Studies on the Mechanism of Estrogen Biosynthesis. III. The Stereochemistry of Aromatization of C$_{19}$ and C$_{18}$ Steroids*

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ABSTRACT: To elucidate the mechanism for oxidative attack in ring A resulting in the production of estrogens, the stereochemistry and cofactor requirements for aromatization of estr-4-ene-3,17-dione (I) were determined and compared with the aromatization characteristics of androst-4-ene-3,17-dione (II). Incubations of [4-^{14}C]II stereoselectively tritiated at C-1β or C-1α with the 10,000*g* supernatant of human term placenta and a reduced nicotinamide–adenine dinucleotide phosphate generating system showed that the 1β-hydrogen was lost in the conversion to estrone. Incubation of [4-^{14}C-1-^{3}H (83% β)]I under similar conditions yielded estrone in which 82% of the tritium had been lost indicating that the 1β-hydrogen is eliminated in this case also and showing that the loss is not dependent on the presence of the C-10 methyl group. Reduced nicotinamide–adenine dinucleotide phosphate and O$_2$ were shown to be obligatory cofactors for aromatization of I as is the case with C$_{19}$ steroids. These results suggest that C$_{18}$ and C$_{19}$ steroids are converted to estrogens in a similar way with regard to desaturation in ring A. Possible mechanisms are discussed. Using washed placental microsomes suspended in 0.05 M phosphate buffer at pH 7.2, 5 mM EDTA stimulated the conversion of 19-norsteroid to estrone and 1β-hydroxyestr-4-ene-3,17-dione by 50%.

The conversion of androstenedione[1] or testosterone to estrogens by human placental preparations requires two general steps: (1) hydroxylation and eventual elimination of the C-10 methyl group, and (2) oxidative attack resulting in double-bond introduction in ring A (Talalay, 1965). The available evidence suggests step 2 occurs after C-19 hydroxylation and before or during cleavage of the C-10,19 bond. No potential intermediate beyond 19-oxoandrostenedione has been isolated, and it is not known how desaturation occurs in ring A to produce estrogen. In a study to elucidate the events occurring in ring A, leading to aromatization, we found evidence, presented in a preliminary communication, that the 1β-equatorial hydrogen is removed. This suggested enzymatic participation or steric interaction of the C-19 group at C-1β (Morato *et al.*, 1962). 19-Nor steroids also serve as estrogen precursors in placental and ovarian preparations (Townsley and Brodie, 1966, 1967) and the absence of the C-19 methyl group makes them useful model compounds for the study of the aromatization reactions in ring A if similar mechanisms are involved. In addition to mechanistic studies, the biochemistry of 19-nor compounds is of interest in itself. They are widely used for contraceptive and chemotherapeutic purposes and are reported to be formed and aromatized in human prostate tissue *in vitro* (Farnsworth, 1966) as well as being naturally occurring in mare follicular fluid (Short, 1960).

This paper presents results of an investigation of the placental metabolism of 19-norandrostenedione, including the stereochemical and cofactor requirements for aromatization, as well as a more detailed study of the stereochemistry of aromatization of androstenedione. The relevance of the data obtained to an understanding of the mechanism of estrogen formation is discussed.

Experimental Section

Materials. [1-^{3}H (83% β)]19-Norandrostenedione, [1-^{3}H (83% β)]androstenedione (Brodie, 1967), and [1-^{3}H (93% α)]androstenedione (Brodie *et al.*, 1962) were prepared as reported. [4-^{14}C]19-Nortestosterone acetate, [4-^{14}C]androstenedione, [6,7-^{3}H]estrone, and [6,7^3-H]estradiol-17β were obtained from New England Nuclear Corp. and their purity was established by chromatography on paper and thin-layer silica gel. [4-^{14}C]19-Norandrostenedione was prepared from [4-^{14}C]19-nortestosterone acetate by refluxing for 1 hr in 2% KOH in methanol–water (1:1, v/v) followed by chromic acid oxidation in acetone–sulfuric acid (Bowers *et al.*, 1953). NADPH, NADP, NAD, NADH, glu-

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[1] Abbreviations used: androstenedione, androst-4-ene-3,17-dione; 19-hydroxyandrostenedione, 19-hydroxyandrost-4-ene-3,17-dione; 1β-hydroxy-19-norandrostenedione, 1β-hydroxyestr-4-ene-3,17-dione; NAD, oxidized nicotinamide–adenine dinucleotide; NADH, reduced NAD; NADP, oxidized nicotinamide–adenine dinucleotide phosphate; NADPH, reduced NADP; 19-norandrostenedione, estr-4-ene-3,17-dione; 19-nortestosterone, 17β-hydroxyestr-4-en-3-one; 19-nortestosterone acetate, 17β-acetoxyestr-4-en-3-one; 19-oxoandrostenedione, 19-oxoandrost-4-ene-3,17-dione; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis[2-(5-phenyloxazolyl)]benzene; ATP, adenosine triphosphate.

cose 6-phosphate, glucose 6-phosphate dehydrogenase (Sigma), and menadione (Hoffmann-LaRoche, Nutley, N. J.) were used as received. Phenazine methosulfate (Sigma) was recrystallized from ethanol before use.

