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THE PREPARATION AND ENZYMATIC C-1,2-DEHYDROGENATION OF ESTR-4-ENE-3,17-DIONE-1-^{3}H (83%-β)

H. J. BRODIE
with the technical assistance of
P. A. WARG

Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts 01545

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Abstract—The preparation of 19-hydroxyandrost-4-ene-3,17-dione-1-^{3}H (83%-β) and estr-4-ene-3,17-dione-1-^{3}H (83%-β) from androst-4-ene-3,17-dione-1-^{3}H (83%-β) is described. Estr-4-ene-3,17-dione-1-^{3}H (83%-β) was converted to estrone using a respiring culture of *B. sphaericus* (ATCC 7055) with loss of 21% of the tritium, showing that the stereospecific preference of the micro-organism's C-1,2-dehydrogenase for C-1α-hydrogen abstraction is the same as for the C-10 methyl analog.

IN PREVIOUS communications the syntheses of 1β- and 1α-tritiated androstenedione (androst-4-ene-3,17-dione) were reported[1] as well as their use in studying the mechanism of estrogen biosynthesis.[2]

It was found that during aromatization of these substrates by human placental microsomes, the C-1β-hydrogen was eliminated suggesting that there was enzymatic or C-19-oxygen participation in its removal.[2]

19-Norandrostenedione (estr-4-ene-3,17-dione) and 19-hydroxyandrostenedione (19-hydroxyandrost-4-ene-3,17-dione) are other important precursors to estrogens in both mammalian and microbiological systems. While the role of the former compound is unknown, the latter compound, or a related derivative, is a very likely intermediate on the major pathway from C-19 compounds to estrogens in humans. The synthesis of these compounds, labelled stereoselectively at C-1, was undertaken to have them available for further studies on the mechanism of aromatization. The procedure described in this paper was chosen mainly because the distribution of tritium could be obtained readily, obviating the necessity of establishing it in the products. In addition, both products as well as other possibly useful ones could be obtained in the same synthetic sequence.

The starting material, testosterone-1-^{3}H (83%-β) was obtained by reduction of 17β-hydroxyandrosta-1,4-dien-3-one with carrier-free tritium using Pd-C catalyst and dioxan.[3] The mixture of Δ^1- and Δ^4-reduced products was chromatographed on a partition column to obtain testosterone-1,2-^{3}H which was refluxed with base to remove tritium at C-2.

Before proceeding with the synthesis, it was necessary to ascertain that the remaining tritium was essentially at C-1 and was stereoselectively oriented in the *beta* position. To do this, a portion of the testosterone-1,2-^{3}H was oxidized to

[1] H. J. Brodie, M. Hayano and M. Gut, *J. Amer. Chem. Soc.* **84**, 3766 (1962).
[2] T. Morato, K. Raab, H. J. Brodie, M. Hayano and R. I. Dorfman, *J. Amer. Chem. Soc.* **84**, 3764 (1962).
[3] cf. P. Osinski and H. Vanderhaeghe, *Rec. Trav. Chim.* **79**, 216 (1960).

androstenedione which was then equilibrated with base to remove labile tritium. This material was reacted with chloranil (2,3,5,6-tetrachloroquinone)[4] to give androsta-4,6-diene-3,17-dione with little loss of tritium. Reaction of the $\Delta^{4,6}$-diene with DDQ (dichlorodicyanoquinone)[5] gave androsta-1,4,6-triene-3,17-dione with tritium loss of 20% (Table, No. 2). (Recrystallization data establishing specific activities of reactants and products for this and the other transformations described are given in the table.) As described in the next paragraph, there is a similar amount (17%) lost when androstenedione-1-^{3}H is converted microbiologically to androsta-1,4-diene-3,17-dione-1-^{3}H and it is no doubt due to stereoselective tritium abstraction at C-1α. On treating the triene with acid, a "dienone-phenol" rearrangement occurred to give 3-acetoxy-1-methyl-estra-1,3,5(10),6-tetraen-17-one in which the C-1-hydrogen (tritium) is displaced by the C-10 methyl group.[1,6] Tritium (95%) was removed in these operations (Table, No. 3). If the original C-1,2-tritiated material contained twice as much tritium,[7] the original catalytic introduction of tritium at C-1,2 probably caused about 3% exchange of tritium into nonenolizing positions. Another batch of androstenedione-1-^{3}H prepared from a separate reduction and carried through the same procedure to the 1-methyl-Δ^4-estrone acetate showed complete loss of tritium.[1] Thus introduction of tritium by catalytic hydrogen exchange during reduction does not appear to occur to any appreciable extent with 17β-hydroxyandrosta-1,4-dien-3-one.

