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# METABOLISM OF ESTRADIOL BY TRUE AND PSEUDOPEROXIDASES

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**Summary**—The activation of $^{14}C$-labeled estradiol by "true" and "pseudo" peroxidases to form conjugates and other products was compared in four model systems using $H_2O_2$, glutathione, $Mn^{2+}$ or irradiated riboflavin. Albumin was used as acceptor except in the glutathione system. The binding of estradiol to glutathione in the presence of the true peroxidases, lacto- or uterine peroxidase (no $H_2O_2$ added), was also examined and the conditions shown to differ from those required with the pseudoperoxidases, microperoxidase or trypsin-digested cytochrome *c*. The conjugates were purified by chromatography after elution from Amberlite XAD-2 and the relative amounts of these products assessed by autoradiography. The ratio of steroid to glutathione in the main water-soluble metabolite formed with lactoperoxidase was found to be approx 1:1 in a double label experiment with [$^{14}C$]estradiol and [$^{3}H$]glutathione. It was also shown, using estradiol labeled with $^{3}H$ in different positions of the steroid molecule, that lactoperoxidase acts non-specifically in catalyzing the formation of glutathionyl conjugates as indicated by the release of $^{3}H_2O$. The possible role of peroxidase and glutathione in the metabolism of estrogens and in the formation of artifactual products is discussed.

## INTRODUCTION

Previous studies [1–6] have shown that peroxidase may play a role in estrogen metabolism in the uterus and will catalyze the conversion of estradiol ($E_2$) to inactive water-soluble products. It has also been demonstrated that glutathione (GSH) is effective in replacing $H_2O_2$, which can be generated from the sulfhydryl group of this tripeptide by peroxidase under aerobic conditions [7, 8]. Thus, GSH which is present in relatively high concentration (2–10 mM) in most cells [9, 10] might, under certain conditions, influence the rate of inactivation of estrogens. In this paper, we have compared the abilities of two "pseudoperoxidases", microperoxidase [11] and protease-digested cytochrome *c*, with those of lactoperoxidase and uterine peroxidase to form water-soluble products from estradiol in the presence of GSH or other agents such as $H_2O_2$, $Mn^{2+}$ or light-sensitized riboflavin. We have also examined the ability of glutathione to couple with the oxidized metabolites of estradiol which arise during incubation with these hemoproteins. Estrogen–glutathione conjugates are known to be formed in the rat both *in vitro* [12, 13] and *in vivo* [14, 15] and this type of peroxidase-catalyzed reaction may therefore account for estrogen metabolism in certain tissue preparations, as well as in the intact animal. It has been suggested [16, 17] that the bioactivation of diethylstilbestrol may involve peroxidase and that the localization of this enzyme could be a factor in the tissue specificity of DES-associated tumors.

## EXPERIMENTAL

### *Reagents*

Lactoperoxidase, cytochrome *c* (type III from horse heart), trypsin, collagenase (type I), estradiol-17$\beta$ and glutathione were purchased from Sigma Chemical Co. (St Louis, MO), riboflavin from General Biochemicals (Chagrin Falls, OH), 2,4-dichlorophenol from Eastman Kodak Co., Rochester, NY, guaiacol and $H_2O_2$ from Fisher Scientific Co., Ottawa, Ontario and microperoxidase and pronase from Calbiochem, San Diego, CA. The glutathionyl conjugate of 2-hydroxyestradiol (S-(2,3,17$\beta$-trihydroxy-1,3(10)-estratrien-4- (or 1)-yl)-glutathione was synthesized as described previously [18]. [4-$^{14}C$]Estradiol (57 mCi/mmol) from New England Nuclear Corp. (Boston, MA) was shown by chromatography and autoradiography to be free of radioactive impurities. It was diluted with carrier to a specific radioactivity of 5–6 mCi/mmol and kept at 4°C in the dark as a stock solution in ethanol (1 mg/ml). Glutathione [glycine-2-$^{3}H$] (5.0 mCi/mmol) from the same source was diluted with carrier to 1.99 mCi/mmol before use. Tritiated estradiol containing the label at C-1, C-2, C-4 or C-6,7 of the steroid was purified and handled as described previously [19]. Silica gel precoated on aluminium sheets (Merck) for thin-layer chromatography and the Amberlite XAD-2 resin were obtained from BDH Chemicals, Toronto, Ontario. The ether used for extraction was free of peroxides;

