

Comparative Studies on the Metabolic Hydrogenation of the Ring A in Testosterone and Its Conjugates by Male Rat Liver *in Vitro*

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Metabolic hydrogenation of the ring A double bond in testosterone and its conjugates were studied by incubating ¹⁴C-testosterone, ¹⁴C-testosterone 17-N-acetylglucosaminide and ³H-testosterone 17-glucosiduronate with 20000 × g or 105000 × g supernatant fluids and microsomal fraction of male rat liver homogenate under carbon monoxide atmosphere or in air. It was demonstrated that in contrast to testosterone the conjugates were not good substrates for microsomal Δ^4 -5 α -hydrogenase as well as for hydroxylases, while they were better substrates for soluble Δ^4 -5 β -hydrogenase than testosterone. These findings are consistent with the observations on *in vivo* metabolism of testosterone and its conjugates in man.

Steroid conjugates were usually considered as end products of steroid metabolism. However, there has been accumulating evidence that not only do these conjugates undergo hydrolysis and reconjugation *in vivo* but that the steroid moiety undergoes metabolic transformations as the conjugate.²⁾ Robel, *et al.*³⁾ compared the metabolic fate of testosterone and testosterone 17-glucosiduronate (3-oxoandrost-4-en-17 β -yl- β -D-glucopyranosiduronate, TGA) following intravenous administration to man. Whereas testosterone was reduced to yield both 5 α - and 5 β -metabolites, TGA yielded only 5 β -metabolites. More recently, Fukushima, *et al.*⁴⁾ observed similar stereoselective transformation to 5 β -metabolites following both intravenous and oral administration of 4-¹⁴C-testosterone 17-N-acetylglucosaminide (3-oxoandrost-4-en-17 β -yl-2'-acetamido-2'-deoxy- β -D-glucopyranoside, TNAG) to man. These studies demonstrated a stereochemical influence of the sugar group at C-17 on the biochemical reduction of the Δ^4 -3-ketone.

It has been well established from enzymic studies using free steroids as substrates that the Δ^4 -5 α -hydrogenase is located in the microsomal fraction and Δ^4 -5 β -hydrogenase is present in the soluble fraction.⁵⁾ The differences in the *in vivo* metabolic pathways of testosterone and its conjugates might be due to enzyme specificity of Δ^4 -hydrogenase or due to the selective accessibility of the substrates to the enzymes. In order to obtain further knowledge on the effect of conjugation at C-17, labeled testosterone, TGA and TNAG have been incubated with cell fractions from male rat liver.

Material and Method

Steroids and Reagents—4-¹⁴C-Testosterone (¹⁴C-T) (40 mCi/mmmole), 4-¹⁴C-testosterone 17-N-acetylglucosaminide (¹⁴C-TNAG) (40 mCi/mmmole) and 1,2-³H-testosterone 17-glucosiduronate (³H-TGA) (25

- 1) Location: *Takada 3-Chome, Toshima-ku, Tokyo.*
- 2) E.E. Baulieu, C. Corpéchet, F. Dray, R. Emiliozzi, M. Lebeau, P. Mauvais-Jarvis, and P. Robel, "Recent Progress in Hormone Research," Vol. 21, ed. by G. Pincus, Academic Press, New York, 1965, p. 411; H.E. Hadd and R.T. Blickenstaff, "Conjugates of Steroid Hormones," Academic Press, New York, 1969; S. Bernstein and S. Solomon (ed.), "Chemical and Biological Aspects of Steroid Conjugation," Springer-Verlag, New York, 1970.
- 3) P. Robel, R. Emiliozzi, and E.E. Baulieu, *J. Biol. Chem.*, **241**, 20, 5879 (1966).
- 4) D.K. Fukushima, M. Matsui, H.L. Bradlow, and L. Hellman, *Steroids*, **19**, 385 (1972).
- 5) R.I. Dorfman and F. Ungar, "Metabolism of Steroid Hormones," Academic Press, New York, 1965, p. 406, and literatures cited therein.

