the case of tissues from the central nervous system such as the brain or pituitary glands, both hydroxylations occur to approximately the same degree.

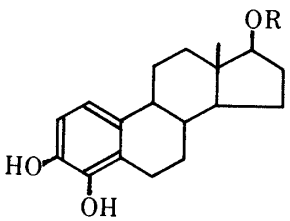
Recently, we have demonstrated the 2-hydroxylation of estradiol 17-sulfate (Ib) by rat



Ia : R=H
Ib : R=SO_3K



IIa : R=H
IIb : R=SO_3K



IIIa : R=H
IIIb : R=SO_3K

Chart 1

liver microsomes in the presence of an NADPH-generating system,[4] and characterized the hydroxylation enzyme.[5] Later, we thoroughly investigated the incubation products to examine whether the 4-hydroxylated derivative of Ib is formed or not. In the present work, we confirmed that 4-hydroxyestradiol 17-sulfate (IIIb) is produced from Ib by rat liver microsomes. This was mainly because a highly sensitive electrochemical detector was employed in the product analyses by high-performance liquid chromatography (HPLC). The identification of the product was further confirmed by comparison with an authentic specimen (IIIb), which was synthesized for this purpose.

Experimental

Chemicals——Catechol estrogens, IIa and IIIa, were prepared from estradiol by the method of Gelbke *et al.*[6] Estradiol 17-sulfate (Ib),[7] estradiol 3,17-disulfate,[7] 2-hydroxyestradiol 17-sulfate (IIb),[8] and 2-hydroxyestradiol-3-methyl-ether 17-sulfate[9] were synthesized by the cited methods. The labeled conjugate, 4-^{14}C-estradiol 17-sulfate was prepared from 4-^{14}C-estradiol (50 mCi/mmol, New England Nuclear, Inc., Boston, Mass., U.S.A.) by the method described in the previous paper.[4] Sep-Pak C_{18} cartridges were obtained from Waters Associates (Milford, MA, U.S.A.). Column-guards were obtained from Millipore Co. (Bedford, Mass., U.S.A.). All other reagents and solvents including Mylase P (activity: unknown) were obtained from Wako Pure Chemicals Ltd. (Tokyo, Japan) and were used without further purification.

Instruments——Melting points were determined on a Kofler-type micro-hot stage (Mitamura, Tokyo) and are uncorrected. Thin-layer chromatography (TLC) was performed with Merck precoated Silicagel 60 F_{254} plates. Preparative TLC was done with the same commercial product (20 × 20 cm; 0.5 mm thickness). The following solvents were used as mobile phases: System A, chloroform–ethyl acetate–acetic acid 100 : 8 : 1 (by vol); system B, ethyl acetate–methanol–water 8 : 2 : 1 (by vol).

Ultraviolet (UV) spectra were measured in methanol with a model 200-20 spectrometer (Hitachi, Tokyo) and are expressed as λ_{max} nm (ε). Infrared spectra (IR, ν_{max}) in KBr disks were recorded on a JASCO IR-2 instrument (Nihon Bunko, Tokyo) and are expressed as cm^{-1}. Nuclear magnetic resonance (NMR) spectra were measured on a JNM FX-100 spectrometer (JEOL, Tokyo) at 100 MHz and chemical shifts are expressed relative to 1% tetramethylsilane as an internal standard. Symbols: s = singlet, d = doublet, t = triplet, and m = multiplet. Mass spectra (MS) were taken by the direct insertion method with a model 9000B mass spectrometer (Shimadzu, Kyoto).

HPLC was carried out on a model 803 chromatograph equipped with a 1205-T UV detector (280 nm), or with an EC-8 electrochemical detector (ECD) at 0.8 V *vs* Ag/AgCl reference electrode (Toyo Soda, Tokyo). A stainless steel column (30 cm × 4 mm, i.d.) packed with TSK Gel ODS-120A (5 μm) (Toyo Soda) was used. The flow rate of the mobile phase was 1.0 ml/min and the column pressure was 130 kg/cm^2. The temperature of the column was maintained at 40 °C in a circulating water bath. Preparative HPLC was carried out on the same machine using a 30 cm × 7.5 mm (i.d.) column packed with the same stationary phase under similar conditions (UV detector). The following solvent was used as the mobile phase: system C, 0.5% $NH_4H_2PO_4$ (pH 3.0)–tetrahydrofuran 5 : 1 (v/v).