To establish the orientation of tritium at the C-1 position, another portion of the androstenedione-1-^{3}H was dehydrogenated at C-1,2 by incubation with a respiring culture of *B sphaericus* (ATCC 7055). The loss of tritium in the androsta-1,4-diene-3,17-dione product was 17% (Table, No. 4). The dehydrogenase preferentially removes the 1α-hydrogen and the loss of tritium essentially represents the amount at that position.[1,8]

The equilibrated testosterone (I), shown by the above operations to contain 83% 1β- and 17% 1α-tritium, was deconjugated to 17β-hydroxyandrost-5-en-3-one by treatment with potassium-t-butoxide in diglyme for three hours followed by rapid acidification with acetic acid.[9] The product consisted of an 80:20 mixture of Δ^5- and Δ^4-androstenediones as judged by the relative intensity of the carbonyl bands at 1710 and 1660 cm^{-1}. Although careful chromatography with ethyl acetate-benzene mixtures on a silica gel column yielded the pure Δ^5-compound, separation was facilitated by reducing the crude material with lithium aluminum hydride to give an 80:20 mixture of Δ^5- and Δ^4-androstene-3,17-diols. Selective dehydration of the allylic hydroxyl with hydrochloric acid in aqueous methanol gave a mixture of 17β-hydroxyandrosta-3,5-diene and unreacted 3β,17β-hydroxyandrost-5-ene (IIa). These were easily separated by silica gel column chromatography.

After acetylation of the Δ^5-diol, the diacetate IIb was converted to 5α-bromo-3,17-

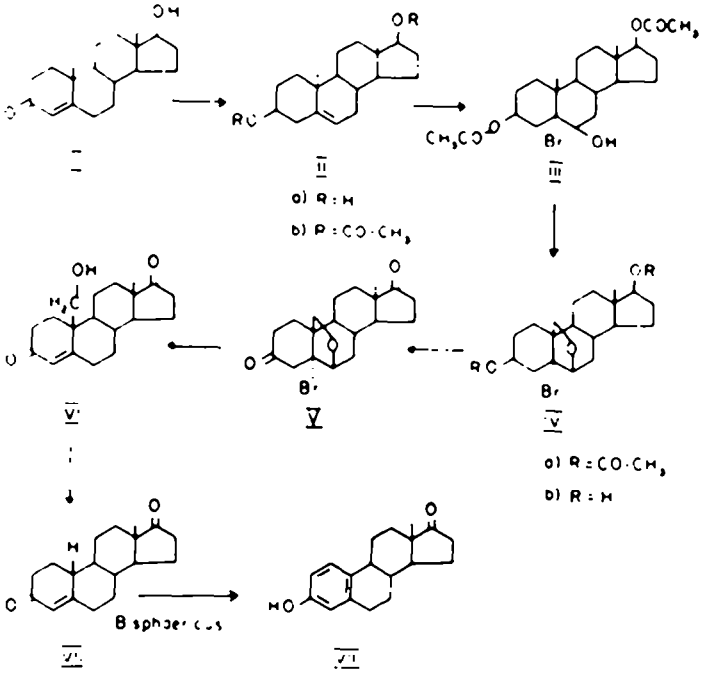
[4] E. J. Agnello and G. D. Laubach, *J. Amer. Chem. Soc.* **82**, 4293 (1960).
[5] D. Burn, D. N. Kirk and V. Petrow, *Proc. Chem. Soc.* 14 (1960).
[6] E. Caspi, P. K. Grover and Y. Shimizu, *J. Amer. Chem. Soc.* **86**, 2463 (1964).
[7] The loss of tritium from androstenedione-1,2-^{3}H on base equilibrium was not determined with this particular preparation. However, other preparations show that 40–60% of the tritium is in exchangeable positions. See Ref. 1 and M. Gut and M. Hayano, *Advances in Tracer Methodology* Edited by S. Rothchild, Vol. I; p. 60. Plenum (1963).
[8] H. J. Ringold, M. Hayano and V. Stefanovic, *J. Biol. Chem.* **238**, 1960 (1963).
[9] H. J. Ringold and S. K. Malhotra, *Tetrahedron Letters* 669 (1962).

