Biochemical Pharmacology, Vol 27, pp 1087–1094 Pergamon Press, 1978 Printed in Great Britain.

OXIDATIVE METABOLITES OF DIETHYLSTILBESTROL IN THE FETAL, NEONATAL AND ADULT MOUSE

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(*Received* 8 *August* 1977; *accepted* 3 *October* 1977)

Abstract—Urinary metabolites of ^{14}C-labeled diethylstilbestrol (DES) in adult female mice were identified by radio gas chromatography and gas chromatography-mass spectrometry as β-dienestrol and ω-hydroxy-derivatives of DES, ψ-DES and dienestrol. The metabolic β-dienestrol does not appear to be formed through a DES epoxide. Most of the metabolites found in adult mice were also detected by mass fragmentography in the whole-body homogenates obtained from mice of age 1, 4 and 8 days, indicating that oxidative metabolism of DES is operative in neonatal mice. Furthermore, extensive conjugation of DES and its metabolites to glucuronic acid was found in adult and neonatal mice. Part of the administered radioactivity was unextractable from tissue macromolecules in neonatal mice. The biotransformation of DES and binding of its metabolites, implies a metabolic activation of this synthetic estrogen that might be associated with its teratogenic and carcinogenic potential for neonatal and fetal mice.

Diethylstilbestrol (DES) has an established history as a carcinogenic compound [1, 2]. Recently, this synthetic estrogen has evoked renewed interest due to its transplacental toxicity in human beings. Cervicovaginal alterations including carcinoma were observed in the female offspring of DES-treated women, as were certain teratogenic changes in the genital tract of their male progeny [3–6]. Many of the DES-induced fetotoxic effects observed in man are also found in mice exposed to DES prenatally or neonatally [7–10].

At present, the mechanism underlying these adverse effects is unknown. Among others, the possibility that DES acts basically like other chemical carcinogens through covalent binding to critical cellular targets has to be considered [11]. Metabolites, which according to their chemical structure can be expected to react with nucleic acids and other cellular constituents, are formed from DES in several species including man and nonhuman primates [12–15]. These reactive metabolites are formed by oxidation of the stilbene molecule and hydroxylation of the aliphatic and aromatic moiety [13]. If metabolic activation of DES is relevant for its fetotoxicity, it should also take place in the fetal and neonatal mouse as a susceptible species. Oxidative metabolism of drugs, however, cannot generally be assumed to occur in neonatal rodents [16–18]. Therefore, the ability of the mouse, particularly of the neonate, to metabolize DES has been studied.

Part of this paper was presented at the First International Congress on Toxicology, Toronto, 1977.

Abbreviations DES—Diethylstilbestrol, 3,4-di-(*p*-hydroxyphenyl)-hex(3)ene ψ-DES—pseudo diethylstilbestrol, 3,4-di-(*p*-hydroxyphenyl)-hex(2)ene, GC—gas chromatography, MS—mass spectrometry, TLC—thin layer chromatography, TMS—trimethylsilyl.

MATERIALS AND METHODS

Substances. Monoethyl-1-[^{14}C]DES was obtained from The Radiochemical Centre, Amersham. Radiochemical purity according to the manufacturer was only 95 per cent. Therefore the ^{14}C-labeled DES was recrystallized with unlabeled *trans*-DES (E. Merck, Darmstadt, West Germany) from benzene to yield a material with a radiochemical and stereochemical purity >99 per cent, as shown by radio-GC and -TLC. Only recrystallized [^{14}C]DES was used in the experiments described in this paper, with specific radioactivities of 1.21 and 0.53 mCi/m-mole for different batches.

The mother liquor of the crystallized [^{14}C]DES was analyzed by radio-GC, and radioactive peaks other than *cis*- and *trans*-DES (retention times 12.4 and 14.6 min, respectively) were noted at 4.0 min (containing 0.5 per cent of the radioactivity of the original solution), 5.8 min (0.3), 17.0 min (0.7), 18.8 min (4.9) and 20.8 min (0.5). The major impurity at 18.8 min could be identified by GC/MS: the mass spectrum with ions at m/e 412 (100 relative intensity), 410 (33), 397 (46), 395 (24), 383 (30) and 381 (20) as well as the retention time is indicative for a β-dienestrol containing 75 atoms per cent ^{14}C. So far, it is unclear whether the impurities are by-products of the radioactive synthesis or result from radiolytic decay. In the recrystallized [^{14}C]DES, none of the five impurities could be found with a limit of detectability at 0.2 per cent.

