Biochem. J. (1972) **126**, 1067–1071
Printed in Great Britain

Metabolism of a Glutathione Conjugate of 2-Hydroxyoestradiol-17β in the Adult Male Rat

By JOHN S. ELCE
Department of Biochemistry, Queen's University, Kingston, Ont., Canada

(*Received* 24 *August* 1971)

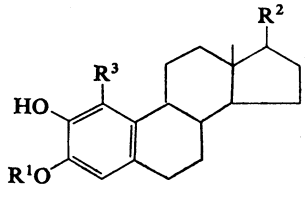
Adult male rats with cannulated or ligated bile ducts were given *S*-(2-hydroxyoestradiol-1-yl)[^{35}S]glutathione, *S*-(2-hydroxy[6,7-$^{3}H_2$]oestradiol-1-yl)glutathione or *S*-(2-hydroxyoestradiol-1-yl)[*glycine*-^{3}H]glutathione by intraperitoneal injection. The recovery of radioactivity in the bile of bile duct-cannulated rats was 33–86% and in the urine of bile duct-ligated rats was 54–105%. Oestrogen thioether derivatives of glutathione, cysteinylglycine, cysteine and *N*-acetylcysteine were isolated from bile; only the *N*-acetylcysteine derivatives could be identified in the urine. The steroid moiety was characterized by microchemical tests before and after treatment with Raney nickel: 2-hydroxyoestradiol-17β was released from the glutathione conjugate, and 2-hydroxyoestrone and 2-hydroxyoestrone 3-methyl ether from the other conjugates. From intact rats the recovery of administered radioactivity was about 15% in the urine and 5% in the faeces over a period of several days and the radioactivity appeared to be largely protein-bound. The results demonstrate that injected oestrogen–glutathione conjugate undergoes conversion into *N*-acetylcysteine derivatives *in vivo*. Oestrogen–glutathione conjugates formed in the intact rat may be excreted in an apparently non-steroidal, possibly protein-bound form, which would not be detected by current analytical techniques.

The formation of conjugates of 2-hydroxyoestradiol-17β [oestra-1,3,5(10)-triene-2,3,17β-triol] with glutathione during incubation of oestradiol-17β with rat liver preparations was demonstrated by Jellinck *et al.* (1967) and by Kuss (1971). This work raised the possibility that oestrogens might follow a mercapturic acid pathway of excretion, hitherto known only for foreign compounds (Boyland & Booth, 1962; Bray *et al.*, 1959). The expected metabolism of *S*-(2-hydroxyoestradiol-1-yl)glutathione (I) to cysteine and *N*-acetylcysteine derivatives has been demonstrated *in vitro* (Elce, 1970). It was therefore decided to establish first whether metabolism of *S*-(2-hydroxyoestradiol-1-yl)glutathione to *N*-acetylcysteine derivatives (mercapturic acids) occurred also *in vivo*. This required the development of new techniques to purify and characterize oestrogen–catechol peptide conjugates from biological fluids; many of the current techniques of oestrogen conjugate analysis are unsatisfactory, since the catechols are highly labile, particularly in alkaline conditions. These techniques and the demonstration of the metabolism of *S*-(2-hydroxyoestradiol-1-yl)glutathione to mercapturic acids *in vivo* are reported in the present paper. This work was a necessary preliminary to the demonstration that 2-hydroxyoestradiol-17β itself is converted into glutathione conjugates *in vivo* (Elce & Harris, 1971).

Experimental

Materials

Non-radioactive conjugates of 2-hydroxyoestradiol with glutathione, cysteine and *N*-acetylcysteine, and *S*-(2-hydroxyoestradiol-1-yl)[^{35}S]glutathione (1.5mCi/mmol) were prepared as described previously (Elce, 1970). 2-Hydroxy[6,7-$^{3}H_2$]oestradiol



	R^1	R^2	R^3
(I)	H	17β-OH	γ-Glu-Cys-Gly
(II)	H	=O	Cys-Gly
(III)	H	=O	Cys
(IV)	CH_3	=O	Cys-Gly
(V)	CH_3	=O	Cys
(VI)	CH_3	=O	*N*-Ac-Cys
(VII)	H	=O	*N*-Ac-Cys

