Metabolism of [^{14}C]Diethylstilboestrol Epoxide by Rat Liver *in vitro*

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1. The *trans*-epoxide of diethylstilboestrol and its pinacolone were synthesized chemically and the pinacolone shown to be formed from the epoxide by a non-enzymic process.
2. [^{14}C]Diethylstilboestrol epoxide was converted by rat liver microsomal fraction into 4'-hydroxypropiofenone by a new type of cleavage reaction involving mono-oxygenase. Conditions for the formation of this metabolite and also water-soluble products were investigated together with the effect of inhibitors. A sex-difference in the conversion of diethylstilboestrol epoxide into 4'-hydroxypropiofenone and to polar and water-soluble products was observed.
3. Diethylstilboestrol epoxide was found to be a relatively stable compound that did not form a glutathione conjugate readily without further microsomal activation. A purified preparation of epoxide hydratase did not enhance its rate of conversion into the pinacolone.
4. Diethylstilboestrol epoxide was found to have about one-tenth the oestrogenic activity of diethylstilboestrol as measured by the increase in uterine weight or the induction of peroxidase in immature rat uteri. It was inactive as a mutagen when tested for its ability to inhibit bacteriophage ϕX174 DNA viral replication.
5. The possible role of diethylstilboestrol epoxide as an intermediate in the metabolism of diethylstilboestrol and in mediating the harmful effects of this synthetic oestrogen is discussed.

The synthetic oestrogen diethylstilboestrol is known to induce tumours in experimental animals (Kirkman & Bacon, 1950) and has also been linked to cases of cancer of the vagina in humans (Herbst *et al.*, 1971) and to hepatic centrilobular necrosis (Krishna *et al.*, 1972). Diethylstilboestrol has also been shown to bind to DNA (Blackburn *et al.*, 1976) and to other tissue macromolecules in a reaction involving cytochrome *P*-450 and NADPH (Krishna *et al.*, 1973). Covalent binding was inhibited by CO and compound SKF 525-A, but was increased by prior treatment of the rats with phenobarbital, which further supports the participation of a mono-oxygenase in the reaction. These findings, together with the known epoxidation of the stilbene double bond during the metabolism *in vivo* of aminostilbene derivatives (Metzler & Newmann, 1977) and the nature of the urinary products of rats treated with diethylstilboestrol (Metzler, 1975), some of which could be formed via diethylstilboestrol epoxide, all provide indirect evidence for the possible conversion of diethylstilboestrol into an epoxide. It was therefore decided to synthesize ^{14}C -labelled as well as unlabelled diethylstilboestrol epoxide to study its chemical and biological properties and its metabolism by rat liver

fractions. It was hoped that these experiments would provide further information about the role of the putative epoxide in the action of diethylstilboestrol.

Experimental

Materials

Reagents. Oestradiol, diethylstilboestrol, hexoestrol [*meso*-3,4-di-(*p*-hydroxyphenyl)hexane], glucose 6-phosphate, 5,5-dithiobis-(2-nitrobenzoic acid), GSH, NADH and NADPH were purchased from Sigma Chemical Co., St. Louis MO, U.S.A. Styrene oxide, cyclohexene oxide and 3-chloroperbenzoic acid were obtained from Aldrich Chemical Co., Milwaukee, WI, U.S.A.; guaiacol and 4'-hydroxypropiofenone were from Eastman Kodak Co., Rochester, NY, U.S.A., and DEAE-cellulose (DE-52) was from Whatman, Maidstone, Kent, U.K. The detergent lubrol 12-A-9 was a gift from I.C.I. Organics Division, Manchester, U.K. and compound SKF-525 A (β -diethylaminoethylidiphenyl-*n*-propyl acetate) was from Dr. A. J. Moriarity, Smith, Kline and French Corp., Montreal, Que., Canada. 3-Chloroperbenzoic acid was purified by the method of Schwartz & Blumbergs (1964) and assayed iodimetrically (Greenspan & MacKellar, 1948).

[4- ^{14}C]Oestradiol (sp. radioactivity 40 mCi/mmol) from Schwarz/Mann, Orangeburg, NY, U.S.A., was shown by chromatography and radioautography to

Abbreviation used: GSH, glutathione, reduced.

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be free of radioactive impurities. It was diluted with carrier to a specific radioactivity of 2–3 mCi/mmol and kept at 4°C in the dark as a stock solution in ethanol (1 mg/ml). [*monoethyl*-1-¹⁴C]Diethylstilboestrol (sp. radioactivity 58 mCi/mmol) was shown by t.l.c. (chloroform/ethyl acetate, 17:3, v/v) and radioautography to contain a major impurity (*cis*-diethylstilboestrol), which was removed after dilution with approx. 100-fold excess of carrier diethylstilboestrol by passage through a column (1.1 cm × 40 cm) of Florisil with hexane/diethyl ether/dichloromethane (4:3:2, by vol.) as eluant.

