[CONTRIBUTION FROM THE RESEARCH LABORATORIES, THE UPJOHN CO.]

Microbiological Transformations of Steroids. XV. Tertiary Hydroxylation of Steroids by Fungi of the Order *Mucorales*[1,2]

BY S. H. EPPSTEIN, P. D. MEISTER, D. H. PETERSON, H. C. MURRAY, H. M. LEIGH OSBORN, A. WEINTRAUB, L. M. REINEKE AND R. C. MEEKS

RECEIVED OCTOBER 31, 1957

Organisms of the order *Mucorales, viz., Mucor parasiticus* (A.T.C.C. 6476), *Mucor griseo-cyanus* (A.T.C.C. 1207) and *Helicostylum piriforme* (A.T.C.C. 8992), were found to oxygenate progesterone, deoxycorticosterone, 11-deoxycortisol and testosterone to the corresponding 14α-hydroxylated steroids. From the dissimilations of deoxycorticosterone and of 11-deoxycortisol, two additional products were isolated to which the formulas of the corresponding 8β(or 9α)-hydroxylated derivatives are tentatively assigned.[3]

Discussion

Previous communications from these laboratories showed that microörganisms of the genus *Rhizopus* oxygenate a number of steroids at positions 6β- and/or 11α-[4] and that *Cunninghamella blakesleeana*[5] can carry out 11β-hydroxylation of 11-deoxycortisol (Reichstein's Substance S) to form cortisol.

Since *Rhizopus* and *Cunninghamella* are genera of the order of *Mucorales* a detailed study of all the families of this order was undertaken in an attempt to determine whether such hydroxylations of steroids was a general feature of the metabolic make-up of the *Mucorales*. In this communication we report the oxygenation of testosterone, progesterone, deoxycorticosterone and 11-deoxycortisol by representatives of the family *Mucoraceae, viz., Mucor parasiticus* (A.T.C.C. 6476) and *M. griseo-cyanus* (A.T.C.C. 1207) and of the family *Thamnidiaceae viz., Helicostylum piriforme* (A.T.C.C. 8992). Some of these results have been disclosed previously[6] but this is the first detailed description of the work.

Deoxycorticosterone.—All three organisms oxygenated deoxycorticosterone (VII) at the 14α-position, to give 14α,21-dihydroxypregn-4-ene-3,20-dione (VIII). Either deoxycorticosterone or its 21-acetate could be employed but the product was always the free alcohol. The tertiary nature of the newly introduced hydroxyl group was established through the ready formation of only the 21-monoacetate (IX) and through resistance of the latter compound to oxidative attack. That this hydroxyl group occupied the 14α-position was proved when compound VIII was degraded to 14α-hydroxyandrostene-3,17-dione (IV) in low yield by oxidation with chromic acid.[7] Compound IV was identical to the 14ξ-hydroxyandrost-4-ene-3,17-dione prepared chemically from 3β-hydroxyandrost-5-en-17-one by St. Andre, *et al.*[8] That the configuration must be 14α- is deduced later in this discussion (*vide infra*).

Further evidence that compound VIII is, indeed, 14α,21-dihydroxypregn-4-ene-3,20-dione later was obtained by utilizing a microbiological removal of the side chain to form 14α-hydroxyandrostenedione. A small amount (20 mg.) of one of the original isolates of VIII was incubated in an erlenmeyer flask with *Penicillium lilacinum* (A.T.C.C. 10114). (For a fuller description of the use of this reaction *vide infra*.) Aliquots of the methylene chloride extract of this fermentation were analyzed by paper chromatography using a variety of solvent systems. In addition to about 14% of the starting material, at least 25% of a material was formed which could not be separated from authentic 14α-hydroxyandrostenedione, but was separated nicely from both 6β-hydroxyandrostenedione and 8β- (or 9α)-hydroxyandrostenedione. About 1% of a third component (presumed to be 14α-hydroxytestosterone) was formed. A comparison of the mobilities of the three hydroxyandrostenediones in the three solvent systems most useful in this case is given in Table I.

TABLE I

RELATIVE MOBILITIES OF 14α-HYDROXYANDROSTENEDIONE AND 8β(OR 9α)-HYDROXYANDROSTENEDIONE BY PAPER CHROMATOGRAPHY

Solvent system[a]	FBF	K-1	B-1
Relative mobility of			
14α-Hydroxyandrostenedione (IV)	0.55[b]	0.79[d]	0.33[e]
8β(or 9α)-Hydroxyandrostenedione (XIX)	.78[c]	1.17[c]	.40[e]
6β-Hydroxyandrostenedione	.74[b]	0.89[d]	.45[e]

[a] FBF = benzene *vs.* formamide, K-1 = cyclohexane–benzene *vs.* propylene glycol; B-1 = toluene–Skellysolve C *vs.* aqueous methanol. For a fuller treatment of these systems with authorship credit *cf.* footnote 20. [b] Compared to 11-ketoprogesterone. [c] Compared to 14α-hydroxyandrostenedione and 6β-hydroxyandrostenedione. [d] Compared to 11α-hydroxyprogesterone. [e] Compared to the solvent front.

A second bioconversion product was isolated from the fermentation of deoxycorticosterone acetate with *M. parasiticus* in approximately 1% yield.

(1) Paper XIV of this series, THIS JOURNAL, **78**, 1512 (1956).

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

(3) Since the completion of this work, other work in progress in these laboratories favors the 9α-hydroxy formulation as probably the correct one. A detailed report by the individuals involved will be submitted at a later date.

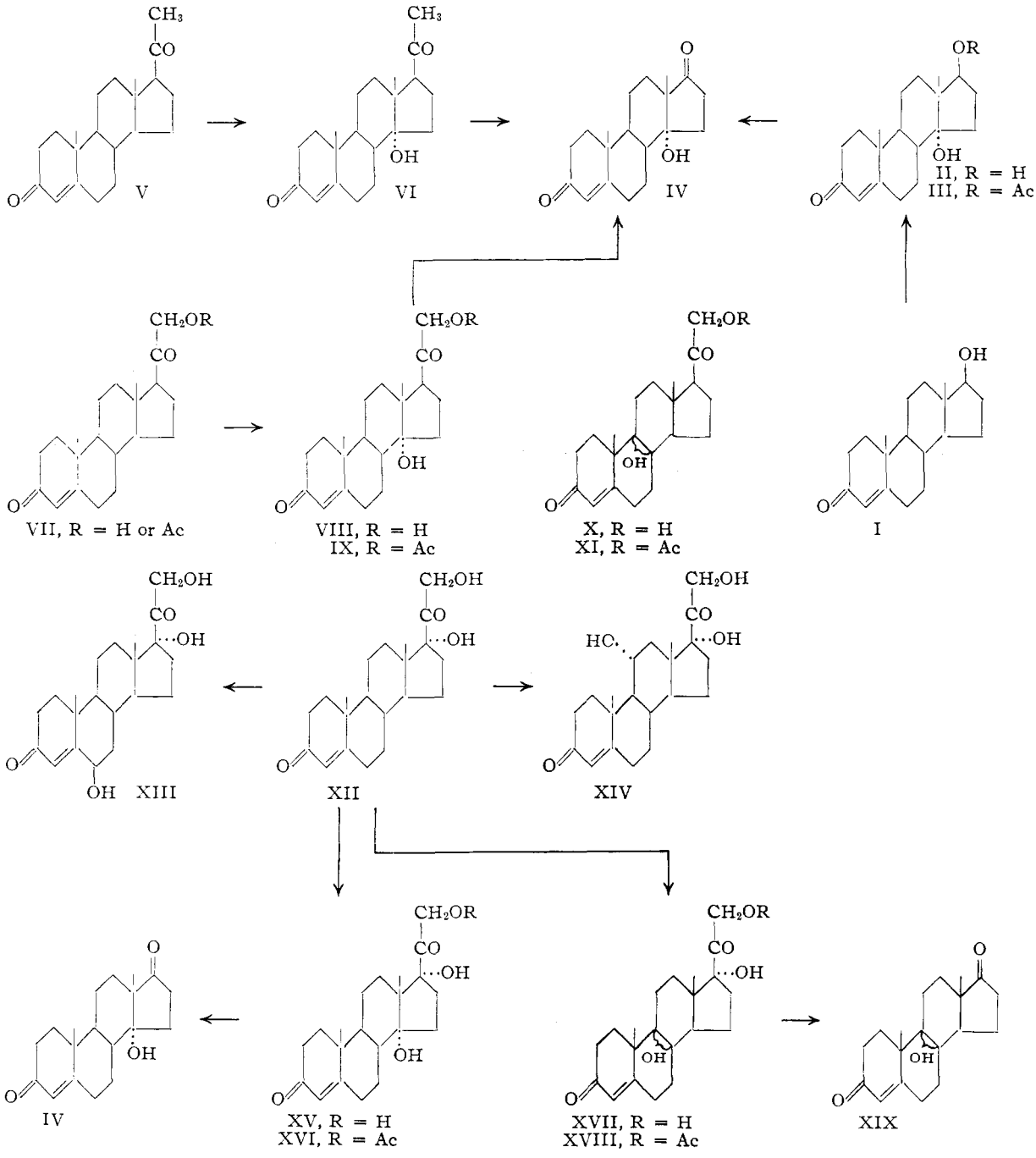
(4) Papers I–VI, X, XI and XIII of this series, THIS JOURNAL, **77**, 4428 (1955).

