

# Endogenous Steroids in the Rat Seminiferous Tubules. Comparison of the Stages of the Epithelial Cycle Isolated by Transillumination-Assisted Microdissection

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A microdissection procedure is described that allows the isolation of segments of rat seminiferous tubules in different stages of the epithelial cycle. This procedure is based on spermatogenic stage-dependent differences in the transillumination pattern along the freshly isolated, unstained seminiferous tubules. It allows collection of segments representing defined stages in amounts sufficient for biochemical studies (5–10 mg wet weight).

Tubular segments at stages I, II–III, IV–V, VI, VIIa–b, VIIc–d, VIII, IX–XI, XII, and XIII–XIV have been used for measurement of endogenous concentrations of testosterone, 5 $\alpha$ -dihydrotestosterone, progesterone, and 17 $\alpha$ -hydroxyprogesterone by radioimmunoassays after Lipidex-5000 chromatography. Testosterone was the most abundant steroid at all stages of the cycle, and its concentration at stage VIII was significantly higher than at any other stage. The concentration of 5 $\alpha$ -dihydrotestosterone was markedly lower and did not exhibit similar differences in distribution. The levels of progesterone and 17 $\alpha$ -hydroxyprogesterone were the same at all stages of the cycle of the seminiferous epithelium.

A number of morphologic and biochemical observations suggest unique changes in the metabolic activity of both germ cells and Sertoli cells at stages VII and VIII of the cycle. The possible androgen control of these changes is discussed.

**Key words:** androgens, seminiferous tubules, rats, stages of spermatogenesis, transillumination.

Developing germ cells in the mammalian seminiferous tubules form associations of constant and predictable composition. In the rat, 14 such associations have been defined (Leblond and

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Clermont, 1952). The cell associations, also called stages, are typically arranged in a wave-like fashion along the seminiferous tubules (Perey et al, 1961). All germ cells have an intimate contact with the Sertoli cells. Indeed, both meiotic and post-meiotic cells are completely surrounded by processes of Sertoli cell cytoplasm. The completion of spermatogenesis is dependent on testicular androgens (Steinberger, 1971) that exert their effects on the developing germ cells primarily, if not exclusively, through the Sertoli cells (Hansson et al, 1978; Fritz, 1978). During spermatogenesis, the synthesis of both DNA and RNA shows considerable variations (Monesi, 1971). It is tempting to think that this variation is regulated by hormones. Molecular mechanisms that could account for this regulation remain unknown, particularly since germ cells themselves may not be the target of direct androgen action (Fritz, 1978; Ritzén et al, 1981b).

Sertoli cells undergo morphologic changes during the cycle of the seminiferous epithelium (Brökelmann, 1963), and their lipid content shows marked variations (Niemi and Kormanen, 1965; Kerr and de Kretser, 1975). These studies, together with recent morphometric analyses (Assaf, 1980), suggest the presence of a functional cycle of the Sertoli cells related to the stage of spermatogenesis. Recently, we have been able to demonstrate its existence in the rat. Thus, we have shown that FSH exhibits differential binding and action, de-

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pending on the seminiferous epithelial stages (Parvinen et al, 1980). In addition, there are stage-dependent differences in the secretion rate of androgen binding protein (ABP) (Ritzén et al, 1981a) with maximal ABP secretion at stage VIII of the cycle. Development of the transillumination-assisted microdissection method permitted biochemical studies of the cycle of the seminiferous epithelium (Parvinen and Vanha-Perttula, 1972). This microdissection method has now been modified for collection of sufficient amounts of material for a variety of biochemical studies. In the seminiferous tubular segments dissected by this procedure, the normal contact between the Sertoli cells and the germ cells remains undisturbed and contamination with Leydig cells can be avoided. Consequently, it is possible to study the function of the Sertoli cells as it relates to various stages of the cycle. In this paper, we present results of measurements of endogenous steroid concentrations in segments of rat seminiferous tubules at different stages of the cycle, with the main emphasis on the levels of active androgens.

### Materials and Methods

#### *Transillumination-Assisted Microdissection of the Seminiferous Tubules*

Twelve young adult (3–5 months) Sprague-Dawley rats were used. The animals were killed by cervical dislocation; the testes were removed, decapsulated, and placed in a cooled phosphate buffered saline (PBS) solution. The seminiferous tubules were separated from the interstitial tissue by manual dissection (Christensen and Mason, 1965) and subjected to transillumination under the stereomicroscope, as described previously (Parvinen and Vanha-Perttula, 1972). On the basis of appearance under transillumination, the following four principal zones were recognized: pale, weak spot, dark spot, and dark (Parvinen and Parvinen, 1978). The

principle of the microdissection is shown in Fig. 1. The isolated seminiferous tubules were cut in 2 mm segments as follows. The pale zone was subdivided into two sections, with the first 2 mm flanking the dark zone representing stages IX–XI and the remainder of the pale zone being assigned to the pool of stage XII. The weak spot zone was also subdivided into two segments, the first representing stages XIII–XIV and the second, stage I. Similarly, the dark spot zone was divided into two parts, the first comprising stages II–III and the second, stages IV–V. The transition between strong spots and homogenous darkness was considered stage VI. Finally, the long dark zone was subdivided into three zones, the first representing stages VIIa–b, the middle, stages VIIc–d, and the last 2 mm before the beginning of the pale zone, stage VIII. The total length of the segments assigned to each of the abovementioned pools was measured using a 1 mm mesh placed beneath the Petri dish. A total of 6 cm (thirty 2 mm segments) of seminiferous tubule fragments could be collected in each pool during 2 to 3 hours of dissection. The wet weight of 1 cm of the tubule is approximately 1 mg. The diameter of fresh (wet) tubules is 350  $\mu\text{m}$ .

