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New Metabolites of 11β-Hydroxy-Δ⁴-androstene-3,17-dione*

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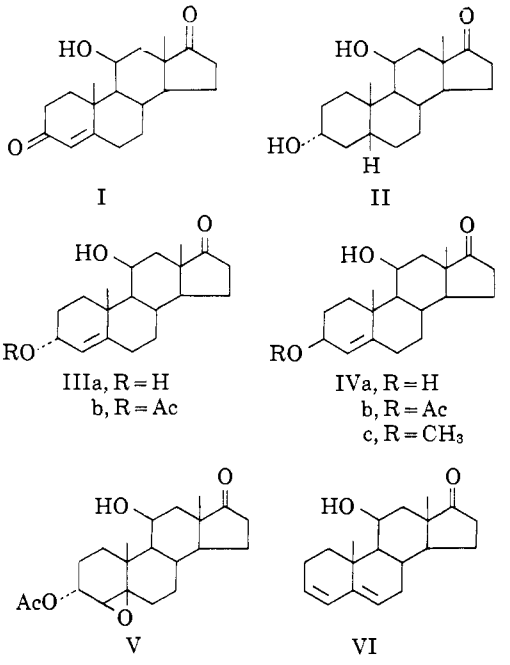
ABSTRACT: 3α,11β-Dihydroxy-Δ⁴-androsten-17-one has been identified as a metabolite of 11β-hydroxy-Δ⁴-androstene-3,17-dione in man. Its epimer, 3β,11β-dihydroxy-Δ⁴-androsten-17-one, was also isolated from urine but it was shown to be derived from the 3α-hydroxy compound during the mild acid conditions of hydrolysis. The unsaturated metabolite was excreted in conjugated form which could be cleaved by treatment at pH 5.0 at 45° for 1 day. Longer periods of incubation resulted in a 1:1 equilibrium mixture of the two epimers.

Studies of the metabolism of labeled 11β-hydroxy-Δ⁴-androstene-3,17-dione (I) demonstrated variable amounts of acid-labile metabolites with the chromatographic mobility of 11β-hydroxyetiocholanolone[1] (II) (Bradlow *et al.*, 1966). These products were observed with normal people and very small amounts were produced by hyperthyroid subjects. The new metabolites were, however, greatly increased in the urine of hypothyroid subjects and in some instances represented as much as 25% of the administered hormone. These metabolites have now been isolated and characterized as 3α,11β-dihydroxy-Δ⁴-androsten-17-one (IIIa) and its 3β-hydroxy epimer IVa. This report is a description of an isolation study made in a representative patient; it is similar in all pertinent details to a number of other investigations of the metabolic fate of 11β-hydroxy-Δ⁴-androstene-3,17-dione given to men and women, in large and small amounts, intravenously or orally.



Procedure

A man with myxedema, V. R., age 67, was given 840 mg of 11β-hydroxy-Δ⁴-androstene-3,17-dione-1,2-^{3}H (16,900 cpm/mg) dissolved in 24 ml of absolute alcohol by continuous intravenous drip at a rate of 2–3 ml/day for 10 days. Urine was collected during administration

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[1] Trivial names and abbreviations used: 11β-hydroxyetiocholanolone, 3α,11β-dihydroxy-5β-androstan-17-one; 11β-hydroxyandrosterone, 3α,11β-dihydroxy-5α-androstan-17-one; tlc, thin layer chromatography.

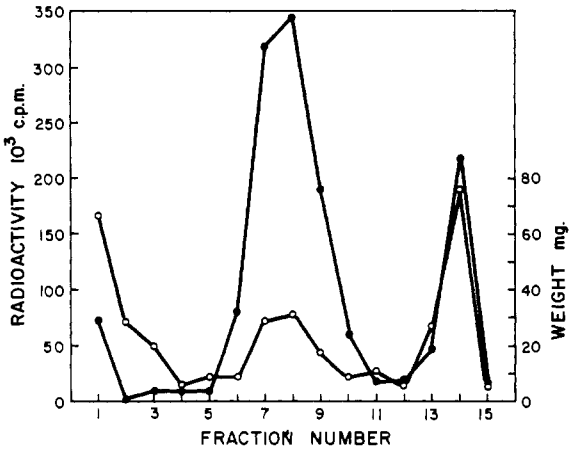

FIGURE 1: Chromatography of neutral steroid extract from patient V. R. on 180 g of Celite 545; solvent system 2,2,4-trimethylpentane–butyl alcohol–methyl alcohol–water (5:2:2:2); 250 ml/fraction per hr. Fractions 13–15 were eluted with absolute ethyl alcohol. ●—●, radioactivity in counts per minute. O—O, weight in milligrams.

