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IN VITRO METABOLISM OF TESTOSTERONE IN SEMINAL VESICLES OF RATS

KEIKO SUZUKI and BUN-ICHI TAMAOKI*

National Institute of Radiological Sciences. Anagawa-4-chrome. Chiba-shi 280. Japan

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SUMMARY

When [4-^{14}C]-testosterone was incubated *in vitro* with cell-free homogenates (800 *g* supernatant fluid) of rat seminal vesicles, 5α-dihydrotestosterone and 5α-androstane-3α,17β-diol were identified as the metabolites. Both the nuclear and microsomal fractions most efficiently converted testosterone to the above 5α-hydrogenated metabolites. When testosterone was pre-incubated with the cytosol fraction (105,000 *g* supernatant fluid), consequent 5α-hydrogenation by the nuclear and microsomal fractions was decreased. If the cytosol fraction was heated at 100°C, the inhibitory effect upon the enzyme activity diminished. 3α-Hydroxysteroid dehydrogenase activity upon 5α-dihydrotestosterone was detected almost exclusively in the cytosol fraction among the subcellular fractions.

NADPH was found as the most preferable cofactor for Δ^4-5α-hydrogenase and 3α-hydroxysteroid dehydrogenase. Δ^4-5α-Hydrogenase and 3α-hydroxysteroid dehydrogenase reduced testosterone and 5α-dihydrotestosterone respectively by the transfer of 4-pro-S-hydrogen of NADPH. Optimal pH of Δ^4-5α-hydrogenase in the nuclear and microsomal fractions was from 5·7 to 5·9 and that of the 3α-hydroxysteroid dehydrogenase in the cytosol fraction was about 6·3. Optimal temperature was around 37°C for Δ^4-5α-hydrogenase in the two subcellular fractions, and 50°C for 3α-hydroxysteroid dehydrogenase. Cu^{2+}, Cd^{2+}, Hg^{2+}, Zn^{2+} and *p*-chloromercuribenzoate markedly inhibited both Δ^4-5α-hydrogenase and 3α-hydroxysteroid dehydrogenase activities, whereas Co^{2+} selectively inhibited Δ^4-5α-hydrogenase activity. Δ^4-5α-Hydrogenase activity was reduced by EDTA and *o*-phenanthroline.

INTRODUCTION

The metabolism of testosterone and related enzymology in the prostate have been extensively investigated by several workers [1–4]. For the seminal vesicle, *in vitro* production of 5α-dihydrotestosterone from testosterone has been studied in tissue slices [5], cell-free homogenates [6] and in the nuclear fraction [7]. 5α-Androstanediol formation from 5α-dihydrotestosterone has only been examined in the supernatant fluid at 100,000 *g* of seminal vesicle homogenates [7].

In this paper, the intracellular distributions, enzyme kinetics and effect of recombination of subcellular components of the enzyme Δ^4-5α-hydrogenase and 3α-hydroxysteroid dehydrogenase in seminal vesicles of rats were examined to clarify the metabolic dynamics and action of androgens in this target organ of androgens.

The following abbreviations of steroids, enzymes and cofactors were used in this text: 5α-androstanediol, 5α-androstane-3α,17β-diol; Δ^4-5α-hydrogenase, 4-en-3-oxosteroid:NADPH 5α-oxidoreductase; 3α-hydroxysteroid dehydrogenase, 3α-hydroxysteroid:NAD(P) oxidoreductase (EC 1.1.1.50); NAD^+ and NADH, nicotinamide-adenine dinucleotide and its reduced form; and $NADP^+$ and NADPH, nicotinamide-adenine dinucleotide phosphate and its reduced form.

* To whom reprint requests and inquiries should be addressed.

EXPERIMENTAL

Subcellular fractionation of seminal vesicles

Male Wistar rats (about 10-weeks-old) bred in this Institute, were decapitated and the seminal vesicles were immediately removed. The isolated organs were freed of adhering fat and other tissues, and the seminal fluid was squeezed out. The tissue was then finely minced with scissors in ice-cold 0·25 M sucrose solution (pH 7·4), ground in a mortar, homogenized in the sucrose solution with a loose-fitting Teflon–glass homogenizer, and finally filtered through Nylon net (100 mesh). The filtrate was centrifuged at 800 *g* for 20 min, and the precipitate was reserved for further preparation of the nuclear fraction. The 800 *g* supernatant fluid (hereafter called the "cell-free homogenates") was further centrifuged at 1,000 *g* for 20 min. The supernatant fluid at 1,000 *g* was again centrifuged at 6,000 *g* for 20 min, the precipitate yielding the mitochondrial fraction. The 6,000 *g* supernatant fluid was spun at 10,000 *g* for 20 min and the supernatant fluid obtained was further centrifuged at 105,000 *g* for 60 min giving a

microsomal fraction as the sediment and cytosol fraction as the supernatant fluid.

