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METABOLISM AND IRREVERSIBLE BINDING OF DIETHYLSTILBESTROL IN THE KIDNEY OF THE SYRIAN GOLDEN HAMSTER*†

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Abstract—Oxidative metabolism of [^{3}H]diethylstilbestrol (DES) and the irreversible binding of reactive [^{3}H]DES metabolites to the macromolecules in kidney slices of the Syrian golden hamster were investigated. Non-extractable binding of [^{3}H]DES to kidney macromolecules was observed after incubating hamster kidney slices under aerobic conditions (95% O_2/5% CO_2), but not under anaerobic conditions (100% nitrogen + 2 mM KCN). A number of oxidative metabolites of [^{3}H]DES were detected in the incubation medium of kidney slices incubated under aerobic, but not anaerobic, conditions The amount of radiolabeled macromolecules formed in male cortical slices under aerobic conditions increased with time of incubation At a medium concentration of 50 nM [^{3}H]DES, 0 08 pmole [^{3}H]DES equiv / mg dry weight at 30 min and 0 19 pmole [^{3}H]DES equiv./mg dry weight at 120 min were observed The amount of irreversible [^{3}H]DES-macromolecular complexes also increased with the concentration of [^{3}H]DES in the incubation medium 1.59 pmole [^{3}H]DES equiv /mg dry weight was formed with 0 5 μM [^{3}H]DES and 21 89 pmole [^{3}H]DES equiv /mg dry weight was formed with 10 μM [^{3}H]DES Non-extractable [^{3}H]DES binding was detected in all the subcellular fractions of hamster kidney with the highest amount in the microsomal and soluble fractions, followed by the mitochondrial and nuclear fractions The macromolecular-[^{3}H]DES complexes were solubilized by proteases but not nucleases, suggesting that [^{3}H]DES irreversible binding is principally to the proteins and not the nucleic acids The cortex as compared with the medulla of the male hamster kidney displayed a 5-fold greater capacity to irreversibly bind [^{3}H]DES metabolites The male hamster renal cortex showed a 2- to 3-fold greater capacity to form irreversible macromolecular-[^{3}H]DES complexes than the female hamster renal cortex These data demonstrate that (1) renal oxidative metabolism of DES results in [^{3}H]DES metabolites binding irreversibly to macromolecules, and (2) the sex and organ site specificity of the [^{3}H]DES-macromolecular binding corresponds with the sex and organ site specificity of renal tumors of the hamster

Diethylstilbestrol (DES), a potent synthetic estrogen, is carcinogenic to humans and animals [1] Male but not female Syrian golden hamsters chronically exposed to DES develop renal tumors with an incidence approaching 100% [2]. Several lines of evidence suggest that oxidative metabolism of DES may play a role in the development of clear cell carcinoma of the male hamster kidney. Formation of the renal tumors is inhibited by treating the hamsters with anti-oxidants or α-napthoflavone, an inhibitor of NADPH-dependent monooxygenases [3–5]. Oxidative metabolites of DES have been detected in the urine of hamsters that were treated with DES [6] Two stable urinary metabolites of E-DES, Z,Z-dienestrol (Z,Z-DIES) and 1-hydroxy-Z,Z-dienestrol (1-OH-Z,Z-DIES), are formed by a tautomerization of unstable quinone-like intermediates. One of these unstable intermediates, DES-4′,4″-quinone, has been synthesized and was found to have a 40-fold greater capacity to irreversibly bind to calf thymus DNA than DES [7]. Li and co-workers [8] have demonstrated that the incidence of hamsters developing renal tumors when treated with natural or synthetic estrogens may be related to the ability of these compounds to act as substrates for enzymes involved in catechol estrogen formation. Catechol estrogens can be oxidized to phenoxy-radical semiquinones and *ortho*-quinones which may react with cellular nucleophiles including proteins and nucleic acids [8, 9]. The DES-induced renal tumors are site-specific, having a cortical tubular origin [10]. The sex-related differences in the susceptibility of hamsters to develop DES-induced renal tumors may be related to a sexual dimorphism of DES metabolism by the hamster kidney [11, 12].

Since oxidative metabolism of DES may play a role in the carcinogenic mechanism through covalent binding to important cellular macromolecules, we investigated the capacity of the hamster kidney slices to metabolize and irreversibly bind [^{3}H]DES.

