Biochemical Pharmacology, Vol. 32, No. 24, pp. 3711–3718, 1983.
Printed in Great Britain.

0006-2952/83 $3.00 + 0.00
Pergamon Press Ltd.

DIETHYLSTILBESTROL (DES) QUINONE: A REACTIVE INTERMEDIATE IN DES METABOLISM*

JOACHIM G. LIEHR,† BEVERLY B. DAGUE, ANNIE M. BALLATORE and JACK HENKIN

Analytical Chemistry Center and Department of Biochemistry and Molecular Biology, University of Texas Medical School at Houston, Houston, TX 77030, U.S.A.

(*Received* 22 *October* 1982; *accepted* 9 *May* 1983)

Abstract—The quinone of E-diethylstilbestrol (DES), a postulated metabolic intermediate derived from DES, has been synthesized by oxidation of DES in chloroform using silver oxide. The reaction product was structurally characterized by infrared, ultraviolet, nuclear magnetic resonance, and mass spectrometry. The product of oxidation of DES by hydrogen peroxide, catalyzed by horseradish peroxidase and also by rat uterine peroxidase, was shown to be identical with synthetic DES quinone based on identical u.v. spectra and on identical decomposition products. DES quinone was stable only in non-protic solvents such as chloroform. In acids, bases or protic solvents, DES quinone rearranged to Z,Z-dienestrol (β-DIES). The half-life of DES quinone in water was approximately 40 min; in methanol it was approximately 70 min. Bacterial mutagenicity (Ames) tests did not indicate that DES quinone had mutagenic or genotoxic activity. However, DES quinone was found to bind to calf thymus DNA without any enzyme mediation at levels significantly above the binding of DES under the same conditions. Based on the binding of DES quinone to DNA, this intermediate must be considered as a possible carcinogenic metabolite of DES.

DES,‡ the synthetic estrogen which was widely used as a food additive for poultry and cattle and also for therapeutic purposes in humans, has been clearly established to be carcinogenic in humans and in rodents [1–4]. It is still not known whether carcinogenesis occurs via some hormonal mechanism or by metabolic activation of DES to an electrophilically reactive, genetically damaging intermediate. DES was not found to be active as a mutagen in bacterial assays such as the Ames test [5, 6] (making it one of the most notable exceptions to the relationship between mutagenic and carcinogenic action [7]), although it was positive in sister chromatid exchange induction [5] after metabolic activation and induced mutations in *Saccharomyces cerevisiae* strains XV185-14C and D5 in the presence of oxidizing agents [8]. DES was found to be as active as benzo[*a*]pyrene in inducing neoplastic cell transformation yet failed to induce mutations at two conventionally studied loci [9]. Studies of the oxidative biotransformation of DES have provided circumstantial evidence for the production of a transient quinone-like reactive intermediate [10–14]. The existence of DES quinone in metabolic reactions has never been clearly established, however, and its carcinogenic potential has not been evaluated, although its chemical synthesis had been reported previously [15]. The present study was undertaken to investigate the postulated DES quinone intermediate, to study its metabolic formation from DES and its reactivity and carcinogenic potential.

* Financial support was provided by the National Institutes of Health (1 RO1 CA 27539).

† Author to whom correspondence and reprint requests should be addressed.

‡ Abbreviations: DES, E-diethylstilbestrol; *cis*-DES, Z-diethylstilbestrol; DES quinone, diethylstilbestrol-4′,4″-quinone; β-DIES, Z,Z-dienestrol; MS, mass spectrometry; GC–MS, gas chromatography–mass spectrometry.

MATERIALS AND METHODS

Chemicals

DES, horseradish peroxidase (190 purpurogallin units/mg of solid), and calf thymus DNA (Type I, sodium salt) were purchased from the Sigma Chemical Co., St. Louis, MO. Silver oxide and hydrogen peroxide (30%) were obtained from the Fisher Scientific Co., Pittsburgh, PA. A sample of *cis*-DES was a gift of Dr. P. Murphy, Eli Lilly & Co., Indianapolis, IN.

Analytical instrumentation

Infrared spectra were recorded using a Perkin–Elmer 710B spectrophotometer. Ultraviolet spectra were recorded on a 8450A Hewlett–Packard high-speed spectrophotometer, scans usually being completed in 5–10 sec. ^{1}H-NMR spectra were obtained using a JEOL, model FX 900, 90 MHz. Mass spectra were recorded using a Finnigan, model 3200, quadrupole GC–MS system combined with an Incos data system. Spectra of underivatized compounds were obtained using the direct inlet system. GC–MS was carried out with trimethylsilylated samples [10% anhydrous pyridine and 90% *N,O*-bis-(trimethylsilyl)-trifluoroacetamide at 60° for 30 min]. A 6 ft × 2 mm glass column packed with 3% OV-1 on Gas-chrom Q 100/120 was used with a temperature program of 200–290° at an increase of 12°/min. Spectra of DES and metabolites were compared with published spectra of authentic material [14, 16].

