Studies on the Reduction of Aromatic Ketones by the Clemmensen Method

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Isolation and Characterization of 18-Hydroxy-17-ketosteroids*

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In the course of numerous metabolic studies with testosterone-4-C^{14} in man, radioactivity was detected in chromatographic areas that contained compounds more polar than either 5α-androstane-3,17-diol or its 5β epimer. In many patients, the amount of this radioactivity was from 1 to 3% of the total administered; with some subjects, however, it represented as much as 10% of the dose. Two dihydroxy-17-ketosteroids have been isolated from these polar fractions, and the structures have been elucidated. 18-Hydroxyetiocholanolone[1] was isolated from the urine of a patient with a metastatic arrhenoblastoma, as well as from the urine of patients given large amounts of testosterone and etiocholanolone. Its 5α epimer, 18-hydroxyandrosterone, was isolated from urine following administration of androsterone.

The patient with an arrhenoblastoma received a tracer dose of testosterone-4-C^{14}. Chromatography of the neutral urinary steroid extract yielded a dihydroxyketone with approximately the same specific activity found for androsterone and etiocholanolone. The material crystallized readily, and the elementary analysis was consistent with the composition $C_{19}H_{30}O_3$. The acetylated product lacked absorption in the hydroxyl-stretching region (3700 to 3500 cm^{-1}); this was taken as evidence that the hydroxyl groups were readily acetylated. The same metabolite was obtained from another patient in approximately 3% yield after the administration of substantial amounts of etiocholanolone. The provisional conclusion drawn from this was that the product was a saturated steroid of the 5β-androstane series, probably with a 3α-hydroxyl group and a ketone at C-17. An absorption band at 1733 cm^{-1} (KBr) was consistent with the presence of a carbonyl group in the 5-membered ring. Absorption at 1407 cm^{-1} (CCl_4 solution; CaF_2 prism) in the acetylated derivative was interpreted as indicative of an unsubstituted methylene group adjacent to the carbonyl. The free compound was recovered unchanged after treatment with periodic acid, and the color reaction with blue tetrazolium was negative. In the quantitative Zimmermann reaction, the compound produced less than 10% of the color equivalent of etiocholanolone. This suggested that there was some interference in the neighborhood of the presumed carbonyl at C-17. Since the optical evidence and negative blue tetrazolium reaction excluded C-16, consideration was given to a C-18 hydroxyl.

Treatment with potassium hydroxide in ethanol yielded 73% of the calculated amount of formaldehyde measured colorimetrically with chromotropic acid. Formaldehyde was identified as the dimedone derivative by melting point and infrared spectrum. The product remaining after alkali treatment yielded a crystalline compound with an infrared spectrum identical with that of an authentic sample of 3α-hydroxy-18-nor-5β,13α-androstan-17-one (2). The metabolite, therefore, must have the structure 3α,18-hydroxy-5β-androstan-17-one (18-hydroxyetiocholanolone). This was confirmed by the molecular rotation difference between the metabolite and etiocholanolone. The value obtained, ΔM_D (18-H $\rightarrow$ OH), was -33 compared to $\Delta M_D - 25$ between 18-hydroxyestrone and estrone. Further evidence for this structure was obtained from its nuclear magnetic resonance spectrum in $CDCl_3$ solution[2] containing tetramethylsilane as reference compound, compared with that of etiocholanolone (Fig. 1). It showed the absence of signal at 51 cycles per second (c.p.s.) ascribed to the protons of the angular C-18 methyl group but still retained the signal at 57 c.p.s. ascribed to the protons of the angular C-19 methyl group. It also showed increased signal flanking at 220 c.p.s., indicative of additional protons on a carbon with an oxygen atom.

