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# Catecholestrogens as Mediators of Carcinogenesis: Correlation of Aromatic Hydroxylation of Estradiol and Its Fluorinated Analogs with Tumor Induction in Syrian Hamsters

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## SUMMARY

17$\beta$-Estradiol is known to induce kidney tumors in male Syrian hamsters when administered chronically, whereas 4-fluoroestradiol does so only after an extended induction period and 2-fluoroestradiol is not carcinogenic; both fluorinated analogs are hormonally active. Because C-4 and C-2 hydroxylations are, respectively, minor and major routes of estrogen metabolism *in vivo*, these observations suggest mediation of tumorigenesis by catecholestrogen metabolites. However, the analogs were reported to undergo oxidative defluorination *in vitro*. We have determined the metabolic fates of estradiol, 2-fluoroestradiol, and 4-fluoroestradiol in male hamsters. [6,7-$^3$H]Estradiol was principally C-2 hydroxylated when given intravenously at either 0.1 $\mu$mol/kg or 50 $\mu$mol/kg; 2-hydroxyestradiol was eliminated in bile and urine, largely as a glucuronide, without undergoing extensive deactivation via *O*-methylation. Alicyclic alcohol metabolites were minor products. [6,7-$^3$H]2-Fluoroestradiol underwent either glucuronylation or sequential dehydrogenation and alicyclic hydroxylation followed by glucuronylation but neither oxidative defluorination nor compensatory C-4 hydroxylation. [6,7-$^3$H]4-Fluoroestradiol was also considerably dehydrogenated to the keto form and glucuronylated. Nevertheless, only 4-fluoroestradiol yielded appreciable quantities of C-2 hydroxylated metabolite at the lower dose; methylation was an insignificant pathway. No defluorinated products were observed. Dehydrogenation of both analogs and alicyclic hydroxylation of the 2-fluoroestrone metabolite were less extensive at the higher dose; all of the polar metabolites of 2-fluoroestradiol in bile, although not those in urine, declined to trace amounts. C-2 hydroxylation of 4-fluoroestradiol was greater at this dose. Thus, the rank order of catechol formation from estradiol and its fluoro analogs observed *in vivo*, unlike that found in microsomal incubations, was consistent with the hypothesis that catechols mediate estrogen-dependent renal carcinogenesis in hamsters.

When $E_2$ and some other steroidal estrogens are administered chronically to male Syrian hamsters, they induce renal tumors with an incidence of 80–100% (1–5). Contributory causal mechanisms founded upon either hormonal activity or metabolic activation have been proposed, although none of them has been substantiated (6). Thus, induction of the tumors, which does not correlate with the hormonal potency of the steroids (2, 3, 7), has been repeatedly but inconclusively (6, 8, 9) attributed to reactive metabolites that damage DNA either directly by adduction (10, 11) or indirectly via redox cycling (12).

Nonetheless, the essential elements of the "reactive metabolite hypothesis" have been confirmed (13–28). Steroidal estrogens are oxidized by enzymes (14, 24), microsomes (13, 15–18), and isolated cells (16) to intermediates that react with cellular macromolecules (13–17); they undergo irreversible binding to protein *in vivo* (15). CE (C-2 and C-4 hydroxyl derivatives), major metabolites of estrogens *in vitro* (13, 16, 25) and *in vivo* (15, 26–28), are formed at the site of carcinogenesis (20, 25) and yield reactive electrophiles upon enzymic oxidation (18, 24), and their *o*-quinone (19, 21, 22) and semiquinone (23) derivatives have been shown to be highly reactive. Finally, exogenous 4-$OHE_2$, although not 2-$OHE_2$, is carcinogenic in male Syrian hamsters (29), possibly because 4-$OHE_2$ undergoes relatively slow metabolic inactivation via *O*-methylation of the catechol function (30). CE may additionally contribute to tumor growth (29) through mitogenic activity (31).

The postulated induction of renal tumors in male Syrian hamsters by CE metabolites was apparently confirmed by the

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**ABBREVIATIONS:** $E_2$, 17$\beta$-estradiol; $\Delta^{9,11}E_1$, 3-hydroxyestra-1,3,5(10),9(11)-tetraen-17-one; CE, catecholestrogen(s); 2-$OHE_2$, 2-hydroxyestradiol; 4-$OHE_2$, 4-hydroxyestradiol; 2-$FE_2$, 2-fluoroestradiol; 4-$FE_2$, 4-fluoroestradiol; 4-F-2$OHE_2$, 4-fluoro-2-hydroxyestradiol; 2-$MeOE_2$, 2-methoxyestradiol; $E_1$, estrone; 2-$OHE_1$, 2-hydroxyestrone; 4-$OHE_1$, 4-hydroxyestrone; 2-$FE_1$, 2-fluoroestrone; 4-$FE_1$, 4-fluoroestrone; 2-$MeOE_1$, 2-methoxyestrone; 2-F-14$OHE_1$, 2-fluoro-14-hydroxyestrone; EIMS, electron impact mass spectrometry; HPLC, high performance liquid chromatography; 4-F-2$OHE_1$, 4-fluoro-2-hydroxyestrone; 2-F-x$OHE_1$, 2-fluoro-*x*-hydroxyestrone; 4-F-2$MeOE_2$, 4-fluoro-2-methoxyestradiol; 4-F-2$MeOE_1$, 4-fluoro-2-methoxyestrone; 2-F-16$\alpha$$OHE_1$, 2-fluoro-16$\alpha$-hydroxyestrone.

complete and partial abolition of the carcinogenicity but not the estrogenicity of $E_2$ after C-2 and C-4 fluorination, respectively (7, 32). Although the metabolic fates of 2- and 4-$FE_2$ in hamsters were not determined, substitution at C-2, by virtue of the strength of the C-F bond, was presumed to block the principal route of aromatic hydroxylation *in vivo* (25, 33). A fluorine at C-4 would be expected to block the minor route of hydroxylation and retard C-2 hydroxylation. Contrary to expectations, hamster liver and kidney microsomes were subsequently shown, although by indirect metabolite analysis, to catalyze oxidative defluorination of 2-$FE_2$ as well as accelerated C-2 hydroxylation of 4-$FE_2$ (34). Such findings were taken as strong evidence against the incremental carcinogenicity of 2-$FE_2$, 4-$FE_2$, and $E_2$ being a corollary of CE formation (6). However, the extent to which the fluoroestradiols underwent aromatic hydroxylation might have been artifactually enhanced, for example by the absence of those conjugation reactions that limit the availability of a substrate for oxidation (15, 35).

To resolve this controversy over structure-metabolism relationships of fluoroestradiols, we have fully characterized the metabolic fates of $E_2$ (26), 2-$FE_2$, and 4-$FE_2$ *in vivo*. An earlier study (36) found that 2-$FE_2$, which was reported to be defluorinated by rat liver microsomes (37), is not subject to defluorination in rats, whereas 4-$FE_2$ undergoes appreciable C-2 hydroxylation. Nevertheless, the C-2 fluorine is rendered susceptible to oxidative displacement by a distal substituent effect of 17$\alpha$-ethynylation (38). The present study has characterized the metabolism of $E_2$ and its A-ring fluorinated analogs in male Syrian hamsters.

