REFERENCES

1. D. S. Rosenblatt, V. M. Whitehead, N. Vera, A. Pottier, M. Dupont and M-J. Vuchich, *Molec. Pharmac.* **14**, 1143 (1978).
2. J. Galivan, *Cancer Res.* **39**, 735 (1979).
3. J. Galivan, *Molec. Pharmac.* **17**, 105 (1980).
4. M. Balinska, J. Galivan and J. K. Coward, *Cancer Res.* **41**, 2751 (1981).
5. D. W. Fry, J. C. Yalowich and I. D. Goldman, *J. biol. Chem.* **257**, 1890 (1982).
6. J. Jolivet, R. L. Schilsky, B. D. Bailey, J. C. Drake, and B. A. Chabner, *J. clin. Invest.* **70**, 351 (1982).
7. J. J. McGuire, P. Hsieh, J. K. Coward and J. R. Bertino, *J. biol. Chem.* **225**, 5776 (1980).
8. D. S. Rosenblatt, V. M. Whitehead, M-J. Vuchich, A. Pottier, N. Vera Matiaszuk and D. Beaulieu, *Molec. Pharmac.* **19**, 87 (1981).
9. M. Balinska, Z. Nimec and J. Galivan, *Archs Biochem. Biophys.* **216**, 466 (1982).
10. M. W. McBurney and G. F. Whitmore, *Cell* **2**, 173 (1974).
11. R. T. Taylor and M. L. Hanna, *Archs Biochem. Biophys.* **181**, 331 (1977).
12. R. E. Richardson, M. J. Healy and P. F. Nixon, *Biochim. biophys. Acta* **585**, 128 (1979).
13. D. G. Priest, M. T. Doig and M. Mangum, in *The Chemistry and Biology of Pteridines* (Ed. J. A. Blair), in press. W. de Gruyter, Hawthorne, NY (1983).
14. J. P. Brown, G. E. Davidson, D. G. Weir and J. M. Scott, *Int. J. Biochem.* **5**, 727 (1974).
15. P. A. Barford, J. A. Blair and M. A. K. Malghani, *Br. J. Cancer* **41**, 816 (1980).
16. B. A. Kamen, P. S. Nylen, B. M. Camitta and J. R. Bertino, *Br. J. Haemat.* **49**, 355 (1981).
17. J. Galivan, G. F. Maley and F. Maley, *Biochemistry* **15**, 356 (1976).
18. P. Nixon and J. Bertino, *Analyt. Biochem.* **43**, 162 (1971).
19. M. Balinska, W. Samsonoff and J. Galivan, *Biochim. biophys. Acta* **721**, 253 (1982).
20. J. J. McGuire and J. R. Bertino, *Molec. cell Biochem.* **38**, 19 (1981).

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Oxidation of [^{14}C]diethylstilbestrol epoxide by uterine peroxidase: a possible protective mechanism

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Previous studies on the binding of diethylstilbestrol (DES) to DNA [1] and other tissue macromolecules [2], together with the known expoxidation of the stilbene double bond during the metabolism *in vivo* of aminostilbene derivatives [3], have provided indirect evidence for the conversion of DES into an epoxide. The nature of the urinary products of rats treated with DES [4], some of which are also formed after *in vitro* incubation of ^{14}C-labeled DES-epoxide with rat liver preparations [5], also supports the proposal that DES-epoxide may be an intermediate in the breakdown of DES. However, it has also been suggested [6] that the bioactivation of DES may involve peroxidase and that the localization of this enzyme could be an additional factor in the tissue specificity of DES-associated tumors. Oxidation of DES *in vitro* by a preparation of peroxidase from horseradish or the mouse uterus yielded reactive metabolites which combined covalently with salmon sperm DNA and bovine serum albumin [7]. It was therefore decided to study the effect of peroxidase on the further metabolism of the putative epoxide of DES which we have synthesized [5] in order to determine the extent of its conversion to water-soluble products and also to identify ether-soluble metabolites. For this purpose, lactoperoxidase and estrogen-induced rat uterine peroxidase [8] were chosen. Both catalyzed the rapid cleavage of DES-epoxide to 4′-hydroxypropiophenone in the presence of H_2O_2.

