more polar solvents, but this material was not used for the following transformation.

The benzene eluted 1.5-g. sample was dissolved in acetic acid and shaken overnight at room temperature with 0.4 g. of platinum oxide catalyst. The product was saponified with 5% aqueous ethanolic potassium hydroxide for 24 hr., concentrated, and water added. Extraction with ether left no residue and acidification of the ether solution followed by extraction with ether, extraction with bicarbonate, acidification, and re-extraction with ether furnished, after drying and evaporation, 0.86 g. of acid.

A 0.55-g. sample of this acid was esterified with ethereal diazomethane solution and the crude ester was fractionated by distillation affording 0.31 g. of material with b.p. 60°/0.01 mm. and 0.20 g. with b.p. 93°/0.01 mm. The lower boiling fraction exhibited no infrared hydroxyl absorption, a strong band at 5.76 μ, a shoulder at 5.85 μ and a relatively weak band at 6.08 μ.

Anal. Calcd. for $C_{11}H_{18}O_3$: C, 66.64; H, 9.15; O, 24.21; C-methyl, 7.57; methoxyl, 15.66. Found: C, 66.81; H, 8.80; O, 24.70; C-methyl, 6.48; methoxyl, 16.30.

As the infrared spectrum indicated the presence of some remaining unsaturated ester, 250 mg. of the above analytical sample was again hydrogenated with acetic acid and platinum oxide at 30° and the ester isolated in the usual way; $\lambda_{max}^{capill.}$ 5.75 μ, no selective ultraviolet absorption. A 200-mg. aliquot of the ester was saponified by heating under reflux for 5 hr. with 5% ethanolic potassium hydroxide affording 120 mg. of acid, which was dissolved in 2.5 cc. of water; 2 drops of phenolphthalein indicator was added followed by 1*N* aqueous sodium hydroxide until a faint pink color persisted after heating to about 80°. Two drops of 0.1*N* hydrochloric acid was added to make the solution slightly acidic followed by the dropwise introduction of 10.5 g. of *S*-benzylthiourea in 2.5 cc. of water. The *S*-benzylthiuronium salt precipitated immediately and, after cooling in ice, it was filtered and recrystallized from water; m.p. 135.5–136.5°.

Anal. Calcd. for $C_{18}H_{26}N_2O_3S$: C, 61.75; H, 7.43; N, 8.00; O, 13.72; S, 9.15. Found: C, 61.55; H, 7.40; N, 8.27; O, 13.64; S, 8.97.

DETROIT, MICH.
MEXICO, D. F.

[CONTRIBUTION FROM THE PROCESS DEVELOPMENT AND RESEARCH DEPARTMENT OF THE SCHERING CORP.]

Microbiological Transformation of Steroids. VIII. 16β-Hydroxylation and Other Transformations of Testosterone by *Wojnowicia graminis*

HERSHEL L. HERZOG, MARGARET JEVNIK GENTLES, ALVIN BASCH, W. COSCARELLI, MARY ELLEN ATNARISH ZEITZ, AND WILLIAM CHARNEY

Received April 11, 1960

Incubation of testosterone with *Wojnowicia graminis* (CBS) affords a variety of transformation products, including 16β-hydroxytestosterone, 16α-hydroxytestosterone, 16-ketotestosterone, 6β-hydroxytestosterone, 6β-hydroxy-4-androstene-3,17-dione, 14α-hydroxy-4-androstene-3,17-dione, 4-androstene-3,17-dione, and two additional, incompletely characterized crystalline products, one of which appears to be hydroxylated at 12α-. From a limited study of the action of the same organism on 4-androstene-3,17-dione, testosterone, and 16α-hydroxytestosterone were isolated. This is the first example of microbiological 16β-hydroxylation.

From the work of McAleer and Dulaney[1] it is known that *Wojnowicia graminis* (CBS) hydroxylates progesterone at C_{21}, affording thereby desoxycorticosterone. We speculated that hydroxylation of a steroidal substrate of only 19 carbon atoms might also occur preferentially at a methyl site rather than a methylene or methylidyne site, and we might thereby effect 18- or 19-hydroxylation. With this in mind we have incubated testosterone (I) with *W. graminis* in a 1% yeast extract-1% cerelose medium for seventy-two hours and isolated the steroidal products by extraction with chloroform.

After very careful chromatography over Florisil and in a toluene–propylene glycol partition system, we were able to isolate nine crystalline transformation products (in addition to recovered starting material), and to identify completely seven of them. These, in order of increasing polarity with respect to partition in the toluene–propylene glycol paper chromatographic system, were: 4-androstene-3,17-dione (II), uncharged testosterone (I), 16-ketotestosterone (III), 6β-hydroxy-4-androstene-3,17-dione (IV), 14α-hydroxy-4-androstene-3,17-dione (V), 16β-hydroxytestosterone (VI), 6β-hydroxytestosterone (VII), and 16α-hydroxytestosterone (VIII).

