WATER-SOLUBLE METABOLITES OF THE ESTROGENS. QUANTITATION OF C-18 TETROLS IN RAT FECES.

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ABSTRACT

Several radioactive estrogens possessing one, two and three hydroxyl groups were injected orally (and in the case of estrone sulfate also intraperitoneally) into adult male rats. The rats were either intact or had ligated or cannulated bile ducts. Two unconjugated estrogen tetrols together represented 21 - 87% of the total metabolites in the intact rat. One of the tetrols was 2-hydroxyestriol (estra-1,3,5(10)-triene-2,3,16α, 17β-tetrol); the other may be estra-1,3,5(10)-triene-2,3,6ξ,17β-tetrol but this was not confirmed. It is concluded that poly-hydroxylated estrogens represent a very large proportion of the previously unidentified water-soluble metabolites of the estrogens in the adult male rat.

INTRODUCTION

It has been repeatedly demonstrated, both in the rat and in man, that a major portion of the radioactive metabolites of injected [^{14}C]-estrogen remains water-soluble even after treatments which are expected to release steroids from conjugation with glucuronic and sulfuric acids. This has continued to be true following improvements in isolation techniques and in the efficiency of hydrolysis of the conjugates. Breuer (1) suggested that some of the following types of compounds might remain in the aqueous phase: (i) unhydrolyzed conjugates of unknown type; (ii) poly-hydroxylated steroids which are highly water-soluble even when unconjugated; (iii) various structures involving enzymic or non-enzymic disruption of the steroid ring system. All of these may include the products formed while the metabolites are still in the excreta *in vivo*, or during isolation. The ring-D α-ketols and especially the ring-A catechols are difficult to manipulate without loss.

Some new types of estrogen conjugate have been detected (2), such as N-acetylglucosaminides, glucosides and glutathione derivatives, but are not quantitatively very important. In the rat, for example, the glutathione conjugates represent less than 10% of the total estrogen

metabolites (3) and in man they could not be detected at all (4). Although certain micro-organisms are capable of opening the steroid ring system (5) there has been no suggestion that this occurs in a mammalian species. Recently, the use of ascorbic acid as an anti-oxidant and of mildly acid conditions (6,7) has been shown to minimize the decomposition of the labile estrogens. Attention is therefore now drawn mainly to the poly-hydroxylated estrogens in the search for quantitatively significant 'water-soluble metabolites'.

In the present work, isotope techniques, extraction procedures and column chromatographic systems suitable for the more polar steroids (8) were employed. As a model system, rat fecal metabolite patterns were studied, since the adult male rat is known to excrete more than 80% of its estrogen metabolites via the feces, and its liver is also known to be particularly active in steroid hydroxylation.

MATERIALS AND METHODS

Adult male hooded rats (250 - 450 g) were obtained from Canadian Breeding Farms and Laboratories Ltd., St. Constant, P.Q. Radioactive compounds and liquid scintillation materials were obtained from New England Nuclear. Radioactivity was determined on a Beckman Unilux II liquid scintillation spectrophotometer. Efficiencies were determined by means of the external standard. In double isotope determinations, the efficiency for ^{14}C was about 55% and for ^{3}H was about 25%. Other chemicals were obtained from Sigma Chemical Co. 2-Hydroxyestradiol was kindly supplied by the Cancer Chemotherapy National Service Centre, Bethesda, Md. Estetrol was a gift from Dr. N.F. Taylor of the Division of Clinical Chemistry, Clinical Research Centre, Middlesex, U.K. Amberlite XAD-2 and Amberlyst-15 were purchased from British Drug Houses Ltd., Toronto. Celite 545 was obtained from Johns-Manville, Toronto, and Ketodase (a beef liver β-glucuronidase extract) from Warner-Lambert, Toronto.

Synthesis of 2-Hydroxyestriol

The method of Nambara *et al.* (9) was modified for synthesis of gram quantities of 2-hydroxyestriol. We are grateful also to Dr. H. Watanabe for details of her experience with this method (10). In summary, estriol was methylated with dimethyl sulfate (11) and then acetylated with acetic anhydride in pyridine to estriol 3-methyl ether 16α,17β-di-acetate. Friedel-Crafts acylation of the di-acetate with $AlCl_3$ and acetyl chloride in a mixture of CH_2Cl_2 and CS_2 yielded the 2-acetyl derivative, m.p. 205 - 208°C, λ_{max} 315 nm (ε 3414), λ_{min} 282 nm (ε 602). The acetyl compound was treated in $CHCl_3$ solution with three equivalents of m-chloro-peroxybenzoic acid for 2 h at room temperature in the dark and then extracted, yielding estriol 3-methyl ether 2,16α,17β-tri-acetate, m.p. 157 - 160°C, λ_{max} 285 nm (ε 1630), 278 nm (ε 1745); analytical figures for $C_{25}H_{32}O_7$, calculated, C 67.55%; H 7.26%; observed, C 67.30%; H 7.04%. The tri-