(5) F. R. Hanson, K. M. Mann, E. D. Nielson, H. V. Anderson, Marie P. Brunner, J. N. Karnemaat, D. R. Colingsworth and W. J. Haines, *ibid.*, **75**, 5369 (1953).

(6) *Cf.* ref. 2; also U. S. Patent 2,602,769.

(7) This oxidation was performed on the assumption that possibly we had 17-isodeoxycortisol (17β,21-dihydroxy-17α-pregn-4 ene-3,20-dione). The purity of compound VIII had been established by paper chromatography so that the compound IV, although formed in low yield, must have come from compound VIII. That the reaction occurred at all is remarkable.

(8) A. F. St. Andre, H. B. MacPhillamy, J. A. Nelson, A. C. Shabica and C. R. Scholz, THIS JOURNAL, **74**, 5506 (1952). We are greatly indebted to Dr. H. B. MacPhillamy, of Ciba Pharmaceutical Products, Inc., Summit, N. J., for a generous gift of this compound.



Paper chromatography showed that this compound was also present in very small quantities in two other bioconversions of deoxycorticosterone, one with *M. griseocyanus* and one with *H. piriforme*. The analytical and spectral data provided evidence that this compound was a monohydroxylated deoxycorticosterone. The tertiary nature of the new hydroxyl group was established by the same criteria as used above. The structure 8β(or 9α),21-dihydroxypregn-4-ene-3,20-dione (X) was tentatively assigned to this compound. [9]

(9) *Cf.* D. Stone, M. Hayano, R. I. Dorfman, O. Hechter, C. R. Robinson and C. Djerassi, THIS JOURNAL, **77**, 3926 (1955), who found this same compound as a dissimilation product of deoxycorticosterone by *Neurospora crassa*.

11-Deoxycortisol.—When the 11-deoxycortisol (Reichstein's Substance S) (XII) was incubated with *H. piriforme*, chromatography of the extractives over Florisil[10] led to the isolation of four products. Besides small amounts of the known compounds 6β,17α,21-trihydroxypregn-4-ene-3,20-dione (XIII) and 11α,17α,21-trihydroxypregn-4-ene-3,-20-dione (XIV) (epicortisol or "epi-F"), two new compounds were obtained as the major oxygenation products. One compound, 14α,17α,21-trihydroxypregn-4-ene-3,20-dione (XV), was isolated in 6.4% yield. Ready acetylation of XV to only the 21-

(10) A synthetic magnesium silicate manufactured by the Floridin Co., Warren, Pa.

monoacetate XVI was indicative of the tertiary nature of the nuclear hydroxyl group. In order to relate compound XV with the above-described 14α-hydroxysteroids, it was oxidatively degraded to 14α-hydroxyandrost-4-ene-3,17-dione (IV) with chromium trioxide.

The predominant product XVII of the bioconversion was isolated in 15% yield. The tertiary nature of the nuclear hydroxyl group was established through the acetylation of XVII to the 21-monoacetate XVIII and by oxidative degradation to a hydroxyandrostenedione (XIX) different from IV. The new hydroxyl group was tentatively assigned to the 8β(or 9α)-position.

The influence exerted by the 17α-hydroxyl group of 11-deoxycortisol on the enzymatic activity of *H. piriforme* is noteworthy. This microörganism produces the 14α-hydroxy-11-deoxycorticosterone from deoxycorticosterone as the predominant product. Any other bioconversion products formed are present only in quantities smaller than 1%. However, if 11-deoxycortisol is subjected to the action of the same microörganism, the oxygenation at position 14 is suppressed in favor of the oxygenation at position 8 (or 9). Thus, 8β(or 9α),17α,21 - trihydroxypregn - 4 - ene - 3,20 - dione (XVII) becomes the major oxygenation product.

Testosterone.—When testosterone (I) was transformed by *M. griseo-cyanus*, the crude extractives of the fermentation medium contained, on the basis of paper chromatography, predominantly one steroidal component. Alumina chromatography and recrystallization of the appropriate fractions led to the isolation of 14α-hydroxytestosterone (II) in 35% yield. Acetylation gave the 17β-monoacetate III and oxidation with two equivalents of chromic acid converted II to 14α-hydroxyandrostenedione (IV).

Progesterone.—The introduction of the 14α-hydroxyl group into progesterone was achieved with either *M. parasiticus*, *M. griseo-cyanus* or *H. piriforme*. The three samples of 14α-hydroxyprogesterone (VI) obtained from the three bioconversions differed from each other significantly in rotation and melting point although all three exhibited the same infrared spectrum (Table II). Paper chromatography disclosed, in each case, only one (identical) material which absorbed ultraviolet light. When the paper strips were examined by the fluorescence technique of Bush[11] a second component was detected. This contaminant exhibited a bright yellow fluorescence (the 14α-hydroxyprogesterone showed no fluorescence under these conditions in our hands). To attempt any sort of quantitation of the unknown by the fluorescence was obviously impossible. However, when 100 mcg. quantities of the three samples of 14α-hydroxyprogesterone were chromatographed, sample A showed only a small and weakly fluorescent spot of contaminant, sample C showed a large and brightly fluorescent spot, while sample B showed no contaminant. The unknown contaminant was not detected by 2,4-dinitrophenylhydrazine reagent. Although the impurity of sample B could not be shown by these tests, the more drastic treatment used in isolating this sample together with the disparity in elementary analysis and the low molecular rotation made this sample suspect. Since the molecular rotatory contribution of the 14α-hydroxy group as calculated from sample A is in best agreement with the values obtained for the other 14α-hydroxysteroids (see Table III), the properties of sample A were presumed to represent the physical constants of 14α-hydroxyprogesterone.

(11) E. I. Bush, *Biochem. J.*, **50**, 370 (1952).

