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Metabolism of Testosterone and Its Conjugates by Female Rat Liver

Michio Matsui, Yuko Kinuyama, Misako Hakozaki,[1a] Fukuo Abe, Masako Kawase, and Masashi Okada[1b]

Kyoritsu College of Pharmacy[1a] *and Tokyo Biochemical Research Institute*[1b]

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An interesting aspect in the study of steroid conjugates is the further metabolism of these compounds without prior hydrolysis.[2] In the preceding papers,[3,4] we compared the metabolic transformation of testosterone,[5] testosterone 17-glucosiduronate (TGA), testosterone 17-N-acetylglucosaminide (TNAG) and testosterone 17-sulfate (TS) by incubation with cell-free preparations of male rat liver, and demonstrated metabolic differences among these compounds. Thus, TGA and TNAG were not good substrates for microsomal Δ^4-5α-hydrogenase as well as for hydroxylases, while they were better substrates for soluble Δ^4-5β-hydrogenase than testosterone. In contrast, TS was metabolized more readily by microsomal Δ^4-5α-hydrogenase than by soluble Δ^4-5β-hydrogenase, but not a good substrate for microsomal hydroxylases. Testosterone was extensively metabolized by the same enzymes.

On the basis of enzymic studies, the sex difference in Δ^4-5α- and Δ^4-5β-hydrogenase activities of rat liver was well proved by using free steroids as substrates.[6] In both sexes, the Δ^4-5α-hydrogenase is located mostly in the microsomal fraction and the Δ^4-5β-hydrogenase is present in the soluble fraction. In female rat liver, the Δ^4-5β-hydrogenase activity is much lower than that of the Δ^4-5α-hydrogenase, thus 3-oxo-Δ^4-steroids being mostly metabolized to 5α-steroids. Quite recently, Schriefers, *et al.*[7] observed sex-specific aglycone patterns by incubating testosterone with rat liver slices, followed by examination of the glucosiduronate fraction. Both 5α- and 5β-steroid glucosiduronates were produced in the male, while only 5α-steroid glucosiduronates were present in the female. In the present paper, the metabolism of testosterone, TGA and TS was studied by incubation with cell fractions from female rat liver, and their metabolic patterns were compared with those of male rat liver.[3,4]

Material and Method

Steroids and Reagents——4-^{14}C-Testosterone(^{14}C-T) (58.8 mCi/mmole), 1,2-^{3}H-testosterone 17-glucosiduronate (^{3}H-TGA) (25 Ci/mmole) and 7-^{3}H-testosterone 17-sulfate (ammonium salt) (^{3}H-TS) (25 Ci/mmole) were purchased from New England Nuclear, Boston. The radiochemical purities of these labeled steroids were confirmed by thin-layer chromatography (TLC) or paper electrophoresis[3] shortly before use. The reference steroids and reagents used in the present work were described in the previous papers.[3,4]

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1) Location: *a) Shibakoen-1-Chome, Minato-ku, Tokyo, 105, Japan*; *b) Takada-3-Chome, Toshima-ku, Tokyo 171, Japan.*
2) S. Bernstein and S. Solomon, (ed.), "Chemical and Biological Aspects of Steroid Conjugation," Springer-Verlag, New York, 1970, and literatures cited therein.
3) M. Matsui, F. Abe, K. Kimura, and M. Okada, *Chem. Pharm. Bull.* (Tokyo), **20**, 1913 (1972).
4) M. Matsui, F. Abe, M. Kunikane, and M. Okada, *Chem. Pharm. Bull.* (Tokyo), **21**, 558 (1973).
5) Following trivial names are used: testosterone, 17β-hydroxyandrost-4-en-3-one; androsterone, 3α-hydroxy-5α-androstan-3-one; testosterone 17-glucosiduronate, 3-oxoandrost-4-en-17β-yl-β-D-glucopyranosiduronate; testosterone 17-N-acetylglucosaminide, 3-oxoandrost-4-en-17β-yl-2′-acetamido-2′-deoxy-β-D-glucopyranoside; testosterone 17-sulfate, 3-oxoandrost-4-en-17β-yl-sulfate.
6) R.I. Dorfman and F. Ungar, "Metabolism of Steroid Hormones," Academic Press, New York, 1965, and literatures cited therein.
7) H. Schriefers, R. Ghraf, and E.R. Lax, *Z. Physiol. Chem.*, **353**, 371 (1972).

