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IN VITRO METABOLISM OF [4-^{14}C]OESTRADIOL-17β BY MOUSE LIVER

T. THORSEN and K. F. STØA
Hormone Laboratory, University of Bergen School of Medicine, Bergen, Norway

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SUMMARY

The metabolism *in vitro* of [4-^{14}C] oestradiol-17β by mouse liver slices has been investigated. Oestrone, 2-methoxy-oestradiol-17β, 2-methoxy-oestrone, 6-oxo-oestradiol-17β, 6-oxo-oestrone and 6α-hydroxy-oestradiol-17β were identified as metabolites. No 16-hydroxylated metabolite was detected. More than 50% of the total metabolites were insoluble in ether at pH 2. A fraction consisting of unidentified acidic metabolites and extractable with ether at low pH amounted to approximately 18% of the total radioactivity.

INTRODUCTION

BIOTRANSFORMATION of phenolic steroids by liver tissue of human and animal origin has been extensively investigated in recent years, and considerable progress has been made in the elucidation of the complex pattern of oestrogen metabolism. The role of the liver in oxidative metabolism of oestrone, oestradiol-17β and oestriol is now well established. It involves hydroxylation at the C-2 position and subsequent methylation, or the formation of C-6 oxygenated oestrogens[1, 2]. In addition, it has been shown that the liver of several species can effect the conversion of oestradiol-17β to 16-oxygenated metabolites[3, 4] and the interconversion of oestrone, oestradiol-17β and oestradiol-17α[5].

Investigation of steroid metabolism in different animal species is of importance from a comparative point of view, and such studies may also give valuable information on the mechanisms and intracellular location of the steroid-metabolizing enzymes. Oestrogen metabolism has been extensively investigated in the domestic animals[6], in the fowl[7, 8], the rabbit[5, 9] and the rat[1, 2, 10, 11]. Although the mouse is a common laboratory animal, very little information is available on its oestrogen metabolism. It was, therefore, considered of interest to study the biotransformation of [4-^{14}C] oestradiol-17β in mouse liver *in vitro*. Some of the dynamic aspects of the oestrogen metabolism were also investigated.

MATERIALS AND METHODS

Steroids. All steroids used were tested for chromatographic purity. Oestrone (OE_1), oestradiol-17β (OE_2), oestriol (OE_3), 2-methoxy-oestrone (2-Me-OE_1), 2-methoxy-oestradiol-17β (2-Me-OE_2) and 6-oxo-oestradiol-17β (6-Oxo-OE_2) were commercially available.* 6α-Hydroxy-oestradiol-17β (6α-OH-OE_2) was obtained by reduction of 6-Oxo-OE_2 with sodium borohydride. According to Wintersteiner and Moore[12] this reduction would give an α-oriented 6-hydroxy-

*Steroids were purchased from the following firms: Oestrone: E. Merck A. G., Darmstadt, Germany. Oestradiol: Koch-Light Laboratories Ltd, Colnbrook, Bucks, England. Oestriol: Sigma Chemical Company, St. Louis, Missouri, U.S.A. 2-Methoxy-oestrone and 2-methoxy-oestradiol: Mann Research Laboratories, Inc., New York, N.Y., U.S.A. 6-Oxo-oestradiol: Ikapharm, Ramat-Gan, Israel.

group. 6-Oxo-oestrone (6-Oxo-OE_1) was made from 6-Oxo-OE_2 by chromic acid oxidation. Both compounds were recrystallized from acetonitrile-ethanol.

Radioactive substrate. [4-^{14}C] oestradiol-17β was obtained from the Radiochemical Centre, Amersham, England, and was tested for radiochemical homogeneity by paper chromatography. No radioactive impurities could be detected.

Detection and measurement of radioactivity. Radioactivities were measured by liquid-scintillation spectrometry using an automated three-channel Packard instrument (Series 3003). The window setting was 50–1000 V with a gain of 6%. Steroid fractions were counted either in toluene containing 4·0 g PPO and 50 mg POPOP per 1000 ml or in the scintillation solution described by Bray[13]. The volume of the scintillator used was 10 ml, and quenching was corrected by external standard.

