J. Steroid Biochem. Molec. Biol. Vol. 42, No. 1, pp. 65–76, 1992
Printed in Great Britain. All rights reserved

0960-0760/92 $5.00 + 0.00
Copyright © 1992 Pergamon Press plc

THE SEXUALLY DIFFERENTIATED METABOLISM OF [6,7-^{3}H]17β-OESTRADIOL IN RATS: MALE-SPECIFIC 15α- AND MALE-SELECTIVE 16α-HYDROXYLATION AND FEMALE-SELECTIVE CATECHOL FORMATION

J. L. MAGGS, P. MORGAN and B. K. PARK*
Department of Pharmacology and Therapeutics, University of Liverpool, Liverpool L69 3BX, England

(Received 10 August 1991)

Summary—The oxygenated-metabolite profiles of exogenous 17β-oestradiol (E_2) in adult male and female Wistar rats have been characterized and major sex-dependent biotransformations observed which correlate with the regioselectivities of known sexually differentiated hepatic *P*450. [6,7-^{3}H]E_2 (27 μg/kg) was given i.v. The metabolites of E_2 were rapidly and extensively excreted in bile (46 and 78% of the dose over 1 and 6 h, respectively). Female rats metabolized E_2 by one major pathway: oxidation to oestrone (E_1) followed by C-2 hydroxylation and *O*-methylation; the principal aglycones (0–1 h bile collections) were E_1 (14%), 2-hydroxyE_1 (2-OHE_1) (42%) and 2-methoxyE_1 (24%). Male rats metabolized E_2 principally by two parallel composite pathways of E_1 hydroxylation which yielded a complex mixture of mono- and di-oxygenated compounds: 15α-OHE_1 (33%), 2,15α-diOHE_1 (7%), and 2-methoxy-15αOHE_1 (14%); 16α-OHE_1 (13%), 2,16α-diOHE_1 (4%) and 2-methoxy-16α-OHE_1 (2%). 15α-Hydroxylation was unique to males. The balance of aromatic and alkyl hydroxylation in males was dose-dependent: at 3 mg/kg, 15α-hydroxylation was decreased approx. 50% in favour of 2-hydroxylation whilst 16α-hydroxylation was largely unaffected.

The male-specific 15α-hydroxylation and male-predominant 16α-hydroxylation of E_1 derived from E_2 *in vivo* may be ascribable to the male-specific isoforms *P*450IIC13 and *P*450IIC11, respectively.

INTRODUCTION

The multiplicity of regioselective and stereospecific steroid hydroxylations in adult rat liver are catalysed by microsomal *P*450 from several gene sub-families [1–7]. Amongst these enzymes are male-specific [3–7] and female-specific [6–8] forms with broad, yet selective, substrate specificities. Additional but non-constitutive *P*450 steroid hydroxylases can be induced in both sexes [1, 6, 9]. Finally, certain constitutive steroid hydroxylases are common to both sexes [6 10]; though they may be preferentially expressed in one sex [11]. The expression of a sexual dimorphism in steroid hydroxylation could be further complicated by *P*450 polymorphism [2, 12–14].

The androgen and progesterone specificities of rat sex-specific and sex-predominant *P*450 are known in detail [1–7, 11–15]; a male-specific hepatic form determines the sexual dimorphism of androgen 16α-hydroxylation [1, 4]. Less is known about the sexual differentiation of oestrogen hydroxylation—especially alkyl hydroxylation—and the involvement of sex-specific *P*450. The metabolism of 17β-oestradiol (E_2) is dominated by dehydrogenation to oestrone (E_1) and subsequent hydroxylation [16]. C-2 hydroxylation, predominant in adult female rats [17–19], is the principal pathway in rat liver microsomes [20, 21]. Several *P*450 catalyse oestrogen 2-hydroxylation [20, 22, 23]; including male-specific forms considered responsible [20] for the sexual dimorphism of the microsomal activity [24, 26].

Male rat hepatic microsomes contain a male-specific *P*450 [1, 4, 22] with oestrogen 16α-hydroxylase activity [15, 22]. Though another major form possessing this activity is present in both sexes [10], male microsomes are more active [27]. Nevertheless, E_2 16α-hydroxylation is a minor [21, 27] and strain-dependent [27] microsomal reaction. Various 16α-hydroxylated metabolites of exogenous oestrogens have been isolated from rat bile [28, 29] but with incomplete qualitative and quantitative

*To whom correspondence should be addressed.

characterization. A sexual dimorphism of oestrogen 16α-hydroxylation *in vivo* has not been reported hitherto. There appears to be no previous evidence that any of the other oestrogen alkyl hydroxylations [30–33] display sexual dimorphism. However, one of the major male-specific, though strain-dependent, hepatic *P*450 [12–14, 22] has oestrogen 15α-hydroxylase activity [15, 22] and thus represents an enzymic potential for a metabolic disparity.

In the present study, the pathways of E_2 hydroxylation in rats, but especially the incompletely described alkyl hydroxylations, have been characterized to determine whether they are corollaries of the regioselectivities of known sex-specific *P*450.

EXPERIMENTAL

Materials

[6,7-^{3}H]17β-oestradiol ([^{3}H]E_2; 48Ci/mmol; radiochemical purity, 99%; NEN Research Products, Fed. Rep. Germany) was re-purified, as a methanol solution (128 μCi/μmol), on a Techopak column (C_{18}, 10 μm; 30 × 0.39 cm i.d.; HPLC Technology, Macclesfield, England) by elution with acetonitrile (20 to 75% over 40 min) in 43 mM ammonium dihydrogen orthophosphate, pH 3.0, at 1.5 ml/min. It was recovered by extraction into ether following evaporation of the acetonitrile under nitrogen. Unlabelled E_2, other oestrogen standards, oestrogen conjugates, β-glucuronidase (Glucurase) and β-glucuronidase-arylsulphohydrolase (Type H-2; 4.2 × 10^3 U/ml arylsulphohydrolase) were from Sigma Chemical Co. (Poole, England). 15α-Hydroxyoestrone (15α-OHE_1) was generously provided by Dr M. Levitz, New York University Medical Centre (NY, USA). Boron tribromide (1 M in dichloromethane) was from Aldrich Chemical Co. (Gillingham, England).