MATERIALS AND METHODS

Chemicals and reagents [Monoethyl-^{3}H]DES (97–120 Ci/mmole) and NCS tissue solubilizer were purchased from Amersham, Arlington Heights, IL The

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[^{3}H]DES was purified by high pressure liquid chromatography (HPLC) Deoxyribonuclease I (EC 3 1 21.1) bovine pancreas (sp. act approximately 2000 Kunitz units/mg protein) and 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES) were obtained from the Sigma Chemical Co , St Louis, MO Pronase, *Strep. griseus* (sp. act. 82000 PUK/g), was provided by the Calbiochem-Behring Corp , La Jolla, CA Ribonuclease A (EC 3.1 27.5) bovine pancreas (sp. act. 4500 units/mg) was supplied by the Worthington Biochemical Co., Freehold, NJ Liquiscint scintillation fluid was obtained from National Diagnostics, Somerville, NJ Methanol (HPLC grade) was purchased from Fisher Scientific, Chicago, IL. DES was supplied by ICN Laboratories, Plainview, NY 4′-Hydroxy-propiophenone was obtained from the Aldrich Chemical Co , Milwaukee, WI. Unlabeled 1-hydroxy-E-DES was provided by Dr. Kenneth Korach, National Institute for Environmental Health Science, Research Triangle Park, NC. Unlabeled E-DES glucuronide, Z,Z-DIES, and 1-hydroxy-Z,Z-DIES triacetate were provided by Dr Manfred Metzler, Institute for Pharmacology and Toxicology, University of Wurzburg, Wurzburg, Federal Republic of Germany. 1-Hydroxy-Z,Z-DIES triacetate was hydrolyzed to 1-hydroxy-Z,Z-DIES according to the method of Metzler [13] All other chemicals were reagent grade or better.

Animals Adult Syrian golden hamsters (110–125 g) were purchased from the Charles River Lakeview Hamster Colony, Wilmington, MA

Preparation and incubation of kidney slices. Hamsters were decapitated and the kidneys were rapidly removed and placed in ice-cold 0.9% NaCl buffered with 10 mM phosphate, pH 7 4 (PBS). Fat and kidney capsules were quickly excised, and 0.5-mm tissue slices were made with a Stadie–Riggs microtome Incubation medium consisted of a modified Krebs–Henseleit buffer: 118 mM NaCl, 4 mM KCl, 1.7 mM KH_2PO_4, 1 2 mM $MgSO_4$, 2.4 mM $CaCl_2$, 24 mM $NaHCO_3$, 25 mM HEPES, and 5 mM glucose The renal medulla was separated from the cortex by dissection. Kidney slices (110–140 mg) were incubated in 5 ml medium containing [^{3}H]DES Incubations were carried out in stoppered 25-ml Erlenmeyer flasks under an atmosphere of 95% O_2 and 5% CO_2 Anaerobic incubations were carried out under an atmosphere of nitrogen in the presence of 2 mM KCN All samples were incubated at 37° in a shaker bath for the time cited.

Determination of tissue and irreversibly bound radioactivity Following incubation, the kidney slices were rinsed with 5 ml of ice-cold PBS and then blotted on filter paper The incubation medium was saved for analysis by HPLC Samples of tissue (5–10 mg) were digested with 0 5 ml NCS and 0.125 ml H_2O After addition of acetic acid, the samples were counted using 10 ml of Liquiscint in a Packard Tri-Carb 300C liquid scintillation spectrometer (Packard Instrument Co Inc , Downers Grove, IL) The remainder of the tissue was homogenized in 3 ml of 5% trichloroacetic acid (TCA) at 4° The TCA-insoluble macromolecules were collected by centrifugation after standing on ice for 1 hr. Each pellet was washed as follows 2 × 2 ml of 5% TCA at 4°, 5 × 2 ml of 80% ethanol at 60°, 3 × 2 ml of absolute ethanol at 60°; 2 × 2 ml of ethanol/ether/chloroform (1/2/1) at 25°; and 2 × 2 ml of ether at 25° Aliquots of wash supernatants were counted to check the efficiency of the procedure. The pellets were air-dried overnight, and samples were solubilized in 0 4 ml NCS and 0.1 ml H_2O, neutralized with acetic acid, and counted in 10 ml of Liquiscint The background binding of [^{3}H]DES to hamster kidney macromolecules was determined employing the following procedure: 110–140 mg of kidney slices was homogenized in 1 ml of 40 mM Tris, 1 mM dithiothreitol, 1 mM EDTA, pH 7 4 Then [^{3}H]DES was added to the homogenate to determine the background or non-specific binding The samples were vortexed, then 1 ml of ice-cold 10% TCA was added, and the samples were vortexed again. After 60 min on ice, the acid-insoluble macromolecules were collected by centrifugation The pellets were washed with organic solvents, dried, and solubilized as previously described.

