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Metabolism of Diethylstilbestrol by Horseradish Peroxidase and Prostaglandin-H Synthase

GENERATION OF A FREE RADICAL INTERMEDIATE AND ITS INTERACTION WITH GLUTATHIONE*

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Diethylstilbestrol is carcinogenic in rodents and in humans and its peroxidatic oxidation *in utero* has been associated with its carcinogenic activity. Horseradish peroxidase-catalyzed oxidation of [^{14}C]diethylstilbestrol and [^{14}C]diethylstilbestrol analogs induced binding of radiolabel to DNA only when the compound contained a free hydroxy group (Metzler, M., and Epe, B. (1984) *Chem. Biol. Interact.* 50, 351–360). We have found that horseradish peroxidase or prostaglandin-H synthase-catalyzed oxidation of diethylstilbestrol in the presence of the spin trap 5,5-dimethyl-1-pyrroline-*N*-oxide caused the generation of an ESR signal indicative of a free radical intermediate (a_N = 14.9 G, a_H = 18.3 G). The identity of the trapped radical could not be identified on the basis of published hyperfine coupling constants, but the observation that horseradish peroxidase-catalyzed oxidation of 1-naphthol produced an identical ESR signal suggests that the radical was either a phenoxy or phenoxy-derived radical. During horseradish peroxidase-catalyzed oxidation of diethylstilbestrol in the presence of glutathione the thiol reduced the diethylstilbestrol radical to generate a thiyl radical. This was shown by a thiol-dependent oxygen uptake during horseradish peroxidase-catalyzed oxidation of diethylstilbestrol and the observation of an ESR signal consistent with 5,5-dimethylpyrroline-*N*-oxide-glutathionyl radical adduct formation. A diethylstilbestrol analog devoid of free hydroxy groups, namely diethylstilbestrol dipropionate, did not produce an ESR signal above control levels during horseradish peroxidase-catalyzed metabolism in the presence of 5,5-dimethylpyrroline-*N*-oxide. Thus, free radicals are formed during peroxidatic oxidation of diethylstilbestrol and must be considered as possible determinants of the genotoxic activity of this compound.

Diethylstilbestrol (DES[1]) is a synthetic estrogen that has been used as a food additive for livestock and, in women, to prevent miscarriages and as a postcoital contraceptive. DES has been shown to be carcinogenic in animals (1) and its use as an abortifacient in humans has been linked to the appearance of genital tract tumors in women whose mothers were treated with the drug (2, 3). Reproductive tract lesions in human males (4–6), and in mice (7) exposed prenatally to DES have also been reported. Furthermore, DES has been shown to cause kidney carcinoma in Syrian hamsters (8) and to induce neoplastic transformation in Syrian hamster embryo fibroblasts *in vitro* (9).

The mechanisms underlying DES carcinogenicity are as yet unresolved but have been thought to involve the estrogenic activity of the compound (1). Recent data, however, from *in vivo* carcinogenicity studies (10, 11) and structure-activity studies on neoplastic transformation *in vitro* (9) suggest that hormonal activity alone is insufficient to explain the carcinogenicity of DES.

DES is metabolized in both animals and man (12, 13) and has been shown to be extensively oxidized to reactive intermediates by peroxidases *in utero* (14). Thus peroxidatic oxidation of DES is a possible determinant of its carcinogenicity. One product of the peroxidase-catalyzed oxidation of DES by uterine peroxidase and horseradish peroxidase has been characterized as DES quinone, which covalently binds to DNA and is thus a possible cause of the carcinogenic activity of DES (15). A recent study showed, however, that horseradish peroxidase-catalyzed metabolism of [^{14}C]DES derivatives that could not form quinones resulted in DNA binding. This study showed that at least one free hydroxy group was required for DNA binding and suggested that the phenoxy free radical derived from DES was the causative agent (16). The formation of free radicals during peroxidase-catalyzed metabolism of DES has, however, never been shown.