Radioactivity on paper strips was detected by scanning on a Frieseke & Hoepfner radio-chromatogram scanner.

Incubation experiments. Male white mice, local strain, weighing 15–20 g, were killed by decapitation. The livers were removed and immediately cooled on ice. Approximately 300 mg portions of liver slices were incubated aerobically for varying periods of time in 5·0 ml of Krebs–Ringer phosphate buffer, pH 7·4, containing 0·02 M glucose, 0·4 μCi of [4-^{14}C] oestradiol-17β and 25 μg of inactive OE_2. The steroids were dissolved in 0·2 ml of propylene glycol. The incubation temperature was 37°C. Incubations were stopped by adding hydrochloric acid to pH 2·0 and immediate freezing. A blank incubation, containing no substrate, was carried through the same procedure. Radioactive substrate and carrier were added immediately prior to extraction in order to determine the extent of methodological losses.

Extraction. After thawing, the content of each incubation flask was diluted to 10 ml with distilled water and filtered. The precipitated insoluble substances and the remaining liver tissue were extracted with hot acetone and the extract filtered and evaporated. The residue was dissolved in 1·0 ml of ethanol and 5·0 ml of distilled water and combined with the incubation fluid. The pH was adjusted to 2·0 and the mixture extracted three times with 30 ml portions of diethyl ether*. A fourth extraction gave no additional radioactivity. After evaporation of the ether, the residues were dissolved in small volumes of ethanol and stored at −15°C.

Paper chromatography. Paper chromatography was carried out on Whatman No. 1 paper. The following systems were used:

(1) Monochlorobenzene/formamide
(2) Cyclohexane/formamide
(3) Chloroform : ethyl acetate (5 : 1)/formamide
(4) Cyclohexane : dioxane : methanol : water (100 : 100 : 50 : 25).

Oestrogens were detected by spraying with a solution containing 1% each of ferric chloride and potassium cyanide in water.

The individual labelled compounds in the ether extracts were purified by fractionation according to the procedure indicated schematically in Fig. 1. Recovery from individual chromatographic steps averaged 85%. Correction was made for methodological losses.

RESULTS

Figure 2 shows the percentage of the radioactivity present in the ether phase

*All solvents were redistilled before use and only peroxide-free ether was employed for extractions.

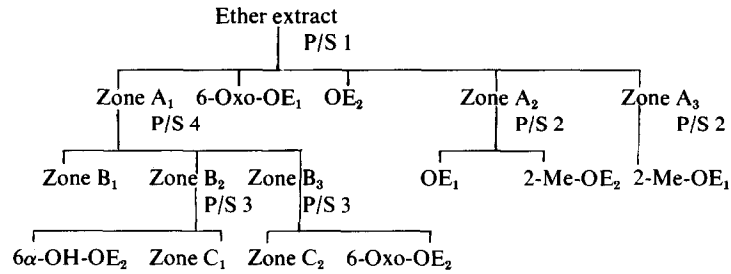

Fig. 1. Scheme for the isolation and identification of the different metabolites of oestradiol-17β. P/S = Partition system. Zones B_1, C_1 and C_2: unidentified metabolites.

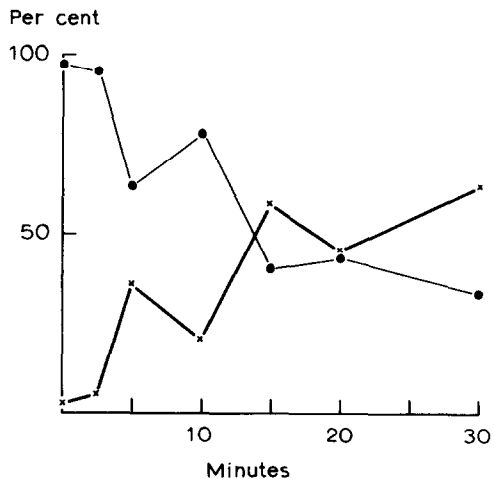

Fig. 2. Distribution of radioactivity between the ether and water phase after different periods of incubation. Ether extraction at pH 2. x–x: Water soluble radioactivity. ●–●: Ether soluble radioactivity.

and the aqueous phase at pH 2 after different periods of incubation. By extending the incubation time increasing amounts of radioactivity were rendered insoluble in ether, and after 30 min more than 50% of the labelled metabolites could not be extracted at pH 2.

