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ENDOGENOUS ANDROGEN CONCENTRATIONS IN NUCLEI ISOLATED FROM SEMINIFEROUS TUBULES OF MATURE RAT TESTES

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SUMMARY

A series of studies measured endogenous androgen concentrations by radioimmunoassay within nuclei prepared from seminiferous tubules of the adult male rat. Testosterone was the principal androgen in the nuclei and occurred in high concentration (0.3 ng/mg DNA). This concentration was maintained even after substantial purification of the nuclei, indicating that the concentrations of testosterone measured were not the result of cytoplasmic contamination. Testosterone was actively retained in these nuclei following hypophysectomy of the animal: nuclear testosterone concentration fell to 20% of that in intact animals, while serum testosterone concentration fell to 3%. About 70% of the testosterone within the nuclei could be extracted with buffers containing KCl. Data from titration of testosterone in the salt extract of nuclei and in their residue generated a regression line which passed through the origin. Initial observations showed that salt-extracted testosterone could be separated into bound and free fractions by chromatography and that the bound portion was approx. 55% of total. It is suggested that this methodology is appropriate for the study of the relation of androgen binding to spermatogenesis in the seminiferous tubules of normal animals.

INTRODUCTION

Testosterone (T) is necessary for the maintenance of spermatogenesis in the male rat [1]. This requirement is met by the high concentrations of T in the fluids which bathe the tubules [2], and is supplied principally by Leydig cells which surround the tubules [3]. T is transported through the blood-testis barrier and into the tubular cells and intracellular lumen either passively or by facilitated transport [4]. How T regulates spermatogenesis and through which cells have not been clearly established. Receptor proteins for T have been identified in both nuclear and cytosol preparations of Sertoli cells [5, 6]. Androgen receptors have been detected in cytosol [7] but not in nuclei [8] prepared from germ cells. The lack of detectable androgen receptors in nuclei of germ cells is puzzling since germ cells have been shown to contain chromatin acceptor sites for T-receptor complexes [9].

The conflicting data on the distribution of androgen receptors may be the result of problems inherent in working with a tissue which normally contains high concentrations of T. Binding of radioactive steroids is minimal in tissue which contain high endogenous concentrations of that steroid. If high endogenous levels of tubular T are reduced by hypophysectomy of the animal, the tubules regress and binding activity may be depleted by proteolysis, as has been shown in regressing prostatic tissue [10]. We therefore decided to study tubular androgen receptors in normal, intact male rats in order to assess the validity of prior observations made by studying uptake of radiolabeled steroids into seminiferous tubules of animals which were either intact or hypophysectomized. Intact animals could best be studied by using radioimmunoassay to measure directly the endogenous T concentration in their tubules. Our initial studies were directed toward the nucleus, which is currently believed to be the primary site of steroid action within the cell [11].

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The abbreviations used are: T, testosterone; DHT, 17β-hydroxy-5α-androstane-3-one; 3α-diol, 5α-androstane-3α,17β-diol; 3β-diol, 5α-androstane-3β,17β-diol; A.diol, chromatographic fraction containing 3α-diol and 3β-diol; ABP, androgen binding protein.

EXPERIMENTAL

Animals

Male Sprague–Dawley rats bred in our colony were caged in pairs in a vivarium maintained at 20 ± 1°C in a light cycle of 12 h of light and 12 h of darkness. The rats were 120–200 days of age at the time of the experiment. Water and Purina rat chow were available to the animals *ad libitum*; adrenalectomized animals received 0.9% saline. All surgery was performed on animals anesthetized with ether. Castrations and adrenalectomies were performed according to standard laboratory procedures. Transauricular

hypophysectomy was performed according to the method of Gay [12]. Sham hypophysectomy consisted of all steps of hypophysectomy except that when the 18 gauge needle pierced the sphenoid bone, the needle was withdrawn. Animals were killed by decapitation and blood samples were collected from the trunk. Testes were immediately removed and placed in ice-cold phosphate-buffered saline, pH = 7.0 (PBS). All subsequent procedures were carried out with the tissue at 4°C.

