Bradlow, H. L. (1968), *Steroids 11*, 265.
Budzikiewicz, H., Djerassi, C., and Williams, D. H. (1964), Structure Elucidation of Natural Products by Mass Spectrometry, Vol. II, San Francisco, Calif., Holden-Day, p 62.
Collins, D. C., Jirku, H., and Layne, D. S. (1968), *J. Biol. Chem., 243*, 2928.
Collins, D. C., Williams, K. I. H., and Layne, D. S. (1967), *Arch. Biochem. Biophys. 121*, 609.
Conchie, J., and Levvy, G. A. (1963), *Methods Carbohydrate Chem. 2*, 342.
Conchie, J., Levvy, G. A., and Marsh, C. A. (1957), *Advan. Carbohydrate Chem. 12*, 173.
Dutton, G. J. (1966), *Arch. Biochem. Biophys. 116*, 399.
Eichenberger, W., and Newman, D. W. (1968), *Biochem. Biophys. Res. Commun. 32*, 366.
Fex, H., Lundvall, K. E., and Olsson, A. (1968), *Acta Chem. Scand. 22(1)*, 254.
Gal, A. E. (1968), *Anal. Biochem. 24*, 452.
Gessner, T., and Vollmer, C. A. (1969), *Fed. Proc. 28*, 545.
Hall, L. D. (1964), *Advan. Carbohydrate Chem. 19*, 51.
Hough, L., and Richardson, A. C. (1967), *in* Rodd's Chemistry of Carbon Compounds, Vol. I^F, Coffrey, S., Ed., Amsterdam, Elsevier, p 327.
Jirku, H., and Layne, D. S. (1965), *Biochemistry 4*, 2126.
Layne, D. S., Sheth, N. A., and Kirdani, R. Y. (1964), *J. Biol. Chem. 239*, 3221.
Lemieux, R. U., and Morgan, A. R. (1965), *Can. J. Chem. 43*, 2199.
Mazurek, M., and Perlin, A. S. (1965), *Can. J. Chem. 43*, 1918.
Prochazka, Z. (1968), *Collection Czech. Chem. Commun. 33*, 4039.
Redemann, C. E., and Niemann, C. (1955), Organic Syntheses, Coll. Vol. III, New York, N. Y., Wiley, p 11.
Smith, J. N., and Turbert, H. (1964), *Biochem. J. 92*, 127.
Williams, R. T. (1967), *in* Biogenesis of Natural Compounds, Bernfeld, P., Ed., 2nd ed, Oxford, Pergamon, p 598.

Stereochemistry of Estrogen Biosynthesis*

Jack Fishman, Henry Guzik, and Donna Dixon

ABSTRACT: The stereoselective synthesis of [2β-^{3}H]- and [2α-^{3}H]androst-4-ene-3,17-diones is described. The orientation of the isotope in each followed from the method of preparation and from dehydrogenation with *Bacillus sphaericus* in which only the 2β-^{3}H was lost. Incubation of each substrate with human placental aromatase preparation established that the hydrogen loss from C-2 in estrogen biosynthesis is β. The recovered starting material showed a gain in tritium indicating the irreversibility of the loss and raising questions regarding the enolization mechanism proposed for this step. The 1β,2β stereochemistry of aromatization was further confirmed by the tritium analysis of estrone and 2-hydroxyestrone derived from [1,2-^{3}H]testosterone by sequential aromatization and 2 hydroxylation.

The *in vivo* transformation of cholesterol to the C-18 female sex hormone estradiol proceeds by way of discrete hormonal stages. The final sequence in this biotransformation which is the conversion of an androgen into estrogen involves the loss of the C-19 methyl group and of a hydrogen atom from both C-1 and C-2. Extensive study of this biosynthetic step has revealed that hydroxylation of the methyl group precedes its expulsion and that the hydrogen loss from C-1 is stereospecifically β (Talalay, 1965; Townsley and Brodie, 1968). The nature and stereochemistry of hydrogen loss from C-2 during the biological aromatization was the purpose of this study.[1]

The design of the experiment required the separate preparation of androst-4-ene-3,17-diones stereospecifically labeled with tritium in the 2α and 2β orientations which would serve as substrates for the enzymatic aromatization. At the outset their preparation was constrained by the requirement that the introduction of the isotope proceed by reactions of known high stereoselectivity. In addition since the desired α-methylene-labeled ketones would be subject to isotope loss or epimerization by an enolization reaction it was essential to limit the opportunity for this by delaying the formation of the C-3 ketone to the final step of the synthetic sequence. These requirements were fulfilled by the reaction sequences depicted in Scheme I. Reaction of 3β-tosyloxy-5α,6α-epoxyandrostan-17-one (**1**) with Li_2CO_3 in dimethylacetamide gave the olefin **2** which on reduction with $LiAlH_4$ led to androst-2-ene-5α,17β-diol (**3**). Epoxidation of **3** provided the 2α,3α-epoxide **4** which on opening with lithium aluminum tritiide gave the 2β-tritioandrostane-3α,5α,17β-triol (**5**). Selective acetylation of the two secondary hydroxyls led to the diacetate **6**; dehy-

