ESTRADIOL 17 β -SULFATE AS A SUBSTRATE FOR 2-HYDROXYLATION ENZYME OF RAT LIVER MICROSOMES (CLINICAL ANALYSIS ON STEROIDS. XX¹⁾)

KAZUHIRO WATANABE AND ITSUO YOSHIZAWA

Hokkaido Institute of Pharmaceutical Sciences, 7-1, Katsuraoka-cho, Otaru, Hokkaido, 047-02, Japan

(Received December 2, 1981)

4-¹⁴C-Estradiol and its 17 β -sulfate were incubated with rat liver microsomes under NADPH-generating system.

Estradiol in liver microsomes from male and female rats was metabolized to multiple kinds of oxidized products including estrone, 2-hydroxyestrone, 2-hydroxyestradiol, and other minor steroids.

Incubation of estradiol 17 β -sulfate was carried out by the same condition, and it was shown that the metabolic pattern between male and female rats was different.

By incubation of estradiol 17 β -sulfate with male rat liver microsomes, 2-hydroxyestradiol 17 β -sulfate was obtained as the sole product (6%). The hydroxylation was shown to occur without cleavage of the conjugate group.

No such regulating effect by conjugate group on 2-hydroxylation of estradiol 17 β -sulfate was observed in liver microsomes from female rats. The amount of 2-hydroxyestradiol 17 β -sulfate formed was only 1%, and other metabolites which were thought to be mono-hydroxylated estradiols were produced as the major products.

The 2-hydroxylated metabolite of estradiol 17 β -sulfate was confirmed by its isolation as a stable form of derivative by the following way. The incubation mixture of massive amount of estradiol 17 β -sulfate was extracted with *n*-butanol. Methylation of the extract with diazomethane, followed by acid-catalized hydrolysis, acetylation, and finally separation by preparative thin-layer chromatography, gave a crystalline material, the spectral properties of which were completely identical with those of the synthetic specimen, 2, 3-dimethoxy-1,3,5(10)-estratrien-17 β -yl acetate.

Keywords—estradiol, estradiol 17 β -sulfate; 2-hydroxyestradiol; metabolism; rat liver microsomes; regulating effect; sexual difference

INTRODUCTION

The 2-hydroxylation of estradiol and estrone to their catechol derivatives is now regarded as a major pathway of estrogen metabolism in laboratory animals ²⁾ as well as in man.³⁾ Recent accumulating evidences have been indicating that catechol estrogens are not merely inactive metabolites but act physiologically as neurotransmitters.⁴⁾ Although 2-hydroxyestrone and 2-hydroxyestradiol, the representative catechol estrogens, are playing important roles in physiological and endocrine actions, there seems to be some differences between their biological activities. 2-Hydroxyestradiol has its own charac-

teristics such as: the specific binding activity to rat anterior pituitary cell membrane,⁵⁾ the stimulatory effect on prolactin release in hypogonadal women,⁶⁾ the binding activity to estrogen receptor of rat mammary tumors induced by dimethylbenz[*a*]anthracene,⁷⁾ the uterotrophic activity,⁸⁾ the competitive activity with catecholamines to rat anterior pituitary cell membrane,⁹⁾ the regulating effect on pituitary LH-secretion,¹⁰⁾ the direct inhibitory effect upon tyrosine hydroxylase,¹¹⁾ and the antagonistic effect on generation of cyclic AMP in hypothalamus.¹²⁾

Previously, we have reported a directive effect

of 17 β -substituent groups on enzymatic *O*-methylation of 2-hydroxyestradiol 17 β -conjugates and also have speculated the possibility of enzymatic 2-hydroxylation for estradiol 17 β -conjugates.¹³⁾ In the present paper, we have undertaken to explore the aromatic hydroxylation pathway and established that estradiol 17 β -sulfate could be substrate for 2-hydroxylation enzyme in rat liver microsomes.

