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IN VITRO METABOLISM OF DIETHYLSTILBESTROL BY HEPATIC, RENAL AND UTERINE MICROSOMES OF RATS AND HAMSTERS

EFFECTS OF DIFFERENT INDUCERS*

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Abstract—In order to elucidate possible differences in the metabolism of the synthetic estrogen diethylstilbestrol (DES) by target and non-target tissues for DES carcinogenicity, the biotransformation of [^{14}C]DES has been studied *in vitro* with hepatic and renal microsomes of male and female hamsters and rats, and from hamster and rat uterus. Of these tissues, only the male hamster kidney is susceptible to the carcinogenic effect of DES. Moreover, the effect of various inducers on the *in vitro* metabolism of DES has been investigated. It was found that male hamster kidney microsomes produced a markedly different pattern of DES metabolites as compared to renal microsomes from female hamster or male and female rats. Pretreatment with phenobarbital markedly increased oxidative DES metabolism by renal microsomes from female rat but not from male rat. Diethylstilbestrol metabolism by hepatic microsomes was different between hamster and rat, but was not sex-dependent and could not be significantly affected by pretreatment with phenobarbital, DES, 3-methylcholanthrene and 7:8-benzoflavone. The differences in DES metabolism between target and non-target organs and its modulation by inducers may help to gain further insight into the mechanism of DES tumorigenesis.

The kidney of the male Syrian golden hamster is uniquely susceptible to the carcinogenic effect of various estrogenic compounds, including the synthetic estrogen diethylstilbestrol (DES)‡: renal adenocarcinoma are obtained with 80–100% incidence within 250 days of subcutaneous administration of DES to male hamsters [2]. Under the same conditions, virtually no tumorigenic effect is illicited by DES on the kidney of intact female hamsters [3] and on the liver of both male and female hamsters [4]. In intact rats of both sexes, neither renal nor hepatic tumors can be induced by DES to a significant extent [5].

The pronounced organotropism of DES carcinogenicity should be exploited to clarify the mechanism of tumorigenesis of DES and other estrogenic compounds, which is largely unknown to date. Although the hormonal property of DES appears to be an important prerequisite for its carcinogenicity, there is growing evidence that oxidative metabolism also plays an important role (for review see [6]). In support of this hypothesis is a recent finding from our laboratory that microsomes from male hamster kidney have a unique capability to metabolize [^{14}C]-DES to reactive products: the amount of radioactivity covalently bound to microsomal protein was 5–10-fold higher for renal microsomes from male hamsters compared to renal microsomes from female hamsters, from male or female rats and for hepatic microsomes from hamsters and rats of either sex [7]. Moreover, it was found that the amount of covalently bound material was significantly increased for renal but not for hepatic microsomes after pretreatment with phenobarbital (PB) [7] but not with DES or 3-methylcholanthrene (MC) or 7:8-benzoflavone (7:8-BF) [8]. However, the structure of the binding metabolite(s) is as yet unknown.

The present study was undertaken to gain information on the metabolites of DES generated by the microsomes from target and non-target tissue for DES carcinogenicity and to investigate the effects of inducers on the microsomal metabolism of DES.

MATERIALS AND METHODS

Chemicals. [2-^{14}C]Diethylstilbestrol (sp. act. 56 mCi/mmole, The Radiochemical Centre, Amersham, U.K.) was shown by radio-HPLC and radio-

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‡ Abbreviations used: DES, diethylstilbestrol, 3:4-bis-(*p*-hydroxyphenyl)hex-3-ene; DIES, dienestrol, 3:4-bis(*p*-hydroxyphenyl)hexa-2:4-diene; E and Z, configurational descriptors (the nomenclature of DES metabolites follows the recommendation of Metzler and McLachlan [1]); PB, phenobarbital; MC, 3-methylcholanthrene; 7:8-BF, 7:8-benzoflavone; TMS, trimethylsilyl; HPLC, high pressure liquid chromatography; GC, gas chromatography; MS, mass spectrometry.

