Anal. Calcd. for $C_7H_{13}N_3 \cdot 2HCl$: Cl, 33.43; N, 19.81. Found: Cl, 33.16; N, 19.50.

The dipicrate was prepared and recrystallized from methanol, m.p. 210°.

Anal. Calcd. for $C_{19}H_{19}N_9O_{14}$: N, 21.10. Found: N, 21.11.

5-(3-Phenylpyrazolyl)-methyl Phenyl Ketone Hydrazone (IX).—To a solution of 5 g. (0.02 mole) of 2,6-diphenyl-4-pyrone[15] in 25 ml. of methanol was added 5 ml. (0.1 mole) of hydrazine hydrate. A mild exothermic reaction took place. The solution was heated under reflux for one hour and then cooled in an ice-bath. The white crystalline solid that separated was collected and recrystallized from methanol. The yield was 2.8 g. (50%), m.p. 175–176°.

Anal. Calcd. for $C_{17}H_{16}N_4$: C, 73.89; H, 5.84; N, 20.28. Found: C, 73.59; H, 6.13; N, 20.17.

3-Phenyl-5-(β-phenyl-β-amino)-ethylpyrazole (XI).—The above hydrazone was hydrogenated under high pressure in ammoniacal methanol solution using Raney nickel catalyst. After removal of the catalyst and solvent, the residue was dissolved in 50 ml. of ethanol and treated with 4.6 g. of picric acid in 100 ml. of ethanol. The resulting picrate, 5.0 g. (66% yield), was recrystallized from ethanol, m.p. 204°.

Anal. Calcd. for $C_{29}H_{23}N_9O_{14}$: C, 48.27; H, 3.21; N, 17.47. Found: C, 48.51; H, 3.34; N, 17.31.

3-Methyl-5-pyrazoleacetic Acid Hydrazide (XIII).—A solution of 12.6 g. (0.1 mole) of 2-hydroxy-6-methyl-4-pyrone[16] in 50 ml. of methanol was treated with 12 ml. (0.22 mole) of hydrazine hydrate. An exothermic reaction took place. After the initial reaction, the solution was heated for a short time and then evaporated to dryness under reduced pressure leaving a crystalline solid. This was washed with ether and air dried. The yield was quantitative. A sample was recrystallized from dioxane and obtained as white needles, m.p. 145°.

Anal. Calcd. for $C_6H_{10}N_4O$: C, 46.74; H, 6.54; N, 36.34. Found: C, 46.80; H, 6.86; N, 36.01.

A solution of 3 g. of the above hydrazide and 19 g. of potassium permanganate in 150 ml. of water was heated on the steam-bath for two hours. The manganese dioxide was removed by filtration and the filtrate was acidified with hydrochloric acid. It was evaporated to dryness under reduced pressure. The residue was extracted with a little warm ethanol, and the filtered extract was evaporated to dryness. The residual solid was recrystallized from a small volume of water. It was identified as 3-methyl-5-pyrazolecarboxylic acid, m.p. 241–242° (lit.[9] m.p. 236–238° dec.).

Anal. Calcd. for $C_5H_6N_2O_2$: C, 47.62; H, 4.80; N, 22.22. Found: C, 47.57; H, 4.91; N, 21.97.

Sodium 3-Methyl-5-pyrazoleacetate (XIV).—A solution of 1.5 g. of 3-methyl-5-pyrazoleacetic acid hydrazide in 20 ml. of 1 *N* sodium hydroxide solution was heated under reflux overnight. The solution was evaporated to dryness, and the residue was extracted with ethanol. When the alcohol solution was diluted with ether, sodium 3-methyl-5-pyrazoleacetate separated as a white crystalline solid, m.p. 196–197°.

Anal. Calcd. for $C_6H_7N_2NaO_2$: N, 17.28. Found: N, 17.37.

INDIANAPOLIS, INDIANA

(15) J. Kalff, *Rec. trav. chim.*, **46**, 594 (1927) [*C. A.*, **22**, 240 (1928)].

(16) J. N. Collie, *J. Chem. Soc.*, **59**, 607 (1891).

[CONTRIBUTION FROM THE RESEARCH LABORATORIES, THE UPJOHN COMPANY]

Microbiological Transformations of Steroids.[1] X. The Oxygenation of Androgens by *Rhizopus*[2]

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The three androgens, 4-androstene-3,17-dione (I), testosterone (VIII) and 17α-methyltestosterone (XV), were subjected to enzymatic transformation by various species of *Rhizopus*. In each case the corresponding 11α-hydroxylated derivative was isolated as the major product and the 6β-hydroxylated derivative as a minor product. In the case of testosterone, 17β-hydroxyandrostane-3,6-dione was also isolated from the fermentation.

Discussion

In the continuation of our survey of steroid oxygenation by fungi, the androgens, 4-androstene-3,17-dione (I), testosterone (VIII) and 17α-methyltestosterone (XV), were added to various species of *Rhizopus*, notably *Rhizopus nigricans*, *Rhizopus arrhizus* and *Rhizopus reflexus*, and the transformation products isolated and characterized. No essential difference was observed among the three species of fungus used so that the conversion of each androgen will be illustrated with a different species of *Rhizopus*. The methods used for the bioconversion and extraction of steroids from the fermentation liquor, as well as the procedures for paper and alumina column chromatography used in the isolation of the steroids, have been described in our earlier communications.[3] Paper chromatography of the methylene dichloride extractives of the androgen fermentations indicated that the steroid substrates were practically completely converted into more polar substances. In each case two new compounds of different polarity were the major conversion products. The more polar compound in each instance was the 11α-hydroxylated steroid, present in greatest amount, and the less polar, the 6β-hydroxylated steroid.

