Cu(II) binding site is composed of the four N-terminal amino acids.

The binding of Cu(II) to the α-NH_2 end of the S-peptide and not to the histidyl residue (residue 12) is interesting in view of the fact that histidyl residues in proteins are known to have high affinity for Cu^{2+} (Breslow, 1964; Breslow and Girotti, 1966), and are often assumed to be the ligands in both synthetic and natural copper–protein complexes. It is interesting to note that recently it has been found (Breslow and Girotti, 1970) that the dominant Cu(II) binding site on RNase at pH 7 is the α-NH_2 terminus, in support of our findings. In the case of the bovine serum albumin–Cu(II) complex where Cu(II) was found to be bound to the α-NH_2 end (Peters, 1960; Peters and Blumenstock, 1967; Shearer *et al.*, 1967), the histidyl residue is part of the copper binding site since it occupies position 3 from the N terminal.

It seems that the $IrCl_6^{2-}$ method can be applied to locate Cu(II) binding sites in proteins. Thus it may be possible to apply the $IrCl_6^{2-}$ to natural copper-containing proteins and thereby locate the copper binding sites, a task not accomplished by the available physical techniques. This type of "affinity labeling" may also help X-ray crystallographers to locate the amino acid residues involved in Cu(II) binding when the crystallography of copper proteins is undertaken. It is however not yet clear whether the $IrCl_6^{2-}$ method will pick up binding sites not involving peptide nitrogens as the immediate ligands of the copper atom.

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Estrogen Biosynthesis. Stereospecific Distribution of Tritium in Testosterone-1α,2α-t_2*

Yoshio Osawa† and Donald G. Spaeth‡

ABSTRACT: The stereospecific distribution of tritium in testosterone-1α,2α-t_2 produced by tris(triphenylphosphine)rhodium chloride catalytic reduction of 17β-hydroxy-1,4-androstadien-3-one with tritium was determined to be 43% at C-1α, 7% at C-1β, 43% at C-2α, and 6% at C-2β. The chemical determination was made by mild and drastic alkali treatment; chloranil followed by 2,3-dichloro-5,6-dicyanobenzoquinone dehydrogenation; and dienone–phenol rearrangement. Biochemical aromatization with human placental microsomes gave 17β-estradiol retaining 87% of the original tritium. Bromination of the 17β-estradiol afforded 2,4-dibromo-17β-estradiol with retention of 44% of the original tritium. The results showing removal of the 1β-equatorial and the 2β-axial hydrogens are compatible with previous work carried out using human placental preparations, but appear not to agree with work done using ovarian tissue. The advantages of using testosterone-1α,2α-t_2 in place of testosterone-1β,2β-t_2 for metabolic studies are discussed.

The interpretation of the results of investigations into the metabolism of testosterone[1] labeled with tritium is dependent upon the stereochemical distribution of the tritium in the steroid. The testosterone-1,2-t_2 previously used has been produced by palladium–charcoal catalytic reduction of 17β-hydroxy-1,4-androstadien-3-one with tritium (Osinski and Vanderhaeghe, 1960). The tritium label has been reported to be distributed 40–60% at C-1 and 60–40% at C-2. Of the tritium at C-1, 75–83% is β oriented, but of the tritium at C-2, only 58% is β (Brodie *et al.*, 1962, 1969a; Brodie, 1967). Placental aromatization of androgens removes the 1β hydrogen (Brodie *et al.*, 1968; Townsley and Brodie, 1968; Morato *et al.*, 1962) and the 2β hydrogen (Fishman *et al.*, 1969; Fishman and Guzik, 1969; Brodie *et al.*, 1969b). A loss of 82% of tritium of testosterone-1β,2β-t_2 was reported in this sequence (Fishman *et al.*, 1969) whereas previously reported data (Brodie *et al.*, 1969a) indicated that 69% of the tritium should have been lost. Therefore, it appears that there is an inconsistency of the distribution of tritium among testosterone-1β,2β-t_2 preparations. Reports that catalytic hydrogenation with tris(triphenylphosphine)rhodium chloride is a cis process (Biellmann and Jung, 1968; Osborn *et al.*, 1966; Jardine *et al.*, 1967) occurring at the α face of 1,4-androstadiene-3,17-dione (Djerassi and Gutzeviller, 1966) sug-

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† Faculty Research Awardee PRA-72 of the American Cancer Society; to whom to address correspondence.