Radioactivities were determined on a Packard Tri-Carb 2650 liquid scintillation spectrometer. Toluene containing 6 g/l of 2,5-diphenyloxazole and 300 mg/l of 1,4-bis(5-phenyloxazol-2-yl)benzene was used as the scintillant. Aqueous samples were counted in Bray's solution.

Potassium 3,4-Dihydroxyestra-1,3,5(10)-trien-17β-yl Sulfate (IIIb)——Chlorosulfonic acid (1.0 ml) was added to 10 ml of dry pyridine, and the mixture was stirred for 15 min at 50 °C, then 120 mg of IIIa was added, and the whole was stirred for 6 h at 50 °C, and for 20 h at room temperature. Complete sulfation of the starting material was confirmed in the following way.

A part (1.0 ml) of the reaction mixture was taken to dryness under reduced pressure to give an oil, which was dissolved in 1.0 ml of water and applied to a Sep-Pak C_{18} cartridge. The cartridge was washed with water (2.0 ml), and the conjugate was obtained by elution with 3.0 ml of methanol. The methanolic solution was used for the following experiments.

1) One-fifth of the solution was treated with ethereal diazomethane, and the mixture was allowed to stand for 20 h in a refrigerator. After removal of the solvent, the oily residue was dissolved in 2 N HCl (5.0 ml) containing 5.0 mg of ascorbic acid (as an antioxidant). The mixture was refluxed for 60 min, followed by extraction with peroxide-free ether (5 × 5 ml). The extract was subjected to TLC using system A as the mobile phase, and only one spot corresponding to authentic IIIa was detected.

2) Half of the methanolic solution was taken to dryness to give an oil, which was dissolved in a mixure (3.0 ml) of pyridine and acetic anhydride (2 : 1). The resultant solution was allowed to stand for 20 h at room temperature, followed by concentration to the residue under reduced pressure. The oily product was dissolved in 1.0 ml of water, and the solution was passed through a Sep-Pak C_{18} cartridge. The cartridge was washed with water (2.0 ml), and the

steroidal conjugate was obtained by elution with methanol (3.0 ml). The IR spectrum of the well-dried material showed no absorption at 1730—1700 cm^{-1} (acetyl group).

3) The rest of the methanolic solution was taken to dryness and the residue was subjected to TLC. After development with system B as the mobile phase, the plate was sprayed with 10% H_2SO_4 and heated. The reddish-orange spot that appeared had a far lower *Rf*-value than that of estradiol 3,17-disulfate.

As it was considered that the starting material had been completely sulfated, the following experiments were undertaken on the reaction mixture. Pyridine was removed under reduced pressure at 50 °C, and the residue was dissolved in 100 ml of acetate buffer (10 mM, pH 6.0) containing Mylase P (3.5 g) and ascorbic acid (100 mg). The mixture was allowed to stand for a week at 37 °C. After filtration, the mixture was extracted with *n*-butanol (5 × 30 ml). The combined extract was washed once with water, followed by concentration under reduced pressure to give the residue, which was applied to a column (20 cm × 1 cm, i.d.) packed with Dowex 50 W ion-exchange resin (× 8, 200—400 mesh, K^+ form), and eluted with water. The eluate was concentrated under reduced pressure below 50 °C to give a white powder (94 mg), which was recrystallized from acetone to afford fine needles (44 mg), mp 230 °C (dec.). *Anal.* Calcd for $C_{18}H_{23}KO_6 \cdot 3H_2O$: C, 46.94; H, 6.35; S, 6.96. Found: C, 46.79; H, 6.55; S, 6.88. UV: 255 (7000, sh), 280 (1900). IR: 3450 (OH), 3000—2850 (CH), 1625 and 1500 (both aromatic), 1230 (OSO_3K). NMR (pyridine-d_5) δ: 7.50 (1H, d, J=10.0 Hz, 1-H), 6.84 (1H, d, J=10.0 Hz, 2-H), 4.85—4.76 (1H, m, 17α-H), 0.96 (3H, s, 18-H).

