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Catechol Formation of Fluoro- and Bromo-Substituted Estradiols by Hamster Liver Microsomes

Evidence for Dehalogenation

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SUMMARY

We have examined the validity of using fluorine-substituted estrogens as probes to assess the significance of 2- and 4-hydroxylation in estrogen-induced carcinogenesis in the hamster. Liver microsomes from castrated hamsters were incubated with 2-fluoro-, 4-fluoro-, or 2,4-difluoroestradiols and analogous bromo-substituted estradiols to determine the extent of 2- and 4-hydroxylation with these substrates. Estrogen 2- and 4-hydroxylase activity was determined by radioenzymatic assay, and the ^{3}H-labeled monomethyl ether products were identified by high performance liquid chromatography. With unsubstituted 17β-estradiol as substrate, 97% of the product formed was 2-hydroxylated, and 3% was 4-hydroxylated. The monosubstituted fluoroestradiols exhibited more than a 2-fold enhanced ability to form catechol estrogens compared with their corresponding bromoestradiols. Data presented herein indicate substantial defluorination when 2-fluoroestradiol was the substrate, which amounted to 36% of the total product formed, and 32% of the rate of 2-hydroxylation found with unsubstituted 17β-estradiol as substrate. Interestingly, the rate of 4-hydroxylation was elevated 20- and 6.7-fold, respectively, when 2-fluoroestradiol and 2,4-difluoroestradiol were the substrates compared to the rate with 17β-estradiol. Moreover, both 4-fluoroestradiol and 2,4-difluoroestradiol exhibited at least a 1.6-fold greater rate of 2-hydroxylation compared with 17β-estradiol. In contrast, the rate of dehalogenation with 2-bromoestradiol was only 12% of that found with 2-fluoroestradiol. No debromination was obtained with 4-bromoestradiol, and essentially no catechols were formed using 2,4-dibromoestradiol as substrate with these hamster liver microsomes. These data clearly provide evidence for defluorination of these substituted estrogens, particularly at the C-2 position, and seriously hamper the use of fluoroestrogens in studies of hormonal carcinogenicity.

INTRODUCTION

In an effort to identify pertinent sites of possible reactive carcinogenic intermediates and to block or reduce the oxidative metabolism of a parent compound, aromatic ring hydrogen has been substituted by a fluorine atom for use as a probe (1–3). Such a fluorine-substituted analogue could result in a reduction of carcinogenic activity or even in a noncarcinogenic derivative, provided the substituted site was essential for carcinogenic activity. On the other hand, if the fluorine substitution is at some other position, then the fluoro analogue could possess the same or even an enhanced carcinogenic potency relative to the unsubstituted parent compound. Several studies, however, have shown the enzymatic replacement of fluorine by a hydroxyl group with a variety of compounds (4–6). Recently, the metabolic defluorination of 6-fluorobenzo(a)pyrene has also been demonstrated (7, 8). These observations therefore emphasize the need for detailed metabolic studies whenever fluorine substitution is used as a probe to determine if metabolism at the substituted position is involved in carcinogenicity of the parent compound.

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Recently, Liehr (9–11) has employed both monofluoro-substituted estradiols and tetrafluorodiethylstilbestrol as probes to investigate the carcinogenic and estrogenic activities involved in estrogen-induced cell transformation in the hamster kidney. Because one is unable to predict if fluorine can be enzymatically displaced from such substituted estrogens in hamster tissues, we have undertaken studies to examine this possibility using both fluoro- and bromo-substituted estradiols with hamster liver microsomes.

MATERIALS AND METHODS

Chemicals and reagents. *S*-[*methyl*-^{3}H]Adenosyl-L-methionine (7.8 Ci/mmol) was obtained from New England Nuclear, Boston, MA. Catechol *O*-methyltransferse (EC 2.1.1.6; porcine liver; specific activity, 2200 units/mg of protein), DMSO[1] (grade I), and NADPH (type III) were provided by Sigma Chemical Co., St. Louis, MO. L-Ascorbic acid was supplied by Behring Diagnostics Co., La Jolla, CA. Hepes buffer (0.1 M), pH 7.4, was purchased from GIBCO Laboratories, Grand Island, NY. HPLC-grade tetrahydrofuran and isopropanol were purchased from Burdick and Jackson Laboratories, Muskegon, MI. Cesium fluoroxysulfate was prepared as described by Thompson and Appelman (12). Sodium methoxide, *N*-bromoacetamide, and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine were supplied by Aldrich Chemical Co., Milwaukee, WI. HPLC-grade methanol, ethyl acetate, *n*-hexane, and *n*-heptane were obtained from Fisher Scientific, Chicago, IL. USP-grade absolute ethanol was purchased from U. S. Industrial Chemicals, New York.

Syntheses of fluoro- and bromo-substituted estradiols. Chromatographic purification of product was performed by the technique of Still *et al.* (13) using Merck silica gel (grade 60, 230–400 mesh, Aldrich Chemical Co.) developed with dichloromethane and 5–10% ethyl acetate in dichloromethane. Bromination of estradiol as described by Zontova *et al.* (14) with 2,4,4,6-tetrabromocyclohexa-2,5-dienone (*I*), synthesized according to Calo *et al.* (15), provided 2- and 4-bromoestradiol. The products were converted to 2- and 4-methoxyestradiol, respectively, with sodium methoxide using the procedure of Rao and Burdett (16). These 2- and 4-monomethyl ethers were further reacted with *I* to yield, respectively, 4-bromo-2-methoxyestradiol (m.p. 165–167°, after chromatographic purification and crystallization from ethanol/water) and 2-bromo-4-methoxyestradiol (m.p. 188–190°, after chromatographic purification and crystallization from ethyl acetate/*n*-hexane). Bromination of estradiol with *N*-bromoacetamide as described by Woodward (17) gave 2,4-dibromoestradiol. The synthesis of 2- and 4-fluoroestradiols was initially described by Zillig and Mueller (18) and has been subsequently modified. The required 4-fluoroestradiol was prepared from 19-nortestosterone using the procedure of Neeman *et al.* (19). Bromination of 4-fluoroestradiol 17-acetate (17) with *I* gave 2-bromo-4-fluoroestradiol 17-acetate (m.p. 163–165°) after chromatographic purification and crystallization from methanol/water. This product was treated with sodium methoxide (15) to yield 2-methoxy-4-fluoroestradiol (m.p. 190–192°) after chromatographic purification and crystallization from dichloromethane/hexane. The required 2-fluoroes-