Ci/mmmole), which were used in this investigation, were described previously.⁴⁾ All the radiochemical purities of these steroids were confirmed by thin-layer chromatography shortly before use. Androst-4-ene-3,17-dione, 5 α -androstane-3,17-dione and 5 β -androstane-3,17-dione were prepared by oxidation of testosterone, androsterone and 3 β -hydroxy-5 β -androstane-17-one with Jones' reagent, respectively. 17 β -Hydroxy-5 α -androstane-3-one and 17 β -hydroxy-5 β -androstane-3-one were obtained by catalytic hydrogenation of testosterone over palladium-on-charcoal.⁶⁾ 5 α -Androstane-3 β ,17 β -diol, 5 β -androstane-3 α ,17 β -diol and 5 β -androstane-3 β ,17 β -diol were synthesized by reduction of 5 α -androstane-3,17-dione, etiocholanolone and 3 β -hydroxy-5 β -androstane-17-one with NaBH₄, respectively. TNAG was prepared as described previously,⁷⁾ while TGA was prepared according to the procedure described by Robel, *et al.*³⁾ 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 Preparation—Male rats of Wistar strain, weighing 250 to 450 g were decapitated and a 20% (w/v) liver homogenate was prepared in ice cold 0.25 M sucrose solution with a Teflon-glass homogenizer. The homogenate was fractionated by differential centrifugation at 2000 $\times g$ for 10 min, 20000 $\times g$ for 45 min, and 105000 $\times g$ for 60 min. The 105000 $\times g$ pellet was suspended in the ice cold sucrose solution and sedimented again by centrifugation at 105000 $\times g$ for 60 min. The resulting 105000 $\times g$ pellet was resuspended in 0.25M sucrose solution to give a concentration equivalent to 0.2 g of fresh liver tissue per ml, and used as microsomal fraction. The 20000 $\times g$ and the 105000 $\times g$ supernatant fluids and the microsomal fraction were used for the incubation experiments, whose protein concentrations were about 19 mg/ml, 16 mg/ml and 1.4 mg/ml respectively, as determined by the procedure of Lowry, *et al.*⁸⁾

Incubation—Three ml of 20000 $\times g$ and 105000 $\times g$ supernatant fluids or microsomal fraction was added to brown glass-stoppered flask containing 3.5 ml of 0.2 M Tris buffer, pH 7.2, 3 mg of NADP, 10 mg of glucose-6-phosphate and 7 units of glucose-6-phosphate dehydrogenase, as described by Stárka, *et al.*⁹⁾ Two incubation procedures were then carried out. One was the incubation under carbon monoxide (CO) atmosphere and the other was the incubation under air. CO treatment¹⁰⁾ was performed in order to suppress hydroxylase activities in microsomes by bubbling CO current into the incubation medium for 2 min, followed by quick addition of labeled steroids and then stoppered. Ethanol solution (45–85 μ l)¹¹⁾ of ¹⁴C-T (100000 dpm), ¹⁴C-TNAG (100000 dpm) or ³H-TGA (200000 dpm) was added to the incubation flask. Incubations were carried out for 5 min at 37° with shaking and were 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 under reduced pressure to give a residue, which was dissolved in water and extracted with ether. The ether extract was washed with water, dried over Na₂SO₄, and evaporated *in vacuo* to afford the free steroid fraction. The aqueous fraction was evaporated under reduced pressure just to eliminate ether, and then passed through XAD-2 (100 g) column. The column was washed with 400 ml of water and eluted with 400 ml of methanol according to the procedure of Bradlow.¹²⁾ The methanol effluent was evaporated *in vacuo* to give the conjugate (or polar) fraction. The free steroid fraction and the conjugate fraction were stored as ethanol and methanol solutions respectively.

Enzymic Hydrolysis—A) Glucosiduronates: The conjugate fraction obtained from the incubation with ³H-TGA was evaporated *in vacuo* to dryness and dissolved in 2.5 ml of water. To the aqueous solution were added 1 ml of 0.5 M acetate buffer, pH 5.2, and 0.5 ml of β -glucuronidase (Ketodase, Warner-Chilcott, 5000 u/ml). They were incubated at 37° for 48 hr and extracted with ether. The ether extract was washed with water, dried over Na₂SO₄, and evaporated under reduced pressure to afford hydrolyzed steroid fraction which was stored as ethanol solution.

