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In Vitro Metabolism of Testosterone Sulfate and Testosterone Tetrahydropyranyl Ether in Male Rat Liver

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In vitro metabolism of ^{3}H-testosterone 17-sulfate (^{3}H-TS) and ^{14}C-testosterone 17-tetrahydropyranyl ether (^{14}C-TP) was studied by incubation with $20000 \times g$ or $105000 \times g$ supernatant and microsomal fractions of male rat liver homogenate under carbon monoxide atmosphere or in air. The results demonstrated that the steroidal ring A of ^{3}H-TS was readily metabolized by microsomal Δ^4-5α-hydrogenase, while ^{14}C-TP was a good substrate for Δ^4-5β-hydrogenase present in the $105000 \times g$ supernatant fraction as well as microsomal Δ^4-5α-hydrogenase. On the other hand, ^{3}H-TS and ^{14}C-TP were not good substrates for microsomal hydroxylases. The influence of the substituent at C-17 of testosterone upon the biotransformation of ring A was discussed.

There is abundant evidence that steroid conjugates can undergo direct metabolic transformations without prior hydrolysis.[2] In the previous paper,[3] *in vitro* metabolism of testosterone, testosterone 17-glucosiduronate (TGA), and testosterone 17-N-acetylglucosaminide (TNAG) has been investigated by incubation with $20000 \times g$ or $105000 \times g$ supernatant fluids and microsomal fraction of male rat liver homogenate under carbon monoxide atmosphere or in air. It has been demonstrated in these experiments that ring A of TGA and TNAG is metabolized with sugars attached and that a glycoside group at C-17 affects the stereochemical course of reduction of the ring A double bond. In contrast to testosterone, the conjugates 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. The predominant formation of 5β-metabolites from the testosterone conjugates is consistent with the observations on their *in vivo* metabolism in man.[4,5] Since Δ^4-5α-hydrogenase is located in the microsomal fraction while Δ^4-5β-hydrogenase is present in the supernatant fraction,[6] the metabolic differences between testosterone and its conjugates might be due to the enzyme specificity of Δ^4-hydrogenases or due to the selective accessibility of the substrates to the enzymes. The present study is designed to obtain further information concerning the influence of the substituent at C-17 of testosterone upon the biochemical hydrogenation of ring A.

Thus, testosterone 17-sulfate (TS) and testosterone 17-tetrahydropyranyl ether (TP) were chosen as a polar (or water soluble) type derivative and a non-polar type derivative

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with an analogous bulky substituent at C-17 to TGA or TNAG, respectively. Conversion of TS into 3-keto-5α-androstane-17β-yl sulfate by incubation with lyophilized microsomal fraction of female rat liver was reported by Wu, *et al.*[7] Their experimental conditions were quite different from ours. In the present work, labeled steroids were incubated with freshly prepared cell fractions of male rat liver.

Material and Method

Steroids and Reagents——4-^{14}C-Testosterone (^{14}C-T) (58.8 mCi/mmole), and 7-^{3}H-testosterone 17-sulfate (ammonium salt) (^{3}H-TS) (25 Ci/mmole) were purchased from New England Nuclear, Boston. 4-^{14}C-Testosterone-17-(2′-tetrahydropyranyl)ether (^{14}C-TP) was prepared by the procedure described for the unlabeled one as follows.[8] To a solution of ^{14}C-T(7.59×10^{6} dpm) and dihydropyran (0.10 ml) in ether (5.0 ml) was added an ether solution of *p*-toluenesulfonic acid monohydrate (1%, 0.05 ml) and the mixture was stood at 25° for 48 hr. The ether solution was treated in the same way as for the unlabeled compound. The product obtained was purified by thin-layer chromatography (TLC) using silica gel GF (Merck) with solvent system benzene–ethanol (10: 1). The TLC plate was developed twice with the same solvent. The zone corresponding to TP, which was less polar than testosterone, was scraped and eluted with AcOEt–MeOH (7: 3), and the solvent was evaporated *in vacuo* to dryness. The residue was dissolved in 99% EtOH (3.0 ml). The product contained radioactivity of 6.09×10^{6} dpm. Purity of ^{14}C-TP was confirmed by adding 25.8 mg of TP[8] (mixture of C-1′ anomers) to ^{14}C-TP (8.12×10^{4} dpm) and recrystallizing the mixture from isopropyl ether to constant specific activity: 2930 dpm/mg, calculated: 3150 dpm/mg (93% purity). Solvolysis of ^{14}C-TP followed by TLC showed 97% of the radioactivity in testosterone fraction. All the radiochemical purities of these labeled steroids were confirmed by TLC or paper electrophoresis[3] shortly before use. The reference steroids used in this investigation were described in the previous paper.[3] Sodium salt of TS was prepared according to the procedure described by Holden, *et al.*[9] Glucose -6-phosphate,glucose-6-phosphate dehydrogenase (grade I), and NADP were purchased from Boehringer Co., Mannheim. All other chemicals were of reagent grade.