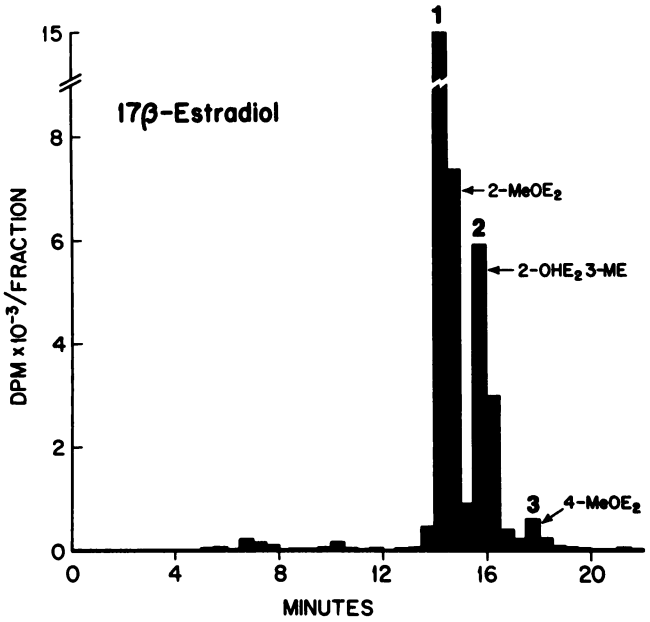

FIG. 1. *Separation by HPLC of the monomethyl ethers of the catechols of 17β-estradiol*

The products were obtained after incubation of liver microsomes (120 μg of protein) derived from castrated hamsters with 17β-estradiol for 10 min at 37°. Identification of the product numbers are indicated in Table 1. Details of the HPLC procedure are presented in Materials and Methods.

[1] The abbreviations used are: DMSO, dimethyl sulfoxide; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high performance liquid chromatography.

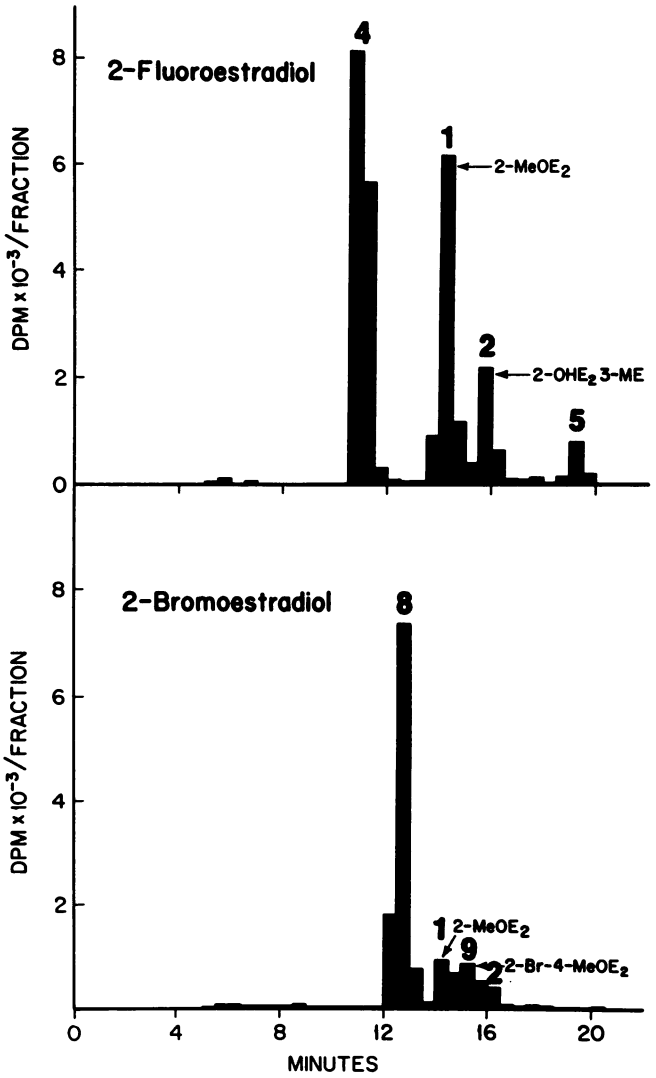

FIG. 2. *Separation of HPLC of the products formed in the radioenzymatic assay of catechol estrogen formation using 2-fluoroestradiol and 2-bromoestradiol as substrates in incubations with castrate hamster liver microsomes (120 μg of protein) for 10 min at 37°. Identification of the product numbers is provided in Table 1.*

