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Activation and Irreversible Binding of Regiospecifically Labeled Catechol Estrogen by Rat Liver Microsomes: Evidence for Differential Cytochrome P-450 Catalyzed Oxidations†

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ABSTRACT: Estradiol and 2-hydroxyestradiol labeled with ^3H at different positions in rings A or B were incubated with male rat liver microsomes, and their oxidative transformation was followed by the transfer of ^3H into ^3H_2O. ^{14}C-Labeled estrogen or catechol estrogen was used to determine the fraction that becomes bound covalently to microsomal protein. The further metabolism of 2-hydroxyestradiol involves activation of the steroid at C-4 and, to a much lesser extent at C-1, by a cytochrome P-450 mediated reaction as indicated by the effects of NADPH, spermine, SKF-525A, and CO in the microsomal system. Glutathione promoted the loss of ^3H from C-4 of either estradiol or 2-hydroxyestradiol but had less effect on this reaction at C-1 and inhibited it at C-6,7. It also abolished the irreversible binding of ^{14}C-labeled estradiol and 2-hydroxyestradiol to microsomal protein. NADPH was needed specifically for glutathione to exert its effect both on the transfer of ^3H into ^3H_2O and on the formation of water-soluble products from catechol estrogen by rat liver microsomes. It could not be replaced by NADP, NAD, or NADH. Ascorbic acid inhibited these enzymatic reactions but did not affect significantly the initial 2-hydroxylation of estradiol. Evidence is also provided for the further hydroxylation of 2-hydroxyestradiol at C-6 (or C-7). These results indicate that cytochrome P-450 activates catechol estrogens by an electron abstraction process.

In recent years, the possibility has emerged that some of the actions of estradiol (E_2) may involve significant contributions from its metabolites (Fishman & Martucci, 1978). In particular, the catechol estrogens formed by 2- or 4-hydroxylation of the parent estrogens have been shown to modulate a number of important endocrine functions when administered in pharmacological doses (MacLusky et al., 1981).

Relatively little is known about the further oxidative metabolism of the catechol estrogens although their conversion to water-soluble products (Jellinck & Smith, 1974) and their irreversible binding to liver microsomal protein in the presence of oxygen and NADPH or a superoxide generating system have been reported (Nelson et al., 1976). The synthesis of 2-hydroxyestradiol (2-OHE_2) labeled regiospecifically with ^3H at C-1, C-4, or C-6,7 has made it possible to obtain information about the site of interaction of the steroid with nucleophiles such as glutathione (GSH) (Jellinck et al., 1984) without having to isolate and characterize the products, and more recently, we have reported on the possible mechanism of such adduct formation (Jellinck et al., 1986). In this paper, we assess the contribution of cytochrome P-450 to the further metabolism of the catechol estrogens at specific carbon atoms of the steroid molecule by rat liver microsomes using agents known to increase or inhibit the activity of this enzyme system. We also report on studies of the role of microsomal monooxygenases on the interaction of [4-^{14}C]2-OHE_2 with GSH and show that this reaction is dependent specifically on NADPH. In addition, GSH was found to prevent the irreversible binding of the catechol estrogen to microsomal protein.

Experimental Procedures

Materials. 2-^3H- and 4-^3H-labeled estradiols were prepared and purified as described previously (Jellinck et al., 1984). [4-^{14}C]E_2 (57 mCi/mmol; New England Nuclear Corp., Boston, MA) was shown by chromatography and autoradiography to be free of radioactive impurities. It was diluted with carrier to a specific activity of 2–3 mCi/mmol and kept

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Table I: Comparative Release of ^3H from C-1 or C-4 of 2-Hydroxyestradiol or Formation of Water-Soluble Metabolites by Rat Liver Microsomes[a]

	[1-^{3}H]2-OHE$_2$			[4-^{3}H]2-OHE$_2$		
experimental conditions	^3H transfer into ^3H_2O (%)	water-soluble radioactivity (%)	^3H-labeled aqueous metabolites (%)	^3H transfer into ^3H_2O (%)	water-soluble radioactivity (%)	^3H-labeled aqueous metabolites (%)
no additions	5.3 ± 0.7	21.7 ± 1.5 (3)	16.4	10.2 ± 0.7	21.0 ± 1.5 (11)	10.8
NADPH	2.2 ± 0.6	8.5 ± 2.3 (4)	6.3	3.3 ± 0.4	9.0 ± 0.8 (5)	5.7
M	15.2 ± 3.2	23.5 ± 3.3 (6)	8.3	19.2 ± 1.9	23.7 ± 2.7 (15)	4.5
M + NADPH	16.4 ± 1.3	35.4 ± 1.9 (6)	19.0	27.6 ± 1.6	37.4 ± 2.2 (15)	9.8
M (boiled)	14.8 ± 3.0	18.9 ± 3.9 (2)	4.1	15.1 ± 1.4	19.7 ± 1.7 (5)	4.6
M (boiled) + NADPH	10.9 ± 0.8	19.4 ± 2.3 (2)	8.5	13.0 ± 0.9	18.4 ± 1.3 (5)	5.4