Animal experiments

Male (220 ± 18 g body wt; mean ± SD, $n = 4$) and female (220 ± 20 g; $n = 4$) Wistar rats were anaesthetized with urethane (1.4 g/kg body wt, in isotonic saline given i.p.) and cannulated via the jugular vein and common bile duct. [^{3}H]E_2 (1–3 μCi) was administered i.v. in saline–polyethylene glycol 200 (1:1, v/v) at a dose of 0.1 μmol/kg (27.2 μg/kg). Bile was collected hourly for 6 h, and assayed for radioactivity [34].

Chromatographic analysis of biliary metabolites

Bile (10–20 μl), deconjugated biliary metabolites (50–80 μl methanol solutions) and co-injected unlabelled standards were analysed on a Techopak column linked to an LKB 2150-2152 pump-controller and a Gilson IIIB u.v. (280 nm) detector. Biliary conjugates (15–50 × 10^3 dpm) were resolved with methanol (20 to 60% over 40 min) in 10 mM ammonium acetate, pH 6.9, and aglycones (5–50 × 10^3 dpm) with acetonitrile (20 to 75% over 40 min) in 43 mM ammonium dihydrogen orthophosphate, pH 3.0. The flow rate was 1.5 ml/min. Pairs of polar ketols and methoxyketols, irresolvable on the C_{18} column, were separated on a diol column (10 μm, 25 × 0.46 cm i.d.; HPLC Technology) with propan-2-ol in hexane (10% for 10 min, then 10–20% over 20 min; flow rate, 1.5 ml/min) after isolation by C_{18}-HPLC. They were recovered from the reversed-phase eluate by ether extraction, and reconstituted in methanol for diol-phase HPLC. Metabolites were quantified by scintillation counting: 30 s eluate fractions were dissolved in 4 ml of scintillant (Aqua Luma Plus; Lumac bv, The Netherlands). Recoveries of chromatographed radioactivity were 90–100%.

Enzymic hydrolysis

Bile (20–50 μl; 20–80 × 10^3 dpm; 50–200 ng equivalent E_2) was incubated with either β-glucuronidase (5 × 10^3 U) or β-glucuronidase-arylsulphohydrolase (approx. 1.0 × 10^3 and 42 U, respectively) in 1.0 ml 0.1 M sodium acetate, pH 5.0, containing 10 mM ascorbate at 37°C for 16 h; the ascorbate prevented oxidative loss of catechol metabolites [35]. Combined ether extracts (5 ml × 2) of the incubations were evaporated under nitrogen, and reconstituted in 100 μl methanol for HPLC.

Isolation and identification of metabolites

Isolation by reversed-phase HPLC. Anaesthetized and cannulated male Wistar rats (350 g) were administered [^{3}H]E_2 (3.0 mg/kg; 5.0 μCi) in 0.3 ml polyethylene glycol 200–isotonic saline (2:1, v/v) given i.v. Bile (0.4–2.7 ml) was collected for 3 h; 68–72% of the dose was recovered. Radiochromatographic analysis of the aglycones revealed substantial dose-related changes [Fig. 2(B and C)]. The metabolite profile combined the male and female low-dose profiles, plus a small amount of E_2, with elevated proportions of the most polar male-specific metabolites [M-α, R_t (^{3}H peak) 7.5 min, and

M-β, R_t 9.0 min]. Three steroids—2-OHE$_1$ (17.5 min), E$_1$ (22.5 min) and 2-methoxyE$_1$ (23.5 min)—were ascribed by co-elution with standards. The remaining fractions, i.e. male-specific M-A (12.0 min) and male-predominant M-B (13.5 min), co-eluted with authentic 15α-OHE$_1$ and 16α-OHE$_1$, respectively, but mass spectrometry (MS) and HPLC showed them to be heterogeneous.

Bile (250 μl; 100 μg equivalent) was incubated with β-glucuronidase-arylsulphohydrolase (2.5 × 10^3 and 105 U, respectively) in 2.5 ml of acetate buffer containing 10 mM ascorbate at 37°C for 16 h. The ether (7 ml × 2)-extracted aglycones, representing 87% of incubated radioactivity, were reconstituted in methanol (500 μl). Portions (70–120 μl; 12–21 μg equivalent) were chromatographed on a Techopak (C_{18}) column. E$_2$, five metabolites and two heterogeneous fractions (83% of chromatographed radioactivity) were eluted with acetonitrile–phosphate buffer, and recovered by ether extraction (2 vol × 2). The isolated metabolites and fractions (1–18 μg) were radiochromatographically homogeneous on the C_{18} column. Aliquots were analysed by electron impact (EI) and chemical ionization (CI) MS.