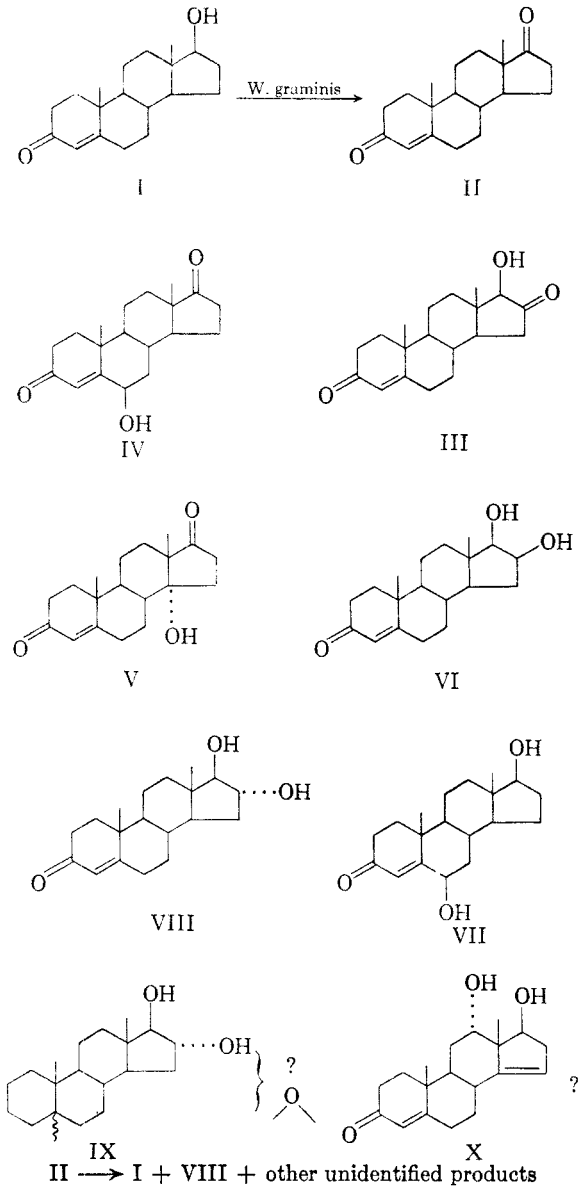
Identifications of I and II were easily accomplished by comparison of melting point, paper chromatographic mobilities and infrared spectra with those of authentic samples.

Our sample of III had m.p. 153–155°, $[\alpha]_D^{24}$ −54° (chloroform), $\lambda_{max}^{CH_3OH}$ 240 mμ (ϵ = 16,600). A five-membered ring carbonyl band was noted in the infrared spectrum at 5.70 μ. Both blue and red tetrazolium reagents gave positive tests with III, indicating the presence of an α-ketol system. Since the ultraviolet spectrum of III did not shift in alkaline solution,[2] the ketol must have been in the D-ring. Meyer and Lindberg[3] describe 16-ketosterosterone, m.p. 152–158°, $[\alpha]_D^{25}$ −52° (chloroform). Upon

(1) W. J. McAleer and E. L. Dulaney, *Arch. Biochem. Biophys.*, 62, 109 (1956).

(2) A. S. Meyer, *J. Org. Chem.*, 20, 1240 (1955).

(3) A. S. Meyer and M. C. Lindberg, *J. Am. Chem. Soc.*, 76, 3033 (1954).



II ⟶ I + VIII + other unidentified products

acetylation III afforded an acetate, m.p. 194–197° $[\alpha]_D^{25}$ −44° (chloroform), with infrared bands at 5.66 μ and 5.74 μ characteristic for the absorptions of a five-membered ring ketone and a neighboring acetate group. Meyer and Lindberg[3] report 16-ketotestosterone acetate, m.p. 195–199°, $[\alpha]_D^{25}$ −29° (chloroform). The alternative possibilities for the structure of III, namely 16α-hydroxy- and 16β-hydroxy-4-androstene-3,17-dione are excluded by a rotational argument.[4]

Compound IV was obtained only in a mixture with nonultraviolet absorbing impurities ($\lambda_{max}^{CH_3OH}$ 236 mμ, (ε = 8,400). From the location of the maximum and the behavior of the ultraviolet spectrum of the mixture on treatment with alkali according to Meyer,[2] an hydroxyl group must be placed at the 6-position in the ultraviolet-absorbing component of the mixture. This ultraviolet-absorbing component had the same migration rate as an authentic sample of 6β-hydroxy-4-androstene-3,17-dione[5] in toluene-propylene glycol paper chromatography.[6] From these data we are fairly sure that 6β-hydroxy-4-androstene-3,17-dione is a product of the fermentation. This conclusion is further reinforced in the subsequent discussion by the isolation and identification of the closely related 6β-hydroxytestosterone (VII).

The next, more polar fermentation product isolated (V) melted at 236–246°, $[\alpha]_D^{24}$ +166° (chloroform), $\lambda_{max}^{CH_3OH}$ 239 mμ (ε = 15,700) (no shift of ultraviolet in alkali), λ_{max}^{Nujol} 2.94 μ and 5.72 μ. From the infrared and ultraviolet data we inferred we were dealing with an hydroxylated 4-androstene-3,17-dione, the new hydroxyl group or groups being in the C or D rings or at C-18. No reaction occurred between V and acetic anhydride in pyridine solution, from which it was inferred that the new hydroxyl group must be at 11β- and/or 14α-. Both 11β-hydroxy-4-androstene-3,17-dione[7] and 14α-hydroxy-4-androstene-3,17-dione[8] are known. From a comparison of the physical constants the latter alternative seemed more likely [11β-ol,[7] m.p. 200°, $[\alpha]_D^{23}$ +220° (chloroform); 14α-ol[8] m.p. 257°, $[\alpha]_D^{25}$ +173° (chloroform)]. Comparison of the infrared spectrum of V with that from an authentic sample of 14α-hydroxy-4-androstene-3,17-dione[9] revealed that the compounds were identical.

