Biosynthesis of Estrogens

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ABSTRACT. The formation of estrogens and phenolic material from a large number of C_{10} C_{18}, C_{19} and C_{21} steroids and related structures has been investigated, employing a human placental microsomal preparation supplemented with TPNH and oxygen. The data from the incubation of 2 C_{14}-labeled androgens are included. There is a discussion of possible biosynthetic pathways for the estrogens.

THE STUDY OF the formation of estrogens from acetate and cholesterol is an important part of the early work on the biosynthesis of these phenolic hormones. Heard *et al.* (1) reported C^{14}-estrone in the urine of a pregnant mare to which CH_3-$C^{14}OONa$ had been administered intravenously, but found no C^{14} in the urinary estrone after cholesterol-4-C^{14} had been given by the same route (2). Werbin *et al.* (3) later demonstrated that cholesterol-4-C^{14} can serve as precursor for estrone in the human. The pathway of cholesterol to C_{19}-steroids is well documented; however, the mechanisms involved in the aromatization of ring A from these androgens are not completely known.

The biologic conversion of androgens to estrogens has been recognized for many years. However, it required the use of radioactive isotope tracer techniques to establish a direct relationship between these two classes of steroids (4–6). The finding by Meyer (7) that bovine adrenal glands are able to introduce a hydroxyl function in the methyl group at C-10 of 4-androstene-3,17-dione was followed by the suggestion that the biologic removal of the angular methyl group would be facilitated by this preliminary oxygenation. The conversion of 19-hydroxy-4-androstene-3,17-dione to estrone by human placental tissue was demonstrated subsequently by Meyer (8), Ryan (9) and Longchampt *et al.* (10). From this hydroxylated intermediate, several alternative pathways are possible for the formation of the phenolic ring A, differing in the oxidation state at which the angular methyl group is eliminated.

Ryan (9, 11) has reported the conversion of androgens to estrogens in a high yield by a system consisting of human placental microsomes, the reduced form of triphosphopyridine nucleotide or a TPNH generating system and oxygen. Many steroids have been investigated with the use of this tissue preparation as possible precursors of the natural estrogens. In the present study, many other interesting structures have been examined for conversion to phenolic substances. This manuscript is a compilation of these data, together with a discussion of the findings.

Materials and Methods

Incubation and Extraction. Human term placentas obtained within 3 hr of delivery were processed according to Ryan's pro-

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cedure (9). The microsomal fractions obtained by differential centrifugation between 10,000 x *g* and 105,000 x *g*, representing 15–20 g of wet tissue or 19 mg of tissue nitrogen, were resuspended in 2.0 ml of 0.1 M phosphate buffer, pH 7.0, and 100 μg of steroid substrate (nonradioactive) was added in 0.1 ml of propylene glycol. The quantities of radioactive steroids incubated are given in the text. Final additions of 3 μmoles of TPN, 5 μmoles of glucose-6-phosphate and 2 units of glucose-6-phosphate dehydrogenase dissolved in phosphate buffer were made, bringing the total volume to 3 ml. The mixture was incubated for 1 hr in 20 ml beakers in a Dubnoff incubator at 37 C, with air as the gas phase. At the end of this period, the incubated mixture was extracted 3 times with 6 volumes of chloroform. The pooled chloroform extracts were filtered and evaporated to dryness.

Analyses of Incubation Products. Paper chromatography of the extracts was carried out on both phenolic and neutral fractions on Whatman No. 1 paper, using the ligroin propylene-glycol system of Savard (12), the toluene propylene-glycol and chloroform-formamide systems of Zaffaroni (13) and the 66% petroleum ether-34% benzene: 80% methanol-H_2O system of Bush (14). The term R_T indicated mobility in cm/hr.

Turnbull's blue reagent [$FeCl_3 - K_3Fe(CN)_6$] was used to detect phenolic compounds on the paper chromatograms. Ultraviolet light absorption and the Zimmermann and isonicotinic acid hydrazide (15) reagents were used to detect the neutral steroids.