In this study we have examined the metabolism of DES by horseradish peroxidase and prostaglandin synthase, a peroxidase that is present in organs susceptible to DES-induced carcinogenesis (17, 18). We show that both of these peroxidatic oxidations proceed via the production of free radical intermediates that can interact with the endogenous protective thiol glutathione (GSH) to produce glutathionyl radicals.

MATERIALS AND METHODS

Chemicals and Enzymes—DES, DES dipropionate, 1-naphthol, horseradish peroxidase Type VI, hydrogen peroxide, arachidonic acid, hematin, and indomethacin were obtained from Sigma. 5-5-Dimethyl-1-pyrroline-*N*-oxide (DMPO) was obtained from Aldrich. *p*-Phenetidine hydrochloride was synthesized as described previously (19). Purified prostaglandin-H synthase was a generous gift from Prof. L. Marnett, Department of Chemistry, Wayne State University, Detroit, MI.

Incubations with Horseradish Peroxidase—The following condi-

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[1] The abbreviations used are: DES, diethylstilbestrol; DMPO, 5,5-dimethylpyrroline-*N*-oxide.

tions were used: hydrogen peroxide (0.1 mM), horseradish peroxidase (3 μg/ml), DES or 1-naphthol (0.1 mM), and in some cases GSH (5 mM). Potassium phosphate buffer (0.1 M, pH = 7.4) was used for all experiments. In experiments using ESR spectroscopy, DMPO (0.1 M) was included in the reaction mixture. Reactions were performed at 25 °C and were initiated by the addition of hydrogen peroxide.

Incubations with Prostaglandin-H Synthase—The following conditions were used: arachidonic acid (0.1 mM) or hydrogen peroxide (0.05 mM), purified prostaglandin-H synthase (equivalent to 12.3 μg of protein), DES (0.1 mM), hematin (1.0 μM), and DMPO (0.1 M). Reactions were performed at 25 °C and were initiated by addition of either arachidonic acid or hydrogen peroxide.

Oxygen Uptake—This was measured using a Clarke electrode in a sample volume of 3 ml at 25 °C.

ESR Spectroscopy—This was performed on a Varian E109E spectrometer at room temperature. Instrument conditions, unless otherwise stated, were power (10 mW), modulation amplitude (1.25 G), time constant (0.064 s), receiver gain (2.5×10^4), and scan time (1 min).

RESULTS

Horseradish peroxidase- and prostaglandin-H synthase-catalyzed oxidation of DES leads to the generation of colored metabolites and the inclusion of GSH in such reactions inhibits color development. This suggests that either GSH reacts with these metabolites or prevents their formation. Horseradish peroxidase-catalyzed co-oxidation of xenobiotics, driven by hydrogen peroxide, is not an oxygen-consuming reaction (20), and there was no immediate oxygen uptake observed during horseradish peroxidase-catalyzed oxidation of DES. Interestingly, a small delayed oxygen uptake was observed (Table I) reflecting, presumably, oxidation of a primary metabolite. In the presence of GSH, however, an immediate oxygen uptake was apparent (Table I) and the incubation mixture remained colorless. A GSH-dependent oxygen uptake was also observed during horseradish peroxidase-catalyzed oxidation of another phenolic compound, 1-naphthol, in the presence of GSH (Table I). We have previously shown that thiol-dependent oxygen uptake occurs during horseradish peroxidase-catalyzed oxidation of both *p*-phenetidine and acetaminophen (21, 22), reactions known to involve the generation of cosubstrate-derived free radicals (22, 23). The mechanism underlying this oxygen consumption involves the reduction of the cosubstrate-derived radical by GSH to generate a thiyl radical that then either reacts with oxygen to form a peroxysulfenyl radical or with GSH to form a glutathionyl anion radical that then autoxidizes to form superoxide (24).