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[1] Abbreviations used are: testosterone, 17β-hydroxy-4-androsten-3-one; E_2 (17β-estradiol), 1,3,5(10)-estratriene-3,17β-diol; chloranil, 2,3,5,6-tetrachlorobenzoquinone; DDQ, 2,3-dichloro-5,6-dicyanobenzoquinone; diglyme, bis(2-methoxyethyl)ether.

TABLE I: Chemical and Biochemical Determination of the Distribution of Tritium in Testosterone-1α,2α-t_2 Produced by Tris-(triphenylphosphine)rhodium Chloride Catalytic Reduction of 17β-Hydroxy-1,4-androstadien-3-one.

Reactant[a]	Method	Product[a]	$^3H/^{14}C$ (dpm)	^3H Remaining (% Original)	^3H Removed (% Original)	Assigned Position
T(R-1)[b]	Drastic alkali	T(R-4)	8.1	51		1α + 1β
					49	2α + 2β
T(R-1)	Mild alkali	T(R-2)	15.1	94		1α + 1β + 2α
					6	2β
T(R-4)	Chloranil dehydrogenation	Δ^6-T	8.0	50		
					<1	
Δ^6-T	DDQ dehydrogenation	$\Delta^{1,6}$-T	1.5	<10		1β
					>40	1α
$\Delta^{1,6}$-T	Dienone–phenol rearrangement	MeΔ^6–E_2Ac_2	0.1	<1		Nonspecific
					<9	1β
T(R-1)	Placental aromatization	E_2-1	13.8	87		1α + 2α
					13	1β + 2β
T(R-2)		E_2-2	13.9	87		1α + 2α
					7	1β
T(R-4)		E_2-4	7.1	44		1α
					7	1β
E_2-1	Bromination	Br_2E_2-1	7.0	44		1α
					43	2α
E_2-2		Br_2E_2-2	7.0	44		1α
					43	2α
E_2-4		Br_2E_2-4	7.1	44		1α

[a] T, testosterone: Δ^6-T, 17β-hydroxy-4,6-androstadien-3-one; $\Delta^{1,6}$-T, 17β-hydroxy-1,4,6-androstatrien-3-one; MeΔ^6-E_2Ac_2, 1-methyl-1,3,5(10),6-estratetraene-3,17β-diol diacetate; E_2, 17β-estradiol; Br_2E_2, 17β-2,4-dibromoestradiol. [b] The $^3H/^{14}C$ (dpm) of T(R-1) was 16.0.

gested that testosterone-1α,2α-t_2 could be synthesized from the appropriate precursor. Such testosterone would be a useful tool for metabolic studies. This paper reports the distribution of tritium at C-1α, C-1β, C-2α, C-2β, and other nonspecific positions in testosterone. It also reports the advantage of working with testosterone that contains very little of its radioactivity in a labile (2β) position and retains the majority of its label after metabolic alterations.

Results

Distribution of Tritium by Chemical Methods. Testosterone produced by using tris(triphenylphosphine)rhodium chloride catalyst was designated R. Refluxing this testosterone (R-1, $^3H/^{14}C$ 16.0) with 2% potassium hydroxide in 50% aqueous methanol resulted in a loss of 49% of the tritium to give R-4 ($^3H/^{14}C$ 8.1) (Table I). Similar treatment of testosterone formed by palladium–charcoal catalysis (P-1, $^3H/^{14}C$ 32.0) showed a loss of 50% tritium to give P-4 ($^3H/^{14}C$ 16.0) (Table II). The amount of tritium removed by mild alkali enolization (Malhotra and Ringold, 1964) was determined. The exchange reaction was carried out for 17 hr at room temperature under nitrogen atmosphere. At 1.5, 5, and 17 hr, the percentage of tritium lost was 4, 5, and 5, respectively. Addition of methanol, followed by refluxing for 60 min increased the loss of tritium to 49%.[2] The tritium lost in the mild alkali treatment was attributed to the 2β position. R-1 afforded R-2 which after purification showed a loss of 6% of tritium ($^3H/^{14}C$ 15.1, Table I). Subtraction of 6% from 49% yields 43% which was estimated as the amount of tritium incorporated into the 2α position.[3]