Structural Confirmation of IIIb——Ethereal diazomethane was added to a methanolic solution (5.0 ml) of 10 mg of IIIb, and the mixture was allowed to stand for 16 h in the refrigerator, then concentrated. The crystalline product obtained was dissolved in 10 ml of 10% H_2SO_4 in 50% aqueous methanol. The solution was refluxed for 3 h, and diluted with 50 ml of 10% H_2SO_4. The mixture was then extracted continuously with ether for 72 h. The organic layer was washed with water, dried (anhydrous Na_2SO_4), and concentrated. The product (5.0 mg) was crystallized from methanol to give fine needles, mp 182—184 °C. UV: 276 (1300), IR: 3500—3450 (OH), 3040—2850 (CH), 1600 and 1490 (both aromatic). NMR (chloroform-*d*) δ: 7.01 (1H, d, J=8.4 Hz, 1-H), 6.75 (1H, d, J=8.7 Hz, 2-H), 3.84 and 3.80 (both 3H, both s, 2 × CH_3O), 3.70—3.65 (1H, m, 17α-H), 0.80 (3H, s, 18-H). MS *m/e*: 316 (M^+). No depression of melting point was observed on admixture of this material with authentic 4-hydroxyestradiol-3,4-dimethyl ether.

Animals——Wistar rats were fed a synthetic diet. All the animals (weighing 200—250 g) were starved for 18 h prior to sacrifice.

Preparation of Microsomes——Rat liver microsomes were prepared by the method of the previous paper,[4] and microsomal protein was determined by the method of Lowry *et al.*[10] using bovine serum albumin as a reference. One g of liver was found to be equivalent to 10.0—12.5 mg of microsomal protein.

Incubation Studies——Incubations were carried out under the following conditions. The ice-cooled reaction vessels contained liver microsomal protein (0.5 ml, 2.0 mg/ml), an NADPH-generating system (NADP, 0.5 mM; glucose 6-phosphate, 5 mM; $MgCl_2$, 5 mM; glucose 6-phosphate dehydrogenase, 0.6 unit/ml), KCl (90 mM), EDTA (0.1 mM) and Ib (200 μM). The mixture was diluted with Tris-HCl buffer (50 mM, pH 7.4) to 3.0 ml final volume and was incubated for 30 min at 37 °C under aerobic conditions. The reaction was terminated by heating the incubation vessels in boiling water for 1 min, followed by addition of ascorbic acid (5.0 mg) as an antioxidant and 2-hydroxyestradiol-3-methyl-ether 17-sulfate (approximately 10 μg) as an internal standard, and finally the mixture was diluted with 10 ml of water. For the control experiment, boiled microsomes (100 °C, for 1 min) were put through the same procedure as described above.

The incubation mixtures were centrifuged at 1500 *g* for 20 min, and the precipitates were suspended in water and again centrifuged. The combined supernatants were passed through Sep-Pak C_{18} cartridges. Each cartridge was washed with water (2.0 ml), and the conjugate fraction was obtained by elution with methanol (4.0 ml). The eluate was passed through a Column-guard (0.45 μm), and the filtrate was evaporated under a nitrogen stream at 40 °C to give the residue, which was dissolved in 200 μl of methanol. A 20 μl aliquot was subjected to ECD–HPLC.