TABLE I

GSH-dependent oxygen uptake observed during the horseradish peroxidase-catalyed oxidation of diethylstilbestrol(A) and 1-naphthol(B)

Complete incubations contained H_2O_2 (0.1 mM), horseradish peroxidase (3 μg/ml), GSH (5 mM), and either DES (0.1 mM, A) or 1-naphthol (0.1 mM, B). Incubations without DES contained an equivalent volume of the solvent dimethyl sulfoxide.

Incubation mixture	Initial rate of oxygen uptake
	nmol/ml/min
A) Complete	18 ± 2 (n = 3)
minus GSH	0[a]
minus DES	0
minus horseradish peroxidase	0
B) Complete	23
minus GSH	0

[a] A delayed rate of oxygen consumption was consistently observed in these reactions (delay 30 s, rate 5 ± 1 nmol/ml/min).

These results imply that free radical species are produced during the peroxidatic oxidation of DES, and we sought to confirm radical formation with ESR spectroscopy. Indeed, in the presence of the spin trap, DMPO, a 6-line paramagnetic signal (a_N = 14.9 G, a_H = 18.3 G) could be detected during horseradish peroxidase-catalyzed oxidation of DES (Fig. 1*A*). Control reactions without DES, in the presence of boiled enzyme, without enzyme, without hydrogen peroxide, or without DMPO produced signals indistinguishable from noise (Fig. 1*B*). This signal was not dependent on the presence of solvent (dimethyl sulfoxide), since control experiments using ethanol, where the solvent was evaporated prior to initiation of the enzyme reaction, still produced the 6-line signal (as in Fig. 1*A*). The ESR signal shown in Fig. 1*A* was short-lived and decayed appreciably within three successive 1-min scans. When the reaction was performed in the absence of DMPO no signal could be detected. Similarly, if the spin trap was added after 15 min of reaction no signal was detected, confirming the transient nature of the radical. The identity of the radical species (Fig. 1*A*) could not be deduced from our data; the hyperfine coupling constants of the observed signal cannot be identified with available published constants (25). We also studied the horseradish peroxidase-catalyzed oxidation of another phenolic compound, 1-naphthol, and found an identical six-line signal, which was enzyme- and cosubstrate-dependent (Fig. 2). Horseradish peroxidase-catalyzed oxidation of DES dipropionate (0.1 mM) and the aromatic amine *p*-phenetidine (0.1–0.5 mM) produced no ESR signals, showing that a free hydroxy group is needed for a cosubstrate to produce this particular radical signal, and that enzyme turnover induced by very efficient cosubstrates such as *p*-phenetidine is not sufficient to generate this signal.

The above experiments show that a free radical species is generated during horseradish peroxidase-catalyzed oxidation of DES. Moreover, the data shown in Table I suggest that such a radical can interact with GSH to form a thiyl radical. This was confirmed using DMPO to trap the glutathionyl radical (26–28) as shown in Fig. 3. An ESR signal identical to the signal observed when glutathione was treated with a hydroxyl radical-generating system (data not shown) was

