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The *in vitro* Conversion of Dehydroepiandrosterone-4-C¹⁴ to Estrogens by Ovarian Tissue*

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After the incubation of equine ovarian follicular tissue with dehydroepiandrosterone-4-C¹⁴, radioactive estradiol, estrone, androstenedione, and testosterone were isolated and identified by the criteria of paper chromatography and recrystallization to constant specific activity. These results provide additional evidence for the existence of an alternate pathway for estrogen biosynthesis from pregnenolone through dehydroepiandrosterone with androstenedione and testosterone as intermediates.

Considerable evidence has accumulated to indicate that the same precursors are utilized in the biosynthesis of estrogens as for adrenal and testicular steroids (Ryan, 1959; Ryan and Smith, 1961; Solomon *et al.*, 1956; Sweat *et al.*, 1960). On the basis of this evidence the following partial biosynthetic pathway for estrogens has been proposed: Pregnenolone → progesterone → 17-hydroxyprogesterone → Δ^4 -androstenedione → estrogens.¹ With the isolation of dehydroepiandrosterone from gonadal tissue, Neher and Wettstein (1960) proposed an alternate pathway from pregnenolone → 17-hydroxypregnenolone → dehydroepiandrosterone → androstenedione. The recent demonstration by Ryan and Smith (1961) that acetate-1-C¹⁴ can be converted to 17-hydroxypregnenolone and dehydroepiandrosterone by ovarian tissue, and the isolation of dehydroepi-

androsterone from ovarian follicular fluid by Short (1961), suggest that this latter route might play a more important role in estrogen synthesis than was previously thought to be the case.

In the present study the conversion of dehydroepiandrosterone-C¹⁴ to estradiol and estrone in excellent yields by equine ovarian follicular tissue *in vitro* has been demonstrated. Testosterone and androstenedione were also isolated and identified as radioactive metabolites.

¹ The following trivial names for steroids are used in this manuscript: androstenedione = 4-androsten-3,17-dione; 19-hydroxyandrostenedione = 4-androsten-19-ol-3,17-dione; dehydroepiandrosterone = 5-androsten-3 β -ol-17-one; estradiol (E₂) = 1,3,5-estratrien-3,17 β -diol; estriol (E₃) = 1,3,5-estratrien-3,16 α ,17 β -triol; estrone (E₁) = 1,3,5-estratrien-3-ol-17-one; progesterone = 4-pregnen-3,20-dione; 17-hydroxyprogesterone = 4-pregnen-17 α -ol-3,20-dione; pregnenolone = 5-pregnen-3 β -ol-20-one; 17-hydroxypregnenolone = 5-pregnen-3 β ,17 α -ol-20-one; testosterone = 4-androsten-17 β -ol-3-one.

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METHODS

Incubation.—Ovaries were obtained from non-pregnant mares at the abattoir and transported on ice to the laboratory within 15 minutes after death. Large follicles were removed and dissected free of stromal tissue, and the fluid was evacuated. The follicle walls were sliced into pieces approximately 3 mm², and 18 g were added to 40 ml of Kreb's phosphosaline buffer, pH 7.4. Sixty-six µg of dehydroepiandrosterone-4-C¹⁴ (1.85×10^6 dpm) in 0.2 ml of propylene glycol were added, and the mixture was incubated under air at 37° for 4 hours with moderate shaking.

Countercurrent Distribution.—To separate the neutral and phenolic fractions, countercurrent distribution was carried out as described by Baggett *et al.* (1956).

Paper Chromatography.—Paper chromatography was carried out by the methods of Bush (1952) and Zaffaroni (1958). Phenolic materials on the paper chromatograms were detected by the method of Barton *et al.* (1952). Androstenedione and testosterone were visualized on paper strips by means of an ultraviolet scanner. Dehydroepiandrosterone was observed as an orange spot when reacted with a mixture of concentrated H₂SO₄ and ethanol (1:1 v/v).