Compound VI melted at 179–182°, $[\alpha]_D^{25}$ +101° (chloroform), $\lambda_{max}^{CH_3OH}$ 241 mμ (ε = 15,900) (no shift of ultraviolet in alkali). No five-membered ring carbonyl band was apparent in the infrared spectrum (hydroxyl and Δ^4-3-keto groups were evident). From the polarity of VI it appeared that two hydroxyl groups were present, both of which had to be in rings C or D. One was probably at 17- as the starting material contained this group. Acetylation of VI with acetic anhydride in pyridine afforded an hydroxyl-free product, from which it was inferred that the second hydroxyl group was not at 11β- or 14α-. A diagnostic cleavage of VI with sodium bismuthate[10] resulted in the formation of at least four new products (paper chromatogram), from which it was concluded that the

(4) $\Delta M^{16H-16\beta OH}$ = −49; $\Delta M^{16H-16\alpha OH}$ = −34 (both values derived from estrone and appropriately substituted derivatives). $\Delta M^{16H-16C=O}$ = −472 (given by Meyer[2] for testosterone to 16-ketotestosterone). The rotation of III as prepared by us is in good agreement with Meyer's value.

(5) D. H. Peterson, *et al.*, *J. Am. Chem. Soc.*, **75**, 5768 (1953).

(6) R. B. Burton, A. Zaffaroni, and E. H. Keutmann, *J. Biol. Chem.*, **188**, 763 (1951).

(7) T. Reichstein, *Helv. Chim. Acta*, **20**, 978 (1937); M. E. Herr and F W. Heyl, *J. Am. Chem. Soc.*, **75**, 5927 (1953).

(8) A. F. St. André, et al., *J. Am. Chem. Soc.*, **74**, 5506 (1953).

(9) Kindly provided by Prof. E. Testa, Lepetit, Milan, Italy.

(10) C. J. W. Brooks and J. K. Norymberski, *Biochem. J.*, **55**, 371 (1953).

second hydroxyl group was at C_{16}. Both 16α-[11] and 16β-hydroxytestosterone[12] are known. The former melts at 191–192°, $[\alpha]_D^{25}$ +80° (chloroform), while the latter melts at 172–173° (no rotation reported). The respective diacetates are also known: 16α- m.p. 139–140°, $[\alpha]_D^{25}$ +12° (chloroform)[11], 16β- m.p. 199° (no rotation reported).[12] The diacetate of VI melted at 201–202.5°, $[\alpha]_D^{25}$ +88° (chloroform). The physical constants of VI are in much better accord with 16β-hydroxytestosterone and the structure was so assigned. A final confirmation was obtained by the conversion of VI into an acetonide with acetone–hydrochloric acid according to Butenandt.[12] The product which resulted melted at 185–187°, while Butenandt reported 183–184°. No acetonide would be expected to form from 16α-hydroxytestosterone. The formation of VI from I is the first microbiological 16β-hydroxylation to be reported.[13]

Compound VII melted at 215–218°, $[\alpha]_D^{25}$ +29° (chloroform), $\lambda_{max}^{CH_3OH}$ 236 mμ (ε = 13,600). The short wave length of the ultraviolet maximum was most consistent with 6β-hydroxylation. This assignment was confirmed by the alkaline ultraviolet spectrum of VII which displayed the characteristic shift.[2] The infrared spectrum contained no five-membered ring carbonyl band from which it was inferred that the 17-hydroxyl group was intact. Analysis was consistent with the empirical formula $C_{19}H_{28}O_3$, a monohydroxylated testosterone. The physical constants of VI were in good agreement with those reported for 6β-hydroxytestosterone[14] [m.p. 216–222°, $[\alpha]_D^{25}$ +32° (chloroform)]. Acetylation of VI afforded an hydroxyl-free product, m.p. 132–135° (phase change 119°). The known diacetate melted at 135–143° (phase change 127°). All the evidence accordingly supports the assigned structure.

Compound VIII, the most polar of the crystalline products isolated, melted at 183–187°, $[\alpha]_D^{25}$ +63° (dioxane), $\lambda_{max}^{CH_3OH}$ mμ (ε = 14,000). The alkaline ultraviolet displayed no shifts in maximum or intensity. It appeared that the 17-hydroxyl was unaltered (no five-membered ring carbonyl in infrared) and that one or more hydroxyl groups had been introduced (polarity). The new group or groups were restricted to C or D ring and C-18 locations by the alkaline ultraviolet measurement. Acetylation in acetic anhydride–pyridine at room temperature afforded an hydroxyl-free product. Hence 11β- and 14α-hydroxylations were excluded. A diagnostic reaction with sodium bismuthate gave evidence for transformation of VIII into a variety of products, indicating thereby that an hydroxyl group had probably been introduced at C_{16}. Monohydroxylation of 16β- was excluded as the infrared spectrum of VIII did not match that of VI. Accordingly 16α-hydroxylation was a most likely alternative. The diacetate of VIII melted at 142–143°, $[\alpha]_D^{25}$ +4° (chloroform). Infrared comparison of the diacetate of VIII with the spectrum from an authentic sample of 16α-hydroxytestosterone 16, 17-diacetate established the structure of VIII unequivocally.[15]

