PITUITARY-OVARIAN ENDOCRINOLOGY

Dorfman & Castro

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HOLDEN-DAY, INC., SAN FRANCISCO: 1963

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Library of Congress Catalog Card No. 63-22194

Printed in the United States of America

FOREWORD

This volume is the record of a Symposium on Endocrine Gynecology sponsored by the Portuguese Cancer Institutue in Lisbon in the summer of 1962. It was my pleasure to participate in these discussions and to help in the editing of this volume. The meeting was a huge success and an excellent meeting ground for the discussions of the newer developments in the field of pituitary-ovarian endocrinology, pure and applied.

This volume outlines most of the important recent advances in endocrine gynecology from aspects of the biochemistry of steroid biosynthesis through new analytical methods which permit a reasonable evaluation of the "androgen burden," some important new reflections on the Stein-Leventhal Syndrome, newer physiological aspects of the gonadotropins, clinical diagnosis and the important advances in sterility and fertility.

I know that I speak for the visiting American and English contributors when I thank our Portuguese Cancer Institute colleagues, one and all, for their many kindnesses. All of us would hope to have the opportunity in the near future to repay their thoughtfulness and fine hospitality.

Shrewsbury, Massachusetts
August, 1963

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I

Ralph I. Dorfman, Ph.D.

BIOSYNTHESIS AND DETECTION OF ESTROGENS

Considerable new information is now available on the biosynthesis and estimation of estrogens. This chapter will be devoted to a discussion of these areas.

BIOSYNTHESIS OF ESTROGENS

The biological conversion of androgens to estrogens and thus the route of estrogen biosynthesis had been suggested for some years [3, 13, 16, 20], but it remained for radioisotopic technics to establish this interrelationship beyond a reasonable doubt [1, 2, 5, 19]. Specifically, the substrate was testosterone and the products were estrone and estradiol-17β.

Acetate-1-C^{14} could also serve as a precursor for estrone in the pregnant mare [6] but in one study cholesterol did not seem to be a proper intermediate [7]. Contrary to this report, Werbin, et al. [18] demonstrated that cholesterol-4-C^{14} could be converted to labeled estrone by an *in vivo* experiment in a human. The pathway of cholesterol to androgens is well documented and the mechanisms involved in the aromatization of ring A have been elucidated.

Meyer [10] discovered that bovine adrenal glands may hydroxylate androst-4-ene-3, 17-dione at carbon 19, and he suggested that the removal of the C-10 angular methyl group was facilitated by this preliminary oxidation. These reactions had been accomplished by chemical means. The conversion of 19-hydroxy-androst-4-ene-3, 17-dione to estrone by human placental tissue has been demonstrated by Meyer [11], Ryan [15] and Longchampt, et al. [9].

Since the first experiments by Meyer [10], [11] efforts of a number of workers in various laboratories including the Worcester Foundation group have been extended towards the elucidation of the individual steps of estrogen biosynthesis in the human. In *in vitro* work, the term

"placenta preparation" was used throughout as the enzyme source since this tissue is easily available and a good reproducible incubation system had been established in the pioneer studies of Ryan [15]. It is expected that all findings with this tissue can be extrapolated to ovarian systems. Innumerable steroid structures were examined with two aims in mind: (1) to find what compounds could contribute estrogenic material, even if a very small percentage, to body pools, and (2) to elucidate the steps in the major pathway of estrogen biosynthesis.

In Table 1-1 are some of the structures which have been tested for aromatization in ring A by the placental enzyme system. Most of the compounds were not converted. There were, however, a few which led to the natural estrogens, estrone and estradiol-17β. 19-Norandrost-4-ene-3, 17-dione was converted in low yield. This substance, which has been shown to be present in large amounts in mare follicular fluid, may be a significant source of body estrogens. Androst-4-ene-3, 17-dione, 19-hydroxyandrost-4-ene-3, 17-dione and 19-oxo-androst-4-ene-3, 17-dione, as well as testosterone, were excellent substrates. These are components of the major pathway of biological estrogen formation. 10β-Carboxy-estr-4-ene-3, 17-dione yielded only a trace of estrogen and this by virtue of its very rapid decarboxylation to 19-norandrost-4-ene-3, 17-dione in the presence of placental tissue. Dehydroepiandrosterone yielded estrone in good yield due to its fast oxidation to androst-4-ene-3, 17-dione. Androst-1, 4-diene-3, 17-dione is another source of estrogens; the question of its natural occurrence in the body is, however, equivocal. Rigorous examination of the utilization of 5α-androst-1-ene-3, 17-dione has been carried out using C^{14}-labeled substrate. The results show that this compound does not give rise to phenolic structures.