## Experimental Procedures

### Materials

[6,7-$^3$H]$E_2$ (45.4 Ci/mmol; radiochemical purity, 99%) and [6,7-$^3$H]$E_1$ (48 Ci/mmol; radiochemical purity, 99%) were obtained from NEN Research Products (Dreieich, Germany). Unlabeled estrogen standards, $\beta$-glucuronidase (Glucurase), and $\beta$-glucuronidase/arylsulfohydrolase (type H-2; 4.2 × $10^3$ units/ml arylsulfohydrolase) were from Sigma Chemical Co. (Poole, Dorset, UK).

### Syntheses

2- and 4-$FE_2$ were prepared as described previously (39) except that 4-$FE_1$ acetate and 4-$FE_2$ were purified on HPLC columns (5-$\mu$m Techsphere silica, 25 cm × 0.8-cm i.d., and 10-$\mu$m Techopak $C_{18}$, 30 cm × 0.39-cm i.d., respectively; HPLC Technology, Macclesfield, Cheshire, UK); 4-$FE_1$ acetate ($R_t$, 19 min) and 4-$FE_2$ ($R_t$, 21.5 min) were eluted with ethyl acetate/cyclohexane (8:92, v/v; flow rate, 1.75 ml/min) and acetonitrile/water (20–75% acetonitrile over 45 min; 1.5 ml/min), respectively. [$^3$H]2- and [$^3$H]4-$FE_2$ were prepared by the method of Morgan *et al.* (36).

### Animal Experiments

Male Syrian golden hamsters (*Mesocricetus auratus*; 110–150-g body weight at time of use) were purchased from Wright's (Essex, UK). They were housed in steel cages and maintained on tap water and a standard rodent diet for at least 4 weeks after purchase. For metabolic studies, they were anesthetized with 90 mg/kg pentobarbitone (60 mg/ml in isotonic saline solution) given intraperitoneally and were cannulated via the jugular vein, urinary bladder, and common bile duct; anesthesia was maintained with intravenous pentobarbitone as required. Low-dose [$^3$H]$E_2$ (2.3–2.6 $\mu$Ci, 200 $\mu$Ci/$\mu$mol), [$^3$H]2-$FE_2$ (2.3–2.8 $\mu$Ci, 100 $\mu$Ci/$\mu$mol), and [$^3$H]4-$FE_2$ (2.2–2.4 $\mu$Ci, 100 $\mu$Ci/$\mu$mol) were administered intravenously at 0.1 $\mu$mol/kg in saline/ethanol (7:3, v/v). High-dose [$^3$H]$E_2$ (2.8–3.3 $\mu$Ci, 0.47 $\mu$Ci/$\mu$mol), [$^3$H]2-$FE_2$ (2.7–3.9 $\mu$Ci, 0.59 $\mu$Ci/$\mu$mol), and [$^3$H]4-$FE_2$ (1.6–2.5 $\mu$Ci, 0.38 $\mu$Ci/$\mu$mol) were administered intravenously at 50 $\mu$mol/kg in polyethylene glycol/saline (4:1, v/v). Bile was collected hourly for 4 hr and urine was collected in one 4-hr collection. The animals were killed on the fourth hour, and their livers were removed. Aliquots of bile, urine, and liver were taken for determination of radioactivity (36).

### Enzymic Hydrolysis

For metabolite analysis, bile (10–100 $\mu$l, 100–150 × $10^3$ dpm) and urine (500–2000 $\mu$l, 100–150 × $10^3$ dpm) were incubated with either $\beta$-glucuronidase (5 × $10^3$ units) or $\beta$-glucuronidase/arylsulfohydrolase (approximately 2 × $10^3$ units and 100 units, respectively), and the liberated steroids were extracted with ether (26).

### Chromatographic Analysis of Aglycon Metabolites

Metabolites extracted from enzymic hydrolysates (20–50-$\mu$l methanol solutions) were analyzed on a Techopak column ($C_{18}$, 10-$\mu$m, 30 cm × 0.39-cm i.d.; HPLC Technology) by elution with acetonitrile (20–75% over 40 min) in 43 mM ammonium dihydrogen orthophosphate, pH 3.0, at 1.5 ml/min. Further analyses were performed on a diol column (10 $\mu$m, 25 cm × 0.46-cm i.d.; HPLC Technology) by elution with propan-2-ol/hexane (1:19, v/v), at 1.5 ml/min. Unlabeled standards were detected at 280 nm. Radiolabeled metabolites were quantified on-line with a Flo One Beta radiometric detector (Canberra Packard, Pangbourne, Berks, UK). Recoveries of chromatographed radioactivity were 80–100%.

### Isolation and Identification of Aglycon Metabolites

**Metabolites of [$^3$H]$E_2$.** Bile (250-$\mu$l aliquots of 0–2-hr pooled collections; 300 $\mu$g equivalents) from high-dosed hamsters was incubated with $\beta$-glucuronidase/arylsulfohydrolase (2.5 × $10^3$ and 105 units, respectively) in 2.5 ml of acetate buffer/ascorbate, and the liberated aglycons were extracted into ether (26). Parent steroid (20.5 min), 2-$OHE_2$ (16 min), 2-$OHE_1$ (17.5 min), 2-$MeOE_2$ (21.5 min), $E_1$ (22.5 min), and 2-$MeOE_1$ (23 min), resolved on a Techopak column as described above, were determined by co-chromatography with standards (Fig. 1A). $E_2$ and four metabolite fractions (10–180 $\mu$g) (2-$MeOE_2$ and $E_1$ were collected in one fraction) were recovered via ether extraction (2 × 2 volumes) of the concentrated eluate. Aliquots were analyzed by EIMS. The mixed fraction corresponding to partially resolved 2-$MeOE_2$ and $E_1$ was separated into two subfractions on a diol column; the first, broader, peak (11 min; 50% of the $^3$H) co-chromatographed with $E_1$ and the second peak (14.0 min) co-chromatographed with 2-$MeOE_2$; they were isolated as radiochromatographically homogeneous components and characterized by EIMS.

Urine (2-ml aliquots; 240 $\mu$g equivalents) from high-dosed hamsters was mixed with 1.0 ml of acetate buffer/ascorbate and incubated with hydrolases as described above. Combined ether extracts (2 × 5 ml), containing 77% of incubated radioactivity, were reconstituted in methanol (500 $\mu$l) for HPLC. The aglycons (approximately 20-$\mu$g aliquots) were fractionated on a Techopak column, recovered (3–70 $\mu$g) from the eluate by ether extraction (2 × 3 volumes), and characterized by EIMS.

**Metabolites of [$^3$H]2-$FE_2$ and [$^3$H]4-$FE_2$.** Bile (150-$\mu$l aliquots of 0–4-hr pooled collections, 220 $\mu$g equivalents) from hamsters given 50 $\mu$mol/kg or 5 $\mu$mol/kg [$^3$H]2-$FE_2$ yielded, upon enzymic hydrolysis, 2-$FE_2$ (60%) and 2-$FE_1$ (25%) but only trace amounts (2–3% of chromatographed radiolabel each) of six polar aglycons. 2-$FE_2$ and its metabolites were isolated by reverse phase HPLC as described above. Urine from the animals given 50 $\mu$mol/kg [$^3$H]2-$FE_2$ contained appreciable quantities (5–27% of deconjugated $^3$H) of two of the polar metabolites, which, together with 2-$FE_2$ and 2-$FE_1$, were liberated from 2.5-ml aliquots (230 $\mu$g equivalents) by enzymic hydrolysis and isolated in the same manner as the urinary metabolites of $E_2$. The purified biliary and urinary fractions (1–40 $\mu$g) were analyzed by EIMS.

