SEX-SPECIFIC 15α-HYDROXYLATION OF SULPHO-CONJUGATED 5α-ANDROSTANE-3α, 17β-DIOL IN LIVER MICROSOMES FROM FEMALE RATS

Jan-Åke GUSTAFSSON and Magnus INGELMAN-SUNDBERG

Department of Chemistry and Department of Germfree Research, Karolinska Institutet, S-104 01 Stockholm 60, Sweden

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1. Introduction

An extensive literature exists on microsomal hydroxylation of steroid hormones in rat liver. Without exception, these studies have been carried out with free, unconjugated substrates. Since the main part of steroids excreted in bile – which constitutes the most important excretory route in rats – is sulpho-conjugated it seemed plausible, however, that steroid sulphates are physiological substrates for the steroid-metabolizing microsomal hydroxylase systems. It was therefore of interest to investigate the microsomal metabolism of a sulpho-conjugated steroid. In this laboratory we have focused our attention to the development and regulation of sexual differences in the hepatic microsomal hydroxylation of 5α-androstane-3α, 17β-diol [1, 2]. This paper is a comparative study on the metabolism of free 5α-androstane-3α, 17β-diol and 5α-androstane-3α, 17β-diol 3, 17-disulphate in rat liver microsomes and describes the sex-specific 15α-hydroxylation of the latter substrate in preparations from female rats.

2. Materials and methods

5α-Androstane-3α, 17β-diol was purchased from Sigma Chemical Company, St. Louis, Mo., USA. 4-[4-^{14}C]Androstene-3, 17-dione (specific radioactivity, 60 mCi/mmole) was purchased from the Radiochemical Centre (Amersham, England). Both steroids were purified and assayed for purity by thin-layer chromatography prior to use. 5α-[4-^{14}C]Androstane-3α, 17β-diol was synthesized from 4-[4-^{14}C]androstene-3, 17-dione as described previously [2]. Labelled and unlabelled 5α-androstane-3α, 17β-diol 3, 17-disulphate was synthesized from the free preparations essentially according to Mumma et al. [3]. The reaction products were separated and purified by chromatography on Sephadex LH-20 using the solvent system chloroform/methanol, 1:1 (v/v), 0.01 M with respect to NaCl [4]. The disulphate fraction was passed through a column of Amberlite XAD-2 [5] which was subsequently washed with water whereupon the steroid conjugate was eluted with methanol. Both the labelled and the unlabelled disulphate were analyzed by thin-layer chromatography using the solvent system ethyl acetate/ethanol/ammonium hydroxide, 5:5:1 (by vol), and were found to have identical mobilities to reference steroid disulphates.

Liver microsomes from male and female rats of the Sprague-Dawley strain were prepared as described previously [6]. The following conditions of incubations were chosen as optimum after kinetic studies had shown that formation of products by microsomes was proportional to time as well as to the amount of microsomes. Four different sets of incubations were carried out for each sex and each substrate. 200,000 cpm of 5α-[4-^{14}C]androstane-3α, 17β-diol was diluted with 685 nmoles of unlabelled 5α-androstane-3α, 17β-diol and dissolved in 100 μl of acetone. In the same way 200,000 cpm of 5α-[4-^{14}C]androstane-3α, 17β-diol 3, 17-disulphate was diluted with 685 nmoles of 5α-androstane-3α, 17β-diol 3, 17-disulphate and dissolved in 100 μl of water. The steroid solution was added to a mixture of about 10 mg microsomal protein, 0.03 μmoles of $MnCl_2$, 4 μmoles of NADP, 12.5 μmoles of isocitrate and 10 μl of isocitrate