Detailed chromatographic analysis of M-B (16 μg) revealed two sub-fractions separated by 0.7 min; base-line resolution of u.v. absorbance but not of radioactivity by fraction collection was achieved. The first-eluted component

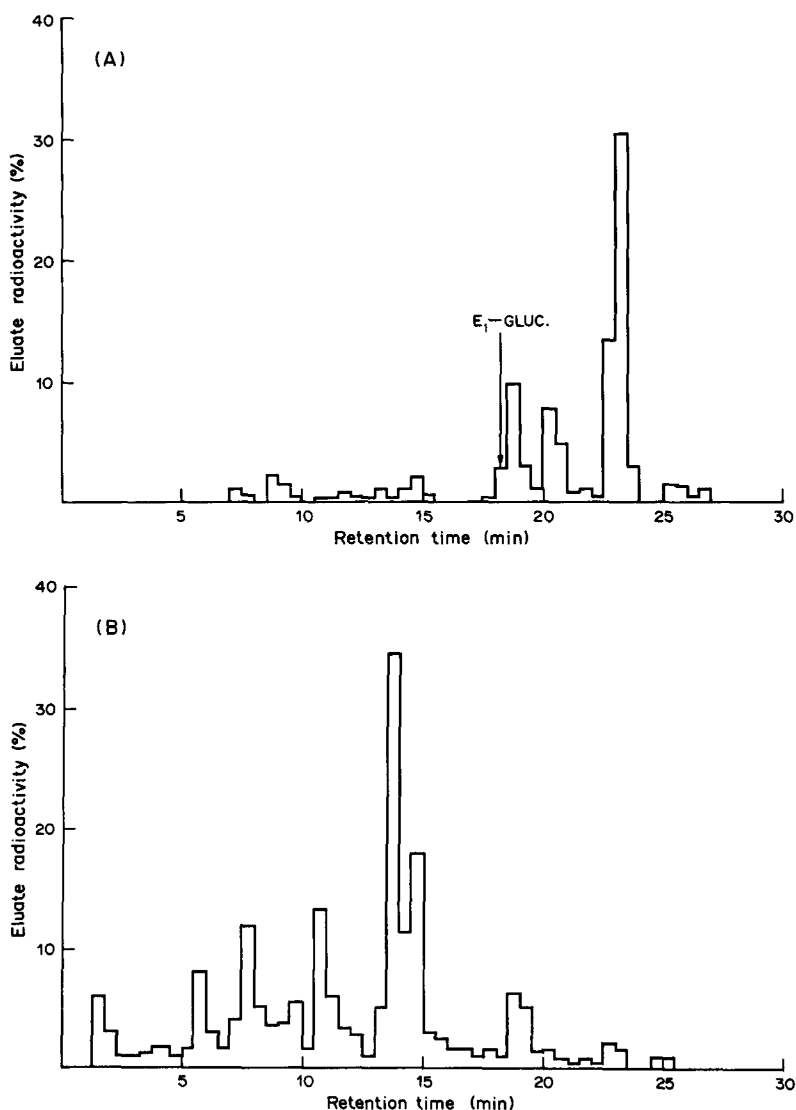

Fig. 1. High-performance liquid radiochromatograms of the biliary metabolites of [^{3}H]E$_2$ (27 μg/kg) in (A) female (B) male rats at 0–1 h. The metabolites and unlabelled standards were resolved on a C_{18} column using a gradient of methanol in ammonium acetate.

exactly co-chromatographed with authentic 16α-OHE$_1$. 16α-OHE$_1$ and its methoxylated co-fraction (*m/z* 316 in spectrum of M-B) were isolated (ratio of radioactivity, 0.95:1.0) and characterized.

Isolation by diol-phase HPLC. EI spectra of M-A indicated 15α-OHE$_1$ [[M]$^{+\cdot}$ at *m/z* 286, relative intensity (RI) 72%]—acquired spectrum was computer-matched with standard's spectrum—and a methoxylated derivative ([M]$^{+\cdot}$ at *m/z* 316; RI 100%). These were irresolvable on the C$_{18}$ column but could be separated (R_t 19.5 and 21.5 min; ratio of radioactivity, 0.75:1.0) on a diol column with propan-2-ol in hexane (10% for 10 min, then 10–20% over 20 min) at 1.5 ml/min; the second-eluted component co-chromatographed with authentic 15α-OHE$_1$. Isolated M-A (21 μg) was fractionated on the diol column, and yielded radiochromatographically homogeneous 15α-OHE$_1$ and methoxylated 15α-OHE$_1$ which was 86% pure. The sub-fractions were analysed by MS.

Chemical demethylation of methoxylated metabolites. Characterization of the methoxylated, i.e. methylated catechol, metabolites and their putative catechol precursors was aided by demethylation with boron tribromide [36]. Authentic 2-methoxylated E$_1$, E$_2$ and 16α-OHE$_2$ (50–100 μg; approx. 0.16–0.33 μmol) were dissolved in 1 ml dichloromethane in a conical glass tube, the head-space flushed with nitrogen, and boron tribromide (10–20 μl; 10–20 μmol) injected via a septum cap. After 3–4 h at 20°C, the reaction was terminated by adding water (2 ml) and the products extracted into ether (6 ml × 2). Extensive (75–80%) or quantitative formation of catechol was established by C$_{18}$-HPLC and MS. 2-methoxyE$_1$, M-A and M-B (20–28 μg), isolated from the bile hydrolysates by C$_{18}$-HPLC, were reacted with boron tribromide (50 μl) for 1.5–2 h. Essentially quantitative demethylation of 2-methoxyE$_1$ and the methoxylated sub-fractions of M-A and M-B was confirmed by HPLC and MS; only traces of (non-polar) side products were formed. 2-OHE$_1$ co-eluted with its standard, and the ketol sub-fractions of M-A and M-B with 15α-OHE$_1$ and 16α-OHE$_1$, respectively. The ketols and the catechols liberated from their co-fractions were isolated (3–6 μg) by C$_{18}$-HPLC for MS.

MS

Standards and metabolites were analysed by direct-probe EI and CI (isobutane) MS using a VG TS-250: electron energy, 70 eV (EI) or 50 eV (CI); filament emission, 500 μA (EI) or 300 μA (CI); source temperature, 180°C; accelerating voltage, 4 × 10^3 V. Spectra were acquired via a VG 11/250J data system over *m/z* 50 (EI)/75 (CI)-600 (scan time, 1 s) at resolution 800.

RESULTS

Excretion of radiolabelled metabolites

The radiolabelled metabolites of i.v.-administered [^{3}H]E$_2$ (27 μg/kg) were rapidly and extensively excreted in bile by both sexes: 51.6 ± 3.2% (mean ± SD, $n = 4$) and 40.6 ± 8.5% of the dose during the first hour by males and females, respectively; 73.8 ± 1.7 and 65.9 ± 4.6% over 3 h; 79.2 ± 2.4 and 76.8 ± 9.0% over 6 h.

