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COMPARISON OF THE DISPOSITION OF DIETHYLSTILBESTROL AND ESTRADIOL IN THE FETAL RAT

CORRELATION WITH TERATOGENIC POTENCY

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Abstract—The dispositions of radiolabeled diethylstilbestrol (DES) and estradiol (E_2) in the fetal rat were compared to determine whether kinetic differences accounted for their differences in teratogenic potency. ^{14}C (from DES) was concentrated in fetal tissues relative to plasma, whereas ^{3}H (from E_2) was largely retained protein-bound in fetal plasma. Both compounds were rapidly metabolized in the fetus (and mother) to less or non-estrogenic products. Fetal levels of E_2 declined faster than those of DES (E_1 was the primary circulating estrogen within 1–3 hr of E_2 injection) so that exposure to unchanged DES was of longer duration than to E_2. The unchanged compounds were retained longer and at higher concentrations in the target genital tissue compared to other tissues. Although these differences were consistent with the potencies, the concentration of the unchanged estrogen in fetal genital tract was lower after a teratogenic dose of DES than after a threshold teratogenic dose of E_2. However, the ^{3}H in fetal plasma and genital tract cytosol at 1 hr after injection of [^{3}H]E_2 at 2 ng or 10 μg/fetus was found to be highly protein-bound. DES competed poorly for these binding sites. It is suggested that the concentration of E_2 which is "free" in the cell (as DES is), rather than the total content, correlates with its teratogenicity. Thus, in the rat, rapid metabolism and extensive protein-binding, both extra- and intracellularly, reduce the teratogenicity of the natural estrogen compared to the synthetic estrogen.

The synthetic estrogen, diethylstilbestrol (DES), is a potent urogenital teratogen in humans [1] and in laboratory animals [2–5]. In contrast, the natural estrogen, estradiol (E_2), induces urogenital malformations (in the rat) only at a maternal-toxic dose [6]. Does this difference indicate that fetal sensitivity to DES is due to some mechanism of interaction unique to DES? Or are there differences in distribution of the active compounds to the fetus, perhaps due to the high-affinity binding of the natural estrogen, but not DES, to the alpha-fetoprotein (AFP) in fetal rat and mouse plasma [7]?

We have shown previously that both estrogens induce the same urogenital malformations when injected directly into day 19 fetal rats [6]. These results suggested that DES and E_2 likely act via the same mechanism, perhaps involving the estrogen receptor, and that they act directly in the fetus (without maternal mediation). However, DES was about an order of magnitude more potent in the fetus, whereas DES and E_2 have comparable uterotrophic activities and receptor affinities in the adult [8]. In the present study, the distribution and metabolism of radiolabeled DES and E_2 were compared to determine whether pharmacokinetic differences accounted for the differences in teratogenic potency. Both direct fetal injections and maternal s.c. injections were used for comparison with the teratology studies [6]. It was not a goal of this study to identify all the metabolites of DES, since this has been documented by other investigators (see reviews by Metzler [9, 10]). Of primary concern was monitoring the disappearance of the parent compounds, since they are the most potent estrogens. Evaluation of metabolic conversion of E_2 in the fetal rat was of particular interest, since published studies have reported placental transfer of radiolabel without identifying possible metabolites [11].

METHODS

Animals. Virgin female Wistar rats (Crl:(WI)Br) (200–250 g) were mated by housing them overnight with males of the same strain. The morning when vaginal plugs were present was designated day 0 of pregnancy. Pregnant females were housed up to three per plastic cage with Pine-Dri shavings and with a 12-hr light/dark cycle, controlled temperature (20–22°), and Purina rat chow and tap water *ad lib.* Experiments were performed on day 19 or 20 of gestation, with pentobarbital anesthesia (40 mg/kg, i.p.).

Maternal injections. For experiments of up to 6 hr duration, the dam was anesthetized, a tracheotomy was performed, and a carotid catheter (PE-50 polyethylene tubing) was inserted for sampling of blood. Labeled estrogens were injected subcutaneously in the nape of the neck. After each 0.3-ml blood sample, volume was replaced with heparized saline. For 24-hr exposures, the dam was not anesthetized; blood was sampled from the tail.

Fetal injections. The uterine horns were exposed

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by laparotomy, and fetuses were injected i.p. through the uterine wall. The uterus was gently replaced into the abdomen, or was left exposed and moistened with saline-soaked gauze. For 24-hr exposures, surgery was performed as previously described [6], so that dams awoke within about 20 min after surgery.

Injection solutions. [Monoethyl-1-^{14}C]-DES (Amersham, 56 mCi/mmole), [4-^{14}C]-estradiol (Amersham, 55 mCi/mmole) and [2,4,6,7-^{3}H]-E_2 (New England Nuclear, 90–116 mCi/mmole), and unlabeled estrogens (Sigma) were checked by high pressure liquid chromatography (HPLC) for >98% purity. Labeled and unlabeled compounds were combined as needed for injections, using a corn oil vehicle. Injections of 0.5 μCi in 5 μl/fetus and 20 μCi/dam in 1 ml/kg, were used at doses indicated in the figures.

