# D-RING OXYGENATION OF 5α-ANDROSTAN-3-ONE IN RABBITS

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Abstract - Metabolism of 5α-androstan-3-one in the rabbit was studied in order to investigate changes in the steroidal ring system. The total crude urinary extract was separated into a non-ketonic and a ketonic fraction and was further purified by column chromatography. 3α- and 3β-Hydroxy-5α-androstan-16-one together with 5α-androstane-3α,16α- and 3β,16α-diol, and 5α-androstane-3β,17α-diol have been isolated and identified by spectroscopic methods and comparison with authentic samples. The results were compared with other metabolic studies of steroid derivatives. From this study it is suggested that metabolic oxidation occurs in positions removed from the initial oxygen function of the monooxygenated steroids.

## INTRODUCTION

Many studies of the relationships between the androgenic and anabolic properties of synthetic 5α-androstane derivatives and their chemical structure have been carried out (1) and their *in vivo* and *in vitro* metabolism has been studied (2). The most characteristic structural features of the compounds investigated have been the presence of an oxygen function in both the 3- and 17-positions of the steroid nucleus. Removal of the 3-oxygen function leads to increased anabolic over androgenic activity (3). The importance of the 17-oxygen function for androgenic and anabolic activity has been generally accepted, particularly for maximum anabolic activity. However, the hydrocarbon 5α-androstane (4), and 5α-androstane-3β,16α-diol (5) both have androgenic activity, thereby indicating that a 17-oxygen function is not an essential requirement.

Metabolic experiments have shown that conversion of the unsubstituted A-ring, e.g. 17α-methyl-5α-androstan-17β-ol (6) and 5-androsten-17-one (7), to 3-oxygenated derivatives occurs. These results suggest that their biological activity is associated with the more usual 3-oxygenated compounds. Similarly, the androgenic and anabolic properties of 17β-hydroxy-5α-androst-2-ene (8) may be due to metabolic conversion to a 3-oxygenated derivative. In order to determine whether metabolic oxidation of the unsubstituted steroidal D-ring occurs, 5α-androstan-3-one (I) was fed to the rabbit.

## EXPERIMENTAL

Apparatus: Melting points were carried out on a Thomas-Hoover capillary apparatus and are uncorrected. Optical rotations were measured in $CHCl_3$ solution at 23° on a Bellingham and Stanley (Model A) Polarimeter. The i.r. spectra were recorded on a Beckman Model 8 spectrophotometer using the KBr disc method. For p.m.r. spectra of II, III and V a Varian XL-100-15 spectrometer was used with accumulations recorded in the CW mode utilizing the Varian 620L Data Machine. The remaining spectra were recorded on a Varian A56/60A instrument. Deuterochloroform as solvent and TMS as internal standard were used. Mass spectra were determined on a Finnigan Quadrapole Model 1015 instrument at 70 ev using a direct probe method by the Chemistry Department, University of Manitoba. Elemental analysis was performed by Pascher and Pascher, Microanalytical Laboratory Bonn, West Germany.

Chromatography: Column chromatography was carried out on basic alumina. Thin-layer chromatography (TLC) was run on silica gel coated glass plates and developed in ethyl acetate: petroleum ether (60°-90°) (1:1). Plates were visualized by spraying with 4% ethanolic sulfuric acid followed by heating at 100°. Gas-liquid chromatography (GLC) was carried out on a Varian Aerograph Ser. 1800 chromatograph equipped with a hydrogen flame ionization detector and a pyrex glass column packed with 2% OV-17 on Chromosorb GHP, 60/80 mesh, internal diameter 2 mm, length 180 cm. The detector temperature was 300°, column temperature 240° and the carrier gas ($N_2$) flow rate was 40 ml/min. All steroids were treated with trimethylsilyl chloride in pyridine before injection so that 1 μl contained 25 μg of steroid. Retention times are relative to 5α-cholestane. The diols II, III and V were present in the relative ratio of 10:3:7, respectively, and the ketols VI and

