RELATIVE RATES OF 2- AND 4-HYDROXYESTROGEN SYNTHESIS ARE DEPENDENT ON BOTH SUBSTRATE AND TISSUE

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1. Introduction

The variety of physiological effects attributed to estrogens, as well as the pathological characteristics of estrogen-sensitive human breast tumors, have prompted numerous studies on the possible metabolic conversion of the parent estrogens to activated metabolites. Most of these efforts have focused on the formation and properties of the catecholestrogens of E_1 and E_2. The enzyme system catalyzing their synthesis is a cytochrome P450-dependent monooxygenase localized primarily in liver, but also found in numerous estrogen-sensitive mammalian tissues [1,2] including human mammary neoplasma [3].

Little information is available on the substrate specificity of this microsomal enzyme system. It is usually described as an estrogen 2-hydroxylase, since derivatives of 2-OHE_1 and 2-OHE_2 are the principal products identified from incubations of E_1 and E_2, respectively, with microsomes from rat tissues [4–7]. The objective of this study was to determine if differences in the structure of estrogens of major therapeutic interest would significantly affect the relative rates of formation of their 2- and 4-hydroxy metabolites. Evidence was also sought for the biosynthesis of catecholestrogens by microsomes from a cultured line of human mammary tumor cells whose growth was known to be stimulated by these hormones [2].

Abbreviations and trivial names: estrone, E_1; 2-hydroxyestrone, 2-OHE_1; 4-hydroxyestrone, 4-OHE_1; estradiol-17β, E_2; 2-hydroxyestradiol-17β, 2-OHE_2; 4-hydroxyestradiol-17β, 4-OHE_2; 11β-methoxy-17α-ethynyl-1,3,5-(10)estratriene-3,17β-diol, moxestrol; 11β-methoxy-17α-ethynyl-1,3,5(10)-estratriene-2,3,17β-triol, 2-OHmoxestrol; *S*-[*methyl*-^{3}H]-adenosyl-L-methionine, [^{3}H]SAM; high-performance liquid chromatography, HPLC

2. Materials and methods

Moxestrol, the 2- and 4-hydroxy derivatives of moxestrol [8] and ethynylestradiol were generously provided by Drs J. P. Raynaud and G. Deltour (Roussel-UCLAF, Romainville). The method in [9] was used for the synthesis of 4-hydroxyequilenin. Other catecholestrogens and their monomethyl ethers were obtained from Steraloids (Wilton NH) or prepared by established procedures [1]. Hepes, NADPH and L-ascorbic acid were purchased from Sigma (St Louis MD). [^{3}H]SAM (13.5 Ci/mmol) was obtained from New England Nuclear (Boston MA). Spectro-analyzed grade dimethylsulfoxide and HPLC grade *n*-heptane were from Fisher Scientific (Fair Lawn NJ) and USP grade absolute ethanol was from US Industrial Chemicals (New York NY).

Radioenzymatic assay: The preparation of catechol-*O*-methyltransferase (EC 2.1.1.6) and the microsomal fractions has been described [5]. Washed microsomes from the livers of adult female baboons were stored at –80°C in 0.25 M sucrose at 30 mg microsomal protein/ml with no loss of activity for at least 1 year. Prior to assay, they were thawed and diluted with 10 mM Hepes buffer (pH 7.4) at 0–4°C. Microsomes from MCF-7 cells [3] were resuspended in this buffer and used within 2 h. Incubations were performed using slight modifications of the procedure in [5]. The incubation mixtures were prepared at 0–4°C and consisted of the following: 300 μl of 10 mM Hepes buffer (pH 7.4) containing 50–500 μg microsomal protein and 22.5 nmol estrogen (in 1 μl dimethylsulfoxide), 15 μl [^{3}H]SAM (7.5 μCi), 30 μl partially purified catechol-*O*-methyltransferase (333 units), 25 μl freshly prepared 6 mM L-ascorbic acid, and 30 μl 1 M $MgCl_2$ in 400 μl. The incubation mixtures

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were then placed in a shaking water bath at 37°C for 5 min. The enzymatic reaction was initiated by the addition of 50 μl of 18 mM NADPH. Blanks consisted of incubation mixtures lacking substrate or containing heat-treated microsomes including substrate, and were not significantly different from each other. Aliquots (80 μl) were withdrawn at time 0 and at 2.5 min intervals for 10 min and added to 1.0 ml 0.05 M borate buffer (pH 10) containing a recovery standard (40 000 dpm) of ^{14}C-labeled 2-methoxyestradiol. A mixture of unlabeled 2- and 4-methoxy derivatives of E_1 and E_2 (10 μg each) was added to provide chromatographic markers. The radioactive monomethyl ethers were extracted into 6 ml *n*-heptane and the radioactivity in 0.5 ml aliquots of the extracts was measured [5]. The total dpm of ^{3}H-labeled monomethyl ethers formed in the reactions was corrected for the recovery of ^{14}C-labeled 2-methoxyestradiol which averaged ~85%. (Use of the 7 other ^{14}C-labeled monomethyl ethers of the catecholestrogens of E_1 or E_2 shown in fig.2 yielded a similar 83–87% recovery.) The remainder of the heptane extracts was evaporated under N_2 and dissolved in 50 μl dichloromethane for analysis by HPLC.