Dienone-phenol rearrangement of 17β-hydroxy-1,4,6-androstatrien-3-one-1-t to replace the tritium at C-1 with the C-19 methyl group would determine the amount of tritium at positions other than C-1 and C-2 (Brodie *et al.*, 1969a). Testosterone (R-4, $^3H/^{14}C$ 8.1) was dehydrogenated with chloranil to give 17β-hydroxy-4,6-androstadien-3-one ($^3H/^{14}C$ 8.0). Further dehydrogenation of this compound

[2] The report of Bordwell and Scamehorn (1968) of the lack of steric hindrance and stereoelectronic control in alkali-induced α-proton exchange from 4,4-disubstituted cyclohexanones would imply that the mild alkali treatment of testosterone would not selectively remove (exchange) the 2β proton (*cf.* Fishman *et al.*, 1969). We observed, however, that the nonalcoholic condition of Malhotra and Ringold (1964) would produce a limited removal of tritium. The difference in the amount of tritium lost upon aromatization of R-1 and R-4 was the same as that removed by mild alkali treatment, suggesting that this process was highly stereoselective. The presence of any amount of methanol (as in the condition used by Bordwell and Scamehorn, 1968) caused an increased loss of tritium which approached 50% of the tritium originally present.

[3] Any tritium present at C-4 and C-6 would also be lost during drastic alkalai treatment and included in this value. However, 2,4-dibromination of estradiol (E_2-1, Table I), which would affect only the values for the 2α and 4 positions, resulted in the same 43% loss. Thus the labeling at C-6 was estimated as negligible. Similarly monobromination of E_2-1 according to Utne *et al.* (1968) gave 4-bromoestradiol (mp 211–212°, λ_{max} 282 nm (ϵ 2560), and 288 nm (ϵ 2520)), which showed no significant loss of tritium ($^3H/^{14}C$ 13.5). Therefore, the labeling at C-4 in the original testosterone was also considered as negligible.

TABLE II: Distribution of Tritium in Testosterone-1,2-t_2. Produced by Tris(triphenylphosphine)rhodium Chloride (R) and Palladium-Charcoal (P) Catalytic Reduction of 17β-Hydroxy-1,4-androstadien-3-one.

Position Containing ^3H	% ^3H R	P	P-B[a]	P-F[b]	Procedure
1α + 1β	51	50	57		Drastic alkali
1α + 2α	87	25	31	18	Aromatization
1β + 2β	13	75	69	82	Aromatization
1α	43	13	13[c]	12[d]	Bromination
2α	43	12	18	6	Calculated
1β	7	38	44	(38)[e]	Calculated
2β	6	37	25	(44)[e]	Calculated
1β/1α	0.16	2.9	3.4	(3.2)[e]	Calculated
2β/2α	0.14	3.1	1.4	(7.3)[e]	Calculated
β/α	0.15	3.0	2.2	4.6	Calculated

[a] Data from Brodie *et al.* (1969a). [b] Data from Fishman *et al.* (1969a). [c] Microbiological dehydrogenation used in place of bromination. [d] Biochemical hydroxylation used in place of bromination. [e] Values calculated on the assumption that the 1β/1α is in agreement with the 1β/1α values determined for P and P-B.

with DDQ yielded 17β-hydroxy-1,4,6-androstatrien-3-one ($^3H/^{14}C$ 1.5) (Table I). Dehydrogenation by DDQ has been reported to be stereoselective for the removal of the 1α hydrogen (Ringold *et al.*, 1962; Brodie *et al.*, 1962). Since the predominant position of tritium is in 1α position, any decrease in the stereospecificity of the reaction would cause a decrease in the amount of tritium lost. Therefore, the 81% loss upon DDQ dehydrogenation represents the minimum amount of tritium located at the 1α position of 17β-hydroxy-4,6-androstadien-3-one. This 81% loss is equivalent to 40% of the original amount of tritium present in R-1. The remaining tritium, 10% of original, is the maximum amount incorporated into 1β position. This value includes any tritium not exchangeable in base and not at C-1 or C-2 positions. Treatment of 17β-hydroxy-1,4,6-androstatrien-3-one ($^3H/^{14}C$ 1.5) with sulfonic acid in acetic anhydride produced 1-methyl-1,3,5(10),6-estratetraene-3,17β-diol diacetate ($^3H/^{14}C$ 0.1). This remaining tritium, less than 1%, not located at C-1 or C-2, is considered nonspecific labeling. The tritium lost during the dienone–phenol rearrangement, 9% of original, is the maximum amount of tritium at C-1β position.