TABLE 1. RECRYSTALLIZATION DATA OF SPECIFIC ACTIVITIES

Reactant	Specific activity dpm/μmole	Product	Specific activity dpm/μmole	Conditions*	% Tritium loss
1. Testosterone	1st 17,360 2nd 16,700	Androst-4-ene-3,17-dione	1st 17,900 2nd 17,100	CrO_3 oxidation and equilibration	≈0
2. Androst-4-ene-3,17-dione	1st 5,860 2nd 5,660 3rd 5,765	Androsta-1,4,6-triene-3,17-dione	1st 4,556 2nd 4,580	Chloranil – DDQ (C)	20
3. Androst-4-ene-3,17-dione	1st 5,860 2nd 5,660 3rd 5,765	3-Acetoxy-1-methyl-estra-1,3,5(10),6-estratetren-17-one	1st 460 2nd 290 3rd 308	Same as above + H^+ (C)	95
4. Androst-4-ene-3,17-dione	1st 8,480 2nd 8,560	Androsta-1,4-diene-3,17-dione	1st 7,210 2nd 7,130	*B. sphaericus* (D)	17
5. Estr-4-ene-3,17-dione	1st 8,362 2nd 8,364	Estrone	1st 6,870 2nd 7,060 3rd 6,640	*B. sphaericus* (K)	21
6. Estrone	1st 1,518 2nd 1,564	Estrone Acetate	1st 1,608 2nd 1,664	Acetylation (K)	
7. Estrone Acetate	1st 1,608 2nd 1,664	Estrone	1st 1,459 2nd 1,630 3rd 1,617	Hydrolysis (K)	≈0

* Letters in brackets refer to section in Experimental.

diacetoxy-6β-hydroxyandrostane (III) with N-bromoacetamide.[10] This compound decomposed on thin-layer plates but could be purified by silica gel column chromatography. It was converted to 5α-bromo-3β,17β-diacetoxy-6,19-epoxyandrostane (IVa) using lead tetraacetate, calcium carbonate and iodine in benzene. Of the many conditions reported for effecting this conversion,[11] the conditions outlined above and detailed in the experimental worked best in our hands. Both the calcium carbonate and iodine contributed to the improvement of yield. The use of UV light (Hanovia-"Barbadas") increased the production of by-products.



Following hydrolysis of the bromoether diacetate IVa with potassium carbonate in aqueous methanol to the corresponding diol IVb, oxidation with chromic oxide in acetic acid gave the 3,17-diketone V which on standing dehydrobrominated to give 6,19-epoxyandrost-4-ene-3,17-dione. The crude bromoether diketone V was cleaved with zinc and acetic acid by the method of Ueberwasser *et al.*[12] to give 19-hydroxy-androst-4-ene-3,17-dione (VI). This was oxidized to the 19-oic acid with chromic oxide and sulfuric acid in acetone and decarboxylated by heating with 2 N methanolic hydrochloric acid[13] to give 19-norandrostenedione VII.

As was described above, when androstenedione-1-^{3}H was dehydrogenated to $\Delta^{1,4}$-androstadienedione with *B. sphaericus* 17% of the tritium was lost, corresponding

[10] V. Grenville, D. K. Patel, V. Petrow, I. A. Staurt-Webb and D. M. Williamson, *J. Chem. Soc.* 4105 (1957).

[11] For a review see K. Heusler and J. Kalvoda, *Angew. Chem.* (Intern. Ed.) 3, 525 (1964).

[12] H. Ueberwasser, K. Heusler, J. Kalvoda, Ch. Meystre, P. Wieland, G. Anner and A. Wettstein, *Helv. Chem. Acta* 46, 344 (1963).

[13] H. Hagiwara, S. Noguchi and M. Nishikawa, *Chem. Pharm. Bull. Tokyo* 8, 84 (1960); *Chem. Abstr.* 55, 3653c (1961).

to the amount in the C-1α-position. In the absence of a C-10 methyl group, C-1,2-dehydrogenation of 19-norandrostenedione gives, at least formally, the $\Delta^{1,4}$-compound, which rapidly isomerizes to estrone (VIII). When 19-norandrostenedione (VII) was incubated with *B. sphaericus*, the estrone isolated had 21% less tritium (Table, No 5). The absence of the C-10 methyl group did not inhibit the enzymatic reaction itself or substantially change the stereochemical requirement for C-1α-hydrogen (tritium) abstraction. It would appear that the enzymatic dehydrogenation is useful in determining the distribution of label at C-1 in 19-nor compounds synthesized by alternate methods involving hydrogen addition to C-1 and in providing information on the stereochemistry of the reactions themselves.