Product Analysis——4-^{14}C-Estradiol 17-sulfate (200 μM, 1.02×10^6 dpm) was incubated under the same conditions as described above, and incubation mixtures of ten experiments were combined. The combined mixture (about 30 ml) was at first extracted continuously with peroxide-free ether for 6 hr. Radioactivities of the ether and aqueous layers were counted. The aqueous layer was then treated with Sep-Pak C_{18} cartridges, and the conjugated fraction, obtained by elution with methanol, was subjected to preparative HPLC using system C. As the 4-hydroxy metabolite could not be detected by the UV detector because of the small content, fractions corresponding to peak 3 were collected by monitoring the retention times. Confirmation of the complete separation of the desired material was obtained by HPLC with ECD. The radioactivity of the separated fraction corresponding to IIIb was 2.5×10^4 dpm. An exact amount of IIIa (approximately 30 mg) and ascorbic acid (50 mg) were added to the above fraction, and the mixture was dissolved in 10% H_2SO_4 (20 ml). The whole was extracted continuously with peroxide-free ether for 72 h. The organic layer was washed with water, dried and concentrated. The residue (27 mg) obtained was then acetylated in the usual way using a mixture (10 ml) of pyridine and acetic anhydride (2:1) to afford the crude acetate. The identity of the product was confirmed by analysis of the IR and NMR spectra, and crystallization from methanol was repeated until constant specific radioactivity was obtained.

Calibration Curve for IIIb——The ECD–HPLC assay method for IIIb used 2-hydroxyestradiol-3-methyl-ether 17-sulfate as an internal standard, and the procedure was similar to that used for IIb, as described previously.[5]

Results

(I) Preparation of 4-Hydroxyestradiol 17-Sulfate (IIIb) and HPLC

For the preparation of IIIb, the following synthetic procedure was employed. 4-Hydroxyestradiol (IIIa) was converted to its trisulfate, which was so difficult to crystallize that it was partially hydrolyzed to the 17-monosulfate (IIIb) by an enzymatic procedure.

It was necessary to check that at least the C-17 hydroxyl group of IIIa was sulfated: this was confirmed by the finding that the trisulfate-like material had no hydroxyl group(s) reacting with diazomethane and/or acetic anhydride in pyridine. Further, although it is not a sufficient proof, the *Rf*-value of the material on TLC was far lower than that of estradiol 3,17-disulfate. The reaction product was then treated with Mylase P to give the desired product IIIb. Structural confirmation of the product was carried out by treatment with diazomethane followed by solvolysis. The product obtained was identical with authentic 4-hydroxyestradiol-3,4-dimethyl ether.

Separation of Ib, IIb and IIIb using several kinds of solvents as mobile phases was then undertaken. When a mixture of 0.5% $NH_4H_2PO_4$ (pH 3.0) and tetrahydrofuran (5 : 1, v/v) was used, these conjugates were satisfactorily separated as shown in Fig. 1(a).

Development of a method for the quantification of IIIb was then undertaken. A calibration curve was constructed by plotting the peak height of IIIb relative to that of the internal standard against the amount of the former, and a satisfactory linearity was observed in the range of 1—50 ng of the catechol.

In order to confirm the validity of the present method for the determination of IIIb, a recovery test was carried out using an authentic sample. A known amount of the conjugate was added to the incubation medium, and the conjugate recovered through the whole clean-up procedure was determined. It is evident from the data in Table I that the recovery of 4-hydroxyestradiol 17-sulfate was satisfactory.

(II) Incubation Studies of Estradiol 17-Sulfate (Ib)

Figure 1(b) shows a chromatogram of the incubation product of Ib with liver microsomes from male rats at the limit of the detection of the UV detector; only two peaks corresponding to the substrate (peak 1) and its 2-hydroxylated product (peak 2) were observed. When ECD

