Biochem. J. (1970) **116**, 913–917
Printed in Great Britain

Metabolism of a Glutathione Conjugate of 2-Hydroxyoestradiol by Rat Liver and Kidney Preparations *in vitro*

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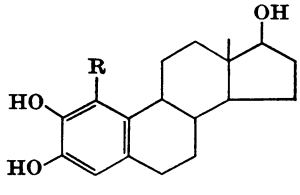
(*Received* 24 *October* 1969)

Adult male rat liver and kidney preparations were incubated with (2-hydroxyoestradiol-1-yl)[^{35}S]glutathione. The glutamic acid and glycine residues were removed by enzymes present in the kidney microsomal fraction; the liver preparations had no effect. The resulting 2-hydroxyoestradiol–cysteine conjugate was acetylated at the α-amino group by both liver and kidney homogenates fortified with acetyl-coenzyme A, but not significantly in the absence of this coenzyme, or by liver or kidney slices. These results suggest that an oestrogen–glutathione conjugate, if formed *in vivo*, would be converted into the corresponding mercapturic acid before excretion.

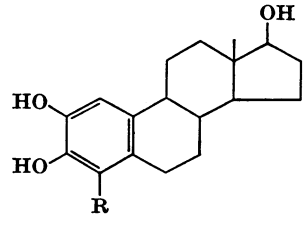
The formation of conjugates of 2-hydroxyoestradiol[oestra-1,3,5(10)-triene-2,3,17β-triol]with glutathione has been demonstrated *in vitro* by incubation of [^{14}C]oestradiol with liver homogenates (Jellinck, Lewis & Boston, 1967; Kuss, 1967, 1968); standard compounds have been synthesized (Jellinck & Elce, 1969) and shown to be identical with some of the products of the incubation experiments (Kuss, 1969). Many foreign compounds are excreted by mammals as mercapturic acids (*N*-acetylcysteine derivatives), and conjugates with glutathione are known to be biological precursors of the mercapturic acids (Boyland & Booth, 1962; Bray, Franklin & James, 1959*a*). It was therefore to be expected that an oestrogen–glutathione conjugate, if formed *in vivo*, might be converted successively into the corresponding cysteinylglycine, cysteine and *N*-acetylcysteine conjugates. To investigate this, the metabolism of (2-hydroxyoestradiol-1-yl)[^{35}S]glutathione (Ia) has been followed in adult male rat tissue preparations. The adult male rat was selected for study since it has been shown *in vitro* that the livers of 300g male rats exhibit a maximum rate of production of oestrogen–glutathione conjugates, a rate much greater than that observed in younger or older male rats or in female rats of any age (Kuss, 1967, 1968). This variation is almost certainly due to the age- and sex-dependence of the rat microsomal steroid hydroxylases (Conney & Burns, 1962; Jellinck & Lucieer, 1965).

EXPERIMENTAL

Materials. 2-Hydroxyoestradiol was kindly supplied by the Cancer Chemotherapy National Service Center (Bethesda, Md., U.S.A.). Conjugates of 2-hydroxyoestradiol with glutathione (Ia and Ib), L-cysteinylglycine (IIa and IIb), L-cysteine (IIIa and IIIb) and *N*-acetyl-L-cysteine (IVa and IVb) were prepared as described by Jellinck & Elce (1969). Following the report by Kuss (1969), the products of these syntheses were reinvestigated. It was found possible to separate the C-1–S and C-4–S isomers of compounds (I) and (IV) on a column (8g, 34cm × 1cm diam.) of DEAE-Sephadex A-25 (acetate



(Ia) R = γ-Glu-Cys-Gly
(IIa) R = Cys-Gly
(IIIa) R = Cys
(IVa) R = *N*-acetyl-Cys



(Ib) R = γ-Glu-Cys-Gly
(IIb) R = Cys-Gly
(IIIb) R = Cys
(IVb) R = *N*-acetyl-Cys

form) packed in 0.5M-acetic acid and run with a gradient of 4.0M-acetic acid (250ml) into 0.5M-acetic acid (250ml). The observed elution volumes were: compounds (IIa), (IIb), (IIIa) and (IIIb), 40–60ml (inseparable); compound (Ia), 120–140ml; compound (Ib), 140–160ml; compound (IVa), 270–290ml; compound (IVb), 290–310ml.

