Journal of Steroid Biochemistry, 1977, Vol. 8, pp. 835–840. Pergamon Press. Printed in Great Britain

A NEW MECHANISM OF *IN VITRO* FORMATION OF CATECHOL ESTROGEN GLUTATHIONE CONJUGATES BY RAT LIVER MICROSOMES*

MITSUTERU NUMAZAWA and TOSHIO NAMBARA
Pharmaceutical Institute, Tohoku University, Sendai, Japan

(*Received* 2 *December* 1976)

SUMMARY

The mechanism of *in vitro* formation of 2-hydroxyesteradiol 1- and 4-glutathione thioethers from estradiol, 2-hydroxy-3-deoxyestradiol, and 2-hydroxyestradiol by rat liver microsomes in the presence of NADPH and glutathione has been studied. The amount ratios of the 1- to 4-glutathione thioether of 2-hydroxyestradiol formed from these three substrates were 2.10, 0.96, and 1.70, respectively. In addition, by periodate oxidation 2-hydroxyestradiol was transformed through the *o*-quinone to the two isomeric thioethers whose ratio was 3.43 and distinctly different from that observed with the microsomal preparation. In the double isotope experiments employing [4-^{14}C]-estradiol and [6,7-^{3}H]-2-hydroxy-3-deoxyestradiol as substrates the ^{3}H/^{14}C values of 2-hydroxyestradiol and its 1- and 4-glutathione thioethers were found not to be identical. These results imply that 2-hydroxyestradiol may be converted to the thioethers by way of the semiquinone rather than the *o*-quinone and a new intermediate, presumably an arene oxide, may participate in the binding with glutathione.

INTRODUCTION

In recent years considerable attention has been drawn to the formation of the glutathione and cysteine conjugates of estrogen. It was found that the water-soluble metabolites are formed when estrone† and estradiol are incubated with the rat liver preparation [1–5]. These polar metabolites were unequivocally characterized to be 1- and 4-monothioethers and 1,4-dithioether of catechol estrogen by direct comparison with the synthetic specimens [6, 7]. It has generally been accepted that binding of estrogen to the sulfur-containing amino acid or peptide requires the sequential reactions involving hydroxylation at C-2 and oxidation into the *o*-quinone or semiquinone [4, 8, 9]. In our previous work, however, it was suggested that a new hypothetical intermediate which differes from the 2-hydroxylated metabolite, may serve as a proximate precursor leading to the thioether [10] and in addition, 2-hydroxy-3-deoxyestradiol, that is a positional isomer of estradiol, is similarly converted to 2-hydroxyestradiol and its glutathione conjugate by rat liver microsomes in the presence of NADPH and glutathione [11]. In this paper we wish to report a new mechanism operative for the formation of glutathione thioethers of 2-hydroxyestradiol from estradiol and 2-hydroxy-3-deoxyestradiol by rat liver microsomes and discuss the metabolic significance of this new route in the living animals.

MATERIALS AND METHODS

Animals. Male Wistar rats weighing 250–300 g were used.

Materials. NADPH, NADP^{+}, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, and glutathione from Sigma Chemical Co. (St. Louis), Amberlite XAD-2 resin from Rohm and Haas (Philadelphia), silica gel H from E. Merck AG (Darmstadt), cellulose powder from Asahi Kasei Kogyo Co. (Tokyo), and [6,7-^{3}H]-estradiol (48 Ci/mmol) and [4-^{14}C]-estradiol (50 mCi/mmol) from the Radiochemical Centre (Amersham) were purchased, respectively. [6,7-^{3}H]-2-Hydroxyestradiol (2.5 mCi/mmol) was enzymatically prepared from [6,7-^{3}H]-estradiol by the method of Marks and Hecker[12]. [6,7-^{3}H]-2-Hydroxy-3-deoxyestradiol (10.8 Ci/mmol) was chemically synthesized from [6,7-^{3}H]-estradiol by the method developed in these laboratories (total yield 28%) [13, 14] and the radiochemical purity was checked by t.l.c. prior to use. 2-Hydroxyestradiol was prepared in the manner as described by Gelbke, *et al.* [15]. Glutathione 1- and 4-thioethers of 2-hydroxyestradiol were synthesized according to the procedure of Kuss[7].