Chromatography. Systems (v/v) used were (1) paper: ligroin (bp 60–90°)–benzene–methanol–water, Bush B_3 (6.67:3.33:4:1), modified Bush B_3 (4:1:4:1); Bush A, ligroin–methanol–water (5:4:1) (Bush, 1952); ligroin–propylene glycol (Savard, 1954); toluene–propylene glycol (Zaffaroni, 1953); and (2) thin-layer chromatography: ether–hexane, system I (13:7), system Ia (1:1), system Ib (3:1); benzene–ethyl acetate, system II (4:1), system IIa (83:17), system IIb (93:7).

Paper chromatography was carried out on Whatman No. 1 grade. Paper involved in fluorometric estimations was prewashed by descending chromatography in methanol for 48 hr and methanol–benzene (1:1, v/v) for 24 hr. Silica gel GF_{254} (Brinkmann) was used for the preparation of thin layers (250 μ) on glass plates. To improve recovery of estrogens, the silica gel was slurried once with ethyl acetate and air dried prior to plate preparation (R. B. Wilcox, personal communication). Standards were located with ultraviolet light (Δ^4-3-one), with iodine vapor (estrogens on thin-layer plates) or by Turnbull's blue reagent (Barton *et al.*, 1952) (estrogens on paper). Radioactivity was located by use of a Vanguard 880 chromatogram scanner fitted with a plate-scanning attachment, Model 885.

Quantification. Measurements of radioactivity were carried out in 10 ml of scintillation fluid by conventional single-label or double-label (Okita *et al.*, 1957) counting in a Packard Tri-Carb Model 314 EX. The scintillation mixture consisted of 4 g of PPO and 116 mg of POPOP (Pilot) in 1 kg of Spectrograde toluene (Matheson). Counter characteristics were: ^{3}H, efficiency, 17.0%, background 23.7 cpm; contribution to ^{14}C channel 0.88/10^3 ^{3}H counts; and ^{14}C, efficiency 37.7%, background 12.8 cpm; contribution to ^{3}H channel 177/10^3 ^{14}C counts. The counting error was 2% for ^{3}H and 5% for ^{14}C.

The mass of Δ^4-3-one substrates for specific activity determinations was measured in methanol using their extinction coefficient at 241 mμ in a Perkin-Elmer spectrophotometer, Model 202. The ^{3}H:^{14}C ratios were verified by crystallizations of diluted samples as shown in the appropriate tables. Estimation of mass of these and diluted, recrystallized products was carried out by direct weighing of approximately 200 μg on a Cahn electrobalance, Model M-10. Estimation of estrone from the experiment with tritiated 19-norandrostenedione was determined by fluorometry according to the general procedure of Bates and Cohen (1950) using an Aminco-Bowman spectrophotometer SPF.

Tissue Preparations. Human term placentas were processed according to the method of Ryan (1959) using deionized water adjusted to pH 7.2 with $NaHCO_3$ as a suspending medium. The subcellular fractions, 10,000*g* supernatants (30-min centrifugation), or microsomes (precipitated by 60-min centrifugation of the 10,000*g* supernatant at 104,000*g*) were stored at −15° until required. No loss of activity was noted over a period of several months. Concentrations of enzymes for incubations are given in enzyme tissue equivalents which are defined as the quantity of supernatants (12 ml) or microsomes obtained from 20 g wet wt of tissue.

Incubations. For standard incubations, the steroid (12–50 μg) in methanol together with 0.1 ml of propylene glycol was added to 50–100-ml erlenmeyer flasks and the methanol was evaporated. The microsomes, resuspended in 2.7 ml of medium, or the supernatants were added to each flask followed by a NADPH-generating system consisting of 3 μmoles of NADPH, 10 μmoles of glucose 6-phosphate, and 1 unit of glucose 6-phosphate dehydrogenase in 0.2 ml of medium per enzyme tissue equiv at pH 7.2. After incubation in air for 1 hr with shaking in a Dubnoff incubator at 37°, the reaction was stopped by the addition of ethyl acetate. The mixtures were extracted with ethyl acetate and the extract was chromatographed in the ligroin–propylene glycol system with a 3-hr overrun to remove nonpolar, nonradioactive impurities. The chromatograms were air dried briefly and redeveloped in the toluene–propylene glycol system. The radioactive 19-nortestosterone–estrone area was eluted and these materials were separated either by base partition between toluene and 1 N KOH followed by chromatography in the Bush B_3 or modified B_3 system or by thin-layer chromatography in system I or II. Other radioactive materials from the toluene–propylene glycol chromatograms were treated as detailed in the Results section.