(2-Hydroxyoestradiol-1-yl)[^{35}S]glutathione was synthesized in the usual manner, from [^{35}S]glutathione obtained from Schwarz BioResearch Inc. (Orangeburg, N.Y., U.S.A.). The product was purified by passage through successive columns of Sephadex G-25 (Jellinck & Elce, 1969) and DEAE-Sephadex A-25 as described above. Most of the subsequent work was carried out either on the material that spontaneously crystallized in the eluate from the Sephadex G-25 column, and that was shown to be composed entirely of the isomer (Ia), or on the material in the peak first eluted from the DEAE-Sephadex column and designated (Ia). This was assumed to be the C-1–S isomer (see the Results and Discussion section). The initial specific radioactivity of the purified product was 129μCi/mmol and suitable corrections were made for radioactive decay during its use.

L-Cysteinylglycine was purchased from International Chemical and Nuclear Corp. (City of Industry, Calif., U.S.A.); CoA (free acid) was obtained from Sigma Chemical Co. (St Louis, Mo., U.S.A.); other chemicals were obtained from Fisher Scientific Co. (Toronto, Ont., Canada). Sephadex G-25 (medium grade), DEAE-Sephadex A-25, Blue Dextran 2000 and the columns were obtained from Pharmacia Ltd. (Montreal, P.Q., Canada). Male hooded rats weighing 225–250g were obtained from Canadian Breeding Farms and Laboratories (St Constant, P.Q., Canada).

Methods. The u.v. spectra were recorded in water or in ethanol solution on a Unicam SP.800 spectrophotometer. Paper chromatograms were run on Whatman no. 1 or 3MM paper in the system butan-1-ol–acetic acid–water (12:3:5, by vol.) by using the ascending technique. Electrophoresis was carried out on a Shandon high-voltage electrophoresis apparatus with Whatman 3MM paper in the buffer system pyridine–acetic acid–water (1:10:89, by vol.), pH3.5, with a current of 90mA and voltage of 3–4kV, giving a voltage gradient of about 60–80V/cm. Radioactivity was measured in vials containing 10ml of toluene–ethanol (3:2, v/v) with 0.4% PPO (2,5-diphenyloxazole) and 0.01% POPOP [1,4-bis-(5-phenyloxazol-2-yl)benzene] (Nuclear–Chicago Corp., Des Plaines, Ill., U.S.A.) in a Nuclear–Chicago series 720 scintillation counter, with channels-ratio correction for efficiencies. When necessary, protein precipitates were redissolved for scintillation counting in the Nuclear–Chicago strong organic base preparation NCS. Radioactivity on chromatograms and electrophoretograms was detected with a Packard 7200 radiochromatogram scanner. Compounds were detected colorimetrically on chromatograms either by spraying with 0.25% (w/v) ninhydrin in acetone, containing a few drops of acetic acid, followed by heating, or by spraying with Folin–Ciocalteu reagent; with this reagent catechols and the thiopyrogallol derivatives used in the present work gave a blue colour without exposure to NH_3 (Mitchell & Davies, 1954; Knuppen, 1962).

Tissue preparations. Male hooded rats weighing about 300g were killed by a blow on the head. The liver and kidneys were excised, rinsed and chopped in ice-cold 0.25M-sucrose and homogenized in 4vol. of 0.25M-sucrose. The homogenate was centrifuged at 8000$g_{av.}$ for 15min at 4°C in a Sorvall Superspeed RC-2 centrifuge to remove nuclei, cell debris and mitochondria; portions of the supernatant, which contained microsomal particles, were used for incubation. When washed microsomes were required, the 8000g supernatant was further centrifuged at 100000$g_{av.}$ for 1h in a Beckman model L ultracentrifuge. The supernatant of this centrifugation (referred to as the 100000g supernatant) was retained, and the pellet was resuspended in 0.25M-sucrose and recentrifuged at 100000$g_{av.}$ for 60min. The resulting pellet was again resuspended in 0.25M-sucrose so that 1ml of the suspension corresponded to 200mg of the original tissue; this preparation is referred to as microsomes. These operations were carried out at 4°C. In some cases the liver and kidneys were homogenized in Krebs–Ringer phosphate buffer, pH7.4, prepared as described by Umbreit, Burris & Stauffer (1964). After incubation of a steroid substrate with cell-free preparations, 10vol. of ethanol was added, and the mixtures were stored for 30min at −20°C and centrifuged to remove the precipitated protein. Portions of this supernatant were subjected to paper chromatography; the bulk of the material was concentrated and purified, if required, by passage through successive columns of Sephadex G-25 and DEAE-Sephadex A-25, the material being detected either by u.v. spectrophotometry at 300nm (Jellinck & Elce, 1969) or by radioactive methods.