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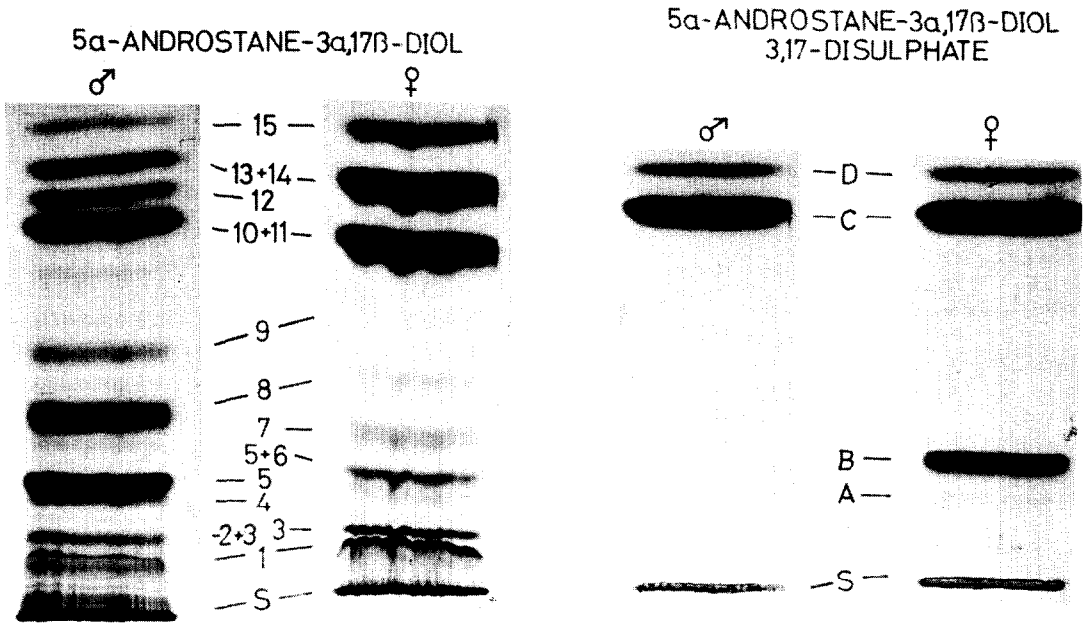

Fig. 1. Thin-layer chromatograms of extracts of incubations of 5α-[4-^{14}C]androstane-3α, 17β-diol and 5α-[4-^{14}C]androstane-3α, 17β-diol 3, 17-disulphate with the microsomal fraction of liver homogenate from male (♂) and female (♀) rats. The solvent system used was chloroform/ethyl acetate, 4:1 (v/v). No steroids were detected in zone S (starting zone). The following steroids were identified in the other zones: 1, 5α-androstane-3α, 7α, 17β-triol; 2, 5α-androstane-3α, 7β, 17β-triol (tentatively identified); 3, 3α, 7α-dihydroxy-5α-androstan-17-one; 4, 5α-androstane-2β, 3α, 17β-triol; 5, 5α-androstane-2α, 3α, 17β-triol; 6, 7α, 17β-dihydroxy-5α-androstan-3-one; 7, 2α, 17β-dihydroxy-5α-androstan-3-one; 8, 5α-androstane-3β, 17β, 18-triol; 9, 5α-androstane-3α, 17β, 18-triol; 10, 5α-androstane-3α, 17β-diol; 11, 5α-androstane-3β, 17β-diol; 12, 3β-hydroxy-5α-androstan-17-one; 13, 3α-hydroxy-5α-androstan-17-one; 14, 17β-hydroxy-5α-androstan-3-one; 15, 5α-androstane-3, 17-dione; A, 5α-androstane-3α, 7β, 17β-triol (tentatively identified); B, 5α-androstane-3α, 15α, 17β-triol; C, 5α-androstane-3α, 17β-diol; D, 3α-hydroxy-5α-androstan-17-one.

dehydrogenase solution in a total volume of 4 ml of Bucher medium [7] (NADP, DL-isocitrate and isocitrate dehydrogenase were purchased from Sigma Chemical Co., St. Louis, Mo., USA). The incubations were carried out for 10 min at 37° and were terminated by the addition of 10 ml of chloroform/methanol, 2:1 (v/v).

Incubations with free 5α-androstane-3α, 17β-diol were worked up in the following way. After filtration of the incubation mixture 0.2 vol of a solution of sodium chloride (0.9%, w/v) were added to the filtrate. The chloroform phase was collected and the solvent was evaporated. The residue was subjected to thin-layer chromatography using the solvent system cyclohexane/ethyl acetate, 4:6 (v/v).

