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INDIRECT EVIDENCE FOR THE METABOLIC DEHALOGENATION OF TETRAFLUORO-DIETHYLSTILBESTROL BY RAT AND HAMSTER LIVER AND KIDNEY MICROSOMES

SPECIES- AND ORGAN-DEPENDENT DIFFERENCES

M. M. HEY,* H HAAF,* J. A MCLACHLAN† and M. METZLER*‡

* Institute of Pharmacology and Toxicology, University of Wurzburg, Versbacher Strasse 9, D-8700 Wurzburg, Federal Republic of Germany, and † Laboratory of Reproductive and Developmental Toxicology, National Institute of Environmental Health Sciences, Research Triangle Park, NC, U S A

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Abstract—In order to assess the significance of the catechol pathway for the carcinogenic activity of diethylstilbestrol (DES), the stability of 3′,5′,3″,5″-tetrafluoro-DES (TF-DES) against metabolic catechol formation was examined *in vitro* A radioenzymatic assay was used for determining the estrogen hydroxylase activity of liver and kidney microsomes from male and female Syrian golden hamsters and from male Wistar rats for the substrates TF-DES, DES, estradiol-17β and 2-fluoro-estradiol-17β With all microsomes tested, catechols were formed from TF-DES to an extent similar to or, in some cases, even exceeding that observed with DES and the steroidal estrogens The estrogen hydroxylase activity measured for the various microsomes depended on the species, organ and substrate Analysis by high performance liquid chromatography showed that four products were formed in the radioenzymatic assay with DES and TF-DES These data demonstrate that the fluorine substitution present in TF-DES does not prevent catechol formation and imply that the catechol pathway must be taken into account as a putative pathway for the metabolic activation of DES

Whereas the carcinogenic potential of the synthetic estrogen, diethylstilbestrol (DES)§ has been well documented [2], the mechanism of DES tumorigenesis remains unknown Recent evidence suggests that nonhormonal events are involved [3], and metabolic activation is thought to play an important role [4] However, it is unclear which of several oxidative pathways known to be operative in DES metabolism is critical for the carcinogenic activity. At present, the two most likely candidates are peroxidative oxidation of DES to Z,Z-dienestrol (Z,Z-DIES) and aromatic hydroxylation to the catechol 3′-hydroxy-DES (Fig 1)

In an attempt to evaluate the importance of catechol formation for DES carcinogenic activity, a fluorinated analogue of DES, 3′,3″,5′,5″-tetrafluoro-DES (TF-DES) was synthesized [5]. The fluorine substitution ortho to the phenolic hydroxyl groups was expected to prevent catechol formation. Indeed, in an *in vivo* study with unlabelled TF-DES, the only metabolite detected in rat bile was the product of the peroxidative pathway, TF-Z,Z-DIES, and no evidence for the formation of defluorinated metabolites was obtained [6]. The fact that TF-DES was still able to induce tumors in the male hamster kidney [7] and to neoplastically transform Syrian hamster embryo fibroblasts in culture [8] suggested that peroxidative metabolism was sufficient for the metabolic activation of the DES molecule to carcinogenic intermediates. However, there is an increasing number of studies demonstrating that fluorine atoms bound to aromatic systems can be metabolically replaced by hydroxyl groups. In particular, the recent findings by LI *et al.* [9] that fluoro- and bromo-substituted estradiol-17β is easily dehalogenated by hamster liver microsomes has led us to examine the stability of TF-DES against aromatic hydroxylation *in vitro* Using microsomes from various tissues and a radioenzymatic assay for the detection of catechols, we have found that catechols are formed from TF-DES by microsomes from the liver and kidney of male rats and of male and female hamsters These observations, together with marked differences in the enzymatic activity for catechol formation between different organs and species, are reported in this communication.

‡ To whom correspondence should be addressed

§ Abbreviations COMT, catechol-*O*-methyltransferase, DES, diethylstilbestrol, 3,4-bis-(*p*-hydroxyphenyl)hex-3-ene, TF-DES, 3′,5′,3″,5″-tetrafluorodiethylstilbestrol, DIES, dienestrol, 3,4-bis-(*p*-hydroxyphenyl)hexa-2,4-diene, E and Z are configurational descriptors, the nomenclature of DES metabolites follows the recommendation of Metzler and McLachlan [1], E_2, estradiol-17β; ESH, estrogen hydroxylase, 2-F-E_2, 2-fluoro-estradiol-17β, HPLC, high pressure liquid chromatography, NADPH, reduced nicotinamide dinucleotide phosphate, SAM, *S*-adenosyl-L-methionine