Materials and Methods

Reagents. Diethylstilbestrol (DES), lactoperoxidase and GSH were purchased from the Sigma Chemical Co., St. Louis, MO, and guaiacol, 2,4-dichlorophenol and 4′-hydroxypropiophenone from Eastman Organics, Rochester, NY. [^{14}C]Diethylstilbestrol epoxide (specific radioactivity 0.49 mCi/mmole) was prepared from [monoethyl-1-^{14}C]diethylstilbestrol (58 mCi/mmole) (Amersham Corp., Arlington Heights, IL) as described previously [5]. Silica gel pre-coated on aluminum sheets for TLC was purchased from Brinkmann Instruments, Rexdale, Ontario. All chemicals were the purest available commercially, and the solvents were redistilled.

Animals. Mature (190–250 g) female Sprague–Dawley rats (Canadian Breeding Laboratories, St. Constant, Quebec) with free access to food (Purina Labena) and water were injected subcutaneously with estradiol (10 μg in 0.2 ml oil) 18 hr before isolation of uterine peroxidase. Uteri, devoid of peroxidase activity, were obtained from immature (23 to 25-day-old) female rats.

Preparation of uterine extracts and incubation. The tissue was dissected free of any adhering fat, blotted, and weighed. It was then cut into small pieces and homogenized in the appropriate volume of 10 mM Tris–HCl (pH 7.2) to give a 5% (w/v) homogenate using a Polytron homogenizer (10-sec burst at speed setting 4.5). After centrifugation at 40,000 g_{av} for 30 min at 4° the pellet was resuspended in a Potter–Elvehjem homogenizer with a Teflon pestle in 10 mM Tris–HCl (pH 7.2) containing 1.2 M NaCl and centrifuged again at 40,000 g_{av} for 30 min to obtain a supernatant fraction (NaCl extract) containing most of the peroxidase in the tissue.

The NaCl extract (1 ml) from 50 mg of uterine tissue was incubated for various time periods with constant shaking at 37° with [^{14}C]diethylstilbestrol epoxide (4.5 μM) or [^{14}C]DES, together with H_2O_2 (0.33 mM) or GSH (1 mM) in 10 mM Tris–HCl, pH 7.2, in a total volume of 4 ml. The

reaction was stopped by extracting the incubation mixture three times with equal volumes of diethyl ether and the combined organic phase was dried over anhydrous Na_2SO_4. The radioactivity in each fraction was then determined by liquid scintillation counting as described previously [9].

Examination of ether-soluble fraction. A portion of the ether extract was examined by TLC (chloroform/ethyl acetate, 9:1, v/v), and radioactive areas were located by autoradiography. The main metabolite of [^{14}C]DES epoxide, in addition to the pinacolone which is formed non-enzymically [5], had the same chromatographic properties as 4′-hydroxypropiophenone and co-crystallized with this compound when analyzed by reverse isotope dilution. The ^{14}C-labeled compound was eluted from the TLC silica gel with absolute ethanol and mixed with non-radioactive 4′-hydroxypropiophenone (50 mg) followed by three crystallizations from 50% aqueous ethanol and determination of the specific radioactivity.

Determination of peroxidase activity. Peroxidase activity was determined at 25° by using guaiacol as substrate [10]. The reaction mixture (3.0 ml) contained guaiacol (13 mM) and H_2O_2 (0.33 mM) in 10 mM Tris–HCl (pH 7.2) and 0.1 to 0.5 ml of the sample. One enzyme unit is defined as the amount of enzyme required to produce an increase of 1 absorbance unit/min, and the results are expressed as units/g wet wt of tissue.

Results

The results (Table 1) show that peroxidase from an extract of estradiol-treated rat uteri will catalyze the conversion of [^{14}C]DES epoxide into water-soluble metabolites and 4′-hydroxypropiophenone in the presence of added H_2O_2. The only other ether-soluble product formed in significant amounts, DES pinacolone, had been shown previously to arise non-enzymatically [5]. The uterine preparation was inactivated by boiling, and no degradation of DES-epoxide occurred in the presence of H_2O_2 alone. Bovine serum albumin did not increase significantly the yield of 4′-hydroxypropiophenone at the expense of other products, but 2,4-dichlorophenol which had been shown previously [11,12] to be required for the conversion of estradiol to water-soluble products by uterine peroxidase also increased the percentage of DES-epoxide radioactivity remaining in the aqueous fraction.

The rate of formation of water-soluble metabolites and 4′-hydroxypropiophenone from [^{14}C]DES-epoxide by lactoperoxidase in the presence of H_2O_2 is shown in Fig. 1.