Measurement of Radioactivity.—Radioactive steroids were detected on paper strips by scanning with an Atomic Accessories Scanogram II. Carbon-14 was measured quantitatively either by liquid scintillation counting with the Packard Tricarb or by solid plate counting with the Nuclear-Chicago Counter, Model C115, with an automatic sample changer and a guard tube for low background counting. The solution used for liquid scintillation contained 4 g of 2,5-diphenyloxazole and 50 mg of 1,4-bis-2-(5-phenyloxazolyl)-benzene per liter of toluene. In liquid scintillation counting an endogenous standard was added so that the radioactivity of each sample was easily expressed as dpm. Solid plate counting was carried out at infinite thinness on copper planchets with a background of less than 3 cpm and an efficiency of 31%. In these latter cases the radioactivity of each sample was expressed in terms of cpm.

Acetylation.—Steroid samples were acetylated with acetic anhydride-pyridine (1:2 v/v) at room temperature in the dark overnight.

Steroid Standards.—All steroid standards used for paper chromatography and added as non-radioactive carrier for crystallization to constant specific activity were obtained from commercial sources. Prior to use they were crystallized at least twice from different solvent systems, and their purity was tested by paper chromatography and by determination of melting point.

EXPERIMENTAL PROCEDURE AND RESULTS

Extraction and Separation into Neutral and Phenolic Fractions.—After incubation the flask

contents were extracted six times with three volumes of chloroform. The pooled chloroform extracts were washed once with 30 ml of distilled H₂O and dried in vacuum. The dried extract was defatted by partitioning between pentane and 90% methanol and separated into neutral and phenolic fractions by partitioning between toluene and 1.0 N NaOH. The neutral fraction contained 566,200 dpm C¹⁴ (30.2% of the dehydroepiandrosterone-C¹⁴) and the phenolic fraction contained 560,800 dpm C¹⁴ (29.9% of the dehydroepiandrosterone-C¹⁴). There were 129,000 dpm C¹⁴ (6.9% of the dehydroepiandrosterone-C¹⁴) left in the residual incubation medium.

Phenolic Fraction.—To obtain better separation of phenols from neutral compounds the crude phenolic fraction was subjected to countercurrent distribution between 1.0 N NaOH and toluene with 14 transfers. As shown in Figure 1, 80% of the radioactivity was found in the first five tubes, with tube #0 having the greatest amount, decreasing progressively to tube #4. The contents of the first five countercurrent tubes were analyzed separately by paper chromatography in isooctane-toluene (25:75 v/v)/methanol-water (80:20 v/v) with estrone, estradiol, and estriol external standards. The chromatograms from countercurrent tubes 0-3 showed three separate peaks of radioactivity, corresponding to estrone (R_F 0.76), estradiol-17β (R_F 0.41), and an origin peak. Countercurrent tube 4 had only two radioactive peaks of low activity, one corresponding to estrone and the other to the origin material.

Identification of Estradiol-17β.—The "estradiol" peaks were eluted separately, dried, and submitted to a second paper chromatography in benzene-formamide. In each instance a single peak of radioactivity was obtained which had the same mobility as estradiol (R_F 0.16). A total of 162,805 dpm was accounted for in these "estradiol" peaks after two chromatographies.

Half of the "estradiol" peak originating from countercurrent tube 0 was chromatographed on a 0.5 × 2.0 cm column of Woelm's Grade I alumina. The radioactive sample was applied to the column in benzene and eluted with 3% ethanol in benzene. Nonradioactive estradiol (m.p. 178-179°) was added to an aliquot of the radioactive sample from alumina chromatography (14,366 dpm C¹⁴). Three successive crystallizations were carried out in different solvent systems with no significant alteration in specific activity of either the crystals or the material in the mother liquor (Table I).

The other half of the "estradiol" peak originating from countercurrent tube 0 was acetylated and chromatographed in hexane-formamide. A radioactive peak corresponding to estradiol diacetate (R_F 0.87) was obtained. The "estradiol diacetate" peak was then chromatographed on alumina as described previously. The sample was applied to the column in a solution of hexane and eluted with 5% methanol in hexane. Non-radioactive estradiol diacetate (m.p. 126-128°)