Preparation of nuclei

Seminiferous tubules which were isolated from the surrounding interstitium by mechanical dissection of decapsulated testes [13] were placed on monofilament nylon grid (mesh opening = 147 μ) and washed with PBS. Tubules from each testis were homogenized in 10 ml of 0.32 M sucrose, 20 mM Tris–HCl, 3 mM Mg acetate, 0.1% Tween 80, pH = 7.4 (0.32 M STM) with a glass–Teflon homogenizer. Tissue was disrupted by 12 strokes (Studies 1–3) or 48 strokes (Studies 3–7) of a motor driven pestle (300 rev./min). The homogenate was filtered through monofilament nylon cloth (mesh opening = 147 μ) and then centrifuged at 800 ***g*** for 15 min. The pellet was resuspended in 6 ml of 0.32 M STM per testis, filtered through monofilament nylon cloth (mesh opening = 74 μ) and recentrifuged. For the preparation of crude nuclear pellets, nuclei were once more resuspended in 0.32 M STM and recentrifuged. To prepare purified pellets, following the second centrifugation, nuclei were resuspended in 0.32 M STM to which was added a sufficient volume of 2.2 M sucrose, 20 mM Tris–HCl, 3.0 mM Mg acetate, pH = 7.4 (2.2 M STM) to make the final sucrose concentration 1.8 M. The nuclei were then sedimented for one h at 20,000 rev./min (113,000 ***g*** max) in a Spinco SW-27 or SW-27.1 rotor. Liver nuclei were prepared from rats castrated and adrenalectomized one week previously; 20 grams of liver were homogenized in 80 ml of 0.32 M STM with 12 thrusts of the motor driven pestle (600 rev./min). The first two filtration and centrifugation steps were identical to those used in the preparation of tubular nuclei. The pellet was resuspended in 0.32 M STM and a sufficient volume of 2.2 M STM was added to make the final sucrose concentration 2.0 M. This suspension was then underlaid with a cushion of 2.2 M STM and the nuclei pelleted for 1 h at 27,000 rev./min (131,000 ***g*** max) in a Spinco SW-27 rotor. The purity of the nuclear preparations was checked by phase contrast microscopy. The crude tubular nuclear pellets contained some cytoplasmic debris, fibrous material and a few intact cells. The purified nuclear pellet contained neither cytoplasmic debris nor intact cells, and very little fibrous material. The liver nuclei preparation appeared to be free of all contamination.

Steroid assay procedures

T concentration was measured in serum by the method of Frankel *et al.* [14]. In the measurement of androgens in tissue fractions, 2,000 c.p.m. of the appropriate tritiated androgen were added to cytosol, salt extracts or disrupted nuclei (see Experimental Protocols). All radioactive steroids were purchased from New England Nuclear Corp.: [1,2,6,7-^{3}H]-T (99 Ci/mmol), [1,2-^{3}H]-DHT (40 Ci/mmol) and [1,2-^{3}H]-3α-diol (40 Ci/mmol) and tested for purity by Sephadex LH-20 column chromatography (see below). Androgens were extracted twice into 3 volumes of hexane–benzene (2:1, V/V). Sensitivity of the assay was estimated by extraction of the appropriate buffer and estimation of its apparent T by radioimmunoassay. Two systems using Sephadex LH-20 for the isolation of androgens were used in these studies. In *System I* (used in Studies 1 and 3), 850 mg of Sephadex LH-20 were swollen overnight in benzene–methanol–water (85:15:0.5, by vol.). The solvent above the gel was then removed and 5 ml of eluting solvent, isooctane–benzene–methanol (95:2.5:2.5, by vol.), were added to the gel. The solvent above the gel was removed and an additional 5 ml of eluting solvent added, an 8 mm × 30 mm column poured and packed with 10 ml of eluting solvent. Use of eluting solvent to wash the gel removed fine particles of Sephadex LH-20 from the slurry which otherwise interfered with the measurement of T isolated in this system. Androgens eluted from the column in the following fractions: DHT (11–20 ml), T (25–34 ml) and 3α-diol (39–54 ml). This system did not separate 3α-diol from 3β-diol. Since there was a 5% overlap between the T and A.diol fractions, only one of the radioactive steroid tracers (2000 c.p.m.) was added alternatively to duplicate samples, overlap was estimated and concentrations of each steroid adjusted accordingly. The sensitivity of the assay for this system was approx. 30 pg/sample. *System II* (used in Studies 2, 4–7) was as described earlier [14]. Sephadex LH-20, 850 mg, was swollen for 1 h in benzene–methanol (85:15, V/V). The slurry solvent was removed, the gel washed with eluting solvent, isooctane–benzen–methanol (90:5:5, by vol.), and the column poured as in System I. T eluted at 14–21 ml; there was slight overlap of T with the other 2 androgens (DHT, 8–14 ml; 3α-diol, 20–26 ml). Since T was found to be the principal androgen in the nuclei of the seminiferous tubules (see Results, Study I) and since System II was more economical to run than System I, System II was used in all the later studies, when only T was measured. Only [^{3}H]-T (2000 c.p.m.) was added to each sample, and the minimal contribution of DHT and A.diol to T concentration was ignored. The sensitivity of the assay of T isolated by System II (10–13 pg) was significantly greater than that in System I. When all 3 androgens were measured by radioimmunoassay, using the antibody to T which has a cross-reactivity of 65% to DHT and 46% to 3α-diol [14], appropriate standards (DHT or 3α-diol) were used for the determination of each androgen.