Subcellular fractionation of male renal cortical slices following incubation with [^{3}H]DES After incubation in 40 ml of medium and rinsing with PBS, 1 0-g renal cortical slices were minced and homogenized in 5 ml of 40 mM Tris, 1 mM dithiothreitol, 1 mM $MgCl_2$, 10 mM KCl, 250 mM sucrose, pH 7 4 (TDMKS) using ten strokes with a motor-driven Teflon pestle tissue grinder The homogenate was centrifuged at 800 *g* for 15 min to obtain the nuclear pellet. The nuclear pellet was resuspended in 2 ml TDMKS and recentrifuged at 800 *g* The supernatant fractions were pooled and centrifuged at 10,000 *g* for 15 min to obtain the mitochondrial pellet The supernatant fraction was centrifuged at 105,000 *g* for 60 min to obtain the microsomal pellet and the cytosol The nuclear and the mitochondrial pellets were resuspended in 2 ml TDMKS, and the microsomal pellet was resuspended in 2 ml TDMKS containing 0.1% Triton X-100. All the above operations were performed at 4°. The macromolecules in each fraction were precipitated by addition of an equal volume of ice-cold 10% TCA for 60 min at 4° and collected by centrifugation The pellets were washed with organic solvents, dried, and solubilized as described in the previous section

Treatment of [^{3}H]DES complexes in subcellular fractions with hydrolytic enzymes Approximately 1 mg of washed and dried macromolecular samples obtained from the subcellular fractions was suspended in 1 ml of buffer with or without enzyme Pronase was dissolved in PBS at a concentration of 0 5 mg/ml. Ribonuclease A (RNase) was dissolved in 0 1 M sodium acetate, pH 5 0. Deoxyribonuclease I (DNase) was dissolved in 15 mM NaCl/0 1 M sodium acetate/5 mM $MgSO_4$, pH 5 0 All buffers contained 0.1% Triton X-100 The Pronase and RNase samples were incubated at 37°, and the DNase samples were incubated at 25° All samples were incubated for 18 hr and then 1 ml of 10% TCA was added to 1 hr at 4°. The TCA-insoluble material was collected on Whatman GF/B glass fiber filters and washed with de-ionized water The filters were placed in scintillation vials, and the trapped material was digested with NCS, neutralized with acetic acid, and counted in 10 ml Liquiscint

HPLC purification of [^{3}H]DES and analysis of incubation medium extracts. The toluene in which the [^{3}H]DES was dissolved was evaporated under a stream of nitrogen and the residue was dissolved in HPLC grade MeOH. The radiolabel was chromatographed using a Varian 5020 Liquid Chromatograph (Varian Associates, Palo Alto, CA) on a Dupont Zorbax ODS analytical column (4.6 mm × 25 cm) (Dupont Co., Wilmington, DE). A guard column packed with Co : Pell ODS 30–38 μm pelicular particles (Whatman Inc , Clifton, NJ) was placed between the injection port and the analytical column The mobile phase consisted of methanol and water and was run as a linear gradient, 40 to 100% methanol in 40 min. A modification was later employed: a linear gradient from 40 to 80% methanol in 35 min, followed by a linear gradient from 80 to 100% methanol in the next 5 min. The flow rate was maintained at 1.0 ml/min The [^{3}H]DES was greater than 99% pure by HPLC and contained a mixture of approximately 4 to 1 E-DES to Z-DES. The tissue incubation medium was acidified to pH 2–3 with 1 N HCl and extracted with 2 vol. of ether/ethyl acetate (1/1). The organic solvents were evaporated under a stream of nitrogen, and the residue was dissolved in methanol and filtered using a Millipore 0.5 μm SR filter (Millipore Corp., Bedford, MA). Samples were eluted employing the same methods as in the purification of [^{3}H]DES. Fractions were collected at 0.5-min intervals with an LKB Ultrarac 7000 fraction collector (LKB Instruments, Inc., Gaithersburg, MD). Aliquots (0.1 ml) were counted in 3 ml of Liquiscint Unlabeled standards were detected by measuring absorbance at 254 nm, and retention times were calculated using a Varian CDS 111L integrator.