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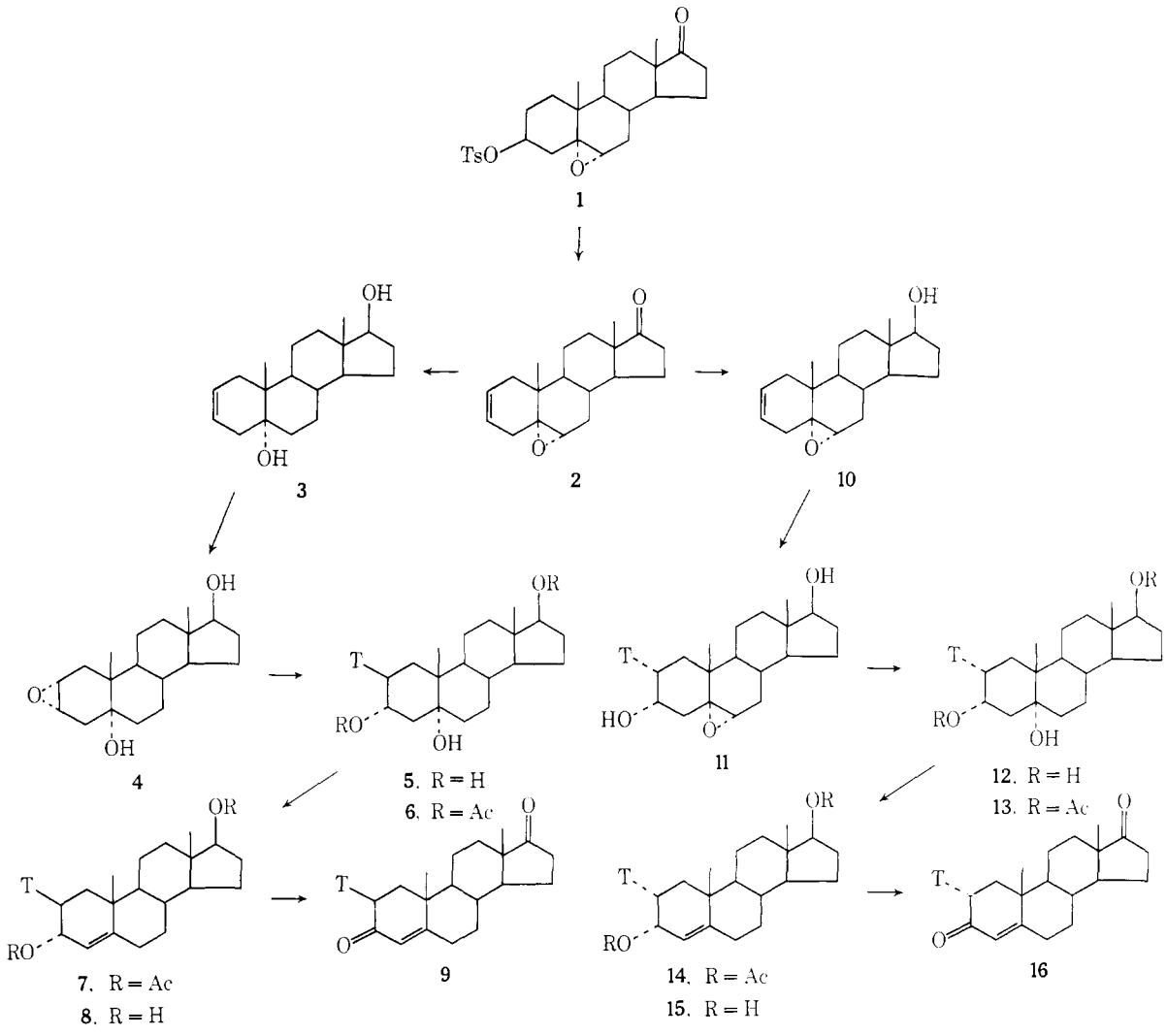
[1] While this manuscript was in preparation a report on the stereochemistry of hydrogen loss at C-2 during aromatization has appeared. Although different procedures were employed, the results obtained were in excellent agreement with those reported in this paper (Brodie *et al.*, 1969).