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Tissue Preparation——After female rats of the Wistar strain weighing 250 to 310 g were decapitated, the liver was homogenized in ice cold 0.25 M sucrose solution and 105000×*g* supernatant fluid and microsomal fraction were prepared as reported earlier,[3] whose protein concentrations were about 17 mg/ml and 1.0 mg/ml respectively, as determined by the procedure of Lowry, *et al.*[8]

Incubation——The incubation was carried out for 5 min at 37° in Tris buffer, pH 7.2, fortified with NADPH regenerating system under carbon monoxide atmosphere or in air as described previously.[3] Ethanol solution (45—64 μl) of ^{14}C-T (100000 dpm), ^{3}H-TGA (200000 dpm) or ^{3}H-TS (200000 dpm) was used as substrate. The incubation was terminated by addition of methylene dichloride, followed by vigorous shaking. The incubation experiment was usually repeated three times using different tissue preparation each time.

Extraction and Isolation of the Metabolites——The incubation medium was extracted with methanol and evaporated *in vacuo* to give a residue, which was dissolved in water and extracted with ether. Recovery of the radioactivity from the incubation medium was 90—100%. Only 1—5% of the radioactivity was extracted with ether and most of the radioactivity remained in the aqueous fraction in the incubations of ^{3}H-TS and ^{3}H-TGA. While in the incubations of ^{14}C-T with 105000×*g* supernatant fluid (in air) and microsomal fraction under carbon monoxide or air 99, 98 or 87% of the radioactivity were extracted with ether respectively. The ether extract was dried over Na_2SO_4 and evaporated *in vacuo* to afford the free steroid fraction. The aqueous fraction was evaporated under reduced pressure just to eliminate ether, and passed through a XAD-2 (100 g) column. The column was washed with 400 ml of water and eluted with 400 ml of methanol as described by Bradlow.[9] The methanol effluent was evaporated *in vacuo* to give the conjugate (or polar) fraction. Paper electrophoresis of this fraction was performed with solvent system pyridine–acetic acid, pH 6.4, as described earlier,[3] confirming that TGA and TS were not hydrolyzed during the incubation with the liver preparations. The conjugate fraction obtained from the incubation with ^{3}H-TGA or ^{3}H-TS was hydrolyzed with β-glucuronidase (Ketodase, Warner-Chilcott) in acetate buffer, pH 5.2, or by solvolysis with acidic ethyl acetate[10] respectively, as reported previously.[3,4] The liberated free steroids were extracted with ether or ethyl acetate. Usually, 95—99% of the radioactivity appeared in the organic phase. The free steroid fraction thus obtained was chromatographed on a thin-layer plate coated with Silica gel GF (Merck), using chloroform–acetone (29: 1) as solvent.[3] The plate was developed three times in the same solvent system. The reference steroids were applied at the edge of the plate. Radioactive spots which were detected with Aloka or Packard Model 7201 autoscanner, were then scraped and eluted with methanol. 5α-Androstane-3α,17β-diol and its 3β-epimer fraction was further separated by TLC on Aluminum oxide G (Merck) with solvent system cyclohexane–chloroform–ethyl acetate (2: 2: 1). Androsterone and 3β-hydroxy-5α-androstan-17-one fraction was purified by silica gel plate with benzene–ethanol (40: 1) as solvent. The recovery of the radioactivity from the plate was usually 90—95%. A portion of the purified metabolite which was tentatively characterized by co-chromatography with reference steroids on TLC, was oxidized with Jone's reagent[11] followed by chromatography on a silica gel plate with chloroform as solvent. These procedures were employed as preliminary characterization.[3] The percentage conversion indicated in the "Result and Discussion" was calculated from the radioactivity of each purified metabolite by TLC, which was corrected from recrystallization data.

