ether was added dropwise over a period of 5 minutes. The ammonia was allowed to evaporate and the other solvents were removed under a jet of nitrogen. The residue was dissolved in ether; the resulting solution was washed with water, decolorized with carbon, dried (sodium sulfate), filtered, and the solvent evaporated. Crystallization of the residue from dilute methanol, then twice from acetone–petroleum ether, b.p. 60–70°, yielded 0.12 g. of 1-methoxy-4-methyl-1,3,5(10)-estratrien-17β-ol (X), m.p. 115–116.5°. Further crystallization from petroleum ether, b.p. 60–70°, raised the m.p. to 116.5–117.5°, $[\alpha]D$ + 185.3°; λ_{max} 278 mμ (ε 1,740), 285 mμ (ε 1,760). This material proved to be identical (m.m.p. and infrared spectra) to a sample of 1-methoxy-4-methyl-1,3,5(10)-estratrien-17β-ol,[17] m.p. 118–118.5°, prepared from 1-hydroxy-4-methyl-1,3,5(10)-estratrien-17-one[16] (which in turn was prepared from 1,4-androstadiene-3,17-dione (II) *via* the dienonephenol rearrangement).

Anal. Calcd. for $C_{20}H_{28}O_2$: C, 79.95; H, 9.39. Found: C, 80.14; H, 9.67.

Acetylation of 50.2 mg. of the above product with acetic anhydride and pyridine, followed by crystallization of the acetate from methanol, yielded 33.5 mg. of 1-methoxy-4-methyl-1,3,5(10)-estratrien-17β-ol acetate (XI), m.p. 148.5–150°. This material was identical (m.m.p. and infrared spectra) with an authentic sample[17] prepared *via* the dienol-phenol rearrangement of 1,4-androstadiene-3,17-dione (II).

Estrone from 19-Hydroxy-4-androstene-3,17-dione.—*Pseudomonas sp.* B40-327 (A.T.C.C. 13262) was grown as a submerged culture for 24 hours in 100 ml. of Difco Nutrient Broth in a 500-ml. erlenmeyer flask. Incubation was at 25° on a rotary shaker operating at 200 r.p.m. through a 2-inch diameter revolution. 19-Hydroxy-4-androstene-3,17-dione (25 mg.) was added to the culture in 1 ml. of acetone and incubation was continued for an additional 24 hours. The culture was extracted twice with methylene chloride and the rich solvent extracts were pooled and reduced to dryness. Paper chromatographic analysis of this residue indicated a 70–80% yield (estimated visually) of material behaving like estrone.

The residue from the fermentation was dissolved in ethyl acetate. The solution was filtered from a small quantity of insoluble material, then evaporated to dryness. Crystallization of the residue from acetone–petroleum ether, b.p. 60–70°, then from dilute acetone yielded 1.4 mg. of estrone (XIV), m.p. 259–262°. Comparison of the infrared spectrum of this material with that of an authentic sample of estrone confirmed identity.

Since the yield of estrone obtained by direct crystallization was so small, all of the mother liquors from the above crystallization were combined, then evaporated to dryness. The residue was dissolved in a mixture of ether and benzene and the estrone isolated by alkaline extraction. In this way an additional 4.6 mg. of estrone, m.p. 259–262°, was obtained. In order to purify it, this estrone was sublimed at 200° and 0.2 mm. pressure. After sublimation this material showed no depression in melting point when mixed with an authentic sample, m.p. and m.m.p. 260–262°

[CONTRIBUTION FROM THE BIOLOGICAL AND CHEMICAL RESEARCH DIVISIONS OF G. D. SEARLE AND CO., CHICAGO 80, ILL.]

Microbiological Transformations. VII. The Hydroxylation of Steroids at C-9

BY R. M. DODSON[1] AND R. D. MUIR

RECEIVED JULY 5, 1961

Incubation of 4-androstene-3,17-dione (I) with *Nocardia sp.* produced 9α-hydroxy-4-androstene-3,17-dione (II) and 3-hydroxy-9,10-seco-1,3,5(10)-androstatriene-9,17-dione (III). The structure of the 9α-hydroxyandrostenedione (II) was established by converting it by fermentation with a species of *Arthrobacter* to the known 9,10-seco-phenol (III). Incubation of progesterone (IV) with *Nocardia sp.* produced 9α-hydroxyprogesterone (V) and 9α-hydroxytestosterone (VI). The structure of the 9α-hydroxyprogesterone (V) was established by its conversion by fermentation with a species of *Arthrobacter* to 9α-hydroxyandrostenedione (II). 9α-Hydroxytestosterone was synthesized by the sodium borohydride reduction of 9α-hydroxyandrostenedione (II). The configuration of the 9α-hydroxyl group in these steroids was determined by the conversion of 9α-hydroxyprogesterone (V) to 3β-hydroxy-3α,9α-epoxy-5β-pregnan-20-one (XIII) on reduction.

