Journal of Steroid Biochemistry, 1977, Vol. 8, pp. 323–328. Pergamon Press. Printed in Great Britain

COMPARATIVE FATE OF TESTOSTERONE AND TESTOSTERONE SULPHATE IN FEMALE RATS: $C_{19}O_2$ AND $C_{19}O_3$ STEROID METABOLITES IN THE BILE

MICHIO MATSUI and YUKO KINUYAMA
Kyoritsu College of Pharmacy, Shibakōen, Minato-ku, Tokyo 105, Japan

(*Received* 4 *October* 1976)

SUMMARY

Comparative fate of [^{14}C]-testosterone and [^{3}H]-testosterone sulphate was investigated in female rats with biliary fistulas. The injected steroids were predominantly excreted in the bile. There were large variations in testosterone metabolites. In the rat with high rate of biliary excretion (the HE rat), androsterone glucosiduronate was the major metabolite present in the bile, whereas monosulphates of 3α,7α- and 3α,11β-dihydroxy-5α-androstan-17-ones and androsterone were the predominant ones in the rat with low rate of biliary excretion (the LE rat). As minor metabolites, monoglucosiduronates of 2α,3α- and 3α,7α-dihydroxy-5α-androstan-17-ones and 5α-androstane-3α,17β-diol, monosulphate of 3α,15α-dihydroxy-5α-androstan-17-one, and disulphates of 5α-androstane-3α,17β-diol, 5α-androstane-3α,7α,17β-triol and 5α-androstane-3α,15β,17β-triol were isolated in both HE and LE rats. In contrast, variations were not so marked in the testosterone sulphate metabolites. 5α-Androstane-3α,17β-diol disulphate and 5α-androstane-3α,7β,17β-triol monosulphate were the major metabolites. In addition, monosulphate of 5α-androstane-3α,17β-diol, and disulphates of 5α-androstane-3α,7β,17β-triol and 5α-androstane-3α,15β,17β-triol were identified. Small amounts of 5α-androstane-3α,17β-diol were found in the sulphoglucosiduronate fraction in both testosterone and testosterone sulphate administered rats.

INTRODUCTION

In a recent publication, we described that disulphates of 5α-androstane-3α,17β-diol and polar hydroxylated steroids constituted the major biliary metabolites of testosterone sulphate in female rats [1]. Gustafsson *et al.* recently reported the 15β-hydroxylation of 5α-androstane-3α,17β-diol 3,17-disulphate in female rat liver microsomes [2]. These results strongly suggest the occurrence of 15β-hydroxylated steroids in the biliary metabolites of testosterone sulphate. In the present paper, [^{14}C]-testosterone and [^{3}H]-testosterone sulphate were administered into female rats and the biliary $C_{19}O_2$ and $C_{19}O_3$ steroid metabolites were identified by gas chromatography–mass spectrometry.

EXPERIMENTAL

Materials. [4-^{14}C]-Testosterone (20.2 mCi/mmol) and [7-^{3}H]-testosterone sulphate (25 Ci/mmol) were purchased from New England Nuclear Corp. The radiochemical purities of these steroids were checked by thin-layer chromatography prior to use. Androsterone, testosterone and 3α,11β-dihydroxy-5α-androstan-17-one were obtained from Sigma Chemical Co. Sodium borohydride reduction of androsterone gave 5α-androstane-3α,17β-diol. Preparation of 5α-androstane-3α,15α,17β-triol and -3α,15β,17β-triol was reported previously [3]. 2α,3α-Dihydroxy-5α-androstan-17-one was prepared by osmium tetroxide oxidation of 5α-androst-2-en-17-one following the procedure of Edwards[4], and separation of the reaction mixture by thin-layer chromatography on silica gel GF (Merck) with solvent system, cyclohexane–ethyl acetate (2:3 v/v) afforded 2α,3α-dihydroxy-5α-androstan-17-one (m.p. 160–163°) and 2β,3β-dihydroxy-5α-androstan-17-one (m.p. 175–181°). Sodium borohydride reduction of these 17-oxo steroids yielded 5α-androstane-2α,3α,17β-triol and -2β,3β,17β-triol, respectively. 5α-Androstane-2β,3α,17β-triol and -2β,3β,17β-triol were prepared as described by Kwok *et al.*[5]. Sodium salt of testosterone sulphate was synthesized according to the procedure described by Holden *et al.*[6].