EXPERIMENTAL

The synthesis of 19-norandrostenedione from androstenedione was first carried out on non-radioactive material. The physical constants for the intermediates and final products reported refer to material obtained in this sequence. The identities of the corresponding radioactive compounds II through V are inferred from the similarity of the reactions employed and chromatographic data. Additional evidence for structures VI and VII was obtained as shown in the Experimental. The physical data reported for the microbiological reactions, equilibrations and estrone acetate formation were obtained directly from the radioactive materials since they were of much lower specific activity.

IR spectra: (KBr) on a Perkin-Elmer "Infracord" Spectrophotometer; UV Spectra: a Cary model 11MS or a Perkin-Elmer model 202 spectrophotometer.

All solvents used were reagent grade or were distilled in glass before use. Steriods were obtained from commercial sources except where noted. They were purified by recrystallization and by TLC and showed one spot in I vapor when chromatographed in thin layers of silica gel.

Radiochemical analyses for specific activities: a Packard model 314–DC liquid scintillation counter on weighed amounts of steroid (100–500 μg) in 10 ml of scintillation fluid. [Scintillation fluid: 4 g PPO (2,5-diphenyloxazole) and 116 mg POPOP[1,4-bis-2-(5-phenylozazolyl)-benzene], both from Pilot Chemicals, Watertown, Mass., in one kg of toluene (Matheson scintillation grade)] Efficiences were determined with an external standard. Test determinations with internal standards on androstenedione and estrone and on residues from paper and thin-layer, silica-gel elutions did not show any quenching effects.

"Thin-layer" plates were prepared with a silica gel slurry (Brinkman GF) formulated according to the distributor's instructions with the applicator opening set at 250 μ. "Thick-layer" plates were prepared from a silica gel slurry of 120 g of absorbent and 260 ml of 34.6% aqueous methanol with the applicator opening set at 1 mm. This formulation prevents cracking of the absorbent layer on drying.

Location of steroids. Δ^4-3-One compounds were located by viewing plates with short wave lengths UV light; others were located with I vapor. In preparative work, only the edges of the band were exposed and their areas were not included in the recovery. This precaution may be omitted for many compounds because iodine vapor is usually non-destructive.

TLC of radioactive material were scanned using the Vanguard 885 plate scanner attachment to the model 880 chromatograph scanner.

Solvent for elution from plates—20% acetone-benzene. Solvent mixtures are given in percent by volume.

A. *Testosterone*-1-^{3}H (83%-β). Testosterone-1,2-^{3}H was prepared by reduction of 17β-hydroxy-androsta-1,4-dien-3-one with carrier-free tritium on 5% Pd–C catalyst.[8] The product was chromatographed on a Bush B-2 celite partition column. The material having the mobility of testerone (100mc) was diluted with 500 μg of testosterone and chromatographed on a thin-layer plate in 30% AcOET benzene. The UV absorbing band corresponding to testosterone was eluted and the eluate was evaporated to dryness. The residue was dissolved in 2% (w/v) KOH in 500 ml of 50% MeOH-water and refluxed under N until no further exchange of tritium with solvent was noted. This was determined by periodically withdrawing aliquots into scintillation vials, acidifying with HCl, evaporating to dryness and then adding scintillation fluid and counting. The testosterone, now tritiated essentially at C-1 (for proof, see Section C) was recovered from the acidified reaction mixture by extraction with

benzene after most of the MeOH was evaporated under red. press. The dried benzene extract was concentrated and chromatographed on a thin-layer plate in 25% acetone–benzene. The eluted UV absorbing band contained 20 mc. It chromatographed as one radioactive zone with the mobility of testosterone in the paper systems ligroin (60–90° always used) -propylene glycol and benzene–ligroin–MeOH–water (4:1:4:1). A portion was diluted with authentic testosterone and crystallized from benzene–hexane to constant specific activity of 16,760 dpm/μmole (Table). Refluxing of this material for 2 hr with KOH in aqueous MeOH as indicated above gave, on isolation and recrystallization, material of constant specific activity of 17,100 dpm/μmole, indicating complete exchange. Proof for the position and distribution of tritium is given in Sections C and D.