The biliary and urinary metabolites of [$^3$H]4-$FE_2$ (50 $\mu$mol/kg) were obtained from 200-$\mu$l (350-$\mu$g equivalents) and 2.5-ml (140-$\mu$g equiva-

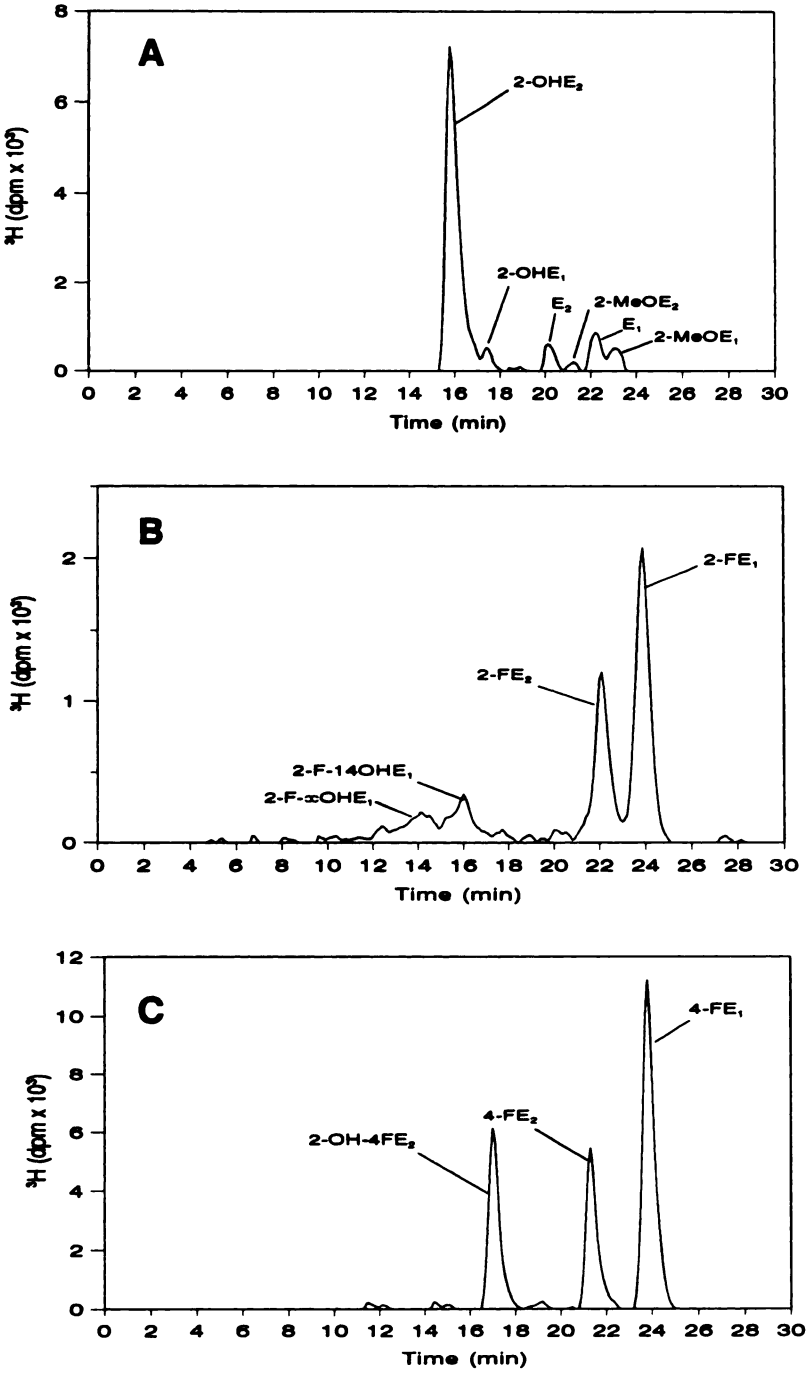


**Fig. 1.** High performance liquid radiochromatograms of the aglycon metabolites of [$^3$H]$E_2$ (A), [$^3$H]2-$FE_2$ (B), and [$^3$H]4-$FE_2$ (C) (all administered at 0.1 μmol/kg, intravenously) recovered from hamster bile. Metabolites and standards were analyzed on a $C_{18}$ column.

lents) aliquots, respectively. Aglycon fractions (2–35 μg) purified from enzymic hydrolysates by reverse phase HPLC were analyzed by EIMS.

### EIMS

Standards and isolated metabolites were analyzed by direct-probe EIMS using a VG TS-250 mass spectrometer (26).

## Results

### Excretion of Radiolabeled Metabolites

The radiolabeled metabolites of intravenously administered [$^3$H]$E_2$, [$^3$H]2-$FE_2$, and [$^3$H]4-$FE_2$ were rapidly excreted in bile, at both high (50 μmol/kg) and low (0.1 μmol/kg) doses, by male hamsters (Table 1). In contrast to the consistent biliary elimination, the recovery of radiolabel in bladder urine over 0–4 hr was highly variable, irrespective of compound and dose; for animals given low-dose [$^3$H]$E_2$, [$^3$H]2-$FE_2$, and [$^3$H]4-$FE_2$ it was 13–22%, 7–14%, and 19–30% of the dose, respectively, and for those given high-dose steroid it was 7–33%, 3–17%, and 1–4%, respectively.

### Tissue Residues of Radiolabeled Material

After 4 hr, only residual quantities of radiolabeled material remained in the livers of all groups studied, i.e., 2.7–9.4%, 3.5–7.5%, and 2.7–6.4% of the dose for hamsters given low-dose [$^3$H]$E_2$, [$^3$H]2-$FE_2$, and [$^3$H]4-$FE_2$, respectively, and 1.2–3.7%, 6.5–11.3%, and 8.3–13.1% for those given a high dose of the

TABLE 1

**Biliary excretion of the metabolites of low- and high-dose [$^3$H]$E_2$, [$^3$H]2-$FE_2$, and [$^3$H]4-$FE_2$ by male Syrian hamsters**

Low-dose (0.1 μmol/kg) and high-dose (50 μmol/kg) steroids were administered intravenously to anesthetized hamsters; bile was collected via a cannula inserted into the common bile duct. Data are mean ± standard error ($n$ = 4).

| Time | Excretion | | | | | |
|---|---|---|---|---|---|---|
| | $E_2$ | | 2-$FE_2$ | | 4-$FE_2$ | |
| | Low | High | Low | High | Low | High |
| hr | % of administered radiolabel | | | | | |
| 0–1 | 21.2 ± 5.9 | 26.1 ± 1.9 | 21.5 ± 2.8 | 21.6 ± 4.9 | 19.1 ± 3.0 | 16.8 ± 5.3 |
| 1–2 | 8.5 ± 1.4 | 12.7 ± 2.6 | 11.5 ± 1.4 | 9.6 ± 1.4 | 11.0 ± 2.8 | 7.0 ± 0.9 |
| 2–3 | 4.8 ± 0.5 | 5.5 ± 1.2 | 5.8 ± 0.9 | 5.8 ± 1.1 | 5.8 ± 0.6 | 4.6 ± 0.4 |
| 3–4 | 2.8 ± 0.6 | 3.2 ± 0.9 | 3.6 ± 0.3 | 4.1 ± 1.5 | 6.3 ± 1.8 | 4.4 ± 0.6 |
| 0–4 | 37.2 ± 7.2 | 47.5 ± 3.0 | 42.4 ± 1.9 | 41.1 ± 8.7 | 42.1 ± 6.6 | 32.8 ± 6.2 |

corresponding steroid. The quantity of radiolabel left in the kidneys was determined only in the case of hamsters given high-dose [$^3$H]$E_2$, when values representing 0.15–2.6% of the dose were obtained.