In addition to the seven products to which structures have been assigned, two additional, apparently pure, crystalline compounds of uncertain identity were isolated. Both of these were intermediate in polarity on paper between VI and VII. The less polar of the two, IX, melted at 195–199°, $[\alpha]_D^{25}$ +10° (dioxane or chloroform), no strong ultraviolet peaks at 220–350 mμ, only hydroxyl bands in functional group region of infrared. The carbon-hydrogen analyses were in fair agreement with the formula $C_{19}H_{30}O_3$. Acetylation of IX with acetic anhydride in pyridine at room temperature afforded an hydroxyl-free derivative, m.p. 134–135°, $[\alpha]_D^{25}$ +12° (chloroform), whose analyses were in fair agreement with a diacetate formulation, $C_{23}H_{34}O_5$. There was no reaction between manganese dioxide and IX. Diagnostic sodium bismuthate cleavage afforded a spectrum of products, resembling that which had been obtained previously with VI and VIII. In acetone–hydrochloric acid IX was transformed into a more polar product containing chlorine. These facts are in accord with the formulation of IX as a 16α,17β-dihydroxysteroid bearing an epoxide group, *e.g.*, 4α,5α-oxidoandrostane-16α, 17β-diol. There is a precedent for the microbiological epoxidation of a double bond.[16] However one cannot say that a clear parallel exists in our case, as the origin of the oxido oxygen is obscure. The possibility that the 3-oxygen is the source of the oxido oxygen has not been excluded.

The second unknown, X, melted at 166–168°, $[\alpha]_D^{25}$ +136° (chloroform), $\lambda_{max}^{CH_3OH}$ 239 mμ (ε = 16,400), with hydroxyl and conjugated carbonyl bands in the infrared and was apparently a monohydroxylated testosterone from its relative polarity with respect to VI and VII. The ultraviolet spectrum did not shift in alkali. Acetylation of X in acetic anhydride–pyridine afforded an hydroxyl-free product. From this evidence hydroxyl substituents were excluded from rings A and B, from 19-,

(11) W. J. Adams, *et al.*, *J. Chem. Soc.*, 297 (1956).

(12) A. Butenandt, J. Schmidt-Thomé, and T. Weiss, *Ber.*, **72**, 417 (1939).

(13) *Cf.* E. Vischer and A. Wettstein, *Adv. Enzym. XX*, 248 (1958).

(14) S. Eppstein, *et al.*, *J. Am. Chem. Soc.*, **76**, 3174 (1954); C. P. Balant and M. Ehrentein, *J. Org. Chem.*, **17**, 1587 (1952).

(15) The measurement and comparison of spectra was carried out by Mrs. Beatrice S. Gallagher of Sloan-Kettering Institute (chart 464, *Infrared Absorption Spectra of Steroids*, Vol. II) to whom we are indebted.

(16) B. M. Bloom and G. M. Shull, *J. Am. Chem. Soc.*, **77**, 5767 (1955).

11β, and 14α. Positions 11α, 15α, 15β, 16α, and 16β were *ostensibly* excluded by comparison of the infrared spectrum of X with those of the appropriate hydroxylated testosterones. The remaining sites for an hydroxyl group seemed therefore to be 12α, 12β, and 18. Measurement of the NMR spectrum[17] of X clearly excluded the 18-position as a site of hydroxylation and was also in accord with a 12α-hydroxyl substitution (equatorial hydrogen at 12-).

The NMR spectrum also contained a band which is interpreted[17] as being consistent only with the presence of a *14,15-unsaturation*. With phosphomolybdic acid reagent under appropriate experimental conditions X gives a characteristic purple color which is given by authentic 14α, 15α, and 15β-hydroxy-4-androstenes and by 4, 14-androstadienes.[18]

Within the framework of a 4,14-androstadiene-17β-ol-3-one structure one may exclude substitution in rings A and B by the alkaline ultraviolet measurement, at 11α, 12β, 18, and 19 by the NMR results, at 11β by the acetylation experiment, and 16α and 16β by diagnostic sodium bismuthate degradation in which no transformation products were formed and starting material was identified. Hence all evidence is consistent with the tentative assignment of X as 4,14-androstadiene-12α,17β-diol-3-one.

We speculate that the C_{14} unsaturation probably arises from dehydration of a 14α-hydroxy intermediate during the fermentation or subsequent work-up. It has already been demonstrated that the organism possesses an enzyme which promotes C_{14}-hydroxylation (V). The properties of a 12α,-14α-dihydroxysteroid might be expected to vary from those usually associated with 14α-hydroxysteroids because of a strong steric interaction between the axial hydroxyl groups with a resultant increased propensity for elimination at C_{14}. The direct microbiological introduction in steroids of unsaturation not conjugated with existing carbonyl or olefinic unsaturation is, so far as we are aware, without precedent and we accordingly prefer to consider this alternative explanation less likely.