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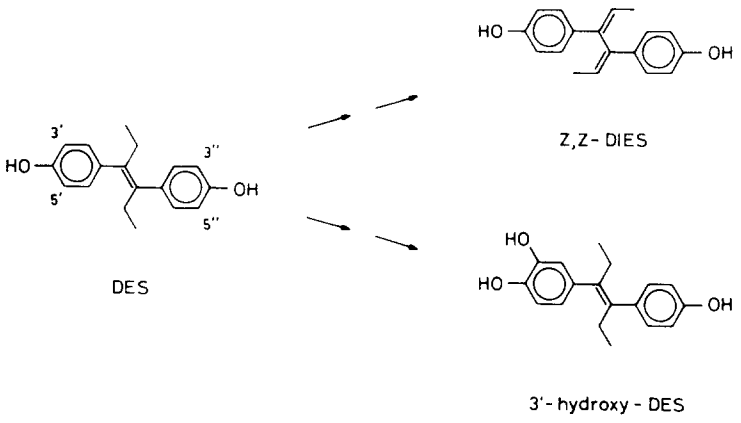

Fig 1 Two putative pathways for the metabolic activation of diethylstilbestrol (DES)

MATERIALS AND METHODS

Chemicals and reagents. *S*-[methyl-^{3}H]-Adenosyl-L-methionine (spec. radioact 15 Ci/mmol) was purchased from the Radiochemical Centre (Amersham, U.K.). DES, estradiol-17β (E_2), 3,4-dihydroxybenzoic acid and NADPH were obtained from Sigma (Munchen, F.R.G) TF-DES, which consisted of a mixture of 73% Z- and 27% E-isomer, was supplied by Dr. J. A McLachlan (National Institute of Environmental Health Sciences, Research Triangle Park, U.S.A). 2-Fluoro-estradiol-17β (2-F-E_2) was kindly provided by Dr J J Li (Veterans Administration Medical Center, Minneapolis, U S A) All chemicals and solvents used were of analytical grade.

Animals and housing Male Wistar rats (340–380 g body wt) and male and female Syrian golden hamsters (125–135 g body wt) were supplied by the Zentralinstitut fur Versuchstierzucht (Hannover, F R.G.). The animals had access to standard lab chow (Altromin R10, Altrogge, Lage/Lippe, F R G) and tap water *ad libitum* They were kept in climatized rooms on a 12-hr light, 12-hr dark cycle and acclimated at least 7 days prior to use.

Preparation of enzymes Catechol-*O*-methyltransferase (COMT) was prepared from the liver of male Wistar rats using the method of Axelrod and Tomchick [10] as modified by Nikodejevic *et al* [11] The final preparation contained 5 2 mg protein/ml, as measured with the BioRad Coomassie Blue reagent [12], and was stored in 1 mM phosphate buffer pH 7.0 at −20° Its activity was determined with 3,4-dihydroxy-benzoic acid, using essentially the assay for estrogen hydroxylase (see below) without microsomes No decrease of enzyme activity was noted for 12 months

Microsomes were prepared from the livers and kidneys of male Wistar rats and Syrian golden hamsters as recently described [13] The final pellet was dissolved in 150 mM Tris–HCl buffer pH 7.4 at a concentration of 2 mg protein/ml Aliquots of 2 ml were quickly frozen in dry ice/acetone and stored at −70°

Estrogen hydroxylase assay The activity of microsomal estrogen hydroxylase (ESH) was determined by the radioenzymatic procedure described by Paul *et al* [14] and Purdy *et al* [15] In our experiments, the incubation mixture consisted of 18.8 μl 0 01 M Tris–HCl buffer pH 7.4, 12 5 μl 1 M $MgCl_2$, 12 5 μl 6 mM ascorbic acid, 6 2 μl COMT preparation (containing 32 μg protein), 150 μl microsomal preparation (containing 300 μg protein), 25 μl [^{3}H]-SAM (5 μCi) and 5 μl 250 mM substrate in dimethylsulfoxide. After pre-incubation with gentle shaking at 37° for 5 min, the enzymatic reaction was started by the addition of 25 μl 18 mM NADPH 100 μl of the incubation mixture were taken just prior to the addition of NADPH and another 100 μl after 10 min They were pipetted into 1 ml 0 5 M borate buffer pH 10.0 to stop the reaction. These solutions were vigorously vortexed for 30 sec after addition of 6 ml *n*-heptane. 10 min later, 3 ml of the heptane layer was transferred into a scintillation vial containing 9 ml Quickszint 212 (Zinsser Analytic Ltd , U K) and the radioactivity determined in a Packard 3390 Liquid Scintillation counter with automatic external standardization. All incubations were carried out in duplicate