SCHEME I



dration of **6** with thionyl chloride gave [2β-^{3}H]androst-4-ene-3α,17β-diol diacetate (**7**). Mild alkaline hydrolysis led to the free diol **8**, which on oxidation with the Jones reagent (Bowers *et al.*, 1953) gave the desired [2β-^{3}H]substrate **9**. Following the introduction of the radioactive isotope in **5**, an aliquot of the product was diluted with carrier and the subsequent reactions were followed by serial specific activity determinations which are given in Table I.

The tritium was introduced into the 2α position also utilizing the epoxy olefin **2** as the starting material. Reduction of **2** with $NaBH_4$ produced the 17β-ol **10** which was reacted with tritiated diborane and oxidized with hydrogen peroxide. A mixture of isomeric diols was obtained from which the desired [2α-^{3}H]5α,6β-epoxyandrostane-3α,17β-diol (**11**) was obtained by preparative thin-layer chromatography. The epoxide in **11** was opened with $LiAlH_4$ to give the triol **12**. The latter was converted into the desired [2α-^{3}H]substrate **16** by a sequence identical with that used before proceeding *via* the [2α-^{3}H]androstane-3α,5α,17β-triol 2,17-diacetate (**13**), [2α-^{3}H] androst-4-ene-3α,17β-diol diacetate (**14**), and the free diol **15**. The isotope-inserting reactions, $LiAlH_4$ opening of the epoxide in **4**, and the hydroboration of the double bond in **10** have been firmly established to be *trans* (Trevoy and Brown, 1949; Mousseron *et al.*, 1952) and *cis* (Brown and Zweifel, 1959), respectively, and could thus be expected to lead to highly stereoselective if not stereospecific isotope orientations, the nature of which follows from the orientation

TABLE I: ^{3}H Specific Activities of Intermediates.

Compound	Sp Act. (cpm × 10^{-3}/mg)	Molar Act. (cpm × 10^{-3}/mM)
[2β-^{3}H]Androstanetriol (**5**)	7.10	2,182
[2β-^{3}H]Androstanetriol diacetate (**6**)	5.47	2,143
[2β-^{3}H]Androstenediol diacetate (**7**)	5.71	2,138
[2β-^{3}H]Androstenediol (**8**)	7.28	2,110
[2β-^{3}H]Androstenedione (**9**)	7.08	2,095
[2α-^{3}H]Androstenediol (**15**)	348	101,000
[2α-^{3}H]Androstenedione (**16**)	359	103,000

TABLE II: Loss of Tritium in *B. sphaericus* Dehydrogenation.

	$^3H^a$	$^{14}C^a$	$^3H/^{14}C$	% ^3H Lost
[2β-^{3}H]Androstenedione	252,000	21,700	11.1	
Androst-1,4-diene-3,17-dione	1,340	6,700	0.2	98
Recovered substrate	124,000	5,900	21.0	
[2α-^{3}H]Androstenedione	151,000	37,700	4.0	
Androst-1,4-diene-3,17-dione	84,000	24,800	3.4	15
Recovered substrate	32,000	2,100	14.2	

[a] Total content in counts per minutes.

TABLE III: Loss of Tritium in Enzymic Aromatization.

	$^3H^a$	$^{14}C^a$	$^3H/^{14}C$	% ^3H Lost
[2β-^{3}H]Androstenedione	1,790,000	188,000	11.1	
Estradiol	11,200	5,900	1.9	83
Estrone	149,000	78,500	1.9	83
Recovered substrate	224,000	18,700	12.0	
[2α-^{3}H]Androstenedione	251,000	43,000	5.9	
Estradiol	105,500	19,100	5.5	8
Estrone				
Recovered substrate	24,000	3,400	7.1	

[a] Total content in counts per minute.

of the hydroxy group generated at C-3. Oxidation to the C-3 ketone was the final step in each synthesis and was carried out under nonenolizing conditions (Fishman, 1965; Ramseyer and Hirschmann, 1967) as confirmed by the constancy of the specific activities of the two products prior to and following the oxidation (Table I).

The orientation of the tritium atom in each of the two labeled androstenediones **9** and **16** was further confirmed by biochemical procedures. Each of the substrates **9** and **16** was admixed with ^{14}C-labeled androst-4-ene-3,17-dione and incubated with *Bacillus sphaericus* in the presence of menadione as an electron acceptor. The products of the reaction and recovered starting material were isolated and separated. Carrier androst-1,4-diene-3,17-dione and androst-4-ene-3,17-dione were added and recrystallized to constant isotope ratio. The stereochemistry of dehydrogenation by this organism has been previously demonstrated to be stereoselectively 1α,2β (Hayano *et al.*, 1961; Ringold *et al.*, 1962, 1963). The results obtained are listed in Table II and demonstrate loss of the 2β isotope but not of the 2α epimer. The specificity of this biochemical reaction confirms the stereoselectivity of the chemical synthesis employed and therefore the orientation of the isotope in the two labeled androstenediones **9** and **16** can be considered to be established. The recovered starting materials from both substrates exhibited a tritium gain, and the reaction thus proceeds with a substantial isotope effect, confirming the results obtained with the deuterated species (Jerussi and Ringold, 1965). The effect of this on the product isotope ratio cannot be calculated precisely but as an approximation it will lead to an overestimation of 10% in the tritium loss giving corrected values of 88 and 5%, respectively.