B) N-Acetylglucosaminides: The conjugate fraction obtained from the incubation with ¹⁴C-TNAG was evaporated *in vacuo* to dryness and dissolved in 10 ml of 0.1 M citrate buffer, pH 4.3. To the aqueous solution was added 10 mg of hyaluronidase from bovine testes (type I, Sigma Chem. Co., St. Louis, Mo.). They were incubated at 37° for 7 days and extracted with ether. The ether extract was washed with water, dried over Na₂SO₄, and evaporated *in vacuo* to give hydrolyzed steroid fraction, which was stored as ethanol solution.

Thin-Layer Chromatography (TLC)—TLC was carried out on 5 \times 20 cm glass plate coated with 0.5 mm thick film of Silica gel GF (Merck) or Aluminum oxide G (Merck). Reference steroids were applied at the edge of the plate. The following solvent systems were used: S-1, chloroform-acetone (29:1); S-2, *n*-

- 6) A.J. Liston, *J. Org. Chem.*, **31**, 2105 (1966).
- 7) G. Sauer, M. Matsui, R. Bloch, J.S. Liang, and D.K. Fukushima, *J. Org. Chem.*, **34**, 3525 (1969).
- 8) O.H. Lowry, N.J. Rosebrough, A.L. Farr, and R.J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
- 9) V.L. Stárka and H. Breuer, *Z. Physiol. Chem.*, **349**, 1698 (1968).
- 10) H. Inano, K. Tsuno, and B. Tamaoki, *Biochemistry*, **9**, 2253 (1970).
- 11) Ethanol contents changing from 45 to 85 μ l had a slight influence on the metabolic patterns of steroids.
- 12) H.L. Bradlow, *Steroids*, **11**, 265 (1968).

hexane-ethyl acetate (1:1); S-3, benzene-ethanol (40:1); S-4, chloroform; S-5, cyclohexane-chloroform-ethyl acetate (2:2:1). The plate was usually developed three times with the same solvent system. The reference steroids with Δ^4 -3-oxo grouping were detected under a short wave ultraviolet lamp. The radioactivity was detected with an Aloka autoscanner. The radioactive spots were then scraped and eluted with methanol. The methanol extract was evaporated *in vacuo* to dryness and stored as ethanol solution. The recovery of the radioactivity from the plate was usually about 90 to 95%. Subsequently, the reference steroids were visualized by spraying the rest of the plate with sulfuric acid, followed by heating.

Paper Electrophoresis—Paper electrophoresis of the conjugate fraction was performed with solvent system pyridine-acetic acid, pH 6.4, using a slight modification (Toyo Roshi No. 51, 12.5 × 26 cm: 2 hr run at 600 V) of the direction of Kornel.¹³⁾ Testosterone, TNAG and TGA were applied as references, which were detected under a short wave ultraviolet lamp. Testosterone stayed at the origin and TGA migrated toward the cathode twice as long as TNAG. The radioactivity was detected with an Aloka autoscanner. The radioactive zones were then cut into pieces and extracted with methanol. The methanol extract was evaporated under reduced pressure and stored as ethanol solution.

Preliminary Characterization—A) Chromium Trioxide Oxidation: A portion of the purified metabolite which was tentatively characterized by co-chromatography with reference steroids on TLC, was oxidized with Jone's reagent.¹⁴⁾ Each 50 μ g of testosterone, androsterone and etiocholanolone was added to the sample as carriers and the oxidation was carried out in 2 ml of acetone solution by adding 2 drops of chromium trioxide reagent (CrO_3 , 2.7 g) and conc. H_2SO_4 (2.3 ml) diluted with water to 10 ml). After standing 15 min at room temperature, the reaction mixture was extracted with ether. The usual work up gave dione fraction, which was chromatographed on silica gel plate with S-4 as solvent.