Biliary metabolites

Female bile contained three major radiolabelled conjugates (R_t 19, 20.5 and 23.5 min) and several minor ones [Fig. 1(A)]. Collectively, the three represented 72–78% of the radiolabel excreted during the first hour; with the principal metabolite (R_t 23.5 min) representing 43–47% (mean, 46%). The conjugate of R_t 19 min co-eluted with authentic E$_1$ β-glucuronide. Male rats formed a greater number of more polar conjugates [Fig. 1 (B)]. Since 75–86 and 81–89% of the biliary radiolabel in both sexes was ether-extractable after incubations with β-glucuronidase and β-glucuronidase-arylsulphohydrolase, respectively, whereas only 4–6% was immediately extractable, it is evident that the conjugates were predominantly glucuronides.

Identification of steroidal metabolites

E$_2$, E$_1$, 2-OHE$_1$ (M$^{+\cdot}$ *m/z* 286, RI 100%) and 2-methoxyE$_1$ (M$^{+\cdot}$ *m/z* 300, RI 100%) isolated from enzymic hydrolysates of bile [Fig. 2(C)] were identified from their EI spectra; which matched those of unlabelled standards (data not shown).

Ketol metabolites. 15α-OHE$_1$ and 16α-OHE$_1$ were identified by EI [Table 1; Figs 3(A) and 4(A), respectively] and CI MS. Their EI spectra matched library spectra [37] and acquired spectra of standards [Fig. 3(A and B)]. Ring-D fragmentation yielded diagnostic ions at characteristic RI: metabolic and authentic 15α-OHE$_1$ gave *m/z* 214 ([M-C$_3$O$_2$H$_4$]$^{+\cdot}$) and *m/z* 213 ([M-C$_3$O$_2$H$_5$]$^{+\cdot}$) in the RI ratio 5:22 and 7:20, respectively, whereas the corresponding ratios for 16α-OHE$_1$ were 34:100 and 42:98. The

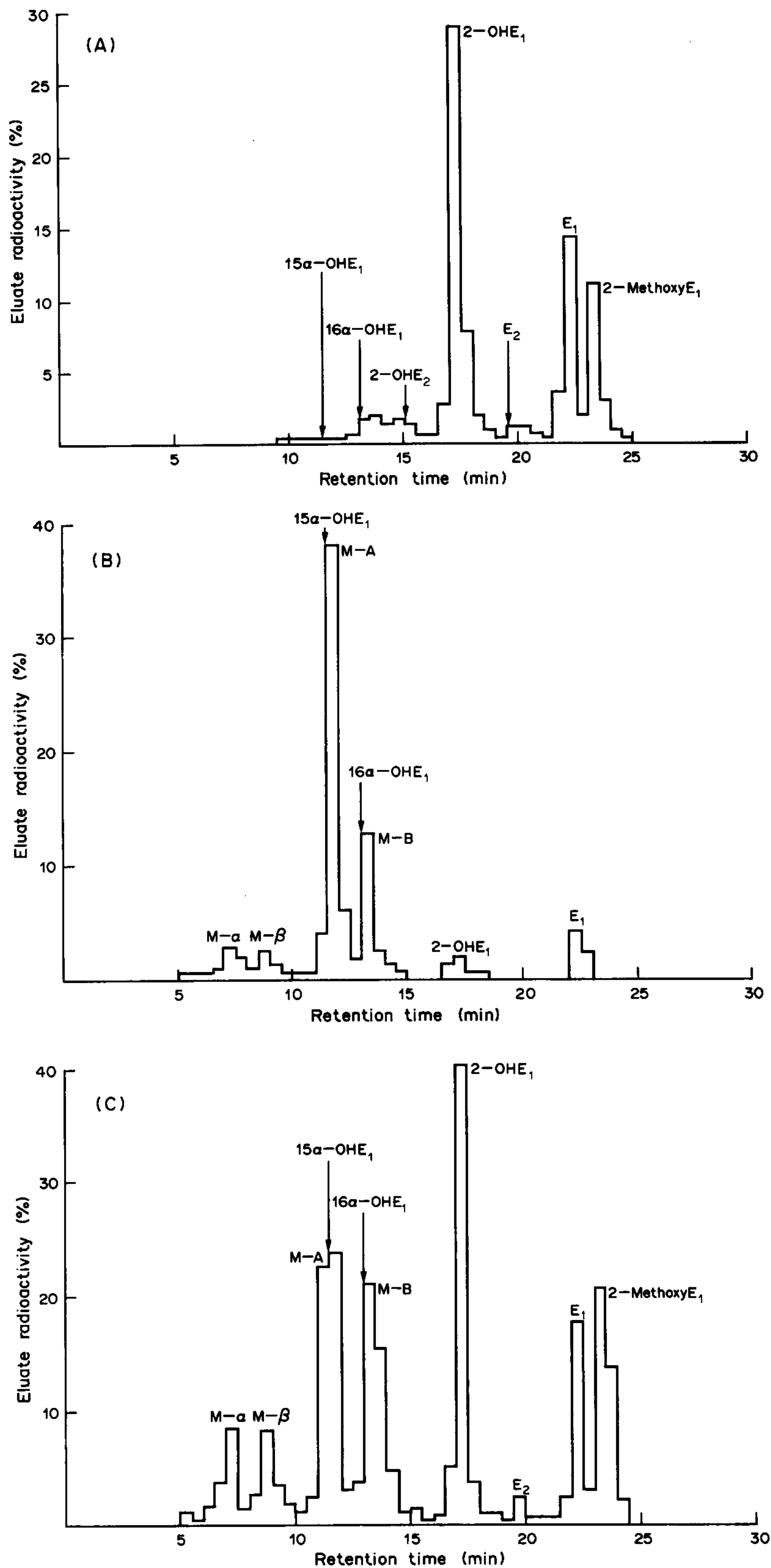

Fig. 2. High-performance liquid radiochromatograms of the phase I metabolites of $[^3H]E_2$ liberated from conjugates in rat bile: (A) females, 27 μg/kg, 0–1 h; (B) males, 27 μg/kg, 0–1 h; (C) males, 3 mg/kg, 0–3 h. The metabolites and unlabelled standards were resolved on a C_{18} column using a gradient of acetonitrile in ammonium dihydrogen orthophosphate.