Silica gel pre-coated on aluminium sheets for t.l.c. was purchased from Brinkman Instruments, Rexdale, Ont., Canada, and NCS solubilizer was from Amersham Corp., Oakville, Ont., Canada. All chemicals were the purest available commercially and the solvents were redistilled. The diethyl ether used for extraction gave a negative test for peroxide.

Animals. Albino Holtzman rats (Canadian Breeding Laboratories, St. Constant, Que., Canada) with free access to food (Purina Labena) and water were used. Mature female (200–220 g) or male (250–400 g) animals were used to obtain liver fractions. For the uterine peroxidase assay, immature (22–25 days old) female rats weighing 60–90 g were given a priming dose of oestradiol (5 µg in 0.2 ml of oil) by subcutaneous injection 4 days before receiving the test compound in 0.5 ml of 0.9% (w/v) NaCl. The animals were then killed 20 h later.

Methods

Determination of ¹³C n.m.r. spectra. ¹³C chemical shifts were obtained on a Varian XL-100-12 n.m.r. spectrometer equipped with a Nicolet Technology Corp. TT-100 Fourier-transform accessory. The spectra were recorded at 25.2 MHz by using broadband proton decoupling. Chemical shifts for 0.1 M solutions in [³H]acetone were measured relative to internal tetramethylsilane with sweep widths of 5000 and 7000 Hz at a probe temperature of 32°C. The analyses were kindly carried out by Dr. G. W. Buchanan, Department of Chemistry, Carleton University, Ottawa, Canada.

Synthesis of diethylstilboestrol epoxide. Diethylstilboestrol (5 g, 18.7 mmol) in chloroform (550 ml) was mixed with an equimolar amount of purified 3-chloroperbenzoic acid in chloroform (50 ml) and the resulting red solution was kept at 22°C in the dark for 24 h before concentration *in vacuo* to approx. 75 ml. The yellow crystals that formed on cooling to 4°C were dissolved in diethyl ether, washed with a saturated solution of NaHCO₃ followed by water and evaporated to dryness under reduced pressure after drying over anhydrous Na₂SO₄. Crystallization (twice) from aqueous ethanol (1:1, v/v) gave diethylstilboestrol epoxide, yield 275 mg; m.p. 102–104°C (decomp.) (Found: C, 71.0; H, 7.3. Calc. for

C₁₈H₂₀O₃·1H₂O: C, 71.5; H, 7.3%). Analysis by t.l.c. on silica gel (chloroform/ethyl acetate, 17:3, v/v) yielded a single product (*R_F* 0.30) that gave a blue colour on spraying with diluted Folin–Ciocalteu phenol reagent followed by exposure to NH₃ vapour (Mitchell & Davies, 1954). The i.r. (KBr disc) and ¹³C n.m.r. ([²H]acetone) spectra of diethylstilboestrol epoxide and of its pinacolone were also determined (see the Results section). The same procedure on a reduced scale was used to obtain 13.6 mg of [¹⁴C]-diethylstilboestrol epoxide (sp. radioactivity 0.97 mCi/mmol) starting from [*monoethyl*-1-¹⁴C]diethylstilboestrol (250 µCi in 1.1 mg) diluted with recrystallized diethylstilboestrol (80 mg, 0.3 mmol).

Synthesis of the pinacolone of diethylstilboestrol [3,3-di-(*p*-hydroxyphenyl)-hexan-4-one]. Conc. H₂SO₄ (150 mg) was added dropwise to a solution of diethylstilboestrol epoxide (2.0 g, 7.1 mmol) in 30 ml of aqueous acetone (1:1, v/v) and shaken for 1 h at 21°C. It was allowed to stand in the dark for 24 h, extracted with diethyl ether and the organic phase washed with a saturated solution of NaHCO₃ followed by water. After drying over anhydrous Na₂SO₄, it was evaporated *in vacuo* to yield a yellow oil from which white crystals were obtained after 1 month at 4°C. They were washed with *n*-pentane and dried *in vacuo* (yield 0.42 g; m.p. 86–88°C) (Found: C, 74.1; H, 7.5. Calc. for C₁₈H₂₀O₃: C, 76.0; H, 7.0%). The i.r. (KBr disc) (at 1683 cm⁻¹) and the ¹³C n.m.r. spectra both showed an oxo carbon absorption. Analysis by t.l.c. (see above) gave a single product (*R_F* 0.21).