B) Girard's Reagent T Separation: A portion of the purified metabolite which was characterized as non-ketonic steroid by TLC, was treated with Girard's reagent T.¹⁵⁾ To 6 ml of 95% ethanol solution were added 0.3 ml of acetic acid and 300 mg of Girard's reagent T. The mixture was refluxed for 1 hr, poured into ice water, adjusted pH to 6.5 and extracted with ether. The ether extract was treated as usual to give non-ketonic fraction. To the aqueous layer were added one tenth volume of conc. HCl and small amount of ether to cover the surface, and kept at room temperature overnight. The aqueous layer was extracted with ether and usual work up gave ketonic fraction. Ketonic and non-ketonic fractions were stored as ethanol solution.

C) Digitonin Precipitation: Androsterone and epiandrosterone or 5α -androstane- $3\alpha,17\beta$ -diol and its 3β -epimer could not be separated from each other by using silica gel plate with solvent system S-1. The approximate ratios of the two pairs of steroids present were obtained by digitonin precipitation according to the procedure of Butt, *et al.*¹⁶⁾

Recrystallization—The identity and the purity of the isolated metabolite were confirmed by adding 20 to 30 mg of the authentic steroid and recrystallizing the mixture from appropriate solvents 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, chloroform-ether; F, benzene-pet. ether; G, acetone-pet. ether; H, benzene-ether; I, cyclohexane; J, ether-pet. ether; K, methanol-ether-pet. ether.

Measurement of Radioactivity—The radioactivity was measured in Nuclear-Chicago Mark I liquid scintillation spectrometer. The sample aliquot, which consisted of 0.5 or 1.0 ml of methanol, ethanol or aqueous solution, was dissolved in 10 ml of the scintillation fluid containing 5 g of PPO or 2,5-diphenyloxazole and 300 mg of 1,4-bis-[2-(4-methyl-5-phenyloxazolyl)]-benzene per liter of toluene. One or 2 ml of BIOSOLV BBS-3(Beckman) was added to 0.5 or 1.0 ml of the aqueous sample respectively to obtain homogeneous counting fluid. The counting efficiency for each sample was determined by reference to a calibration curve plotted from a set of quenched standards using external ^{133}Ba source and a channel ratio method. Efficiency of ^{14}C and ^3H counting was about 80 and 40% respectively. All the radioactivities are expressed in dpm.

Result

Incubation with 20000 × g Supernatant Fluid under Carbon Monoxide Atmosphere

The incubation medium was treated with CO current just before addition of the substrates to suppress the hydroxylase activities and to identify the metabolites of testosterone and its conjugates as C_{19}O_2 steroids. It was observed that the amount of hydroxylated metabolites increased with a elapsed time of incubation.

13) L. Kornel, *J. Clin. Endocrinol.*, **24**, 956 (1964).

14) C. Djerassi, R.R. Engle, and A. Bowers, *J. Org. Chem.*, **21**, 1547 (1956).

15) A. Girard and G. Sandulesco, *Helv. Chim. Acta*, **19**, 1095 (1936).

16) W.R. Butt, A.A. Henly, and J.O.R. Morris, *Biochem. J.*, **42**, 477 (1948).