From fermentations with 4-androstene-3,17-dione (I), 11α-hydroxy-4-androstene-3,17-dione (II) and 6β-hydroxy-4-androstene-3,17-dione (III) were isolated. The configuration of II was established by oxidation to adrenosterone (IV).[4] The melting point of II (225–227°) differed greatly from that of 11β-hydroxy-4-androstene-3,17-dione (189–191°) reported by Reichstein.[5] The ease of acetylation of the hydroxyl on carbon number 11 of compound II and its contribution to the molecular rotation established its α orientation.[6]

(1) Paper IX of this series, THIS JOURNAL, **75**, 5768 (1953).

(2) Presented in part before the Division of Biological Chemistry at the 123rd Meeting of the American Chemical Society, Los Angeles, California, March 15–19, 1953 (Division of Biological Chemistry, Abstract 5C).

(3) Paper I of this series by D. H. Peterson, H. C. Murray, S. H. Eppstein, L. M. Reineke, A. Weintraub, P. D. Meister and H. M. Leigh, THIS JOURNAL, **74**, 5933 (1952).

(4) T. Reichstein, *Helv. Chim. Acta*, **20**, 953 (1937).

(5) T. Reichstein, *ibid.*, **20**, 978 (1937).

(6) Since the completion of this work a fuller characterization of 11β-hydroxy-4-androstene-3,17-dione has been reported by R. W. Jeanloz, *et al.* (*J. Biol. Chem.*, **203**, 453 (1953)). Their compound, m.p. 197–199.5°, $[\alpha]D$ +207.6° (chloroform), did not acetylate with acetic anhydride in pyridine.

TABLE I

Parent compound	M_D Unsubstituted I	M_D 6β-Hydroxy II	M_D 6β-Acetoxy III	ΔM_D II-I	ΔM_D III-I	ΔM_D III-II
21-Hydroxy-4-pregnene-3,20-dione	611	350		−261		
21-Acetoxy-4-pregnene-3,20-dione	677	438	470	−239	−207	32
17α,21-Dihydroxy-4-pregnene-3,20-dione	440 (Meth.)	212 (Eth.)		−228		
17α-Hydroxyprogesterone	346	21	40	−325	−306	19
4-Androstene-3,20-dione	570	330	381	−240	−189	51
Testosterone	330[10]	97		−233		
17α-Methyltestosterone	266	3		−263		

Oxidation of III yielded 4-androsten-3,6,17-trione (VI).[7] The β-orientation of the hydroxyl on carbon number 6 of compound III was established by comparison of the molecular rotation increments of the 6-hydroxyl and its acetoxy derivative VII with the corresponding values in the 6β-hydroxy-C_{21}-steroids (Table I). That III was 6β-hydroxy-4-androstene-3,17-dione was later confirmed by comparison of the infrared spectrum with that of an authentic sample.[8]

From fermentations with testosterone (VIII), 11α-hydroxytestosterone (IX), 6β-hydroxytestosterone (X) and 17β-hydroxyandrostane-3,6-dione (XI) were isolated. Oxidation of IX to adrenosterone (IV) proved that IX was an 11-oxygenated testosterone; the ease with which IX formed a diacetate XII proved the α-orientation of the hydroxyl on carbon number 11. The molecular rotation (Table II) is in harmony with this assignment.

TABLE II

Parent compound	M_D Unsubstituted I	M_D 11α-Hydroxy II	M_D 11-Keto III	ΔM_D III-I	ΔM_D III-II	ΔM_D II-I
4-Androstene-3,17-dione	570	490	902	332	412	−80
Testosterone	330[10]	283	677	347	394	−47
17α-Methyltestosterone	266	210	567	301	357	−56

That the less polar new compound X was a 6-hydroxylated testosterone was shown by its oxidation to 4-androsten-3,6,17-trione (VI); confirmation was found in the shift of the ultraviolet absorption maximum which is typical of 6β-hydroxy steroids and in the sulfuric acid-catalyzed rearrangement of X (in acetic acid solution) to yield 17β-acetoxyandrostane-3,6-dione (XIV).[9] The contribution to the molecular rotation by the 6-hydroxyl is compatible with the 6β orientation (Table I). Complete confirmation was obtained by the selective oxidation of the 17β-hydroxyl group of X to yield 6β-hydroxy-4-androstene-3,17-dione (III). Reaction of X with acetic anhydride in pyridine produced the diacetate XIII.

The degree of reactivity of the secondary 17β-hydroxyl group was surprising. Thus 17-acylation occurred (with concomitant molecular rearrangement in the A:B rings to produce XIV) when sulfuric acid was added to an acetic acid solution of X; the primary 21-hydroxyl group of 6α-hydroxy-11-desoxycorticosterone does not acylate under these conditions,[9] nor does the secondary 11α-hydroxyl of 6β,11α-dihydroxyprogesterone.[11] Subsequently it was found that testosterone itself readily acylates under similar conditions.[11] Hence an activation by the 6β-hydroxyl group is not involved.

Another bioconversion product of testosterone isolated not only *via* alumina chromatography but also by direct crystallization[12] showed no light absorption in the region of 240 mμ. The compound was shown to be 17β-hydroxyandrostane-3,6-dione (XI). The structure of XI was proved by acetylation to XIV. Since the conditions of fermentation and isolation do not lead to a high concentration of either hydroxyl or hydrogen ions, the rearrangement of X to XI apparently was induced by the microörganism.

Fermentations of 17α-methyltestosterone (XV) led to the isolation of 11α-hydroxy-17α-methyltestosterone (XVI) and 6β-hydroxy-17α-methyltestosterone (XVII). Oxidation of XVI and XVII yielded, respectively, 11-keto-17α-methyltestosterone (XVIII) and 6-keto-17α-methyltestosterone (XX). Reaction of XVI and of XVII with acetic anhydride in pyridine produced the 11α-acetoxy and 6β-acetoxy derivatives, respectively, of 17α-methyltestosterone.

The configurations of XVI and of XVII were assigned on the basis of the following considerations.