Syntheses

DES quinone. DES (0.03 mmole, 8.0 mg) and 80 mg of silver oxide in 1.2 ml of chloroform (anhydrous) were stirred at room temperature for 20 min. The mixture was then filtered through celite and this solution was used for spectroscopic measurements. Ultraviolet: (H_2O) λ_{max} 310, 345 nm; (CH_3OH) λ_{max} 296, 354 nm. Infrared: ($CHCl_3$) 1640 cm^{-1} (unsaturated carbonyl). MS: *m/z* 266 molecular ion; the mass spectrum is identical with that of β-DIES [16]. ^{1}H-NMR: ($CDCl_3$, 90 MHz) δ 1.16 (t, 6H, CH_3); 2.2–3.3 (m, 4H, —CH_2—); 6.38 (m, 4H, =CH—C=O); 6.98 (dd, 2H, C<u>H</u>=CH—CO); 7.70 (dd, 2<u>H</u>=CH—CO).

β-DIES. β-DIES was synthesized from DES by oxidation with lead tetraacetate and subsequent rearrangement of the resulting DES quinone with aqueous sodium carbonate solution as described by von Euler and Adler [15]. The product was identified by u.v. and MS using underivatized material and also a trimethylsilylated derivative in GC–MS measurements.

Half-life of DES quinone

A DES quinone solution was prepared as described above by oxidation of DES using silver oxide except that methanol was used as solvent. The reaction mixture was stirred vigorously at 23° for 1 min and then filtered through celite. The decomposition of DES quinone in methanol was studied by measuring u.v. spectra at regular intervals. The decrease in absorption at 354 nm was recorded as a function of time. The percentage of remaining DES quinone vs time was plotted.

For the measurements of the half-life of DES quinone in water at 23°, a fresh DES quinone solution in methanol was prepared as described above. This solution was diluted with water to a final concentration of 10% methanol/90% water. Measurements of the half-life were carried out and plotted as was done for the decomposition in methanol.

In vitro *binding of DES quinone to DNA*

The methods of Blobstein *et al.* [17] and Jennette *et al.* [18] served as models for these studies. Radiolabeled DES and DES quinone solutions were prepared containing 15 μCi [monoethyl-1-^{14}C]DES (Amersham, 56 mCi/mmole) and 1.25 mg unlabeled DES per ml in $CHCl_3$/acetone (1/9, v/v). The radiolabeled DES quinone was prepared from a dried mixture of unlabeled and [^{14}C]DES suspended in $CHCl_3$ by oxidation with Ag_2O (8 times the amount of DES by weight) as described above. Acetone (9 vol.) was added at the end of the 30-min reaction period, and the suspension was centrifuged briefly. The DES quinone solution was transferred free of Ag_2O to a clean vial. All solvents were purchased from Burdick & Jackson, distilled-in-glass, and were stored over molecular sieves (Fisher, Davison Type 3A). Calf thymus DNA was dissolved at 2.5 mg DNA/ml water by heat denaturation in a boiling water bath for 15 min followed by quick-cooling in a salt–ice bath.

For the reaction of DNA with DES or DES quinone, 0.4 ml of acetone was added to 0.4 ml of heat-denatured DNA solution followed by 0.2 ml of the DES or DES quinone solution. The reactions were heated in a 37° water bath in tightly capped vials protected from light. At the end of the reaction period, the organic solvents were removed from the solutions by a stream of N_2, 0.6 ml of water was added, and the samples were extracted ten times with 2.0 ml of diethyl ether. After extraction, the volume of the samples was brought to 1.0 ml with water and 0.1 ml of 2 M sodium acetate buffer (pH 5) was added. Cold absolute ethanol (2.5 vol.) was added to the chilled samples and the DNA was precipitated overnight at −20°. The DNA was pelleted by centrifugation at 10,000 rpm in a Sorvall SS34 rotor at 4° for 20 min, redissolved in 1.0 ml of H_2O and 0.1 ml of 2 M sodium acetate buffer (pH 5) with heating, and precipitated with ethanol as before. A total of six precipitations was used to ensure that the DNA was clean of DES quinone breakdown products not extracted by diethyl ether and to reduce the amount of any DES quinone simply intercalated into the DNA molecule. The final DNA pellet was dissolved in water by heating in a boiling water bath. The DNA concentration was determined by u.v. absorbance measurements at 260 nm using an ε_{260} of 8.5×10^3 M^{-1} cm^{-1} [19]. Bound radioactivity, measured in Liquiscint (National Diagnostics) using a Beckmann LS-233 liquid scintillation counter, was expressed as dpm/μmole DNA (50% counting efficiency).

Peroxidase reactions with DES

Production of DES quinone from DES by horseradish peroxidase was examined by the method of Liao and Williams-Ashman [10]. For the reaction, 0.02 ml of 1.5 mg DES/ml ethanol was dissolved in 3.0 ml of 0.08 M Tris–HCL (pH 7.4) containing 0.33 mM hydrogen peroxide. Horseradish peroxidase was added to a final concentration of 0.13 purpurogallin units/ml reaction mixture in the sample cuvette. Ten-second u.v.-visible absorption scans were taken of the reaction at various times after the addition of enzyme.