All solvents used in extraction and chromatography were reagent grade, and were purified before use, as

described earlier [14], except isooctane, which was spectrograde quality (Eastman Chemical Co.) and used without further purification. Water was glass-distilled twice, and then further purified by Millipore Milli-Q reagent grade water system. In both systems, the chromatographically-isolated steroid samples were immediately dried under purified nitrogen, resuspended in benzene and aliquots taken for the estimation of recovery and for androgen measurement. Within 8 h, the samples were analyzed by radioimmunoassay [14]. No further delay was allowed between the chromatographic step and the assay of the samples, since extended storage of the samples was found to decrease the sensitivity of the assay. Aliquots from a pool of serum from male rats were measured in quadruplicate in each assay carried out in the course of these studies. Intra- and interassay coefficients of variation for 10 assays were 3 and 14% respectively.

Other procedures

DNA concentration was measured using the diphenylamine reaction [15] and salmon sperm DNA (Sigma Chemical Co.) as standard. Protein concentration in cytosol was measured, following precipitation of the sample with 10% trichloroacetic acid, by the method of Lowry *et al.*[16]. Protein concentration in salt extracts was estimated by measuring the absorbance of the sample at 280 nm and 260 nm [17] in a Gilford Model 250 spectrophotometer. Statistical analysis was made by analysis of variance [18].

Experimental protocols

Study 1: Measurement of T, DHT and A.diol in crude nuclear pellets. The purpose of this study was to determine the ratio of the androgens present in the nuclei of seminiferous tubules. Crude nuclear pellets were prepared from tubules of 4 testes. These pellets were pooled, suspended in 16 ml of water and nuclei disrupted by sonication (6 × 15 s at 60 cycles/s) in a Labline Ultratip Labsonic System (Model 9100). Radioactively labeled steroid tracer (2000 c.p.m. of [^{3}H]-DHT and 2000 c.p.m. of either [^{3}H]-T or [^{3}H]-3α-diol) was added to 4 ml of the solubilized nuclei, and the samples were processed in quadruplicate for the analysis of DHT, T and A.diol concentrations, using Sephadex LH-20 column System I for isolation of the steroids.

Study 2: The retention of T and DHT in the nuclei of seminiferous tubules following hypophysectomy. The purpose of this study was to compare, following hypophysectomy, the changes in the T concentration in seminiferous tubules with changes in plasma T concentration. Sham-hypophysectomized ($n = 5$) and hypophysectomized ($n = 5$) animals were killed 4 days after surgery, and trunk blood collected. The sella turcica of each hypophysectomized animal was examined macroscopically to insure that no pituitary fragments remained. Crude nuclear pellets were prepared as described earlier and extracted with 4 ml of 0.4 M KCl, 20 mM Tris–HCl, pH = 7.4 (0.4 M KTH). After a 15 min incubation at 0°C, the nuclei were pelleted at 30,000 ***g*** for 15 min. Tubular cytosol was also prepared from the supernatant obtained from the first nuclear precipitation. This supernatant was recentrifuged for one h at 108,000 ***g*** in a Spinco T40 rotor. Testosterone was measured in serum, and T and DHT in cytosol and salt extracts of nuclei from each animal. T and DHT were isolated chromatographically using Sephadex LH-20 column System II.