1. By analogy with other steroid bioconversions where we have found that the *Rhizopi* preferentially oxygenate the 6β- and 11α-positions and that the 6β-compound is less polar than the 11α-compound, XVI should be 11α-hydroxy-17α-methyltestosterone and XVII should be 6β-hydroxy-17α-methyltestosterone.

2. The ultraviolet absorption spectrum of XVII ($\lambda_{max}^{alc.}$ 238, ε 13,550) suggested a 6β-hydroxy steroid.[13a] Further presumptive evidence that XVII was a 6-hydroxyl compound was obtained by adding a catalytic amount of sulfuric acid to an acetic acid solution of XVII; a new compound XXII was formed which did not absorb light in the region of 240 mμ, and whose infrared spectrum

(7) A. Butenandt and B. Riegel, *Ber.*, **69**, 1163 (1936).

(8) We wish to thank Dr. M. Ehrenstein for this sample of 6β-hydroxy-4-androstene-3,17-dione.

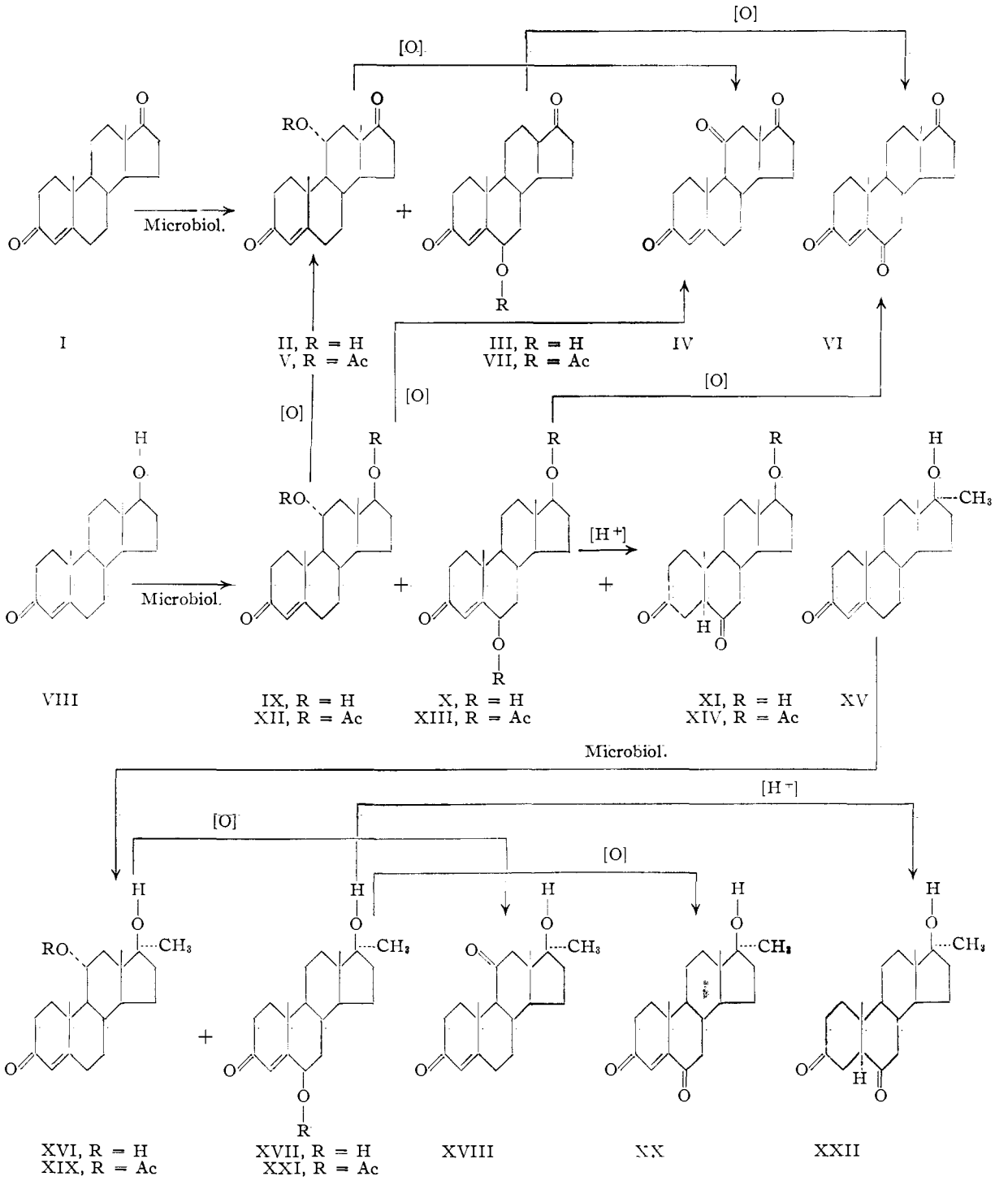
(9) This type of rearrangement has been discussed by P. T. Herzig and M. Ehrenstein, *J. Org. Chem.*, **16**, 1050 (1951).

(10) F. Sondheimer, *et al.* (ref. 15), determined the optical rotations in chloroform of various hormones for molecular rotation calculations and reported for testosterone $[\alpha]^{20}D$ +109°. David, *et al.* (*Z. physiol. Chem.*, **233**, 281 (1935)) gave a value of 109° (alcohol). The values determined by us are: $[\alpha]^{23}D$ +114.5° (*c* 1.079 in $CHCl_3$) and +108.8° (*c* 1.104 in 95% ethanol).

(11) Unpublished data, these laboratories.

(12) This compound, as well as IX and X, was obtained by fractional crystallization from ethyl acetate of the crude methylene dichloride extractives by Dr. R. P. Holysz of these laboratories.