(0.78mCi/mol) was prepared as described by Jellinck & Brown (1971) and used for preparation of *S*-(2-hydroxy[6,7-$^3H_2$]oestradiol-1-yl)glutathione (0.27mCi/mmol) in the usual manner. *S*-(2-Hydroxyoestradiol-1-yl)[*glycine*-^{3}H]glutathione was prepared from L-glutathione labelled with [2-^{3}H]glycine, supplied by New England Nuclear Corp. (Boston, Mass., U.S.A.). [^{3}H]Oestradiol was obtained from Amersham Searle (Toronto, Canada), [^{35}S]glutathione from Schwarz BioResearch Inc. (Orangeberg, N.Y., U.S.A.) and Amberlite XAD-2 (a neutral cross-linked polymer) from Rohm and Haas (West Hill, Ont., Canada). Radioactivity was measured in a toluene–ethanol scintillator solution (Elce, 1970) in a Nuclear-Chicago Unilux II liquid-scintillation spectrometer. Efficiencies of counting were measured by using channels-ratio curves generated by the external standard; ^3H_2O and [^{14}C]hexadecane were used to calibrate the machine (Buckley, 1971). Areas of radioactivity on thin-layer chromatograms were located with a Varian Aerograph model 6000 t.l.c. scanner.

Animals

Adult male Sprague–Dawley and hooded rats were obtained from Canadian Breeding Farms and Laboratories (St. Constant, P.Q., Canada) and were used when they weighed nearly 300g (Kuss, 1971). The bile ducts were cannulated or ligated under ether anaesthesia. The steroid was injected at about 1h after operation and the bile was collected over the next 6h, the rats being maintained supine by intramuscular injection of the veterinary tranquillizer Atravet (0.1mg/kg; acepromazine maleate; Ayerst Laboratories, Montreal, P.Q., Canada). In bile-duct-ligated rats, the steroid was injected after operation during ether anaesthesia, and the rats were provided with water *ad lib.* for 24–48h. In all cases the steroids were injected intraperitoneally in solution in 1–2ml of dimethyl sulphoxide – propan-2-ol – 0.3M-NaCl (3:7:10, by vol.). Bile and urine were collected into 0.5 or 10ml respectively of 0.5M-acetic acid containing ascorbic acid (1mg/ml). Every solvent and column eluent in the entire process, unless otherwise stated, contained 5.6μM-ascorbic acid and the pH was maintained at 3.5–4.0 whenever possible.

Methods

Amberlite XAD-2 (Bradlow, 1968) was washed with methanol and then with water, and the columns (27cm×2.2cm) were packed in 0.1M-acetic acid. Bile and urine were diluted with 0.1M-acetic acid and applied to the column, which was eluted first with 0.1M-acetic acid (200ml) and then with ethanol. The ethanolic eluate from the Amberlite XAD-2 resin was evaporated to dryness and the residue dissolved in 0.1M-sodium acetate buffer, pH5 (10ml). This solution was applied to a column (35cm×2.5cm) of Sephadex G-25 packed in, and eluted with, 0.1M-acetic acid. The greater part of the radioactivity was eluted between 230 and 380ml. This portion of the column eluate was evaporated to dryness and the residue applied in 0.5M-acetic acid (10ml) to a DEAE-Sephadex column (60cm×1.1cm) (Fig. 1) packed in 0.5M-acetic acid, and eluted with a gradient of 4M-acetic acid (250ml) supplied to a reservoir containing 0.5M-acetic acid (250ml). The peaks observed in the eluate of this column were sufficiently pure for microchemical tests and chromatographic identification of their contents.