Preparation of liver fractions and incubation. The rats were anaesthetized with CO₂ and were killed by cervical dislocation; the liver was removed, washed with 0.25 M-sucrose and weighed. The tissue was then minced with scissors and one part by weight of tissue in one part by volume of 0.25 M-sucrose was homogenized in a Potter–Elvehjem homogenizer with a Teflon pestle (10 strokes at 700 rev./min). After dilution to a final concentration of 50 mg (wet wt.) of liver per ml of sucrose solution, the homogenate was centrifuged at 8000 *g_{av}* for 15 min in a Spinco model L ultracentrifuge (no. 50 titanium rotor). Microsomal fraction was obtained from this postmitochondrial supernatant fraction by centrifugation at 100 000 *g_{av}* for 1 h followed by two washings with 0.25 M-sucrose.

The liver preparations (1 ml) were incubated for various time periods with constant shaking under O₂ at 37°C with [¹⁴C]diethylstilboestrol epoxide (4.5 µM) or other radioactive substrates and NADPH (0.3 mM) in 0.1 M-potassium phosphate, pH 7.4, in a total volume of 4 ml. In some experiments, tissue concentration and other variables were examined. Anaerobic incubations were carried out in evacuated Thunberg tubes after bubbling N₂ through both the tissue preparation and the substrate solution in the

side arm before mixing them. In the experiments with CO , the gas was bubbled through the incubation mixture at times 0, 10 and 20 min during incubation in the dark.

The reaction was stopped by extracting the incubation mixture three times with equal volumes of diethyl ether saturated with phosphate buffer and the combined organic phase was dried over anhydrous Na_2SO_4 . The radioactivity in each fraction was then determined by liquid-scintillation counting as described previously (Jellinck & McNabb, 1977).

Examination of diethyl ether-soluble fraction. A portion of the diethyl ether extract was examined by t.l.c. (chloroform/ethyl acetate, 17:3, v/v) and radioactive areas were located by radioautography. Two products (R_F 0–0.06 and 0.39) in addition to the pinacolone were obtained from diethylstilboestrol epoxide. The more polar metabolite(s) was not identified, but the other product, which in male rats accounted for about 20% of the total radioactivity in the diethyl ether fraction, had the same chromatographic properties as 4'-hydroxypropio-phenone and co-crystallized with this compound when analysed by reverse isotope dilution. The ^{14}C -labelled compound (18000 c.p.m.) was eluted from the t.l.c. silica gel with absolute ethanol and mixed with non-radioactive 4'-hydroxypropio-phenone (50 mg) followed by three crystallizations from aqueous ethanol without decrease in specific radioactivity (335, 334 and 343 c.p.m./mg). No ^{14}C radioactivity was retained when 4'-hydroxypropio-phenone was mixed with [^{14}C]diethylstilboestrol epoxide and recrystallized.

Examination of aqueous fraction. Protein in a portion (2 ml) of the aqueous fraction from which any residual diethyl ether had been removed with N_2 was precipitated by the addition of 1 M-HCl (0.5 ml) and 1.5 M-trichloroacetic acid (1.5 ml). After 1 h, the suspension was centrifuged at 1000g for 5 min and the precipitate was washed once with trichloroacetic acid (1 ml) and twice with ethanol (2 ml). It was dissolved in NCS solubilizer and 0.1 ml portions were counted for radioactivity.

The results are the means for at least two experiments

Assay of epoxide hydratase activity. Epoxide hydratase activity was determined by the method of Oesch (1974) by using [^{14}C]diethylstilboestrol epoxide as substrate. The enzyme was partially purified on a DEAE-cellulose column after extraction from rat liver microsomal fraction with 1% (w/v) Lubrol as described by Knowles & Burchell (1977). The epoxide (5 μg) in acetonitrile (0.1 ml) was incubated for various time periods at 37°C with 0.2 ml of the enzyme preparation and 0.7 ml of 0.1 M-Tris/HCl, pH 9.0, followed by extraction twice with ethyl acetate (3 ml). Analyses by t.l.c. on silica gel (benzene/ethyl acetate/chloroform, 1:1:1, by vol.) revealed

only one radioactive product (diethylstilboestrol pinacolone, R_F 0.52) after radioautography. It was scraped off the plate directly into a vial and its ^{14}C radioactivity was determined by scintillation counting in Omnifluor (New England Nuclear).

Epoxide hydratase activity was also estimated qualitatively by using styrene oxide as substrate (Oesch *et al.*, 1971). Portions (0.01–0.02 ml) of the enzyme preparation diluted to 0.1 ml with 0.5 M-Tris/HCl, pH 9.0, were incubated for 10 min at 37°C with styrene oxide (0.8 μmol) in acetonitrile (0.2 ml) and water (0.1 ml) followed by extraction of the product (styrene glycol) from the aqueous phase into ethyl acetate (2 ml). It was then examined by t.l.c. on silica gel (60 F_{254} plate; Brinkman Instruments) with ethyl acetate/chloroform (1:1, v/v) and the location of the only product, styrene glycol (R_F 0.24), was determined by its u.v. fluorescence and by exposure to I_2 vapour.