Recrystallization——The identity and purity of the isolated metabolite were confirmed by adding 15 to 25 mg of an authentic steroid and recrystallizing the mixture from the appropriate solvent to constant specific activity. The following solvent systems were used for recrystallization: A, methanol; B, acetone-*n*-hexane; C, ethyl acetate–*n*-hexane; D, chloroform–petroleum ether; E, benzene–petroleum ether; F, ether–petroleum ether; G, ethyl acetate–cyclohexane; H, ethanol–*n*-hexane; I, ethanol–petroleum ether.

Measurement of Radioactivity——The radioactivity was measured in Nuclear–Chicago Mark I or Aloka LSC-502 liquid scintillation spectrometer, as described previously.[3] All the radioactivities are expressed in dpm.

Result and Discussion

It is apparent from the present study that TGA and TS undergo substantial metabolic transformation as conjugate by the female rat liver preparations as does testosterone.

The incubation of testosterone, TGA and TS with 105000×*g* supernatant fluid in air showed that they were good substrates for Δ^4-hydrogenases and 3α-hydroxysteroid oxidoreductase (Table I). Testosterone was metabolized to hydroxylated polar steroids (5%),[12]

8) O.H. Lowry, N.J. Rosebrough, A.L. Farr, and R.J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
9) H.L. Bradlow, *Steroids*, **11**, 265 (1968).
10) R. Vihko, *Acta Endocrinol., Suppl.*, **No. 109**, (1966).
11) C. Djerassi, R.R. Engle, and A. Bowers, *J. Org. Chem.*, **21**, 1547 (1956).
12) The fraction remained near the starting line on TLC plate was named as such. It is reasonably presumed that this fraction is mainly composed of hydroxylated testosterone metabolites, although their identification was not tried.

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5β-androstane-$3\alpha,17\beta$-diol (15%) and 5α-androstane-$3\alpha,17\beta$-diol (28%), 42% of testosterone being recovered unchanged. TS gave the similar type of metabolic pattern to testosterone after solvolysis: hydroxylated polar steroids (4%), 5β-androstane-$3\alpha,17\beta$-diol (14%), 5α-androstane-$3\alpha,17\beta$-diol (41%). The increased formation of the 5α-steroid resulted in the reduced recovery of unchanged testosterone (16%), in comparison with the incubation of testosterone. In contrast, TGA produced 5β-androstane-$3\alpha,17\beta$-diol (78%) in high yield and hydroxylated polar steroids (7%) after hydrolysis with β-glucuronidase. Formation of 5α-steroid was not observed when testosterone and TS were incubated with the $105000\times g$ supernatant fluid of male rat liver in the same condition.[3,4] Presence of Δ^4-5α-hydrogenase as well as Δ^4-5β-hydrogenase in the $105000\times g$ supernatant fluid of female rat liver was reported by Forchielli, *et al.*,[13] using androst-4-ene-3,17-dione as substrate. However, the predominant production of 5β-steroid with TGA in the incubation was quite consistent with the observation on male rat liver.[3] These findings indicate that TGA is quite readily metabolized by the Δ^4-5β-hydrogenase. Enzymic mechanism for the stereospecific hydrogenation of the Δ^4-double bond of TGA is not clear. It could be explained analogously according to Gerhards, *et al.*[14] that the bulky and polar sugar group attached at 17β-position of testosterone makes the α-side of the steroidal ring A approach preferentially to the surface of the 5β-enzyme, followed by the transfer of the hydride ion from coenzyme NADPH to the β-side of the Δ^4-double bond, though there is no definite evidence that the same 5β-enzyme can reduce both testosterone and TGA.