We have recently reported the preparation of 3-hydroxy - 9,10 - seco - 1,3,5(10) - androstatriene-9,17-dione (III)[2] by the incubation of 4-androstene-3,17-dione (I) with a species of *Pseudomonas*. Knowledge of the structure of this compound, III, enabled us to establish definitively the structure of 9α-hydroxy-4-androstene-3,17-dione (II), 9α-hydroxytestosterone (VI) and 9α-hydroxyprogesterone (V) obtained from fermentations using species of *Nocardia*.[3]

Fermentation of 4-androstene-3,17-dione (I), by the methods previously described,[4] with a species of *Nocardia*, A.T.C.C. 13259, isolated from soil, produced 3-hydroxy-9,10-seco-1,3,5(10)-androstatriene-9,17-dione (III) and a monohydroxy-4-androstene-3,17-dione (II), m.p. 222–223.5°. The ultraviolet and infrared spectra of compound II confirmed the presence of the new hydroxyl group, of the 3-keto group conjugated with the 4,5-double bond and of the 17-keto group. The failure of compound II to acetylate, when treated with pyridine and acetic anhydride, indicated that the hydroxyl group occupied either a tertiary position (C-8,9 or 14) or the 11β-position. This information, in conjunction with the isolation of 3-hydroxy-9,10-seco-1,3,5(10)-androstatriene-9,17-dione (III) from the fermentation, made the 9-position the most probable site for the new hydroxyl group.

Fermentation of compound II with a species of *Arthrobacter* (Searle B22-9), known to convert 4-androstene-3,17-dione to 1,4-androstadiene-3,17-dione in excellent yield, gave 3-hydroxy-9,10-seco-1,3,5(10)-androstatriene-9,17-dione (III). The latter compound was purified as its acetate, which proved to be identical in all respects with the 3-acetoxy-9,10-seco-1,3,5(10)-androstatriene-9,17-dione reported previously.[2] Thus, compound II was proved to be 9-hydroxy-4-androstene-3,17-dione.[5]

(1) Department of Chemistry, University of Minnesota, Minneapolis 14, Minn.

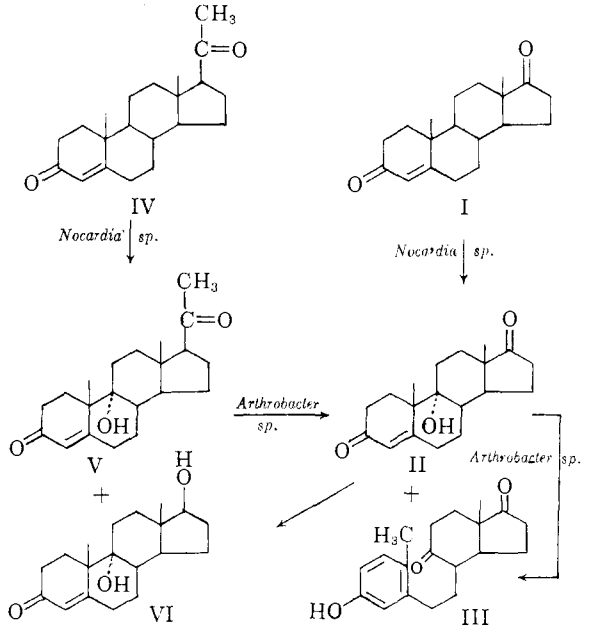
(2) R. M. Dodson and R. D. Muir, *J. Am. Chem. Soc.*, **83**, 4627 (1961); R. M. Dodson and R. D. Muir, *ibid.*, **80**, 5004 (1958).

(3) A preliminary communication on the 9α-hydroxylation of androstenedione by *Nocardia sp.*, A.T.C.C. 13259 (G. D. Searle A20-10) appeared in the *J. Am. Chem. Soc.*, **80**, 6148 (1958). Since the completion of the work described in this paper, the conversion of progesterone to 9α-hydroxyprogesterone using a species of *Nocardia* has been reported by C. J. Sih and F. L. Weisenborn, *ibid.*, **82**, 2653 (1960).

(4) D. H. Peterson, H. C. Murray, S. H. Eppstein, L. M. Reineke, A. Weintraub, P. D. Meister and H. M. Leigh, *ibid.*, **74**, 5933 (1952).

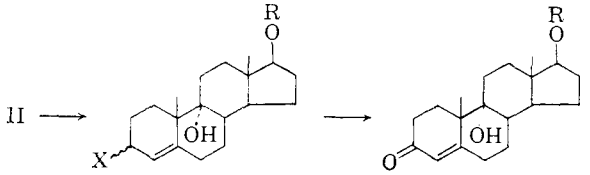
(5) The specific rotation of the previously described 8β(or 9α)-hydroxy-4-androstene-3,17-dione, m.p. 214–217°, $[\alpha]^{23}D$ 165° ($CHCl_3$),

Incubation of progesterone (IV) with *Nocardia*[6] *sp.*, A.T.C.C. 13934, produced 9α-hydroxyprogesterone (V) and 9α-hydroxytestosterone (VI). The structure of 9α-hydroxyprogesterone[7] (V)



was established by converting it, by fermentation with an *Arthrobacter sp.*, Searle B20–27, to the well defined 9α-hydroxy-4-androstene-3,17-dione (II).