Anaerobic incubations were carried out in Warburg flasks with 0.2 ml of alkaline pyrogallol in the center well absorbed on filter paper (40% KOH–25% pyrogallol, 10:1, v/v). The steroid and cofactors were placed in the sidearm and the suspended microsomes were placed in the main chamber. The flask was evacuated, flushed with oxygen-free nitrogen three times, and then sealed with screw clamps on rubber tubing attached to hollow stoppers in the sidearm and body of the flask. After mixing the components and incubating for 1 hr at 37°, the flask was placed in Dry Ice–acetone to freeze the contents. Ethyl acetate then was injected through the rubber tubing on the sidearm with a hypodermic syringe and the flask was stored at 4° overnight before exposing the contents to oxygen. Aerobic control experiments and aerobic incubations with other cofactors were carried out in a similar manner except for the nitrogen introduction step and the use of pyrogallol.

Estimation of Per Cent Conversion. The amounts of ^{14}C radioactivity in the estrone and 1β-hydroxy-19-norandrostenedione zones recovered from toluene–propylene glycol chromatograms were compared with the total amount of radioactivity elutable from the paper. Any contributions of 19-nortestosterone or testosterone to the estrone fraction, or estradiol-17β to the 1β-hydroxy-19-norandrostenedione fraction, were determined by subsequent thin-layer chromatography in system II or Ib, respectively (method A). For the cofactor experiments appropriate amounts of [6,7-^{3}H]estrone and [6,7-^{3}H]estradiol-17β were added to the ethyl acetate quenched incubation mixture after enzyme activity was destroyed (16 hr at 4°) and the fraction of tritium recovered after a given purification step was taken as a measure of recovery (method B). Both methods gave similar results.

Results

General Considerations. Preliminary experiments on the aromatization of 19-norandrostenedione resulted in much lower yields of estrogen than those obtained by others. Subsequently it was shown that previous yield values probably included significant contributions from another metabolite, 1β-hydroxy-19-norandrostenedione, which is converted to estrone under the base-partition purification procedures used (Townsley and Brodie, 1966). Because of the limited amount of 1β-tritiated 19-norandrostenedione substrate available, the incubation conditions were investigated in an attempt to improve the conversion. It was found that the supernatant, after 1-hr incubation, gave only one phenolic product, estrone, whereas the microsomes gave a slightly poorer yield of total estrogen which often contained significant amounts of both estrone and estradiol-17β. Use of the supernatant facilitated analysis since it did not necessitate subsequent chemical steps to convert the estrogens to one product. 19-Norandrostenedione appears to saturate the enzyme at a much lower concentration than does androstenedione and per cent conversion was markedly improved by using less substrate. Using 1 enzyme equiv of supernatant and 100 μg of 19-norandrostenedione only a 4.5% conversion was obtained, whereas with 12.5 μg of substrate a 24% conversion was realized. Later in the work it was noted that deionized water gave enzyme preparations of higher activity and the presence of EDTA also was beneficial. Duplicate incubations of 12 μg of [^{14}C]19-nortestosterone were carried out with a microsomal preparation prepared in deionized water and suspended in 0.05 M phosphate buffer in the presence and absence of 5 mM EDTA. The set without EDTA had conversions to estrone of 12.4 and 11.0%, whereas the set with EDTA had conversions of 17.5 and 17.8%, representing a 50% stimulation (estimation method B). This is in contrast to Ryan's experience with the C_{19} steroids where little effect on estrogen biosynthesis was noted with 1 mM EDTA.

No other variables tested were found to influence the per cent conversion. Microsomal preparations, prepared in distilled water and adjusted to pH 7.2 with $NaHCO_3$, were equally as active as those prepared using 0.05 M phosphate buffer alone or containing 0.25 M sucrose and 0.04 M nicotinamide. With the 10,000*g* supernatant prepared in phosphate–sucrose–nicotinamide medium, the addition of 10 μmoles of ATP and 5 μmoles of NAD was equally as effective as the standard concentration of exogenous NADPH-generating system for converting 19-nortestosterone, 19-norandrostenedione, and androstenedione to estrogen.

Incubations. C_{19} SUBSTRATES. The results of incubating [4-^{14}C-1-^{3}H (83% β)]androstenedione with the placental supernatant are given in Table I. After scanning the toluene–propylene glycol chromatogram for radioactivity, the area corresponding to estrone was eluted and yielded 74% of the original ^{14}C incubated. When an aliquot of the eluate was analyzed by thin-layer chromatography in system II, the radioactivity was found only in the estrone area and the ^{3}H:^{14}C ratio was 10.1:1, showing that 83% of the tritium was lost in this transformation (starting ratio, 59:1) and indicating that the 1β label is eliminated. Base partition of another aliquot followed by chroma-

TABLE I: Incubation of [4-^{14}C-1-^{3}H (83% β)]Androstenedione with Placental Supernatant.[a]