Abbreviations: LP, lactoperoxidase; MP, microperoxidase; PP, pseudoperoxidase; UP, uterine peroxidase; DCP, 2,4-dichlorophenol; $E_2$, estradiol-17$\beta$; GSH, glutathione, ESG, glutathione conjugate of 2-hydroxyestradiol-17$\beta$.

all other chemicals were the purest available commercially.

*Preparation of uterine peroxidase*

This enzyme was extracted from the uteri of mature female Sprague–Dawley rats (150–220 g) from Canadian Breeding Laboratories, St Constant, Quebec as described previously [20]. The animals were given a subcutaneous injection of estradiol (10 $\mu$g in 0.2 ml sesame oil) 20 h before killing by cervical dislocation. The uteri were dissected free of any adhering fat, blotted and weighed. They were 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 s burst of speed setting 4.5) which was then centrifuged at 40,000 $g_{av}$ for 30 min at 4°C. 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 at 40,000 $g_{av}$ for 30 min to obtain a supernatant fraction containing most of the peroxidase activity in the uterus. This was determined using guaiacol as substrate [21]. One enzyme unit is defined as the amount of enzyme required to produce an increase of 1 absorbance unit/min.

*Preparation of pseudoperoxidase*

Horse heart cytochrome *c* (20 mg) was incubated with constant shaking for 1 h at 38°C with pronase (2 mg) in 2 ml of 0.1 M sodium phosphate buffer, pH 7.2. Its peroxidase activity, which was absent before digestion, was determined by the guaiacol method, and it was kept frozen at −20°C. Digestion with trypsin provided a pseudoperoxidase with the same activity but collagenase was ineffective.

*Incubation and extraction*

Generally, the different peroxidases were incubated with constant shaking at 38°C in 4 ml of 0.1 M sodium phosphate buffer, pH 7.4 with [4-$^{14}$C]$E_2$ (1.84 $\mu$M) and $H_2O_2$ or other activated oxygen generating system for the time periods indicated in Table 1 which were optimum for lactoperoxidase in each system [8]. A 60 W household tungsten lamp was used to illuminate the tubes containing riboflavin and bovine serum albumin (2.5 mg/ml) was used as water-soluble acceptor for the metabolites of $E_2$ except in the glutathione system. The mixture after incubation was extracted three times with equal volumes of diethyl ether and the combined organic phase dried over anhydrous $Na_2SO_4$. It was evaporated to dryness under $N_2$ at 40°C and the residue dissolved in ethanol (1 ml). The radioactivity in the ether-soluble and extracted aqueous fractions was then determined in a Beckman LS 7500 scintillation spectrometer as described previously [4].

*Examination of the aqueous fraction*

The steroid–glutathione conjugates in the aqueous fraction were extracted by the method of Bradlow [22]. The ether-extracted aqueous fraction (3.5 ml) was applied to a column of Amberlite XAD-2 resin (1 × 20 cm) which had been previously washed with methanol and $H_2O$. Prior to application any ether in the aqueous fraction was removed by bubbling $N_2$ through the solution for 10 min. The column was eluted with $H_2O$ (80–100 ml) and pressure applied to remove excess $H_2O$ prior to elution with methanol (20–30 ml) at a flow rate of approx 1 ml/min. Fractions (2 ml) were collected and those which contained radioactivity were pooled, evaporated to dryness under $N_2$ at 40°C and the residue dissolved in a small volume of methanol for subsequent analysis by TLC in butanol–acetic acid–water (4:1:1, by vol). The glutathione conjugate of 2-hydroxyestradiol used as standard was visualized by spraying with diluted Folin–Ciocalteu reagent (1:3, v/v) followed by exposure to ammonia vapor. Glutathione was detected with ninhydrin. Radioactive material was located by autoradiography [4] and quantitated by counting the appropriate area on the silica gel after removal by scraping.

