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Estrogenic activity *in vivo* and *in vitro* of some diethylstilbestrol metabolites and analogs

(diethylstilbestrol metabolism/estrogen receptor/mouse uterus/hormonal toxicity/structure–activity)

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ABSTRACT **The diethylstilbestrol (DES) metabolite (β-dienestrol), which had been identified in mouse, rat, monkey, and human urine, and two proposed metabolic intermediates (diethylstilbestrol α,α'-epoxide and α,α'-dihydroxy DES) were synthesized and their estrogenic activities determined. In addition, three DES analogs, α-dienestrol, DES-dihydroxy diethyl phenanthrene (DES-phenanthrene), and 1-(α-ethyl, 4α-hydroxyphenyl)indanyl-5-ol (indanyl-DES), were studied. Estrogenic activities of the compounds *in vivo* were determined by the immature mouse uterine weight bioassay; *in vitro*, their estradiol receptor binding activity (competitive equilibrium binding, sucrose gradient analysis, and association rate inhibition assays) was determined. Results of the mouse uterine weight bioassay gave the following order of estrogenicity: DES > α-dienestrol ≥ DES-epoxide > indanyl-DES > dihydroxy DES > β-dienestrol > DES-phenanthrene. Results of competitive equilibrium binding analyses of these compounds with estradiol-17β for the mouse uterine cytosol receptor followed the same order seen for the bioassay, except for indanyl-DES. DES, indanyl-DES, and α-dienestrol had the greatest affinities (K_a values approximately 0.5–19.1 × 10^{10} M^{-1}), while DES-phenanthrene had the lowest (K_a = 3.5 × 10^7 M^{-1} ± 1.2). Sucrose gradient analysis of the above competition preparations illustrated the displacement of [^{3}H]estradiol from the receptor peak. This displacement was receptor specific and concentration dependent and correlated with the equilibrium binding concentrations. In addition, the most hormonally active substances demonstrated the greatest rate inhibition in the estradiol cytosol receptor association rate reaction (V_0). The rank order of estrogenicity of the compounds determined in this study should be useful in evaluating alternative metabolic pathways of DES as well as distinguishing biologically active metabolites from relatively inactive ones.**

Diethylstilbestrol (DES) is a nonsteroidal estrogen first synthesized and described by Dodds in 1938 (1). It has significant oral activity as an estrogenic substance and invokes a strong hormonal response equivalent to an injected dose of the steroidal estradiol-17β.

In recent years, reports by Herbst and colleagues (2) have linked *in utero* exposure to DES with subsequent development of vaginal clear cell adenocarcinoma in a number of female offspring. A detailed review by Gunning of the human studies has been published (3). Investigations in our laboratory (4, 5) have been concerned with the development of an animal model system for studying this effect of *in utero* exposure.

Pharmacokinetic studies with radiolabeled DES in the laboratory mouse model (6) have shown that this compound is localized in the fetal reproductive tract and associated structures. The metabolic pathways for DES are still unknown, but recent work has illustrated tentative metabolic schemes by identifying the structures of oxidative metabolites of DES from rat and hamster (7) or human (8) urine. Engel and associates (9) have shown evidence for an additional pathway, describing DES ring catechol formation using *in vitro* studies with rat liver microsomes. The question of whether the carcinogenic nature of DES resides in the DES molecule itself or in one of its metabolites or metabolic intermediates remains to be answered. This study describes our observations of the estrogenic activities of some DES metabolites *in vivo* and *in vitro* and discusses their importance in understanding the metabolism and action of DES.

METHODS AND MATERIALS

Animals. Adult female CD-1 mice were obtained from Charles River Breeding Laboratories (Wilmington, MA). Immature CD-1 mice used in the bioassay experiments were derived from Charles River stock at the National Institute of Environmental Health Sciences and weaned at 21 days of age. Animals were housed in a controlled temperature room (70–72°F; 21–22°C) with 14-hr light and 10-hr dark periods. Standard laboratory feed and water were given ad lib.