acetate was hydrolyzed with hot alcoholic KOH under N_2, yielding 2-hydroxyestriol 3-methyl ether, m.p. 272 - 275°C, λ_{max} 286 nm (ε 3312), λ_{min} 253 nm (ε 350) (12). The methyl ether was cleaved with BBr_3 in CH_2Cl_2 at -80°C (13) to yield 2-hydroxyestriol, m.p. 255 - 265°C, λ_{max} 288 nm (ε 3171) (14) The overall yield of 2-hydroxyestriol from estriol was 17%. This demethylation procedure was much superior to treatment with pyridine hydrochloride and represents an improvement of the procedure of Nambara *et al.* (9). The use of Fremy's salt (15) is ideal for production of mg quantities of 2-hydroxylated estrogens, but was not found suitable for an increase in scale owing to the necessity for column chromatographic separation of the products.

Radioactive catechol estrogens were prepared by the method of Jellinck & Brown (16) using mushroom tyrosinase. [4-^{14}C]Estrone sulfate sodium salt was prepared by the method of Levitz (17).

Thin layer chromatography (TLC) was carried out on 0.25 mm layers of Silica Gel G (Macherey-Nagel), using some of the solvent systems listed by Lisboa (18). The steroids were detected by spraying the plates with Folin-Ciocalteau reagent (10% v/v in water) followed by exposure of the plates to NH_3 vapour; or by spraying the plates with 2% H_2SO_4 in 50% (v/v) aqu. ethanol followed by heating at 85°C for 10 - 15 min. Radioactive steroids were detected by means of a Varian Aerograph Berthold scanner. Paper chromatography was performed on Whatman 3MM paper impregnated with formamide, using cyclohexane, chloroform, or chloroform-ethyl acetate (5:1, v/v) as the mobile phase (19).

Throughout all the extraction procedures and during paper and column chromatography, all aqueous solutions were 0.1M in acetic acid and contained ascorbic acid.

Amberlite XAD-2 resin was washed thoroughly with methanol and acetone and used as described by Bradlow (20). Amberlyst-15 in the NH_4^+ form was used as described by Gustafsson *et al.* (21); the samples were dissolved in 10 ml of methanol-chloroform (4:1, v/v) and applied to a column of 5 g of Amberlyst-15; the columns were eluted with 100 ml of the same solvent mixture, collecting 10 ml fractions. The principal radioactive fractions eluted from the Amberlyst-15 column were evaporated to dryness and the residue dissolved in 1 ml of methanol and 19 ml of 0.1M-acetic acid (containing also 1 mg/ml ascorbic acid) for subsequent extraction by 3 x 10 ml each of hexane, methylene dichloride and n-butanol.

Celite 545 was washed in 6M-HCl, water and methanol (22). Three systems of Celite partition column chromatography were employed (Table 1). For each column, 100 mg of ascorbic acid were added per 10 ml of stationary phase for mixing with the appropriate amount (2:3, v/w) of dry Celite. The moistened Celite was packed into the column as described by Siiteri (23) and mobile phase initially drawn through under vacuum. The sample was dissolved in the minimum volume of stationary phase, which was then mixed with Celite and packed onto the top of the column. The eluate was collected in 10 ml fractions.

Estrogen conjugates were chromatographed on columns of DEAE-Sephadex A25 (10 g, 1x50 cm) with a linear gradient of 500 ml of 0.1M-acetic acid

and 500 ml of 0.8M-NaCl in 0.1M-acetic acid (24), the solutions also containing 1 µg/ml of ascorbic acid.

Table 1. Celite column chromatography systems

System	Phase	Composition
1	Stationary	Lower phase from H_2O-acetic acid-methanol-hexane (4:1:46:50)
	Mobile	Upper phase from the above mixture
	Gradient	Mobile phase-methylene dichloride (1:1)
		5 g of Celite, 1x20 cm column. The column was eluted with 200 ml of mobile phase, followed by a gradient consisting of 250 ml of mobile phase and 210 ml of gradient phase (22).
2	Stationary	Lower phase from benzene-ethyl acetate-methanol-H_2O (13:7:11:9)
	Mobile	Upper phase from benzene-methanol-H_2O (6:5:4)
	Gradient	Ethyl acetate
		18 g of Celite, 1x56 cm column. The column was eluted with a gradient consisting of 150 ml of mobile phase and 150 ml of gradient phase (system 12 of Jirku *et al.* (8)).
3	Stationary	Lower phase from benzene-hexane-methanol-acetone-H_2O (8:8:6:5:10)
	Mobile	Upper phase from the above mixture
	Gradient	Ethyl acetate-benzene (1:3)
		18 g of Celite, 1x56 cm column. The column was eluted with a gradient consisting of 400 ml of mobile phase and 400 ml of gradient phase (system 15 of Jirku *et al.* (8)).