metabolites' spectra were distinct from those of 14-OHE_1, 12β-OHE_1, 11β-OHE_1, 6β-OHE_1, 16-keto-17βE_2, 16-keto-17αE_2 and 6-keto-17βE_2 [37]. These identifications were supported by CI MS. Both metabolites and their standards gave base-peak $[M+1]^+$ at m/z 287. The pseudomolecular ions underwent regioselective dehydration [39]: the RI ratio [M + 1-18]: [M + 1] for 15α- and 16α-OHE_1 was 68:100 and 21:100, respectively.

Hydroxyketol (catechol) metabolites. M-α and M-β [Fig. 2(B)] gave dioxygenated $M^{+\cdot}$ at m/z 302 [Figs 3(C) and 4(B)]. Under CI conditions, m/z 303 ($[M+1]^+$) and m/z 285 ($[M+1\text{-}18]^+$) were obtained; as with the ketols, the more polar metabolite, i.e. M-α, underwent greater dehydration. M-α and M-β were deduced to be C-2 catechol, rather than cycloalkanol, derivatives of 15α- and 16α-OHE_1, respectively, from EI spectra and by comparison with the catechols liberated from the methoxylated ketols. Thus the peaks at m/z 227, 229 and 230 (Table 1) are ring-D fragmentation ions for catechols [37, 38], i.e. analogues of m/z 211, 213 and 214 in the ketols' spectra, and the greater fragmentation of M-β is typical of a 16-OH ketol. Those at m/z 162, 173, 175 and 188 are catechol fragments containing rings A and B [37] whilst the peaks at m/z 123, 131 and 136 appear to be oxygenated ring-A fragments [28, 37]. A structural connection between the dioxygenated polar metabolites and the methoxyketols was conclusively established by demethylation; boron tribromide effects quantitative demethylation of aryl methylethers under mild conditions [36]. The catechols obtained from 2-methoxy-15$\alpha$$OHE_1$ and 2-methoxy-16$\alpha$$OHE_1$ (*vide infra*) co-eluted with M-α and M-β, respectively, and yielded corresponding EI and CI spectra.

Methoxyketol (methylated catechol) metabolites. Putative 2-methoxy-15$\alpha$$OHE_1$ and 2-methoxy-16$\alpha$$OHE_1$ were isolated by diol- and C_{18}-HPLC, respectively; the site of methoxylation was indicated by the predominance of C-2 hydroxylation. Their EI spectra [Table 1; Figs 3(D) and 4(C)] contained the fragments diagnostic of 2-methoxyoestrogens [37, 40], including 2-methoxy-16$\alpha$$OHE_1$ [28, 29], e.g. m/z 137, 176, 189, 202, 215, 229, 243 and 244. The peaks at m/z 243 (16α-OH isomer only) and 244, analogues of m/z 213 and 214, respectively, in the ketols' spectra, had RI indicative of 15α- and 16α-hydroxylated isomers. CI spectra contained pseudomolecular base peaks at m/z 317 and dehydration fragments at m/z 299. The RI ratio $[M\text{-}H_2O]$:[M + 1] for the methoxylated co-fractions of 15α-OHE_1 and 16α-OHE_1–28:100 and 11:100, respectively—conformed with those for the corresponding ketols.

Schematic metabolic pathways based upon the above identifications are shown in Fig. 5. However, they represent the minimum number of reactions considered necessary to explain the sexual differentiation of metabolism rather than an attempt to incorporate every possible transformation.

Quantitation of steroidal metabolites

The phase EI metabolites of low-dose $[^3H]E_2$ (27 μg/kg) displayed an unambiguous sexual dimorphism [Table 1; Fig. 2 (A and B)]. Unchanged E_2 was either absent or present in only trace amounts (approx. 1% deconjugated radioactivity). The major metabolites formed by females, i.e. E_1, 2-OHE_1 and 2-methoxyE_1, represented 80% of the radiolabel liberated from conjugates in 0–1 h bile collections. Whilst

Table 1. Molecular ions and principal fragments of the polar metabolites of $[^3H]E_2$ isolated from rat bile

Metabolite	Ions (RI)
15α-OHE_1	286 ($M^{+\cdot}$,100) 268 (9), 240 (8), 225 (7), 214 (22), 213 (5), 211 (7), 199 (8), 186 (36), 172 (17), 159 (67), 157 (18), 146 (45), 133 (21), 120 (13).
2-OH-15$\alpha$$OHE_1$	302 ($M^{+\cdot}$,100), 284 (32), 242 (6), 230 (11), 227 (16), 215 (8), 213 (9), 202 (12), 188 (11), 175 (24), 173 (28), 162 (24), 136 (11).
2-Methoxy-15$\alpha$$OHE_1$	316($M^{+\cdot}$,100),244 (21), 230 (4), 216 (12), 202 (4), 189 (19), 187 (12), 176 (27), 159 (17), 150 (11), 137 (17).
16α-OHE_1	286($M^{+\cdot}$,100),258 (8), 214 (100), 213 (34), 201 (18), 199 (20), 186 (11), 172 (54), 159 (46), 157 (23), 146 (33), 133 (22), 115 (15).
2-OH-16$\alpha$$OHE_1$	302($M^{+\cdot}$,100), 230 (54), 229 (46), 215 (18), 202 (16), 188 (35), 175 (33), 173 (52), 162 (21), 161 (32), 131 (29), 123 (23).
2-Methoxy-16$\alpha$$OHE_1$	316($M^{+\cdot}$,100), 288 (3), 244 (36), 243 (14), 229 (8), 215 (4), 202 (15), 189 (14), 187 (12), 176 (11), 163 (8), 145 (7), 137 (26).