The structure of the 9α-hydroxytestosterone (VI) was established by its synthesis, *via* two different routes, from 9α-hydroxy-4-androstene-3,17-dione (II). Reduction of 9α-hydroxy-4-androstene-3,17-dione (II) with excess sodium borohydride produced a mixture of 4-androstene-3,9α,-17β-triols (VIII) epimeric at C-3. This mixture could not be resolved into its isomers by direct crystallization. Oxidation of a portion of this mixture with manganese dioxide[8] suspended in benzene produced 9α-hydroxytestosterone (VI) identical with that obtained from the fermentation of progesterone (IV). Pure 3β,17β-diacetoxy-4-androsten-9α-ol (IX) could be obtained either by repeated crystallization or by chromatography of the mixture produced by acetylation of VIII. Chromatography of this acetylated mixture also produced some 17β-acetoxy-4-androstene-3β,9α-diol (X). The location of the acetyl group in X was confirmed by its conversion to 9α-hydroxytestosterone acetate (VII) with manganese dioxide in benzene. Pure 4-androstene-3β,9α,17β-triol (XI) was easily obtained from X by basic hydrolysis of the 17-ester. The configurations of the groups



at C-3 in compounds IX, X and XI were assigned first, on the basis of the fact that the sodium borohydride reduction would be expected to produce largely the 3β-isomer[9] and, second, on the basis of the molecular rotatory difference between the rotation of 4-androstene-3β,9α,17β-triol (XI) and 9α-hydroxytestosterone (VI), ΔM_D [(3β-OH) − (3C=O)] = −200° ($CHCl_3$). The corresponding value derived from 4-cholesten-3β-ol[10] and 4-cholesten-3-one[10] was ΔM_D[(3β-OH) − (3C=O)] = −167°. 4-Cholesten-3α-ol[10] yielded a positive value, ΔM_D [(3α-OH) − (3C=O)] = +124°. Since both IX and X can be hydrolyzed to XI, this also established their configuration.

9α-Hydroxytestosterone (VI) can best be prepared from 9α-hydroxyandrostenedione (II) by the selective reduction of the 17-carbonyl group using a limited quantity of sodium borohydride for a short period of time at low temperature.[11] The 9α-hydroxytestosterone (VI) so obtained was readily separated from the unreduced 9α-hydroxyandrostenedione (II) by chromatography.

Configuration of the 9-Hydroxyl Group.—The newly introduced hydroxyl group was initially assigned the 9α-configuration on the basis of its molecular rotatory contribution (see Table I). Recent evidence has shown that a microbiologically introduced hydroxyl group has the same configuration as the hydrogen atom replaced.[12] If this is

obtained *via* the hydroxylation of 11-deoxycortisol with *Helicostylum piriforme*, does not agree with that obtained by us, [α]D 182° [see 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, *ibid.*, **80**, 3382 (1958)]. However, since this same organism definitely hydroxylated 11β,21-dihydroxypregna-4,17(20)-dien-3-one at the 9α-position, it is probable that our 9α-hydroxy-4-androstene-3,17-dione differs from the 8β(or 9α)-hydroxy-4-androstene-3,17-dione described by Eppstein and co-workers only in its state of purity [see A. R. Hanze, O. K. Sebek and H. C. Murray, *J. Org. Chem.*, **25**, 1968 (1960)].

(6) Searle number A20-13.

(7) The preparation of 9α-hydroxyprogesterone by the fermentation of progesterone with *Circinella sp.* [A. Schubert, D. Onken, R. Siebert and K. Heller, *Chem. Ber.*, **91**, 2549 (1958)] and with *Streptomyces aureofaciens*, NRRL 2209 [D. Perlman, J. D. Dutcher, J. Fried and E. O. Titus, U. S. Patent 2,840,578, June 24, 1958] has also been described. Since the rotation of our compound [α]D +188°, differed from that reported by Schubert, [α]D +204°, or Perlman, [α]D +202°, and since the properties of the reduction products of the 9α-hydroxyprogesterone prepared by us differed markedly from those described by Schubert, we felt it necessary to relate our 9α-hydroxyprogesterone to the well established 9α-hydroxy-4-androstene-3,17-dione. The rotation of our 9α-hydroxyprogesterone is in excellent agreement with that recently reported by Sih and Weisenborn (ref. 2). ADDED IN PROOF.—We have been informed by Dr. Schubert that correction of their calculations gave [α]D +187° ($CHCl_3$) for 9α-hydroxyprogesterone and [α]D +131° ($CHCl_3$) for "9α-hydroxypregnandion-3,20," m.p. 152–155°. A sample of their 9α-hydroxyprogesterone was identical with ours (m.m.p.).

(8) O. Mancera, G. Rosenkranz and F. Sondheimer, *J. Chem. Soc.*, 2189 (1953); F. Sondheimer, C. Amendolla and G. Rosenkranz, *J. Am. Chem. Soc.*, **75**, 5930 (1953); R. M. Evans, *Quart. Revs.*, **13**, 61 (1959).

(9) W. G. Dauben, R. A. Micheli and J. F. Eastham, *J. Am. Chem. Soc.*, **74**, 3852 (1952); Pl. A. Plattner, H. Heusser and A. B. Kulkarni, *Helv. Chim. Acta*, **32**, 265 (1949).

(10) The rotations of these compounds were taken from, J. P. Mathieu and A. Petit, "Constants Selectionees Pouvoir Rotatoire Natural. I. Steroids," Masson et Cie, Editeurs, 1956. The rotation of 4-cholesten-3α-ol is only recorded in benzene. However, since the rotation of 4-cholesten-3β-ol is practically identical in either benzene ([α]D +44°) or chloroform ([α]D +44.5°), this should introduce very little error into the molecular rotatory differences.

(11) J. K. Norymberski and G. F. Woods, *J. Chem. Soc.*, 3426 (1955).