B. *Androst-4-ene*-3,17-*dione*-1-^{3}H (83%-β). Testosterone-1-^{3}H (365 mg, 17,100 dpm/μmole) was dissolved in 35 ml acetone and oxidized to androstenedione-1-^{3}H with 5·25 ml of 8N chromic acid in H_2SO_4. The reaction was carried out at 4° for 30 min and after warming the mixture to room temp, the product was isolated in the usual way.[14] The crude material was equilibrated with methanolic KOHaq as detailed in Section A. Chromatography on thick-layer plates, elution of the UV absorbing, androstenedione zone and recrystallization as above gave material of constant specific activity of 17,580 dpm/μmole. The IR spectrum and m.p. agreed with authentic material. Proof for the position and distribution of tritium is given in Sections C and D.

C. 3-*Acetoxy*-1-*methyl*-*estra*-1,3,5(10),6-*estratetraen*-17-*one* via *androsta*-1,4,6-*triene*-3,17-*dione*-1-^{3}H. A portion of the androst-4-ene-3,17-dione-1-^{3}H prepared above was diluted with non-radioactive material to give a specific activity of 5765 dpm/μmole. To 150 mg of this dissolved in 13 ml of t-butyl alcohol, was added 544 mg of chloranil (2,3,5,6-tetrachloroquinone).[4] The mixture was refluxed for 3 hr and then evaporated to dryness and taken up in chf. The chf extract was washed with water, 5% NaOHaq and again with water, 3 times each. After evaporating the solvent, the residue (119 mg) was placed on "thick-layer" plates and chromatographed in 25% AcOEt-benzene. The main UV absorbing band, with an R_f similar to androstenedione, was eluted to give 90 mg of crystals, which showed UV absorbance at 283 mμ. Recrystallization gave material with a specific activity of 5480 dpm/μmole, m.p. 168–169°; ε ~ 26,000. This material (67 mg) and DDQ (2,3-dichloro-5,6-dicyanoquinone 356 mg) was added to 10 ml of purified[15] dioxan and the mixture was heated under reflux for 30 hr, cooled and filtered. The residue was washed with dichloromethane and the filtrate was extracted with the same solvent. The combined organic extracts were washed with water, 5% NaOHaq and finally with water 3 times each. After removal of the solvent by evaporation, the residue was chromatographed on a thick-layer plate in 30% AcOEt–benzene. Elution of the main UV absorbing zone gave 45 mg of androsta-1,4,6-triene-3,17-dione-1-^{3}H with characteristic absorption maxima at 222, 256 and 298 mμ.[16] This was recrystallized to a constant specific activity of 4580 dpm/μmole (Table), m.p. 151–152·5° lit.[17] 152–153°; loss of tritium from androst-4-ene-3,17-dione, 20%.

To 2 ml of Ac_2O was added 40 mg of androsta-1,4,6-triene-3,17-dione and 10 mg of *p*-toluenesulfonic acid. The mixture was heated on a steam bath for 5 hr, cooled and then treated with water. A ppt of 3-acetoxy-1-methyl-estra-1,3,5(10),6-tetraen-17-one formed, which was collected and recrystallized from MeOH to a constant specific activity of 308 dpm/μmole. 95% loss of tritium from androst-4-ene-3,17-dione-1-^{3}H, λ_{max}^{MeOH} 222, 265 mμ; m.p. 151–154°, lit.[16] m.p. 152–153; λ_{max}^{EtOH} 222, 264 mμ.

D. *Androsta*-1,4-*diene*-3,17-*dione*-1-^{3}H *using* B. sphaericus. A portion of testosterone-1-^{3}H (Section A) was diluted with authentic material, oxidized to androst-4-ene-3,17,-dione-1-^{3}H as in Section B, and then recrystallized to a constant specific activity of 8560 dpm/μmole. A starter culture of 1 ml of *B sphaericus* (ATCC 7055) was added to a sterile soln of 400 ml of distilled water containing 0·3% yeast extract (Difco) and 0·5% "N-Z Case" peptone (Sheffield). After 48 hr of agitation on a rotary shaker at 30°, 58 mg of the steroid in 3 ml of 50% EtOH–propylene glycol was added and incubated with the respiring organism for 24 hr. After adding 200 ml AcOEt with shaking, the mixture was filtered through a double layer of cheese cloth. The residue was extracted with AcOEt and discarded. The aqueous layer was extracted 3 times with 200 ml portions of AcOEt and the combined organic extracts

[14] K. Bowden, I. M. Heilbron, E. R. H. Jones and B. E. L. Weedon, *J. Chem. Soc.* 39 (1946).

[15] L. Fieser, *Experiments in Organic Chemistry* (3rd Edition) p. 285. Heath, Boston (1955).

