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PROPERTIES OF ENZYME SYSTEMS INVOLVED IN THE FORMATION OF CATECHOL ESTROGEN GLUTATHIONE CONJUGATES IN RAT LIVER MICROSOMES*

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Abstract—The properties of enzyme systems involved in the formation of the glutathione 1- and 4-thioethers of catechol estrogen from estradiol, 2-hydroxy-3-deoxyestradiol, or 2-hydroxyestradiol in rat liver microsomes have been investigated. Molecular oxygen was essential and NADPH was preferable as a cofactor to obtain the maximum activity with the three substrates. The presence of carbon monoxide suppressed the formation of the thioethers where the CO/O_2 ratios needed for 50 per cent inhibition of the bioconversion were 2.1 to 3.6. This inhibitory effect was reversed almost completely by illumination with white light. SKF-525A inhibited considerably the formation of the glutathione conjugates. Pretreatment with phenobarbital stimulated the formation of the thioethers from the three substrates by 100–220 per cent, whereas administration of 3-methylcholanthrene did not exert any significant influence. The storage of frozen microsomes resulted in a marked decrease in the enzyme activity: the initial activity was depressed to 50 per cent at 24 hr for catechol estrogen and at 4–5 days for phenol estrogens. The NADH-dependent enzyme activities were also inhibited by both SKF-525A and CO; the CO inhibition was reversed by light irradiation. It is evident from these data that cytochrome P-450 participated in both NADPH- and NADH-dependent formation of 2-hydroxyestradiol glutathione thioethers and two different enzyme systems are involved in this biotransformation between phenol estrogens and catechol estrogen.

Estrone† and estradiol are converted by rat liver microsomes to glutathione 1- and 4-thioethers of catechol estrogen and protein-bound metabolites under aerobic conditions [1–6]. The binding of estrogen with the sulfur-containing peptide or protein requires the sequential reactions involving hydroxylation at C-2, further oxidation into a semiquinone radical and nonenzymatic coupling with the sulfhydryl group [4, 7, 8]. Recently, we have demonstrated that 2-hydroxy-3-deoxyestradiol, which is a positional isomer of estradiol, is similarly transformed into 2-hydroxyestradiol and the binding metabolites [9], and a new hypothetic intermediate, presumably an arene-oxide, serves as a proximate precursor leading to the binding metabolites [10, 11]. However, the properties of the enzyme systems associated with the formation of the glutathione and protein conjugates still remains unclear, although an attempt at elucidation has been made by Marks and Hecker [12]. It appears to be of interest to clarify whether the conversion of estrogen into the binding metabolites is catalyzed by a mixed function oxidase or not. This paper describes the properties of the enzyme systems which are involved in the transformation of 2-hydroxyestradiol, estradiol and 2-hydroxy-3-deoxyestradiol into the glutathione thioethers of catechol estrogen, in comparison with the cytochrome P450-dependent mixed function oxidase system in rat liver microsomes.

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

† The following trivial names are used in the text: 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; 2-hydroexyestradiol 1-SG, *S*-(2,3,17β-trihydroxy-1,3,5(10)-estratrien-1-yl)-glutathione; 2-hydroxyestradiol 4-SG *S*-(2,3,17β-trihydroxy-1,3,5(10)-estratrien-4-yl)-glutathione.

MATERIALS AND METHODS

Animals. Male Wistar rats (10- to 13-weeks-old) weighing 250–300 g were used except for the induction experiments in which immature rats weighing 90–110 g were employed. The immature rats were injected intraperitoneally with either phenobarbital in saline (0.25 ml) at a dose of 80 mg/kg body weight or 3-methylcholanthrene in corn oil (0.25 ml) at a dose of 40 mg/kg body weight once daily for 4 days. The animals were fasted for the last 18 hr and sacrificed 24 hr after the last injection. The control rats were injected with the appropriate vehicles.

Materials. NADPH, NADH, $NADP^+$, NAD^+, glucose, glucose oxidase and glutathione were purchased from Sigma Chemical Co. (St. Louis, MO), Amberlite XAD-2 resin from Rohm & Haas Co. (Philadelphia, PA), Soluene 100 from Packard Instrument Co. (Downers Grove, IL), silica gel 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). [6,7-^{3}H]2-hydroxyestradiol (250 mCi/m-mole) was prepared enzymatically from [6,7-^{3}H]estradiol according to the procedure of Marks and Hecker [13]. [6,7-^{3}H]2-Hydroxy-3-deoxyestradiol (10 mCi/m-mole) was synthesized chemically from [6,7-^{3}H]estradiol by the methods

developed in these laboratories (total yield 28 per cent) [14, 15] and the radiochemical purity was checked by thin-layer chromatography (t.l.c.) prior to use. 2-Hydroxyestradiol 1-SG and 4-SG were synthesized according to the procedure of Kuss [5]. SKF-525A was kindly donated by Smith Kline & French Laboratories (Philadelphia, PA).