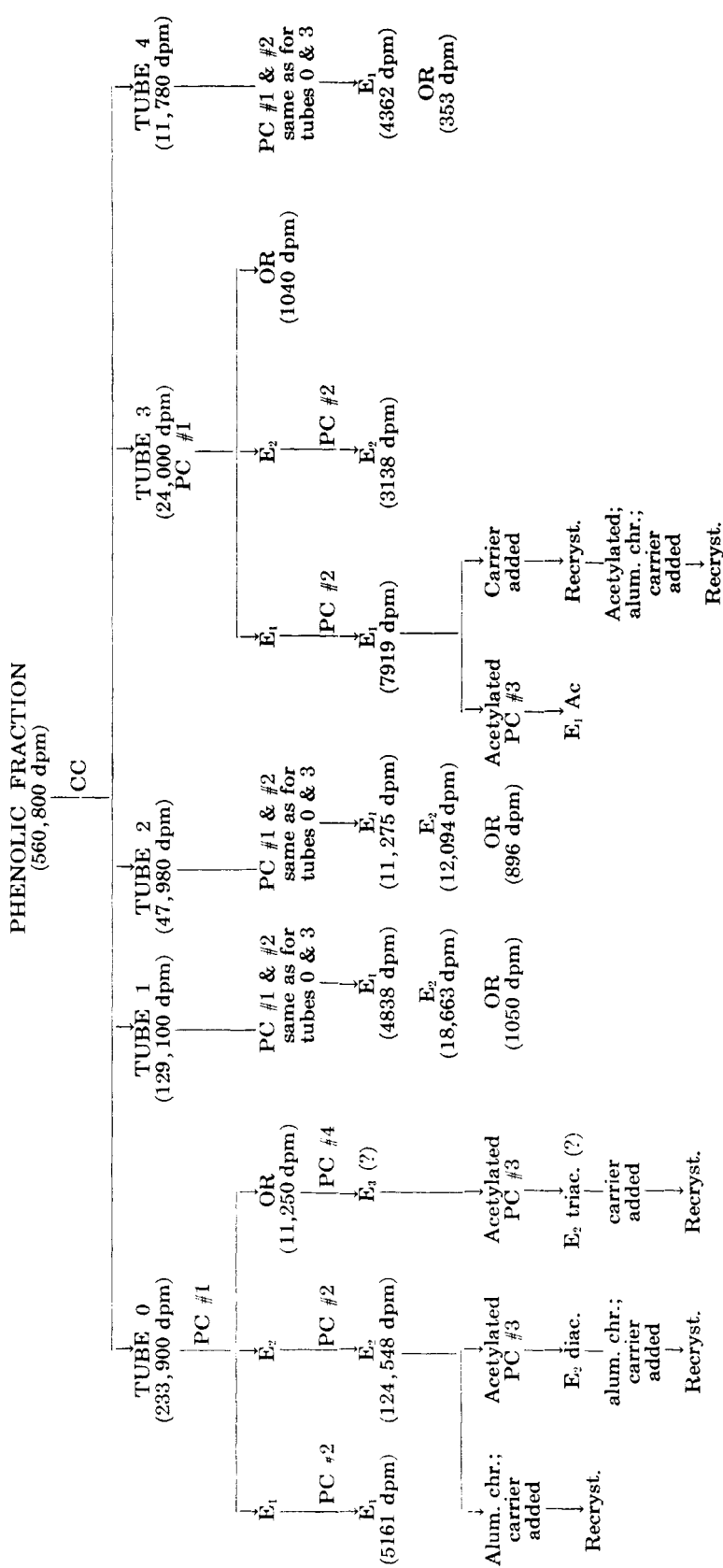


FIG. 1.—Outline of method of analysis for the phenolic fraction. CC = Counter-current distribution in toluene-1.0 N NaOH with 14 transfers. PC #1 = Paper chromatography in isooctane-toluene (25:75 v/v)/methanol-H₂O (80:20 v/v). PC #2 = Paper chromatography in benzene-formamide. PC #3 = Paper chromatography in hexane-formamide. PC #4 = Paper chromatography in CHCl₃-formamide. Alum. Chr. = Alumina chromatography.