Only the ether-extractable compounds have been further investigated. By successive chromatography the following substances were isolated (Fig. 1): 2-methoxy-oestrone, 2-methoxy-oestradiol-17β, oestrone, oestradiol-17β, 6-oxo-oestrone, 6-oxo-oestradiol-17β and 6α-hydroxy-oestradiol-17β. In Table 1 are listed the R_F-values of the isolated radioactive steroids together with the R_F-values of the corresponding authentic reference compounds. The identity of the isolated steroids was confirmed by recrystallization to constant specific activity (Table 2).

Of the labelled metabolites separated by paper chromatography, three fractions were not identified, as shown in Fig. 1. The most polar fraction isolated in

Table 1. Chromatographic mobilities of oestrogens extracted from the incubation mixture by diethyl ether at pH 2·0. R_F values obtained by different chromatographic systems. Figures in parentheses: R_F values of authentic steroid standards

Steroid	Paper partition chromatography system			
	1	2	3	4
6α-OH-OE_2	0·00(0·00)		0·22(0·22)	0·15(0·12)
6-Oxo-OE_2	0·00(0·05)		0·63(0·63)	0·23(0·25)
6-Oxo-OE_1	0·19(0·19)			
OE_2	0·27(0·30)			
OE_1	0·72(0·71)	0·07(0·06)		
2-Me-OE_2	0·72(0·78)	0·12(0·12)		
2-Me-OE_1	0·90(0·86)	0·26(0·28)		

Table 2. Reverse isotope dilution of isolated steroids. The figures represent c.p.m. per mg. Amount of authentic carrier added: at least 20 mg. Solvents used in crystallizations: acetonitrile or acetonitrile: ethanol

Steroid	Crystallization No.	Crystals	Mother liquor
	Starting material	1630	
	1	1580	2230
2-Me-OE_1	2	1510	1480
	3	1495	1569
	Starting material	563	
	1	438	694
2-Me-OE_2	2	513	452
	3	486	579
	Starting material	3518	
	1	3861	3472
OE_1	2	3791	3901
	3	3810	3786
	Starting material	6810	
	1	6631	7016
OE_2	2	6822	7218
	3	6636	6809
	Starting material	789	
	1	688	819
6-Oxo-OE_1	2	671	684
	3	708	751
	Starting material	810	
	1	711	911
6-Oxo-OE_2	2	685	710
	3	634	681
	Starting material	3765	
	1	3510	4115
6α-OH-OE_2	2	3620	3492
	3	3466	3680

system No. 1 (zone A_1), corresponding in mobility to oestriol, revealed three radioactive zones (B_1, B_2 and B_3) in system No. 4.

The fraction contained in zone B_1, which remained on the starting line in all systems used, was readily extractable from ether with 8% (w/v) sodium bicarbon-

ate solution. It could be extracted with ether from aqueous solution only at a pH-value below 3 and has been termed the "acid fraction".

Zone B_2 had a mobility corresponding to 6α-hydroxy-oestradiol-17β. By chromatography in system No. 3, this zone was found to contain a main fraction with the same mobility as 6α-hydroxy-oestradiol-17β. A minor unidentified contaminant was isolated in zone C_1. Zone B_3 consisted of almost equal parts of 6-oxo-oestradiol-17β and a substance ("Zone C_2") which has not been identified.