Thin-layer chromatography. t.l.c. Was carried out on a glass plate coated with a layer (0.25 mm. thick) of cellulose and of silica gel H impregnated with ascorbic acid [16] employing the following solvent

*Part CXXII of "Studies on Steroids" by T. Nambara; Part CXXI: H. Hosoda, K. Yamashita, S. Ikegawa, and T. Nambara, *Chem. Pharm. Bull.* (Tokyo), in press.

† The following trivial names are used in this paper: estrone = 3-hydroxy-1,3,5(10)-estratrien-17-one; estradiol = 1,3,5(10)-estratriene-3,17β-diol; 2-hydroxy-3-deoxyestradiol = 1,3,5(10)-estratriene-2,17β-diol; 2-hydroxyestradiol = 1,3,5(10)-estratriene-2,3,17β-triol.

systems: TL-1, hexane–ethyl acetate (1:1, v/v); TL-2, benzene–ether (1:1, v/v); TL-3, 0.2 N acetic acid–0.1 N NH_4OH (14.7:20, v/v). The adsorbent was scraped off into each 0.5 cm. width and radioactivity of the eluate from each section was counted.

Chemical synthesis of glutathione thioethers of [6,7-^{3}H]-2-hydroxyestradiol. To a solution of [6,7-^{3}H]-2-hydroxyestradiol (2 mg, 1.4×10^5 d.p.m.) in acetic acid (0.5 ml) was added sodium metaperiodate (5 mg) in water (1 ml) and the solution was stirred vigorously for 1 min at room temperature. The resulting solution was extracted with chloroform (1 ml × 3) and the extract was washed with water (1 ml × 3). To this organic layer was added glutathione (2.10 or 20 mg) in 50% acetic acid (1 ml) and the mixture was shaken vigorously for 10 min at room temperature. The resulting solution was extracted with water (2 ml × 3) and the aq. layer was combined and evaporated to dryness *in vacuo*. The residue was redissolved in water, percolated through a column packed with Amberlite XAD-2 resin (50 ml), and washed with water (50 ml). Elution with methanol (50 ml), followed by evaporation of the effluent under the reduced pressure gave glutathione thioethers of [6,7-^{3}H]-2-hydroxyestradiol.

Radioactivity counting. Counting was carried out on a Packard Tri-Carb Model 3380 liquid scintillation spectrometer employing Bray's scintillator [17]. Correction for quenching was made by the automatic external standard method.

Preparation of microsomes. The rat was sacrificed by stunning and decapitation and liver was immediately removed and chilled on ice. All subsequent procedures were carried out at 0–4°. The tissue was weighed, finely minced with scissors, homogenized with 4-fold vol. of an ice-cold 1.15% KCl solution by a Potter–Elvehjem homogenizer with a Teflon pestle, and centrifuged at 10,000 *g* for 30 min. The supernatant was carefully transferred and centrifuged at 105,000 *g* for 60 min by a Hitachi Model 40P ultracentrifuge. The microsomal pellet separated was washed with 1.15% KCl twice and then gently resuspended in 1.15% KCl in such a way that 1 ml was equivalent to 4 mg of protein. Microsomal protein was determined by the method of Lowry, *et al.*[18] using bovine serum albumin as a reference.