The 5α epimer of the above metabolite, 18-hydroxyandrosterone, was isolated in crystalline form from extracts of pooled urines after administration of large amounts of androsterone to several patients with no overt disorders of steroid production. The molecular rotation difference, ΔM_D (18-H $\rightarrow$ OH), between the metabolite and androsterone was -22, in good agreement with -33 and -25 for the etiocholanolone and estrone series, respectively. As expected, the metabolite had a greater mobility on paper chromatography than its 5β epimer. Treatment with base afforded 84% of the calculated amount of formaldehyde. The product remaining after the base treatment was chromato-

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[1] The systematic names in accordance with the recent recommendation of the International Union of Pure and Applied Chemistry are given below for the trivial names employed in this report: 18-hydroxyetiocholanolone, 3α,18-dihydroxy-5β-androstan-17-one; 18-hydroxyandrosterone, 3α,18-dihydroxy-5α-androstan-17-one; etiocholanolone, 3α-hydroxy-5β-androstan-17-one; androsterone, 3α-hydroxy-5α-androstan-17-one; 18-hydroxyestrone, 3,18-dihydroxyestra-1,3,5(10)-trien-17-one; estrone, 3-hydroxyestra-1,3,5(10)-trien-17-one; aldosterone, 18,21-dihydroxy-11β,18-oxidopregn-4-ene-3,20-dione; 11β-hydroxyetiocholanolone, 3α,11β-dihydroxy-5β-androstan-17-one.

[2] We are indebted to Le Roy F. Johnson, Applications Laboratory, Instruments Division, Varian Associates, Palo Alto, California, for the determination and interpretation of the spectrum.

graphed on paper and was found to be more polar than androsterone. The difference in mobility between the product and androsterone was comparable to that between 3α-hydroxy-18-nor-5β,13α-androstan-17-one and etiocholanolone and, therefore, the deformylated product was in all probability 3α-hydroxy-18-nor-5α,13α-androstan-17-one. Studies designed to corroborate these identifications by syntheses of the compounds are now in progress.