Tissue Preparations——Male rats of Wistar strain, weighing 200 to 420 g were decapitated and 20000 $\times g$ supernatant fluid, 105000$\times g$ supernatant fluid, and microsomal fraction were prepared as reported earlier.[3]

Incubation——The incubation was carried out for 5 min at 37° under CO atmosphere or in air as described previously.[3] Ethanol solution (43—49 μl) of ^{14}C-T (100000 dpm), ^{14}C-TP (100000 dpm), or ^{3}H-TS (200000 dpm) was used as substrate.

Extraction and Isolation of Metabolites ——This was performed by the procedure described previously[3] to obtain the free steroid fraction and the conjugate (or polar) fraction.

Solvolysis——A) Sulfates: The conjugate fraction obtained from the incubation with ^{3}H-TS was evaporated *in vacuo* to dryness and the residue was treated with acidic ethyl acetate (50 ml) at 37° for 16 hr according to the procedure of Vihko.[10] The ethyl acetate phase was washed with 5% $NaHCO_3$ and water, and dried over anhyd. Na_2SO_4. Evaporation of the solvent *in vacuo* gave the hydrolyzed steroid fraction which was stored as ethanol solution.

B) Tetrahydropyranyl Ethers: The ether extract or the free steroid fraction obtained from the incubation with ^{14}C-TP was evaporated under reduced pressure to dryness and the residue was hydrolyzed as described by Ott, *et al.*[8] Thus, to the residue were added 95% EtOH (7.0 ml), water (0.40 ml), and conc. HCl (0.10 ml), and the mixture was refluxed for 1.5 hr. The resultant solution was evaporated *in vacuo* to about 3 ml, poured into water, and extracted with ether. The ether extract was washed with 5% $NaHCO_3$ and water, and dried over anhyd. Na_2SO_4. Evaporation of the solvent under reduced pressure gave the hydrolyzed steroid fraction, which was stored as ethanol solution.

Thin-Layer Chromatography (TLC)——TLC was performed as reported earlier.[3]

Paper Electrophoresis——Paper electrophoresis of the conjugate fraction was done according to the procedure described in the previous paper.[3] Testosterone, TGA, and TS were used as references. TS migrated closer toward the cathode than TGA.

Preliminary Characterization——Chromium trioxide oxidation and Girard's Reagent T separation were carried out as described previously.[3]

Recrystallization——The identity and purity of the isolated metabolites were confirmed by adding 14 to 20 mg of the authentic steroids and recrystallizing the mixtures from appropriate solvents to constant

7) H.L.C. Wu and M. Mason, *Steroids*, **5**, 45 (1965).
8) A.C. Ott, M.F. Murray, and R.L. Pederson, *J. Am. Chem. Soc.*, **74**, 1239 (1952).
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specific activity. The following solvent systems were used for recrystallization: A, methanol; B, acetone–hexane; C, ethyl acetate–hexane; D, chloroform–petroleum ether; E, chloroform–ether; F, benzene–petroleum ether.

Measurement of Radioactivity——The radioactivity was measured as described earlier.[3]

Result

Incubation with 20000 × *g* Supernatant Fraction under Carbon Monoxide Atmosphere

Hydroxylase activities present in the supernatant fraction were suppressed by treatment with CO current just before addition of the substrates. ^{14}C-T was also incubated in order to ascertain that the incubation did proceed as reported previously.[3]