An extract of rat uterus with peroxidase activity was prepared according to the method of Lyttle and DeSombre [20] from ovariectomized estrogen-induced rats. Peroxidative activity for the extract [in 10 mM Tris–HCl (pH 7.2) containing 0.5 M $CaCl_2$] was determined by the modified guaiacol assay [20] to be 34 units/ml or 110 units/mg protein. Protein was determined by the Bio Rad method [21] utilizing as a standard crystalline bovine serum albumin (Sigma, A7638) dissolved in 10 mM Tris–HCl (pH 7.2) containing 0.5 M $CaCl_2$. This tissue extract was assayed for the ability to convert DES to DES quinone by the addition of 0.01 ml of 1.0 mg DES/ml ethanol to a 0.99 ml reaction solution containing 0.2 ml of tissue extract, 0.76 ml of 0.15 M Tris–HCl (pH 7.4), and 0.033 ml of 10 mM H_2O_2. Ultraviolet-visible absorbance scans (2 sec) were taken at various times after the addition of DES to the reaction mixture. A chloroform extraction of the reaction mixture (Scaled up 10-fold) yielded a product which was identified by GC–MS (after trimethylsilylation) to be β-DIES.

Bacterial assays

The mutagenicity tests were performed as described by Ames *et al.* [22–24]. A mammalian metabolizing system for the activation of test compound was not used. DES quinone (in 50 μl of a mixture of 92% acetone and 8% chloroform) was added to 0.1 ml of log phase *Salmonella typhimurium* (histidine-dependent) bacterial strains TA 100, TA 1535, TA 1537, or TA 8414 in 2.0 ml of warm soft agar (0.6%). The solution was mixed and poured onto Vogel agar plates. After solidification of the top soft agar, the plates were incubated at 37° for 72 hr. The plates were then scored for his^+ revertants and compared with controls for which test compound was omitted. Results were expressed as the range of the number of colonies counted on three test plates. At concentrations of ≥300 μg DES quinone/plate a significant reduction of the background lawn was observed. These latter counts were, therefore, considered unreliable.

Tests for DNA-modifying activity were carried out as described by Rosenkranz and Leifer [25]. *Escherichia coli* strains pol A^+ and pol A_1^- were allowed to reach early exponential growth phase (approximately 2×10^8 cells/ml). The cultures were then diluted to a density of approximately 20,000 cells/ml. Test compounds in 17 μl of tetrahydrofuran were added to 5 ml of culture in test tubes. The tubes were shaken to mix the contents and then incubated at 37° for 1 hr. Aliquots (100 μl) of the treated cultures were spread onto the surface of HA agar plates, and the plates were incubated at 37° for 48 hr. Colonies were counted, and the results were expressed as the survival index, which is the ratio of the percent survivors pol A_1^- to the percent survivors pol A^+. The survival indices obtained from tests with DES quinone at various concentrations (10–100 μg/μg test mixture) were compared with those obtained from experiments with 4-nitroquinoline-*N*-oxide.

Stability of DES in biological fluids

Solutions of 100 μg *trans*-DES or *cis*-DES in 20 μl ethanol were diluted with 0.5 ml of human urine or plasma, obtained from a healthy male. The bottles were kept at 37° for 20 hr. The mixtures were extracted twice with ether. The organic phases were concentrated, dried and trimethylsilylated, and the contents were analyzed by GC–MS.

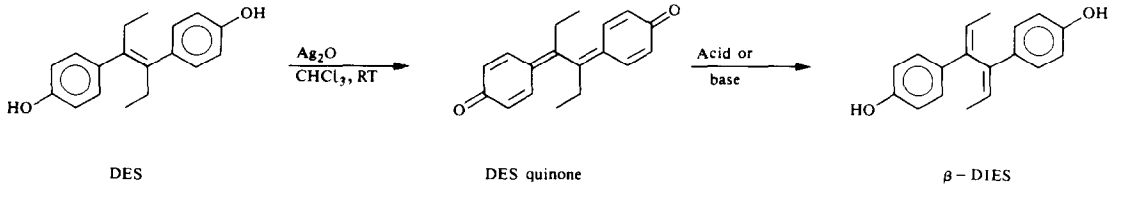
RESULTS

Synthesis and spectra

In initial attempts to synthesize DES quinone (Scheme 1), the procedure of von Euler and Adler [15] was followed. Probably due to the acetic acid formed in this reaction, a mixture of β-DIES and DES quinone was always isolated. Continuous conversion of DES quinone to β-DIES occurred during chromatographic isolation procedures. Therefore, a new improved synthesis of DES quinone was designed to be carried out in the absence of any acids, bases, and even protic solvents. Briefly, DES was oxidized with silver oxide in ethanol-free chloroform. The mixture was stirred vigorously at room temperature for 20 min and then filtered. DES quinone was found to be stable at room temperature for at least 3 days in this solvent. Upon standing in the dark at 23° for longer periods, slow conversion of DES quinone to β-DIES was observed. Therefore, all spectroscopic measurements were carried out with freshly prepared solutions. The mass spectrum of DES quinone was identical with that of β-DIES. Since samples introduced into the mass spectrometer by direct insertion have to be vaporized before ionization can take place, it is likely that isomerization of DES quinone to β-DIES took place during evaporation in the instrument. The mass spectrum, nevertheless, shows that oxidation to a species of molecular weight 266 had occurred. The unique i.r. and u.v. spectra, which are different from those of DES or β-DIES, and the evidence for intact ethyl side chains in the ^{1}H-NMR spectrum provided strong support for the proposed DES quinone structure (Scheme 1). It was not possible to obtain DES quinone in crystallized form. All studies of its reactivity, therefore, were carried out with DES quinone solutions. Since proton signals other than those reported were absent in the ^{1}H-NMR spectrum, almost quantitative conversion (>95% yield) of DES to DES quinone was assumed.