Enzyme assay. The incubation studies were undertaken with the following two systems: (A) the microsomal preparation (0.5 ml), NADPH (3 μmol), and glutathione (0.4 μmol) dissolved in 0.05 M Tris–HCl buffer (pH 7.4) (0.7 ml), and [6,7-^{3}H]-2-hydroxy-3-deoxyestradiol (0.2 μmol), [6,7-^{3}H]-estradiol (0.2 μmol), or [6,7-^{3}H]-2-hydroxyestradiol (0.2 μmol) dissolved in 50% aq. methanol (0.1 ml); (B) the microsomal preparation (0.5 ml), $NADP^+$ (4 μmol), glucose-6-phosphate (50 μmol), glutathione (10 μmol), and glucose-6-phosphate dehydrogenase (10 units) dissolved in 0.05 M Tris–HCl buffer (pH 7.4) (1.0 ml), [6,7-^{3}H]-2-hydroxy-3-deoxyestradiol (0.2 μmol) and [4-^{14}C]-estradiol (0.2 μmol) in 50% aq. methanol (0.2 ml). Incubation was carried out for 30 min at 37° under the aerobic conditions. After a definite period 1 N HCl (2 ml) was added to terminate the reaction and the solution was cooled immediately in an ice-bath. To the incubation mixture were added 2-hydroxyestradiol (500 μg) and its 1- and 4-glutathione thioethers (500 μg) as carriers together with ascorbic acid (2 mg) to prevent oxidative degradation of catechol estrogens. The reaction mixture was brought to 10% trichloroacetic acid solution and then centrifuged at 3,000 rev./min for 20 min to separate the protein-bound metabolites. The sediment was washed with 10% trichloroacetic acid (2 ml), ethanol (2 ml), and ethyl acetate (2 ml), successively. The supernatant was in turn extracted with ethyl acetate (3 ml × 3) to divide into the lipophilic and water-soluble fractions. Separation of these three fractions was performed according to the method previously established with a slight modification [1, 19]. The water-soluble fraction was purified by column chromatography on Amberlite XAD-2 resin. The reliability of this procedure has been demonstrated in the preceding paper [11]. The ^{3}H-labeled 2-hydroxyestradiol and its glutathione conjugate added to the incubation mixture with boiled microsomes were recovered at the rate of 81.0 and 90.5% (means of three determinations), respectively.

Identification and quantitation of 2-hydroxyestradiol and its glutathione thioethers. 2-Hydroxyestradiol obtained from the lipophilic fraction was identified by t.l.c., g.l.c.–mass spectrometry, and reverse isotope dilution technique [11]. The structures of glutathione thioethers of 2-hydroxyestradiol were confirmed by leading to 2-hydroxyestradiol by desulfurization with Raney nickel, followed by characterization of the steroid aglycone liberated by t.l.c. and reverse dilution analysis [11]. For semiquantitative analysis of the lipophilic fraction the extract was dissolved in a small vol. of methanol, applied to a silica gel thin-layer plate as a strip (2 × 0.3 cm.), and developed. The radioactivity of the zone (TL-1 0.35, TL-2 0.55) corresponding to 2-hydroxyestradiol on the chromatogram developed with two solvent systems was measured and the ^{3}H/^{14}C value was obtained. The partially purified water-soluble fraction was submitted to t.l.c. on the cellulose plate with the solvent system TL-3 in the manner as described above. The radioactivity was detectable solely on the zones corresponding to glutathione 1- and 4-thioethers of 2-hydroxyestradiol. Counting of the radioactivity provided the molar ratio of these two conjugates and their ^{3}H/^{14}C values.

RESULTS

Formation of glutathione thioethers of 2-hydroxyestradiol from estradiol and 2-hydroxy-3-deoxyestradiol by rat liver microsomes

Aerobic incubation of labeled estradiol and 2-hydroxy-3-deoxyestradiol with rat liver microsomes in