Tissue analyses. At the end of the exposure period, maternal blood and tissues were sampled (liver, kidney, adrenal, heart, lung, GI, injection site). The uterus was removed, and fetuses, placentae, and membranes were dissected. Fetuses were decapitated for blood collection from neck vessels. The fetal plasma and tissue samples (liver, kidney, adrenal, heart, lung, GI, reproductive tract) were pooled from each litter, frozen in liquid nitrogen, and stored at −15°.

Total radioactivity was measured in duplicate plasma and tissue samples after digestion in NCS (Amersham), using a TM Analytic 6881 scintillation spectrometer with dpm correction. Liquiscint (National Diagnostics) and 100 μl of acetic acid (to reduce chemiluminescence) were added to each sample.

Plasma and tissue radioactivities were analyzed further by HPLC. Homogenates in methanol–ethanol (1:1, MeOH:EtOH) were centrifuged for 15 min at 12,000 *g* (Sorvall RC-5B), and the supernatant fraction was removed for evaporation under nitrogen. Supernatant fractions were pooled from at least three re-extractions with MeOH:EtOH, and the final pellets were dissolved in NCS for counting radioactivity.

After evaporation of the supernatant fractions to dryness, 50–100 μl of MeOH containing unlabeled standards was added to each sample tube, for analysis on a Varian 5060 HPLC. A pre-column was packed with Microparticulate MCH monomeric 10 μm (Varian), and the main column (250 × 4 mm, Hibar EC, Merck) contained 10 μm RP-18 Lichrosorb. E_2 and metabolites were eluted with 50% acetonitrile in water; DES and metabolites were eluted with MeOH/water (12-min gradient 25%–65% MeOH, then isocratic 65%); they were monitored using a Varichrome UV detector at 217 and 254 nm respectively. Unlabeled compounds eluted at the indicated times (in min): estrone (E_1, 12.5), E_2 (11.0), estriol (4.8), and E_1-sulfate (1.8); or DES (*trans*-isomer, 19.2; *cis*-isomer, 24.2), DES-glucuronide (5.5), and *p*-hydroxypropiophenone (10.5). All fractions (0.2 to 1-min intervals) were counted. Recovery of ^{3}H and ^{14}C from the column was 88 ± 5 and 87 ± 5% respectively. The limit of detection for HPLC analyses, at the specific activities used for fetal injections, was about 2 pmoles for [^{14}C]-DES, 5 pmoles for [^{3}H]E_2 (10 μg/fetus), and 50 pmoles for [^{3}H]E_2 (100 μg/fetus). For maternal injections, it was about 15 pmoles for both compounds.

Assay of estrogen binding to total protein in fetal tissues. To further characterize the measured radioactivity in the fetus, cytosols and plasma were assayed [12] with hydroxylapatite (HAP) to determine to what extent radioactivity was protein-bound. Fetuses were injected with [^{3}H]-E_2 at 1 hr before sampling of tissues and plasmas. Cytosols were prepared from homogenates of fetal reproductive tracts or liver (40 mM Tris, 1.5 mM EDTA, 50 mM KCl, pH 7.2) by centrifugation at 100,000 *g* for 60 min (Beckman L5-65 Ultracentrifuge). The pellet (unwashed) contained 5–10% of total tissue radioactivity. Aliquots of cytosol or plasma, containing approximately 40–200 μg protein, were incubated on ice for 2 hr with or without unlabeled E_2 or DES. (The proportion of protein-bound ^{3}H was found to be comparable whether incubated with unlabeled ligands for 1–4 hr or overnight.) A slurry of HAP (Bio-rad, DNA grade, 40% HAP by volume) in Tris buffer was added to each tube. After incubation on ice for 45 min, HAP was pelleted by a 5-min centrifugation (1500 *g*), and the supernatant fraction was removed. This pellet was washed four times, and the final pellet was extracted twice with 1 ml of EtOH. Radioactivity was measured in washes and EtOH extracts; the percentage of total radioactivity which was in the EtOH represented protein-bound material. In control tubes with no protein, 99.4 to 99.8% of the added [^{3}H]E_2 was removed by the four buffer washes.

RESULTS

Maternal injections. The time course of total radioactivity in maternal plasma during 3 hr following s.c. injection is shown in Fig. 1. Activity associated with

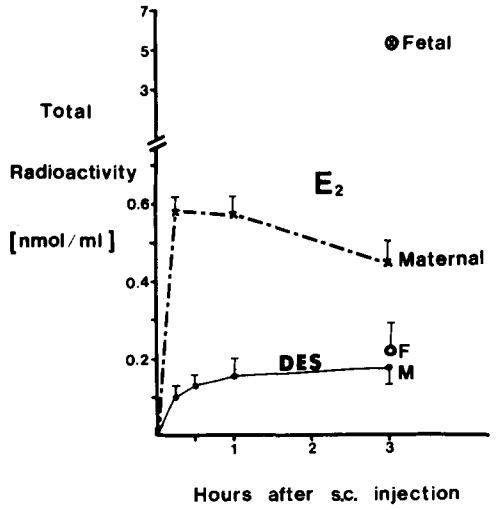

Fig. 1. Concentration of total radioactivity in maternal (M) and fetal (F) rat plasma during 3 hr following s.c. injection of [^{14}C]DES (●——●) or [^{14}C]E_2 (×–·–×), 2.5 mg/kg. Each point is the mean ± S.D. for three to four mothers.