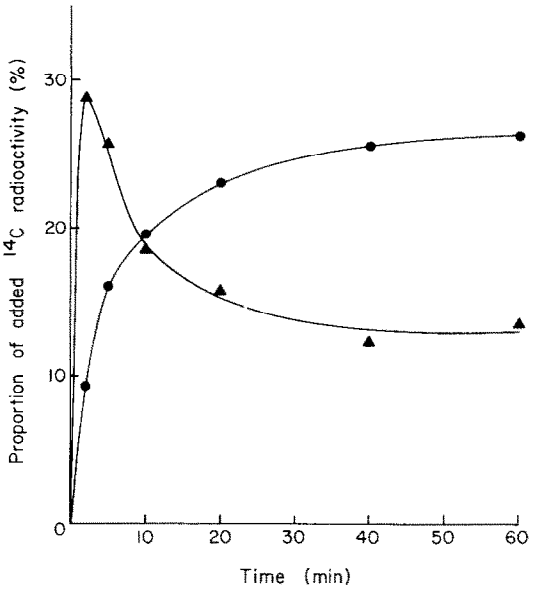

Fig. 1. Rate of conversion of [^{14}C]DES-epoxide to 4′-hydroxypropiophenone and water-soluble products by lactoperoxidase. Lactoperoxidase (12.5 μg/ml) was incubated for various time periods with [^{14}C]DES-epoxide (4.5 μM) and H_2O_2 (0.3 mM) before extraction with ether and separation of the products by TLC as described in the text. The radioactive area corresponding to 4′-hydroxypropiophenone was located by autoradiography and eluted with ethanol for ^{14}C-radioactivity determination. The yield of water-soluble products (●) or 4′-hydroxypropiophenone (▲) is shown. Values are the means of 3–5 experiments, with S.E.M. ± 10–15%.

Similar kinetics were observed with uterine peroxidase but the rates of product formation varied with the guaiacol oxidizing activity of the preparation. Under similar incubation conditions, very little (<1%) peroxidase-catalyzed conversion of [^{14}C]DES to 4′-hydroxypropiophenone was observed. Chromatography and reverse isotope dilution were used to identify 4′-hydroxypropiophenone in the

Table 1. Percent conversion of [^{14}C]DES-epoxide to water-soluble products and to 4′-hydroxypropiophenone by uterine peroxidase under various experimental conditions*

Additions to uterine peroxidase	Percent of added ^{14}C-radioactivity	
	Aqueous fraction	4′-Hydroxypropiophenone
	5.2 ± 0.5	8.5 ± 0.9
H_2O_2	13.4 ± 1.7	39.8 ± 2.5
H_2O_2 + albumin	13.6 ± 1.4	46.2 ± 1.8
H_2O_2 + DCP	27.8 ± 4.3	29.3 ± 0.9
H_2O_2 + DCP + albumin	29.2 ± 1.0	34.8 ± 1.3
H_2O_2, peroxidase omitted	1.8 ± 0.7	2.1 ± 0.24
H_2O_2, peroxidase boiled	4.7 (4.2–5.3)	4.4 (2.9–5.8)
H_2O_2, uterine extract from immature rats	2.6 (2.4–2.7)	4.2 (3.7–4.7)

* NaCl (1.2 M) extracts of uterine tissue (50 mg) (peroxidase activity: 2.3 to 2.7 guaiacol units) from estrogen-treated rats were incubated for 30 min with [^{14}C]DES-epoxide (4.5 μM) and one or more of the following: H_2O_2 (0.3 mM), 2,4-dichlorophenol (DCP) (0.25 mM), bovine serum albumin (10 mg) in 4 ml of 10 mM Tris–HCl, pH 7.2. Estradiol (10 μg in 0.2 ml oil) was injected s.c. 18 hr before the animals were killed. Other experimental conditions are given in the text. Results are the means of two experiments (range given) or the means for 5–7 experiments (± S.E.M.)

ether-soluble fraction. Following the addition of non-radioactive 4′-hydroxypropiophenone, the specific radioactivity (158, 164 and 167 cpm/mg) remained constant after three crystallizations from aqueous ethanol.

Discussion

The synthetic estrogen DES is known to exert toxic effects in animals and humans [13–15] and, recently, has been shown to induce neoplastic transformation in Syrian hamster embryo cells in the absence of gene mutations [16]. DES is also known to induce carcinoma in male Syrian hamster kidneys [17] which led to the suggestion [6] that the unique susceptibility of this organ to DES may be due to its content of peroxidase, an enzyme not found in the rat or mouse kidney. Peroxidase has been shown by Metzler and McLachlan [7] to catalyze the conversion of DES to β-dienestrol, a major *in vivo* metabolite in several mammalian species. They proposed a reaction involving the quinone [18] or semiquinone intermediate which could also bind covalently to nucleic acids and proteins. Earlier, Metzler [4] had provided indirect evidence for the formation of a DES-epoxide intermediate *in vivo* which is readily converted either directly [5] or via the vicinal diol [19] by rat liver microsomes to 4′-hydroxypropiophenone. This cleavage product has been identified as a urinary metabolite following the administration of DES [20].