Distribution of ^3H by Biochemical Methods. To substantiate the distribution pattern elucidated by chemical methods, testosterone (untreated, R-1 and P-1; mild alkali treated, R-2; and drastic alkali treated, R-4 and P-4) was converted into E_2 with the aromatase in human placental microsomes (Ryan, 1959). The results are presented in Tables I and II. Assuming that the aromatization mechanism is completely stereospecific for the 1β,2β hydrogen removal, the loss of 13% tritium in the conversion of R-1 into E_2 is attributed to be the amount of tritium located in the 1β plus 2β positions. R-2 and R-4, which contain no tritium at 2β, lost 7% tritium during aromatization. Therefore the 1β position contains 7% of the tritium, and the 2β position contains 6% (Table I). P-1 lost 75% ($^3H/^{14}C$ decreased from 32.0 to 8.1), and P-4 lost 38% of original tritium ($^3H/^{14}C$ decreased from 16.0 to 3.8) under the same condition. This, in addition to drastic alkali treatment, indicated that the palladium–charcoal hydrogenation introduced 37% of the tritium at 2β, 38% at 1β, 13% at 2α, and 12% at 1α.

The tritium at C-2 and C-1 in E_2 produced above corresponds to the tritium at C-2α and C-1α of the substrate testosterone. Displacement of the C-2 hydrogen would give the amount of tritium located at the 2α position. Bromination of E_2 produced from R-1 and R-2 forming 2,4-dibromo-E_2 caused the $^3H/^{14}C$ to be halved (Table I). This showed that tritium was equally distributed between the 1α and 2α positions, and that testosterone produced by the homogeneous (tris(triphenylphosphine)rhodium chloride) catalysis contained 43% tritium at 2α.[3] The remaining 44% of the tritium is located at the 1α and nonspecific sites. Similar bromination of E_2 derived from P-1 decreased the $^3H/^{14}C$ from 8.1 to 4.2, indicating a 12% loss of the original tritium. This represents an incorporation of 12% tritium at 2α, and 13% at the 1α plus nonspecific positions under the heterogeneous (palladium–charcoal) catalysis. The bromination of E_2 derived from R-4 and P-4 should not change the $^3H/^{14}C$ since all C-2 tritium had been removed by base equilibration prior to aromatization. Upon bromination of these materials, no change was observed. 2,4-Dibromo-E_2 derived from P-4 contained 13% of the original tritium, and that derived from R-4 contained 44%. Acetylation of E_2 and 2,4-dibromo-E_2 caused no change in the $^3H/^{14}C$ or in the specific activity, establishing that none of the nonspecific tritium was located at the hydroxyl hydrogens.

The stereospecificity of the catalytic tritiation is indicated by the ratio of tritium located in the β face to the tritium in the α face. Testosterone-1α,2α-t_2 produced by homogeneous catalytic reduction had a 1β/1α of 0.15 and a 2β/2α of 0.16. Heterogeneous catalytic reduction produced testosterone-1β,2β-t_2 with a 1β/1α of 2.9 and a 2β/2α of 3.1.

Discussion

Summarizing the chemical and biochemical data for testosterone-1α,2α-t_2, it was concluded that C-1α and C-2α each contained 43%; C-1β, 7%; C-2β, 6% and the nonspecific positions, 1% of the original amount of tritium. The results obtained by the chemical methods except for those by DDQ dehydrogenation are in excellent agreement with that derived from the biochemical method. Brodie *et al.* (1962) reported that DDQ dehydrogenation at C-1α of 5α-androstane-3,17-dione was 96% stereospecific compared to microbiological dehydrogenation with *Bacillus sphaericus*. Our results showed DDQ dehydrogenation of 17β-hydroxy-4,6-androstadien-3-one to be 92% stereospecific relative to the microsomal aromatization. The excellent agreement of the results by the independent methods would indicate that the chemical and biochemical reactions except for DDQ dehydrogenation are probably completely stereoselective and therefore the actual distribution of tritium is that given above.