Spectra were obtained by direct-probe EI.

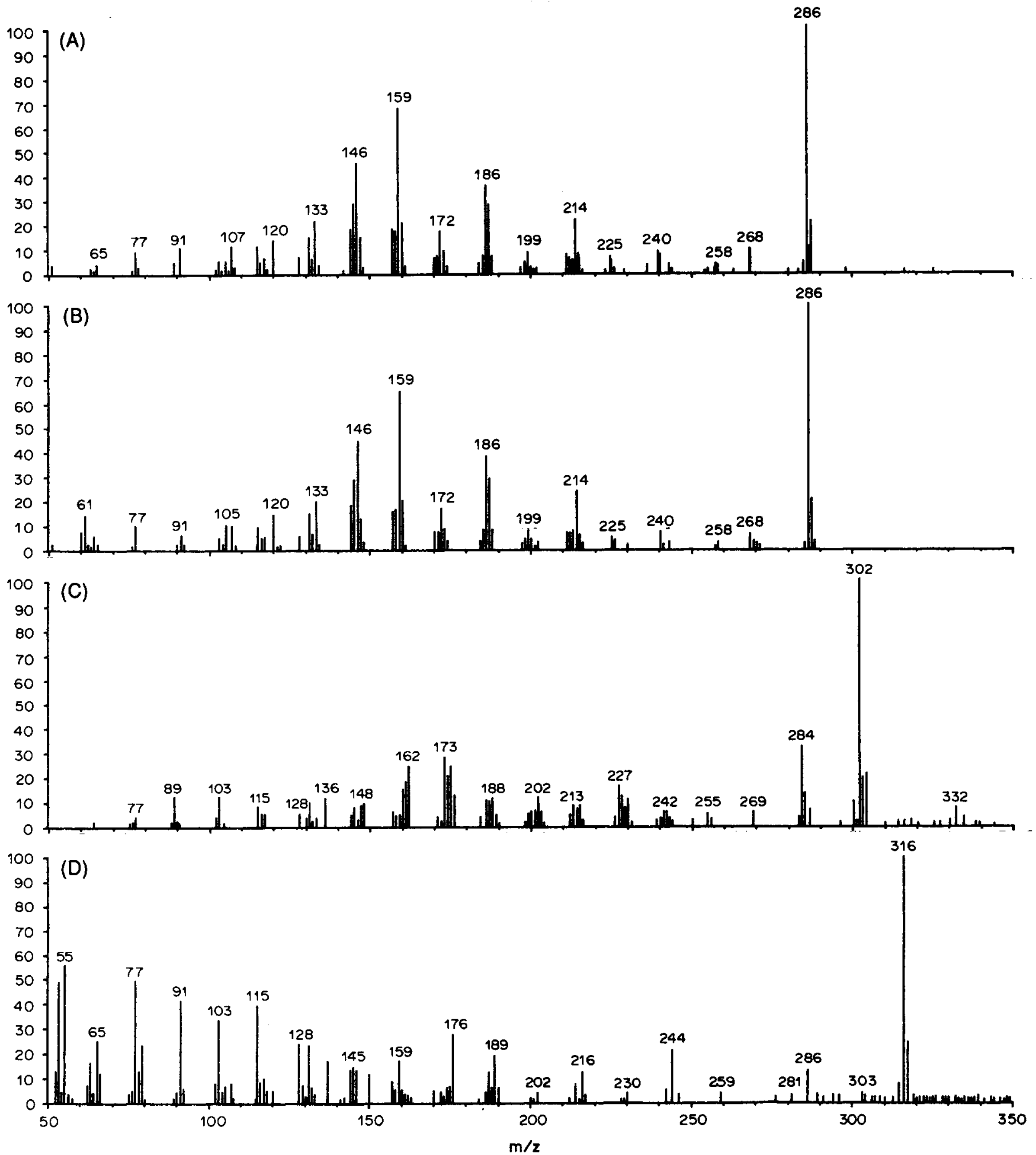

Fig. 3. Direct-probe electron impact mass spectra of 15α-hydroxylated metabolites of [^{3}H]E_2 formed in male rats: (A) 15α-OHE$_1$; (B) authentic 15α-OHE$_1$; (C) M-α[2-OH-15αOHE$_1$]; and (D) 2-methoxy-15αOHE$_1$. Metabolites were isolated from enzymic hydrolysates of bile by HPLC.

E_1 and 2-OHE$_1$ were consistent though minor products in males, 2-methoxyE$_1$ was only a trace metabolite (0–1.5% of deconjugated radiolabel). 16α-OHE$_1$ and, by chromatographic implication, 2-methoxy-16αOHE$_1$, were minor female metabolites but 15α-OHE$_1$ and 2-methoxy-15αOHE$_1$, and both the catechol intermediates, appeared to be unique to males. Male-specific, i.e. 15α-oxygenated, and male-predominant, i.e. 16α-oxygenated, products collectively represented an estimated 54 and 19.5%, respectively, of the males' phase I metabolites (Table 2). Chromatographic (C_{18}-HPLC) comparison of the metabolites with unlabelled standards indicated that the following were not present in appreciable quantities:

2,16α-diOHE$_2$ (2-OH oestriol; R_t 6.5 min), 16α-OHE$_2$ (9.5 min), 2-methoxy-16αOHE$_2$ (10.0 min) 6- and 16-ketoE$_2$ (12.5 min; standards not resolved), 4-OHE$_1$ (18.5 min) and 2-methoxyE$_2$ (20.5 min). 6β-OHE$_2$ co-chromatographed with M-β [2-OH-16αOHE$_1$; Fig. 2(B)] but the latter's mass spectra clearly indicated a molecular weight of 302. 2-OHE$_2$ (15.5 min) may have been formed in trace amounts by female rats [Fig. 2(A)].