The results of a series of incubations of [4-^{14}C] oestradiol-17β with mouse liver slices are depicted in Fig. 3. The plotted values represent individual compounds as per cent of the total radioactivity in each incubation mixture. The results show that the amount of [4-^{14}C] oestradiol-17β decreases rapidly during the first 5 min of incubation. After a preliminary plateau at about 25% of the original radioactivity, the disappearance of oestradiol-17β from the incubation medium follows an exponential curve. During the first 10 min the amount of oestrone increases to a maximum of about 25 per cent of the total radioactivity. At this maximum an equilibrium is attained between oestradiol-17β and oestrone. From now on they both disappear at an equal rate.

The amount of 6α-hydroxy-oestradiol-17β varies between 4·2 and 8·4% during the entire incubation period, the values displaying a slightly biphasic form with a second maximum at 20 min. The 2-methoxy oestrone curve has also two maxima, one after 5–10 min of incubation and the other after 30 min. The values for this steroid vary about 5%. The concentration of 2-methoxy-oestradiol-17β reaches

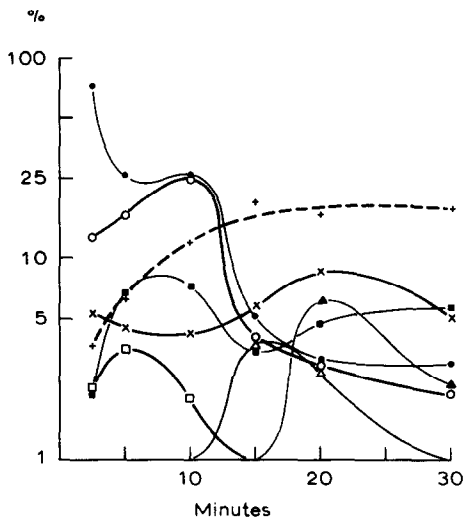

Fig. 3. Formation of pH 2 ether-soluble metabolites of [4-^{14}C] oestradiol-17β by mouse liver slices. Abscissa: incubation time. Ordinate: per cent of total radioactivity added to the incubation mixture on logarithmic scale. ●–●: oestradiol-17β. ○–○: oestrone. x–x: 6α-hydroxy-oestradiol-17β. △–△: 6-oxo-oestrone. ▲–▲: 6-oxo-oestradiol-17β. □–□: 2-methoxy-oestradiol-17β. ■–■: 2-methoxy-oestrone. +--+: acid fraction.

a maximum of 3·6% after 5 min and then falls to zero after 15 min. On the other hand, 6-oxo-oestrone (maximum concentration 3·9%) and 6-oxo-oestradiol-17β (maximum concentration 6%) were present in the media only after an incubation time of 15 and 20 min respectively. The amount of radioactivity present in zone B_1 (Fig. 1) (the "acid fraction") reaches a maximum of about 18% of the total radioactivity at 15 min and then remains constant.

DISCUSSION

The only investigation of oestradiol-17β metabolism in mouse liver known by the authors is the work of Rumney and Mueller[14, 15]. In these studies it was demonstrated that mouse liver microsomes in the presence of NADPH converted oestradiol-17β to some 6-oxygenated compounds which were considered to be 6β-hydroxyoestradiol-17β, 6β-hydroxyoestrone and 6-oxo-oestradiol-17β.

As shown by several authors[16, 17], oestrogen metabolism in the rat is influenced by the sex and age of the animal. In this work young male mice in the weight range 15–20 g have been used. It has been shown[16] that the water-soluble oestrogen metabolites formed by rat liver differ from the normal sulphate and glucosiduronate conjugates found in other species, and that the liver microsomes from male rats convert a higher proportion of oestrone and oestradiol into these products than do similar preparations from female rats. In accordance with these findings, mouse liver slices produced water soluble metabolites, the amounts of which increased rapidly with increasing incubation time. After 30 min incubation, only about 45% of the radioactivity could be extracted with ether at pH 2. In the rat, Jellinck *et al.*[18] found that some of the water soluble metabolites were firmly bound to protein, and the formation of oestrogen–gluthathione conjugates and other thio-ethers of 2-hydroxy-oestrone and 2-hydroxy-oestradiol has recently been demonstrated[10, 18, 19]. Although no experimental data exist on this point, it may be considered a possibility that water-soluble compounds of the same types are also present in the mouse liver incubation media.