Secondly, the picture of the major biosynthetic pathway of estrogen formation from the androgens, androst-4-ene-3, 17-dione and testosterone, is now essentially complete. It has been known for many years that both the above C_{19}-steroids are transformed to estrogens; many tracer studies with C^{14}-labeled precursors have been documented. The next step was the definite establishment of the 19-hydroxylated derivative as an intermediate in the biosynthesis, following up initial experiments where 19-hydroxyandrost-4-ene-3, 17-dione was observed to be converted to estrone at a faster rate than androst-4-ene-3, 17-dione. Confirmation of its formation by placental tissue and its transformation, in turn, to estrone was obtained through the isolation of this oxygenated derivative in incubations with C^{14}-labeled androst-4-ene-3, 17-dione. The next series of experiments were conducted when 19-oxo-androst-4-ene-3, 17-dione and 10β-carobxy-estr-4-ene-3, 17-dione became available. The 19-oxo compound was found to be converted even more rapidly than the 19-hydroxy derivative. The carboxylic acid, however, yielded only a trace of estrone, which subsequent examination revealed arose from its decarboxylated product,

Table 1-1. Conversion of Various Steroids to Estrogens*

	Per cent conversion
C_{18}	
19-Norandrost-4-ene-3,17-dione	10
17β-Hydroxy-5α,10β-estran-3-one	0
5α,10α-Estrane-3,17-dione	0
17α-Ethynyl-10β,17β-dihydroxyandrost-4-ene-3,17-dione	0
17α-Ethynyl-17β-hydroxy-19-nor(10β)-androst-5(10)-en-3-one	0
C_{19}	
Androst-4-ene-3,17-dione	40
19-Hydroxyandrost-4-ene-3,17-dione	60
19-Oxo-androst-4-ene-3,17-dione	80
10β-Carboxy-estr-4-ene-3,17-dione	1
17β-Hydroxyandrost-4-en-3-one (testosterone)	40
3β-Hydroxyandrost-5-en-3-one (dehydroepiandro-sterone)	30
Androsta-1,4-diene-3,17-dione	25
5α-Androst-1-ene-3,17-dione	0
1α-Hydroxyandrost-4-ene-3,17-dione	0
1α,3β-Dihydroxyandrost-5-en-17-one	0
1α-Methyl-17β-hydroxyandrost-4-en-3-one	0
2β-Hydroxyandrost-4-ene-3,17-dione	10
2-Hydroxymethylene-17α-methyl-17β-hydroxyandrost-4-en-3-one	0
2βMethyl-17β-hydroxyandrost-4-en-3-one	0
2-Formyl-17α-methyl-17β-hydroxyandrosta-1,4-dien-3-one	0
Androsta-4,6-diene-3,17-dione	0
Androsta-1,4,6-triene-3,17-dione	0
Androst-4-ene-3,11,17-trione	0
11β-Hydroxyandrost-4-ene-3,17-dione	0
11α-Hydroxyandrost-4-ene-3,17-dione	40
6α-Fluoro-17β-hydroxyandrost-4-en-3-one	0
6β-Fluoro-17β-hydroxyandrost-4-en-3-one	0
5α-Androstane-3,17-dione (androsterone)	0
9α-Fluoroandrosta-1,4-diene-3,17-dione	35
17β-Acetoxyandrosta-4,7-dien-3-one	15
C_{21}	
Pregn-4-ene-3,20-dione (progesterone)	0
17α,19,21-Trihydroxypregn-3-ene-3,20-dione	0
6β-Fluoro-16α-methyl-21-acetoxy-11β,17α-dihydroxypregnane-1,4-diene-3,20-dione	0

*Gual, et al. [4]

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19-norandrost-4-ene-3, 17-dione. Examination of the co-factor requirements for the aromatization of each of the two 19-oxygenated structures above showed that both TPNH and oxygen were essential (Table 1-2).

Table 1-2. Cofactor and Oxygen Requirement for Aromatization*

	Gaseous phase	Cofactor	Per cent conversion to estrogen
10β-Carboxy-estr-4-ene-3,17-dione	N_2	DPN	0
"	N_2	TPN	0
"	N_2	-	0
"	Air	TPNH	5
19-Norandrost-4-ene-3,17-dione	Air	TPNH	5
19-Oxo-androst-4-ene-3,17-dione	N_2	DPN	0
"	N_2	TPN	0
"	N_2	-	0
"	Air	TPNH	100
"	"	DPNH	0
"	"	TPN	0
"	N_2	TPNH	5
19-Hydroxyandrost-4-ene-3,17-dione	Air	TPNH	50-60
"	"	TPN	0
"	"	DPNH	0
"	N_2	TPNH	0
Androst-4-ene-3,17-dione	Air	TPNH	30-40
"	N_2	TPNH	0

DPN, DPNH, or TPN (Sigma) 2.5 μMoles; TPNH regenerating system: TPN 2.5 μMoles, gluc-6-P (Sigma) 4.3 μMoles, gluc-6-P dehydrogenase (Sigma) 0.5 K.U.

