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DRUG–PROTEIN CONJUGATES—V

SEX-LINKED DIFFERENCES IN THE METABOLISM AND IRREVERSIBLE BINDING OF 17α-ETHINYLESTRADIOL IN THE RAT*

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Abstract—Sex-linked differences in the disposition, biotransformation, excretion and irreversible binding of [6,7-^{3}H]17α-ethinylestradiol ([^{3}H]EE_2) in Wistar rats have been observed. Three hours after i.v. administration of [^{3}H]EE_2 (5 μg/kg) the livers of males contained twice as much ^{3}H-labelled material as those of females. The biliary metabolites were largely glucuronides in both sexes, but males also excreted arylsulphates. The principal metabolites liberated from biliary conjugates by enzymes were 2-hydroxyEE_2 and 2-methoxyEE_2 in females and males, respectively. Biliary elimination of ^{3}H over 3 hr was slightly greater in males ($P < 0.05$). Radiolabelled material was irreversibly bound to hepatic microsomal and soluble protein. The material bound to microsomes represented 0.24 ± 0.07% (mean ± S.D.) of the dose in males and 0.56 ± 0.10% in females ($P < 0.001$). Oxygenation of the steroid D-ring was not indicated, and 2-hydroxyEE_2 appears to be the precursor of the reactive metabolite. The metabolic basis of the sex-linked difference in irreversible binding is discussed.

17α-Ethinylestradiol (EE_2), the estrogenic component of most combined oral contraceptive preparations, undergoes metabolic activation *in vivo* to form a reactive metabolite(s) that binds irreversibly to cellular macromolecules [1–5]. *In vitro* studies have indicated that EE_2 is metabolized to an *o*-quinone or *o*-semiquinone via 2-hydroxyethinylestradiol (2-$OHEE_2$) [6, 7]. The EE_2 *o*-semiquinone generated by hepatic microsomes reacts non-enzymically with thiol groups of microsomal proteins, soluble proteins and peptides [7, 8]. Hydroxylation at C-2 is the major pathway of EE_2 metabolism in the rat [4, 9, 10]. However, the extensive binding of activated metabolite(s) observed *in vitro* is largely prevented *in vivo* by catechol *O*-methylation [3, 11, 12], sulphation and glucuronylation (Fig. 1). The foregoing details relating to EE_2 are analogous to findings made with respect to estrone and estradiol [13].

Although irreversible binding to macromolecules is only a minor route of EE_2 metabolism *in vivo*, it may still have toxicological implications. The effects of binding of reactive compounds might be to cause functional changes. One possibility is that the moiety of a reactive metabolite irreversibly bound to protein acts as an antigenic determinant. This may be the case with EE_2: Beaumont *et al.* [14, 15] have found anti-EE_2 antibodies in some women taking oral contraceptives, and claim that the presence of immune complexes in the blood of such women is associated with a greater incidence of vascular disease. However, it would appear from the reports of other workers [16, 17] that the immunotoxicity of EE_2 in women has yet to be firmly established. As a continuation of our studies of EE_2–protein conjugate formation *in vivo* [4, 5] and *in vitro* [7], we have further investigated the sex-linked differences in metabolism and irreversible binding of EE_2 in rats, which we briefly reported in a recent communication [18].

MATERIALS AND METHODS

Materials. [6,7-^{3}H]17α-Ethinylestradiol ([^{3}H]EE_2), sp. act. 55 Ci/mmole, was obtained from New England Nuclear Corp. (F.R.G.). It was radiochemically homogeneous when analysed by HPLC. *S*-Adenosyl-L-[methyl-^{14}C]methionine, sp. act. 59 mCi/mmole, was obtained from Amersham International (Amersham, Bucks, U.K.). Reference estrogens were from previously described sources [5]. The 2-hydroxyethinylestradiol (2-$OHEE_2$) used as a substrate for catechol-*O*-methyltransferase and 4-hydroxyethinylestradiol (4-$OHEE_2$) were prepared by the method of Stubenrauch and Knuppen [19]. *S*-Adenosyl-L-methionine (chloride salt, grade II) and 3,4-dihydroxybenzoic acid (DHBA) were purchased from Sigma (London) Chemical Co. (Poole, Dorset, U.K.). Arylsulphohydrolase (type VIII from abalone), β-glucuronidase (type B-3 from bovine liver) and a *Helix pomatia* preparation containing both enzyme activities were also from Sigma. HPLC, grade solvents were from Rathburn Chemicals Ltd. (Walkerburn, Peeblesshire, Scotland).