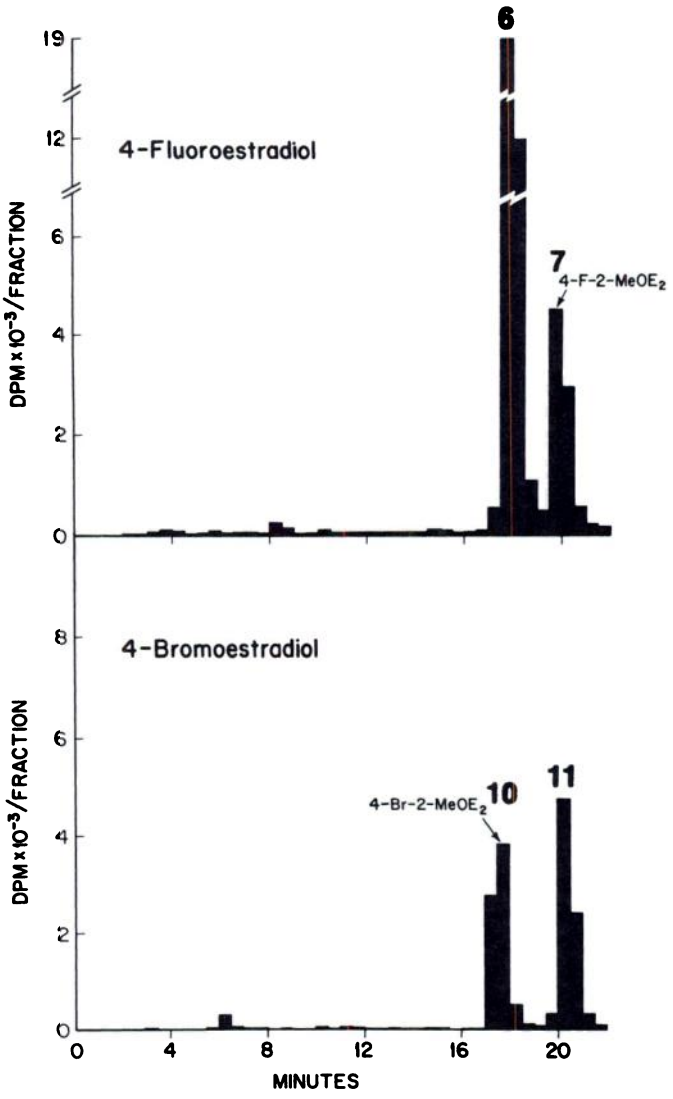

FIG. 3. *Separation by HPLC of the monomethyl ethers of halogen-substituted catechol estrogens of 4-fluoroestradiol and 4-bromoestradiol following incubation with liver microsomes of castrated hamsters for 10 min at 37°*

Identification of the product numbers is shown in Table 1.

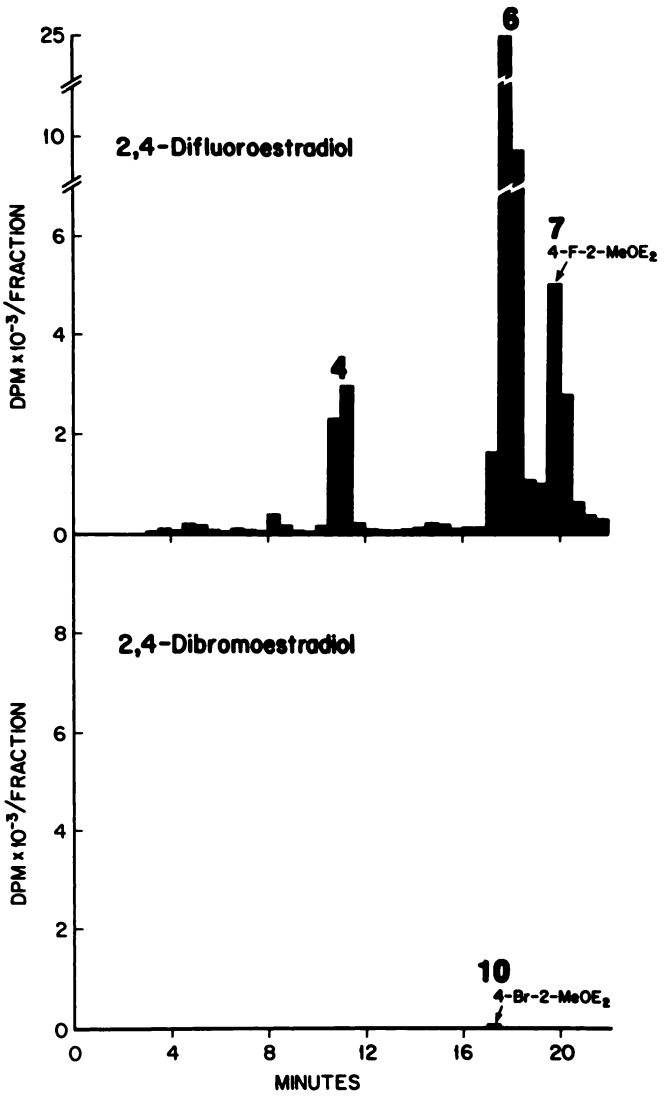

FIG. 4. *Separation of HPLC of the monomethyl ethers of catechols from 2,4-difluoroestradiol and 2,4-dibromoestradiol formed in incubations with castrate hamster liver microsomes (120 μg of protein) for 10 min at 37°*

Identification of the product numbers is presented in Table 1.

tradiol was prepared by fluorination of estrone with cesium fluoroxysulfate (*II*) as described by Appelman *et al.* (20) to yield 2-fluoroestrone, which was reduced with sodium borohydride to provide 2-fluoroestradiol (20, 21). Fluorination of the above 4-fluoroestradiol 17-acetate with *II*, followed by chromatographic purification and crystallization from aqueous methanol, gave 2,4-fluoroestradiol 17-acetate (m.p. 205–207°). This intermediate was hydrolyzed for 16 hr with aqueous potassium hydroxide in ethanol under nitrogen to provide 2,4-difluoroestradiol (m.p. 208–209°) after chromatographic purification and crystallization from aqueous methanol. The structures of the products described above were confirmed by mass spectroscopy and nuclear magnetic resonance spectroscopy (^1H, and also ^{13}C and ^{19}F for 2,4-difluoroestradiol 17-acetate).

