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METABOLISM OF TESTOSTERONE AND DIHYDROTESTOSTERONE IN SUBCELLULAR FRACTIONS OF SEMINIFEROUS TUBULES AT THE TIME OF INITIATION OF THE MEIOTIC DIVISION

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SUMMARY

Homogenates of seminiferous tubules isolated from 26-day-old rats were fractionated by ultracentrifugation and the fractions incubated with [^{3}H]-testosterone and [^{3}H]-dihydrotestosterone to study the subcellular distribution of testosterone oxidoreductases. Total tissue homogenates showed active 5α-reductase and 3α-hydroxysteroid dehydrogenase activities and little 3β-hydroxysteroid dehydrogenase. 3α-Hydroxysteroid dehydrogenase was mainly located in the soluble fraction of the cells while 3β-hydroxysteroid dehydrogenase and 5α-reductase activities were found to be present in the particulate fractions of the cytoplasm and in the nuclei. This distribution of enzyme activities is similar to that reported for other androgen target tissues. The highly active soluble 3α-hydroxysteroid dehydrogenase is probably responsible for the high concentrations of 5α-androstan-3α,17β-diol that we had previously found in the seminiferous tubules at the time of initiation of the meiotic division.

INTRODUCTION

Reduction of testosterone (T) to dihydrotestosterone (DHT) has been postulated as one of the stages of the mechanism of action of androgens in target tissue. NADPH-dependent 4-ene-3-ketosteroid 5α-oxidoreductases (5α-reductase) have been described in the nuclear and microsomal fractions of several androgen-responsive tissues, such as prostate [1, 2], and seminal vesicles [3, 4]. They have also been found localized in these fractions in kidney [5, 6], liver [7] and pituitary [8]. We had previously reported that seminiferous tubules of developing rats show very active 5α-reductase and 3α-hydroxysteroid dehydrogenase activities with maximal activity at the time of initiation of the meiotic division [9]. This coincided with the maximal tubular concentration of 5α-androstane-3α,17β-diol (3α-DIOL) [10]. It was the purpose of this work to study the subcellular distribution of these reductases in seminiferous tubules of developing rats.

EXPERIMENTAL

Radioactive steroids: [7α-^{3}H]-T, S.A. 24.8 Ci/mmol; [1,2-^{3}H]-DHT, S.A. 44 Ci/mmol; [4-^{14}C]-T, S.A. 57.6 mCi/mmol; and [4^{14}C]-DHT, S.A. 50.9 mCi/mmol were purchased from New England Nuclear. [4-^{14}C]-3α-DIOL was prepared by incubation of immature testes with [4-^{14}C]-T as already published [9]. [4-^{14}C]-5α-androstane-3β,17β-diol (3β-DIOL) was prepared by sodium borohydride reduction of [4-^{14}C]-DHT. All radioactive steroids were purified by paper chromatography.

Preparation and incubation of subcellular fractions from seminiferous tubules

Male Wistar rats, aged 26 days, were used. Animals were sacrificed by cervical dislocation. The testes were weighed and tubules and interstitial tissue were separated as previously described [11]. Tubules were washed twice with Krebs–Ringer phosphate buffer pH 7.4, and once with 0.88 M Sucrose, 50 mM Tris–HCl buffer, pH 7.4. Isolated tubules were homogenized in 2 vol. of 0.88 M sucrose, 50 mM Tris–HCl buffer pH 7.4 and filtered through Nitex 23.

Aliquots of the homogenate, diluted with buffer to obtain a 0.25 M sucrose concentration, were kept for incubation, DNA and protein determinations. The remaining was centrifuged at 800 *g* for 15 min in an International refrigerated centrifuge. The unpurified nuclear fraction was resuspended and centrifuged twice. The resulting pellet was suspended in 7.5 ml of 2.0 M sucrose, the suspension, divided into three portions, was layered on tubes containing 2.5 ml of 2.2 M sucrose, and centrifuged in a SW 65 rotor (Beckman) for 60 min at 60000 *g* [12]. The supernatant from this centrifugation was decanted and the walls of the tubes were cleaned. The 3 pellets were combined and resuspended in 3.3 ml of 0.25 M sucrose 50 mM Tris–HCl (nuclear fraction).