[a] The ^3H-labeled steroids (8 μM) were incubated for 30 min at 37 °C in 4 mL of Tris-HCl (0.1 M), pH 7.4, with male rat liver microsomes (M) (from 50 mg of tissue) or in the presence or absence of NADPH (0.14 mM). The amount of ^3H_2O formed during the reaction and the amount of ^3H radioactivity remaining in the ether-extracted aqueous fractions were determined as described under Experimental Procedures. Data are presented as the mean ± SE or ± range when $n = 2$. Numbers in parentheses are the number of experiments.

at 4 °C in the dark as a stock solution in ethanol (1 mg/mL). [6,7-^{3}H]E_2 (52 Ci/mmol) was purchased from the same supplier while [1-^{3}H]E_2 was prepared from [1,2α-^{3}H] testosterone by aromatization, after removal of ^3H from C-2 of the steroid (Jellinck et al., 1984). [1-^{3}H]2-OHE$_2$ and [4-^{3}H]2-OHE$_2$ were prepared enzymatically from the ^3H-labeled parent estrogen by the tyrosinase method (Jellinck & Brown, 1971). Tyrosinase (EC 1.14.18.1, 2000 units/mg), NADPH, and spermine tetrahydrochloride were purchased from Sigma Chemical Co. (St. Louis, MO); all chemicals were of the purest grade available commercially. SKF-525A was a gift from Smith, Kline, and French Canada, Mississauga, Ontario.

Treatment of Animals and Fractionation of Liver. Mature male (300–400 g) Sprague-Dawley rats (Charles River Breeding Laboratories, Wilmington, MA) were used. The animals were killed by cervical dislocation after CO_2 anesthesia, the livers were perfused with cold 0.15 M KCl to remove hemoglobin, and a 10% (w/v) homogenate was prepared in 0.25 M sucrose with a Potter-Elvejhem homogenizer with a Teflon pestle. It was centrifuged at 8000*g* for 15 min, and a microsomal fraction was obtained from the supernatant by centrifuging at 105000*g* for 1 h, washing the pellet by redispersal in sucrose and centrifugation, and resuspending it in sucrose. The microsomes, derived from 200 mg of original tissue/mL, could be stored at −70 °C without loss of hydroxylating activity for several months.

Conditions of Incubation. ^3H- or ^{14}C-labeled E_2 [8 μM; (4.7–7.0) × 10^5 dpm] was incubated for 30 min with constant shaking at 37 °C with the resuspended microsomes (105000*g* pellet) from 50 mg of liver (~0.8 mg of protein) and NADPH (0.14 mM) in 0.1 M Tris-HCl buffer, pH 7.4, in a total volume of 4 mL. NADH (0.67 mM) was used for the synthesis of the catechol estrogen by tyrosinase (0.2 mg), while in the experiments with CO the gas was bubbled through the mixture for 0.5 min before incubation in the dark. After extraction 3 times with equal volumes of diethyl ether, any residual solvent was removed from the aqueous fraction by a stream of N_2, and the radioactivity in 0.2 mL was determined in a Beckman LS 7500 scintillation counter.

^3H_2O Formation and Irreversible Binding to Microsomes. The formation of ^3H_2O was determined from the difference between the ^3H radioactivity in 0.5 mL of the original aqueous fraction and that in the residue from the same sample redissolved in 0.5 mL of H_2O after evaporation to dryness in a fume hood at room temperature. This method gave results identical with those obtained by lyophilization (Jellinck et al., 1984). The amount of substrate which is converted to products that have reacted at positions other than the one bearing the ^3H label and which are not extractable by ether was assessed by subtracting the amount of ^3H_2O formed from the total water-soluble ^3H radioactivity. To measure irreversible binding of ^{14}C-labeled metabolites of E_2 to liver microsomes, 1 mL of HCl (1 M) and 2 mL of trichloroacetic acid (1.5 M) were added to 2 mL of the extracted aqueous medium, and the mixture, after being stirred by vortex, was allowed to stand for 1 h at 25 °C. It was spun at 1000*g* for 15 min and the precipitate washed with trichloroacetic acid (1 mL) followed by ethanol (2 × 1 mL) by repeated stirring and sedimentation at 1000*g*. The residue was dissolved in NCS tissue solubilizer (Amersham), and 0.2 mL of this solution was counted. Values are expressed as means ± SEM or as means (range: <10%) when experiments were repeated only once.