TABLE II

COMPARISON OF THREE SAMPLES OF 14α-HYDROXYPROGESTERONE

Sample	Organism used	[α]D	14α-OH Δ [M]D	M.p., °C.	Analyses, % C	% H	Infrared spectrum
A	*M. griseo-cyanus*	200°	+33	180–187	76.50	9.31	Same
B	*M. parasiticus*	188	− 7	195–200.5	77.02	9.55	Same
C	*H. piriforme*	215	+82	191–199	76.39	9.36	Same
				Calcd.	76.40	9.15	

The structure proof of 14α-hydroxyprogesterone by conventional chemical means would have required a number of steps either for the degradation of the 14α-hydroxyprogesterone to 14α-hydroxyandrostenedione or for the conversion of any of the other 14α-hydroxysteroids into compound VI. A short cut to this problem was found in the microbiological degradation of C_{21}-steroids by certain *Penicillia*, *Aspergilli* and *Gliocladia*.[12] When 14α-hydroxyprogesterone was incubated with *Penicillium lilacinum* (A.T.C.C. 10114), 14α-hydroxyandrostene-3,17-dione (IV) was isolated in 5% yield. Although the yield was low, this represented the major conversion product since considerable starting material was left.

Discussion of the Structural Assignments

14α-Hydroxysteroids.—The 14α-position of the microbiologically introduced hydroxyl function in compounds II, VI, VIII and XV has been deduced from the following observations: 14α-Hydroxyandrost-4-ene-3,17-dione (IV) is, as briefly pointed out above, identical in all physical details to 14ξ-hydroxyandrost-4-ene-3,17-dione which was prepared by St. Andre, *et al.*[8] The fact that 14-hydroxy - 21 - acetoxypregn - 4 - ene - 3,20 - dione (IX) is completely different from 14β-hydroxy-21-acetoxypregn-4-ene-3,20-dione as prepared by Lardon[13] determines the α-configuration of the hydroxyl function in compound IX and hence, in view of the above described degradations, in compounds II, VI, VIII and XV.

The molecular rotatory contribution of the 14α-hydroxyl group in 14α-hydroxyandrost-4-ene-3,17-dione (Table III) is markedly lower than the contributions observed in the case of the corresponding testosterone, progesterone or deoxycorticosterone derivative. This is presumably a vicinal action of the 17-ketone on the 14-hydroxyl group.

Since the completion of this work other 14α-hydroxysteroids have been reported by Agnello, *et al.*[14] In order to compare the molecular rotatory contributions of the 14α-hydroxyl groups in these

(12) Paper IX of this series, THIS JOURNAL, **75**, 5786 (1953).

(13) A. Lardon, *Helv. Chim. Acta*, **32**, 1517 (1949).

(14) E. J. Agnello, B. L. Bloom and G. D. Laubach, THIS JOURNAL, **77**, 4684 (1955).

TABLE III

MOLECULAR ROTATION INCREMENTS OF HYDROXYL GROUPS IN POSITIONS 14α, 14β AND 8β(OR 9α)

Parent compound	[M]D[a] I Unsubstd.	II 14α-Hydroxy	III 14β-Hydroxy	IV 8β(or 9α)-Hydroxy	Δ [M]D II-I	Δ [M]D III-I	Δ [M]D IV-I
11-Deoxycortisol	+426	+562	...	+388	+122	...	−53
	+440	(MeOH)		(Di)			
Deoxycorticosterone	+611	+658	...	+578	+47	...	−33
Deoxycorticosterone Acetate	+677	+746	+481	+688	+69	−196	+11
Progesterone	+628	+661	+446	...	+33	−182	..
Testosterone	+330 ($CHCl_3$)	+378	...	...	+52	...	..
Androstenedione	+570	+527	...	+499	−42	...	−71
Cortisone	+742 (Di)	+790[b] (Di)	...	...	+48	...	..
Cortisone acetate	+869 (Di)	+992[b] (Di)	...	...	+123	...	..
Cortisol	+589 (EtOH)	+693[b] (EtOH)	...	...	+95	...	..
Cortisol acetate	+708 (EtOH)	+618[b] (EtOH)	...	...	−90	...	..
Cortisol acetate	+691[c] (Di)	+710[c] (Di)	...	...	+19	...	..

[a] All rotations in chloroform except where otherwise mentioned (EtOH = ethanol, MeOH = methanol, Di = dioxane). Calculated from the data of Agnello, *et al.*[14] [c] Personal communication from Dr. Agnello *via* Dr. Bloom.

TABLE IV

PAPER CHROMATOGRAPHIC MOBILITIES OF 6β-HYDROXY-, 11α-HYDROXY- AND 14α-HYDROXYSTEROIDS

Solvent system[a]	Parent compound	Mobility[b] of deriv., cm. I 6β-OH	II 11β-OH	III 14α-OH	Ratio I/II	Ratio I/III	Ratio II/III
PTF	Testosterone	10.5	6.1	13.9	1.72	0.76	0.44
PT	Androstenedione	36.0	23.0	28.0	1.57	1.32	.82
PT	Progesterone	68.0	36.0	68.0	1.89	1.0	.53
PTF	Deoxycorticosterone	34.8	17.3	34.8	2.01	1.0	.50
PTF	17α,21-Dihydroxy-4-pregnene-3,20-dione	2.26	1.22	17.2	1.85	0.13	.07

[a] PT, propylene glycol *vs.* toluene system of Zaffaroni and Burton[15]; PTF, as above, with the amount of glycol halved by dilution with methanol followed by evaporation of the diluent from the paper. [b] Comparisons of actual mobilities are valid only within the horizontal rows.

compounds with the results reported above, the rotations of the corresponding 14α-hydroxysteroids were determined by us in the solvents which Agnello, *et al.*, used for the 14α-hydroxysteroids. With the exception of 14α-hydroxycortisol 21-acetate, the results are in reasonable agreement with the contributions of the 14α-hydroxyl group obtained by us. Dr. Agnello kindly sent us a revised rotation for the 14α-hydroxycortisol acetate which brings this compound in closer agreement to the others. These comparisons are given in Table III.

Paper chromatographic mobilities (Table IV) reveal another interesting detail which indirectly supports the assignment of the 14α-position to the microbiologically introduced hydroxyl group in compounds II, VI, VIII and XV. If the ratios of the mobilities of the 6β-hydroxy-, 11α-hydroxy- and 14α-hydroxysteroids are compared, it is evident that 14α,17α,21-trihydroxypregn-4-ene-3,20-dione (XV) moves 6 to 10 times faster than would be expected. Since 14α-hydroxy-11-deoxycorticosterone (VIII) shows the average mobility expected of a 14α-hydroxysteroid, the presence of the 17α-hydroxyl group in compound XV is presumed to be responsible for the drastic change in the polarity of XV. This effect might be satisfactorily explained by virtue of the axial (with respect to ring C) relationship of the 14α- and the 17α-hydroxyl groups.[16] The diaxial 1,3-glycol group in XV appears to have an entirely different effect on the polarity than the 14α-hydroxyl group alone in VIII, which is not at all surprising.

Another observation may, perhaps, be interpreted to support this argument. Bloom and Shull[17] stated that the 14α,15α-epoxide of 11-deoxycortisol is less polar (as judged by paper chromatography) than is 11-deoxycortisol itself. Generalizing from some twenty examples, we have concluded that the epoxide group only slightly increased the polarity of the parent compound (for example, 16α,17α-epoxydeoxycorticosterone has a mobility less than that of deoxycorticosterone but greater than that of 11-deoxycortisol). One might expect that the physicochemical behavior of a *cis*-1-hydroxy-3-epoxy group would be similar to that of a diaxial 1,3-glycol; *i.e.*, among other effects there should be a decrease in polarity and, since the contribution to the polarity of the molecule furnished by the epoxide is considerably less than that furnished by the corresponding hydroxyl (14α-, in this case), the net result of this interaction is a polarity less than that of the parent steroid.