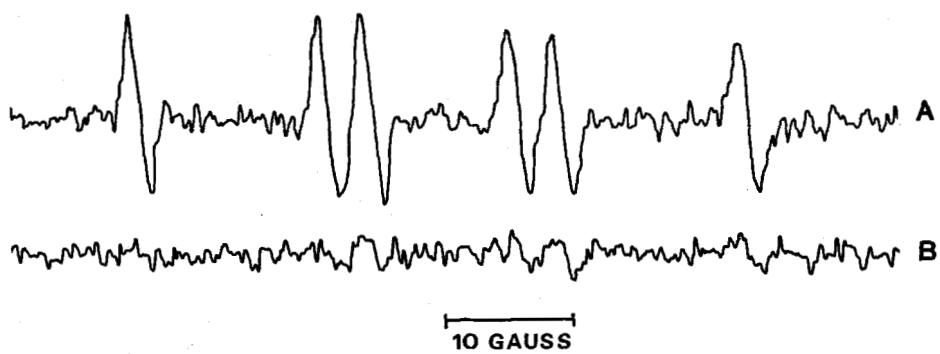

FIG. 1. **ESR spectra observed during horseradish peroxidase-catalyzed oxidation of DES in the presence of DMPO.** *A*, DES added as a dimethyl sulfoxide solution (0.1 mM), horseradish peroxidase (3 μg/ml), hydrogen peroxide (0.1 mM), and DMPO (0.1 M); *B*, as in *A*, but with DES omitted from the dimethyl sulfoxide. Similar spectra were seen when horseradish peroxidase was omitted, when hydrogen peroxide was omitted, when DMPO was omitted, or when boiled enzyme was substituted for horseradish peroxidase.

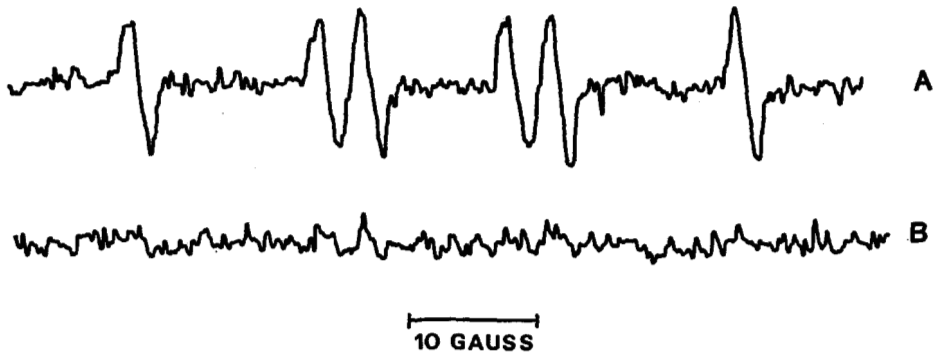

FIG. 2. **ESR spectra observed during horseradish peroxidase-catalyzed oxidation of 1-naphthol in the presence of DMPO.** *A*, 1-naphthol in dimethyl sulfoxide (0.1 mM), horseradish peroxidase (3 μg/ml), hydrogen peroxide (0.1 mM), and DMPO (0.1 M); *B*, as in *A* but with 1-naphthol omitted from the dimethyl sulfoxide. A similar spectrum was observed when horseradish peroxidase was omitted.