Hot acid hydrolysis of estrogen conjugates was performed in the reducing conditions described by Gelbke & Knuppen (25). Ketodase hydrolysis of glucuronide conjugates was performed as described by Keith & Williams (26) in the presence of 0.05M-ascorbic acid. Control incubations were performed which lacked Ketodase. The activity of the enzyme was assayed before and after incubation by means of phenolphthalein glucuronide (27).

Treatment of the rats and administration of estrogens

For ligation, the bile ducts were tied twice and cut. For bile collection, the bile duct was cannulated with vinyl tubing which was taken out through the back of the rat to a glass receiver secured with tape onto the shoulders of the rat.

The animals, in metabolic cages, were given free access to water at all times. Food was withdrawn only for the 24 h following administration of steroid. Urine and feces were collected separately, daily for up to three days. The excreta fell immediately on voiding into flasks containing 10 ml of 0.5M-acetic acid and 100 mg of ascorbic acid. Bile samples were collected into 2 ml of the same solution.

For oral injection, the steroid was dissolved in the minimum volume of ethanol (0.2 - 0.5 ml), the solution made up to 1 ml with water, and administered to the rat under light ether anesthesia, by means of a stomach tube. The tube and syringes were washed with ethanol and the radioactivity in the wash determined. For intraperitoneal (i.p.) injection, the steroid was dissolved in 1 ml of 10% ethanolic saline (ethanol-0.9% NaCl, w/v (1:9)).

Extraction procedures

The feces collected each day were homogenized in ethanol and the mixture centrifuged. For combustion, 100 mg of dried ethanol-insoluble material were mixed well with KIO_3 (2 g) and $K_2Cr_2O_7$ (1 g) followed by addition of 9 ml of conc.H_2SO_4-conc.H_3PO_4 (1:1, v/v), and brisk heating under N_2 (28). The CO_2 produced was absorbed in Cellosolve-ethanolamine (1:1,v/v). The method was calibrated on each occasion by combustion of standard [^{14}C]methyl methacrylate tablets mixed with non-radioactive feces, and was found to give a yield of 60 - 80% of the theoretical.

The ethanolic supernatant was evaporated to dryness and the residue redissolved in 20% (v/v) aqu. methanol and left for 2 days at -20°C. The soluble material was passed through an Amberlyst-15 column followed by solvent extraction. The hexane- and methylene dichloride-soluble material was chromatographed on Celite in system 1; the n-butanol-soluble material was chromatographed on Celite successively in systems 2 and 3. The radioactivity in the urine from intact rats was determined but not analyzed.

The urine and bile samples from rats with ligated or cannulated bile ducts were passed through an XAD-2 column followed either by hot acid hydrolysis or by ion-exchange chromatography on DEAE Sephadex A25 and Ketodase hydrolysis. The products from either treatment were further purified by solvent extraction and Celite chromatography as described for the fecal extracts. The surgically modified rats passed little or no feces.

Treatment of data and corrections for recovery

Authentic radioactive standards of conjugates of 2-hydroxyestradiol and of 2-hydroxyestriol were not available (and the number of possible alternatives is daunting). In an effort to prepare such standards,

[^{14}C]2-hydroxyestriol was incubated with UDP-glucuronic acid and liver preparations from mouse, guinea pig and rat (29), but poor yields of mixtures of several conjugate forms were obtained (as judged by DEAE-Sephadex chromatography). It was therefore necessary to use free [^{3}H]-estrogens as recovery tracers.

Two types of correction were applied to the excretion data, and the implicit assumptions are described later: (i) The amounts of radioactivity (^{14}C dpm) in various extracts and eluted chromatographic peaks were expressed as percentages either of the amount initially present in the excreta or of the amount initially administered to the rat. In general, 300 - 500 µg of [^{3}H]2-hydroxyestriol were added to the excreta to act both as internal standard and also as carrier. The percentage of this added [^{3}H]2-hydroxyestriol recovered in the final step of the extraction was used to correct for losses of ^{14}C during the extraction; this provided an estimate of the amount of [^{14}C]2-hydroxyestriol which had initially been present in the sample of excreta. (ii) As a further correction, the recovery of orally administered radioactive free 2-hydroxyestriol in unaltered form in the feces, in one animal, could be used to correct for losses of [^{14}C]2-hydroxyestriol formed *in vivo* from injected precursors in another animal. It is recognized that this latter type of recovery calculation can give only approximate results.