Monoethyl-1-[^{14}C]DES epoxide was synthesized by a modification of the method of Wessely *et al.* [19]: 68 mg (0.25 m-mole) [^{14}C]DES (spec.radioact. 82 μCi/m-mole) were dissolved in a 0.5% solution

of perbenzoic acid in diethylether (14 ml, 0.5 m-mole). After 16 hr in the dark at ambient temperature, the organic phase was extracted with 10% aqueous Na_2CO_3 solution (5 ml) and water (5 ml), dried over Na_2SO_4, and evaporated. The residue was dissolved in ethanol (1 ml), and water (0.6 ml) was added. After 20 hr at 4°, the crystals were collected and dried in the dark at room temperature and atmospheric pressure to yield 30 mg (0.10 m-mole) of [^{14}C]DES epoxide. The product gave a single peak as a TMS derivative in GC which clearly separated from DES and other possible reaction products (Fig. 1). Furthermore, it has a characteristic mass spectrum, and upon treatment with 0.1 M aqueous H_2SO_4-dioxane 1:1 v/v (12 hr at ambient temperature) was completely converted into 3,3-di(*p*-hydroxyphenyl)-hexan(4)on), as characterized by its GC retention time and mass spectrum [19] (Figs. 1 and 2).

3,4-Di(*p*-hydroxyphenyl)-3,4-dihydroxy-hexane (α, β-dihydroxy-hexestrol) was prepared according to Dodds *et al.* [20] and consisted of a 1:1 mixture of the erythro- and threo-isomer, as shown by GC of the TMS derivatives. All other compounds were synthesized or purchased as described previously [13, 15]. The β-dienestrol (isodienestrol) used as a reference substance had a m.p. of 181/2°.

Animals and administration of labeled compounds. CD-1 mice were used at NIEHS and Swiss NMRI mice in Würzburg. No difference with respect to metabolism of DES was noted between the two strains. [^{14}C]DES was administered orally to adult female mice by gastric intubation (0.2 ml/mouse) of a solution containing 10 mg [^{14}C]DES/ml propane-1,2-diol. [^{14}C]DES epoxide was dissolved in propane-1,2-diol–0.9% saline 1:1 v/v (1 mg/ml) and 0.2 ml were injected intraperitoneally (i.p.) and intravenously (i.v.) (tail vein). The DES epoxide was shown by GC to be stable in this solution for at least 15 min. For neonatal mice, a solution with a concentration of 1 mg [^{14}C]DES–ml propane-1,2-diol–0.9% saline (1:1 v/v) was used and volumes of 20, 25 and 50 μl were injected i.p. into neonates of age 1, 4 and 8 days, respectively. Neonatal mice of both sexes from the same litter were used in each experiment.

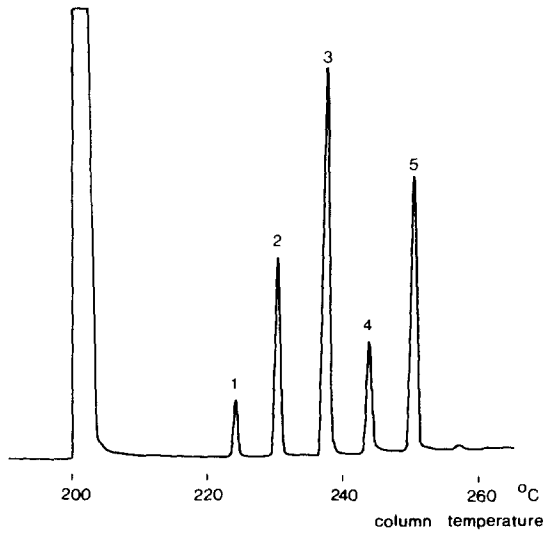

Fig. 1. GC of reference compounds after trimethylsilylation. 1 *cis*-DES, 2 *trans*-DES, 3 DES epoxide, 4 β-dienestrol, 5 3,3-di (*p*-hydroxyphenyl)hexan(4)on. GC was carried out on a glass column packed with 3% OV-225 on GasChrom Q 100/120 mesh. Carrier gas He 30 ml/min. Temperatures; column oven 200–260° with 4°/min, injector 250°, detector (FID) 270°.

Extraction and analysis of metabolites. Collection of urine and extraction of metabolites was carried out as described for rats and hamsters [13]. Neonatal and fetal mice were homogenized in water using an ultra-turrax homogenizer (Janke and Kunkel KG, West Germany). Ethanol was added to the homogenate to a final concentration of 80% ethanol and the precipitate removed by centrifugation and washed successively with ethanol, ether–ethanol 3:1 v/v and ether by thoroughly resuspending the sediment in each of the solvents. The remaining precipitate was dried at ambient temperature and aliquots of 200–400 mg were combusted in a Packard Sample Oxidizer 306 for radioactivity measurement. The combined extracts were evaporated under reduced pressure, water was added and the metabolites were extracted twice by a 2-fold vol. of ether–ethanol 3:1 v/v from the aqueous phase after saturation with ammonium sulfate. 96–98 per cent of the radioactivity were consistently found in the combined organic phases and 2–4 per cent were left in the aqueous phase after extraction.