8β(or 9α)-Hydroxysteroids.—The nuclear hydroxyl introduced in compounds X, XVII and XIX is presumed to be the same. Although compounds X and XVII have never been directly related to each other by degradation, the molecular rotatory increments show clearly that the hydroxyl group in all probability occupies the same position in all three compounds. The increment for the hydroxyandrostenedione does not differ to any great extent from the increments for compounds X and

(15) A. Zaffaroni and R. B. Burton, *J. Biol. Chem.*, **193**, 749 (1951).

(16) D. H. R. Barton, *J. Chem. Soc.*, 1027 (1953); *Experientia*, **6**, 316 (1950).

(17) B. M. Bloom and G. M. Shull, THIS JOURNAL, **77**, 5767 (1955)

XVII which would indicate that the center bearing this hydroxyl group is outside any vicinal effect of the 17-keto group. This new hydroxyl group must be in either the 8β- and 9α-position if we assume that ring inversion has not taken place (and until evidence is furnished to the contrary this is the logical assumption). The scant information regarding the increments of hydroxyl groups in position 9α- and 8β- does not allow a definite location of this group at the present time (*cf.* footnote 3).

Experimental[18]

A. Conversion of Deoxycorticosterone Acetate (VII) to 14α-Hydroxy-11-deoxycorticosterone (VIII). 1. By *M. griseo-cyanus* (A.T.C.C. 1207).—*M. griseo-cyanus* was grown on 4 liters of medium H.[19] After 24 hours 1.13 g. of deoxycorticosterone acetate (equivalent to 1 g. of deoxycorticosterone) was added in 100 ml. of acetone and the fermentation continued for another 24 hours. The final *p*H was 3.6. The methylene chloride extract of the beer yielded 2.5 g. of oily crystals. Paper chromatography of this material showed complete utilization of the substrate with production of a main component VIII with a mobility similar to that of 11-deoxycortisol. Two components moving near cortisone and cortisol were also present in very minor amounts

The solvent-free extract was triturated exhaustively with Skellysolve B and then with ether, leaving 640 mg. of brown crystals. These were dissolved in 12 ml. of hot methanol, the solution filtered and then cooled in the refrigerator to yield 300 mg. of white crystals, m.p. 175–176° (softening at 173°). Two more recrystallizations of the material from methanol did not alter the melting point. The infrared spectrum of this compound in chloroform indicated retention of the conjugated ketone and the ketol side chain and indicated extra hydroxyl. When recrystallized twice more from methanol, this sample analyzed for a monohydroxydeoxycorticosterone (VIII) solvated with methanol, m.p. 175–176°. No impurity was detected by paper chromatography until a level of 400 mcg. of steroid was employed. At this level approximately 1 mcg. of an impurity was noted which absorbed ultraviolet light and reduced silver diamine and which moved more slowly than 6β,21-dihydroxypregn-4-ene-3,20-dione in the benzene–formamide system of Zaffaroni.[15] Other solvent systems added nothing to this picture.[20]

Anal. Calcd. for $C_{21}H_{30}O_4CH_3OH$: C, 69.81; H, 9.05. Found: C, 70.37; H, 8.70.

Recrystallization from acetone afforded crystals with the analysis: Calcd. for $C_{21}H_{30}O_4$: C, 72.80; H, 8.73. Found: C, 72.2; H, 8.60.

Another preparation of the same compound which had never come in contact with methanol was recrystallized from acetone throughout. The constants of compound VIII were m.p. 167–170.5°, $[\alpha]^{23}D$ +190° (*c* 0.334 in chloroform); infrared spectrum: hydroxyl absorption at 3519, 3461 and 3322 cm.$^{-1}$; non-conjugated carbonyl absorption at 1720, 1706 cm.$^{-1}$; conjugated carbonyl at 1679, 1656 cm.$^{-1}$; conjugated carbon double bond at 1615 cm.$^{-1}$.

Anal. Calcd. for $C_{21}H_{30}O_4$: C, 72.80; H, 8.73. Found: C, 72.83; H, 8.48.

Acetylation of 14α,21-Dihydroxypregn-4-ene-3,20-dione (VIII).—14α-Hydroxy-21-acetoxypregn-4-ene-3,20-dione (IX) was prepared readily in 85% yield by reaction with acetic anhydride–pyridine (1:1) at room temperature for 16 hours. It was recrystallized from acetone–hexane (1:1). The melting point of the product was 158–161°, $[\alpha]^{23}D$ 192° (*c* 0.535 in chloroform). The infrared spectrum indicated that one hydroxyl group had not been acetylated; infrared spectrum: hydroxyl absorption at 3449 cm.$^{-1}$, 21-acetate carbonyl at 1751 cm.$^{-1}$, 20-ketone at 1722 cm.$^{-1}$, conjugated ketone at 1664 cm.$^{-1}$, conjugated carbon double bond at 1614 cm.$^{-1}$, acetate carbon–oxygen at 1234 cm.$^{-1}$.

Anal. Calcd. for $C_{23}H_{32}O_5$: C, 71.10; H, 8.30. Found: C, 71.00; H, 8.71.

Oxidation of 14α-Hydroxy-11-deoxycorticosterone (VIII) to 14α-Hydroxyandrostene-3,17-dione (IV).—Attempts to oxidize the free hydroxyl group of IX with the theoretical amount of chromium trioxide in acetic acid at room temperature for 4 hours yielded only starting material.

14α-Hydroxy-11-deoxycorticosterone (150 mg.) was dissolved in 25 ml. of redistilled acetic acid; 250 mg. of chromium trioxide in 25 ml. of acetic acid was added and the mixture allowed to stand at room temperature for 48 hours. (The oxidation was slow as determined by color change.) The mixture was evaporated to dryness *in vacuo* at room temperature and extracted with methylene chloride. The extract was washed with bicarbonate and water, dried with Na_2SO_4 and evaporated to yield 20 mg. of a product which was fractionated over alumina by our usual procedure; the fractions eluted with benzene–ether (3:1) contained 15.8 mg. of a product which was crystallized from acetone to yield 3.6 mg. of impure crystals, m.p. 242–245.5°. The infrared spectrum clearly indicated this compound to be identical to 14α-hydroxyandrost-4-ene-3,17-dione (IV).

2. By *M. parasiticus* (A.T.C.C. 6476).—*M. parasiticus* was grown in 4 five-gallon bottles, each containing 12 liters of medium H. After 24 hours, 11 g. of substrate VII was distributed between the four bottles. The bioconversion was continued in bottles A and B for 24 hours and in bottles C and D for 48 hours. The extracts of the four bottles were investigated individually by paper chromatography.

Experiment	Cmpd. VIII, %	Cmpd. X, %
A	44	1
B	40	4
C	75	3
D	72	3

The four extracts were combined to give 27.35 g. of solids. These were dissolved in 500 ml. of ethylene dichloride and chromatographed over 900 g. of Florisil. Fractions of 1400-ml. volume were eluted, using ethylene dichloride containing increasing quantities of acetone from 0 to 100%.

Fractions 10–17 (9, 11.2 and 16.6% acetone), containing the major bioconversion product (paper chromatography), were combined. After evaporation of the solvent the residue was crystallized from 20 ml. of methanol, yielding 4.06 g. of crystalline 14α,21-dihydroxypregn-4-ene-3,20-dione (VIII). A sample was recrystallized from acetone to give crystals melting at 174–179°. The infrared spectrum identified this compound as the solvated 14α-hydroxy-11-deoxycorticosterone (VIII) described above.