For large-scale preparation of 2-hydroxyoestradiol–L-[^{35}S]cysteine, the oestrogen–[^{35}S]glutathione conjugate (Ia, 10mg) was incubated for 1h under N_2 in a mixture containing 8ml of the 8000g supernatant derived from 1.5g of rat kidney homogenized in 8ml of 0.25M- sucrose, 20ml of 0.1M-sodium–potassium phosphate buffer, pH7.4, and sodium ascorbate at a final concentration of 1.0mM. After incubation, 64ml of ethanol was added and the resulting precipitate was removed by centrifugation; the supernatant was concentrated and applied to a column of Sephadex G-25 as described above. The portion of the eluate that contained both radioactivity and 300nm-absorbing material was concentrated and applied to a column of DEAE-Sephadex A-25 packed and run with 0.1M-acetic acid. The (2-hydroxyoestradiol-1-yl)-L-[^{35}S]cysteine was eluted between 30 and 70ml. This solution was concentrated and stored at 4°C, in the presence of 1mM-sodium ascorbate.

For experiments with tissue slices, the liver and kidney were sliced with a Stadie–Riggs tissue slicer (A. H. Thomas Co., Philadelphia, Pa., U.S.A.), which gave slices about 0.5mm thick. Slices of the outer layers were discarded and only the inner tissue was used. After incubation the slices were homogenized in the incubation medium and the resulting homogenates treated as described for the cell-free experiments.

Acetyl-CoA was synthesized by the method described by White, Jenne & Evans (1969) with slight modifications (D. V. Hendrick, personal communication). A 15mg portion of CoA (free acid) was dissolved in 2ml of ice-cold glass-distilled water; 0.2ml of 1.0M-$KHCO_3$ was added followed by 0.5ml of freshly prepared ice-cold diluted acetic anhydride (prepared by mixing 0.94ml of freshly distilled acetic anhydride with 99ml of ice-cold glass-

distilled water). The mixture was kept at 0°C for 30min. Ice-cold 0.1M-HCl (1.6ml) was then added, the pH was adjusted to 6.0 with 1.0M-$KHCO_3$ and the volume adjusted to a total of 5.0ml. Unchanged acetic anhydride was removed by extraction with ether (2×4ml). A spot test with alkaline sodium nitroprusside indicated the absence of free thiol groups. The concentration of acetyl-CoA was assumed to be 3.0mM, which includes an allowance for impurities in the starting material. The preparation was stored at −20°C.

Throughout this work, identification of the metabolites was based on coincidence of radioactivity with Folin–Ciocalteu- or ninhydrin-positive responses in the expected positions relative to standards on paper chromatograms and electrophoretograms. Further evidence of identity was derived from the occurrence of radioactivity and of 300nm-absorbing material in the characteristic positions in the eluates from DEAE-Sephadex columns.

RESULTS AND DISCUSSION

After separation of the isomers (Ia) and (Ib) of 2-hydroxyoestradiol–glutathione on a DEAE-Sephadex column (see the Experimental section), the n.m.r. spectra of the two compounds were recorded in the manner described by Jellinck & Elce (1969). The results were qualitatively in agreement with those of Kuss (1969). The evidence for the assignment of C-1–S and C-4–S structures to the isomers (Ia) and (Ib) was given by Kuss (1969), and is based on the extrapolation of the results that Fishman & Liang (1968) obtained with oestrogen–catechols. Although this extrapolation from catechols to the thiopyrogallols of the present work seems reasonable, it does not constitute a rigorous proof of structure, and the assignment of the C-1–S structure to the isomer (Ia) of 2-hydroxyoestradiol–glutathione and of the C-4–S structure to the isomer (Ib) is still tentative.

Conversion of (2-hydroxyoestradiol-1-yl[^{35}S]glutathione (Ia) into (2-hydroxyoestradiol-1-yl)-L-[^{35}S]-cysteine (IIIa). The results (Table 1) indicate that the glutathione conjugate (Ia) was metabolized to the corresponding cysteine derivative (IIIa) by enzymes present in the microsomal fraction of kidney homogenates but not by enzymes present in the 8000*g* supernatant of liver homogenates. The alternative isomer (Ib) appeared to undergo similar metabolism. The conversion was at least 95% complete, as estimated by the radioactive peaks on the chromatograms. Recoveries of radioactivity in the aqueous ethanolic supernatants averaged 81% (68–102%), and in the protein pellets 2.5% (1.6–3.3%); these values were not significantly altered by any of the control conditions, except by the absence of ascorbate, which slightly lowered recoveries. Ascorbate was included to protect the catechols from oxidation (Jellinck & Perry, 1967).