MATERIALS AND METHODS

Chemicals — The labeled steroid, 4-¹⁴C-estradiol (55 mCi/mmol) was obtained from New England Nuclear, Inc. (Boston, Mass., USA) and used without further purification. Radioactive estradiol 17 β -sulfate was prepared from 4-¹⁴C-estradiol by the method of Kirdani,¹⁴⁾ and its radiochemical purity was 99.8–100.3%. Estradiol was purchased from Steraloids Inc. (Wilton, N.H., USA) and 2-hydroxyestradiol was obtained in this laboratory according to the previous paper.¹⁵⁾ The 17 β -sulfate of 2-hydroxyestradiol was prepared as reported in the previous paper.¹⁶⁾ Glucose-6-phosphate, NADP, NADPH, and glucose-6-phosphate dehydrogenase, were obtained from Oriental Yeast, Inc. (Osaka, Japan). All other reagents and solvents were obtained from commercial sources.

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

Preparation of Microsomes — The rats were sacrificed by decapitation, and after perfusion with cold 1.15% KCl, the liver was immediately removed and chilled on ice. All the subsequent procedures were carried out at 0–4°C. The tissue was weighed and washed with 1.15% KCl and homogenized with 2-fold volume of an ice-cold 1.15% KCl in 50 mM of Tris-HCl buffer (pH 7.4) by a Teflon-glass Potter-Elvehjem homogenizer to give 33% (w/v) homogenate. The homogenate was centrifuged at 10000 $\times g$ for 20 min in Hitachi model 18 PR-3 automatic high speed refrigerated centrifuge. The supernatant was carefully transferred and centrifuged at 105000 $\times g$ for 60 min by using a Hitachi model

65-P ultracentrifuge. The microsomal pellet separated was suspended in 5 ml of Tris-HCl buffer (pH 7.4). The microsomal protein was determined by the method of Lowry *et al.*¹⁷⁾ using bovine serum albumin as a reference. One g liver was found to be equivalent to 10.0–12.5 mg of protein.

Thin-Layer Chromatography — Thin-layer chromatography (TLC) was carried out with Merck precoated silicagel 60-F₂₅₄ plates. The preparative thin-layer chromatographic plates were made from Wako-gel B-5F (Wako Chemicals, Tokyo, Japan) by the usual way having a thickness 0.75 mm. Following solvents (v/v) were used for developing.

system A: benzene–acetone (98 : 2)

system B: chloroform–ethyl acetate–acetic acid (100 : 8 : 1)

system C: ethyl acetate–methanol–water (50 : 6 : 1)

system D: methanol–water–acetic acid (75 : 20 : 5)

Incubations — The incubation studies were carried out with following conditions. Ice-cold reaction vessels contained liver microsomal protein (2.0 mg/ml); radioactive substrates (estradiol: 6.20×10^5 dpm, 0.511 μ mol, estradiol 17 β -sulfate: 5.86×10^5 dpm, 0.502 μ mol); a NADPH-generating system (NADP: 0.5mM, G-6-P: 5 mM, MgCl₂: 5 mM, G-6-P dehydrogenase: 0.6 unit/ml); KCl (90 mM); EDTA (0.1mM). The mixture was diluted with Tris-HCl buffer (pH 7.4, 50 mM) to 2.0 ml as a final volume. The mixture was incubated for 30 min at 37°C under aerobic condition. The reaction was terminated by heating the incubation vessels into boiling water for 30 s, followed by addition of ascorbic acid (100 mg) as antioxidant, and finally was diluted with 20 ml of water. For control experiment, the incubation was employed using boiled microsomes (100°C for 10 min) by the completely same procedure as described above.

Extraction and Hydrolysis — In the work of 4-¹⁴C-estradiol, the incubation mixture was diluted with two kinds of carrier steroids, estradiol and 2-hydroxyestradiol (30–40 mg for

each), followed by continuous extraction with ether for 72 h.

In the experiment using estradiol 17 β -sulfate as a substrate, the reaction mixture was extracted with ether (10 ml $\times$ 5) to remove free steroid if produced. After dilution with the similar amount of two carriers as described, the aqueous layer was acidified to 10% H₂SO₄, followed by extraction with ether for 72 h. The extract was used for the following experiment. Aqueous layer obtained was assayed for the determination of radioactivity.

Acetylation, Separation, and Crystallization

—The ether extract was washed with water and dried over anhydrous Na₂SO₄, and evaporated. The crystalline residue obtained was acetylated with acetic anhydride in pyridine to give a product, which was submitted to preparative TLC using system A by multiple runnings. The two separated bands corresponding to estradiol diacetate and 2-hydroxyestradiol triacetate were scraped off and extracted with ethyl acetate. The structures of the acetates were confirmed by nuclear magnetic resonance spectra and crystallization from methanol to constant specific radioactivity.