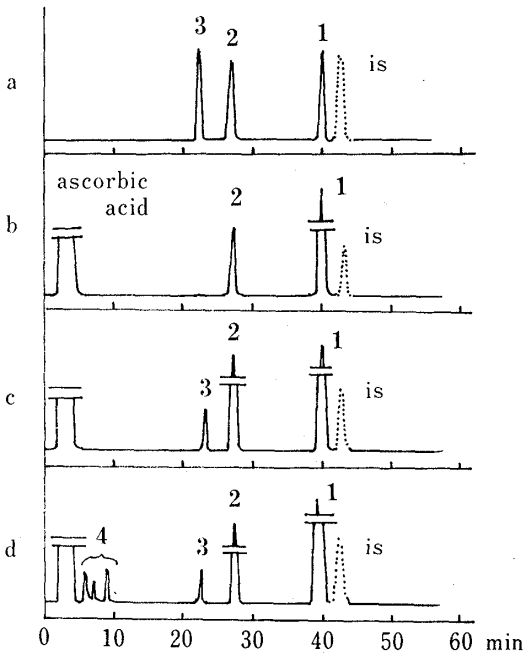


Fig. 1. High-Performance Liquid Chromatograms of Authentic Conjugates (a) and Incubation Products of Estradiol 17-Sulfate with Liver Microsomes from Male (b and c) and Female (d) Rats Using an Electrochemical Detector (a, c and d) and a UV Detector (b)

1, estradiol 17-sulfate; **2**, 2-hydroxyestradiol 17-sulfate; **3**, 4-hydroxyestradiol 17-sulfate; **4**, group composed of metabolites hydroxylated at 6β, 7β, and 16α of the substrate; is, 2-hydroxyestradiol-3-methyl-ether 17-sulfate (internal standard).

TABLE I. Recoveries of 4-Hydroxyestradiol 17-Sulfate from the Incubation Medium and after the Whole Clean-up Procedure[a]

Added amount (μg)	10	5	2	0.2
Recovery (%)	99.7±3.6	98.2±2.0	96.0±1.5	101.3±2.6

a) Steroid was dissolved in the incubation medium (3.0 ml) and the mixture was immediately heated for 1 min in boiling water, followed by the same treatment as described in the text. Each value is expressed as the mean±S.D. ($n=6$).

TABLE II. Isotope Contents of 4-Hydroxyestradiol 17-Sulfate Produced from 4-^{14}C-Estradiol 17-Sulfate as Determined by the Reverse Isotope Dilution Method[a]

No.	Crystallized from	Specific radioactivity (dpm/mg)	
1	Methanol	900	
2	Methanol	790	
3	Methanol	750	770 (Mean)
4	Methanol	760	

a) Crystallizations were done with 4-hydroxyestradiol triacetate and specific radioactivities are shown as free steroid.

TABLE III. Amounts of 2-Hydroxyestradiol 17-Sulfate (IIb) and 4-Hydroxyestradiol 17-Sulfate (IIIb) Produced from Estradiol 17-Sulfate by Rat Liver Microsomes in the Presence of an NADPH-Generating System[a]

Sex	IIb	IIIb
Male	15.03±1.43	0.23±0.01
Female	8.75±0.97	0.14±0.02

a) Figures represent % of the substrate employed and mean±S.D. ($n=6$).

was used with the same sample, on the other hand, an additional peak (peak 3) could be detected at the same position as that of authentic IIIb, as shown in Fig. 1(c). By using the same detector, the third peak was also detected in the incubation product with liver microsomes from female rats, together with other minor products (peak 4) as shown in Fig. 1(d).[11]

To confirm that peak 3 is composed of IIIb, the following experiments were undertaken. The incubation product of the radioactive substrate was extracted with ether to remove free steroid. Less than 1% of the radioactivity was detected in the ether-soluble fraction, whereas over 98% of the radioactivity remained in the aqueous layer. The aqueous layer was subjected to preparative HPLC to give a product corresponding to peak 3 (IIIb), the total radioactivity of which was 2.5×10^4 dpm. The separated conjugate was solvolyzed for analysis by the reverse isotope dilution method (Table II). The total radioactivity obtained from the final steroid was 2.3×10^4 dpm.

Determination of the catechols produced from Ib was then undertaken by ECD–HPLC. The amounts of 2- and 4-hydroxy metabolites (IIb and IIIb) formed by liver microsomes from male and female rats are shown in Table III.