A) **^{3}H-Testosterone Sulfate**——Recovery of the radioactivity from the incubation medium was 97%, 1% of which appeared in the ether extract and 99% remained in the aqueous fraction. Examination of the latter by paper electrophoresis showed that a single radioactive peak moved very close to TS. After solvolysis, 93% of the radioactivity of the conjugate fraction appeared in the ethyl acetate fraction. These results indicated that the sulfate was not hydrolyzed during the incubation with the supernatant fraction. The ethyl acetate extract was separated by TLC on silica gel GF with solvent system $CHCl_3$–acetone (29:1) to give a fraction containing hydroxylated polar steroids (HPS-fraction)[11] (4%), 5β-androstane-3α, 17β-diol (2%), and a mixture of 5α-androstane-3α, 17β-diol and its 3β-epimer (91%). The mixture was further separated by TLC on aluminum oxide G(Merck) with solvent system cyclohexane–$CHCl_3$–AcOEt (2:2:1) to afford 5α-androstane-3α, 17β-diol (58%) and 5α-androstane-3β, 17β-diol (37%). Preliminary characterization of the main metabolites supported the above assignment. The identification of each metabolite was finally made by recrystallization with the authentic steroid to constant specific activity, as shown in Table I. Percentage conversion (%) indicated in Table I was calculated from the radioactivity of each purified metabolite by TLC, which was corrected from recrystallization data.

B) **^{14}C-Testosterone Tetrahydropyranyl Ether**——The radioactivity was quantitatively recovered from the incubation medium. Ninety-eight percent of the radioactivity appeared in the ether extract and 2% was in the aqueous fraction. Examination of the former by TLC on silica gel GF as described above revealed that 88% of the radioactivity appeared in the fraction less polar than testosterone. While, solvolysis of the ether extract followed by TLC as described above showed that 94% of the radioactivity appeared in the fraction more polar than testosterone; HPS-fraction (5%), 5β-androstane-3α, 17β-diol (39%), fraction containing 5α-androstane-3α, 17β-diol and its 3β-epimer (50%). The latter fraction was further purified as described above to afford 5α-androstane-3α, 17β-diol (81%) and 5α-androstane-3β, 17β-diol (14%). In contrast, ^{14}C-T yielded appreciable amounts of 17-oxosteroids (androsterone, epiandrosterone and etiocholanolone) in a similar ratio reported earlier.[3] These results clearly demonstrated that a slight cleavage of the ether linkage in ^{14}C-TP occurred during the incubation with the supernatant fraction. Identification of the above metabolites was made by the preliminary characterization and recrystallization with the authentic steroids. These results are summarized in Table I, in which percentage conversion was calculated as described above.

Incubation with 20000 × *g* Supernatant Fraction under Air

^{3}H-TS and ^{14}C-TP were metabolized under air in the analogous way to that under CO atmosphere. However, formation of the HPS-fraction from ^{3}H-TS and ^{14}C-TP in air was

11) For convenience' sake, the fraction remained near the starting line on TLC plate was named as such, when the ethyl acetate or ether extracts obtained after solvolysis were separated by TLC. It is reasonably presumed that this fraction is principally composed of hydroxylated testosterone metabolites, although identification of these steroids was not tried, since it was a minor fraction.

increased considerably to 11 and 19% as compared with that under CO atmosphere, which was 4 and 5%, respectively. These results are shown in Table I.

Incubation with 105000×*g* Supernatant Fraction under Air

Hydroxylases are mostly localized in microsomal fraction, therefore, all the incubations with 105000×*g* supernatant fraction were performed in air. ^{14}C-T was also incubated for the comparison purpose.

A) ^{3}H-Testosterone Sulfate——Recovery of the radioactivity from the incubation medium was 97%. Only 1% of the radioactivity appeared in the ether extract, while 99% remained in the aqueous fraction. Paper electrophoresis of the latter revealed a single radioactive peak migrating in TS fraction. Following solvolysis of the conjugate fraction, 97% of the radioactivity appeared in the ethyl acetate fraction. These results indicated that the conjugate was not cleaved during the incubation with 105000×*g* supernatant fraction. The ethyl acetate extract was separated by TLC to give the HPS-fraction (6%), 5β-androstane-3α, 17β-diol (57%), and testosterone (26%). Identification of the principal metabolites was performed as usual. These results are given in Table I.

B) ^{14}C-Testosterone Tetrahydropyranyl Ether——Recovery of the radioactivity from the incubation medium was 98%, which was quantitatively extracted with ether. The ether extract was chromatographed on silica gel plate as described above. The scan of the radioactive areas showed that 94% of the radioactivity appeared in the fraction less polar than testosterone. On the other hand, solvolysis of the ether extract followed by TLC afforded the HPS-fraction (4%), 5β-androstane-3α, 17β-diol (84%), and testosterone (6%). These results indicated that the ether linkage of TP was slightly cleaved during the incubation with the supernatant fraction. Identification of the principal metabolite, 5β-androstane-3α, 17β-diol, was carried out as described above and is indicated in Table I.