Other procedures. The peroxidase activity of uterine extracts was measured either by their ability to convert [4- ^{14}C]oestradiol into water-soluble products as described previously (Lyttle & Jellinck, 1972) or more directly by using guaiacol as substrate (Himmelhoch *et al.*, 1969). The reaction mixture contained guaiacol (13 mM) and H_2O_2 (0.33 mM) in 0.01 M-sodium phosphate buffer (pH 7.0) and 0.05–0.2 ml of the peroxidase extract, depending on its activity. The linear increase A_{470} resulting from the oxidation of guaiacol was followed in a Unicam SP. 800 recording spectrophotometer. These two biochemical methods, which show a good correlation with uterotrophic activity, have been used previously to determine the oestrogenic activity of a number of compounds (Jellinck & Newcombe, 1977). Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard. A sample of diethylstilboestrol epoxide was kindly tested by Dr. R. G. Harvey, Ben May Laboratory for Cancer Research, University of Chicago, IL, U.S.A., for its ability to inhibit bacteriophage ϕX174 DNA viral replication by the procedure of Hsu *et al.* (1977).

Results

Characterization of diethylstilboestrol epoxide and diethylstilboestrol pinacolone

The ^{13}C n.m.r. spectral characteristics of the carbon atoms of diethylstilboestrol epoxide and diethylstilboestrol pinacolone ($\pm$ S.E.M.) are given in Table 1. The spectra were consistent with a *trans*-epoxide and a ketone respectively. The experimental δ (p.p.m.) of 72.8 was close to the calculated value (δ 72.9) for *trans*-diethylstilboestrol epoxide obtained by adding the shielding values (Paulson *et al.*, 1975) to the observed epoxide carbon absorption of *trans*-stilbene oxide. The corresponding δ value for *cis*-

Table 1. ^{13}C chemical shifts for diethylstilboestrol epoxide and diethylstilboestrol pinacolone
Results are means ± 0.1 s.e.m.

Diethylstilboestrol epoxide

Diethylstilboestrol pinacolone

δ (p.p.m.) from tetramethylsilane

Position	Diethylstilboestrol epoxide	Diethylstilboestrol pinacolone
1	131.3	133.5
2	128.9	131.2
3	115.6	115.7
4	157.2	156.9
72.8
211.4
28.4	32.5, 30.9
9.3	9.9, 9.5
65.8

diethylstilboestrol epoxide would be 70.2. The i.r. spectrum showed absorption at 1240cm^{-1} (epoxide), 910cm^{-1} (*trans*-epoxide) and a broad band at 3240cm^{-1} indicating the presence of water.

Conversion of [^{14}C]diethylstilboestrol epoxide to water-soluble products by rat liver fractions

The effect of cofactors on the conversion of [^{14}C]diethylstilboestrol epoxide and oestradiol to water-soluble products is given in Table 2. The concentrations of protein in the 8000g supernatant and in the microsomal pellet derived from 1g (wet wt.) of liver were 94mg and 34mg respectively.

NADPH or an NADPH-generating system was required to obtain maximum yield of water-soluble metabolites of these compounds by the 8000g supernatant fraction of rat liver and this coenzyme was more effective than NADH. The yield was lower with the resuspended microsomal pellet, but could be increased by the addition of GSH when NADPH was present. The lack of such effect by GSH with the 8000g supernatant fraction is due, presumably, to the high endogenous concentration (6–8mM) of this tripeptide in liver cytosol known to contain glutathione *S*-transferases.

A marked sex-difference in the conversion into water-soluble products was observed with both oestradiol and diethylstilboestrol epoxide, but only

Table 2. Cofactor requirement for the conversion of ^{14}C -labelled diethylstilboestrol epoxide and oestradiol to water-soluble products by rat liver fractions from male and female rats

Subcellular fractions derived from rat liver (50mg wet wt.) were incubated for 30min at 37°C with radioactive substrates ($4.5\mu\text{M}$) and NADH (0.3mM) or NADPH (0.3mM) or GSH (1mM) in 0.1M-potassium phosphate, pH 7.4, followed by extraction with diethyl ether as described in the text. Results are means for three experiments (range given) or the means for four to six experiments ($\pm$ s.e.m.).