*Determination of $^3H_2O$ release by lactoperoxidase*

$^3$H-Labeled $E_2$ (2.5–4.0 × $10^5$ dpm in 37 nmol) were incubated for 1 h at 37°C in 4 ml of 0.1 M Tris–HCl, pH 7.4 with lactoperoxidase (50 $\mu$g) and GSH (2 mM). A portion (1 ml) of the solution was then mixed with a suspension of 1% (w/v) charcoal (Norit A, Fisher Scientific Co.) in buffer (1 ml) and allowed to stand on ice for 10 min before pelleting the charcoal by centrifugation at 1000 *g* for 10 min at 4°C. A 1 ml sample of the supernatant was frozen in acetone–dry ice, lyophilized and 0.5 ml of the condensed liquid counted as described previously [19].

## RESULTS

Previous studies have shown that, under aerobic conditions, glutathione, $Mn^{2+}$ or riboflavin + light were effective in promoting the conversion of $^{14}$C-labeled $E_2$ by lactoperoxidase (LP) to water-soluble protein- or glutathione-bound products even in the total absence of added $H_2O_2$. In the present work we have extended these findings by comparing these reactions with those catalyzed by uterine peroxidase (UP) and microperoxidase (MP) under various conditions.

A number of differences were observed (Table 1). The LP-catalyzed conversion of $E_2$ was inhibited at the higher concentration of $H_2O_2$ in contrast to UP and MP, while 2,4-dichlorophenol (DCP) abolished this inhibition and yet was required to obtain high yields of products with UP (see Discussion). In the glutathione system, DCP had no effect on the LP-catalyzed reaction but enhanced the effect of UP and was inhibitory in the LP + $Mn^{2+}$ and the riboflavin systems. Boiling the enzymes abolished the catalytic activities of LP and UP in all the systems except the

Table 1. Conversion of [4-$^{14}$C]estradiol to water-soluble products by lactoperoxidase, microperoxidase and uterine peroxidase in the presence of $H_2O_2$, glutathione, $Mn^{2+}$ or riboflavin under various conditions

| Enzyme and conditions | % $^{14}$C in water-soluble products[a] | | | | |
|---|---|---|---|---|---|
| | $H_2O_2$ (5 μM) | $H_2O_2$ (0.33 mM) | Glutathione | $Mn^{2+}$ | Riboflavin plus light |
| — | 1.1 ± 0.1 | 1.2 ± 0.1 | 1.0 ± 1.0 | 1.8 ± 0.2 | 43.4 ± 2.9[b] |
| LP | 43.1 ± 4.2 | 17.4 ± 1.1 | 42.2 ± 5.2 | 52.4 ± 1.7 | 66.1 ± 0.5 |
| LP + DCP | 9.1 ± 0.7 | 33.2 ± 3.9 | 46.2 ± 2.0 | 17.0 ± 0.8 | 17.2 ± 1.7 |
| LP (boiled) | 2.6 ± 0.8 | 1.5 ± 0.2 | 1.8 ± 0.5 | 2.9 ± 0.5 | 42.8 ± 5.2 |
| UP | 5.1 ± 1.0 | 12.5 ± 0.9 | 19.5 ± 0.9 | 8.1 ± 1.7 | 49.6 ± 0.6 |
| UP + DCP | 4.3 ± 0.8 | 63.0 ± 2.3 | 40.1 ± 2.5 | 8.2 ± 2.0 | 20.9 ± 1.6 |
| UP (boiled) | 2.8 ± 1.4 | 2.2 ± 0.6 | 2.9 ± 1.7 | 3.0 ± 0.7 | 44.1 ± 4.3 |
| UP (boiled) + DCP | 2.3 ± 0.2 | 3.1 ± 0.2 | 3.4 ± 0.4 | 4.0 ± 0.2 | 12.4 ± 0.4 |
| MP | 48.0 ± 4.9 | 80.0 ± 1.2 | 12.8 ± 0.4 | 36.2 ± 0.8 | 63.3 ± 0.7 |
| MP (boiled) | 42.2 ± 4.4 | 82.0 ± 1.8 | 13.8 ± 0.5 | 36.0 ± 0.6 | 62.6 ± 2.7 |
| PP | 45.8 | 65.1 ± 7.6 | 16.9 | — | — |
| PP (boiled) | 54.1 | 69.7 ± 77.0 | 14.3 | — | — |
| Cytochrome *c* | 0.6 | 2.8 ± 0.7 | 1.4 | — | — |