### Biliary and Urinary Metabolites

The metabolites of both doses of [$^3$H]$E_2$ and its fluoro analogs were eliminated in bile and urine principally as glucuronides. Nonconjugated steroids, i.e., those that were immediately extractable into ether, constituted 7–25% of total radiolabeled material. After incubation with β-glucuronidase, approximately 75% of this material was extracted from bile and urine. A mixture of β-glucuronidase and arylsulfohydrolase effected approximately the same hydrolysis of biliary and urinary conjugates as did β-glucuronidase alone; 75–85% of the radiolabel was extracted into ether.

### Identification of Metabolites

**$E_2$.** $E_2$, 2-$OHE_2$, 2-$MeOE_2$, $E_1$, 2-$OHE_1$, and 2-$MeOE_1$ isolated from enzymic hydrolysates of bile (Fig. 1A) and urine (Fig. 2A) were identified by co-chromatography with unlabeled standards and by matching of their EIMS spectra with library (40) spectra (data not shown). No C-4 CE metabolites were observed; standards of 2/4-$OHE_2$ and 2/4-$OHE_1$ were resolved (with 0.7 min between isomers) by the HPLC system employed. The metabolite fraction that coeluted with $E_1$ on $C_{18}$ and diol columns yielded, after its isolation by diol-phase HPLC, an additional molecular ion at $m/z$ 268; $m/z$ 270 ($E_1$) and $m/z$ 268 were obtained at a ratio of relative intensities of 1.0:0.23. The minor component was ascribed to $\Delta^{9,11}E_1$ by library match; diagnostic peaks were present at $m/z$ 268, 253, 235, 210, and 197.

**2-$FE_2$.** Unchanged 2-$FE_2$ recovered from bile and urine (Figs. 1B and 2B) was identified by co-chromatography with standard and by EIMS (Table 2). 2-$FE_1$ isolated from bile and urine was identified by EIMS. The major polar metabolites of 2-$FE_2$, i.e., 2-F-x$OHE_1$ and 2-F-14$OHE_1$ (Figs. 1B and 2B), were, at the higher dose, eliminated in quantifiable amounts only in urine (Tables 3 and 4). Nevertheless, the more polar of the two could be isolated from bile and urine. It yielded the molecular ion ($m/z$ 304) of an hydroxyfluoroestrone and fragments indicative of functionalization in either ring D or ring C; thus, $m/z$ 164, $m/z$ 177, and $m/z$ 190 are fluoro analogs of the characteristic steroid ions $m/z$ 146 (rings A and B), $m/z$ 159 (rings A and B plus C-11), and $m/z$ 172 (rings A and B plus C-11 and C-12) (41), respectively. However, the position of the alicyclic hydroxyl group could not be assigned precisely. This product (2-F-x$OHE_1$) co-chromatographed on a $C_{18}$ column with the more polar of the two hydroxyfluoroestrone metabolites of 2-$FE_2$ formed by male rats (36) but gave a different mass spectrum. The second polar aglycon isolated from hamster urine, which yielded a base peak at $m/z$ 288, was not exactly coincident with the corresponding rat biliary metabolite (36). Detailed chromatographic analysis, using a slower gradient of acetonitrile (20–70% over 45 min), revealed that the hamster metabolite eluted between 2-$OHE_2$ and 4-$OHE_2$. By comparison with library spectra of monohydroxyl derivatives of $E_1$ and $E_2$ (40), it was preliminarily characterized as 2-F-14$OHE_1$; this tertiary alcohol estrogen, unlike B-, C-, and D-ring secondary alcohols, undergoes a distinctive facile loss of oxygen from the molecular ion (relative intensities of M−16 for 14-$OHE_1$ and the metabolite were 4% and 3%, respectively).

**4-$FE_2$.** 4-$FE_2$ and 4-$FE_1$ isolated from bile and urine (Figs. 1C and 2C) were identified by co-chromatography and EIMS and by EIMS, respectively (Table 2). The mass spectra of the two major polar metabolites eliminated in bile and urine contained molecular ions and numerous fragments diagnostic of ring A hydroxylation; they were assigned as 4-F-2$OHE_2$ and 4-F-2$OHE_1$. The radiochemically homogeneous fractions corresponding to 4-$FE_2$ and 4-$FE_1$ yielded additionally the molecular ions of 4-F-2$MeOE_2$ ($m/z$ 320) and 4-F-2$MeOE_1$ ($m/z$ 318), respectively. However, neither of the putative methoxy derivatives was chromatographically distinguishable, even though 4-F-2$MeOE_1$ (36), 2-$MeOE_2$, and 2-$MeOE_1$ (Figs. 1A and 2A) were resolved from their parent compounds under the conditions used for metabolite profiling. It was concluded that these derivatives were present in no more than trace amounts.

A number of minor (2–5% each of radiolabel eluted from the $C_{18}$ column) polar metabolites ($R_t$ < 18 min) were isolated from the urine of hamsters that were administered [$^3$H]$E_2$ and from the bile of those that received [$^3$H]2-$FE_2$. The relatively short retention times of the metabolites indicated that they were products of either alicyclic hydroxylation or a combination of alicyclic and aromatic hydroxylation (26). Those formed from $E_2$ yielded putative molecular ions at $m/z$ 286, 288, 304, 316, and 318, but no specific structures could be assigned. A similar situation obtained with most of the metabolites of 2-$FE_2$ (putative $M^{+\cdot}$ at $m/z$ 302, 304, and 306), although the product of $R_t$ 17 min ($M^{+\cdot}$ at $m/z$ 304) gave prominent fragment ions indicative of C-16 hydroxylation, i.e., $m/z$ 232 (86%), 231 (34%), 217 (11%), 190 (54%), 177 (30%), 175 (31%), and 164 (39%); 2-F-16α$OHE_1$ has been identified as a biliary metabolite of 2-$FE_2$ in male rats (36).