Examination of the Organic Phase. The organic extract, after drying over anhydrous Na_2SO_4, was evaporated to dryness under N_2 and the residue dissolved in a few drops of ethanol. It was applied together with a drop of ascorbic acid (50 mM) in methanol–acetic acid (98:2) and carrier 2-OHE$_2$ (10 μg) onto silica gel precoated on aluminum sheets (Merck) and examined by TLC using cyclohexane–ethyl acetate–ethanol (10:9:1) followed by autoradiography.

Results

Comparative Loss of ^3H from C-1, C-4, or C-6,7 of 2-Hydroxyestradiol. A major problem in studying the further metabolism of 2-hydroxyestradiol formed from the parent estrogen by the cytochrome P-450 system of liver microsomes is its rapid nonenzymatic oxidation as illustrated in Table I. Even without the addition of microsomes a considerable amount of ^3H_2O is released during incubation of either [1-^{3}H]- or [4-^{3}H]2-OHE$_2$ with the formation of products which are no longer extractable with ether. The nonenzymatic reaction was inhibited by NADPH, acting presumably as an antioxidant, but this effect was less pronounced with boiled microsomes.

Liver microsomes increased the yield of ^3H_2O and water-soluble products with both [1-^{3}H]- and [4-^{3}H]2-OHE$_2$, but an increase in the formation of ^3H_2O when NADPH was added was only observed with [4-^{3}H]2-OHE$_2$ which indicates preferential activation at C-4 with concomitant loss of ^3H from this position of the steroid. The effect of NADPH is even greater than indicated because the nontissue control value was lower in the presence of the cofactor than when NADPH is omitted.

The involvement of cytochrome P-450 in the reaction is supported by the increased generation of ^3H_2O from [4-^{3}H]-2-OHE$_2$ brought about by spermine in the presence of NADPH and the decrease to the values observed with unfortified microsomes when SKF-525A or CO was present

Table II: Effect of Spermine, Glutathione, SKF-525A, Ascorbic Acid, and CO on Release of ^3H or Formation of Water-Soluble Metabolites from ^3H-Labeled 2-Hydroxyestradiol by Rat Liver Microsomes[a]

additions	^3H transfer into ^3H_2O (%)	water-soluble radioactivity (%)	^3H-labeled aqueous metabolites (%)
[1-^3H]2-OHE_2			
none	2.2 ± 0.6 (5.3 ± 0.7)[b]	8.5 ± 2.3 (21.7 ± 1.5)[b]	6.3
M	16.4 ± 1.3 (15.2 ± 3.2)[b]	35.4 ± 1.9 (23.5 ± 3.3)[b]	20.0
M + Sp	20.0	43.2	23.2
M + GSH	20.6 ± 4.9 (4.8 ± 2.4)	72.5 ± 2.5 (12.0 ± 1.7)	51.9
M + SKF-525A	11.8 ± 1.0	21.7 ± 0.9	9.9
M + Asc	2.2	7.6	5.4
M + CO	5.6	15.5	9.9
[4-^3H]2-OHE_2			
none	3.3 ± 0.4 (10.2 ± 0.7)[b]	9.0 ± 0.8 (21.0 ± 1.5)[b]	5.7
M	26.9 ± 1.6 (16.9 ± 1.1)	36.6 ± 2.1 (21.0 ± 1.0)	9.7
M + Sp	32.3 ± 2.4 (16.7 ± 1.6)	46.0 ± 3.3 (20.6 ± 1.7)	13.7
M + GSH	38.3 ± 2.9 (11.1 ± 1.2)	71.9 ± 1.3 (18.6 ± 1.4)	33.6
M + SKF-525A	15.5 ± 0.7 (14.1 ± 2.1)	23.0 ± 1.9 (17.8 ± 1.6)	7.5
M + Asc	3.0 (1.0)	7.9 (2.0)	4.9
M + CO	7.6	8.9	1.3

[a] The ^3H-labeled steroids (8 μM) were incubated with male rat liver microsomes in the presence of NADPH (0.14 mM), spermine (0.3 mM), glutathione (2 mM), SKF-525A (0.2 mM), ascorbic acid (1 mM), or CO under the same conditions as in Table I. Data are presented as mean ± SE (n = 5) or as a mean only when n = 2. Values in parentheses were obtained in the absence of NADPH. [b] From Table I.