and for 4 days following the end of administration and combined as follows: days 1–5, 6–10, and 11–14. The steroid conjugates were hydrolyzed by two successive treatments with β-glucuronidase at 38° followed by continuous ether extraction at pH 5.0 for 48 hr after each incubation.

The neutral steroid extract (1.74×10^6 cpm) obtained after incubation with Ketodase[2] of urine of days 1–5 was employed for isolation. This extract was put on a column of 180 g of Celite 545 containing 90 ml of the lower phase of solvent system 2,2,4-trimethylpentane–butyl alcohol–water–methyl alcohol (5:2:2:2). The column was developed with the upper phase and 250-ml fractions were collected each hour. A chart of the chromatogram is recorded in Figure 1.

3β,11β-Dihydroxy-Δ⁴-androsten-17-one (IVa). Fraction 9 contained 18 mg and 190,000 cpm; it was chromatographed on a thin layer of silica gel GF with ether. The plate was sprayed with water in order to detect the steroids present and the area containing 3β,11β-dihydroxy-Δ⁴-androsten-17-one (R_F 0.33) was eluted with methyl alcohol–chloroform to yield 8 mg of product. Recrystallization from acetone–ether yielded 5 mg of 3β,11β-dihydroxy-Δ⁴-androsten-17-one (IVa), mp 182–185°; 16,500 cpm/mg; R_F 0.16 on tlc in ethyl acetate–cyclohexane (1:1). The infrared spectrum was identical with that of the synthetic sample (Fukushima *et al.*, 1966). The 3-monoacetate IVb, prepared with acetic anhydride and pyridine and after recrystallization from cyclohexane, melted at 158–159°; 14,400 cpm/mg corresponding to 16,500 cpm/mg for the free steroid; R_F 0.41 with ethyl acetate–cyclohexane (1:1). The infrared spectrum was identical with that of the synthetic sample (Fukushima *et al.*, 1966).

The 3α-hydroxy-Δ⁴-epimer of IVa, R_F 0.23 in ether, was detected in fraction 9 but not in sufficient amount for isolation. Small amounts of 11β-hydroxy-Δ⁴-androstene-3,17-dione (I) and 3α,11β-dihydroxy-5β-androstan-17-one (II) were also detected in this fraction.

3α,11β-Dihydroxy-Δ⁴-androsten-17-one (IIIa) and 3α-11β-Dihydroxy-5β-androstan-17-one (II). Fraction 7 contained 29 mg and 318,000 cpm; it was combined with 11 mg containing 115,000 cpm from fraction 8 and chromatographed on eight sheets of Whatman no. 1 paper (18 × 118 cm) in the system toluene–2,2,4-trimethylpentane–methyl alcohol–water (5:3:4:1) for 16 hr at 24°. Reference samples of 11β-hydroxyandrosterone and 11β-hydroxyetiocholanolone were chromatographed on either side of the extract. After chromatography two narrow strips were cut out from the middle portion of the paper and one stained with the Zimmermann reagent and the other with 77% sulfuric acid reagent. The areas corresponding to both the purple stain with Zimmermann reagent and a pink stain with sulfuric acid contained 3α,11β-dihydroxy-Δ⁴-androsten-17-one; these were eluted with acetone–ethyl alcohol and combined to give 25 mg, 235,000 cpm. These eluates contained 11β-hydroxyetiocholanolone as well. Recrystallizations from acetone of the combined eluates afforded 4 mg of 11β-hydroxyetiocholanolone (II), mp 231–232°; 16,000 cpm/mg; calculated from the specific activity of the administered steroid, 16,700 cpm/mg.