Animals, dosing, tissue distribution of radioactivity and measurement of irreversibly bound radioactivity in vivo. Male (256 ± 7 g body wt; mean ± S.D., $n = 4$) and female (221 ± 7 g body wt; $n = 5$) rats of the Wistar strain were purchased from Bantin and Kingman Ltd. (Hull, U.K.). They were maintained

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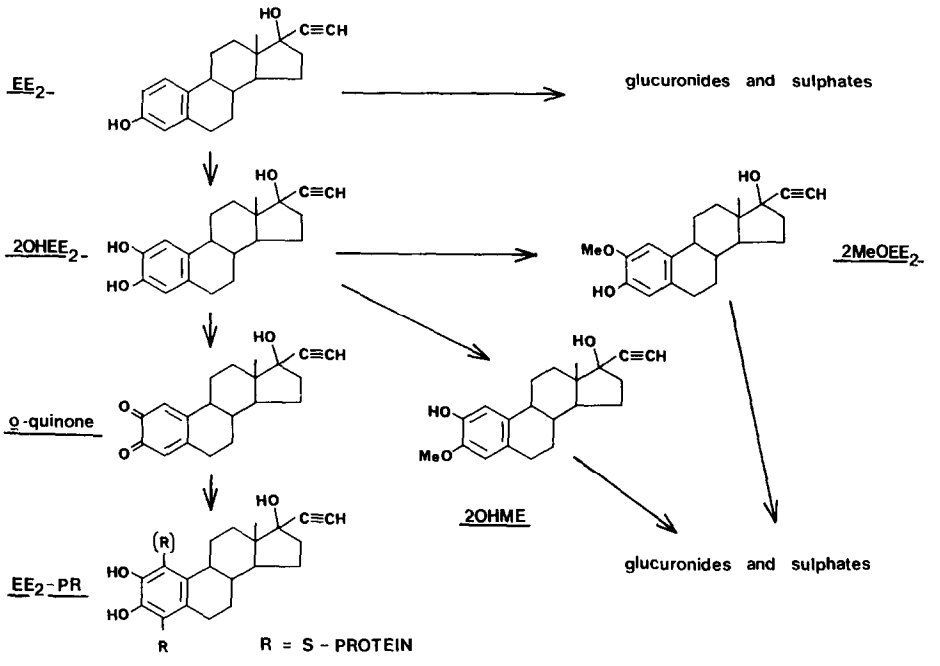

Fig. 1. Principal pathways of the metabolism of EE_2 in the rat. EE_2 = Ethinylestradiol; 2-$OHEE_2$ = 2-hydroxyethinylestradiol; 2-$MeOEE_2$ = 2-methoxyethinylestradiol; 2-OHME = 2-hydroxymestranol; EE_2-PR = ethinylestradiol–protein conjugate(s).

on a pellet diet (41 Labsure Animal Diets, Rank Hovis McDougall, Poole, Dorset, U.K.) and tap water *ad lib*. The rats were anaesthetized with urethane (1.4 g/kg body wt in 0.15 M NaCl) administered intraperitoneally, and their jugular veins and common bile ducts were cannulated. [^{3}H]EE_2 (4 μCi/μg) was given intravenously dissolved in 0.15 M NaCl–ethanol (9:1, by vol.) at a dose of 5 μg/kg body wt. Bile was collected for 3 hr and stored at −30° until analysed.

The rats were killed by cervical dislocation. Blood was obtained by cardiac puncture. The rats' livers (males, 8.89 ± 0.63 g, 3.5 ± 0.2 % body wt; females, 6.04 ± 0.47 g, 2.7 ± 0.2 % body wt), kidneys, hearts, lungs, brains, ovaries and testes were excised. Samples (50 mg) of the organs were solubilized with NCS® solubilizer (Amersham Corp., Arlington Heights, IL) according to the manufacturers' recommendations. Following digestion, glacial acetic acid was added to neutralize the solubilizer base and aliquots were taken for measurement of radioactivity by liquid scintillation counting.

The remainders of the livers were homogenized in 2 vols. of ice-cold iso-osmotic KCl solution, pH 8.0. Microsomal and cytosolic fractions were prepared as described previously [4]. Protein concentrations were determined by the method of Lowry *et al.* [20] using bovine serum albumin as a standard. Radiolabelled material irreversibly bound to the microsomes and cytosolic protein was measured by exhaustive solvent extraction as described by Maggs *et al.* [4].