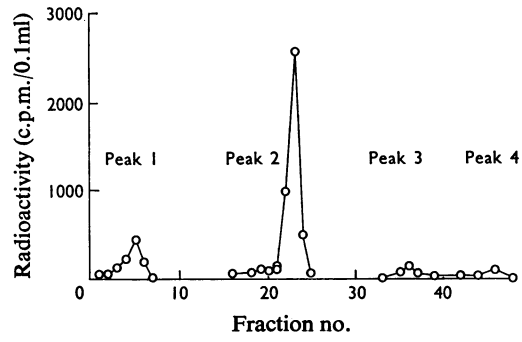


Fig. 1. *Elution of radioactivity derived from injected S-(2-hydroxyoestradiol-1-yl)[^{35}S]glutathione in a bile-duct-cannulated rat*

The ethanol eluate of the bile from Amberlite XAD-2 column was chromatographed on Sephadex G-25 and then on a column (60cm×1.1cm) of DEAE-Sephadex A-25 in 0.5M-acetic acid. Elution was by a gradient of 4M-acetic acid (250ml) supplied to a reservoir containing 0.5M-acetic acid (250ml); 10ml fractions were collected.

The various compounds were characterized by paper chromatography and high-voltage electrophoresis as described previously (Elce, 1970) and by t.l.c. on silica gel in the system butan-1-ol–acetic acid–water (4:1:1, by vol.). Free amino groups were detected with a ninhydrin spray. A useful but not entirely reliable distinction could be made between the response of the catechols and of the catechol monomethyl ethers in their response to Folin–Ciocalteu reagent before and after exposure to NH_3 (Knuppen, 1962), and in their tendency to autoxidize on t.l.c. plates. Compounds containing bivalent sulphur were located with the $K_2Cr_2O_7$–$AgNO_3$ spray of Knight & Young (1958), and the presence of 17-oxo groups was demonstrated with alkaline *m*-dinitrobenzene (Bush, 1961). Amino acids were determined in a Beckman model 120C amino acid analyser after hydrolysis of the sample with 5.7M-HCl

for 16h *in vacuo* at 110°C; about 85% recovery of glycine and glutamic acid was observed from standard *S*-(2-hydroxyoestradiol-1-yl)glutathione, but cysteine was represented in only 10–20% total yield by poorly defined peaks with the retention times of serine, alanine and cystine. To obtain the steroid free of sulphur and attached amino acids, the conjugates in dilute acetic acid solution were shaken overnight at 4°C in the dark with fresh Raney nickel (Fieser & Fieser, 1967), followed by filtration and extraction with an organic solvent. The liberated steroids were characterized by standard chromatographic criteria and in some cases by crystallization with added carrier to constant specific radioactivity.

Results and Discussion

The recoveries of radioactivity in the bile or urine and in discrete peaks in the eluate of the DEAE-Sephadex column are shown in Table 1. The pattern of radioactivity in the eluate is illustrated for a typical case in Fig. 1. Table 1 shows that in the bile duct-cannulation experiments the relative amounts of the various peaks did not vary in any regular manner with doses of 0.3–24mg, which were the limits imposed by available radioactivity and by solubility. The greater part of the injected dose was always recovered unchanged as *S*-(2-hydroxyoestradiol-1-yl)glutathione (I) (peak 2, Fig. 1); the sum of cysteine, cysteinylglycine (peak 1, compounds II–V) and *N*-acetylcysteine (peak 3, compound VI, and peak 4, compound VII) metabolites varied between one-fifth and one-half of the unchanged material. The recovery of radioactivity within the first 6h after injection of *S*-(2-hydroxyoestradiol-1-yl)[^{35}S]-glutathione is shown in Table 2, and indicates a very rapid excretion of the radioactivity into the bile. In these experiments the urine was not collected.

In contrast, when the bile duct was ligated and the steroids were retained within the animal for a longer time, no unchanged *S*-(2-hydroxyoestradiol-1-yl)-glutathione could be detected in the urine (Table 1), and almost complete conversion into mercapturic acids (peaks 3 and 4) was observed.