The mother liquors from the saturated metabolite were combined and acetylated with acetic anhydride and pyridine at room temperature to give 20 mg and 170,000 cpm. A solution of this acetate and 12 mg of *m*-chloroperbenzoic acid in 1.2 ml of chloroform was allowed to stand overnight at room temperature. The reaction mixture was extracted with ether, washed with dilute base and water and, after drying over sodium sulfate, the solvent was evaporated to give 19 mg of product. This was chromatographed on six sheets of Whatman no. 1 paper (18 × 118 cm) in the system 2,2,4-trimethylpentane–methyl alcohol–water (5:4:1) for 16 hr. 11β-Hydroxyetiocholanolone acetate was used as reference compound and two narrow strips were cut from the middle portion and one stained with Zimmermann and the other with sulfuric acid reagent. The areas with the mobility of 11β-hydroxyetiocholanolone acetate (60–80 cm) which stained only with the Zimmermann reagent and not with sulfuric acid were eluted to give 5 mg and 26,000 cpm. A more polar area (12–17 cm from origin) that stained both with Zimmermann reagent and with sulfuric acid was eluted with chloroform–methyl alcohol to give 8 mg and 73,000 cpm. The presence of two substances was detected in this extract by tlc with ethyl acetate–cyclohexane (1:1), R_F 0.38 and 0.34. The mixture of epimeric oxides was, therefore, rechromatographed on two sheets of Whatman no. 1 paper (18 × 118 cm) for 21 hr in the system used above. The principal product had the mobility of 3α-acetoxy-4β,5β-oxido-11β-hydroxyandrostan-17-one (V) (18–23

[2] Ketodase obtained from the Warner-Chilcott Laboratories, New York, N. Y.

cm from origin). These areas were eluted to give 3 mg, 43,000 cpm. Recrystallization from ether gave fine needles of 3α-acetoxy-4β,5β-oxido-11β-hydroxyandrostan-17-one (V), 2 mg, mp 178–180°, 188°; 15,800 cpm/mg calculated to I; R_F 0.37 tlc with ethyl acetate–cyclohexane (1:1). The melting point was not depressed on mixture with the synthetic sample (Fukushima *et al.*, 1966) and the infrared spectra of the two samples were identical.

Studies of Hydrolytic Conditions. A woman with myxedema was given orally 1.0 g of 11β-hydroxy-Δ⁴-androstene-3,17-dione-1,2-³H (11,700 cpm/mg) over 5 hr in 220 ml of aqueous suspension. Urine was collected for 24 hr following the first ingestion. This day's urine contained 7.72×10^6 cpm corresponding to 660 mg of the ingested hormone. The urine (30%) was brought to pH 9 and extracted with ethyl acetate and with ether. These extracts contained little radioactivity and insignificant amounts of 3-hydroxy-Δ⁴-steroids. The residual urine was acidified and buffered at pH 5 with acetate and divided into six equal portions. Each portion was treated sequentially as shown in Table I. After each treatment listed, the urine was extracted with ethyl acetate which was then washed with dilute base and with water; these aqueous extracts were discarded. The portion of urine was then subjected to the next treatment listed after nitrogen was bubbled through to remove the prior solvents.

An aliquot of each extract was chromatographed on thin layer silica gel G in ethyl acetate. The chromatogram was stained with 77% sulfuric acid and the amount of each of the epimeric 3,11β-dihydroxy-Δ⁴-androsten-17-ones was estimated by visual comparison with known amounts of reference steroids. Addition of saturated metabolites to the allylic alcohols did not affect the visual estimation by the pink color formed, because the saturated analogs require heating to develop a color and this is different from that seen with the unsaturated steroids. Only an insignificant amount of the dehydration product 11β-hydroxy-Δ³,⁵-androstadien-17-one (VI) was observed in any of the extracts. Aliquots of some of the extracts listed in Table I were chromatographed on Whatman no. 1 paper (18 × 118 cm) in the system toluene–2,2,4-trimethylpentane–methyl alcohol–water (5:3:4:1) for 17 hr at 24°. A 1-cm strip was cut from the chromatogram and stained with 77% sulfuric acid. The two main areas staining pink corresponded to 3α,11β-dihydroxy-Δ⁴-androsten-17-one (64–68 cm from origin) and its 3β-epimer (53–59 cm from origin). These areas were eluted and counted (Table I). It should be noted that, although all the fractions were examined by paper chromatography and were counted for tritium radioactivity, only the essentially homogeneous areas are recorded in Table I. The relatively good agreement between the estimates of mass from the color reaction and the estimates from specific activity lends assurance that the extracts where radioactivity is recorded were essentially free of saturated metabolites. When the conjugated metabolites were subjected to enzymatic hydrolysis the area containing 3α,11β-dihydroxy-Δ⁴-androsten-17-one may contain a great deal of radioactive 3α,11β-dihydroxy-5β-androstan-17-one.