Animals and treatment. Adult castrated male Syrian golden hamsters (LAK:LVG, noninbred strain) weighing 85–95 g were purchased from Charles River Lakeview Hamster Colony, Wilmington, MA. All animals were acclimated at least 1 week on a 12:12 light/dark cycle prior to use.

Microsomal preparation. Liver microsomes from castrated animals were prepared by a method described by Holtzman *et al.* (22). Briefly, male hamsters were killed by decapitation, and their livers were removed immediately and rinsed. Thereafter, the tissues were minced, blotted on filter paper, weighed, and homogenized in chilled glass-Teflon tissue homogenizers with 3 volumes/g of 150 mM KCl, 50 mM Tris (HCl), pH 7.4. Liver homogenates were centrifuged at 9,000 × *g* for 15 min, and the resultant supernatant fractions were then subjected to centrifugation in a Spinco L2-65B ultracentrifuge for 45 min at 165,000 × *g*. The microsomal fractions, free of glycogen, were washed in 1.0 ml of KCl (Tris-HCl) homogenizing buffer, pH 7.4, containing 50% glycerol and 0.01% butylated hydroxytoluene and gently homogenized in small conical all-glass tissue homogenizers. The microsomes were stored in aliquots (100 μl) under N_2 at −15° at a protein concentration of 12–15 mg/ml. Upon thawing, the microsomes were diluted in Hepes buffer, pH 7.4, to a protein concentration of 400–500 μg/ml just prior to use.

Estrogen hydroxylase assay. Estrogen 2- and 4-hydroxylase activity was determined by the radioenzymatic assay of Paul *et al.* (23) as modified by Purdy *et al.* (24). Briefly, the incubation mixture consisted of 40 μl of 10 mM Hepes buffer, pH 7.4, 20 μl of 6 mM ascorbic acid, 100 units of catechol *O*-methyltransferase in 20 μl of Hepes buffer, 300 μl of microsomes, 50 μl of *S*-[^3H]adenosyl-L-methionine (2.56 μCi), and

TABLE 1

Analysis of catechol monomethyl ethers by HPLC (system A)

Substrate	Product No.[a]	^{3}H-labeled monomethyl ether[b]	R_t	^{3}H in products[c]	
				2-Hydroxy	4-Hydroxy
			min	*dpm* × 10^{-2}	
17β-Estradiol	1	2-Methoxyestradiol	14.3	206	
	2	2-Hydroxyestradiol 3-methyl ether	15.8	68	
	3	4-Methoxyestradiol	17.7		8
2-Fluoroestradiol	4	(2-Fluoro-4-hydroxyestradiol 3-methyl ether)	11.0		145
	1	2-Methoxyestradiol	14.3	67	
	2	2-Hydroxyestradiol 3-methyl ether	15.8	21	
	5	(2-Fluoro-4-methoxyestradiol)	19.2		11
4-Fluoroestradiol	6	(4-Fluoro-2-hydroxyestradiol 3-methyl ether)	17.9	377	
	7	4-Fluoro-2-methoxyestradiol	19.7	94	
2,4-Difluoroestradiol	4	(2-Fluoro-4-hydroxyestradiol 3-methyl ether)	11.0		54
	6	(4-Fluoro-2-hydroxyestradiol 3-methyl ether)	17.9	346	
	7	4-Fluoro-2-methoxyestradiol	19.7	81	
2-Bromoestradiol	8	(2-Bromo-4-hydroxyestradiol 3-methyl ether)	12.7		101
	1	2-Methoxyestradiol	14.5	7	
	9	2-Bromo-4-methoxyestradiol	15.2		13
	2	2-Hydroxyestradiol 3-methyl ether	16.0	4	
4-Bromoestradiol	10	4-Bromo-2-methoxyestradiol	17.5	101	
	11	(4-Bromo-2-hydroxyestradiol 3-methyl ether)	20.3	102	
2,4-Dibromoestradiol	10	4-Bromo-2-methoxyestradiol	17.5	1	

[a] See Figs. 1–4 for designation of product numbers.

[b] The products shown without parentheses have the same retention times as authentic reference standards. No reference standards were available for the products shown within parentheses. In the case of 6, 8, and 11, the ^{3}H-labeled monomethyl ethers were identified by formation of their dimethyl ethers (see Table 2). The structures of 4 and 5 are discussed in Results.

[c] Data represent the mean of triplicate determinations.

TABLE 2

Identification of monomethyl ether products from Table 1 by methylation and HPLC in system B

Substrate	Product No.[a]	^{3}H-labeled dimethyl ether[b]	R_t
			min
17β-Estradiol	3	4-Methoxyestradiol 3-methyl ether	19.2
	1,2	2-Methoxyestradiol 3-methyl ether	23.1
2-Fluoroestradiol	4,5	(2-Fluoro-4-methoxyestradiol 3-methyl ether)	17.4
	1,2	2-Methoxyestradiol 3-methyl ether	23.1
4-Fluoroestradiol	6,7	4-Fluoro-2-methoxyestradiol 3-methyl ether	21.3
2,4-Difluoroestradiol	4	(2-Fluoro-4-methoxyestradiol 3-methyl ether)	17.4
	6,7	4-Fluoro-2-methoxyestradiol 3-methyl ether	21.3
2-Bromoestradiol	8,9	2-Bromo-4-methoxyestradiol 3-methyl ether	17.3
	1,2	2-Methoxyestradiol 3-methyl ether	23.1
4-Bromoestradiol	10,11	4-Bromo-2-methoxyestradiol 3-methyl ether	21.6
2,4-Dibromoestradiol	10	4-Bromo-2-methoxyestradiol 3-methyl ether	21.6

[a] See Table 1 for the description of the monomethyl ether product numbers.

