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PROPERTIES OF ESTRADIOL 2-HYDROXYLASE AND 2-HYDROXY-3-DEOXYESTRADIOL 3-HYDROXYLASE IN RAT LIVER*

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SUMMARY

The properties of enzyme systems involved in the formation of catechol estrogen from estradiol and 2-hydroxy-3-deoxyestradiol in rat liver microsomes have been investigated. Molecular oxygen dissolved in the incubation medium was enough for the occurrence of 2- and 3-hydroxylations. The presence of carbon monoxide suppressed the formation of catechol estrogen from the two substrates where the CO/O_2 ratios needed for 50% inhibition of the bioconversion were 12.0 and 14.1, respectively. The inhibitory effect was reversed almost completely by illumination with white light. Pretreatment with phenobarbital and 3-methylcholanthrene did not exert any significant change in the activities of 2- and 3-hydroxylases, but increased the content of cytochrome P-450 or P-448 in liver microsomes. The storage of microsomal preparation in the frozen state resulted in a marked decrease in the hydroxylase activities. These results strongly imply that a similar cytochrome P-450 system would be operative in the formation of catechol estrogen from estradiol and 2-hydroxy-3-deoxyestradiol in rat liver microsomes.

INTRODUCTION

Considerable attention has recently been focused on the physiological significance of 2-hydroxylation in the catabolism of estrogens [1–6]. As an initial biotransformation of estrone† and estradiol in living animals 2-hydroxylation precedes ring D hydroxylation [1, 6], and the resulting catechol estrogen is further converted into the *O*-methyl ether [7, 8] and the glutathione conjugate [3, 9]. Evidence accumulated in recent years has revealed that the behavioral and neuroendocrine effects exerted by estradiol are associated with the formation of the 2-hydroxylated metabolites [10–15]. In a previous paper of this series we reported that 2-hydroxy-3-deoxyestradiol, a positional isomer of estradiol, undergoes hydroxylation solely at C-3 to yield 2-hydroxyestradiol by rat liver microsomes [16]. Despite the great importance of catechol estrogens, little is known about the properties of estradiol 2-hydroxylase. In addition, the characteristics of 2-hydroxy-3-deoxyestradiol 3-hydroxylase also still remain unclear. The present paper describes the comparative studies on the properties of both aromatic hydroxylases in rat liver microsomes.

* Part CXXXIX of *Studies on Steroids* by T. Nambara; Part CXXXVIII: H. Kimura, N. Suzuki, T. Sato, J. Goto and T. Nambara, *J. Lipid Res.*, submitted.

† The following trivial names are used in this paper: estrone = 3-hydroxy-1,3,5(10)-estratrien-17-one; estradiol = 1,3,5(10)-estratriene-3,17β-diol; 2-hydroxy-3-deoxyestradiol = 1,3,5(10)-estratriene-2,17β-diol; 2-hydroxyestradiol = 1,3,5(10)-estratriene-2,3,17β-triol; estriol = 1,3,5(10)-estratriene-3,16α,17β-triol.

MATERIALS AND METHODS

Animals and pretreatment with drugs. Male Wistar rats (10- to 13-weeks-old) weighing 250–300 g were used except for the induction experiments in which immature male rats weighing 90–120 g were employed. The immature rats were injected intraperitoneally with phenobarbital (80 mg/kg body weight) in saline (0.25 ml), 3-methylcholanthrene (40 mg/kg body weight) in corn oil (0.25 ml) once daily for 4 days, or clomiphene (10 mg/kg body weight) in saline (0.20 ml) once daily for 5 days. The animals were fasted for the last 24 h prior to sacrifice. The control rats were treated with injection of the appropriate vehicles.

Materials. NADPH, glucose and glucose oxidase were purchased from Sigma Chemical Co. (St. Louis, MO), silica gel H from E. Merck AG (Darmstadt, West Germany), Soluene 100 from Packard Instrument (Downers Grove, Il.), and [6,7-^{3}H]-estradiol (48 Ci/mmol) and [4-^{14}C]-estradiol (54 mCi/mmol) from the Radiochemical Centre (Amersham, England), respectively. [6,7-^{3}H]-2-Hydroxy-3-deoxyestradiol (10.8 μCi/mmol) was chemically synthesized from [6,7-^{3}H]-estradiol by methods developed in these laboratories [17, 18]. The radiochemical purity of these ^{3}H-labeled steroids was checked by thin-layer chromatography (t.l.c.) prior to use. 2-Hydroxyestradiol was prepared in the manner described by Gelbke *et al.*[19].

