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COMPARATIVE STUDIES ON THE METABOLISM OF OESTRADIOL-17β AND 2-HYDROXY-OESTRADIOL-17β IN MAN *IN VITRO* AND *IN VIVO*

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SUMMARY

[4-^{14}C]-Oestradiol was incubated with liver slices of two male subjects. Under the protection of ascorbic acid the metabolites formed were separated into the free steroid, sulphate, glucuronide and thioether fractions. Using different chromatographic and hydrolytic procedures the metabolites were isolated and identified by various microchemical reactions. Apart from oestradiol (31%) and oestrone (31%) the 2-substituted oestrogens 2-hydroxy-oestradiol (5·3%), 2-hydroxyoestrone (2·6%), 2-hydroxyoestrone 2-methyl ether (2·2%) and 2-hydroxyoestradiol 2-methyl ether (2·2%) were found to be the main metabolites (13%). They were present as free steroids (7·3%), sulphates (4·8%), glucuronides (0·3%) and thioethers (0·1%). Conjugation of the catechol oestrogens with either sulphuric acid or with glucuronic acid was only found at the 2-hydroxy group. The amount of ring B and ring D hydroxylated compounds did not exceed 3·8 and 0·5%, respectively. This means that the metabolism of oestradiol mainly proceeds *via* 2-hydroxylation and further metabolism of the 2-hydroxylated oestrogens.

The intermediary metabolism of catechol oestrogens formed after incubation of oestradiol was essentially the same as that after direct incubation of [4-^{14}C]-2-hydroxyoestradiol.

When [4-^{14}C]-oestradiol was administered orally to the same two male subjects 4 weeks after the liver excision the following excretion pattern of metabolites was found in the 72 h urine: 2-substituted oestrogens were the main catabolic products (11%), followed by oestrone and oestradiol (8·0%) and 16α-hydroxylated compounds (6·4%). All metabolites were mainly present as glucuronides.

INTRODUCTION

It has been believed for a long time that the 16α-hydroxylation is the main reaction in the catabolism of oestrogens in human liver. First doubts of the validity of this assumption arose from the findings of Fishman and co-workers[1, 2] that after administration of radioactive oestradiol to man "hydroxylation of the 2-position assumes a quantitative importance which equals or even surpasses 16α-hydroxylation as a metabolic pathway".

Our investigations[3, 4] of the urinary excretion of endogenous 2-hydroxyoestrogens, confirmed the *in vivo* experiments of Fishman. It was shown that the excretion of 2-hydroxyoestrone†—only one representative of the catechol estrogens—equaled or even exceeded the excretion of oestriol in the urines of male and female subjects[5].

Using new analytical procedures we were able to demonstrate that oestradiol is predominantly metabolized in rat liver *in vitro* to 2-hydroxylated compounds which are then partly hydroxylated at the C-atom 16[6]. It was of interest to find out whether the liver is of equal importance for 2-hydroxylation in man.

Therefore, the metabolism of oestradiol in human liver *in vitro* and after oral administration to man *in vivo* was comparatively investigated.

EXPERIMENTAL

Detailed information on steroids and chemicals, buffers and solutions is given l.c.[6].

In vitro *studies*. Pieces (approx. 2 g) of healthy human liver were obtained from the surgery unit from