[b] The products shown without parentheses have the same retention times as the unlabeled products obtained from treatment of the authentic reference monomethyl ethers with diazomethane as described in Materials and Methods. No unlabeled standard was available for 2-fluoro-4-methoxyestradiol 3-methyl ether.

5 μl of estrogen substrate (50 μM) in DMSO. The mixture was preincubated for 5 min at 37° with gentle agitation. The enzymatic reaction was initiated by the addition of 50 μl of 18 mM NADPH; the final incubation volume was 0.505 ml. Aliquots (100 μl) were taken after 5 min just prior to the addition of NADPH and at 10 min. To each aliquot was added 1.0 ml of 0.05 M borate buffer, pH 10, which contained 20 μg of 2-methoxyestradiol and 4-methoxyestradiol as recovery standards. The radiolabeled monomethyl ethers were extracted with 7 ml of *n*-hexane. Samples of the *n*-hexane extract were assessed for radioactivity, and the remainder was evaporated under N_2 and stored at −80° for subsequent HPLC analyses. Control incubations containing boiled microsomes or lacking either NADPH or substrate were also subjected to the above procedure. The picomoles of catechol formed were calculated from the disintegrations/min of the ^{3}H-labeled monomethyl ethers in the *n*-hexane extracts above that of the controls

TABLE 3

Relative rates of catechol estrogen formation using 17β-estradiol and halogen-substituted derivatives as substrates with liver microsomes of castrated hamsters

Substrate	Total catechol formation[a]	Average ratio of 2-hydroxy:4-hydroxy products[b]	Calculated average of relative rates of activities[c]	
			2-Hydroxylase	4-Hydroxylase
		%		
17β-Estradiol	5.6 ± 0.9	97:3	5.4	0.17
2-Fluoroestradiol	5.5 ± 0.5	36:64	2.0	3.5
4-Fluoroestradiol	9.7 ± 1.0	100:0	9.7	ND[d]
2,4-Difluoroestradiol	9.3 ± 1.6	89:11	8.3	1.0
2-Bromoestradiol	2.5 ± 0.2	6:94	0.15	2.4
4-Bromoestradiol	5.2 ± 0.4	100:0	5.2	ND
2,4-Dibromoestradiol	0.03 ± 0.01	100:0	0.03	ND

[a] Estrogen substrates were incubated with hamster liver microsomes (150 μg of protein) for 10 min at 37°. Total catechol formation is expressed as picomoles of catechol monomethyl ethers formed/min/mg of protein ± SE ($n = 4$).

[b] Calculated from the data in Table 1 for the 2-hydroxy and 4-hydroxy monomethyl ethers.

[c] Calculated from the rates of total catechol formation times the average ratio shown here of 2-hydroxy or 4-hydroxy monomethyl ether products.

[d] ND, not detectable.

divided by the disintegrations/min/pmol of the *S*-[^{3}H]adenosyl-L-methionine (24).

HPLC (system A) of estrogen monomethyl ethers. Separation of the ^{3}H-labeled monomethyl ethers of catechol estrogens was accomplished using a Hewlett-Packard model 1084B liquid chromatograph equipped with a variable UV detector set at 284 nm. Each sample was dissolved in 40 μl of tetrahydrofuran, and a 20-μl aliquot was injected on a 0.46 × 30 cm Chromegabond Diol column (ES Industries, Marlton, NJ). Estrogen samples were eluted isocratically with 4% isopropanol in *n*-hexane for 5 min, after which a linear gradient from 4–8% isopropanol in *n*-hexane was applied for the next 15 min. The flow rate was held constant at 2.0 ml/min, and column temperature was maintained at 40°. A fraction collector (SuperRac model 2211, LKB, Rockville, MD) was used to collect 0.5-min fractions for determination of radioactivity. After elution of each sample, the column was washed 10 min with 100% isopropanol and re-equilibrated for 10 min with 4% isopropanol in *n*-hexane before applying the next sample. Retention times of the methylated catechol estrogens were compared to those of the authentic monomethyl ethers of catechol estrogens and the fluoro- and bromo-substituted catechol estrogens as indicated.

Formation of HPLC (system B) of estrogen dimethyl ethers. Aliquots of the individual ^{3}H-labeled monomethyl ethers purified by HPLC were evaporated under N_2, dissolved in 0.5 ml of methanol, and cooled to 0°. An ice-cold ether solution of diazomethane (2 ml), prepared from *N*-methyl-*N'*-nitro-*N*-nitroguanidine as described by Fales *et al.* (25), was added. The reactions proceeded overnight at 4°, and the mixtures were then evaporated under N_2 at room temperature in a well ventilated hood. The dimethyl ether products were subjected to HPLC using a 0.46 × 50 cm Chromegabond Diol column equilibrated with 1.25% ethanol in *n*-heptane at a flow rate of 2.0 ml/min and column temperature of 25–28°. After injection of the sample in 50 μl of dichloromethane, the column was eluted using a linear gradient from 1.25–11.25% ethanol in *n*-heptane over 80 min. After elution of the sample, the column was washed for 15 min with absolute ethanol and re-equilibrated with 1.25% ethanol in *n*-heptane before the next chromatogram. The retention times of the ^{3}H-labeled dimethyl ethers were compared with those of unlabeled dimethyl ethers similarly prepared from authentic reference standards of the monomethyl ethers.

Radioactivity and protein determinations. Total radioactivity in the initial aliquots and radioactivity in the collected fractions was measured after the addition of 8 ml of Liquifluor/toluene (42.5:1000, New England Nuclear). All samples were counted at 5° in a Packard Tri-Carb model 4640 liquid scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, IL) with a counting efficiency of about 58% for tritium. Protein concentration of the microsomal fractions was determined by the method of Lowry *et al.* (26), using bovine serum albumin as a standard.