In one case [^{3}H]2-hydroxyestriol was administered simultaneously with [^{14}C]estrone sulfate, and the conversion of [^{14}C]estrone sulfate to [^{14}C]2-hydroxyestriol was calculated from the isotope ratios of the injected dose and of the [^{3}H,^{14}C]2-hydroxyestriol extracted and purified from the feces.

RESULTS

Bile duct-ligated rats

Since the results of this part of the work were largely negative with respect to 2-hydroxyestriol production, little detail is reported.

[^{14}C]2-Hydroxyestriol (0.6 µCi, 244 µg) was administered orally to a rat with a ligated bile duct. The recovery of radioactivity in the urine was 91.2% in 44 h. The urine was passed through XAD-2 resin and [^{3}H]2-hydroxyestriol (0.45 µCi, 450 µg) was added to the ethanol eluate, followed by acid hydrolysis, solvent partition and column chromatography. In the 2-hydroxyestriol peak eluted from a Celite column in system 3, the recoveries were 26.8% of the ^{14}C originally present in the urine and 49.2% of the ^{3}H added. It may therefore be calculated that at least 54.5% of the ^{14}C present in the urine was in a form readily converted to free [^{14}C]2-hydroxyestriol by hot acid hydrolysis.

[^{14}C]2-Hydroxyestriol (0.5 µCi, 211 µg) was administered orally to a second bile duct-ligated rat. The recovery of ^{14}C was 78.2% in 26 h. Part of the urine, after the XAD-2 column, was chromatographed directly

on Celite in system 3, and part was purified on DEAE Sephadex and subjected to Ketodase hydrolysis. It was shown that about 6.4% of the ^{14}C present in the urine was in the form of free [^{14}C]2-hydroxyestriol, and at least 42% was present as a monoglucuronide of 2-hydroxyestriol. This is to be compared with the value of 54.5% arrived at using hot acid hydrolysis.

When [^{14}C]estrone sulfate (2.4 μCi, 4.38 mg) was injected orally into a bile duct-ligated rat, only 23.2% of the dose was recovered in 31 h. No analysis was performed. Other experiments also suggested that intestinal absorption of estrone sulfate was inhibited in the absence of bile while the more polar steroids such as 2-hydroxyestriol were still efficiently absorbed.

[^{14}C]Estriol (0.5 μCi, 2.3 mg) and [^{3}H]2-hydroxyestradiol (1.0 μCi, 1.26 mg) were injected simultaneously into a bile duct-ligated rat. The recoveries of radioactivity in the urine in 46 h were 92.2% and 94.5% respectively. After the normal extraction process and Ketodase hydrolysis, it appeared that less than 2% of the injected estriol and none of the injected 2-hydroxyestradiol had been converted to 2-hydroxyestriol.

Bile duct-cannulated rats

A recovery of 38% of orally injected [^{14}C]2-hydroxyestriol (0.7 μCi, 305 μg) was obtained in the bile in 24 h. The bile contained mono-glucuronides of two radioactive compounds, which were shown to be probably 2-methoxyestriol (3% of the injected dose) and 2-hydroxyestriol (positively identified; 10.7% of the injected dose).

The recovery of orally injected [^{14}C]estrone sulfate (2.5 μCi, 4.5 mg) was only 4.3% in the bile in 22 h. In another rat after i.p. injection of [^{14}C]estrone sulfate (1.0 μCi, 1.9 mg), the recovery in the bile was 60% in 18 h. [^{3}H]2-Hydroxyestriol was added to this bile and after Ketodase hydrolysis 0.4% of the injected ^{14}C and 60% of the added ^{3}H were recovered in the 2-hydroxyestriol peak eluted from the Celite column in system 3.

Intact rats - fecal excretion patterns

Five different [^{14}C]-labelled estrogens and in one case also [^{3}H]-2-hydroxyestriol were administered orally to a total of ten rats, the rats being numbered as shown in Table 2, which lists the doses and

Table 2. Recoveries of administered radioactivity in the excreta of intact rats. Data are given as total percentages of the injected doses over 3 days.