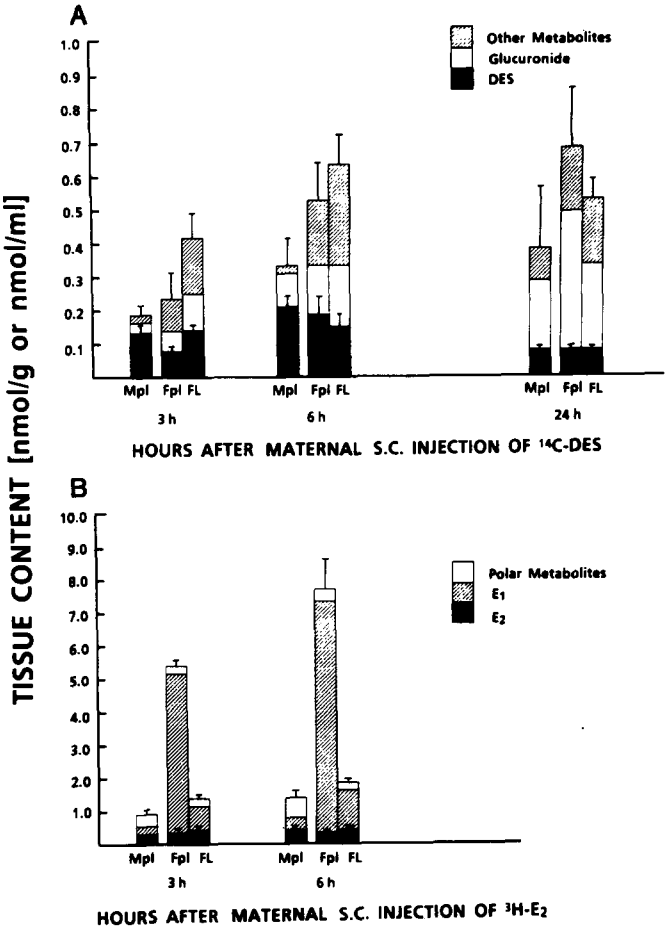

Fig. 2. Distribution of metabolites of DES (A) and E_2 (B) in maternal plasma (Mpl), fetal plasma (Fpl), and fetal liver (FL) following s.c. injection of [^{14}C]DES or [^{14}C]E_2, 2.5 mg/kg. Bars are mean ± S.D. for three to four litters. Data for E_2 at 24 hr have been omitted because of the problem of release of ^3H_2O, as discussed in the text. "Other metabolites" and "polar metabolites" refer to the pooled activity in peaks not co-eluting with the other indicated compounds.

E_2 initially reached levels about five times that of DES; by 24 hr (data not shown), they were comparable. At 3 hr, the fetal:maternal ratio of activity was about 1 for DES, compared to 10 for E_2-associated activity. DES was taken up to a greater extent than was E_2 by both fetal (Fig. 2) and maternal tissues. Although only fetal liver concentrations are included in Fig. 2, ^{3}H (from E_2) was uniformly distributed among the other fetal tissues, except the fetal reproductive tract, where levels were about twice those in liver at 3–6 hr; ^{3}H remained largely in the fetal plasma (tissue:fetal plasma ratios were 0.2–0.3). Concentrations of ^{14}C (from DES) in fetal liver, GI tract (+ contents), and adrenal exceeded the fetal plasma level; tissue:plasma ratios for the other fetal tissues were 0.6–0.9.

Both DES and E_2 were metabolized extensively, and fetal plasma contained high proportions of metabolites. Even so, the concentration of unchanged DES in fetal plasma was significantly higher ($P < 0.05$) at 6 hr than at 3 hr (Fig. 2A). E_2 comprised a small percentage of total ^{3}H even by 3 hr (Fig. 2B); the predominant component (90%) was estrone (E_1). Thus, the fetal exposure to DES was of longer duration than to E_2.

The data for E_2 in Fig. 1 are from three experiments using [^{14}C]E_2. When [^{3}H]E_2 was administered to dams, ^{3}H levels in maternal plasma continued to increase at least to 24 hr. However, by 24 hr, 90 and 65% of the ^{3}H in maternal and fetal plasma, respectively, was "volatile" (lost during evaporation of MeOH:EtOH extract). The volatile ^{3}H was presumed to be ^3H_2O since (1) it was trapped by condensing the evaporate, (2) its concentration equilibrated with total body water, e.g. across the placenta, and (3) the nonvolatile component in maternal and fetal plasma and fetal liver was of equal concentration to ^{14}C when [^{14}C]E_2 was administered. Release of ^{3}H likely occurred when [^{3}H]E_2 was metabolized at positions 2 or 4 (these hydroxylated and methoxylated products have been reported in rats *in vivo*, primarily as conjugates [13, 14]). Thus, the ^{3}H levels measured at 6–24 hr did not represent actual circulating steroid concentrations. This disparity was greatest in the maternal compartment. In the fetal compartment, which was of greatest interest in