The control experiments using boiled microsomes showed no product formation, and the substrate was recovered quantitatively.

Discussion

Based on the high-performance liquid chromatographic behavior, and also the results of reverse isotope dilution analysis of the solvolyzed product, it seems evident that 4-hydroxylation of Ib by rat liver microsomes occurred without cleavage of the 17-sulfate group. As less than 1% of the radioactivity was detected in the ether extract from the incubation mixture of labeled substrate, while over 98% remained in the aqueous layer, it is not likely that any resulfurylation of the hydrolyzed metabolites could have taken place. It is also clear that 4-hydroxylation occurred enzymatically, because no hydroxylated products were formed in the control experiments using boiled microsomes.

The formation of IIIb was verified in the present investigation, mainly because of the use of an ECD instead of a UV detector. The detection limit of IIIb with the UV detector was 150 ng (*S/N* 4.2, at full scale), which is far greater than the amount of IIIb in the incubation mixture. When ECD was employed, on the other hand, IIIb could be detected at 200 pg (*S/N* 3.6, at 1 nA full scale).

Because only the 2- and 4-hydroxylated derivatives of Ib (IIb and IIIb) are available, it was necessary to confirm that peak 3 is composed of only 4-hydroxyestradiol 17-sulfate. This was demonstrated by the isolation of the corresponding radioactive metabolite from labeled substrate by preparative HPLC, followed by solvolysis to the free steroid for analysis by the reverse isotope dilution method (Table II). The total radioactivities of the isolated conjugate and its solvolyzed product were 2.5×10^4 and 2.3×10^4 dpm, respectively (*i.e.*, essentially equal). It may be concluded, therefore, that peak 3 in the chromatogram of the incubation product is composed of only IIIb.

On the basis of these results and also the linearity of the calibration curve for IIIb as described above, quantification of IIIb by ECD–HPLC was possible. The results in Table III were obtained when Ib was incubated at the substrate concentration of 200 μM, that is approximately twice the K_m value (85.5 μM) of estradiol 17-sulfate 2-hydroxylase.[5] From Table III, it may be deduced that the extents of both hydroxylations of Ib by liver microsomes from female rats are about half of the corresponding hydroxylations by microsomes from male rats, and the ratio of 2- and 4-hydroxylation is about 60 : 1 in both sexes.

Recently, Purdy *et al.*[12] have reported that the ratio of 2- and 4-hydroxylations of estrogen substrates depends not only on the sources of microsomes but also on the substrate structure. They found that the relative ratio was markedly altered by structural alterations of estrogens, especially in the B- and C-rings. Alterations of the D-ring structure did not significantly change the ratio of the hydroxylations.

In this respect, the present result is in accordance with that of Purdy *et al.*, and shows that 4-hydroxylation of estradiol was not influenced by the sulfate group at the C-17 position, because the ratio of 2- and 4-hydroxylations of free estradiol by male rat liver microsomes was approximately 50 : 1.[3] Introduction of a sulfate group at C-17 of the estradiol molecule, however, suppressed the random hydroxylations of the substrate in male rats.[4,5] These results indicate that, in estradiol metabolism by liver microsomes from male rats, the C-17 sulfate group has no significant influence on the production ratio of 2- and 4-hydroxylated metabolites, but has a marked influence upon the regiospecific formation of catechol estrogens.

In the present investigation, it was also confirmed that there is a significant sex difference in the metabolism of Ib. In contrast to the liver microsomes from male rats where only catechols were formed, microsomes from female rats gave multiple kinds of hydroxylated products including 6β,7β, and 16α-hydroxylated products.[11] This difference could have important consequences in terms of the biological effects produced by catechol estrogens and other hydroxylated estrogens.

Acknowledgement The authors are indebted to the staff of the Analytical Center of Hokkaido University (Sapporo) for elemental analyses. This work was supported in part by a grant from the Ministry of Educations, Science and Culture.

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