14α-HYDROXYLATION OF TESTOSTERONE AND ANDROST-4-ENE-3,17-DIONE IN RAT LIVER

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The formation of 14α-hydroxylated steroids of various classes has been described in the plants and microorganisms. However, 14α-hydroxylation in mammalian tissues has been reported only by Knuppen et al. [1] and by Loke and Cheng-Yong [2], who isolated and identified 14α-hydroxyoestrone after incubation of oestrone with ox adrenal glands and procine adrenals, respectively.

Our previous investigations [3–5] showed that testosterone and *epi*testosterone incubated with rat liver microsomes and slices yielded a large number of polar metabolites. In one of these studies [3] material was found which had the properties of 3-keto-Δ^4-steroids substituted with a tertiary hydroxyl group. The present paper describes the complete characterization of 14α-hydroxytestosterone and the tentative identification of 14α-hydroxyandrost-4-ene-3,17-dione as products after incubations of testosterone and androst-4-ene-3,17-dione.

In typical experiments, 200 μg of testosterone or androst-4-ene-3,17-dione (dissolved in 0.05 ml propylene glycol) were incubated with about 200 mg of sliced liver tissue from male Wistar rats in 5 ml Ringer-Krebs phosphate buffer with glucose (20 mM), pH 7.4, for 30 min at 37° under oxygen atmosphere. The combined incubations of androst-4-ene-3,17-dione (total amount incubated 5.2 mg) and testosterone (4.8 mg) were extracted with ethyl acetate. The extracts were evaporated to dryness and were subjected to thin-layer chromatography on Kieselgel HF_{254} (E. Merck AG, Darmstadt, Germany) in the system dichloromethane/methanol, 95:5 (v/v). The zones corresponding to authentic 14α-hydroxytestosterone (R_F 0.14) and 14α-hydroxyandrost-4-ene-3,17-dione (R_F 0.52), localized by ultraviolet absorption, were scraped out, eluted with methanol and rechromatographed on Whatman No. 1 paper in the Bush B5 system (benzene/methanol/water, 2:1:1). The spots corresponding to authentic standards (14α-hydroxytestosterone, R_F 0.42; 14α-hydroxyandrost-4-ene-3,17-dione, R_F 0.68) were eluted. Aliquots of these eluates were used for combined gas chromatography-mass spectrometry. The remaining material, pooled from several series of experiments, was acetylated by treatment with acetic anhydridepyridine at room temperature overnight and was then further purified by thin-layer chromatography in the system benzene/ethanol, 85:15 (v/v). The substances giving spots with R_F values of 0.44 and 0.55, respectively (14α-hydroxytestosterone, 0.37; 14α-hydroxytestosterone 17β-acetate, 0.55; 14α-hydroxyandrost-4-ene-3,17-dione, 0.45) were eluted. Both compounds gave ultraviolet absorption spectra which showed a maximum at 242 nm. Oxidation of the acetylated substance by chromic acid in acetone yielded unchanged material, m.p. 251–254° and 197–199°, respectively, with chromatographic and staining properties of 14α-hydroxyandrost-4-ene-3,17-dione and 14α-hydroxytestosterone 17β-acetate.

The results of the gas chromatography-mass spectrometry analyses are shown in table 1 and fig. 1. The compounds were analyzed as trimethylsilyl ethers and *O*-methyloxime trimethylsilyl ether derivatives using the conditions described previously [5]. The mass spectra and retention times of the derivatives of 14α-hydroxytestosterone and the compound isolated from the incubations were identical. The 14α-hydroxy group was not silylated with hexamethyldisil-

Table 1

Relative retention times (cholestane = 1.00) on SE-30 and QF-1 columns of derivatives of 14α-hydroxytestosterone (A^4-14α,17β-ol-3-one), 14α-hydroxyandrost-4-ene-3,17-dione (A^4-14α-ol-3,17-one) and metabolites isolated from the incubations.

Compound	1.5% SE-30		3% QF-1	
	TMS *	MO-TMS *	TMS	MO-TMS
A^4-14α,17β-ol-3-one	1.00	1.03	4.40	1.70
Metabolite	**	1.02	4.40	1.70
A^4-14α-ol-3,17-one	0.93	1.00	**	2.08
Metabolite	0.97 ***	**	**	2.13

* TMS: trimethylsilyl ether; MO-TMS: *O*-methyloxime trimethylsilyl ether.
** No peak obtained.
*** See text.