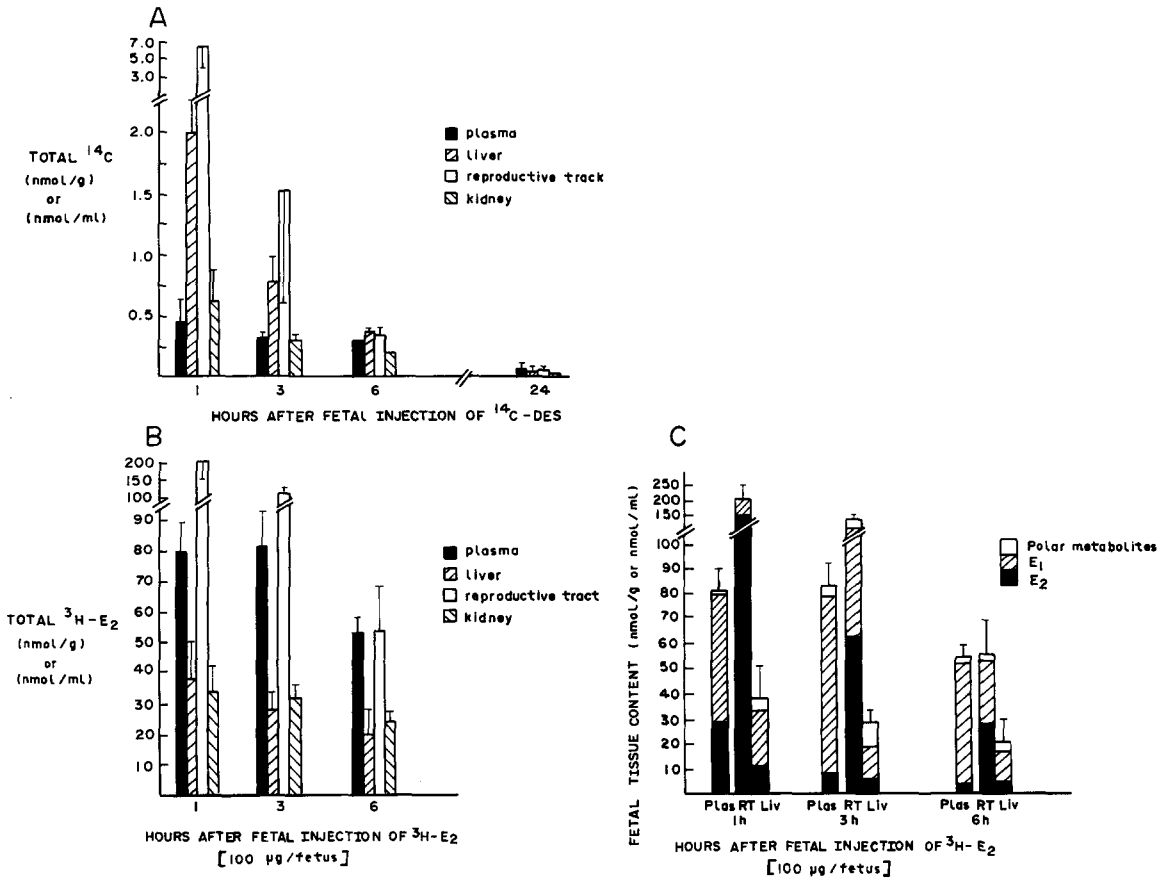

Fig. 3. (A) Concentration of total ^{14}C in fetal plasma and tissues after fetal injection of [^{14}C]DES, 2.4 µg/fetus. Bars are the mean ± S.D. (except when too small to show on figure) for three to six litters. Male and female reproductive tract were not significantly different, so values were pooled. (B) Concentration of total ^{3}H in fetal plasma and tissues after fetal injection of [^{3}H]E_2, 100 µg/fetus. Bars are the mean ± S.D. for four to six litters. At a dose of 10 µg/fetus, distribution was comparable; tissue levels were about one-tenth of those shown. (C) Distribution of metabolites of E_2 in fetal plasma (Plas), reproductive tract (RT), and liver (Liv) following fetal injection of [^{3}H]E_2, 100 µg/fetus. Bars are the mean ± S.D. for three to six litters. Similar results were obtained at 10 µg/fetus.

the remainder of these studies, at 1–3 hr (after fetal or maternal injection), recovery of ^{3}H was 95–100%, indicating that formation of volatile ^{3}H was negligible. Therefore, the observations and calculations using [^{3}H]E_2 data were considered to be valid without correction for the later, gradual change in specific activity.

Fetal injections. ^{14}C (from DES) was concentrated by fetal tissues to a greater extent than from maternal injections, especially by the reproductive tract (Fig. 3A). On the other hand, ^{3}H (from E_2) was largely retained in fetal plasma; tissue:plasma concentration ratios exceeded 1 only in fetal reproductive tissues at a dose of either 10 or 100 µg/fetus (Fig. 3B). Consistent with the above pattern, it was also observed that, following fetal injection of [^{3}H]E_2 at 10 µg/fetus (or maternal injection), fetal plasma ^{3}H was 90–94% protein-bound; at 100 µg/fetus, ^{3}H was about 80% protein-bound (determined by dextran-charcoal precipitation; see also Fig. 6); only approximately 15% of ^{14}C (from DES) was protein-bound (data not shown).