The nuclear fraction was prepared according to the method of Maggio *et al.* [8], with minor modifications. The 800 *g* precipitate was re-suspended in 0·88 M sucrose solution (pH 7·4), containing 1·5 mM $CaCl_2$. Five ml of 1·8 M sucrose solution were gently layered over 5 ml of 2·2 M sucrose solution in 30 ml centrifuge tubes. Both solutions contained 0·5 mM $CaCl_2$ and were adjusted at pH 7·4. Twenty ml of the tissue suspension in 0·88 M sucrose solution was then gently layered over the discontinuous sucrose gradients. The tubes were centrifuged at 33,000 *g* for 90 min in a swinging bucket rotor (no. SB-110, model B-60, International Equipment Co., Mass. U.S.A.). The 33,000 *g* precipitate is hereafter referred to as the nuclear fraction. The nuclear, mitochondrial and microsomal pellets were washed with the isotonic sucrose solution to remove the cytosol fraction and re-suspended in isotonic sucrose solution. Centrifugations were carried out at 0 C and the other procedures at temperatures less than 5°C.

Specimens of the different organella fractions were examined under an electron microscope (model HU-11D-S, Hitachi, Japan), according to the method previously described [9].

Steroids and cofactors

[4-^{14}C]-Testosterone (S.A. 58·2 mCi/mmol) was purchased from the Radiochemical Centre (Amersham, England). [4-^{14}C]-5α-Dihydrotestosterone was enzymatically prepared from [4-^{14}C]-testosterone by the nuclear fraction of rat ventral prostate and its identity was firmly established by thin-layer chromatography and re-crystallization [4]. Radiochemical purities of labelled steroids were confirmed by thin-layer chromatography just before use. Radioactive steroids were appropriately diluted with corresponding non-radioactive steroids in order to saturate the enzymes. NADPH and $NADP^+$ were obtained from Boehringer, Mannheim, Germany and NADH and NAD^+ from Sigma Chemical Co., St. Louis, Mo., U.S.A.

[4-^{3}H]-$NADP^+$ (29·0 μCi/mmol) was synthesized from $NADP^+$ (Boehringer) by exchange reaction with ^3H_2O in the presence of NaCN by the method of San Pietro *et al.* [10] and Krakow *et al.* [11]. [4-*pro*-*R*-^{3}H]-NADPH ($2·68 \times 10^4$ c.p.m./mg) was enzymatically prepared from [4-^{3}H]-$NADP^+$ with glucose-6-phosphate (Sigma) and glucose-6-phosphate dehydrogenase (Sigma), and [4-*pro*-*S*-^{3}H]-NADPH ($2·72 \times 10^4$ c.p.m./mg) was obtained from [4-^{3}H]-$NADP^+$ with isocitrate (Sigma) and isocitrate dehydrogenase (Sigma), according to the method of Abul-Hajj [12]. After incubation, the reaction mixtures were heated at 70°C for 2 min to inactivate the dehydrogenase activities and were subsequently employed as the labelled cofactors.

Incubation

Each incubation flask contained the following, unless otherwise mentioned: steroid dissolved in 2 drops of propylene glycol, enzyme preparation (2·1 43·2 mg of protein) and cofactor (NADPH, 240 or 480 μM final concentration) in 0·25 M sucrose solution buffered at pH 7·4 with 10 mM Tris–HCl, containing 1 mM $MgCl_2$. The final vol. of each incubation mixture was adjusted to 5 ml. Testosterone (2·22 nmol, $5·0 \times 10^4$ c.p.m.) was added per flask as the substrate for the assay of Δ^4-5α-hydrogenase, and 5α-dihydrotestosterone (34·4 nmol, $1·8 \times 10^4$ c.p.m.) for the assay of 3α-hydroxysteroid dehydrogenase. Incubation was carried out at 37°C for 60 min in air with constant shaking. Under this condition, appreciable amounts of substrate remained unchanged at the end of incubation, indicating substrate saturation of the enzyme systems.