Apart from oestradiol-17β and oestrone, three main groups of metabolites have been identified in the ether extractable fraction. These are the 2-oxygenated metabolites, the 6-oxygenated metabolites and the "acid" fraction, which is extractable from ether with aqueous sodium bicarbonate. It is also interesting to note that no formation of 16-hydroxylated metabolites could be demonstrated in these experiments. This is in contrast to the results of other groups of investigators, who found a certain degree of 16α-hydroxylation of oestradiol-17β and oestrone in studies with rat liver slices[20] and microsomes[2, 21]. This discrepancy may be explained by species difference. However, it should be pointed out that Jellinck and Garland[22] did not find any ether-soluble 16-hydroxylated metabolites after incubation of oestradiol-17β with rat liver microsomes.

A dynamic pattern of oestradiol metabolism in mouse liver slices *in vitro* has been made possible by the carrying out of experiments with different incubation times. This pattern displays the following main features: During the first ten min equilibrium is attained between oestrone and oestradiol, which subsequently disappear at an equal rate. The 2-oxygenated metabolites are abundant during the first 10 min period and appear to arise from 2-hydroxylation of both oestradiol and oestrone. After 10 min all 2-methoxy-oestradiol formed is converted into 2-methoxy-oestrone. No 2-hydroxy metabolite could be detected, which means either that the 2-methyl transferase system is very efficient or that the 2-hydroxy

metabolites are rapidly converted into water soluble compounds, e.g. gluthathione derivatives, or acid substances. The 6α-hydroxy-oestradiol-17β curve of Fig. 3 is biphasic. This probably indicates that 6α-hydroxy-oestradiol-17β is formed during the early incubation period by direct hydroxylation of oestradiol. Later, part of this metabolite originates from hydrogenation of 6-oxo-oestradiol-17β, and 6α-hydroxy-oestrone (Fig. 4).

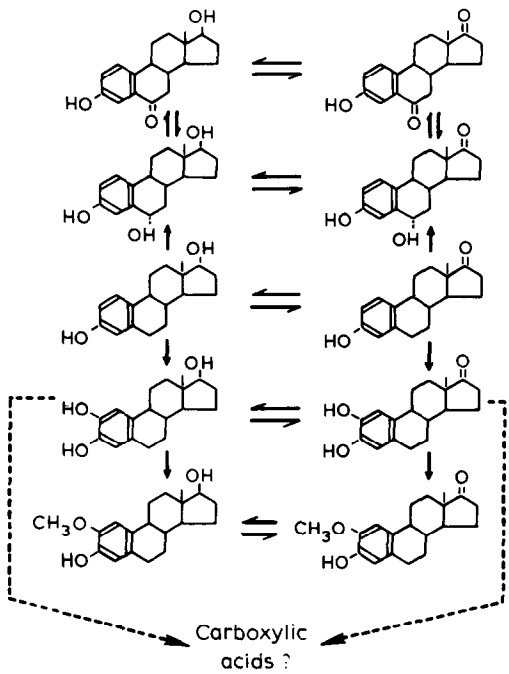

Fig. 4. Concept of the metabolism of oestradiol-17β in mouse liver *in vitro*.