RESULTS

Time dependence of [^{3}H]DES irreversible binding to hamster renal cortical slices Renal cortical slices from male and female hamsters were incubated with 50 nM [^{3}H]DES for 30, 60, 90, or 120 min under either 95% O_2/5% CO_2 or 100% nitrogen in the presence of 2 mM KCN. The amount of [^{3}H]DES associated with male hamster renal cortical macromolecules increased with incubation time, reaching 0.187 pmole [^{3}H]DES equiv./mg dry weight at 120 min under aerobic conditions (Fig. 1). The amount of irreversibly bound [^{3}H]DES in female cortical slices was 0.083 pmole [^{3}H]DES equiv./mg dry weight at 120 min under aerobic conditions. This was less than one-half the amount of irreversibly bound [^{3}H]DES observed in the male (Fig. 1) The amount of irreversible binding of [^{3}H]DES to kidney slices under anaerobic conditions was approximately 10–15% of the amount of binding observed under aerobic conditions (Fig. 1). The increase in [^{3}H]DES irreversible binding was not due to an increase of radioactivity in the tissue with time. Tissue levels of radiolabel remained constant, approximately 1.0 pmole [^{3}H]DES equiv./mg wet weight, between 30 and 120 min of incubation irrespective of the sex of the animal or the incubation conditions (Fig 1). The amount of radioactivity in the tissue after incubation was equivalent to approximately 50% of the [^{3}H]DES in the incubation medium at time zero. Measurements of radioactivity in the incubation medium before and after incubation correspondingly showed approximately a 50% decrease in radioactivity. Thus, for determining the nonspecific or background binding of [^{3}H]DES, one-half the amount of [^{3}H]DES used in the incubations was added directly to homogenates of the male or female hamster renal cortical slices. The tissue was washed as described in Methods and the remaining radioactivity was measured. For male and female hamster renal cortex, the background binding was 0.019 and 0.017 pmole [^{3}H]DES equiv /mg dry weight respectively. These values were subtracted from the total amount of irreversibly bound [^{3}H]DES.

Concentration dependence of [^{3}H]DES irreversible binding to hamster renal cortical slices Male and female renal cortical slices were incubated for 90 min with increasing concentrations of [^{3}H]DES up to 10 μM under aerobic and anaerobic conditions The

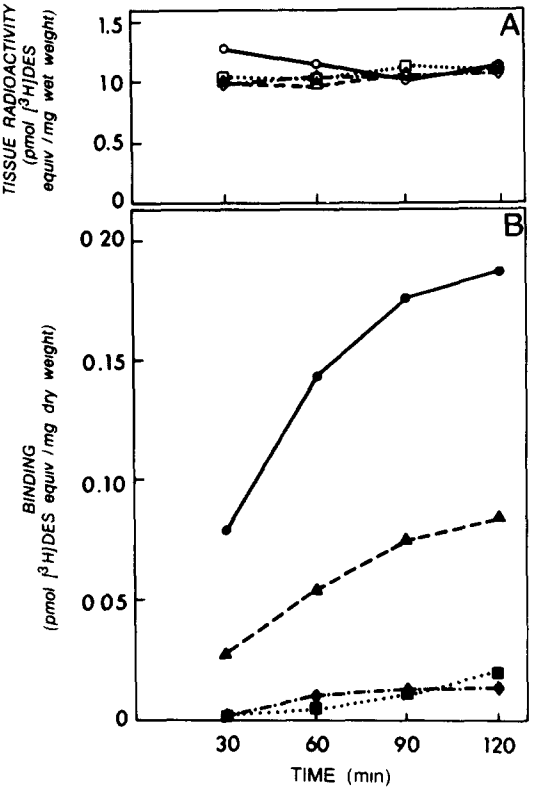

Fig 1 Effect of incubation time on irreversible binding of [^{3}H]DES metabolites to hamster renal cortical macromolecules. Cortical slices from male and female hamsters were incubated with 50 nM [^{3}H]DES at 37° under either aerobic (95% O_2/5% CO_2) or anaerobic (2 mM KCN + 100% N_2) conditions for the times cited (A) The tissue content of radioactivity was determined for each time point for (○) male, aerobic, (△) female, aerobic, (□) male, anaerobic, and (◇) female, anaerobic samples (B) The specific activity of irreversible binding of [^{3}H]DES was determined for each time point for (●) male, aerobic, (▲) female, aerobic, (■) male, anaerobic, and (◆) female, anaerobic samples The background values, 0 019 and 0.017 pmole [^{3}H]DES equiv /mg dry weight, were subtracted from each male and female sample respectively Values shown are the averages of duplicate samples