Separation of extracted metabolites by chromatography on alumina, enzymatic hydrolysis of glucuronides, liquid scintillation counting and radio-GC and -TLC were performed as reported previously [13, 15]. Briefly, extracts were applied to the alumina column in ethanolic solution and successively [13, 15]. Briefly, extracts were applied to an alumina column in ethanolic solution and successively eluted with 95% ethanol (to yield unconjugated metabolites), water (sulfates), a phosphate/citrate buffer of pH 6 (glucuronides), a 2 M sodium acetate buffer and 20% formic acid. When metabolites were recovered from TLC plates, the corresponding zones were scraped off within 10 min after the run, and the silica gel filled into small glass columns and eluted with ethyl acetate. GC/MS was carried out on a Varian CH 7 mass spectrometer combined with a Varian 2700 gas chromatograph, and the data were handled with a Varian SS 100 MS data system. Quantitation of metabolites in neonatal and fetal mice was achieved by mass fragmentography, i.e. operating the GC/MS in the selected ion monitoring mode. A LKB 9000 mass spectrometer was used for this purpose at NIEHS. Standards for quantitation were either made from synthetic reference compounds (for DES and β-dienestrol) or using a mixture of the ω-hydroxylated metabolites isolated from the urinary glucuronides of adult mice. Taking into account the specific radioactivity of the metabolites (assumed to be identical with the parent [^{14}C]DES) and knowing the composition of the mixture (determined by radio-GC), the absolute amount of each metabolite in the standard could be calculated from the radioactivity.

RESULTS

Metabolites of DES in the urine and plasma of adult mice. Urinary metabolites. The 24-hr urine collected after oral administration of [^{14}C]DES to four adult female mice at a dosage of 100 mg/kg contained 7.5 and 9.6%, respectively, of the administered radioactivity in two experiments. Upon chromatography on alumina, 74.9 and 72.2 per cent of the urinary radioactivity appeared in the glucuronide fraction (see Methods). After incubation of this fraction with β-glucuronidase, more than 80 per cent of the radioactivity was extractable into ether and separated into two zones upon TLC in benzene–ethyl acetate 1:1 v/v. The less polar zone (zone 2, R_f 0.5–0.6) migrated like DES and accounted for about 80 per cent of the radioactivity on the plate, while the other zone (zone 1, R_f 0.3–0.4) contained 20 per cent.

The radioactivity of both zones was further analyzed by radio-GC and GC/MS. DES was shown to account for only 30 per cent of zone 2, and the major metabolite in this zone (metabolite E, 57 per cent) was identified as β-dienestrol (isodienestrol) according to its mass spectrum and cochromatography with authentic reference substance in GC (Fig. 2). Zone 1 separated into five major peaks upon radio-GC (Fig. 3). One of them (metabolite J, approximately 22 per cent) had identical retention time and mass spectrum with synthetic ω-hydroxy-dienestrol (Fig. 2). The remaining four radioactive peaks consisted of two pairs with identical mass spectra and were therefore assumed to represent the *cis*- and *trans*-isomers of two metabolites designated H and