Rat	Compound injected	Amount injected µC	Amount injected mg	Urine	Feces Ethanol soluble	Feces Ethanol insoluble	Total recovery
1	[^{14}C]E_1S	1.7	3.08	12.4	46.8	46.4	105.6
2	[^{14}C]E_1S	1.7	3.13	11.8	32.6	20.5	64.9
3	[^{14}C]E_1S	1.3	2.37	10.0	58.0	41.5	109.5
	& [^{3}H]$2HOE_3$	1.6	0.70	6.7	50.6	--*	--
4	[^{14}C]E_2	1.9	1.84	11.9	51.0	39.8	102.7
5	[^{14}C]E_2	2.0	2.01	11.3	49.6	36.0	96.9
6	[^{14}C]$2HOE_2$	0.9	1.18	9.3	42.1	56.9	108.3
7	[^{14}C]$2HOE_2$	0.9	1.11	8.7	42.3	38.9	89.9
8	[^{14}C]E_3	0.6	3 µg	9.3	54.8	41.1	105.2
9	[^{14}C]$2HOE_3$	0.8	0.36	4.6	25.3	37.9	67.8
10	[^{14}C]$2HOE_3$	0.5	0.22	9.2	46.3	40.4	95.9
	[^{14}C]E_2 added to feces				96.0		
	[^{14}C]$2HOE_2$ added to feces				79.0		
	[^{14}C]$2HOE_3$ added to feces				71.0		

* Collection of [^{3}H]water after combustion was not attempted.

percentage recoveries. The amounts of radioactivity in the ethanol supernatant and in the ethanol-insoluble residue were usually approximately equal. It should be noted that the more polar estrogens simply added to feces and then immediately extracted could not be entirely recovered in the ethanol supernatant. Extraction of the feces with water alone, or extraction with methanol under reflux, did not improve the recoveries of soluble radioactivity. It was assumed that estrogen conjugates would be soluble in the ethanol-dilute acetic acid-ascorbic acid to which the feces were exposed, but conjugated estrogens were not detectable in the soluble fecal extracts, so that Ketodase- or other hydrolysis was not necessary.

The distribution of fecal ethanol-soluble radioactivity during solvent partition is shown in Table 3, in order to give some idea of the relative polarities of the metabolites.

Table 3. Distribution of fecal radioactivity after extraction with organic solvents. The amounts of radioactivity in each extract are expressed as percentages of the total radioactivity present in the fecal ethanol-soluble supernatant.

Rat #	Compound injected	Hexane	Methylene chloride	n-Butanol	Aqueous
1	[^{14}C]E_1S	3.1	31.0	31.6	2.3
2	[^{14}C]E_1S	0.8	20.2	50.8	7.6
3	[^{14}C]E_1S	2.8	44.5	39.9	3.6
	& [^{3}H]2HOE_3	5.9	10.2	75.9	6.0
4	[^{14}C]E_2	0.6	31.7	51.9	4.1
5	[^{14}C]E_2	0.6	26.2	60.5	4.2
6	[^{14}C]2HOE_2	0.8	29.4	48.8	4.8
7	[^{14}C]2HOE_2	0.9	39.0	41.7	4.4
8	[^{14}C]E_3	0.7	10.8	56.1	10.2
9	[^{14}C]2HOE_3	2.1	10.8	64.0	2.3
10	[^{14}C]2HOE_3	0	8.4	64.5	2.3

The hexane- and methylene dichloride-extracted metabolites were usually identified only by their positions of elution from the Celite column in system 1, with respect to standard compounds, except for 2-hydroxyestradiol which was studied in more detail.

The amounts of radioactivity recovered in the 2-hydroxyestradiol peak from Celite columns in system 1 are given in Table 4. It may be seen that estrone sulfate and estradiol were converted to fecal 2-hydroxy estradiol in the range of 4.3 - 9.8%, while injected 2-hydroxyestradiol was itself recovered unchanged in the feces to the extent of 9.1 and 13.9%. From these figures, and making certain assumptions which are discussed later, two extreme values can be obtained for the conversion of estrone and estradiol to 2-hydroxyestradiol *in vivo*: 4.3/13.9, i.e. approximately 31%; and 9.8/9.1, i.e. 100% (Table 7, see Discussion).

Table 4. Recoveries of radioactivity in the 2-hydroxyestradiol peak eluted from Celite in system 1. The data in column (a) are the % of the injected doses which were recovered in this peak. The data in column (b) are obtained by dividing those in column (a) by 0.39, since the recovery from the Celite column in system 1 of [^{3}H]2-hydroxy-estradiol added to feces was 39%. The figures in column (b) therefore represent the % conversion of the injected steroids to fecal 2-hydroxy-estradiol.