The investigations into steroid metabolism, especially those concerning the mechanism of estrogen biosynthesis have been hindered by the use of the 4-ene-3-one steroids tritiated mostly in 1β and 2β positions. The extreme lability of the 2β tritium can complicate interpretations of the results. To avoid this difficulty one can remove all radioactivity from the C-2 position by treatment with alkali. This produces steroids labeled at C-1, and naturally these do not afford any information regarding events at C-2. Interpretations of

reactions using steroids labeled predominantly in the 1α,2α positions would produce only a slight error in the assigned distribution, even if a complete loss of the 2β tritium occurred during handling. The retention of most of the tritium after aromatization of the 1α,2α-labeled substrate is also advantageous to metabolic studies involving estrogen biosynthesis (*cf.* Fishman *et al.*, 1969). The use of 1α,2α-deuterated androstenedione (Djerassi and Gutzviller, 1966) is not suitable for metabolic studies because of the relatively large quantities required for mass spectrometric analysis.

The controversy that is found in the literature (Brodie *et al.*, 1969a; Fishman *et al.*, 1969) concerning the distribution of tritium in testosterone-1,2-t_2 and the mechanism of hydrogenation of the 1,4-dien-3-one using palladium–charcoal catalyst may be resolved by the work reported here. The 50% distribution between C-1 and C-2, and the similar 1β/1α and 2β/2α ratios for testosterone P (Table II) are readily interpreted as supporting simultaneous 1,2-cis addition to the C-1 double bond. Brodie *et al.* (1969a) proposed that their observed differences between 1β/1α and 2β/2α distribution (Table II, P-B) is indicative of 1,4 addition to the 1-ene-3-one system under palladium–charcoal catalysis. Since the 1β/1α ratio for P (2.9) and P-B (3.4) are similar, and our experiments have indicated the extreme lability of the 2β tritium, we suggest that possibly some of the tritium was lost from P-B during purification. Testosterone, P-1 ($^3H/^{14}C$ 32.0), which had been purified through paper chromatography using the Zaffaroni system (*cf.* Experimental Section) and on drastic alkali treatment had given P-4 ($^3H/^{14}C$ 16.0), lost 5 and 13% of tritium during paper chromatography using Whatman No. 1 and 2 papers developed with the Bush system. The $^3H/^{14}C$ were determined after elution of the total peak area. The loss in Bush system may be caused by alkali contained in the paper. The testosterone which had lost 13% of tritium by paper chromatography showed only 16% of original tritium at C-2β when analyzed by the mild alkali treatment. This indicates that the tritium loss during purification could be attributable to the unstable 2β position. In fact, commercial testosterone-1,2-t_2, claimed to be 97% radiochemically pure, retained 56% of tritium by drastic alkali treatment. This preparation probably had lost some tritium from 2β before the distribution study. This corresponds to 57% at C-1 of P-B. An assumption was made that the tritium at C-1 in P-B was originally 50% of total, instead of the reported 57%, and the difference was attributed to loss of a portion of the 2β-tritium. The adjusted distribution then would be 39% at C-1β, 34% at C-2β, 11% at C-1α, and 16% at C-2α. The data presented under P-F in Table II are quoted from Fishman *et al.* (1969). The values in parentheses were calculated on the basis that the 1β/1α ratio of P-F was similar to the 1β/1α ratios of P and P-B. Thus, three preparations of testosterone-1β,2β-t_2 studied independently are in reasonable agreement on the distribution of tritium resulting from 1,2-cis addition preferentially to the β side.

The stereoselective attack on the α face of the steroid by the homogeneous catalyst (R) and the β face by the heterogeneous catalyst (P) in the tritiation of 17β-hydroxy-1,4-androstadien-3-one may be explained by the conformation of the substrate and the relative size of the active catalysts. The molecular structure of 17β-hydroxy-1,4-androstadien-3-one elucidated by a crystal structure determination (Dr. W. L. Duax, private communication) is shown in Figure 1 as it would appear approaching a planar surface either α or β to ring A. The dihedral angle of 128° between the least-squares plane of ring A and the least-squares plane of C-6 through C-17 indicates that ring A of this steroid is bent extraordinarily downward. As seen readily from the figure, the D ring would prevent proximity of the α side of the C-1 double bond to the activated tritium on the large surface of the insoluble palladium catalyst. The homogeneous Wilkinson catalyst, on the other hand, being a dissolved organic molecule, is probably not influenced by ring D and therefore able to attack the α side of the ring A.