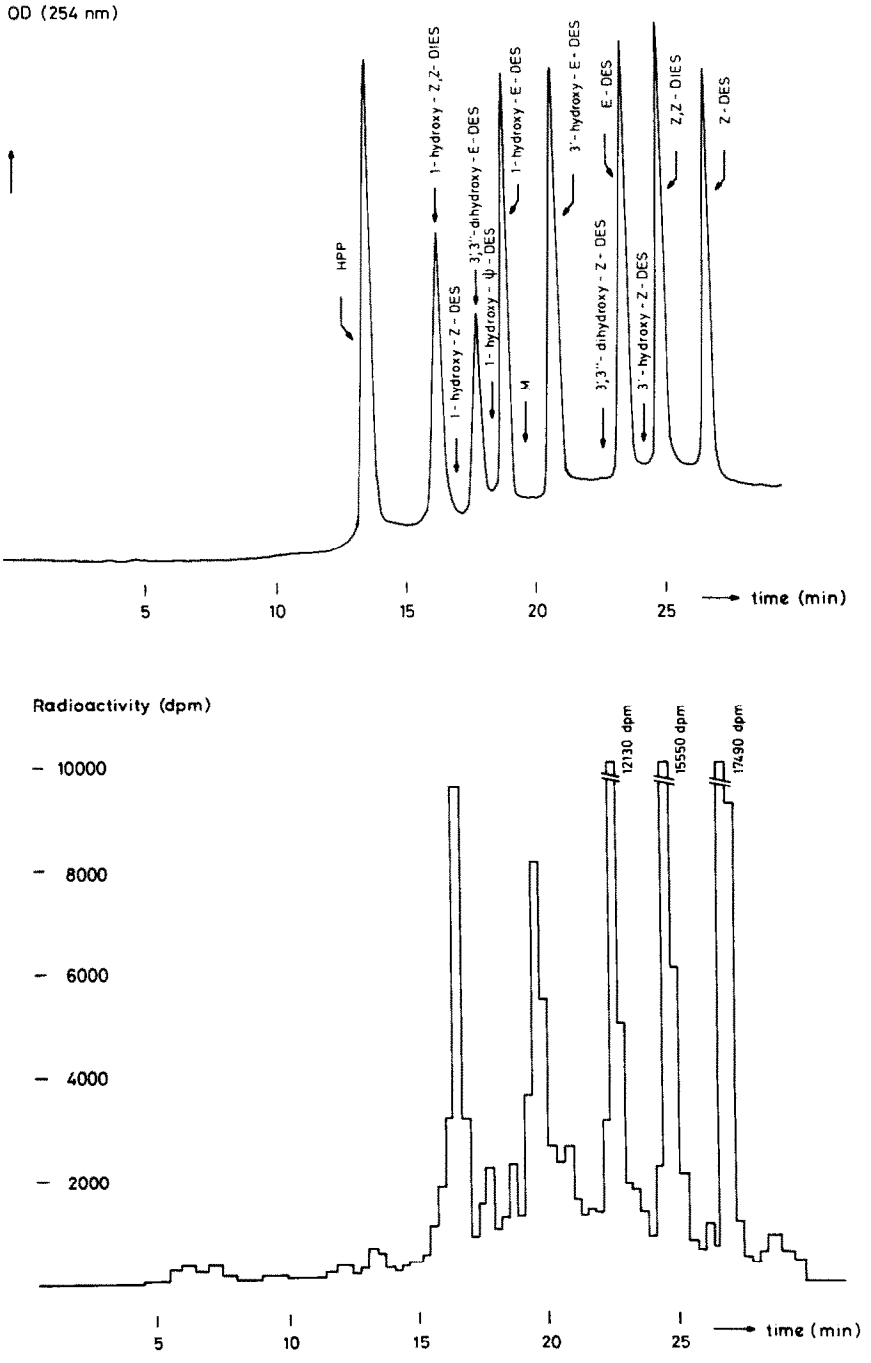

Fig. 1. HPLC separation of DES metabolites. Upper chart: Reference compounds. Arrows without O.D. indicate retention time of the *Z*-isomers. Lower chart: Metabolites of [^{14}C]DES from hepatic microsomes of untreated male rats.

GC to be of 99% radiochemical purity and to consist of 82% *E*- and 18% *Z*-isomer. Unlabeled *E*-DES and 4'-hydroxypropiophenone were obtained from Merck (Darmstadt, F.R.G.), MC, 7:8-BF and NADPH from Serva (Heidelberg, F.R.G.) and PB (sodium salt) from Sigma (Munich, F.R.G.). All chemicals were of analytical grade.

Z:*Z*-Dienestrol and 1-hydroxy-*Z*:*Z*-DIES were prepared in our laboratory as previously described [9]. 1-Hydroxy-*E*-DES, 3'-hydroxy-*E*-DES and 3':3''-dihydroxy-*E*-DES were kindly provided by Dr. John A. McLachlan (National Institute of Environmental Health Sciences, Research Triangle Park, U.S.A.) and *Z*-DES by Dr. P. Murphy (Lilly Research Laboratories, Indianapolis, U.S.A.). 1-Hydroxy-*Z*-DES and 3'-hydroxy-*Z*-DES were obtained from the respective *E*-isomers through exposure to daylight for 2 days in ethanolic solution and purified by HPLC.