5α-Androstane-2α,3α,17β-triol. To a solution of 2α,17β-diacetoxy-5α-androstan-3-one (154 mg, m.p. 187–192°)[7] in methanol (10 ml) was added $NaBH_4$ (200 mg) with cooling in ice-water. After 0.5 h, the solution was poured into water, and extracted with ethyl acetate. The organic phase was washed with water, dried and evaporated *in vacuo*. The residue (170 mg) was separated by silica gel GF plate with solvent system, chloroform–acetone (20:1 v/v), and crystallized from acetone to afford 2α,17β-diacetoxy-5α-androstan-3β-ol (R_F 0.38, m.p. 201–208°, 47 mg) and the corresponding 3α-epimer (R_F 0.52, m.p. 230–236°, 30 mg). The configuration of the 3-hydroxy group was established by the nuclear magnetic resonance spectra determined for solutions in deuteriochloroform with tetramethylsilane as internal standard. The 3α-ol gave the H-3 signal at 4.0 ppm as narrow multiplet, while the 3β-epimer showed the signal at 3.8 ppm as broad multiplet [3]. 2α,17β-Diacetoxy-5α-androstan-3α-ol (30 mg) was dissolved in 10% NaOH-methanol (2 ml) and allowed to stand at room

temperature for 2 days. The mixture was extracted with chloroform, washed with water, dried, and evaporated to give a residue, which was crystallized from methanol–ether to afford 5α-androstane-2α,3α,17β-triol (14 mg), m.p. 239–242° (Found: C, 72.8; H, 10.4. $C_{19}H_{32}O_3 \cdot 0.25\ H_2O$ requires C, 72.9; H, 10.5%). Alkaline hydrolysis of 2α,17β-diacetoxy-5α-androstan-3β-ol (33 mg) gave 5α-androstane-2α,3β,17β-triol (15 mg), m.p. 174–178°/195–198° (Found: C, 70.4; H, 10.3. $C_{19}H_{32}O_3 \cdot H_2O$ requires C, 69.9; H, 10.5%).

5α-Androstane-3α,7α,17β-triol. Hydrolysis of 3α,17β-diacetoxy-5α-androstan-7α-ol (20 mg, m.p. 246–248°) [8] in 10% NaOH-methanol (7 ml) afforded 5α-androstane-3α,7α,17β-triol (crystallized from acetone) (8 mg), m.p. 100–103°/175–177° (Found: C, 69.5; H, 10.2. $C_{19}H_{32}O_3 \cdot H_2O$ requires C, 69.9; H, 10.5%).

5α-Androstane-3α,7β,17β-triol. 3α-Hydroxy-5α-androstane-7,17-dione (80 mg, m.p. 214–219°) [8] was dissolved in dry ether (10 ml) and tetrahydrofuran (2 ml). A solution of $LiAlH_4$ (80 mg) in dry ether (5 ml) was added portionwise with cooling in ice-water and allowed to stand at room temperature for 1 h. The excess $LiAlH_4$ was destroyed by addition of 2N H_2SO_4. The mixture was extracted with chloroform, washed with water, aqueous 5% sodium bicarbonate and water, and dried. Evaporation left a residue (80 mg), which was crystallized from acetone four times to give 5α-androstane-3α,7β,17β-triol (5 mg), m.p. 156–158° (Found: C, 73.3; H, 10.6. $C_{19}H_{32}O_3 \cdot 0.25\ H_2O$ requires C, 72.9; H, 10.5%).

Conditions of animal experiments. The common bile duct was cannulated in female rats of the Wistar strain (150–270 g: Matsumoto Experimental Animal Lab., Tokyo, Japan) as described previously [9]. After operation, the rat was kept in a restraining cage with free access to water and food pellets. Ethanol solution of [^{14}C]-testosterone (0.18 ml, 0.80 μCi, 3.82 μmol) or [^{3}H]-testosterone sulphate (0.14 ml, 0.70 μCi, 3.27 μmol) was diluted with 0.17 or 0.26 ml of saline, respectively, and injected intraperitoneally 18–23 h after operation into female rats. Bile was collected at 0–1, 1–2, 2–4, 4–6, 6–24, and 24–48 h.