		Sp Act. (dpm/μmole)	
Compound	^{3}H:^{14}C (dpm)	^{3}H	^{14}C
1. Substrate	59	2.33×10^{6}	3.95×10^{4}
Diluted and recrystallized	62	8827	142
2nd crystallization	60	8576	143
Residue	60	8812	146
2. Crude incubation extract	17		
3. Estrone after 1st chromatogram	10.2		
4. Thin-layer chromatography of 3	10.1		
5. Base partition of 3	9.9		
6. Modified B_3 of 4	9.5		
7. Dilution and crystallization of 6	9.8	478	49
8. Acetylation of 7	9.6	411	43
9. CCD of 3			
Tube 47	9.6		
Tube 51 (peak)	9.5		
Tube 55	9.9		
10. Recovered substrate, 1st chromatogram	95		
11. Bush A of 10	76		
12. Thin-layer chromatography of 11	64		
13. Dilution and crystallization of 12	59		
Residue	69		
2nd crystallization	55	4577	83
Residue	64		
3rd crystallization	55	4542	83
Residue	54	4763	89

[a] Supernatant was prepared by homogenization in water. Each of 20 100-ml erlenmeyer flasks contained 1 enzyme tissue equiv of enzyme 56 μg of substrate, and the standard concentrations of the NADPH-generating system in a total volume of 12.3 ml.

tography in the modified B$_3$ system failed to change the ^{3}H:^{14}C ratio significantly. When a portion of the material with the mobility of estrone from the modified B$_3$ chromatography was diluted and crystallized from methanol a ratio of 9.8:1 was obtained which did not change significantly on mild acetylation (pyridine and acetic anhydride, overnight at room temperature) followed by thin-layer chromatography in system IIb. A 95-transfer countercurrent distribution of the base-partitioned material in carbon tetrachloride–methanol–water (20:14:6, v/v) (Gallagher *et al.*, 1958) gave a symmetrical radioactive peak with a K 1.16 value (theoretical value 1.25) corresponding to estrone. Aliquots of the combined phases in each tube were used to locate the radioactivity and, as shown in the table, the ^{3}H:^{14}C ratios in these samples agreed with those obtained after recrystallization to constant activity.

The radioactive material with R_F corresponding to unconverted androstenedione on the toluene–propylene glycol chromatogram was eluted (3250 dpm of ^{14}C, 2.1% recovery) and rechromatographed in the Bush A system. The radioactive zone corresponding to androstenedione was eluted and analyzed by thin-layer chromatography in system II. The ultraviolet-positive radioactive area which had the mobility of androstenedione was eluted, diluted with 5 mg of androstenedione, and recrystallized from benzene–hexane to constant specific activity. A value of 55:1 in ^{3}H:^{14}C ratio was obtained, which agrees closely with that of the starting material (59:1).

A similar incubation using [4-^{14}C-1-^{3}H (93% α)]-androstenedione as substrate gave estrone from the toluene–propylene glycol chromatogram with a ^{3}H:^{14}C ratio of 21.2:1, representing a 6% loss of tritium from this essentially 1α-labeled substrate (Table II). Further purification by base partition followed by chromatography in the modified Bush B$_3$ system did not change the ratio significantly. These steps had been shown to give a constant ratio for the incubation with the 1β-labeled substrate (Table I). Thus the 1α label is not removed in the transformation of estrone and the results together with those using the 1β-labeled material confirm the conclusion of our previous study with microsomes (Morato *et al.*, 1962) that the 1β-hydrogen is removed in the aromatization of androstenedione.

TABLE II: Incubation of [4-^{14}C-1-^{3}H (93% α)]Androstenedione with Placental Supernatant.[a]

	^{3}H:^{14}C (dpm)
1. Androstenedione (substrate)	22.5
Diluted and recrystallized	23.3
2. Estrone from toluene–propylene glycol	21.2
Estrone after base partition	20.9
Estrone after modified B$_3$ of 2	21.6

[a] Conditions for incubation were as given in footnotes to Table I except that each of ten flasks contained 47.9 μg of substrate, sp act. 1.92 × 10^{6} dpm/μmole, ^{3}H; 8.52 × 10^{4} dpm/μmole, ^{14}C.