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† The following abbreviations and trivial names have been used: E_1, oestrone = 3-hydroxy-1,3,5(10)-oestratrien-17-one; E_2, oestradiol = 1,3,5(10)-oestratriene-3,17β-diol; 2-OHE_1, 2-hydroxyoestrone = 2,3-dihydroxy-1,3,5(10)-oestratrien-17-one; 2-OHE_2, 2-hydroxyoestradiol = 1,3,5(10)-oestratriene-2,3,17β-triol; 2-OHE_1 2-Me, 2-hydroxyoestrone 2-methyl ether = 2,3-dihydroxy-1,3,5(10)-oestratrien-17-one 2-methyl ether; 2-OHE_1 3-Me, 2-hydroxyoestrone 3-methyl ether = 2,3-dihydroxy-1,3,5(10)-oestratrien-17-one 3-methyl ether; 2-OHE_2 2-Me, 2-hydroxyoestradiol 2-methyl ether = 1,3,5(10)-oestratriene-2,3,17β-triol 2-methyl ether; 2-OHE_2 3-Me, 2-hydroxyoestradiol 3-methyl ether = 1,3,5(10)-oestratriene-2,3,17β-triol 3-methyl ether; 6α-OHE_1, 6α-hydroxyoestrone = 3,6α-dihydroxy-1,3,5(10)-oestratrien-17-one; 6β-OHE_1, 6β-hydroxyoestrone = 3,6β-dihydroxy-1,3,5(10)-oestratrien-17-one; 7α-OHE_1, 7α-hydroxyoestrone = 3,7α-dihydroxy-1,3,5(10)-oestratrien-17-one; 7β-OHE_1, 7β-hydroxyoestrone = 3,7β-dihydroxy-1,3,5(10)-oestratrien-17-one; 6α-OHE_2, 6α-hydroxyoestradiol = 1,3,5(10)-oestratriene-3,6α,17β-triol; 6β-OHE_2, 6β-hydroxyoestradiol = 1,3,5(10)-oestratriene-3,6β,17β-triol; 7α-OHE_2, 7α-hydroxyoestradiol = 1,3,5(10)-oestratriene-3,7α,17β-triol; 7β-OHE_2, 7β-hydroxyoestradiol = 1,3,5(10)-oestratriene-3,7β,17β-triol; 16α-OHE_1, 3,16α-dihydroxy-1,3,5(10)-oestratrien-17-one; E_3, oestriol = 1,3,5(10)-oestratriene-3,16α,17β-triol; 2-OHE_3, 2-hydroxyoestriol = 1,3,5(10)-oestratriene-2,3,16α,17β-tetrol; E_1 3-S = oestrone 3-sulphate.

two male subjects with *ulcera duodeni*: B.O. 33 years and F.M. 24 years. At the time of excision both subjects were under narcotics (neurolept-analgesia: 0·5 g Epontol, 20 mg Dihydrobenzperidol, 0·7 mg Fentanyl) for about 1 h. The liver pieces were macroscopically and microscopically normal.

One hundred nmol (5 μCi) [4-^{14}C]-oestradiol or [4-^{14}C]-2-OHE$_2$ dissolved in 50 μl of ethanol were incubated with 750 mg of human liver slices in 5 ml of a modified Krebs–Ringer–Henseleit buffer. Incubations were carried out for 1h at 37 C under O_2.

For the work-up procedure, the separation of the metabolites formed and the final identification see l.c.[6].

In vivo studies. Both subjects B.O. and F.M. received 200 nmol [4-^{14}C]-oestradiol (1.0 μCi) orally 4 weeks after the liver excision. The subjects were without drugs for at least two weeks, and the gastro-intestinal as well as liver and kidney function were obviously normal.

24 h-Urines were collected separately for 3 days, assayed for radioactivity, combined and worked-up in 100 ml portions. The free steroid fractions were obtained by extraction with three 100 ml portions of ethyl acetate. The further working-up procedure of the free steroid fraction and of the conjugate fraction was performed as described l.c.[6].

RESULTS

Metabolism of oestradiol and 2-hydroxyoestradiol in vitro. After incubation of 2-OHE$_2$ or oestradiol with human liver slices the incubation mixtures of 2 separate incubations were combined and divided into lipophile, water-soluble and protein-bound fractions. The recovery of radioactivity in the different fractions is given in Table 1.

Characterization and identification of metabolites found in the lipophile fraction. Characterization of metabolites was done by multiple paper- and thin-layer chromatography, identification by microchemical reactions and mass spectrometry, resp. (for details see[6]).

After incubation of 2-OHE$_2$ the following metabolites were found: 2-OHE$_1$, 2-OHE$_2$, 2-OHE$_1$ 2-Me, 2-OHE$_1$ 3-Me, 2-OHE$_2$ 2-Me and 2-OHE$_2$ 3-Me; the incubation with oestradiol yielded in addition to the metabolites found after incubation of 2-OHE$_2$ the following steroids: E$_1$, E$_2$, 6α-OHE$_1$, 6β-OHE$_1$, 7α-OHE$_1$, 7β-OHE$_1$, 6α-OHE$_2$, 6β-OHE$_2$, 7α-OHE$_2$, 7β-OHE$_2$, 16α-OHE$_1$ and E$_3$. The quantitative evaluation is given in Table 2.