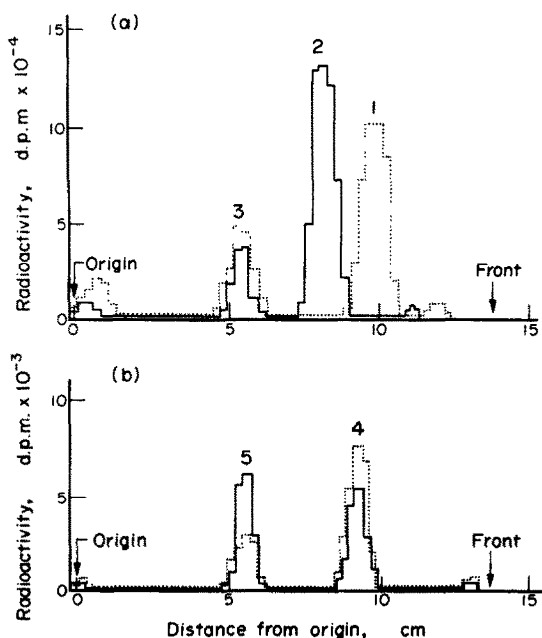

Fig. 1. Thin-layer chromatogram of lipophilic (a) and water-soluble (b) metabolites formed from estradiol and 2-hydroxy-3-deoxyestradiol by rat liver microsomes. [6,7-^{3}H]-Estradiol (0.2 μmol, 0.5 μCi) or [6,7-^{3}H]-2-hydroxy-3-deoxyestradiol (0.2 μmol, 0.5 μCi) was incubated with rat liver microsomes under the condition of system A in the text. Solvent system: a, hexane–ethyl acetate (3:2, v/v); b, 0.2 N acetic acid–0.1 N NH_4OH (14.7:20, v/v). 1: estradiol, 2: 2-hydroxy-3-deoxyestradiol, 3: 2-hydroxyestradiol, 4: 2-hydroxyestradiol 1-glutathione thioether, 5: 2-hydroxyestradiol 4-glutathione thioether. ——: from 2-hydroxy-3-deoxyestradiol, -----: from estradiol.

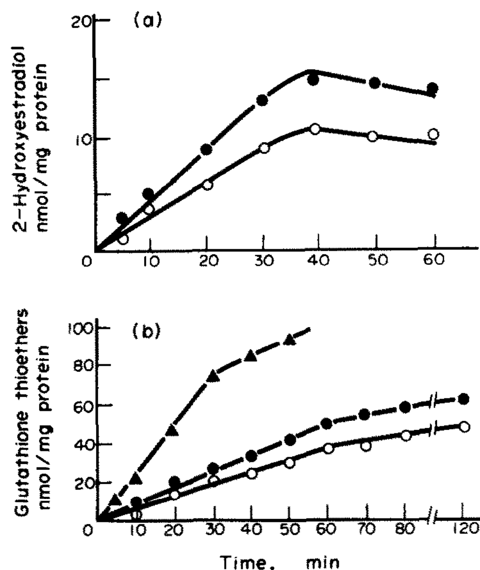

Fig. 2. Effect of incubation time on the formations of 2-hydroxyestradiol (a) and its glutathione thioethers (b). [6,7-^{3}H]-Estradiol (0.2 μmol, 0.5 μCi), [6,7-^{3}H]-2-hydroxy-3-deoxyestradiol (0.2 μmol, 0.5 μCi), or [6,7-^{3}H]-2-hydroxyestradiol (0.2 μmol, 0.5 μCi) was incubated with rat liver microsomes under the condition of system A in the text. ●——●: from estradiol, ○——○: from 2-hydroxy-3-deoxyestradiol, ▲——▲: from 2-hydroxyestradiol.

the presence of glutathione and NADPH resulted in incorporation of the radioactivity into the transformation products, 2-hydroxyestradiol and glutathione thioethers. The radiochromatograms of the products and unchanged substrates are illustrated in Fig. 1. As can be seen in Fig. 2, the yields of catechol estrogen and its conjugates increased with the incubation time linearly up to 30 min and 60 min, respectively. The amounts of these metabolites formed from 2-hydroxy-3-deoxyestradiol were much less than those from estradiol under the identical conditions. In addition, incubation of 2-hydroxyestradiol under the conditions as the above provided the glutathione conjugates whose yields increased with the incubation period up to 25 min and were much higher than those from other two substrates.

Determination of the ratio of isomeric glutathione thioethers of 2-hydroxyestradiol formed from estradiol, 2-hydroxy-3-deoxyestradiol, and 2-hydroxyestradiol

In our previous work the *in vitro* and *in vivo* experiments with the rat suggested that 2-hydroxyestrogen thioethers are not solely produced *via* the catechol and a new hypothetical species derived from estradiol also may serve as a proximate precursor in the formation of these conjugates [10]. In order to clarify the formation mechanism the amount ratio of two isomeric glutathione thioethers enzymatically produced from estradiol, 2-hydroxy-3-deoxyestradiol, or 2-hydroxyestradiol, was determined. The ratios of the 1- to 4-thioether derived from estradiol and 2-hydroxy-3-deoxyestradiol were found to be 2.10 and 0.96, respectively (Table 1). The addition of non-labeled 2-hydroxyestradiol to the incubation mixture caused