The two substrates **9** and **16** containing added ^{14}C-labeled material were incubated separately with the human placental aromatase preparation (Ryan, 1959). The aromatic products and recovered starting materials were isolated, diluted with carrier, and recrystallized until constant isotope ratios were achieved. The results and calculated tritium loss given in Table III establish that the hydrogen loss at C-2 during aromatization is highly stereoselective and is β. The recovered starting materials from both incubations were enriched in tritium indicating the presence of primary and secondary isotope effects and the irreversible nature of the hydrogen loss at C-2. The recovery of the starting materials was in each case only about 10%, so that the isotope effects in this case unlike the *B. sphaericus* dehydrogenations have little influence on the product isotope ratio and hence on the calculated tritium loss. The isotope analysis of estrogens produced from commercially available testosterone or androstenedione labeled with tritium at C-1 and C-2 provided an opportunity to test the results obtained above. These materials which are frequently used as estrogen precursors in aromatization studies are produced by the heterogeneous catalytic reduction with tritium of the ring A dienone structure. This procedure has been demonstrated to result in C-1 tritium being approximately 83% β oriented (Brodie *et al.*, 1962). As catalytic hydrogenation involves *cis* addition the tritium at C-2 in these compounds should also be 83% β. Estrogens derived from such substrates by a 1β,2β hydrogen loss should therefore exhibit an 83% tritium loss. Further metabolism of estrone to 2-hydroxyestrone should yield a product with 91% of the tritium lost. Since it has been demonstrated that the human placental homogenate possesses estrone-2-hydroxylating capacity (Fishman and Dixon, 1967), such a sequential tritium loss experiment proved feasible. Incubation of [1,2-^{3}H]testosterone admixed with ^{14}C-labeled material with placental homogenate produced the results given in Table IV. The estrone isolated exhibited an 82% tritium loss, while 2-hydroxyestrone lacked 88% of the initially present tritium.

Discussion

The exact nature and sequence of the events involved in the biological aromatization of androst-4-ene-3,17-dione to estrone have not as yet been established. Available evidence suggests strongly that hydroxylation of the angular methyl group is an initial and requisite step (Wilcox and Engel, 1965). The subsequent oxidative changes in ring A are at the present time conjectural. The question whether the hydrogen loss from C-1 and C-2 is linked or whether these are distinct and separate steps with that at C-1 being connected with the expulsion of the angular substituent is of particular relevance. Current concepts of the mechanism imply that the hydrogen loss at C-2 occurs first by enolization and is followed by expulsion of the C-19 fragment in association with the removal of the 1β hydrogen (Morato *et al.*, 1962; Townsley and Brodie, 1968). The demonstration that the 2β hydrogen is preferentially removed would appear to lend support to the enolization mechanism since it is the β

TABLE IV: Loss of Tritium in Placental Aromatization and 2 Hydroxylation.

	$^3H^a$	$^{14}C^a$	$^3H/^{14}C$	% ^3H Lost
[1,2-^{3}H]-Testosterone	10.4×10^6	0.3×10^6	34.6	
Estradiol	110,000	17,500	6.3	82
Estrone	840,000	127,000	6.6	81
2-Hydroxy-estrone	23,600	5,500	4.3	88

[a] Total content in counts per minute.

and axial hydrogen which would be expected to be more susceptible to abstraction in enol formation (Corey and Sneen, 1956).[2] Such an enolization, however, if unaccompanied by other driving forces, should be a reversible process, as is the case with the *B. sphaericus* dehydrogenation in the absence of electron acceptors (Stefanovic *et al.*, 1962). Such is not the case here as evidenced by the lack of isotope exchange in the recovered starting material. This then would suggest that if enolization is involved in the loss of hydrogen at C-2 it is concerted with the other steps or succeeds them so that the driving force of the aromatization precludes reversal of the enolization.