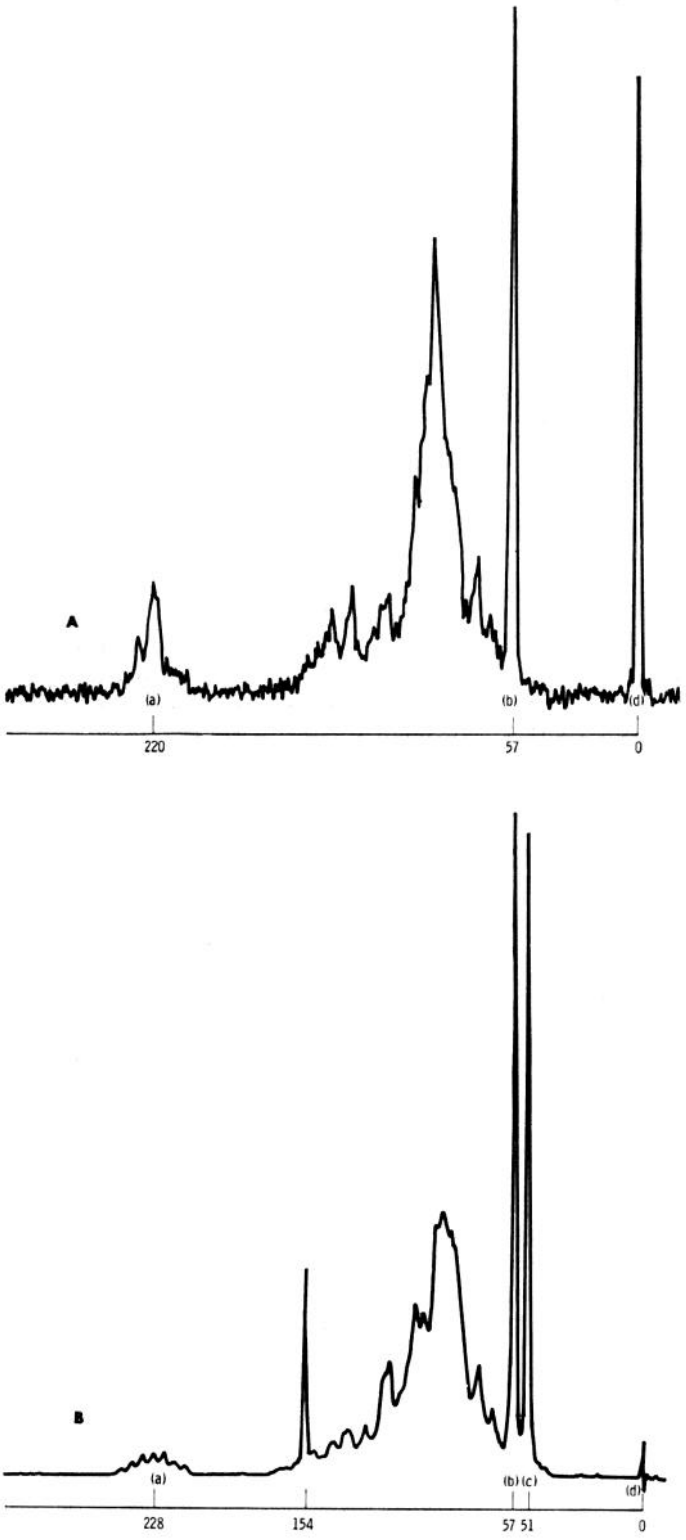

FIG. 1. Nuclear magnetic resonance spectra of 18-hydroxyetiocholanolone (*A*) and etiocholanolone (*B*). (*a*), Hydrogen attached to a carbon bearing an oxygen atom; (*b*), Hydrogen atoms of C-19 methyl; (*c*), Hydrogen atoms of C-18 methyl; and (*d*), Temtraethylsilane.

Hydroxylation of C_{21} steroids at C-18 is a well known biochemical reaction of the adrenal tissues, with the formation of aldosterone a prime example. The isolation of 18-hydroxyestrone from human pregnancy urine has been reported by Loke *et al.* (3). It was suggested that the compound was of adrenal origin since these investigators were able to demonstrate 18-hydroxylation of estrone with beef adrenal homogenate. The present results suggest, however, that 18-hydroxylation can also occur in extra-adrenal site(s), since the products isolated were formed after parental administration of preformed metabolites and from androgens produced by an arrhenoblastoma that exhibited none of the characteristic steroidogenesis of adrenal tissue. It appears unlikely that the 18-hydroxylation of these substances occurred in the adrenal gland since such a small amount would circulate through that organ.

The possibility that microbiological hydroxylation occurred at C-18 during enterohepatic circulation can be disregarded, since studies in this laboratory as well as by others have shown that enterohepatic circulation plays a negligible role in the metabolism of testosterone (4, 5).

Steroid production of the androgen-producing tumor has been reported in detail in another communication (6). During the testosterone-4-C^{14} administration period, the daily endogenous production of androsterone and etiocholanolone was 13 and 100 mg, respectively. The metabolites of 11-oxygenated hormones were produced in normal amounts so there was no unusual function of the adrenal glands in this patient. Approximately 30 mg of 18-hydroxyetiocholanolone per day were isolated from the urine extracts.

EXPERIMENTAL PROCEDURE[3]

Isolation of 18-Hydroxyetiocholanolone

From Endogenous Precursor—Subject H (arrhenoblastoma) received 2.42×10^6 c.p.m. of testosterone-4-C^{14} intravenously, and urine was collected for 2 days (6). The first 24-hour urine sample (1.46×10^6 c.p.m.) was treated with β-glucuronidase[4] in the usual manner and extracted with ether (7). The residual urine and the alkaline plus aqueous washes were combined, acidified to 1 N with sulfuric acid, and boiled for 30 minutes. The mixture was then cooled and extracted with ether. The neutral extracts obtained from the enzyme hydrolysis (6.90×10^5 c.p.m.) and the acid hydrolysis (5.13×10^5 c.p.m.) were combined and treated with Girard's Reagent T to yield a ketonic fraction (9.50×10^5 c.p.m.). The second 24-hour urine sample (6.05×10^4 c.p.m.) was adjusted to 1 N with sulfuric acid and boiled for 30 minutes. The neutral extract (3.45×10^4 c.p.m.) and the ketonic fraction were prepared in the usual way (7).