Thin-layer chromatography. Thin-layer chromatography was carried out on a plate coated with a layer (0.25 mm thick) of silica gel G and cellulose employing the following systems: TL-I *n*-butanol–acetic acid–water (4:1:1, v/v); TL-II 0.2 N acetic acid–0.1 N NH_4OH (14.7:20, v/v, pH 5.0), 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 silica gel and cellulose in scintillator liquid.

Radioactivity counting. Counting was carried out on a Packard Tri-Carb model 3380 liquid scintillation spectrometer employing Bray's scintillator [16]. Correction for quenching was made by the automatic external standard method.

Preparation of microsomes. The rats were sacrificed by decapitation, and the livers were 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 fraction was carefully transferred and centrifuged at 105,000 *g* for 60 min by a Hitachi model 40P ultracentrifuge. The separated microsomal pellet 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 4 mg protein. 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 10–15 mg protein.

Enzyme assay. In the assay for the formation of the glutathione thioethers, the standard reaction mixture contained the following: a microsomal preparation (0.5 ml) (equivalent to 2 mg protein), NADPH (4 μmoles), glutathione (0.4 μmole), ^{3}H-labeled substrate (0.2 μmole, 0.5 μCi) dissolved in 50% methanol (0.2 ml) and sufficient 0.05 M Tris–HCl buffer (pH 7.4) to make the total volume 1.5 ml. Incubation was carried out in a test tube at 37° for 20 min under aerobic conditions. When the effect of carbon monoxide (CO) and requirement for oxygen (O_2) were examined, the microsomal preparation and a solution containing both glutathione and cofactor were slowly flushed for 10 min with an appropriate gas mixture and then were pipetted separately into the main room 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 were irradiated with white light from a tungsten lamp (100 W) at a distance of 3 cm. After addition of 1 N HCl (2 ml), ascorbic acid (2 mg) and 2-hydroxyestradiol 1- and 4-SG (500 μg), the incubation mixture was brought to a 10% trichloroacetic acid solution and centrifuged at 3000 *g* for 20 min for removal of 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 assayed for radioactivity. The supernatant fraction was extracted with ethyl acetate (3 ml × 3). The aqueous phase was adjusted to pH 5.0 with 2 N NH_4OH, gently poured onto a column packed with Amberlite XAD-2 resin (20 ml) and percolated. The column was washed with distilled water (50 ml) and then eluted with methanol. The methanolic fraction was evaporated *in vacuo* below 50°, and the residue was dissolved in 0.5 N acetic acid (water-soluble fraction) and the radioactivity counted. The reliability of this procedure was demonstrated in preceding papers [9, 11]. The ^{3}H-labeled glutathione thioethers of catechol estrogen added to the incubation mixture with boiled microsomes were recovered at the rate of 81.0 per cent (mean of three determinations).

RESULTS

Incubation of the ^{3}H-labeled substrate, estradiol, 2-hydroxy-3-deoxyestradiol or 2-hydroxyestradiol with rat liver microsomes in the presence of NADPH and glutathione under aerobic conditions provided 2-hydroxyestradiol 1-SG and 4-SG. Approximately 86 per cent of the radioactivity in the water-soluble fraction was found at the zones corresponding to 2-hydroxyestradiol 1-SG (R_f TL-I 0.65, TL-II 0.62) and 4-SG (R_f TL-I 0.65, TL-II 0.45). The yields of the glutathione conjugates formed from the three substrates rose with the increasing concentration of glutathione up to 0.12–0.15 mM and then reached a plateau. After incubation with the denatured microsomal preparation, 1.8 and 3.8 per cent of the labeled substrates were recovered in the water-soluble and protein fractions respectively. The radioactivity incorporated into the protein fraction was 2.4 per cent when glutathione and NADPH were eliminated from the incubation mixture.

