2-Hydroxylation of Estradiol by Human Placental Microsomes*

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ABSTRACT: Microsomes prepared from human term placentas converted estradiol-^{14}C to 2-hydroxyestrone-^{14}C in yields of 2–12%. Except for 17β-dehydrogenase activity no other transformations of the phenolic steroids could be demonstrated. The use of androstenedione-^{14}C as substrate did not result in any increase in the amount of 2-hydroxylated phenolic steroids.

The 2-hydroxylating enzyme is assumed to be located in the microsomes only since incubations with the 10,000g placental supernatant fraction did not produce any increase in 2-hydroxylation.

The microsomal fraction of the human placenta, which was first studied by Ryan (1959), has been used extensively for the conversion of C_{19} neutral steroids to the C_{18} phenolic female sex hormones. The nature of this significant enzymic conversion has been studied intensively (Morato *et al.*, 1962; Axelrod *et al.*, 1965), and although much progress has been made in the elucidation of the sequence of events leading to aromatization, some essential features still remain unclear. The necessary use of the impure particulate enzyme preparation for the study of the aromatization reaction has been attended with some problems. Chief among these problems is the lack of information on any further alterations of the estrogens by the placental preparation. A detailed study of the transformation of the female sex hormone by the human placental microsome preparation was, therefore, necessary prior to further investigation of the aromatization reaction.

Experimental Section

Tissue Preparation. Human term placentas were placed on ice immediately after delivery, and the subsequent work-up was carried out at 4°. The placenta was dissected free of fetal membranes and large blood vessels, chopped into small pieces, weighed, and homogenized for 1 min in the following buffer: 0.05 M phosphate at pH 7.0, 0.25 M sucrose, and 0.04 M nicotinamide (Ryan, 1959). In some experiments 0.05 M Tris buffer at pH 7.2 was used instead. One volume of buffer to three parts of tissue by weight was used. The placental homogenate was centrifuged at 10,000g for 30 min to give a "10,000g supernatant fraction" and a pellet which was discarded. Microsomes were prepared from the 10,000g supernatant by centrifugation at 100,000g for 60 min. The microsomal pellet obtained from 350 g of tissue (wet weight of an average size placenta) was suspended in 30 ml of the original buffer. The placental preparations were stored at −20° until used.

Incubation Procedures. The radioactive steroid substrate was incubated with the enzyme preparation and a NADPH[1]-generating system consisting of NADP, glucose 6-phosphate, and glucose 6-phosphate dehydrogenase. For the microsomal incubations 5.0 ml of microsomes (90 mg of protein) was used, and the NADPH-generating system consisted of NADP (12 μmoles), glucose 6-phosphate (40 μmoles), and glucose 6-phosphate dehydrogenase (5 Kornberg units) in a total volume of 5.61 ml. The 10,000g supernatant fraction (23 ml or approximately 15% of the total supernatant fraction of the placenta) was incubated with the radioactive substrate and a NADPH-generating system of NADP (12 μmoles), glucose 6-phosphate (120 μmoles), and glucose 6-phosphate dehydrogenase (10 Kornberg units) in a total volume of 24.02 ml.

The steroid substrates used were androst-4-ene-3,17-dione-4-^{14}C (sp act. 0.158 mc/mg) and estradiol-4-^{14}C (sp act. 0.116 mc/mg). The steroid was dried down in a test tube, and the additions were made therein. The mixture was incubated for 1 hr in a 37° water bath with air as the gas phase. The reaction was stopped, the carrier steroids dissolved in ether were added, and the mixture was acidified immediately. Then the incubation mixture was thoroughly extracted with ether.

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[1] Abbreviations used: NADP, nicotinamide-adenine dinucleotide phosphate; NADPH, reduced nicotinamide-adenine dinucleotide phosphate; PPO, 2,5-diphenyloxazole; dimethyl-POPOP, 1,4-bis-2-(4-methyl-5-phenyloxazolyl)benzene. Trivial names will be used for 3-hydroxyestra-1,3,5(10)-trien-17-one, estrone; 3,17β-dihydroxyestra-1,3,5(10)-triene, estradiol-17β; 3,16α,17β-trihydroxyestra-1,3,5(10)-triene, estriol; 2,3-dihydroxyestra-1,3,5(10)-triene-17-one, 2-hydroxyestrone; 2,3,17β-trihydroxyestra-1,3,5(10)-triene, 2-hydroxyestradiol; 2,3-dihydroxyestra-1,3,5(10)-triene-17-one-2-methyl ether, 2-methoxyestrone; 2,3,17β-trihydroxyestra-1,3,5(10)-triene-2-methyl ether, 2-methoxyestradiol; 3,6α,17β-trihydroxyestra-1,3,5(10)-triene, 6α-hydroxyestradiol-17β; androst-4-ene-3,17-dione, androstenedione.