In this paper, we have demonstrated that DES-expoxide is also readily oxidized by lactoperoxidase or rat uterine peroxidase to 4′-hydroxypropiophenone, a compound not known to have toxic properties. In contrast, very little, if any, DES was converted to 4′-hydroxypropiophenone under identical conditions. Thus, expoxidation of DES leads to a product which can be broken down more readily than the parent compound either by liver microsomes or by peroxidase to a non-toxic metabolite and, in this situation, peroxidase would have a protective role. This is in contrast to its metabolic activation of DES which results in considerable irreversible binding to DNA or albumin and conversion to β-dienestrol [7], a metabolite, also formed in the mouse, which does not appear to go through an epoxide intermediate [21]. Our suggestion that peroxidase might also have a protective role is supported by the findings that epoxidation of a related compound, *trans*-4-acetylaminostilbene, provides protection against its activation to a mutagen [22].

In summary, DES-epoxide was readily cleaved *in vitro* to 4′-hydroxypropiophenone in the presence of either lactoperoxidase or estrogen-induced uterine peroxidase. It is proposed that the toxic effects of DES in various tissues of different species may be influenced, among other factors, by its conversion to DES-epoxide as well as by the amount of peroxidase present. This enzyme could either activate DES or inactivate it, depending on the extent to which DES is converted to its epoxide in the intact animal. These results, however, do not exclude the possibility that some of the epoxide may also be converted to more reactive and, possibly, harmful products.

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REFERENCES

1. G. M. Blackburn, M. H. Thompson and W. S. King, *Biochem. J.* **158**, 643 (1976).
2. G. Krishna, E. Docks, G. U. Corsini, J. Gillette and B. B. Broche, *Fedn Proc.* **32**, 319 (1973).
3. M. Metzler and H. G. Newman, *Xenobiotica* **7**, 117 (1977).
4. M. Metzler, *Biochem. Pharmac.* **24**, 1449 (1975).
5. P. H. Jellinck and J. H. Bowen, *Biochem. J.* **185**, 129 (1980).
6. J. A. McLachlan, M. Metzler and J. C. Lamb, *Life Sci.* **23**, 2521 (1978).
7. M. Metzler and J. A. McLachlan, *Biochem. biophys. Res. Commun.* **85**, 874 (1978).
8. C. R. Lyttle and P. H. Jellinck, *Biochem. J.* **127**, 481 (1972).
9. P. H. Jellinck and T. McNabb, *Steroids* **29**, 525 (1977).
10. S. R. Himmelhoch, W. H. Evans, M. G. Mage and E. A. Peterson, *Biochemistry* **8**, 914 (1967).
11. C. R. Lyttle, T. McNabb and P. H. Jellinck, *Can. J. Biochem.* **51**, 1066 (1973).
12. T. McNabb, J. Sproul and P. H. Jellinck, *Can. J. Biochem.* **53**, 855 (1975).
13. A. L. Herbst, H. Ulfelder and D. C. Poskanzer, *New Engl. J. Med.* **284**, 423 (1971).
14. J. A. McLachlan, *J. Toxic. environ. Hlth* **2**, 527 (1977).
15. H. Vorherr, R. H. Messer, U. F. Vorherr, S. W. Jordan and M. Kornfeld, *Biochem. Pharmac.* **28**, 1865 (1979).
16. J. C. Barrett, A. Wong and J. A. McLachlan, *Science* **212**, 1402 (1981).
17. H. Kirkman and R. L. Bacon, *Cancer Res.* **10**, 122 (1950).
18. S. Liao and H. G. Williams-Ashman, *Biochim. biophys. Acta* **59**, 705 (1962).
19. P. Laskowski and C. Chen, *Archs Biochem. Biophys.* **202**, 318 (1980).
20. M. Metzler, *J. Toxic. environ. Hlth* (Suppl. 1,) 21 (1976).
21. M. Metzler and J. A. McLachlan, *Biochem. Pharmac.* **27**, 1087 (1978).
22. H. R. Glatt, M. Metzler, H-G. Neumann and F. Oesch, *Biochem. biophys. Res. Commun.* **73**, 1025 (1976).