Analysis of the biliary metabolites of [^{3}H]EE_2: *high-performance liquid chromatography and enzymic hydrolysis*. Samples (20–25 μl) of the 0–3 hr bile collections and the radiolabelled steroids liberated from conjugates by enzymes were routinely analysed by reversed-phase HPLC. The bile was injected onto the column without prior treatment. An octadecyl-bonded silica column (Partisil 10/25 ODS-2, 25 cm × 0.46 cm. i.d., Whatman Inc., Clifton, NJ) was employed under ambient conditions. The chromatographic methods used have been previously described in detail [4, 5].

During an earlier study [4], it was observed that estrone (E_1) and D-homoestrone (D-HomoE_1) co-chromatograph with EE_2 and 2-hydroxymestranol (3-*O*-methylether of 2-$OHEE_2$), respectively, on the reversed-phase column. Although E_1 and D-HomoE_1 are excreted metabolites of EE_2 in some mammals [21–23], they were not detected when rat biliary metabolites were deconjugated and the freed steroids analysed by mass spectrometry [4]. In order to confirm this finding, and to separate 4-$OHEE_2$ from 2-$OHEE_2$—the isomeric cathechols co-elute from the reversed-phase column (unpublished observation)—a normal-phase column was utilized. The technique was based on that described by Williams and Goldzieher [22, 23]. In brief, deconjugated biliary metabolites were injected onto a Lichrosorb Hibar® diol column (25 cm × 0.4 cm i.d., 10 μm particles; E. Merck, Darmstadt, F.R.G.), and eluted with a linear gradient of propan-2-ol in heptane (5–20% at 0.4%/min). Eluate fractions (1 ml) from the columns were dissolved in 4 ml of scintillant (Scintillator 299®, Packard–Becker B.V., Groningen, The Netherlands) and assayed for ^{3}H in an Intertechnique SL30 scintillation spectrometer. Quench correction was by external standardization. Efficiencies of counting were about 30%. Results are expressed in dpm.

Portions of bile (50 μl, *ca* 300–600 × 10^3 dpm ^{3}H)

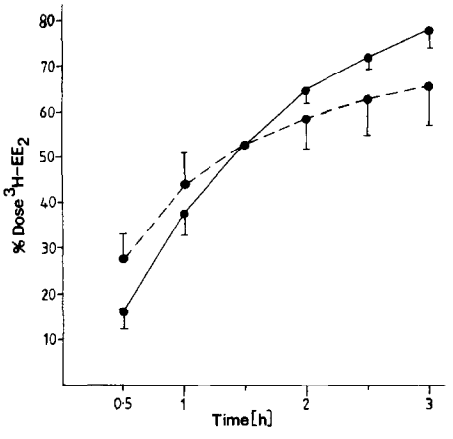

Fig. 2. Cumulative excretion of radioactivity in bile by cannulated male (●—●) and female (●--●) Wistar rats administered 5 μg/kg [6,7-^{3}H]EE$_2$ i.v. under anaesthesia. Points represent means, bars indicate S.D. (males, $n = 4$; females, $n = 5$); S.D. was 4 and 7 at 1.5 hr for males and females, respectively.

were mixed with 0.1 M sodium acetate buffer, pH 5.0, which contained ascorbate (5 or 10 mM) to prevent oxidative loss of catechol estrogen [10, 24]. The buffered solutions were either immediately extracted with peroxide-free ether (2 × 4 ml) or first incubated with enzymes as previously reported [4]. Three and sixteen hour incubations were performed in the presence of 5 and 10 mM ascorbate, respectively; 10 mM ascorbate inhibits β-glucuronidase to a negligible extent [10, 25].

Determination of irreversibly bound radioactivity in vitro. Microsomes were prepared from the livers of male and female Wistar rats, incubated with [^{3}H]EE$_2$ (0.25 mM), and irreversibly bound ^{3}H was determined by previously described methods [4, 7]. Protein concentrations were measured as above.

Enzyme assays. Hepatic microsomal cytochrome P-450 contents were determined by the method of Omura and Sato [26].

For the assay of cytosolic catechol-*O*-methyltransferase (COMT) (EC 2.1.1.6), 105,000 *g* supernatant fractions were prepared from the livers of male and female Wistar rats. Enzyme activity was measured by the method of Borchardt [27] using DHBA and 2-OHEE$_2$ as substrates; the latter has a very high affinity for COMT [12].