Table I. Identification by Recrystallization of Radioactive Metabolites Obtained by Incubation with $105000\times g$ Supernatant Fluid in Air

Steroid incubated	Metabolite	Recrystallization 1st Solvent[a]	1st Specific activity (dpm/mg)	2nd Solvent	2nd Specific activity (dpm/mg)	3rd Solvent	3rd Specific activity (dpm/mg)	Percentage conversion[b] (%)
^{14}C-T[c]	hydroxylated polar steroids							5± 2
	5β-androstane-$3\alpha,17\beta$-diol	A	656	A	598	A	649	15± 6
	5α-androstane-$3\alpha,17\beta$-diol	B	2060	C	2060	D	2100	28± 9
	testosterone	B	1070	C	1110	E	1100	42± 7
^{3}H-TGA[d]	hydroxylated polar steroids							7± 1
	5β-androstane-$3\alpha,17\beta$-diol	A	2060	A	1980	A	1990	78± 2
^{3}H-TS[e]	hydroxylated polar steroids							4± 1
	5β-androstane-$3\alpha,17\beta$-diol	A	1540	A	1460	A	1400	14± 5
	5α-androstane-$3\alpha,17\beta$-diol	B	2180	C	2180	D	2230	41+11
	testosterone	E	1900	B	1610	C	1670	16± 5

a) *cf.* "Material and Method" in the text
b) mean and standard error of three incubations
c) 4-^{14}C-testosterone
d) 1,2-^{3}H-testosterone 17-glucosiduronate
e) 7-^{3}H-testosterone 17-sulfate

The incubation of testosterone, TGA and TS with microsomal fraction in air revealed that they were metabolized by Δ^4-5α-hydrogenase, 3α-hydroxysteroid oxidoreductase as well as hydroxylase (Table II). Testosterone was transformed in 88% yield to hydroxylated polar steroids, as was observed with male rat liver.[3] TGA and TS were metabolized as conjugate and their metabolites were determined after hydrolysis. Thus, TS was converted to hydroxylated polar steroids (47%), 17β-hydroxy-5α-androstan-3-one (16%) and 5α-androstane-$3\alpha,17\beta$-diol (12%). Only 3% of testosterone was recovered unchanged. While, TGA gave

13) E. Forchielli, S. Ramachandran, and H.J. Ringold, *Steroids*, **1**, 157 (1963).
14) E. Gerhards, G. Raspé, and R. Wiechert, *Arzneim. Forsch.*, **17**, 431 (1967).

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Table II. Identification by Recrystallization of Radioactive Metabolites Obtained by Incubation with Microsomal Fraction in Air[a]

Steroid incubated	Metabolite	Recrystallization 1st Solvent	1st Specific activity (dpm/mg)	2nd Solvent	2nd Specific activity (dpm/mg)	3rd Solvent	3rd Specific activity (dpm/mg)	Percentage conversion (%)
^{14}C-T	hydroxylated polar steroids							88±5
^{3}H-TGA	hydroxylated polar steroids							7±1
	5α-androstane-3α,17β-diol	B	693	C	667	D	668	17±3
	testosterone	B	2510	C	2470	E	2510	57±3
^{3}H-TS	hydroxylated polar steroids							47±5
	5α-androstane-3α,17β-diol	B	345	C	343	D	353	12±1
	testosterone	B	537	C	349	E	368	3±1
	17β-hydroxy-5α-androstan-3-one	G	1820	H	1780	I	1710	16±5

a) See the legends of Table I.

hydroxylated polar steroids (7%) and 5α-androstane-3α,17β-diol (17%), 57% of testosterone being recovered unchanged in this case. Furthermore, most of the metabolites of testosterone, TGA and TS were compared after incubation with microsomal fraction under carbon monoxide atmosphere whereby hydroxylase activities were suppressed (Table III). Testosterone produced hydroxylated polar steroids (16%), 5α-androstane-3α,17β-diol (38%) and androsterone (27%). TS was transformed to hydroxylated polar steroids (22%), 5α-androstane-3α,17β-diol (40%) and 17β-hydroxy-5α-androstan-3-one (13%), only a small amount (3%) of testosterone being recovered. On the other hand, TGA gave essentially a similar result to that of the incubation in air: hydroxylated polar steroids (4%), 5α-androstane-3α,17β-diol (11%), and testosterone (68%).