The *cis* nature of the C-1,2 dehydrogenation in the aromatization can also be conceived as supporting an alternative mechanistic version. This involves a C-1,2 dehydrogenation followed by the expulsion of the C-19 fragment by a process illustrated in Figure 1. The intermediacy of 19-oxo derivatives in the aromatization has been supported by recent evidence (Akhtar and Skinner, 1969) and dehydrogenation at other sites of the steroid molecule have been shown to proceed *via cis* elimination (Paliokas and Schroepfer, 1968). In contrast C-1,2 dehydrogenation with *B. sphaericus* which involves an enolization step exhibits *trans* stereochemistry. Enzymic dehydrogenations have been suggested to proceed *via* initial hydroxylation and subsequent dehydration. This possibility has been considered in connection with the aromatization sequence and 2β-hydroxyandrost-4-ene-3,17-dione has been tried as an aromatization substrate and found not very effective (Gual *et al.*, 1962). A more suitable test for this hypothesis however would be the aromatization of a 2β,19-dihydroxy derivative of testosterone or androstenedione which has as yet not been attempted. Additional work is clearly necessary to determine the sequence of these primary steps and to distinguish between the alternative aromatization pathways.

The results obtained from [1,2-^{3}H]testosterone are in excellent agreement with those predicted from the 1β,2β stereochemistry of aromatization. Further, they focus attention on the care which should be exercised in calculating the percentage conversion of these frequently used precursors

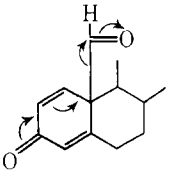


FIGURE 1

into estrogens and 2-oxygenated estrogens both in *in vitro* and *in vivo* experiments.[3]

Experimental Procedures[4]

5α,6α-Expoxyandrost-2-en-17-one (**2**). A solution of 7.6 g of 3β-tosyloxy-5α,6α-epoxyandrostan-17-one (**1**) in 350 ml of dimethylacetamide was refluxed for 3.5 hr with a 7.6 g of lithium carbonate. After cooling the inorganic material was filtered and washed with ethyl acetate. Following addition of 400 ml of ethyl acetate the filtrate was washed with water and the organic layer was dried and evaporated and the residue was chromatographed on 350 g of acid-washed alumina. Elution with ethyl ether–benzene (1:4) gave 2.37 g of the olefin **2**. Elution with chloroform afforded 0.89 g of 3α-acetoxy-5α,6α-epoxyandrostan-17-one which apparently resulted from the reaction with acetate ions obtained from the solvent.

Recrystallization from ethanol afforded pure 5α,6α-epoxyandrost-2-en-17-one (**2**), mp 150–153°. *Anal.* Calcd for $C_{19}H_{26}O_2$: C, 79.68; H, 9.15. Found: C, 79.42; H, 8.87. The nuclear magnetic resonance spectra showed ($CDCl_3$): 49, 53 Hz (18-, 19-CH_3) singlets; 180 Hz (6β-H) doublet; 340 Hz (2,3-H) broad singlet.

A solution of 500 mg of the epoxide **2** in 100 ml of dioxane was refluxed overnight with excess $LiAlH_4$. After cooling the excess reagent was decomposed by the careful addition of ice-cold water. The reaction mixture was diluted with water and extracted with $CHCl_3$ which was washed with water, dried, and evaporated. The residue crystallized from acetone–petroleum ether (bp 30–60°) to give 400 mg of androst-2-ene-5α,17β-diol (**3**): mp 158–160° (lit. (Klimstra, 1966) mp 160–162°). *Anal.* Calcd for $C_{19}H_{30}O_2$: C, 78.57; H, 10.41. Found: C, 78.91; H, 10.19. The nuclear magnetic resonance spectra showed ($CDCl_3$): 44, 53 Hz (18-, 19-CH_3) singlets; 339 Hz (2,3-H) broad singlet.

Epoxidation of **3**. A solution of 190 mg of **3** in 10 ml of chloroform was allowed to stand overnight with 140 mg of *m*-chloroperbenzoic acid. The reaction was diluted with 50 ml of chloroform which was washed with sodium bicarbonate solution then water, dried, and evaporated. The residue was crystallized from acetone–petroleum ether to give 160 mg of 2α,3α-epoxyandrostane-5α,17β-diol (**4**), mp 195–198°. *Anal.* Calcd for $C_{19}H_{30}O_3$: C, 74.47; H, 9.87. Found: C,

[2] A recent report by Bordwell and Scamehorn (1968) suggests that the accepted concepts of steric and stereoelectronic control of enolization may require revision.

[3] The isotope in 1,2-^{3}H substrates produced by *homogeneous* catalytic reduction would have mostly the opposite (α) orientation and hence would retain most of the tritium on aromatization. Knowledge of the synthetic method used in producing these substrates is therefore essential to a rational calculation of androgen into estrogen conversion.

[4] The nuclear magnetic resonance spectra were obtained on a Varian A60 instrument. They were run at 60 Mc and the chemical shifts are given in hertz downfield from tetramethylsilane as an internal standard.