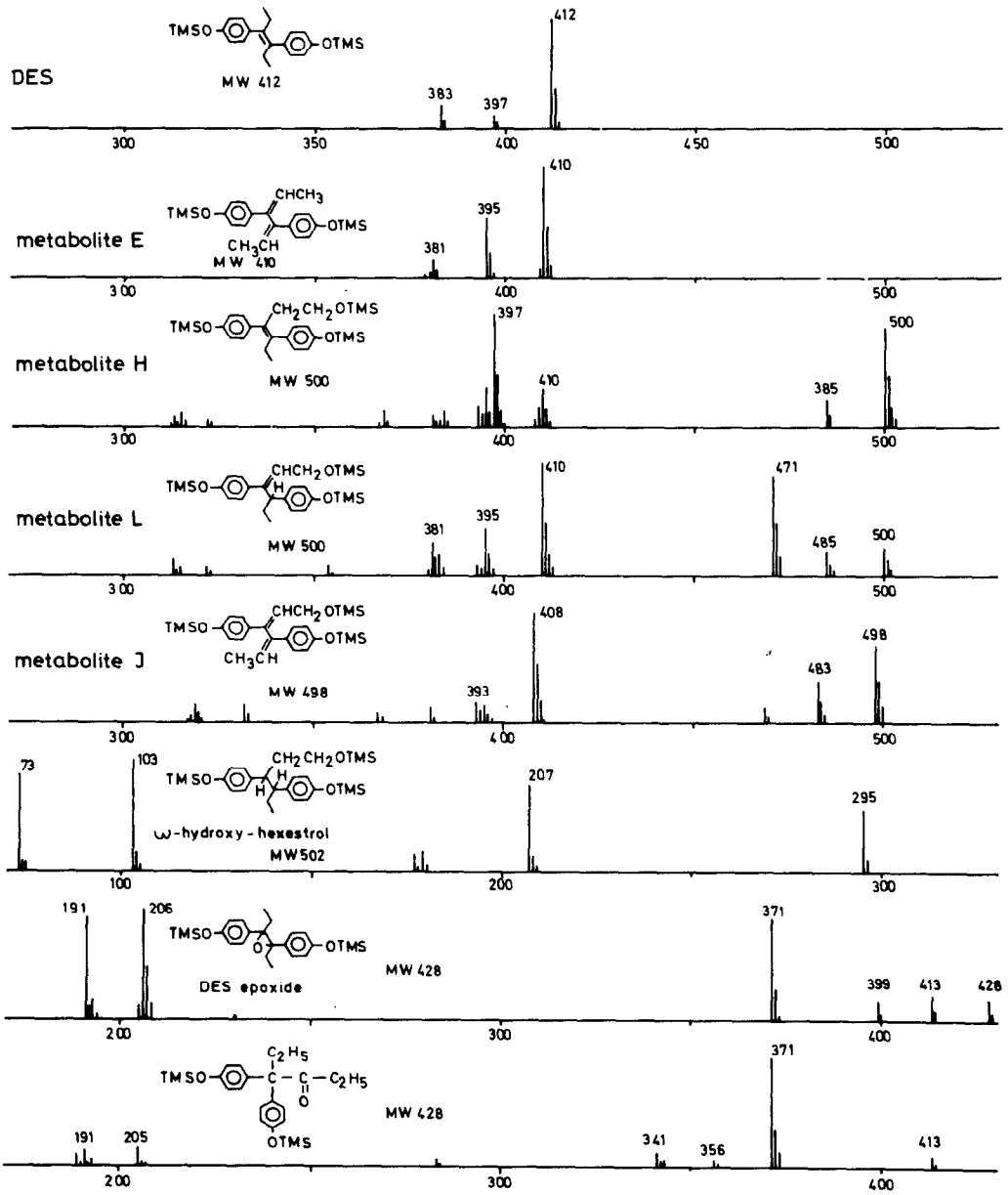

Fig. 2. Electron impact mass spectra (70 eV) of DES metabolites and reference compounds (TMS derivatives).

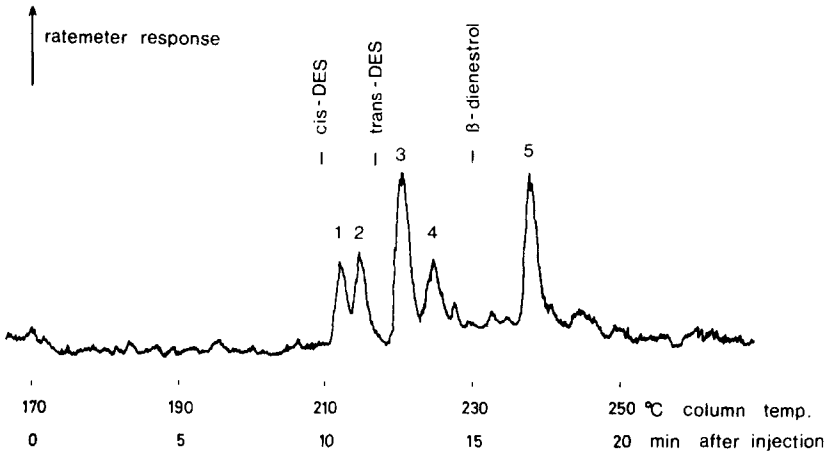

Fig. 3. Radio-GC of hydroxylated DES metabolites, as obtained from the urinary glucuronide fraction after hydrolysis and TLC of the aglycons. 1 and 2 *cis*- and *trans*-ω-hydroxy-ψ-DES, 3 and 4 *cis*- and *trans*-ω-hydroxy-DES, 5 ω-hydroxy-dienestrol.