VII in the ratio of 7:3 by comparison of GLC peak areas.
5α-Androstan-3-one: 5α-Androstan-3-one (m.p. 99.5-100°; $[\alpha]_D$ + 21°, C,1, dioxane (lit. (9) m.p. 104.5-105.5°; $[\alpha]_D$ +18°, C,~1, dioxane) was prepared by Wolff-Kishner reduction (10) of 3β-hydroxy-5α-androstan-17-one followed by Jones' oxidation (11) and was chromatographically pure (TLC, GLC).
Administration of 5α-androstan-3-one (I): No gross alteration in the appearance, food intake or behaviour of the dosed animals was observed.
Two mature male albino rabbits, maintained on a Purina rabbit chow diet and water ad libitum, were housed singly in cages designed for efficient separation of urine and faeces. A controlled illumination environment of 12 hours light and 12 hours darkness was maintained. The animals were each dosed three times at two-day intervals with a finely divided slurry of 5α-androstan-3-one (1 g) in propylene glycol (12.5 ml) by oral administration. Urine was collected for a total of seven days. A total of 4 l of urine was collected under a thin layer of toluene and stored at -5°. Similar results were obtained in three experiments using different animals.
Isolation of neutral steroids: A portion (2 l) of urine was acidified to 1N with concentrated hydrochloric acid and refluxed for 30 min. The cooled solution was extracted with ether and the combined ether layers were washed with aqueous 2N-sodium hydroxide, water until neutral, and saturated sodium chloride. After drying over anhydrous sodium sulfate the solvent was removed at reduced pressure to give a total crude residue (792 mg). Equivalent control experiments gave 116 mg and 126 mg of crude residue with and without propylene glycol, respectively. Analogous experiments administering a dose (3x) of 100 mg showed only quantitative differences in the relative intensities of the five major metabolites on GLC. Urine from animals dosed orally twice a day (1 g in 5 ml of water) for two days with neomycin before administration of 100 mg dose (3x) as above showed an identical GLC trace for the five major peaks as in the equivalent experiment without neomycin treatment. An identical GLC pattern was obtained when the urine was adjusted to pH 5 with HOAc and incubated with calf-liver β-glucuronidase (800 FU/ml) at 37° for 72 hours and extracted as before.
Separation of ketonic and non-ketonic materials: A solution of the crude urine extract (1.12 g) and Girard T reagent (0.75 g) in 1M-acetic acid in 95% ethanol (12.5 ml) was refluxed for 1 hr. The reaction was cooled, then poured into excess cold saturated aqueous sodium bicarbonate. After extraction with ether the ether layers were washed with bicarbonate solution, then with saturated sodium chloride, dried over sodium sulfate and evaporated to dryness at reduced pressure to give a crude non-ketonic fraction (700 mg). The combined sodium bicarbonate layers were acidified with concentrated hydrochloric acid (20 ml) and heated on the steam-bath for ½ hour. The reaction was cooled, extracted with ether to give a crude ketonic fraction (410 mg).

Non-ketonic fraction: The non-ketonic fraction showed the presence of three peaks on GLC (RRT, 0.34, 0.43, 0.47). Chromatography over alumina yielded two major fractions on elution with ether and ether-methanol.

The least polar substance ($R_f$, 0.28; RRT, 0.34) was recrystallized from either methanol or ethyl acetate to give 5α-androstane-3β,17α-diol (II) (24 mg), m.p. 210-211° (lit. (12) m.p. 214°); i.r. ν max 3333 (OH), 1044, 1076 (CO) $cm^{-1}$; p.m.r. δ: 0.655, s, (C-13 methyl), 0.815, s, (C-10 methyl), 3.60, m, (3αH), 3.72, d, J = 6Hz (17β-H) ppm; m.s. parent peak 292. Comparison of the i.r. and m.s. spectra of this material with an authentic sample showed them to be identical. A mixed melting point was not depressed.

The more polar fraction showed two peaks on GLC (RRT, 0.43, 0.47) but were unseparated on TLC ($R_f$, 0.20). Recrystallization from methanol gave 5α-androstane-3α,16α-diol (III) (67 mg), m.p. 212°; $[\alpha]_D$ - 12.5° (C.1, dioxane); RRT, 0.43; i.r. ν max 3279, 3400 (OH), 1001, 1042 (CO); p.m.r. δ: 0.725, s, (C-13 methyl), 0.805, s, (C-10 methyl), 4.07, s, (3βH), 4.49, m, (16βH) ppm; m.s. parent peak 292.

Found: C, 78.17; H, 10.91. $C_{19}H_{32}O_2$ requires C, 78.03; H, 11.03.