Animals and pretreatment. Syrian golden hamsters

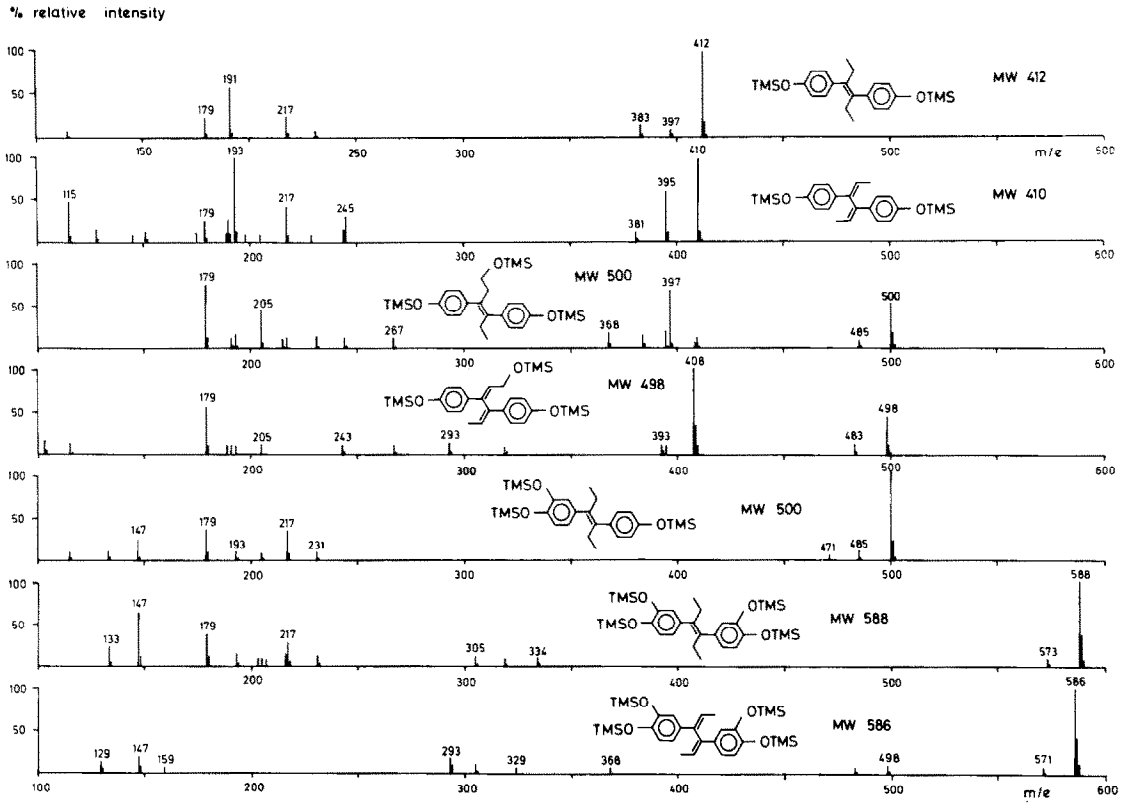

Fig. 2. Mass spectra of the *in vitro* metabolites of DES. Names of the metabolites (from top to bottom) are: *E*-DES, *Z*:*Z*-DIES, 1-hydroxy-*E*-DES, 1-hydroxy-*Z*:*Z*-DIES, 3′-hydroxy-*E*-DES, 3′:3″-dihydroxy-*E*-DES, 3′:3″-dihydroxy-*Z*:*Z*-DIES. Synthetic reference compounds were available for all metabolites except 3′:3″-dihydroxy-*Z*:*Z*-DIES and had mass spectra identical with those of the metabolites.

and Wistar rats were obtained from the Zentralinstitut für Versuchstiere (Hannover, F.R.G.). The animals had access to standard lab chow (Altromin R10, Altrogge, Lage/Lippe, F.R.G.) and tapwater *ad libitum*. For pretreatment with PB, the substance was added to the drinking water at 0.2% for 3 days. In addition, an 8% solution of PB in saline was injected intraperitoneally into rats (220 g body wt, dose 100 mg PB/kg body wt) and into hamsters (130 g body wt, dose 60 mg/kg body wt) on day 1 and 3. For pretreatment with *E*-DES, a 2.5% DES solution in propan-1:2-diol was orally administered to the animals at a dose of 15 mg DES/kg body wt every 24 hr for 3 days. Animals pretreated with MC at a dose of 20 mg/kg body wt obtained intraperitoneal injections of a 0.5% MC solution in tricapryline (Roth, Karlsruhe, F.R.G.) every day for 3 days, while 7:8-BF was administered at a concentration of 0.4% with the ground lab chow for 3 days.

Preparation of microsomes and incubation with diethylstilbestrol. The animals were sacrificed by cervical dislocation after 72 hr of pretreatment. Livers, kidneys and uteri were removed, rinsed and placed on ice. The liver was perfused with 150 mM Tris–Cl buffer pH 7.4 through the portal vein. Homogenization was carried out for 3 × 10 sec at 1000 rpm in the same buffer (5 ml/g tissue) at 0° with an Ultra-Turrax TP 18/2 (IKA-Werk, Staufen, F.R.G.). The homogenate was centrifuged at 4° and 9000 *g* for 20 min and the supernatant centrifuged at 4° and 105,000 *g* for 60 min. The microsomal pellet was resuspended in the same volume of Tris buffer and centrifuged again at 105,000 *g* for 60 min. Finally the pellet was suspended with gentle homogenization in Tris buffer and used immediately for the incubations (see below). Protein was determined according to the method of Lowry *et al.* [10].

Incubation mixtures contained 24, 9 or 10 mg of hepatic, renal or uterine microsomes, respectively, at a concentration of 2 mg microsomal protein/ml 150 mM Tris buffer pH 7.4. The amount of microsomal protein corresponded to 1.5 g of tissue in all cases. Two nanomoles of [^{14}C]DES (1 μg/μl ethanol) and 100 nmoles NADPH (Serva, Heidelberg, F.R.G.) were added per milligram microsomal protein and the mixture was shaken gently at 37°. In control experiments NADPH was omitted. The incubation was stopped after 30 min by adding a 5-fold volume of methanol/acetone (1:1, v/v), which contained 0.4% ascorbic acid and 0.5% acetic acid. The precipitated protein was sedimented at 2000 *g* for 20 min and washed successively with 40 ml each of methanol, methanol/diethyl ether (1:1, v/v) and diethyl ether by suspending the precipitate thoroughly in each of the solvents prior to cen-