[3] Melting points were determined on a micro hot stage and are corrected. Optical rotations were determined in ethanol. Infrared spectra were determined on Perkin-Elmer model 21 spectrophotometers; 4000 to 2750 cm^{-1} calcium fluoride prism, carbon disulfide; 1800 to 1600 cm^{-1} and 1500 to 1280 cm^{-1} calcium fluoride prism, carbon tetrachloride; 1300 to 650 cm^{-1} sodium chloride prism, carbon disulfide. The same prisms were employed with potassium bromide dispersions.

[4] The enzyme, β-glucuronidase, used for hydrolysis, was obtained from the Warner-Chilcott Laboratories, a division of Warner-Lambert Pharmaceutical Company, New York; it is commercially available under the trade name Ketodase.

Ninety per cent of the ketonic fraction from the 2 days was combined and chromatographed on 80 g of silica gel containing 32 ml of ethanol. Elution with 1-liter volumes of 1% ethanol in petroleum ether-methylene chloride (7:3) was followed by 2% ethanol in methylene chloride. The latter eluate contained 61 mg of 18-hydroxyetiocholanolone, 9.50×10^4 c.p.m. or 1600 c.p.m. per mg. Androsterone and etiocholanolone isolated from the combined extracts had 1700 and 1750 c.p.m. per mg, respectively, as described elsewhere (6). Recrystallization of the combined fractions from acetone yielded the dihydroxyketone, m.p. 206–208°.

From Exogenous Testosterone—Subject K (cancer of prostate) received 1.8 g of testosterone-4-C^{14} (1730 c.p.m. per mg) in divided doses over 10 days. The urine, which was collected for 10 days and pooled, contained 1.27×10^6 c.p.m. The urine was adjusted to 1 N with sulfuric acid and boiled for 30 minutes. The neutral extract, prepared in the usual way, contained 8.35×10^5 c.p.m. Treatment of the extract with Girard's Reagent T yielded 6.83×10^5 c.p.m. in the ketonic fraction. The ketonic fraction was chromatographed on 80 g of silica gel containing 32 ml of ethanol with 1-liter volumes as before. Elution with 2% ethanol in methylene chloride yielded 69 mg of crystalline material, 1.33×10^5 c.p.m., which was shown to be 18-hydroxyetiocholanolone by infrared spectrometry and paper chromatography in System A. Two recrystallizations from acetone afforded an analytical sample of 18-hydroxyetiocholanolone, m.p. 206–208°.

From Exogenous Etiocholanolone—Subject M (reticulum cell sarcoma) received a daily intramuscular injection of 50 mg of etiocholanolone in benzyl alcohol-sesame oil for 48 days. Urine collected during the injection period was acidified to 1 N with sulfuric acid and boiled for 30 minutes. Extraction with ether and preparation of the neutral extract yielded 3.41 g. Treatment with Girard's Reagent T afforded 1.65 g of ketonic material. This fraction was chromatographed on 80 g of silica gel containing 32 ml of ethanol with 1-liter volumes as before and 18-hydroxyetiocholanolone was eluted with 2% ethanol in methylene chloride. Recrystallization of the steroid (127 mg) from acetone and ethyl acetate afforded 29 mg of 18-hydroxyetiocholanolone, m.p. 213–216°. Chromatography of the mother liquors on 20 g of silica gel and elution with ether-benzene (7:3) and ether afforded 35 mg of the 18-hydroxy derivative accompanied by reddish oil. Washes with cold methylene chloride and recrystallization from ethyl acetate yielded a further 14 mg of the 18-hydroxy metabolite, m.p. 205–209°. An additional 17 mg were obtained by chromatography of the mother liquors and adjacent chromatographic fractions on Whatman No. 1 paper (18 × 118 cm) in System A for 65 hours.