Study 3: The effect of nuclear purification on salt-extractable T. The purpose of this study was to determine if the high levels of T measured in salt extracts of tubular nuclei could be attributed to cytoplasmic contaminants. Crude nuclear pellets were prepared as in Study 1, while purified nuclear pellets were prepared from tissue disrupted with 12 or 48 strokes of the pestle. Each pellet was extracted with 4 ml of 0.4 M KTH. Testosterone was isolated using Sephadex LH-20 column System I.

Study 4: Titration of T in salt extracts and residual nuclei from tubules and liver by radioimmunoassay. There was a possibility that the T concentration measured in our assay was artifactual, and represented the contribution of non-steroidal elements in the tissue which inhibited the binding of radioactive T to the antibody for T. Purified tubular nuclei prepared from 6 testes were extracted with 24 ml of 0.4 M KCl, 20 mM Tris–HCl, 1.5 mM EDTA, pH = 7.4 (0.4 M KTE). Purified liver nuclei from male rats castrated and adrenalectomized for 1 week were extracted with 32 ml of 0.4 M KTE. The residual nuclear pellets of both tissues were resuspended in 20 ml of 0.4 M KTE and the nuclei disrupted with ultrasound as in Study 2. Duplicate samples with a volume of 2, 3 and 4 ml of salt extract or nuclear homogenate were processed by Sephadex LH-20 System II for isolation of T.

Studies 5, 6: Efficiency of extraction of T from tubular nuclei with buffers containing KCl. Two studies were conducted to determine whether T could be quantitatively extracted from nuclei with buffers containing KCl. In Study 5, purified nuclei from 2 pools of tubules were (A) extracted with 4 ml of 0.4 M KTE, or (B) resuspended in 2 ml of hypotonic buffer, 20 mM Tris–HCl, 1.5 mM EDTA, pH = 7.4 (TE) to which was added, 2 ml of 0.8 M KCl, 20 mM Tris–HCl, 1.5 mM EDTA, pH = 7.4 (0.8 M KTE) or (C) resuspended in 2 ml of hypotonic buffer, 20 mM Tris–HCl, pH = 7.4 (TH) to which was added 2 ml of 0.8 M KCl, 20 mM Tris–HCl, pH = 7.4 (0.8 M KTH). The nuclei were incubated at 0°C for 30 min before centrifugation. The residual nuclear pellet was disrupted with ultrasound as before. The effect of EDTA on the ability of KCl to extract T was studied because of the finding of Roy and McEwen[19] that EDTA inhibited the extraction of [^{3}H]-estradiol–receptor complexes from brain cell nuclei.

In Study 6, the effect of repeated extraction of nuclei with buffers containing KCl was examined. Puri-

fied nuclei were lysed with (D) 2 ml of TE, the nuclei pelleted and this pellet extracted with 2 ml of 0.8 M KTE. The two supernatants were pooled, and the pellet was reextracted with 4 ml of 0.4 M KTE. Nuclei were also extracted with (E) 4 ml of 0.4 M KTE and reextracted with 2 ml of the same buffer. Purified nuclei were also extracted with (F) 2 ml of 0.4 M KTE and reextracted with 2 ml of that buffer. In all cases, nuclei were incubated for 30 min during the first salt extraction and 15 min during the second. T was isolated by Sephadex LH-20 column System II.

Study 7: Separation of bound and free T. A pool of 0.4 M KTE extract was prepared from purified tubular nuclei. Five thousand c.p.m. of [^{3}H]-T were added to 3 ml of this extract. The sample was applied to a column (12 ml bed volume) of Sephadex LH-20 swollen in 0.4 M KTE and twenty 3 ml fractions collected. T was isolated on Sephadex LH-20 column System II and its concentration was measured by radioimmunoassay in the unchromatographed crude extract, bound T in void volume (fractions 2,3), free T (fractions 9,10) and column buffer containing no authentic T (fractions 5,6). Recovery of free T was estimated from the percent recovery of the original 5000 c.p.m. of [^{3}H]-T added to the sample. Recovery of T in the bound T fraction was estimated by percent recovery of protein concentration in crude extract on Sephadex LH-20 in 0.4 M KTE, after which radioactive tracer was added to monitor further procedural losses.