Table III: Effect of Coenzymes, SKF-525A, and Carbon Monoxide on Release of ^3H or Formation of Water-Soluble Metabolites from [4-^3H]-2-Hydroxyestradiol by Liver Microsomes in the Presence of Glutathione[a]

additions to microsomes + GSH	^3H transfer into ^3H_2O (%)	water-soluble radioactivity (%)	^3H-labeled aqueous metabolites (%)
none	2.3	9.3	7.0
NAD	11.2	24.8	13.6
NADH	10.4	22.3	11.9
NADP	6.8	15.9	9.1
NADPH	42.1	75.6	33.5
NADPH + SKF-525A	10.5	20.7	10.2
NADPH + CO	4.3	8.8	4.5

[a] The ^3H-labeled steroid (8 μM) was incubated with male rat liver microsomes and GSH (2 mM) in the presence of nicotinamide coenzymes (0.14 mM), SKF-525A (0.2 mM), or carbon monoxide under the same conditions as in Table I. Values are the means of two experiments.

(Table II). The rates of ^3H_2O formation from [4-^3H]2-OHE_2 in the presence of glutathione (GSH) were shown to be higher than those from [4-^3H]E_2, and both reactions required NADPH (Figure 1). GSH produced a significant increase in the release of ^3H_2O (26.9 → 38.3%) and the formation of water-soluble products from [4-^3H]2-OHE_2 (Table II). This reaction was also dependent on NADPH with virtually no increase over control values being observed when the reduced coenzyme was replaced by NADP, NAD, or NADH. It was also inhibited by SKF-525A and CO (Table III). The formation of ^3H_2O was less marked (16.4 → 20.6%) with [1-^3H]2-OHE_2 as substrate (Table II).

Less ^3H_2O was formed from [6,7-^3H]2-OHE_2 than from the ring A labeled catechol estrogens, and GSH decreased rather than increased the loss of ^3H from the 6,7-labeled catechol estrogen. SKF-525A and ascorbic acid still acted as inhibitors (Table IV). It was also shown, with ^{14}C-labeled E_2 and 2-OHE_2, that GSH abolished irreversible binding of these compounds to microsomal protein (Table V).

Comparative Loss of ^3H from C-2, C-4, or C-6,7 of Estradiol. With [2-^3H]E_2 where loss of ^3H represents hydroxylation almost exclusively, spermine increased and SKF-525A or CO decreased this reaction, while GSH and ascorbic acid had little effect. With this labeled substrate the yield of ^3H_2O and the amount of ^3H radioactivity remaining in the aqueous fraction were almost the same, in contrast to the values observed with estradiol containing the tritium label in other positions of the ring (Tables IV and VI). When [4-

Table IV: Effect of Spermine, Glutathione, SKF-525A, and Ascorbic Acid on Release of ^3H or Formation of Water-Soluble Metabolites from Estradiol and 2-Hydroxyestradiol Labeled with ^3H at C-6,7 by Rat Liver Microsomes[a]

additions	^3H transfer into ^3H_2O (%)	water-soluble radioactivity (%)	^3H-labeled aqueous metabolites (%)
[6,7-^3H]E_2			
none	0 (0)	2.0 (2.1)	2.0
M	6.0 (2.1)	21.0 (7.4)	15.0
M + Sp	8.7	33.2	24.5
M + GSH	2.2	37.6	35.4
M + SKF-525A	1.5	6.4	4.9
M + Asc	2.6	8.2	5.6
[6,7-^3H]2-OHE_2			
none	0 (5.8)	7.7 (24.6)	7.7
M	13.7 (9.1)	40.1 (22.3)	26.4
M + Sp	16.3	44.6	28.3
M + GSH	8.8	73.4	64.6
M + SKF-525A	4.4	23.9	19.5
M + Asc	8.1	15.2	7.1

[a] The ^3H-labeled steroids (8 μM) were incubated with male rat liver microsomes under the same conditions as in Table II. Data are the means of two experiments. Values in parentheses were obtained in the absence of NADPH.

Table V: Formation of Water-Soluble Products and Irreversible Binding of ^{14}C-Labeled Estradiol and 2-Hydroxyestradiol to Liver Microsomes[a]

additions	^{14}C radioactivity in aqueous fraction (%)	irreversible binding of ^{14}C radioactivity to microsomal protein (%)
[4-^{14}C]E_2		
M	39.0 (3.9)	19.1 (0.6)
M + Sp	44.9 (3.9)	21.1 (0.4)
M + GSH	45.1 (5.1)	0.4 (0.1)
M + SKF-525A	5.5 (4.0)	0.9 (0.4)
[4-^{14}C]2-OHE_2		
M	39.5 (16.8)	20.4 (11.1)
M + Sp	49.8 (18.4)	25.7 (14.8)
M + GSH	75.7 (14.9)	0.6 (0.4)
M + SKF-525A	12.8 (13.5)	9.6 (11.5)

[a] The ^{14}C-labeled steroid (8 μM) was incubated with male rat liver microsomes in the presence of spermine, glutathione, or SKF-525A under the same conditions as in Table II. Values are the means of three experiments, and those in parentheses were obtained in the absence of NADPH.