Pre-incubation of testosterone with cytosol fraction

For pre-incubation studies, testosterone in 2 drops of propylene glycol was mixed with 2 ml of cytosol fraction of seminal vesicles and incubated for 20 min at 37°C without cofactor. Subsequently, the enzyme preparation and cofactor were added and further incubated for 60 min at 37 C. In other experiments, the cytosol fraction was first heated in a boiling water bath for 20 min and then centrifuged at 10,000 *g* for 20 min to remove precipitated denatured protein. The supernatant fluid was then referred to as "heated cytosol fraction" and also used in pre-incubation studies in the same manner as described above.

Extraction of radioactive steroids from the incubation mixtures, separation of each metabolite and measurement of radioactivity were carried out as previously reported [13].

Identification of metabolites

The following criteria were employed for identification of radioactive metabolites, in comparison with authentic preparations: (1) identical mobility on thin-layer chromatograms using different solvent systems; (2) identical behaviour against several chemical reagents; (3) constant specific activity of crystals on repeated crystallization with authentic preparation.

Expression of enzyme activity

Specific activity of the enzymes was expressed as the sum of identified products (pmol) formed by the enzyme for 60 min per mg protein. Protein content in each subcellular fraction was measured by the copper–Folin method [14].

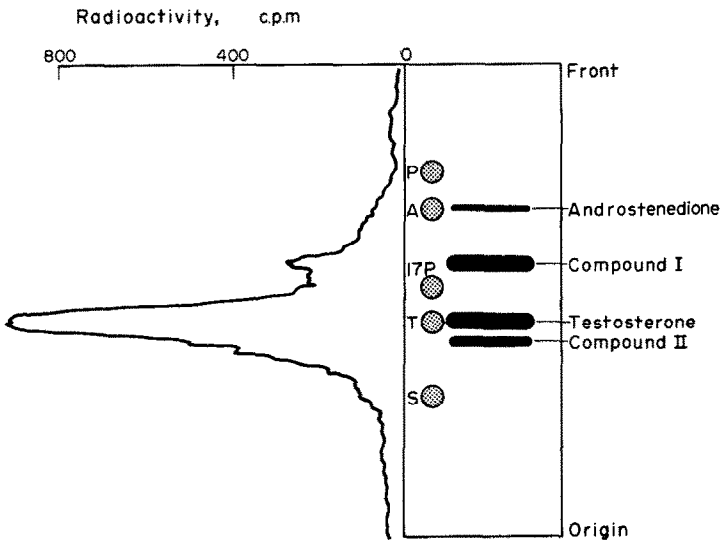

Fig. 1. Radioscannogram (left) and a sketch of a radioautogram (right) of the metabolites, which were transformed from [4-^{14}C]-testosterone by the cell-free homogenates of rat seminal vesicles.

Mobilities of Compounds I and II on thin-layer chromatograms were identical to those of 5α-dihydrotestosterone and 5α-androstane-3α,17β-diol, respectively. The developing system for the thin-layer chromatography was benzene–acetone (4:1, v/v).

▬ : Radioactive areas detected by radioautography.

▬ : Spots of marker 4-en-3-oxosteroids. P, A, 17P, T and S represent progesterone, androstenedione, 17α-hydroxyprogesterone, testosterone and 11-deoxycortisol, respectively.

Influence of metal ions and metabolic inhibitors on the testosterone metabolizing enzymes

Enzyme reactions were carried out in the presence of various metal cations, metal chelating agents, mercaptide-forming agents, etc. As the sources of heavy metal ions, $MnSO_4$, $FeSO_4.(NH_4)_2SO_4$, $CoCl_2$, $CdSO_4$, $CuSO_4$, $HgCl_2$, $Sr(NO_3)_2$ and CsCl were used. As metal chelating agents, disodium EDTA, α,α′-dipyridyl and *o*-phenanthroline were examined. *p*-Chloromercuribenzoate (*p*-CMB) and monoiodoacetamide were used as mercaptide-forming and thiol-alkylating reagents. All concentrations were 1×10^{-3} M. The pH of incubation mixtures containing inorganic salts or disodium EDTA was adjusted to pH 7·4. Since α,α′-dipyridyl and *o*-phenanthroline were insoluble in the sucrose solution, these compounds were previously dissolved in 0·1 ml of ethanol before being added to the incubation mixture. In this case, the control also contained 0·1 ml of ethanol in the incubation mixture.