L (41 and 37 per cent). Metabolite H according to its mass spectrum has the structure of an ω-hydroxylated DES (Fig. 2). For metabolite L, the mass spectrum shows the same molecular ion but a different fragmentation pattern, implying the structure of the allylic hydroxy compound ω-hydroxy- ψ-DES (Fig. 2).

To substantiate the structures of metabolites H, L and J, a mixture of them as obtained from the TLC zone 1 was hydrogenated in ethanol with 10 per cent Pd on charcoal as a catalyst for 15 min. The radio gas chromatogram of the product revealed two major and two minor peaks, which were identified through their mass spectra as the diastereomeric forms of ω-hydroxy-hexestrol and hexestrol, respectively (Fig. 2). When synthetic ω-hydroxy-dienestrol was hydrogenated under the same conditions, a similar mixture of hexestrol and ω-hydroxy-hexestrol was obtained.

In a control experiment, the stability of DES in mouse urine was tested. 150 μg of the same [^{14}C]DES as used for the metabolic study was added to the 24-hr urine of four untreated mice and kept under the conditions of urine collection for 5 days. Radioactivity was almost completely (97 per cent) eluted from the alumina column into the fraction of unconjugated material and in the subsequent TLC migrated quantitatively in zone 2. *Cis*- and *trans*-DES were the only radioactive peaks in the radio gas chromatogram of zone 2 under conditions, where less than 1 per cent of other labeled compounds would have been detected. Similarly, no radioactive peaks other than those from DES were found by radio-GC in the ether extract of the glucuronide fraction, to which 100 μg of [^{14}C]DES were added prior to hydrolysis. When the ether extract was analyzed by TLC, 99.2 per cent of the radioactivity was in zone 2 (R_f 0.44–0.75; R_f DES 0.6) and less than 0.5 per cent was in zone 1 (R_f 0.10–0.44). Mass fragmentographic analysis of the material in zone 2 showed that only 0.2–0.3 per cent of the radioactivity was associated with β-dienestrol.

Plasma metabolites. Blood was collected 3 hr after oral administration of [^{14}C]DES to five adult female mice and the plasma was found to contain 0.05 per cent of the administered radioactivity. Glucuronides represented 63 per cent of the plasma radioactivity. The minute amounts precluded radio-GC, but by mass fragmentography DES (m/e 412 and 397) and metabolite E (m/e 410 and 395) were found in a ratio of about 1:1 in the glucuronide fraction, whereas metabolites H and L (m/e 500) were not present in significant amounts.

Metabolites of DES in neonatal and fetal mice. [^{14}C]DES was injected i.p. into neonatal mice of different ages and the radioactivity extracted from the wholebody homogenate after 2 hr was chromatographed on alumina (Table 1). Since the extraction procedure was almost quantitative (see Methods), but only 71–82 per cent of the administered radioactivity were found in the total extract and 1–6 per cent in the precipitated material, some of the radioactivity was apparently excreted within 2 hr. In 1-day old neonates, the extracted radioactivity was about equally distributed among unconjugated metabolites, glucuronides and more polar material, which was only partially eluted from the alumina column. The chemical nature of this material is as yet unknown. Its proportion appears to increase with the age of the mice (Table 1). Thus, it constitutes half of the extracted radioactivity in 4-day old neonates and two-thirds in 8-day old mice, while the proportions of unconjugated radioactivity and glucuronides decrease concomitantly.

In a control experiment, [^{14}C]DES in an amount equal to that used for injection was added to the vial prior to the homogenation of untreated 4-day old neonates. Extraction of the radioactivity from the homogenate and recovery in the fraction of unconjugated material was almost quantitative (Table 1).

After i.p. injection of [^{14}C]DES at a dose of 16 μg/g into a near-term pregnant mouse, 9.9 per cent of the administered radioactivity was found in the extract of the fetuses after 2 hr. The extracted radioactivity was attributed predominantly (84 per cent) to unconjugated material, but a significant proportion (6 per cent) was found in the glucuronide fraction.

Since contamination with lipophilic endogeneous compounds was much higher in the fraction of the

Table 1. Conjugation and unextractable binding of [^{14}C]DES by neonatal mice

Age of mice, days	Percent of administered radioactivity: Total extract*	Fraction of unconjugated metabolites	Fraction of glucuronides	Other conjugates†	Extracted sediment
1	71.3	24.5	22.5	24.3	N.M.‡
	75.0	26.6	19.1	29.3	1.2
4	81.8	18.5	15.9	47.4	3.1
	80.0	27.9	11.9	38.4	4.4
8	76.5	10.8	13.1	52.6	N.M.
	73.6	10.9	13.1	49.6	5.8
Control 4	97.7	95.9	<0.5	1.3	0.2

[^{14}C]DES (1 μg/μl propane-1.2-diol–0.9% saline 1:1 v/v) was injected i.p. at a dose of 10 μg/g. 12, 8, and 4 mice of age 1, 4, and 8 days were used for each experiment in order to get comparable amounts of tissue and radioactivity. The mice were sacrificed after 2 hr and extracts chromatographed on alumina (see Methods)

* Accounting for 96–98 per cent of the radioactivity in the supernatent of the whole body homogenate.