HPLC was performed using an automated gradient system (Model 273, Waters Assoc., Milford MA) equipped with system controller (model 720), sample processor (WISP model 710B), data module (model 730), a 280 nm UV detector (model 1203, Lab. Data Control, Riviera Beach FL), and a fraction collector (MultiRac, model 2111, LKB, Rockville MD). A 0.46 × 50 cm Chromegabond Diol column (E. S. Industries, Marlton NJ) was equilibrated with 1.25% ethanol in *n*-heptane. All flow rates were 2 ml/min. After injection of the sample in 50 μl dichloromethane the column was developed using a linear gradient of 1.25–8.75% ethanol in *n*-heptane over 1 h. Fractions of the eluate were collected at 0.5 min intervals and used for determination of radioactivity. The column was washed for 15 min with absolute ethanol and re-equilibrated for 15 min with 1.25% ethanol in *n*-heptane prior to injection of the next sample.

Standard curves were prepared by dissolving authentic catecholestrogens in dimethylsulfoxide, diluting to the appropriate concentration in 150 μl cold Hepes buffer, and incubating in the absence of microsomes and NADPH [10]. A recovery standard of ^{14}C-labeled 2-methoxyestradiol was used to correct for losses during extraction and chromatography.

3. Results and discussion

Monomethyl ether derivatization [10] of the catecholestrogens produced by microsomes has been used to prevent their further A ring oxidation and irreversible binding to microsomal protein [1]. HPLC separated the different pairs of monoethyl ether derivatives of the individual 2- and 4-hydroxyestrogens [1] and eliminated interfering products. Fig.1 demonstrates that similar yields were obtained for the linear formation of the ^{3}H-labeled monomethyl ethers of 2-OHE_1, 2-OHE_2, 4-OHE_1, 4-OHE_2, and 2-OHmoxestrol. These results are in accord with the established substrate specificity of rat liver catechol-*O*-methyltransferase [11]. The separation of these monomethyl ethers by HPLC is shown in fig.2. The resolution of peaks I–IV and peaks V–VIII allows the simultaneous measurement of the relative rates of formation of the catecholestrogens from E_1 or E_2. Peak IX is an unresolved mixture of the 2- and 3-monomethyl ethers of 2-OHmoxestrol which has been included as an internal standard. Aliquots (8.3%) of the blanks before HPLC averaged ~600 dpm. After HPLC of the remainder of the extracts of the blanks, the total amount of tritium in the areas of the individual monomethyl ethers (fig.2) averaged only ~120 dpm.

The formation of 2- and 4-hydroxyestrogen monomethyl ethers from separate incubations of E_1

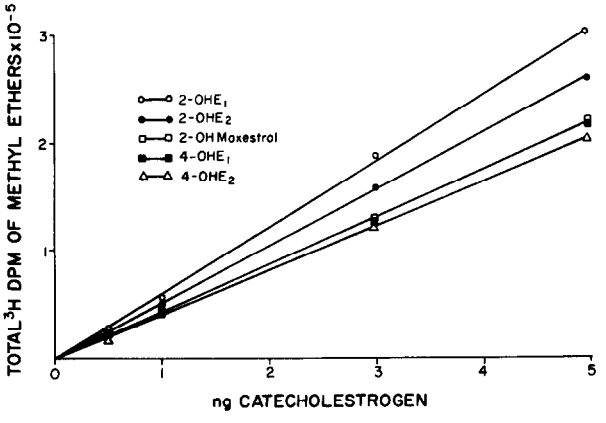

Fig.1. Standard curves for the radioenzymatic assay of catecholestrogens. The total ^{3}H dpm of the monomethyl ethers obtained after HPLC and correction for recovery (av. 85%) is shown as a function of ng catecholestrogen used in the assay.

or E_2 with microsomes from baboon liver and from cultured MCF-7 cells is illustrated in fig.2. The predominance of the ^{3}H-labeled monomethyl ethers of 2-OHE_1 (90%) and 2-OHE_2 (93%) with these primate microsomes is similar to results obtained with rat liver microsomes [5–7]. An 87% average recovery of the ^{3}H-labeled monomethyl ethers was obtained when 5 ng of the authentic 2- and 4-hydroxy metabolites of E_1 and E_2 (fig.1) were added to these incubations, demonstrating that there is not a selective depletion of the 4-hydroxy products by other pathways. The yield (fig.2) of 4-OHE_1 and 4-OHE_2 increased from 10% and 6%, respectively, with hepatic microsomes, to 28% and 14%, respectively, with the MCF-7 cell microsomes. Thus, the proportion of 4-hydroxyestrogen formation was nearly 3 times greater with microsomes from MCF-7 cells than with hepatic microsomes.