Table 1. Recovery of radioactivity in the different fractions after incubation of oestradiol and 2-OHE$_2$ with human liver slices. Percentages given refer to incubated radioactivity; each figure represents the mean of two incubations

Steroid incubated	Subject	Lipophilic fraction	Water-soluble fraction	Protein-bound fraction	Total
E$_2$	B.O.	65	26	1.7	92
	F.M.	67	18	1.6	87
2-OHE$_2$	B.O.	31	53	7.6	92
	F.M.	44	35	6.4	85

Characterization and identification of metabolites found in the water-soluble fraction. After incubation of both 2-OHE$_2$ and oestradiol the radioactivity present in the water-soluble fraction could be separated by high-voltage paper electrophoresis into three radioactive zones corresponding in mobility to steroid-sulphates (K1), steroid-glucuronides (K2) and steroid-thioethers (K3); moreover radioactive material was found at the origin of the electropherogram which contained partly radioactive garbage, partly unidentified conjugates and partly free steroids spontaneously formed during the working-up procedure. The recovery of radioactivity in the different zones is given in Table 3.

Treatment of the three fractions with acid, enzyme (Ketodase) or Raney–Nickel supported the idea that zone K1 contained sulphoconjugates, zone K2 glucuronides, and zone K3 glutathione thioethers.

For the identification of the steroid moiety the sulphates and glucuronides were hydrolysed under reducing conditions with acid and the thioethers were treated with Raney–Nickel under the protection of ascorbic acid in an atmosphere of N_2. The free steroids obtained were separated and identified as described for the lipophilic fraction.

From the sulphate fraction the following steroids were liberated in the incubation experiment with 2-OHE$_2$: 2-OHE$_1$, 2-OHE$_2$, 2-OHE$_1$ 2-Me, 2-OHE$_1$

Table 2. Distribution of radioactive metabolites in the different lipophilic and water-soluble fractions after incubation of oestradiol and 2-OHE$_2$ with human liver slices. Percentages given refer to incubated radioactivity and represent the mean of two incubations; subject B.O. → normal figures, subject F.M. → italics. $S_1 = E_2$ incubated substrate, S_2 = 2-OHE$_2$ incubated substrate; nd = not detected

Metabolites found	Lipophilic fraction		Sulfate fraction		Glucuronide fraction		Thioether fraction		Total	
	S_1	S_2	S_1	S_2	S_1	S_2	S_1	S_2	S_1	S_2
E$_1$	29.3	nd	1.6	nd	0.31	nd	nd	nd	31	nd
	27.1	*nd*	*2.3*	*nd*	*0.41*	*nd*	*nd*	*nd*	*30*	*nd*
E$_2$	19.0	nd	1.3	nd	1.64	nd	nd	nd	22	nd
	33.4	*nd*	*3.8*	*nd*	*3.40*	*nd*	*nd*	*nd*	*40*	*nd*
2-OHE$_1$	1.2	7.9	1.8	3.5	0.13	0.7	0.06	0.48	3.2	11.6
	1.0	*8.3*	*0.9*	*0.7*	*0.12*	*1.3*	*nd*	*0.30*	*2.0*	*10.6*
2-OHE$_2$	4.0	13.1	2.2	6.9	0.22	1.72	0.04	0.48	6.5	22.2
	2.7	*8.4*	*1.2*	*2.6*	*0.20*	*6.1*	*nd*	*1.50*	*4.1*	*18.8*
2-OHE$_1$ 2-Me	1.5	3.5	0.61	0.67	nd	<0.01	nd	<0.01	2.1	4.2
	1.8	*2.5*	*0.40*	*0.22*	*nd*	*0.06*	*nd*	*nd*	*2.2*	*2.8*
2-OHE$_1$ 3-Me	nd	nd	0.07	0.04	nd	<0.01	nd	<0.01	0.08	0.05
	0.1	*nd*	*nd*	*nd*	*nd*	*0.05*	*nd*	*nd*	*0.1*	*0.05*
2-OHE$_2$ 2-Me	0.6	2.5	0.8	1.06	0.03	0.09	<0.01	0.03	1.4	3.7
	1.5	*2,3*	*1.5*	*1.71*	*0.03*	*0.25*	*nd*	*nd*	*3.0*	*4.3*
2-OHE$_2$ 3-Me	nd	nd	0.06	0.11	nd	0.04	<0.01	0.01	0.07	0.16
	0.03	*nd*	*nd*	*nd*	*0.01*	*0.02*	*nd*	*nd*	*0.03*	*0.02*
6/7-OHE$_1$	3.5	nd	0.16	nd	nd	nd	nd	nd	3.7	nd
	0.1	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*0.1*	*nd*
6/7-OHE$_2$	3.1	nd	<0.01	nd	<0.01	nd	nd	nd	3.1	nd
	0.6	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*0.6*	*nd*
16α-OHE$_1$	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
	0.2	*nd*	*0.05*	*nd*	*0.05*	*nd*	*nd*	*nd*	*0.3*	*nd*
E$_3$	nd	nd	nd	nd	<0.01	nd	nd	nd	<0.01	nd
	0.25	*nd*	*0.1*	*nd*	*0.2*	*nd*	*nd*	*nd*	*0.6*	*nd*
2-OHE$_3$	<0.01	<0.01	nd	nd	nd	nd	nd	nd	<0.01	<0.01
	nd	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*