RESULTS

Metabolism of testosterone in cell-free homogenates of seminal vesicles

When the cell-free homogenates of rat seminal vesicles were incubated with [4-^{14}C]-testosterone, two main metabolites were obtained besides androstenedione by t.l.c. The less polar of the two was designated Compound I and the other Compound II, as illustrated in Fig. 1. Mobilities of Compounds I and II on thin-layer chromatograms were identical to those of 5α-dihydrotestosterone and 5α-androstane-3α,17β-diol. When Compounds I and II were oxidized by 0·5% CrO_3 in 90% aqueous acetic acid solution, a steroid which had an identical mobility to 5α-androstane-3,17-dione on thin-layer chromatograms was obtained as the major reaction product in both cases. When subjected to acetylation, the product obtained from Compound I was identical to 5α-dihydrotestosterone 17β-acetate, while the product of Compound II was identical to 5α-androstanediol 3α,17β-diacetate. Finally, after repeated crystallization of radioactive Compounds I and II with authentic preparations of 5α-dihydrotestosterone and 5α-androstanediol respectively, from different solvent systems, constant specific activities of crystals were obtained (Table 1).

When [4-^{14}C]-5α-dihydrotestosterone was incubated as the substrate, the major metabolite was identified as 5α-androstane-3α,17β-diol by the same procedures as described above.

Morphological examination of organella fractions

Each organella fraction was morphologically examined under an electron microscope, as shown in Figs. 2–5. The nuclear fraction consisted mainly of nuclei (Fig. 2), although some of the nuclear membrane were

Table 1. Identification of Compounds I and II by recrystallization with 5α-dihydrotestosterone and 5α-androstane-3α,17β-diol

	Compound I (crystallized with 5α-dihydrotestosterone)		Compound II (crystallized with 5α-androstane-3α,17β-diol	
	Specific activity of crystal (c.p.m./mg)	Solvents	Specific activity of crystal (c.p.m./mg)	Solvents
1st	657	Dichloromethane–*n*-Heptane	1099	Ethanol–water
2nd	658	Chloroform–*n*-Heptane	1029	Tetrahydrofuran–*n*-Heptane
3rd	642	Dioxane–*n*-Heptane	958	Methanol–water
4th	655	Ethylacetate–*n*-Heptane	955	Ethylacetate–*n*-Heptane
Calculated specific activity	651		1041	

ruptured probably due to the drastic change of osmotic pressure during purification. In the mitochondrial fraction, mitochondria were observed as the main constituent and the cristae were noted but some membrane structures derived from the endoplasmic reticula were also observed (Fig. 3). The microsomal fraction mainly consisted of homogeneous membranes with ribosomal particles on the surface (Fig. 4).

Intracellular distribution of Δ^4-5α-*hydrogenase*

Among the subcellular fractions of seminal vesicles, the highest specific activity of Δ^4-5α-hydrogenase was detected in the microsomal fraction followed by the nuclear fraction (Table 2). In another experiment, however, the specific activity of the enzyme in the nuclear fraction was found to be higher than that in the microsomal fraction (see Table 3). Some enzyme activity was detected in the mitochondrial fraction, but the cytosol fraction was nearly devoid of enzyme activity. The ratio of 5α-androstanediol to 5α-dihydrotestosterone was highest in the cytosol fraction, followed by the microsomal and mitochondrial fractions, and finally very low in the nuclear fraction. For the 5α-hydrogenation of testosterone, NADPH was preferred over NADH as cofactor in both the nuclear and microsomal fractions.

Intracellular distribution of 3α-*hydroxysteroid dehydrogenase*

[4-^{14}C]-5α-Dihydrotestosterone was most efficiently converted to 5α-androstanediol in the cytosol fraction, as expressed in terms of specific activity (Table 2).

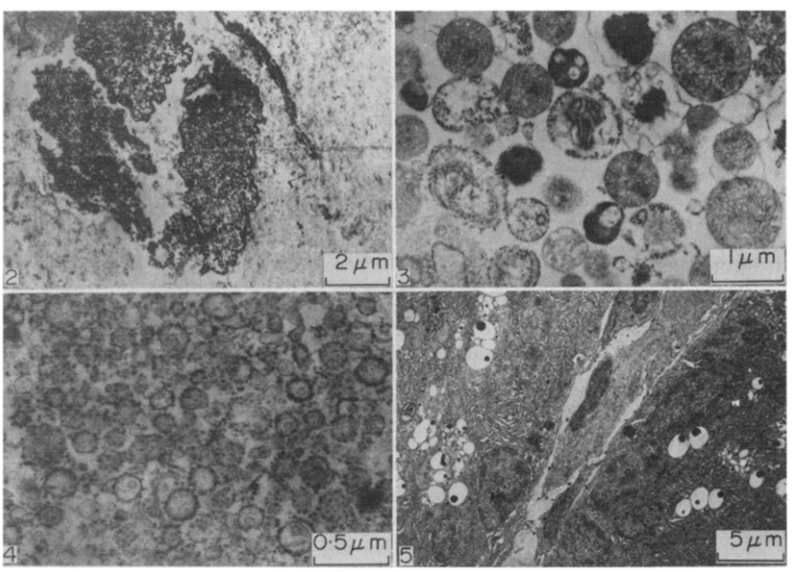

Fig. 2. Electron micrograph of the nuclear fraction of rat seminal vesicle.