The male and female metabolite profiles remained qualitatively unchanged throughout the 3 h after dosing and the proportion of each metabolite in males was essentially constant. All the quantitative changes observed in females were slight and inconsistent.

The regioselective hydroxylation of E$_1$ formed from E$_2$ in males was dose-dependent between 27 μg/kg and 3 mg/kg [Fig. 2(B and C)]: the proportions of the hydroxylations at C-2, C-15 and C-16, estimated from a combination of diol- and C$_{18}$-HPLC analyses, changed from approx. 29:53:18 (Table 2), respectively, to 59:24:17; 15α-hydroxylation declined approx. 50% in favour of 2-hydroxylation whilst 16α-hydroxylation was largely unaffected. Consequently, the major metabolites at 3 mg/kg were 2-OHE$_1$ (20% radiolabel), 2-methoxyE$_1$ (15%), 15α-OHE$_1$ (12%), 2-methoxy-15αOHE$_1$ (10%) and 16α-OHE$_1$ (10%).

DISCUSSION

The present studies have identified and quantified the hitherto poorly characterized components of the "polar" fraction of E$_2$s biliary metabolites in rats [28]. A sexual

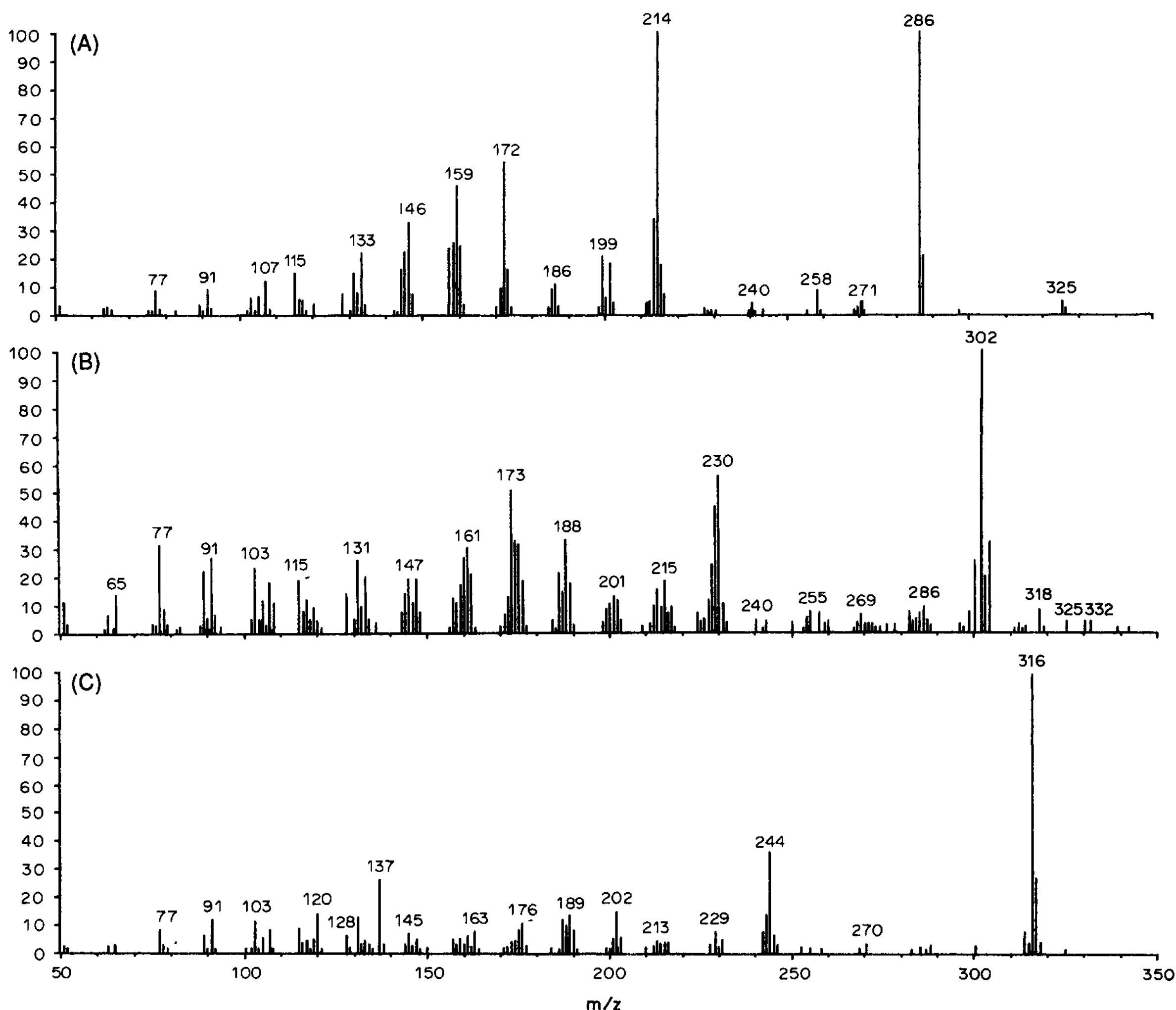

Fig. 4. Direct-probe electron impact mass spectra of 16α-hydroxylated metabolites of [^{3}H]E$_2$ formed in male rats: (A) 16α-OHE$_1$; (B) M-β[2-OH-16αOHE$_1$]; and (C) 2-methoxy-16αOHE$_1$. Metabolites were isolated from enzymic hydrolysates of bile by HPLC.