The enzyme activities in the formation of the glutathione conjugates of catechol estrogen from the three substrates were measured in the presence of various cofactors (Table 1). The most pertinent cofactor was NADPH and its optimal concentration was 4 mM in all the experiments. In addition, NADH also served as a cofactor and was one-third as potent as NADPH. In order to clarify whether O_2 is required for the NADPH-dependent formation of the thioethers, the three substrates were incubated with liver microsomes in a nitrogen gas (N_2) atmosphere. The amounts of the glutathione thioethers yielded from phenol and catechol estrogens under these conditions decreased to 5 and 25 per cent respectively. When the glucose–glucose oxidase system [18] was added to the incubation mixture for removal of O_2 dissolved in medium, the enzyme activity was lost almost entirely (Table 2). The influence of CO on the NADPH-dependent biotransformation by liver microsomes was then explored. The inhibitory

Table 1. Cofactor requirement for the formation of glutathione thioethers of 2-hydroxyestradiol from estradiol, 2-hydroxy-3-deoxyestradiol or 2-hydroxyestradiol with rat liver microsomes*

Cofactor	Estradiol	2-Hydroxy-3-deoxyestradiol	2-Hydroxyestradiol
	(pmoles/mg protein/min)		
NADPH	350 ± 30	270 ± 13	472 ± 22
NADH	115 ± 3	97 ± 5	155 ± 12
$NADP^+$	18 ± 1	15 ± 1	33 ± 5
NAD^+	3 ± 0	7 ± 1	47 ± 3
None	3 ± 0	3 ± 0	23 ± 2

*Microsomes (equivalent to 2 mg protein), ^{3}H-labeled steroid (0.2 μmole), glutathione (0.4 μmole) and cofactor (4 μmoles) were incubated under air at 37° for 30 min. Values are expressed as mean ±S.D. (n = 6).

Table 2. Requirement of O_2 for the formation of glutathione thioethers of 2-hydroxyestradiol from estradiol, 2-hydroxy-3-deoxyestradiol or 2-hydroxyestradiol with rat liver microsomes*

Gas phase	Estradiol	2-Hydroxy-3-deoxyestradiol	2-Hydroxyestradiol
	(pmoles/mg protein/min)		
Air	305 ± 15	200 ± 17	423 ± 28
N_2	15 ± 1	8 ± 0	107 ± 8
N_2 + glucose	25 ± 2	—	103 ± 10
N_2 + glucose oxidase	30 ± 2	—	63 ± 3
N_2 + glucose + glucose oxidase	3 ± 0	2 ± 0	13 ± 1

*The standard mixture was incubated under aerobic (air) or anaerobic conditions at 37° for 30 min. Ten mg glucose and 100 units glucose oxidase were used. Values are expressed as mean ±S.D. (n = 4).

effects of CO on the formation of the glutathione conjugates observed with the three substrates are listed in Table 3. It is evident from the data that the phenol and catechol substrates were susceptible to CO to almost the same degree. However, a slight difference was seen in the partition constant (K), the ratio of CO to O_2 required for 50 per cent inhibition, between 2-hydroxyestradiol and other substrates (Table 4). The results imply that the enzyme systems involved in aryl hydroxylation and glutathione conjugation would not necessarily be identical with respect to CO sensitivity.

Table 4. Ratio of CO to O_2 required for 50 per cent inhibition of the formation of glutathione thioethers of 2-hydroxyestradiol from estradiol, 2-hydroxy-3-deoxyestradiol or 2-hydroxyestradiol with rat liver microsomes*

Substrate	K
Estradiol	2.2
2-Hydroxy-3-deoxyestradiol	2.1
2-Hydroxyestradiol	3.6

* The partition constant (K), the ratio of CO to O_2 needed to produce 50 per cent inhibition, was calculated from the mean value for two levels of concentration of CO (see Table 3).

It has already been demonstrated that white light of a tungsten lamp is as effective as monochromatic light of 450 nm in reversing the inhibition of cytochrome P450-dependent reactions by CO [19]. The three substrates were incubated in the presence of white light and in the dark under the O_2/N_2 (7:93, v/v) and $O_2/CO/N_2$ (7:65:28, v/v) gas mixtures respectively. It is evident from the data in Table 3 that white light did not cause any significant change in the formation of the thioethers without CO, but brought about the partial reversal of the inhibitory effects due to CO.