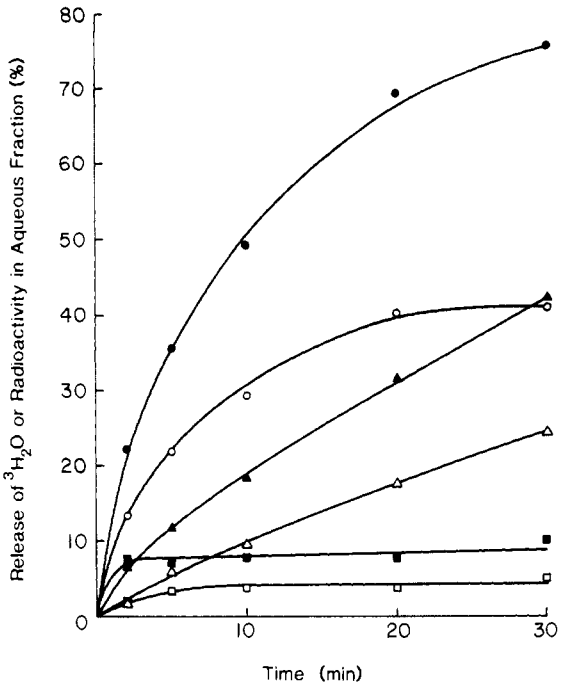

FIGURE 1: Effect of NADPH on the rate of ^3H_2O release and formation of water-soluble products from tritium-labeled estradiol and 2-hydroxyestradiol by male rat liver microsomes in the presence of glutathione. The steroids (8 μM) were incubated at 37 °C for different time periods with male rat liver microsomes (about 0.8 mg of protein), GSH (2 mM), and NADPH (0.14 mM) in 4 mL of 0.1 M Tris-HCl, pH 7.4. A portion was evaporated to dryness to measure ^3H_2O release (open symbols), and the remainder was extracted with ether before determining radioactivity in the residual aqueous medium (closed symbols). Loss of ^3H from [4-^{3}H]E_2 with NADPH omitted was <5%. [4-^{3}H]2-OHE_2 + GSH + NADPH (○, ●); [4-^{3}H]E_2 + GSH + NADPH (△, ▲); [4-^{3}H]2-OHE_2 + GSH (□, ■).

Table VI: Effect of Spermine, Glutathione, SKF-525A, Ascorbic Acid, and CO on Release of ^3H or Formation of Water-Soluble Metabolites from ^3H-Labeled Estradiol by Rat Liver Microsomes[a]

additions	^3H transfer into ^3H_2O (%)	water-soluble radioactivity (%)	^3H-labeled aqueous metabolites (%)
[2-^{3}H]E_2			
none	4.2 (4.1)	8.9 (9.0)	4.7
M	54.8 (6.6)	62.6 (13.5)	7.8
M + Sp	68.9 (5.0)	76.3 (12.7)	7.4
M + GSH	59.3 (6.1)	68.8 (14.6)	9.5
M + SKF-525A	7.8 (6.0)	14.0 (12.0)	6.2
M + Asc	48.1 (1.8)	55.0 (9.1)	6.9
M + CO	2.1	3.9	1.7
[4-^{3}H]E_2			
none	2.1 (1.8)	4.0 (3.8)	1.9
M	20.3 (4.1)	27.2 (8.7)	6.9
M + Sp	25.5 (3.5)	34.1 (7.4)	8.6
M + GSH	31.1 (5.2)	44.9 (10.8)	13.8
M + SKF-525A	2.9 (3.2)	7.6 (6.5)	4.7
M + Asc	4.9 (2.6)	8.7 (5.4)	3.8
M + CO	4.9	9.2	4.3

[a] The ^3H-labeled steroids (8 μM) were incubated with male rat liver microsomes in the presence of NADPH, spermine, glutathione, SkF-525A, ascorbic acid, or CO under the same conditions as in Table II.

^3H]E_2 was incubated with the microsomes, both the release of ^3H_2O and water-soluble product formation were increased by spermine and GSH and decreased by SKF-525A, ascorbic acid, or CO. However, GSH was inhibitory with [6,7-^{3}H]E_2 as substrate. ^3H_2O formation from [4-^{3}H]E_2, not accounted for by 4-hydroxylation, is due primarily to conjugation with protein or GSH, but with [6,7-^{3}H]E_2 it presumably represents hydroxylation only because the 6,7-position is unlikely to react with nucleophiles with loss of ^3H_2O.