A) ^{14}C -Testosterone—The radioactivity was quantitatively recovered by extracting the incubation medium with methanol. Eighty-five percent of the radioactivity appeared in the ether extract and 15% was in the aqueous fraction. The ether extract was chromatographed on silica gel plate with solvent system S-1. The scan of the radioactive areas revealed the presence of several metabolites corresponding to hydroxylated polar steroids (20%), 5β -androstane- $3\alpha,17\beta$ -diol (10%), 5α -androstane- $3\alpha,17\beta$ -diol and its 3β -epimer (33%), testosterone and etiocholanolone (7%), androsterone and epiandrosterone (28%). 5α -Androstane- $3\alpha,17\beta$ -diol and its 3β -epimer fraction was further separated by TLC on aluminum oxide G with solvent system S-5 to afford 5α -androstane- $3\alpha,17\beta$ -diol (69%) and 5α -androstane- $3\beta,17\beta$ -diol (20%). Testosterone and etiocholanolone fraction was chromatographed on silica gel plate with S-2 as solvent and yielded testosterone (39%) and etiocholanolone (52%). Androsterone and epiandrosterone fraction was purified by silica gel plate with S-3 as solvent and gave epiandrosterone (22%) and androsterone (53%). Preliminary characterization of each metabolite, as described in "Materials and Methods," supported the above assignment. The identity of each metabolite was finally established by recrystallization with the authentic steroid to constant specific activity, as shown in Table I. The 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 N-Acetylglucosaminide—The recovery of the radioactivity from the incubation medium was about 96%. Two percent of the radioactivity appeared in the ether extract and 98% was in the aqueous fraction. Examination of the conjugate fraction by paper electrophoresis revealed that a single radioactive peak migrated very close to TNAG. Hydrolysis of the conjugate fraction with hyaluronidase type I made 90% of the radioactivity to appear in the ether extract. These results indicated that very little N-acetylglucosamine was cleaved from the steroid during incubation of the conjugate with the $20000\times g$ supernatant fraction. The ether extract was chromatographed on silica gel plate with S-1 as solvent, affording the metabolites corresponding to polar hydroxylated steroids (5%), 5β -androstane- $3\alpha,17\beta$ -diol (85%) and testosterone and etiocholanolone (1%). The preliminary characterization and recrystallization with the authentic steroid confirmed the predominant conversion of the steroid moiety testosterone into 5β -androstane- $3\alpha,17\beta$ -diol, as shown in Table I. The percentage conversion was calculated from the radioactivity of the purified free steroids by TLC, which was corrected by recrystallization data.

C) ^3H -Testosterone Glucosiduronate—The recovery of the radioactivity from the incubation medium was about 90%. Five percent of the radioactivity appeared in the ether extract and 95% remained in the aqueous fraction. Examination of the conjugate fraction by paper electrophoresis showed that a single radioactive peak moved very close to TGA. After β -glucuronidase (Ketodase) hydrolysis of the conjugate fraction, 96% of the radioactivity appeared in the ether extract. These results demonstrated that only a few percent of the glucosiduronate was hydrolyzed during the incubation with the rat liver preparation. The ether extract was chromatographed on silica gel plate with solvent system S-1 to afford the metabolites corresponding to polar hydroxylated steroids (4%) and 5β -androstane- $3\alpha,17\beta$ -diol (93%). The preliminary characterization and recrystallization demonstrated the predominant conversion of steroid moiety testosterone into 5β -androstane- $3\alpha,17\beta$ -diol, as indicated in Table I.

Incubation with $20000\times g$ Supernatant Fluid under Air

The recovery of the radioactivity from the incubation of ^{14}C -T was quantitative and 60% of the radioactivity was obtained in the ether extract. Testosterone was metabolized to polar hydroxylated steroids due to microsomal hydroxylases. Ninety-five percent of the radioactivity of the ether extract remained at the origin on TLC. These results were consistent with the metabolic studies of testosterone and related steroids in the rat liver.^{9,17)} Therefore,

17) J. Chamberlain, N. Jagarinec, and P. Ofner, *Steroids, Suppl.*, **2**, 1 (1965).

the identification of the metabolites was not made. On the other hand, ^{14}C -TNAG and ^3H -TGA were metabolized exactly in the same way in air as under CO atmosphere. They were converted exclusively into 5β -androstane- $3\alpha,17\beta$ -diol 17-glycosides. These results are summarized in Table II.