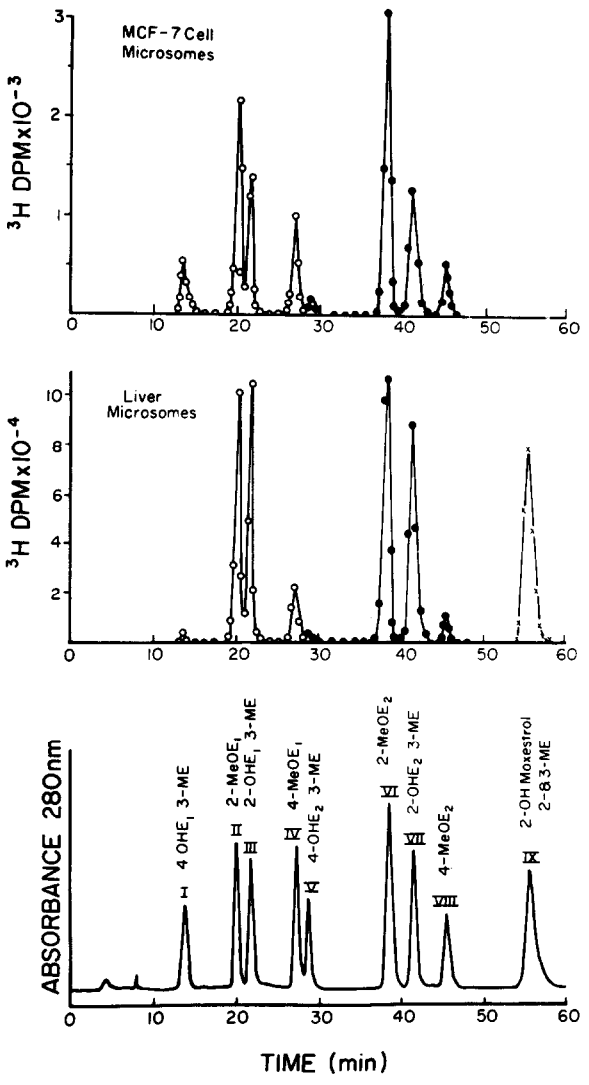

Fig.2. Separation of the monomethyl ethers of catecholestrogens by HPLC. In the lower curve the authentic 2-methoxy (2-MeO), 4-methoxy (4-MeO), or 3-methyl (3-ME) monomethyl ethers of the catecholestrogens shown in fig.1 were used. The two upper curves are chromatograms of the products of the assay of baboon liver microsomes (50 μg protein) and MCF-7 cell microsomes (500 μg protein) using 50 μM E_1 (○——○) and E_2 (●——●) as substrates, and an internal standard of 2-OHmoxestrol (×——×). The relative retention times for the labeled and unlabeled monomethyl ethers were identical.

The relative rates of total catecholestrogen formation from 6 different estrogen substrates by baboon liver microsomes are shown in fig.3. A yield of 40 pmol total product (12×10^5 dpm ^{3}H) represents a 40% yield from [^{3}H]SAM but only a 1% yield from the unlabeled estrogen substrate which is present in saturating concentration in these assays. Ethynylestradiol was the most active substrate, and a linear rate was obtained with a 20-fold reduction in microsomal protein (not shown). With moxestrol, there was a 40-fold reduction in the initial rate of total catecholestrogen formation compared to ethynylestradiol due to a marked inhibition of 2-hydroxyestrogen synthesis by the neighboring 11β-methoxy group of this potent estrogen. The relative rate of catecholestrogen formation with E_1, which was 62% of the rate with E_2, was the same as that reported for

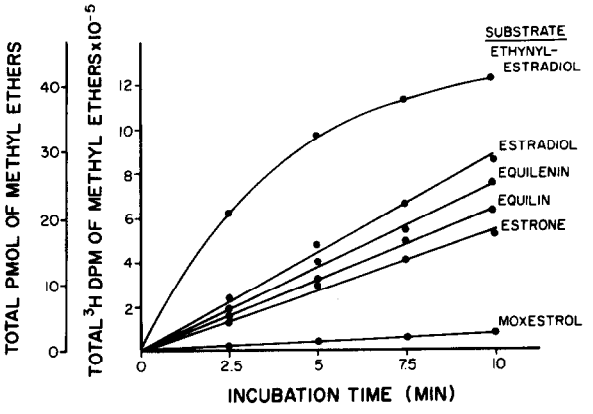

Fig.3. Radioenzymatic assay of 50 μM estrogens with baboon liver microsomes (50 μg protein). The total pmol and ^{3}H dpm of the monomethyl ethers obtained in the heptane extracts after correction for recovery is shown as a function of incubation time (min).