(12) M. Hayano, M. Gut, R. I. Dorfman, O. K. Sebek and D. H. Peterson, *J. Am. Chem. Soc.*, **80**, 2336 (1958); E. J. Corey, G. A.

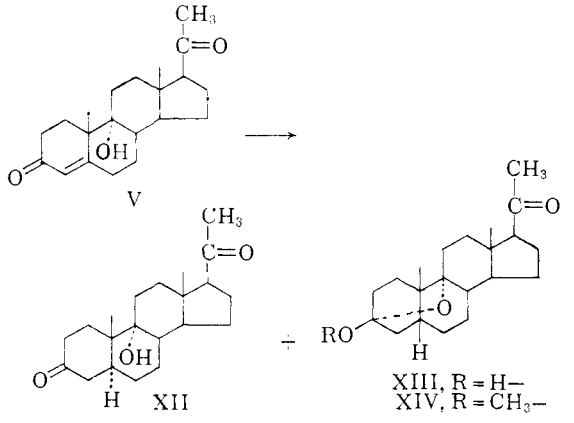
TABLE I[a]

Compound	M_D	ΔM_D [(9α-OH) − (9-H)]
3β-Acetoxyergostan-9α-ol[b]	− 4.61	−31
9α-Hydroxy-4-androstene-3,17-dione	+550	−18
9α-Hydroxytestosterone	+316	−24
4-Androstene-3β,9α,17β-triol	+116	−25[c]
9α-Hydroxyprogesterone	+609	−26
9α-Hydroxy-5α-pregnane-3,20-dione	+345	−38

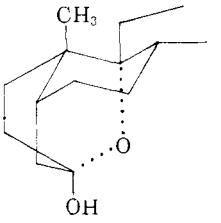
[a] Rotations of the corresponding 9α-H compounds obtained from J. P. Mathieu and A. Petit (ref. 10). [b] A. S. Hallsworth and H. B. Henbest, *J. Chem. Soc.*, 4604 (1957). [c] The rotation of 4-androstene-3β,17β-diol was determined in ethanol (ref. 10).

also true in this instance, it would necessitate a 9α-configuration for this hydroxyl group.

Definitive proof of the 9α-configuration of the newly introduced hydroxyl group was obtained from a study of the products of reduction of 9α-hydroxyprogesterone (V). Hydrogenation of a solution of V in methanol using a palladium-on-carbon catalyst yielded 9α-hydroxy-5α-pregnane-3,20-dione (XII) (10% yield) and 3β-hydroxy-3α,-9α-epoxy-5β-pregnan-20-one (XIII) (68% yield).[13] A comparison of the infrared spectra of XII and XIII determined in chloroform at similar con-



centrations clearly indicated (from the intensity of the carbonyl absorption at 5.87 μ) the absence of a second carbonyl group in compound XIII. Treatment of a solution of 3β-hydroxy-3α,9α-epoxy-5β-pregnan-20-one (XIII) in methanol with a small quantity of *p*-toluenesulfonic acid gave 3β-methoxy-3α,9α-epoxy-5β-pregnan-20-one (XIV) in quantitative yield. The formation of a 3α,9α-epoxide in a steroid with a 3-ketone, a 9α-hydroxyl group and a *cis* A:B ring juncture is not without precedence.[14] The structure of compound XIII definitely establishes the configuration of the 9α-hydroxyl group. A 9β-hydroxyl group could not possibly interact with either the 3- or the 20-carbonyl. This proof of the configuration of the hydroxyl group at C-9 also provides further evidence that a microbiologically introduced hydroxyl group has the same configuration as the hydrogen atom replaced.



XIII

Experimental[15]

Fermentation of 4-Androstene-3,17-dione (I) with Nocardia sp. A.T.C.C. 13259.—*Nocardia sp.* A.T.C.C. 13259 was grown as a submerged culture in a stainless steel fermentor in approximately 350 l. of medium containing 800 g. of Difco Nutrient Broth, 200 g. of Difco Yeast Extract and 15 g. of Dow Corning Antifoam AF. The culture was agitated by a stirrer operating at 170 r.p.m. and was aerated with 30 l.p.m. of sterile air which entered through a sparger located below the agitator. The temperature was maintained at 25°. After a growth period of 22.5 hours, 125 g. of 4-androstene-3,17-dione in 1 l. of acetone was added and incubation was continued for an additional 22.5 hours. The culture was extracted twice, each time with approximately one-half volume of methylene chloride. The solvent extracts were pooled and concentrated under reduced pressure.

The methylene chloride solution (approx. 700 ml.) was heated to boiling then filtered to free it from a small quantity of insoluble material. It was then evaporated to dryness. The residue was triturated with 150 ml. of ether; the suspension was cooled; and the product was separated by filtration and washed on the filter with 50 ml. of ether. The product was resuspended in 200 ml. of ether, the lumps were broken up, and the suspension refiltered. Crystallization of the residue from acetone–cyclohexane, after decolorization with carbon in acetone, followed by crystallization from dilute methanol, yielded 31.22 g. of 9α-hydroxy-4-androstene-3,17-dione (II), m.p. 222–223.5°. The sample for analysis was crystallized again from acetone–cyclohexane; m.p. 223.5–224.5°, λ_{max} 241 mμ (ε 15,400); λ_{max}^{KBr} 2.89, 5.73, 6.04 and 6.20 μ; $[\alpha]_D$ +182.8°, +180.9°, +183.5°.