Table 1. The ratio of 1- to 4- glutathione thioether of 2-hydroxyestradiol formed from estradiol, 2-hydroxy-3-deoxyestradiol, or 2-hydroxyestradiol with rat liver microsomes

Substrate	Ratio of 1- to 4-glutathione thioether Mean	Range
Estradiol	2.10	2.08–2.15 (8)
2-Hydroxy-3-deoxyestradiol	0.96	0.88–1.05 (8)
2-Hydroxyestradiol	1.70	1.65–1.75 (6)

Microsomes (equivalent to 2 mg protein), ^{3}H-labeled steroid (0.2 μmol), and NADPH (4 μmol) were incubated at 37° for 30 min. Incubated radioactivity: [6,7-^{3}H]-estradiol 0.5 μCi; [6,7-^{3}H]-2-hydroxy-3-deoxyestradiol 0.5 μCi; [6,7-^{3}H]-2-hydroxyestradiol 0.1 μCi. Figures in parentheses represent the number of experiments.

Table 2. Effect of non-radioactive 2-hydroxyestradiol on incorporation of the radioactivity into the water-soluble metabolites and on the ratio of 1- to 4-glutathione thioether of 2-hydroxyestradiol formed from estradiol and 2-hydroxy-3-deoxyestradiol by rat liver microsomes

Substrate	2-Hydroxyestradiol added (μmol)	Radioactivity converted to water-soluble metabolites (%)	Ratio of 1- to 4-glutathione thioether	
			Mean	Range
Estradiol	0	100	2.10*	
	0.025	52	2.45	2.40–2.50 (6)
	0.10	40	3.51	3.42–3.65 (6)
2-Hydroxy-3-deoxy-estradiol	0	100	0.96*	
	0.025	62	0.70	0.65–0.75 (6)
	0.10	45	0.41	0.40–0.42 (4)

* Taken from Table 1.

To the incubation mixture (system A in the text) 2-hydroxyestradiol (0.025 or 0.1 μmol) was added and incubated at 37° for 30 min. [6,7-^{3}H]-Estradiol (0.2 μmol, 0.5 μCi) or [6,7-^{3}H]-2-hydroxy-3-deoxyestradiol (0.2 μmol, 0.5 μCi) was used as a substrate. Figures in parentheses represent the number of experiments.

a marked decrease in the yield of radioactive water-soluble metabolites together with an alteration of the ratio of two isomeric glutathione conjugates. When estradiol was used as a substrate, the ratio was elevated from 2.10 to 3.51 along with the increasing amount of 2-hydroxyestradiol added. In sharp contrast the value decreased from 0.96 to 0.41 in the case of 2-hydroxy-3-deoxyestradiol (Table 2). 2-Hydroxyestradiol being incubated with rat liver microsomes under the above conditions, the ratio in the yield of 1- to 4-glutathione thioether was 1.70, distinctly different from the values obtained with estradiol and 2-hydroxy-3-deoxyestradiol.

From these data it seems likely that a pathway by which the 1-thioether is formed from a new proximate precursor derivable from estradiol may be more dominant than that from 2-hydroxyestradiol, and also the 4-thioether may be produced from 2-hydroxy-3-deoxyestradiol *via* the analogous intermediate more preferentially than from the catechol. A marked change in the ratio of the two isomeric thioethers caused by the addition of 2-hydroxyestradiol might be ascribable to the diminished contribution of the biosynthetic route *via* catechol estrogen. If the glutathione conjugates were formed solely through 2-hydroxyestradiol by rat liver microsomes, the ratios of the isomeric glutathione conjugates produced from estradiol, 2-hydroxy-3-deoxyestradiol, and 2-hydroxyestradiol should be equivalent. Moreover, the isomeric thioethers chemically synthesized from the catechol through the *o*-quinone by periodate oxidation showed a value of approx. 3.4, which differed obviously from that obtained by the enzymatic reaction (Table 3). These results strongly imply that the enzymatic formation of the glutathione conjugates of catechol estrogen does not nesessarily take a course by way of the *o*-quinone.