HPLC Analysis of methyl ethers. Heptane extracts (12 ml) from duplicate experiments were combined, evaporated to dryness *in vacuo* and redissolved in 20 μl ethanol HPLC analysis was carried out on a RP-18 column (DuPont, Wilmington, U S A) at 42° with a flow rate of 1 ml/min. Solvent A was water/methanol 8:2 (v/v) and solvent B was methanol A solvent gradient changing linearly from 45 to 100% B in 30 min was used, and the eluate collected in 0 3-ml fractions for measurement of radioactivity

RESULTS

Kinetics of microsomal ESH from various tissues In order to ensure first-order kinetics for the ESH assay, the microsomal preparations from the liver and kidney of male rat and of male and female hamster were subjected to kinetic studies using DES as substrate. Protein concentrations were varied between 80 and 640 μg/ml and substrate concentrations between 5 and 250 μM As an example, the data obtained with kidney microsomes of male rats are shown in Fig 2 With all microsomal preparations used in this study, the production of catechol, as indicated by the consumption of SAM, was linear over a range of 80–400 μg protein per ml

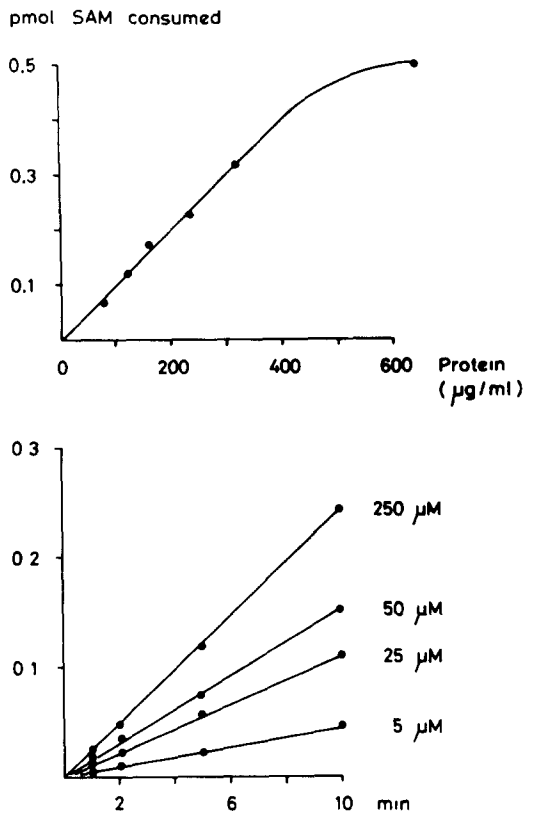

Fig 2 Kinetic studies with male rat kidney microsomes and DES Upper chart. linearity of hydroxylation at varying microsomal protein concentration (substrate concentration 250 μM) Lower chart: linearity of hydroxylation at different substrate concentration (incubations contained 300 μg microsomal protein)

and over 10 min for a substrate concentration ranging from 5 to 250 μM

Product formation from various estrogens. The radioenzymatic assay for catechol formation was performed with liver and kidney microsomes from male rats and male and female hamsters. In addition to TF-DES, three other substrates, namely DES, E_2 and 2-F-E_2 were studied. DES was used as a positive control for TF-DES, because the catechol formation of DES in this assay has been previously reported [16–19] E_2 and 2-F-E_2, which are also known to yield catechols [9, 19], were included for quantitative comparison of the ESH activity of the different microsomes.

In complete incubations, formation of catechols, as indicated by a significant amount of heptane-extractable radioactivity, was found with all substances, including TF-DES, and with all microsomal preparations. Prior to the addition of NADPH, the heptane-extractable radioactivity was as low as in control experiments where the estrogens were omitted For example, in a typical incubation with DES and rat liver microsomes, the amount of heptane-extractable radioactivity was 18×10^3 dpm when NADPH was omitted (control value), but 133×10^3 in the presence of NADPH. With other substrates, the increase in heptane-extractable radioactivity caused by NADPH was even more pronounced This indicates that the compounds are not directly methylated but require hydroxylation prior to methylation.