TABLE I
 CRYSTALLIZATION OF PHENOLIC METABOLITES TO CONSTANT SPECIFIC ACTIVITY

Substance	Solvent for Crystallization	Specific Activity (cpm/mg)
A. Estradiol		
1. Pool ^a		694
2. First crystallization	Aqueous ethanol	662
Mother liquor		688
3. Second crystallization	CHCl ₃ /cyclohexane	628
Mother liquor		680
4. Third crystallization	Aqueous acetone	623
Mother liquor		644
B. Estradiol diacetate		
1. Pool ^a		87
2. First crystallization	Aqueous ethanol	90
3. Second crystallization	Aqueous acetone	85
C. Estrone		
1. Pool ^a		184
2. First crystallization	Aqueous ethanol	158
3. Second crystallization	CHCl ₃ /cyclohexane	142
4. Third crystallization	Aqueous acetone	145
D. Estrone acetate		
1. Pool ^a		44
2. First crystallization	Aqueous ethanol	46
Mother liquor		51
3. Second crystallization	Aqueous acetone	43
Mother liquor		55
4. Third crystallization	Aqueous ethanol	45
Mother liquor		43

^a Radioactive sample + nonradioactive carrier.

was added to a fraction from alumina chromatography and the mixture was recrystallized to constant specific activity as shown in Table I.

Identification of Estrone.—The radioactive material corresponding to estrone from the first paper chromatography for each of the countercurrent tubes 0–4 was eluted and rechromatographed separately in benzene-formamide (Fig. 1). Each chromatogram had a single radioactive peak identical in mobility with the external estrone standard. The sum of the radioactivity in the estrone peaks in the five chromatograms amounted to 33,555 dpm C¹⁴.

The aliquot of the "estrone" peak originating from countercurrent tube 3 was acetylated and chromatographed on paper in hexane-formamide. A single radioactive peak identical in mobility to estrone acetate was obtained.

Nonradioactive estrone (m.p. 255–257°) was added to a second aliquot of the "estrone" peak from the second paper chromatography, and three successive crystallizations to constant specific activity in different solvents were carried out as indicated in Table I. The crystals from the final crystallization from aqueous acetone were acetylated in the usual manner and chromatographed on alumina. The sample was applied to the column in hexane and eluted with 5% methanol in hexane. Authentic estrone acetate (m.p. 126–127°) was added to the radioactive eluate and the mixture crystallized to constant specific activity (Table I).

Attempt to Identify Estriol.—As shown in Figure 1, each of the first five tubes from the countercurrent distribution contained a highly polar radioactive phenolic material which remained at the origin on paper chromatography in isooctane-toluene (25:75 v/v)/MeOH-H₂O (80:20 v/v). In this solvent system estriol has an R_F of 0.03, and it could have been in this origin material. To investigate this possibility the origin material from countercurrent tube 0 (11,250 dpm C¹⁴) was eluted and rerun on paper in CHCl₃-formamide. Two radioactive peaks, one staying at the origin and one moving identically with the estriol standard, were observed. The "estriol" peak was acetylated and rerun in hexane-formamide. A single radioactive peak was observed which corresponded to the estriol triacetate standard. To this radioactive material nonradioactive estriol triacetate (m.p. = 128–130°) was added. The pooled material weighed 33.2 mg, with a total of 1726 dpm C¹⁴. With the first crystallization from heptane the specific activity fell from 52 to 21 dpm/mg. The first crop of crystals was recrystallized from 80% methanol, and the specific activity fell further to 12 dpm/mg. A third successive crystallization was carried out from aqueous acetone, and the specific activity remained at 13 dpm/mg. There was insufficient material for further identification.

Neutral Fraction.—The neutral fraction (566,200 dpm C¹⁴) was first chromatographed on paper in hexane-benzene (1:1 v/v), formamide

TABLE II
CRYSTALLIZATION OF NEUTRAL METABOLITES TO CONSTANT SPECIFIC ACTIVITY

Substance	Solvent for Crystallization	Specific Activity (dpm/mg)
A. Testosterone acetate		
1. Pool ^a	None	435
2. First crystallization	Aqueous ethanol	387
3. Second crystallization	Aqueous acetone	392
4. Third crystallization	Pentane	426
B. Δ^4 -Androstenedione		
1. Pool ^a	None	2552
2. First crystallization	Aqueous methanol	2648
Mother liquor		2766
3. Second crystallization	Aqueous acetone	2306
Mother liquor		2657
4. Third crystallization	Hexane	2543
Mother liquor		2640

^a Radioactive sample + nonradioactive carrier.

with testosterone, androstenedione, and dehydroepiandrosterone as exogenous standards. Scanning revealed four radioactive peaks: A small one at the origin, a second peak with an R_F of 0.4 (the same as the testosterone), a third peak corresponding to dehydroepiandrosterone (R_F of 0.52), and a fourth peak identical with androstenedione (R_F 0.66).