In order to obtain further evidences for the new formation mechanism a double isotope experiment using [6,7-^{3}H]-2-hydroxy-3-deoxyestradiol and [4-^{14}C]-estradiol as substrates was undertaken. The ^{3}H- and ^{14}C-labeled substrates ($^3H/^{14}C = 23.3$) were incubated simultaneously with rat liver microsomes in the presence of NADPH and glutathione. Then 2-hydroxyestradiol and its 1- and 4-glutathione thioethers formed were separated from the lipophilic and water-soluble fractions, respectively. The $^3H/^{14}C$ ratios were found to be 10.7 for 2-hydroxyestradiol, 8.4 for the 1-thioethers, and 20.9 for the 4-thioether (Table 4). It is evident from the data that the value of 2-hydroxyestradiol is not identical with those of its glutathione conjugates. Furthermore, there can be seen a significant difference in the $^3H/^{14}C$ value between the two isomeric conjugates. These evidences lent a support to the assumption that the new mechanism mentioned above may participate in the formation of the glutathione conjugates of catechol estrogen.

Table 3. The ratio of 1- to 4-glutathione thioether of 2-hydroxyestradiol formed from 2-hydroxyestradiol by chemical reaction

Molar ratio of glutathione to 2-hydroxyestradiol	Ratio of 1- to 4-glutathione thioether*
1.0	3.42
5.0	3.25
10.0	3.64

* Mean value of duplicate determinations.

[6,7-^{3}H]-2-Hydroxyestradiol (2 mg, 1.4×10^5 d.p.m.) was oxidized with sodium metaperiodate to the *o*-quinone which in turn was condensed with various amounts of glutathione.

Table 4. The $^3H/^{14}C$ values of 2-hydroxyestradiol and its 1- and 4-glutathione thioethers formed from [4-^{14}C]-estradiol and [6,7-^3H]-2-hydroxy-3-deoxyestradiol by rat liver microsomes. Microsomes (equivalent to 2 mg protein), [4-^{14}C]-estradiol (0.2 μmol, 0.1 μCi), [6,7-^3H]-2-hydroxy-3-deoxyestradiol (0.2 μmol, 2.33 μCi), and NADPH-generating system were incubated at 37° for 30 min under the condition of system B in the text.

Exptl. No.	$^3H/^{14}C$ value		
	2-Hydroxyestradiol	1-Glutathione thioether	4-Glutathione thioether
1	10.3	7.9	18.1
2	10.5	8.1	24.0
3	11.2	8.9	19.8
4	11.1	8.7	21.6
Mean	10.7	8.4	20.9

DISCUSSION

It is generally accepted that the estrogen metabolites bound with protein or glutathione are formed oxidatively from the catechol [2–10]. Since the typical *o*-quinone reagent and basic amino acid fail to trap the radioactive products derived from labeled estrone and 2-hydroxyestrone and inhibit labeling of the thiol compound and protein in the microsomal system, it has been supposed that a reactive species formed by oxidation is not an *o*-quinone [8, 20], but an *o*-semiquinone radical [4, 9, 20]. In the present studies the ratio of 1- to 4-glutathione thioether produced from 2-hydroxyestradiol by rat liver microsomes has proved to be different from that obtainable by the chemical synthesis *via* the *o*-quinone. These results lend a support to the assumption that a semiquinone-type intermediate may participate in the formation of the glutathione conjugate of catechol estrogen.

It is sufficiently substantiated that NADPH-dependent mixed function oxidase is responsible for the oxidative metabolism of hormonal steroids as well as various foreign compounds [21–25]. Carcinogenic polycyclic hydrocarbons are metabolized by oxygenase into the K-region epoxide and diol-epoxide to exert the biological activities including mutagenicity [26–35]. Moreover, the arene oxide is further converted to the glutathione conjugate, *trans*-dihydrodiol, and phenolic compound in the biological system [2, 36–40]. Booth, *et al.*[41] reported that the amounts of these three metabolites formed from polynuclear aromatic hydrocarbons are significantly depressed by the presence of estradiol probably due to the competitive inhibition against the formation of the arene oxide. There can be seen close similarities in the mode of biotransformation between the phenolic steroid and polycyclic hydrocarbon as judged from the inhibitory effect of the aromatic steroid. The present results together with the previous findings imply that the arene oxide may possibly be an important intermediate in the formation of the glutathione thioether of estrogens and in another word a new mechanism involving the participation of the arene oxide may also be operative. As illustrated in Fig. 3, epoxidation of the aromatic ring of a phenolic steroid and subsequent conjugation with glutathione, not through a semiquinone intermediate, may be an alternative route leading to the glutathione conjugate of catechol estrogen.