TABLE I. Identification by Recrystallization of Radioactive Metabolites obtained by Incubation with $20000 \times g$ Supernatant under Carbon Monoxide Atmosphere

Steroid incubated	Metabolite	Recrystallization						Percentage conversion (%)
		1st		2nd		3rd		
		Sol-vent ^{a)}	Specific activity (dpm/mg)	Sol-vent	Specific activity (dpm/mg)	Sol-vent	Specific activity (dpm/mg)	
^{14}C -T ^{b)}	5β -androstane- $3\alpha,17\beta$ -diol	A	645	A	605	A	593	6 (4—10) ^{c)}
	5α -androstane- $3\alpha,17\beta$ -diol	B	1510	C	1590	D	1550	23 (22—26)
	5α -androstane- $3\beta,17\beta$ -diol	B	514	C	511	E	500	6 (6—7)
	testosterone	B	299	C	321	F	304	2 (2—3)
	etiocholanolone	G	445	H	428	I	429	5 (2—6)
	androsterone	B	894	J	885	C	878	13 (10—15)
	epiandrosterone	B	515	J	516	C	514	5 (4—6)
^{14}C -TNAG ^{d)}	5β -androstane- $3\alpha,17\beta$ -diol	A	1560	A	1510	A	1590	82 (81—84)
^3H -TGA ^{e)}	5β -androstane- $3\alpha,17\beta$ -diol	A	2820	A	2780	A	2780	89 (89—89)

- a) cf. "Material and Method" in the text
 b) $4\text{-}^{14}\text{C}$ -testosterone
 c) mean and range of three incubations
 d) $4\text{-}^{14}\text{C}$ -testosterone 17-N-acetylglucosaminide
 e) $1,2\text{-}^3\text{H}$ -testosterone 17-glucosiduronate

TABLE II. Identification by Recrystallization of Radioactive Metabolites obtained by Incubation with Supernatants and Microsomes under Air

Incubation medium	Steroid incubated	Metabolite	Recrystallization						Percentage conversion (%)
			1st		2nd		3rd		
			Sol-vent ^{a)}	Specific activity (dpm/mg)	Sol-vent	Specific activity (dpm/mg)	Sol-vent	Specific activity (dpm/mg)	
$20000 \times g$ Supernatant	^{14}C -TNAG ^{b)}	5β -androstane- $3\alpha,17\beta$ -diol	A	1350	A	1390	A	1390	89 (88—89) ^{c)}
	^3H -TGA ^{d)}	5β -androstane- $3\alpha,17\beta$ -diol	A	3050	A	3160	A	3090	89 (86—94)
$105000 \times g$ Supernatant	^{14}C -T ^{e)}	5β -androstane- $3\alpha,17\beta$ -diol	A	1430	A	1370	A	1350	43 (39—47)
		testosterone	B	1060	C	1030	F	1010	17 (17—18)
		etiocholanolone	G	280	H	265	I	250	3 (3—3)
	^{14}C -TNAG	5β -androstane- $3\alpha,17\beta$ -diol	A	1600	A	1600	A	1610	85 (84—87)
	^3H -TGA	5β -androstane- $3\alpha,17\beta$ -diol	A	2820	A	2810	A	2770	89 (87—90)
Microsomes	^{14}C -TNAG	testosterone	B	1330	C	1330	F	1350	83 (81—84)
	^3H -TGA	testosterone	B	2200	C	2210	F	2190	82 (80—83)

- a) cf. "Material and Method" in the text
 b) $4\text{-}^{14}\text{C}$ -testosterone 17-N-acetylglucosaminide
 c) mean and range of three incubations
 d) $1,2\text{-}^3\text{H}$ -testosterone 17-glucosiduronate
 e) $4\text{-}^{14}\text{C}$ -testosterone

Incubation with 105000×g Supernatant Fluid under Air

Since hydroxylases are almost absent in 105000×g supernatant, all the incubations with this enzyme preparation were performed under air.

A) ¹⁴C-Testosterone—The recovery of the radioactivity from the incubation medium was quantitative. Ninety-five percent of the radioactivity appeared in the ether extract. The ether extract was chromatographed as described above to give the metabolites corresponding to polar hydroxylated steroids (16%), 5β-androstane-3α,17β-diol (53%), and testosterone and etiocholanolone (27%). Testosterone and etiocholanolone fraction was separated as described above to yield testosterone (68%) and etiocholanolone (16%). The identification of the metabolites was carried out in the above mentioned way. These results are summarized in Table II.