Rat	Compound injected	% injected dose in Celite peak (a)	% injected dose in feces (b)
1	[^{14}C]E_1S	3.8	9.8
2	[^{14}C]E_1S	1.8	4.7
3	[^{14}C]E_1S	1.7	4.3
4	[^{14}C]E_2	3.6	9.1
5	[^{14}C]E_2	1.8	4.7
6	[^{14}C]2HOE_2	3.6	9.1
7	[^{14}C]2HOE_2	5.4	13.9
	[^{3}H]2HOE_2 *added* to feces	39.0	

The n-butanol-extracted metabolites derived from the fecal ethanol-soluble radioactivity usually gave rise to two peaks of radioactivity on Celite in system 2, designated peak 2.1 (eluted between 70 and 110 ml) and peak 2.2 (110 - 170 ml). Peak 2.1 was obtained following estrone sulfate, estradiol or estriol injection, but was absent following 2-hydroxyestradiol or 2-hydroxyestriol injection. It was not further analysed. The radioactive material in peak 2.2, which was always observed, was rechromatographed on Celite in system 3; the only significant radioactive peaks were designated 3.1 (370 - 420 ml) and 3.2 (450 - 500 ml). It was shown that peak 3.1 was composed largely of 2-hydroxyestriol: crystallization of 47,000 dpm ^{14}C of peak 3.1 material with 39.7 mg carrier 2-hydroxyestriol (predicted value, 1175 dpm/mg) gave values of the specific radioactivity in successive crystals of 838, 798, 755, 779 dpm/mg. The structure of the principal component of peak 3.2 has not been determined. Both peaks 3.1 and 3.2 were obtained following injection of estrone sulfate, estradiol and 2-hydroxyestradiol; peak 3.2 was absent following injection of estriol and 2-hydroxyestriol. The R_f values and elution positions of the peak 3.2 material and of various standards are listed in Table 5.

Table 5. R_f values (in the system cyclohexane-ethyl acetate-ethanol (9:9:1) on TLC) and elution position from the Celite column in system 3 of the peak 3.2 material and of some reference compounds.

Compound	R_f	Celite, system 3 (ml)
17-epi-E_3	0.66	--
16-epi-E_3	0.60	30 - 70
4HO-16-epiE_3*	0.54	80 - 100
2HO-16-epiE_3*	0.46	110 - 140
16,17-epiE_3	0.38	--
E_3	0.29	140 - 160
2HOE_3	0.24	370 - 420
E_4	0.21	330 - 400
6α-HOE_3	0.08	> 800
Peak 3.2	0.20	450 - 510

* Prepared by the method of Gelbke *et al.* (15)

The evidence of the relative mobilities, and the fact that peak 3.2 was not formed from estriol or 2-hydroxyestriol, suggest that it is a tetrol with hydroxyl groups at C2, C3, C17 and at one other position not including C16. It is referred to subsequently as the unknown tetrol.

The amounts of radioactivity recovered in peaks 3.1 and 3.2 from Celite columns in system 3 are given in Table 6.

Table 6. Recoveries of radioactivity in the 2-hydroxyestriol peak (3.1) and in the unknown tetrol peak (3.2) eluted from Celite in system 3. The data in columns (a) and (b) are the percentages of the injected doses which were recovered in these peaks. The data in columns (c) and (d) are obtained by dividing those in column (a) and (b) by 0.45, since the recovery to peak 3.1 of [^{3}H]2-hydroxyestriol added to feces was 45%; the assumption has been made that the behaviour of the unknown tetrol was sufficiently similar to that of 2-hydroxyestriol to apply the same recovery corrections. The figures in columns (c) and (d) therefore represent the percentage conversion of the injected steroids to fecal 2-hydroxyestriol and unknown tetrol, respectively.

Rat	Steroid injected	% injected dose in Celite peaks		% injected dose in feces	
		3.1 (a)	3.2 (b)	fecal 2HOE$_3$ (c)	fecal unknown tetrol (d)
1	[^{14}C]E$_1$S	1.4	1.8	3.0	3.9
2	[^{14}C]E$_1$S	2.3	2.2	5.1	5.0
3	[^{14}C]E$_1$S	3.3	1.2	7.2	2.7
	[^{3}H]2HOE$_3$	15.7	-	34.8	-
4	[^{14}C]E$_2$	4.3	3.3	9.6	8.1
5	[^{14}C]E$_2$	5.0	5.0	11.2	11.2
6	[^{14}C]2HOE$_2$	1.2	2.8	2.7	6.3
7	[^{14}C]2HOE$_2$	1.2	2.5	2.8	5.3
8	[^{14}C]E$_3$	3.6	nil	8.0	nil
9	[^{14}C]2HOE$_3$	8.5	-	18.7	-
10	[^{14}C]2HOE$_3$	11.4	-	25.4	-
[^{3}H]2HOE$_3$ added to feces		45.0			