Several attempts have been made to determine the chemical nature of the compounds associated with radioactivity excreted in the urine of intact rats that had received *S*-(2-hydroxyoestradiol-1-yl)[^{35}S]glutathione. The recovery in the urine over several days was about 15% and in the faeces about 5%; thereafter excretion was negligible. Approximately 80% of the radioactivity in the urine passed through an Amberlite XAD-2 column in the aqueous phase. Since all known steroids and steroid conjugates are retained by this resin and since fission of the thioether was considered an unlikely metabolic process, the behaviour of the ^{35}S on the Amberlite XAD-2 column suggested that

Table 1. *Recovery of radioactivity in the bile or urine, and in discrete peaks in the eluate of the DEAE-Sephadex column*

The recoveries are expressed as percentages of the radioactivity injected as *S*-(2-hydroxyoestradiol-1-yl)[^{35}S]-glutathione or *S*-(2-hydroxyoestradiol-1-yl)[*glycine*-^{3}H]glutathione and appearing in bile in 6h or urine in 24h. For details see the Experimental section.

Radioactive isotope	Dose of oestradiol conjugate (mg)	Recovery of radioactivity (% of dose)				
		In bile	In column eluate			
			Peak 1	Peak 2	Peak 3	Peak 4
Bile-duct-cannulation experiments						
glycine-^{3}H+^{35}S	17.0	60 (^{3}H)	0.7	36	0	0
		74 (^{35}S)	6.1	36	2.1	1.6
^{35}S	24.0	76	8.1	31	2.8	5.7
	22.0	89	7.0	66.4	3.8	6.2
	17.0	75	6.1	36	2.1	1.6
	6.8	33	2.0	19.4	10.7	
	0.31	80	7.4	49.5	8.8	12.0
S-(2-Hydroxy[6,7-$^{3}H_2$]oestradiol-1-yl)glutathione added to bile	1.0	—	0	84	0	0
Bile-duct-ligation experiments		In urine				
^{35}S	3.0	54	0.4	0	6.7	11.8
	3.6	105	0.2	0	12.8	17.3
	8.2	90	7.4	0	13.9	34.6

Table 2. *Excretion of bile and recovery of radioactivity*

The recoveries of radioactivity in bile are expressed as percentages of the injected dose during each of the 6h after injection.

	Rat 1		Rat 2	
Time (h)	Wt. of bile (g)	Radio-activity (% of dose)	Wt. of bile (g)	Radio-activity (% of dose)
0–1	0.51	28.1	0.87	27.4
1–2	0.54	32.3	0.71	20.9
2–3	0.65	18.0	0.66	14.7
3–4	0.59	6.8	0.61	9.0
4–5	0.64	3.7	0.64	2.8
5–6	—	—	0.64	0.1
Total		88.9		74.9

most of the ^{35}S was present in metabolites that had lost all steroidal character, or which while still steroidal were covalently bound to protein or to some other water-soluble carrier molecule whose properties predominated on the Amberlite XAD-2 column.

The character of the amino acid side chain could, in general, be deduced from the composition of the eluate of the DEAE-Sephadex column, from paper chromatography and high-voltage electrophoresis. Amino acid analysis after acid hydrolysis was followed by ninhydrin and potassium dichromate–silver nitrate sprays. Similarly the nature of the steroid moiety was established by the evidence of Folin–Ciocalteu and alkaline *m*-dinitrobenzene sprays, and the mobilities of the product of Raney nickel treatment.

The position of peak 1 (Fig. 1) is characteristic of both cysteinylglycine and cysteine–oestrogen derivatives. The result of simultaneous injection of *S*-(2-hydroxyoestradiol-1-yl)[*glycine*-^{3}H]glutathione and *S*-(2-hydroxyoestradiol-1-yl)[^{35}S]glutathione (Table 1) suggested that peak 1 contained about 89% cysteine and 11% cysteinylglycine derivatives. The results of amino acid analysis were qualitatively in agreement with this. After an injection of *S*-(2-hydroxy[6,7-$^{3}H_2$]-oestradiol-1-yl)glutathione and purification of the metabolites, material eluted in peak 1 was treated with Raney nickel and later was filtered and extracted with an organic solvent. The recovery of radioactivity in the organic extract was almost quantitative. T.l.c. of a small portion of this extract in benzene–ethanol (19:1, v/v) indicated the presence of 2-hydroxyoestrone [2,3-dihydroxyoestra-1,3,5(10)-trien-17-one], but the major component had a mobility on paper in formamide–cyclohexane equal to that of 2-hydroxyoestrone 3-methyl ether and different from that of 2-methoxyoestrone (Knuppen & Breuer, 1966). The remainder of the organic extract was reduced with sodium borohydride, after addition of 2-hydroxyoestradiol 3-methyl ether (31mg) (Nambara *et al.*, 1970), and the product recrystallized to constant specific radioactivity with respect to ^{3}H.