Oxidation of 5α-androstane-3α,16α-diol (III) with Jones reagent (11) gave 5α-androstane-3,16-dione (IV), m.p. 159-161° (lit. (13) m.p. 157-159°); i.r. ν max 1700 (6-membered ring (C=O) 1736 (5-membered ring C=O). Mixed melting point with an authentic sample showed no depression.

Repeated chromatography of the mother liquor after isolation of II and III over alumina gave 5α-androstane-3β,16α-diol (V) (35 mg), m.p. 186-188° (lit. (14) m.p. 192-193°); RRT, 0.47; i.r. ν max 3390, 3425 (OH), 1040 (CO); p.m.r. δ: 0.72, s, (C-13 methyl), 0.82, s, (C-10 methyl), 3.60, m, (3αH), 4.47, m, (16βH) ppm; m.s. parent peak 292. Mixed melting point with an authentic sample was undepressed.

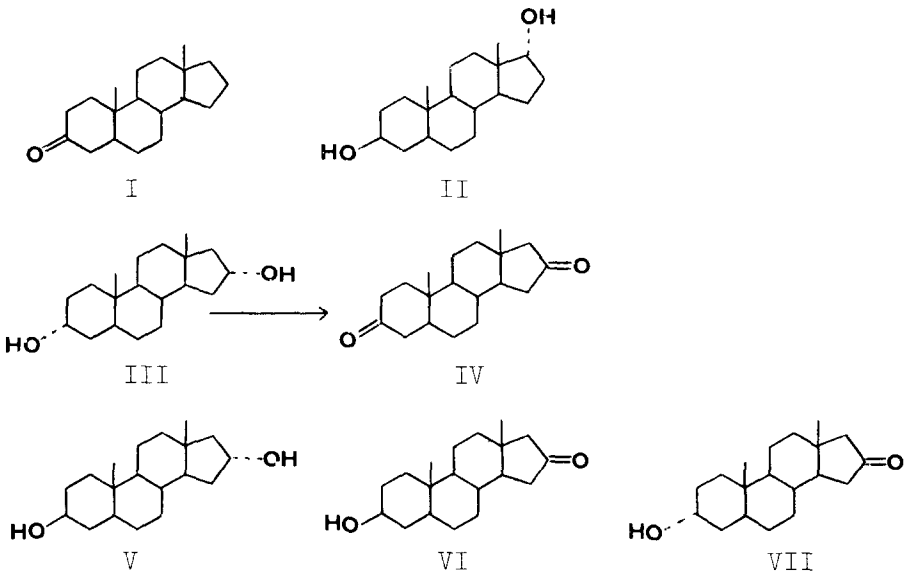
Ketonic fraction: The crude ketonic fraction showed on GLC two major peaks (RRT, 0.52, 0.68). Chromatography over alumina gave on elution with benzene two major fractions. The more polar material ($R_f$, 0.41) on recrystallization from methanol gave 3β-hydroxy-5α-androstan-16-one (VI) (16 mg), m.p. 186-187° (lit. (15) m.p. 186-187°); i.r. ν max 3509 (OH), 1733 (5-membered ring C=O), 1050, 1080 (CO); p.m.r. δ: 0.865, s, (C-13 methyl), 0.89, s, (C-10 methyl), 3.65, m, band width at half-height, 22 Hz (3αH). Mixed melting point with an authentic sample was not depressed. Comparison of i.r. and m.s. spectra showed them to be identical.

The less polar material ($R_f$, 0.47) yielded on crystallization from methanol 3α-hydroxy-5α-androstan-16-one (VII), m.p. 151-152.5° (13 mg), (lit. (16) m.p. 153.5-154.5°; i.r. ν max 3570 (OH), 1733 (5-membered ring C=O), 1000, 1032 (CO); p.m.r. δ: 0.83, s, (C-13 methyl) 0.89, s, (C-10 methyl), 4.06, m, band width at half-height, 7Hz, (3βH). Mixed melting point with an authentic sample was not depressed and spectral comparisons (i.r. and m.s.) were identical.