RESULTS

Identification of monomethyl ether products. The radioenzymatic assay of estrogen 2- and 4-hydroxylase activity used in this study yields a mixture of the 2-hydroxy- and 4-hydroxycatechol monomethyl ethers of the parent estrogens, which must be resolved in order to identify the products and thereby determine the relative rates of 2- and 4-hydroxylation of different estrogen substrates. The results of the HPLC (system A) of products with 17β-estradiol and the A-ring-halogenated estradiols as substrates, shown in Figs. 1 to 4, are summarized in Table 1. The analysis of product formation by HPLC with the 2-, 4-, and 2,4-halogenated estradiols is more complex. Of the 11 products shown in Table 1, all but products 4 and 5 were identified by formation of their dimethyl ethers and HPLC in system B (Table 2). We have been unable to synthesize 2-fluoro-4-methoxyestradiol or its 3-methyl ether. However, product 4 is presumed to be 2-fluoro-4-hydroxyestradiol 3-methyl ether and product 5 is presumed to be 2-fluoro-4-methoxyestradiol since 1) they were obtained from both 2-fluoroestradiol and 2,4-difluoroestradiol as substrates; 2) they were not found using 17β-estradiol or 4-fluoroestradiol as substrates; 3) after reaction with diazomethane, they both were converted to a derivative with the same retention time in system B (Table 2); and 4) the 4-methoxy derivatives of estrone or 17β-estradiol are more polar than their isomeric 4-hydroxy-3-methyl ethers in this HPLC system (24).

Product formation from fluoro-substituted estradiols with hamster liver microsomes. When unsubstituted 17β-estradiol was used as substrate with hepatic microsomes of male hamsters, the monomethyl ethers of 2-hydroxyestradiol constituted approximately 97% of the total catechol estrogen formation by estrogen 2- and 4-hydroxylase activity (Table 3, Fig. 1). Products 1 and 2 obtained by defluorination of 2-fluoroestradiol amounted

to 36% of the total product formation with this halogenated estrogen substrate and about 32% of the rate of 2-hydroxylation found with unsubstituted 17β-estradiol. Interestingly, the rate of 4-hydroxylation was nearly 20-fold higher with 2-fluoroestradiol than with 17β-estradiol (Table 3, Fig. 3). Moreover, when 4-fluoroestradiol was the substrate for estrogen 2- and 4-hydroxylase, the rate of 2-hydroxylation was approximately 1.7-fold greater than that found with 17β-estradiol. However, no evidence of defluorination was obtained with 4-fluoroestradiol as substrate. With 2,4-difluoroestradiol as substrate, 89% of product formation occurred by defluorination at C-2, and the remaining 11% was due to an apparent loss of fluorine at C-4 (Table 1, Fig. 4). Consequently, a substantial increase in both 2- and 4-hydroxylated products was observed with 2,4-difluoroestradiol as substrate compared to the products obtained from unsubstituted 17β-estradiol.

Product formation from bromo-substituted estradiols with hamster liver microsomes. The data in Table 1 also provide the analysis of the catechol monomethyl ethers obtained using the bromoestradiols as substrates. All of the monobrominated products 8, 9, 10, and 11 and the debrominated products 1 and 2 were further characterized by HPLC in system B after formation of their dimethyl ethers (Table 2). Products 1 and 2, obtained by debromination with 2-bromoestradiol as substrate, amounted to only 9% of the total product formation of the monomethyl ethers (Table 1, Fig. 2). The amount of debromination with 2-bromoestradiol was only 12% of that found with 2-fluoroestradiol (Table 1). An appreciable increase in 4-hydroxylation was also found with this halogen-substituted estradiol substrate. The rate of 2-hydroxylation of 4-bromoestradiol was essentially the same as that found with unsubstituted 17β-estradiol, but was only 43% of that found using 4-fluoroestradiol (Table 1, Fig. 3). No debromination was observed with 4-bromoestradiol as substrate, and essentially no products were obtained using 2,4-dibromoestradiol as substrate with these hamster liver microsomes (Table 1, Fig. 4).

DISCUSSION

The ratio of 2- to 4-hydroxylation for castrated male hamster liver microsomes with unsubstituted 17β-estradiol as a substrate is similar to that reported for hamster renal microsomes (27). For the monohalogenated substrates compared with 17β-estradiol, 2-halosubstitution decreases the net rate of 2-hydroxylase activity and enhances the rate of 4-hydroxylase activity. The present findings, however, clearly demonstrate substantial defluorination when 2-fluoroestradiol is used as a substrate, but only slight dehalogenation with 2-bromoestradiol. It appears that 4-monohalo substitution abolishes the rate of 4-hydroxylase activity. When the 4-substituent is bromine, 2-hydroxylase activity is slightly reduced; when the substituent is fluorine, 2-hydroxylase activity is appreciably increased. It was observed that 2,4-difluoroestradiol is a better substrate than 17β-estradiol for both estrogen 2- and 4-hydroxylase activities, whereas 2,4-dibromoestradiol is essentially inactive as a substrate. These results may be due to the inability of the dibromo compound to bind to these microsomal P-450 monooxygenases because of the presence of two bromine atoms, which have much larger van der Waals radii than do fluorine atoms. Similar results with these dihalogenated substrates have been found with other mammalian liver microsomes.[2] The much lower net rate of 2-dehalogenation of 2-bromoestradiol compared with that of 2-fluoroestradiol may also be due to such a steric effect. The nearly 2-fold increase in the rate of catechol formation from 4-fluoro- and 2,4-difluoroestradiol over that from unsubstituted 17β-estradiol may be analogous to the 2- to 3-fold increase observed with 6-fluorobenzo(a)pyrene over that with benzo(a)pyrene (7, 8) in the rate of 7,8-diol formation.