TABLE I: Hydrolysis of Δ⁴-3-Hydroxysteroid Conjugates.

Sequential Treatment[a]	Time (days)	Temp (°C)	Amt Present (mg) from Tlc[b] 3α-Δ⁴[c]	3β-Δ⁴[d]	from ³H[i] 3α-Δ⁴	3β-Δ⁴
pH 5	1	45	3	1	2.4	0.7
	5	38	tr[e]	tr	...	...
	6	38	0	0	...	...
pH 5	5	38	2	2	1.3	1.7
Ketodase at pH 5	7	38	0	0	...	...
pH 5	12	38	2	2	1.4	1.5
Ketodase at pH 5[h]	1	45	3	1	*f*	
	5	38	tr	tr		
	6	38	0	0		
H. pomatia at pH 5.0[g]	1	38	3	1	*f*	
	5	38	0	0		
	6	38	0	0		
Ketodase at pH 5[h]	5	38	2	2	*f*	
	7	38	0	0		

[a] The neutral steroid extract (5%) from 24-hr urine was used for each study. [b] The weights were estimated by visual comparison of the color formed with sulfuric acid with that of the reference compound. [c] 3α-Δ⁴, 3α,11β-dihydroxy-Δ⁴-androsten-17-one. [d] 3β-Δ⁴, 3β,-11β-dihydroxy-Δ⁴-androsten-17-one. [e] tr, trace. [f] Radioactivity of the areas was determined but the calculated weights are not recorded since in these extracts the saturated metabolites are also present. No attempts were made to separate the Δ⁴-3-hydroxysteroids from these mixtures. [g] 1000 units of *Helix pomatia*/ml of urine. No additional enzyme was added after the initial incubation. [h] 300 units of Ketodase/ml of urine. No additional β-glucuronidase was added after the initial incubation. [i] The weights were calculated from radioactivity eluted from the paper chromatogram areas containing the metabolites based on the specific activity, 11,700 cpm/mg, of the administered steroid. *Cf.* Experimental Section.

The melting points were taken on a micro hot stage and are corrected. Infrared spectra were determined in potassium bromide dispersion on a Beckman IR-9 spectrophotometer. Radioactivity was determined in an automated Packard Tricarb liquid scintillation counter. The samples were counted in toluene using the scintillant mixture previously described (Fukushima *et al.*, 1963). Quenching was corrected by internal standards.

Discussion

This study has demonstrated that the acid-labile metabolites of 11β-hydroxy-Δ⁴-androstene-3,17-dione with chromatographic properties like that of 11β-hydroxyetiocholanolone are 3α,11β-dihydroxy-Δ⁴-androsten-17-one (IIIa) and its 3β-epimer IVa. 3β,11β-Dihydroxy-Δ⁴-androsten-17-one, the most polar of all the 3,11β-dihydroxy-17-keto steroids by partition chromatography on Celite, was readily separated from the other hormonal metabolites. Further chromatography on thin layer silica gel afforded pure crystalline IVa, identified by infrared spectrum, specific radioactivity, melting point, and R_F values. The isolation and characterization of 3α,11β-dihydroxy-Δ⁴-androsten-17-one (IIIa) proved to be more difficult. The metabolite IIIa as well as the monoacetate IIIb had almost the same mobility as 11β-hydroxyetiocholanolone (II) and its acetate in all the systems studied. Characterization of IIIa was finally achieved by the formation of the 4,5-oxidoacetate derivative V which was readily separated from contaminants. The oxide V was identical with the synthetic product (Fukushima *et al.*, 1966) and had the same specific radioactivity as the administered steroid. 11β-Hydroxyetiocholanolone (II) was isolated for comparison of the specific radioactivity and, as expected, this was the same as both the hormone administered and the Δ⁴-metabolites.