Fig. 3. The mitochondrial fraction of the same.

Fig. 4. The microsomal fraction of the same.

Fig. 5. Intact seminal vesicle of rat.

Table 2. Intracellular distributions of Δ^4-5α-hydrogenase and 3α-hydroxysteroid dehydrogenase in rat seminal vesicle

Subcellular fraction	Specific activity (pmol products for 60 min/mg protein) Δ^4-5α-Hydrogenase*	3α-Hydroxysteroid dehydrogenase
Nuclear fraction	26·4 (0·13)†	$3{\cdot}4 \times 10^2$
Mitochondrial fraction	18·0 (0·32)	$2{\cdot}9 \times 10^2$
Microsomal fraction	42·5 (0·37)	$2{\cdot}4 \times 10^2$
Cytosol fraction	2·4 (4·17)	$17{\cdot}0 \times 10^2$

Final concentration of NADPH was 480 μM.

* Δ^4-5α-Hydrogenase activity is expressed as the sum of 5α-dihydrotestosterone and 5α-androstanediol produced from testosterone.

† Ratio of 5α-androstanediol to 5α-dihydrotestosterone.

Using the cytosol fraction as the source of 3α-hydroxysteroid dehydrogenase, the preferred cofactor for this enzyme was NADPH, when compared with NADH, $NADP^+$ and NAD^+. However, $NADP^+$ was almost as efficient as its reduced form. After spectrometric analysis, it was found that, when $NADP^+$ (1·2 μmol) was incubated alone with the cytosol fraction, $NADP^+$ was enzymatically transformed to NADPH (0·45 μmol after 40 min).

Influence of the cytosol fraction on Δ^4-5α-hydrogenase

When [^{14}C]-testosterone was pre-incubated with 2 ml of the native cytosol fraction at 37°C for 20 min and then re-incubated with the nuclear or microsomal fraction in the presence of NADPH, Δ^4-5α-hydrogenase activities in the two fractions decreased in comparison with those without cytosol fraction (Table 3). When the amount of pre-incubated native cytosol fraction was increased, the inhibitory effect upon the Δ^4-5α-hydrogenase in the microsomal fraction was proportionally enhanced. By heating the cytosol fraction at 100°C, the inhibitory effect of the cytosol fraction diminished considerably (Table 3). In the case of pre-incubation with the native cytosol fraction, the relative production of 5α-androstanediol from 5α-dihydrotestosterone was increased but, if heated cytosol was used, the ratio remained similar to the value of the control without the cytosol fraction.

Characteristics of Δ^4-5α-hydrogenase and 3α-hydroxysteroid dehydrogenase

When incubations were carried out at 37°C in solutions of different pH buffered with 0·1 M citric acid and 0·2 M Na_2HPO_4, Δ^4-5α-hydrogenases in the nuclear and microsomal fractions exhibited an optimal pH range between 5·7 to 5·9, while 3α-hydroxysteroid dehydrogenase showed a pH optimum around 6·3 (Fig. 6).

By incubating at various temperatures, in pH 7·4, the highest production of 5α-dihydrotestosterone from testosterone by nuclear or microsomal Δ^4-5α-hydrogenase was observed around 37°C, and the highest activity of 3α-hydroxysteroid dehydrogenase was observed at 50°C (Fig. 7).

Michaelis constants (K_m) of the enzymes were estimated by the Lineweaver–Burk plot [15]. The Michaelis constants (K_m) for testosterone of the nuclear and microsomal Δ^4-5α-hydrogenase were 1·10 and 0·70 μM, respectively. K_m for 5α-dihydrotestosterone of the 3α-hydroxysteroid dehydrogenase in the cytosol fraction was 9·64 μM.

Table 3. Influence of pre-incubation of testosterone with the native or heated cytosol fractions upon Δ^4-5α-hydrogenase activity

Pre-incubation	Specific activity of Δ^4-5α-hydrogenase (pmol products for 60 min/mg protein) Nuclear fraction	Microsomal fraction
with native cytosol*	18·4 (6·25)‡	13·3 (0·71)‡
with the heated cytosol†	26·5 (0·18)	17·7 (0·30)
without cytosol	24·9 (0·16)	19·9 (0·59)

Final concentration of NADPH was 240 μM.