The supernatant from the original 800 *g* spin was centrifuged at 15000 *g* for 30 min. The pellet (enriched

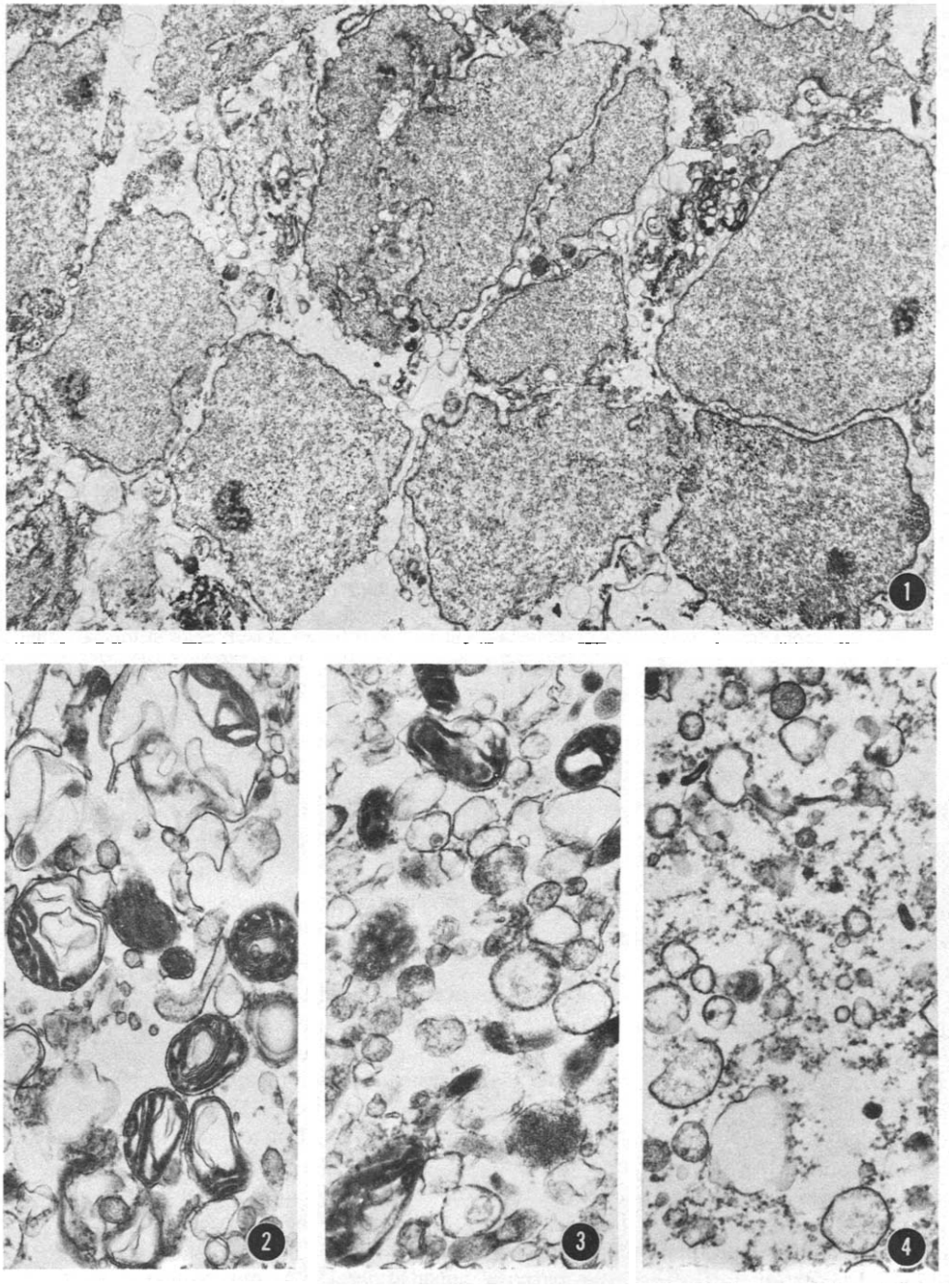

Fig. 1. Electron microscopy picture of the nuclear fraction showing a predominant nuclear population. Intact nuclei, as well as fragments of nuclear material could be seen. In addition, there is a contamination of cytoplasmic structures and fragments of the tubular wall, but in no case this extra-nuclear material was more than 20–30% of the total.

Fig. 2. Suspension of the 15000 *g* pellet containing numerous mitochondria of the spermatocyte and spermatid type, some of them altered, and membranous profiles presumably belonging to the outer mitochondrial membrane. In addition, numerous smaller vesicular components related to the endoplasmic reticulum and lysosomes as well as free or attached ribosomes, can be seen in this fraction.