Lactoperoxidase (LP), microperoxidase (MP), pseudoperoxidase (PP), cytochrome *c*, each at 12.5 μg/ml, or uterine peroxidase (UP) from 50 mg tissue were incubated at 38°C with [4-$^{14}$C]estradiol (1.84 μM) and either $MnCl_2$ (1.0 μM) or riboflavin (30 μm) for 1 h, glutathione (2 mM) for 20 min or $H_2O_2$ (5 μM or 0.33 mM) for 10 min) in 0.1 M sodium phosphate (pH 7.4) before extraction with ether. Albumin (2.5 mg/ml) was used as acceptor except in the glutathione system. Other conditions as described in the text. Incubation with the enzymes alone yielded only unchanged estradiol.

[a]Values are the means of 4–6 experiments ± SE unless indicated otherwise.

[b]Riboflavin—no light: 2.7 ± 0.3; riboflavin—no light + UP: 6.4; riboflavin + light + DCP: 10.3 ± 0.7.

one involving light sensitized riboflavin while, as expected, this treatment had no effect on the activity of microperoxidase. No change in activity was observed after boiling pronase-digested cytochrome *c* which gave similar percentage yields of water-soluble $E_2$ metabolites as microperoxidase on incubation with $H_2O_2$ or glutathione. Native cytochrome *c* was devoid of activity. Riboflavin catalyzed the reaction even in the absence of enzyme but this effect was inhibited by DCP and virtually abolished in the absence of light.

The effect of different concentrations of glutathione (GSH) on the formation of $^{14}$C-labeled water-soluble metabolites of $E_2$ by LP and by UP in the presence and absence of DCP is shown in Fig. 1. In all cases, a maximum value was reached at 1–2 mM GSH but higher concentrations (> 5 mM) were inhibitory. Again, the need for DCP in the UP-catalyzed reaction was demonstrated. With MP and digested cytochrome *c* (pseudoperoxidase), a much lower concentration optimum (0.05–0.2 mM) for GSH was found (Fig. 2), which in the case of MP was influenced by enzyme concentration.