8β(or 9α),21-Dihydroxypregn-4-ene-3,20-dione (X).—The fractions of the above chromatogram which were eluted with 50% acetone in ethylene dichloride were combined (2.66 g.), the solvent removed by evaporation, and the residue dissolved in 150 ml. of ethylene dichloride and chromatographed over 150 g. of Florisil. Fractions of 250-ml. volume were collected, using as solvents ethylene dichloride containing increasing amounts of acetone.

Fractions 10 and 11 (25% acetone) eluted 795 mg. of material (crystalline after removal of solvent) which was recrystallized from 10 ml. of acetone. The concentrated solution was diluted with petroleum ether until crystallization set in. After two recrystallizations, 134.5 mg. of pure 8β(or 9α),21-dihydroxypregn-4-ene-3,20-dione (X), having a melting point of 180–183°, was obtained. The infrared spectrum showed that compound X was not identical to any previously prepared hydroxy-deoxycorticosterone; λ_{max}^{alc} 243 mμ (*E* 14,300), $[\alpha]^{23}D$ 167° (*c* 0.709 in chloroform); infrared spectrum: hydroxyl absorption at 3425 cm.$^{-1}$, 20-ketone at 1703 cm.$^{-1}$, conjugated ketone at 1667 cm.$^{-1}$, conjugated carbon double bond at 1619 cm.$^{-1}$.

Anal. Calcd. for $C_{21}H_{30}O_4$: C, 72.80; H, 8.73. Found: C, 72.38; H, 8.64.

Acetylation of X to Form the 21-Monoacetate (XI).—8β(or 9α)-Hydroxy-21-acetoxypregn-4-ene-3,20-dione (XI) was prepared in the conventional manner by overnight reaction with pyridine–acetic anhydride (1:1). The crystals, prepared from 44.5 mg. of compound X, were recrystallized from acetone–Skellysolve B to give 37.6 mg. of the acetate, m.p. 212–214°, λ_{max}^{alc} 243 mμ (*E* 15,150), $[\alpha]^{23}D$ 177° (*c*

(18) All melting points were taken on a Fisher–Johns block and are uncorrected.

(19) Description of the media and general conditions of growth, conversion and extraction are given in paper I of this series, THIS JOURNAL, **74**, 5933 (1952).

(20) For a fuller characterization of VIII by paper chromatography, *cf.* L. M. Reineke, *Anal. Chem.*, **28**, 1853 (1956).

0.834 in chloroform). The infrared spectrum indicated that one hydroxyl group was not acetylated; infrared spectrum: hydroxyl absorption at 3397 cm.$^{-1}$, 21-acetate carbonyl at 1749 cm.$^{-1}$, 20-ketone at 1718 cm.$^{-1}$, conjugated ketone at 1639 cm.$^{-1}$, conjugated carbon double bond at 1608 cm.$^{-1}$, acetate carbon–oxygen at 1234 cm.$^{-1}$.

Anal. Calcd. for $C_{23}H_{32}O_5$: C, 71.10; H, 8.30. Found: C, 71.14; H, 8.08.

Attempted Oxidation of Compound XI.—When 18 mg. of 8β(or 9α)-hydroxy-21-acetoxypregn-4-ene-3,20-dione was dissolved in 3 ml. of glacial acetic acid and mixed with a solution of 5 mg. of chromium trioxide in 2 ml. of 90% acetic acid at room temperature for 5 hours, 17.0 mg. of crystals was recovered. These, upon recrystallization from ethyl acetate–Skellysolve B, gave 11.5 mg. of crystals with a melting point of 209–212°. The infrared spectrum of this compound was identical to the spectrum of the starting material.

3. By *H. piriforme* (A.T.C.C. 8992).—*H. piriforme* was grown on 12 l. of medium H for 25 hours under our standard conditions. Then a solution of 3 g. of deoxycorticosterone acetate in 200 ml. of acetone was added, and the fermentation was continued for 24 hours. The usual extraction procedure yielded 5.52 g. of an oily product. This was shown by paper chromatography to contain traces of X, traces of a substance with a mobility close to that of cortisone and 50% of 14α,21-dihydroxypregn-4-ene-3,20-dione. The extractive was dissolved in 200 ml. of ethylene dichloride and chromatographed over 400 g. of Florisil. Fractions of 650 ml. were collected, using increasing quantities of acetone in ethylene dichloride as eluents.

Fractions 13–17 (9 and 16.6% acetone) contained the bulk of the main conversion product. These fractions were combined, the solvent removed, and the residue dissolved in 20 ml. of methanol. After concentration of the methanolic solution, ether was added. Upon cooling in the refrigerator an amorphous precipitate formed which was separated by filtration. The filtrate immediately crystallized. Two crystallizations yielded 590 mg. of product having a melting point of 167–173.5°. The infrared spectrum confirmed the identity of this compound with 14α-hydroxydeoxycorticosterone as produced by the *Mucors*, λ_{max}^{alc} 242 mμ (*E* 15,800).

B. Conversion of 11-Deoxycortisol (XII) to 6β,17α,21-Trihydroxypregn-4-ene-3,20-dione (XIII), 11α,17α,21-Trihydroxypregn-4-ene-3,20-dione (XIV), 14α,17α,21-Trihydroxypregn-4-ene-3,20-dione (XV) and 8α(or 9β),17α,21-Trihydroxypregn-4-ene-3,20-dione (XVII).—*H. piriforme* was grown on 240 liters of medium H for 24 hours. Then 32 g. of 11-deoxycortisol (XII) dissolved in 2 liters of alcohol was added. After an incubation period of 48 hours the fermentation broth was separated into mycelium and beer filtrate which were extracted separately with methylene chloride. Paper chromatographic analysis showed that the extract obtained from the mycelial cake contained various steroids in trace amounts. It was, therefore, discarded. The extract of the beer filtrate consisted of six components, two of which were present in concentration of 25–30%. The solids (120 g.) of this extract were dissolved in 2.8 l. of ethylene dichloride and chromatographed over 1.8 kg. of Florisil using increasing quantities of acetone in ethylene dichloride as eluents. Paper chromatography of each fraction showed that the 11 and 16.6% acetone eluates consisted largely of one component. These fractions were, therefore, combined to give fraction A. The two 25% acetone eluates were shown to contain mostly 6β,17α,21-trihydroxypregn-4-ene-3,20-dione. These two fractions were combined to give fraction B. The 50% acetone eluates were composed of 11-epicortisol and a new compound in a ratio of 1:3. They were combined to give fraction C. In the 100% acetone eluates 11-epicortisol was the major component.

Isolation of 14α,17α,21-Trihydroxypregn-4-ene-3,20-dione (XV).—Fraction A of the above chromatogram (5.32 g.) was dissolved in 100 ml. of methanol and the solution concentrated until crystallization set in. After refrigeration for one hour, filtration gave 2.15 g. of crystals (6.4% yield) with a melting point of 225–232°. This compound was identified as 14α,17α,21-trihydroxypregn-4-ene-3,20-dione (XV) (*vide infra*). A small sample was recrystallized to give material melting at 234–237°. Further recrystallization did not raise the melting point. The infrared spectrum showed that the compound was not identical to any known compound of the $C_{21}H_{30}O_5$ series; $[\alpha]^{23}D$ 155° (*c* 1.13 in methanol); infrared spectrum: hydroxyl at 3549, 3372 cm.$^{-1}$, 20-ketone at 1704 cm.$^{-1}$, conjugated ketone at 1650 cm.$^{-1}$, conjugated carbon double bond at 1613 cm.$^{-1}$.