B) ¹⁴C-Testosterone N-Acetylglucosaminide—The recovery of the radioactivity from the incubation medium was about 93%. One percent of the radioactivity appeared in the ether extract and 99% remained in the aqueous fraction. Paper electrophoresis of the conjugate fraction showed that a single radioactive peak moved very near to TNAG. Following hydrolysis of the conjugate fraction with hyaluronidase type I, 83% of the radioactivity appeared in the ether extract. These results indicated that the conjugate was not cleaved during the incubation with the 105000×g supernatant of the rat liver. TLC of the ether extract afforded the metabolites corresponding to polar hydroxylated steroids (4%) and 5β-androstane-3α,17β-diol (90%). The identification of the principal metabolite was performed as usual and is shown in Table II.

C) ³H-Testosterone Glucosiduronate—The recovery of the radioactivity from the incubation medium was 90%. Five percent of the radioactivity appeared in the ether extract and 95% was in the aqueous fraction. Paper electrophoresis of the conjugate fraction revealed a single radioactive peak migrating very close to TGA. Following hydrolysis of the conjugate fraction with Ketodase, 95% of the radioactivity was obtained in the ether extract. The separation of the metabolites in the ether extract by TLC showed the presence of metabolites corresponding to polar hydroxylated steroids (1%) and 5β-androstane-3α,17β-diol (94%). The identification of the principal metabolite was performed in the usual way and is shown in Table II.

Incubation with Microsomal Fraction under Carbon Monoxide Atmosphere

A) ¹⁴C-Testosterone—The recovery of the radioactivity from the incubation medium was quantitative and 95% of the radioactivity was obtained in the ether extract. TLC of the ether extract showed the presence of the metabolites corresponding to polar hydroxylated steroids (18%), 5α-androstane-3α,17β-diol and its 3β-epimer (28%), and androsterone and epiandrosterone (48%). The preliminary characterization and the identification of the metabolites were carried out as above and the results are summarized in Table III.

B) ¹⁴C-Testosterone N-Acetylglucosaminide—The recovery from the incubation medium was quantitative. Only 1% of the radioactivity was in the ether extract and 99% remained in the aqueous fraction. Examination of the conjugate fraction by paper electrophoresis showed that a single radioactive peak moved in the TNAG area. Following hydrolysis of the conjugate fraction with hyaluronidase type I, 95% of the radioactivity was extracted with ether. Metabolites corresponding to polar hydroxylated steroids (4%) and testosterone (83%) were obtained by TLC. The identification of testosterone was done as usual and is shown in Table III. Most of the substrate remained unchanged during the incubation with the microsomal enzyme preparation.

C) ³H-Testosterone Glucosiduronate—The recovery from the incubation medium was about 95%. Five percent of the radioactivity was obtained in the ether extract and 95% remained in the aqueous fraction. Paper electrophoresis of the conjugate fraction showed that a single radioactive peak appeared in the TGA area. Following Ketodase hydrolysis

of the conjugate fraction, 96% of the radioactivity was obtained in the ether extract, which afforded the metabolites corresponding to polar hydroxylated steroids (2%) and testosterone (92%). The identification of the recovered testosterone was carried out as described above and is shown in Table III. Most of the substrate was recovered unchanged in this incubation with the rat liver microsomes.

TABLE III. Identification by Recrystallization of Radioactive Metabolites by Incubation with Microsomes under Carbon Monoxide Atmosphere