The nature of the products formed from $E_2$ in the

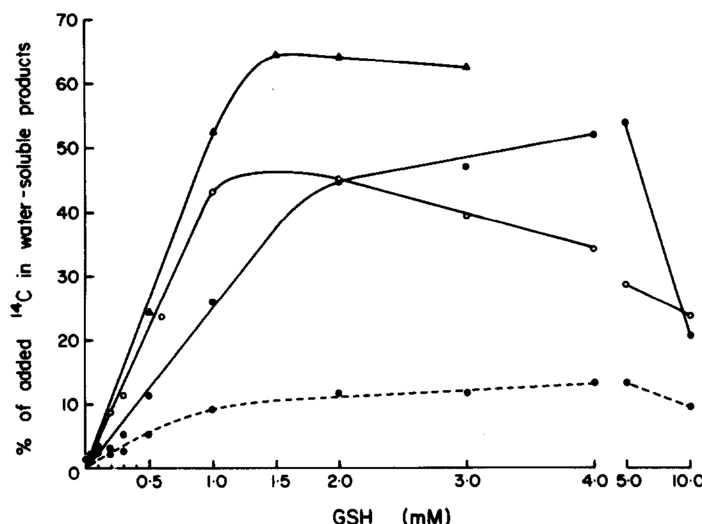


Fig. 1. Effect of glutathione concentration on the conversion of [4-$^{14}$C]estradiol to water-soluble products by lactoperoxidase and uterine peroxidase. [4-$^{14}$C]estradiol (1.84 μM] was incubated for 20 min with lactoperoxidase at 12.5 μg/ml (○) or 25 μg/ml (▲), or with uterine peroxidase (●) from 100 mg of tissue in the presence (——) and absence (---) of DCP (0.25 mM) and various concentrations of glutathione (GSH) before extraction with ether. Other conditions as described in the text. Values are the means of 3 experiments.

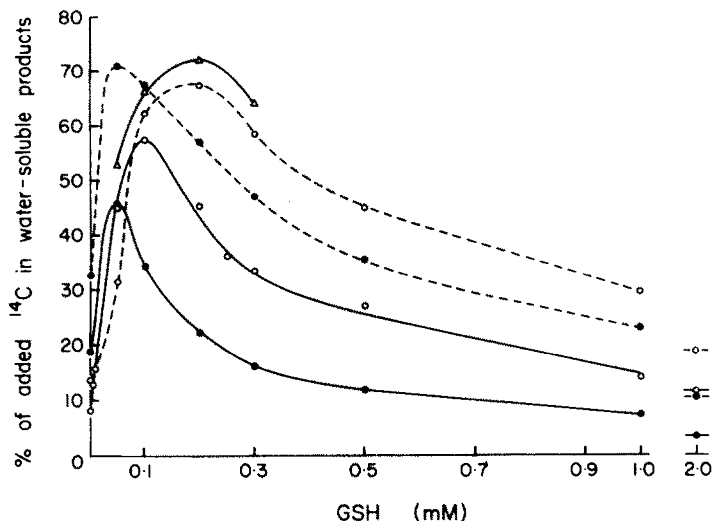


Fig. 2. Effect of glutathione concentration on the conversion of [4-$^{14}$C]estradiol to water-soluble products by microperoxidase and pseudoperoxidase. [4-$^{14}$C]Estradiol (1.84 $\mu$M) was incubated with microperoxidase (○) or pseudoperoxidase (●) at 12.5 $\mu$g/ml (——) or 25 $\mu$g/ml (---). Microperoxidase was also incubated at 50 $\mu$g/ml (△). Other conditions as in Fig. 1.

GSH system was also investigated for the 4 different peroxidases, using Amberlite XAD-2 followed by TLC in butanol–acetic acid–water (4:1:1, by vol) to separate conjugates. The location of the products was determined by autoradiography (Fig. 3). Uterine peroxidase gave rise to a compound (I) isopolar with the glutathione conjugate of 2-hydroxyestradiol (ESG) and it was also the main product with lactoperoxidase. However, a less polar metabolite (II) was also formed with LP, MP and PP and predominated with MP and PP which also gave rise to other products nearer to the origin. The ratio of compound I to II was 4.0 with LP, 0.31 with MP and 0.12 with PP.

The association of glutathione with the steroid in approximately a 1:1 ratio was demonstrated for the main LP metabolite (I) by incubating [4-$^{14}$C]estradiol (50 $\mu$g) with $^{3}$H-labeled glutathione (2 mM) and lactoperoxidase. The molecular ratio was calculated from the $^{3}$H and $^{14}$C content of the purified conjugate using labeled $E_2$ and GSH of known specific radioactivity. The $^{3}$H to $^{14}$C ratio in the minor metabolite (II) was less consistent and no integral value could be assigned to it. Lactoperoxidase was able to release $^{3}H_2O$ from all the labeled positions in ring A of estradiol in the presence of glutathione but this reaction did not occur at C-6 or 7 (Table 2).