76.64; H, 9.66. The nuclear magnetic resonance spectra showed ($CDCl_3$): 43, 55 Hz (18-, 19-CH_3) singlets; 199 Hz (2β,3β-H) broad singlet.

LiAlH^3H_4 Reduction of **4**. A solution of 10 mg of **4** in 2 ml of tetrahydrofuran was stirred overnight at room temperature with 2.7 mg of tritiated lithium aluminum hydride (5 mCi/mg). Following the addition of two drops of cold water, 10 ml of 8% sulfuric acid was added and the reaction was extracted with chloroform. The organic layer was washed with water, dried, and evaporated. The residue was purified by preparative thin-layer chromatography on silica gel in the system ethyl acetate–cyclohexane (1:1) to give 7 mg of [2β-^{3}H]-androstane-3α,5α,17β-triol (**5**) containing 25.7×10^6 cpm.

An aliquot of this material containing 24.2×10^4 cpm was diluted with 47 mg of inert triol **5** (Williams *et al.*, 1963) and recrystallized to constant specific activity (Table I). This material of low specific activity was carried through all the reactions done on the high specific activity material described below. The specific activity at the various stages was obtained, and the material also served to determine physical constants which could not be obtained from the small quantities available from the high specific activity synthesis.

[2β-^{3}H]Androst-4-ene-3,17-dione (**9**). [2β-^{3}H]Androstane-3α,5α,17β-triol (**5**) (7.0 mg) containing 25.5×10^6 cpm was diluted with 7.5 mg of inert material, dissolved in 1 ml of pyridine, and allowed to stand with 0.5 ml of acetic anhydride overnight. Removal of the solvents under vacuum gave the crystalline diacetate **6**, mp 195–198° (Williams *et al.*, 1963), which without further purification was dissolved in 0.3 ml of pyridine to which 0.1 ml of thionyl chloride was added at 0°. After standing 10 min at 15°, cold water was added and the material was extracted with chloroform, which was washed with $NaHCO_3$ solution, dried, and evaporated. The residue was purified by preparative thin-layer chromatography on silica gel in ethyl acetate–cyclohexane (3:7) to give androst-4-ene-3α,17β-diol diacetate (**7**): mp 134–136° from methanol, lit. (D. K. Fukushima, 1969, personal communication) mp 134–136°; nuclear magnetic resonance spectra showed ($CDCl_3$): 49, 60 Hz (18-, 19-CH_3) singlets; 122 Hz (β3-, 17-acetate methyls) singlet; 324 Hz (4-H) multiplet.

The diacetate **7** was dissolved in 0.25 ml of methanol, 1.3 ml of 5% methanolic KOH, and 0.15 ml of water, and allowed to stand overnight at room temperature. Dilution with water and extraction with a large quantity of ethyl acetate gave 4 mg of crystalline androst-4-ene-3α,17β-diol (**8**): mp 209–218° from methanol, lit. (D. K. Fukushima, 1969, personal communication) mp 212–218°.

The free diol **8** was dissolved in 25 ml of acetone, cooled to 0°, and treated with three drops of the Jones reagent. After standing at 0° for 7 min, water was added and the reaction mixture was extracted with chloroform. The organic layer was washed well with water, dried, and evaporated and the residue of 3.8 mg was purified by preparative thin-layer chromatography on silica gel in ethyl acetate–cyclohexane (1:1). The [2β-^{3}H]androstenedione (**9**) located by its ultraviolet absorption was eluted to yield 3.2 mg of material containing 6.67×10^6 cpm.

Reverse isotope dilution of this material indicated an isotopic purity of 96.7%.

5α,6α-Epoxyandrost-2-en-17β-ol (**10**). A solution of 280 mg of 5α,6α-epoxyandrost-2-ene-17-one (**2**) in 30 ml of ethanol was allowed to stand for 1 hr with an excess of sodium borohydride. Dilution with 5% sulfuric acid and extraction with chloroform gave 240 mg of **10**, mp 139–143°. *Anal.* Calcd for $C_{19}H_{28}O_2$: C, 79.12; H, 9.79. Found: C, 79.32; H, 9.69.

[2α-^{3}H]Androst-4-ene-3,17-dione (**16**). Tritiated diborane generated from the slow dropwise addition of 51 mg of [^{3}H]sodium borohydride (1.3 mCi/mg) in 3 ml of diglyme to 0.3 ml of boron trifluoride etherate in 2 ml of diglyme was passed through a solution of 23 mg of 5α,6α-epoxyandrost-2-en-17β-ol (**10**) in 5 ml of tetrahydrofuran. The addition took 1 hr in a nitrogen atmosphere. Following another hour at room temperature the reaction was cooled and 0.5 ml of water was added followed by 0.2 ml of 10% aqueous NaOH and 1.5 ml of 30% H_2O_2. After standing for 1 hr at room temperature, the reaction was diluted with water and extracted with chloroform, which was washed well with water, dried, and evaporated. The residue was separated by preparative thin-layer chromatography in ethyl acetate–cyclohexane (7:3) and the area corresponding to 5α,6α-epoxyandrostane-3α,17β-diol (**11**) was eluted. The 2 mg of material thus obtained was diluted with 28.9 mg of carrier and recrystallized from methanol to a constant specific activity of 1.12×10^6 cpm/mg.