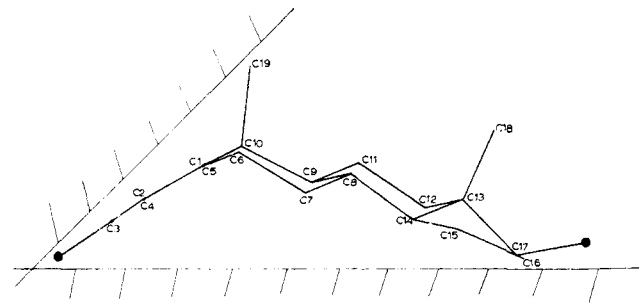

FIGURE 1: The molecular structure of 17β-hydroxy-1,4-androstadien-3-one projected parallel to the C(5)–C(1) vector situated on planar surfaces either α or β to the steroid.

It is interesting to note that Bell and Kodicek (1970) reported 38–44% 1α and 16–22% 1β-tritium labeling with heterogeneous catalysis and 47–49% 1α and 15–17% 1β with homogeneous catalysis of 1,4-cholestadien-3-one. They suggested that the change of side chain causes a sufficient difference in the accessibility of the α face, accounting for the inverted stereoselectivity of labeling at C-1 of 17β-hydroxy-1,4-androstadien-3-one and 1,4-cholestadien-3-one with heterogeneous catalyst. Although the conformation of the cholesterol side chain is difficult to ascertain it is unlikely that the side chain alters the ring conformation or gives a steric hindrance on the β side of ring A to retard the β side approach to the catalyst. It appears rather to indicate that the difference of polarity between the oxygen and the hydrocarbon group at C-17 influences the affinity of that end of the substrate for the catalyst, accounting for the difference in stereoselectivity.

Axelrod and Goldzieher (1962) reported the C-1α,C-2β removal from androgens during aromatization with normal ovaries based on 50% retention of tritium. Though the assignment of the labeling was on the basis of a wrong assumption (Morato *et al.*, 1962), the results are not reconciled with the C-1β,C-2β-elimination mechanism which occurs in placental aromatization. It was further complicated by the report based on the metabolism of testosterone-1β,2β-t_2 (Axelrod and Goldzieher, 1965) that there are possibly two different mechanisms of dehydrogenation with polycystic ovaries. Experiments using testosterone-1α,2α-t_2 are in progress in an attempt to clarify the difference between placental and ovarian aromatases. Our preliminary results indicate that ovarian aromatization also involves removal of the C-1β and C-2β hydrogens.

Experimental Section

Testosterone-1α,2α-t_2 (R) was prepared through cooperation of Dr. E. A. Evans of Amersham–Searle Inc., using tris(triphenylphosphine)rhodium chloride as the catalyst for the reduction of 17β-hydroxy-1,4-androstadiene-3-one. Testosterone-4-^{14}C and testosterone-1β,2β-t_2 (P) formed by palladium–charcoal catalytic reduction were purchased from

New England Nuclear Corp. and from Amersham–Searle Inc. After mixing portions of R and P with testosterone-4-^{14}C, the testosterone was purified by paper chromatography using Zaffaroni system described below. After scanning the chromatogram with an Actigraph III (Nuclear-Chicago Corp.), the peak area was eluted with methanol. The eluent was kept as a stock solution and the $^{3}H/^{14}C$ was determined. The purity was further determined by crystallization to a constant specific activity to be more than 95%. The radioactivity of all samples was determined by use of a Parkard Tri-Carb liquid scintillation spectrometer Model 3375 and a scintillation fluid consisting of 30 g of naphthalene, 500 ml of dioxane, 100 ml of 2-ethoxyethanol, and 25 ml of Packard 25× concentrated liquid scintillator (3.125 g of 2,5-diphenyloxazole and 0.0625 g of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene). Samples were counted for sufficient time to reach to more than 10,000 gross counts so that the standard error in the tritium channel was less than 1%. The ultraviolet spectra were obtained in 95% ethanol using a Cary-14 spectrophotometer. The infrared spectra were determined as KBr micropellets using a Perkin-Elmer spectrophotometer, Model 421. Melting points were obtained in capillary tubes on a Mel-Temp apparatus and are uncorrected.

Paper Chromatography. ZAFFARONI SYSTEM. Whatman No. 1 paper was dipped in formamide–methanol (1:1, v/v) before sample was applied, and development was carried out with benzene–hexane (1:1, v/v) at room temperature.