RESULTS

Study 1: Measurement of T, DHT and A.diol in crude nuclear pellets

T concentration in nuclei from the seminiferous tubules was significantly greater than the concentration of DHT and A.diol ($P < 0.01$). T comprised 63% of the total androgen measured (Table 1).

Study 2: The retention of T and DHT in the seminiferous tubules following hypophysectomy

Four days following hypophysectomy or sham-hypophysectomy, serum T concentrations in the hypophysectomized animals were 3% of the levels in sham-hypophysectomized animals ($P < 0.05$) (Table 2). T and DHT levels in the tubular nuclei and cytosol of hypophysectomized animals were reduced to about 20% of the levels in sham-hypophysectomized animals ($P < 0.05$). These decreases in androgen concentration in the nuclei and cytosol following hypophysectomy were significantly different from the decrease in plasma T concentration ($P < 0.05$).

Study 3: The effect of nuclear purification on salt-extractable T

Increased purification of the nuclei reduced the amount of DNA recovered in the samples ($P < 0.05$), but did not alter the specific concentration of T, as ng T/mg DNA, in the nuclei (Table 3). Since purified nuclei were free of contaminants and since 48 thrusts of the pestle resulted in somewhat greater DNA yields, in all subsequent studies, purified nuclei were prepared from tissue disrupted by 48 thrusts of the pestle.

Study 4: Titration of T in salt extracts and residual nuclei from tubules and liver by radioimmunoassay

Protein concentrations in the salt extracts of tubular and liver nuclei were identical. Data generated from the titration of T by radioimmunoassay of the salt extract and nuclear residue prepared from the seminiferous tubules fit linear regression lines ($r = 0.99$) which passed through the origin (Fig. 1). In the titration of T concentration in salt extracts and nuclear residue from liver, only in 4 ml of salt extract were both duplicate measurements greater than the sensitivity of the assay.

Studies 5, 6: Efficiency of extraction of T from tubular nuclei with buffers containing KCl

In Study 5, suspension of the nuclei in hypotonic buffers before the addition of buffers containing 0.8 M KCl did not substantially improve the extraction of T from nuclei (Table 4, methods A–C). The presence of EDTA in the buffers did not affect the efficacy of extraction. In Study 6, repeated extraction of nuclei did increase the yield of salt-extracted T, especially

Table 1. The concentration of three androgens in homogenates of nuclei isolated from seminiferous tubules

Androgen	pg/4 ml of nuclear homogenate	% of total androgen content
17β-Hydroxy-5α-androstane-3-one	97.4 ± 10.3	14%
Testosterone	435.4 ± 61.0	63%
5α-Androstan-3α,17β-diol plus 5α-androstan-3β,17β-diol	159.5 ± 52.0	23%

Seminiferous tubules were isolated from 4 testes and crude nuclear pellets prepared, resuspended in 16 ml of water, and homogenized with ultrasound. 4 ml aliquots of this homogenate were extracted into organic solvents, steroids isolated by LH-20 chromatography (System I) and the concentration of the steroids measured by radioimmunoassay. Data are presented as mean ± SE.

Table 2. Rentention of testosterone (T) and 17β-hydroxy-5α-androstane-3-one (DHT) in seminiferous tubules following hypophysectomy

Group	Serum T (ng/ml)	Tubular cytosol T(ng/mg ptn)	Tubular cytosol DHT (ng/mg ptn)	Tubular nuclei T(ng/mg DNA)	Tubular nuclei DHT(ng/mg DNA)
Sham hpx	1.79 ± 0.49	0.783 ± 0.27	0.041 ± 0.014	0.267 ± 0.05	0.014 ± 0.002
Hpx	0.05 ± 0.04	0.149 ± 0.03	0.008 ± 0.001	0.057 ± 0.01	0.003 ± 0.001
Hpx/Sham hpx	'0.03	0.19	0.19	0.21	0.21

Animals were either hypophysectomized (hpx) (n = 5) or sham hypophysectomized (n = 5). Four days later, the animals were killed, seminiferous tubules isolated from the testes of each animal, and androgen content measured in serum, tubular cytosol and salt extracts of tubular nuclei. Data are presented as mean ± SE.