Evidence for Further Hydroxylation of 2-Hydroxyestradiol. Examination of the ether extract by TLC followed by autoradiography revealed only small amounts of a radioactive product (A) more polar than 2-hydroxyestradiol after incubation of the ^{14}C-labeled catechol estrogen in the presence of ascorbic acid to prevent the oxidative degradation of labile steroids. Extraction of the incubation mixture with more polar solvents such as ethyl acetate or dichloromethane–methanol (Bligh & Dyer, 1959) did not yield any other radioactive products. Metabolite A, which was also formed from E_2, had the same TLC mobility in cyclohexane–ethyl acetate as (16α,17β)-estriol and was not hydroxylated at C-4 because the ^3H to ^{14}C ratio was not decreased significantly (4.9 → 4.7) when it was isolated after incubation of a mixture of [4-^{3}H]2-OHE_2 and [4-^{14}C]2-OHE_2 with rat liver microsomes. Any 2,4-dihydroxyestrogen (pyrogallol type) formed by further hydroxylation of [4-^{3}H]2-OHE_2 would have lost all its ^3H from C-4, would be more polar, and therefore would probably not be extractable from the aqueous medium by the organic solvents used. The evidence points to metabolite A being the catechol estrogen bearing an oxygen atom at C-6 (or possibly C-7) because of the loss of ^3H observed when [6,7-^{3}H]2-OHE_2 was incubated with rat liver microsomes (Table IV). In addition, the 6- (or 7-) ketocatechol derivative would have the expected R_f value, and 6β-hydroxy- and 6-ketoestradiol are known metabolites of E_2 (Mueller & Rumney, 1957). An attempt to synthesize 6-keto-2-hydroxyestradiol from 6-ketoestradiol by the tyrosinase method (Jellinck & Brown, 1971) was unsuccessful because of the stringent specificity of this enzyme toward substituted estrogens (unpublished results).

Discussion

The evidence presented in this paper points to the need for further enzymatic activation of 2-hydroxyestradiol as part of the mechanism of conjugation with nucleophiles in the microsomal preparation.

We have shown that the addition of NADPH increases the release of ^3H_2O from [4-^{3}H]2-OHE_2 but not from [1-^{3}H]2-OHE_2 over that observed with rat liver microsomes alone and that SKF-525A, an inhibitor of cytochrome P-450 catalyzed hydroxylation reactions (Axelrod et al., 1954), or CO, which reacts with reduced hemoproteins (Estabrook, 1984), prevents this increase. Spermine, which stabilizes components involved in microsomal hydroxylation (Jellinck & Perry, 1967; Brown & Jellinck, 1971) also produced an increase in the release of ^3H_2O and formation of water-soluble products from [4-^{3}H]-2-OHE_2 with a much smaller effect being observed with [1-^{3}H]2-OHE_2. The decrease in ^3H loss with SKF-525A or CO and the increase produced by spermine were also observed when the parent estrogen labeled with ^3H at either C-2 or C-4 was used as substrate. In this case, the ^3H has been shown to be lost from C-2 primarily during 2-hydroxylation and removed from C-4 mainly after catechol estrogen formation during binding to microsomal protein, GSH, or other nucleophiles (Jellinck et al., 1984, 1985). The additional formation of some 2,4-dihydroxyestradiol (pyrogallol type) from 2-OHE_2 cannot be excluded, and this compound has been shown previously (Stubenrauch et al., 1976) to be synthesized by liver preparations from its methoxy derivatives.

The lack of significant reaction at C-1 of estrogens (Jellinck et al., 1986) or catechol estrogens, as shown in this paper, and the known preference of C-2 over C-4 hydroxylation by rat liver microsomes (MacLusky et al., 1981) make it possible to get an estimate of the extent of conjugation with microsomal protein at different positions in the aromatic ring by use of