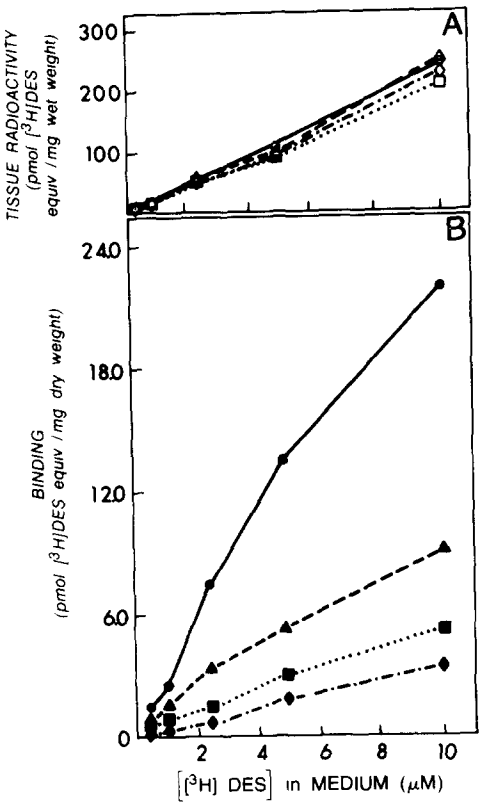

Fig 2 Effect of [^{3}H]DES concentration on the irreversible binding of [^{3}H]DES metabolites to hamster renal cortical macromolecules Cortical slices from male and female hamsters were incubated with 0 5 to 10 μM [^{3}H]DES for 90 min at 37° under aerobic (95% O_2/5% CO_2) or anaerobic (2 mM KCN + 100% N_2) conditions (A) After the incubation the content of tissue radioactivity was determined for (○) male, aerobic, (△) female, aerobic, (□) male, anaerobic, and (◇) female, anaerobic samples (B) The amount of irreversible binding was determined for (●) male, aerobic, (▲) female, aerobic, (■) male, anaerobic, and (◆) female, anaerobic samples Since background binding was not determined at each concentration, none was subtracted Values shown are the averages of duplicate samples

amount of [^{3}H]DES irreversibly bound to hamster renal cortical macromolecules increased nonlinearly with respect to [^{3}H]DES concentration in the medium Male hamster cortical slices incubated under aerobic conditions had the highest amount of irreversible binding at all concentrations 2- to 3-fold higher than female cortical slices under aerobic conditions, 3- to 4-fold higher for male cortical slices under anaerobic conditions, and 6- to 8-fold higher than female cortical slices under anaerobic conditions (Fig 2) Tissue levels of radioactivity increased linearly with respect to [^{3}H]DES concentration and were not greatly affected by either the sex of the animal or the incubation conditions (Fig 2)

Irreversible binding of [^{3}H]*DES by male hamster kidney slices under aerobic and anaerobic conditions* Renal cortical slices incubated with 10 μM [^{3}H]DES for 90 min had a 5-fold higher amount of non-extractable [^{3}H]DES in the acid-insoluble fraction as compared with the medullary tissue slices, and a 1 5-fold higher amount of irreversibly bound [^{3}H]DES as compared with slices containing both cortical and medullary elements (Table 1) Incubation of the slices with 2 mM KCN under nitrogen inhibited 92% of the irreversible binding of [^{3}H]DES as compared with slices incubated under aerobic conditions (Table 1). Background binding was determined and subtracted: 3.19, 3 35, and 3 32 pmole [^{3}H]DES equiv / mg dry weight for cortex, medulla and medulla and cortex respectively The difference in values obtained for the cortex between the two experiments (5 9 vs 18 6) may have resulted from a loss of viability due to the extra time that was necessary to obtain the medullary slices in the former experiment

HPLC analysis of extracts The [^{3}H]DES was metabolized extensively by male hamster kidney slices incubated under an atmosphere of oxygen, several radioactive peaks corresponding to oxidative metabolites of E-DES were found Radioactive species that co-eluted with Z,Z-DIES, 1-OH-Z,Z-DIES, 1-OH-E-DES, and 4′-hydroxypropiophenone were detected (Fig 3A) Conjugation of [^{3}H]DES was confirmed by the presence of a radioactive peak

Table 1 Irreversible binding of [^{3}H]DES by hamster kidney slices under aerobic and anaerobic incubation conditions

Kidney section	Incubation conditions	Irreversible binding (pmole [^{3}H]DES equiv /mg dry weight)
Medulla	95% O_2/5% CO_2	1 2
Cortex	95% O_2/5% CO_2	5 9
Medulla	2 mM KCN + 100% N_2	0 4
Cortex	2 mM KCN + 100% N_2	0 5
Medulla and cortex	95% O_2/5% CO_2	12 3
Cortex	95% O_2/5% CO_2	18 6
Medulla and cortex	2 mM KCN + 100% N_2	1 3
Cortex	2 mM KCN + 100% N_2	1 4