Identification of 18-Hydroxyetiocholanolone—The analytical sample of 18-hydroxyetiocholanolone had m.p. 206–208° and 214.5–216.5°; $[\alpha]_D^{28}$ +93.5°; the infrared spectrum is shown in Fig. 2.

$C_{19}H_{30}O_3$

Calculated: C 74.48, H 9.87
Found: C 74.45, H 9.83

When chromatographed on Whatman No. 1 paper (18 × 118 cm) for 65 hours at 23° in System A the metabolite traveled 47 to 51 cm; under the same conditions for 44 hours, it moved to 38 to 42 cm from the origin, while 11β-hydroxyetiocholanolone traveled 88 to 93 cm. Under the standard conditions for the Zimmermann reaction (8), the compound formed only 10% of the color of the same weight of androsterone. Negative reactions were obtained with tetranitromethane and blue tetrazolium reagent. The compound was recovered unchanged after heating under reflux with 1 N sulfuric acid in 30% ethanol. Acetylation with pyridine and acetic anhydride at room temperature afforded a diacetoxyketone which could not be crystallized even after purification by chromatography and sublimation. The oily diacetate had the following principal absorption bands: $\nu_{max}^{CS_2, CCl_4}$ 1744, 1450, 1407, 1237, and 1030 cm^{-1}. Other methods of acetylation also yielded only oily products.

The nuclear magnetic resonance spectrum of 18-hydroxyetiocholanolone in $CDCl_3$ solution taken on the Varian HR-60 spectrometer operating at 60 megacycles was compared with that of etiocholanolone (Fig. 1).

A solution of 63.5 μg of the 18-hydroxy metabolite in 0.5 ml of ethanol was added to 0.5 ml of a solution of 50 mg of potassium hydroxide in 95% ethanol. The mixture was allowed to stand at 23° for 3 hours, at which time 0.2 ml of this mixture and 0.2 ml of 5 N sulfuric acid were placed in the outer ring of a Conway diffusion dish. The inner ring contained 0.8 ml of 0.2% chromotropic acid in 30 N sulfuric acid. The formaldehyde was allowed to diffuse for 16 hours at 23°. The chromotropic acid was removed and heated at 100° for 30 minutes, and the color was read at 570 mμ. Reagent blanks and standard formaldehyde solution were measured simultaneously. Under these conditions, 73% of the calculated amount of formaldehyde was released from 18-hydroxyetiocholanolone.

Approximately 10 mg of the dihydroxyketone were treated with alkali under the above conditions, and the alkaline solution was extracted with chloroform. The chloroform solution was washed with water and dried, and the solvent was evaporated. Chromatography of the oily product on alumina and elution with ethyl acetate-benzene (1:19) gave 3 mg of crystalline material. Recrystallization from acetone-petroleum ether gave 3α-hydroxy-18-nor-5β,13α-androstan-17-one, m.p. 152–154°, which had an infrared spectrum (Fig. 3) identical with that of an authentic sample, m.p. 150–151° (2) kindly supplied by Professor W. S. Johnson and Dr. K. L. Williamson Chromatography on Whatman No. 1 paper (18 × 118 cm) in System B