Incubation with Microsomal Fraction under Carbon Monoxide Atmosphere

A) ^{3}H-Testosterone Sulfate——Recovery of the radioactivity from the incubation medium was 96%. Only 1% of the radioactivity was extracted with ether and 99% remained in the aqueous fraction. Paper electrophoresis of the latter revealed a single radioactive peak migrating very close to TS. Following solvolysis of the conjugate fraction, 98% of the radioactivity appeared in ethyl acetate fraction. Separation by TLC afforded the HPS-fraction (5%) and a mixture of 5α-androstane-3α, 17β-diol and its 3β-epimer (91%). The mixture was further separated as described above to give 5α-androstane-3α, 17β-diol (1%) and 5α-androstane-3β, 17β-diol (96%). The preliminary characterization and the identification of the main metabolite were carried out in the usual way and the results are summarized in Table I.

B) ^{14}C-Testosterone Tetrahydropyranyl Ether——The radioactivity was quantitatively recovered from the incubation medium. Ninety-eight percent of the radioactivity appeared in the ether extract and 2% remained in the aqueous fraction. Examination of the former by TLC showed that 95% of the radioactivity appeared in the fraction less polar than testosterone. While, solvolysis of the ether extract followed by TLC gave the HPS-fraction (4%) and a fraction containing 5α-androstane-3α, 17β-diol and its 3β-epimer (89%). The latter fraction was further purified by TLC to afford 5α-androstane-3α, 17β-diol (31%) and 5α-androstane-3β, 17β-diol (63%). Identification of the main metabolites was done as usual and the results are shown in Table I.

Incubation with Microsomal Fraction under Air

^{3}H-TS and ^{14}C-TP were metabolized in air virtually in the similar way to that under CO atmosphere, with a slight increase of the HPS-fraction (6—10%). The results are given in Table I.

Table I. Identification by Recrystallization of Radioactive Metabolites

Incubation medium	Substrate	CO[a]	Metabolite	Recrystallization 1st Solvent[b]	1st dpm/mg	2nd Solvent	2nd dpm/mg	3rd Solvent	3rd dpm/mg	Percentage conversion[c] (%)
$20000 \times g$ Supernatant fraction	^{3}H-TS[d]	+	5α-androstane-3α, 17β-diol	B	2290	C	2210	D	2270	48
		+	5α-androstane-3β, 17β-diol	B	1730	C	1650	E	1760	33
		+	HPS-fraction[11]							4
		−	5α-androstane-3α, 17β-diol	B	1900	C	1680	D	1720	32
		−	5α-androstane-3β, 17β-diol	B	3550	C	3430	E	3410	37
		−	HPS-fraction							11
	^{14}C-TP[e]	+	5β-androstane-3α, 17β-diol	A	2420	A	2490	A	2490	38
		+	5α-androstane-3α, 17β-diol	D	2680	C	2670	B	2700	38
		+	5α-androstane-3β, 17β-diol	C	423	B	448	E	432	7
		+	HPS-fraction							5
		−	5β-androstane-3α, 17β-diol	A	1400	A	1450	A	1420	24
		−	5α-androstane-3α, 17β-diol	D	2580	C	2490	B	2570	36
		−	5α-androstane-3β, 17β-diol	E	434	C	433	B	439	8
		−	HPS-fraction							19
$105000 \times g$ Supernatant fraction	^{3}H-TS	−	5β-androstane-3α, 17β-diol	A	3180	A	3070	A	3080	50
		−	testosterone	B	1050	C	1000	F	1030	20
		−	HPS-fraction							6
	^{14}C-TP	−	5β-androstane-3α, 17β-diol	A	1680	A	1670	A	1670	80
		−	HPS-fraction							4
Microsomal fraction	^{3}H-TS	+	5α-androstane-3β, 17β-diol	B	4470	C	4230	E	4310	87
		+	HPS-fraction							5
		−	5α-androstane-3β, 17β-diol	B	2930	C	2840	E	2800	86
		−	HPS-fraction							6
	^{14}C-TP	+	5α-androstane-3α, 17β-diol	B	1300	C	1360	D	1360	21
		+	5α-androstane-3β, 17β-diol	B	3280	C	3130	E	3150	56
		+	HPS-fraction							4
		−	5α-androstane-3α, 17β-diol	B	709	C	714	D	710	15
		−	5α-androstane-3β, 17β-diol	B	3060	C	3040	E	3030	59
		−	HPS-fraction							10

a) CO+ : under carbon monoxide atmosphere; CO− : under air
b) *cf.* "Material and Method" in the text
c) average of three incubations
d) ^{3}H-testosterone 17-sulfate
e) ^{14}C-testosterone 17-tetrahydropyranyl ether