Fig. 3. Suspension of the 105000 *g* pellet showing a heterogenous picture with microsomes and other cytoplasmatic components, mainly mitochondria.

Fig. 4. A different zone of the 105000 *g* pellet, showing areas with vacuolar structures with isolated round vesicles, some cisternae with ribosomes attached to them, as well as numerous free ribosomes.

in mitochondria) was saved. The new supernatant was diluted 3 times with 50 mM Tris–HCl buffer, pH 7.4 and then centrifuged at 105000 *g* for 60 min. Pellet (enriched in microsomes) and supernatant (cytosol) were saved.

The pellets from the last two centrifugations were resuspended in 6 vol. of testicular weight (w/v) with 0.25 M sucrose, 50 mM Tris–HCl buffer. The various fractions (whole homogenate, nuclei, suspension of 15000 *g* pellet, suspension 105000 *g* pellet and cytosol) were analyzed for protein, DNA and steroid enzyme activities.

Fragments of the 3 pellets were fixed by immersion in phosphate-buffered glutaraldehyde, post-fixed in phosphate-buffered osmium and embedded in Maraglass, previous "in block" staining with uranyl acetate. Thin sections exhibiting silver to pale gold interference colors were cut in a Porter Blum MT 2 Ultramicrotome and double stained with uranyl acetate and lead citrate.

Grids were examined and pictures were taken in a Phillips 200 Electron Microscope. Representative pictures of the 3 fractions examined are shown in Figs. 1–4. For incubation, 0.25 ml aliquots were taken from each fraction and made up to a final vol. of 0.5 ml with 0.25 M sucrose 50 mM Tris–HCl containing 1×10^{-4} M NADPH, and [7α-^{3}H]-T (2×10^5 d.p.m., 7×10^{-9} M) or [1,2-^{3}H]-DHT (4×10^5 d.p.m., 7.6×10^{-9} M). In some instances, a NADPH generating system consisting of $NADP^+$ 10^{-4} M, glucose-6-phosphate dehydrogenase, 2.5 IU per ml, and glucose-6-phosphate, 10^{-3} M was used, instead of NADPH. Incubation times were 5, 10, 20 or 30 min and temperature 31°. For the estimation of apparent K_M value of 3α-hydroxysteroid dehydrogenase the assay mixture contained 0.1 ml of total cytosol, 6.2×10^{-9} M [1,2-^{3}H]-DHT; 5, 10, 20, 30, 50, 100, 150 and 200 ng of nonradioactive substrate and 2.4×10^{-4} M NADPH in a final vol. of 0.5 ml. Incubation time was 10 min.

The reaction was stopped by the addition of 1 ml of methanol to each tube. Known amounts of ^{14}C tracers and 50 μg of non radioactive standards were added. The steroids were extracted with methanol: dichloromethane (1:1, v/v). Whatman No. 3 paper chromatography was carried out with the system of solvents methanol:water:hexane (90:10:100, by vol.) for 8 h at room temperature. Three areas of the paper, corresponding to the following steroids were eluted with methanol: area 1; 3β-DIOL, area 2, T and 3α-DIOL: area 3, DHT. The methanol was evaporated to dryness. All these compounds were acetylated for 15 h at room temperature using acetic anhydride–pyridine (1:1, v/v). T monoacetate and 3α-DIOL diacetate were then separated by silica-gel thin layer chromatography (t.l.c.) with the system of solvents dichloromethane:benzene:methanol (68:68:1.4, by vol.).

DHT acetate and 3β-DIOL diacetate were also submitted to t.l.c. with the same system of solvents. The corresponding areas were eluted. Aliquots of the samples were kept for recrystallization. ^{14}C and ^{3}H radioactivity was determined using a Packard Tri-Carb liquid Scintillation Spectrometer model 3001. Percentage distribution of ^{3}H radioactivity added remaining as the substrate or converted to various products was calculated using ^{14}C tracer recoveries to correct for losses. Constant ^{3}H/^{14}C ratios after three successive recrystallizations were observed in the main metabolic products for all experiments. Proteins were determined by the method of Lowry[13] and DNA by the method of Burton[14].