Table 3. Recovery of radioactivity in the different conjugate fractions after incubation of oestradiol and 2-OHE_2 with human liver slices. Percentages given refer to incubated radioactivity; each figure represents the mean of two incubations

Steroid incubated	Subject	Sulfate fraction	Glucuronide fraction	Thioether fraction	Total
E_2	B.O.	9.4	3.3	0.9	14
	F.M.	11	4.3	0.4	16
2-OHE_2	B.O.	14	3.5	1.9	19
	F.M.	7.0	11	7.2	25

3-Me, 2-OHE_2 2-Me and 2-OHE_2 3-Me; the incubation of oestradiol yielded in addition to the metabolites found after incubation of 2-OHE_2 the following steroids: E_1, E_2 and traces of ring-B-hydroxylated derivatives of E_1 and E_2.

From the glucuronide fraction the following steroids were liberated in the incubation experiment with 2-OHE_2: 2-OHE_1, 2-OHE_2, 2-OHE_1 2-Me, 2-OHE_1 3-Me, 2-OHE_2 2-Me and 2-OHE_2 3-Me; the incubation with oestradiol yielded in addition to the metabolites found after incubation of 2-OHE_2 the steroids: E_1, E_2 and traces of E_3 and 6- resp. 7-hydroxylated derivatives of E_2.

Further investigations were carried out to localize both the positions of the sulphuric and glucuronic acid residues on the steroid molecule: portions of fractions K1 and K2 were hydrolysed with hot acid, i.e. the first portion without treatment with diazomethane and the second portion after treatment with an excess of diazomethane. The steroids liberated were purified by paper chromatography and quantitated. The amount of monomethyl ethers found without treatment with diazomethane were then subtracted from the amount of monomethyl ethers found after treatment with diazomethane and the following results were derived: catechol oestrogens are predominantly conjugated with sulphuric and glucuronic acid at position 2; conjugation at position 3 was demonstrated only for 2-monomethyl ethers. This result was found after incubation of oestradiol as well as after incubation of 2-OHE_2.

From the thioether fraction the following steroids were liberated after incubation of oestradiol: 2-OHE_1, 2-OHE_2, 2-OHE_2 2-Me and 2-OHE_2 3-Me; after incubation of 2-OHE_2 in addition the monomethyl ethers of 2-OHE_1 were found.

To obtain more information on the thioether conjugates portions of fraction K3 were subjected to high-voltage paper electrophoresis for 4 h. Two zones of radioactivity were detected corresponding in mobility to the 1- and 4-glutathione thioethers of 2-hydroxyoestrogens. The 1-glutathione thioether was associated with about 60% and the 4-glutathione thioether with about 40% of the radioactivity of zone K3.

The quantitative distribution of the steroids liberated of the water-soluble fraction is given in Table 2.

Metabolism of oestradiol in man in vivo. After oral administration of oestradiol 42% and 45%, respectively, of the administered radioactivity was excreted in the 72 h urine: the first 24 h voiding contained 31% (33%), the second 7% (8%) and the third 4·1% (3·9%).

After separation of the lipophile and water-soluble metabolites the lipophile steroids were characterized and identified as described for the *in vitro* experiment and the water-soluble fraction was further subjected to high-voltage paper electrophoresis. Two radioactive zones, one corresponding in mobility to steroid-sulphates (K1) and one to steroid-glucuronides (K2) were detected. In contrast to the *in vitro* studies no radioactivity was found in the position of the glutathione thioethers. Recovery and distribution of radioactivity is given in Table 4. After treatment of the conjugate fraction K1 with hot acid and K2 with Ketodase, characterization and identification of the steroid moieties was achieved according to the *in vitro* incubation experiment.