regiospecifically labeled steroids. Binding to carbon atoms not bearing a ^3H label can be determined by measuring the difference between the amount of ^3H radioactivity remaining in the aqueous fraction after extraction with ether and the amount of ^3H_2O formed. Thus, when [4-^{3}H]2-OHE$_2$ was incubated with liver microsomes in the presence of NADPH (Table I), this difference (9.8%) indicates binding to positions on the steroid devoid of radioactive label with the formation of products that are not extracted by ether. This approaches the amount (16.4%) of ^3H released from [1-^{3}H]2-OHE$_2$ under the same conditions of incubation and implies that binding to molecules not occurring at C-4 involves primarily C-1. Conversely, with [1-^{3}H]2-OHE$_2$, the difference in water-soluble ^3H radioactivity and ^3H_2O (19.0%) can be compared to ^3H_2O formed (27.6%) from [4-^{3}H]2-OHE$_2$. However, in both cases, other types of reactions such as polymerization after further oxidation can contribute to loss of ^3H from the molecule. These values will depend on experimental conditions and are high in the absence of tissue or NADPH. Similar comparisons also show that microsomes give higher yields of ^3H-bearing aqueous metabolites when they are incubated with [1-^{3}H]2-OHE$_2$ rather than with [4-^{3}H]2-OHE$_2$ in the presence of NADPH and GSH (Table II), indicating preferential binding of this tripeptide to C-4 of the steroid. With [2-^{3}H]E$_2$, the difference between ^3H release and water-soluble radioactivity probably represents binding at C-1 after formation of the 4-hydroxylated estrogen ([2-^{3}H]4-OHE$_2$), because binding to microsomal nucleophiles would not be expected to occur readily at C-6 or C-7, outside the aromatic ring, and the C-1 or C-4 conjugates of 2-OHE$_2$ would not contain ^3H.

The rate and extent of water-soluble product formation in the presence of GSH depended on whether [4-^{3}H]E$_2$ or [4-^{3}H]2-OHE$_2$ was used as substrate, and the higher yields of aqueous metabolites from the latter was confirmed with ^{14}C-labeled steroids as substrates. This difference in rate is due presumably to the lower initial concentration of the catechol estrogen when it is generated from the parent estrogen. It does not imply that added catechol estrogen is treated differently by the liver cytochrome P-450 system than when it is formed from estradiol, which supports our recent findings that adduct formation with GSH does not occur during the process of catechol estrogen biosynthesis (Jellinck et al., 1986). The binding of both [4-^{14}C]E$_2$ and [4-^{14}C]2-OHE$_2$ to microsomal protein, as expected, was prevented by the addition of glutathione, with which it is known to react preferentially (Jellinck et al., 1965).

Thus, all the evidence described above points to the further enzymatic activation of 2-OHE$_2$ at C-4 and to a lesser extent at C-1. The effect of ascorbic acid in inhibiting the release of ^3H_2O from [1-^{3}H]2-OHE$_2$ and [4-^{3}H]2-OHE$_2$ without affecting significantly the 2-hydroxylation of E$_2$ points to the need for a different type of oxidation prior to conjugation of the catechol estrogen with GSH or protein. It is proposed that estradiol undergoes a sequential cytochrome P-450 catalyzed oxidation in which the classical monooxygenase step which is not affected by ascorbate is followed by electron abstraction yielding a product whose subsequent reactions are terminated by ascorbate. Whether both oxidative steps are exerted by the same or different cytochrome P-450 isozymes has not been established. Conversion of 2-OHE$_2$ to a semiquinone could result in radical generation formed during the attainment of the equilibrium between the catechol and quinone (Kalyanaraman et al., 1984; Abul-Hajj, 1985), and such a mechanism has been proposed for the cytochrome P-450 mediated redox cycling of estrogens (Liehr et al., 1986). In addition, cytochrome P-450 has been shown to generate free radicals by hydrogen abstraction during its action on the desaturation of branched-chain fatty acids (Rettie et al., 1987) although this may not apply to an aromatic system.

Ascorbic acid, which is also readily converted to a free-radical form (Yamazaki, 1962) and is an excellent free-radical scavenger (Bielski et al., 1975; Suhara et al., 1987), would interfere with the formation of the estrogen semiquinone intermediate. However, it should not inhibit the 2- (or 4-) hydroxylation of estradiol because ascorbate has been shown to protect cytochrome P-450 catalyzed steroid hydroxylations from inactivation by O_2^- generated during the reaction (Suhara et al., 1987). Further evidence supporting quinoid forms in the covalent binding of estrogen metabolites to hamster liver microsomal proteins and its inhibition by ascorbic acid has been reported recently (Haaf et al., 1987).