Cofactor	Proportion of added ^{14}C remaining in aqueous medium after extraction with diethyl ether (%)			
	[^{14}C]Diethylstilboestrol epoxide		[^{14}C]Oestradiol	
	Male	Female	Male	Female
8000g supernatant	8.7 (7.7–10.6)	10.1 (6.6–12.0)	3.7 (2.6–4.9)	2.8 (1.9–3.7)
+NADH	15.2 $\pm$ 0.9	13.7 (11.6–15.6)	13.7 (11.6–15.5)	8.2 (8.1–8.3)
+NADPH	51.1 $\pm$ 1.7	30.7 $\pm$ 4.1	59.1 (56.4–61.8)	32.0 (29.9–34.1)
100000g pellet	9.5 $\pm$ 1.8	8.6 (6.4–10.8)	2.9 (2.5–3.2)	12.0 (10.5–13.5)
+NADH	13.9 $\pm$ 2.5	12.5 (10.8–14.8)	10.2 (8.2–13.5)	6.5 (5.3–7.6)
+NADPH	26.2 $\pm$ 1.9	20.7 $\pm$ 3.9	19.0 $\pm$ 1.8	10.4 (8.8–11.9)
+GSH (1mM)	6.4 (6.2–6.5)	8.4 (7.6–9.1)	4.1 (3.1–5.1)	3.1 (2.9–3.1)
+NADPH+GSH	58.0 (56.2–59.8)	34.8 (30.9–38.7)	58.6 $\pm$ 3.2	21.6 (20.9–22.3)
100000g supernatant	7.0 $\pm$ 0.7	6.2 (5.2–7.4)	2.7 (2.5–2.9)	2.6 (2.5–2.7)
+NADH	5.0 (4.8–5.1)	6.9 (5.3–8.4)	3.1 (2.5–3.6)	2.6 (2.3–2.8)
+NADPH	6.6 $\pm$ 0.7	7.1 (4.2–10.0)	4.2 (2.3–5.4)	3.0 (2.2–3.8)
+GSH	11.2 $\pm$ 1.4	9.0 (4.3–11.6)	3.7 (2.4–5.4)	3.3 (3.1–3.4)
+NADPH+GSH	11.5 $\pm$ 1.6	10.7 (6.5–13.5)	4.4 (2.6–5.4)	3.5 (2.6–4.3)

the latter compound gave rise to any significant amounts of these metabolites when incubated with the 100000g supernatant (cytosol) fraction of liver. This suggests that some conjugation with glutathione could occur with diethylstilboestrol epoxide without microsomal activation.

The rate of conversion of [¹⁴C]diethylstilboestrol epoxide to water-soluble derivatives by the 8000g and 100000g supernatant fractions of male rat liver in the presence or absence of GSH or NADPH is shown in Fig. 1. It was found to be linear for 15–20 min and only a small fraction of the products was bound to protein. The rate of formation of the water-soluble metabolites was higher in male than in female rat liver microsomal fraction (Table 2 and Fig. 2).

Conversion of [¹⁴C]diethylstilboestrol epoxide to diethyl ether-soluble metabolites by rat liver microsomal fraction

4'-Hydroxypropiophenone was one of the major diethyl ether-soluble metabolites of diethylstilboestrol epoxide formed during incubation of diethylstilboestrol epoxide with male rat liver microsomal fraction under aerobic conditions in the presence of NADPH (Fig. 2a). The other main product, diethylstilboestrol pinacolone, was shown to arise non-enzymically, because it was still formed readily on incubation with boiled microsomal fraction or in the absence of tissue. A preparation of epoxide

Fig. 1. Effect of GSH on the conversion of [¹⁴C]diethylstilboestrol epoxide to water-soluble products by male rat liver fractions

The 8000g supernatant was incubated for various time periods with [¹⁴C]diethylstilboestrol epoxide (4.5μM), NADPH (0.3mM) and GSH (10mM) (●) or in the absence of GSH (○) or NADPH (Δ) before extraction with diethyl ether. The cytosol (100000g supernatant) was incubated with (■) or without (□) GSH, with NADPH omitted. Values are corrected for ¹⁴C radioactivity (3–6%) not removed from liver fractions at time zero.

Table 3. Effect of inhibitors on the metabolism of diethylstilboestrol epoxide by rat liver microsomal fraction
Male rat liver microsomal fraction was incubated at 37°C for 30 min with [¹⁴C]diethylstilboestrol epoxide (4.5 μM) and NADPH (0.3 mM) in the presence and absence of inhibitor. Other conditions are described in the text. Values are means for three experiments (range given) or means for four to six experiments (±s.e.m.)