At least six additional ultraviolet absorbing products, all more polar than VIII, were observed by paper chromatographic analysis. No attempt was made to characterize these.

From a more limited study of the fermentation of 4-androstene-3,17-dione (II), with the same organism under the same conditions, testosterone (I) and 16α-hydroxytestosterone were identified by the techniques already described. Hence *W. graminis* appears to have an enzyme (or enzymes) which can effect reversible oxidation-reduction at C_{17}.

(17) We are indebted to Dr. James Shoolery of Varian Associates, Palo Alto, Calif., for the measurement (with 2 mg. of substance) and interpretation of this spectrum.

(18) If spraying and drying are carried out according to the procedure given under the experimental heading, *Sodium bismuthate oxidation of IX*, and the colors are read *immediately* after being developed the following results are noted: olive green—IV and VII; blue-green—VI, VIII; blue—I, II, III, IX; purple—V, X, 15α-hydroxy-4-androstene-3,17-dione, 15β-hydroxy-4-androstene-3,17-dione, 4,14-pregnadiene-17α,21-diol-3,20-dione 21-acetate.

EXPERIMENTAL[19]

Fermentation of testosterone (I) *with Wojnowicia graminis* (*CBS*). *Wojnowicia graminis* (CBS) was maintained on a medium prepared from 10 g. of Difco yeast extract, 10 g. of cerelose, 20 g. of Difco agar, and 1.0 l. of tap water.

Inoculum for the fermentation was prepared by seeding a 300-ml. Erlenmeyer flask, containing 100 ml. of the aforementioned medium without agar, from an agar slant. The inoculum was incubated on a rotary shaker at 28° for 72–96 hr.

One hundred 300-ml. Erlenmeyer flasks, each containing 100 ml. of the forementioned medium without agar, were sterilized and inoculated with the 72–96-hr. growth culture. After 48–72 hr. on a rotary shaker (300 strokes/min.) at 28° good growth of the culture was obtained and thereupon there was added to each flask 0.025 g. of testosterone (I) dissolved in 1.0 ml. of dimethylformamide. Shaking was continued for 72 hr., when most of the testosterone had been consumed as measured by paper chromatography in the toluene–propylene glycol system.

The contents of all flasks were then pooled and extracted exhaustively with ethyl acetate. The residue remaining from removal of the ethyl acetate by distillation *in vacuo* was taken up in a minimum volume of methylene chloride and transferred to a 200-g. Florisil column. The fractions (175–200 ml.) were collected according to the following pattern:

Pool No.	Fraction No.	Eluting Solvent	
—	1–3	Hexane	
A	5–15	Ether	
	16–25	Ether-methylene chloride (1:1)	
B	26–34	Methylene chloride	
C	35–39	Methanol-methylene chloride	(1:99)
D	40–49	" " "	(2:98)
E	50–61	" " "	(4.96)
F	62–65	" " "	(5.95)
G	66–70	" " "	(10.90)

Pool A was crystallized from ether-hexane affording two crops of tan rosettes: 1st crop 0.124 g., m.p. 207–210°; 2nd crop 0.052 g., m.p. 185–195°. Combined crops 1 and 2 were chromatographed on 20 g. of Florisil and eluted with methylene chloride with progressively increasing amounts of methanol. From the methylene chloride eluates 0.050 g. of *testosterone* (I) was isolated, m.p. 146–150°, whose infrared spectrum was identical with that of an authentic sample. Fractions collected with 1% methanol-5% methanol contained crystals, m.p. >205°. Recrystallization from acetone-hexane afforded 0.081 g. of 6β-hydroxytestosterone (VII), m.p. 205°, resolidify, remelt 215–218°, $[\alpha]_D^{25}$ +29° (chloroform), $\lambda_{max}^{CH_3OH}$ 236 mμ (ϵ = 13,600), λ_{max}^{Nujol} 3.0 μ (hydroxyl), 6.02 μ (3-carbonyl), 6.15 μ (Δ^4).

Anal. Calcd. for $C_{19}H_{28}O_3$: C, 74.96; H, 9.27. Found: C, 74.99; H, 9.40.

Acetylation of VII (0.017 g.) with 0.2 ml. of acetic anhydride and 0.1 ml. pyridine at room temperature overnight

(19) All melting points are corrected. Analyses and optical data are by the Physical Chemistry Department of these laboratories. We are indebted to Mr. Richard Wayne for the interpretation of the infrared spectra.

afforded, after water precipitation, 0.020 g. of crystalline diacetate, m.p. 119°, resolidify, remelt 132–138°; $\lambda_{max}^{CH_3OH}$ 235 mμ (ε = 13,200), λ_{max}^{Nujol} 5.74 μ (acetate carbonyl), 5.95 μ (3-carbonyl), 6.16 μ (Δ⁴), 8.10 μ (C—O—C of acetate). The product was paper chromatographically homogeneous in ligroin-propylene glycol.