### Metabolite Profiles and Pathways

**$E_2$.** 2-$OHE_2$ was the principal metabolite of $E_2$ in bile (Fig. 1A) and urine (Fig. 2A) at both 0.1 μmol/kg and 50 μmol/kg

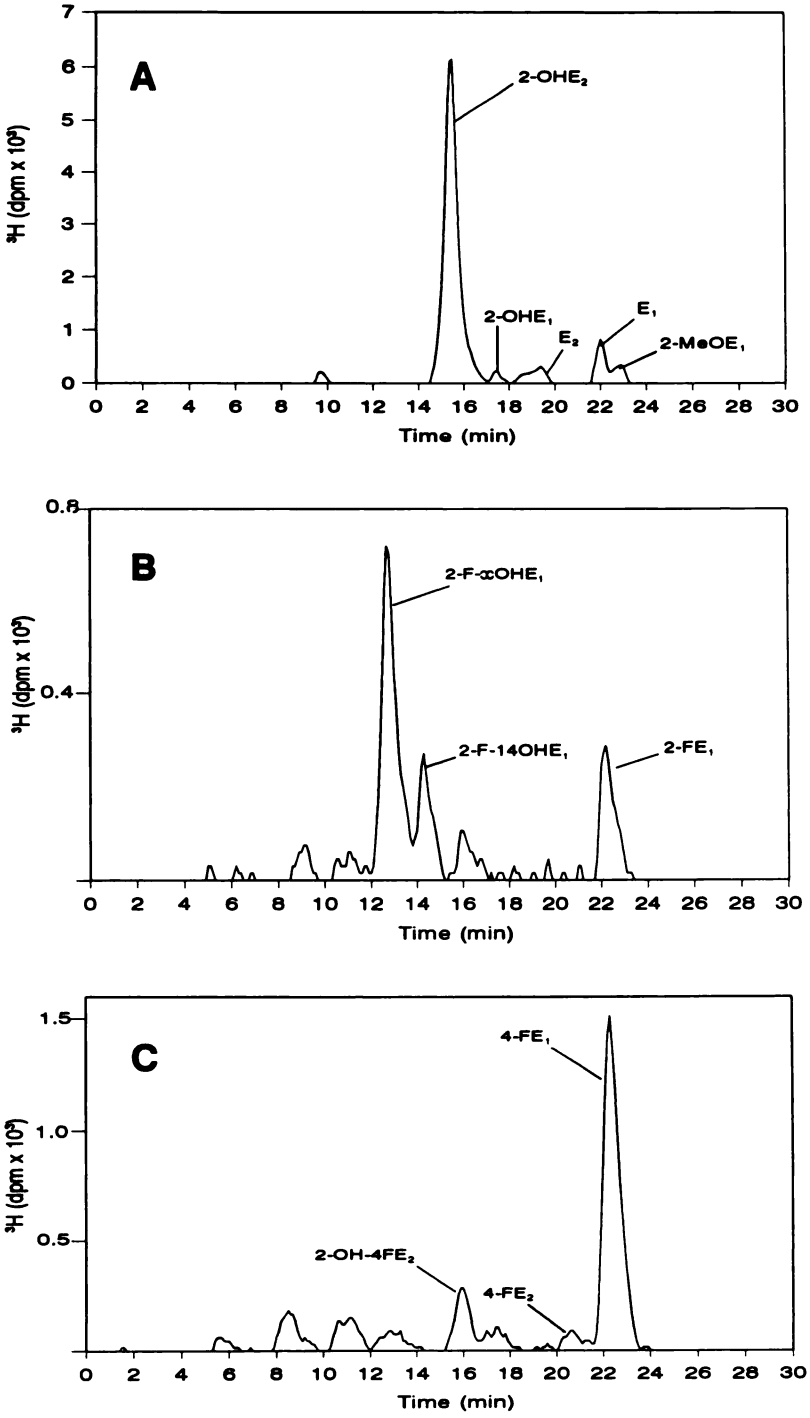


**Fig. 2.** High performance liquid radiochromatograms of the aglycon metabolites of [$^3$H]$E_2$ (A), [$^3$H]2-$FE_2$ (B), and [$^3$H]4-$FE_2$ (C) (all administered at 0.1 $\mu$mol/kg, intravenously) recovered from hamster urine. Metabolites and standards were analyzed on a $C_{18}$ column.

(Tables 3 and 4); only in urine did the proportion of 2-$OHE_2$ decrease with the 500-fold increase in dose. The percentage of administered radiolabel eliminated in urine displayed considerable interindividual variation but was not discernibly related to either the identities or proportions of the labeled compounds recovered from urine. The notable features of the biotransformation of $E_2$ in hamsters were the modest extent of dehydrogenation, the much lower fractional C-2 hydroxylation of $E_1$ versus $E_2$, and the inconsiderable (approximately 5–20%) C-2-*O*-methylation of 2-$OHE_2$ and 2-$OHE_1$ (Table 5). Although 2-$OHE_1$ was the lesser CE, it yielded as much biliary methylated catechol as did 2-$OHE_2$; 2-$MeOE_2$ was not found in urine. $\Delta^{9,11}E_1$, isolated in combination with $E_1$ as a minor biliary fraction (Table 3), was not detected in urine. A minor product of $E_1$ metabolism in hamster liver microsomes that was separated from $E_1$ on tandem $C_{18}$ columns was assigned as $\Delta^{9,11}E_1$ (25). At both doses, the metabolite profiles were characterized by considerable quantitative variations between individual hamsters. Thus, at 0.1 $\mu$mol/kg, two animals eliminated less 2-$OHE_2$ but more 2-$MeOE_2$ and 2-$MeOE_1$ in bile. Despite these disparities in the proportions of certain metabolites, the sums of C-2 oxygenated products were less variable (Table 5).

**2-$FE_2$.** 2-$FE_2$ was refractory to oxidative defluorination; neither 2-$OHE_2$ nor 2-$OHE_1$ was found among its metabolites.

TABLE 2

**Molecular ions and principal fragments of the metabolites of [$^3$H]2-$FE_2$ and [$^3$H]4-$FE_2$ in male hamsters**

Samples of 2-$FE_2$ and 4-$FE_2$ and their metabolites isolated from bile and urine were analyzed by direct-probe EIMS.

| Steroids and metabolites | Ions (relative intensity) |
|---|---|
| | *m/z* |
| 2-$FE_2$[a] | 290 ($M^{+\cdot}$, 100), 272 (10), 246 (9), 232 (16), 231 (79), 217 (10), 204 (14), 190 (28), 178 (74), 164 (36), 151 (39), 125 (21) |
| 2-$FE_1$[a] | 288 ($M^{+\cdot}$, 100), 260 (6), 244 (17), 232 (24), 231 (37), 217 (11), 203 (18), 190 (33), 178 (29), 164 (29), 151 (21), 125 (13) |
| 2-FxO$HE_1$[b] | 304 ($M^{+\cdot}$, 100), 286 (23), 259 (9), 245 (14), 231 (14), 229 (14), 215 (13), 204 (19), 203 (14), 190 (27), 177 (27), 175 (25), 164 (72) |
| 2-F-14O$HE_1$[a] | 304 ($M^{+\cdot}$, 3), 288 (100), 229 (21), 215 (4), 188 (19), 176 (39), 162 (20) |
| 4-$FE_2$[a] | 290 ($M^{+\cdot}$, 100), 272 (8), 246 (10), 232 (18), 231 (89), 217 (11), 204 (15), 190 (30), 178 (79), 164 (35), 151 (34), 125 (15) |
| 4-F-2O$HE_2$[b] | 306 ($M^{+\cdot}$, 100), 288 (4), 262 (5), 248 (7), 247 (32), 233 (5), 231 (6), 220 (5), 206 (10), 194 (28), 180 (11), 178 (15), 167 (11), 141 (13) |
| 4-$FE_1$[a] | 288 ($M^{+\cdot}$, 100), 260 (4), 244 (19), 232 (24), 231 (40), 217 (12), 203 (20), 190 (36), 178 (31), 164 (31), 151 (20), 125 (11) |
| 4-F-2O$HE_1$[b] | 304 ($M^{+\cdot}$, 100), 286 (12), 260 (10), 248 (14), 247 (39), 231 (15), 206 (16), 194 (30), 180 (12), 178 (28), 167 (12), 141 (7) |

[a] Spectrum of sample isolated from urine.
[b] Spectrum of sample isolated from bile.