Steroid incubated	Metabolite	Recrystallization						Percentage conversion (%)
		1st		2nd		3rd		
		Sol-vent ^{a)}	Specific activity (dpm/mg)	Sol-vent ^{a)}	Specific activity (dpm/mg)	Sol-vent ^{a)}	Specific activity (dpm/mg)	
¹⁴ C-T ^{b)}	5 α -androstande-3 β ,17 β -diol	C	1410	B	1350	E	1370	22 (21—24) ^{c)}
	epiandrosterone	K	1490	J	1450	B	1460	40 (35—47)
¹⁴ C-TNAG ^{d)}	testosterone	B	1630	C	1630	F	1690	79 (76—83)
³ H-TGA ^{e)}	testosterone	B	1410	C	1370	F	1380	78 (77—79)

a) cf. "Material and Method" in the text

b) 4-¹⁴C-testosterone

c) mean and range of three incubations

d) 4-¹⁴C-testosterone 17-N-acetylglucosaminide

e) 1,2-³H-testosterone 17-glucosiduronate

Incubation with Microsomal Fraction under Air

The recovery of the radioactivity from the incubation of ¹⁴C-T was quantitative and 70% of the radioactivity was obtained in the ether extract. Testosterone was metabolized to polar hydroxylated steroids. Ninety-five percent of the radioactivity in the ether extract remained at the origin on TLC. Therefore, the identification of the metabolites was not performed. In contrast, ¹⁴C-TNAG and ³H-TGA behaved virtually in the same manner as incubations under CO atmosphere, with most of the substrates being recovered unchanged. These results are shown in Table II.

Discussion

It is quite evident from the present results that ring A of testosterone can undergo metabolic transformation with sugar groups attached at C-17 and that the glycosidic group at C-17 affects the stereochemical course of reduction of ring A double bond. The differences between testosterone and its conjugates in the *in vitro* metabolic hydrogenation of the ring A were clearly demonstrated by incubation with 20000 \times g supernatant fluid of male rat liver homogenate which contains both Δ^4 -5 α - and Δ^4 -5 β -hydrogenases. Thus the incubation of TNAG and TGA in 20000 \times g supernatant fluid of male rat liver homogenate yielded only the 5 β -metabolite, 5 β -androstande-3 α ,17 β -diol with the sugar moiety still attached, in very high yield. No evidence for the formation of 5 α -metabolites was obtained. While testosterone was converted by the supernatant fluid to both 5 α - and 5 β -metabolites. The sole conversion of the testosterone conjugates to the 5 β -metabolites may be due to (1) the water solubility of the conjugates which allow more ready access to the soluble Δ^4 -5 β -hydrogenase so that they are preferentially reduced by this enzyme and to (2) the sugar moiety which binds to the microsomes in such a fashion to inhibit approach of the ring A of testosterone to the microsomal Δ^4 -5 α -hydrogenase.

The studies with 105000 \times g supernatant fluid containing the soluble 5 β -hydrogenase provide evidence that the water soluble testosterone conjugates are good substrates for the enzyme. The conjugates were metabolized in over 80% yield to 5 β -androstande-3 α ,17 β -diol

without hydrolysis of the glycosidic linkage whereas testosterone afforded less than 65% yield; no 5α -metabolites were observed in the enzyme preparation. In contrast, the testosterone conjugates were poor substrates for the microsomal Δ^4 - 5α -hydrogenase being almost quantitatively recovered unchanged. The activity of the microsomal preparation was demonstrated by high conversion of testosterone to its 5α -metabolites. The conjugates were further demonstrated to be poor substrates for other microsomal enzymes such as the hydroxylases which transformed the unconjugated testosterone almost completely to polar compounds when the incubation was carried out in air.

The extension of the present *in vitro* findings may be made to the observations on the predominant formation of 5β -compounds in man following administration of testosterone conjugates.⁴⁾ The water soluble conjugates are also poor substrates for the human microsomal 5α -hydrogenase and are therefore rapidly reduced by the "soluble" 5β -hydrogenase before any hydrolysis of the sugar group can take place. Should the *in vivo* hydrolysis of the glycosidic linkage precede reduction of ring A, the resulting testosterone can be metabolized to the 5α -compounds as well as the 5β -metabolites.

In order to obtain further information concerning the influence of the substituent at C-17 on the biochemical reduction of ring A, metabolic transformations of several testosterone derivatives by rat liver preparations are under current investigation. Moreover, in view of the fact⁵⁾ that the microsomal fraction of female rat liver contains more Δ^4 - 5α -hydrogenase than that of male rat liver, we are going to make an *in vitro* study with female rat liver preparations.

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