These allylic metabolites are readily dehydrated by acid or by column chromatography on silica gel or acid-washed alumina to 11β-hydroxy-Δ³,⁵-androstadien-17-one (Rothman and Wall, 1960). The diene was previously isolated by Slaunwhite and his co-workers following administration of large amounts of 11β-hydroxy-Δ⁴-androstene-3,17-dione to normal subjects (Neeman *et al.*, 1960, Slaunwhite *et al.*, 1964). Their work-up of the urine from these subjects involved dehydrating conditions and they recognized that the diene was an artifact. From the ready formation of the diene these authors concluded that the precursor was probably 3α,11β-dihydroxy-Δ⁴-androsten-17-one.

Although both epimers of 3,11β-dihydroxy-Δ⁴-androsten-17-one were obtained from the urine following β-glucuronidase incubation in the present study, there is very good presumptive evidence that the unsaturated metabolite was excreted principally as the 3α-epimer. In the study of hydrolytic conditions for conjugate cleavage, the 3α-isomer was obtained as the predominant epimer on incubation of the urine with or without β-glucuronidase at pH 5.0 provided the time of exposure to the acidic medium was short. On more prolonged treatment at this mildly acid pH the equilibrium mixture (1:1) of the allylic alcohols was obtained regardless of the presence or absence of enzyme. Under no conditions was the β-isomer found to predominate. These facts provide evidence for the conclusion that the principal if not the exclusive allylic metabolite excreted in the urine was the conjugated form of the 3α-epimer IIIa.

In the accompanying paper (Fukushima *et al.*, 1966) the interconversion of 3α,11β-dihydroxy-Δ⁴-androsten-17-one and its 3β-epimer in aqueous acidic solution has been studied. It was demonstrated that they can be interconverted without dehydration at pH 5.0 and 38°. The equilibrium mixture (1:1) was achieved by the sixth day from the 3α-hydroxy compound whereas equilibrium was not reached until the 10th day with the 3β-hydroxy epimer.

The unsaturated metabolite(s) have been shown to be excreted principally in conjugated form. Only insignificant amounts of allylic alcohol could be detected by the sulfuric acid stain on thin layer chromatography of the extract obtained after ethyl acetate extraction of urine at pH 9.0. The metabolite(s) were cleaved from their conjugated forms by incubation at pH 5.0; addition of β-glucuronidase or sulfatase was not necessary and failed to improve the yield compared with treatment at pH 5.0 alone. Essentially all of the conjugates were cleaved in 1 day at 45° at pH 5.0. Under the hydrolytic conditions studied, the last-mentioned treatment afforded the best yield of these metabolites and, more important still, there was no evidence for dehydration and artifactual formation of 11β-hydroxy-Δ³,⁵-androstadien-17-one (VI). At lower pH there would be increased possibility of both dehydration and epimerization with no advantage in yield of these particular metabolites (Fukushima *et al.*, 1966).

The conjugated form of the allylic metabolite(s) is as yet unknown. Slaunwhite *et al.* (1964) have reported that treatment with β-glucuronidase at pH 7.5 yielded an ether extract having only end absorption in the ultraviolet which upon treatment with acid yielded material with an ultraviolet spectrum typical of the Δ³,⁵-diene. If these results can be taken as evidence for a glucoside linkage, it is clear from the present study that exposure to pH 5.0 alone, without enzyme, is an equally efficient means for the hydrolysis of this linkage without dehydration of the steroid. In the accompanying study of the chemistry of these allylic alcohols it has been shown that the 3-methyl ether of 3β,11β-dihydroxy-Δ⁴-androsten-17-one can be cleaved to a mixture of the epimeric alcohols in aqueous solution at pH 5.0 and 38°. Since the methyl ether is predictably more stable than the corresponding glucoside, the ready cleavage of the conjugate by mild acid treatment would be consistent with a glucosiduronate. However, it seems equally certain that a sulfate ester of 3α,11β-dihydroxy-Δ⁴-androsten-17-one would be at least as readily split at pH 5.0. Finally, short of isolation of the conjugate in pure form, it is clear that not even a tentative conclusion about the nature of the conjugate can be drawn on the present evidence. This interesting problem invites further study.