Anal. Calcd. for $C_{21}H_{30}O_5$: C, 69.58; H, 8.34. Found: C, 69.42, 69.66; H, 8.71, 8.40.

Acetylation of XV.—The 21-monoacetate (XVI) was prepared by dissolving 44.2 mg. of 14α,17α,21-trihydroxypregn-4-ene-3,20-dione in 4 ml. of absolute pyridine–acetic anhydride (1:1). When the reaction mixture was worked up after 18 hours, 55.5 mg. of a crystalline residue was obtained. Two recrystallizations from acetone yielded 36.0 mg. of the acetate XVI, melting at 232–235°, $[\alpha]^{23}D$ 167° (*c* 0.591 in chloroform). The infrared spectrum indicated that one hydroxyl had been acetylated; infrared spectrum: hydroxyl at 3485, 3362 cm.$^{-1}$; 21-acetate carbonyl and 20-ketone at 1748, 1735 cm.$^{-1}$; conjugated ketone at 1667 cm.$^{-1}$; conjugated carbon double bond at 1618 cm.$^{-1}$; acetate carbon–oxygen at 1279, 1244 cm.$^{-1}$.

Anal. Calcd. for $C_{23}H_{32}O_6$: C, 68.29; H, 7.89. Found: C, 68.19; H, 8.07.

Oxidation of 14α,17α,21-Trihydroxypregn-4-ene-3,20-dione (XV) to 14α-Hydroxyandrost-4-ene-3,17-dione (IV).—Compound XV (129.5 mg.) was dissolved in 10 ml. of glacial acetic acid and oxidized by adding dropwise a solution of 103.5 mg. of chromic anhydride in 1 ml. of water and 5 ml. of acetic acid. After 20 hours at room temperature the reaction mixture was diluted with methanol (25 ml.), concentrated *in vacuo*, diluted with 70 ml. of water and extracted with ether–methylene chloride (5:1).

The combined extracts were washed once with water, twice with 5% sodium hydroxide, then four times with water. The extract was dried over sodium sulfate and concentrated to yield 71.7 mg. of crystals. These were recrystallized twice from 2 ml. of acetone. Crystallization was induced by adding hexane to the boiling solution. The final product had a melting point of 259–265°. Its infrared spectrum was identical to that of 14α-hydroxyandrost-4-ene-3,17-dione.

Isolation of 6β,17α,21-Trihydroxypregn-4-ene-3,20-dione (XIII).—Fraction B of the above chromatogram (2.46 g.) was recrystallized twice from methanol to give 750.5 mg. of crystals (2% yield), melting at 228–232°; when this compound was mixed with 6β,17α,21-trihydroxypregn-4-ene-3,20-dione the melting point was 225–230°. The infrared spectrum showed that this compound was identical to 6β,17α,21-trihydroxypregn-4-ene-3,20-dione (XIII) as isolated from the dissimilation by *Rhizopus nigricans*.[4] The mother liquors of this crystallization were shown (paper chromatography) to consist of about equal amounts of compound XIII and compound XV.

Isolation of 8β(or 9α),17α,21-Trihydroxypregn-4-ene-3,20-dione (XVI).—Fraction C of the above Florisil chromatogram (7.43 g.) was dissolved in 100 ml. of methanol. The solution was concentrated to incipient crystallization. On filtration, 3.627 g. of crystals melting at 242–248° was obtained. From the mother liquor of this crystallization a second crop of crystals (1.33 g.), melting at 240–245° was obtained. The total yield was 14.8%. A sample was twice recrystallized from methanol to give pure 8β(or 9α),17β,21-trihydroxypregn-4-ene-3,20-dione (XVII), melting at 248–252°. The melting point of this compound is subject to fluctuations in the range of 242–252°, $[\alpha]^{23}D$ 107° (*c* 0.946 in dioxane). The infrared spectrum characterized compound XVII as a new steroid of the $C_{21}H_{30}O_5$ group; infrared spectrum: hydroxyl absorption at 3555 cm.$^{-1}$; 20-ketone at 1719, 1699 cm.$^{-1}$; conjugated ketone at 1637 cm.$^{-1}$; conjugated carbon double bond at 1618 cm.$^{-1}$.

Anal. Calcd. for $C_{21}H_{30}O_5$: C, 69.58; H, 8.34. Found: C, 69.46; H, 8.46.

Acetylation of XVII.—The 21-monoacetate (XVIII) was prepared from 30.6 mg. of compound XVII with acetic anhydride—pyridine at room temperature. The reaction was allowed to proceed overnight. The crude acetate (34.5 mg.) was recrystallized twice from acetone to give crystals melting at 245–246° or 255–258°. The infrared spectrum indicated acetylation of one hydroxyl; infrared spectrum: hydroxyl absorption at 3555 cm.$^{-1}$, 21-acetate at 1750 cm.$^{-1}$, 20-ketone at 1718 cm.$^{-1}$, conjugated ketone at 1646 cm.$^{-1}$, conjugated carbon double bond at 1616 cm.$^{-1}$, acetate ester linkage at 1241 cm.$^{-1}$.

Anal. Calcd. for $C_{23}H_{32}O_6$: C, 68.29; H, 7.98. Found: C, 68.18; H, 8.24.

Oxidation of Compound XVII to 8β(or 9α)-Hydroxyandrost-4-ene-3,17-dione (XIX).—Compound XVII (60.0 mg.) was dissolved in 5 ml. of glacial acetic acid. To this a solution of 50.7 mg. of chromium trioxide in 3 ml. of 95% acetic acid was added dropwise. After 20 hours at room temperature the solution was diluted with 20 ml. of methanol and concentrated to dryness *in vacuo* in a water-bath at 50°. The residue was taken up in water and extracted with ether–chloroform (10:1). The extracts were washed once with water, once with 5% sodium hydroxide and four times with water. Drying over sodium sulfate followed by evaporation of solvent yielded 38.7 mg. of a crystalline product which, after recrystallization from acetone, had a melting point of 214–217°, $[\alpha]^{23}D$ 165° (*c* 0.80 in chloroform). The infrared spectrum showed this compound to be a new hydroxyandrostenedione. It was tentatively assigned the structure of 8β (or 9α)-hydroxyandrost-4-ene-3,17-dione (XIX), λ_{max}^{alc} 242 mμ (*E* 14,400); infrared spectrum: hydroxyl absorption at 3415 cm.$^{-1}$, 17-ketone at 1725 cm.$^{-1}$, conjugated ketone at 1655 cm.$^{-1}$, conjugated carbon double bond at 1613 cm.$^{-1}$.

Anal. Calcd. for $C_{19}H_{26}O_3$: C, 75.46; H, 8.66. Found: C, 75.49; H, 9.21.

Isolation of 11α,17α,21-Trihydroxypregn-4-ene-3,20-dione (11-Epicortisol) (XIV).—Fraction 20 of the Florisil chromatogram (2.587 g.) was triturated with 5 ml. of methanol. Decantation of the supernatant solution gave 1.03 g. of a mixture of compounds (paper chromatography). The mixture was dissolved in 150 ml. of ethylene dichloride and chromatographed over 80 g. of Florisil. The 50% acetone eluates were shown by paper chromatography to consist largely of the 11-epimer of cortisol. One of these fractions (67.5 mg.) was dissolved in methanol and concentrated to give a first crop (10 mg.) of crystals melting at 205–224°. From the mother liquors of this crystallization a second crop (25 mg.) was obtained, having a melting point of 195–210°. These crystals were recrystallized from methanol to give 15 mg. of 11α,17α,21-trihydroxypregn-4-ene-3,20-dione (XIV), melting at 206–211°. The infrared spectrum identified this compound as 11-epicortisol.