In recent reports (9, 11), Liehr has proposed a separation of estrogenicity from carcinogenicity in the estrogen-induced renal carcinoma model system, when he used 2- and 4-fluorinated estradiols. This conclusion is based mainly on the finding that 2-fluoroestradiol did not induce renal carcinomas in hamsters despite its evident estrogenic activity. He suggested from these data that the prevention of renal tumorigenesis is due to the inability of this fluorinated estrogen to form catechols. Our present findings do not necessarily support Liehr's conclusion, in light of our more detailed metabolic studies. Although appreciable amounts of 2-hydroxylation and significantly elevated amounts of 4-hydroxylation occur in the hamster liver when 2-fluoroestradiol is used as a substrate, the lack of carcinogenic activity seen with this haloestradiol may be related to other factors in its metabolism which have not previously been considered. For instance, when exogenous catechol estrogens are administered, present evidence indicates a rapid clearance and inactivation of these compounds (28, 29). This would have a profound effect on the realization of their ultimate biologic and potential carcinogenic responses. When mean clearance rates of catechols were compared with that of 17β-estradiol, a rapid inactivation of 2-hydroxyestrogens was observed, whereas 4-hydroxyestrogens were cleared at a less rapid rate (30). The rapid clearance of catechol estrogens has been attributed in part to the high activity of catechol *O*-methyltransferase in red blood cells (31). Consistent with these considerations is our finding[3] that there was no evidence of renal tumors when castrated male hamsters were treated with 2-hydroxyestradiol under conditions that produce 100% incidence of renal carcinomas with either 17β-estradiol or diethylstilbestrol (32). Therefore, significant defluorination of 2-fluoroestradiol in the hamster liver to yield 2-hydroxyestradiol is still unlikely to contribute to the expression of carcinogenic activity. In this regard, it should be pointed out that hamster kidney estrogen hydroxylase activity is more than 8-fold lower than that obtained with the liver mixed function oxidases with this estrogen substrate (26), and the rate of dehalogenation with 2-fluoroestradiol is correspondingly low as expected.[4] On the other hand, Liehr (9, 11) has reported that hamsters treated with 4-fluoroestradiol show an

[2] R. H. Purdy, unpublished data.
[3] J. J. Li, unpublished data.
[4] S. A. Li, unpublished results.

appreciable incidence of renal tumors. The substantial increase in 2-hydroxylation apparent with 4-fluoroestradiol as a substrate over that obtained with unsubstituted 17β-estradiol may be a pertinent factor in the carcinogenic activity of 4-fluoroestradiol. An important question, which has not been sufficiently addressed, is whether the fluorine atom remaining on the catechol estrogen products of such substituted estrogens could have pertinent effects in the metabolism and subsequent carcinogenic activity of these compounds.

The striking increase in both 2- and 4-hydroxylated fluorinated products of 2,4-difluoroestradiol and marked defluorination of tetrafluorodiethylstilbestrol mainly to the 3′-diethylstilbestrol catechol[5] is consistent with the observation that tetrafluorodiethylstilbestrol possesses substantial carcinogenic activity in the hamster kidney (10). Since we have previously provided evidence for an intimate relationship between hormonal and carcinogenic properties of estrogens that results in renal cell transformation in the hamster (32, 33), it is clear that additional information is needed concerning the metabolic and hormonal activities of these halogen-substituted estrogens and their halogen-containing metabolic products before their tumorigenic effects can be properly assessed.