It is noteworthy that the absorption, metabolic transformation, and excretion of end products of a rather large amount of hormone given orally to a myxedema patient were so efficient. In the first 24 hr of study in which 11β-hydroxy-Δ⁴-androstene-3,17-dione was ingested during a 5-hr period, 66% was recovered in urine. The quantity recovered as 3α(+3β?),11β-dihydroxy-Δ⁴-androsten-17-one was 8% of the amount given. This finding is pertinent in view of the diminished

metabolic rate and other evidence that intestinal absorption of many substances is impaired in myxedema.

Acknowledgments

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Synthesis and Reactions of 3,11β-Dihydroxy-Δ^4-androsten-17-one*

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ABSTRACT: 3α,11β-Dihydroxy-Δ^4-androsten-17-one and its 3β-epimer have been synthesized from cortisol by reduction with lithium aluminum hydride followed by periodate oxidation in dioxane. The interconversion of the epimers was studied at various pH values and time periods. The equilibrium mixture of 1:1 could be achieved at pH 4 or 5 without dehydration to the $\Delta^{3,5}$-diene.

The four isomeric 4,5-oxides were prepared and characterized. 3β-Methoxy-11β-hydroxy-Δ^4-androsten-17-one was obtained when cortisol was reduced with sodium borohydride and oxidized with periodate in methanol solution. The interconversion of the 3-methoxy-Δ^4- and 3-hydroxy-Δ^4-steroids has been studied.

In the preceding paper the isolation and characterization of 3α,11β-dihydroxy-Δ^4-androsten-17-one (Ia) and its 3β-epimer IIa as metabolites of 11β-hydroxy-Δ^4-androstene-3,17-dione (III) in man were reported (Fukushima *et al.*, 1966). The present study describes the partial synthesis of these two allylic alcohols and some of their derivatives.

Cortisol (IV) was chosen as the starting material since it has the required 11β-hydroxyl group, the Δ^4-3-keto group which can be readily reduced to the Δ^4-3-hydroxy epimers, and a side chain which can easily be converted to the 17-ketone. Reduction with lithium aluminum hydride yielded the unsaturated pentol which, without isolation, was oxidized with periodate buffered at pH 6.5. The oxidized product contained a mixture of I, II, and 11β-hydroxy-Δ^4-androstene-3,17-dione (III). 3β,11β-Dihydroxy-Δ^4-androsten-17-one (II) was readily separated by Celite chromatography whereas more difficulty was encountered in the purification of the 3α-epimer I. Losses were incurred by the ready dehydration, allylic rearrangement, and apparent oxidation to the ketone of the 3α-hydroxyl group (Ward *et al.*, 1965). Most of the Δ^4-3-keto steroid III was formed because of incomplete reduction of the unsaturated ketone of hydrocortisone under the present condition.

The orientation of the C-3 hydroxyl group of I and II was assigned on the basis of molecular rotatory difference between the Δ^4-3-keto and the 3-hydroxy-Δ^4-steroids and by nuclear magnetic resonance spectrometry. The transformation to the 3β-epimer has been reported to be accompanied by a large negative molecular rotation change, *ca.* -170 for the 3β-OH and -295 for the 3β-acetate (Nes and Kim, 1963; Table I). Therefore, the allylic alcohol with molecular rotatory differences of -250 and -384, respectively, has been assigned as the 3β-epimer II and its acetate IIb (Table I). The nuclear magnetic spectra of IIa and its acetate IIb confirm the β orientation of the C-3 substituent of II. The alcohol IIa in pyridine exhibited a doublet at δ = 5.27 ppm (J = 4 cps) for the vinyl

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