Radio Thin-Layer Chromatoscanning of the Product —The incubation mixture of estradiol was diluted with 100 μ g of 2-hydroxyestradiol and 1 g of ascorbic acid, followed by continuous extraction with ether for 48 h. The ether layer was washed with water, dried over anhydrous Na₂SO₄, and evaporated. The extract was submitted to TLC using system B by multiple runnings. After development, the plate was run on radio thin-layer chromatoscanner. As for the metabolite(s) of estradiol 17 β -sulfate, the incubation mixture was diluted with authentic 2-hydroxyestradiol 17 β -sulfate (100 μ g) and ascorbic acid (1 g), followed by extraction with *n*-butanol (5 ml $\times$ 7). The combined extract was once washed with water and evaporated to give a residue, which was submitted to TLC using system C by multiple runnings and radioscanning. For investigating the contents of hydroxylated metabolite(s) of estradiol 17 β -sulfate, the following experiments were done. To the incubation

mixture of estradiol 17 β -sulfate were added 2-hydroxyestradiol (100 μ g) and ascorbic acid (1 g). The mixture was then acidified to 10% H₂SO₄ and extracted continuously with ether for 48 h. The extract was washed with water, dried over anhydrous Na₂SO₄, and evaporated. The residue obtained was afforded to TLC using system B by multiple runnings, followed by radioscanning. After scanning, the TLC plate was sprayed with 10% H₂SO₄ and heated at 140°C to develop the color of authentic steroids developed concurrently.

Instruments —Melting points were determined on a Kofler micro-hot stage and are uncorrected. Nuclear magnetic resonance (NMR) spectra were measured in a pulsed Fourier-transform mode on a JNM FX-100 (Jeol, Tokyo, Japan). The chemical shifts are expressed relative to 1% tetramethylsilane as an internal standard. Ultraviolet (UV) spectra were measured with model 200–20 spectrophotometer with X-Y typed recorder (Hitachi, Tokyo, Japan). Infrared (IR) spectra in KBr disks were recorded by Jasco IR-2 (Nihon Bunko, Tokyo, Japan). Mass spectra (MS) were taken with 9000 B (Shimadzu, Kyoto, Japan) by direct insertion method. The radio thin-layer chromatoscanner used was Aloka JTC-203 (Nihon Musen, Tokyo, Japan).

Counting of Radioactivity —Radioactivities were determined on a Packard Tri-Carb 2650 scintillation spectrometer. Toluene containing 6 g/l of 2,5-diphenyloxazole and 300 mg/l of 1,4-bis [2-(5-phenyloxazole)]-benzene was used as scintillant. Aqueous samples were counted in Bray's system.

Product Isolation —Seventy mg of estradiol 17 β -sulfate (1.02×10^6 dpm) was dissolved in 2 ml of dimethylsulfoxide. The solution was divided into ten equal aliquots, which were incubated under the same conditions as described except total volume 20 ml for each. After incubation, the mixtures were combined and extracted with *n*-butanol (50 ml $\times$ 5). The combined extract was washed with water and evaporated under reduced pressure at 50°C. The residue obtained was dissolved in 30 ml of methanol con-

taining ethereal diazomethane. The solution was allowed to stand for 16 h in a refrigerator and evaporated. The crystalline product was then dissolved in 10 ml of 10% H₂SO₄. The solution was once heated at 60°C for 1 h, then extracted with ether for 72 h. The ether layer was washed with water, dried over anhydrous Na₂SO₄, and evaporated. The product was acetylated by the usual way to give a crystalline material, which was subjected to preparative TLC using system D by runnings twice. Two separated bands were scraped off and extracted with ethyl acetate. From the upper band, 36 mg of crystalline material was obtained, which was identified as 3-methoxy-1,3,5(10)-estratrien-17 β -yl acetate by melting point determination and instrumental analyses. From the lower band, 4 mg of an oily material was obtained, crystallization of which once from methanol gave 1.6 mg of 2,3-dimethoxy-1,3,5(10)-estratrien-17 β -yl acetate as fine needles, mp 182.5–185°C (179–182°C,¹⁸) 183–184.5°C¹⁹). No depression of mixed melting point of this material with authentic compound was observed.²⁰ The specific radioactivity of this material was 12900 dpm/mg. NMR (CDCl₃) δ in ppm: 6.81 (1H, s, C₁-H), 6.59 (1H, s, C₄-H), 4.70 (1H, t, J =8.0 Hz, 17 α -H), 3.86 and 3.85 (both 3H, both s, 2 $\times$ CH₃O), 2.07 (3H, s, CH₃COO), 0.84 (3H, s, C₁₈-CH₃). MS m/e : 358 (M⁺). UV, $\lambda_{\text{max}}^{\text{EtOH}}$ nm (ϵ): 284.6 (3640) [285.3 (3830)]¹⁹. IR, ν_{max} cm⁻¹: 2910, 2845, 1724, 1505. The spectrum was completely identical with that of authentic specimen.