When the heptane-extractable radioactivity of the assays with DES and TF-DES and male hamster hepatic and renal microsomes were analyzed by HPLC, four distinct radioactive peaks were found in all cases (e.g. Fig. 3). Thus far, these products were not identified due to the lack of reference compounds.

Microsomal ESH activity in rat and hamster liver and kidney. A quantitative account of the ESH activity for DES, TF-DES, E_2 and 2-F-E_2 in male rat and hamster liver and kidney is given in Fig. 4. It is obvious that the ESH activity depends on substrate, organ, and species. For liver ESH of both rat and hamster, E_2 and 2-F-E_2 are better substrates than DES or TF-DES Rat and hamster liver show approximately the same activity for DES, but differ for TF-DES and E_2. Remarkably, hamster liver microsomes are more active than rat liver microsomes for TF-DES. The same species difference is observed for kidney microsomes and TF-DES (Fig. 4, right-hand side). Surprisingly, TF-DES is an even better substrate for renal hamster ESH than DES and E_2

In a preliminary study, the ESH activity from female hamster liver and kidney was determined with the same four substrates (data not shown) Liver microsomes from female hamsters proved to show approximately half the activity of those from male hamsters for each substrate. The same was found with kidney microsomes for DES, E_2 and 2-F-E_2; however, for TF-DES, male and female hamster renal microsomes showed the same ESH activity.

DISCUSSION

The radiochemical assay using microsomes/NADPH for hydroxylation and catechol-*O*-methyltransferase/*S*-[methyl-^{3}H]adenosyl-L-methionine for conversion of catechols into radiolabelled methyl ethers is an established method for detecting *ortho*-hydroxylation of phenols. Accordingly, this assay is commonly employed for measuring the formation of catechols from estrogens, which is catalyzed by microsomal estrogen hydroxylase (ESH), a cytochrome P-450 multisubstrate monooxygenase. For example, several laboratories have previously shown that the synthetic estrogen diethylstilbestrol (DES) is a substrate of microsomal ESH from rat liver [16–18] and from male hamster and rat kidney [19].

The aim of the present study was to find out whether fluorine substitution of the positions *ortho* to the hydroxyl groups of DES prevents catechol formation or not. The results clearly show that 3′,5′,3″,5″-tetrafluoro-DES (TF-DES) is a substrate of microsomal ESH from male and female hamster liver and kidney and also from male rat liver and kidney *in vitro*. The reaction requires NADPH, indicating that a hydroxylation step is involved and precluding that unchanged TF-DES is directly methylated by COMT. According to HPLC analysis, four major products are formed from TF-DES in the ESH assay (Fig. 3). It is tempting to speculate that they are derived from the E- and Z-TF-DES by defluor-

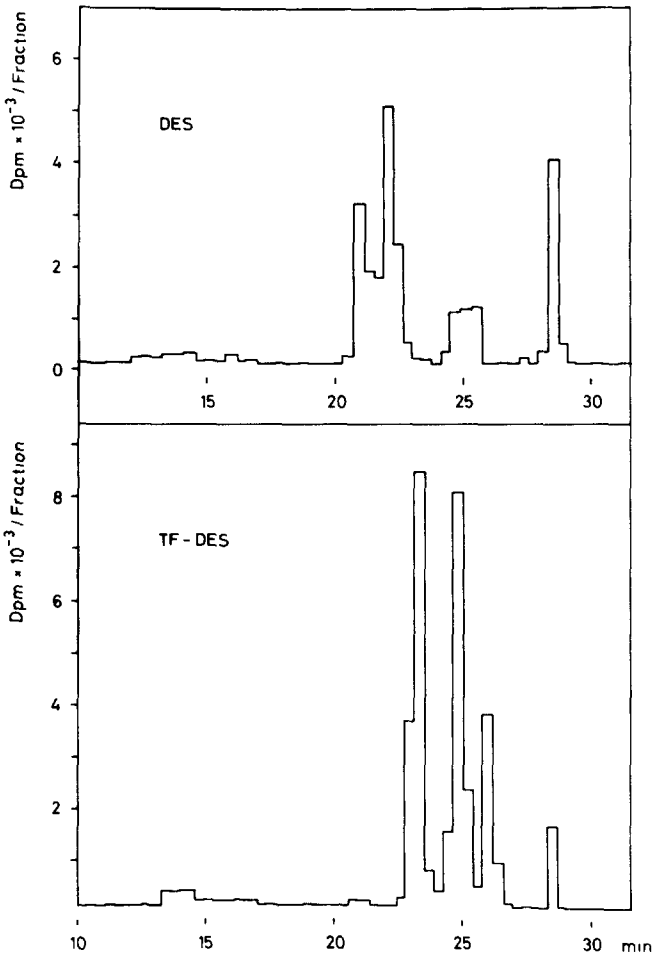

Fig 3 HPLC separation of the heptane-extractable products formed in the radioenzymatic assay using DES (upper chart) and TF-DES (lower chart) with hamster liver microsomes