Extraction and purification of biliary metabolites. Biles obtained at 0–6 and 6–24 h were worked up separately. The bile fraction was extracted with ether. The ether extract was washed with water, dried, and evaporated *in vacuo*. The aqueous fraction was evaporated *in vacuo* to a vol. of 20 ml and then passed through a column packed with Amberlite XAD-2 resin (100 g). The column was washed with 100 ml of water, followed by elution with 400 ml of methanol [10]. The methanol effluent was evaporated *in vacuo* to give the conjugate fraction. The conjugate fraction was dissolved in 3 ml of chloroform–methanol (1:1 v/v), 0.01 M with respect to NaCl, and applied on Sephadex LH-20 (20 g) column [11]. The column was eluted with 200 ml of the same solvent system, followed by 200 ml of methanol and yielded monoglucosiduronate, monosulphate, and diconjugate fractions.

Hydrolysis of conjugate fractions. The hydrolytic procedures have been described previously in detail [9]. The monoglucosiduronate fraction was hydrolyzed by incubation with β-glucuronidase (Ketodase). The monosulphate fraction was solvolyzed in acidified ethyl acetate. The diconjugate fraction was hydrolyzed by solvolysis and by incubation with β-glucuronidase.

Thin-layer chromatography (TLC). The liberated steroids were purified by TLC on plates coated with silica gel GF (Merck), using chloroform–acetone (29:1) as solvent. Radioactive zones were detected with a Packard Model 7201 autoscanner, scraped, and eluted with methanol as previously described [9]. Polar steroid fractions remained near the starting line on the TLC plate were further separated by silica gel GF plate with solvent system cyclohexane-ethyl acetate (2:3 v/v) and afforded polar steroids (probably $C_{19}O_4$, etc.) remained near the origin, 5α-androstane-3α,15α,17β-triol, 3α,7α-dihydroxy-5α-androstan-17-one (or 5α-androstane-3α,7β,17β-triol), 5α-androstane-3α,15β,17β-triol, 2α,3α-dihydroxy-5α-androstan-17-one, 3α,15α-dihydroxy-5α-androstan-17-one, and 3α,11β-dihydroxy-5α-androstan-17-one. In general, TLC plate was developed three or four times in the same solvent system.

Sodium borohydride reduction. A portion of the purified metabolite, which was tentatively identified as 17-oxo-$C_{19}O_3$ steroid, was dissolved in 1 ml of methanol and reduced with 20 mg of $NaBH_4$ under cooling in ice-water. After 1 h, the solution was poured into water, and extracted with chloroform. The extract was washed with water and dried. Evaporation gave androstanetriol, which was submitted to gas chromatographic and gas chromatography–mass spectrometric analyses.

Gas chromatography (GC) and gas chromatography–mass spectrometry (GC–MS). Each purified metabolite was trimethylsilylated as described by Lisboa *et al.* [12] and analyzed by GC and GC–MS. GC was performed on a Shimadzu GC-4BM flame ionization chromatograph using 0.5% CHDMS (2.0 m × 3 mm; column 200°C; detector and flash heater 240°C) and 1.5% SE-30 (1.5 m × 3 mm; column 230°C; detector and flash heater 250°C) as the stationary phase. The column was eluted with nitrogen at a rate of 40 ml/min. Relative retention times were calculated relative to 5α-cholestane. In general, the peaks were quantitated by peak-height measurement, using known amounts of 5α-cholestane as internal standard. GC–MS was carried out on a JEOL JMS-D100 spectrometer equipped with 1.5% SE-30 column (2.0 m × 3 mm; column 260°C; detector and flash heater 270°C). Helium gas flow-rate was about 30 ml/min. The temperatures of the molecular separator and ion source were 200°C. Mass spectra were recorded with a bombarding electron energy of 24 eV and filament current of 300 μA.

Measurement of radioactivity. The radioactivity was counted with an Aloka LSC-502 liquid scintillation

Table 1. Biliary excretion of radioactivity (% dose) following intraperitoneal injection of [^{14}C]-testosterone and [^{3}H]-testosterone sulphate into female rats*

Bile (h)	[^{14}C]-Testosterone		[^{3}H]-Testosterone sulphate	
	HE rat†	LE rat‡	HE rat†	LE rat‡
0–1	30.7 (26.0–33.3)§	13.2 (8.5, 17.8)‖	31.1 (27.6–34.7)§	13.5 (9.7, 17.3)‖
1–2	19.5 (18.2–20.8)	16.8 (11.7, 21.8)	18.8 (17.6–21.2)	16.1 (11.1, 21.1)
2–4	19.1 (17.4–21.4)	18.1 (13.6, 22.5)	14.9 (11.1–18.6)	15.7 (13.6, 17.7)
4–6	7.7 (7.3–8.0)	9.1 (7.5, 10.6)	3.9 (3.0–4.9)	4.4 (5.1, 3.6)
6–24	11.4 (7.0–19.5)	15.7 (18.4, 12.9)	2.4 (2.1–2.7)	3.4 (4.8, 2.0)
Total	88.4 (83.9–94.2)	72.7 (59.7, 85.6)	71.0 (61.5–78.2)	53.0 (44.3, 61.7)