19-NOR SUBSTRATES. Results of incubation of [4-^{14}C-1-^{3}H (83% β)]19-norandrostenedione are given in Table III. Analysis of the toluene–propylene glycol chromatogram of the incubation extract showed that the crude extract was separated into five radioactive zones. Three of these corresponded to 1β-hydroxy-19-

TABLE III: Incubation of [4-^{14}C-1-^{3}H (83% β)]19-Norandrostenedione with Placental Supernatant.[a]

Compound	^{3}H:^{14}C (dpm)	Sp Act. (dpm/μmole) ^{3}H	^{14}C
1. Substrate	59	4.66 × 10^{6}	7.9 × 10^{4}
Diluted and crystallized	60	5062	84
2nd crystallization	58	4959	86
3rd crystallization	59	4750	91
Residue	59	4994	85
2. Crude extract	41		
3. Materials from 1st chromatogram			
Zone 1 (51, 0.13)[b]	13		
Zone 2 (3, 0.21)	90		
Zone 3 (32, 0.38)	28		
Zone 4 (6, 0.48)	93		
Zone 5 (8, 1.0)	115		
4. Estrone, base partition of zone 3	10.2		
5. Modified B$_3$ of 4	10.9		
6. Thin-layer chromatography of 4	11.3		
7. Dilution and crystallization of 4	10.5	1346	127
2nd crystallization	10.6	1520	143
Residue	10.2	1518	148
8. Recovered substrate			
Dilution and crystallization	119	4175	35
Residue	97	4829	50
2nd crystallization	130	4015	31
Residue	117	4330	37
3rd crystallization	127	3935	31
Residue	123	4072	33
4th crystallization	126	3917	31
Residue	124	3967	32

[a] Conditions for incubation were as given in footnote to Table I except that each of 12 flasks contained 22.2 μg of substrate. [b] First figure is the per cent of ^{14}C in the zone relative to the total amount of ^{14}C eluted from the paper. Second figure is the mobility relative to 19-norandrostenedione.

norandrostenedione (zone 1), estrone (zone 3), and starting material (zone 5). Zone 3, after elution, was diluted with 100 μg of estrone and, following base partition, the base-soluble fraction was chromatographed in the modified Bush B_3 system. The single radioactive zone corresponding to estrone was eluted and the $^3H:^{14}C$ ratio was found to be 10.9:1. This ratio agreed closely with that obtained after the base partition (10.2:1) and also with values obtained when portions of the base-partitioned material was analyzed by thin-layer chromatography in system II or if it was diluted with estrone and recrystallized to constant specific activity and ratio (10.6:1). These ratios show that 82% of the tritium was lost when the starting material was converted to estrone. Since the tritium label in the starting material was at C-1 (83% β), the 1β label is lost in the transformation.

Zone 5 from the toluene–propylene glycol paper was diluted with 100 μg of authentic 19-norandrostenedione and was analyzed by thin-layer chromatography in system IIa as developing solvent. The single, radioactive ultraviolet-positive area corresponding to 19-norandrostenedione was eluted, diluted with authentic material, and recrystallized to constant specific activity. The ratio, 126:1, showed an increase in tritium specific activity in the recovered starting material of 114%. This is in contrast to results obtained with androstenedione in which no positive isotope effect was noted and these differences will be commented on in the Discussion section.

Zone 4, which had a slightly higher R_F than estrone, after rechromatography in the modified Bush B_3 system separated into two radioactive peaks. The larger one, R_{estrone} 0.59, contained 90% of the radioactiviy and had a ratio of 118:1, twice that of the starting material. This indicates that the compound was formed late in the incubation after the $^3H:^{14}C$ ratio of substrate had increased. Although this material had the R_F of 2α-hydroxy-19-norandrostenedione in the Bush B_3 system and formed an acetate which ran with 2α-acetoxy-19-norandrostenedione in the Bush A and modified B_3 system, it was not converted to estrone under the acid conditions which convert 2α-hydroxy- or 1β-hydroxy-19-norandrostenedione to this material. The decreased polarity of the acid-treated material in the modified B_3 system (R_{esterone} 1.9) indicated that the hydroxyl group had been in an activated position other than C-1 or C-2. Based on what is known concerning the enzyme capabilities of the placenta, this would indicate that the metabolite was hydroxylated at C-6 (Cedard and Knuppen, 1965). Zone 2 was not investigated further.

Zone 1 has been extensively investigated in other placental incubations by the present authors and has been shown to be 1β-hydroxy-19-norandrostenedione (Townsley and Brodie, 1966). In this incubation it had a $^3H:^{14}C$ ratio of 13:1, indicating that the 1β label had been lost in its formation. This was expected since there is a direct replacement of hydrogen by a hydroxyl group in enzymatic hydroxylations (Hayano, 1962). Being a β-hydroxy ketone, treatment with base effected dehydration to give estrone which had a ratio of 8.6:1 after B_3 chromatography, dilution, and crystallization (85% loss of ^3H). The ratio of the production of 1β-hydroxy-19-norandrostenedione to estrone was 1.6:1.