* Morato, et al. [12]

It thus appeared that the sequence of steps from the C_{19}-compounds to the C_{18}-phenols was: androst-4-ene-3, 17-dione → 19-hydroxyandrost-4-ene-3, 17-dione → 19-oxo-androst-4-ene-3, 17-dione → estrone. Examination of the nature of the 1-carbon moiety, the 19-carbon atom, eliminated in the course of the reaction was next undertaken. Theoretically, via a mechanism of the reverse aldol condensation type, the direct aromatization of the 19-hydroxy structure would yield formaldehyde, and the 19-oxo compound, formic acid. Experimental results obtained can be briefly summarized: when androst-4-ene-3, 17-dione was the substrate, only formaldehyde appeared in measurable quantities;

when 19-hydroxyandrost-4-ene-3, 17-dione was the substrate, both formaldehyde and formic acid appeared; and when 19-oxo-androst-4-ene-3, 17-dione was the substrate, only formic acid appeared. All possible controls of the incubation system and analysis procedures have been run. All blanks were negative. No inter-conversion of formaldehyde and formic acid occurred in the placental system. In no instance was formaldehyde or formic acid formed other than as a product of placental incubations with the steroids above. Steroid fractions were analyzed by paper chromatography. Both estrone and estradiol-17β were always found as products.

It would appear from these results that in the intact animal the following situation may obtain: estrogen can arise from both the 19-hydroxy and 19-oxo structures directly, and it is highly probable that the same enzyme catalyzes the aromatization of both substrates. A provision for the oxidation of the 19-hydroxy function to the 19-oxo grouping is present in the placenta and has been noted. This transformation probably requires TPN, which is always present in this system. It is esthetically pleasing to visualize a situation where TPNH can be internally generated at the site of estrogen formation at the moment when it is needed to catalyze the final step in the biosynthetic sequence. In this respect another source of the reduced or oxidized pyridine nucleotide is in the 17-dehydrogenase reaction which is TPN linked. The biosynthetic steps are summarized in Fig. 1-1. The elucidation of these mechanisms sets the proper stage for studies of control of estrogen biosynthesis under normal and pathological conditions.

DETERMINATION OF PLASMA ESTROGENS

Elegant methods for the determination of estrogens in human urine have been developed and have recently been reviewed [14]. This review also deals with plasma estrogen levels. Of the various plasma methods, however, only two appear to approach the required sensitivity needed to analyze plasma from non-pregnant human subjects. One of these, developed by Svendsen [17], had a sensitivity of about 0.002 μg for estrone and estradiol-17β in plasma. The method is based on the double isotopic derivative principle using S^{35} and I^{131}. Plasma, derived from heparinized blood, is extracted with chloroform and the estrone and estradiol-17β purified, esterified by means of p-iodobenzenesulphonyl chloride, purification of the esters by paper chromatography and determining radioactivities. Using this method, preliminary plasma values are given for normal women, patients with primary and secondary amenorrhea, and pregnant women.

The second method has been developed in the Worcester Foundation laboratory [8] and is briefly described. Plasma, after adding an

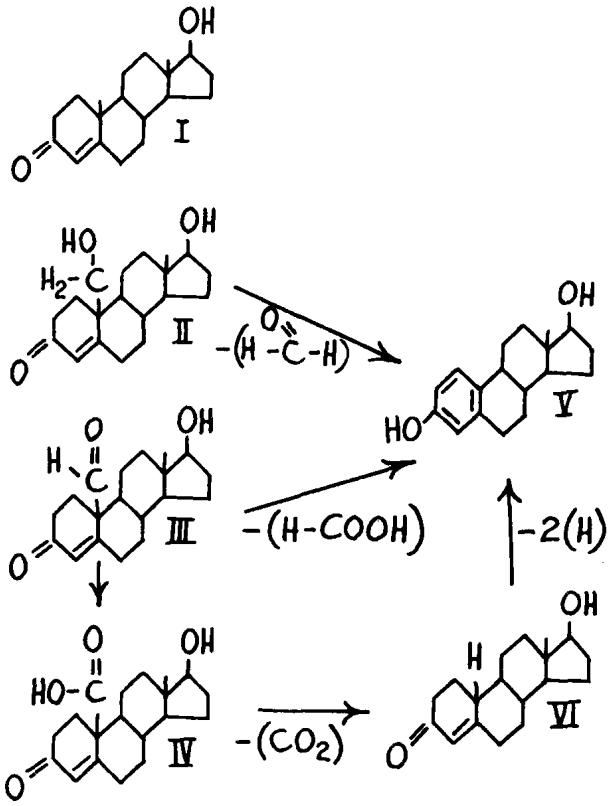

Fig. 1-1. Biosynthesis of Estrogens

I. Testosterone
II. 19-Hydroxytestosterone
III. 19-Oxotestosterone
IV. 10-Carboxy-19-nortestosterone
V. Estradiol-17β
VI. 19-Nortestosterone