TABLE 3

**Biliary metabolites of [$^3$H]$E_2$, [$^3$H]2-$FE_2$, and [$^3$H]4-$FE_2$ in male Syrian hamsters**

Steroids were administered intravenously to anesthetized hamsters. Conjugates in pooled 0-4-hr bile collections were hydrolyzed with enzymes, and the aglycons were chromatographed on a $C_{18}$ column. Compounds were characterized by direct-probe EIMS. Values are mean ± standard deviation ($n$ = 4) of percentage of chromatographed $^3$H label.

| Steroids and metabolites | Amounts of compounds | |
|---|---|---|
| | 0.1 μmol/kg | 50 μmol/kg |
| | % of $^3$H | |
| $E_2$ | 7.6 ± 2.7 | 10.9 ± 3.6 |
| 2-$OHE_2$ | 60.0 ± 19.6 | 62.4 ± 8.3 |
| 2-Me$OE_2$ | 4.6 ± 4.0 | 2.4 ± 0.6 |
| $E_1$ and $\Delta^{9,11}E_1$[a] | 8.3 ± 2.7 | 1.3 ± 0.8 |
| 2-$OHE_1$ | 4.3 ± 1.5 | 15.0 ± 12.4 |
| 2-Me$OE_1$ | 9.9 ± 8.3 | 2.2 ± 0.6 |
| 2-$FE_2$ | 30.7 ± 2.4 | 61.6 ± 9.5 |
| 2-$FE_1$ | 43.8 ± 6.5 | 25.1 ± 3.6 |
| 2-F-14O$HE_1$ | 10.3 ± 3.5 | NQ[b] |
| 2-F-xO$HE_1$[c] | 5.0 ± 2.5 | NQ |
| 4-$FE_2$ | 22.8 ± 3.4 | 28.8 ± 1.0 |
| 4-F-2O$HE_2$ | 27.8 ± 5.5 | 43.2 ± 8.2 |
| 4-$FE_1$ | 44.0 ± 5.4 | 23.4 ± 6.3 |
| 4-F-2O$HE_1$ | 1.6 ± 0.5 | 3.9 ± 2.8 |

[a] Irresolvable on either a $C_{18}$ column (acetonitrile phosphate buffer eluent) or a diol column; partial resolution when the isolated fraction was eluted from the former with methanol water (40% to 70%, in 40 min), giving approximately 4:1 $E_1$:$\Delta^{9,11}E_1$ at the higher dose.
[b] NQ, not quantifiable.
[c] Position of alicyclic hydroxyl group uncertain.

TABLE 4

**Urinary metabolites of [$^3$H]$E_1$, [$^3$H]2-$FE_2$, and [$^3$H]4-$FE_2$ in male Syrian hamsters**

Steroids were administered intravenously to anesthetized hamsters. Conjugates in 0–4-hr urine samples were hydrolyzed with enzymes, and the aglycons were chromatographed on a $C_{18}$ column. Compounds were characterized by direct-probe EIMS.

| Steroids and metabolites | Amounts of compounds | |
|---|---|---|
| | 0.1 μmol/kg[a] | 50 μmol/kg[b] |
| | % of $^3$H | |
| $E_2$ | 8.9 ± 7.4 | 13.8 ± 3.8 |
| 2-$OHE_2$ | 63.1 ± 20.6 | 30.9 ± 5.4 |
| $E_1$ | 7.3 ± 1.6 | 5.5 ± 0.8 |
| 2-$OHE_1$ | 5.2 ± 3.1 | 16.5 ± 6.1 |
| 2-Me$OE_1$ | 3.9 ± 2.8 | 5.5 ± 1.0 |
| 2-$FE_2$ | 2.8 ± 2.2 | 26.8 ± 9.8 |
| 2-$FE_1$ | 27.8 ± 11.2 | 24.5 ± 7.5 |
| 2-F-14O$HE_1$ | 14.0 ± 3.6 | 11.5 ± 5.7 |
| 2-F-xO$HE_1$[c] | 33.7 ± 13.0 | 13.0 ± 9.6 |
| 4-$FE_2$ | 4.3 ± 2.4 | 20.1 ± 7.0 |
| 4-F-2O$HE_2$ | 10.3 ± 1.7 | 13.9 ± 1.7 |
| 4-$FE_1$ | 56.4 ± 4.6 | 54.2 ± 3.5 |
| 4-F-2O$HE_1$ | 3.4 ± 1.0 | 2.4 ± 1.0 |

[a] Mean ± standard deviation ($n$ = 4) of percentage of chromatographed $^3$H label.
[b] Mean ± standard deviation ($n$ = 3) of percentage of chromatographed $^3$H label, except for 2-$FE_2$ ($n$ = 4).
[c] Position of alicyclic hydroxyl group uncertain.

In marked contrast to $E_2$, 2-$FE_2$ underwent substantial dehydrogenation and alicyclic hydroxylation (Tables 3 and 4). The latter was apparently conditioned by the former; only 2-$FE_1$, the principal metabolite of 2-$FE_2$, gave rise to products of alicyclic hydroxylation (2-F-14O$HE_1$ and 2-F-xO$HE_1$) in quantities sufficient for characterization. Not only, as with $E_2$, were considerable interindividual variations in metabolite proportions encountered, but greater differences between the biliary and urinary profiles and larger dose-related effects were also seen. In particular, 2-$FE_2$, representing ~30% and ~3% of biliary and urinary radiolabel, respectively, at 0.1 μmol/kg, was excreted in 2-fold and 10-fold higher proportions, respectively, at 50 μmol/kg. Whereas in bile this was accompanied by a decline of 2-$FE_1$ and the near disappearance of the other metabolites, in urine it was primarily balanced by a selective fall in the fraction of 2-F-xO$HE_1$ (Table 4).

**4-$FE_2$.** 4-$FE_2$ was refractory to oxidative defluorination (neither 4-$OHE_2$ nor 4-$OHE_1$ was found among its metabolites) but underwent appreciable C-2 hydroxylation. Shared features of the metabolism of the fluorinated analogs at the 0.1 μmol/kg dose were a higher proportion of parent steroid in bile and extensive dehydrogenation. In common with $E_2$, 4-$FE_2$ was apparently a better substrate for C-2 hydroxylation than was its 17-keto metabolite; for example, at 0.1 μmol/kg the ratio of catechol to phenol in bile was 0.8–1.6:1 and 0.02–0.06:1 for 4-$FE_2$ and 4-$FE_1$, respectively; the corresponding values for urine were 1.5–5.6:1 and 0.03–0.07:1. The noteworthy differences between the biotransformation of 4-$FE_2$ and $E_2$ were the greater C-2 hydroxylation of $E_2$, the insignificant *O*-methylation of the catechol products of 4-$FE_2$, and the excretion of those catechols in smaller proportions in urine versus bile at both doses (Table 5). 4-$FE_2$ was metabolized to several uncharacterized polar compounds that were eliminated via urine but not bile (Figs. 1C and 2C) and were formed in much smaller quantities at the higher dose. As with 2-$FE_2$, this reduction in polar metabolites was coincident with higher levels of parent steroid and unchanged levels of 17-keto metabolite (Table 4). The major dose-