Renal tissue slices from male hamsters were incubated with 10 μM [^{3}H]DES for 90 min at 37° under aerobic conditions (95% O_2/5% CO_2) or anaerobic conditions (2 mM KCN under 100% N_2) Following incubation, the amount of irreversible binding was determined Values are means of duplicate samples Background values of 3 19, 3 35 and 3 32 pmole [^{3}H]DES equiv /mg dry weight have been subtracted for cortex, medulla and medulla and cortex respectively

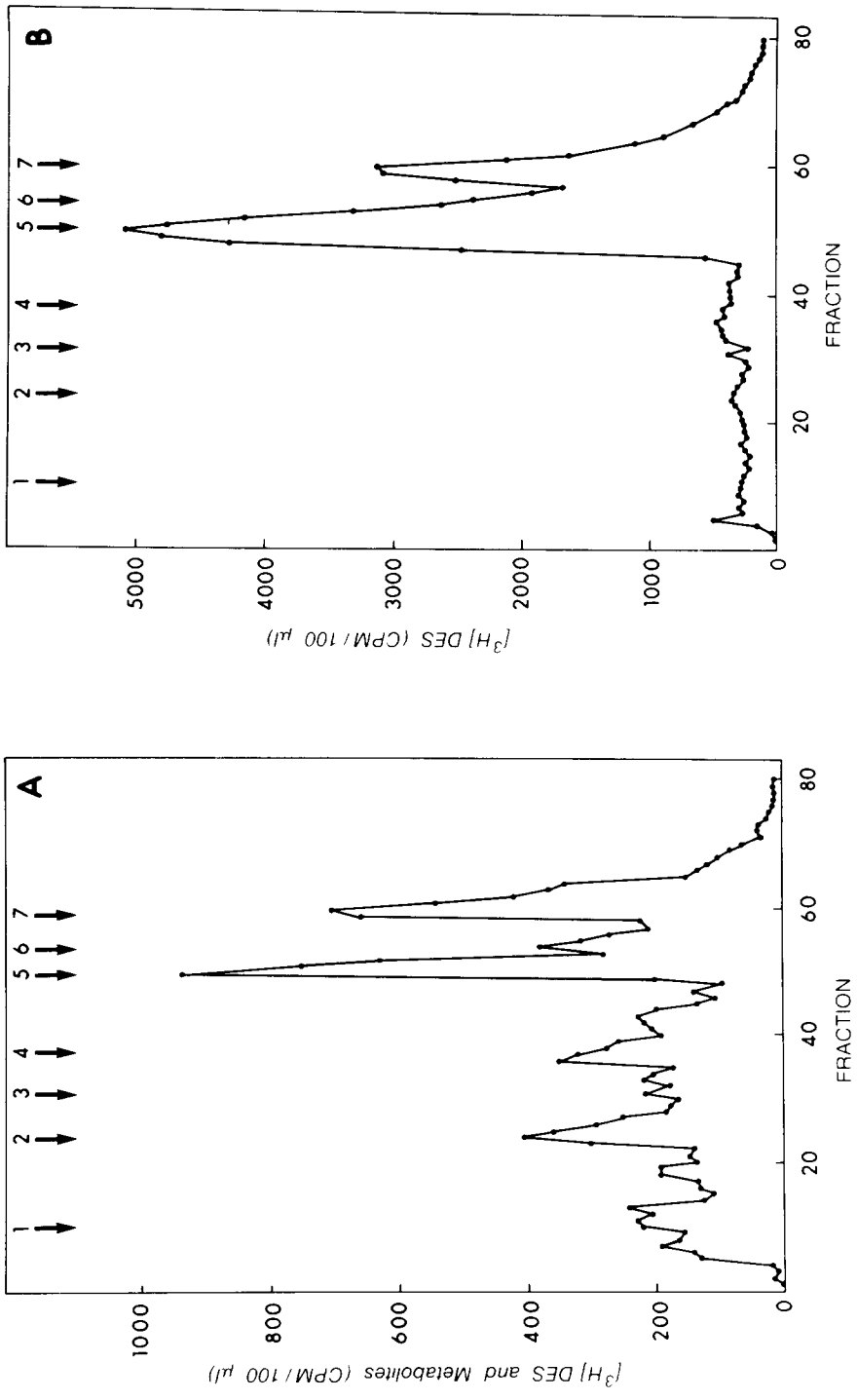

Fig. 3. Metabolic transformation of the [^{3}H]DES by male hamster kidney slices containing cortical and medullary elements under aerobic and anaerobic incubation conditions. The HPLC analyses of the extract from an aerobic (A) and an anaerobic (B) kidney slice incubation were performed on a Dupont Zorbax ODS column. The mobile phase was a linear gradient from 40% MeOH (H_2O) to 100% MeOH in 40 min at a flow rate of 1.0 ml/min. Fractions were collected every 0.5 min. The arrows indicate the following reference compounds: (1) E-DES glucuronide, (2) 4′-hydroxypropiophenone, (3) 1-hydroxy-Z,Z-DIES, (4) 1-hydroxy-E-DES, (5) E-DES, (6) Z,Z-DIES, and (7) Z-DES.