Steroids and Chemicals. All buffer reagents and other chemicals were of analytical grade and obtained from commercial sources. DES and estradiol were purchased from Steraloids Inc. (Wilton, NH) and checked for purity by appropriate thin-layer chromatography. [^{3}H]Estradiol-17β (specific activity 110 Ci/mmol) was purchased from New England Nuclear Corp. (Boston, MA) and brought to greater than 98% radiochemical purity by thin-layer chromatography in benzene/ethyl ether (1:1). Stock ethanolic solutions of [^{3}H]estradiol were diluted to the required specific activity with unlabeled estradiol and constituted in Tris/EDTA/glycerol (10 mM Tris·HCl/1.5 mM tetrasodium EDTA/10% glycerol) at pH 8.0 at 4°. The ethanol concentrations of the Tris/EDTA/glycerol solutions used in the incubations were <2%. The DES metabolites and analogs used in this study were synthesized and characterized as described elsewhere (ref. 8; M. Metzler, unpublished data). The 3,6-dihydroxy-9,10-diethyl phenanthrene (DES-phenanthrene) was synthesized by the procedure of Hugelshofer *et al.* (10). All compounds were stable under the assay conditions used.

Bioassay Experiments. The DES metabolites were dissolved in propylene glycol/H_2O (1:2) and injected subcutaneously at a constant volume of 0.01 ml/g of body weight. CD-1 mice were injected (dose range, 10 μg/kg to 2000 μg/kg) for three consecutive days starting on day 23 of age. Groups of 5–19 animals were killed on the fourth day by cervical dislocation and the uteri were rapidly removed, trimmed, and weighed. The logarithmic dose-response data were analyzed by a curve

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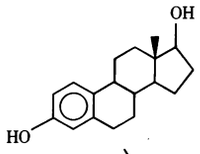
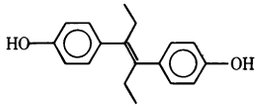
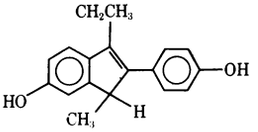
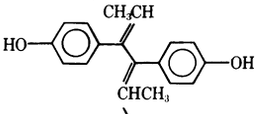
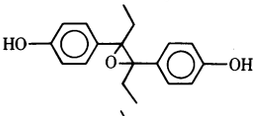
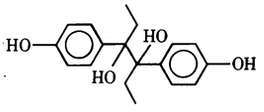
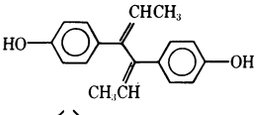
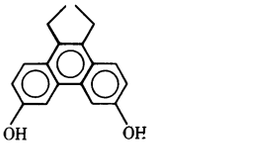
Abbreviation: DES, diethylstilbestrol.

Table 1. Estrogenicity of DES and some related compounds

Compound	Structure	Competitive equilibrium binding C_{50}*	Apparent K_a†	Dose to double uterine weight, μg/kg‡
Estradiol-17β		1.6 ± 0.5	$1.5 \times 10^{10} \pm 0.3$	5.0
DES		2 ± 0.3	$1.0 \times 10^{10} \pm 0.8$	7.0 ± 2.5
Indanyl-DES		2 ± 0.4	$9.1 \times 10^{9} \pm 1.8$	107.4 ± 13.8
α-Dienestrol		5 ± 0.8	$4.2 \times 10^{9} \pm 0.6$	14.2 ± 5.7
DES-epoxide		17 ± 2	$1.0 \times 10^{9} \pm 0.2$	14.1 ± 2.2
Dihydroxy DES		334 ± 92	$7.3 \times 10^{7} \pm 4.2$	3,374 ± 763
β-Dienestrol		367 ± 72	$4.8 \times 10^{7} \pm 0.8$	15,000
DES-phenanthrene		600 ± 173	$3.5 \times 10^{7} \pm 1.2$	>15,000

* Cytosol prepared from CD-1 mouse uterus; expressed as the nM concentration necessary to occupy 50% of the receptor binding sites.
† Apparent K_a value calculated from the C_{50} value by the method of Korenman (14).
‡ The dose of the compound required to cause a 2-fold increase in the uterine weight/body weight ratio as compared to controls.

fitting technique, and the *in vivo* response was expressed as the dose required to cause a doubling of the uterine weight/body weight ratio.