RESULTS

Metabolism of [^{3}H]-T and [^{3}H]-DHT following incubation of the homogenate and subcellular fractions of seminiferous tubules in 26-day-old rats

Percentage conversions of [^{3}H]-T to DHT, 3α-DIOL and 3β-DIOL at different incubation times in the homogenate and in the soluble fraction are shown in Fig. 5. In the homogenate, rapid metabolization of the substrate was shown by the fact that 60% of it was metabolized during the first 5 min of incubation. The sum of the three 5α-hydrogenated products accounted for practically all of the radioactivity metabolized at any time. During the first 5 min, DHT was the main metabolite. Later on, DHT decreased and 3α-DIOL became the main metabolic product at 10, 20 and 30 min. 3β-DIOL also showed some early accumulation and subsequent decline. In the cytosol, T was poorly metabolized and only small amounts of the reduced metabolites could be detected.

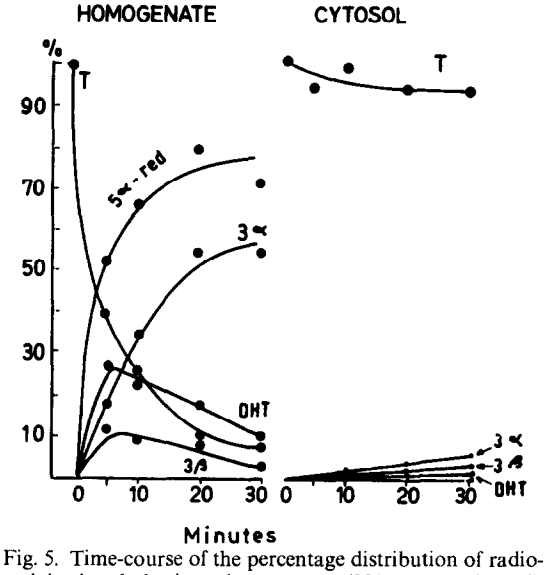

Fig. 5. Time-course of the percentage distribution of radioactivity in whole tissue homogenate (201 mg proteins, aliquot incubated 1.57 mg of proteins) and cytosol (93.6 mg proteins, aliquot incubated 0.78 mg) from 26-day-old rat seminiferous tubules incubated with [^{3}H]-T (7.6×10^{-9} M) in presence of NADPH (1×10^{-4} M). 5α-red: sum of DHT, 3α-DIOL and 3β-DIOL. 3α:3α-DIOL. 3β:3β-DIOL.

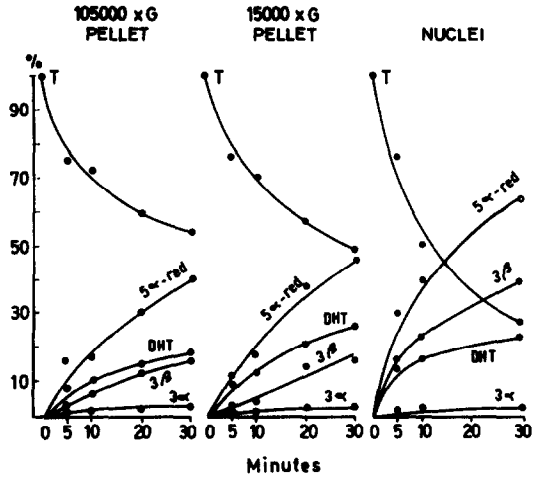

Fig. 6. Time-course of the percentage distribution of radioactivity in the 105000 *g* pellet (19.6 mg proteins, aliquot incubated 0.175 mg); 15000 *g* pellet (28 mg proteins, aliquot incubated 0.25 mg) and the nuclear fraction (61 mg proteins, aliquot incubated 1.12 mg; total nuclear proteins were corrected for losses according to DNA recovery) obtained from 26-day-old rat seminiferous tubules after incubation with [^{3}H]-T (7.6×10^{-9} M) in the presence of NADPH (1×10^{-4} M).

The time-course of the metabolism of [^{3}H]-T by 15000 *g* pellet, 105000 *g* pellet and nuclear fractions is shown in Fig. 6. 5α-reductase activity was present in all fractions but the pattern of metabolism was different from that of the homogenate. DHT and 3β-DIOL were the main metabolic products with little formation of 3α-DIOL.

A second experiment using [^{3}H]-T and [^{3}H]-DHT as substrates was carried out. Homogenate and subcellular fractions were incubated for 30 min. As it can be observed in Fig. 7, 3α-DIOL was the main metabolite formed from [^{3}H]-T and [^{3}H]-DHT in the total tissue homogenate incubation confirming the previous results. In the cytosol, T was not metabolized while practically all DHT was converted to 3α-DIOL. In the 15000 *g* pellet, 105000 *g* pellet, and nuclear fractions, most of T was converted to 5α-reduced products.