BUSH SYSTEM. Samples were applied to Whatman No. 1 or 2 paper which was then equilibrated at 37° for 72 hr in an atmosphere of methanol–water (85:15, v/v) saturated with heptane, and developed for 4 hr at 37° with heptane saturated with methanol–water (85:15, v/v). Steroids were detected by radioactivity, ultraviolet absorption, and by 1% $K_3Fe(CN)_6$–1% $FeCl_3$ (1:1, v/v).

Mild Alkali Treatment. Metallic sodium (26 mg) was dissolved with stirring in a solution of 0.5 ml of water in 4 ml of diglyme freshly distilled over lithium aluminum hydride. This solution was purged with N_2 for 20 min and added to 2 ml of diglyme containing R-1 ($^{3}H/^{14}C$ 16.0, 1.2×10^{6} dpm of ^{14}C) from which methanol had been removed as an azeotropic mixture with benzene. The combined solution was stirred at room temperature under an atmosphere of nitrogen. Aliquots (25 μl) were placed in scintillation counting vials, acidified with hydrochloric acid, dried at 50° with N_2, dissolved in a few milliliters of water, dried, and the $^{3}H/^{14}C$ was determined. After the $^{3}H/^{14}C$ (15.1) had not changed for three consecutive aliquots (1.5, 5, 17 hr), the reaction was terminated by rapid neutralization and flash evaporation. Testosterone with 91% of the original ^{14}C was recovered after paper chromatography using the Zaffaroni system.

Drastic Alkali Treatment. To 10 ml of 2% potassium hydroxide in 50% aqueous methanol solution, purged with N_2 was added R-1 ($^{3}H/^{14}C$ 16.0, 90,000 dpm of ^{14}C) or P-1 ($^{3}H/^{14}C$ 32.0, 350,000 dpm of ^{14}C). The solution was refluxed for 3 hr, and aliquots were taken and treated as above. The solution was neutralized with 2 N hydrochloric acid, and then concentrated by evaporation at 50° with N_2. The aqueous remainder was extracted four times with 8 ml of benzene. The combined benzene extract was subjected to paper chromatography using the Zaffaroni system. Elution of testosterone with methanol yielded 95% of the original ^{14}C (R-4, $^{3}H/^{14}C$ 8.1 and P-4, $^{3}H/^{14}C$ 16.0).

Chloranil Dehydrogenation. To a solution of 400 mg of R-4 ($^{3}H/^{14}C$ 8.1, 240,000 dpm of ^{14}C/mmole) in 35 ml of *tert*-butyl alcohol was added 1.45 g of chloranil and the mixture was refluxed for 3 hr. The solvent was removed by flash evaporation and the residue taken up in 20 ml of chloroform. After filtration, the chloroform was washed three times with water, three times with 5% aqueous sodium hydroxide, three times with water, and concentrated. Silica gel thin-layer chromatography (1.0 mm) developed with 25% ethanol in benzene, and two crystallizations from aqueous ethanol gave 130 mg of 17β-hydroxy-4,6-androstadien-3-one: mp 200–202°; λ_{max} 284 nm (ϵ 25,000) (lit. mp 204–205°; λ_{max}^{EtOH} 284 nm (ϵ 26,300), Djerassi *et al.*, 1950a); $^{3}H/^{14}C$ 8.0, 235,000 dpm of ^{14}C/mmole.

DDQ Dehydrogenation. To 128 mg of 17β-hydroxy-4,6-androstadien-3-one ($^{3}H/^{14}C$ 8.0, 235,000 dpm of ^{14}C/mmole) dissolved in 20 ml of dioxane purified according to Fieser (1955) was added 712 mg of DDQ. After refluxing for 26 hr the solution was cooled, filtered, and dried with N_2 stream at 50°. The material was taken up in methylene chloride, washed four times with water, three times with 5% aqueous sodium hydroxide, four times with water. The residue from evaporation of the solvent was chromatographed on silica gel thin-layer chromatography (0.75 mm) developed with 30% ethyl acetate in benzene. Two ultraviolet-absorbing bands were detected. The slower moving band, after two recrystallizations from acetone–hexane afforded 14 mg of 17β-hydroxy-1,4,6-androstatrien-3-one: mp 145–147°: λ_{max} 299 nm (ϵ 12,500), 252 nm (ϵ 8800) (lit. mp 143–149 and 156–158° (Neudert and Ropke, 1965); 151–153°, λ_{max}^{EtOH} 299 nm (ϵ 13,000), 256 nm (ϵ 9500) (Djerassi *et al.*, 1950a)); $^{3}H/^{14}C$ 1.5, 232,000 dpm of ^{14}C/mmole.