Inhibitor or other conditions	Concentration (mM)	Proportion of total ¹⁴ C radioactivity in water-soluble fraction (%)	Proportion of diethyl ether-soluble ¹⁴ C radioactivity in diethylstilboestrol epoxide and products (%)			
			Polar compound(s)	Diethylstilboestrol pinacolone	Diethylstilboestrol epoxide	4'-Hydroxypropiophenone
Boiled microsomal fraction	—	23.4 ± 1.5	3.2 ± 0.7	43.4 ± 6.9	31.8 ± 3.0	21.7 ± 4.0
CO	—	13.4 ± 1.8	0.7 ± 0.2	50.6 ± 2.7	43.5 ± 2.6	5.3 ± 0.7
Anaerobic conditions	—	18.4 ± 1.0	2.9 ± 0.7	49.1 ± 4.2	37.8 ± 2.5	10.2 ± 1.9
p-Hydroxymercuribenzoate	—	13.1	2.3	56.9	33.3	7.5
NaCN	0.1	13.4 ± 1.5	2.1 ± 0.5	48.4 ± 6.1	42.4 ± 5.5	7.2 ± 1.3
KCN	1	19.2 (13.2–25.2)	11.1 (5.5–16.6)	32.3 (28.2–36.3)	30.9 (27.4–34.4)	25.8 (19.6–31.9)
Ascorbate	1	24.8 (23.9–25.6)	10.9 (9.2–12.5)	34.5 (25.9–43.0)	27.5 (24.5–30.4)	27.2 (23.3–31.1)
Compound SKF-525A	1.0	12.4 ± 0.8	28.8 ± 5.1	32.8 ± 4.6	22.0 ± 2.4	16.5 ± 1.3
Thioglycollate	0.1	17.8 ± 1.9	5.7 ± 1.3	43.9 ± 6.2	32.4 ± 3.7	15.3 ± 1.4
	1	18.8 ± 1.8	3.9 ± 0.6	39.9 ± 6.5	47.0 ± 5.1	8.7 ± 1.9
	10	4.5 (4.1–4.9)	3.0 (1.2–4.7)	36.0 (28.0–44.0)	56.4 (53.2–63.6)	2.7 (1.6–3.7)
GSH	1	29.8 ± 3.6	3.7 ± 0.6	41.9 ± 5.6	33.3 ± 3.4	24.8 ± 4.3
	10	39.1 ± 5.4	4.8 ± 1.4	37.2 ± 5.3	32.5 ± 4.4	25.6 ± 3.7
	50	61.2 (58.2–64.2)	21.9 (16.2–27.5)	17.6 (16.9–18.2)	22.6 (18.4–26.7)	38.0 (28.8–47.2)
	1	58.4 ± 4.4	8.5 ± 2.6	27.8 ± 4.9	29.9 ± 3.0	33.8 ± 1.7

Fig. 2. Rate of conversion of [^{14}C]diethylstilboestrol epoxide to its metabolites by male and female rat liver microsomal fractions

The diethyl ether-soluble products formed after incubation of the microsomes (100000g pellet) from male (a) and female (b) rat liver with [^{14}C]diethylstilboestrol epoxide under the conditions described in Fig. 1 were separated by t.l.c. The radioactive areas located by radioautography were then eluted with ethanol and ^{14}C radioactivity was determined. Results

hydratase, which possibly could give rise to the diol intermediate in a pinacol-pinacolone rearrangement, did not enhance the reaction. Thus, incubating [^{14}C]diethylstilboestrol epoxide ($4.5\mu\text{M}$) at 37°C in 0.1 M-potassium phosphate, pH 7.4, for various time periods in the presence of a purified epoxide hydratase preparation, followed by extraction with ethyl acetate, t.l.c. and radioautography, did not result in an increase in the rate of formation of diethylstilboestrol pinacolone over that of the control without the enzyme. A small amount of diethylstilboestrol epoxide was converted into polar metabolites, which may be the precursors of the water-soluble products.

The rate of formation of 4'-hydroxypropio-phenone by liver microsomal fraction from female rats was lower than from male rats and more diethylstilboestrol pinacolone was found (Fig. 2b).

Effect of inhibitors

The effect of various metabolic inhibitors and other substances on the conversion of [^{14}C]diethylstilboestrol epoxide to aqueous and diethyl ether-soluble metabolites is shown in Table 3. No inhibition was observed with either cyanide or azide, but the yield of water-soluble products and also that of 4'-hydroxypropio-phenone was decreased considerably under anaerobic conditions, or if the microsomal fraction was boiled. The thiol reagent *p*-hydroxy-mercuribenzoate, CO and compound SKF-525A, which interacts with cytochrome *P*-450, also acted as inhibitors, but thioglycollate and GSH increased the yield of water-soluble products as well as that of the polar diethyl ether-soluble metabolites. The formation of this last group of compounds was also enhanced by ascorbate. None of the inhibitors had any marked effect on the conversion of diethylstilboestrol epoxide to its pinacolone.