## DISCUSSION

In the previous studies [23] we have shown that several tissues which possess high peroxidase activity are able to convert [4-$^{14}$C]estradiol to inactive water-soluble products in the presence of $H_2O_2$. However, in contrast to peroxidase from the uteri of estrogen-treated rats, preparations from lung and spleen retained most of their activity even after boiling indicating a "pseudoperoxidase" reaction catalyzed by hemoproteins. We have also demonstrated [8] that $H_2O_2$ could be replaced by glutathione, $Mn^{2+}$ or light-sensitized riboflavin in a model estradiol-oxidizing system using lactoperoxidase.

It was therefore considered worthwhile to examine whether uterine peroxidase would behave like lactoperoxidase particularly in the presence of glutathione, and whether pseudoperoxidases prepared from cytochrome *c* by proteolytic digestion were also capable of inactivating estrogen by conversion to water-soluble products. Degraded hemoproteins may occur during tissue damage and necrosis, while heme or other metal complexes could be responsible for some of the non-specific hydroxylation and related reactions observed in incubations of estradiol with brain subcellular fractions [24]. In this tissue, both estradiol 2- and 4-hydroxylase activities are low, allowing non-specific reactions to be major contributors to the end-products and thus obscuring the results of enzymatic studies.

Thus, a knowledge of the mechanisms involved in the non-specific oxidation of estradiol would be useful for developing methods to control the formation of artefacts during tissue incubations with estrogens. Determining the role of glutathione is also important because this tripeptide, which is present in high concentrations in most tissues [9, 10], forms conjugates with estrogens both *in vitro* [12, 13] and *in vivo* [14, 15] and can activate estradiol in the presence of peroxidase under aerobic conditions [7, 8].

In the present work, we have demonstrated that microperoxidase, a hemepeptide with a molecular weight of only 1900 [11], as well as pronase- or trypsin-digested cytochrome *c*, catalyzes the conversion of [4-$^{14}$C]estradiol to water-soluble products in high percentage yields in all the systems tested. In

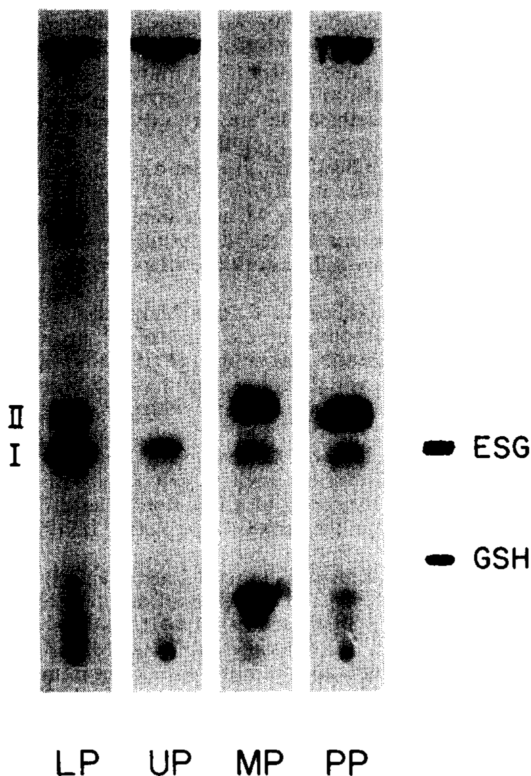


Fig. 3. Autoradiogram of products formed from [4-$^{14}$C]estradiol after incubation with lactoperoxidase (LP), uterine peroxidase (UP), microperoxidase (MP) or pseudoperoxidase (PP) with glutathione (GSH). The water-soluble conjugates were adsorbed onto Amberlite XAD-2 and after elution with methanol were separated by TLC in butanol–acetic acid–water (4:1:1, by vol). The GSH and ESG (glutathione conjugate of 2-hydroxyestradiol) markers were visualized as described in the text.