TABLE 5

**C-2 oxygenated metabolites and CE of [$^3$H]$E_2$ and [$^3$H]4-$FE_2$ eliminated in bile and urine of male Syrian hamsters**

| Steroids and metabolites | Amounts of compounds | | | |
|---|---|---|---|---|
| | 0.1 μmol/kg | | 50 μmol/kg | |
| | Bile[a] | Urine[a] | Bile[a] | Urine[b] |
| | % of $^3$H | | | |
| $E_2$ | | | | |
| C-2-Oxygenated[c] | 78.7 ± 8.6 | 72.3 ± 16.2 | 81.9 ± 4.9 | 52.8 ± 0.7 |
| Catechols | 64.2 ± 20.2 | 68.4 ± 18.4 | 77.4 ± 5.7 | 47.3 ± 0.7 |
| Methylation index[d] | 0.80 ± 0.18 | 0.94 ± 0.05 | 0.95 ± 0.02 | 0.90 ± 0.02 |
| 4-$FE_2$ | | | | |
| C-2-Oxygenated/catechols[e] | 31.7 ± 6.3 | 13.6 ± 2.7 | 47.1 ± 5.8 | 16.3 ± 1.3 |

[a] Mean ± standard deviation ($n$ = 4) of percentage of chromatographed $^3$H label.
[b] Mean ± standard deviation ($n$ = 3) of percentage of chromatographed $^3$H label.
[c] Catechols plus $O$-methylated catechols.
[d] Catechol:catechol + methylated catechol.
[e] Methylation of catechols insignificant.

dependent changes in the biliary metabolite profile were a fall in 4-$FE_1$ and a rise in 4-F-2$OHE_2$ without, as occurred with 2-$FE_2$, an elevation of parent fluoroestrogen (Table 3).

Schematic representations of the principal metabolic pathways of $E_2$ and its fluoro analogs are shown in Fig. 3.

## Discussion

Introduction of fluorine into the C-2 and C-4 positions of $E_2$ had a profound effect on the metabolism of the steroid by male Syrian hamsters. In conformity with the decreasing rank order of their ability to induce renal tumors (7, 32), 4-$FE_2$, which has a longer induction period than $E_2$ (7), yielded less biliary and urinary CE than did $E_2$ (Table 5), whereas noncarcinogenic 2-$FE_2$ was refractory to oxidative defluorination and did not undergo discernible C-4 hydroxylation. Because CE are subject to modest $O$-methylation of the catechol function in hamsters, the rank orders of total C-2 oxygenated products in bile and urine are corollaries of aromatic hydroxylation.

Certain features of the biotransformation by hamsters of $E_2$ and its fluoro analogs were predictable from reaction rates determined *in vitro*, primarily the rapid C-2 hydroxylation of $E_2$ in hepatic microsomes (34) and the slow $O$-methylation of CE by fractions from hamster liver and red blood cells (30). However, disparities with respect to oxidative defluorination and hydroxylations at unsubstituted aromatic positions revealed that such reactions are subject to enhancement *in vitro* (34). Thus, 2- and 4-$FE_2$ are rapidly converted to 2- and 4-$OHE_2$, respectively, in hamster liver microsomes, $E_2$ and 4-$FE_2$ are good substrates for C-4 hydroxylation in hamster kidney microsomes, C-2 hydroxylation in microsomes from both organs is appreciably enhanced by a C-4 fluorine, and C-4 hydroxylation in the renal microsomes is likewise enhanced by a *meta*-fluorine. None of these pathways and substituent effects was observed *in vivo*. Consequently, previous attempts to rationalize the structure-metabolism-carcinogenicity relationships of $E_2$ and its fluoro analogs have resorted to additional metabolic, and also hormonal, factors to compensate for inconsistencies between CE formation *in vitro* and renal carcinogenicity (34). The rapid conversion of noncarcinogenic 2-$FE_2$ to CE was considered to be counterbalanced by rapid $O$-methylation. The enhancement of C-2 and C-4 hydroxylation effected by the C-4 fluorine, which yields an intermediate (4-F-2$OHE_2$) and a poor (4-$OHE_2$) substrate for $O$-methylation, was judged to be the basis of the carcinogenicity of 4-$FE_2$, but to reconcile

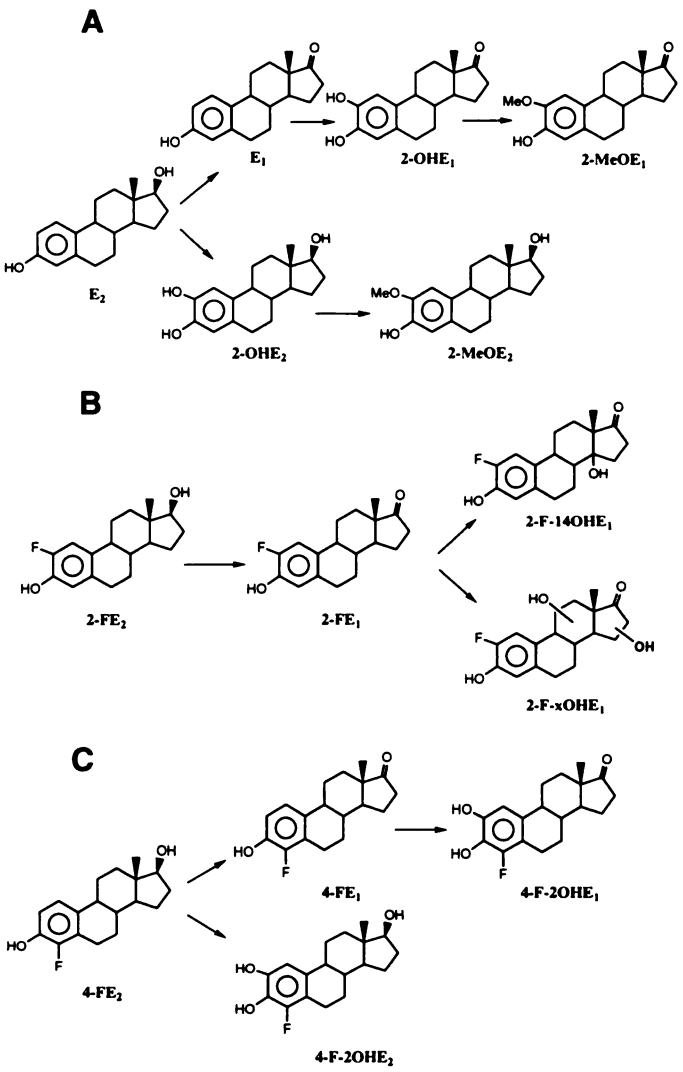


**Fig. 3.** Principal biotransformations of $E_2$ (A), 2-$FE_2$ (B), and 4-$FE_2$ (C) in male Syrian hamsters; 2-$OHE_2$, 2-$FE_1$, and 4-$FE_1$, respectively, were the major phase I metabolites at doses of 0.1 μmol/kg (Tables 3 and 4). Parent steroids and metabolites were largely conjugated with glucuronic acid before excretion.

the greater yield of CE from 4-$FE_2$, versus $E_2$, with the prolonged latency of this carcinogenicity, it was thought necessary to introduce the possibility that enhanced turnover of 4-$FE_2$ reduces the estrogenic effect of the analog (34). Hormonal actions are certainly required for cell transformation and growth of the renal tumors (1, 2, 6, 7, 32) and may explain why 17$\alpha$-ethynylestradiol, which in hamster liver microsomes undergoes extensive hydroxylation to a poorly methylated C-2 CE (42), is weakly carcinogenic in hamster kidneys (2, 4, 8). Nevertheless, because formation of CE from 2-$FE_2$ *in vivo* was evidently insignificant and 4-$FE_2$ yielded appreciably less CE than did $E_2$, there is presently no compelling experimental justification for qualifying the proposition that the carcinogenic activity of $E_2$ and its fluoro analogs reflects their biotransformation to CE (7). The simplest metabolic explanation for the artifactual acceleration of the aromatic hydroxylation of fluoroestrogens *in vitro* is the absence of conjugative pathways that limit the availability of substrate *in vivo* (35).