Thin-layer chromatography. T.l.c. was carried out on a glass plate coated with a layer (0.25 mm thick) of silica gel H impregnated with ascorbic acid [20]

employing hexane–ethyl acetate (1:1, V/V) as a developing solvent.

Radioactivity counting. Counting was carried out on a Packard Tri-Carb Model 3380 liquid scintillation spectrometer employing the Bray's scintillator [21]. Correction for quenching was made by the automatic external standard method.

Preparation of microsomes. The rats were sacrificed by stunning and decapitation, and the liver was immediately removed and chilled on ice. All the subsequent procedures were carried out at 0–4°C. The tissue was weighed, finely minced with scissors, homogenized with a 4-fold volume of an ice-cold 1.15% KCl solution by a Potter–Elvehjem homogenizer with a Teflon pestle, 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 Beckman Model L5-65 ultracentrifuge. The microsomal pellet was washed with 1.15% KCl twice to remove endogenous soluble sulfhydryl compounds and then gently resuspended in 1.15% KCl in such a way that 1 ml was equivalent to 4 mg of protein. Microsomal protein was determined by the method of Lowry *et al.* [22] using bovine serum albumin as a reference. One gram of liver was equivalent to 10–15 mg of protein.

Spectrometry. The amount of the carbon monoxide (CO)-binding hemoprotein was determined by the method previously established [23]. The CO difference spectrum of the reduced hemoprotein was recorded on a Hitachi Model 124 spectrophotometer. The concentration of cytochrome P-450 and P-448 in rat liver microsomes was calculated by the use of an extinction coefficient of 91 $mM^{-1} \cdot cm^{-1}$ on the difference in absorbance between the Soret maximum and 490 nm.

Enzyme assay. In the assay for the formation of 2-hydroxyestradiol the standard reaction mixture contained the following: a microsomal preparation (0.5 ml), NADPH (4 μmol), [6,7-^{3}H]-estradiol (0.5 μCi) or [6,7-^{3}H]-2-hydroxy-3-deoxyestradiol (0.5 μCi) dissolved in 50% (V/V) aq. methanol (0.1 ml) and sufficient 0.05M Tri–HCl buffer (pH 7.4) to make the total volume 1.2 ml. Incubation was carried out at 37°C under aerobic conditions. When the effect of CO and requirement for molecular oxygen (O_2) were examined, the microsomal preparation and a solution containing the cofactor were slowly flushed for 10 min with an appropriate gas mixture and then were pipetted separately into the main chamber containing a substrate and the hollow stopper of a Thunberg tube which was previously evacuated and refilled with an appropriate gas mixture. In the illumination experiments the Thunberg tubes containing reaction mixtures were irradiated with white light from a tungsten lamp (100 watts) at a distance of 3 cm [24]. After addition of 1N HCl (2 ml) to terminate the reaction, the incubation mixture was cooled immediately in an ice-bath. To the incubation mixture were added 2-hydroxyestradiol (500 μg) as a carrier and [4-^{14}C]-estradiol (20,000 d.p.m.) as an internal standard together with ascorbic acid (2 mg) to prevent oxidative degradation of catechol estrogen. The reaction mixture was centrifuged at 3,000 *g* for 20 min to separate the protein-bound metabolites. The sediment was washed with water (2 ml), ethanol (2 ml), and ethyl acetate (2 ml), successively. The supernatant was in turn extracted with ethyl acetate (3 ml × 3) to separate the water-soluble fraction. A portion of the protein-bound metabolites dissolved in Soluene 100 and the water-soluble metabolites was submitted to radioactivity counting, respectively. The combined organic layer was evaporated to dryness *in vacuo*, and the residue obtained was dissolved in methanol (lipophilic fraction). The recovery of total radioactivity from these three fractions was found to be almost 100%.