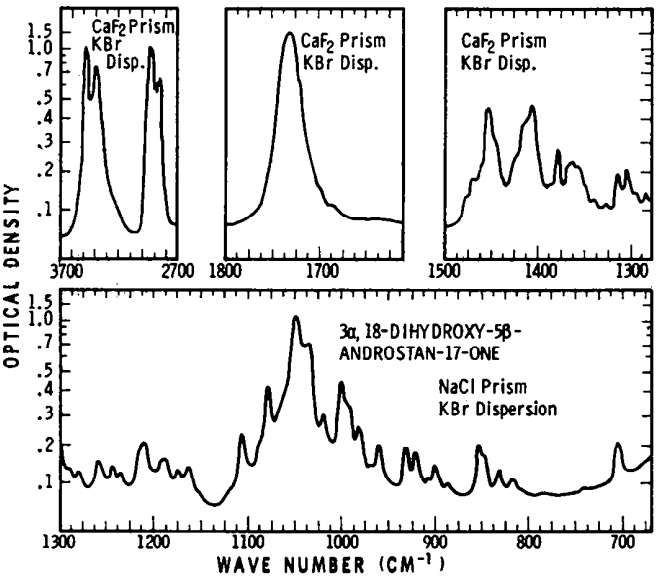

FIG. 2. Spectrum of 3α,18-dihydroxy-5β-androstan-17-one (potassium bromide dispersion).

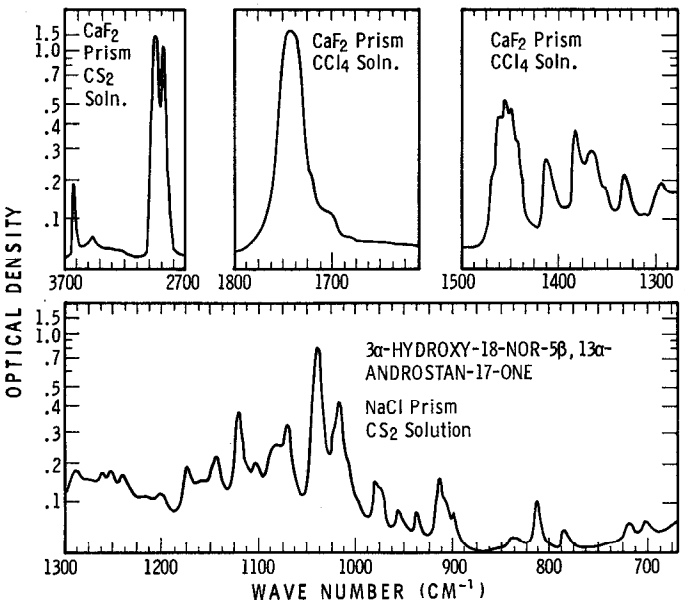

FIG. 3. Spectrum of 3α-hydroxy-18-nor-5β,13α-androstan-17-one in solution.

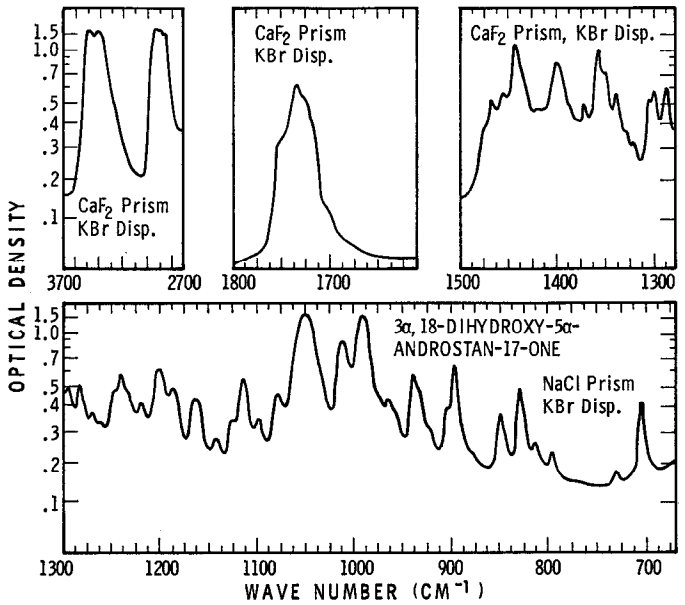

FIG. 4. Spectrum of 3α,18-dihydroxy-5α-androstan-17-one (potassium bromide dispersion).

for 40 hours at 23° showed that it moved 47 to 50 cm, whereas etiocholanolone and androsterone moved 56 to 59.5 and 69 to 72.5 cm, respectively. The alkaline solution remaining after extraction with chloroform was acidified and distilled. Approximately 15 ml of distillate were trapped in a 10-ml solution of 15 mg of dimedone in 50% ethanol. On standing, needles of formaldehyde-dimedone were formed, m.p. 189–190°; the infrared spectrum was identical with that of an authentic sample, m.p. 189–190°.