insignificant weight but significant number of counts of estrone-6,7-H^3 and estradiol-17β,6,7-H^3, was extracted with a mixture of chloroform: ether (3:1). The residue, after solvent distillation, was dissolved in 70 per cent methanol, placed in a deep freeze overnight, centrifuged, and the supernatant partitioned against petroleum ether. The phenolic material of the 70 per cent methanol fraction was subjected to paper chromatography in the Bush B3 system. The zones containing estrone and estradiol-17β were rechromatographed in the Bush BA and B1 systems, respectively. The quantity of estrogens was determined by a fluorescence method, and the radioactivity (for recovery correction) in a scintillation counter.

The recovery of estradiol-17β and estrone was 62 per cent on a weight basis and 66 per cent on the basis of H^3. The sensitivity of the method using 50 ml of plasma was of the order of 0.002 μg of each of the estrogens.

In a woman studied just at the end of the menstrual flow and at the mid-cycle, about three times as much estrogen was found at the later time (Table 1-3). The mean estrone values for men and women were of the same order in our small number of subjects, but the mean value of 0.031 μg for estradiol-17β for women was considerably higher than the mean value of 0.003 μg for the men. Svendsen [17] reported on

Table 1-3. The Concentration of Estrone and Estradiol-17β in Various Plasmas

Subjects or patients	Age (range)	No. of subjects	μg/100 ml.	
			Estrone	Estradiol-17β
Normal women	18-33	4	0.015 (0.008-0.028)	0.031 (0.013-0.056)
Normal women			0.106 mid-cycle	0.023
			0.024 (day of end of menstrual flow)	0.009
Normal men	22-28	5	0.023 (0.010-0.036)	0.003 (<0.002-0.006)
Pregnancy 7th Month		1	1.03	0.55
9th Month		1	0.57	0.98
Granulosa cell tumor	30	1	0.33-0.37	0.18-0.19
Adrenal adenoma			0.128	0.076
Hirsutism Polycystic ovaries		7	0.070 (0.004-0.134)	0.021 (0.015-0.044)
Steer adrenal vein plasma (lyophilized)		2	0.100	0.028
			0.020	0.032
Women with ovarian dermoid cyst			0.998	0.085
Adrenal adenoma			0.035 before surgery	0.012
			0.005 after surgery	0.004

estrone and estradiol-17β titers in the plasma of normal women by a double isotope method employing S^{35} and I^{131}. These investigators found slightly more estradiol-17β than estrone and the mean of five samples was 0.024 μg of estrone and 0.037 μg of estradiol-17β. On a preliminary basis there appears to be a reasonable correspondence between the two methods.

The expected increases in both estrogens were found in the two pregnancy samples and on two samples from a patient who had a granulosa cell tumor.

Steer adrenal vein plasma from two different animals indicated estrone at the levels of 0.100 and 0.020 μg, and estradiol-17β was present at the 0.028 and 0.032 μg levels.

Two adrenal adenoma patients were studied, one with an elevated value for the estrogens and a second with normal values. The one patient with values in the normal range showed a drop from 0.035 to 0.005 μg per 100 ml plasma for estrone and a decrease from 0.012 to 0.004 μg for estradiol-17β after removal of the adenoma.

Seven subjects with hirsutism due to polycystic ovaries had a mean estradiol-17β concentration of 0.021 μg per 100 ml of plasma (range 0.015-0.044) which did not seem to vary significantly from that found for the limited observations for normal women, but the estrone value of 0.070 μg per 100 ml plasma for the hirsute women appeared to be significantly higher than that for normal women.

SUMMARY

The bulk, if not all, of estrogen biosynthesis proceeds through a route involving grossly acetate → cholesterol → androgen → estrogen. If testosterone is the example of the androgen, then the following pathways are indicated: testosterone → 19-hydroxytestosterone → estradiol-17β + formaldehyde; 19 hydroxytestosterone → 19-oxotestosterone → estradiol-17β + formic acid; 19 oxotestosterone → 10-carboxy-19-nortestosterone → 19-nortestosterone → estradiol-17β.

Estrone and estradiol-17β may be determined with a sensitivity of 0.002 μg using a combination of paper chromatographic, phosphoric acid fluorescence, and radioisotopic labeled estrogens for recovery correction. The same sensitivity may be attained using a double isotope method. The estrone and estradiol-17β concentration of plasma of normal menstruating women was 0.015 μg (0.008-0.028) and 0.031 μg (0.013-0.056) per 100 ml, while the concentration of these steroids in men's plasma was 0.023 μg (0.010-0.036) and 0.003 μg (<0.002-0.006) per 100 ml, respectively.

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