The following steroids were found in the lipophilic fraction: E_1, E_2, 2OHE_1, 2-OHE_2, E_3, in the sulphate fraction: E_1, E_2, 2-OHE_1, 2-OHE_1 2-Me, 2-OHE_1 3-Me, traces of ring B- hydroxylated derivatives of E_1, $16\alpha\text{-OHE}_1$ and E_3, in the glucuronide fraction: E_1, E_2, 2-OHE_1, 2-OHE_2, 2-OHE_1 2-Me, 2-OHE_1 3-Me and E_3. The quantitative distribution of the metabolites identified is given in Table 5.

DISCUSSION

The results described in this paper confirm those obtained after incubation of oestradiol and 2-hydroxyoestradiol with rat liver slices[6]. Although under the same incubation conditions higher amounts of oestradiol and 2-hydroxyoestradiol were transformed by rat liver (recovery of $E_2 = 5\%$, of $2\text{-OHE}_2 = 14\%$) as compared to human liver (recovery of $E_2 = 31\%$, of $2\text{-OHE}_2 = 20\%$), the metabolic patterns did not differ significantly. As shown in the Fig. 2-substituted oestrogens were, apart from oestrone, the main products of oestradiol metabolism followed by ring B hydroxylated oestrogens, whereas metabolites hydroxylated at C-atom 16 were only detected in minor quantities.

The possibility cannot be ruled out that the extremely low 16α-hydroxylation found in the *in vitro* experiments might be due to an inhibitory effect of drugs; as described in methods, both subjects had received narcotics at the time of liver excision. This assumption is fortified by a recent report of Femino

Table 4. Recovery of radioactivity in the free steroid and the different conjugate fractions after oral administration of oestradiol to man. Percentages given refer to ingested radioactivity

Subject	Free steroid fraction	Sulfate fraction	Glucuronide fraction	Total
B.O.	2.1	5.6	23	31
F.M.	2.0	7.1	28	37

Table 5. Excretion of radioactive metabolites in urine and their distribution on the different lipophilic and water-soluble fractions after oral administration of oestradiol to man. Percentages given refer to ingested radioactivity. nd = not detected

Metabolites found	Subject	Lipophilic fraction	Sulfate fraction	Glucuronide fraction	Total
E_1	B.O.	0.20	0.25	4.60	5.1
	F.M.	0.30	0.40	5.30	6.0
E_2	B.O.	0.14	0.25	1.80	2.2
	F.M.	0.16	0.35	2.20	2.7
2-OHE$_1$	B.O.	0.08	1.25	6.30	7.6
	F.M.	0.10	1.50	9.90	11.5
2-OHE$_2$	B.O.	0.06	nd	0.02	0.1
	F.M.	0.07	0.03	0.40	0.5
2-OHE$_1$ 2-Me	B.O.	nd	0.60	0.05	0.7
	F.M.	nd	0.80	0.70	1.5
2-OHE$_1$ 3-Me	B.O.	nd	0.10	0.03	0.1
	F.M.	nd	0.10	0.10	0.2
6/7-OHE$_1$	B.O.	nd	0.05	nd	0.1
	F.M.	nd	0.07	0.02	0.1
16α-OHE$_1$	B.O.	nd	0.13	0.18	0.3
	F.M.	nd	0.42	1.90	2.3
E_3	B.O.	0.04	1.70	3.10	4.8
	F.M.	0.03	0.80	4.50	5.3

et al.[7] that drugs as ethinyloestradiol may inhibit 16α- but not 2-hydroxylation and furthermore by the results of the *in vivo* experiments described in our paper which were performed four weeks after drug administration: this time higher amounts of 16α-substituted oestrogens, markedly lower amounts of ring B hydroxylated compounds were found; catechol oestrogens were again the main metabolic products.

In human liver *in vitro*, as previously demonstrated for rat liver [6], non-2-substituted phenolic steroids as E_1, E_2 and ring B and D hydroxylated compounds were predominantly present as free steroids, whereas the 2-substituted oestrogens were found both in the free steroid (7·3%) and the conjugate fraction (5·2%). Essentially the same results were obtained in the *in vivo* experiments* although, *in vivo*, free steroids were of minor quantitative importance (2% of the dose administered).