Table 1
Catecholestrogen formation by baboon liver microsomes

Substrate	Method of analysis	% [^{3}H]Monomethyl ethers of		Average ratio 2-OH:4-OH product
		2-OH Product	4-OH Product	
Ethynylestradiol	A	93.2–95.4	4.5– 5.2	20:1
Estradiol	A	92.1–95.4	4.3– 7.5	16:1
Equilenin	B	–	76.1–81.4	<0.3:1
Equilin	C	67.1–69.7	27.5–29.0	2.4:1
Estrone	A	88.0–91.1	8.0–12.1	9:1
Moxestrol	A	68.9–71.3	25.9–28.6	2.5:1

Substrates (50 μM) were incubated for 10 min with baboon liver microsomes (50 μg protein). The results are shown for the range of 3 different incubations as analyzed (A) by comparison with retention times of authentic standards, (B) by comparison with retention times of monomethyl ethers obtained from incubation of 4-hydroxyequilenin with catechol-*O*-methyltransferase and [^{3}H]SAM, and (C) by comparison with the retention times of authentic monomethyl ethers of 2-OHE$_1$ and 4-OHE$_1$. The double bond in ring B of the equilin series caused a uniform 2 min increase in retention time over that of the analogous estrane

rat liver microsomes [6]. The rates with the 17-keto equine estrogens equilin and equilenin were greater than the rate with E_1.

The relative ratios of 2- to 4-hydroxyestrogen formation from these 6 estrogen substrates are shown in table 1. A 20:1 ratio was obtained with ethynylestradiol and a 16:1 ratio with E_2. This ratio decreased with structural alterations in ring D (E_1, 9:1) and ring C (moxestrol, 2.5:1). The above changes in the ratio of catecholestrogen products parallel the relative rates reaction shown in fig.2. However, this generalization does not apply for all substrates. Comparison of results with E_1 and equilin shows that the unconjugated double bond in ring B of equilin resulted in an ~3-fold increase in the amount of 4-hydroxyequilin formation compared to 4-OHE$_1$ formed from E_1. In the case of equilenin, where rings A and B are both aromatic, the major catecholestrogen formed was the 4-hydroxy product in 78% average yield. Structural alterations of estrogens in the B and C rings can therefore markedly alter the relative ratio of 2- and 4-hydroxyestrogen synthesis by hepatic microsomes. This is further illustrated by results with 11β-ethylestradiol (not shown) where a 1.9:1 ratio was obtained *vs* the 16:1 ratio with E_2 (table 1).

These data show that the relative rates of formation of 2- and 4-hydroxyestrogens depend not only on the source of the microsomal activating system, but also on the estrogen substrate. This finding would have important consequences where there is a significant difference in the biological effects produced by 2- and 4-hydroxyestrogens [12–14].

Since estrogen binding to the estrogen receptor of human breast tumor cells is being extensively investigated in other laboratories, it is noteworthy that microsomes from continuously cultured MCF-7 cells showed a significant rate of formation of catecholestrogens. Microsomes from samples of normal breast tissue are devoid of this activity [3]. Many human breast tumors are also capable of forming E_1 and E_2 from androstenedione and testosterone in vitro, including cultured MCF-7 cells [15]. Markedly elevated levels of 2-OHE$_2$ have been found in the urine of some patients with breast cancer; these decrease to very low levels several days after removal of the tumors (S. L. Cohen and P. Ho, personal communication). 2-OHE$_2$ stimulates the growth of MCF-7 cells and binds to the estrogen receptor [2]. The clinical significance of the estrogen-responsive breast tumor as a paraendocrine organ [16] has not yet been established.

In a study of the neoplastic transformation of Balb/c 3T3 cells by E_2 and ethynylestradiol, we found that microsomes from these mouse fibroblast cells also catalyzed the formation of catecholestrogens from these substrates [17]. In contrast, moxestrol did not induce transformation of these cells and was also a poor substrate for microsomal oxidation, in agreement with the data obtained with hepatic microsomes (fig.2). These results are consistent with the mecha-

nism that has been proposed for isomeric 1,2- and 4,5-epoxides of E_2 as intermediates in the formation of catecholestrogens and as transforming agents [18]. By analogy with the metabolism of carcinogenic polycyclic aromatic hydrocarbons, the relative rates of formation of such intermediates would be expected to depend on both the tissue from which the microsomes were derived, and also on functional groups of the estrogen substrate which could influence this type of metabolic activation.

Acknowledgements

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