Table 1. *Conversion of 2-hydroxyoestradiol–glutathione into 2-hydroxyoestradiol–L-cysteine.*

Tissues were homogenized and centrifuged in 4 vol. of 0.25M-sucrose. Except where otherwise indicated, the incubation tubes contained 0.1M-sodium–potassium phosphate buffer, pH7.4, 1.0mM-sodium ascorbate, 0.5mM-(2-hydroxyoestradiol-1-yl)[^{35}S]glutathione and 0.5ml of tissue preparation corresponding in each case to 100mg of the original tissue, in a total volume of 2ml. The tubes were gassed with N_2, closed and incubated at 37°C for 1h and then subjected to ethanol precipitation. Portions of the aqueous-ethanolic supernatant were used for paper chromatography. Standard 2-hydroxyoestradiol–glutathione (Ia) had R_F0.56, and standard 2-hydroxyoestradiol–cysteine (IIIa) had R_F0.72. +, Both radioactivity and a Folin–Ciocalteu-positive spot at the given position.

	Paper chromatography	
Tissue	R_F 0.56	R_F 0.72
Kidney		
8000*g* supernatant	−	+
8000*g* supernatant (no ascorbate)	−	+
8000*g* supernatant (under O_2)	−	+
None	+	−
8000*g* supernatant (no substrate)	−	−
8000*g* supernatant (incubated for 24h)	−	+
None (incubated for 24h)	+	−
boiled 8000*g* supernatant	+	−
8000*g* supernatant*	−	+
100000*g* supernatant	+	−
Microsomes	−	+
Microsomes (no ascorbate)	−	+
Boiled microsomes	+	−
Liver		
8000*g* supernatant	+	−
Boiled 8000*g* supernatant	+	−

* The sample contained the alternative 2-hydroxyoestradiol–glutathione isomer (Ib) in place of the isomer (Ia). The isomers are not separable by paper chromatography.

Since no chromatographic distinction can at present be made between *S*-(2-hydroxyoestradiol-1-yl)–cysteinylglycine (IIa) and *S*-(2-hydroxyoestradiol-1-yl)–L-cysteine (IIIa), it was necessary to establish the nature of the material appearing on the paper chromatograms with R_F0.72. For this purpose the aqueous-ethanolic fractions from several incubations with the 8000*g* supernatant of kidney were combined, concentrated and purified by passage through successive columns of Sephadex G-25 and DEAE-Sephadex A-25. Hydrolysis of suitable quantities of the resulting material at 100°C with 2M-hydrochloric acid for 1h or with 6M-hydrochloric acid for 16h released no detectable glycine;

the milder hydrolysis yielded only unchanged 2-hydroxyoestradiol–cysteine (IIIa), but the stronger hydrolysis destroyed the oestrogen moiety and yielded only cystine (Jellinck & Elce, 1969).

Two of the sequence of four enzymes that catalyses the biosynthesis of mercapturic acids are probably identical with the known glutathionase (γ-glutamyl transpeptidase) and cysteinylglycinase (L-cysteinylglycine hydrolase; EC3.4.3.5). This has most recently been indicated for some *S*-benzylglutathione derivatives by Suga, Kumaoka & Akagi (1966). The location in kidney microsomes of the enzymes involved in conversion of the 2-hydroxyoestradiol–glutathione conjugate (Ia) into the cysteine conjugate (IIIa) points to a similar identity, since Revel & Ball (1959) showed that, in the kidney, the glutathionase and cysteinylglycinase were microsome-bound. At present, owing to the difficulties of chromatographic separation, the sequence and relative rates of removal of the glutamate and glycine residues from the 2-hydroxyoestradiol–glutathione conjugate have not been studied.

Acetylation of (2-hydroxyoestradiol-1-yl)-L-[^{35}S]-cysteine (IIIa) to N-acetyl-(2-hydroxyoestradiol-1-yl)-L-[^{35}S]cysteine (IVa). The substrate for this phase of the work, 2-hydroxyoestradiol–[^{35}S]cysteine (IIIa), was prepared by large-scale incubation of the [^{35}S]glutathione conjugate (Ia) with kidney 8000*g* supernatant, followed by purification on columns of Sephadex G-25 and DEAE-Sephadex A-25. The purity and identity of the product were checked by paper chromatography, paper electrophoresis and acid hydrolysis. The material was found to be unstable and could be stored only as a solution (1mg/ml) in 0.5M-acetic acid containing 1.0mM-sodium ascorbate. It was necessary to evaporate samples of this solution to dryness just before use and to redissolve the residue in the appropriate buffer.