As was observed following maternal injection, DES and E_2 were rapidly metabolized following fetal injection. The fetal concentration of E_2 decreased faster than total ^{3}H (Fig. 3C). Fetal reproductive tract contained the highest proportion of unchanged E_2 (75–80% at 1 hr). Similarly, DES was particularly localized in fetal reproductive tissue: ^{14}C was more than 10-fold concentrated compared to plasma at 1 hr, and 85% was unchanged DES at 1 and 3 hr post-injection compared to 31% in plasma.

In Fig. 4, concentrations of total radioactivity and of parent E_2 and DES in fetal reproductive tissue are compared. The concentrations of total ^{3}H as well as E_2 at the threshold teratogenic dose* of 10 µg E_2/fetus were greater than total ^{14}C and DES at the teratogenic dose of 2.4 µg/fetus (Fig. 4A). The teratogenic dose of 100 µg E_2/fetus produced tissue

* The doses of E_2 of 10 µg/fetus and 2.5 mg/kg to the dam are referred to as "threshold teratogenic" doses since they elicit a low incidence (10–15%) of the mildest grade of urogenital malformations. DES at 2.4 µg/fetus produces more severe malformations and at higher incidences (≥ 75%) [6].

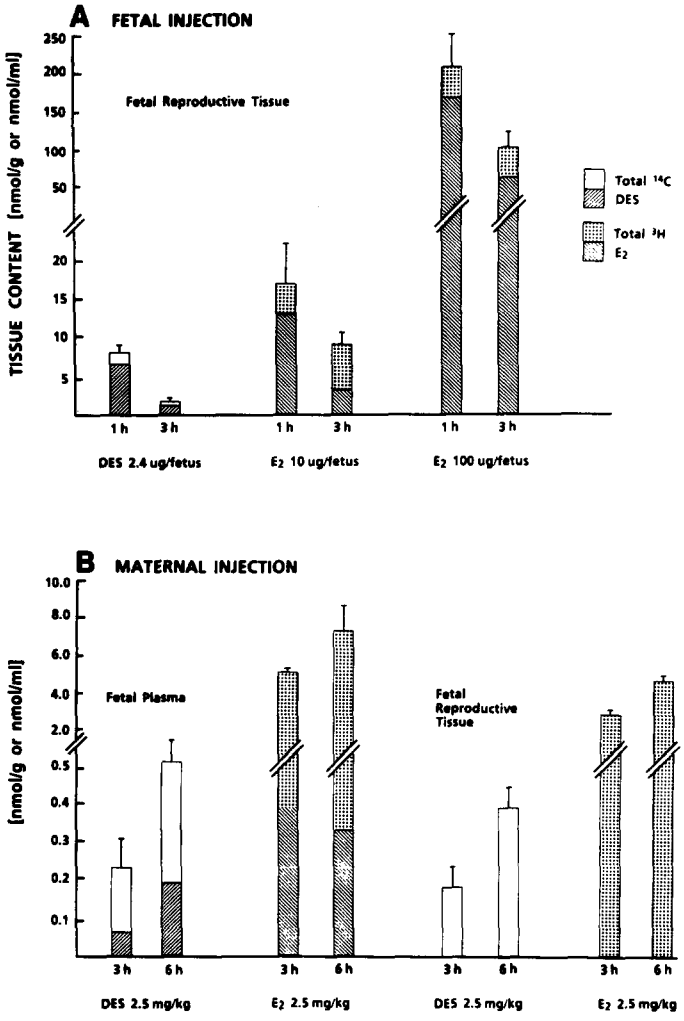

Fig. 4. Concentrations of total radioactivity and of parent estrogen in fetal reproductive tissue and plasma following fetal (A) or maternal (B) injection of [^{14}C]DES or [^{3}H]E_2. Bars are the mean ± S.D. for three to six litters.

concentrations of $E_2 > 20$ times higher than DES. Similarly, following maternal injection at 2.5 mg/kg, more E_2 than DES was present in reproductive tissue and fetal plasma (Fig. 4B). Only total ^{3}H and ^{14}C are indicated for the tissue, since the level of radioactivity was too low for analysis by HPLC. However, it is likely that the proportions of parent compound were higher in this tissue than in fetal plasma, as they were following fetal injection (Fig. 3C). Even assuming conservatively that they were equal, concentrations of E_2 greatly exceeded those of DES. Note that at this dose to the dam, DES is highly teratogenic whereas E_2 is a threshold teratogenic dose [6]. Thus, while the observed differences in tissue distribution of E_2 and DES were consistent with the differences in teratogenic potency, the actual tissue concentrations of parent compounds did not directly correlate with teratogenicity.

This disparity in tissue concentrations could reflect a difference in fetal tissue sensitivity to the synthetic versus natural estrogen at the molecular level, or could be due to an intracellular difference in kinetics or availability. To further characterize the tissue estrogen content, binding of ^{3}H to total cytosolic protein was measured in fetal reproductive tissue, liver, and plasma at 1 hr after [^{3}H]E_2 injection into fetuses. Note that a significant conversion of E_2 to E_1 occurred within 1 hr (Fig. 3C), but E_1 and E_2 were found to have similar affinities for plasma proteins (Ref. 7, and unpublished data).