DES quinone formation by enzymes

The oxidation of DES catalyzed by horseradish peroxidase was carried out according to the procedure of Liao and Williams-Ashman [10] using approximately the same amounts of materials and the same reaction conditions. A similar increase in u.v. absorption at 310 nm and 345 nm was obtained within a few minutes after addition of peroxidase (Fig. 1). The absorption maxima and the shape of the absorption curve were found to be identical with those of synthetic DES quinone in water. Furthermore, addition of synthetic DES quinone to the horseradish peroxidase catalyzed oxidation reaction resulted in identical u.v. absorption characteristics with regard to absorption maxima and the shape of the absorption curve. Identical decomposition products (β-DIES) and identical u.v. absorption characteristics in these experiments were taken as evidence



Scheme 1. Synthesis and rearrangement of DES quinone.

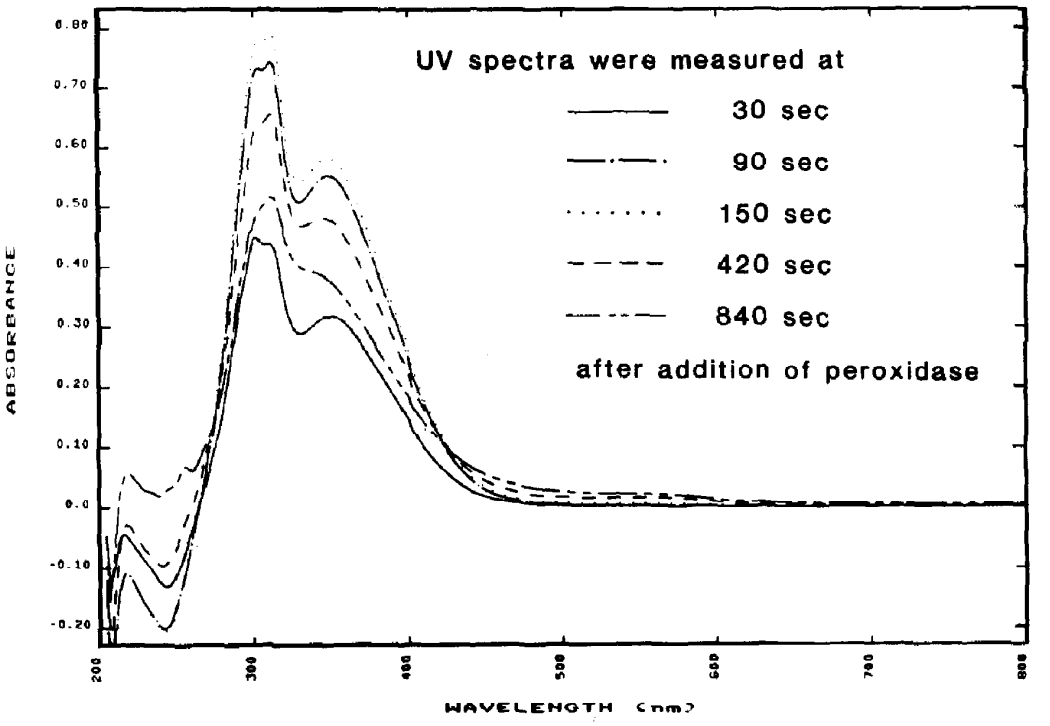


Fig. 1. Horseradish peroxidase catalyzed oxidation of DES to DES quinone.