* [^{14}C]-Testosterone was pre-incubated with 2 ml of cytosol fraction at 37°C for 20 min and after cofactor and enzyme preparation were added, the mixture was incubated again at 37°C for 60 min.

† The cytosol fraction was heated for 20 min in a boiling water bath, and the denatured protein was removed by centrifugation at 10.000 *g* for 20 min. The supernatant fluid was subjected to preincubation.

‡ Ratio of 5α-androstanediol to 5α-dihydrotestosterone.

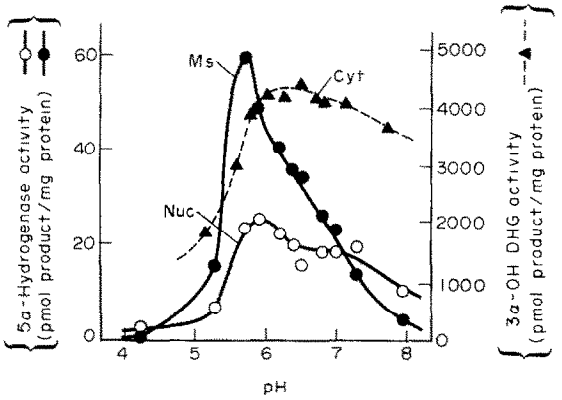

Fig. 6. pH dependency of 5α-hydrogenase and 3α-hydroxysteroid dehydrogenase of rat seminal vesicles. The microsomal (Ms, 8·52 mg/flask) and nuclear (Nuc, 1·20 mg/flask) fractions of the seminal vesicles were incubated with testosterone at 37 C at various pH (from 4–8) buffered with citric acid and Na_2HPO_4, in the presence of NADPH (240 μM for 60 min. The cytosol fraction (Cyt, 7·52 mg/flask) was incubated with 5α-dihydrotestosterone under the same condition as stated above. 5α-Hydrogenase activity was expressed as the amount of 5α-hydrogenated steroids produced for 60 min in pmol/mg protein (left ordinate), while 3α-hydroxysteroid dehydrogenase activity was expressed in the amount of 5α-androstane-3α,17β-diol produced for 60 min in pmol/mg protein (right ordinate). More details were described in the text.

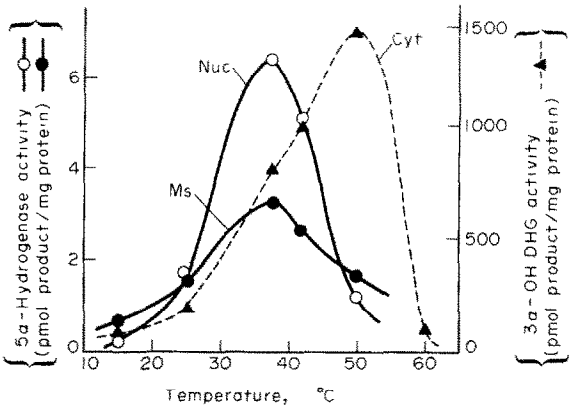

Fig. 7. Optimal temperature of Δ^4-5α-hydrogenase and 3α-hydroxysteroid dehydrogenase of rat seminal vesicles. Temperature dependency of 5α-hydrogenase and 3α-hydroxysteroid dehydrogenase of rat seminal vesicles. The nuclear (Nuc, 1·20 mg/flask) and microsomal (Ms, 8·52 mg/flask) fractions of the seminal vesicles were incubated with testosterone and the cytosol fraction (Cyt, 7·52 mg/flask) was incubated with 5α-dihydrotestosterone at pH 7·4 at various temperatures for 60 min in the presence of NADPH (240 μM). The enzyme activities were expressed in the same unit as employed in Fig. 6.

Influence of metal ions and metabolic inhibitors

Among the metal ions, Cu^{2+}, Ca^{2+} and Hg^{2+} at 1×10^{-3} M inhibited Δ^4-5α-hydrogenase and 3α-hydroxysteroid dehydrogenase almost completely (Table 4). Zn^{2+} and Co^{2+} inhibited Δ^4-5α-hydrogenase activity to a lesser but still considerable extent. Zn^{2+} reduced the 3α-hydroxysteroid dehydrogenase activity as well. Mn^{2+} and Fe^{2+} moderately reduced Δ^4-5α-hydrogenase activity. On the other hand, Co^{2+}, Mn^{2+} and Fe^{2+} had no effect on 3α-hydroxysteroid dehydrogenase. Sr^{2+}, Cs^+ and Ba^{2+} showed no effect on the activities of both enzymes.