† Calculated as the difference between totally extractable radioactivity and the radioactivity attributed to unconjugated metabolites and glucuronides. For further discussion see text.

‡ N.M. = not measured.

unconjugated metabolites as compared to the glucuronides, the glucuronide fractions listed in Table 1 were used to search for metabolites of DES. After enzymic hydrolysis and extraction with ether, part of the extract was analyzed by TLC and the radioactivity located on the plate by autoradiography. The major portion migrated like DES and dienestrol in all of the glucuronide fractions, but there always was another radioactive band on the plate at about half the R_f of DES, implying the presence of a hydroxylated metabolite. The radioactivity in this zone, which appeared to increase with increasing age of the neonates (zone 1, Table 2) could be identified and quantitated by mass fragmentography as metabolites L (ω-hydroxy-ψ-DES, m/e 500, 471 and 410) and H (ω-hydroxy-DES, m/e 500 and 397). In zone 2, mainly DES and some metabolite E (β-dienestrol) were found.

Again control experiments were carried out to preclude that the metabolites listed in Table 2 are artefacts formed during workup procedures. Thus, the fraction of unconjugated material from the control experiment given in Table 1 was analyzed by TLC. All the radioactivity was found in zone 2, and no β-dienestrol (<0.1 per cent) could be detected in this zone by mass fragmentography. However, when [^{14}C]DES (30 μg) was added to the glucuronide fraction of untreated neonatal mice prior to hydrolysis, and TLC zone 2 was analyzed, 0.9 and 1.4 per cent of the radioactivity of this zone was found to be β-dienestrol in two experiments (control 1 and 2, Table 2). TLC zone 1 did not contain significant amounts of radioactivity in the control experiments, and no radioactive bands were seen in the autoradiogram after prolonged exposure (Table 2).

Failure to detect biotransformation of DES epoxide into dienestrol. In order to establish the role of DES epoxide and its hydrolysis product, hexestrol-α,β-diol, as possible precursors of metabolic β-dienestrol, the epoxide and the diol were administered to adult female mice at a dose of 10 mg/kg, and the glucuronide fractions of the pooled urine of four

Table 2. Metabolites of [^{14}C]DES in the glucuronide fraction of neonatal mice

Age of mice, days	Percent of the glucuronide radioactivity in: Zone 1	Zone 2	Metabolite: H	L	E	DES
1	11	89	1	8	1	88
4	23	77	5	15	4	73
8	28	72	8	18	8	64
Control 1	1.5	98.5	N.D.*	N.D.	1.4	97
Control 2	0.4	99.6	N.D.	N.D.	0.9	98

The glucuronide fractions were hydrolyzed and the aglycons separated by TLC on silica gel with benzene–ethylacetate 1:1 v/v into zone 1 (R_f 0.20–0.45) and zone 2 (R_f 0.45–0.70). DES and metabolites E, H and L were identified in the ethylacetate extracts of the TLC zones by mass fragmentography. In the control experiments, [^{14}C]DES was added prior to hydrolysis to the glucuronide fraction of 4-day old untreated mice.

* N.D. = not detected (<0.1%).

mice were analyzed for β-dienestrol. Both substances were injected i.v. and i.p. in separate experiments. The use of ^{14}C-labeled DES epoxide (see Methods) permitted a quantitative study of the fate of this compound. Thus, 31 and 32 per cent of the dose were found in the 24-hr urine after i.v. and i.p. injection, respectively, and 58 and 51 per cent of the urinary radioactivity were in the glucuronide fractions. Analysis of this fraction, however, by radio-GC and mass fragmentography did not provide any evidence for β-dienestrol in amounts exceeding 0.1 per cent of the radioactive material in these fractions. Instead, the rearrangement product of the DER epoxide was seen as the major peak in the radio gas chromatogram together with a small peak of 4'-hydroxypropiophenone. These two compounds were also found in the sulfate fractions of both experiments, which were eluted from the alumina column between unconjugated material and glucuronides (see Methods), and hydrolyzed by glucuronidase/steroid sulfatase. They contained 28–37 per cent of the urinary radioactivity, mostly as 4'-hydroxypropiophenone (74–87 per cent) together with 13–17 per cent 3,3-di (*p*-hydroxyphenyl)hexan(4)on. These compounds were also identified by GC/MS. It is interesting to note, that the urinary sulfate fraction in the metabolism studies with [^{14}C]DES usually contained only 1–2 per cent of the excreted radioactivity.