Saponification — About 0.1 mg of the above product was dissolved in 2 ml of 0.1 N KOH in 90% methanol, and the mixture was allowed to stand overnight at room temperature. The solution was diluted with 30 ml of ether and washed with water, and dried over anhydrous Na₂SO₄. The oily product by MS showed a base peak at m/e 316, corresponding to the parent ion of 2,3-dimethoxyestra-1,3,5(10)-trien 17 β -ol.

RESULTS

Aromatic 2-hydroxylation of estradiol and its 17 β -sulfate with rat liver microsomes under

NADPH-generating system was investigated.

Regarding the metabolite(s) of these substrates, the following qualitative experiments were carried out. The ether-soluble fraction from the incubation mixture of free 4-¹⁴C-estradiol, and *n*-butanol extract from the incubation mixture of conjugated 4-¹⁴C-estradiol were afforded to radio thin-layer chromatoscanner. The results on free steroid are shown in Fig. 1.

On the metabolites of estradiol in male rats, five radioactive peaks are detected and four of them are assigned as estrone (1), estradiol (2), 2-

FIG. 1. Thin-Layer Chromatogram (Above) of Authentic Steroids and Radioscannograms (Below) of the Incubation Products of 4-¹⁴C-Estradiol (A; Male, B; Female), and of the Hydrolyzates of Incubation Products of 4-¹⁴C-Estradiol 17 β -Sulfate (C; Male, D; Female), with Rat Liver Microsomes under NADPH-generating System
1, estrone; 2, estradiol; 3, 2-hydroxyestrone; 4, 2-hydroxyestradiol; 6, 6 α - and 6 β -hydroxyestradiol; 7, estriol.

The products were developed concurrently with the authentic steroids by using a mixture of chloroform-methanol-acetic acid (100:8:1) by multiple runnings.

hydroxyestrone (3), and 2-hydroxyestradiol (4), by comparison with authentic steroids developed concurrently. The rest of radioactive peak (5) is unknown.

Similarly on the metabolites of the same substrate in female rats, another radioactive peaks (6 and 7) are detected, and their *R_f*-values are very close to those of such triols as estriol, 6 α -, and 6 β -hydroxyestradiols developed concurrently.

The chromatograms of *n*-butanol extract from the incubation mixture of estradiol 17 β -sulfate are shown in Fig. 2. In male rats, only one radioactive peak appeared at the same position with authentic 2-hydroxyestradiol 17 β -sulfate (9). In female rats, on the other hand, additional radioactive peak (10) is detected at lower *R_f*-value posi-

tion than 2-hydroxyestradiol 17 β -sulfate.

For the confirmation of the product from estradiol 17 β -sulfate, the massive amount of low radioactive estradiol 17 β -sulfate was incubated and the metabolite was extracted with *n*-butanol, followed by treatment with diazomethane, acid-catalyzed hydrolysis, acetylation by the usual way, and finally the separation by preparative TLC. The structure of the product thus obtained was assigned as 2,3-dimethoxy-1,3,5(10)-estratrien-17 β -yl acetate by the IR, NMR and mass spectra. The specific radioactivity of this product was 13900 dpm/mg (calculated as free steroid), which is nearly equal to that of the substrate (15700 dpm/mg as free steroid).

As for the contents of the metabolite(s) of estradiol 17 β -sulfate by both sexes, the radio thin-layer chromatograms of the hydrolyzate are shown in Fig. 1. Only 2-hydroxyestradiol was detected as the sole metabolite of the sulfate in liver microsomes from male rats. While in that from female rats, multiple kinds of products are detected, in which 2-hydroxyestradiol is rather minor product. The major products are thought to be such triols as estriol, 6 α -, and 6 β -hydroxyestradiols by their chromatographic behaviors.