When a tracer quantity of [^{3}H]E_2 was injected (8 pmoles/fetus), ^{3}H in fetal genital tract cytosol was 65–70% protein-bound, in fetal liver 50%, and in fetal plasma 90% (Fig. 5). Increasing concentrations of unlabeled E_2 added *in vitro*, but not DES, caused displacement of the bound ^{3}H. (Note that the indicated quantities of ligand were 8,000- to 30,000-fold excess over the ^{3}H present; concentrations were chosen to be in the range observed *in vivo*.) Clearly, DES was not an effective competitor for [^{3}H]E_2 binding sites.

In cytosols prepared after injection of [^{3}H]E_2 at

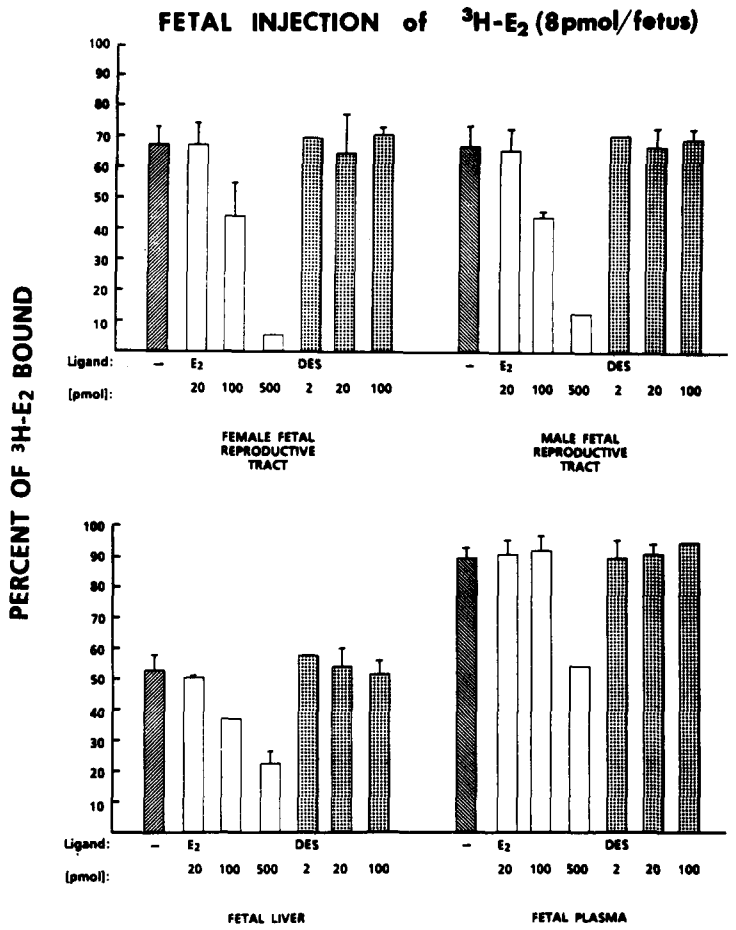

Fig. 5. Binding of ^{3}H to total protein in cytosol and plasma from fetuses 1 hr after injection with tracer [^{3}H]E$_2$. Cytosols and plasmas (40–200 μg protein) were incubated on ice for 2 hr with and without unlabeled E$_2$ or DES as indicated. Protein-bound activity was measured using hydroxylapatite, as described in the text. Bars are the mean ± S.D. for three to four litters.

10 μg/fetus, the degree of ^{3}H binding in fetal genital tract was slightly lower than from tracer ^{3}H injections (only significant in female); plasma binding was equal, and in liver it was significantly lower ($P < 0.05$) (Fig. 6). Thus, the reproductive tissue and plasma have high-capacity estrogen binding. In these cytosols, 100- to 400-fold excess E$_2$ and DES caused significant displacement of bound ^{3}H. E$_2$ (at 10 nmoles/tube = 2 μmoles/ml plasma) displaced 91% of protein-bound ^{3}H in plasma. At this concentration, even DES caused displacement (40%) of bound ^{3}H. "Normalized" binding of ^{3}H (per mg total protein in the cytosol) was more than 10-fold greater in fetal reproductive tract than liver (see Fig. 6). Although liver yielded 1.6 times more protein/mg tissue, only a small component had affinity for E$_2$ and E$_1$. Liver also contained 25% conjugates (Fig. 3C), which would not likely be protein-bound; however, this cannot account for the 10-fold less binding compared to reproductive tissue. In five of six experiments, normalized binding in fetal plasma was higher than in either tissue, although the mean values were not significantly different because of the large variance (Fig. 6).

DISCUSSION

The dispositional differences observed between DES and E$_2$ were consistent with their differing teratogenic potencies. Fetal:maternal plasma concentration ratios were several-fold greater for E$_2$ than for DES but fetal tissues accumulated DES, whereas fetal tissue:plasma ratios for E$_2$ were less than 1 except in genital tract. Both compounds were metabolized to non- or less estrogenic products, and in both cases parent compound was retained longest and at highest concentration in the fetal genital tissue. Other studies which evaluated the fetal distribution of DES used i.v. injection or i.v. infusion [15, 16]; these protocols produced fetal reproductive tissue levels up to 10-fold higher than in fetal plasma, whereas in the present study, using maternal s.c. injection, fetal plasma and reproductive tissue levels were comparable (Fig. 4B). The s.c. dose may be metabolized faster than it reaches the fetus. Compared to DES, E$_2$ was more rapidly metabolized (primarily to E$_1$), resulting in a shorter duration of exposure to E$_2$.