Estradiol Receptor Binding Experiments. Uteri were rapidly removed, weighed, and homogenized in ice-cold Tris/EDTA/glycerol. This homogenate was centrifuged at 1000 × *g* for 20 min; the supernatant was decanted and centrifuged at 105,000 × *g* for 60 min. This high-speed supernatant (cytosol) was used for all cytoplasmic binding studies. Saturation binding analyses of cytosol preparations were performed as described (11) and the data were computed by Lineweaver–Burk analysis (12). When similar experiments were performed to establish whether the interaction was competitive or noncompetitive, the DES analog was added at the stated concentration to each incubation tube concomitantly with the [^{3}H]estradiol. Association rate analyses were performed with uterine cytosol that was reacted with [^{3}H]estradiol; unlabeled competitors were preincubated with the cytosol for 2 hr, as described (13). All competitive equilibrium binding experiments were performed as before (13) and the results were analyzed according to Korenman (14).

RESULTS

Competition equilibrium binding experiments for the estradiol cytosol receptor gave a series of sigmoidal competition curves that varied according to the strength of the interaction. From these data apparent equilibrium affinity constants (K_a) could be calculated (13) for the different DES metabolites and analogs; these are listed in Table 1. All compounds studied gave K_a values within a 300-fold range (3.3×10^7–9.1×10^9 M^{-1}). Estradiol and DES have similar apparent affinities for the estradiol receptor sites. Indanyl-DES and α-dienestrol had K_a values similar to that of DES. DES-epoxide also exhibited significant binding to the estradiol cytosol receptor. The other compounds, such as dihydroxy DES and β-dienestrol, had quite low affinities. The phenanthrene derivative of DES was only weakly estrogenic. Binding saturation studies in the presence of competitors at four different concentrations were analyzed by a Lineweaver–Burk plot; each metabolite showed competitive inhibition with no indication of covalent binding.

The doses (in μg/kg) of DES analogs that caused a 2-fold increase in the weight of the immature mouse uterus were used