RESULTS

Excretion and distribution of radioactivity

Intravenous administration of [^{3}H]EE$_2$ to male and female Wistar rats was in both sexes followed by rapid excretion of radioactivity in bile (Fig. 2). Although males excreted more radioactivity over 3 hr than females (Table 1), the 0–0.5 hr elimination was significantly ($P < 0.05$) greater in the latter.

Measurement of radioactivity in the rats' tissues at 3 hr revealed that only the livers contained more than trace amounts (Table 1). Blood did not contain measurable quantities. The residual radioactivity of whole male livers was approximately twice that of female livers; expressed as % dose/g wet liver it was 2.0 ± 0.5 and 1.3 ± 0.4, respectively. Total liver wt as a percentage of body wt was significantly ($P < 0.001$) greater in males. The total recovery in the organs which were examined was 19.6 ± 4.1% of the dose with males and 9.7 ± 1.7% with females.

Irreversible binding of radioactivity in vivo *and* in vitro

Radiolabelled material was irreversibly bound to microsomes and cytosolic protein isolated from the male and female livers (Table 2). A significantly greater percentage of the dose was bound to the fractions from females. The sex-linked difference was greater with soluble than microsomal protein: it was approximately three-fold and two-fold, respectively. However, when the irreversible binding was expressed as dpm/mg protein, this order was reversed. The sex-linked disparity in irreversible binding to rat hepatic microsomes noted *in vivo* was not expressed when [^{3}H]EE$_2$ was incubated with microsomes *in vitro* (Fig. 3). This observation accorded with the finding that the hepatic microsomal cytochrome P-450 contents of males and females (0.45 ± 0.08 nmole/mg protein and 0.41 ± 0.09 nmole/mg, respectively) were not significantly different.

COMT activity in hepatic cytosol fractions

The activities of male and female hepatic cytosol COMT towards DHBA and 2-OHEE$_2$ are shown in Table 4. No sex-linked differences were observed.

Analysis of biliary metabolites

Reversed-phase HPLC analysis of whole bile from the male and female rats resolved up to seven ^{3}H-

Table 1. Distribution and excretion of radioactivity following administration of [^{3}H]EE$_2$ to male and female rats

Sex	Excretion of ^{3}H in bile (0–3 hr)	Distribution of ^{3}H in Organs (3 hr after administration)						
		Liver	Kidneys	Brain	Lung	Testes/ Ovaries	Spleen	Heart
Male	78 ± 4	17 ± 4	0.5 ± 0.1	0.4 ± 0.3	0.4 ± 0.1	0.4 ± 0.1	0.1 ± 0.0	0.1 ± 0.0
Female	66 ± 8*	8 ± 2†	0.5 ± 0.2	0.7 ± 0.1	0.4 ± 0.1	0.0	0.1 ± 0.0	0.1 ± 0.0

Data represent mean % of dose ± S.D. (males, $n = 4$; females, $n = 5$). [^{3}H]EE$_2$ was administered i.v. (5 μg/kg, 20 μCi/kg) to bile duct-cannulated anaesthetized rats and bile was collected for 3 hr. The tissue distribution of ^{3}H was determined at 3 hr.

*$P < 0.05$, †$P < 0.001$ (different from males).

Table 2. Irreversible binding of radioactivity in livers of male and female rats administered [^{3}H]EE_2

Sex	% Total hepatic ^{3}H which was irreversibly bound	Irreversible binding of ^{3}H to subcellular fractions: Microsomes, Total (% dose)	Microsomes, dpm/mg protein	Soluble protein, Total (% dose)	Soluble protein, dpm/mg protein
Male	3.8 ± 1.2	0.24 ± 0.07	136 ± 34	0.41 ± 0.13	80 ± 31
Female	24.0 ± 7.0*	0.56 ± 0.10†	513 ± 50†	1.37 ± 0.49*	263 ± 102*

Data represent mean ± S.D. (males, $n = 4$; females, $n = 5$). [^{3}H]EE_2 was administered i.v. (5 μg/kg, 20 μCi/kg) to bile duct-cannulated anaesthetized rats. Irreversible binding of ^{3}H-labelled metabolites to hepatic microsomes and soluble protein was determined by exhaustive extraction following removal of liver at 3 hr.
*$P < 0.01$, †$P < 0.001$ (different from males).

labelled components. No sex-linked qualitative differences were seen. The pattern of ^{3}H peaks was essentially the same as that previously found in bile from male rats [4]. However, qualitative and quantitative inter-individual variations were observed with both sexes. There was a sex-linked difference with respect to the proportion of the peak eluting at 13 min: females, 23 ± 6% eluate ^{3}H($n = 4$); males 10 ± 4%. Earlier studies employing enzymic hydrolysis and HPLC [4] have shown this peak to be largely comprised of conjugated 2-$OHEE_2$.