An incubation of [1-^3H (83% β)]19-norandrostenedione was carried out in a similar fashion with microsomes using nine flasks each containing 12.5 μg of substrate, sp act. 2.94 × 10^6 dpm/μmole, and 2 enzyme equiv of microsomes suspended in a total volume of 6 ml at pH 7.2. The microsomes were prepared in 1% KCl and washed three times in the same medium prior to suspension in the buffer. The residues from extraction of the combined incubations were analyzed by thin-layer chromatography in system II. The material in the estrone areas was eluted and after rechromatography in system Ia followed by base partition, the base-soluble material was chromatographed in the modified Bush B_3 system. The eluate of the single radioactive zone which corresponded to estrone was filtered through a fine sintered-glass funnel and the mass for specific activity determination estimated by fluorometry. A value of 4.86 × 10^5 dpm/μmole was obtained representing a loss of tritium of 83% from starting material. This value is the same as that obtained from the experiment with double-labeled substrate and supernatant at a similar purification stage for which constancy of specific activity was subsequently demonstrated.

Incubations with Cofactors. After microsomal incubation of [4-^{14}C]19-norandrostenedione with various cofactors and in one case in the absence of oxygen, 40 μg each of 19-norandrostenedione, 19-nortestosterone, 1β-hydroxy-19-norandrostenedione, and 1β-hydroxy-19-nortestosterone was added to the incubation mixture. In addition, 21,627 dpm of [6,7-^3H]estrone and 8746 dpm of [6,7-^3H]estradiol-17β of negligible mass were also added. The mean recovery of radioactivity after extraction was 96% for ^{14}C and 100% for ^3H. Zones corresponding to estrone and 17β-estradiol were eluted from the toluene–propylene glycol chromatograms and were analyzed by thin-layer chromatography in systems II and Ib, respectively. The areas corresponding in R_F to estrone, 19-nortestosterone (system II), estradiol-17β, and 1β-hydroxy-19-norandrostenedione (system Ib) were eluted giving a recovery of tritium at this stage of 64–75%. The results are recorded in Table IV. Whereas good conversion to estrone was achieved in triplicate incubations in the presence of NADPH and oxygen (18–25%), no conversion to estrogen was achieved with NADPH when a nitrogen atmosphere was substituted for air. Similar aerobic incubations with NADP, NAD, and NADH also were without effect. Menadione and phenazine methosulfate also were incubated with this substrate and the placental microsomes. These materials are electron acceptors for C-1,2 dehydrogenation reactions in preparations from microorganisms (Stefanovic *et al.*, 1963, Sih and Rahim, 1963). However, they were completely inactive as acceptors for reactions involving the 19-nor steroid and no evidence of any metabolism was obtained. As was expected, 1β-hydroxy-19-norandrostenedione was produced only

TABLE IV: Effect of Cofactors on the Metabolism of [4-^{14}C]19-Norandrostenedione by Placental Microsomes.[a]

	Products		
Cofactor	Estrone	17β-Estradiol (% conversion)	1β-Hydroxy-19-norandrostenedione (% yield)
NADPH-generating	25	<2	28
system + air	24	<2	34
	18	<2	30
NADPH-generating	0	0	0
system	0	0	0
+nitrogen	0	0	0

[a] Similar incubations with NADP (8 μmoles), NADH (8 μmoles), NAD (8 μmoles), phenazine methosulfate (5 μmoles), menadione (1.2 μmoles), and without cofactors yielded none of the above products. Each flask contained 13.6 μg of [4-^{14}C]19-norandrostenedione, specific activity 2550 dpm/μg, and 1 enzyme tissue equiv of microsomes prepared in water and suspended in 3 ml of 0.05 M phosphate buffer.

when NADPH and oxygen were present. The yield of this material after thin-layer chromatography ranged from 28 to 34%, an average conversion of 44% based on the recovery for estrogens at the same purification stage.

Discussion

The results obtained for the incubations of androstenedione and 19-norandrostenedione with placental supernatant strongly suggest that similar mechanisms are involved in ring A oxidation to achieve aromatization. When [4-^{14}C-1-^{3}H (83% β)]androstenedione and [4-^{14}C-1-^{3}H (93% α)]androstenedione were converted to estrone, 82% of the tritium was lost with the first compound, and only 6% was lost with the second, showing that the 1β-hydrogen was eliminated in these transformations. Conversion of [4-^{14}C-1-^{3}H (83% β)]19-norandrostenedione to estrone with the same preparation also showed that the 1β label is eliminated since 82% of the tritium was lost. The final values obtained for the purified estrone were essentially achieved after chromatography (paper or thin-layer chromatography) and base partition followed by another chromatography step on paper, and are similar to results obtained at this purification stage using microsomes and C$_{19}$-tritiated substrates (Morato *et al.*, 1962) or tritiated 19-norandrostenedione (this paper). The mass of product estrone for specific activity determination was estimated using the Kober procedure or sulfuric acid fluorometry, respectively. These methods are thus shown to be adequate for this purpose and could be methods of choice in some circumstances, *e.g.*, when difficult syntheses of potential estrogen precursors are involved.