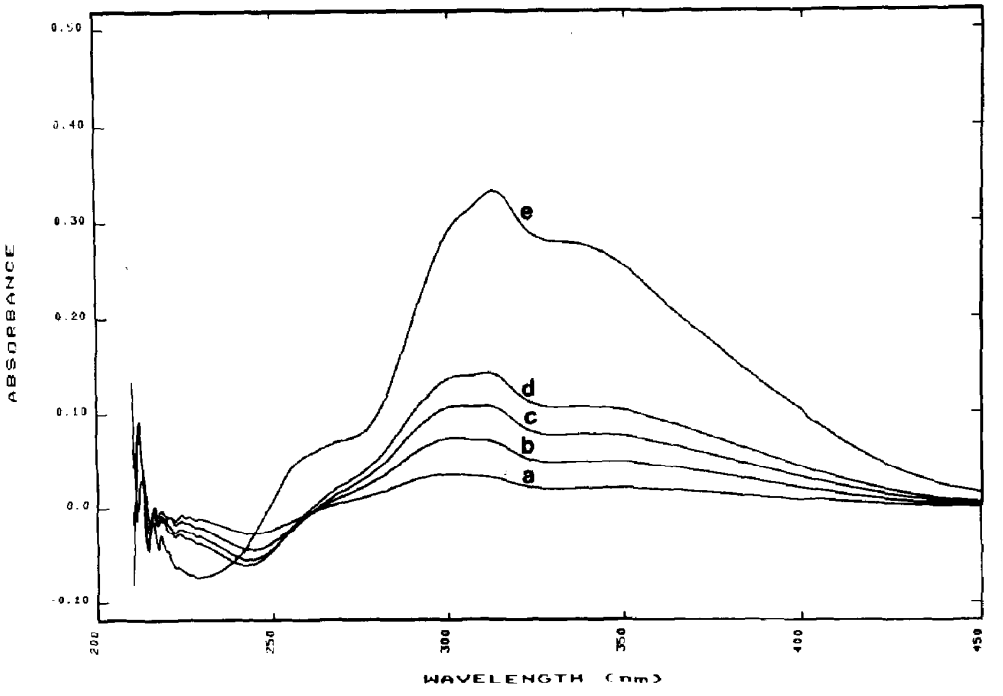


Fig. 2. Rat uterine peroxidase catalyzed oxidation of DES to DES quinone. Ultraviolet spectra were recorded at various times after addition of DES. The following difference spectra were plotted: (a) 2 min—20 sec, (b) 4 min—20 sec, (c) 6 min—20 sec, (d) 8 min—20 sec, and (e) 22 min—20 sec.

that the product of oxidation of DES catalyzed by horseradish peroxidase was DES quinone.

The oxidation of DES quinone catalyzed by estrogen-induced rat uterine peroxidase was also investigated. Uterine peroxidase of rats treated with estradiol was isolated, and its peroxidase activity was determined according to the procedures of Lyttle and DeSombre [20]. The rat uterine peroxidase catalyzed oxidation of DES was carried out essentially as described for the horseradish peroxidase catalyzed reaction [10] except that 0.12 M Tris buffer was used instead of 0.08 M Tris. Ultraviolet spectra obtained during the reaction were recorded. In the spectrum recorded 22 min after addition of DES, increases in absorption at 310 nm and 345 nm were observed. The difference spectrum obtained by subtracting the spectrum recorded 20 sec after addition of DES from the spectrum recorded 22 min after addition of DES clearly shows absorption maxima at 310 nm and 345 nm, identical with those obtained from synthetic DES quinone (Fig. 2). The ultimate product in the oxidation was identified by GC–MS methods as β-DIES. Rat uterine peroxidase thus catalyzed the oxidation of DES to DES quinone.

Reactivity of DES quinone

The reactivity of DES quinone and its carcinogenic potential were studed by determining its chemical stability in various solvents, by measuring its binding capability to DNA, and by testing its mutagenic potential in the Ames test. von Euler and Adler [15] have reported that DES quinone is unstable and, when exposed to acids or bases, readily rearranges mainly into β-DIES, one of the three sterically possible DIES isomers. In fact, DES quinone was found to be unstable in protic solvents such as water and methanol. In water its half-life at 23° was determined to be approximately 40 min and, in methanol its half-life was found to be about 70 min (Fig. 3). Its rearrangement to β-DIES in these solvents occurred at room temperature and probably was solvent-mediated. DES quinone was found to be quite stable in chloroform. Decomposition in this solvent was not measurable during a 72-hr period at room temperature.

Table 1. Binding of ^{14}C-labeled DES quinone to calf thymus DNA

Compound	Reaction time (hr)	Binding (dpm/μmole DNA)
[^{14}C]DES	0.5	34 ± 4*
	2	35 ± 3
	20	47 ± 9
[^{14}C]DES quinone	0.5	2100 ± 200
	2	1500 ± 200
	20	1100 ± 100

* Value are means ± S.D.

Synthetic DES quinone was found to bind to DNA. Calf thymus DNA and ^{14}C-labeled DES quinone in an acetone/water solvent system were allowed to react for varying lengths of time (Table 1). The solutions were extracted ten times with diethyl ether, and the DNA was then precipitated six times by addition of ethanol. Non-extractable radioactivity in the solubilized DNA was measured