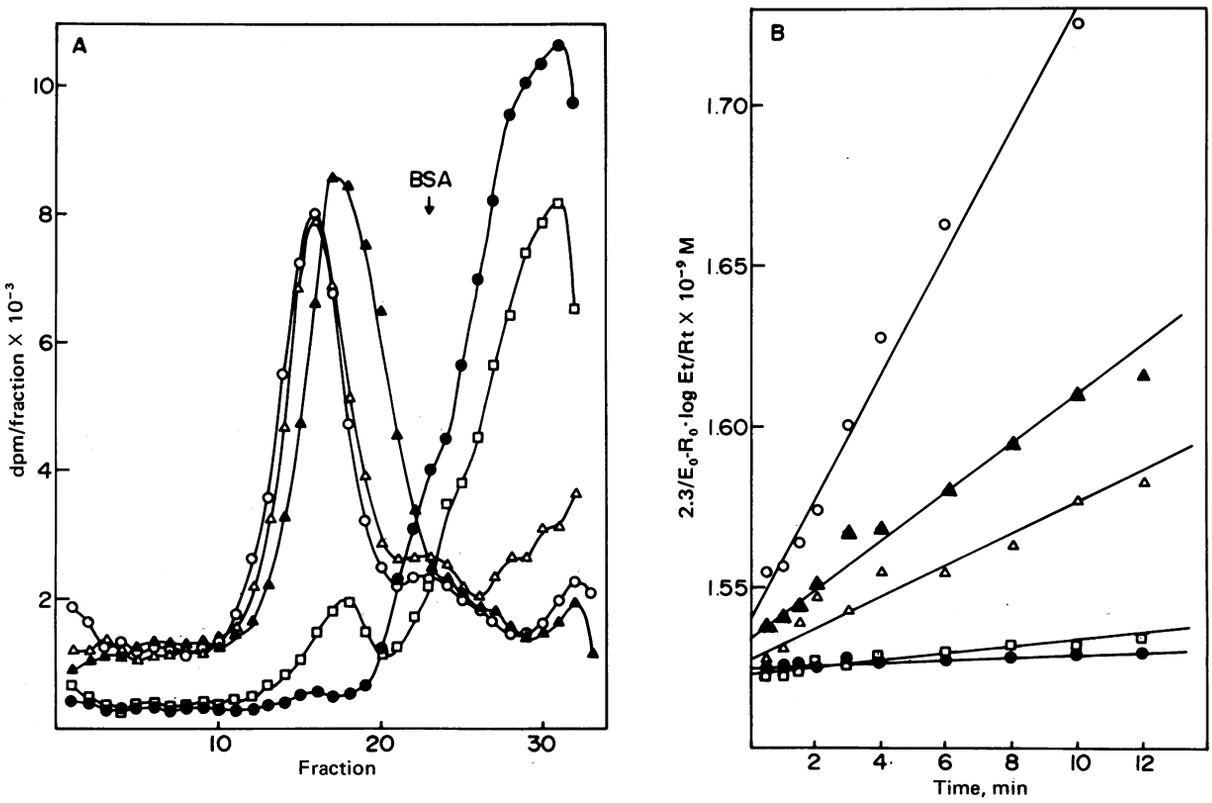


FIG. 1. (*A*) Aliquots of cytosol (300 μl) were incubated for 2 hr at 4° with 150 nmol of various competitors: (○) no competitor, (●) DES, (□) α-dienestrol, (△) β-dienestrol, and (▲) DES-phenanthrene. After the preincubation with competitors, 1.5 nmol of [^{3}H]estradiol was added and incubation was continued at 4° for 3 hr. Aliquots of 300 μl were then layered on chilled, preformed 5–20% sucrose gradients and centrifuged at 225,000 × *g* for 17 hr. A 200-μg sample of bovine serum albumin (BSA) was processed along with the samples, and its position is marked in the figure. (*B*) Equal 1-ml volumes of cytosol (1.26 mg/ml) were preincubated for 3 hr at 4° with 50 nmol of the same competitors used in *A*. Then 0.5 nmol of [^{3}H]estradiol was added to start the reaction and the procedure followed was as described (13).

as indexes of *in vivo* estrogenicity and are also presented in Table 1. Those compounds with high affinities (10^9 or greater) for the cytoplasmic estradiol receptor (DES, indanyl-DES, α-dienestrol, and DES-epoxide) were also estrogenic *in vivo* at doses of approximately 100 μg/kg or less. Analogs with affinities 2 orders of magnitude less than α-dienestrol (dihydroxy DES, β-dienestrol, and DES-phenanthrene) were also weakly estrogenic in the bioassay test; these compounds required doses in the mg/kg range for uterine weight doubling and, for DES-phenanthrene, this end-point was not reached even at 15 mg/kg.

The Kendall's tau rank correlation (15) between receptor binding and *in vivo* activity was 0.76 ($P = 0.004$) when indanyl-DES was included in the analysis, and 0.90 ($P = 0.002$) when the data were analyzed excluding this compound.

Evidence that the competition was receptor specific was demonstrated by sucrose gradient sedimentation profiles of cytosol samples that had been incubated with [^{3}H]estradiol and various competitors (Fig. 1*A*). The sedimentation properties (approximately 6 S) of the receptor were not altered by the presence of different DES analogs in the preparation. Other studies gave a dose-response of cytoplasmic receptor binding with a proportional drop in the size of the receptor peak as a function of increasing concentrations of competitor.