The other important similarity in the aromatization of C$_{19}$ steroids and 19-norandrostenedione is that all compounds tested, even C$_{19}$ steroids with hydroxyl or aldehyde groupings at C-19 (Morato *et al.*, 1961), require NADPH and oxygen, the usual cofactors for enzymatic hydroxylation. Menadione and phenazine methosulfate, electron acceptors for dehydrogenases, were ineffective in converting 19-norandrostenedione to estrogen. To explain the loss at C-1β and the requirement for these cofactors for the C$_{19}$ oxygenated steroids, we suggested that there is oxidative attack at either the C-19 oxygen (in which case the resultant C-19 oxonium ion is sterically oriented for abstraction of the 1β-hydride ion) or at C-1β (Morato *et al.*, 1962). The data reported here, showing that a compound *without* a C-19 structure, *i.e.*, 19-norandrostenedione, has the same stereochemical and cofactor requirements for estrogen formation make C-19 oxygen attack unlikely and leave C-1β as the probable reactive site for both 19-nor and C$_{19}$ oxygenated precursors. This is supported by a consideration of the ^{3}H:^{14}C ratio in recovered 19-norandrostenedione. From the reasonable assumption that the initial oxidative attack to produce an activated complex is a rate-determining step, the 114% increase in the ^{3}H:^{14}C ratio in recovered 19-norandrostenedione is evidence that attack is at that position. However, until the relationship of 1β-hydroxyandrostenedione formation to estrogen production is resolved, it cannot be said how much of the isotope effect noted is due to the latter. The absence of an isotope effect in the conversion of [4-^{14}C-1-^{3}H (83% β)]androstenedione to estrone is additional evidence that 19-hydroxylation is the first step in estrogen formation from this substrate. This C-19 attack should be little affected by tritium at the 1β position, and so no change in starting material ratio is noted. Other evidence for C-19 hydroxylation being the first step in aromatization is based on the relative ease of conversion of 19-hydroxy- and 19-oxoandrostenediones as compared to androstenedione (Morato *et al.*, 1961) and on kinetic data (Wilcox and Engel, 1965). Hydrogen loss from C-2 can occur readily by enolization through attachment of an electrophilic protein group to the 3-ketone position when the substrate is bound to the active enzyme site as shown in Figure 1 (structure A) (*cf.* also Hayano *et al.*, 1961; Morato *et al.*, 1962). Such a structure would be energetically favored for subsequent anion elimination at C-1β and serves to explain the aromatization of C$_{19}$ and 19-nor steroids by a common mechanism as regards desaturation in ring A. As indicated, depending on whether the substrate A is a 19-nor steroid or an oxygenated C$_{19}$ compound [CH(OH)$_2$ = hydrated aldehyde] the by-product R of the aromatization is a proton or, in addition, formaldehyde (Breuer and Grill, 1961) or formic acid (Axelrod *et al.*, 1965). In the absence of any evidence to the contrary, it may be assumed that one enzyme system is involved in the transformations involving C-1β for the substrates indicated since it is

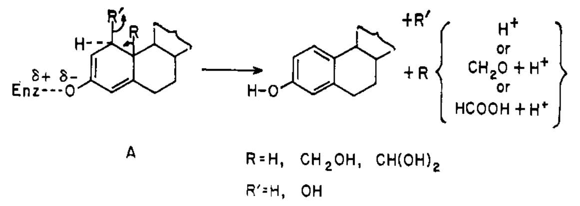

FIGURE 1: Common mechanisms for ring A oxidation in the aromatization of C_{19} and C_{18} steroids.

not necessary to postulate that the elimination of R requires enzymatic intervention.

The mechanism of 1β-hydrogen elimination remains an interesting problem. From the previous discussion, the isolation of 1β-hydroxy-19-norandrostenedione would suggest that this is a likely intermediate for aromatization. In the context of Figure 1 it means that the 1β-hydrogen is replaced by a 1β-hydroxyl group which then is eliminated as a hydroxide ion. However, as was found with 2α-hydroxy-19-norandrostenedione and 10β-hydroxy-19-nortestosterone there was insignificant conversion of this or the corresponding C-17 reduced material to estrogens under conditions where a 20% conversion of 19-norandrostenedione occurred. The 2β-hydroxy isomer, although a less likely possibility as a precursor (Gual *et al.*, 1962), remains to be tested. In the case of the 1β-hydroxy compounds, a consideration of nuclear magnetic resonance data (H. J. Brodie and J. D. Townsley, unpublished observations) indicates that the 1β-hydroxy group is axial rather than equatorial. This is also the case with the $\Delta^{5(10)}$-3α-ol 19-nor steroids (Levine *et al.*, 1966). Presumably when formed on the enzyme surface, the 1β-hydroxy group could be in the usual (for C_{19} steroids) equatorial conformation suitable for subsequent enzymatic dehydration while bound. Another interesting possibility suggested by the resemblance of the aromatase and hydroxylation reaction is that the 1β-hydroxy compound and estrogen are formed concurrently by attack of activated oxygen, conveniently designated as OH^+, at C-1β to produce a common transition state capable of collapsing to yield both products as shown in Figure 2. Recent work on fatty acid transformations (Gurr and Bloch, 1966) suggest that there may be a class of hitherto undefined dehydrogenases capable of introducing carbon-carbon double bond and having the characteristic cofactor requirements of the mixed function oxidase, thus suggesting a hydroxylation–dehydration mechanism. In this case the enzyme from algae was unable to effect dehydration of the expected hydroxylated intermediate. The aromatase has the characteristics of this "dehydrogenase" and in addition it is capable of forming the 1β-hydroxy compound whereas formation of the expected hydroxylated intermediate has not been shown with the plant system. This may be due to the relative importance of pathway 1 to pathway 2 in various systems (see figure) and that because 19-norandrostenedione is not the preferred substrate for aromatization, pathway 2 could predominate in this case. Final classification of these enzymes must await elucidation of the mechanisms involved.