Figures for conversion of the injected steroids to these two tetrols may be obtained by calculations similar to those used to 2-hydroxyestradiol. It may be seen that the conversions to fecal 2-hydroxyestriol were: estrone sulfate 3 - 7%; estradiol 9.6 - 11.2%; 2-hydroxyestradiol 2.7%; estriol 8%. In the case of rat 3, the $^{3}H/^{14}C$ ratio of the injected dose was 1.27:1 and of the purified peak 3.1 material was 5.90:1; these ratios lead to a value for the conversion of estrone sulfate to 2-hydroxyestriol *in vivo* of 21.5%. Injected 2-hydroxyestriol itself was recovered unchanged in the feces to the extent of 18.7, 25.4 and 34.8%. Taking the average of these three figures, 26.3%, and again making certain assumptions, the following ranges are suggested for conversion of the injected steroids to 2-hydroxyestriol *in vivo*: estrone sulfate 11 - 22%; estradiol 37 - 44%; 2-hydroxyestradiol 10%; estriol 30% (Table 7). If the unknown tetrol behaved similarly, the ranges of conversion would be: estrone sulfate 10 - 19%; estradiol 31 - 43%; 2-hydroxyestradiol 20 - 24%; estriol zero.

Table 7. Approximate range of % conversion of injected steroids to 2-hydroxyestradiol, 2-hydroxyestriol and to the unknown tetrol *in vivo* (i.e. within the animal and prior to any subsequent biochemical or analytical loss).

Compound injected	*In vivo* conversion to:			
	$2HOE_2$	$2HOE_3$	unknown tetrol	total tetrols
$[^{14}C]E_1S$	31 - 100	11 - 22	10 - 19	21 - 43
$[^{14}C]E_2$	31 - 100	37 - 44	31 - 43	68 - 87
$[^{14}C]2HOE_2$		10	20 - 24	30 - 34
$[^{14}C]E_3$		30	zero	30

DISCUSSION

As a result of the attempts of many workers to characterize the total range of estrogen metabolites, and to identify the so-called water-soluble metabolites, it became apparent that the highly polar estrogens, even in unconjugated form, might have a greater significance

than had been realized, and that their importance could have been overlooked because of their instability and high water solubility. Our efforts were therefore directed to a study of 2-hydroxyestriol and similar compounds as *in vivo* metabolites.

Synthesis of 2-hydroxyestriol was reported by several groups in about 1960 (12,14). Its formation *in vitro* was observed by King (30) who incubated estriol with rat liver slices, and by Wortmann *et al.* (31) who incubated estradiol with rat liver microsomes. 2-Hydroxyestriol was observed as one product of 2-hydroxyestradiol infusion by Axelrod *et al.* (32), and Sisenwine *et al.* (33) observed the homologous 2-hydroxy-18-homoestriol on injection of 18-homoestriol into the dog. The only observation of 2-hydroxyestriol as an endogenous metabolite was made by Gelbke & Knuppen (25), in human pregnancy urine.

Of the other possible estrogen tetrols, 6α-hydroxyestriol was observed *in vitro* by Breuer *et al.* (34) but has not been further studied. The unfortunately-named 'estetrol' (E_4, the 3,15α,16α,17β-tetra-hydroxy compound) is found in the human fetus and neonate but not in adult man (35). We have largely ignored in this work the methoxy derivatives, such as 2-methoxyestriol and 2-methoxy-16α-hydroxyestrone (10), since these compounds are not as polar as the tetrols, although, being catechol derivatives, they are prone to non-enzymic degradation to highly polar materials. Some 15-hydroxy- and 18-hydroxy-estriols of pregnancy urine have recently been reported (39).

In the present work, the experiments performed on surgically-modified rats showed that injected 2-hydroxyestriol was excreted with little change and that its metabolites could readily be isolated and quantitated in urine and bile. Orally injected 2-hydroxyestriol was efficiently absorbed from the digestive tract and was secreted into urine or bile largely as a mono-glucuronide. About one quarter of the material had been modified, probably by methylation of one hydroxyl group. No detectable 2-hydroxyestriol formation occurred, however, from injected 2-hydroxyestradiol and less than 2% from estriol in a bile duct-ligated rat, and the formation of 2-hydroxyestriol from injected estrone sulfate was less than 1% in a bile duct-cannulated rat.

On the other hand, much 2-hydroxyestriol was formed from all of the precursors administered to intact rats. This could be the result either