The mother liquor from the crystallization of Pool A was chromatographed over 150 g. of Johns Manville Chromosorb-W which was prepared with 150 ml. of the aqueous phase from the system made from 400 parts of ethyl acetate, 100 parts of ligroin, 600 parts of methanol, and 500 parts of water. Following pretreatment of the Chromosorb with the aqueous phase the resulting powder was packed with vibration in a 4-cm. (diameter) column to the height of 27.5 cm. The column was wetted throughout with the ethyl acetate phase and a small layer of refined sand was then floated down onto the Chromosorb. The mother liquor residue from Pool A was dissolved in 5 ml. of the ethyl acetate-ligroin phase, which was then equilibrated with 5.0 ml. of the aqueous phase. To the two-phase system was added 5.0 g. of Chromosorb-W and the resulting damp powder was vibrated onto the layer of damp sand. Additional ethyl acetate–ligroin phase was added to wet the newly added Chromosorb, and then another layer of sand was floated onto the Chromosorb. The column was then eluted with the ethyl acetate–ligroin phase and 100-ml. fractions were collected. From fractions 6–9, after crystallization from methylene chloride–hexane, there resulted 0.334 g. of *testosterone* (I). From fractions 10–14 there was isolated, after two crystallizations from acetone-hexane, 0.079 g. of *unknown epoxydiol* (IX), m.p. 196–199°, $[\alpha]_D^{25}$ +10° (dioxane), +10° (chloroform), λ_{max}^{Nujol} 3.08 μ (hydroxyl).

Anal. Calcd. for $C_{19}H_{30}O_3$: C, 74.47; H, 9.87. Found: C, 75.32, H, 10.28.

Acetylation of the epoxydiol (IX) (0.026 g.) in 0.2 ml. of pyridine with 0.2 ml. of acetic anhydride at room temperature overnight afforded after water precipitation, 0.029 g. of crystalline diacetate, m.p. 132–134°. Recrystallization afforded 0.019 g., m.p. 134–135°, $[\alpha]_D^{25}$ +12° (chloroform), λ_{max}^{Nujol} 5.75 μ (acetate carbonyl), 8.04 μ (C—O—C of acetate).

Anal. Calcd. for $C_{23}H_{34}O_5$: C, 70.74; H, 8.78. Found: C, 71.55; H, 8.67.

Manganese dioxide oxidation of the epoxydiol (IX) was attempted by dissolving 0.013 g. of IX in 1.0 ml. of chloroform and adding 0.033 g. of activated manganese dioxide.[20] After agitation of the mixture overnight at room temperature, the manganese dioxide was removed by filtration and the filter cake was washed carefully with chloroform. The combined filtrates were concentrated to a crystalline residue, m.p. 194–199° which did not display any significant ultraviolet absorption in the 220–350 mμ region. The crystalline product was identical with starting material by comparison of infrared spectra.

A solution of 0.010 g. of IX in 1.0 ml. of *acetone-hydrochloric acid* (99 parts acetone: 1 part concd. hydrochloric acid) was allowed to stand at room temperature overnight. Water was added and the acetone was removed by slow evaporation in a stream of air. There resulted 0.0055 g. of rosettes, m.p. 110–117°, which moved as a single spot (phosphomolybdic acid staining) in toluene–propylene glycol paper chromatography with Rf 0.06, whereas starting material had Rf 0.14, and as a single spot in toluene-dioxane (78:22) propylene glycol, Rf 0.19 whereas starting material had Rf 0.33.

Anal. Calcd. for $C_{19}H_{31}O_3Cl$ (Sample size 0.00166 g.): Cl, 10.34. Found: Cl, 7.43.

Sodium bismuthate oxidation of IX was carried out by stirring a solution of 0.007 g. of IX in 1.0 ml. of acetic acid and 1.0 ml. of water with 0.14 g. of sodium bismuthate overnight at room temperature. The insoluble material was removed by filtration and a portion of the filtrate was examined paper chromatographically in toluene–propylene glycol. The dried paper chromatogram was sprayed with a 10% solution of phosphomolybdic acid in methanol and the chromatogram was then dried out 60–70° for 10–15 min. The product of reaction showed the presence of four compounds which stained, one of which was starting material, one slower-moving, and two faster-moving components.

From fractions 17–25 there was isolated by crystallization from acetone-hexane 0.021 g. of crude *6β-hydroxytestosterone* (VII), m.p. 185–205°. The infrared spectrum was in reasonable agreement with that of the purer material isolated earlier.

Pool B was partitioned over 60 g. of Chromosorb which had been treated with 60 ml. of propylene glycol saturated with toluene. The damp Chromosorb was then added slowly to the column which was half full of toluene saturated with propylene glycol. Refined sand was added to cover the Chromosorb and the excess solvent was pushed out with nitrogen under slight pressure. Thereupon a solution of *B* in 10.0 ml. of toluene saturated with propylene glycol was added to the column and the liquid level was lowered to the surface of the sand by draining off excess toluene from the bottom of the column. The column was then eluted with toluene saturated with propylene glycol and 20 ml. fractions were collected. Recrystallization of fractions 5–7 from ether-hexane afforded 0.024 g. of *4-androstene-3,17-dione,* (II) m.p. 160–167°, whose infrared spectrum matched that of an authentic sample.

Recrystallization of fractions 13–15 from ether-hexane gave 0.060 g. of *testosterone* (I), m.p. 150–152°.