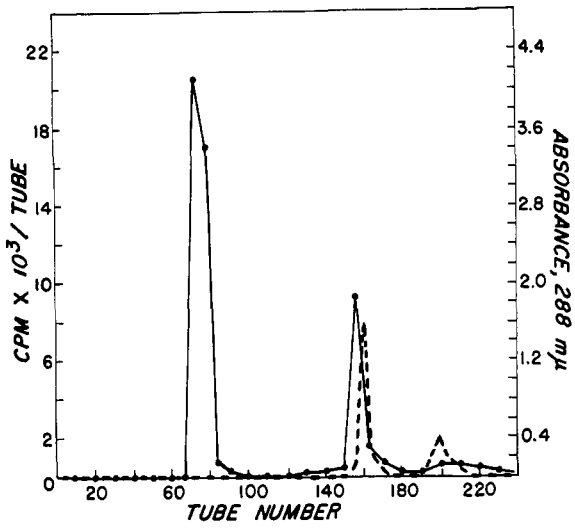

FIGURE 1: Partition column separation of products of estradiol-^{14}C incubation with added inert 2-hydroxyestrone and 2-hydroxyestradiol-17β. The solid line represents radioactivity. The broken line represents absorption at 288 mμ.

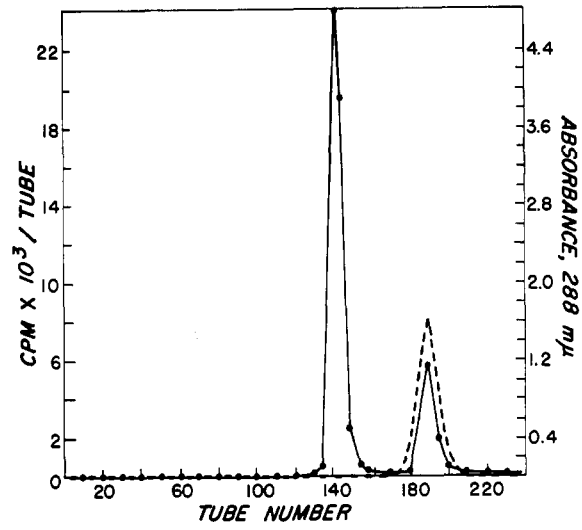

FIGURE 2: Partition column separation of products of incubation with estradiol-^{14}C, with addition of 2-hydroxyestrone and 2-hydroxyestradiol-17β followed by $LiAlH_4$ reduction. The solid line represents radioactivity, and the broken line represents absorption at 288 mμ.

In four separate control experiments microsomes and 10,000*g* supernatant fractions which had been heated to 100° for 5–10 min were incubated with androstenedione-^{14}C and estradiol-^{14}C. In no instance was any radioactivity found in the carrier 2-hydroxyestrone or 2-hydroxyestradiol.

Partition chromatography was done on a column of acid-washed Hyflo-Supercel (Celite) with 90% methanol as the stationary phase and isooctane (2,2,4-trimethylpentane) as the mobile phase. The gradient elution was provided by 25 and 100% 1,2-dichloroethane. Fractions were 10 ml. The following steroids were collected: estrone (fractions 50–70, radioactive peak at tube 55), estradiol plus 2-hydroxyestrone (fractions 131–153, radioactive peak at tube 142), and 2-hydroxyestradiol (fractions 200–226, radioactive peak at tube 213).

Further Purification. Acetylated derivatives were prepared by dissolving the steroid in 0.5 ml of pyridine with the addition of 0.2 ml of acetic anhydride followed by storage overnight at room temperature. Thin layer chromatography was carried out on silica gel G utilizing solvent systems comprised of ethyl acetate–cyclohexane (A, 30:70 or B, 50:50). Estradiol diacetate was recrystallized from petroleum ether (bp 30–60°), 2-hydroxyestrone diacetate from an acetone–petroleum ether mixture, and 2-hydroxyestradiol triacetate from methanol.