The incubation mixture obtained after incubation of 5α-androstane-3α, 17β-diol 3, 17-disulphate was diluted with 100 ml of chloroform/methanol, 2:1 (v/v), and the suspension was allowed to stand in an ultrasonic water bath for about 20 min. After centrifugation at 2,000 *g* for 30 min the supernatant was decanted and taken to dryness under reduced pressure. The residue was chromatographed on a column of Sephadex LH-20 prepared in and eluted with chloroform/methanol, 1:1 (v/v), 0.01 M with respect to NaCl. The disulphate fraction was evaporated to dryness and the residue was solvolyzed in acidified ethyl acetate. The liberated steroids were subjected to thin-layer chromatography using the solvent system cyclohexane/ethyl acetate, 4:6 (v/v) (cf. above).

The chromatoplates were autoradiographed with an exposure time of eight days. The radioactive zones on the thin-layer chromatographic plates were determined exactly from the X-ray film and were scraped off, eluted with methanol and aliquots were measured for radioactivity in a Packard Liquid Scintillation Spectrometer, Model 2425. The rest of the methanol extracts of the radioactive zones from corresponding incubations were pooled and evaporated to dryness, (trimethyl) silylated and anlayzed

Table 1
Metabolism of 5α-[4-^{14}C]androstane-3α, 17β-diol in liver microsomes from male (♂) and female (♀) rats.

Compound	♂	♀
	(nmoles of metabolites formed/mg protein/min)	
5α-Androstane-3α, 17β, 18-triol+ 5α-Androstane-3β, 17β, 18-triol	1.22 ± 0.30	−*
2α, 17β-Dihydroxy-5α-androstan-3-one+ 5α-Androstane-2α, 3α, 17β-triol	0.93 ± 0.27	0.26 ± 0.10
5α-Androstane-2β, 3α, 17β-triol	0.40 ± 0.15	−*
5α-Androstane-3α, 7β, 17β-triol[1]	0.10 ± 0.01	−*
7α, 17β-Dihydroxy-5α-androstan-3-one+ 3α, 7α-Dihydroxy-5α-androstan-17-one+ 5α-Androstane-3α, 7α, 17β-triol	0.80 ± 0.07	0.99 ± 0.07

[1] Tentatively identified.
* Less than 0.03.

The conversions were calculated from the amounts of radioactivity in the different zones of the thin-layer chromatograms. The values listed are the means ± S.D. Four male and four female rats were used.

by gas chromatography—mass spectrometry employing an LKB 9000 instrument. 1.5% SE-30 and 1% OV-17 were used as stationary phases.

3. Results

Fig. 1 shows the thin-layer chromatograms obtained after incubation of free and sulpho-conjugated 5α-androstane-3α, 17β-diol with liver microsomes from male and female rats. The steroid metabolites formed after incubation of the free substrate have been identified in other studies [8–10] and the quantitations of these compounds are summarized in table 1. Incubation of 5α-androstane-3α, 17β-diol 3, 17-disulphate with the liver microsomal fraction from female rats resulted in the formation of two metabolites. The silyl ether of the predominant steroid gave the mass spectrum shown in fig. 2. This is very similar to that of 5α-androstane-3α, 15α, 17β-triol silyl ether; the t_R (retention time relative to cholestane) of 0.71 on SE-30 excluded a 5α-androstane-3β, 15α, 17β-triol (t_R = 1.04) and a 5α-androstane-3β, 15β, 17β-triol (t_R = 0.93) configuration (cf. [8]). When the isolated metabolite was chromatographed on OV-17 it separated from 5α-androstane-3α, 15β, 17β-triol (t_R = 0.43) but was eluted together with 5α-androstane-3α, 15α, 17β-triol (t_R = 0.46). Based on these considerations the major metabolite formed was identified as 5α-androstane-3α, 15α, 17β-triol. The minor metabolite formed had a t_R of 0.72 and gave a mass spectrum similar to that of the silyl ether of 5α-androstane-3α, 7α, 17β-triol [8] but this configuration (t_R = 0.62) was excluded as was 5α-androstane-3β, 7α, 17β-triol (t_R = 0.67) and 5α-androstane-3β, 7β, 17β-triol (t_R = 0.94). Based on mass spectrometric and gas chromatographic considerations the isolated steroid was tentatively identified as 5α-androstane-3α, 7β, 17β-triol. No hydroxylated metabolites were found after incubation of 5α-androstane-3α, 17β-diol 3,17-disulphate with liver microsomes from male rats.