[16] C. Djerassi, G. Rosenkranz, J. Romo, St. Kaufmann and J. Pataki, *J. Amer. Chem. Soc.* 72, 4534 (1950).

[17] C. Djerassi, G. Rosenkranz, J. Romo, J. Pataki and St. Kaufmann, *J. Amer. Chem. Soc.* 72, 4540 (1950).

were washed with 2% $NaHCO_3$aq and then with water. The dried soln was evaporated and the resultant residue was placed on a thick-layer plate and developed in 20% AcOEt–benzene. The UV absorbing zone corresponding to androsta-1,4-diene-3,17-dione was eluted and rechromatographed in the same system. No starting compound was noted and recovery from the second chromatography was 26 mg; yield 45%. Recrystallization from benzene–hexane gave material of constant specific activity of 7130 dpm/μmole (Table); 17% loss of tritium. The IR spectrum was identical with that of authentic androsta-1,4-diene-3,17-dione.

E. *3β,17β-Dihydroxyandrost-5-ene-1-^{3}H (83%-β)* (IIa). Testosterone-1-^{3}H (83%-β) prepared as in Section A was diluted with non-radioactive material to give an approximate specific activity of 19·6 mc/mole. The steroid (200 mg) was added to 500 mg of potassium butoxide in 2 ml of diglyme [bis(2-methoxymethyl)ether]. After being stirred in N for 2 hr, the mixture was cooled to 5° and treated with 10 ml of ice-cold 50% AcOH. The thick mustard-colored mixture clarified and after 5 min a cream-colored ppt formed. This was collected by vacuum filtration and was washed with small amounts of water. The IR spectrum showed a strong peak at 1710 cm^{-1} (3-one, ring A saturated; relative % T ~ 10) and a weak peak at 1650 cm^{-1} (Δ^4-3-one; relative % T ~ 28) along with a OH peak at 3350 cm^{-1}. The estimated deconjugation was greater than 90% as judged by comparison of the spectrum of material from trial experiments with prepared mixtures. A sample of the pure Δ^4-3-one for the comparison was obtained by silica gel column chromatography using AcOEt–benzene as the developing medium. The material, on standing in a stoppered vial at 4° for one week, isomerized about 50% to the Δ^4-3-one structure.

A soln of the mixture of Δ^4- and Δ^4-3-one in 25% diethyl ether–THF was added dropwise to 10 ml of an ether soln of LAH (assay[18] ~1·1 mmole/ml) under anhydrous conditions. After complete addition, the heterogeneous mass was stirred for 2·5 hr. The excess hydride then was decomposed by the slow addition of a sat Na_2SO_4aq with cooling. After removal of most of the organic layer by decantation, the residue was diluted with 3N HCl and extracted with warm (40–50°) AcOEt. Evaporation of the combined organic extracts yielded a solid which was a mixture of two materials as judged by a TLC developed in 30% benzene–AcOEt and treated with I vapor. The main zone, R_f = 0·55, had the mobility of 3β,17β-dihydroxyandrost-5-ene and the minor zone, R_f = 0·59, corresponded to the mobility of the Δ^4-isomer. The IR spectrum of the mixture showed peaks at 3430 and 3000 cm^{-1} (OH) and no absorption band in the carbonyl region. It was warmed to 50–60° for 3 hr in 40 ml of 2N HCl in 25% MeOH–water. After adjusting the pH to 6 and extracting with AcOEt and evaporating, the residue was chromatographed on a silica gel column developed with AcOEt–benzene mixtures. Compound IIa was eluted in the 90% AcOEt–benzene fraction. The IR spectrum, m.p. (181–183°) and running rate on thin-layer plates (70% AcOEt–benzene) agreed with that of authentic standard. The diol IIa was added to 2 ml of 50% pyridine–Ac_2O and allowed to stand overnight. Addition of MeOH and, after 0·5 hr, evaporation and purification on thick-layer plates in 50% AcOEt–benzene gave IIb (72 mg) 36% overall yield from I. The IR spectrum, m.p. and the chromatographic data above agreed with that of authentic material.