Radioactive Counting. Samples containing ^{14}C were counted either in a Tracerlab SC 50 gas-flow automatic sample changer as infinitely thin solid mounts or in a Nuclear-Chicago Unilux liquid scintillation counter. For toluene-soluble samples toluene containing 6 g/l. of PPO and 300 mg/l. of dimethyl-POPOP was used as the scintillant. Aqueous samples were counted in diotol, a solvent composed of 1 l. of dioxane, 1 l. of toluene, 600 ml of methanol, 208 g of naphthalene, 13 g of PPO, and 260 mg of dimethyl-POPOP.

Results

An initial inspection of the products of incubation of the microsome preparation with estradiol-^{14}C was carried out by gradient elution chromatography of the initial ether-soluble extract. Two radioactive areas corresponding to estrone and estradiol were obtained as the sole products. While this result seemingly excluded the presence of other products, it failed to eliminate the possibility of 2-hydroxylation. These labile catechol products could have been destroyed prior or during the chromatography, and moreover, the polarity of 2-hydroxyestrone is virtually identical with that of estradiol (Fishman *et al.*, 1960). It was thus possible that the peak ascribed to estradiol contained 2-hydroxyestrone. To resolve this question, weighed amounts of inert 2-hydroxyestrone and 2-hydroxyestradiol were added in the organic solvent used to terminate the incubation. The incubation mixture was then extracted with ether without any intervening alkali step, since this is essential for the preservation of the catechols. The ether-soluble extract was then submitted to gradient elution partition chromatography on Celite using 90% methanol as the stationary phase and isooctane with a gradient of dichloroethane as the eluents (Engel *et al.*, 1961).

The radioactive material was located by counting, and the inert compounds were located by their absorption at 288 mμ in the ultraviolet spectrum. The results of such a chromatogram are shown in Figure 1. The

TABLE I: Representative Reverse Isotope Dilution Data.

	Cpm	Free Steroid Added (mg)	Crystallization 1	2	3	4
Estradiol diacetate	39,000	21.3	1,628[a]	1,583	1,555	1,566
2-Hydroxyestrone diacetate	39,000	18.2	303	268	258	262
2-Hydroxyestradiol triacetate	14,500[b]	27.4	490	447	422	431

[a] Expressed as counts per minute per milligram of free compound. [b] From $LiAlH_4$-reduction experiment.

TABLE II: Microsome (100,000*g*) Incubations.

Substrate	Products Estrone	Estradiol	2-OH Estrone	2-OH Estradiol
Estradiol-^{14}C, 221,000	128,000[d] (75.2)[a]	34,000 (20.0)	4,600 (2.7)	~240 ~(0.1)
Estradiol-^{14}C,[b] 458,000	—	325,000 (95.5)	—	11,800 (3.3)
Androstenedione-^{14}C, 840,000	71,000 (16.2)	360,000 (82.2)	5,200 (1.2)	—
Androstenedione-^{14}C,[b] 550,000	—	310,000 (98.2)	—	5,100 (1.6)
Estradiol-^{14}C,[c] 221,000	—	180,000 (100.0)	—	—

[a] Values in parentheses are percentages of total phenolic products. [b] Extract was reduced with $LiAlH_4$ before separation procedure. [c] Enzyme preparation was heated at 100° for 10 min. [d] Radioactivity is expressed as counts per minute.

fractions containing the 2-hydroxyestrone were combined, a known amount of estradiol-17β was added, and the mixture was acetylated with acetic anhydride and pyridine. The 2-hydroxyestrone diacetate and estradiol diacetate were then separated by preparative thin layer chromatography, and each was recrystallized to constant specific activity. Similarly the fractions containing 2-hydroxyestradiol were combined and acetylated, and the triacetate was purified by preparative thin layer chromatography and then recrystallized to constant specific activity. The total radioactivity associated with estrone was obtained by counting the combined estrone fractions since these were shown to contain no other compounds. In another experiment, incubation with estradiol-^{14}C was followed by the addition of inert 2-hydroxyestrone and 2-hydroxyestradiol. The whole reaction mixture was then reduced with $LiAlH_4$ and then submitted to partition chromatography. Only two areas corresponding to estradiol and 2-hydroxyestradiol were then observed (Figure 2). The latter fractions were combined, acetylated, and crystallized to constant specific activity; and the total extent of 2-hydroxylation was thus estimated. The specific activities of a representative set of successive recrystallizations of the pertinent compounds are listed in Table I. The results of all three experiments are listed in Table II where they are compared with a control study in which the microsomes had been heated to 100° prior to incubation.