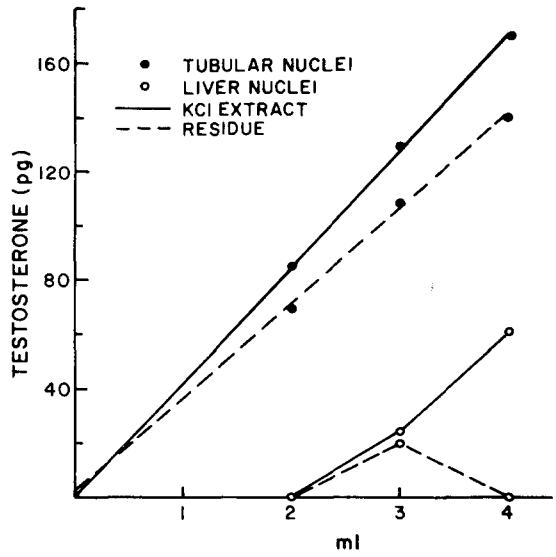

Fig. 1. Titration of testosterone in salt extracts and residual nuclei from tubules and liver by radioimmunoassay. Each point is the mean of 2 determinations.

in nuclei that were first lysed and then extracted with 0.8 M KTE (Table 4, methods D–F). However, 0.8 M KCl extraction solubilized 43% of the DNA in the nuclei (method D), while 0.4 M KCl extraction solubilized none of the DNA (methods E,F). The amount of T extracted was independent of the volume of 0.4 M KTE used (Table 4, compare E to F, first extraction). None of the procedures quantitatively extracted T from the nuclei.

Study 7: Separation of bound and free T

Bound T was completely isolated from free T by the Sephadex LH-20 column slurried in 0.4 M KTE. None of the radioactive T which was originally added to the sample was observed in the void volume, while all protein eluted in the void volume. The column itself did not interfere with the sensitivity of the radioimmunoassay. The sum of the bound and free hormone concentrations was a 15% overestimation of the T concentration in the original salt extract. About 55% of the T concentration in the 0.4 M KTE extract was protein-bound (Table 5).

Table 3. The effect of nuclear purification on concentrations of testosterone (T) and DNA

Sample	mg DNA per sample	ng T per sample	ng T per mg DNA
Crude homogenate—10 ml of tubular homogenate in 0.32 M sucrose, 20 mM Tris-HCl, 3 mM Mg Acetate, 0.1% Tween-80, pH = 7.4 (n = 4).	1.813 ± 0.382	14.873 ± 5.715	9.313 ± 3.308
0.4 M KCl extracts of crude nuclear pellets prepared from tissue disrupted with 12 strokes of the pestle (n = 4).	1.686 ± 0.124	0.555 ± 0.131	0.318 ± 0.060
0.4 M KCl extracts of purified nuclei prepared from tissue disrupted with 12 strokes of the pestle (n = 4).	0.659 ± 0.208	0.103 ± 0.047	0.497 ± 0.430
0.4 M KCl extracts of purified nuclei prepared from tissue disrupted with 48 strokes of the pestle (n = 4).	0.873 ± 0.089	0.615 ± 0.248	0.695 ± 0.228

Four pools of seminiferous tubules were prepared. Crude nuclear pellets or purified nuclear pellets were prepared from each pool (see text). Tissue was disrupted with either 12 or 48 strokes of the pestle. Nuclei were extracted with 0.4 M KCl, 20 mM Tris-HCl, pH = 7.4, and testosterone concentration of this extract was measured by radioimmunoassay. DNA concentration in the residual nuclear pellet was also measured. Data are presented as mean ± SE.

Table 4. Efficacy of extraction of testosterone (T) from purified tubular nuclei with buffers containing KCl