The much smaller increase in ^3H_2O formation from [1-^{3}H]2-OHE$_2$ than from [4-^{3}H]2-OHE$_2$ produced by GSH in the presence of NADPH is in agreement with the lesser effect of this coenzyme on liver microsomes to increase ^3H release from C-1 of 2-OHE$_2$ (Table I). Whether the regioselectivity of the enzyme-generated covalent binding of the catechol estrogen to nucleophiles reflects preferential radical formation at C-4 by the monooxygenase as a consequence of the stereospecificity of enzyme–substrate interaction or a thermodynamic preference for radical localization at C-4 over C-1 has not been ascertained. However, it is more likely due to the former because the nonenzymatic reactions (no enzyme, boiled microsomes, or NADPH omitted) showed little distinction in ^3H transfer into ^3H_2O between [1-^{3}H]2-OHE$_2$ and [4-^{3}H]2-OHE$_2$ (Table I). Stereochemical arguments can also be presented for preferential binding at C-4 because C-1 is sterically hindered by the C-9–C-11 carbon–carbon bond. With different thiols, the ratio of C-1 to C-4 conjugates has been shown to depend on their physicochemical characteristics (Abul-Hajj & Cisek, 1986) and may reflect the nonregiospecific generation of reactive species by nonenzymatic oxidative processes.

It has been proposed that conjugation with GSH provides a protective mechanism against cellular damage and that the ability of catechol estrogens to bring about neoplastic transformation in mammalian cells in culture (Purdy, 1984) and also their carcinogenic action in the hamster kidney (Li et al., 1985) is related to their known ability to bind irreversibly to proteins and other key macromolecules. Very recently, Liehr and co-workers (Liehr et al., 1987) have shown that cytochrome P-450–mixed-function oxidase is the enzyme system most closely associated with estrogen-induced DNA damage preceding renal carcinoma in the hamster. Therefore, it is of importance to determine the mechanism of these particular interactions, which may also involve superoxide anion radicals, hydrogen peroxide, hydroxyl radicals, or singlet oxygen (Coon, 1981; Estabrook, 1984). In this respect, it is of interest that the catechol estrogens have been shown to be even more potent inhibitors of membrane lipid peroxidation than α-tocopherol (Nakano et al., 1987).

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Registry No. E$_2$, 50-28-2; 2-OHE$_2$, 362-05-0; NADPH, 53-57-6; GSH, 70-18-8; cytochrome P-450, 9035-51-2.

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Characterization of the Combining Site of Mouse Myeloma Protein M315

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ABSTRACT: The interaction of M315 with 2,4-dinitrophenyl haptens was studied. 2,4-Dinitroaniline (DNP-NH_2) showed maximum affinity to M315 at about pH 4. The pH dependence of the association constant of DNP-NH_2 to M315 showed three transitions at pH 4.7, at pH 7.2, and below pH 9, respectively. Since the DNP-NH_2 molecule has no charged group in this pH range, the transitions were explained in terms of amino acid residues with ionizable side chains in M315. Judging from the pK values and the effect of succinylation, these transitions were concluded to be related to ionizations of carboxyl, imidazole, and phenol groups, respectively. Measurement of the fluorescence of affinity-labeled M315 suggested that the transition at pH 4.7 reflected an equilibrium between two forms of M315 with different conformations of the combining site. The contribution of the amino acid sequence on the light (L) chain to the interaction with haptens was studied by use of antibodies (Abs) reconstituted from the heavy chain of M315 (H^{315}) and either a homologous or a heterologous L chain. The reconstituted heterologous Ab ($H^{315}L^{952}$) showed similar pH dependence of binding to DNP-NH_2 to that of the homologous Ab ($H^{315}L^{315}$). Moreover, the two Abs showed no appreciable difference in binding to DNP-haptens of different sizes. These results suggested that the difference in the amino acid sequences of L^{315} and L^{952}, which originated by a somatic hypermutation, has little effect on the ligand binding. The inability of the somatic hypermutation to increase the affinity of M315 to DNP-haptens, which are artificial antigens, was explained in terms of lack of stimulation and selection by DNP, because M315 is a myeloma protein secreted by naturally occurring plasmacytoma, MOPC-315.

The interaction of the mouse myeloma protein M315 with haptens has been studied in detail (Haselkorn et al., 1974). The three-dimensional structure of M315 has not yet been reported, so Padlan et al. (1976) constructed a model of its combining site with hapten on the basis of results of chemical modification and kinetic studies on the interaction of M315 and DNP-haptens,[1] assuming that it has the same backbone

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[1] Abbreviations: DNP, 2,4-dinitrophenyl; DNP-NH_2, 2,4-dinitroaniline; DNP-Gly, (2,4-dinitrophenyl)glycine; DNP-Ala, (2,4-dinitrophenyl)-β-alanine; DNP-But, γ-[(2,4-dinitrophenyl)amino]butyric acid; DNP-Cap, ϵ-[(2,4-dinitrophenyl)amino]caproic acid; CDR, complementarity-determining region; CD, circular dichroism; Q_{obsd}, observed quenching; Q_{max}, maximum quenching; K, association constant; H^{315}, heavy chain of M315; L^{315}, light chain of M315; L^{952}, light chain of T952.

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