* Dose: [^{14}C]-testosterone (0.80 μCi, 3.82 μmol); [^{3}H]-testosterone sulphate (0.70 μCi, 3.27 μmol).
† Rats with high rate of biliary excretion (n = 3).
‡ Rats with low rate of biliary excretion (n = 2).
§ Mean and range (in round brackets).
‖ Mean and results of two determinations (in round brackets).

spectrometer in a toluene medium as previously described [9]. Efficiencies of ^{14}C and ^{3}H counting were about 80 and 40%, respectively.

RESULTS

Distribution of radioactivity. The biliary excretion of the ^{14}C and ^{3}H following intraperitoneal injection of [^{14}C]-testosterone and [^{3}H]-testosterone sulphate is shown in Table 1. The mean recoveries of the administered ^{14}C and ^{3}H in the bile were about 82 and 64% during the first 24 h, respectively. Less than 1% of the radioactivity was excreted in the 24–48 h bile. There were variations in the biliary excretion rate among [^{14}C]-testosterone or [^{3}H]-testosterone sulphate injected rats. Based on the biliary excretion rate, rats were divided into two groups. One group (the HE rat) eliminated about 31% of the radioactivity during the first h, whereas another group (the LE rat) excreted only 13% of the radioactivity in the first h. The HE rat excreted more radioactivity in the 24 h than the LE rat. Biles obtained at 0–6 h were worked up separately and the 6–24 h biles were combined in each group and analyzed.

Extraction of each bile fraction with ether resulted in the quantitative recovery of the radioactivity in the aqueous fraction, which was then passed through Amberlite XAD-2 column, followed by elution with methanol. The recoveries of the ^{14}C and ^{3}H in the methanol effluent (conjugate fraction) were about 98 and 91%, respectively. The conjugate fraction was chromatographed on Sephadex LH-20 and afforded monoglucosiduronate, monosulphate, and diconjugate fractions (Table 2). There were large variations in the constitution of these conjugates in the [^{14}C]-testosterone administered rats. As for the 0–6 h bile, the monoglucosiduronate was the major conjugate in the HE rat, whereas the monosulphate was mainly excreted in the LE rat. In the 6–24 h bile of the HE rat, smaller amount of the monoglucosiduronate and

Table 2. Distribution of biliary radioactivity (% dose) in various fractions

Bile (h)	Fraction	[^{14}C]-Testosterone		[^{3}H]-Testosterone sulphate	
		HE rat*	LE rat†	HE rat*	LE rat†
0–6	Monoglucosiduronate	49.7 (48.2–51.7)‡	6.3 (4.9, 7.7)§	0	0
		[65.7 (62.7–70.6)]‖	[11.6 (12.1, 11.0)]	[0]	[0]
	Monosulphate	15.1 (8.5–20.0)	33.3 (24.7, 41.8)	17.6 (12.7–22.0)‡	16.8 (13.1, 20.5)§
		[19.8 (11.6–25.5)]	[60.8 (61.6, 59.9)]	[29.7 (19.8–40.7)]	[35.3 (35.7, 34.9)]
	Diconjugate	10.3 (8.6–12.2)	14.6 (10.1, 19.1)	41.8 (30.9–50.4)	30.1 (22.8, 37.3)
		[13.6 (10.9–16.7)]	[26.2 (25.1, 27.3)]	[68.4 (57.1–78.5)]	[62.9 (62.2, 63.6)]
6–24¶	Monoglucosiduronate	4.0	0.4	0	
		[35.4]	[2.5]	[0]	
	Monosulphate	4.8	12.3	0.9	
		[42.5]	[79.1]	[34.4]	
	Diconjugate	2.3	2.4	1.6	
		[20.7]	[15.5]	[59.3]	

* Rats with high rate of biliary excretion (n = 3).
† Rats with low rate of biliary excretion (n = 2).
‡ Mean and range (round brackets).
§ Mean and results of two determinations (round brackets).
‖ Values in square brackets indicate % of the total radioactivity excreted in each bile fraction.
¶ Values are obtained from the pooled bile fractions and indicated as mean values per rat.

increased proportion of the monosulphate were excreted. In contrast, little variation was observed in the [^{3}H]-testosterone sulphate administered rats. Diconjugate fraction constituted the major conjugate present in the bile in both HE and LE rats.