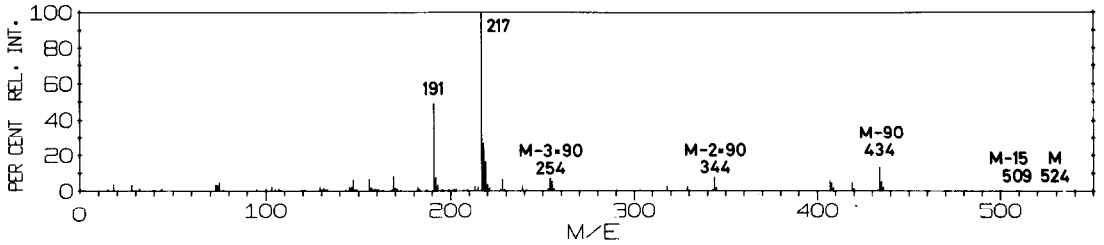

Fig. 2. Mass spectrum of the silyl ether of the major metabolite isolated after incubation of 5α-androstane-3α, 17β-diol 3, 17-disulphate with liver microsomes from female rats. The compound was identified as 5α-androstane-3α, 15α, 17β-triol. Abbreviation: M = molecular ion.

Table 2
Metabolism of 5α-[4-^{14}C]androstane-3α, 17β-diol 3, 17-disulphate in liver microsomes from male (♂) and female (♀) rats.

Compound	♂	♀
	nmoles of metabolites formed/mg protein/min	
5α-Androstane-3α, 15α, 17β-triol	−*	0.52 ± 0.10
5α-Androstane-3α, 7β, 17β-triol[1]	−*	0.05 ± 0.02

[1] Tentatively identified.
* Less than 0.03.

The conversions were calculated from the amounts of radioactivity in the different zones of the thin-layer chromatograms obtained after analysis of the solvolysed incubation extract. The values listed are the means ± S.D. and are expressed in terms of free steroid. Four male and four female rats were used.

4. Discussion

The behaviour of the microsomal metabolites of 5α-androstane-3α, 17β-diol 3, 17-disulphate on Sephadex LH-20 and their cleavage by solvolysis in acidified ethyl acetate indicate that the steroid was metabolized as a disulphate. Less than 1% of the incubated radioactivity was eluted from the Sephadex column in the free steroid and the steroid monosulphate fractions indicating that the microsomal sulphatase activity [11] was of little importance under the experimental conditions used. Since the sulphurylase activity is localized in the 105,000 *g* supernatant fraction [12] it is not probable that any resulphurylation of hydrolyzed metabolites could have taken place.

The present results clearly demonstrate that 5α-androstane-3α, 17β-diol 3, 17-disulphate is metabolized in rat liver microsomes in a totally different way than the free steroid. Male rats were unable to hydroxylate the disulphate in measurable amounts whereas female rats hydroxylated the conjugated substrate mainly in position 15α and to a smaller extent in position 7, probably 7β. With the unconjugated substrate the situation is the reverse: male rats are much more efficient in hydroxylating 5α-androstane-3α, 17β-diol than female rats. Thus, the sexual difference in microsomal hydroxylation becomes reversed when the substrate is changed from the free to the sulphoconjugated steroid. To our knowledge this is the first example of much more efficient hydroxylation in female as compared to male rat liver and the results stimulate further work on the regulation of the 15α-hydroxylase system active on 5α-androstane-3α, 17β-diol 3, 17-disulphate. Interestingly, the 21-sulphate of 3α, 11β, 15α, 21-tetrahydroxy-5α-pregnan-20-one is a specifically female metabolite of corticosterone in rats [13]; male rats excrete practically no hydroxylated corticosterone metabolites [14]. In view of the present findings the explanation of this may be that the 21-sulphate of corticosterone or a reduced metabolite of corticosterone is 15α-hydroxylated by a microsomal hydroxylase system active in female but not in male rat liver.

The present results show that a steroid sulphate may act as substrate for some of the microsomal hydroxylase systems. The physiological importance of this is difficult to evaluate at the present time but the question may be raised whether microsomal hydroxylation of sulphurylated substrates also occurs in drug metabolism.

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