Identification of Testosterone.—The "testosterone" peak from the first paper chromatography of the neutral fraction was eluted, and paper chromatography in hexane-benzene (1:1 v/v)/formamide was repeated after the addition of 100 μ g of non-radioactive testosterone to permit visualization with an ultraviolet scanner. A single radioactive peak was obtained which coincided with the area of ultraviolet absorption. The area of radioactivity was eluted and acetylated. The entire acetylated sample was then chromatographed on paper in hexane-formamide. A single radioactive peak with an R_F of 0.65 which absorbed ultraviolet light was observed.

The radioactive sample was then crystallized to constant specific activity in three different solvent systems after the addition of nonradioactive testosterone acetate (Table II).

Identification of Dehydroepiandrosterone.—The "dehydroepiandrosterone" peak from the first paper chromatography of the neutral fraction was acetylated and subjected to two additional paper chromatographies as shown in Figure 2. Each time a single radioactive peak was obtained, corresponding with the exogenous dehydroepiandrosterone acetate standard.

Identification of Δ^4 -Androstenedione.—The radioactive peak from the first paper chromatogram, which appeared to be androstenedione, was acetylated and submitted to paper chromatography again in hexane-benzene (1:1 v/v)/formamide. Scanning revealed a single radioactive peak with the same R_F as the exogenous androstenedione standard (0.68). Two more successive paper

chromatographies in different solvent systems revealed a single radioactive compound which behaved like androstenedione. Nonradioactive androstenedione (m.p. 175–176°) was then added, and crystallization from three different solvent systems was carried out. As shown in Table II, there was no significant alteration in specific activity in either the crystals or the material in the mother liquors throughout this procedure.

DISCUSSION

In this study the *in vitro* conversion of dehydroepiandrosterone to estrone, estradiol, androstenedione, and testosterone by equine ovarian follicular tissue has been demonstrated by paper chromatography and by crystallization to constant specific activity. In Table III an attempt has been made to quantitate the per cent conversion of dehydroepiandrosterone to these products. To compensate for losses in purification, per cent conversion was estimated by determining the relative areas under the radioactive peaks on the first paper chromatograms of the neutral and phenolic fractions respectively.

Estradiol was the major metabolic product, comprising 23.5% of the starting material and exceeding the amount of estrone formed by a factor of 4. This observation is compatible with the concept that estradiol is the primary hormone produced by the ovarian follicle.

Recently there has been a renewed interest in the possible synthesis of estriol by ovarian tissue (Wotiz *et al.*, 1956; Warren and Salhanick, 1961; Ryan and Smith, 1961; Zander, 1958; Short, 1960). In this study an attempt was made to isolate estriol. A phenolic compound was obtained which chromatographed like estriol in both the free and acetylated forms. However, the specific activity fell sharply upon recrystallization, indicating that estriol was not present in