These observations were also consistent with the

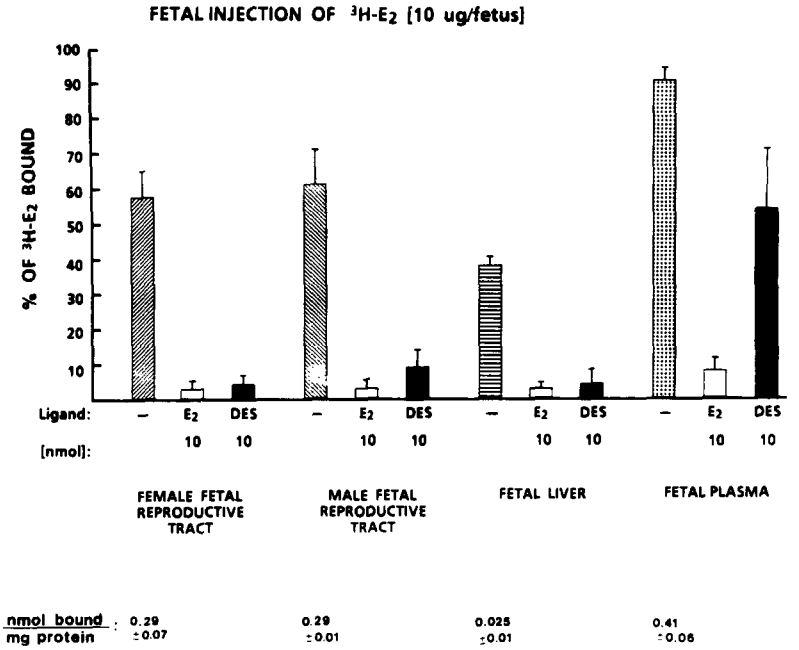

Fig. 6. Binding of ^{3}H to total cytosolic and plasma protein, measured as in Fig. 5, from fetuses injected with [^{3}H]E$_2$, 10 μg/fetus. Numbers below each tissue represent total nmoles of ^{3}H bound normalized per mg of total protein in the assay tube. All data are mean ± S.D., N = 4.

binding of E_2 (and E_1), but not DES, by alpha-fetoprotein (AFP) in fetal plasma [7, 17]. Binding of drugs to plasma proteins generally renders them less available for uptake into tissues and for transplacental movement [18]. In this case, because the effective (free) level of E_2 is maintained low by AFP in the fetus but not in the mother, E_2 rapidly moves from mother to fetus down its concentration gradient. DES, which primarily circulates non-protein-bound, diffuses more readily into tissues, and more rapidly decreases its maternal:fetal plasma transplacental concentration gradient. LeGuern *et al.* [19] also found approximately 5-fold and 15-fold greater transfer of [^{3}H]E$_2$ to the fetal rat compared to [^{3}H]DES or [^{3}H]moxestrol, respectively, 90 min after s.c. injection (only plasma and amniotic fluid were analyzed). The [^{3}H]E$_2$ was associated with AFP, as verified by polyacrylamide gel electrophoresis. All of these observations indicate that the potential for fetal rat tissue exposure to DES is greater than for E_2. Consistent with this explanation is the observation that in monkeys, which lack AFP, transplacental kinetics of total radioactivity from DES and E_2 are quite similar [20]. Both cross the placenta to about the same degree following i.v. administration, and remain elevated in fetal compared to maternal plasma from 20 min to the end of the 2-hr experiments. However, unchanged DES reaches the fetus while E_2 does not because E_2 is rapidly converted to E_1 by the mother or placenta.

Oxidative metabolites and conjugates of DES were also detected in fetal rats after an i.v. infusion of [^{14}C]DES [16] and in fetal mice after an i.v. injection [15]. These metabolites are produced by fetal liver and genital tract incubated *in vitro* [21, 22], although to a much lesser degree than by maternal liver; this may account for the increasing proportion of DES metabolites in the fetus compared to the mother (Fig. 2), since the polar products do not likely cross the placenta back to the mother. It has been suggested that metabolic activation/covalent binding mediates the transplacental carcinogenicity of DES [9] (as well as the renal carcinogenicity of estradiol (in the hamster) [23]), although it is difficult to isolate estrogenic from metabolic contributions to carcinogenicity. DES is also active in sister chromatid exchange assays [24, 25]. However, these carcinogenic interactions may differ from those inducing malformations.

Similarly, E_2 was readily metabolized by fetal liver *in vitro*, yielding E_1 and conjugates; fetal genital tract also produced E_1 from E_2 (unpublished observations). Thus, rapid metabolism in both mother and fetus, in conjunction with protein binding of E_2, may serve to "protect" the fetus from the natural estrogen. However, it has not been proven that some aspect of this oxidative metabolism is not involved in teratogenicity. In the present studies, the parent estrogens were assumed to be the teratogenic agents, since their metabolites have little or no estrogenic activity, at least in adult uterus [8].