The effects of various agents on the formation of the glutathione thioethers from estradiol, 2-hydroxy-3-deoxyestradiol or 2-hydroxyestradiol with rat liver microsomes were examined (Table 5). SKF-525A markedly decreased the enzyme activity with the three substrates, while cyanide exerted a lesser effect. Hydrogen peroxide (H_2O_2) did not exhibit any significant effect on the formation of the glutathione conjugates.

The influences of pretreatment with phenobarbital and 3-methylcholanthrene on the production of the glutathione thioethers were also investigated. The results obtained are collected in

Table 3. Effects of CO on the formation of glutathione thioethers of 2-hydroxyestradiol from estradiol, 2-hydroxy-3-deoxyestradiol or 2-hydroxyestradiol with rat liver microsomes and reversal of the inhibition by illumination with white light*

Gas phase	Estradiol	2-Hydroxy-3-deoxyestradiol	2-Hydroxyestradiol
	(pmoles/mg protein/min)		
O_2/N_2 (7:93)	255 ± 20	208 ± 17	335 ± 27
O_2/N_2 (7:93)	268 ± 27†	—	347 ± 33†
$O_2/CO/N_2$ (7:28:65)	101 ± 8	68 ± 8	150 ± 18
$O_2/CO/N_2$ (7:65:28)	40 ± 5	27 ± 4	91 ± 13
$O_2/CO/N_2$ (7:65:28)	225 ± 31†	161 ± 17†	259 ± 38†

* The standard mixture was incubated in the dark or under light illumination at 37° for 30 min under various gas conditions. Values are expressed as mean ±S.D. (n = 4).

† Results when the mixtures were irradiated with white light (see text).

Table 5. Effects of inhibitors on the formation of glutathione thioethers of 2-hydroxyestradiol from estradiol, 2-hydroxy-3-deoxyestradiol or 2-hydroxyestradiol with rat liver microsomes*

Inhibitor	Estradiol	2-Hydroxy-3-deoxyestradiol	2-Hydroxyestradiol
None	100	100	100
KCN, 1.0 mM	74†	70†	68†
SKF-525A			
0.035 mM	27†	—	44†
0.075 mM	23†	26†	32†
H_2O_2,			
0.01 mM	96	101	98
Norepinephrine			
0.05 mM	—	—	93
0.10 mM	—	—	93
0.15 mM	—	—	86

* The standard mixture was incubated with or without the inhibitor at 37° for 30 min. Each number is the mean of four determinations. The amounts of the thioethers formed from estradiol, 2-hydroxy-3-deoxyestradiol or 2-hydroxyestradiol without the inhibitor were 308, 230 or 435 pmoles/mg of protein/min and were taken as 100 per cent, respectively.

† $P < 0.01$.

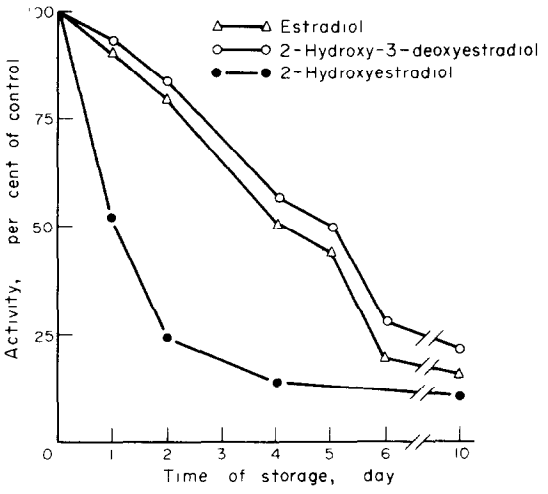

Fig. 1. Effects of freezing time of microsomes on the formation of glutathione thioethers of 2-hydroxyestradiol from estradiol, 2-hydroxy-3-deoxyestradiol or 2-hydroxyestradiol. Microsomes were stored at −20° under N_2 gas. The enzyme assay was carried out as described in Table 1.

Table 6. Administration of phenobarbital increased the formation of the thioethers from the three substrates by 200–320 per cent. On the other hand, pretreatment with 3-methylcholanthrene did not significantly change the amount of the binding metabolites produced from the three substrates. As for the ratio of 2-hydroxyestradiol glutathione 1- to 4-thioether derived from the two phenol substrates, no remarkable change was brought about by pretreatment with phenobarbital.

The rate of conversion into the glutathione conjugates from estradiol and 2-hydroxy-3-deoxyestradiol decreased to approximately 50 per cent when the microsomal preparation was stored at −20° in a N_2 gas atmosphere for 4–5 days. In contrast, the enzyme activity involved in the formation of the binding metabolites from 2-hydroxyestradiol decreased by half after being frozen for only 24 hr (Fig. 1).