It was concluded that peak 1 contained predominantly *S*-(2-hydroxyoestrone 3-methyl ether-1-yl)cysteine (V), together with an indeterminate mixture of compounds (II), (III) and (IV).

In peak 2 *S*-(2-hydroxyoestradiol-1-yl)glutathione was the only detectable component. Its character was confirmed by crystallization to constant specific radioactivity both as the conjugate and as 2-hydroxyoestradiol after nickel treatment.

Evidence similar to that described above indicated that peaks 3 and 4 contained respectively *N*-acetyl-*S*-(2-hydroxyoestrone 3-methyl ether-1-yl)cysteine and *N*-acetyl-*S*-(2-hydroxyoestrone-1-yl)cysteine as their only detectable components.

The results described above demonstrated that the expected metabolism of the glutathione conjugate via cysteinylglycine and cysteine to *N*-acetylcysteine derivatives (mercapturic acids) had occurred *in vivo*, accompanied by complete oxidation of the 17β-hydroxyl group and by partial methylation of the C-3 phenolic group. The oxidation and methylation steps occurred only after removal of at least the glutamic acid moiety. The stereospecific nature of the methylation is in agreement with the findings *in vitro* of R. Knuppen (personal communication) that rat liver catechol *O*-methyltransferase (EC 2.1.1.6) will catalyse methylation of *S*-(2-hydroxyoestradiol-1-or-4-yl)cysteine only at the phenolic group remote from the thioether.

The recent demonstration that 2-hydroxyoestradiol is converted into glutathione conjugates in the rat lends further support to the idea that oestrogen mercapturic acids may be present among the water-soluble metabolites of the oestrogens (Elce & Harris, 1971).

The time and skill of Dr. T. G. Flynn in performing the bile cannulations and the interest and support of Professor P. H. Jellinck are most gratefully acknowledged. Considerable labour was saved by the generous provision of 2-hydroxyoestradiol by the National Cancer Chemotherapy Service (Bethesda, Md., U.S.A.). 2-Methoxyoestrone was kindly supplied by Dr. D. N. Kirk from the Medical Research Council Steroid Reference Collection (Westfield College, London N.W.3, U.K.). This work was supported by grant no. MA 3396 from the Medical Research Council of Canada, of which J.S.E. is a scholar.

References

Boyland, E. & Booth, J. (1962) *Annu. Rev. Pharmacol.* **2**, 129

Bradlow, H. L. (1968) *Steroids* **11**, 265

Bray, H. G., Franklin, T. J. & James, S. P. (1959) *Biochem. J.* **71**, 690

Buckley, J. T. (1971) *Int. J. Appl. Radiat. Isotop.* **22**, 41

Bush, I. E. (1961) *The Chromatography of Steroids*, p. 375, Pergamon Press, Oxford

Elce, J. S. (1970) *Biochem. J.* **116**, 913

Elce, J. S. & Harris, J. (1971) *Steroids* **18**, 583

Fieser, L. F. & Fieser, M. (1967) *Reagents for Organic Synthesis*, vol. 1, p. 729, John Wiley and Sons, New York

Jellinck, P. H. & Brown, B. J. (1971) *Steroids* **17**, 133

Jellinck, P. H., Lewis, J. & Boston, F. (1967) *Steroids* **10**, 329

Knight, R. H. & Young, L. (1958) *Biochem. J.* **70**, 111

Knuppen, R. (1962) *Z. Vitam. Hormon. Fermentforsch.* **12**, 355

Knuppen, R. & Breuer, H. (1966) *Hoppe-Seyler's Z. Physiol. Chem.* **346**, 114

Kuss, E. (1971) *Hoppe-Seyler's Z. Physiol. Chem.* **352**, 817

Nambara, T., Honma, S. & Akiyama, S. (1970) *Chem. Pharm. Bull.* **18**, 474