By crystallization to constant specific activity the identity of the isolated 6α-hydroxyoestradiol-17β obviously is established. This is in contrast to Mueller and Rumney[15] who claimed to have demonstrated the formation of 6β-hydroxy-oestrogens by mouse liver microsomes. However, it should be pointed out that these authors have made their identification experiments with a reference standard prepared in the same way as the reference standard used in the present study, viz. by reduction of 6-oxo-oestradiol-17β by $NaBH_2$. By this procedure the 6α-configuration is formed rather than the 6β-configuration[12]. On the basis of the present work, a definite conclusion with respect to the configuration of the 6-hydroxylated oestradiol primarily formed is hardly possible. Breuer *et al.*[23] have found that rat liver microsomes convert oestradiol-17β to 6α- and 6β-hydroxy-oestradiol-17β in the ratio approximately 5 : 1. The results of the present investigation do not exclude that minor amounts of the β-hydroxy epimer are formed. Although the incubation mixture was frozen immediately after acidification to pH 2 and kept in the frozen state until extraction, the possibility cannot be ruled out that an epimerization of the 6-hydroxy groups of 6α- and 6β-hydroxy-oestrogens may have taken place.

The nature of the "acid metabolites" has not yet been established. The fraction is extractable with ether from an acid aqueous phase and readily distributes to sodium bicarbonate solution from ether[24]. In addition, the fraction is stable to hydrochloric acid and enzyme hydrolysis (unpublished results). At present, work is being done in this laboratory in order to elucidate the chemical structure of these compounds. Preliminary evidence indicates that they may be degradation products of the steroid structure, possibly originating from fission of ring A of the oestrogen molecule.

REFERENCES

1. H. Breuer, L. Nocke and R. Knuppen: *Hoppe-Seyler's Z. physiol. Chem.* **315** (1959) 72.
2. S. C. Brooks, L. Horn and J. P. Horowitz: *Biochim. biophys. Acta* **104** (1965) 250.
3. L. L. Engel, B. Baggett and M. Halla: *Biochim. biophys. Acta* **30** (1958) 435.
4. H. Breuer: *Arzneimittelforschung* **9** (1959) 667.
5. H. Breuer and G. Pangels: *Acta endocr. (Kbh.)* **33** (1960) 532.
6. W. Velle: In *Progress in Endocrinology* (Edited by C. Gual and F. J. G. Ebling). Excerpta Medica Foundation, Amsterdam (1969) pp. 905–909.
7. L. Ainsworth, A. L. Carter and R. H. Common: *Can. J. Biochem. Physiol.* **40** (1962) 123.
8. H. R. Raud and R. Hobkirk: *Can. J. Biochem.* **46** (1968) 759.
9. D. C. Collins, K. I. H. Williams and D. S. Layne: *Arch. biochem. Biophys.* **121** (1967) 609.
10. E. Kuss: *Hoppe-Seyler's Z. physiol. Chem.* **348** (1967) 1707.
11. F. Marks and E. Hecker: *Hoppe-Seyler's Z. physiol. Chem.* **350** (1969) 69.
12. O. Wintersteiner and M. Moore: *J. Am. chem. Soc.* **81** (1959) 442.
13. G. A. Bray: *Anal. Biochem.* **1** (1960) 279.
14. G. Rumney: *Fedn. Proc.* **15** (1956) 343.
15. G. C. Mueller and G. Rumney: *J. Am. chem. Soc.* **79** (1957) 1004.
16. P. H. Jellinck and I. Lucieer: *J. Endocr.* **32** (1965) 91.
17. E. Kuss: *Hoppe-Seyler's Z. physiol. Chem.* **350** (1969) 95.
18. P. H. Jellinck, J. Lewis and F. Boston: *Steroids* **10** (1967) 329.
19. F. Marks and E. Hecker: *Hoppe-Seyler's Z. physiol. Chem.* **349** (1968) 523.
20. L. Savu, O. Crépy, O. Judas and M. A. Ducret: *Bull. Soc. Chim. biol. (Paris)* **51** (1969) 1297.
21. G. Pangels and H. Breuer: *Naturwissenschaften* **49** (1962) 106.
22. P. H. Jellinck and M. Garland: *J. Endocr.* **45** (1969) 75.
23. H. Breuer, R. Knuppen and G. Pangels: *Biochim. biophys. Acta* **65** (1962) 1.
24. T. Thorsen and K. F. Støa: *Acta endocr. (Kbh.) Suppl.* **138** (1969) 81.