The formation of the thioethers from the three substrates was examined with the use of NADH as a cofactor under a variety of conditions. The enzyme activity was inhibited completely by the addition of the glucose–glucose oxidase system under anaerobic conditions and was remarkably affected by SKF-525A (Table 7). In addition, it was suppressed to a certain extent by CO where the K value was of the same order as that observed with NADPH, and the inhibition was reversed in part by white light (Table 8).

DISCUSSION

Estradiol, 2-hydroxy-3-deoxyestradiol and 2-hydroxyestradiol were converted into glutathione 1- and 4-thioethers of catechol estrogen by rat liver microsomes in the presence of glutathione. The present study has demonstrated that different cytochrome P-450 systems would be involved in the formation of the glutathione conjugates be-

Table 6. Effects of pretreatment with phenobarbital and 3-methylcholanthrene on the formation of 2-hydroxyestradiol glutathione 1- and 4-thioethers from estradiol, 2-hydroxy-3-deoxyestradiol or 2-hydroxyestradiol with rat liver microsomes

Pretreatment	Glutathione thioethers* (pmoles/mg protein/min)			Ratio of 1- to 4-thioether†	
	Estradiol	2-Hydroxy-3-deoxyestradiol	2-Hydroxyestradiol	Estradiol	2-Hydroxy-3-deoxyestradiol
3-Methylcholanthrene					
Control	272 ± 4	191 ± 12	325 ± 14	—	—
Treatment	281 ± 17	195 ± 32	285 ± 27	—	—
Phenobarbital					
Control	256 ± 14	168 ± 18	308 ± 22	2.10 ± 0.4	0.96 ± 0.1
Treatment	807 ± 42‡	442 ± 40‡	608 ± 47‡	2.26 ± 0.6	0.89 ± 0.2

* Mean ±S. D. ($n = 6$).

† Mean ±S. D. ($n = 4$).

‡ $P < 0.01$.

Table 7. NADH-dependent formation of glutathione thioethers of 2-hydroxyestradiol from estradiol, 2-hydroxy-3-deoxyestradiol or 2-hydroxyestradiol with rat liver microsomes under various conditions*

Gas phase	Inhibitor	Estradiol	2-Hydroxy-3-deoxyestradiol	2-Hydroxyestradiol
Air	None	100	100	100
Air	SKF-525A (0.075 mM)	21	16	30
N_2	None	12	8	16
N_2	Glucose + glucose oxidase	3	1	4

* Microsomes (equivalent to 2 mg protein), ^{3}H-labeled steroid (0.2 μmole), glutathione (0.4 μmole) and NADH (4 μmoles) were incubated at 37° for 30 min under the conditions described in Tables 2 and 3. The amounts of the thioethers formed from estradiol, 2-hydroxy-3-deoxyestradiol or 2-hydroxyestradiol were 88, 77 or 152 pmoles/mg of protein/min and were taken as 100 per cent respectively.

Table 8. Effects of CO on the NADH-dependent formation of glutathione thioethers of 2-hydroxyestradiol from estradiol, 2-hydroxy-3-deoxyestradiol or 2-hydroxyestradiol and reversal of the inhibition by illumination with white light*

Gas phase	Estradiol	2-Hydroxy-3-deoxyestradiol	2-Hydroxyestradiol
O_2/N_2 (7:93)	97 ± 3	70 ± 7	135 ± 7
$O_2/CO/N_2$ (7:65:28)	23 ± 4 (3.1)†	20 ± 3 (3.4)	47 ± 5 (1.3)
$O_2/CO/N_2$ (7:65:28)	85 ± 13‡	68 ± 8‡	120 ± 19‡

* ^{3}H-Labeled steroid was incubated in the dark or under light illumination as described in Table 3. The K value was obtained by the method described in Table 4. Values are expressed as mean ±S. D. (n = 4).