Recrystallization of fractions 18 and 19 from acetone-ether-hexane yielded 0.046 g. of *impure 6β-hydroxy-4-androstene-3,17-dione* (IV), m.p. 160–167°, $\lambda_{max}^{CH_3OH}$ mμ (ε = 8,400), alkaline ultraviolet[2]-typical 6β-hydroxy-3-keto-Δ⁴-steroid pattern. The product had only *ca.* 65% of the expected ultraviolet intensity.[5] However the nature of the saturated impurities could not be ascertained by staining the paper chromatogram. The only product spot had the same migration rate in toluene–propylene glycol as that of authentic 6β-hydroxy-4-androstene-3,17-dione. The infrared spectrum of IV was very similar to that of authentic 6β-hydroxy-4-androstene-3,17-dione.

Recrystallization of fractions 21–24 from acetone-ether-hexane gave 0.045 g. of *14α-hydroxy-4-androstene-3,17-dione,* (V), m.p. 246–252°, $[\alpha]_D^{25}$ +166° (chloroform), $\lambda_{max}^{CH_3OH}$ 239 mμ, (ε = 15,700), λ_{max}^{Nujol} 2.94 μ (hydroxyl), 5.72 μ (17-carbonyl), 6.02 μ (3-carbonyl), 6.20 μ (Δ⁴).

Attempted acetylation of V in acetic anhydride–pyridine at room temperature gave only starting material.

Comparison of V with an authentic sample of 14α-hydroxy-4-androstene-3,17-dione, m.p. 257–260°, $[\alpha]_D^{25}$ +171° (chloroform) kindly provided by Prof. E. Testa revealed that the two samples had superimposable infrared spectra.

Recrystallization of fractions 36–46 from acetone-ether-hexane afforded 0.033 g. of *16β-hydroxytestosterone* (VI), m.p. 179–182°, $[\alpha]_D^{25}$ +101° (chloroform), +94° (dioxane), $\lambda_{max}^{CH_3OH}$ 241 mμ (ε = 15,900), λ_{max}^{Nujol} 3.08 μ (hydroxyl), 6.10 μ (3-ketone), 6.22 μ (Δ⁴) (another polymorphic variety was also observed).

Anal. Calcd. for $C_{19}H_{28}O_3$: C, 74.96; H, 9.27. Found: C, 75.06; H, 9.71.

Acetylation of VI in acetic anhydride-pyridine at room temperature overnight afforded a *diacetate,* which after crystallization from acetone-hexane, melted at 201–202.5°, $[\alpha]_D^{25}$ +88° (chloroform), $\lambda_{max}^{CH_3OH}$ 239 mμ (ε = 16,700), λ_{max}^{Nujol} 5.78 μ (acetate carbonyl), 6.02 μ (3-carbonyl), 6.22 μ (Δ⁴), 7.92 and 8.10 μ (C—O—C of acetate).

Anal. Calcd. for $C_{23}H_{32}O_5$. $1/2C_6H_{14}$: C, 72.35; H, 9.11 Found: C, 72.53; H, 8.82.

Sodium bismuthate oxidation of VI was carried out with a solution of 0.002 g. of VI in 0.2 ml. of acetic acid and 0.2 ml. of water to which was added 0.020 g. of sodium bis-

(20) O. Mancera, G. Rosenkranz, and F. Sondheimer, *J. Chem. Soc.,* 2189 (1953); acetone was substituted for methanol in the washing of the manganese dioxide.

muthate. After overnight agitation at room temperature, the insoluble salts were removed by filtration and the filtrate and washes were extracted with methylene chloride. Paper chromatographic examination of the extract in toluene–propylene glycol showed that starting material was entirely consumed and that two, more polar, ultraviolet-absorbing products and one, less polar, ultraviolet-absorbing product were formed.

An acetonide of VI was prepared by dissolving 0.010 g. of VI in 9.9 ml. of acetone and 0.1 ml. of concd. hydrochloric acid and allowing the reaction mixture to stand at room temperature overnight. By water precipitation 0.009 g. of the 16,17-acetonide of VI, m.p. 185–187°, λ_{max}^{Nujol} 5.96 μ (3-carbonyl), 6.16 μ (Δ^4), 8.00 μ (C—O—C of acetonide), was obtained.

Pool D was chromatographed over 150 g. of Chromosorb W using the ethyl acetate-ligroin-methanol-water system described previously.

Fractions 12–15, after crystallization from ether-acetone-hexane, afforded 0.035 g. of *16β-hydroxytestosterone (VI)*, m.p. 175–180°, and 0.029 g. of second crop VI, m.p. 165–175°.

The mother liquors from fractions 12–15 and the main portion of fractions 17–19 contained mixtures which were further resolved.

Chromatography of the mother liquors from 12–15 with a toluene-propylene glycol-Chromosorb W system afforded, from fractions 7 and 8, 0.081 g. of 16-ketotestosterone (III), m.p. 153–155°, $[\alpha]_D^{25}$ −54° (chloroform), $\lambda_{max}^{CH_3OH}$ 240 mμ (ϵ = 16,600) (no shift in alkali), λ_{max}^{Nujol} 2.95 μ (hydroxyl), 5.70 μ (5-membered ring carbonyl), 5.98 μ (3-carbonyl) 6.18 μ (Δ^4).