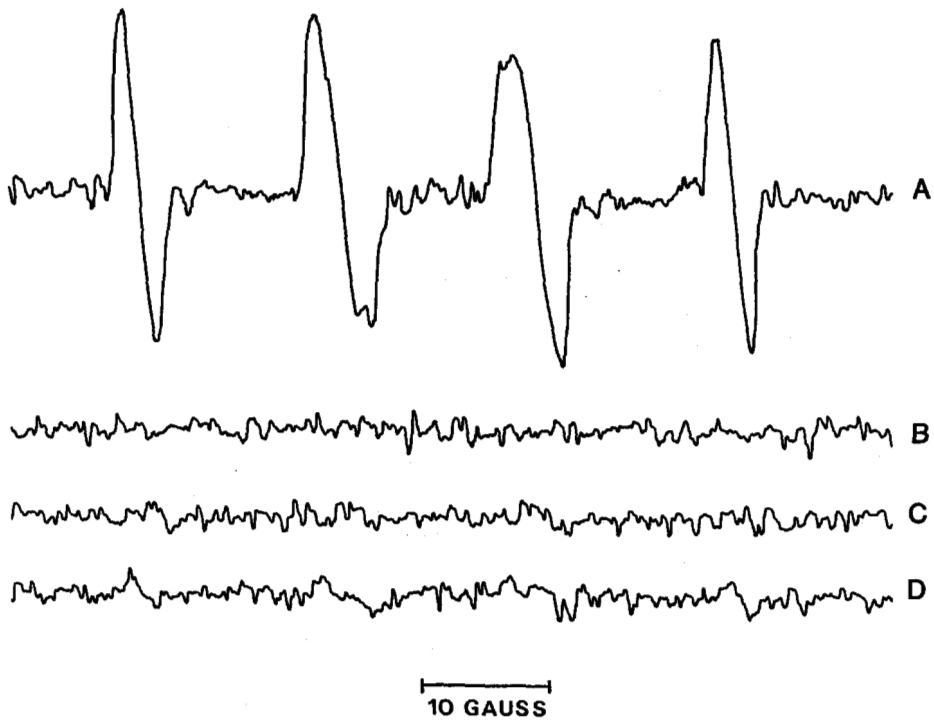

FIG. 3. **ESR spectra resulting from the horseradish peroxidase-catalyzed oxidation of DES in the presence of GSH and DMPO.** *A*, as in Fig. 1*A*, but with 5 mM GSH added; *B*, as in *A* but with horseradish peroxidase omitted; *C*, DES omitted from the dimethyl sulfoxide; *D*, GSH added after incubating a GSH-free reaction mixture for 15 min.

observed during the horseradish peroxidase-catalyzed oxidation of DES in the presence of DMPO and GSH (Fig. 3*A*), which was stable up to 3 min after initiation of the reaction and then subsequently declined. This signal was enzyme- and DES-dependent (Fig. 3, *B* and *C*) and was not produced if GSH was omitted from the initial reaction mixture or if it was added to the reaction after 15 min (Fig. 3*D*).

To show that these reactions occurred in the presence of a biologically relevant peroxidase, the ESR investigations were repeated using prostaglandin-H synthase rather than horseradish peroxidase. Fig. 4*A* shows that the 6-line signal obtained during horseradish peroxidase-catalyzed oxidation was also obtained during prostaglandin-H synthase-catalyzed metabolism of DES in the presence of arachidonic acid. A small signal with identical coupling constants to the previous signal was also obtained in the absence of DES (Fig. 4*B*) and was presumably due to turnover of some component associated with the reaction mixture by the enzyme. The generation of the intense 6-line ESR signal was arachidonate- and prostaglandin-H synthase-dependent (Fig. 4, *C* and *D*), and was almost totally inhibited by indomethacin (Fig. 4*E*), an inhibitor of prostaglandin-H synthase. That the hydroperoxidase component of prostaglandin-H synthase was responsible for oxidation of DES to radical products is shown by the experiments described in the legend to Fig. 5. These demonstrate that hydrogen peroxide could substitute for arachidonic acid as the substrate for prostaglandin-H synthase. Controls without DES (Fig. 5*B*), enzyme (Fig. 5*C*), or hydrogen peroxide (Fig. 5*D*) produced signals of much lower intensity than those obtained with the complete incubation system (Fig. 5*A*). Furthermore, the possibility of nonenzymatic processes causing the generation of such a radical species was discounted as incubations containing boiled enzyme produced no signal (Fig. 5*E*). As in the case of horseradish peroxidase, prostaglandin-H synthase-catalyzed oxidation of DES dipropionate (0.1 mM) or *p*-phenetidine (0.1–0.5 mM) did not induce the formation of ESR signals above control levels (Fig. 5, *F* and *G*).[2]

DISCUSSION

Peroxidase-catalyzed oxidation of DES occurs in all *in vitro* systems in which DES has been found to be genotoxic (16,

[2] To ensure that the ESR signal observed during prostaglandin H synthase-catalyzed metabolism of DES was not the result of a marked increase in enzyme turnover, arachidonate-stimulated, prostaglandin H synthase-dependent oxygen uptake (using conditions identical to those used in the ESR experiments) was measured. The initial rate of oxygen uptake observed, indicative of enzyme turnover, was increased in the presence of both *p*-phenetidine (0.1 mM) and DES (0.1 mM) by only 12 and 6%, respectively, of the control value.