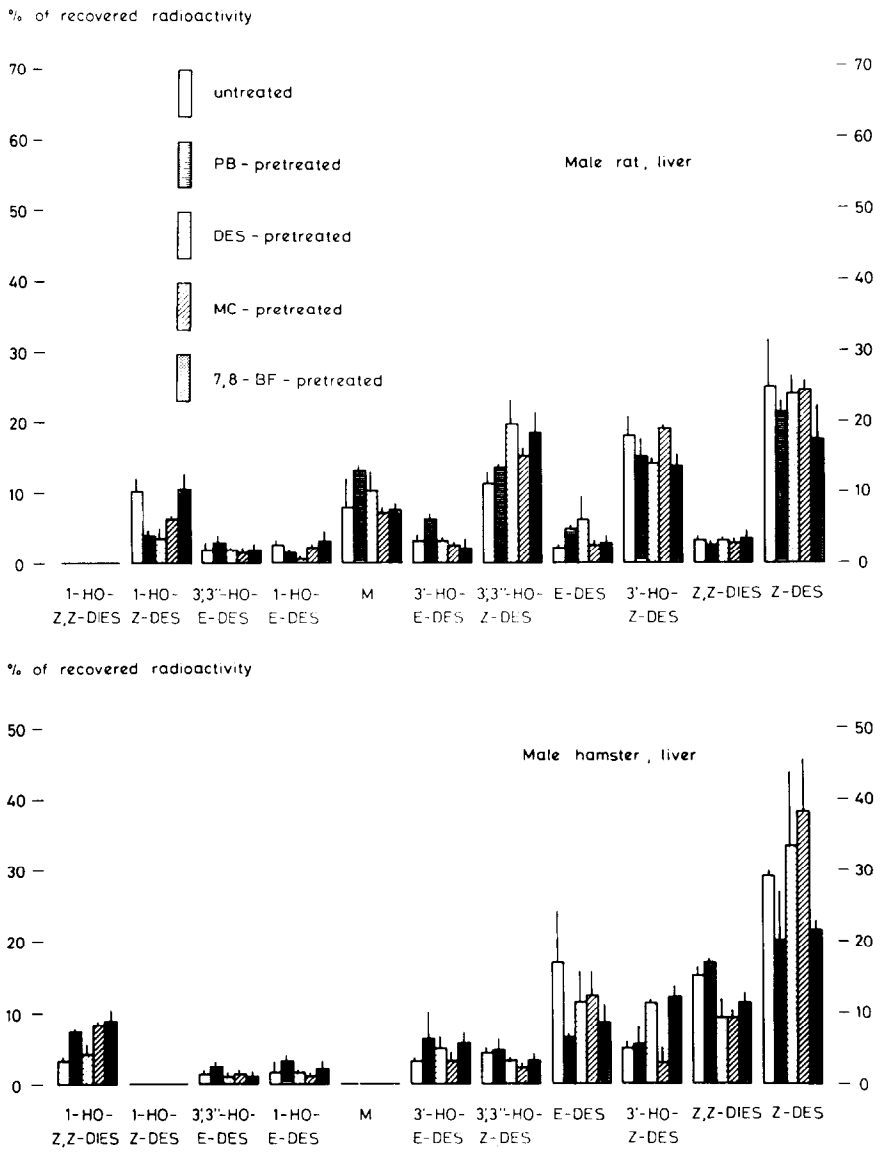

Fig. 3. Effects of inducers on the metabolism of [^{14}C]DES by hepatic microsomes from male rats (upper chart) and male hamsters (lower chart). Data represent mean of at least two experiments with microsomes from different animals. Metabolite "M" has been tentatively identified as 3':3''-dihydroxy-*Z*:*Z*-DIES (see text).

trifugation. The combined solvents were evaporated *in vacuo* and the residue dissolved in 50 ml water. Following extraction with 3 × 100 ml each of diethyl ether, the extract was concentrated to dryness under reduced pressure and redissolved in 300 μl methanol for HPLC analysis. For the identification of DES-metabolites by GC/MS, [^{14}C]DES of sp. act. 5 mCi/mmole was incubated with male rat hepatic microsomes from 6 g of tissue in order to obtain sufficient amounts of metabolites.

Separation and identification of metabolites by high performance liquid chromatography and gas chromatography/mass spectrometry. High performance liquid chromatography was carried out using a Waters high performance liquid chromatograph. A 25 cm × 4.6 mm-Zorbax ODS column packed with a RP-18 phase (Du Pont, Wilmington, U.S.A.) was operated at 42° with a flow rate of 1 ml/min and a solvent gradient (solvent A, water-methanol 8:2 v/v; solvent B, methanol) changing from 25 to 100% B in 30 min. The eluate was monitored by u.v. detector at 254 nm and collected in 0.3-min fractions for measuring radioactivity in a model 460 cd liquid scintillation counter (Packard Instruments, Frankfurt, F.R.G.).

High performance liquid chromatography fractions used for GC/MS analysis were evaporated to dryness under reduced pressure and derivatized with *O*:*N*-bis-(trimethylsilyl)acetamide. Gas chromatography/mass spectrometry was performed on a Finningan 4510 GC/MS/DS, using a fused-silica capillary column (30 m × 0.25 mm i.d.) with a chemically