F. *3β,17β-Diacetoxy-5α-bromo-6β-hydroxyandrostane-1-^{3}H (83%-β)* (III). To 72 mg of IIb was added 428 mg of authentic material to give specific activity of 2·8 mc/mmole. This diluted material was dissolved in 10 ml of purified[18] dioxan containing 0·25 ml of 0·3M perchloric acid. To the cooled (15°), stirred mixture was added 44 mg of N-bromoacetamide in 3 portions at 10 min intervals. After 30 min, the dark yellow soln was poured into 5% (W/V) of Na_2SO_3 in ice water. A white ppt formed which was taken up with 80 ml portions of cold dichloromethane. The extract was washed twice with cold 2% $NaHCO_3$aq and with ice water until neutral to pH paper. After drying and concentrating the extract to an oil, trituration with cold ether gave crystals, yield 607 mg, 96%. This was used directly for the next reaction. Preliminary preparations appeared to be over 90% pure as judged by inspection of thin-layer plates in I after development in AcOEt–benzene. On elution and re-chromatography, multiple zones appeared indicating decomposition. A sample was purified on a silica gel column developed with AcOEt–benzene. The material in the 40% eluate was recrystallized from CH_2Cl_2–hexane. The m.p. (155–158°, lit.[19] 159°) IR and NMR spectrum [τ · 4·58 (3α-H), 5·75 (6α-H), 8·65 (19-CH_3), 9·19 (18-CH_3)] and thin-layer chromatographic running rate agreed with an authentic sample.[19]

[18] Reduction with Complex Metal Hydrides, N. G. Gaylord, page 9, Interscience Publishers, New York City (1956).

[19] As noted in the Acknowledgement section, the compound was obtained from Dr. M. Akhtar, then of the Institute for Research and Medicine, Cambridge, Massachusetts.

G. *5α-Bromo-3β,17β-diacetoxy-6,19-epoxyandrostane-1-³H* (83%-β) (IVa). To 135 ml of dry, freshly distilled benzene was added 1·2 g anhydrous $CaCO_3$ and 3·3 g lead tetraacetate (dried over KOH under red press.). After refluxing for 15 min 800 mg of III and 1·1 g of I were added and the reflux was continued for an additional 2·25 hr. After cooling and filtering, the soln was washed with 5% $Na_2S_2O_3$ and then with water. Drying and evaporating the solvent gave a residue which was chromatographed on two thick-layer silica gel plates in 50% ether–hexane. The broad bands, which became visible by exposing the edges to I vapor (R_f ~ 0·38), were eluted with 20% MeOH–acetone; yield 432 mg; 54%. The IR spectrum, m.p. (177–179°; lit.[50] ~178–180°) and chromatographic mobility in the above system agreed with that of an authentic sample.[10]

H. *5α-Bromo-3β,17β-dihydroxy-6β,19-epoxyandrostane-1-³H* (83%-β) (IVb)[50]. To 294 mg K_2CO_3 in 30 ml 20% MeOHaq was added 432 mg of IVa. The mixture was flushed with N and stirred for 36 hr. After neutralizing with 0·2N HCl and removing most of the MeOH by evaporation, the aqueous mixture was extracted with CH_2Cl_2 and AcOEt. Evaporation yielded crystals, m.p. 234–236°, lit. 236–237°) The IR spectrum showed a strong broad OH band at 3400 cm^{-1} and no band for acetate. TLC in 20% acetone–benzene showed one spot in I vapor, R_f ~ 0·2, yield 320 mg, 90%.

I. *19-Hydroxyandrost-4-ene-3,17-dione-1-³H* (83%-β) (VI). To 10 ml of a 0·91N chromic acid soln [3 g CrO_3/100 ml of 0·25% AcOHaq] stirred at 15° was added 320 mg of IVb. The rate of oxidation was estimated by titrating aliquots with $Na_2S_2O_3$ and I. After 0·5 hr, when oxidation appeared to be complete, 1% $Na_2S_2O_3$ was added and the resultant green soln was extracted with benzene. The extract was neutralized by washing with solns of Na_2CO_3 and $NaHCO_3$ and finally with water. After drying over Na_2SO_4, evaporation of the benzene at low temp gave a yellow oil which crystallized on trituration with ether. The IR spectrum showed a very strong peak at 1750 cm^{-1} attributable to the unresolved 3-keto and 17-keto groups and a weak band at 1495 cm^{-1} characteristic of the 6,19-ether structure. On standing for a day, even in the cold, the material turned brown indicating decomposition with Br evolution. It was assigned the structure of V and was used immediately for the following reaction.