contrast to lactoperoxidase or uterine peroxidase, boiling the pseudoperoxidases did not affect their catalytic activity in the presence of $H_2O_2$ or GSH. Incubating the peroxidases alone yielded only unchanged estradiol. The pseudoperoxidases could also use $Mn^{2+}$ or irradiated riboflavin to activate estradiol but were less effective than LP or UP at the higher concentrations (1–4 mM) of GSH. 2,4-Dichlorophenol has been shown previously [8, 25, 26] to activate peroxidases and to protect them from inactivation by the products of the reaction but to be inhibitory at higher concentrations. It may be significant that the optimum concentration of GSH required for the true peroxidases is within the range that exists in normal tissues [9, 10]. The concentration of estradiol (1.84 $\mu$M) in the model system was chosen to enable us to follow easily the fate of the steroid by autoradiography. It is likely that the activation of estradiol and subsequent conjugation of the products would be more pronounced at lower, more physiological, levels of estrogen but, *in vivo*, many other factors would also have to be considered.

The nature of the water-soluble metabolites formed from estradiol by lactoperoxidase in the presence of glutathione was also investigated. It was shown that they consisted of at least two types of glutathionyl conjugates of which the major product (I), with a steroid to glutathione ratio of 1, was also formed by uterine peroxidase while the other metabolite (II) was formed predominantly by micro- and pseudoperoxidase. Further analysis to determine the position of attachment of glutathione to the estradiol by measuring $^3H_2O$ release from different tritiated isomers after lyophilization of a portion of the incubation mixture showed that it could occur at C-1, C-2 or C-4 in the aromatic nucleus of the steroid. These findings suggest that lactoperoxidase acts non-specifically, producing a variety of steroid–glutathionyl conjugates, presumably from free radical ring A intermediates, in contrast to the situation with tyrosinase where conjugation with GSH occurs only at C-1 and C-4 after specific hydroxylation of C-2 of estradiol by this enzyme [27].

The results raise the possibility that the small quantities of estradiol–glutathione conjugates detected in the rat *in vivo* [14, 15] may originate from highly reactive estrogen free radicals produced either by true peroxidases or by pseudoperoxidases present in tissues such as the spleen or lung [23]. The pseudoperoxidases might be formed from hemoproteins by proteolysis and may also arise during the preparation of subcellular fractions and incubation under certain conditions. With glutathione, no added $H_2O_2$ is required for the reaction because it can be generated from the thiol by peroxidase [7], but $H_2O_2$ could be formed from purines by xanthine oxidase in tissues such as the intestine and uterus (unpublished data). Presumably, the reaction with glutathione is protec-

Table 2. Release of $^3H_2O$ from $^3$H-labeled estradiol by lactoperoxidase in the presence of glutathione

| | % Radioactivity released as $^3H_2O$[a] | | | |
|---|---|---|---|---|
| Position of $^3$H | No enzyme | Lactoperoxidase | GSH | Lactoperoxidase + GSH |
| C-1 | 0.2 | 0.2 | 0.2 | 5.7 ± 0.5 |
| C-2 | 0.6 | 0.6 | 0.4 | 10.0 ± 0.4 |
| C-4 | 0.6 | 1.2 | 1.7 | 6.9 ± 0.8 |
| C-6, 7 | 0.2 | 0.3 | 0.3 | 0.4 ± 0.1 |

$^3$H-labeled $E_2$ (2.5–4.0 × $10^5$ dpm in 37 nmol) was incubated for 1 h at 37°C in 4 ml of 0.1 M Tris–HCl, pH 7.4 in the presence or absence of lactoperoxidase (50 $\mu$g) or GSH (2 mM). A portion (1 ml) was then lyophilized after treatment with charcoal and $^3H_2O$ radioactivity was determined. Other conditions as described in the text.

[a]Values are the means of 2 experiments or 5 experiments ± SE.

tive in nature preventing peroxidase-catalyzed binding of estrogens to DNA and protein which has been proposed to account for some of the toxic effects of diethylstilbestrol [16, 17].

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