The above material (21 mg) was dissolved in 25 ml of tetrahydrofuran and stirred overnight at room temperature with excess $LiAlH_4$. The reaction was worked up in the usual manner to give 14 mg of [2α-^{3}H]androstane-3α,5α-17β-triol (**12**). The subsequent steps *via* [2α-^{3}H]androstane-3α,5α,17β-triol 3,17-diacetate (**13**), [2α-^{3}H]androst-4-ene-3α,17β-diol diacetate (**14**), and [2α-^{3}H]androst-4-ene-3α,17β-diol (**15**) to [2α-^{3}H]-androst-4-ene-3,17-dione (**16**) were carried out in a completely analogous manner to those in the 2β-^{3}H series. An aliquot of product **15** was diluted with carrier material and recrystallized to constant specific activity of 34.8×10^4 cpm/mg upon oxidation to **16** the specific activity was 35.9×10^4 cpm/mg.

Placental Incubations. [2β-^{3}H]Androst-4-ene-3,17-dione (**9**) (1.79×10^6 cpm) was mixed with [^{14}C]androst-4-ene-3,17-dione (0.188×10^6 cpm) to a total weight of 0.2 mg. To this substrate was added 5 ml of placental microsome preparation, 10 μmoles of NADP, 120 μmoles of glucose 6-phosphate, and 9.6 K units of glucose 6-phosphate dehydrogenase. The incubation was carried out in air in Tris buffer at pH 6.9 at 37° for 1 hr. The reaction was terminated by the addition of 10 ml of acetone containing 15 mg each of androst-4-ene-3,17-dione, estrone, and estradiol. The mixture was acidified to pH 5 with dilute sulfuric acid and extracted well with ether which was dried and evaporated. The residue was submitted to preparative thin-layer chromatography and developed first in ethyl acetate–cyclohexane (3:7) followed by ethyl acetate–cyclohexane (1:1), and the appropriate bands were eluted. In order to separate androstenedione and estradiol completely, the two were combined, acetylated, and separated by preparative thin-layer chromatography in ethyl acetate–cyclohexane (3:7). All three compounds were recrystallized until constant isotope ratios were achieved.

The incubation with [2α-^{3}H]androst-4-ene-3,17-dione (**16**) (2.51×10^5 cpm), containing [^{14}C]androst-4-ene-3,17-dione (0.43×10^5 cpm), total weight 0.2 mg, was carried out exactly as above.

Incubation with [1,2-^{3}H]Testosterone. The incubation was repeated as above using as the substrate commercially avail-

able [1,2-^{3}H]testosterone (10.4×10^6 cpm) mixed with [^{14}C]-testosterone (0.30×10^6 cpm), total weight 0.22 mg. The incubation was terminated by the addition of 26.6 mg of 2-hydroxyestrone in acetone, and was then extracted continuously with ether for 24 hr. The organic layer was dried and evaporated and the residue was chromatographed on Celite with 90% methanol as the stationary phase and isooctane as the mobile phase. An elution gradient was provided by 25 and 100% 1,2-dichloroethane (Engel *et al.*, 1961). The fractions were 10 ml, and aliquots were removed for counting.

To fractions 50–73 containing estrone, 21.0 mg of inert estrone was added, and the mixture was acetylated and recrystallized from acetone–petroleum ether to constant isotope ratio. Fractions 74–115 contained 2-hydroxyestrone and estradiol. After addition of 11.3 mg of estradiol, the mixture was acetylated and estradiol diacetate and 2-hydroxyestrone diacetate were separated by preparative thin-layer chromatography on silica gel in ethyl acetate–petroleum ether (1:1). Following elution of the appropriate zones the two compounds were recrystallized until constant isotope ratio was achieved.