Identification and quantitation of 2-hydroxyestradiol. When the lipophilic fraction was applied to t.l.c., the radioactivity was located in the zones corresponding to the substrate (R_F 0.55), 2-hydroxyestradiol (R_F 0.35), and the polar metabolites (R_F 0.12). The amount of the polar metabolites yielded from estradiol was almost equal to that of 2-hydroxyestradiol under standard conditions. Approximately one-third of the polar metabolites were characterized as estriol by the reverse isotope dilution technique. In contrast the amount of the polar metabolites yielded from the other substrates was less than 10% of 2-hydroxyestradiol. For quantitative determination of 2-hydroxy-

Table 1. Requirement of O_2 for the formation of 2-hydroxyestradiol from estradiol and 2-hydroxy-3-deoxyestradiol with rat liver microsomes

Condition	Estradiol	2-Hydroxyestradiol 2-Hydroxy-3-deoxyestradiol (nmol/mg protein/min*)
Air	0.24 ± 0.02	0.16 ± 0.01
N_2	0.23 ± 0.02	0.20 ± 0.02
+ Glucose	0.31 ± 0.04	0.19 ± 0.02
+ Glucose oxidase	0.20 ± 0.01	0.14 ± 0.01
+ Glucose + Glucose oxidase	0.005 ± 0.002	0.007 ± 0.005

* Mean ± S.D. (n = 6).

The standard mixture (see text) was incubated under aerobic or anaerobic conditions at 37°C for 30 min. Ten milligrams of glucose and 100 units of glucose oxidase were used.

Table 2. Effects of CO on the formation of 2-hydroxyestradiol from estradiol and 2-hydroxy-3-deoxyestradiol with rat liver microsomes

Gas phase	2-Hydroxyestradiol (nmol/mg protein/min*) Estradiol	2-Hydroxy-3-deoxyestradiol (nmol/mg protein/min*)
O_2/N_2 (7:93)	0.20 ± 0.02 (100)†	0.15 ± 0.03 (100)
$O_2/CO/N_2$ (7:28:65)	0.14 ± 0.01 (70)	0.12 ± 0.01 (80)
$O_2/CO/N_2$ (7:63:30)	0.12 ± 0.01 (60)	0.09 ± 0.01 (60)

* Mean ± S.D. ($n = 6$).

† Per cent of control values in parentheses.

The standard mixture was incubated in the dark at 37°C for 30 min under various gas conditions.

estradiol the zones corresponding to estradiol and 2-hydroxyestradiol were scraped off and then submitted to radioactivity counting. 2-Hydroxy-3-deoxyestradiol showed the same mobility on t.l.c. as estradiol when developed in this solvent system. The amounts of yielded 2-hydroxyestradiol and unchanged substrates were determined in correction to the recovery rate of [^{14}C]-estradiol added as an internal standard. In the preliminary experiments, [^{3}H]-2-hydroxyestradiol and [^{14}C]-estradiol added to the incubation mixture with boiled microsomes were recovered at the rates of 81.0% and 84.5% (mean of three determinations), respectively. 2-Hydroxyestradiol obtained from the lipophilic fraction was identified by means of t.l.c., gas chromatography–mass spectrometry and reverse isotope dilution technique [16]. The isotopic purity of 2-hydroxyestradiol recovered from the thin-layer chromatogram was found to be more than 93%.

RESULTS

Incubation of labeled estradiol and 2-hydroxy-3-deoxyestradiol with rat liver microsomes in the presence of NADPH under aerobic conditions resulted in the formation of 2-hydroxyestradiol. The reaction rate was linearly raised up with the increasing amounts of the microsomal preparation (up to 3 mg) and the substrate (up to 100 nmol) and with the incubation time (up to 30 min) for both substrates. Based upon these data aerobic incubation of a mixture consisting of 200 nmol of the substrate, 2 mg of the microsomal preparation, and NADPH at 37°C for 30 min was chosen as standard assay condition. The conversion rates of estradiol to catechol estrogen, the water-soluble metabolites and the protein-bound metabolites were 6–8, 1–2 and 5–7%, respectively under standard conditions. On the other hand those of 2-hydroxy-3-deoxyestradiol to the three metabolites were 4–5, 1 and 3–5%.

In order to clarify whether O_2 is required for the NADPH-dependent formation of catechol estrogen the two substrates were incubated in a nitrogen gas (N_2) atmosphere. No difference in the amount of the product was observed between air and N_2 gas conditions. However, when the glucose–glucose oxidase system [25] was added to the incubation mixture for removal of O_2 dissolved in medium, the enzyme activity was almost entirely lost (Table 1). The formation of the water-soluble and protein-bound metabolites was also inhibited under anaerobic conditions.

The influences of CO on the formation of 2-hydroxyestradiol from the two substrates by rat liver microsomes in the dark were then explored. The inhibitory effects of CO on the formation of catechol estrogen observed with the two substrates are listed in Table 2. It is evident from the data that 2- and 3-hydroxylases were susceptible to CO to nearly the same degree. The partition constant (K), the ratio of CO to O_2 required for 50% inhibition, was not significantly different between these two substrates: $K = 12.0$ for estradiol and $K = 14.1$ for 2-hydroxy-3-deoxyestradiol. The formation of other metabolites was inhibited more effectively by CO than that of catechol estrogen.