Anal. Calcd. for $C_{19}H_{26}O_3$: C, 75.46; H, 8.67. Found: C, 75.76; H, 8.58.

All of the mother liquors from the above triturations and crystallizations were combined, diluted with salt water and extracted with 600 ml. of benzene. The organic layer was separated, washed with 1.0 l. of water, then extracted with 1.0 l. of a 10% sodium hydroxide solution in two portions. The organic solution was finally washed with 250 ml. of water. Because of emulsion formation, all three aqueous extracts were back-extracted with ether. Recovery of the product in the organic extract by evaporation of the solution, followed by crystallization of the residue from acetone–cyclohexane then aqueous methanol, gave an additional 8.73 g. of 9α-hydroxy-4-androstene-3,17-dione (II), m.p. 222.5–223.5°.

Neutralization of the aqueous sodium hydroxide extracts obtained above with acetic acid precipitated 45.70 g. of 3-hydroxy-9,10-seco-1,3,5(10)-androstatriene-9,17-dione (III), m.p. 116–123°. Since the ketophenol III is difficult to purify by direct crystallization, a 0.50-g. portion of the above material was acetylated with pyridine and acetic anhydride. This gave, after crystallization from methanol, 0.23 g. of 3-acetoxy-9,10-seco-1,3,5(10)-androstatriene-9,17-dione, m.-

Gregoriou and D. H. Peterson, *ibid.*, **80**, 2338 (1958); M. Hayano, M. Gut, R. I. Dorfman, A. Schubert and R. Siebert, *Biochim. Biophys. Acta*, **32**, 269 (1959).

(13) While the m.p.'s of these two compounds (XII and XIII) agree reasonably well with those reported by Schubert and co-workers (ref. 7), the rotations of both and the structure of XIII differ from those previously reported. Our rotation of XII is consistent with that obtained from other 9α-hydroxysteroid (see Table II).

(14) L. F. Fieser, H. Heymann and S. Rajagopalan, *J. Am. Chem. Soc.*, **72**, 2306 (1950); see also V. R. Mattox, R. B. Turner, L. L. Engel, B. F. McKenzie, W. F. McGuckin and E. C. Kendall, *J. Biol. Chem.*, **164**, 569 (1946); **166**, 345 (1946).

(15) All melting points were taken on a Fisher–Johns melting point apparatus. Unless stated differently, the rotations were taken in chloroform at 24 ± 2° and the ultraviolet spectra in methanol. We are indebted to Drs. R. T. Dillon and H. W. Sause of the Analytical Division of G. D. Searle and Co. for the analytical and optical data reported.

p. 145.5–147.5°, identical in all respects (infrared and m.m.p.) with that previously described.[2]

In order to find any other neutral products that may have been present, the mother liquors from the second isolation of 9α-hydroxyandrostenedione were evaporated to dryness and the residue (18.1 g.) was chromatographed on silica gel. From the chromatogram a small quantity of 4-androstene-3,17-dione, m.p. 172–173.5°, 0.64 g. of relatively pure 3-hydroxy-9,10-seco-1,3,5(10)-androstatriene-9,17-dione (III), m.p. 129–133°, and 2.42 g. of 9α-hydroxyandrostenedione (II), m.p. 218–222.5°, were obtained. A very small quantity of an, as yet, unidentified hydroxyandrostenedione, m.p. 215–222°, was also obtained, but the quantity was too small to permit satisfactory purification.

Attempted Acetylation of 9α-Hydroxy-4-androstene-3,17-dione (II).—A 0.10-g. portion of the monohydroxyandrostenedione, m.p. 222–223.5°, was dissolved in 2.00 ml. of pyridine and 2.00 ml. of acetic anhydride, then allowed to stand overnight at room temperature. No precipitate formed when the solution was diluted with ice and water. The resulting solution was extracted with three 25-ml. portions of ether, and the ether solution was washed with dilute aqueous sodium bicarbonate until free of acid. The ether solution was then dried over sodium sulfate, filtered, evaporated to dryness, and the residue was crystallized from acetone–petroleum ether (b.p. 60–70°) to give 62 mg. of unchanged starting material, m.p. 221–222.5° (identity established by infrared and m.m.p.).

Conversion of 9α-Hydroxy-4-androstene-3,17-dione (II) **to 3-Acetoxy-9,10-seco-1,3,5(10)-androstatriene-9,17-dione.**—Fourteen 500-ml. erlenmeyer flasks, each containing 100 ml. of Difco Nutrient Broth, were inoculated with a culture of *Arthrobacter sp.*, B-22-9, and were incubated at 25° on a rotary shaker operating through a 2-inch diameter circle at 200 r.p.m. After 24 hours, 25 mg. of 9α-hydroxy-4-androstene-3,17-dione (II) in 1.0 ml. of acetone were added to each flask, and incubation was continued for an additional 8 hours. The cultures were pooled and extracted twice with 1500-ml. portions of methylene chloride.