B. sphaericus Incubations. A starter culture of *B. sphaericus* (ATC 7055) was added to 50 ml of a sterile H_2O solution containing 0.3% yeast extract (Fisher) and 0.5% acetone peptone and agitated at 30° for 35 hr. After centrifuging at 3000*g* for 5 min at 0°, the resulting pellet was added to 6 ml of a 0.03 M phosphate buffer solution (pH 6.9) and sonicated for 5 min. The resultant solution was centrifuged for 15 min at 6000*g* and the supernatant was kept at 0° until used in the incubations. The [2β-^{3}H]androst-4-ene-3,17-dione (**9**) (2.25×10^5 cpm) and [^{14}C]androst-4-ene-3,17-dione (0.22×10^5 cpm), total weight 0.2 mg in 0.2 ml of ethanol–propylene glycol (3:1) and 0.5 mg of menadione in 0.1 ml of ethanol, were added to 5 ml of the above cell-free extract. Following agitation at room temperature for 30 min the reaction was stopped by the addition of ethyl acetate containing 15 mg each of androst-4-ene-3,17-dione and androst-1,4-diene-3,17-dione. Extraction with ethyl acetate, drying, and evaporation provided a crystalline mixture which was separated by preparative thin-layer chromatography in ethyl acetate–cyclohexane (1:1). The product and starting material were recrystallized from acetone–petroleum ether until constant isotope ratios were achieved.

The incubation was repeated exactly as above using [2α-^{3}H]androst-4-ene-3,17-dione (**16**) (14.91×10^5 cpm) and [^{14}C]-androst-4-ene-3,17-dione (3.97×10^5 cpm).

Counting was carried out on a Packard Tri-Carb liquid scintillation counter. Each sample was counted in triplicate and sufficient counts were allowed to accumulate to permit an accuracy of $\pm 5\%$. Counting efficiency was 91.0% for ^{14}C and 50.2% for ^{3}H.

References

Akhtar, M., and Skinner, S. J. M. (1969), *Biochem. J. 109*, 318.

Bordwell, F. G., and Scamehorn, R. B. (1968), *J. Am. Chem. Soc. 90*, 6749.

Bowers, A., Halsall, T. G., Jones, E. R. H., and Lemmin, A. J. (1953), *J. Chem. Soc.*, 2548.

Brodie, H. J., Hayano, M., and Gut, M. (1962), *J. Am. Chem. Soc. 84*, 3766.

Brodie, K., Kripolani, J., and Possanza, H. (1969), *J. Am. Chem. Soc. 91*, 1241.

Brown, H. C., and Zweifel, G. (1959), *J. Am. Chem. Soc. 81*, 247.

Corey, E. J., and Sneen, R. A. (1956), *J. Am. Chem. Soc. 78*, 6271.

Engel, L. L., Cameron, C. B., Stoffyn, A., Alexander, J. A., Klein, O., and Trofimow, N. D. (1961), *Anal. Biochem. 2*, 114.

Fishman, J. (1965), *J. Am. Chem. Soc. 87*, 3455.

Fishman, J., and Dixon, D. (1967), *Biochemistry 6*, 1683.

Fishman, J., and Guzik, H. (1969), *J. Am. Chem. Soc. 91*, 2805.

Gual, C., Morato, T., Hayano, M., Gut, M., and Dorfman, R. I. (1962), *Endocrinology 71*, 920.

Hayano, M., Ringold, H. J., Stefanovic, V., Gut, M., and Dorfman, R. I. (1961), *Biochem. Biophys. Res. Commun. 4*, 454.

Jerussi, R., and Ringold, H. J. (1965), *Biochemistry 4*, 2113.

Klimstra, P. D. (1966), U. S. Patent 3,271,425.

Morato, T., Raab, K., Brodie, H. J., Hayano, M., and Dorfman, R. I. (1962), *J. Am. Chem. Soc. 84*, 3764.

Mousseron, M., Jacquier, R., Mousseron-Canet, M., and Zagdoun, R. (1952), *Bull. Soc. Chim. France 19*, 1042.

Paliokas, A. M., and Schroepfer, B. J. (1968), *J. Biol. Chem. 243*, 453.

Ramseyer, J., and Hirschmann, H. (1967), *J. Org. Chem. 32*, 1850.

Ringold, H. J., Hayano, M., and Stefanovic, V. (1963), *J. Biol. Chem. 238*, 1960.

Ringold, H. J., and Turner, A. (1962), *Chem. Ind. (London)*, 211.

Ryan, K. J. (1959), *J. Biol. Chem. 234*, 268.

Stefanovic, V., Hayano, M., and Dorfman, R. I. (1962), *Biochim. Biophys. Acta 71*, 429.

Talalay, P. (1965), *Ann. Rev. Biochem. 34*, 347.

Townsley, J. D., and Brodie, J. (1968), *Biochemistry 7*, 33.

Trevoy, L. W., and Brown, W. G. (1949), *J. Am. Chem. Soc. 71*, 1675.

Wilcox, R. B., and Engel, L. L. (1965), *Steroids, Suppl. 1*, 49.

Williams, K. I. H., Rosenfeld, R. S., Smulowitz, M., and Fukushima, D. K. (1963), *Steroids 1*, 377.