C. Conversion of Testosterone (I) to 14α-Hydroxytestosterone (II) by *M. griseo-cyanus* (A.T.C.C. 1207).—*M. griseo-cyanus* was grown on 12 l. of medium H for 23 hours. Then 4.0 g. of testosterone was added in acetone and the fermentation continued for 23 hours. Extraction with methylene chloride, followed by solvent removal, yielded an oily extractive weighing 6.73 g. Paper chromatography showed a major component (about 40%) and two minor components. The extractives were dissolved in 100 ml. of benzene and chromatographed over 340 g. of alumina. The column was eluted with two 100-ml. portions of each of the following solvents: benzene, benzene–ether 1:1, ether, 5% chloroform in ether, 10% chloroform in ether, 20% chloroform in ether, 50% chloroform in ether, chloroform, 5% acetone in chloroform, 10% acetone in chloroform, 20% acetone in chloroform, 50% acetone in chloroform, acetone, 5% methanol in acetone, 10% methanol in acetone, methanol.

The fractions which were shown by papergram to contain the major bioconversion product (chloroform–acetone mixtures) were individually triturated with a mixture of 1 ml. of acetone and 3 ml. of ether. After decantation of the solvent the crystalline residues were combined, and recrystallized first from acetone and thereafter twice from methanol. Addition of ether was necessary to induce crystallization. The mother liquors of these crystallizations were worked up exhaustively to give a combined crop of 1.577 g. of 14α,17β-dihydroxyandrost-4-ene-3-one (II) (37.5% yield) melting at 183.5–186°, $[\alpha]^{22}D$ 124° (*c* 0.974 in chloroform). Despite sublimation at 0.1 mm. and 160° or prolonged drying at 80° and 0.01 mm., it was impossible to obtain an accurate carbon determination. The infrared spectrum indicated that this compound contained more hydroxyl absorption than the starting material; λ_{max}^{alc} 242 mμ (*E* 14,600); infrared spectrum: hydroxyl absorption at 3467 cm.$^{-1}$, conjugated ketone at 1649 cm.$^{-1}$, conjugated carbon double bond at 1611 cm.$^{-1}$.

Anal. Calcd. for $C_{19}H_{28}O_2$: C, 74.96; H, 9.27. Found: C, 74.38; H, 9.26.

Acetylation of II to the 17β-Monoacetate III.—The acetate III was prepared from 65 mg. of 14α-hydroxytestosterone by reaction overnight at room temperature with acetic anhydride–pyridine (1:1). From this was isolated 64 mg. of crystals which were twice recrystallized from acetone–ether to give 38 mg. of III, melting at 198–200°, $[\alpha]^{22}D$ 114° (*c* 0.775 in chloroform), λ_{max}^{alc} 241 mμ (*E* 16,300). The infrared spectrum showed that one hydroxyl group had not been esterified; infrared spectrum: hydroxyl absorption at 3616 cm.$^{-1}$, acetate carbonyl at 1730 cm.$^{-1}$, conjugated ketone at 1668 cm.$^{-1}$, conjugated carbon double bond at 1617 cm.$^{-1}$, acetate ester linkage at 1283 cm.$^{-1}$.

Anal. Calcd. for $C_{21}H_{30}O_4$: C, 72.80; H, 8.73. Found: C, 72.99; H, 8.66.

Oxidation of 14α-Hydroxytestosterone (II) to 14α-Hydroxyandrostenedione (IV).—14α-Hydroxytestosterone (510 mg.) was dissolved in 8 ml. of glacial acetic acid. To the cooled solution, 130.0 mg. of chromium trioxide (calculated for 1 oxygen, 107.2 mg.) in 2.5 ml. of 75% acetic acid was added dropwise. The reaction mixture was allowed to stand at room temperature for 8 hours. After dilution with 10 ml. of methanol, the solution was concentrated under reduced pressure. When the residue was taken up in 100 ml. of water, a crystalline precipitate was formed which did not dissolve when ether was added. The crystals (90 mg.) were separated by filtration (to be combined with the main product as obtained by extraction). The filtrate was extracted with ether–chloroform (2:1). The extracts were washed with two portions of 5% sodium bicarbonate, then with water and dried over anhydrous sodium sulfate. Upon concentration the extracts gave 410 mg. of crystals. The total product (500 mg.) was recrystallized twice from methanol to give 417 mg. of 14α-hydroxyandrostenedione (IV), melting at 260–262°, λ_{max}^{alc} 241 mμ (*E* 16,100), $[\alpha]^{23}D$ 171° (*c* 0.660 in chloroform). This product was identical, by infrared spectroscopy, to authentic 14α-hydroxyandrostenedione.

Anal. Calcd. for $C_{19}H_{26}O_3$: C, 75.46; H, 8.66. Found: C, 75.21; H, 8.64.

D. Preparation of 14α-Hydroxyprogesterone (VI) from Progesterone (V). 1. By *M. griseo-cyanus* (A.T.C.C. 1207).—*M. griseo-cyanus* was grown on 12 l. of medium H for 24 hours. Then 3 g. of progesterone (V) in alcohol was added, and the fermentation was continued for 24 hours. The extraction with methylene chloride gave 4.81 g. of solids. Trituration with Skellysolve B left 3.322 g. of insoluble material. This, then, was triturated with ether. The ether-insoluble material (1.72 g.) was dissolved in 100 ml. of benzene and chromatographed over 85 g. of alumina. Elution was carried out with two 170-ml. portions of the following solvents: benzene, benzene–5% ether, benzene–10% ether, benzene–50% ether, ether, ether–5% chloroform, ether–10% chloroform; then four 170-ml. portions of ether–50% chloroform and of chloroform, then one 170-ml. portion each of chloroform–5% acetone, acetone and methanol. Paper chromatography indicated that the first two chloroform fractions contained the major conversion product. The solids from these two fractions (513 mg.) were crystallized from acetone to give 382.5 mg. of crystalline material, melting at 179–188°. A sample was recrystallized from 2 ml. of methanol to give 14α-hydroxyprogesterone (VI), m.p. 180–187°, $[\alpha]^{24}D$ 200° (*c* 0.4857 in chloroform); infrared absorptions: hydroxyl at 3543 cm.$^{-1}$, non-conjugated carbonyl at 1692 cm.$^{-1}$ (shoulder at 1708 cm.$^{-1}$), conjugated carbonyl at 1675 cm.$^{-1}$, conjugated carbon–carbon double bond at 1625 cm.$^{-1}$, numerous maxima from 1457 to 678 cm.$^{-1}$.

Anal. Calcd. for $C_{21}H_{30}O_3$: C, 76.40; H, 9.15. Found: C, 76.50, 76.27; H, 9.31, 9.34.

2. By *M. parasiticus* (A.T.C.C. 6476).—*M. parasiticus* was grown on 12 l. of medium H for 20 hours. Then 3 g. of progesterone was added and the culture incubated for 48 hours. Extraction in the usual manner with methylene chloride followed by evaporation of solvent gave 4.667 g. of oily extractives. This was dissolved in 150 ml. of benzene and chromatographed over 220 g. of alumina. Fractions were eluted with 200-ml. volumes of the following solvents: benzene, benzene–5% ether, benzene–20% ether, benzene–50% ether, ether, ether–5% chloroform, ether–10% chloroform, ether–20% chloroform (2 fractions in each instance); ether–50% chloroform, chloroform (3 fractions in each instance); chloroform–5% acetone, chloroform–10% acetone, chloroform–5% acetone (2 fractions in each instance); acetone and methanol (1 fraction each time).