Dienone–Phenol Rearrangement. To 5.8 mg of 17β-hydroxy-1,4,6-androstatrien-3-one ($^{3}H/^{14}C$ 1.5, 232,000 dpm of ^{14}C/mmole) dissolved in 1 ml of acetic anhydride was added 3.9 mg of *p*-toluenesulfonic acid. After heating with steam for 5.5 hr, and dilution with 2 ml of water, the flocculant precipitate which appeared upon cooling was collected by centrifugation. Recrystallization from hexane yielded 7.2 mg of 3,17β-diacetoxy-1-methylestra-1,3,5(10),6-tetraene: mp 117–118°; λ_{max} 264 nm (ϵ 9500) (lit. mp 116–117°; λ_{max}^{EtOH} 264 nm (ϵ 9100) (Djerassi *et al.*, 1950b)); $^{3}H/^{14}C$ 0.1, 240,000 dpm of ^{14}C/mmole.

Aromatization. A modification of Ryan's (1959) procedure was used to aromatize testosterone to E_2. To a solution of R-1 ($^{3}H/^{14}C$ 16.0, 240,000 dpm of ^{14}C) in 2 drops of propylene glycol was added 2 mg of human placental microsomes homogenized in 1 ml of 0.067 M disodium monopotassium phosphate buffer (pH 7.4) containing 0.5 mg of NADPH. The incubation was carried out at 37° for 1.5 hr. Water (1 ml) was added and the suspension was extracted three times with 5 ml of ethyl acetate. E_2 (25 μg) was added to the extract which was then chromatographed on Whatman No. 1 paper with Zaffaroni system. E_2($^{3}H/^{14}C$ 13.9, 74,000 dpm of ^{14}C) was eluted with methanol. Additional E_2 (100 mg) was added to the methanolic eluate and recrystallized from aqueous methanol to a constant specific activity (206,000 dpm of ^{14}C/mmole, $^{3}H/^{14}C$ 13.8).

Bromination of 17β-Estradiol. Bromine–methanol (1:5, v/v) was added to a solution of 47 mg of the E_2 obtained above in 4 ml of methanol, until the bromine color persisted for 5 min (20 drops). The solution was concentrated to 1 ml with N_2 stream and the product precipitated with addition of 5 ml of water. Recrystallization of the precipitate from 70% ethanol gave 52 mg of 2,4-dibromo-E_2 ($^{3}H/^{14}C$ 7.0, 221,000 dpm of ^{14}C/mmole): mp 215–218° (lit. mp 218–219°, Slaunwhite and Neely, 1962; 223–226°, Utne *et al.*, 1968; 215–216°, Woodward, 1940).

2,4-Dibromo-17β-estradiol Diacetate. To 52 mg of 2,4-dibromo-E_2 (mp 223–225°, prepared as described above) dissolved in 0.7 ml of pyridine was added 0.3 ml of acetic anhydride. After standing at room temperature for 18 hr the solution was evaporated with N_2 stream to 0.1 ml. Water (4 ml) was added, and the crystals were collected by centrifugation. Recrystallization of the product from 3 ml of ethanol afforded 51 mg of 2,4-dibromo-E_2 diacetate: mp 179–180°; ν^{KBr} 1723 (17-acetate) and 1778 cm^{-1} (3-acetate); λ_{max} 275 nm (ϵ 615), λ_{sh} 280 nm (ϵ 550) (lit. mp 167–168°; $\lambda_{max}^{alcohol}$ 273 nm (ϵ 750), Slaunwhite and Neely, 1962). The nuclear magnetic resonance spectrum in $CDCl_3$ (60 MHz, Varian A-60) had peaks at δ 7.51 (S, C-1H), 2.38 (S, 3-acetate Me), 2.06 (S, 17-acetate Me), and 0.82 (S, 18-Me). Similar treatment of radioactive 2,4-dibromo-E_2 (34 mg, $^3H/^{14}C$ 7.0, 221,000 dpm of ^{14}C/mmole) gave 37 mg of 2,4-dibromo-E_2 diacetate: mp 174–176°, $^3H/^{14}C$ 7.0, 227,000 dpm of ^{14}C/mmole. This showed no mixture melting point depression with the authentic material described above.

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