Hydrolysis of conjugates. Hydrolysis of the conjugate fractions of the 0–6 h bile gave the following results. After β-glucuronidase hydrolysis of the monoglucosiduronate fraction from the [^{14}C]-testosterone administered rat, approximately 93 and 66% of the radioactivity were extracted with ether in the HE and LE rats, respectively. By solvolysis of the monosulphate fraction, about 90 and 93% of the ^{14}C were extracted with ethyl acetate in the HE and LE rats, respectively. The corresponding figures for the [^{3}H]-testosterone sulphate administered rat were 90 and 76%, respectively. By solvolysis of the diconjugate fraction, approximately 63% of the ^{14}C and 79% of the ^{3}H appeared in the ethyl acetate extract in both HE and LE rats. These results indicated that diconjugates predominantly consisted of disulphates. Following solvolysis, the resultant aqueous fraction was hydrolyzed with β-glucuronidase. About 63 and 22% of the ^{14}C were extracted with ether in the HE and LE rats, respectively. The corresponding figures for the [^{3}H]-testosterone sulphate administered rat were 28 and 29%, respectively. By direct hydrolysis of the diconjugate fraction with β-glucuronidase, no radioactivity was extracted with ether. From these results, it became apparent that the diconjugate contained the sulphoglucosiduronate as minor conjugate. As for the 6–24 h bile fractions, hydrolytic rates were usually similar to those of the 0–6 h bile of the LE rat.

Identification of steroids. The liberated steroids were separated by TLC and purified metabolites were trimethylsilylated and analyzed by GC and GC–MS (Table 3). The identified metabolites provided relative retention times and mass spectra identical with those of the respective reference steroids. 3α,7α- and 3α,15α-Dihydroxy-5α-androstan-17-ones were not available in this laboratory. The metabolites characterized as such had quite similar relative retention times on SE-30 and mass spectra to these steroids reported by Gustafsson *et al.*[13, 14]. Definitive identification was done by sodium borohydride reduction of these metabolites and by comparison of the resulting androstanetriols with 5α-androstane-3α,7α,17β-triol and -3α,15α,17β-triol as shown in Table 3. 2α,3α-Dihydroxy-5α-androstan-17-one was identified in the monoglucosiduronate fraction of the [^{14}C]-testosterone administered rat, by direct comparison with an authentic sample. The configuration of the 2- and 3-hydroxy groups was unequivocally established by comparison of the sodium borohydride reduction product with four isomeric 5α-androstane-2,3,17β-triols. GC of the trimethylsilyl derivatives on CHDMS (or SE-30) gave relative retention times of 0.49 (0.80), 0.49 (0.80), 0.79 (1.08), 0.41 (0.77), and 0.70 (1.08) for the reduction product, 5α-androstane-2α,3α,17β-triol, -2α,3β,17β-triol, -2β,3α,17β-triol, and -2β,3β,17β-triol, respectively. These trimethylsilyl derivatives afforded very similar mass spectra.

Table 4 gives the percentage conversions of the identified metabolites, which were calculated from the injected dose. In the HE rat, testosterone was metabolized predominantly to androsterone glucosiduronate. In addition to this, small amounts of monoglucosiduronates of 2α,3α- and 3α,7α-dihydroxy-5α-androstan-17-ones and 5α-androstane-3α,17β-diol, monosulphates of 3α,7α-, 3α,11β- and 3α,15α-dihydroxy-5α-

Table 3. Gas chromatographic and mass spectrometric data of trimethylsilyl derivatives of metabolites obtained by hydrolysis of biliary conjugates