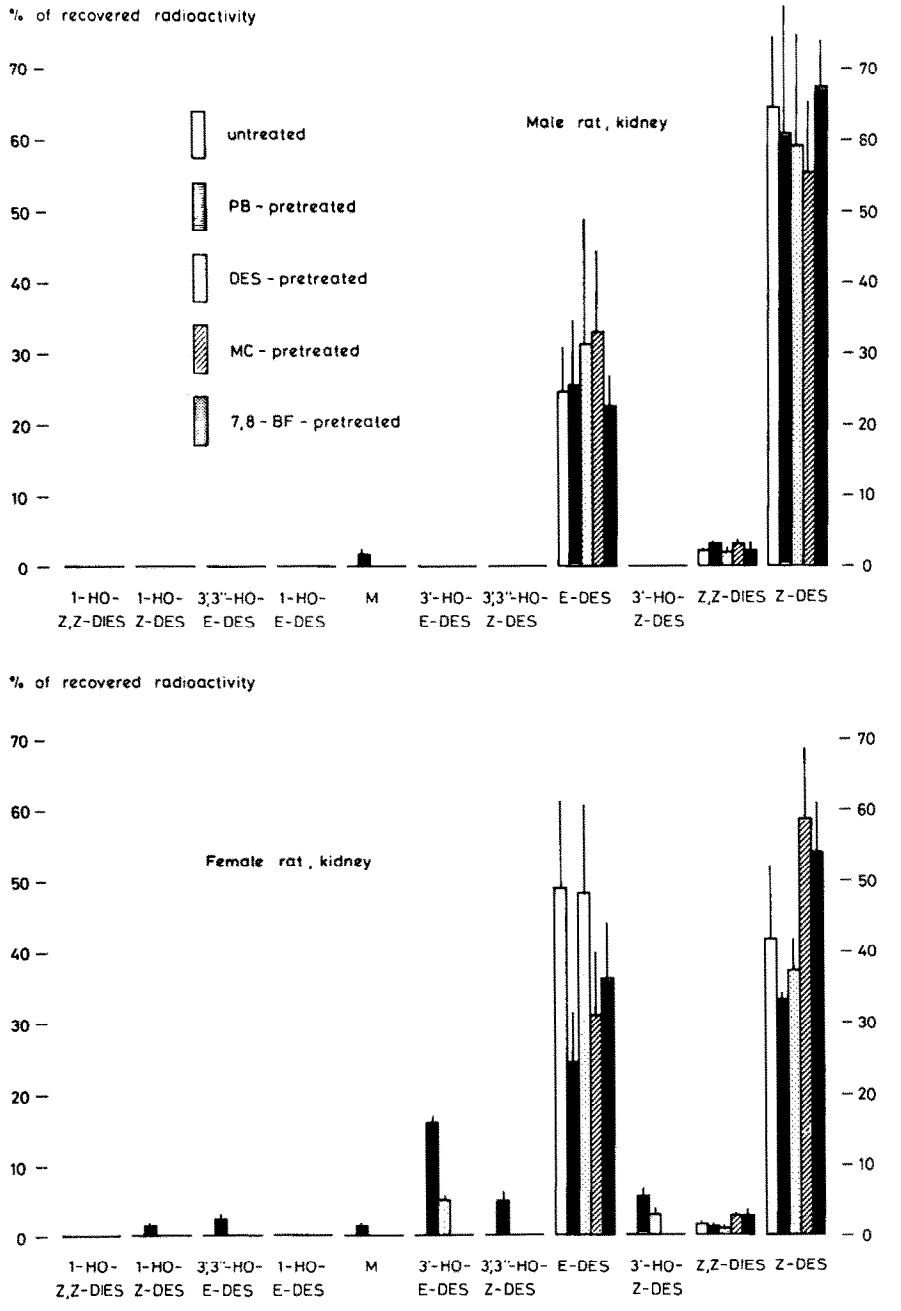

Fig. 4. Effects of inducers on the metabolism of [^{14}C]DES by renal microsomes from male rats (upper chart) and female rats (lower chart). See legend of Fig. 3 for further details.

bonded OV-1 phase of 0.25 μm thickness for separation. The column was heated from 160 to 260° in 30 min, while the injector and transfer line were set to 280°. Electron-impact mass spectra were taken at an electron energy of 70 eV.

RESULTS

Identification of microsomal metabolites

Incubations of [^{14}C]DES with hepatic microsomes from male rats proved to yield the largest number of metabolites (see below) and were therefore used for the identification. Metabolites were first separated by HPLC and then identified by GC/MS. An efficient HPLC separation (Fig. 1) was achieved by using a modification of a previously published method [11].

Gas chromatography/mass spectrometry of the HPLC fractions led to the identification of 10 microsomal DES metabolites (Fig. 2): *Z*-DES, the *E*- and *Z*-isomers of 1-hydroxy-DES, 3′-hydroxy-DES and 3′:3′-dihydroxy-DES, as well as *Z*:*Z*-dienestrol (*Z*:*Z*-DIES) and its 1-hydroxy-derivative were unambiguously identified through their mass spectra