Reversed-phase HPLC and mass spectrometry have previously enabled identification of the following steroids, as tritiated derivatives, in enzymic hydrolysates of male rat bile [4, 5]: EE_2, 2-$OHEE_2$, 16-hydroxyethinylestradiol (16-$OHEE_2$), 2-methoxyethinylestradiol (2-$MeOEE_2$) and 2-hydroxymestranol (2-OHME). Glucuronylated 2-$MeOEE_2$ was the principal biliary metabolite of EE_2 in male rats. When bile from males and females was incubated with *H. pomatia* extract *ca* 60–70% of the metabolites were hydrolysed (Table 3). In males the hydrolysable conjugates were largely glucuronides but included about 20% as arylsulphates, whereas in females they were almost entirely glucuronides.

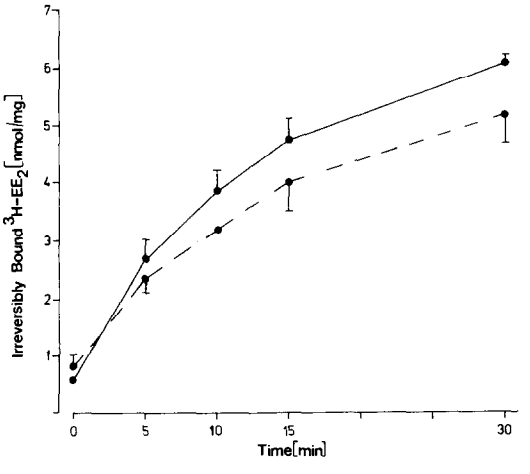

Fig. 3. Irreversible binding of radioactivity to male (●—●) and female (●--●) hepatic microsomes incubated with 0.25 mM [6,7-^{3}H]EE_2. Data are expressed as nmole of bound tritiated steroid per mg microsomal protein. Irreversible binding was measured by exhaustive extraction. Points represent means, bars indicate S.D. ($n = 4$); bar omitted when S.D. < 0.1.

Analysis of the deconjugated steroids by reversed-phase HPLC did not reveal any qualitative sex-linked differences in the metabolite profile (Fig. 4). There were, however, a number of significant quantitative differences in the β-glucuronidase–arylsulphohydrolase fraction, but none in the β-glucuronidase fraction. The most notable difference was a two-fold greater proportion of 2-$OHEE_2$ in the female profile (Table 5); hence glucuronylated 2-$OHEE_2$ was the principal metabolite in females. This was largely balanced by a smaller proportion of the ^{3}H peak eluting at 6 min, previously shown by mass spectrometry to be heterogeneous in male rats [4]. The female metabolite profile also included a smaller proportion of unmetabolized EE_2. Although there was a sex-linked difference in respect of 2-$OHEE_2$, there was none with its 2-*O*-methyl ether. The sex-linked disproportion in 2-$OHEE_2$ revealed by reversed-phase HPLC and reported here was confirmed using the bonded-normal-phase column (unpublished data). Analysis of the deconjugated metabolites on this column demonstrated that neither E_1 nor D-HomoE_1 (retention times *ca* 7 min) was excreted in bile by male and female rats (Fig. 5); additionally, no estradiol was observed. However, a minor peak of radioactivity—representing *ca* 2–4% of eluate ^{3}H—which co-chromatographed with 4-$OHEE_2$ was found in the metabolite profile of both sexes. Inspection of reversed-phase and bonded-normal-phase chromatograms of male metabolites suggested that the heterogeneous peak was resolved into two components by the diol column (Figs. 4 and 5). Their retention times, relative to those of EE_2 and 2-$OHEE_2$, indicated that they were polar metabolites possessing more than three oxygen-containing moieties. In contrast, female rats excreted only one of the polar metabolites (Fig. 5).