(13) (a) L. Dorfman, *Chem. Revs.*, **53**, 47 (1953); (b) Dr. J. L. Johnson of these laboratories will elaborate on this sequence in a forthcoming publication.



showed only hydroxyl and non-conjugated ketone as oxygen functions.

3. Oxidation of XVII yielded XX which showed shifts in ultraviolet[13a] and infrared[13b] spectra which correlate with steroids having the Δ^4-3,6-dione structure. (The conjugated carbonyl absorption in infrared shifted from 1616 to 1602 cm.$^{-1}$; in ultraviolet the $\lambda_{max}^{alc.}$ shifted from 238 to 252 mμ.) In contrast, the ketone formed by oxidation of XVI showed no such shifts in spectrum.

4. The contribution to the molecular rotation by the new hydroxyl in XVI and the ketone derived from it in XVIII is compatible with the assignment of 11α-hydroxy-17α-methyltestosterone to compound XVI (Table II). The contribution to the molecular rotation by the new hydroxyl in XVII is compatible with the assignment of 6β-hydroxy-17α-methyltestosterone to XVII (Table I).

Experimental

Isolation of 11α-Hydroxy-4-androstene-3,17-dione (II) and 6β-Hydroxy-4-androstene-3,17-dione (III) from a Bioconversion of 4-Androstene-3,17-dione (I) by *Rhizopus*

arrhizus (A.T.C.C. 11145). **11α-Hydroxy-4-androstene-3,17-dione (II).**—The methylene dichloride extractives of a fermentation of 2 g. of I by *Rhizopus arrhizus* were chromatographed over 100 g. of alumina by increasing the polarity of the elution solvent from benzene through ether, chloroform, acetone and methanol. The solids in the chloroform and chloroform–acetone (19:1) eluates were crystallized from acetone–chloroform to yield 660.5 mg. of a new steroid, m.p. 216–222°. Recrystallization from ethyl acetate yielded 11α-hydroxy-4-androstene-3,17-dione (II), m.p. 225–227°, $[\alpha]_D$ +162° (*c* 1.415 in $CHCl_3$), $\lambda_{max}^{alc.}$ 242 mμ, ε 15,300. *Anal.* Calcd. for $C_{19}H_{26}O_3$: C, 75.46; H, 8.67. Found: C, 75.68; H, 8.53.

Oxidation of 11α-Hydroxy-4-androstene-3,17-dione (II) to Adrenosterone (IV).—To 35 mg. of II, dissolved in 2 ml. of acetic acid, was added 10 mg. of chromium trioxide in 1.2 ml. of 80% acetic acid. After 5 hours standing at room temperature the excess reagent was destroyed with methanol and the solution extracted with ether. The ether extract was washed with sodium bicarbonate and water and evaporated to yield 27 mg. of crystals. These were twice recrystallized from methylene dichloride–Skellysolve B to give 15 mg. of adrenosterone (IV), m.p. 219–220°, identified by mixed melting point and infrared spectrum. *Anal.* Calcd. for $C_{19}H_{24}O_3$: C, 75.97; H, 8.05. Found: C, 75.61; H, 8.10.

11α-Acetoxy-4-androstene-3,17-dione (V).—Thirty-four mg. of sublimed and recrystallized 11α-hydroxy-4-androstene-3,17-dione (II), m.p. 226–228°, was dissolved in 1.5 ml. of pyridine. Two ml. of acetic anhydride was added with cooling and the mixture refrigerated overnight. The solution was diluted with ice-water and extracted three times with 50-ml. portions of ether. The ether extract was washed twice with 5% hydrochloric acid, then water, 4 times with sodium bicarbonate (cooling under the water tap) and 4 times with water. The neutral ether solution was dried with sodium sulfate and the solvent evaporated in an air stream. The extractives weighed 45 mg. Crystallization of this material could not be achieved. Its infrared spectrum with no free hydroxyl absorption, 17-ketone and ester carbonyl at 1736 cm.$^{-1}$, acetate C–O at 1244 cm.$^{-1}$, conjugated ketone at 1670 cm.$^{-1}$, and conjugated double bond at 1612 cm.$^{-1}$ confirmed the structure of 11α-acetoxy-4-androstene-3,20-dione.

6β-Hydroxy-4-androstene-3,17-dione (III).—The ether, ether–chloroform (19:1) and ether–chloroform (9:1) eluates of the alumina chromatogram (*vide supra*) yielded a steroid less polar than II. These fractions were individually dissolved in a few drops of acetone followed by dropwise addition of ether to induce crystallization. The mother liquors were decanted and the crystalline residues combined and recrystallized from acetone–ether to give 177 mg.; m.p. 170–185°. Repeated recrystallizations yielded 6β-hydroxy-4-androstene-3,17-dione (III), m.p. 191–194°, $[\alpha]_D$ +99°[14] (*c* 0.738 in $CHCl_3$) $\lambda_{max}^{alc.}$ 237 mμ, ε 12,700. The infrared spectrum was identical with that of an authentic sample of III. *Anal.* Calcd. for $C_{19}H_{26}O_3$: C, 75.46; H, 8.67. Found: C, 75.31; H, 8.77.

6β-Acetoxy-4-androstene-3,17-dione (VII).—Forty-nine mg. of III was treated with 1 ml. of acetic anhydride–pyridine (1:1) and allowed to stand overnight. Dilution with 45 ml. of water yielded crystals. These were recrystallized from 0.5 ml. of methanol to yield 6β-acetoxy-4-androstene-3,17-dione (VII), m.p. 200–204°, $[\alpha]_D$ +112° (*c* 1.07 in $CHCl_3$) $\lambda_{max}^{alc.}$ 235 mμ, ε 13,200. The infrared spectrum was identical with that of an authentic sample.