The methylene chloride extract was evaporated to dryness, and the residue was dissolved in 100 ml. of ether. This ether solution was extracted with 100 ml. of 10% aqueous sodium hydroxide in two portions, then washed with water. All three aqueous extracts were back extracted with ether, then individually neutralized with acetic acid. The oily suspension that formed in the first two extracts crystallized on stirring. The resulting crystals were separated by filtration and washed with water to give 161.4 mg. of 3-hydroxy-9,10-seco-1,3,5(10)-androstatriene-9,17-dione (III), m.p. 123–129°. This material was acetylated, then purified by crystallization from dilute acetone and from acetone–cyclohexane. The acetate so obtained, m.p. 143.5–146°, was identical in all respects (m.p., m.m.p., infrared spectra) with that previously described.[2]

Fermentation of Progesterone (IV) **with *Nocardia sp.* (Searle A20-13).**—*Nocardia sp.* A20-13 (A.T.C.C. 13934) was grown as a submerged culture in a stainless steel fermentor in 30 l. of medium containing 200 g. of Difco Nutrient Broth, 50 g. of dried brewer's yeast and 10 ml. of antifoam oil. The culture was agitated by means of a paddle type stirrer operating at 200 r.p.m. and was aerated with 10 l.p.m. of sterile air which entered through a sparger located below the agitator. The incubation temperature was 25°. After an initial growth period of 26 hours, 10 g. of progesterone in 250 ml. of acetone was added and incubation was continued for 18 hours. The culture was extracted with two one-half volume portions of methylene chloride, and the rich solvent fractions were pooled.

The methylene chloride solution was evaporated to dryness, and the residue was chromatographed on 1.5 kg. of silica gel. After washing the column with 10% ethyl acetate in benzene, elution of the column with 15% ethyl acetate in benzene gave a small quantity of progesterone, m.p. and m.m.p. 128.5–131.5°. Further elution of the column with 20% ethyl acetate in benzene yielded a small amount of 4-androstene-3,17-dione, m.p. and m.m.p. 170–172°. The column was then washed with mixtures of ethyl acetate in benzene containing 25%, 30% and 35% ethyl acetate. Elution of the column with 40% ethyl acetate in benzene gave 4.088 g. of 9α-hydroxyprogesterone, which by crystallization from dilute acetone yielded 3.50 g. of relatively pure material, m.p. 191–193.5°. Analytically pure 9α-hydroxyprogesterone (V) was obtained by crystallizations from acetone–cyclohexane and dilute methanol; m.p. 193–194.5°; $[\alpha]_D$ +185°, +188°; λ_{max} 241.5 mμ (ε 15,200).

Anal. Calcd. for $C_{21}H_{30}O_3$: C, 76.32; H, 9.15. Found: C, 76.41; H, 9.17.

The column was next washed with solutions of 50% and 60% ethyl acetate in benzene. Elution with 75% ethyl acetate in benzene and 100% ethyl acetate gave 302 mg. of 9α-hydroxytestosterone (VI), which, after crystallizations from acetone–cyclohexane, dilute acetone, then acetone–cyclohexane, yielded 145.3 mg. of pure material, m.p. 197–199°, $[\alpha]_D$ + 104°, λ_{max} 241.5 mμ (ε 15,300).

Anal. Calcd. for $C_{19}H_{28}O_3$: C, 74.96; H, 9.27. Found: C, 74.71; H, 8.69.

Conversion of 9α-Hydroxyprogesterone (V) **to 9α-Hydroxy-4-androstene-3,17-dione** (II).—Forty 500-ml. erlenmeyer flasks, each containing 100 ml. of Difco Nutrient Broth, were inoculated with *Arthrobacter sp.*, Searle B20-27, an organism known to be capable of converting progesterone to 4-androstene-3,17-dione (I), and were incubated on a rotary shaker as described above. After a growth period of 24 hours, 25 mg. of 9α-hydroxyprogesterone in 1.0 ml. of acetone was added to each flask and incubation was continued for 16 hours. The cultures were pooled and extracted twice with methylene chloride.

The methylene chloride solution was evaporated to dryness, and the residue was triturated with ether, then separated by filtration. The remaining solid was extracted with 75 ml. of boiling methanol and the resulting solution filtered. On evaporation of the filtrate to small volume, 0.48 g. of 9α-hydroxy-4-androstene-3,17-dione (II), m.p. 222–223.5°, separated. Crystallization of this material from acetone–cyclohexane yielded 0.40 g. of pure 9α-hydroxyandrostenedione, m.p. 223–224°, which proved to be identical (m.m.p. and infrared spectra) with that described above.

9α-Hydroxytestosterone (VI).—To a solution of 5.00 g. of 9α-hydroxy-4-androstene-3,17-dione (II) in 40 ml. of warm ethanol was added a solution of 2.50 g. of sodium borohydride in 30 ml. of water and 30 ml. of ethanol. After 1.5 hours, the excess sodium borohydride was decomposed with dilute acetic acid. The resulting solution was diluted to 500 ml. with water, then extracted with 500 ml. of methylene chloride in four portions. The methylene chloride solution was washed with water, dried over sodium sulfate, filtered, then evaporated to dryness. The residue was dissolved in 300 ml. of benzene.

A 60-ml. portion of the above solution (containing the equivalent of 1.00 g. of 9α-hydroxyandrostenedione) was diluted to 150 ml. with benzene, then stirred with 10.0 g. of manganese dioxide at room temperature for 6.75 hours. The manganese dioxide was separated by filtration, then washed with ethyl acetate and with acetone. The filtrate was evaporated to dryness under vacuum, and the residue was crystallized from acetone–cyclohexane to yield 0.48 g. of beautiful needles, m.p. 183°, resolidified then melted 198–204°. Crystallization of this material from very dilute methanol gave 0.24 g. of 9α-hydroxytestosterone, m.p. 194°, resolidified then m.p. 207–209°. Since the compound was not only solvated when crystallized from aqueous solvents but also possessed a double melting point, the analytical sample was prepared by chromatography on silica gel followed by crystallization from acetone–cyclohexane. The pure 9α-hydroxytestosterone so obtained melted at 198–200°, $[\alpha]_D$ +104°, λ_{max} 242 mμ (ε 15,300); λ_{max}^{KBr} 2.90, 6.02 and 6.22 μ. The infrared spectrum of this material was identical with that of the 9α-hydroxytestosterone obtained from the fermentation of progesterone.