Ether–10% chloroform eluted the main conversion product (1.839 g.), as shown by paper chromatography. The

product was dissolved in 20 ml. of acetone, filtered, concentrated to half of the original volume and diluted with ether to initiate crystallization. This procedure gave 585.5 mg. of crystals melting at 182–194°. Eighty-five mg. of this product was sublimed at 165°, at a pressure of 0.05 mm. The sublimate was recrystallized from 3 ml. of acetone–hexane to give 14α-hydroxyprogesterone, m.p. 195–200°, $[\alpha]^{23}D$ 188° (*c* 1.036 in chloroform). The infrared spectrum was identical to that of compound VI obtained by the dissimilation with *M. griseo-cyanus*.

Anal. Calcd. for $C_{21}H_{30}O_4$: C, 76.40; H, 9.15. Found: C, 77.02; H, 9.55.

3. By *H. piriforme* (A.T.C.C. 8992).—Progesterone (6 g.) was incubated with *H. piriforme* in 12 liters of medium H for 27 hours. The usual extraction of the beer with methylene chloride gave 8.313 g. of residue. This was dissolved in 600 ml. of benzene containing 10% ether and chromatographed over 300 g. of alumina. Fractions (600 ml.) were eluted with the following solvents: benzene–10% ether, ether (2 fractions each); ether–5% chloroform, ether–10% chloroform, ether–50% chloroform, chloroform (3 fractions in each instance); chloroform–5% acetone, chloroform–10% acetone, chloroform–50% acetone, acetone and methanol (2 fractions each time). Based on paper chromatographic findings the eluates of ether–50% chloroform and the first eluate of chloroform were combined (1.269 g.) and recrystallized from 15 ml. of ethyl acetate to give 680 mg. of crystalline material. A sample was repeatedly recrystallized from methanol to give 14α-hydroxyprogesterone, melting at 191–199°, $[\alpha]^{23}D$ 215° (*c* 0.693 in chloroform). The infrared spectrum was identical to that of the other two samples of compound VI described above.

Anal. Calcd. for $C_{21}H_{30}O_3$: C, 76.40; H, 9.15. Found: C, 76.39, 76.06; H, 9.36, 9.25.

Attempted Oxidation and Acetylation of 14α-Hydroxyprogesterone (VI).—14α-Hydroxyprogesterone (88 mg.) was dissolved in 1.6 ml. of chlorobenzene with warming. After cooling in an ice-bath a solution of 0.0532 g. of sodium dichromate dihydrate dissolved in 0.8 ml. of water and 0.135 ml. of sulfuric acid was added to the cooled steroid solution. The mixture was stirred vigorously for 2 hours in an ice-bath. The reaction mixture was diluted with water (making the acetic acid concentration less than 10%) and benzene added to bring the organic layer to the surface. The benzene layer was washed with water, 5% sodium bicarbonate solution then again with water, and was dried with anhydrous sodium sulfate. The crystalline material obtained by evaporation of the solvent was shown by infrared spectrum to be starting material.

When a sample of 14α-hydroxyprogesterone (50 mg.) was subjected to the conditions of mild acetylation with pyridine and acetic anhydride (1:1), standing overnight at room temperature, only starting material could be recovered.

Degradation of 14α-Hydroxyprogesterone (VI) to 14α-Hydroxyandrost-4-ene-3,17-dione (IV) by *P. lilacinum* (A.T.C.C. 10114).—To each of twenty erlenmeyer flasks (250-ml. capacity), each containing 100 ml. of medium E[21] were added spores of *P. lilacinum* and 10 mg. of compound VI. The flasks were closed with sterile gauze pads and then shaken for 72 hours at 25°. The fermentations were pooled and extracted with methylene chloride. Paper chromatography indicated that 20% of substrate and 20% of 14α-hydroxyandrostenedione were present in the extract (785 mg.). The extractives were dissolved in 35 ml. of benzene and chromatographed over 40 g. of alumina. The same sequence of solvents as described above was used. Fraction 24 (chloroform) weighed 103.0 mg. and consisted (by paper chromatography) of 22% of IV and 34% of substrate VI. Fraction 25 (acetone) weighing 116.5 mg. had a composition of 16% of IV and 1% of VI. This fraction was crystallized from 1 ml. of ethyl acetate by slow evaporation of the solvent. The product was then triturated with 2 ml. of ether and a few drops of acetone to give 20 mg. of crystals, m.p. 252–258°, identified by infrared spectrum as 14α-hydroxyandrostenedione (IV).

Acknowledgment.—We are grateful to Dr. J. L. Johnson and his associates for all spectrographic analyses; to W. A. Struck and associates for optical rotations and microanalyses; to Jennie M. Noteboom, Henrietta Triemstra, Hester Woltersom, Irene Pratt, G. Staffen and J. R. Heald for technical assistance.

(21) Medium E: cornsteep, 20 g.; potassium dihydrogen phosphate, 1 g.; Cerelose, 30 g.; sodium nitrate, 2 g.; magnesium sulfate, 0.5 g.; potassium chloride, 0.2 g.; ferrous sulfate, 0.01 g.; sodium acetate, 2 g.; distilled water to 1 liter.

KALAMAZOO, MICH.

[CONTRIBUTION FROM THE STERLING-WINTHROP RESEARCH INSTITUTE]

D-Homosteroids. II. 17a-Ethinyl-D-homoetiocholane-3α,17a-diol-11-ones and Related Compounds[1]

BY R. O. CLINTON, R. G. CHRISTIANSEN, H. C. NEUMANN AND S. C. LASKOWSKI

RECEIVED OCTOBER 31, 1957

The Nef reaction with D-homoetiocholan-3α-ol-11,17a-dione gave a mixture of 17aα-ethinyl-D-homoetiocholane-3α,17aβ-diol-11-one and its 17a-epimer. The configuration of the two C_{17a}-epimers has been established by means of both physical and chemical evidence. Additionally, a number of compounds derived from or related to the ethinyl-epimers have been prepared; certain of these possess noteworthy endocrinological activity.

The initial approach to the synthesis of D-homopregn-4-ene-17aα,21-diol-3,11,20-trione 21-acetate (D-homocortisone acetate)[2] involved the introduction of an ethinyl group into D-homoetiocholan-3α-ol-11,17a-dione at the C^{17a}-position by means of the Nef reaction, followed by suitable transformation of the ethinyl group into the desired cortical side chain.[3]

Models of the normal (five-membered D-ring) steroid and of the D-homo (six-membered D-ring) steroid indicate a greater steric hindrance toward rearward approach (α-attack) in the latter system, due primarily to the effects of the axial hydrogens on C_{12}, C_{14} and C_{16}. Chemical reduction of the C_{17a}-ketone group, involving a normal equilibration reaction, gave predominantly the expected 17aβ-hydroxy (equatorial) epimer.[1] Although the Nef reaction may also be regarded as an equilibration reaction, steric effects prevent front-side attack in the normal steroids, and the 17β-ethinyl epimer is obtained in only 0.3% yield.[4] Similar effects have been noted with ethoxyacetylene.[5] In the D-homo

(1) Paper I, THIS JOURNAL, **79**, 6475 (1957).

(2) To be published.

(3) *Cf.* R. M. Dodson, P. B. Sollman and B. Riegel, THIS JOURNAL, **75**, 5132 (1953).

(4) T. Reichstein and C. Meystre, *Helv. Chim. Acta*, **22**, 728 (1939).

(5) H. Heusser, K. Eichenberger and P. A. Plattner, *ibid.*, **33**, 370 (1950).