Isolation of 11α-Hydroxytestosterone (IX), 6β-Hydroxytestosterone (X) and 17β-Hydroxyandrostane-3,6-dione (XI) from Fermentations of Testosterone (VIII) by *Rhizopus reflexus* (ATCC 1225). 11α-Hydroxytestosterone (IX).—The methylene chloride extractives (8.1 g.) of a fermentation of 4 g. of VIII were dissolved in benzene and chromatographed over 275 g. of alumina by increasing the polarity of the elution solvent from benzene through ether, chloroform, acetone and methanol. The fractions eluted by chloroform–acetone (1:1) through acetone, weighing 1.9 g., were combined and crystallized from ethyl acetate to give 1.82 g. of 11α-hydroxytestosterone (IX), melting at 181–184°. Repeated crystallizations of an aliquot yielded material melting at 181–181.5°, $[\alpha]_D$ +93° (*c* 1.205 in $CHCl_3$), $\lambda_{max}^{alc.}$ 243, ε 14,275. *Anal.* Calcd. for $C_{19}H_{28}O_3$: C, 74.96; H, 9.27. Found: C, 74.69; H, 9.26. The infrared spectrum showed hydroxyl absorption at 3461 and 3354 cm.$^{-1}$; conjugated carbonyl at 1654 cm.$^{-1}$ and conjugated carbon–carbon double bond at 1604 cm.$^{-1}$.

11α,17β-Diacetoxy-4-androsten-3-one (XII).—From the reaction of 50 mg. of IX with acetic anhydride in pyridine 34 mg. of crystalline product was obtained on diluting with water. Recrystallization from 1 ml. of methanol by addition of 5 ml. of water yielded 22 mg. of 11α,17β-diacetoxy-4-androsten-3-one (XII), m.p. 201–203°, $[\alpha]_D$ +56° (*c* 1.09 in $CHCl_3$). *Anal.* Calcd. for $C_{23}H_{32}O_5$: C, 71.10; H, 8.30. Found: C, 71.19; H, 8.46. The infrared spectrum showed no hydroxyl absorption bands, but acetate ester absorption at 1257 and 1732 cm.$^{-1}$.

Oxidation of 11α-Hydroxytestosterone (IX) to Adrenosterone (IV).—Fifty mg. of IX was dissolved in 2 ml. of benzene and 1 ml. of acetic acid and cooled in an ice-bath. To this was added 66 mg. of sodium dichromate in 1 ml. of cold acetic acid–benzene (2:1) and the reaction mixture stirred for 4 hours at 0°. It was then diluted with 20 ml. of cold water and extracted 4 times with 10-ml. portions of ether. The combined extracts were washed with 10 ml. of 2% sodium hydroxide and twice with 5 ml. of water. The washed extract was dried with sodium sulfate and the solvent evaporated at room temperature. The extractives (44 mg.) were crystallized from 0.5 ml. of ethyl acetate to give 20 mg. of IV, m.p. 213–217°, identified by infrared spectrum as 4-androstene-3,11,17-trione (adrenosterone).

6β-Hydroxytestosterone (X).—Those fractions of the alumina chromatogram eluted by ether–chloroform (1:1) through chloroform–acetone (19:1), with a combined weight of 3.2 g., were dissolved in 60% methanol and chromatographed over a column of activated carbon (Darco G60) mixed with Celite 1:2. Solvents beginning with methanol and changing in polarity from methanol through acetone and methylene dichloride were used for elution. The fractions forming a peak in the methylene dichloride eluates were combined (1.4 g.) and crystallized from 5 ml. of ethyl acetate to give 0.436 mg. of product. Two recrystallizations from 5 ml. of acetone yielded 0.248 g. of purified 6β-hydroxytestosterone (X), m.p. 216–222°, $[\alpha]_D$ +32° (*c* 1.3 in $CHCl_3$), $\lambda_{max}^{alc.}$ 238 mμ, ε 13,700. *Anal.* Calcd. for $C_{19}H_{28}O_3$: C, 74.96; H, 9.27. Found: C, 75.35; H, 9.32. The infrared spectrum showed the following absorption bands: hydroxyl at 3555, 3490 and 3425 cm.$^{-1}$; conjugated carbonyl, 1660 cm.$^{-1}$; conjugated carbon–carbon double bond at 1618 cm.$^{-1}$.

Oxidation of 6β-Hydroxytestosterone (X) to 4-Androstene-3,6,17-trione (VI).—Two hundred mg. of X was dissolved in 5.2 ml. of acetic acid–benzene (1:1) and cooled in an ice-bath. To this was added 261.2 mg. of sodium dichromate in 2.6 ml. of acetic acid. After 2 hours of stirring at 0° the reaction mixture was diluted with 45 ml. of cold water and extracted 5 times with 10-ml. portions of methylene dichloride. The combined methylene dichloride extracts were washed twice with 5-ml. portions of 2% sodium carbonate and twice with water, dried over sodium sulfate and concentrated at room temperature to give 187 mg. of crystals, m.p. 160–169°. Paper chromatography showed two components to be present, one moving like 4-androstene-3,6,17-trione and one like 6β-hydroxyandrostenedione. The benzene solution of the material was chromatographed over 4 g. of alumina. Although only one peak appeared in the eluates, the material (67.7 mg.) eluted by benzene–ether (9:1) through benzene–ether (1:1) crystallized from ethyl acetate as bipyramids (29 mg., m.p. 200–210°), whereas the material (63.4 mg.) eluted by ether through ether–chloroform (1:1) crystallized as prisms (32 mg., m.p. 170–190°). The bipyramids were recrystallized from 1 ml. of acetone by dropwise addition of Skellysolve B to yield 21 mg. of 4-androstene-3,6,17-trione (VI), m.p. 220–224°, confirmed by infrared spectrum. The prisms, similarly recrystallized, yielded 25 mg. of slightly impure 6β-hydroxy-4-androstene-3,17-dione (III) confirmed by infrared spectrum.