Metabolite	Fraction*		GC(RRT)†		GC–MS (*m/e*)‡										
	[^{14}C]-T	[^{3}H]-TS	CHDMS	SE-30	M^+	BP	Other prominent ions								
androsterone	G,S		0.73	0.39	362	272	271	347	215	257	129	155			
5α-androstane-3α,17β-diol	G,D	S,D	0.30	0.49	436	241	256	129	346	215	331	148			
2α,3α-dihydroxy-5α-androstan-17-one	G		1.02	0.64	450	271	435	253	143	129	142	147	182	155	
3α,7α-dihydroxy-5α-androstan-17-one	G,S		0.79	0.57	450	270	360	271	255	213	243	129	121	332	243
3α,11β-dihydroxy-5α-androstan-17-one	S		1.10	0.69	450	156	199	184	186	157	360	394			
3α,15α-dihydroxy-5α-androstan-17-one	S		0.88	0.65	450	143	270	145	216	106	360				
5α-androstane-2α,3α,17β-triol	R		0.49	0.80	524	255	129	345	509	434	344	143	142	419	
5α-androstane-3α,7α,17β-triol	D,R		0.33	0.62	524	393	344	254	255	394	434	239	213		
5α-androstane-3α,7β,17β-triol		S,D	0.46	0.76	524	434	435	184	344	254	129	393	169	239	
5α-androstane-3α,15α,17β-triol	R		0.49	0.71	524	217	191	218	219	169	434	192	254	344	228
5α-androstane-3α,15β,17β-triol	D	D	0.34	0.70	524	217	191	218	219	434	169	192	228	344	254

* [^{14}C]-T = [^{14}C]-testosterone, [^{3}H]-TS = [^{3}H]-testosterone sulphate, G = monoglucosiduronate, S = monosulphate, D = diconjugate, R = metabolite, tentatively assigned as 17-oxo-$C_{19}O_3$ steroid was reduced with $NaBH_4$ and subjected to GC–MS.

† Gas chromatographic conditions are described in 'EXPERIMENTAL'. RRT = relative retention time.

‡ Gas chromatography–mass spectrometric conditions are described in 'EXPERIMENTAL'. M^+ = molecular ion. BP = base peak.

Table 4. Metabolites (% dose) present in the 0–6 h bile following intraperitoneal administration of [^{14}C]-testosterone and [^{3}H]-testosterone sulphate in female rats

Fraction	Metabolite	[^{14}C]-Testosterone		[^{3}H]-Testosterone sulphate	
		HE rat*	LE rat†	HE rat*	LE rat†
Monogluco-siduronate	androsterone	27.6 (25.1–29.0)‡	trace§		
	5α-androstane-3α,17β-diol	trace	trace		
	2α,3α-dihydroxy-5α-androstan-17-one	2.1 (1.9–2.5)	trace		
	3α,7α-dihydroxy-5α-androstan-17-one	4.9 (4.1–6.1)	trace		
Monosul-phate	androsterone	1.8 (1.2–2.6)	4.6 (2.9, 6.3)‖		
	5α-androstane-3α,17β-diol			trace	trace
	3α,7α-dihydroxy-5α-androstan-17-one	4.3 (2.9–5.2)	9.7 (7.5, 11.9)		
	3α,11β-dihydroxy-5α-androstan-17-one	2.9 (1.6–4.0)	6.9 (6.8, 7.0)		
	3α,15α-dihydroxy-5α-androstan-17-one	trace	1.1 (1.2, 1.0)		
	5α-androstane-3α,7β,17β-triol			6.2 (3.1–11.8)	4.0 (3.1, 4.9)
Disulphate	5α-androstane-3α,17β-diol	2.9 (1.0–4.5)	4.0 (2.5, 5.4)	9.5 (7.0–11.7)	5.4 (4.5, 6.4)
	5α-androstane-3α,7α,17β-triol	trace	trace		
	5α-androstane-3α,7β,17β-triol			1.2 (0.8–1.7)	trace
	5α-androstane-3α,15β,17β-triol	trace	trace	1.0 (0.7–1.4)	trace
Sulphoglu-cosiduronate	5α-androstane-3α,17β-diol	1.8¶		1.7¶	

* Rats with high rate of biliary excretion (n = 3).
† Rats with low rate of biliary excretion (n = 2).
‡ Mean and range (in round brackets).
§ Metabolites less than 1% of the injected dose.
‖ Mean and results of two determinations (in round brackets).
¶ Values are obtained from the pooled fractions and indicated as mean values per rat.

androstan-17-ones and androsterone, and disulphates of 5α-androstane-3α,17β-diol, 5α-androstane-3α,7α, 17β-triol and 5α-androstane-3α,15β,17β-triol were identified. In contrast, the major metabolites in the LE rat were monosulphates of 3α,7α- and 3α,11β-dihydroxy-5α-androstan-17-ones and androsterone as shown in Table 4. 5α-Androstane-3α,17β-diol and 5α-androstane-3α,7β,17β-triol were the major metabolites of testosterone sulphate in both HE and LE rats, appearing in the di- and monosulphate fractions, respectively. As minor metabolites, monosulphate of 5α-androstane-3α,17β-diol, and disulphates of 5α-androstane-3α,7β,17β-triol and -3α,15β,17β-triol were identified. Small amounts of 5α-androstane-3α,17β-diol were isolated in the sulphoglucosiduronate fraction in both testosterone and testosterone sulphate administered rats. There were no substantial differences in the metabolites between the 0–6 and 6–24 h bile fractions. Very small amounts (less than 1% of the injected dose) of these metabolites identified in the 0–6 h fraction were also isolated in the 6–24 h bile.