Δ^4-5α-Hydrogenase activity was fairly reduced by EDTA and *o*-phenanthroline, whereas the chelating agents had no influence on 3α-hydroxysteroid dehydrogenase. *p*-CMB inhibited both enzymes markedly, while monoiodoacetamide only inhibited 3α-hydroxysteroid dehydrogenase.

Stereospecificity of hydrogen transfer from NADPH to steroids by microsomal Δ^4-5α-hydrogenase and cytosol 3α-hydroxysteroid dehydrogenase

[4-^{14}C]-Testosterone (3·5 μmol. $1·0 \times 10^4$ c.p.m.) was incubated with the microsomal fraction (43·2 mg protein) at 37°C for 90 min in the presence of [4-*pro*-*R*-^{3}H] or [4-*pro*-*S*-^{3}H]-NADPH. The 5α-dihydrotestosterone and 5α-androstane-3α,17β-diol produced were combined and oxidized by 0·5% CrO_3 in 90% aqueous acetic acid solution. The tritium and carbon-14 in the 5α-androstane-3,17-dione thus obtained were measured. The $^3H/^{14}C$ ratio of the steroid was 4·58, when [4-*pro*-*S*-^{3}H]-NADPH was employed as the hydrogen donor, while the ratio was 0·20 in the case of [4-*pro*-*R*-^{3}H] NADPH.

[4-^{14}C]-5α-Dihydrotestosterone (3·5 μmol. $5·0 \times 10^3$ c.p.m.) was incubated with the cytosol fraction (33·2 mg protein) at 37°C for 60 min in the presence of the above two types of tritiated NADPH. The $^3H/^{14}C$-ratio of 5α-androstane-3α,17β-diol was 5·17 when [4-*pro*-*S*-^{3}H] NADPH was employed as the cofactor. On the other hand, the ratio was 0·84 in the case of [4-*pro*-*R*-^{3}H] NADPH. The $^3H/^{14}C$ ratio of 5α-androstane-3α,17β-diol obtained in the presence of [4-*pro*-*S*-^{3}H] NADPH remained unchanged when the steroid was acetylated with the mixture of acetic anhydride and pyridine (1:1, v/v). However, the tritium content dropped almost to zero, when oxidized with CrO_3 in aqueous acetic acid solution.

DISCUSSION

The intracellular distributions of Δ^4-5α-hydrogenase and 3α-hydroxysteroid dehydrogenase in the seminal vesicle were similar to those of rat prostate, which has been established as another target organ of androgens

Table 4. Influence of metal ions, metal chelating agents and metabolic inhibitors upon Δ^4-5α-hydrogenase and 3α-hydroxysteroid dehydrogenase activities

Heavy metal ions, metal chelating agents and metabolic inhibitors (1×10^{-3} M)	Percentage of enzyme activity Δ^4-5α-Hydrogenase		3α-Hydroxysteroid dehydrogenase
	Nuclear fraction (%)	Microsomal fraction (%)	(%)
None	100	100	100
Cu^{2+}	3	1	7
Cd^{2+}	3	3	7
Hg^{2+}	6	1	5
Zn^{2+}	17	12	17
Co^{2+}	24	23	103
Mn^{2+}	73	57	108
Fe^{2+}	76	36	103
Sr^{2+}	79	98	109
Cs^{+}	100	100	103
Ba^{2+}	100	90	96
EDTA	45	29	99
o-Phenanthroline	45	75	106
α,α'-Dipyridyl	85	86	90
p-Chloromercuribenzoate	29	11	5
Monoiodoacetamide	100	88	68

[2, 4]. When testosterone was incubated with the subcellular fractions, the ratio of 5α-androstanediol to 5α-dihydrotestosterone (see the figures in parentheses in Table 2) was considered as an index of relative activity of 3α-hydroxysteroid dehydrogenase present in each fraction. An extremely high ratio was observed in the cytosol fraction, being consistent with the distribution of 3α-hydroxysteroid dehydrogenase in which 5α-dihydrotestosterone was used as the substrate.

In the seminal vesicles of castrated rats, binding of [^{3}H]-testosterone to soluble macromolecules has been shown both *in vivo* and *in vitro* in the 100,000 *g* supernatant fluid of the homogenates [16, 17]. When testosterone was pre-incubated with native cytosol fraction, subsequent incubation of the pre-incubated mixture with the nuclear and microsomal fractions resulted in a marked decrease of 5α-hydrogenation of testosterone (Table 3). It is suggested that during pre-incubation with the cytosol fraction, some of the testosterone is bound to certain heat-labile macromolecules in the cytosol and this testosterone-macromolecule complex is not as easily accessible to the Δ^4-5α-hydrogenase in the nuclear and microsomal fraction as the free form. Organ specificity of the macromolecule which shows affinity for testosterone and other possible modes of inhibition of Δ^4-5α-hydrogenase are to be examined further.