For the experiments with hexestrol-α,β-diol, no radioactively labeled substance was available, and therefore quantitative data for the excretion and conjugation could not be obtained. However, mass fragmentographic analysis of the glucuronide fractions from the 24-hr urine after i.p. and i.v. injection failed to give any indication for the presence of β-dienestrol. The unchanged diol could be found by GC in these fractions in amounts corresponding to 2–3 per cent of the dose.

DISCUSSION

In a recent paper on the fate of DES in the pregnant mouse, a detailed investigation of the pharmacokinetics of this synthetic hormone has been reported, but no metabolites other than the DES glucuronide were identified [21]. In the present study, another four metabolites have been identified in mouse urine. They were named E, H, L and J and are derived from the DES molecule through oxidative pathways (Fig. 4). Metabolites E and J were unambiguously identified as β-dienestrol and ω-hydroxy-dienestrol, respectively, by comparison with authentic reference compounds in gas chromatography and mass spectrometry. For metabolites H and L, no synthetic reference compounds are yet available, but mass spectrometric evidence and chemical derivatization through catalytical hydrogenation very strongly suggest the structures of ω-hydroxy-DES (metabolite H) and ω-hydroxy-ψ-DES (metabolite L) (Fig. 2). Compound E, H and J have also been found as DES metabolites in several other species whereas metabolite L so far appears to be unique for the mouse [13, 15]. Recently, ψ-DES has been identified as an impurity of commercial ^{14}C-labeled DES [22]. This compound, however, was not present in our batch of [^{14}C]DES according to radio-GC, where ψ-DES clearly separates from *cis*- and *trans*-DES. Furthermore, our labeled material has been considerably diluted with unlabeled DES and recrystallized prior to use. The ratio of radioactivity to mass, as determined by radio-GC, was the same for metabolite L and the other metabolites. This is additional evidence that metabolite L does not originate from an impurity of the labeled DES.

In neonatal mice, DES is rapidly metabolized both by conjugation reactions and by oxidative pathways. Thus, even in 1-day old neonates, only 25 per cent of the dose could be recovered unconjugated after 2 hr (Table 1). Glucuronides represent a considerable proportion of the conjugates, but other types of conjugates appear to be formed. This is concluded from the fact that a major portion of the extractable radioactivity can only be eluted from the alumina column with very polar solvents like diluted formic acid, and some radioactivity can not be eluted at all. The nature of this polar material, which is also found in large amounts in extracts from tissues like liver and kidney, but not from urine or plasma, is under investigation.

The ability of neonatal mice to metabolize DES by oxidative pathways is clearly demonstrated by the formation of dienestrol and ω-hydroxy-derivatives of DES and ψ-DES (metabolites E, H and L, Table 2). Although a small amount of β-dienestrol can be formed from DES during hydrolysis of the glucuronides, this does not appear to account for the total amount of metabolite E found in 4-day and 8-day old mice (Table 2). As was shown by the control experiments, compounds H and L are not formed during processing of the glucuronide fraction (Table 2).

Since the amounts of identified metabolites accounted for roughly all the radioactivity in the glucuronide fraction, no attempt was made to search for other metabolites like the aromatic hydroxy- and methoxy-derivatives of DES and dienestrol [13]. Thus the possibility that small amounts of these metabolites are formed cannot be ruled out.

Metabolites of DES were also present in the near-term fetuses of mice treated with [^{14}C]DES. Thus,

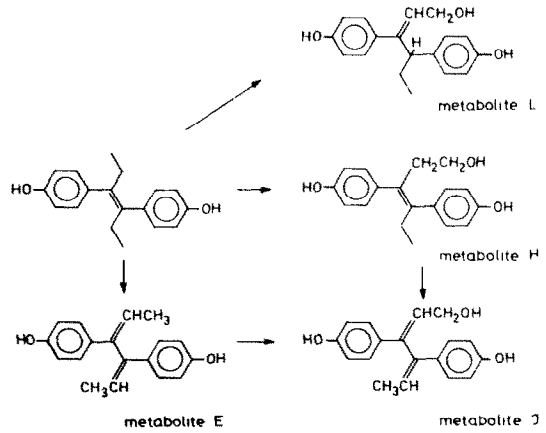

Fig 4. Oxidative metabolism of DES in the mouse.