To 2·5 g of Zn dust, which had been treated for 10 min with 5% AcOH and dried on a porous plate, was added 10 ml AcOH and the crude V. The mixture was heated for 30 min on a steam bath, cooled, filtered and evaporated to dryness under red press. The residue was taken up in CH_2Cl_2 and washed with $NaHCO_3$aq and water and then dried. TLC in 25% acetone–benzene showed a UV absorbing zone with the mobility of VI. Elution of this band gave 77 μc; overall yield, 2·05% from IIb. Due to low conversion in the last step, the yield was 20% of what was expected from experience gained in preliminary experiments.

In the non-radioactive preparation, the IR spectrum of the product was identical to that of an authentic material.[51] In addition to the chromatographic data above, the radioactive material had the mobility of 19-hydroxyandrostene-dione on paper in the systems toulene–propylene glycol and Bush B-3 (Ligroin–benzene–MeOH–water, 6 67 : 3 33 .8 :2). Co-crystallization with authentic 19-hydroxyandrostenedione from benzene–hexane did not remove the radioactivity. The material was converted to VIII under the same oxidation conditions as was 19-hydroxyandrostenedione (Section J).

J. *Estr-4-ene-3,17-dione-1-³H* (83%-β) (VII).[10] To 5·4 mg (50 μc) of VI in 2 ml acetone was added 0·1 ml of chromic acid in acetone. After 0·5 hr at 10°, an excess of isopropyl alcohol was added and the resultant green mixture was diluted with an equal volume of benzene and washed 2 times with sat $(NH_4)_2SO_4$aq. The dried organic extract was evaporated to give the 19-oic acid (3450 cm^{-1} broad) as an oil. This was dissolved in 10 ml of 17% conc. HCl in MeOH and after heating under reflux for 1 hr, the mixture was cooled, neutralized and extracted with AcOEt. On evaporation to dryness the residue was chromatographed on thick-layer plates in 20% AcOEt–benzene. The UV absorbing band corresponding to estr-4-ene-3, 17-dione was eluted and recrystallized from benzene–hexane. The IR spectrum and m.p. agreed with that of authentic material. The radioactive material showed one main radioactive peak having the mobility of authentic material in the thin-layer system above and in the paper systems ligroin–propylene glycol and Bush A (heptane–MeOH–water: 5:4:1). When a portion of the radioactive material was diluted with authentic estr-4-ene-3,17-dione and recrystallized from benzene–hexane, the radioactivity did not separate from the crystals (Table).

[50] J. Kalvoda, K. Heusler, H. Uberwasser, G. Anner and A. Wettstein, *Helv. Chem. Acta* **46**, 1361 (1963).

[51] As noted in the Acknowledgement section, this compound was obtained from Drs. A. Bowers and R. I. Dorfman of Syntex, Inc

K. *Estrone from estr-4-ene-3,17-dione-1-^{3}H (83%-β).* A culture of *B. sphaericus* was grown as described in Section D. To the 48 hr growth was added 60 mg of estr-4-ene-3,17-dione-1-^{3}H (83%-β) (specific activity—8364 dpm/μmole) dissolved in 50% EtOH-propylene glycol. The culture was incubated for an additional 24 hr and the crude products were isolated as described. The residue from the AcOEt extract was placed on thick-layer plates and developed in 2% EtOH-chf. The areas corresponding to estrone was eluted to give 30 mg. This was rechromatographed on plates in 50% ether-hexane. A scan for radioactivity showed only one peak and its mobility corresponded to that of estrone. The radioactive zones were eluted and the solid obtained was recrystallized from EtOH to constant specific acitivity (6640 dpm/μmole; 21% loss of tritium: see Table).

The estrone was diluted with authentic material to give a constant specific activity of 1564 dpm/μmole (Table). To 35 mg of this material was added 2 ml of 50% pyridine-Ac_2O and the mixture was allowed to stand at room temp in a stoppered flask for 48 hr. After the addition of 2 ml of MeOH, the mixture was allowed to stand for 0·5 hr and was then evaporated to dryness. The residue was chromatographed on thin-layer plates in 7% AcOEt-benzene. The estrone acetate band, located with I vapor using a standard, was eluted and the 24 mg obtained was recrystallized from EtOH to a constant specific activity of 1664 dpm/μmole. This material was hydrolysed in 10 ml of 2% KOH (W/V) in 50% MeOH-water for 3 hr under N. The mixture was neutralized to pH 6–7 and after concentration to remove the MeOH it was extracted with ether. After drying and evaporating the solvent, the residue was recrystallized from EtOH to a constant specific activity of 1617 dpm/μmole (no loss). The IR spectrum agreed with that of authentic material.

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