It is sufficiently substantiated that white light of a tungsten lamp is as effective as monochromatic light of 450 nm in reversing the inhibition of the cytochrome P-450-dependent reactions by CO [24]. Estradiol and 2-hydroxy-3-deoxyestradiol were incubated with rat liver microsomes in the presence of white light and in the dark under the O_2/N_2 (7:93) and $O_2/CO/N_2$ (7:63:30) gas mixture, respectively. As illustrated in Fig. 1, white light did not cause any remarkable change in the formation of catechol estrogen without CO, but brought about almost the complete reversal of the inhibitory effect due to CO. The inhibition of aryl hydroxylase by CO and its reversal by the light illumination were similarly observed with the two substrates. The inhibitory effects due to CO on the formation of other metabolites were also almost completely recovered by illumination.

The effects of pretreatment with phenobarbital, 3-methylcholanthrene, and clomiphene on the formation of 2-hydroxyestradiol by rat liver microsomes were also examined (Table 3). Administration of the former two increased the content of cytochrome P-450 and P-448 in liver microsomes by 420% and 210%, respectively, but did not exert any significant change in the yield of 2-hydroxyestradiol from estradiol and 2-hydroxy-3-deoxyestradiol. Pretreatment

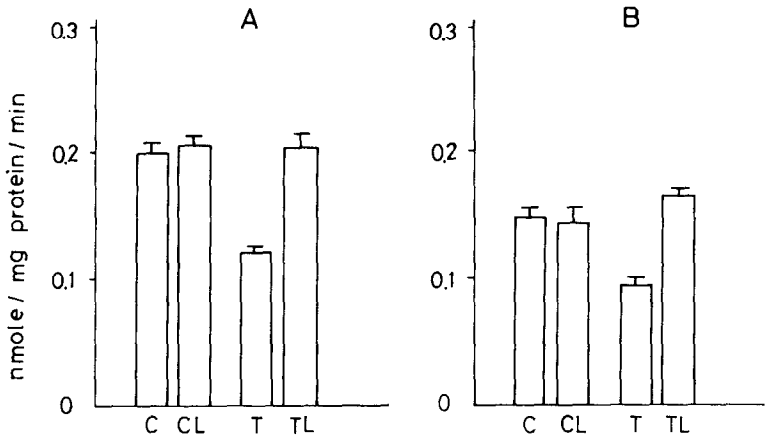

Fig. 1. Effects of illumination with white light on CO inhibition of the formation of 2-hydroxyestradiol from estradiol and 2-hydroxy-3-deoxyestradiol by rat liver microsomes. The standard mixture was incubated in the dark or under light illumination at 37°C for 30 min. The procedure used for irradiation is given in the text. A: estradiol. B: 2-hydroxy-3-deoxyestradiol. C: O_2/N_2(7:93). T: $O_2/CO/N_2$(7:63:30). L: irradiated with a tungsten lamp.

with clomiphene affected neither the content of cytochrome P-450 nor the activity of estadiol 2-hydroxylase. As for the formation of other metabolites administration of phenobarbital caused approximately 50% increase, but other pretreatments did not exert any influence.

The effects of various agents on the *in vitro* formation of the catechol from estradiol and 2-hydroxy-3-deoxyestradiol were also investigated (Table 4). *p*-Chloromercuribenzoate depressed the yielded amount of 2-hydroxyestradiol from the two substrates significantly while SKF-525A did to a certain extent. The radioactivity incorporated into the water-soluble and protein-bound fractions was diminished by these two chemicals. On the other hand cyanide and catalase did not show any remarkable influence on the formation of catechol estrogen and other metabolites.

The rate of the catechol formation from estradiol and 2-hydroxy-3-deoxyestradiol decreased to approximately 50% when the microsomal preparation was stored at −20°C in a N_2 gas atmosphere for 5 to 6 days (Fig. 2). The formation of other metabolites was similarly depressed by freezing storage.

DISCUSSION

In earlier papers hydroxylation of estradiol at C-2 by liver [3, 27, 28], placenta [29], and brain [30, 31] was discussed, but the characteristics of 2-hydroxylase have, so far, been little investigated. Marks and Hecker [32] showed that CO inhibits the formation of 2-hydroxyestrone from estrone by rat liver microsomes. However, the sensitivity of 2-hydroxylase to CO observed by these investigators is extremely low as compared with the present result. This may be ascribable to inaccurate quantitation of 2-hydroxyestrone because it is susceptible to oxidative decomposition [20] and in part converted into 2-hydroxyestradiol [33].