As opposed to the first experiment, some 3α-hydroxysteroid dehydrogenase activity was observed in the particulate fractions. However, this was not found in a third experiment in which incubation of 105000 *g* pellet and nuclei was carried out. Cytosol from 26-day-old rat seminiferous tubules was incubated for 30 min in two additional experiments (experiments 4 and 5) using a NADPH-generating system in which a high yield of 3α-DIOL was obtained from DHT. Incubations were also carried out in homogenate and subcellular fractions of sexually mature rats using [^{3}H]-T as precursor. Poor metabolization of the substrate precluded drawing any conclusions from these experiments.

For K_M measurement of 3α-hydroxysteroid dehydrogenase, initial velocities of the enzyme catalized reaction were calculated from the percentage of the 3-ketosteroid reduced. Double reciprocal plots, analyzed by the method of least squares were used to estimate the apparent K_M value. Results are shown in Fig. 8.

DISCUSSION

The extremely active capacity of the maturing rat testis to convert T into 3α-DIOL has been recognized for some time [15]. We have described that this conversion takes place mainly in the seminiferous tubules at the time of initiation of the meiotic division (20–26 days of age). This has been confirmed by others [16, 17] and furthermore, the activity has been localized in the Sertoli cells [18]. However, others have found formation of 3α-DIOL in the interstitial tissue [19]. Since it has been reported that 5α-reductase activity is membrane bound and localized in mic-

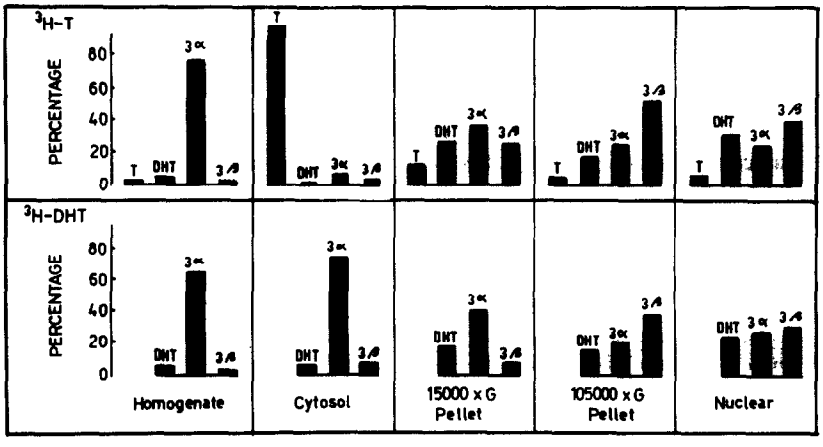

Fig. 7. Percentage distribution of radioactivity in various subcellular fractions after incubation with [^{3}H]-T or [^{3}H]-DHT for 30 min in presence of NADPH (1×10^{-4} M). Homogenate (130 mg proteins, aliquot incubated 2 mg), cytosol (57.4 mg proteins, aliquot incubated 0.96 mg), 15000 *g* pellet (22.8 mg proteins, aliquot incubated 0.36 mg), 105000 *g* pellet (14.7 mg proteins, aliquot incubated 0.23 mg), nuclear fraction (73.7 mg proteins, aliquot incubated 1.72 mg). 3α:3α-DIOL, 3β:3β-DIOL.