To further show that these competitors were interacting with the receptor, we analyzed the effect of these compounds on the association rate reaction of estradiol and the cytoplasmic receptor. Data demonstrating the effect of certain competitors on the association rate plot are shown in Fig. 1*B*. DES or α-dienestrol gave no association rate since these compounds had competed for the receptor binding sites. Experiments with estradiol-17β gave similar results. β-Dienestrol had some effect at this concentration but it was not as dramatic as that of the DES or α-dienestrol samples. DES-phenanthrene was not effective at all. Other experiments, not shown, demonstrated that the effect of the compounds on the reaction is dose dependent and that there is a proportional decrease in the rate as the concentration of competitor is increased.

DISCUSSION

The above data clearly show that some DES metabolites and analogs are estrogenic. Results from the mouse uterine cytoplasmic receptor binding show that this estrogenic activity is due to their ability to interact with the receptor. The equilibrium binding assay does not determine whether the compounds interact with the receptor in a competitive or noncompetitive manner. Those compounds that were checked by the second binding analysis (Lineweaver–Burke) clearly showed competitive interaction. Association rate inhibition experiments support and demonstrate the same pattern, since this technique describes binding only at the receptor site (16). There is an apparent correlation in the ranking of compounds from *in vivo* experiments and the *in vitro* receptor binding experiments. Some compounds that possess good estrogenic activity also show strong receptor binding, while those that bind poorly are also biologically ineffective. It is well documented that in the mechanism of hormone action there is a step involving binding of the hormone to an intracellular receptor (17). This study demonstrates that certain DES metabolites and analogs possess this cytosol receptor binding ability.

Use of the immature mouse uterine weight bioassay as an *in vivo* correlate with the receptor studies is valid since Clark *et al.* (18) have shown a relationship between receptor binding and uterine biological response. There is the possibility in the *in vivo* experiments that certain compounds (e.g., β-dienestrol and dihydroxy DES) may have increased metabolism or decreased entry into the endometrial cells. This could cause a misinterpretation, since, in either case, they would give the appearance of being biologically inactive. However, the fact that neither of these two compounds binds well to the receptor makes it unlikely that they could be biologically active if they did enter the cell. The same argument holds true for the possibility that certain DES compounds could not translocate the steroid receptor complex to the nucleus (19). Those compounds that bind the cytoplasmic receptor poorly will not be able to generate an effective nuclear receptor complex, which is derived from the cytoplasmic receptor and requires hormone binding (19).

Conversely, if a compound adequately interacted with the cytoplasmic receptor but was inadequately translocated to the nucleus, there would be a submaximal biological response. Indanyl-DES may be an example of such a compound since its biological activity is significantly less than predicted from its relatively high affinity for the estradiol receptor. Preliminary studies of the translocation process (K. S. Korach *et al.*, unpublished data) suggest that indanyl-DES does not retain the complex in the nucleus as long as DES.

DES-phenanthrene arises from photocyclization of *cis*-DES (20). This process is not unlike that seen for vitamin D_3 produced by UV irradiation in the skin from relatively inactive 7-dehydrocholesterol (21). Consequently, topically applied estrogenic materials (e.g., beauty creams) could become photoactivated. The possibility that this process may cause or increase certain types of cancer is now under study. However, the low estrogenicity of DES-phenanthrene suggests that estrogen-responsive tissues would not represent the major targets for this compound.

DES metabolism may proceed via two routes. One route could produce hormonally inactive metabolites such as β-dienestrol. The other pathway could produce compounds such as DES-epoxide, which still retain a considerable amount of estrogenic activity. Evidence for such a bilateral metabolism of certain substances has been documented (22). If DES undergoes such metabolism, then large doses or repeated ingestion could produce compounds in the body that are hormonally active and/or carcinogenic. This study has only attempted to determine the hormonal activity of these compounds and does not concern their carcinogenic properties.

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