## RESULTS

Isolation of the neutral steroidal fraction by ether extraction from the acid hydrolyzed urine of rabbits fed 5α-androstan-3-one (I) gave a seven fold increase in weight when compared with a blank experiment. Thin-layer chromatography indicated the presence of oxygenated steroidal material not present in the control urinary extracts of higher polarity than either the initial 3-oxo-steroid or the 3α- and 3β-alcohol derivatives. Column chromatography showed that the total crude urinary extract consists of a minimum of 70% of material corresponding (by TLC) to the five substances isolated. GLC analysis showed that the five peaks corresponding to the substances isolated consists of a minimum of 85% of the material eluted in that retention time range. The total crude urinary extract was separated into a non-ketonic and a ketonic fraction by treatment with Girard T reagent. The ketonic and non-ketonic fractions contained two and three substances, respectively, in the same proportions and with identical $R_f$ and RRT as the unseparated material. A 7:4 ratio of non-ketonic to ketonic material was obtained. Only those substances showing $R_f$ values in the range of diol and ketol androstane derivatives were identified.

The non-ketonic fraction was further separated by column chromatography into three crystalline substances which showed only hydroxylic functional group absorption in their i.r. spectra and parent peaks in the mass spectra corresponding to dihydroxy compounds. Computer averaged p.m.r. spectra in




$CDCl_3$ of these poorly soluble compounds allowed assignment of the structures on the basis of the chemical shifts of the methyl groups at C-10 and C-13 and the signals for the protons attached to the hydroxyl bearing atoms (17). The stereochemistry of the hydroxyl groups are assigned from the characteristic chemical shifts and splitting patterns of the down-field protons and their band width at half-height (18). Identification of the known alcohols, 5α-androstane-3β,17α-diol (II), and 5α-androstane-3β,16α-diol (V), was confirmed by direct comparison with authentic samples. The third diol, on spectral evidence, was 5α-androstane-3α,16α-diol (III) which was further characterized by oxidation to the known 5α-androstane-3,16-dione (IV).

The ketonic fraction could be separated into two major components by TLC and GLC. Chromatographic separation on an alumina column gave 3α- and 3β-hydroxy-5α-androstan-16-one in

the proportion of 3:7 which were identified on the basis of their spectral properties and confirmed by comparison with authentic samples.

## DISCUSSION

Oxidation of the unsubstituted A-ring of 17α-methyl-5α-androstan-17β-ol occurs in the 3-position. Here the thermodynamically less stable axial (3α) alcohol as well as the equatorial (3β) epimer was isolated after incubation with rabbit liver homogenate (6). Metabolism of 5-androsten-17-one by rabbits gave both axial and equatorial alcohols at carbon 3 and the equatorial alcohol at carbon 2, hydroxylation occurring at the less sterically hindered carbon atoms removed from the A/B ring junction (7). In the present work, oxidation of the D-ring of 5α-androstan-3-one in rabbits has given alcohols in both the 16α- and 17α-positions, oxidation occurring on the α-face adjacent to and removed from the C/D ring junction, respectively. Twice as much oxidation has occurred at the 16-position compared with the more sterically hindered 17-position.

From molecular models it can be seen that oxidative attack on the steroid molecule has occurred in positions furthest removed from the initial oxygen function. In 17α-methyl-5α-androstan-17β-ol (6), oxidation occurred in the 3-position which is the carbon atom furthest removed from the initial 17-oxygen function. 5-Androsten-17-one (7) was oxidized at the 2- and 3-positions remote from the 17-oxygen function. Similarly, the oxygenated derivatives isolated from metabolism of 5α-androstan-3-one show that oxidation has occurred at the 16- and

17-positions which are the atoms furthest removed from the 3-oxygen function. Oxidation at the position distant from the initial polar group is consistent with the suggested non-polar environment (19) of the active site of the liver microsomal monooxygenase system. No evidence of substances monohydroxylated at other positions of the hydrocarbon skeleton was observed. These results indicate a high degree of selectivity in the area of enzymatic attack for the neutral urinary excreted steroids. The normal quantity of endogenous androstanes expected to be excreted in the urine over the collection period is less than 0.5% of the total steroidal material isolated (20). These results can be compared with the active site requirements for hydroxylation of monocyclic, dicyclic and tricyclic ring systems proposed by Robertson and Dunstan (21).

The involvement of intestinal microorganisms in the formation of the substances isolated has not been fully determined. However, experiments in animals pretreated with neomycin in order to sterilize the gut showed no significant change in the nature or quantity of compounds isolated. Further, considering the relatively large amount of conversion occurring, liver metabolism is the most probable source of these compounds. 16α-Hydroxylation of steroids has been shown to occur in liver microsomal preparations from germ-free rats (22).

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