Direct fetal injections were originally chosen in order to administer a sufficiently large dose of E_2 to overcome binding by AFP, as a test of the hypothesis that E_2 could elicit the same malformations as DES if an adequate amount reached the fetal tissues [6]. This hypothesis was demonstrated; an effective dose of E_2 was 100 μg/fetus, and 10 μg/fetus was a threshold dose. The 10 μg dose produced plasma ^{3}H concentrations at 1 hr of approximately 9 nmoles/

ml (3 nmoles/ml was E_2, 5 nmoles/ml was E_1) (Fig. 3) compared to endogenous E_1 and E_2 concentrations of about 9–15 and 0.8–4 pmoles/ml, respectively, in the late gestation rat fetus (unpublished observations; [26]). Levels of E_2 above 10 nmoles/ml markedly reduced [^{3}H]E_2 bound by fetal plasma *in vitro* (E. C. Henry and R. K. Miller, submitted). Therefore, 10 nmoles/ml appeared to be a saturating level of E_2 in fetal plasma. At 1 hr after the 100 μg dose, plasma ^{3}H was 80 nmoles/ml (27 nmoles/ml was E_2) which clearly exceeded that saturating level. These values are consistent with the reported capacity of fetal rat AFP of 20–70 nmoles/ml, and K_d for E_2 in the 10 nM range (10 pmoles/ml) [7, 27–30]. Thus, the teratogenic dose of E_2 did exceed the capacity of AFP, apparently leaving sufficient free E_2 available for uptake into fetal tissues; the threshold dose of E_2 just reached the saturation level for AFP.

By comparison, ^{14}C was only 0.3 to 0.4 nmole/ml (0.1 nmole/ml was DES) following [^{14}C]DES injection at the teratogenic dose of 2.4 μg/fetus (Fig. 4). At this concentration, DES is unlikely to interact with AFP, since DES competed with [^{3}H]E_2 bound to fetal plasma *in vitro* only at concentrations 25-fold higher than concentrations of E_2 which competed (data not shown). Therefore, the small amount of ^{14}C which was protein-bound following *in vivo* exposure to [^{14}C]DES may have represented binding to plasma proteins other than AFP, such as albumin, whose affinities for the two estrogens are more comparable.

In spite of the consistencies between disposition and teratogenicity of these estrogens, the actual target tissue concentrations of E_2 (Fig. 4) and the calculated fetal tissue exposures (nmoles·hr/g) to E_2 were much greater than for DES. However, DES and E_2 in fetal tissues were not "biologically equivalent", since E_2 was highly protein-bound intracellularly (Fig. 5). DES was a poor competitor for this binding (as it was for plasma-bound E_2), especially at the concentrations observed following *in vivo* injections. Note that the HAP assay measures binding (covalent and noncovalent) to total protein, not specifically receptor binding. In fact, receptor-bound activity is likely a very small fraction of this total. Covalently bound (nonextractable) radiolabel is also a very small proportion of the total ([16]; unpublished observations). It is not known whether the remainder reflects primarily intracellular AFP, and/or AFP from plasma present in tissue homogenate [29], or other proteins with higher affinity for E_2 than for DES. These data suggest that the quantity of E_2 which is "free" in the cell (as DES is) may determine its teratogenicity. Indeed, concentrations of E_2 in fetal genital tract *in vivo* at a teratogenic dose (Fig. 4) equalled or exceeded the concentrations of E_2 which caused displacement of protein-bound ^{3}H *in vitro*. Similarly, E_2 concentrations which did not affect [^{3}H]E_2 binding *in vitro* were lower than *in vivo* teratogenic levels (Fig. 5). Thus, DES and E_2 may be of comparable potency at the molecular level, when these dispositional and protein binding differences are taken into account. Perhaps a high level of the "free" estrogens is necessary to enable continuous receptor occupation.

These results are analogous to results in rhesus monkeys, in which the synthetic glucocorticoid, triamcinolone acetonide (TAC), causes various developmental deficits, whereas the natural hormone, cortisol, presents little hazard [31]. TAC crosses the placenta to a greater degree than cortisol and is resistant to metabolism in the fetus [32]. Cortisol is rapidly metabolized to inactive compounds, resulting in a lower fetal exposure to cortisol than to TAC. The potency of the natural hormone, cortisol, is also reduced by its binding in maternal plasma to corticosteroid binding globulin, which does not bind TAC.

Our observations are supportive of the hypothesis that the teratogenicity of DES and E_2 is mediated by their estrogenic activity. The estrogen receptor may be involved, although these experiments did not address the question of whether the fetal receptor, like the adult receptor, has comparable affinity for the synthetic and natural estrogens.

In summary, these studies demonstrated that the difference in the teratogenic potencies of DES and E_2 reflects their differing disposition and kinetics in the fetus. Rapid metabolism to non- or less estrogenic products, and high-affinity, high-capacity protein binding both extra- and intracellularly reduce the potency of the natural estrogen. Any synthetic compounds which have estrogenic activity are likely to pose a greater teratogenic risk than the natural estrogens.

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