Production of a large quantity (47%) of hydroxylated polar steroids from TS in the incubation under air is in marked contrast to that obtained with male rat liver,[4] whereby

Table III. Identification by Recrystallization of Radioactive Metabolites Obtained by Incubation with Microsomal Fraction under Carbon Monoxide Atmosphere[a]

Steroid incubated	Metabolite	Recrystallization 1st Solvent	1st Specific activity (dpm/mg)	2nd Solvent	2nd Specific activity (dpm/mg)	3rd Solvent	3rd Specific activity (dpm/mg)	Percentage conversion (%)
^{14}C-T	hydroxylated polar steroids							16± 7
	5α-androstane-3α,17β-diol	B	1550	C	1520	D	1580	38±10
	androsterone	B	831	F	886	C	884	27± 7
^{3}H-TGA	hydroxylated polar steroids							4± 1
	5α-androstane-3α,17β-diol	B	315	C	316	D	316	11± 4
	testosterone	B	1020	C	1020	E	1020	68± 5
^{3}H-TS	hydroxylated polar steroids							22± 8
	5α-androstane-3α,17β-diol	B	1770	C	1810	D	1750	40± 5
	testosterone	B	347	C	325	E	320	3± 1
	17β-hydroxy-5α-androstan-3-one	G	1470	H	1540	I	1520	13± 7

a) See the legends of Table I.

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only 6% of TS was converted into hydroxylated polar steroids. Jacobson, *et al.*[15] had reported that testosterone was more readily hydroxylated by the microsomal fraction of male rat liver than that of the female. Possible interpretation of these striking discrepancies observed between testosterone and TS in both sexes is not available at present. In view of the fact that some steroid sulfates can serve as an active intermediate in the metabolism,[2] these results may be of any physiological significance of TS or related steroid sulfates in the female rat, but await further elucidation.

Formation of 17β-hydroxy-5α-androstan-3-one (dihydrotestosterone), a potent androgen, was demonstrated only when TS was incubated with the microsomal fraction of female rat liver. The catabolic sequence of 3-oxo-Δ^4-steroids is well established to proceed mainly *via* 4,5-dihydro-3-oxosteroids to saturated 3-hydroxysteroids by the consecutive action of Δ^4-hydrogenases and 3-oxosteroid oxidoreductases.[5] Thus, in most cases, 3-oxo-5α-steroids were preferentially reduced to 3-hydroxy-5α-steroids in our incubation condition.

The sex difference in the Δ^4-5α-hydrogenase activity was clearly demonstrated further by the fact that the 5α-enzyme of female rat liver produced 5α-steroid from TGA though in a small amount, whereas the 5α-enzyme from male rat liver did not yield any 5α-metabolite.[3] Finally, metabolism of testosterone also indicated sex-specific patterns of microsomal 3-oxosteroid oxidoreductase activities, since testosterone was transformed to 3α-hydroxy-5α-steroid by the female, while it was converted to 3β-hydroxy-5α-steroid by the male.[3] These results are in good agreement with the perfusion experiment of testosterone with the rat liver.[16]

15) M. Jacobson and R. Kuntzman, *Steroids*, **13**, 327 (1969).
16) H. Schriefers, W. Cremer, and M. Otto, *Z. Physiol. Chem.*, **348**, 183 (1967).

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Changes in Catecholamine Levels of Mouse Brain during Oscillation-stress

CHI-HO LEE, AKIRA MORITA, HIROSHI SAITO, and KEIJIRO TAKAGI

Department of Chemical Pharmacology, Faculty of Pharmaceutical Sciences, University of Tokyo[1]

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It has been demonstrated that increased sympathetic activity associated with stressful conditions increases synthesis of norepinephrine (NE) and epinephrine (Epi) in various tissues.[2,3] These conditions include: intense muscular exercise,[3] immobilization, [4,5] revolving drum,[6] changes in environmental temperature[2,7,8] and chronic electroshock sessions.[9,10]

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4) H. Corrodi, K. Fuxe, and T. Hokfelt, *Life Sci.*, **7**, 107 (1968).
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