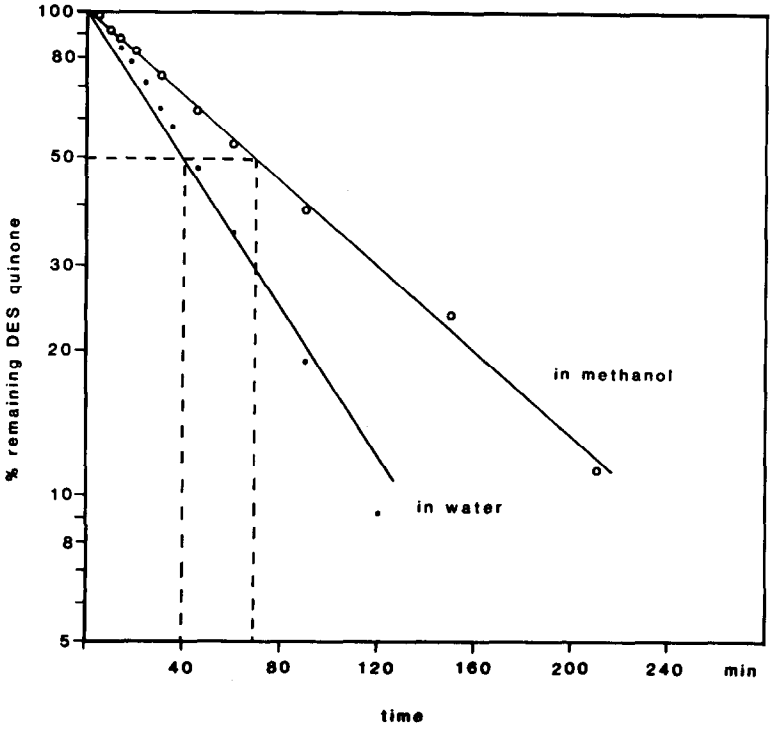


Fig. 3. Half-life of DES quinone in methanol (○) and in water (●).

Table 2. Reversion of his⁻ *S. typhimurium* to histidine prototrophy

Compound (μg/plate)		His⁺ colonies per plate (range) TA 100	TA 1535	TA 1537	TA 8414
Solvent control		141–199	43–61	28–49	ND*
DES quinone	(100)	150–183	52–80	24–42	46–67
	(200)	157–178	59–102	24–68	45–65
	(300)	161–183	64–108	25–36	31–52[T.P]†
	(400)	190–345[P.T]	56–59[T]	39–200[T]	ND
					39–72[T.P]

* Not determined.
† T: severe toxicity to background lawn was observed; and P: a precipitation on the test plate was observed.

and found to be significantly above non-extractable radioactivity levels in DNA treated with ^{14}C-labeled DES. With increasing reaction time, binding of DES quinone to DNA was found to decrease. At a reaction time of 20 hr, binding was approximately one-half of that found at a reaction time of 30 min. It is probable that initially both stable and unstable DES quinone-DNA adducts are formed. The unstable adduct may decompose with time and, therefore, at long reaction times only the stable form of binding may persist. The nature of the binding is presently being investigated.

Synthetic DES quinone was tested in the Ames bacterial assay to obtain an estimate of its mutagenic potential. Since DES quinone was shown to be an activated intermediate in metabolic reactions of DES, microsomal S9 fractions were not used to activate the test compound. Various bacterial tester strains were thus directly exposed to DES quinone. The strains used were *S. typhimurium* TA 100, TA 1537, TA 1535, and TA 8414. DES quinone solutions in 50 μl of acetone/chloroform (92%/8%) at various concentrations (100–400 μg/plate) were added to the bacteria in soft agar, and the mixtures were poured onto agar plates and incubated at 37° for 3 days. At concentrations of 100–200 μg DES quinone/plate, no increase in the number of revertant colonies were found over that for controls treated with solvent (Table 2). Severe toxicity to the bacterial cultures was encountered at concentrations of 300 μg DES quinone/plate or higher. Toxic effects manifested themselves as a reduction in background bacterial lawn, and results from such plates were therefore not considered reliable.

Tests for DNA-modifying activity were also carried out using *E. coli* strains pol A^+/pol A_1^-. The procedure used for testing was that of Rosenkranz and Leifer [25]. DES quinone concentrations used were 10–300 μg/culture test. In these experiments, as in the Ames assays using several *S. typhimurium* strains, no significant DNA modifying activity of DES quinone was detected. The survival index was not significantly different from 1, independent of the DES quinone concentration used in the mixtures. The positive control in this experiment, 4-nitroquinoline-*N*-oxide, showed strong genotoxicity effects. At a concentration of 10 μg of control compound/culture, the survival index was calculated to be approximately 0.1.

Stability of DES in biological fluids

The stability of *cis*-DES and *trans*-DES in human plasma and human urine kept at 37° for 20 hr was measured to determine the extent of *cis–trans* isomerization under biological conditions, i.e. in metabolism studies. *Trans*-DES did not measurably equilibrate in these experiments (Table 3). A small amount of the added *cis*-DES, approximately 5–6%, was converted to more stable *trans*-DES. White and Ludwig [26] had observed that isomerization to the *cis–trans* DES equilibrium mixture may be inhibited by antioxidants. The presence of antioxidants in biological fluids may account for the inhibition of equilibration observed here.