Method of extraction	Pool No.	T in extract (pg)	T in residual nuclei (pg)	Summation of extracted T as percent total nuclear T
A. Extraction with 4 ml of 0.4 M KTE	1	125.0	96.7	56%
	2	104.0	136.0	43%
B. Suspension of nuclei in 2 ml TE followed by the addition of 2 ml 0.8 M KTE	1	98.4	110.9	47%
	2	100.4	58.7	63%
C. Suspension of nuclei in 2 ml TH followed by the addition of 2 ml 0.8 M KTH	1	82.1	57.9	59%
	2	94.1	92.9	50%
D. Lysis of nuclei with 2 ml of TE. Extraction of pellet with 2 ml 0.8 M KTE	3	123.2		
	4	80.3		
Reextraction of nuclei with 4 ml 0.4 M KTE	3	83.7	54.5	79%
	4	52.4	48.9	73%
E. Extraction of nuclei with 4 ml 0.4 M KTE	3	80.8		
	4	68.9		
Reextraction with 2 ml 0.4 M KTE	3	34.5	76.6	60%
	4	36.7	48.6	68%
F. Extraction of nuclei with 2 ml 0.4 M KTE	3	88.4		
	4	69.5		
Reextraction with 2 ml 0.4 M KTE	3	55.1	68.3	68%
	4	50.7	58.5	67%

Two studies were conducted. In Study 5 (see text) nuclei were extracted in the normal manner (A) or resuspended in hypotonic buffers and then brought to 0.4 M KCl with buffers containing KCl, with (B) or without (C) EDTA. In Study 6, nuclei were extracted twice. T concentration in salt extracts and nuclei were measured by radioimmunoassay. *0.4 M KTE* = 0.4 M KCl, 20 mM Tris–HCl, 1.5 mM EDTA, pH = 7.4. *TE* = 20 mM Tris–HCl, 1.5 mM EDTA, pH = 7.4. *0.8 M KTE* = 0.8 M KCl, 20 mM Tris–HCl, 1.5 mM EDTA, pH = 7.4. *TH* = 20 mM Tris–HCl, pH = 7.4. *0.8 M KTH* = 0.8 M KCl, 20 mM Tris–HCl, pH = 7.4. *Total Nuclear T* = Extracted T + Residual T. In D,E and F, extracted T represents sum of 2 extractions.

DISCUSSION

We have outlined in this report appropriate methods for studying endogenous T concentrations within nuclei isolated from the seminiferous tubules. It is apparent that T is the predominant androgen within tubular nuclei (Study 1, Table 1). Tubular nuclei retain T following hypophysectomy to a much greater extent than does plasma (Study 2, Table 2). The concentrations of T which we measured in the nuclei are due neither to the presence of cytoplasmic contaminants nor to non-steroidal tissue components (Studies 3,4; Table 3, Fig. 1). Up to 80% of T concentration in nuclei can be quantitatively extracted into buffers containing KCl (Studies 5,6). However, the use of buffers containing a concentration of 0.8 M KCl

Table 5. Isolation and measurement of bound and free testosterone (T) in salt extracts of purified tubular nuclei

Sample	pg T/ml of original salt extract	mg protein/ml of sample
Original salt extract	67.0 pg/ml	0.594 mg/ml
Bound T, corrected for protein dilution	41.0 pg/ml	0.503 mg/ml
Free T	34.9 pg/ml	0.000 mg/ml
Bound T + Free T	75.9 pg/ml	

Purified tubular nuclei were prepared (see text) and extracted with 0.4 M KCl (0.4 M KTE, see Table 4). 5000 c.p.m. [^{3}H]-testosterone were added to 3 ml of this extract which was layered on a Sephadex LH-20 column slurried in 0.4 M KCl buffer.

or greater is inappropriate because of the solubilization of DNA by the KCl (Study 6). Thus, a yield of 70% can be approached by appropriate use of a 0.4 M KCl extraction. Our preliminary observations indicate that about 55% of the salt-extractable T is protein-bound (Study 7, Table 5).

Four ml of salt extract of liver nuclei appeared to contain a sufficient quantity of non-steroidal substances to interfere with the radioimmunoassay. Although the *apparent* quantity of T that was measured in liver nuclei from castrated-adrenalectomized male rats was insignificant when compared to the quantity of T measured in extracts of tubular nuclei, similar non-steroidal tissue contaminants might become important in the examination of nuclei which contain very low concentrations of T. Thus, validation of the presence of T concentration in nuclei from a particular cell fraction of the seminiferous tubules must entail not only a positive measurement of T by radioimmunoassay, but also a dose response pattern in the radioimmunoassay similar to that generated by a heterogeneous collection of tubular nuclei. The possibility that the low T concentrations measured in liver nuclei represent a persistent retention of authentic T cannot be ignored, however.