Measurement and Identification of Radioactive Samples. Radioactive samples were counted in duplicate in a Packard Tri-Carb liquid scintillation spectrometer, in a scintillation medium of toluene, diphenyloxazole and bisphenyloxazolylbenzene for the period of time necessary to ensure an accuracy of ±2%. Values are reported as disintegrations per minute corrected for background. Radioactive spots on paper chromatograms were detected with a Nuclear-Chicago Model C-100A chromatogram scanner and a D-47 micromil window counter. The radiochemical purity and identity of labeled compounds were established by one or more of the following techniques: reverse isotopic dilution and crystallization to constant specific activity, paper chromatography in various systems, countercurrent distribution and formation of derivatives.

Control Experiments. Controls consisted of 1) the incubated mixture extracted at zero time and 2) a tissue blank incubated for 1 hr without the steroid. No detectable amount of phenolic compounds was found in either.

Results

Incubation of Radioactive Steroids. The transformation of the following radioactive steroids[1] to estrogens was examined: 5 μc 17β-hydroxy-19-nor-4-androsten-3-one-4-C^{14} (SA 12 mc/mM), 1.0 μc 17β-hydroxy-4-androsten-3-one-4-C^{14} (SA 22 mc/mM), 1.0 μc 4-androstene-3,17-dione-4-C^{14} (SA 5.6 mc/mM) and 2.0 μc 5α-androst-1-ene-3,17-dione-4-C^{14} (SA 12 mc/mM). The description and data from the first 2 experiments are presented below. The experiment carried out with 4-androstene-3,17-dione-4-C^{14} has been published elsewhere (10) and describes the isolation, identification and reincubation of 19-hydroxy-4-androstene-3,17-dione-C^{14} formed therefrom to labeled estrone. Because of the varied reports in the literature of the transformation of 5α-androst-1-ene-3,17-dione to estrone, a very careful examination of this structure as a possible precursor to the estrogens was made with the use of labeled material. Since no radioactivity appeared in the traces of estrogens isolated, it can be concluded that this compound is not aromatized by the human placenta. The results confirm those of Ofner *et al.* (16).

Incubation of 17β-Hydroxy-19-nor-4-androsten-3-one-4-C^{14} (19-Nortestosterone). To the crude extract from the incubation containing 2.6×10^6 dpm were added 1 mg each of carrier estrone and

[1] The purity of labeled steroids was checked by UV and infrared spectra, paper chromatography, and crystallization to constant specific activity.

TABLE 1. Specific activity of estradiol-17β-C^{14} formed from 19-nortestosterone-C^{14}

Successive recrystallizations	Compound recovered, mg	Total counts, dpm	Specific activity, dpm/mg
1	33.9	31,800	938
2	26.9	26,600	988
3	19.1	18,400	962
4	15.6	14,400	923
5	13.9	13,100	942

estradiol. A partition between toluene and 1N NaOH was then carried out, yielding phenolic and neutral fractions. The phenolic fraction, containing 4.2 $\times 10^5$ dpm, was subjected to an eight transfer countercurrent distribution (mobile phase, toluene; stationary phase, 1N NaOH). The pool of tubes 0, 1, 2 and 3 contained 2.2 $\times 10^5$ dpm, amounting to 12% over-all yield, after correcting for losses incurred during the purification procedures. An aliquot of this fraction was chromatographed for 12 hr in the toluene-propylene glycol system and two radioactive spots were detected (Zones E-2 and E-1) which corresponded to those of standard estradiol-17β and estrone. Zone E-2 was rechromatographed for 24 hours in the toluene-propylene glycol system. Its R_T corresponded to that of estradiol-17β. The addition of 40 mg of estradiol-17β, followed by recrystallizations from methanol, resulted in material with constant specific activities ranging from 922 to 988 (Table 1).

The neutral fraction, after 96 hr of ligroin-propylene glycol paper chromatography, yielded two major radioactive zones. The migration rate of the principal zone corresponded to that of 19-nortestosterone. Eighty mg of carrier 19-nortestosterone was added to the eluate and the constancy of specific activity was established by recrystallization (Table 2). The identities of other radioactive neutral and phenolic materials observed in the paper chromatograms were not established.