Acetylation of III (0.023 g.) in 0.2 ml. of pyridine and 0.2 ml. of acetic anhydride at room temperature overnight gave, on water precipitation, 0.022 g. of acetate, m.p. 194–197°, $[\alpha]_D^{25}$ −44° (chloroform), $\lambda_{max}^{CH_3OH}$ 239 mμ (ϵ = 17,000), λ_{max}^{Nujol} 5.66 μ (D-ring carbonyl), 5.74 μ (acetate carbonyl), 6.02 μ (3-carbonyl), 6.18 μ (Δ^4), 8.15 μ (C—O—C of acetate).

From fractions 11–14, on crystallization from acetone-hexane-ether, there was isolated 0.014 g. of *14α-hydroxy-4-androstene-3,17-dione*, m.p. 236–244°, identified by its infrared spectrum.

From fractions 24–32 there was isolated, on crystallization from acetone-hexane, 0.062 g. of *16β-hydroxytestosterone* (VI), m.p. 179–180°, identified by infrared spectrum.

Rechromatography of fractions 17–19 from Pool D in toluene–propylene glycol–Chromosorb W afforded in fractions 30–35, on crystallization from acetone-ether-hexane, 0.014 g. of *X (tentatively 4,14-androstadiene-12α,17β-diol-3-one)*, m.p. 166–168°, $[\alpha]_D^{25}$ +136° (chloroform), $\lambda_{max}^{CH_3OH}$ 239 mμ (ϵ = 16,400), λ_{max}^{Nujol} 3.00 μ (hydroxyl), 5.98 μ (3-carbonyl), 6.18 μ (Δ^4).

Acetylation of X in acetic anhydride–pyridine at room temperature afforded an acetate, m.p. 137–140° (with prior melting and resolidification 90–120°), λ_{max}^{Nujol} 5.74 μ (acetate carbonyl), 5.96 μ (3-carbonyl), 6.18 μ (Δ^4).

Qualitative sodium bismuthate reaction with X as described previously afforded paper chromatographic evidence (toluene–propylene glycol) for unchanged starting material alone.

The NMR spectrum of X, kindly measured with 2.0 mg. of substance by Dr. James Shoolery of Varian Associates, displayed the following significant bands [at 60 megacycles and with respect to an internal tetramethylsilane standard (=O)]: 68 cps (18-methyl), 74 cps (19-methyl), multiplet 223 cps (17α-hydrogen), 265 cps (12β-hydrogen), triplet at 329 cps (vinyl hydrogen at 15− on a Δ^{14}-unsaturation), 345 cps (vinyl hydrogen at 4−). The interpretation and assignments are by Dr. Shoolery.

Recrystallization of Pool E from acetone-hexane gave 1.04 g. of *16α-hydroxytestosterone* (VIII), m.p. 183–187°, $[\alpha]_D^{25}$ +63° (dioxane), $\lambda_{max}^{CH_3OH}$ 240 mμ (ϵ = 14,000) (no shift in alkali), λ_{max}^{Nujol} 2.95 μ (hydroxyl), 3.18 μ (associated hydroxyl), 6.08 μ, (3-carbonyl), 6.22 (Δ^4).

Acetylation of 0.100 g. of VIII in 1.0 ml. of pyridine and 1.0 ml. of acetic anhydride on the steam bath for 1 hr. followed by overnight standing at room temperature afforded, after water precipitation, 0.106 g. of the *diacetate of* VIII, m.p. 140–141°. Recrystallization from ether raised the melting point to 142–143°, $[\alpha]_D^{25}$ +4° (chloroform), $\lambda_{max}^{CH_3OH}$ 240 mμ (ϵ = 16,400). The infrared spectrum of the diacetate of VIII matched the spectrum of an authentic sample of 16α-hydroxytestosterone 16,17-diacetate as measured by Mrs. Beatrice Gallagher.[15]

Qualitative sodium bismuthate degradation of VIII as described previously showed some uncharged starting material, one, more polar, ultraviolet-absorbing product and two, less polar, ultraviolet-absorbing products in a toluene–propylene glycol chromatogram.

Pools F and G showed the presence of six additional ultraviolet absorbing products more polar than VIII, which were not further investigated.

Fermentation of 4-androstene-3,17-dione (II) *with W. graminis.* From the fermentation of 0.250 g. of II essentially as described for I, the ethyl acetate extracts were concentrated and the residue chromatographed on 50 g. of Florisil. Elution with 10% ether-in-hexane through 50% ether-hexane afforded a series of *testosterone* (I), fractions totalling 0.103 g. Recrystallization from ether-hexane afforded 0.058 g., m.p. 144–147°, whose infrared spectrum was identical with that of authentic testosterone.

Elution was continued with ether, 50% ether–methylene chloride, methylene chloride, 1% methanol–methylene chloride, and 5% methanol–methylene chloride.

Rechromatography of the 5% methanol fractions over 2.0 g. of Florisil afforded in the methylene chloride fractions 0.003 g. of VIII, identical with that described previously by infrared comparison.

No further studies were made with the intermediate fractions of the first column. A number of ultraviolet-absorbing transformation products were observed from the paper chromatographic analysis of the various fractions.

BLOOMFIELD, N. J.