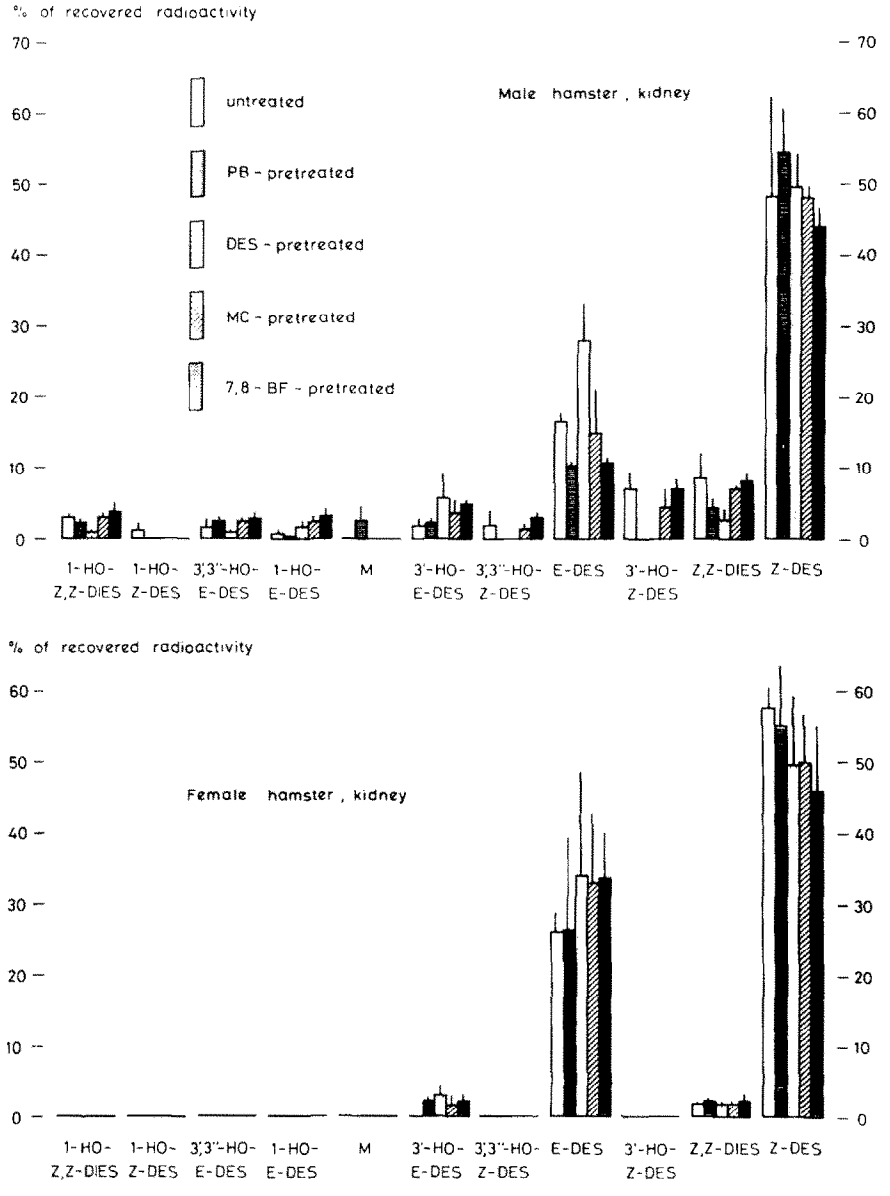

Fig. 5. Effects of inducers on the metabolism of [^{14}C]DES by the renal microsomes from male hamsters (upper chart) and female hamsters (lower chart). See legend of Fig. 3 for further details.

and cochromatography with authentic reference substances in GC and HPLC. Furthermore, one HPLC zone contained a metabolite which, according to its mass spectrum, should have the structure of a *Z*:*Z*-DIES with two aromatic hydroxy groups, presumably 3′-3″-dihydroxy-*Z*,*Z*-DIES. However, this structural assignment must be considered preliminary because a synthetic reference compound is not yet available; the metabolite is therefore termed "M" in the following Figs 3–6.

Metabolic pattern with microsomes from various organs and effect of inducers

For a comparison of the pattern of DES metabolites generated by hepatic and renal microsomes from male and female hamsters and rats, a standard incubation procedure was used. In particular, the ratio of DES to microsomal protein and to NADPH was kept constant. The soluble metabolites remaining after precipitation of the microsomal protein were purified through extraction with diethyl ether from the aqueous phase (see Materials and Methods). Ether-extractable radioactivity accounted for 95, 98 and 99% of the soluble radioactivity in the incubations with hepatic, renal and uterine microsomes, respectively. The ether-extractable metabolites were then separated by HPLC and quantified according to the radioactivity in the various HPLC fractions. Unlabelled reference compounds were added to the sample prior to HPLC for unambiguous identification of metabolites.

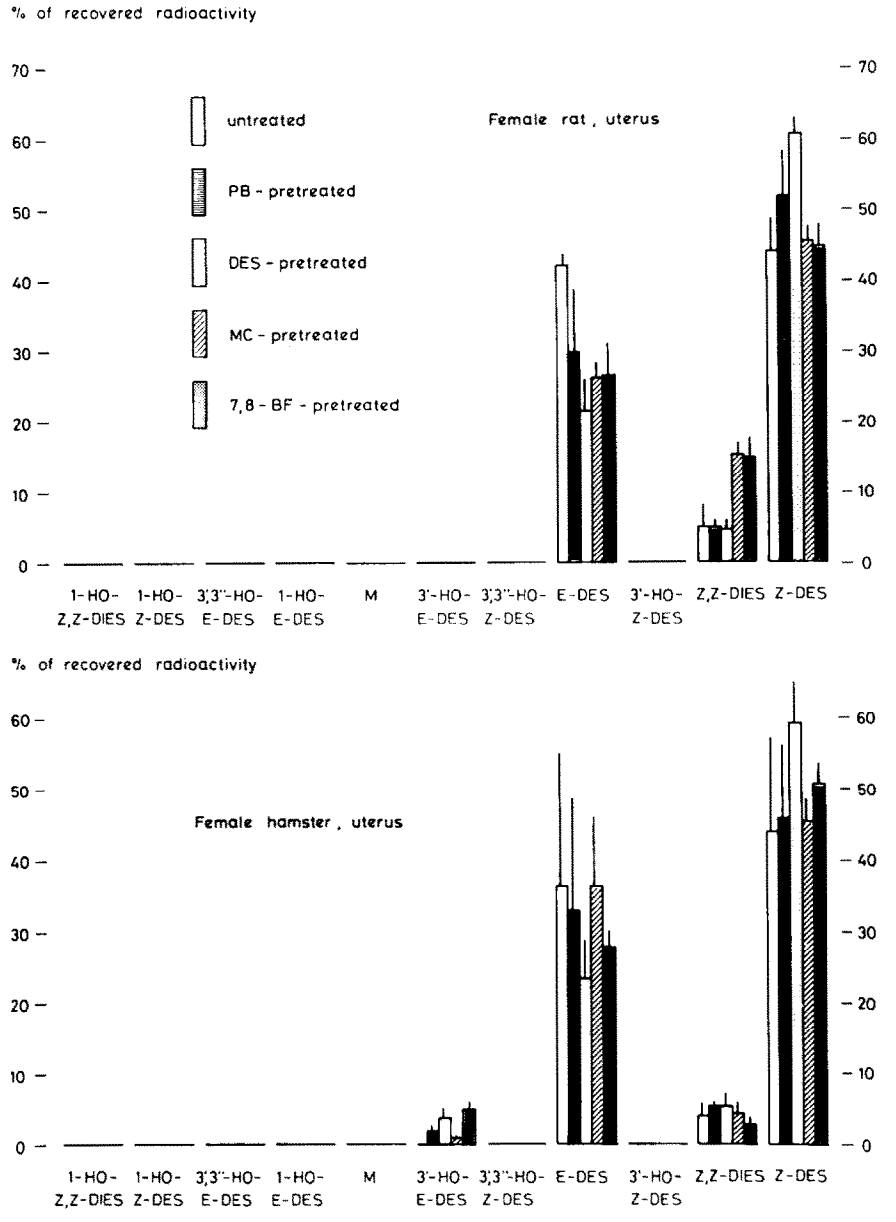

Fig. 6. Effects of inducers on the metabolism of [^{14}C]DES by uterine microsomes from rats (upper chart) and hamsters (lower chart). See legend of Fig. 3 for further details.