Incubation of 17β-hydroxy-4-androsten-3-one-4-C^{14} (testosterone) and subsequent processing for the estrogens were carried out essentially as described above for 19-nortestosterone. Both estrone and estradiol were noted, totaling an approximate yield of 60%. Carrier estradiol was added to paper eluate zones corresponding to this steroid and recrystallizations were made to constant specific activity.

Incubation of Nonradioactive Steroids.[2] All steroids tested for aromatization are listed in Table 3. A complete work-up of a pooled sample from several 100 μg incubations of 4-androstene-3,17-dione was carried out. The residue from the chloroform extract of the incubate was partitioned between pentane and 90% methanol in water, and the methanolic fraction was processed as described by Baggett *et al.* (5) for a separation of the neutral and phenolic compounds.

The latter fraction was subjected to a 49 transfer countercurrent distribution between 70% methanol-H_2O, as the mobile upper phase, and carbon tetrachloride as the lower. Quantitation of phenolic structures in each tube was

[2] We wish to express our gratitude to Syntex, S. A., for the major part of the steroids used in this study.

TABLE 2. Specific activity of 19-nortestosterone-C^{14}

Successive recrystallization	Compound recovered, mg	Total counts, dpm	Specific activity, dpm/mg
1	50.7	53,760	1060
2	46.2	49,600	1071
3	41.0	40,560	989
4	36.3	35,560	979

made with an antimony trichloride-nitrobenzene color test (17). A compound was detected with a partition coefficient (K) of 1.3, which is equal to that of estrone. In the final 8 tubes there were at least 2 more polar phenolic materials. The contents of tubes 22 to 34 were analyzed for ultraviolet absorption. The spectra obtained showed λ_{max}^{MeOH} 279 mμ, ε 2,300 with a shoulder at 286 mμ. On paper chromatography, the mobility of this material was found to be 4 cm/hr in the toluene-propylene glycol system, a rate identical with that of authentic estrone. When tested with the $FeCl_3$-$K_3Fe(CN)_6$ reagent, it gave the

TABLE 3. Conversion of various compounds to phenolic structures

Substrate	% conversion	Phenolic Products
C_{17}		
10-Hydroxymethyl-$\Delta^{1(9)}$-octal-2-one	0	
10-Carboxy-$\Delta^{1(9)}$-octal-2-one	0	
C_{18}		
19-Nor-4-androstene-3,17-dione	10	Estrone, Estradiol
17β-Hydroxy-5α-10β-estrane-3-one	0	
5α,10α-Estrane-3,17-dione	0	
17α-Ethynyl-10β,17β-dihydroxy-4-androstene-3,17-dione	0	
17α-Ethynyl-17β-hydroxy-19-nor-5(10)-androsten-3-one	0	
B-Nor-17β-hydroxy-4-androsten-3-one	30	Probably B-norestradiol
C_{19}		
4-androstene-3,17-dione	60	Estrone, Estradiol
19-Hydroxy-4-androstene-3,17-dione	80	Estrone, Estradiol
17β-Hydroxy-4-androsten-3-one (testosterone)	60	Estrone, Estradiol
3β-Hydroxy-5-androsten-3-one (dehydroepiandrosterone)	40	Estrone, Estradiol
1,4-Androstadiene-3,17-dione	35	Estrone, Estradiol
5α-Androst-1-ene-3,17-dione	0	
5α-Androstane-3,17-dione	0	
1α-Hydroxy-4-androstene-3,17-dione	0	
1α,3β-Dihydroxy-5-androsten-17-one	0	
1α-Methyl-17β-hydroxy-4-androsten-3-one	0	
2β-Hydroxy-4-androstene-3,17-dione	10	Probably 2-hydroxyestrone
2-Hydroxymethylene-17α-methyl-17β-hydroxy-4-androsten-3-one	0	
2β-Methyl-17β-hydroxy-4-androsten-3-one	0	
2-Formyl-17α-methyl-17β-hydroxy-1,4-androstadien-3-one	0	
4,6-Androstadiene-3,17-dione	0	
1,4,6-Androstatriene-3,17-dione	0	
4-Androstene-3,11,17-trione	0	
11β-Hydroxy-4-androstene-3,17-dione	0	
11α-Hydroxy-4-androstene-3,17-dione	60	Probably 11α-hydroxyestrone
6α-Fluoro-17β-hydroxy-4-androsten-3-one	0	
6β-Fluoro-17β-hydroxy-4-androsten-3-one	0	
9α-Fluoro-1,4-androstadiene-3,17-dione	35	Probably 9α-fluoroestrone
9α,17β-Dihydroxy-4-androsten-3-one	30	Probably 9α-hydroxyestradiol
C_{21}		
4-Pregnene-3,20-dione (Progesterone)	0	
17α,17β,21-Trihydroxy-4-pregnene-3,20-dione	0	
17α,19,21-Trihydroxy-4-pregnene-3,20-dione	0	
6β-Fluoro-16α-methyl-21-acetoxy-11β,17α-dihydroxy-1,4-pregnadiene-3,20-dione	0	