DISCUSSION

DES quinone had been postulated [12, 27] to be a metabolic intermediate derived from DES. The structural assignment for DES quinone reported here was based on the molecular weight of the product (mol. wt 266) of oxidation of DES (mol. wt 268) in conjunction with its ethyl side chains remaining intact as shown by its ^1H-NMR spectrum. For the terminal methyl groups of *cis*- or *trans*-DES, triplets at 0.93

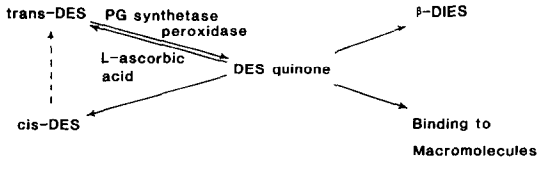
Table 3. Stability of DES in biological fluids

Compound added to fluid	Product distribution found after 20 hr at 37° Urine *cis*-DES (%)	Urine *trans*-DES (%)	Plasma *cis*-DES (%)	Plasma *trans*-DES (%)
trans-DES	<0.5	>99.5	<0.5	>99.5
cis-DES	93.8	6.2	95.3	4.7

or 0.77 ppm (in acetone-d_6; downfield from tetramethylsilane), respectively, had been reported [28]. A more downfield methyl triplet at 1.66 ppm was obtained for the oxidation product of DES, indicating an intact ethyl side chain of this material of molecular weight 266. In contrast, the methyl group of β-DIES is split by the methine proton into a doublet at 1.66 ppm [28]. Furthermore, the ^{1}H-NMR spectrum of the oxidation product did not contain any signals indicative of hydroxyl protons reported [28] for stilbene estrogens (8.17, 2H, s, *trans*-DES; 7.97, 2H, s, *cis*-DES; 8.18, 2H, s, β-DIES). Based on the spectra reported here and previously by Metzler and McLachlan [28], the oxidation product of DES was identified as DES quinone. A detailed structural investigation of this material by high resolution ^{1}H- and ^{13}C-NMR and comparison of its structural features with those of DES will be reported elsewhere.

The unique spectroscopic characteristics of DES quinone were used to identify quinone formation by enzymes. Liao and Williams-Ashman [10] found that horseradish peroxidase catalyzed the oxidation of DES by hydrogen peroxide to an unidentified substance absorbing at 310 and 345 nm within a few minutes after addition of the enzyme. The authors postulated the formation of DES quinone in this reaction. The data presented here identify DES quinone as the oxidation product formed from DES in a horseradish or uterine peroxidase catalyzed reaction.

The short half-life of DES quinone and its facile rearrangement to β-DIES explain the elusiveness of this intermediate and the failure to prove its existence in *in vivo* experiments. DES quinone, nevertheless, may play a central role in DES metabolism judged by the quantities of β-DIES identified *in vivo* or *in vitro* [14] (Scheme 2). Peroxidase, an estrogen-inducible enzyme preferentially found in tissues depending on estrogen for growth [20, 29], readily converted DES in DES quinone. Recently, Degen and McLachlan showed that prostaglandin endoperoxide synthetase, which is also estrogen-inducible [30] and is known [31, 32] to occur in organs susceptible to DES-induced carcinogenesis, was capable of cooxidizing DES to the corresponding DES quinone (G. H. Degen and J. A. McLachlan, personal communication). DES quinone is reduced [33] to a 1:1 mixture of *cis*-DES and *trans*-DES using such endogenous reducing substances as L-ascorbic acid. It is conceivable, however, that *cis*-DES, which has been found [14, 34] in DES metabolism experiments *in vivo* and *in vitro*, may be formed by other enzymic or non-enzymic endogenous reduction processes rather than via *cis/trans* isomerization reactions [26].



Scheme 2. Metabolic formation and reactions of DES quinone.

Whatever the pathway of formation, the stabilities of *cis*- and trans-DES in biological fluids suggest that *cis*-DES is a true DES metabolite and not a product of equilibration of the administered *trans*-DES. The duration of *in vivo* metabolism experiments allows for a small amount of *cis*-DES to isomerize to *trans*-DES; however, the major amount of metabolic *cis*-DES formed probably can be isolated as such. To estimate the amount of quinone formed from administered DES, the amounts of β-DIES and (of at least a portion) of *cis*-DES and an equal amount of *trans*-DES must be added. Such rough estimates suggest that DES quinone may be a major intermediate derived from DES. Correspondingly, in most species only small quantities of other oxidative metabolites were identified [14].

It is postulated that DES quinone is (one of) the carcinogenic intermediate(s) derived from DES. The capability of DES quinone to bind to DNA is thought to support this hypothesis. Previously, peroxidase-mediated binding of DES to DNA and also to protein was taken as evidence [12] for the toxic action of the postulated DES quinone intermediate.

Negative results in the bacterial mutagenicity assays of DES quinone were not surprising. DES has been considered a carcinogen, although bacterial testing with or without metabolic activation did not establish this substance as mutagenic or genotoxic [5]. No mutagenic activity was found even when peroxidase was used for metabolic activation [35]. Why estrogens are not mutagenic in bacterial test systems remains unclear.

Acknowledgements—The authors would like to thank Mr. Paul J. Terracciano and Mrs. Tsen-Ying Fan for their skillful operation of the mass spectrometer. The bacterial assays were carried out in the laboratory of Dr. Thomas S. Matney, University of Texas Graduate School of Biomedical Sciences, Houston, TX. Dr. Matney's helpful discussions and constant encouragement were deeply appreciated.