The cytochrome P-450-dependent enzyme system in the hepatic endoplasmic reticulum is of particular interest in the broad substrate specificity. In recent years it is becoming a popular view that the specificity is attributable to the presence of multi forms of cytochrome P-450 systems which differ from one another in their physical and catalytic properties [34–37]. Several workers indicated that the substrate specificity would be associated with the regulation of oxidative metabolism of steroids [36–41]. Haugen *et al.*[36] demonstrated the existence of four different types of cytochrome P-450 systems at least which are involved in hydroxylation of testosterone at the 6β-,7α- and 16α-positions. As for the hepatic metabolism of testosterone in the rat 16α-hydroxylase is induced by administration of phenobarbital, while

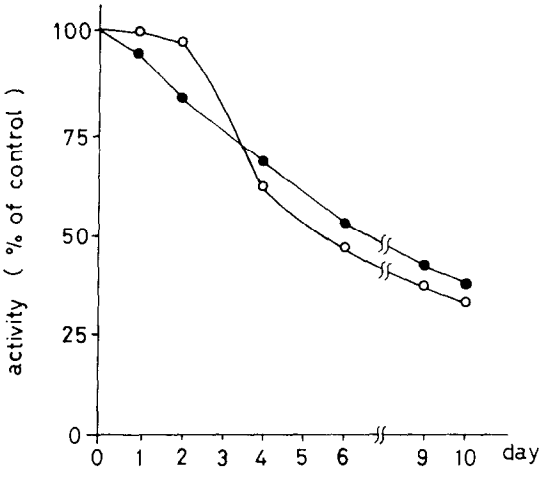

Fig. 2 Effects of freezing time of microsomal preparation on the formation of 2-hydroxyestradiol from estradiol and 2-hydroxy-3-deoxyestradiol. The enzyme assay was carried out with the standard mixture. ○—○: estradiol, ●—●: 2-hydroxy-3-deoxyestradiol.

Table 3. Effects of pretreatment with phenobarbital, 3-methylcholanthrene and clomiphene on the formation of 2-hydroxyestradiol from estradiol and 2-hydroxy-3-deoxyestradiol with rat liver microsomes

Pretreatment	Cytochrome P-450 or P-448 (nmol/mg protein)	Estradiol		2-Hydroxy-3-deoxyestradiol	
		Substrate recovered (nmol*)	2-Hydroxyestradiol formed (nmol/mg protein/min)†	Substrate recovered (nmol*)	2-Hydroxyestradiol formed (nmol/mg protein/min)†
Phenobarbital					
control	0.67	178	0.13 ± 0.01	188	0.08 ± 0.00
treatment	2.80	169	0.13 ± 0.01	185	0.07 ± 0.01
3-Methylcholanthrene					
control	0.63	177	0.14 ± 0.01	188	0.07 ± 0.00
treatment	1.31	177	0.15 ± 0.01	188	0.07 ± 0.01
Clomiphene					
control	0.59	173	0.16 ± 0.03	—	—
treatment	0.54	174	0.15 ± 0.03	—	—

* Mean value of six determinations.
†Mean ± S.D. ($n = 6$).
Male immature rats were administered with phenobarbital (80 mg/kg/day), 3-methylcholanthrene (40 mg/kg/day) or clomiphene (10 mg/kg/day). The assay was carried out by the standard method.

Table 4. Effects of inhibitors and other agents on the formation of 2-hydroxyestradiol from estradiol and 2-hydroxy-3-deoxyestradiol with rat liver microsomes

Inhibitor	Estradiol		2-Hydroxy-3-deoxyestradiol	
	Substrate recovered	2-Hydroxyestradiol formed	Substrate recovered	2-Hydroxyestradiol formed
None	100	100	100	100
SKF-525A				
0.07 mM	107	81	103	72
0.14 mM	116	39	106	41
0.28 mM	119	27	107	32
Catalase (50 μg)	98	109	100	102
p-Chloromercuribenzoate (0.4 mM)	125	9	112	6
KCN (1.0 mM)	100	99	100	96
Aminopyrine (0.15 mM)	101	97	101	94
Testosterone (0.15 mM)	100	110	100	99

The standard mixture was incubated with or without the agent at 37°C for 30 min. The amounts of 2-hydroxyestradiol formed from estradiol or 2-hydroxy-3-deoxyestradiol without the inhibitor were 0.26 or 0.17 nmol/mg protein/min and those of the two substrates were 157 or 177 nmol. These values were taken as 100%, respectively.