Within the conjugate fraction sulpho-conjugates predominated in the *in vitro* experiments, whereas glucuronides were the main excretion products *in vivo*. Of special interest is the finding that in the *in vitro* experiments with human liver the 2-hydroxylated oestrogens were sulpho-conjugated and glucuronidated only at the C-atom 2. Only when the conjugation at C-atom 2 was hindered by other substituents—as in the case of 2-monomethyl ethers—sulpho-conjugation and glucuronidation at the C-atom 3 occurred.

* In this context it should be mentioned that absorption of orally administered oestradiol is a rapid and complete process [Ball, unpublished results] and that the intestinal flora does not play a measurable role in the metabolism of oestradiol prior to absorption [cf. also l.c.8].

These results are in accordance with the *in vitro* experiments with rat liver[6] and the *in vivo* experiments with rats and hamsters[9] whereas in humans *in vivo* glucuronidation is believed to occur only at the C-atom 3[10]. The assumption of Williams[9] that species differences in the structure of urinary 2-hydroxyoestrone glucuronides reside in different transferases of humans and rodents can be excluded as far as the liver is concerned.

In view of our findings the biosynthetic reaction scheme of catechol oestrogen formation—i.e. $E_2 \rightarrow E_1$ 3-S $\rightarrow$ 2-OHE$_1$ 3-S, as postulated by Fishman [11]—must be looked upon with reserve since

1. no 2-OHE$_1$ 3-S but only 2-OHE$_1$ 2-S could be detected in the *in vitro* experiments with human and rat liver,

2. additional experiments carried out with E_1 3-S as substrate [unpublished results] resulted in much less catechol oestrogen conjugates than incubation with E_2, and

3. the metabolic pattern of E_2 incubations corresponded to that of 2-OHE$_2$ especially with respect to the conjugate fraction.

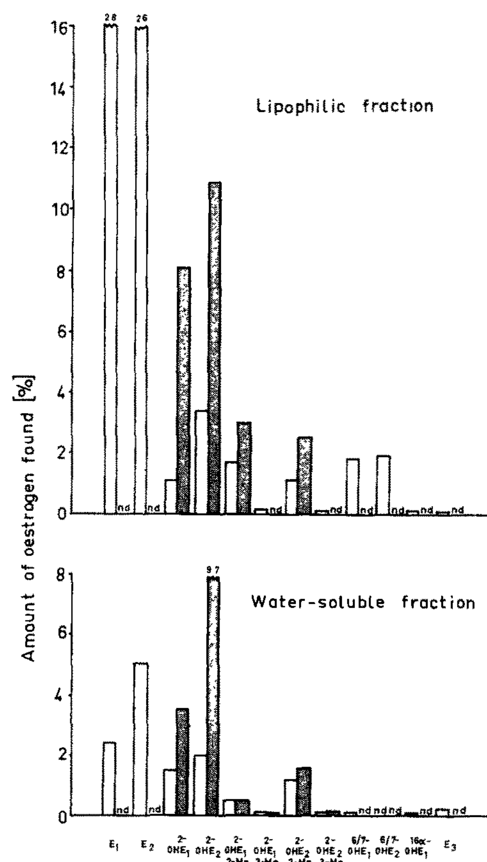

Fig. 1. Distribution pattern of metabolites present in the lipophilic and water-soluble fractions after incubation of oestradiol and 2-OHE$_2$ with human liver slices. Percentages given refer to incubated radioactivity; each figure represents the mean of four incubations.
□, Metabolites found after incubation of E_2.
■, Metabolites found after incubation of 2-OHE$_2$.

So, E_1 3-S does not seem to be an obligatory intermediate in the biosynthesis of catechol oestrogens neither *in vitro* nor *in vivo*[10]. Finally it should be mentioned that after incubation of E_2 and 2-OHE_2 79% and 40%, respectively, of the metabolites were identified. Approx. 5% and 20%, respectively, of the radioactivity were associated with distinct metabolites which were not definitely identified. First attempts at identification indicate that ring A polyhydroxylated compounds may contribute to these unidentified metabolites. It is suggested that ring A polyhydroxylated compounds may play an important role in the further metabolism especially of 2-hydroxyestradiol.

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