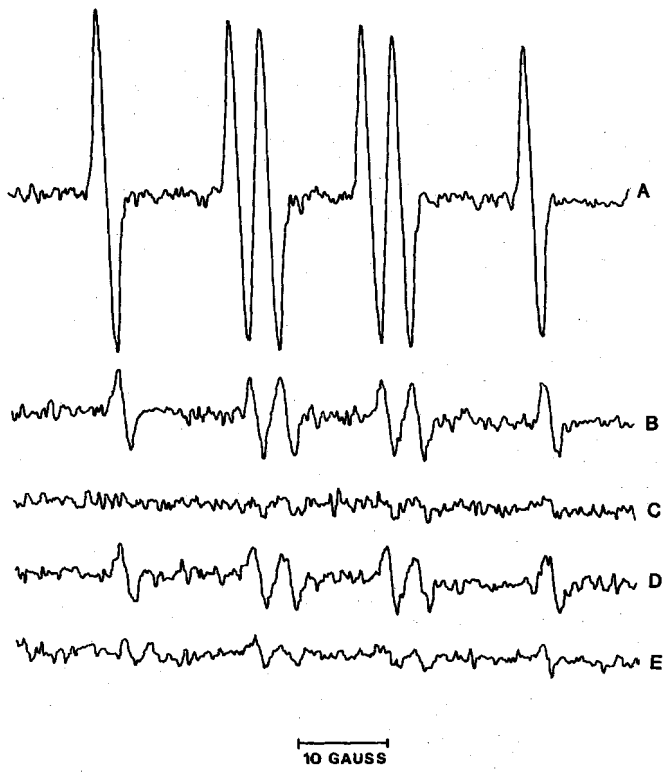

FIG. 4. **ESR spectra observed during arachidonic acid-dependent prostaglandin-H synthase-catalyzed oxidation of DES in the presence of DMPO.** *A*, DES dissolved in dimethyl sulfoxide (0.1 mM), arachidonic acid (0.1 mM), purified prostaglandin-H synthase (12.3 μg/ml protein), hematin (1.0 μM), and DMPO (0.1 M); *B*, as in *A* but with DES omitted from the dimethyl sulfoxide; *C*, arachidonic acid omitted; *D*, prostaglandin-H synthase omitted; *E*, as in *A* but with indomethacin (0.1 mM) added.