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REFERENCES

1. Miller, E. C., T. L. Fletcher, A. Margreth, and J. A. Miller. The carcinogenicities of derivatives of fluorene and biphenyl: fluoro derivatives as probes for active sites in 2-acetylaminofluorene. *Cancer Res.* **22**:1002–1014 (1962).
2. Hecht, S. S., E. J. LaVoie, V. Bedenko, L. Pingaro, S. Katayama, D. Hoffmann, D. J. Sarcella, E. Boger, and R. E. Lehr. Reduction of tumorigenicity of dihydrodiol formation by fluorine substitution in the angular rings of dibenz(a,i)pyrene. *Cancer Res.* **41**:4341–4345 (1981).
3. Oravec, C. T., F. B. Daniel, and L. K. Wong. Comparative metabolism of 7,12-dimethylbenz[α]anthracene and its non-carcinogenic 2-fluoro analogue by Syrian hamster embryo cells. *Cancer Lett.* **21**:43–55 (1983).
4. Kaufman, S. The enzymic conversion of 4-fluorophenylalanine to tyrosine. *Biochim. Biophys. Acta* **51**:619–621 (1961).
5. Daly, J. W., G. Guroff, S. Udenfriend, and B. Witkop. Hydroxylation of alkyl and halogen substituted anilines and acetanilides by microsomal hydroxylases. *Biochem. Pharmacol.* **17**:31–36 (1968).
6. Scribner, J. D., N. K. Scribner, and G. Koponen. Metabolism and nucleic acid binding of 7-fluoro-2-acetamidofluorene in rats: oxidative defluorination and apparent dissociation from hepatocarcinogenesis of 8(N-arylamide)guanine adducts on DNA. *Chem. Biol. Interact.* **40**:27–43 (1982).
7. Buhler, D. R., F. Unlu, D. R. Thakker, T. J. Slaga, A. H. Conney, A. W. Wood, R. L. Chang, W. Levin, and D. M. Jerina. Effect of a 6-fluoro substituent on the metabolism and biological activity of benzo(a)pyrene. *Cancer Res.* **43**:1541–1549 (1983).
8. Buening, M. K., W. Levin, A. W. Wood, R. L. Chang, I. Agranat, M. Rabinovitz, D. R. Buhler, H. D. Mah, O. Hernandez, R. B. Simpson, D. M. Jerina, A. H. Conney, E. C. Miller, and J. A. Miller. Fluorine substitution as a probe for the role of the 6-position of benzo(a)pyrene in carcinogenesis. *J. Natl. Cancer Inst.* **71**:309–315 (1983).
9. Liehr, J. G. 2-Fluoroestradiol: separation of estrogenicity from carcinogenicity. *Mol. Pharmacol.* **23**:278–281 (1983).
10. Liehr, J. G., A. M. Ballatore, J. A. McLachlan, and D. A. Sirbasku. Mechanism of diethylstilbestrol carcinogenicity as studied with the fluorinated analogue E-3′,3″,5′,5″-tetrafluorodiethylstilbestrol. *Cancer Res.* **43**:2678–2682 (1983).
11. Liehr, J. G. Modulation of estrogen-induced carcinogenesis by chemical modifications. *Arch. Toxicol.* **55**:119–122 (1984).
12. Thompson, R. C., and E. H. Appelman. Aqueous fluoroxysulfate: decomposition and some chemical reactions. *Inorg. Chem.* **19**:3248–3253 (1980).
13. Still, W. C., M. Kahn, and A. Mitra. Rapid chromatographic technique for preparative separation with moderate resolution. *J. Org. Chem.* **43**:2923–2925 (1978).
14. Zontova, V. N., V. M. Rzheznikov, and K. K. Pivnitsky. An improved synthesis of 2- and 4-bromoestradiols. *Steroids* **25**:827–828 (1975).
15. Calo, V., F. Ciminale, L. Lopez, and P. E. Todesco. A selective bromination of aromatic amines. *J. Chem. Soc. Chem. Commun.* 3652–3653 (1971).
16. Rao, P. N., and J. E. Burdett, Jr. A novel, two-step synthesis of 2-methoxyestradiol. *Synthesis* 168–169 (1977).
17. Woodward, R. B. 2,4-Dibromo-α-oestradiol. *J. Am. Chem. Soc.* **62**:1625–1626 (1940).
18. Zillig, W., and G. C. Mueller. Preparation and biological evaluation of fluorine derivatives of estradiol. *Fed. Proc.* **15**:503–504 (1956).
19. Neeman, M., Y. Osawa, and T. Mukai. Regiospecific syntheses of modified steroid hormones. II. 4-Fluoro-17β-oestradiol and -oestrone. *J. Chem. Soc. Perkin Trans.* **1**:2297–2300 (1972).
20. Appelman, E. H., L. J. Basile, and R. Hayatsu. The reaction of fluoroxysulfate, SO_4F^-, with aromatic compounds: catalysis by acids. *Tetrahedron* **40**:189–197 (1984).
21. Neeman, M., T. Mukae, J. S. O'Grodnick, and A. L. Rendall. Regiospecific syntheses of modified steroid hormones. III. 2-Fluoro-oestrone and -17β-oestradiol. *J. Chem. Soc. Perkin Trans.* **1**:2300–2302 (1972).
22. Holtzman, J. L., B. H. Rumack, and R. R. Erickson. Spectrophotometric and radiometric studies on the *in vivo* binding of phenobarbital to hepatic microsomes. *Arch. Biochem. Biophys.* **173**:710–719 (1976).
23. Paul, S. M., J. Axelrod, and E. J. Diliberto, Jr. Catechol estrogen forming enzyme of brain: demonstration of a cytochrome P-450 monooxygenase. *Endocrinology* **101**:1604–1610 (1977).
24. Purdy, R. H., P. H. Moore, Jr., M. C. Williams, J. W. Goldzieher, and S. M. Paul. Relative rates of 2- and 4-hydroxyestrogen synthesis are dependent on both substrate and tissue. *FEBS Lett.* **138**:40–44 (1982).
25. Fales, H. M., T. M. Jaouni, and J. F. Babashak. Simple device for preparing ethereal diazomethane without resorting to codistillation. *Anal. Chem.* **45**:2302–2303 (1973).
26. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275 (1951).
27. Li, S. A., J. K. Klicka, and J. J. Li. Estrogen 2- and 4-hydroxylase activity, catechol estrogen formation, and implications for estrogen carcinogenesis in the hamster kidney. *Cancer Res.* **45**:181–185 (1985).
28. Merriam, G. R., 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. Endocrinol. Metab.* **51**:1211–1213 (1980).
29. Longcope, C., A. Femino, C. Flood, and K. I. H. Williams. Metabolic clearance rates and conversion ratios of [^{2}H]2hydroxyestrone in normal men. *J. Clin. Endocrinol. Metab.* **54**:374–380 (1982).
30. Ball, P., G. Emons, H. Kayser, and J. Teichmann. Metabolic clearance rates of catechol estrogens in rats. *Endocrinology* **113**:1781–1783 (1983).
31. Cohn, C. K., D. L. Dunner, and J. Axelrod. Reduced catechol-O-methyltransferase activity in red blood cells of women with primary affective disorder. *Science* **170**:11323–11324 (1970).
32. Li, J. J., S. A. Li, J. K. Klicka, J. A. Parsons, and L. K. T. Lam. Relative carcinogenic activity of various synthetic and natural estrogens in the Syrian hamster kidney. *Cancer Res.* **43**:5200–5204 (1983).
33. Li, J. J., and S. A. Li. Estrogen-induced tumorigenesis in hamsters: roles for hormonal and carcinogenic activities. *Arch. Toxicol.* **55**:110–118 (1984).

[5] R. H. Purdy, J. J. Li, unpublished data.

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