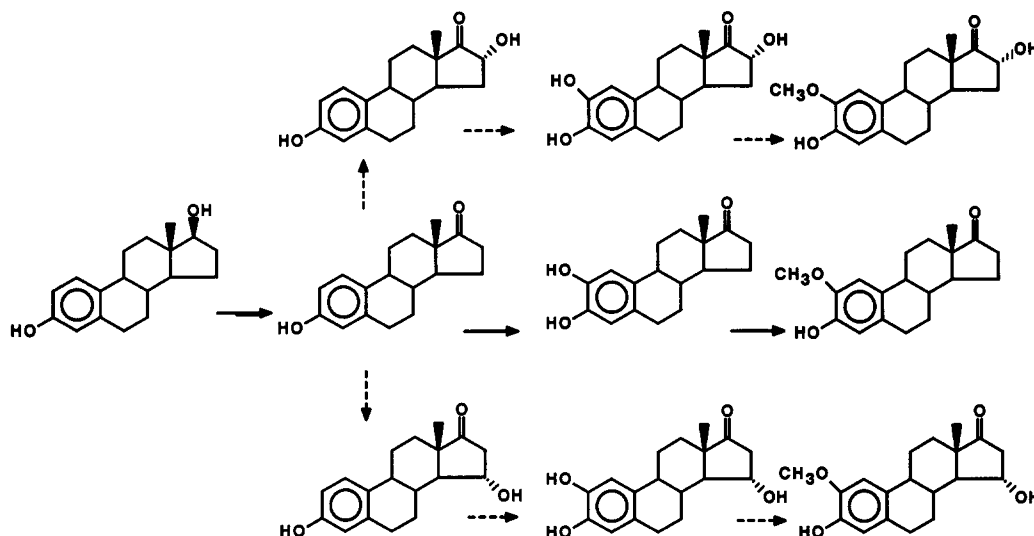

Fig. 5. Scheme of proposed major pathways of E_2 metabolism in male (- - -→) and female (——→) rats at a dose of 27 μg/kg. E_1, 2-OHE$_1$ and 2-methoxyE$_1$ were the major metabolites in females; 15α-OHE$_1$, 16α-OHE$_1$ and 2-methoxy-15αOHE$_1$ the major metabolites in males.

differentiation of alkyl hydroxylations—predictable in essence from the known regioselectivities of male-specific hepatic *P*450 [15, 22]—was established. They, rather than the previously emphasized aromatic hydroxylations [16], were the basis of a sexual dimorphism of E_2 metabolism.

The high ratio of aromatic (C-2) to alkyl (C-16α) hydroxylation in female rats approximately reflects the relative enzyme activities of rat hepatic microsomes [21, 24, 27], and yielded a profile of C-2 oxygenated metabolites similar to those described previously [18, 19]. In contrast, and despite the 2.5- to 7-fold higher oestrogen C-2 hydroxylase activity of male microsomes [20, 26], alkyl hydroxylations, i.e. C-15α and C-16α, either alone or in combination with C-2 hydroxylation, were dominant in males: the ratio of ring-D to ring-A oxygenated products was approx. 71:29 and 5:67 in males and females, respectively.

Table 2. The metabolites of [^{3}H]E_2 (27 μg/kg) liberated from conjugates in the bile of male and female rats

	% Radioactivity (mean ± SD, n = 4)	
Metabolite	Males	Females
Oestrone (E_1)	6.7 ± 1.0	13.5 ± 3.4
2-OHE$_1$	3.0 ± 0.7	42.4 ± 3.6
2-MethoxyE$_1$	0–1.5	24.3 ± 6.1
Fraction M-A	47.2 ± 0.3	0
15α-OHE$_1$[a]	33	
2-OH-15αOE$_1$ (M-α)	6.7 ± 0.9	0
2-Methoxy-15αOHE$_1$[a]	14	0
Fraction M-B	17.1 ± 1.3[b]	5.2 ± 2.5
16α-OHE$_1$[a]	13	ND
2-OH-16αOHE$_1$ (M-β)	4.4 ± 0.6	0
2-Methoxy-16αOHE$_1$[a]	2	ND

Biliary conjugates (0–1 h collections) were hydrolyzed with enzymes. Ether-extracted metabolites were quantified by radiometric C_{18}-HPLC except as indicated. ND = not determined.

[a]Metabolite quantified by diol-phase radiometric HPLC after isolation of relevant heterogeneous metabolite fraction from hydrolysate of pooled bile by reversed-phase HPLC (mean 2/3 analyses).

[b]Comprised of two minor, more polar, sub-fractions in addition to 16α-OHE$_1$ and 2-methoxy-16αOHE$_1$.

16α-OHE$_1$ and 2-methoxy-16αOHE$_1$ were known but previously unquantified metabolites of exogenous E_2 in rats [29]. The male-predominant expression of 16α-hydroxylation *in vivo* accords with the 2-fold greater 16α-hydroxylase activity of hepatic microsomes from male Wistar rats [27]. Although partial sexual dimorphisms could arise from sex-predominant expression of a single *P*450 form [11], the present case can be explained by the combined activities of a male-specific *P*450 and a non-specific form. The latter, *P*450$_{CM/F}$ [10], is constitutively present in the livers of both male and female Sprague–Dawley rats and actively catalyses 2- and 16α-hydroxylation of E_2; it represents approx. 10–20% of the *P*450 in hepatic microsomes from both sexes [10]. Male-predominant 16α-hydroxylation would result from the greater oestrogen 16α-hydroxylase activity [15, 22] of male-specific *P*450IIC11 (equivalent preparations have been termed RLM5 [15], UT-2 [22], *P*450h, UT-A, or *P*4502c [1–4]), a multi-functional steroid hydroxylase [3-6, 22], which is induced at puberty exclusively in males [1] and represents approx. 50% of spectrally-detectable *P*450 in male rat liver microsomes [5]. *P*450IIC11 additionally catalyses androgen 16α-hydroxylation [4] and oestrogen 2-hydroxylation [15, 22], and is