Discussion

In the previous paper,[3] we compared the metabolism of testosterone and its conjugates (TGA and TNAG) by incubation with male rat liver preparations and demonstrated the differences in their metabolic patterns. Thus, in contrast to testosterone the conjugates 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 order to obtain further information concerning the influence of a polar and a non-polar or a bulky substituent at C-17 of testosterone upon the metabolic hydrogenation of ring A, *in vitro* metabolism of TS and TP was investigated. TS was chosen as a water soluble conjugate like TGA or TNAG, while TP was selected as a substrate having a non-polar and bulky group at C-17. The tetrahydropyranyl ether has a pyranoside ring structure like sugar but does not have any hydroxyl group at C-2′ to C-5′, thus being non-polar. The present study demonstrated that TS and TP can be extensively metabolized to 5α- and 5β-$C_{19}O_2$ steroids with sulfate or tetrahydropyranyl group attached at C-17 intact and that they are metabolized in a somewhat different way from one another as well as from testosterone, TGA and TNAG.[3]

The metabolic differences among these compounds were clearly demonstrated in the incubation with $20000 \times g$ supernatant fraction of male rat liver homogenate containing both Δ^4-5α- and Δ^4-5β-hydrogenases. In contrast to the predominant formation of 5β-androstane-3α, 17β-diol from TGA and TNAG under CO atmosphere,[3] TS was metabolized primarily to 5α-androstane-3α, 17β-diol and its 3β-epimer, conversion to 5β-androstane-3α, 17β-diol being very little. While TP was transformed mainly to 5α-androstane-3α, 17β-diol and 5β-androstane-3α, 17β-diol in a similar proportion, 5α-androstane-3β, 17β-diol being a minor metabolite (Table I). From its metabolic pattern, therefore, TP is situated midway between testosterone and TGA or TNAG. The bulky substituent at C-17 alone is not powerful enough to produce a directive influence on the hydrogenation of ring A double bond. Being bulky as wel as polar seems to be required for it, as was found in the cases of TGA and TNAG, where the sugar groups might have a stereochemical influence on the hydrogenation in such a way to inhibit the approach of the steroidal ring A to microsomal Δ^4-5α-hydrogenase.

The incubation with $105000 \times g$ supernatant fraction containing Δ^4-5β-hydrogenase revealed that TP was metabolized in 80% yield to 5β-androstane-3α, 17β-diol. On the other hand, TS was metabolized in 50% yield to 5β-androstane-3α, 17β-diol, 20% of testosterone being recovered (Table I). These results suggested that TP is a better substrate for Δ^4-5β-hydrogenase than TS and are consistent with those of the incubation with $20000 \times g$ supernatant fraction described above.

The incubation of TS and TP with microsomal fraction under CO atmosphere demonstrated that both compounds were good substrates for Δ^4-5α-hydrogenase. 5α-Androstane-3β, 17β-diol was a sole metabolite with TS, while TP yielded 5α-androstane-3β, 17β-diol as a major product and its 3α-epimer as a minor one (Table I). Since TGA and TNAG were recovered almost unchanged in the incubation with microsomal fraction,[3] it should be noticed that TS, though a water soluble conjugate like TGA or TNAG, is quite different in its metabolic behaviors from these conjugates. On the other hand, TS as well as TP were poor substrates like TGA and TNAG[3] for other microsomal enzymes, such as hydroxylases, because the HPS-fractions produced from TS and TP by incubation in air with microsomal fraction were found to be little (6—10%) whereas testosterone was converted by similar incubation to the HPS-fraction in 95% yield.[3] For accurate evaluation of these findings, however, many problems concerning substrate specificity and multiplicity of the enzymes, precise localization of the enzymes in organelle, and the membrane transport system should be clarified.

In vivo metabolism of ^{14}C-TS was investigated by Baulieu, *et al.*,[2] who injected ^{14}C-TS into a normal man, whereby only 3.5% of the injected steroid conjugate was recovered unchanged from the urine and any labeled 5α- or 5β-metabolites could not be isolated from urinary conjugates. The observed differences between *in vivo* and *in vitro* studies might be ascribable to the species difference or membrane permeability of TS, but awaits further elucidation.