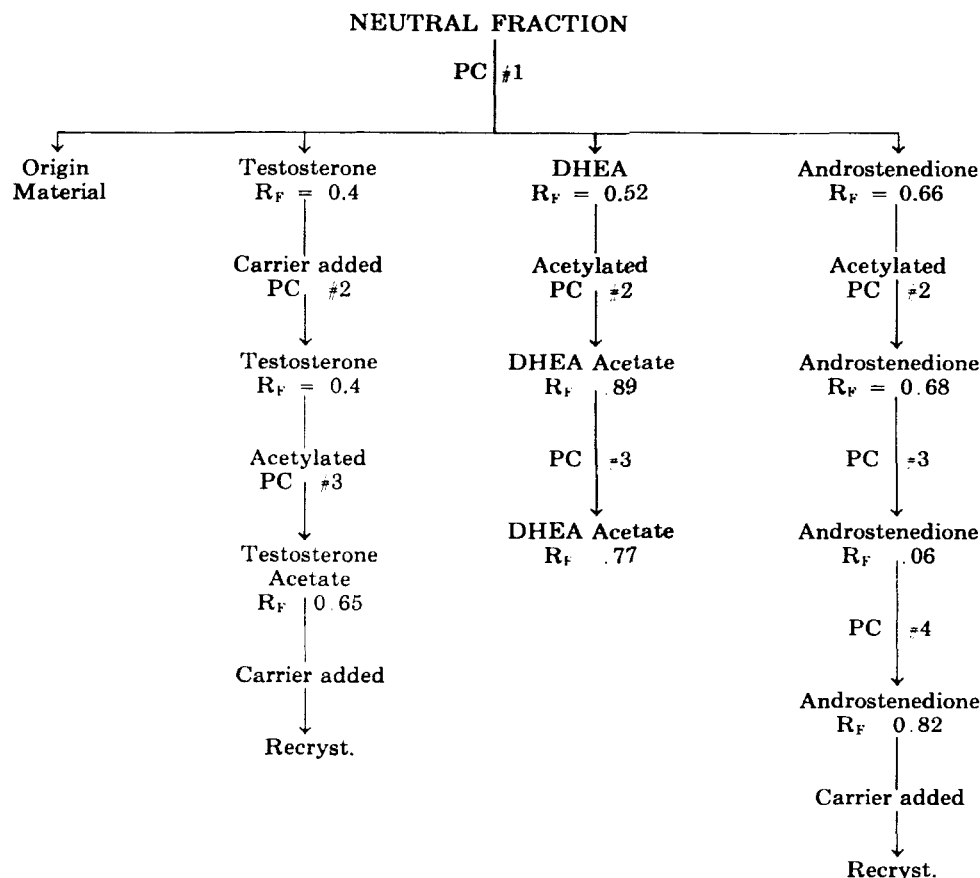


FIG. 2.—Outline of method of analyses for the neutral fraction. PC #1 = Paper chromatography in hexane-benzene (1:1 v/v)-formamide. PC #2 = Paper chromatography in hexane-benzene (1:1 v/v)-formamide. PC #3 = Paper chromatography in hexane-formamide. PC #4 = Paper chromatography in benzene-formamide. DHEA = Dehydroepiandrosterone.

TABLE III
DISTRIBUTION OF RADIOACTIVITY AFTER INCUBATION
OF DEHYDROEPIANDROSTERONE- C^{14} WITH OVARIAN
TISSUE

	% Conversion from Dehydroepi- androsterone- C^{14}
Phenolic fraction	29.9
Estradiol-17 β	23.5
Estrone	5.5
Origin material	0.9
Neutral fraction	30.2
Δ^4 -Androstenedione	15.0
Origin material	7.3
Testosterone	5.4
Dehydroepiandrosterone	2.5

significant amounts in spite of the chromatographic evidence.

Possible pathways for the conversion of dehydroepiandrosterone to estrogens are outlined in Figure 3. Because androstenedione has been isolated from normal ovarian tissue and after incubation with a number of estrogen precursors, it has been generally accepted as an intermediate in

estrogen biosynthesis. Testosterone has not enjoyed the same acceptance, even though its conversion to estrogen by ovarian tissue is well established (Baggett *et al.*, 1956; Wotiz *et al.*, 1956). There are two principal reasons for this: (1) Testosterone has never been isolated from normal ovarian tissue, and (2) several investigators have failed to isolate testosterone from ovarian tissue incubations with a variety of estrogen precursors. A limited review of the literature reveals that only one other laboratory has reported the synthesis of testosterone by normal ovarian tissue (Axelrod and Goldzieher, 1961). It has been demonstrated that neoplastic and polycystic ovarian tissue from patients with virilization can produce testosterone (Savard *et al.*, 1961; O'Donnell and McCaig, 1959).