Analysis of the control biles did not afford any identifiable C_{19}-steroids.

DISCUSSION

In a previous paper, we demonstrated that testosterone and testosterone sulphate were extensively metabolized in the rat [1]. The major portion of the metabolites was excreted in the bile. Testosterone was metabolized to androsterone glucosiduronate and polar hydroxylated steroid monoglucosiduronates, monosulphates, and disulphates. As minor metabolites, androsterone and 5α-androstane-3α17β-diol were isolated in the mono- and disulphate fractions, respectively. In contrast, testosterone sulphate was mainly biotransformed into 5α-androstane-3α,17β-diol disulphate and polar hydroxylated steroid mono- and disulphates. These results are in good accord with the present study, though the nature of polar hydroxylated steroids were not elucidated in the previous paper. Despite these studies being performed after a rather severe surgical intervention, the results seem to provide interesting information on the metabolism of testosterone and testosterone sulphate. This paper presents a detailed study on the comparative fate of testosterone and testosterone sulphate in female rats, with particular emphasis on the $C_{19}O_3$ steroid conjugates.

There were large variations in testosterone metabolism in female rats. The major metabolic pathway of testosterone in the HE rat was via formation of androsterone glucosiduronate, while the main pathway in the LE rat was via formation of monosulphates of androsterone and 7α- and 11β-hydroxylated androsterones. In a separate paper, we described quite similar variations in androsterone metabolism in female rats [15]. Siiteri *et al.*[16] demonstrated that androsterone glucosiduronate was rapidly excreted unchanged in urine in humans. Thus, we speculated that UDP-glucuronyltransferase enzyme might be very active in the HE rat. The administered androsterone should be predominantly converted into androsterone glucosiduronate and subsequently excreted in the bile. On the other hand, low activity

of UDP-glucuronyltransferase or high activity of sulphotransferase in the LE rat might permit further metabolism of androsterone or androsterone sulphate. In previous studies, we demonstrated that the catabolic route of testosterone involving the initial conjugation with sulphuric acid or glucuronic acid should be a very minor pathway in female rats [1, 9]. The present study shows that testosterone metabolites were very similar to those of androsterone. In the HE rat, testosterone should be converted by consecutive action of Δ^4-5α-hydrogenase, 3α- and 17β-hydroxysteroid-oxido-reductases to androsterone and subsequently conjugated with glucuronic acid. Thus, variations in testosterone and androsterone metabolism in female rats seem to be regulated by similar biochemical mechanism. 2α,3α-Dihydroxy-5α-androstan-17-one was identified in the monoglucosiduronate fraction in both HE and LE rats. To our knowledge, this is the first identification of this steroid as a monoglucosiduronate in rat bile. Recently, Gustafsson *et al.* described specific 15β-hydroxylating enzyme active on 5α-androstane-3α,17β-diol 3,17-disulphate in female rat liver microsomes [2]. Isolation of disulphates of 5α-androstane-3α,17β-diol and 5α-androstane-3α,15β,17β-triol should imply an *in vivo* 15β-hydroxylation of 5α-androstane-3α,17β-diol 3,17-disulphate in female rats.