Isolation of 18-Hydroxyandrosterone

A pool of urine representing 40 days was obtained from patients after daily intramuscular administration of 50 mg of androsterone in benzyl alcohol-sesame oil. The disorders of these patients were as follows: dystrophia myotonica, hypertension, breast cancer, hypercholesterolemia, myxedema, rheumatoid arthritis, and polycythemia vera. The neutral steroid extract was prepared in the usual manner after hydrolysis with β-glucuronidase. The steroids were separated with Girard's Reagent T into ketonic and nonketonic fractions. The ketonic fraction was chromatographed on 80 g of silica gel containing 32 ml of ethanol with 1-liter volumes as before. Elution with 2% ethanol in methylene chloride afforded fractions containing 18-hydroxyandrosterone as judged by paper chromatography. Those fractions were combined and rechromatographed on 30 g of silica gel. Elution with ethyl acetate-benzene (2:3) afforded 50 mg of oil containing the 18-hydroxylated metabolite. Further purification by paper chromatography in System A and recrystallization from ethyl acetate gave 8 mg of needles of 18-hydroxyandrosterone, m.p. 184.5–188°; $[\alpha]_D^{23}$ +83.4°; for the infrared spectrum, see Fig. 4.

When chromatographed on Whatman No. 1 paper (18 × 118 cm) in System A at 23° for 65 hours, the metabolite moved 72 to 77 cm, whereas its 5β epimer moved 47 to 51 cm. The dihydroxyketone formed only 10% of the color of the same weight of androsterone in the micro-Zimmermann reaction. It did not form a precipitate with digitonin. Acetylation with pyridine and acetic anhydride at room temperature afforded an oily diacetate with the following principal absorption bands in its infrared spectrum:[5] $\nu_{max}^{CS_2, CCl_4}$ 1740, 1449, and 1443 cm^{-1}.

Formaldehyde produced by the alkaline treatment of 86.6 μg of 18-hydroxyandrosterone was measured as described for the 5β epimer. It was found that 84% of the calculated amount of formaldehyde was released. The alkaline solution remaining from this reaction was extracted with ethyl acetate. The organic layer was washed with water and dried, and the solvent was evaporated. The residue was chromatographed on Whatman No. 1 paper (18 × 57 cm) in System C for 18 hours. 3α-Hydroxy-18-nor-5α,13α-androstan-17-one obtained from the alkali treatment moved 16 to 22 cm, whereas etiocholanolone and androsterone traveled 17 to 21 cm and 23 to 28 cm, respectively.

Systems for Paper Chromatography—System A, 2,2,4-trimethylpentane-toluene (3:5) and methanol-water (4:1); System B, 2,2,4-trimethylpentane-methanol-water (10:8:2); System C, 2,2,4-trimethylpentane-methanol-water (10:10:1).

Radioactivity measurements were made on a Tracerlab SC-50 automatic sample changer. Most extracts and pure products were in the "infinitely thin" range, on nickel-plated steel planchets. With urines or bulky extracts, appropriate corrections to "infinite thinness" were made. Radioactivity on paper chromatograms was detected with an Aquebogue paper chromatogram scanner as previously described (4).

SUMMARY

Two 18-hydroxylated C_{19}-17-ketosteroid metabolites have been isolated from human urine and characterized. 18-Hydroxyetiocholanolone was obtained from a patient with arrhenoblastoma and from patients given large amounts of testosterone or etiocholanolone. Its 5α epimer, 18-hydroxyandrosterone,

[5] The infrared spectrum proved to be identical with an unknown metabolite isolated by Dr. John J. Schneider, Jefferson Medical College, Philadelphia, Pennsylvania, following oral administration of androsterone.

was isolated after androsterone administration. The results suggest that 18-hydroxylation of steroids can occur in extra-adrenal sites.

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