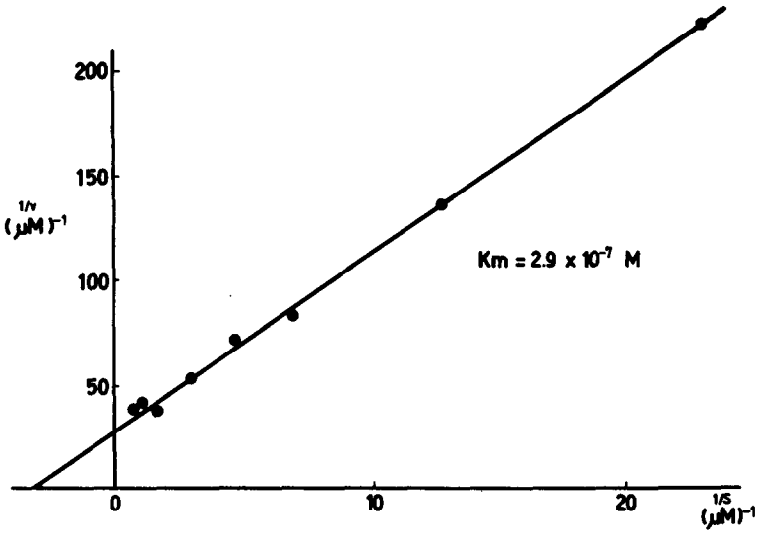

Fig. 8. Double reciprocal plot for the estimation of apparent K_M value of 3α-hydroxysteroid dehydrogenase in cytosol fraction of seminiferous tubules.

rosomal and nuclear fractions of target tissue cells [20] while 3α-hydroxysteroid dehydrogenase has been described to be present in the soluble fraction [21] we became interested in studying the distribution of the two enzymes in tubular cells of 26-day-old rats. Incubation of homogenates of 26-day-old rat seminiferous tubules with [^{3}H]-T in the presence of NADPH resulted in 60% conversion to 3α-DIOL after 30 min. This was very similar to our previous results incubating intact seminiferous tubules without addition of cofactors [9]. In examining the time-course of the conversion in the homogenate, a rapid 5α-reduction of [^{3}H]-T was put in evidence.

3α-hydroxysteroid dehydrogenation velocity was slower resulting in an early accumulation of [^{3}H]-DHT with subsequent decline by conversion into 3α-DIOL. This was probably due to the fact that T is a poor substrate of 3α-hydroxysteroid dehydrogenase. Little 3β-hydroxysteroid dehydrogenase activity was detected in the homogenate. Analysis of the products isolated after incubating the cell sap revealed little transformation of [^{3}H]-T into reduced products indicating absence of 5α-reductase in the cytosol. Incubation with suspensions of the 15000 *g* sediment, rich in mitochondria, the 105000 *g* pellet, rich in microsomes, and with the nuclear fraction showed a completely different pattern of metabolism with presence of active 5α-reductase and 3β-hydroxysteroid dehydrogenase and less 3α-hydroxysteroid dehydrogenase activities. Electron microscopic analysis of the particulate fractions revealed that both the 15,000 *g* and the 105,000 pellets were quite heterogenous. This might explain the similar metabolic pattern observed in these two fractions.

The nuclear fraction seemed to be reasonably pure. It was concluded that T, upon entering the tubular cells of these rats, is first reduced to DHT in the reticulum endothelium or outer nuclear membrane. Subsequent reduction to 3α-DIOL is an extranuclear event. Since 3α-DIOL is the main androgen present in seminiferous tubules at the completion of the initial meiosis [10] it would be of interest to study if it is concentrated inside the nucleus, where, if present, it would have to enter as such. These studies are being carried out at present.

The apparent K_M for 3α-hydroxysteroid dehydrogenase determined in the cytosol fraction of seminal vesicles by Suzuki *et al.*[22] was 9.64 μM which is almost two orders of magnitude higher than our K_M value determined in the cytosol fraction of seminiferous tubules. The latter agrees with the apparent K_M value in prostatic cytosol reported by Taurog *et al.*[23]. Therefore, it is possible that 3α-hydroxysteroid dehydrogenases present in prostate and seminiferous tubules have a higher affinity for DHT than that of seminal vesicles.

In view of the high concentration of 3α-DIOL in the seminiferous tubules at the time of the first meiotic division (90 ng/100 mg protein vs 18 for T and DHT) we had previously speculated that 3α-DIOL might play a role in initiation of meiosis [10]. Other authors had suggested that conversion of T to 3α-DIOL could be a mechanism of androgen inactivation in maturing rats [24, 25]. This seems to be supported by the finding of absence of binding of 3α-DIOL to the nuclear receptor of the testis [26] and lack of penetration into nuclei [27]. These studies however were not correlated with the stage of development of spermatogenesis.

Furthermore, meiosis is an androgen-dependent step and it does not seem reasonable that the biologic activity of androgens should be inactivated at this time of development. It has been observed that 3α-DIOL is able to stimulate the estradiol-depressed initiation of meiotic division in maturing rats [28], but whether this was a direct effect of 3α-DIOL or an action mediated through its conversion into DHT could not be established [29]. Which is the role of

3α-DIOL during development of the germinal epithelium is not known yet.

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