Table 2 Subcellular distribution of irreversibly bound [^{3}H]DES

Subcellular fraction	Irreversible binding (pmole [^{3}H]DES equiv /mg dry weight)
Microsomal	21 9
Cytosolic	18 9
Mitochondrial	15 2
Nuclear	12 8

Male hamster renal cortical slices were incubated with 10 μM [^{3}H]DES for 90 min at 37° under 95% O_2/5% CO_2 Subcellular fractions were isolated and the macromolecules were precipitated, exhaustively extracted with organic solvents and solubilized for determination of irreversible binding A background of 3 2 pmole [^{3}H]DES equiv /mg dry weight was subtracted

that co-eluted with E-DES glucuronide Several other radioactive peaks were present but have not been identified. However, [^{3}H]DES was not metabolized by male hamster kidney slices that were incubated in the presence of KCN under an atmosphere of nitrogen (Fig. 3B). Only two radioactive peaks were observed which corresponded to the geometrical isomers of DES. HPLC analysis of incubation medium containing 10 μM unlabeled DES after 90 min under 95% O_2/5% CO_2 showed that only E-DES and Z-DES were present, indicating that no oxidative species were produced under these conditions (data not shown)

Estimation of [^{3}H]*DES irreversible binding in subcellular fractions of hamster renal cortex and the effect of hydrolytic enzymes on* [^{3}H]*DES complexes.* Male hamster renal cortical slices were incubated with 10 μM [^{3}H]DES for 90 min Following subcellular fractionation, the TCA-insoluble macromolecules were isolated for determination of irreversible binding. Non-extractable binding of [^{3}H]DES was detected in all the subcellular fractions (Table 2) The highest specific activity was found in the microsomal fraction (21 9 pmole [^{3}H]DES equiv./mg dry weight) while the nuclear fraction had the lowest specific activity (12 8 pmole [^{3}H]DES equiv /mg dry weight). The [^{3}H]DES-macromolecular complexes obtained from the subcellular fractions were incubated overnight in the appropriate buffer with or without Pronase, RNase, or DNase Pronase was effective in solubilizing the bound [^{3}H]DES while the nucleases were not (Table 3)

DISCUSSION

In this report, we demonstrate that oxidative metabolism of [^{3}H]DES resulted in irreversible binding of DES metabolites to hamster kidney macromolecules. The *in vitro* incubation of kidney slices with [^{3}H]DES was found to be useful for studies of DES metabolism and formation of irreversible complexes between DES and cellular macromolecules The concentration of [^{3}H]DES in the incubation medium was known and relatively constant during the tissue incubation; the extrarenal DES metabolism was eliminated and the metabolites of DES were readily recovered from the incubation medium or tissue.

The concentration of DES complexes formed with kidney macromolecules was linear with respect to medium concentration of [^{3}H]DES up to 2.5 μM Above a 2 5 μM concentration of DES in the medium, the association of radioactivity with the macromolecules was non-linear, suggesting saturability of the DES-metabolizing enzymes or that availability of co-factors may have been limiting Correspondingly, the formation of DES-macromolecular complexes was linear with duration of incubation up to 60–90 min The deviation from linearity of [^{3}H]DES associated with the macromolecules beyond 90 min may have been due to co-factor availability or tissue viability.

Our results demonstrate that oxidative metabolism of DES was required to observe the non-extractable binding of the DES metabolites The metabolism of DES and its non-extractable binding were inhibited when the hamster kidney slices were incubated under

Table 3 Action of hydrolytic enzymes of irreversibly bound [^{3}H]DES

	Nuclear	Mitochondrial	Microsomal	Cytosolic
+ Pronase	1,702*	3,164	5,307	1,023
Buffer control	5,388	8,192	12,828	11,610
% of Buffer control	31 6	38 6	41 4	8 8
+ DNase I	6,570	8,082	9,454	7,859
Buffer control	5,991	7,399	8,241	7,413
% of Buffer control	109.7	109 2	114 7	106 0
+ RNase A	6,747	7,399	10,694	7,728
Buffer control	6,885	6,994	9,741	8,103
% of Buffer control	98 0	105 8	109 8	95 4