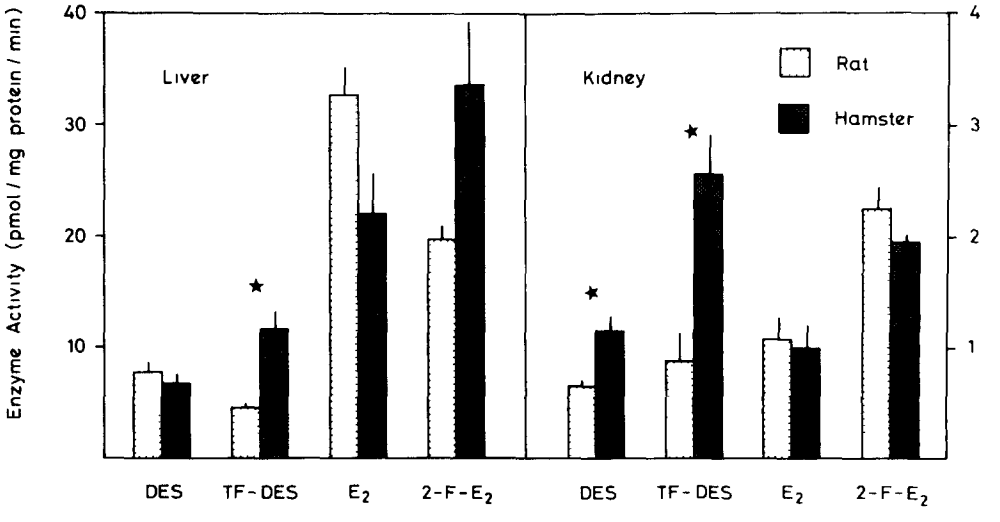

Fig 4 Activity of estrogen hydroxylase for different substrates in hepatic and renal microsomes from male rats and hamsters Columns represent mean ± standard deviation of measurements from two animals carried out in triplicate ★ Different at a significance level of 5% (according to Student's *t*-test)

ination of the 3′-position in one or both aromatic rings, leading to 3′-hydroxy-5′,3″,5″-trifluoro-DES and 3′,3″-dihydroxy-5′,5″-difluoro-DES, respectively. However, whether a defluorination of TF-DES does indeed occur or whether fluorine is retained in the molecule and shifted to the 2′-position must await structural identification of the products. Therefore, the present finding can only be considered as indirect evidence for the metabolic defluorination of TF-DES by microsomal ESH. For fluorinated and brominated estradiol-17β, a recent study has shown that catechol formation by hamster liver microsomes is indeed associated with dehalogenation [9].

Independent of the molecular mechanism of catechol formation, it is of interest to note that fluorinated estrogens are sometimes as good as substrates for ESH or even better than the parent estrogens. This has recently been demonstrated by Li *et al.* [9], who showed that total catechol formation by male hamster hepatic microsomes was the same for estradiol-17β (E_2) and 2-fluoro-estradiol-17β (2-F-E_2). Our observations (Fig. 4) are in accordance with this finding. TF-DES is a markedly better substrate than DES for hamster hepatic ESH but not rat hepatic ESH. With kidney microsomes of rat and hamster, on the other hand, the fluorinated derivatives of both E_2 and DES were invariably better substrates than the parent compounds (Fig. 4).

The fact that TF-DES is a good substrate of microsomal ESH may have implications for its mechanism of carcinogenesis. In addition to peroxidative oxidation, the formation of a catechol must be taken into consideration as a potential route for metabolic activation of TF-DES just as for DES itself (Fig. 1). However, it should be emphasized that catechol formation of TF-DES has so far only been demonstrated with microsomes *in vitro*, and it is unknown at present whether this pathway plays a role in the *in-vivo* metabolism of TF-DES. In order to clarify this question, detailed studies on the metabolic fate of radiolabelled TF-DES in rat and hamster *in vivo* are presently in progress in our laboratory.

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