7α-hydroxylase activity is elevated by pretreatment with 3-methylcholanthrene [36, 41]. Gustafsson and his co-worker[37] reported that the multi forms of cytochrome P-450 systems in rat liver microsomes catalyze the different hydroxylation reactions of 5α-androstane-3α,17β-diol and its disulfate. In the present study estradiol 2-hydroxylase (K = 12.0) and 2-hydroxy-3-deoxyestradiol 3-hydroxylase (K = 14.1) have proved to be much less sensitive to CO than hydroxylases (K = 1–3) of testosterone [39], bile acid [4], and 5α-androstane-3α,17β-diol disulfate [40] and lyase of cholesterol [24]. Transformation into 2-hydroxyestradiol was not significantly depressed under the anaerobic conditions where the formation of glutathione conjugate from catechol estrogen is markedly suppressed [42]. Molecular oxygen dissolved in the incubation medium was enough to promote aromatic hydroxylations. In sharp contrast to 6β- and 7α-hydroxylases of testosterone and bile acids [39, 41] the activities of both 2- and 3-hydroxylases in rat liver were significantly depressed by storage in the frozen state. As was expected the content of cytochrome P-450 or P-448 was raised by pretreatment with phenobarbital and 3-methylcholanthrene. However, these inducers did not exert any remarkable effect on the formation of 2-hydroxyestradiol. The hepatic microsomal enzymes involved in 7α-hydroxylation of cholesterol [43] and 15β-hydroxylation of 5α-androstane-3α,17β-diol disulfate [40] are not significantly influenced by pretreatment, indicating the participation of different hydroxylases from the drug-metabolizing enzyme in these bioconversions. Any inhibitory effects on the *in vitro* formation of catechol estrogen were not observed with testosterone and aminopyrine. In order to confirm the reliability of the assay procedure for catechol estrogen the yielded amount of the water-soluble and protein-bound metabolites was to be estimated. The formation of these metabolites, however, was completely comparable to that observed in the previous study using 2-hydroxyestradiol as a substrate [42].

The present results together with the previous findings imply that hydroxylation of estradiol and 2-hydroxy-3-deoxyestradiol in ring A would be specific and regulated by different mechanisms from those for hydroxylation of testosterone and bile acids and that the different enzyme systems would be involved between the formation of catechol estrogen and its glutathione conjugate. It is well known that hydroxylations of estrogen at C-2 and C-16 are competitive and compensatory transformations in human [44]. Considerable attention has recently been focused to a finding that neurotransmitters are inhibited both *in vivo* and *in vitro* by catechol estrogen [10, 11]. The highly elevated production rate of catechol estrogen in brain may be associated with the prolonged effect of neurotransmitters. Luttge *et al.*[15] suggested a possible role of 2-hydroxyestradiol in the control of sexual behaviors in female rats. In addition, Naftolin *et al.*[13] demonstrated that the systematic administration of 2-hydroxyestrone to immature rats gives rise to a dramatic increase in the serum LH level. The potential role of catechol estrogen in the estradiol-mediated gonadotropin control prompted us to examine whether clomiphene used for inducing ovulation in the clinical states [45] may stimulate hydroxylation of estradiol at C-2 in liver. Any significant change in the activity of estradiol 2-hydroxylase in liver microsomes was not observed when clomiphene was given to immature rats.

The results obtained show that both isomeric phenolic steroids, estradiol and 2-hydroxy-3-deoxyestradiol, are converted into 2-hydroxyestradiol by rat liver microsomes in the presence of NADPH where the mixed function oxidase system catalyzed by cytochrome P-450 is operative. The properties of 2-hydroxy-3-deoxyestradiol 3-hydroxylase appear to be closely related to those of estradiol 2-hydroxylase although the activity of the former is much lower than that of the latter in rat liver. In connection with the present results an interesting paper by Paul *et al.*[46] appeared recently, describing the cytochrome P-450 characteristics of the brain 2-hydroxylase whose sensitivity to CO is different from that of the liver. 2-Hydroxy-3-deoxyestradiol exhibits no estrogenic activity, but its mode of biotransformation is quite similar to that of estradiol. In these respects the biological activity of this modified steroid in living animals is a fertile field for further investigation.

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