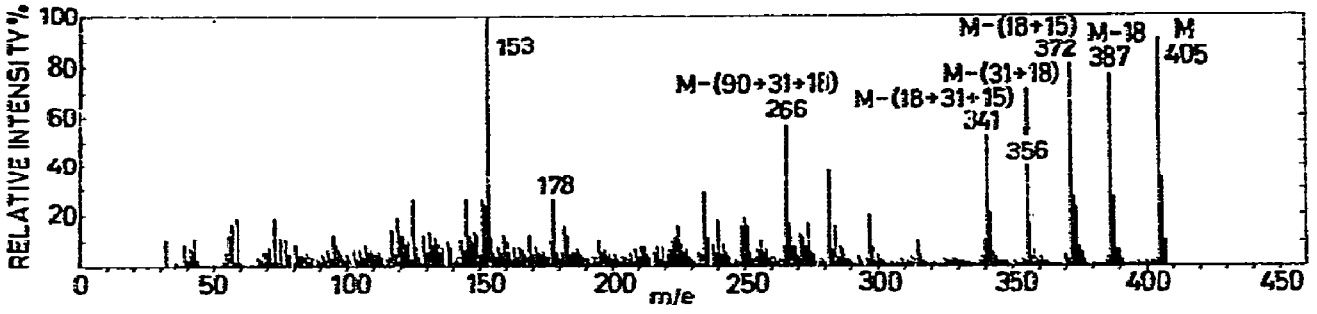

Fig. 1. Mass spectrum of the *O*-methyloxime trimethylsilyl ether derivative of the metabolite isolated from incubations of androst-4-ene-3,17-dione with rat liver slices.

azane/trimethylchlorosilane in pyridine and prominent peaks at m/e 358 (M−18) and 268 (M-(90+18)) were seen in the spectra of the trimethylsilyl ethers. The loss of water is also evident in the spectrum shown in fig. 1. The origin of the ion of mass 153 is not known but it is likely to be analogous to the ion of mass 124 in 3-keto-Δ^4-steroids [6].

Gas chromatography-mass spectrometry did not give unequivocal proof for the presence of 14α-hydroxyandrost-4-ene-3,17-dione. A small peak with the approximate retention time of the reference compound was noted in two analyses (table 1). The mass spectra showed all the major peaks seen in spectra of the reference compound but the intensities of the spectra were too low for a detailed analysis. It is possible that the derivatives of 14α-hydroxyandrost-4-ene-3,17-dione, when present in small amounts, were degraded on the gas chromatography column.

In another experiment, 200 μg of 4-^{14}C-androst-4-ene-3,17-dione (specific activity 1.4 mC/mmol) was incubated with a lyophilized microsomal fraction of male rat liver (corresponding to 1 g of tissue) in tris-maleate buffer, pH 7.4, with an NADPH-generating system added [2]. After the incubation 200 μg each of authentic 14α-hydroxytestosterone and 14α-hydroxyandrost-4-ene-3,17-dione were added and the steroids were extracted and purified as in previous experiments. As an additional step in the purification the acetate was hydrolyzed and the product was chromatographed on paper using Bush's system B5. The specific activities of the steroids were determined after each step in the purification procedure (table 2) The amount of steroid was determined by measurement of absorption at 242 nm. Radioactivity was

Table 2

Specific activities in the purification of 14α-hydroxylated steroids formed after incubation of 1 μC 4-^{14}C-androst-4-ene-3,17-dione with rat liver microsomes. Total radioactivity extracted from the media was 1835000 dpm.

Procedure *	14α-hydroxytestosterone dpm/100 μg	14α-hydroxyandrost-4-ene-3,17-dione dpm/100 μg
Thin-layer chromatography	8320	12650
Paper chromatography	3690	8685
Acetylation, thin-layer chromatography	2670	3630
Hydrolysis, paper chromatography	2480	3540

* See text.

Table 3

Yields of 14α-hydroxylated metabolites after incubation of testosterone and androst-4-ene-3,17-dione (200 μg each) with rat liver slices. Yields in μg/g tissue as determined by ultraviolet absorption. Some contamination with other ultraviolet absorbing material with identical mobility in the solvent systems used cannot be excluded.

Precursor	A^4-14α,17β-ol-3-one	A^4-14α-ol-3,17-one
A^4-17β-ol-3-one	2.4	0.4
A^4-3,17-one	0.6	1.9

measured on aliquots in a Nuclear-Chicago liquid scintillation spectrometer.

The presence of a tertiary hydroxyl group in the metabolites studied was suggested by a) failure of acetylation, b) failure of oxidation. Thin-layer and paper chromatography as well as melting point determinations and ultraviolet spectroscopy indicated that the two isolated metabolites were 14α-hydroxytestosterone and 14α-hydroxyandrost-4-ene-3,17-dione. Gas chromatography-mass spectrometry permitted a complete characterization of one of the metabolites (14α-hydroxytestosterone) while the other (14α-hydroxyandrost-4-ene-3,17-dione) was tentatively identified. The yields of formation of the two metabolites per 1 g of tissue are shown in table 3.

The hydroxylation reaction described in this paper is a further example of the capacity of liver tissue to hydroxylate steroids *in vitro*. The importance of 14α-hydroxylation in rat liver *in vivo* must be determined in order to elucidate the possible physiological meaning of this reaction.

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