Washed and dried TCA-insoluble material obtained from subcellular fractions of male hamster renal cortical slices incubated with [^{3}H]DES was suspended in the appropriate buffer PBS (pH 7 4) ± 0 5 mg Pronase, 15 mM NaCl, 100 mM sodium acetate, 5 mM $MgSO_4$ (pH 5 0) ± 0 2 mg DNase I, or 100 mM sodium acetate (pH 5 0) ± 0 2 mg RNase A All suspension buffers contained 0 1% Triton X-100 The Pronase and RNase A samples and controls were incubated at 37°, and the DNase I samples and controls were incubated at 25° All samples were incubated for 18 hr after which ice-cold 10% TCA was added, and the precipitate was collected on glass-fiber filters, washed, solubilized and counted

* cpm/mg TCA-insoluble material

anaerobic conditions. In addition, the NADPH-dependent monooxygenase inhibitors SKF 525-A and metyrapone were observed to decrease the production of oxidative metabolites of [^{3}H]DES and consequently inhibit irreversible binding of [^{3}H]DES metabolites to cellular macromolecules.* These findings are in agreement with the *in vivo* observations of Li and Li [3, 4]; they reported that hamsters fed diets containing either BHA, an anti-oxidant, or α-napthoflavone, an inhibitor of NADPH-dependent monooxygenases, have a reduced incidence of DES-induced renal tumors as compared with hamsters on a regular diet Similarly, Liehr [5] reported that hamsters given ascorbic acid, an anti-oxidant, also have a reduced incidence of DES-induced renal tumors as compared with hamsters that do not receive ascorbic acid.

Examination of the subcellular fractions of male hamster cortical slices incubated with [^{3}H]DES showed that non-extractable binding was detectable in all four subcellular fractions. The microsomal fraction was found to have the highest specific activity of non-extractable [^{3}H]DES binding. This is not surprising since two enzymatic systems capable of metabolizing DES to a reactive intermediate are in the microsomal fraction. NADPH-dependent microsomal enzymes possess the capability to catalyze the irreversible binding of DES to macromolecules *in vitro* [14]. Prostaglandin synthetase and other microsomal peroxidases have also been shown to catalyze the irreversible binding of DES to macromolecules *in vitro* [15–22]. To observe covalent binding of DES in the other subcellular fractions, the reactive intermediate must be sufficiently stable to reach these targets. DES-4′,4″-quinone was found to have a half-life of 40 min in 10% aqueous methanol at 23° [23] In the cell the half-life would be shorter, but still probably long enough to reach various targets within the cell.

Treatment of the irreversibly bound [^{3}H]DES macromolecular complexes with hydrolytic enzymes (protease, RNase and DNase) indicated that only the protease degraded the radiolabeled macromolecular complexes. These data indicate that DES metabolites were binding principally to protein, but do not exclude the possibility of DES metabolites covalently binding to nucleic acids. However, it may not be necessary for DES to covalently bind to DNA in order to induce renal tumors in hamsters. It has been shown *in vitro* that DES is not genotoxic [24, 25], but still induces neoplastic and morphological transformations such as sister-chromatid exchange, aneuploidy, and unscheduled DNA synthesis [26–29] Recent findings have shown that exogenous metabolic activation of DES increased unscheduled DNA synthesis [30] and that the frequency of sister-chromatid exchanges induced by DES was higher in metabolically active hepatoma cell lines than in metabolically inactive fibroblast cell lines [31].

When non-extractable binding of [^{3}H]DES to macromolecules from the anatomically distinct regions of the kidney was measured, we found that the cortex had a much higher level of DES complexes as compared with the medulla. This correlates with the observation of Kirkman and Robbins [10] that the DES-induced renal tumor in hamsters has a cortical tubular origin. Our finding that male hamster kidney irreversibly bound more [^{3}H]DES than did female hamster kidney is relevant to the finding of Kirkman [11] that female hamsters exposed to DES do not develop the renal carcinomas. Our results with kidney slices have been corroborated by the *in vitro* demonstration by Haaf and Metzler [12] that male hamster kidney microsomal protein had five to ten times higher non-extractable binding of DES as compared with microsomal protein from non-target tissues (female hamster, male and female rat livers and kidneys). The difference in the non-extractable binding of [^{3}H]DES could have arisen from a difference in the levels of enzymes involved in the oxidative metabolism of DES

This work has shown that the hamster kidney can oxidatively metabolize DES, causing irreversible binding of a reactive intermediate with cellular macromolecules. The pattern of non-extractable binding of DES observed by us corresponds to sex- and site-differences of tumor induction by DES DES may be covalently binding to a protein involved in alteration of gene expression, for example the estrogen receptor or other nuclear regulatory proteins. Whether DES-adduct formation with critical cellular proteins results in cellular transformation remains to be tested.

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