Enzymic hydroxylation at C-2 of C_{19} steroids has been suggested as a possible intermediate step in the aromatization sequence (Dorfman, 1956), and incubation of the placental preparation with 2-hydroxylated C_{19} precursors has led to unidentified aromatic substances which could be 2-hydroxyestrogens (Ryan, 1960; Gual *et al.*, 1962). In an effort to determine whether the placental microsomes are capable of hydroxylating at C-2 prior to aromatization, the foregoing experiments were repeated using androstenedione-^{14}C as the substrate for the incubation with the addition of 2-hydroxyestrone upon termination of the reaction. The results of these experiments are also listed in Table II.

In order to locate more precisely the enzyme responsible for the C-2 hydroxylation, incubations with estradiol-^{14}C and androstenedione-^{14}C were repeated using the 10,000*g* supernatant fraction which contains both microsomal and soluble enzymes. These incubations were extracted, separated, and purified exactly as those previously described, and the results are listed in Table III. For comparison the results from an incubation of the microsomal fraction from the same placenta are also included in the same table. Finally, in view of the evidence that the 2-hydroxylating enzyme found in rat liver is inhibited by phosphate (Marks and Hecker, 1966), a 10,000*g* supernatant preparation prepared with Tris buffer instead of phosphate buffer was incubated with estradiol-^{14}C. The results of this experiment

TABLE III: 10,000g Incubations.

Substrate	Products			
	Estrone	Estradiol	2-OH-Estrone	2-OH-Estradiol
Estradiol-^{14}C, 550,000	251,000[e] (63.5)[d]	137,000 (34.8)	5,600 (1.4)	–
Estradiol-^{14}C,[a] 550,000	128,000 (48.2)	103,000 (38.9)	31,000 (11.7)	–
Estradiol-^{14}C,[b] 221,000	128,000 (80.0)	22,000 (13.7)	4,600 (2.9)	~250 ~(0.2)
Androstenedione-^{14}C, 465,000	50,000 (20.4)	186,000 (76.1)	5,500 (2.3)	–
Estradiol-^{14}C,[c] 221,000	–	193,000 (100.0)	–	–

[a] Tris buffer used. [b] Microsomes (100,000g) used only. [c] Control, enzyme preparation was heated at 100° for 10 min. [d] Values in parentheses are percentage of total phenolic products extracted. [e] Radioactivity is expressed as counts per minute.

are also given in Table III and show the great enhancement of activity using this buffer.

Discussion

It is apparent that hydroxylation of the phenolic estrogen at C-2 is a significant metabolic activity of human placental microsomes. Indeed, with the exception of the ubiquitous 17β-dehydrogenase, it appears to be the only placental microsomal activity with estrogen substrate specificity.[2] The 2-hydroxylase enzyme seems to be solely associated with the microsomes since the incubations with 10,000g supernatant listed in Table III failed to result in any increase of 2-hydroxylated products when compared to the microsome preparation alone. The substantial increase in 2-hydroxylation when the Tris-buffered preparation was used demonstrates phosphate inhibition of the 2-hydroxylase, in agreement with the results observed in rat liver 2-hydroxylase (Marks and Hecker, 1966). It must be emphasized that the yield of 2-hydroxyestrone as obtained in these experiments must represent a minimal value due to the apparent substantial protein binding. Indeed, of the amounts of inert 2-hydroxyestrone added to the incubation mixtures only about 50% was ever recovered, despite the simple work-up procedure. This is lower than the recoveries when the material is added to a 3-day urine collection and reisolated after a protracted multistep work-up. Despite these limitations the extent of 2-hydroxylation by the placental microsomes is substantial, equalling as much as 12% of ether-extractable radioactivity under the conditions used.