The quantification of 2-hydroxyestradiol from both substrates was done by reverse isotope dilution method; the results of which are summarized in Table I. The conversion of estradiol to 2-hydroxyestradiol in the liver microsomes from male rats was about 22%, while in that from female rats was 4%. This tendency is in good accordance with reported one.²⁰⁾ The same 2-hydroxylation of estradiol 17 β -sulfate in liver microsomes from male and female rats were, 6% and 1%, respectively.

The control experiment using boiled microsomes showed no product formation and quantitative amount of substrate was recovered from the ether extract.

DISCUSSIONS

The behaviour of the microsomal metabolites of estradiol 17 β -sulfate on radio thin-layer chromatogram and their cleavage by solvolysis in

FIG. 2. Thin-Layer Chromatogram (Above) of Authentic Steroids and Radioscannograms (Below) of *n*-Butanol Extracts from the Incubation Mixture of 4-¹⁴C-Estradiol 17 β -Sulfate with Rat Liver Microsomes under NADPH-generating System (E; Male, F; Female)

8, estradiol 17 β -sulfate; 9, 2-hydroxyestradiol 17 β -sulfate.

The extracts were developed concurrently with authentic steroids by using a mixture of ethylacetate-methanol-water (50:6:1) by multiple runnings.

acidified ether indicate that the sulfate was metabolized without hydrolysis. Less than 1% of the incubated radioactivity was detected in ether extract from the incubation mixture of estradiol 17 β -sulfate. This is indicating that the microsomal sulfatase activity was of little importance under the experimental conditions used. Since the sulphurylase activity is localized in 105000 $\times$ g supernatant fraction,²¹⁾ it may be possible that resulphurylation of hydrolyzed metabolites could have taken place. The amount of this rejugation, however, may be negligible one, because less than 1% of the radioactivity was detected in the aqueous layer of the incubation mixture of estradiol.

The present results on estradiol 17 β -sulfate are different from those reported by Gustafsson and Ingelman-Sandberg.²²⁾ They have identified 6 α , 6 β , 7 β , and 15 β -hydroxy estradiols as the major metabolites of the same substrate by female rat liver microsomes. Probably, the polar metabolites detected in the chromatogram as shown in Fig. 1 (D) should correspond to these steroids. Further, they have reported no formation of 2-hydroxyestradiol. While in male rats, they have found no detectable amount of the metabolite. The

divergence in the occurrence of 2-hydroxylation on estradiol 17 β -sulfate between these authors and ours is not clear. The possible causes may be the difference of the methodology employed. We have added ascorbic acid to the reaction mixture as antioxidant after finishing the incubation, because catechol estrogens are extremely labile. Further, the product has been quantified by reverse isotope dilution method, whereas they have detected the metabolites by gas chromatography-mass spectrometry.

The present results clearly demonstrate the remarkable sexual difference in the metabolism of estradiol 17 β -sulfate. Male rats were able to hydroxylate estradiol 17 β -sulfate in only position 2 in considerable amount, whereas female rats hydroxylated the conjugated substrate in many positions including 2 and to a smaller extent of 2-hydroxylation.

With the unconjugated substrate, male rats are much more effective in oxidizing estradiol than female rats. However, the metabolic features between male and female rats are substantially the same. Thus, it may be obvious that the sexual difference in microsomal 2-hydroxylation becomes remarkable when substrate is changed

TABLE I. *Quantification of 2-Hydroxyestradiol Formed and Estradiol Recovered from the Incubation Products of 4-¹⁴C-Estradiol and 4-¹⁴C-Estradiol 17 β -Sulfate with Rat Liver Microsomes^{a)}*

Substrate ^{b)}	Sex	Ether extract		Aqueous layer	
		2-OH-Estradiol	Estradiol	2-OH-Estradiol	Estradiol
Estradiol (1) ^{c)}	Male	0	103.4	<1.0	
Estradiol (4)	Male	21.8 $\pm$ 13.4	58.5 $\pm$ 17.1	<1.2	
Estradiol					
17 β -sulfate (4)	Male	<1.0		6.2 $\pm$ 1.8	89.4 $\pm$ 11.4
Estradiol (4)	Female	3.9 $\pm$ 1.7	81.9 $\pm$ 9.0	<1.0	
Estradiol					
17 β -sulfate (4)	Female	<0.5		1.0 $\pm$ 0.3	87.5 $\pm$ 10.1

a) Numbers in Table are showing the % radioactivity incubated. The radioactivities were determined on 2-hydroxyestradiol triacetate and estradiol diacetate, and are expressed as their free steroids. The products of 4-¹⁴C-estradiol 17 β -sulfate were obtained after solvolysis of the incubation product. 2-OH-estradiol=2-hydroxyestradiol. b) Numbers in parentheses indicate the number of experiment. c) Incubation with boiled microsomes.