of liver or of bacterial metabolism, or both. It is well established that intestinal micro-organisms are capable of deconjugating steroids, but there is as yet no report that they are active in steroid hydroxylation. The intestinal bacteria are largely anaerobic and at least in the case of the bile acids cause dehydroxylation (36,37). Some incubations were carried out of estradiol and 2-hydroxyestradiol with rat caecal contents homogenized in water or in buffer. Apart from oxidation to the 17-ketone, estradiol was not affected; 2-hydroxyestradiol was not affected in the presence of ascorbic acid, but was completely unrecoverable in its absence. This was presumed to be a result of autoxidation to the quinone followed by random reaction and polymerization. If bacterial action is not involved, the 2-hydroxyestriol must be formed in the liver on repeated turns of the entero-hepatic circulation. The conversion of estrone sulfate to 2-hydroxyestriol in the bile duct-cannulated rat was less than 1%. But it is known that 2-hydroxyestrone and 2-methoxyestrone glucuronides are the major biliary metabolites of injected estrone (10,38). It is possible that 2-hydroxyestrone 2- or 3-glucuronide returned to the liver in the portal circulation is a specific precursor for 16α-hydroxylation and reduction of the 17-ketone. It is also possible that the failure to observe significant formation of 2-hydroxyestriol in the surgically-modified rats was due to disturbance of liver function by the operation. When finally a conjugated form of 2-hydroxyestriol, probably a monoglucuronide, is secreted in the bile of the intact rat, its conjugation must protect it from reabsorption (since free 2-hydroxyestriol is efficiently absorbed). The conjugation is ultimately removed, presumably by bacterial action, since no conjugates were detectable in the feces.

The amounts of 2-hydroxyestriol finally isolated in peak 3.1 eluted from the Celite columns in system 3 were in the range of 1 - 5% of the injected doses of estrone sulfate, estradiol, 2-hydroxyestradiol and estriol. These amounts were corrected for procedural losses back to the amounts actually present in the feces by virtue of the known recovery of [^{3}H]2-hydroxyestriol added to feces. Column (c) in Table 6 (and correspondingly column (b) in Table 4) was obtained in this way. Among several sources of inaccuracy at this level, one of the greatest is the fact that 2-hydroxyestriol simply added to feces and instantly extracted was recovered to the extent only of 71%, in spite of extreme care and the use of ascorbic acid as an anti-oxidant. The mechanism of this loss

is not understood, and therefore the completeness of mixing between the added [^{3}H]2-hydroxyestriol and the [^{14}C]2-hydroxyestriol already present in the feces cannot be guaranteed.

Since the recovery to the feces of orally injected 2-hydroxyestriol was in the range of 19 - 35%, it is clear that to obtain values for the conversion of injected precursors to 2-hydroxyestriol *in vivo*, some sort of upward correction of the values for conversion to fecal 2-hydroxyestriol is necessary. One source of loss of endogenous 2-hydroxyestriol that is very difficult to correct for is the autoxidation or other degradation which may occur while the steroid is in passage down the intestines. The only obvious approach to this problem is the measurement of the recovery of injected 2-hydroxyestriol, followed by the assumption that this injected 2-hydroxyestriol is rapidly and completely mixed with endogenous 2-hydroxyestriol. It is not completely clear whether endogenous 2-hydroxyestriol arises in the liver or as result of bacterial action in the intestine, but since orally administered 2-hydroxyestriol is taken up efficiently and secreted in high yield *via* the bile, and ultimately excreted in the feces, it seems probable that the assumed mixing occurs, though not necessarily with the completeness usually ascribed to a single 'biological pool'. If the mixing is indeed complete, then the figures in Table 7 become valid. It may be noted that conversions of estrone sulfate (21 - 43%) and of estradiol (68 - 87%) to 2-hydroxyestriol were not equal. These two steroids are usually assumed to be in biological equilibrium *in vivo*, but this presumably was not the case at the dose levels of 2 - 3 mg employed here.

Much of what has been written applies equally to the unknown tetrol, although the losses of this compound may have been disproportionately larger, since non-radioactive carrier compound was not added at any time. The most probable structure for this tetrol is the 2,3,6ξ,17β-tetrahydroxy compound, but standards have not yet been obtained to investigate this.

At the very least it is clear that these two highly water-soluble, unconjugated, estrogen tetrols, which have received up till now very little attention, together represent a very important part, in the range of 21 - 87%, of the terminal metabolites of the primary estrogens in the adult male rat.

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NOMENCLATURE

E_1S	estrone sulfate	3-hydroxyestra-1,3,5(10)-trien-17-one 3-sulfate ester
E_2	estradiol	estra-1,3,5(10)-triene-3,17β-diol
$2HOE_2$	2-hydroxyestradiol	estra-1,3,5(10)-triene-2,3,17β-triol
E_3	estriol	estra-1,3,5(10)-triene-3,16α,17β-triol
E_4	estetrol	estra-1,3,5(10)-triene-3,15α,16α,17β-tetrol
$2HOE_3$	2-hydroxyestriol	estra-1,3,5(10)-triene-2,3,16α,17β-tetrol
$6\alpha\text{-}HOE_3$	6α-hydroxyestriol	estra-1,3,5(10)-triene-3,6α,16α,17β-tetrol
$2HO\text{-}16\text{-}epiE_3$		estra-1,3,5(10)-triene-2,3,16β,17β-tetrol
$4HO\text{-}16\text{-}epiE_3$		estra-1,3,5(10)-triene-3,4,16β,17β-tetrol

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