Prior to this work two reports offered indirect evidence for the presence of 2-hydroxylation by the human placenta. Following perfusion of a placenta with estradiol-^{14}C, a less than 0.1% yield of a material tentatively identified as 2-methoxyestrone was obtained (Troen, 1961). Incubation of a placental mince with estradiol or estrone in the presence of methionine, labeled with ^{14}C in the methyl group, gave also about 0.1% yield of radioactive 2-methoxyestrogens (Lucis, 1965). It is noteworthy that in none of the present incubations, whether with microsomes or supernatant, was it possible to detect any 2-methoxyestrone. In view of the above results this is most likely due to the lack of the necessary cofactors in the subcellular fractions. Further, it is evident that the very small amount of 2-hydroxyestradiol found is not at all in proportion to the amount of estradiol available. Similar disproportionation between 2-methoxyestrone and 2-methoxyestradiol has been found by Lucis (1965) who suggests that it is particularly evident in placental tissue.

In the androstenedione-^{14}C incubations the yield of 2-hydroxyestrogens as a fraction of the aromatized products is the same or lower than when aromatic precursors are used. From this it is concluded that C-2 hydroxylation of the neutral ring A and subsequent aromatization to 2-hydroxyestrogens is an unlikely route of synthesis.

In none of the incubations was it possible to detect the presence of either 6α- or 16α-hydroxylated products. 6α-Hydroxyestradiol has been shown to be a minor product of estradiol perfusion through the placenta (Cédard and Knuppen, 1965; Alonso and Troen, 1966). The presence of 16α-hydroxylating capacity by human placenta has been in dispute. The polar product of placental perfusion initially identified as estriol (Varangot *et al.*, 1962) has now been shown to be 6α-hydroxyestradiol-17β (Cédard and Knuppen, 1965). The evidence now strongly favors absence of estrogen 16α-hydroxylating activity in the human placenta, and the large production of estriol in pregnancy probably derives from a 16α-hydroxylated neutral precursor (Siiteri and MacDonald, 1966).

Although results obtained in this work may be unique to the "*in vitro*" conditions used they suggest the presence of substantial estrogen 2-hydroxylating capacity in the placenta and raise the question of the excretion of 2-hydroxyestrogens in pregnancy. The erratic excretion of 2-methoxyestrone in pregnancy has

[2] Incubations of the 10,000g supernatant with estradiol-^{14}C gave an additional radioactive area which did not correspond to any known metabolites. This product is now being further investigated.

been noted and discussed (Hobkirk and Nilsen, 1962). However, the excretion of this metabolite need not be related to the presence of the 2-hydroxy precursors since other factors affecting the catechol-*o*-methyltransfer reaction may be involved. Information on the excretion of 2-hydroxyestrogens in pregnancy, at present unavailable, may be of substantial importance.

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References

Alonso, C., and Troen, P. (1966), *Biochemistry 5*, 337.
Axelrod, L. R., Matthijssen, C., Rao, P. N., and Goldzieher, J. W. (1965), *Acta Endocrinol. 48*, 383.
Cédard, L., and Knuppen, R. (1965), *Steroids 6*, 307.
Dorfman, R. I. (1956), *Am. J. Med. 21*, 679.
Engel, L. L., Cameron, C. B., Stoffyn, A., Alexander, J. A., Klein, O., and Trofimow, N. D. (1961), *Anal. Biochem. 2*, 114.
Fishman, J., Cox, R. I., and Gallagher, T. F. (1960), *Arch. Biochem. Biophys. 90*, 318.
Gual, C., Morato, T., Hayano, M., Gut, M., and Dorfman, R. I. (1962), *Endocrinology 71*, 920.
Hobkirk, R., and Nilsen, M. (1962), *J. Clin. Endocrinol. 22*, 134.
Lucis, O. J. (1965), *Steroids 6*, 307.
Marks, F., and Hecker, E. (1966), *Z. Physiol. Chem. 345*, 22.
Morato, T., Raab, K., Brodie, H. J., Hayano, M., and Dorfman, R. I. (1962), *J. Am. Chem. Soc. 84*, 3764.
Ryan, K. J. (1959), *J. Biol. Chem. 234*, 268.
Ryan, K. J. (1960), *Acta Endocrinol., Suppl. 51*, 697.
Siiteri, P. K., and MacDonald, P. C. (1966), *J. Clin. Endocrinol. 26*, 751.
Troen, P. (1961), *J. Clin. Endocrinol. 21*, 895.
Varangot, J., Cédard, L., and Yanotti, S. (1962), Excerpta Medica International Congress Series 51, Abstract 396, Milan.