DISCUSSION

The disposition, biotransformation and excretion of EE_2 in rats display several sex-linked differences, certain of which may provide an explanation of the sex-linked difference in the steroid's irreversible binding. Four mechanisms for the generation of reactive metabolites from estrogens have been proposed: (a) peroxidase-catalysed oxidation: (b) oxygenation of an ethinyl group producing an oxirene:

Table 3. Enzymic hydrolysis of the biliary metabolites of [^{3}H]EE_2 from male and female rats

	Recovery of incubated radioactivity (mean ± S.D., n = 4)			
	Males		Females	
Incubation	Total recovery	Recovery in ether	Total recovery	Recovery in ether
Buffer (0 hr)	96 ± 1	7 ± 2	98 ± 9	8 ± 2
Buffer (16 hr)	82 ± 4	10 ± 1	82 ± 2	9 ± 2
H. pomatia extract	95 ± 6	67 ± 6*	86 ± 6	52 ± 6
β-Glucuronidase	91 ± 2	42 ± 3	97 ± 13	54 ± 10
Arylsulphohydrolase	100 ± 5	26 ± 1†	91 ± 8	13 ± 5

[^{3}H]EE_2 was administered i.v. (5 μg/kg) to bile duct-cannulated anaesthetized rats. Samples of bile (0–3 hr) were either mixed with buffer, pH 5.0, containing ascorbate (10 mM) and extracted with ether, or first incubated with enzymes in the presence of ascorbate (3 hr, 5 mM; 16 hr, 10 mM).

* Different from females: $P < 0.05$; † different from females: $P < 0.01$.

(c) epoxidation of the A-ring; and (d) oxidation to an *o*-quinone or *o*-semiquinone via a catechol. Although peroxidase catalyses the metabolism and covalent binding of estrogens to protein *in vitro* [8], this does not appear to occur *in vivo* [28]. Ethinyl steroids undergo oxidation to reactive metabolites—possibly oxirenes [29]—which may either combine with the haem group of cytochrome P-450 [30] and nucleophilic protein moieties, or undergo rearrangements to stable compounds. Oxidative D-homoannulation of EE_2 to D-HomoE_1 occurs in rabbits [21], and de-ethinylation to E_1 is observed in mouse and baboon *in vitro* preparations [31]. Nevertheless, *in vitro* studies [6] and the present work indicate that these are insignificant routes of EE_2 metabolism in the rat. Epoxidation may contribute to the formation of reactive metabolites of estrogens *in vitro* [32], but there appears to be no evidence for this reaction *in vivo*. Collectively, the available data [4, 5, 7, 13] indicate that 2-OHEE_2 is the precursor of EE_2's reactive metabolite in the rat.

An explanation for the greater irreversible binding to female rats' hepatic protein is suggested by the finding that female rats excrete a higher proportion of 2-OHEE_2 conjugates in bile than male rats. This