Selective Oxidation of 6β-Hydroxytestosterone (X) to 6β-Hydroxy-4-androstene-3,17-dione (III).—To 400 mg. of slightly impure X dissolved in 10.4 ml. of benzene–acetic acid (1:1) was added 261.2 mg. of sodium dichromate in 2.6 ml. of acetic acid. The mixture was stirred at 0° for 2 hours, then diluted with 65 ml. of cold water, and extracted 4 times with 25-ml. portions of methylene dichloride. The

(14) The higher optical rotation (+109.2°) reported by C. P. Balant and M. Ehrenstein, *J. Org. Chem.*, **17**, 1587 (1952), is probably more nearly correct and has been used in our calculations.

combined extracts were washed with 10% sodium carbonate, then with 1% hydrochloric acid, followed by water. The methylene dichloride extract was dried with anhydrous sodium sulfate and the solvent evaporated at room temperature. The residue was crystallized from ethyl acetate by addition of Skellysolve B to yield 273.4 mg. (68% yield) of 6β-hydroxy-4-androstene-3,20-dione (III).

Rearrangement of 6β-Hydroxytestosterone (X) to 17β-Acetoxyandrostane-3,6-dione (XIV).—Ten mg. of X was dissolved in 1 ml. of acetic acid; 2 drops of 10% sulfuric acid in acetic acid was added. After 24 hours at room temperature the solution was diluted to 10 ml. with water, made slightly alkaline with sodium carbonate, saturated with sodium chloride and extracted 3 times with 10-ml. portions of ethyl acetate. The combined extracts were washed with water and dried with sodium sulfate. After evaporation of the solvent at room temperature the residue was crystallized from 0.3 ml. of acetone by dropwise addition of Skellysolve B to yield 5 mg. of 17β-acetoxyandrostane-3,6-dione (XIV), m.p. 185–186°. The infrared spectrum showed loss of conjugation, appearance of a new ketone, and complete acetylation of the hydroxyl on carbon 17.

The specific rotation of −17° (*c* 0.9 in $CHCl_3$) determined on identical material made by another method (*vide infra*) together with the infrared spectrum and the known rearrangement of 6-hydroxy-Δ^4-3-ketones establishes the structure of XIV.

6β,17β-Diacetoxy-4-androsten-3-one (XIII).—One ml. of acetic anhydride was added to 100 mg. of 6β-hydroxytestosterone (X) dissolved in 2 ml. of pyridine. After 24 hours at room temperature the solution was diluted with 75 ml. of water and cooled in the refrigerator for one hour. The crystals formed were washed with three 5-ml. portions of cold water and dried *in vacuo* at 40° to give 101 mg., m.p. 136–141°. Recrystallization from 0.5 ml. of ethyl acetate by dropwise addition of ether yielded 74 mg., m.p. 127–131°, which resolidified at 135° and melted at 141–143°. Infrared analysis indicated the absence of hydroxyl with acetate absorption bands at 1232 and 1737 cm.$^{-1}$. Several recrystallizations did not change the double melting point of the 6β,17β-diacetoxy-4-androstene-3-one (XIII), $[\alpha]_D$ −1° (*c* 0.869 in $CHCl_3$). *Anal.* Calcd. for $C_{23}H_{32}O_5$: C, 71.10; H, 8.30. Found: C, 71.39; H, 8.26.

Isolation of 17β-Hydroxyandrostane-3,6-dione (XI).—Seventy-two g. of testosterone was fermented with *Rhizopus reflexus* and the extractives separated over alumina in the manner already described. The fractions eluted by ether–chloroform (19:1) through chloroform–acetone (9:1), weighing 59 g., showed practically no ultraviolet absorption and contained a considerable quantity of *l*-leucyl-*l*-proline anhydride. An aliquot of 0.6 g. was rechromatographed over 30 g. of alumina. The bulk of the column eluates was mainly *l*-leucyl-*l*-proline anhydride. Two fractions (benzene–ether, 1:1, and ether), weighing 188 mg. combined, were dissolved in 1 ml. of dimethylformamide. Dropwise addition of water induced crystallization, wt. 25 mg., m.p. 210–225°. The infrared spectrum showed hydroxyl absorption at 3581 cm.$^{-1}$ and ketone absorption at 1706 cm.$^{-1}$, with a trace of conjugated carbonyl absorption.

From a similar bioconversion of 72 g. of testosterone direct crystallization[12] from ethyl acetate (after the 6β-hydroxytestosterone crystallized out) yielded 4.675 g. of material, m.p. 230–233°, identical to the above by infrared spectrum. Recrystallization from methylene dichloride (10 ml./g.) by addition of 5 volumes of ether yielded 17β-hydroxyandrostane-3,6-dione (XI), m.p. 233–234°, $[\alpha]_D$ −9°[15] (*c* 1.08 in $CHCl_3$). *Anal.* Calcd. for $C_{19}H_{28}O_3$: C, 74.96; H, 9.27. Found: C, 74.76; H, 9.03.

Acetylation of 100 mg. of XI with acetic anhydride in pyridine yielded 91.3 mg. of 17β-acetoxyandrostane-3,6-dione (XIV), m.p. 185–186°, $[\alpha]_D$ −17° (*c* 0.904 in $CHCl_3$). *Anal.* Calcd. for $C_{21}H_{30}O_4$: C, 72.80; H, 8.73. Found: C, 72.88; H, 8.55. The infrared spectrum of this material and that obtained by rearrangement of 6β-hydroxytestosterone (*vide supra*) were identical.