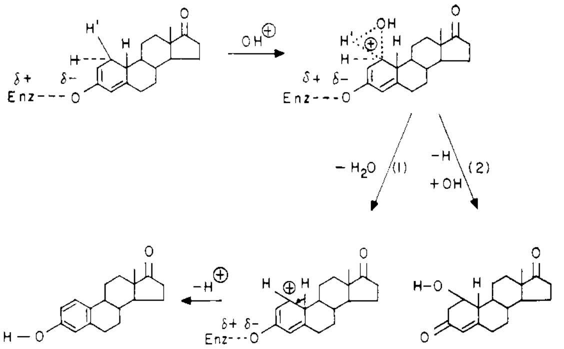

FIGURE 2: Mechanism for production of estrone and 1β-hydroxy-19-norandrostenedione through a common intermediate.

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The Specificity of Dipeptidyl Transferase*

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ABSTRACT: The kinetics of the hydrolytic action of highly purified beef spleen dipeptidyl transferase on a series of dipeptide esters have been determined by automatic titration. The results confirm and extend earlier conclusions regarding the specificity of the enzyme. Glycyl-(β-phenyl)L-lactic acid methyl ester was synthesized and was found to be cleaved by the enzyme to yield the depsipeptide, showing that the amide bond of suitable dipeptide esters (*e.g.*, Gly-Phe-OMe) can be replaced by an ester linkage in substrates of dipeptidyl transferase. It was also found that diazoacetylglycine ethyl ester is resistant to enzymic hydrolysis, whereas Gly-Gly-OEt is readily cleaved. This diazo compound, as well as diazoacetyl-L-phenylalanine ethyl ester (and its D isomer), do not inactivate the enzyme under the conditions of these studies.

Dipeptidyl transferase (formerly termed cathepsin C), an enzyme abundant in extracts of animal tissues, exhibits unusual specificity in its catalysis of reactions at amide and ester bonds. From earlier studies in this laboratory (Wiggans *et al.*, 1954; Izumiya and Fruton, 1956; Fruton and Mycek, 1956) and those of other investigators (Planta *et al.*, 1964), it became evident that the enzyme is specific for the activation of the terminal carbonyl group of a dipeptidyl derivative, provided that the dipeptidyl unit has a free α-amino (or α-imino) group and is composed of L-α-amino acid (or glycyl) residues. In its action on such dipeptide amides or esters, the enzyme showed preference for substrates in which the activated carbonyl group was contributed by an L-amino acid bearing a hydrophobic side chain (Phe, Tyr, Trp, Leu), although dipeptide esters such as Gly-Gly-OEt were hydrolyzed at an appreciable rate. The amino-terminal amino acid residue could be varied considerably; substrates having glycyl, L-prolyl, or various α-L-amino acid residues (but not lysyl) as amino-terminal components were cleaved readily. It was suggested (Izumiya and Fruton, 1956; Fruton, 1957) that, in the interaction of a substrate with the enzyme, the backbone of the dipeptidyl group assumes a conformation approximating a hexagonal structure. This hypothesis is consistent with the observed similarity in the rates of cleavage of pairs of substrates such as the ethyl esters of D-alanyl-L-tyrosine and α-aminoisobutyryl-L-tyrosine.

As has been shown previously (Jones *et al.*, 1952; Fruton *et al.*, 1953; Würz *et al.*, 1962; Nilsson and Fruton, 1964), dipeptidyl transferase is an exceptionally efficient catalyst of reactions in which the dipeptidyl group of a suitable substrate is transferred to an amine. With nucleophiles such as hydroxylamine, hydroxamic acids are formed; this reaction has provided a convenient method for the rapid assay of enzyme preparations (de la Haba *et al.*, 1959; Metrione *et al.*, 1966). Of more general interest is the fact that the enzyme readily catalyzes polymerization reactions in which dipeptidyl units are added to a growing oligopeptide chain to form products whose chain length is determined by their solubility in aqueous media. Since the relative extent of reaction of the activated dipeptidyl unit with either

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