The ether-extractable DES metabolites obtained with hepatic microsomes from male rats and hamsters are depicted in Fig. 3. The blank bars represent microsomal metabolites of untreated animals. With rat hepatic microsomes, *E*-DES was virtually completely metabolized under the conditions used; the *E*- and *Z*-isomers of 3'-hydroxy-DES, 3':3''-dihydroxy-DES, 1-hydroxy-DES, and "M" were formed as major products, whereas only very small amounts of *Z*:*Z*-DIES were detected. *Z*-diethylstilbestrol accounted for approximately 25%; however, since 20% of *Z*-DES was already present in the starting material (see Methods and Materials), it cannot be decided in this case whether the *Z*-DES is a true metabolite or unchanged starting material.

Pretreatment with PB, DES, MC and 7:8-BF did not markedly change the pattern of male rat hepatic microsomal DES metabolites (Fig. 3). Hepatic microsomes from female Wistar rats gave virtually the same pattern (data not shown), which was also not affected by pretreatment of the animals.

A somewhat different profile of DES metabolites was observed with hepatic microsomes from male hamsters (Fig. 3, lower chart). In addition to unmetabolized *E*-DES, there were significant amounts of *Z*:*Z*-DIES, whereas the quantity of hydroxylated metabolites appeared to be smaller with hamster microsomes as compared to rat microsomes. Female hamster liver microsomes produced the same pattern of metabolites (data not shown) as

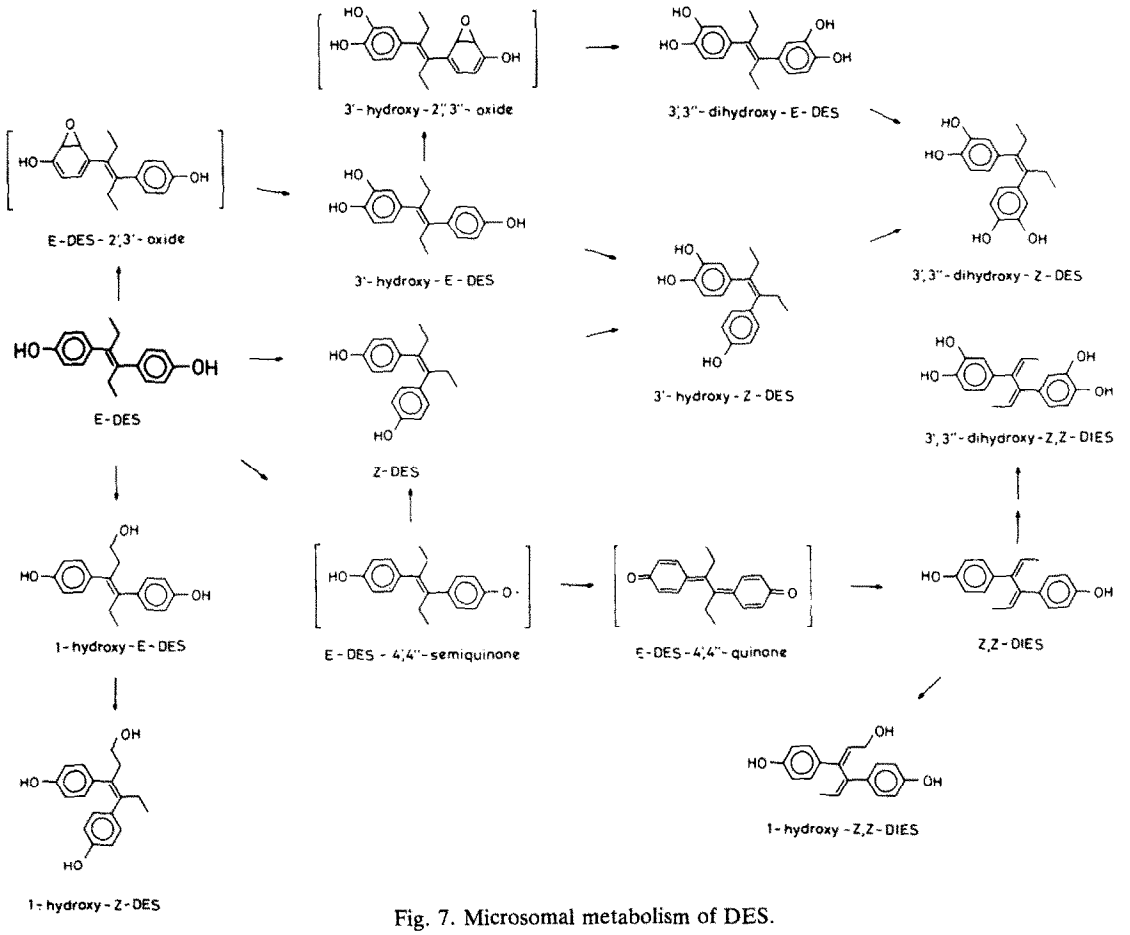

Fig. 7. Microsomal metabolism of DES.

found with males and pretreatment did not markedly influence the metabolic profile of hamster hepatic microsomes of either sex.