Biological activity of diethylstilboestrol epoxide and related compounds

The ability of diethylstilboestrol epoxide and related compounds to evoke an oestrogenic response as measured by the increase in uterine weight or the induction of peroxidase in immature rat uteri is shown in Table 4. Diethylstilboestrol epoxide was found to have about one-tenth the oestrogenic activity of diethylstilboestrol or hexoestrol, whereas the pinacolone of diethylstilboestrol was devoid of any activity even at a dose of $50\mu\text{g}/\text{rat}$. The epoxide was inactive as mutagen. It did not inhibit bacteriophage ϕX174 DNA viral replication at twice the dose at

for water-soluble products (▲), 4'-hydroxypropio-phenone (○), diethylstilboestrol pinacolone (Δ), diethylstilboestrol epoxide (●) and polar products (■) are shown. Values are means for two experiments.

Table 4. *Oestrogenic activity of diethylstilboestrol epoxide and related compounds*

Oestrogen-primed immature rats were given a single subcutaneous injection of the test compound in oil (0.2ml) and killed 20h later. Oestrogenic activity was determined by the increase in uterine weight and peroxidase induction was measured by the conversion of [^{14}C]oestradiol into water-soluble products after incubation for 30min with uterine extracts and by the oxidation of guaiacol. Values are means for two experiments with pooled uteri from five rats.

Compound	Dose (μg)	Uterine wet wt. (mg)	Proportion of added radio-activity remaining in aqueous medium after extraction with diethyl ether (%)	Rate of oxidation of guaiacol ($\Delta A_{470}/\text{min}$ per g of tissue)
Control	—	39.0 ± 8.5	12.3	0
Hexoestrol	1	77.6 ± 9.4	39.8	5.6
	5	82.8 ± 11.4	40.4	6.4
Diethylstilboestrol	1	73.5 ± 7.1	42.7	3.2
	5	70.3 ± 7.5	45.2	4.6
Diethylstilboestrol epoxide	5	43.3 ± 9.7	22.1	1.0
	10	75.8 ± 12.7	41.1	4.0
	25	77.8 ± 12.4	46.8	5.4
	50	79.7 ± 11.2	50.9	6.6
Diethylstilboestrol pinacolone	25	41.7 ± 4.2	11.6	0
	50	45.6 ± 6.3	9.6	0
4'-Hydroxypropiophenone	100	42.4 ± 8.7	12.4	0

which benzo[a]pyrene anti-diol epoxide produces >99% inhibition (R. G. Harvey, personal communication).

Discussion

Metzler & McLachlan (1978) reported the synthesis of diethylstilboestrol epoxide from diethylstilboestrol and perbenzoic acid by a modification of the method of Wesseley *et al.* (1940) and identified their product on the basis of a characteristic mass spectrum and ease of rearrangement to the pinacolone [3,3-di-(*p*-hydroxyphenyl)hexan-4-one]. In our studies, diethylstilboestrol epoxide was synthesized by using 4-chloroperbenzoic acid and the ^{13}C n.m.r. chemical shifts indicated a *trans* configuration of the oxiran ring. Other characteristics of the epoxide including m.p., elemental analysis, i.r. and behaviour on t.l.c. were also determined.

Liver was chosen to study the metabolic fate of diethylstilboestrol epoxide because this organ is readily fractionated into subcellular components containing mono-oxygenase, epoxide hydratase and glutathione *S*-transferase, which are primarily responsible for the further metabolism and detoxification of epoxides (Jerina & Daly, 1974; Jakoby & Keen, 1977).

Diethylstilboestrol epoxide, like oestradiol, required O_2 and NADPH for maximum conversion into water-soluble products by rat liver microsomal fractions and a marked sex-difference in metabolism was also observed. However, unlike oestradiol, diethylstilboestrol epoxide showed some reaction with GSH without microsomal activation when incubated with the soluble (cytosol) fraction of rat

liver known to contain glutathione *S*-transferases (Hayakawa *et al.*, 1975; Kaplowitz *et al.*, 1976; Smith *et al.*, 1977). The postmitochondrial supernatant gave the same yield of water-soluble products as the cytosol when NADPH was omitted, which suggests that the difference in yield by this microsomal fraction in the presence of coenzyme is due to metabolites other than the GSH conjugate. The rate of non-enzymic conjugation of the epoxide with GSH was very low.