Isolation of 11α-Hydroxy-17α-methyltestosterone (XVI) and 6β-Hydroxy-17α-methyltestosterone (XVII) from Fermentations of 17α-Methyltestosterone (XV) with *Rhizopus nigricans* (A.T.C.C. 6227b).—The methylene dichloride extracts of the fermentation of 6 g. of XV were combined and the solvent removed by evaporation at room temperature to yield a partly crystalline gum. On taking the residue up in 80 ml. of benzene the crystals failed to dissolve. The benzene solution was fractionated over alumina. The material eluted by chloroform–acetone (19:1) through acetone was combined (4.1 g.) and crystallized from 10 ml. of acetone by addition of Skellysolve B to turbidity to yield 2 g. of 11α-hydroxy-17α-methyltestosterone (XVI), m.p. 160–161.5°. From the mother liquors an additional gram of XVI, m.p. 159–162°, was obtained. *Anal.* Calcd. for $C_{20}H_{30}O_3$: C, 75.43; H, 9.50. Found: C, 75.40; H, 9.45; $[\alpha]_D$ +66° (*c* 1.1 in chloroform), +59° (*c* 0.83 in ethanol), $\lambda_{max}^{alc.}$ 243, ϵ 14,700. The infrared spectrum showed absorption bands as follows: hydroxyl, 3467, 3382 cm.$^{-1}$; conjugated ketone, 1647 cm.$^{-1}$; conjugated carbon–carbon double bond, 1609 cm.$^{-1}$.

11α-Acetoxy-1α-methyltestosterone (XIX).—From 70 mg. of 11α-hydroxy-17α-methyltestosterone (XVI) by acetylation with acetic anhydride in pyridine, 78.3 mg. of crystalline product was obtained. This was recrystallized once from ether by dropwise addition of hexane and once from ethyl acetate by dropwise addition of hexane to give 23 mg. of 11α-acetoxy-17α-methyltestosterone (XIX), m.p. 152–155°, $[\alpha]_D$ +51° (*c* 0.6296 in $CHCl_3$). *Anal.* Calcd. for $C_{22}H_{32}O_4$: C, 73.30; H, 8.95. Found: C, 73.55; H, 8.83.

11-Keto-17α-methyltestosterone (XVIII).—11α-Hydroxy-17α-methyltestosterone (XVI) (90.5 mg.) was dissolved in 2 ml. of acetic acid. To this 21.7 mg. of chromium trioxide dissolved in 2 ml. of 80% acetic acid was added and the solution allowed to stand for 5 hours at room temperature. It was then diluted with 5 ml. of methanol and concentrated *in vacuo*. The residue, suspended in water, was extracted 3 times with 15-ml. portions of ether. The combined ether extracts were washed twice with 5% sodium bicarbonate and three times with water. The ether extract was then dried with sodium sulfate and the solvent evaporated to give 88.8 mg. of crystals. These were recrystallized from about 0.5 ml. of methanol by dropwise addition of water to yield 11-keto-17α-methyltestosterone (XVIII), m.p. 171–173°, $\lambda_{max}^{alc.}$ 239 mμ, ϵ 13,300, $[\alpha]_D$ +179° (*c* 1.15 in $CHCl_3$). *Anal.* Calcd. for $C_{20}H_{28}O_3$: C, 75.91; H, 8.92. Found: C, 75.90; H, 9.01. The infrared spectrum was in conformity with the structure given.

6β-Hydroxy-17α-methyltestosterone (XVII).—The benzene-insoluble crystals from the methylene dichloride extractives of the fermentation described above were recrystallized from 10 ml. of ethyl acetate to yield 500 mg. of crystals, m.p. 220–235°. This was chromatographed over an alumina column, and the peak fractions (362 mg.) crystallized from 3 ml. of acetone to yield 260 mg. of 6β-hydroxy-17α-methyltestosterone (XVII), m.p. 252–253° (after several transition points), $\lambda_{max}^{alc.}$ 238 mμ, ϵ 13,600, $[\alpha]_D$ +3° (*c* 0.365 in $CHCl_3$). *Anal.* Calcd. for $C_{20}H_{30}O_3$: C, 75.43; H, 9.50. Found: C, 75.61; H, 9.45. The infrared spectrum showed the following absorption peaks: hydroxyl, 3575, 3533 cm.$^{-1}$; conjugated ketone, 1672 cm.$^{-1}$, conjugated carbon–carbon double bond, 1616 cm.$^{-1}$.

6-Keto-17α-methyltestosterone (XX).—To 50 mg. of 6β-hydroxy-17α-methyltestosterone (XVII) dissolved in 1 ml. of acetic acid was added 15.7 mg. of chromium trioxide dissolved in 4 ml. of acetic acid containing 0.02 ml. of water. After 16 hours at room temperature the excess oxidizing agent was discharged with 10 drops of methanol. The reaction mixture was diluted with 45 ml. of water and extracted 3 times with 15-ml. portions of methylene dichloride. The methylene dichloride extracts were combined, washed with sodium bicarbonate and water, dried with sodium sulfate and the solvent evaporated at room temperature to yield 62 mg. of an oily product. This was chromatographed over alumina and the peak fractions crystallized from acetone with the aid of Skellysolve B. Repeated crystallizations from acetone or ethyl acetate failed to give a product of good melting point. The best preparation melted at 110–120°. All crystal fractions showed the same infrared and ultraviolet absorption spectra, namely, hydroxyl absorption

(15) Since the completion of this work, F. Sondheimer, St. Kaufmann, J. Romo, H. Martinez and G. Rosenkranz (THIS JOURNAL, **75**, 4712 (1953)) have reported $[\alpha]_D$ +9° for 17β-hydroxyandrostane-3,6-dione in contrast to our value of −9°. The contribution to the molecular rotation by androstane, 3-ketone, 6-ketone and 17β-hydroxyl (D. H. R. Barton and W. Klyne, *Chem. and Ind.*, 755 (1948)) total −17°. The molecular rotation derived from our value of $[\alpha]_D$ −9° is −29°, but with the value of $[\alpha]_D$ +9° given by Sondheimer, *et al.*, the $[M]_D$ is +27°. Perhaps this is a typographical error on the part of these authors.

at 3380 cm.$^{-1}$; conjugated ketone at 1682 cm.$^{-1}$; conjugated carbon–carbon double bond at 1602 cm.$^{-1}$, $\lambda_{max}^{alc.}$ 251 mμ, ε 8,350; flexure at 314 mμ, ε 606.