tween catechol estrogen and two isomeric phenol estrogens. The role of cytochrome P450 as a terminal oxidase in the biotransformation of steroids and other foreign compounds has been sufficiently substantiated [20–24]. The substrate specificity is ascribable to the existence of multiple forms of cytochrom P450. Lu *et al.* [25] reported that cytochrome P448 derived from 3-methylcholanthrene-treated rats and cytochrome P-450 from phenobarbital-treated rats were equally potent for 6β- and 7α-hydroxylation of testosterone, whereas the former was only 10–20 per cent as potent as the latter for 16α-hydroxylation. Recently, Haugen *et al.* [23] demonstrated that cytochrome P450 LM_2 induced in rabbit liver microsomes by pretreatment with phenobarbital is previously associated with 16α-hydroxylation while cytochrome P450 $LM_{1,7}$ is involved in 6β-hydroxylation. The 16α-hydroxylation was more susceptible to CO than other steroid hydroxylases [26]. Storage of a frozen liver microsomal suspension significantly decreased its 16α-hydroxylase activity but resulted in a smaller change in other hydroxylase activities [27].

In the present study, pretreatment with phenobarbital increased by 200–320 per cent the formation of glutathione conjugates from catechol estrogen by rat liver microsomes. Pretreatment with 3-methylcholanthrene was without effect. In addition, this enzyme activity was markedly decreased by freezing the microsomal suspension and was highly sensitive to CO. These results and the previous findings suggest that the cytochrome P450 system associated with conjugation of 2-hydroxyestradiol with glutathione through the formation of a semiquinone radical may be similar to testosterone 16α-hydroxylase.

Results obtained using estradiol and 2-hydroxy-3-deoxyestradiol as substrates were markedly different from those obtained with catechol estrogen. This appears to be ascribable to the difference in the metabolic routes between the two substrates, phenol and catechol estrogen. The formation of the glutathione conjugates from phenol estrogen takes two different routes. One involves the participation of 2-hydroxyestradiol as an intermediate and the other proceeds through an arene-oxide which also serves as an intermediate leading to catechol estrogen [11]. The enzyme system involved in the bioconversion of the two phenol estrogens was more stable against freezing storage and more effectively inhibited by CO and SKF-525A than that involved in the metabolism of 2-hydroxyestradiol. The increased rates of formation of the glutathione conjugates caused by the pretreatment with phenobarbital were nearly equal for the three substrates. The relative amounts of glutathione 1- to 4-thioether formed were not significantly influenced by administration of the inducer. Marks and Hecker [12] demonstrated that the formation of the glutathione thioethers from estrone was inhibited by CO more effectively than that from 2-hydroxyestrone with rat liver microsomes. These results and the previous findings imply that oxidation of the phenol steroids yielding catechol estrogen or arene-oxide may be catalyzed by the cytochrome P450 system which differs from the enzyme system involved in the formation of the semiquinone radical.

NADPH is usually the most efficient electron

donor for the oxygenase system in hepatic microsomes. Although the hydroxylation rate is somewhat slow, NADH is also capable of serving as an electron donor for the cytochrome P450 system [28–30]. It is generally accepted that liver microsomes contain two electron transport systems and cytochrome P450 can be reduced by NADH through the electron carriers in the cytochrome b_5 chain [31–33]. Recently, Wollenberg *et al.* [34] reported that 2-hydroxyestradiol inhibited the NADPH-dependent reduction of cytochrome P450 and cytochrome b_5 as an uncoupler of microsomal electron transport. Considering these results it can be supposed that the formation of the glutathione conjugates by the cytochrome P450 system is also fortified by NADH. The relatively higher NADH dependency compared with that of other steroid hydroxylases may be associated with the action of 2-hydroxyestradiol as an uncoupler of electron transport.

Complete inhibition of the enzyme activity caused by addition of the glucose–glucose oxidase system might not be due to H_2O_2 produced during incubation, because H_2O_2 added to the incubation mixture did not exert any significant influence on the formation of the glutathione conjugates.

Scheulen *et al.* [35] reported that rat liver microsomes catalyzed the NADPH-dependent irreversible binding of dopa and dopamine metabolites to microsomal protein where the participation of cytochrome P450 was not necessarily required. In the present study it has been demonstrated that 2-hydroxyestradiol was converted into the glutathione conjugates by cytochrome P450 in rat liver microsomes and the conjugation activity of catechol estrogen was not significantly inhibited by norepinephrine. These results strongly imply that different enzyme systems may be involved in the formation of the conjugated metabolites between catecholamine and catechol estrogen.

Considering the close similarities in the mode of biotransformation between aromatic steroid and polycyclic hydrocarbon [11] and between catechol estrogen and catecholamine [36, 37], elucidation of biological activities other than the non-endocrine action seems to be a fertile field for further investigation.

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