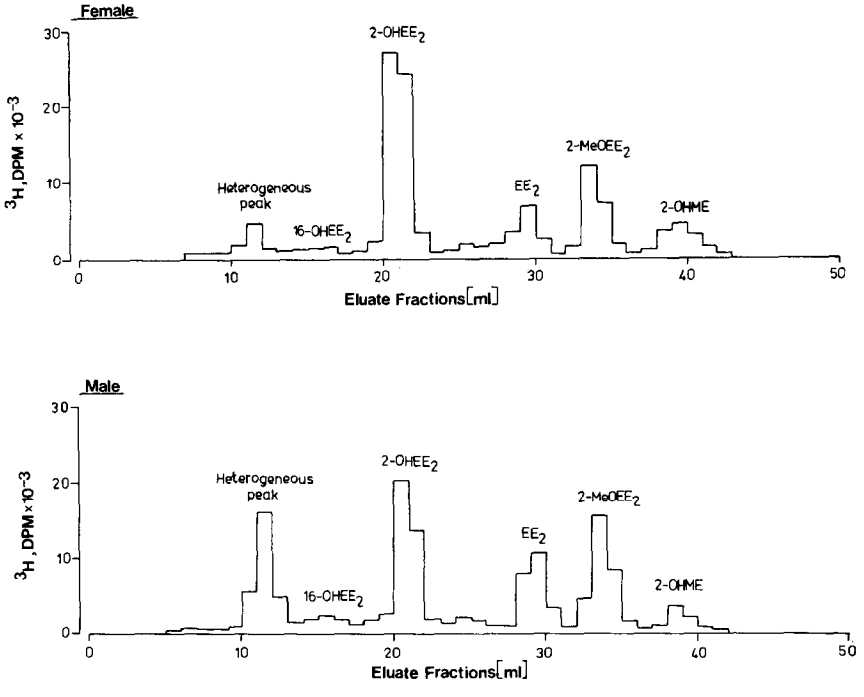

Fig. 4. Reversed-phase high performance liquid chromatograms of enzymically deconjugated biliary metabolites of [6,7-^{3}H]EE_2 from male and female Wistar rats. The conditions for the separation and the peak assignments have been described previously [4]. 2-OHME represents 2-hydroxymestranol. Bile (0–3 hr sample) from bile duct-cannulated rats dosed i.v. was incubated with β-glucuronidase–arylsulphohydrolase, and the deconjugated metabolites were extracted into ether.

Table 4. Catechol-*O*-methyltransferase activity in hepatic cytosol fractions of male and female rats

Substrate	Activity (nmol mg^{-1} protein min^{-1}) Male	Female
2,3-Dihydroxybenzoic acid	4.0 ± 0.2	3.8 ± 0.4
2-$OHEE_2$	0.88 ± 0.18	0.90 ± 0.09

COMT activity was measured in 105,000 *g* supernatant fractions of homogenates of male and female livers. Cytosolic protein (1 mg) was incubated with 0.05 μCi *S*-adenosyl-L-[methyl-^{14}C]methionine (1 mM), dithiothreitol (4 mM), $MgCl_2$ (1.4 mM) and either 3,4-dihydroxybenzoic acid (2 mM) or 2-$OHEE_2$ (0.1 mM) in 100 mM Tris–HCl, pH 7.5, for 15 min; total volume of incubations was 0.25 ml. Data expressed as means ± S.D. ($n = 4$).

implies a higher intracellular 2-$OHEE_2$ concentration in female rat liver. However, the hepatic estrogen-2-hydroxylase activity of male rat liver is six times that of female rat liver [33], and therefore cannot be the determinant of the sex-linked differences in excretion of 2-$OHEE_2$ and irreversible binding. Moreover, from the absence of a significant difference between the rates at which male and female rat liver microsomes metabolize EE_2 to bound material *in vitro*, it may be deduced that the supply of reactive metabolite *in vivo* is not solely regulated by this pathway. An alternative explanation is that irreversible binding is determined by reactions which deactivate either 2-$OHEE_2$ or its reactive metabolite. Catechol *O*-methylation [3], glucuronylation and sulphation might all play a part in the removal of 2-$OHEE_2$, whilst the reactive metabolite might be deactivated by either reduction to 2-$OHEE_2$ or formation of glutathione conjugates [7—9].

The results of the present and previous studies [4, 5] reveal that 2-$OHEE_2$ formed in male rats undergoes extensive methylation prior to conjugation with either glucuronic acid or sulphate. Examination of the deconjugated biliary metabolites (Table 5) indicates that a smaller proportion of 2-$OHEE_2$ is excreted as methylated products in female rats. However, this is not due to sex-linked differences in the activity of hepatic cytosolic COMT in the Wistar rats (Table 4). Furthermore, other workers have not detected sex-linked differences in COMT activity in either Wistar–Furth or Fischer rats [34]. However, the sex-linked difference in the excretion of methylated 2$OHEE_2$ metabolites may be explained by the fact that hepatic estrogen-*O*-demethylase activity is higher in male rats than in female rats [35].

Although the proportion of EE_2 excreted unchanged except for conjugation was only slightly greater in males, the total of 2-hydroxylation products (2-$OHEE_2$, 2-MeOEE_2 and 2-OHME) was considerably lower in males (50 ± 7%) than in females (71 ± 3%). The polar metabolites which eluted together from an ODS column largely accounted for this difference. Although the identities of these polar metabolites remain to be determined, preliminary data [4] suggest that they represent further modifications of 2-$OHEE_2$, e.g. dihydroxymethoxyEE_2. A number of poly-oxygenated polar estrogen metabolites have been identified both *in vitro* and *in vivo* [13].

Glucuronylation and sulphation of 2-$OHEE_2$ will be partly responsible for minimizing irreversible binding *in vivo*. The enzyme-hydrolysable fraction of female rat bile was almost entirely composed of glucuronides. There were no significant sex-linked differences between the aglycone fractions, the lower overall proportion of 2-$OHEE_2$ in male rat bile stemming from the previously observed low proportion of catechol in its sulphate fraction [4]. The finding that female rats excrete little or no sulphated metabolites of EE_2 conforms with the observation that male Wistar rats have a higher hepatic phenolsulphotransferase activity than females [36].