Uniquely among laboratory rodents, male Syrian hamsters treated with various estrogens for 8–9 months develop kidney tumors at an incidence approaching 100% (2, 8); tumor induction has been consistently demonstrated in several studies over four decades (1, 2, 4, 7, 8) and to all appearances represents an inherent susceptibility. Aspects of the biotransformation of $E_2$ suggest the basis for an elaboration of the hypothesis that CE production contributes to this exceptional susceptibility. In comparison with male rats, which metabolize $E_2$ extensively via dehydrogenation followed by either sequential or exclusive aromatic (C-2) and alicyclic (C-15 and C-16) hydroxylation (26), hamsters transform $E_2$ to larger amounts of CE, which undergo less *O*-methylation; this occurs at the expense of both dehydrogenation and D-ring hydroxylation. C-2-oxygenated metabolites constitute ~80% and ~31% (26) of radiolabel recovered from the bile of hamsters and male rats, respectively, but the corresponding values for total CE, i.e., 41–81% and ~14%, imply that the hamster tissues can be exposed to disproportionately higher levels of CE. The greater formation of CE by hamsters is not a corollary of hepatic estrogen hydroxylase activity, because the activity is slightly higher in rat liver microsomes (33). In contrast, the less extensive methylation of CE by hamsters corresponds to the extremely low catechol-*O*-methyltransferase activities found in the liver and blood, but not the kidneys, of this species (30, 42). Alicyclic hydroxylation pathways, which are prominent in male rats (26) but of minor importance in hamsters, might, hypothetically, restrict CE production by yielding poor substrates for estrogen 2-hydroxylase.

The extent to which the hamster kidney cells are exposed to CE is naturally exaggerated as a consequence of substantial urinary excretion of estrogen metabolites (26–28). Whether the urinary metabolites are generated partly within the kidney remains to be determined. Although the slow aromatic hydroxylation of $E_2$ in hamster renal microsomes (20, 25, 34) weighs against such a possibility, dissimilarities of the biliary and urinary metabolite profiles, together with differential dose-related metabolic effects, may be expressions of a renal contribution to the urinary metabolites. Certainly the approximate equality of the C-2 and C-4 hydroxylations of $E_2$ by male hamster kidney microsomes (43) had no detected corollary *in vivo*. Renal C-4 hydroxylation, notwithstanding its low rate *in vitro*, has been assigned a crucial if hypothetical role in tumor induction (43); 4-$OHE_2$ formed *in situ* and protected from deactivation by the inhibitory action of 2-$OHE_2$ on catechol-*O*-methyltransferase might thereby exert a disproportionate carcinogenic effect. Nonetheless, any such 4-$OHE_2$ eliminated in urine was evidently present at levels too low to be detected with the methods presently employed. The liver, possessing a far greater capacity for estrogen C-2 hydroxylation (25, 34), appears a more likely source of urinary CE. The low activity of catechol-*O*-methyltransferase in hamster erythrocytes implies that relatively little hepatic CE entering the blood would undergo methylation. However, the lack of kidney tumors in hamsters treated with 2-$OHE_2$, which has been attributed to rapid inactivation via methylation (29), is not easily reconciled with this proposition. It is conceivable that hepatic CE enter the blood as glucuronic acid and sulfate conjugates (44), consequently being protected from methylation, and are released enzymically within the kidney. Several analyses of the role of metabolism in estrogen-induced renal carcinogenesis have assigned modulatory actions to CE *O*-methylation (30, 34, 42). However, when $E_2$ biotransformation *in vivo* is examined, the modest extent of this reaction appears inconsistent with the operation of a crucial determinant; indeed, paradoxically, 4-$FE_2$ manifests a prolonged latency of tumorigenesis while yielding CE that undergo insignificant methylation *in vivo*. The failure of $E_2$ to induce hepatic tumors in hamsters (1), despite exposing the liver to the great quantities of 2-$OHE_2$ excreted into bile, has been linked to the much higher detoxifying enzyme activities of the liver (34).

An early study on the urinary metabolites of $E_2$ in female Syrian hamsters (27, 28) identified 2-$OHE_1$ and 2-$OHE_2$, in reverse order to that presently found, as the major and minor CE products, respectively, and observed a very high ratio (~15:1) of 2-$OHE_1$ to 2-$MeOE_1$. Because estrogens do not induce renal tumors in female hamsters under normal circumstances, i.e., when antagonists are present (1), it must be presumed that CE-mediated tumor induction is conditioned by the hormonal environment.

The metabolic fate of 2-$FE_2$ in male hamsters, unlike that of $E_2$, is similar to the fate of the steroid in male rats. In rats, dehydrogenation remains almost quantitative, whereas the two pathways of alicyclic hydroxylation are collectively less extensive (26, 36). In hamsters, dehydrogenation becomes much more pronounced and two previously latent alicyclic hydroxylations are expressed. Neither species is able to catalyze appreciable C-4 hydroxylation of 2-$FE_2$ and 2-$FE_1$. The metabolic influences of C-4 fluorination display a marked species dependency; C-2 hydroxylation is suppressed completely in male rats (36) but only partially in hamsters. The absence from hamsters, and from female rats, of highly competitive alicyclic pathways may be the principal reason for retention of considerable C-2 hydroxylation. However, hamsters, unlike female rats (36), cannot *O*-methylate more than trace amounts of 4-fluoro-2-hydroxyestrogens.

The present findings are consistent with the original presumption of Liehr (7) that the renal carcinogenicity of $E_2$ and its ring-A fluoro analogs closely reflects their ability to be converted to CE. Although these findings are in accord with the hypothesis that CE mediate tumor induction, they neither define nor circumscribe any particular mechanism(s) of action. The failures to correlate rates of CE formation in microsomes with the incidence of hamster kidney tumors (6, 8) led inevit-

ably to doubts about a determinative role for estrogen metabolites in tumorigenesis at this site (6). With respect to the series $E_2$, 2-$FE_2$, and 4-$FE_2$, at least, it is possible to obtain a reasonable correlation when metabolism in the whole animal is considered. Crucially, rational structure-metabolism relationships are observed *in vivo*; although fluorine can act as a good leaving group (38), the strength of the C-F bond militates against direct insertion of oxygen, and the extreme electronegativity of fluorine would be expected to retard *meta* (C-2 or C-4) hydroxylation via deactivation of the aromatic system. Therefore, in hamsters, dehydrogenation and glucuronylation supplant C-2 hydroxylation wholly (2-$FE_2$) or partly (4-$FE_2$) and are the principal biotransformations of the fluoroestrogens *in vivo*.

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