from the free to the sulphoconjugated steroids. To our knowledge this is the first example of the regulatory effect upon 2-hydroxylation in estradiol metabolism.

The presence of 2-hydroxylation enzyme in rat is known to be present in liver, kidney, lung,²³⁾ brain,²⁴⁾ testis, adrenal, pituitary, heart, placenta,²⁵⁾ uterus,²⁶⁾ and also in central nervous system.²⁷⁾ Thus, the present results show that estradiol 17 β -sulfate may act as substrate for some of the other microsomal 2-hydroxylase system.

From the accumulating evidences of catechol estrogens on neuroendocrine organs,⁴⁾ and also the specific functions of estradiol 17 β -sulfate such as inhibitory effect upon placental aspartate aminotransferase,²⁸⁾ and stimulatory effect on protein synthesis in muscle microsomes,²⁹⁾ the present results on conjugated estradiol are interesting ones. Recent reports on the rapid metabolic clearance of catechol estrogens³⁰⁾ and the rapid turnover in brain³¹⁾ are pointing the existence of the further pathway of catechol estrogen to another form of metabolite.

REFERENCES

- 1) Part XIX: I.Yoshizawa, E.Kamiya and S.Itoh: Separation and estimation of guaiacol estrogens by high performance liquid chromatography, *Jap. J. Clin. Chem.*, **10**, 155–159 (1981).
- 2) P.Ball, H.Hoppen and R.Knuppen: Metabolism of oestradiol-17 β and 2-hydroxyoestradiol-17 β in rat liver slices, *Z. Physiol. Chem.*, **355**, 1451–1462, (1974).
- 3) J.Fishman, H.Guzik and L.Hellman: Aromatic ring hydroxylation of estradiol in man, *Biochemistry*, **9**, 1593–1598 (1970).
- 4) J.Fishman: The catechol estrogens, *Neuroendocrinology*, **22**, 363–374 (1977); H.P.Gelbke, P.Ball and R.Knuppen: 2-hydroxyoestrogens chemistry, biogenesis, metabolism and physiological significance, *Adv. Steroid Biochem. Pharmacol.*, **6**, 81–154 (1977); P.Ball and R.Knuppen, *Acta endocrinol.*, **93** (supplementum No. 232), 1–127 (1980).
- 5) J.M.Schaeffer, S.Stevens, R.G.Smith and A.J.W.Hsueh: Binding of 2-hydroxyestradiol to rat anterior pituitary cell membranes, *J. Biol. Chem.*, **255**, 9838–9843 (1980).
- 6) E.Y.Adashi, R.F.Casper, J.Fishman and S.S.C.Yen: Stimulatory effect of 2-hydroxyestradiol on prolactin release in hypogonadal women, *J. Clin. Endocr. Metab.*, **51**, 413–415 (1980).
- 7) Y.J.Abul-Hajj: Binding of catechol estrogens to the estrogen receptor of dimethylbenz[a]anthracene induced mammary tumors, *J. Steroid Biochem.*, **13**, 83–88 (1980).
- 8) C.P.Martucci and J.Fishman: Impact of continuously administered catechol estrogens on uterine growth and luteinizing hormone secretion, *Endocrinology*, **105**, 1288–1292 (1979).
- 9) J.M.Schaeffer and A.J.W.Hsueh: 2-Hydroxyestradiol interaction with dopamine receptor binding in rat anterior pituitary, *J. Biol. Chem.*, **254**, 5606–5608 (1979).
- 10) N.Parvizi and F.Ellendorff: Catechol estrogens and the control of LH-secretion, *J. Steroid Biochem.*, **9**, 881 (1978).
- 11) T.Lloyd and J.Weisz: Direct inhibition of tyrosine hydroxylase activity by catechol estrogens, *J. Biol. Chem.*, **253**, 4841–4843 (1978).
- 12) S.M.Paul and P.Skolnick: Catechol oestrogens inhibit oestrogen elicited accumulation of hypothalamic cyclic AMP suggesting role as endogenous anti-oestrogens, *Nature*, **266**, 559–561 (1977).
- 13) I.Yoshizawa, A.Nakagawa, E.Kamiya, S.Itoh and T.Ogiso: Evidence of the directive effect of 17 β -conjugate group on the enzymatic O-methylation of catechol estrogen, *J. Pharm. Dyn.*, **3**, 317–319 (1980).
- 14) R.Y.Kirdani: The synthesis and characterization of estradiol sulfates, *Steroids*, **6**, 845–853 (1965).
- 15) I.Yoshizawa, M.Tamura and M.Kimura: Simple preparation of catechol estrogen and its derivatives, *Chem. Pharm. Bull.*, **20**, 1842–1843 (1972).
- 16) I.Yoshizawa, A.Nakagawa, E.Kamiya and S.Itoh: Clinical analysis on steroids. XIV. Preparation of 2-hydroxyestradiol 17 β -conjugates, *Yakugaku Zasshi*, **100**, 867–870 (1980).
- 17) O.H.Lowry, N.J.Rosebrough, A.L.Farr and R.J.Randall: Protein measurement with the Folin phenol reagent, *J. Biol. Chem.*, **193**, 265–275 (1951).
- 18) J.Fishman, M.Tomasz and R.Lehman: Catechol derivatives of estrogens, *J. Org. Chem.*, **25**, 585–588 (1960).
- 19) Synthesized in this laboratory according to the method by J.Fishman *et al.*¹⁸⁾
- 20) M.Numazawa, N.Soeda, S.Moro and T.Nambara: Metabolism of 2-hydroxy-3-deoxyestradiol by rat liver microsomes, *Biochem. Pharmacol.*, **26**, 769–773 (1977).
- 21) Y.Nose and F.Lipmann: Separation of steroid sulfokinases, *J. Biol. Chem.*, **233**, 1348–1351 (1958).
- 22) J.-Å.Gustafsson and M.Engelmann-Sundberg: Regulation and substrate specificity of a steroid sulfate-specific hydroxylase system in female rat liver