REFERENCES

1. A. L. Herbst, H. Uhlfelder and D. C. Poskanzer, *New Engl. J. Med.* **284**, 878 (1971).
2. A. L. Herbst, P. Cole, T. Colton, S. J. Robboy and R. E. Scully, *Am. J. Obstet. Gynec.* **128**, 43 (1977).
3. J. A. McLachlan and R. L. Dixon, in *Advances in Modern Toxicology* (Eds. M. A. Mehlman, R. E. Shapiro and H. Blumenthal). Vol. 1, p. 423. John Wiley, New York (1976).
4. *Evaluation of the Carcinogenic Risk of Chemicals to Humans*, vol. 21, p. 173. International Agency for Research on Cancer, Lyon, France (1979).
5. H. W. Rüdiger, F. Haenisch, M. Metzler, F. Oesch and H. R. Glatt, *Nature, Lond.* **281**, 392 (1979).
6. M. Metzler, R. Gottschlich and J. A. McLachlan, in *Estrogens in the Environment* (ED. J. A. McLachlan), p. 293. Elsevier/North Holland, Amsterdam (1980).
7. B. N. Ames, *Science* **204**, 587 (1979).
8. R. D. Mehta and R. C. von Borstel, *Mutation Res.* **92**, 49 (1982).
9. J. C. Barrett, A. Wong and J. A. McLachlan, *Science* **212** 1402 (1981).
10. S. Liao and H. G. Williams-Ashman, *Biochim. biophys. Acta.* **59**, 705 (1962).
11. M. Metzler and J. A. McLachlan, *Archs Toxic. Suppl.* **2**, 275 (1979).

12. M. Metzler and J. A. McLachlan, *Biochem. biophys. Res. Commun.* **85**, 874 (1978).
13. M. Metzler and J. A. McLachlan, in *Role of Pharmacokinetics in Prenatal and Perinatal Toxicology* (Eds. D. Neubert, H. J. Merker, H. Nau and J. Langman), p. 157. Georg Thieme, Stuttgart, Germany (1978).
14. M. Metzler, *CRC Crit. Rev. Biochem.* **10**, 171 (1981).
15. H. von Euler and E. Adler, in *The Svedberg*, 1884 $\frac{30}{8}$ 1944 (Eds. A. Jiselins and K. O. Pedersen), Almqvist & Wiksells, p. 246. Uppsala, Sweden (1944).
16. L. L. Engel, P. J. Marshall, J. C. Orr, V. N. Reinhold and P. Carter, *Biomed. Mass Spectrom.* **5**, 582 (1978).
17. S. H. Blobstein, I. B. Weinstein, D. Grunberger, J. Weisgras and R. G. Harvey, *Biochemistry* **14**, 3451 (1975).
18. K. W. Jennette, A. M. Jeffrey, S. H. Blobstein, F. A. Beland, R. G. Harvey and I. B. Weinstein, *Biochemistry* **16**, 932 (1977).
19. I. B. Weinstein, A. M. Jeffrey, K. W. Jennette, S. H. Blobstein, R. G. Harvey, C. Harris, H. Autrup, H. Kasai and K. Nakanishi, *Science* **193**, 592 (1976).
20. C. R. Lyttle and E. R. DeSombre, *Proc. natn. Acad. Sci. U.S.A.* **74**, 3162 (1977).
21. M. M. Bradford, *Analyt. Biochem.* **72**, 248 (1976).
22. B. N. Ames, W. E. Durston, E. Yamasaki and F. D. Lee, *Proc. natn. Acad. Sci. U.S.A.* **70**, 2281 (1973).
23. B. N. Ames, F. D. Lee and W. E. Durston, *Proc. natn. Acad. Sci. U.S.A.* **70**, 782 (1973).
24. B. N. Ames, J. McCann and E. Yamasaki, *Mutation Res.* **31**, 347 (1975).
25. H. S. Rosenkranz and Z. Leifer, in *Chemical Mutagens, Principles and Methods for Their Detection* (Eds. F. J. DeSerres and A. Hollaender), Vol. 6, p. 113. Plenum Press, New York (1980).
26. W. A. White and N. H. Ludwig, *J. agric. Fd Chem.* **19**, 388 (1971).
27. E. C. Horning, J. P. Thenot and E. D. Helton, *J. Toxic. environ. Hlth* **4**, 341 (1978).
28. M. Metzler and J. A. McLachlan, *J. environ. Path. Toxic.* **2**, 259 (1978).
29. C. R. Lyttle and E. R. DeSombre, *Nature, Lond.* **268**, 337 (1977).
30. W. C. Chang, J. Nakao, H. Orimo, H. H. Tai and S. Murota, *Fedn Proc.* **41**, 1442 (1982).
31. B. B. Davis, M. B. Mattamal and T. V. Zenser, *Nephron* **27**, 187 (1981).
32. M. H. Abel and D. T. Baird, *Endocrinology* **106**, 1599 (1979).
33. J. G. Liehr, W. J. Wheeler and A. M. Ballatore, in *Proceedings of the First International Symposium on the Modulation and Mediation of Cancer by Vitamins* (Eds. F. L. Meyskens and K. N. Prasad), in press. S. Karger, Basel, Switzerland (1983).
34. M. Metzler and J. A. McLachlan, *Biochem. Pharmac.* **27**, 1087 (1978).
35. H. R. Glatt, M. Metzler and F. Oesch, *Mutation Res.* **67**, 113 (1979).