It was assumed that epoxide hydratase present in the liver preparation would be involved in the formation of diethylstilboestrol pinacolone. The activity of this enzyme could then be followed by measuring the rate of appearance of the pinacolone after the rapid rearrangement of any diethylstilboestrol diol produced. However, in these experiments, the diol intermediate was not detected and diethylstilboestrol pinacolone was shown to be formed by a non-enzymic process. It has been reported previously (Watabe *et al.*, 1971) that *cis*-stilbene epoxide is 800 times more active as a substrate for epoxide hydratase than the *trans*-isomer, and Oesch (1972) has shown that increased steric bulk around the oxiran ring such as in *trans*-disubstituted or -tetrasubstituted epoxides prevented its approach to the active site of the enzyme. The 'bay-region' epoxides of certain polycyclic aromatic hydrocarbons, which are known to be potent carcinogens, have also been found to be refractory to the action of epoxide hydratase (Wood *et al.*, 1977). The high rate of non-enzymic formation of the inactive pinacolone would tend to compensate for the lack of the partially purified enzyme to catalyse the hydration of *trans*-diethylstilboestrol epoxide. The enzymic hydration of an arene oxide,

Fig. 3. Proposed scheme for the metabolism of diethylstilboestrol epoxide by rat liver microsomal fraction
Reaction 2 is non-enzymic.

such as toluene 3,4-epoxide, could not be demonstrated in microsomal preparations because of its rapid isomerization to *p*-cresol (Daly *et al.*, 1972).

Analysis of the diethyl ether-soluble products formed from diethylstilboestrol epoxide revealed the pinacolone, 4'-hydroxypropiofenone and a third more polar unidentified metabolite(s) near the origin on t.l.c., which increased in yield when ascorbate was added to the incubation mixture (reaction 1, Fig. 3). This last compound(s) could possibly be the ring-hydroxylated derivative(s) that ultimately combines with GSH and protein to form water-soluble products.

The presence of 4'-hydroxypropiofenone in high yield is in accord with results of Metzler (1976), who found it as a urinary metabolite of diethylstilboestrol in male rats and also when diethylstilboestrol epoxide was injected into mice (Metzler & McLachlan, 1978). However, our results with liver microsomal fraction show that 4'-hydroxypropiofenone need not be formed from diethylstilboestrol epoxide *in vivo* by intestinal micro-organisms during the enterohepatic circulation of diethylstilboestrol (Hanahan *et al.*, 1953) as proposed by Metzler (1976). Rather, they suggest that the epoxide is a true intermediate formed by the liver during the metabolism of diethylstilboestrol. They also demonstrate that a major reaction in the metabolism of diethylstilboestrol epoxide by liver microsomal fraction involves its cleavage to 4'-hydroxypropiofenone. The subcellular location, the absolute requirement for NADPH and O₂, the sex-difference and the inhibitory effect of CO and compound SKF-525A together with the lack of inhibition by KCN point to a mono-oxygenase-catalysed reaction involving cytochrome *P*-450 (Mannering, 1971) with

cleavage of the C-C bond of the oxiran ring to give two molecules of 4'-hydroxypropiofenone (reaction 3, Fig. 3). This type of reaction, as far as is known, has not been described previously in a mammalian system, but further work will be required to substantiate its generality and exact mechanism. However, it must occur readily because even incubating diethylstilboestrol epoxide with boiled liver microsomal fraction gave rise to some 4'-hydroxypropiofenone.

The results of these studies support the proposal by Metzler (1975) that diethylstilboestrol epoxide may be formed in the liver as a reactive intermediate of diethylstilboestrol and they are summarized in Fig. 3. They also confirm the findings (Korach *et al.*, 1978) that diethylstilboestrol epoxide retains some of the original oestrogenic activity of diethylstilboestrol. The ability of diethylstilboestrol epoxide to induce peroxidase in the immature rat uterus provides some evidence that this molecule may be transported into the nucleus to react with the transcriptional apparatus of the cell. It may therefore also be able to interact with oestrogen receptors in the liver, which have been shown to be similar to those found in the uterus (Beers & Rosner, 1977; Eisenfeld, 1977).

The relatively poor reactivity of diethylstilboestrol epoxide with a nucleophilic acceptor such as GSH, or other macromolecules in the absence of NADPH, and its inability to inhibit bacteriophage ϕ X174 DNA viral replication, a test for mutagenic activity, cast doubt on the role of this intermediate in the carcinogenic action of diethylstilboestrol. These harmful effects are more likely to be mediated by the arene oxide of diethylstilboestrol or by some other reactive derivative such as the *p*-quinone or *p*-semiquinone

radical. However, the possibility that diethylstilboestrol is also converted into *cis*-diethylstilboestrol epoxide *in vivo* and that this product may differ from the *trans*-isomer, both in its attack by epoxide hydratase and its mutagenic and oestrogenic properties, cannot be excluded.

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