Anal. Calcd. for $C_{19}H_{28}O_3$: C, 74.96; H, 9.27. Found: C, 74.87; H, 9.62.

9α-Hydroxytestosterone Acetate (VII).—Acetylation of 9α-hydroxytestosterone with acetic anhydride and pyridine at room temperature gave, after crystallization of the product from dilute acetone and acetone–petroleum ether (b.p. 60–70°), 9α-hydroxytestosterone acetate, m.p. 209.5–211°, λ_{max} 241.5 mμ (ε 15,000); λ_{max}^{KBr} 2.84, 5.79, 5.98, 6.19 and 7.90 μ.

Anal. Calcd. for $C_{21}H_{30}O_4$: C, 72.80; H, 8.73. Found: C, 72.62; H, 8.48.

3β,17β-Diacetoxy-4-androsten-9α-ol (IX).—The benzene solution of 4-androstene-3ξ,9α,17β-triol (VIII), prepared in the above experiment, on standing deposited 2.33 g. of crystalline material, m.p. 119°, resolidified then m.p. *ca.* 160°. Crystallization of this material from acetone–cyclohexane, then from water raised its melting point to 163–168° but failed to give pure material. Acetylation of 1.66 g. of this material using 20 ml. of pyridine and 5.0 ml. of acetic anhydride at room temperature for 1.25 hours produced, after dilution of the reaction mixture with ice and water and repeated crystallization of the product from dilute acetone, 392 mg. of 3β,17β-diacetoxy-4-androsten-9α-ol, m.p. 162–164.5°, $[\alpha]_D$ +4.0°; λ_{max}^{KBr} 2.86, 5.72, 5.81, 6.02(w), 7.95 and 8.05 μ.

Anal. Calcd. for $C_{23}H_{34}O_5$: C, 70.75; H, 8.78. Found: C, 70.44; H, 8.41.

17β-Acetoxy-4-androstene-3β,9α-diol (X).—The benzene, acetone–cyclohexane and water mother liquors from the crystallization of 4-androstene-3ξ,9α,17β-triol (VIII) were evaporated to dryness, and the residue (2.20 g.) was acetylated by treatment with 20 ml. of pyridine and 5.0 ml. of acetic anhydride for 1.25 hours at room temperature. The product was isolated by dilution of the reaction mixture with water, extraction with methylene chloride, and evaporation of the organic solution under vacuum. The acetylated product was combined with the acetylated material from the acetone–water mother liquors from the crystallization of 3β,17β-diacetoxy-4-androsten-9α-ol, and the total material (3.86 g.) chromatographed on 380 g. of silica gel. Elution of the column with 10% ethyl acetate in benzene produced 0.618 g. of crude 3β,17β-diacetoxy-4-androsten-9α-ol which, after crystallization from dilute acetone, melted at 162–164° (277 mg.). The column was washed with 20% ethyl acetate in benzene. Elution with 30% ethyl acetate in benzene yielded 608 mg. of crystalline material, which, after crystallization from dilute acetone, then acetone–cyclohexane, gave 301 mg. of 17β-acetoxy-4-androstene-3β,9α-diol, m.p. 214.5–216.5°, $[\alpha]_D$ +33.9°; λ_{max}^{KBr} 2.91, 5.82, 6.02(w) and 7.90 μ.

Anal. Calcd. for $C_{21}H_{32}O_4$: C, 72.38; H, 9.25. Found: C, 72.18; H, 9.17.

4-Androstene-3β,9α,17β-triol (XI).—17β-Acetoxy-4-androstene-3β,9α-diol (100 mg.) in 5 ml. of methanol containing 0.20 g. of sodium hydroxide was allowed to stand at room temperature for 3 hours. The solution was neutralized with acetic acid, diluted with water, and the product extracted from it with methylene chloride. The residue from the evaporation of the methylene chloride extract, after crystallization from cyclohexane–acetone, yielded 108 mg. of a highly solvated 4-androstene-3β,9α,17β-triol (XI), m.p. 177–179°. This compound melted completely, with bubbling, when put on a melting-point block preheated to 125°. It then resolidified and melted 177.5–179°. The rotation and infrared spectra were determined on the previously dried analytical sample; $[\alpha]_D$ +38°; λ_{max}^{KBr} 2.91 and 6.00(w) μ.

Anal. Calcd. for $C_{19}H_{30}O_3$: C, 74.47; H, 9.87. Found: C, 74.28; H, 9.82.