The ultimate reaction which may regulate irreversible binding of EE_2's reactive metabolite is formation of thioethers. *o*-Quinones and *o*-semiquinones derived from catechol estrogens react spontaneously with glutathione [13], and cysteine conjugates are formed from EE_2 *in vitro* [7]. More-

Table 5. Reversed-phase HPLC of deconjugated biliary metabolites of [^{3}H]EE_2 from male and female rats

Component	Males		Females	
	β-Glucuronidase –arylsulphohydrolase	β-Glucuronidase	β-Glucuronidase –arylsulphohydrolase	β-Glucuronidase
Heterogeneous	18 ± 7	7 ± 2	7 ± 1*	6 ± 1
16-$OHEE_2$	5 ± 1	5 ± 1	3 ± 1	3 ± 1
2-$OHEE_2$	20 ± 6	28 ± 8	43 ± 1†	39 ± 7
EE_2	14 ± 1	15 ± 3	10 ± 1‡	14 ± 5
2-MeOEE_2	25 ± 7	24 ± 7	20 ± 3	19 ± 2
2-OHME§	5 ± 1	7 ± 7	8 ± 1	5 ± 1

[^{3}H]EE_2 was administered i.v. (5 μg/kg) to bile duct-cannulated anaesthetized rats. Bile samples (0–3 hr) were incubated with enzymes and the metabolites were extracted into ether. The heterogeneous component appears to be comprised of two co-eluting components in males.

*$P < 0.05$

†$P < 0.001$

‡$P \pm 0.005$ (different from moles)

§ 2-Hydroxymestranol.

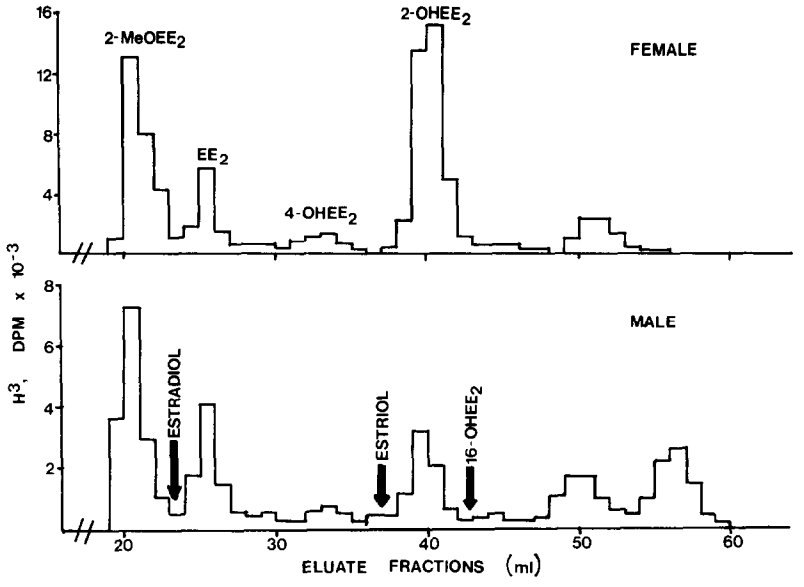

Fig. 5. Bonded-normal-phase high performance liquid chromatograms of enzymically deconjugated biliary metabolites of [6,7-^{3}H]EE_2 from male and female Wistar rats. The chromatographic details are given in the text. Peak assignments are based on co-chromatography with authentic unlabelled standards. Bile (0–3 hr sample) from bile duct-cannulated rats dosed i.v. was incubated with β-glucuronidase–arylsulphohydrolase, and the deconjugated metabolites were extracted into ether.

over, estrogen thioethers are minor biliary metabolites of 2-hydroxy-estradiol given intraperitoneally to male rats [37]. However, when male rats were given multiple doses of L-[^{35}S]cysteine and subsequently administered unlabelled EE_2 no conclusive evidence for the excretion of ^{35}S-labelled EE_2 metabolites in bile was obtained [5].

In summary, irreversible binding of EE_2's reactive metabolite is only a minor route of the steroid's metabolism in male and female rats. The reactions likely to be responsible for the sex-related differences in binding have been delineated, but an assessment of their quantitative significance will require further study.

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