The problem of whether the aromatic steroid, in particular the contraceptive drug, exerts the carcino-

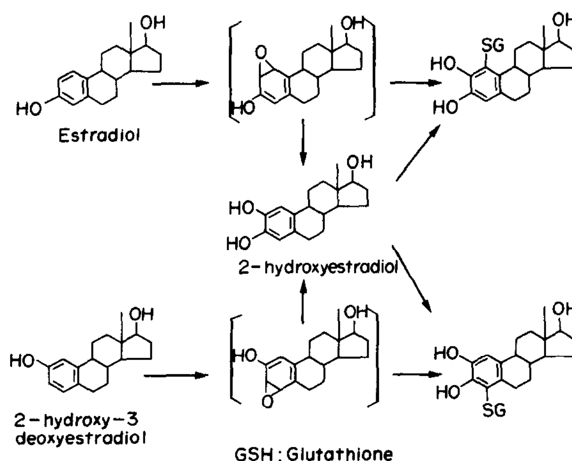

Fig. 3. Proposed mechanism for the formation of 2-hydroxyestradiol glutathione thioethers from estradiol and 2-hydroxy-3-deoxyestradiol by rat liver microsomes.

genic effects or not still remains unclear. Although the possible carcinogenicity of estrogens has been denied [42–46], more detailed studies are required to arrive at the definite conclusion [47–50]. Considering the metabolic fate of the phenolic steroid and polycyclic aromatic hydrocarbon involving the arene oxide, elucidation of the carcinogenic mechanism from this point of view is a fertile field for further investigation.

Acknowledgements—This work was supported in part by a Grant-in-Aid for the Scientific Research from the Ministry of Education, Science and Culture, Japan, which is gratefully acknowledged.