9α-Hydroxytestosterone Acetate (VII) from 17β-Acetoxy-4-androstene-3β,9α-diol (X).—A solution of 160 mg. of 17β-acetoxy-4-androstene-3β,9α-diol (X) in 10 ml. of benzene was stirred for 7 hours with 2.00 g. of manganese dioxide. The resulting suspension was filtered, and the residue was washed on the filter with acetone. Evaporation of the filtrate to dryness and crystallization of the product from aqueous acetone and acetone–petroleum ether (b.p. 60–70°) yielded 66.5 mg. of 9α-hydroxytestosterone acetate, m.p. 210–211°, identical in all respects (m.m.p. and infrared spectra) with that prepared above.

9α-Hydroxytestosterone (VI) by Selective Reduction of 9α-Hydroxy-4-androstene-3,17-dione (II).—A solution of 5.00 g. (16.5 mmoles) of 9α-hydroxyandrostenedione (II) and 1.00 g. (25 mmoles) of sodium hydroxide in 1 liter of methanol was cooled in an ice-bath to 2–4° with stirring. Then 1.00 g. (26.4 mmoles) of sodium borohydride in 20 ml. of water was slowly added, and the resulting solution was stirred at 2–4° for 1 hour. The excess sodium borohydride was decomposed by the addition of 10 ml. of acetic acid. The methanol was removed from the reaction mixture by distillation under vacuum. The residue was partitioned between water and methylene chloride, and the methylene chloride solution was dried over sodium sulfate and evaporated to dryness. The product was then chromatographed on 420 g. of silica gel. After the elution of 0.746 g. of 9α-hydroxyandrostenedione with 35% ethyl acetate in benzene, 2.91 g. of 9α-hydroxytestosterone (VI) was eluted with the same solvent. Crystallization of this material from acetone–cyclohexane, with recrystallization of the second crop from dilute acetone, yielded 2.49 g. of 9α-hydroxytestosterone (VI), m.p. 196–199°. A second determination of the melting point of this material showed melting at 199°, resolidification, then melting 212–213.5°. This material proved to be identical (m.m.p. and infrared spectra) with those samples described above.

9α-Hydroxy-5α-pregnane-3,20-dione (XII) and 3β-Hydroxy-3α,9α-epoxy-5β-pregnane-20-one (XIII).—A solution of 1.025 g. of 9α-hydroxyprogesterone in 50 ml. of methanol was stirred under hydrogen at atmospheric pressure and 23° with 100 mg. of 5% palladium-on-carbon. In three hours 1.05 molar equivalents of hydrogen had been absorbed. The suspension was filtered and the filtrate was evaporated to dryness. Crystallization of the residue from acetone yielded three crops of material (0.17 g.), m.p. 220–250°. Extraction of this material with boiling petroleum ether (b.p. 60–70°) followed by crystallization of the insoluble product from dilute acetone yielded 103 mg. of 9α-hydroxy-5α-pregnane-3,20-dione, m.p. 256–260°, $[\alpha]_D$ +103.8; $\lambda_{max}^{CHCl_3}$ 2.64(w), 5.84 and 7.34 μ (no strong band in the 9–10 μ region) [reported[7] 252–253°, $[\alpha]^{25}_D$ 207° ($CHCl_3$)].

Anal. Calcd. for $C_{21}H_{32}O_3$: C, 75.86; H, 9.70. Found: C, 75.84; H, 9.66.

The acetone mother liquors from the first three crops of 9α-hydroxy-5α-pregnane-3,20-dione (above) were heated to boiling and diluted with water until cloudy. From this solution 0.68 g. of 3β-hydroxy-3α,9α-epoxy-5β-pregnan-20-one, m.p. 149–152.5°, was obtained. Crystallizations from acetone–cyclohexane and petroleum ether (b.p. 60–70°) produced analytically pure material, m.p. 155–156.5°, $[\alpha]_D$ +129°; $\lambda_{max}^{CHCl_3}$ 2.78, 5.87, 7.34, 9.72 and 9.92 μ (3,9-0-). Schubert and co-workers[7] report m.p. 152–155°, $[\alpha]^{25}_D$ +207° ($CHCl_3$), for 9α-hydroxypregnanedione-3,20.

Anal. Calcd. for $C_{21}H_{32}O_3$: C, 75.86; H, 9.70. Found: C, 76.11; H, 9.73.

3β-Methoxy-3α,9α-epoxy-5β-pregnan-20-one (XIV).—A solution of 2.00 g. of 3β-hydroxy-3α,9α-epoxy-5β-pregnan-20-one in 10 ml. of methanol was treated with 0.50 g. of *p*-toluenesulfonic acid, then allowed to stand overnight at room temperature. Solid potassium carbonate was added, and the suspension was stirred until basic. The product was precipitated by the addition of ice and water, then separated by filtration to give 2.09 g. of material, m.p. 126–129°. Crystallization of 1.09 g. of this material from dilute methanol yielded 0.91 g. of 3β-methoxy-3α,9α-epoxy-5β-pregnan-20-one, m.p. 131–132.5°, $[\alpha]^{24}_D$ +115.3°; $\lambda_{max}^{CHCl_3}$ 5.85 (20 C=O), 9.09 equatorial CH_3O—),[16] 9.74 and 9.97 μ (3,9-0—). A sample of this same material prepared previously on a smaller scale melted at 77–78°, $[\alpha]_D$ +115.5°. The infrared spectra of the two samples determined in chloroform were identical.

Anal. Calcd. for $C_{22}H_{34}O_3$: C, 76.26; H, 9.89. Found: C, 76.29; H, 9.84.

(16) D. N. Jones and G. H. R. Summers, *J. Chem. Soc.*, 2594 (1959).