Androstenedione is usually regarded as an obligatory intermediate in estrogen biosynthesis, with subsequent aromatization to estrone, which in turn is reduced to estradiol by a 17 β -estradiol dehydrogenase. Since ovarian tissue has been shown to possess both a nonspecific 17 β -hydroxysteroid dehydrogenase as well as a relatively specific 17 β -estradiol dehydrogenase, estradiol could be produced by the aromatization of testo-

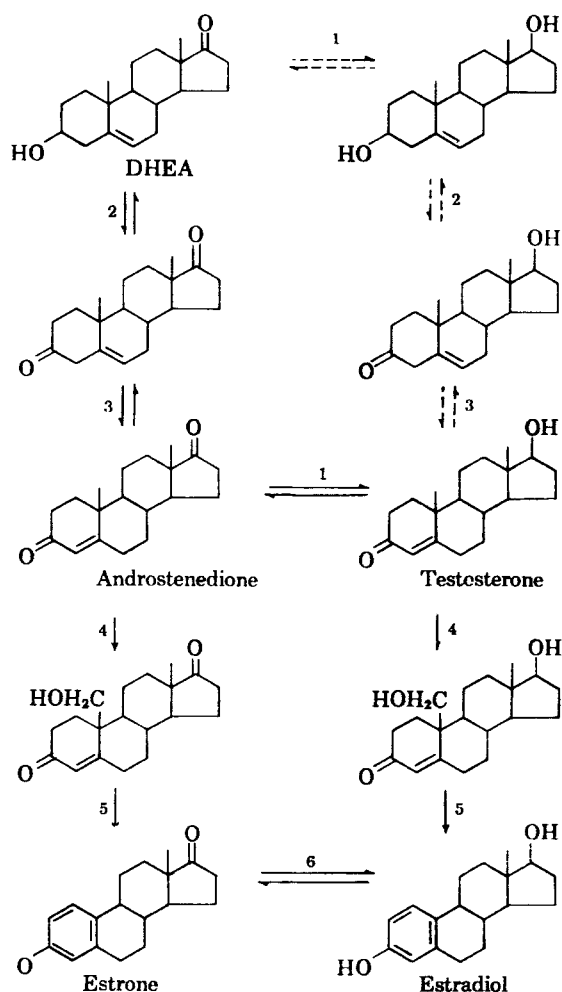


FIG. 3.—Enzyme systems are numbered as follows: (1) 17 β -hydroxysteroid dehydrogenase, (2) 3 β -hydroxysteroid dehydrogenase, (3) Δ^4 -steroid isomerase, (4) 19-hydroxylase, (5) unknown systems for aromatization of ring A, and (6) 17 β -estradiol dehydrogenase. Established reactions are indicated by solid arrows and possible but unestablished reactions by dotted arrows.

sterone without going through estrone. Testosterone could arise from the reduction of androstenedione by the 17 β -hydroxysteroid dehydrogenase, or androstenedione could be bypassed entirely if the 17-ketone of dehydroepiandrosterone were reduced by the 17 β -hydroxysteroid dehydrogenase before the 3-hydroxyl group was oxidized (Fig. 3).

There is good evidence that androstenedione can be aromatized by ovarian tissue, and 19-hydroxyandrostenedione has been shown to be an intermediate. Ryan (1959) has demonstrated

that the aromatization of testosterone can be carried out by placental tissue, although no intermediates were identified. He found that if placental microsomes were washed well prior to incubation only estradiol could be obtained from testosterone and estrone from androstenedione.

Since hydroxylation of C-19 is probably the first step in aromatization and is irreversible (Samuels, 1960), a highly active 19-hydroxylase is probably an absolute requirement for estrogen biosynthesis. Whether it proceeds via androstenedione or testosterone would depend primarily upon the relative activities of the 19-hydroxylase and the 17 β -hydroxysteroid dehydrogenase systems. In this regard, substrate specificity would be a factor as well as the relative concentrations of reduced and oxidized pyridine nucleotides, which have been shown to be co-factors in these oxidation reduction reactions.

In the authors' opinion there is insufficient evidence at the present time for deciding which of the alternate pathways is operating in estrogen biosynthesis by ovarian tissue, and all must be considered until further experimentation clarifies the situation.

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