characteristic blue color, indicating the presence of a phenol. Infrared analysis of the isolated material was performed on a methylene dichloride deposited film. The spectra showed maxima at 3,300, 1,740, 1,580 and 1,500 cm^{-1}. Comparison with the spectrum of an authentic sample of estrone established identity of the product from the incubation as this phenol. Additional verification was also obtained in the mouse uterine weights bio-assay.

The products of the incubation of other steroids were analyzed by paper chromatography. Narrow strips were taken for development with the $FeCl_3$-$K_3Fe(CN)_6$ reagent. Where estrone and estradiol-17β were formed, quantitation was made of the eluate of the remainder of the paper strip with the $SbCl_3$ color test (17). Neutral compounds were measured with the Callow modification of the Zimmermann reaction (18). Aromatized products from incubations of steroid structures not expected to yield estrone or estradiol were localized with the $FeCl_3$-$K_3Fe(CN)_6$ reagent and the yields were estimated visually.

Discussion

A number of pertinent facts and conclusions concerning the human placental tissue biosynthesis of estrogens may be drawn from the results of the incubation of various steroid structures. That an unsaturated A ring is necessary for the aromatization process was seen in the lack of transformation of ring A saturated substances. Conversion of dehydroepiandrosterone to estrone proceeds due to the action of 3β-hydroxy-dehydrogenase on the Δ^5-3β-hydroxy group, yielding the Δ^4-3-keto structure. The Δ^1-3-keto structure was not transformed. $\Delta^{1,4}$-Dienones and the 19-nor (10β) compound were converted slowly. The presence of axial substituents at carbon 11 interferes with ring A aromatization. Aromatization takes place readily in the presence of an 11α-hydroxy group, as it did also in structures with substituents at the 9α position. The reaction proceeded slowly with a 2β-hydroxylated substrate. C_{10} cyclic structure analogues to rings A and B of C_{19} androgens were not aromatized by the placental preparation, indicating that rings C and D are necessary structural requirements for these enzymes. A compound with a five-membered ring B was readily converted. Not all C_{21} steroids tested were transformed, despite the presence of an oxygen function at C-19 in one instance.

It is now established that the major pathway from androgens to estrogens involves an initial hydroxylation at C-19. Oxidation of the primary alcohol to an aldehyde may occur, yielding a structure more readily transformed to a ring A phenol (19). Further oxidation to a carboxylic group has not been observed with placental microsomes. 10β-Carboxy-4-estrene-3,17-dione itself was noted to be decarboxylated rapidly by this tissue to the 19-nor structure, which in turn can be converted enzymatically to estrone, in small yield, in the presence of oxygen and TPNH.

There has been considerable discussion about the possibility that Δ^1-dehydrogenation is a discrete reaction in the biologic sequence to estrogens (20–22). While this reaction is well known in many microorganisms, it has not been demonstrated to proceed adequately in mammalian tissue.

The liberation of HCHO in the final step prior to aromatization has been proposed. And indeed, stoichiometric ratios of this substance and estrone have been found after incubations of androstenedione and 19-hydroxyandrostenedione (21). A mechanism for the formation of HCHO from the latter steroid has been outlined in previous publications

(19, 23). *Via* the same mechanism, HCOOH would be expected from 19-oxo-androstenedione. Investigations are now in progress to clarify this facet and other questions concerning the enzymology of estrogen synthesis.

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