The conversion of $NADP^+$ to NADPH by the cytosol fraction suggests the presence of glucose-6-phosphate and its dehydrogenase system and probably also isocitrate and its dehydrogenase [18]. These two enzymes have been reported to be concentrated in the cytosol fraction of the liver [19].

In rat seminal vesicles, Δ^4-5α-hydrogenase showed a pH optimum around 5·8 and 3α-hydroxysteroid dehydrogenase had a broad range of optimal pH around 6·3, while in the prostate of the same animal, the optimal pH of Δ^4-5α-hydrogenase was 7·0 [4, 20]. The highest activity of 3α-hydroxysteroid dehydrogenase was observed at 50°C in seminal vesicles. Similarly, optimal temperatures for 3α-hydroxysteroid dehydrogenase in the cytosol of rat prostate [21], 17β-hydroxysteroid dehydrogenase in the microsomes of rat testes [22], and 20α-hydroxysteroid dehydrogenase in the cytosol of porcine testes [23] have also been reported to be around 50°C.

Grant *et al.* [24] reported that $ZnCl_2$ (5×10^{-5} M) inhibited Δ^4-5α-hydrogenase originating from the nuclei of human hyperplastic prostatic tissue, whereas concentrations of Cd^{2+}, Hg^{2+} and Mn^{2+} up to 5×10^{-4} M did not inhibit. In our experiments, however, the metal ions, in concentrations of 1×10^{-3} M showed various degrees of inhibition of Δ^4-5α-hydrogenase in rat seminal vesicle (Table 4). Since EDTA and *o*-phenanthroline showed an inhibitory effect on Δ^4-5α-hydrogenase, it is suggested that this enzyme requires certain metal cations for demonstration of its full activity. From the inhibitory effects of *p*-CMB on Δ^4-5α-hydrogenase and 3α-hydroxysteroid dehydrogenase, it can be concluded that the activities of the two enzymes are closely related to the SH groups present in the molecule of these enzymes.

In the present experiments, similarity of enzyme characteristics between the nuclear and microsomal Δ^4-5α-hydrogenases suggests that the site of biosynthesis of the two enzymes could be closely related. Recently, Δ^4-5α-hydrogenase in rat prostate has been reported to be localized in the outer membrane of the nuclei [25, 26] and also relatively in the rough-surfaced endoplasmic reticula [26]. The outer membrane of the nuclei has structures morphologically similar to the granular endoplasmic reticula [27]. It can be noted in the electron micrograph of intact seminal vesicle cell (Fig. 5) that there is an abundance of granular endoplasmic reticula and that the microsomal fraction (Fig. 4) consists mainly of fragments of rough-surfaced endoplasmic reticula, which are regarded as a site of protein synthesis.

Stereospecific transfer of 4-*pro*-*S*-hydrogen of NADPH has been established in the reduction of testosterone to 5α-dihydrotestosterone catalyzed by microsomal 5α-hydrogenase in rat seminal vesicles. This is in agreement with results obtained on 5α-hydrogenase in the microsomal fraction of rat liver [14, 28]. Similarly, in the present experiment, 4-*pro*-*S*-hydrogen of NADPH was transferred to 5α-androstane-3α,17β-diol by cytosol 3α-hydroxysteroid dehydrogenase, coinciding with the results on 3α-hydroxysteroid dehydrogenase in *Pseudomonas testosteroni* [29]. It is interesting to note that the 3α-hydroxysteroid dehydrogenase activity of cortisone reductase from *Streptomyces* spp., which converts 3-oxo-C_{19}-steroid to a 3α-hydroxy compound, utilizes the 4-*pro*-*S*-hydrogen of NADH [30]. On the other hand, this enzyme in rat liver cytosol was reported to utilize 4-*pro*-*R*-hydrogen of NADPH for reduction of C_{19}-, C_{21}-, C_{24}- and C_{27}-steroids [31]. The fact that tritium in the 5α-androstane-3α,17β-diol which was transferred from NADPH by the 3α-hydroxysteroid dehydrogenase was lost by oxidation, but remained after acetylation suggests that tritium in the 5α-androstane-3α,17β-diol was present as 3β-hydrogen.

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