- microsomes, *J. Biol. Chem.*, **250**, 3451–3458 (1975).
- 23) M.Numazawa, Y.Kiyono and T.Nambara: 2-Hydroxylation of estradiol by rat kidney and lung, *J. Steroid Biochem.*, **13**, 1101–1105 (1980).
- 24) R.L.Barbieri, J.A.Canick and K.J.Ryan: Estrogen 2-hydroxylase: activity in rat tissues, *Steroids*, **32**, 529–538 (1978).
- 25) A.R.Hoffman, S.M.Paul and J.Axelrod: Estrogen-2-hydroxylase in the rat, *Biochem. Pharmacol.*, **29**, 83–87 (1980).
- 26) V.V.R.Reddy, P.Hanjani and R.Rajan: Synthesis of catechol estrogens by human uterus and leiomyoma, *Steroids*, **37**, 195–203 (1981).
- 27) J.Fishman and B.Norton: Catechol estrogen formation in the central nervous system of the rat: *Endocrinology*, **96**, 1054–1059 (1975).
- 28) M.O.Pulkkinen and K.Willman: *In vitro* regulation of human placental aspartate aminotransferase by sulfate esters of estrogens, *Steroids*, **8**, 51–59 (1966).
- 29) W.C.DeLoecker, S.C.Brooks and F.DeWever: The stimulation *in vitro* of protein synthesis in muscle microsomal supernatant by estradiol-3, 17 β -disulfate, *Biochim. Biophys. Acta*, **119**, 655–657 (1966).
- 30) G.R.Merriam, D.D.Brandon, S.Kono, S.E.Davis, D.L.Loriaux and M.B.Lipsett: Rapid metabolic clearance of the catechol estrogen 2-hydroxyestrone, *J. Clin. Endocr. Metab.*, **51**, 1211–1213 (1980).
- 31) J.Fishman and C.Martucci: Absence of measurable 2-hydroxyestrone in the rat brain: evidence for rapid turnover, *J. Clin. Endocr. Metab.*, **49**, 940–942 (1979).