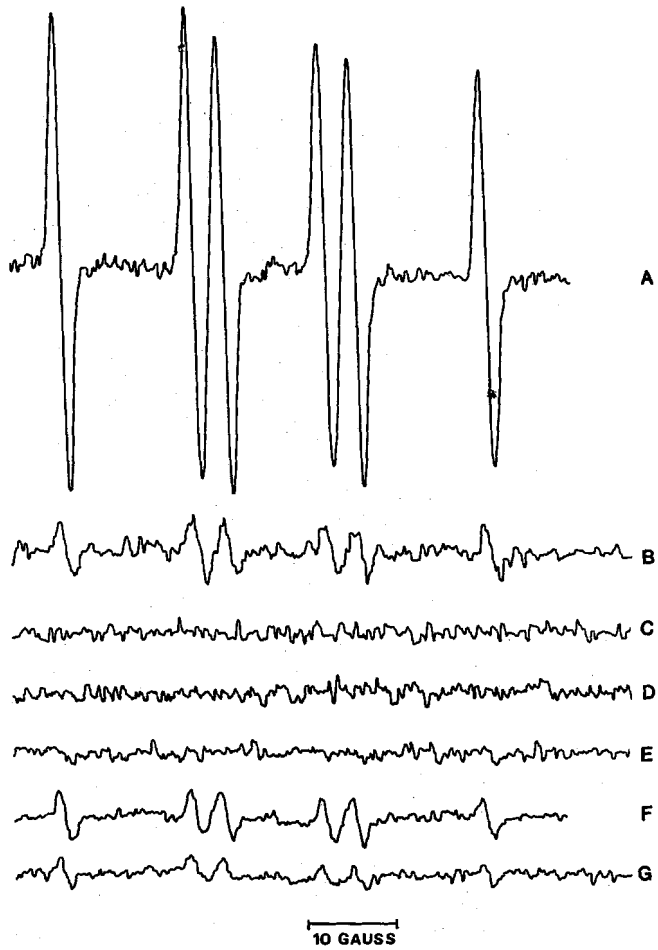

FIG. 5. **ESR spectra observed during hydrogen peroxide-dependent prostaglandin-H synthase catalyzed oxidation of DES in the presence of DMPO.** *A*, conditions as in 4*A* but the reaction was initiated by adding hydrogen peroxide (0.05 mM); *B*, as in *A* but with DES missing from the dimethyl sulfoxide; *C*, without prostaglandin-H synthase; *D*, without hydrogen peroxide; *E*, boiled enzyme; *F*, with DES diproprionate in place of DES; *G*, *p*-phenetidine HCl (0.1 mM) in place of DES.

29) and also in organs susceptible to DES carcinogenicity (30). One product of peroxidase-catalyzed oxidation of DES, DES-quinone, binds to DNA and has therefore been proposed as a possible carcinogenic metabolite of DES (15). A recent study, however, has shown that quinone formation is not necessary for the binding of structural analogs of DES to DNA during peroxidase-catalyzed metabolism (16). The latter study concluded that one free hydroxy group was necessary for the induction of DNA binding during peroxidatic oxidation of analogs of DES and proposed the semiquinone radical of DES as the putative genotoxic agent. Since the generation of such a species during peroxidase-catalyzed oxidation of DES has never been shown, we investigated whether peroxidatic oxidation of DES occurred via the generation of free radical intermediates.

Our results show that both horseradish peroxidase- and prostaglandin-H synthase-catalyzed oxidation of DES cause the formation of a radical species that can be trapped with DMPO. The identity of this radical species cannot be deduced from our data, but the fact that 1-naphthol produces an identical species suggests that the radical is either a phenoxy radical or is derived from a phenoxy radical. The hyperfine coupling constants observed in this study are substantially smaller than constants that have been published for DMPO-phenyl radical adducts (31). Thus this data is not conclusive proof that the 6-line ESR signal obtained during horseradish peroxidase- or prostaglandin-H synthase-catalyzed metabolism of DES in the presence of DMPO is indicative of a DMPO-DES adduct. Attempts to characterize the putative DMPO-DES adduct by mass spectrometry are in progress. The generation of a thiyl radical during horseradish peroxidase-catalyzed oxidation of DES in the presence of GSH is further evidence of the one electron mechanism operative in these reactions and shows that the DES radical can interact with other biological constituents. The observation that prostaglandin-H synthase catalyzes the metabolism of DES to free radical species may be particularly relevant as prostaglandin-H synthase is present in many organs that are susceptible to DES-induced carcinogenesis (17, 18).

In summary, our data show that radical species are produced during peroxidatic oxidation of DES and therefore could be a determinant of the carcinogenic potency of DES either directly or as a result of further reactions such as redox cycling (32) or lipid peroxidation processes (33). As one free hydroxy group has been shown to be sufficient for the induction of DNA binding by DES analogs it seems probable that phenoxy radicals or their rearrangement products can bind to DNA. This is not likely to be the only mechanism eliciting DNA binding, as peroxidatic oxidation also produces the genotoxic, two-electron oxidized product—DES quinone. Whether DES radicals or quinones are more important for the induction of genotoxic effects must await further work, but it seems plausible, as both species are formed during peroxidatic oxidations, that the mechanism of DES-induced genotoxicity could involve both compounds.

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