6β-Acetoxy-17α-methyltestosterone (XXI).—Fifty mg. of 6β-hydroxy-17α-methyltestosterone (XVII) was allowed to react with 1.1 equivalents of acetic anhydride in pyridine for 16 hours. The reaction mixture was diluted with water and extracted with ether. The ether extract was washed with *N* hydrochloric acid, sodium bicarbonate solution, and water and dried with sodium sulfate. After evaporation of the ether the residue (59 mg.) was crystallized from a few drops of acetone by adding 1 drop of Skellysolve B to yield 27.5 mg. of starting material XVII. The residue in the mother liquors was subjected to alumina chromatography and the peak fractions (27 mg.) combined. Several recrystallizations from 0.2 ml. of methanol by addition of a few drops of water yielded 16 mg. of crystals, m.p. 125–143°. Recrystallization from 2 ml. of ethyl acetate plus 2 drops of ether did not improve the melting point. The infrared absorption spectrum indicated that the material was 6β-acetoxy-17α-methyltestosterone (XXI).

17β-Hydroxy-17α-methylandrostane-3,6-dione (XXII).—Five drops of 10% sulfuric acid in acetic acid was added to a solution of 26 mg. of 6β-hydroxy-17α-methyltestosterone (XVII) in 2.5 ml. of acetic acid. After 96 hours at room temperature the solution was made slightly alkaline with *N* sodium carbonate solution and extracted 4 times with 25-ml. portions of methylene dichloride. The methylene dichloride extract was washed with water, dried over sodium sulfate and the solvent removed at room temperature to yield 29 mg. of product. This was crystallized from 0.2 ml. of acetone by addition of 3 drops of Skellysolve B to give 9 mg. of 17β-hydroxy-17α-methylandrostane-3,6-dione (XXII), m.p. 180–187.5°. The infrared spectrum showed the following absorption bands: hydroxyl, 3410 cm.$^{-1}$; non-conjugated ketone, 1707 cm.$^{-1}$.

By alumina chromatography of the mother liquors a second material (7.7 mg.) was obtained, m.p. 136–150°. Infrared analysis indicated that this was the 17-anhydro derivative of XXII; non-conjugated ketone, 1712 cm.$^{-1}$; no hydroxyl absorption.

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KALAMAZOO, MICHIGAN

[CONTRIBUTION FROM THE RESEARCH LABORATORIES OF THE UPJOHN COMPANY]

Chemical Studies with 11-Oxygenated Steroids. V. A One-Step Oxidation–Halogenation of 3-Hydroxysteroids

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21-Acetoxy-3α,17α-dihydroxypregnane-11,20-dione is converted to 3-keto-4-halo compounds in one step by reaction with N-haloamides, hypohalous acids and *t*-butyl hypochlorite in aqueous *t*-butyl alcohol.

In the course of work on the synthesis of 11-dehydro-17α-hydroxycorticosterone acetate (cortisone acetate) and of 17α-hydroxycorticosterone (hydrocortisone) it became of interest to develop a procedure for the conversion of 3-hydroxysteroids to 3-ketosteroids in high yields. Since many of the steroids under investigation contained the sensitive dihydroxyacetone side chain at C-17, the use of an oxidizing agent such as chromic acid was not considered suitable since it is known that this type of reagent will degrade the side chain to the 17-ketones.[1] For this reason we directed our attention to the use of N-bromoacetamide (NBA), N-bromosuccinimide (NBS), N-chlorosuccinimide (NCS) and similar oxidants, which are mild oxidants for alcohols.[2]

Similar research was in progress at this time in another laboratory, and recently Hershberg and coworkers[3] reported their findings on the use of two of the above reagents (NBS and NBA) in the oxidation and bromination of 21-acetoxy-3α,17α-dihydroxypregnane-11,20-dione (I) to give 21-acetoxy-4-bromo-17α-hydroxypregnane-3,11,20-trione (IIIa). More recently there was reported the oxidation–chlorination of 3-hydroxysteroids[4] using principally *t*-butyl hypochlorite. We had previously[5] reported the use of this reagent in the oxidation–chlorination of 3α,11α,17α-trihydroxypregnane 20-one to 4-chloro-11α,17α-dihydroxypregnane-3,20-dione and of 3α,17α-dihydroxypregnane-11,20-dione to 4-chloro-17α-hydroxypregnane-3,11,-20-trione. However, since our reaction conditions and results are somewhat different from the above references, we wish to record them at this time.

The oxidation of I was initially investigated under two different reaction conditions. In one case the reaction solvent was anhydrous *t*-butyl alcohol containing 3% pyridine, whereas in the other the *t*-butyl alcohol contained 3% water. In both cases the reactions were run in ruby low actinic glassware, a 100% excess of oxidizing agent was used, and the reaction was followed by titration of the iodine liberated by the active halogen in an aliquot of the reaction mixture. In *t*-butyl alcohol–pyridine solution using NBA and NBS the reaction leveled off after 24 hours at one mole equivalent consumed. Crystallization occurred spontaneously and was completed by the addition of water containing sodium sulfite, resulting in 21-acetoxy-17α-hydroxypregnane-3,11,20-trione (II) in 90–93% yield in a high state of purity. When the reaction period was extended, some bromination occurred. However, in aqueous *t*-butyl alcohol solution using NBA or NBS, 1.6 mole equivalents was consumed in 16

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(2) H. Reich and T. Reichstein, *ibid.*, **26**, 562 (1943); L. H. Sarett, THIS JOURNAL, **71**, 1165 (1949); L. F. Fieser and S. Rajagopalan, *ibid.*, **71**, 3935 (1949); **72**, 5530 (1950).

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