In control incubations of [^{14}C]DES without microsomes or with rat and hamster hepatic microsomes in the absence of NADPH none of the hydroxylated metabolites, but 1–2% of the *Z*:*Z*-DIES were found. The values given for metabolic *Z*:*Z*-DIES are corrected accordingly.

Renal microsomes from untreated male and female rats produced only large amounts of *Z*-DES, very little *Z*:*Z*-DIES and none of the hydroxylated products (Fig. 4). However, pretreatment with PB and, to a lesser extent DES, enabled renal microsomes from female but not from male rats to hydroxylate DES (Fig. 4). 3-Methylcholanthrene and 7:8-BF did not have this effect.

In contrast to rat kidney microsomes, the renal microsomes from male hamster were capable of forming a variety of hydroxylated DES metabolites even without pretreatment (Fig. 5). Kidney microsomes from untreated female hamsters lacked this capacity, but did gain it to a very small extent through pretreatment (Fig. 5).

Finally, the pattern of uterine microsomal DES metabolites is given in Fig. 6. Both untreated rat and hamster gave only *Z*-DES, little *Z*:*Z*-DIES and no hydroxylated products. Pretreatment with MC and 7:8-BF stimulated formation of *Z*:*Z*-DIES with rat uterine microsomes, whereas aromatic hydroxylation was slightly induced in hamster uterine microsomes by PB, DES, MC and 7:8-BF.

DISCUSSION

In contrast to the *in vivo* metabolism of DES, which has been extensively studied in various animal species and also in humans (for review see [6]), very little data are as yet available on the nature of DES metabolites generated by microsomes. Engel *et al.* [12] incubated DES with rat liver microsomes and tentatively identified a dihydroxy-DES and a dihydroxy-DIES by means of MS. In incubations fortified with *S*-adenosyl-L-methionine and catechol-*O*-methyltransferase, a monomethoxy-DES was detected [12, 13]. The catechol structure of the primary metabolite 3'-hydroxy-DES was later confirmed [14].

3'-Hydroxy-DES, 3':3''-dihydroxy-DES, and a dihydroxy-DIES were also found as DES metabolites in the present study together with 1-hydroxy-DES, *Z*:*Z*-DIES, and 1-hydroxy-*Z*:*Z*-DIES. The *E*- and *Z*-isomers of DES and its hydroxy metabolites were determined separately. All metabolites except dihydroxy-DIES were unambiguously identified through comparison with authentic reference compounds.

A metabolic scheme for the microsomal metabolism of DES is derived from the identified products (Fig. 7). The oxidative pathways operative *in vivo*, viz. oxidation of DES to *Z*:*Z*-DIES and aromatic and aliphatic hydroxylation of DES and *Z*:*Z*-DIES are the same as established for the *in vivo* metabolism of DES [6].

An interesting feature of the *in vitro* metabolites not noted previously with the *in vivo* products is the formation of large amounts of *Z*-isomers. Although the stilbene molecule does isomerize under the influence of light, this mechanism cannot account for the large quantities. Therefore it must be assumed that the isomerization is, at least in part, an enzymatic process. This is particularly obvious in the case of male rat renal microsomes, where up to 70% of the total microsomal products were present as *Z*-DES. The mechanism of this isomerization and the enzyme(s) involved are unknown to date. One possibility would be a phenoxy intermediate generated by a one-electron oxidation (Fig. 7). It has recently been reported that prostaglandin synthase, an enzyme with known peroxidase activity, can catalyse the isomerization of *E*- to *Z*-DES *in vitro* [15].

The profile of microsomal DES metabolites appears to strongly depend on species, sex and organ. For example, hepatic microsomes from male or female rats produce a metabolic pattern different from that obtained with hepatic microsomes from male or female hamsters (Fig. 3). Similarly the metabolic profile of rat hepatic microsomes differed from that of rat renal microsomes (Figs 3 and 4). Sex differences were noted between renal microsomes from male and female hamsters (Fig. 5). This is particularly interesting because of the difference in susceptibility for the carcinogenic effect of DES. The male hamster kidney, which is susceptible, is able to metabolize DES oxidatively, in contrast to the non-susceptible female hamster kidney and the male and female rat kidney.

If the oxidative metabolism of DES in the male hamster kidney is of relevance for the organotropism of DES carcinogenicity, the question arises as to why the livers of rat and hamster, which form the same metabolites as the male hamster kidney, are not susceptible. Although this question cannot be clearly answered at present, it may be speculated that reactive metabolites are more efficiently inactivated by the liver *in vivo* as compared to the kidney, e.g. by conjugation with glucuronic acid. It is interesting to note in this context that prolonged treatment with 7:8-BF, which may interfere with this deactivation, renders the hamster liver susceptible to DES carcinogenicity [16].

Pretreatment with different inducers revealed a species-, sex- and organ-specific response; whereas hepatic microsomes from both male and female rats and hamsters were not markedly affected by PB, DES, MC and 7:8-BF with respect to their pattern of DES metabolites, renal microsomes from female but not from male rats showed a pronounced response to pretreatment with PB even after a short-term pretreatment (Fig. 4).

The pronounced differences in the ability of microsomes from different organs to metabolize DES and their modulation by certain inducers, as demonstrated in the present study, can possibly be used to elucidate further the relevance of oxidative metabolism of DES for its carcinogenicity. For example, it should prove interesting to investigate whether treatment with PB, which obviously induces oxidative metabolism of DES in the kidneys of female rats and hamsters, will render these organs susceptible to the carcinogenic effect of DES. Studies along these lines are in progress in our laboratory.

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