In contrast to testosterone, variations in the metabolites of testosterone sulphate were not so marked. The major biliary metabolite was 5α-androstane-3α,17β-diol 3,17-disulphate in both HE and LE rats. Production of 5α-androstane-3α,17β-diol 3,17-disulphate was, however, about two times more in the HE rat than in the LE rat. *In vitro* studies with rat liver enzymes demonstrated that testosterone sulphate could be metabolized without hydrolysis of the ester linkage by Δ^4-5α- and Δ^4-5β-hydrogenases as well as 3α- and 3β-hydroxysteroid-oxido-reductases [17–20]. Thus, testosterone sulphate must undergo direct metabolism by liver microsomal Δ^4-5α-hydrogenase and 3α-hydroxysteroid-oxido-reductase to 5α-androstane-3α,17β-diol 17-sulphate, which must be consecutively conjugated with sulphuric acid to the 3,17-disulphate by the sulphotransferase located in the soluble fraction of the liver cell. Gustafsson *et al.*[2] described the 7β- and 15β-hydroxylation of 5α-androstane-3β,17β-diol 17-sulphate in female rat liver microsomes, whereas 5α-androstane-3β,17β-diol 3,17-disulphate and the corresponding 3α-epimer afforded solely the respective 15β-hydroxylated metabolites. 5α-Androstane-3α,17β-diol 3-sulphate and 17-sulphates of testosterone and 17β-hydroxy-5α-androstan-3-one were not hydroxylated by microsomal enzymes. Based on these observations, the precursor of mono- and disulphates of 5α-androstane-3α,7β,17β-triol, unique metabolites of testosterone sulphate, should be 5α-androstane-3α,17β-diol 17-sulphate. By analogy, 5α-androstane-3α,17β-diol 3,17-disulphate (and 17-sulphate) must be converted into 15β-hydroxylated metabolite. Sole production of 17β-hydroxy steroids indicates little occurrence of hydrolysis of the 17-sulphate group of testosterone sulphate *in vivo*.

From the present study, marked differences in metabolism between testosterone and testosterone sulphate were demonstrated in both $C_{19}O_2$ and $C_{19}O_3$ steroid metabolites. Testosterone was predominantly converted into 17-oxo steroids, whereas testosterone sulphate was metabolized to 17β-hydroxy steroids. An investigation of the levels and activities of various enzymes involved in testosterone metabolism may be of interest for obtaining further insight into the regulatory mechanism responsible for large variations in testosterone metabolites.

Acknowledgement—This work was supported in part by a grant from the Ministry of Education of Japan.

REFERENCES

1. Matsui M., Kinuyama Y. and Hakozaki M.: *Steroids* **25** (1975) 637–648.
2. Gustafsson J. A. and Ingelman-Sundberg M.: *J. biol. Chem.* **249** (1974) 1940–1945; *Ibid.* **250** (1975) 3451–3458.
3. Matsui M. and Kinuyama Y.: *J. Chem. Soc. Perkin 1* (1976) 1429–1432.
4. Edwards J.: *U.S.* 3066138 (1962); *Chem. Abstr.* **60** (1964) 608–609.
5. Kwok R. and Wolff M. F.: *J. org. Chem.* **28** (1963) 423–427.
6. Holden G. W., Levi I. and Bromley R.: *J. Am. chem. Soc.* **71** (1949) 3844.
7. Fürer B., Julia S. and Papantoniou C. P.: *Bull. Soc. chim. Fr.* (1966) 3407–3410.
8. Joska J., Fajkos J. and Sorm F.: *Coll. Czech. chem. Comm.* **26** (1961) 1646–1657.
9. Matsui M., Kinuyama Y. and Hakozaki M.: *Steroids* **24** (1974) 557–573.
10. Matsui M., Hakozaki M. and Kinuyama Y.: *J. Chromatogr.* **115** (1975) 625–628.
11. Laatikainen T. and Vihko R.: *Steroids* **14** (1969) 119–131.
12. Lisboa B. P., Gustafsson J. A. and Sjövall J.: *Eur. J. Biochem.* **4** (1968) 496–505.
13. Gustafsson J. A. and Sjövall J.: *Eur. J. Biochem.* **6** (1968) 227–235.
14. Gustafsson J. A. and Lisboa B. P.: *Eur. J. Biochem.* **16** (1970) 475–480.
15. Matsui M. and Hakozaki M.: *J. steroid Biochem.* **8** (1977) 319–322.
16. Siiteri P. K. and Lieberman S.: *Biochemistry* **2** (1963) 1171–1177.
17. Wu H. L. C. and Mason M.: *Steroids* **5** (1965) 45–56.
18. Matsui M., Abe F., Kunikane M. and Okada M.: *Chem. Pharm. Bull., Tokyo* **21** (1973) 558–564.
19. Matsui M., Kinuyama Y., Hakozaki M., Abe F., Kawase M. and Okada M.: *Chem. Pharm. Bull., Tokyo* **21** (1973) 2764–2768.
20. Ingelman-Sundberg M.: *Biochim. biopys. Acta* **431** (1976) 592–602.