β-dienestrol was identified by mass fragmentography, and a radioactive substance with the R_f of an ω-hydroxylated metabolite was seen on the TLC autoradiogram of the glucuronide fraction. From the study *in vivo*, however, no implication can be made of the site where the oxidative metabolites are formed, because metabolites found in the fetus may have been transferred from the maternal organism. Therefore, only incubations *in vitro* of isolated fetal tissues can provide information on the activity of the respective enzymes. Significant levels of UDP-glucuronyltransferase with DES as substrate have been demonstrated in fetal mouse liver recently, but no such studies appear to exist on the oxidative metabolism of DES in fetal tissues [21].

Studies in neonatal animals provide means to exclude the maternal metabolism in a situation *in vivo*. The ability of neonatal rodents to metabolize drugs appears to depend largely on the species and on the particular substrate [18]. If the adverse effects of DES in the neonatal mouse are induced by reactive metabolites, the capability to oxidize this drug is an important consideration; another would be electrophilic reactivity of the toxic metabolites. In this study, the formation of oxidative DES metabolites is demonstrated to occur in the neonatal mouse. Moreover, reactivity of the metabolites is implicated, since a considerable proportion of the injected radioactivity was unextractable from the precipitated macromolecules (Table 1). Formation of reactive DES metabolites *in vitro* has been reported recently by Blackburn *et al.* [23], who found that [^{3}H]DES binds to nucleic acids after activation by a liver microsomal system. In one of our laboratories it was shown that the allylic hydroxy compound ω-hydroxy-dienestrol (metabolite J) after acetylation can alkylate 4-(*p*-nitrobenzyl) pyridine and also reacts with the nucleosides adenosine, guanosine, cytidine and uridine [24]. Metabolite L, which is also an allylic hydroxy compound, may exhibit a reactivity similar to ω-hydroxy-dienestrol.

The epoxide of DES has also been proposed as a reactive metabolite. Its intermediate existence was concluded from the formation of dienestrol [12]. The findings reported in this paper, however, cast some doubt on this hypothetical pathway. Neither pure ^{14}C-labeled DES epoxide nor its hydrolysis product, hexestrol-α,β-diol gave rise to detectable amounts of dienestrol when injected into mice i.p. or i.v. Instead, the DES epoxide was rearranged to 3,3-di-(*p*-hydroxyphenyl)-hexan(4)on and cleaved to 4′-hydroxypropiophenone *in vivo*. The cleavage product 4′-hydroxypropiophenone has also been found as an urinary metabolite of DES in male rats, but whether it unambiguously indicates an epoxide intermediate or originates from another yet unknown oxidative pathway remains to be clarified [13].

If the possibility is excluded that exogeneously administered and endogeneously formed DES epoxide give rise to different metabolites, a precursor other than the epoxide has to be postulated for metabolite E. Several possible pathways have been discussed previously, and another one which is particularly interesting, should be added [13]. DES might be oxidized to a *p*-quinone, which can easily tautomerize to β-dienestrol. This oxidation has been achieved chemically and also enzymatically using peroxidase from horse radish [25]. The reaction can possibly be mediated by cytochrome P450, because this enzyme has been shown to function as a peroxidase in some cases [27, 28]. On the other hand, it is well established that estrogen target organs contain peroxidases. In fact, these enzymes are preferentially located in tissues depending on estrogens for growth, and they can be induced by estrogens [29–32]. Thus, it appears possible that oxidation of DES can also occur in the tissue of the Müllerian duct, where the biochemical lesion leading to teratogenic and carcinogenic manifestation takes place [4, 7, 8, 10]. The oxidation of DES by peroxidase to the *p*-quinone presumably proceeds through a *p*-semiquinone radical. Both these intermediates must be considered reactive molecules capable of binding to proteins and nucleic acids, as is known from studies with the respective ortho-quinones of steroid estrogens [33, 34]. Work is now in progress in our laboratory to demonstrate whether a *p*-quinone is actually formed in mammalian DES metabolism and to study its reactivity towards nucleic acids and other cellular macromolecules.

Acknowledgements—This study was supported in part by the Deutsche Forschungsgemeinschaft. We would like to thank Dr. R. L. Dixon (NIEHS) and Dr. H.-G. Neumann (Würzburg) for their interest and support. The excellent technical assistance provided by Mrs. E. Stein (Würzburg) in animal work and chromatographic procedures, by Mrs. J. Colberg (Würzburg) Dr. J. R. Hass (NIEHS) in mass spectrometry, and by Mrs. H. Raabe in chemical syntheses is most gratefully acknowledged. We also thank the R. Pfleger Foundation (Bamberg, West Germany) for providing the data system for our mass spectrometer.

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