REFERENCES

1. Hecker E. and Zayed S. M. A. D.: *Hoppe-Seyler's Z. physiol. Chem.* **325** (1961) 209–223.
2. Jellinck P. H., Lewis J. and Boston F.: *Steroids* **10** (1967) 329–346.
3. Kuss E.: *Hoppe-Seyler's Z. physiol. Chem.* **348** (1967) 1707–1708.
4. Marks F. and Hecker E.: *Biochim. biophys. Acta* **187** (1969) 250–265.
5. Hoppen H.-O., Siekmann L. and Breuer H.: *Hoppe-Seyler's Z. physiol. Chem.* **355** (1974) 1305–1315.
6. Kuss E.: *Hoppe-Seyler's Z. physiol. Chem.* **350** (1969) 95–97.
7. Kuss E.: *Hoppe-Seyler's Z. physiol. Chem.* **352** (1971) 817–836.
8. Jellinck P. H. and Irwin L.: *Biochim. biophys. Acta* **78** (1963) 778–780.
9. Hecker E., Walter G. and Marks F.: *Biochim. biophys. Acta* **111** (1965) 546–548.
10. Numazawa M., Tanaka Y. and Nambara T.: *Chem. Pharm. Bull.* (Tokyo) **22** (1974) 663–668.
11. Numazawa M., Soeda N., Moro S. and Nambara T.: *Biochem. Pharmac.* **26** (1977) 769–773.
12. Marks F. and Hecker E.: *Biochim. biophys. Acta* **144** (1967) 690–691.
13. Nambara T., Honma S. and Akiyama S.: *Chem. Pharm. Bull.* (Tokyo) **18** (1970) 474–480.
14. Nambara T., Numazawa M. and Akiyama S.: *Chem. Pharm. Bull.* (Tokyo) **19** (1971) 153–158.
15. Gelbke H. P., Haupt O. and Knuppen R.: *Steroids* **21** (1973) 205–218.
16. Gelbke H. P. and Knuppen R.: *J. Chromatog.* **71** (1972) 465–471.
17. Bray G. S.: *Analyt. Biochem.* **1** (1960) 279–285.
18. Lowry O. H., Rosenbrough N. J., Farr A. L. and Randall R. J.: *J. biol. Chem.* **193** (1951) 265–275.
19. Marks F. and Hecker E.: *Hoppe-Seyler's Z. physiol. Chem.* **349** (1968) 523–532.
20. Marks F. and Hecker E.: *Hoppe-Seyler's Z. physiol. Chem.* **345** (1966) 22–40.
21. Conney A. H. and Klutch A.: *J. biol. Chem.* **238** (1963) 1611–1617.
22. Conney A. H.: *Pharmacol. Rev.* **19** (1967) 317–366.
23. Kato R., Takahashi A. and Omori Y.: *Life Sci.* **7** (1968) 915–920.
24. Haugen D. A., Van Der Hoeven T. A. and Coon M. J.: *J. biol. Chem.* **250** (1975) 3567–3570.
25. Gustafsson J.-A. and Ingelman-Sunderberg M.: *Eur. J. Biochem.* **64** (1976) 35–43.
26. Jerina D. M., Daly J. W., Witkop B., Zaltzman-Nirenberg P. and Udenfriend S.: *Archs Biochem. Biophys.* **128** (1968) 176–183.
27. Jerina D. M., Daly J. W., Witkop B., Zaltzman-Nirenberg P. and Udenfriend S.: *Biochemistry* **9** (1970) 147–156.
28. Selkirk J. K., Huberman E. and Heidelberger C.: *Biochem. biophys. Res. Commun.* **43** (1971) 1010–1016.
29. Grover P. L., Hewer A. and Sims P.: *FEBS Lett.* **18** (1971) 76–80.
30. Grover P. L., Hewer A. and Sims P.: *Biochem. Pharmac.* **21** (1972) 2713–2726.
31. Jerina D. M. and Daly J. W.: *Science* **185** (1974) 573–582.
32. Wiebel F. J., Selkirk J. K., Gelboin H. V., Haugen D. A., Van Der Hoeven T. A. and Coon M. J.: *Proc. natn. Acad. Sci. U.S.A.* **72** (1975) 3917–3920.
33. Sims P., Grover P. L., Swiasland A., Pal K. and Hewer A.: *Nature* **252** (1974) 326–328.
34. Booth J. and Sims P.: *FEBS Lett.* **47** (1974) 30–33.
35. Huberman E., Sachs L., Yang S. K. and Gelboin H. V.: *Proc. natn. Acad. Sci. U.S.A.* **73** (1976) 607–611.
36. Boyland E. and Williams K.: *Biochem. J.* **94** (1965) 190–197.
37. Jerina D. M., Daly J., Zaltzman-Nirenberg P., Witkop B. and Udenfriend S.: *Archs Biochem. Biophys.* **128** (1968) 176–183.
38. Pandov H. and Sims P.: *Biochem. Pharmac.* **19** (1970) 255–273.
39. Oesh F. and Daly J.: *Biochim. biophys. Acta* **227** (1971) 692–697.
40. Sims P.: *Biochem. J.* **105** (1967) 591–598.
41. Booth J., Keysell G. R. and Sims P.: *Biochem. Pharmac.* **23** (1974) 735–744.
42. Larson J. A.: *Obstet. Gynecol.* **3** (1954) 551–572.
43. Geller W.: *Mod. Treat.* **5** (1968) 564–570.
44. Rogers J.: *New Eng. J. Med.* **280** (1969) 364–367.
45. Breuer H.: *Symp. Deut. Ges. Endokrinol.* **13** (1969) 81–93.
46. Wynder E. L. and Shneiderman M. A.: *J. natn. Cancer Inst.* **51** (1973) 729–731.
47. Bolt H. M. and Kappas A.: *J. steroid Biochem.* **5** (1974) 179–184.
48. Hertz R. and Bailar C.: *Excerpta Med.* Internat. Congr. Ser. **132** (1967) 841.
49. Sturgis S. H.: *New Engl. J. Med.* **281** (1969) 1014–1015.
50. Hecker E.: *Z. Krebsforsch.* **78** (1972) 99–122.