The cysteine conjugate (IIIa) was incubated with the 8000*g* supernatant of liver and kidney in the presence of acetyl-CoA according to the method of White *et al.* (1969). The results (Table 2) indicate that both liver and kidney 8000*g* supernatants possess an enzyme capable of catalysing the acetylation of 2-hydroxyoestradiol–cysteine (IIIa) at the α-amino group in the presence of added acetyl-CoA. To determine the degree of acetylation, the bulk of the aqueous ethanolic supernatants was concentrated and applied directly to columns of DEAE-Sephadex A-25, and the elution of radioactivity measured. The position of the eluted peaks provided confirmation of the nature of the incubation products, namely unchanged 2-hydroxyoestradiol–cysteine (IIIa) and 2-hydroxyoestradiol–*N*-acetylcysteine (IVa). The overall recovery of radioactivity in the two peaks was about 60% of the starting material; this low overall recovery may in part by due to difficulties with the stability and purity of the 2-hydroxyoestradiol–cysteine. The

Table 2. *Acetylation of 2-hydroxyoestradiol–L-cysteine*

Tissues were homogenized and centrifuged in 4 vol. of Krebs–Ringer phosphate buffer. The incubation tubes contained 1.0mM-sodium ascorbate (in addition to that present in the stored substrate solution), 0.5mM-acetyl-CoA, 0.3mM-(2-hydroxyoestradiol-1-yl)-L-[^{35}S]cysteine (IIIa) and 1ml of 8000*g* supernatant corresponding to 200mg of tissue, in a total volume of 1.5ml. The tubes were incubated open to the air at 37°C for 2h and then subjected to ethanol precipitation. Portions of the aqueous-ethanolic supernatant were used for paper chromatography. Standard 2-hydroxyoestradiol–cysteine (IIIa) had R_F 0.72 and standard 2-hydroxyoestradiol–*N*-acetylcysteine (IVa) had R_F 0.92. +, Both radioactivity and a Folin–Ciocalteu-positive spot at the given position. No suggestion of relative intensities is intended.

	Paper chromatography	
Tissue	R_F 0.72	R_F 0.92
Liver		
8000*g* supernatant	+	+
8000*g* supernatant (no acetyl-CoA)	+	–
None	+	–
Kidney		
8000*g* supernatant	+	+

Table 3. *Acetylation of S-substituted cysteines by rat liver and kidney*

Tissue	Substrate (*S*-R-cysteine) R-	Yield of acetylation	Reference
Liver and kidney slices	*p*-Bromophenyl-	Partial	Gutman & Wood (1951)
	(*S*-Benzylhomocysteine)	10%	Gutman & Wood (1951)
Liver slices and kidney homogenate + boiled liver	1,2-Dihydro-2-hydroxy-naphthyl-	Partial	Booth, Boyland & Sims (1960)
Kidney slices	1,2,3,4-Tetrahydro-2-hydroxynaphthyl-	No yield reported	Booth, Boyland, Sato & Sims (1960)
Liver slices (kidney slices did not acetylate)	Butyl-	6%	Bray *et al.* (1959*b*)
	p-Chlorobenzyl-	8%	Bray *et al.* (1959*b*)

8000*g* supernatant of both liver and kidney catalysed the acetylation of about 40% of the substrate as measured by the ratio of radioactivity in these two peaks. The acetylation of various *S*-subsituted cysteines by rat tissue preparations *in vitro* has been reported by several groups of workers (Table 3). The reported yields suggest that 2-hydroxyoestradiol–cysteine (IIIa) is a reasonable substrate for the acetylating systems of both kidney and liver.

In the absence of acetyl-CoA very little or no acetylation was observed with either liver or kidney slices fortified with glucose as described by Bray, Franklin & James, (1959*b*), kidney homogenates with added boiled liver supernatant (Booth *et al.* 1960), liver slices fortified with glutamate, or liver homogenates with added ATP.

It is a pleasure to acknowledge the support and advice of Dr P. H. Jellinck. I am grateful to Miss Anne Nicholls for cheerful technical assistance and to Dr C. E. Bird for permission to use his Packard radiochromatogram scanner. Considerable labour was saved by the generous provision of 2-hydroxyoestradiol by the National Cancer Chemotherapy Service. This work was supported by Grant no. MA 3396 from the Medical Research Council of Canada.

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