Our finding that T is the principal androgen in the nuclei of seminiferous tubules is in agreement with previous reports that there is little 5α-reductase activity in the conversion of T to DHT in the mature rat testes [20–22], either in interstitial cells or seminiferous tubules [22]. Our observation that there is more A.diol than DHT in the nuclei of seminiferous tubules is also consistent with the report that 3α/β-hydroxysteroid dehydrogenase activity in conversion of DHT to A.diol is high in the mature rat testis [21]. It is interesting, however, that in the report cited [21], A.diol was not in the bound form in testicular nuclei. Whether the source of these reduced products is the interstitial cells or seminiferous tubules is not known; however, both compartments are competent [22].

The retention of T in the seminiferous tubules following hypophysectomy is similar to the finding of Harris and Bartke[23] that T was retained in rete testis fluid following hypophysectomy of the animal. We cannot assume that retention in the nucleus was due only to binding of T in the nucleus, since both cytosol and nuclear fractions of the tubules retained the same proportion of T concentration. The T concentration might have been in equilibrium between the extracellular (luminal) compartment and the cytoplasmic and nuclear compartments of the cell. How T was retained in the seminiferous tubules is uncertain. While ABP, which is present in the lumen of the seminiferous tubules, has a high affinity for DHT and T [24], it has been argued that ABP is not necessary for the maintenance of the normal concentrations of T in luminal fluids [25]. Whether ABP is important in maintaining T concentration in the tubules of hypophysectomized animals is not known. Androgen receptors which are present in the Sertoli cells [5] and possibly in the germinal cells [26] might also contribute to the retention of T within the seminiferous tubules.

A residual T concentration remains in nuclei prepared from seminiferous tubules (Study 6), even after repeated salt extraction. Resistance to salt extraction has been observed in receptor-steroid complexes in nuclei of rat uteri [27], interstitial cells of Leydig[28], mouse kidney [29] and Sertoli cells [6]. Clark and Peck[27] have suggested that such salt-resistant steroid–receptor complexes are bound to acceptor sites on the chromatin. We are currently investigating whether the residual T concentration we measure in nuclei is indeed salt-resistant and whether it is bound to chromatin. Lysis of the nuclei followed by extraction with 0.8 M KTE appears to solubilize more nuclear T than does extraction with 0.4 M KTE. The concurrent solubilization, however, of both DNA and steroid makes the technique of extraction with 0.8 M KTE inappropriate for the determination of the specific concentration of T (as ng/mg DNA) in the nuclear pellet. Further, in the fractionation of free and bound T, we have found that the presence of DNA in the sample severely retards the flow rate of the Sephadex LH-20 column in 0.4 M KTE (unpublished observations).

Both bound and free T can be isolated by chromatography of the salt extract of nuclei on Sephadex LH-20 in 0.4 M KTE. The nature of these 2 fractions is uncertain. We found, in Study 7, that one-half of the 0.4 M KTE-extracted steroid was protein-bound. Throughout our studies, total T concentration in the extracts was 0.2–0.4 pmol T/mg protein. Thus, we assume that bound T concentration was 0.1–0.2 pmol/mg extracted protein. However, these concentrations are far greater than those which have been reported by Mulder *et al.*[21] in protein-bound [^{3}H]-testosterone in salt extracts of nuclei prepared from mature rats hypophysectomized for 19 days (0.03 pmol/mg extracted protein). The difference between our results may be due to the fact that these authors used crude nuclear preparations. It has been our experience (unpublished observations) that the ratio of the concentration of protein to the concentration of T in KCl extracts of crude nuclear preparations is 3 times greater than what is observed in extracts of purified nuclei. Meanwhile, "free" T is probably not free in the cell, but rather associated by low affinity, nonspecific interaction with proteins, lipids and lipoproteins. Salt extraction or chromatography on LH-20 columns would probably disrupt such low affinity interactions [30].

Although our methods for the study of T in tubular nuclei are laborious, we believe them to be the most adequate that are currently available. Our methodology may be useful in the study of steroid-receptor complexes in any organ which is the main site of synthesis of that steroid, and in which a nuclear exchange assay has not been achieved. As a result of these studies, it is possible to study the distribution

of at least 3 forms of T (free, bound [specific and nonspecific] and non-extracable) in germ cells, and their role in the regulation of spermatogenesis.

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