#### *Histologic Control of the Isolation Procedure*

For morphologic analysis and photomicrography, the precise stage was first determined by phase contrast microscopy (Söderström and Parvinen, 1976). Subsequently, the samples were quickly photographed in a drop of PBS, fixed in Bouin's fluid, dehydrated, embedded in paraffin, sectioned at 5  $\mu\text{m}$ , stained with PAS-hematoxylin, and photographed again. For controlling the accuracy of the transillumination, pools of 18 to 38 tubular segments were isolated without phase contrast microscopy and processed for histologic analysis as described above.

#### *Analysis of Steroid Diffusion*

The effect of temperature on the rate of diffusion of testosterone from the testicular tissue into the dissection medium was studied by comparing dissection at room temperature (22 C) and at 1.5 C. The Petri dish with PBS was cooled by placing it on the top of a transparent plate

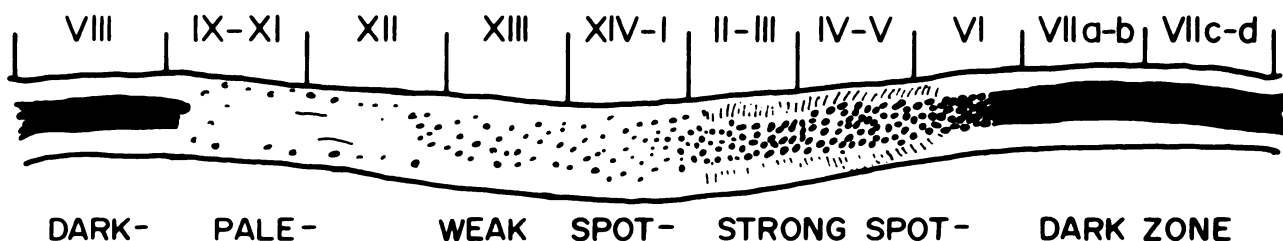


Fig. 1. The principle of the transillumination-assisted microdissection technique for isolation of rat seminiferous tubular segments at defined stages of the epithelial cycle. The point most easily recognized is the abrupt disappearance of the dark absorption at stage VIII, which is due to spermiation. The pale, weak spot, and strong spot zones are each subdivided into two portions, whereas the dark absorption zone can usually be separated into three different subzones. The isolated tubule is usually cut into 2 mm segments, measured by a 1 mm mesh below the dissection vessel, and assigned to the ten pools described in the picture.

from the electrophoresis apparatus (LKB 2117 Multiphor; LKB Bromma, Sweden), which had a circulation of cold ( $-6\text{ C}$ ) ethanol. As a cooling device, an electronic thermoregulator (MGW Lauda K4R; Dr. R. Wobser, KG, West Germany) was used. Different experimental conditions are listed in Table 1.

#### Reagents for Steroid Analysis

Non-radioactive steroids were obtained from Ikaparm (Ramat-Gan, Israel) or from Steraloids, Inc. (Pawling, NY, USA), and were used without further purification. (1,2- $^3\text{H}$ )17 $\alpha$ -hydroxyprogesterone (40 Ci/mmol) was obtained from NEN Chemicals GMBH (D-6072 Dreichenhein, West Germany) and (1,2,4,5,6,7- $^3\text{H}$ )5 $\alpha$ -dihydrotestosterone (107 Ci/mmol) was obtained from the Radiochemical Centre (Amersham, UK). Before use, the tritiated steroids were purified on Lipidex-5000<sup>®</sup> (hydroxylalkoxypropyl Sephadex, obtained from Packard-Becker, P.V., Chemical Operations, Groningen, The Netherlands) microcolumns. The characteristics of the antisera and the specifications of other reagents and solvents used in the assays have been reported previously (Apter et al, 1976; Hammond et al, 1977b).

#### Steroid Assays

After collection of tissue samples, the dissection medium was removed; the samples were frozen in liquid nitrogen and lyophilized. Before extraction, 1 ml of physiologic saline containing 4000 cpm of  $^3\text{H}$ -testosterone was added for monitoring recovery. The unconjugated steroids were extracted twice with 2 ml of ether-ethyl acetate (9:1, v:v). The organic phases were combined and dried under the stream of nitrogen. The residue was dissolved in two 0.2 ml portions of petroleum ether-chloroform (97:3, v:v) and applied onto Lipidex-5000 microcolumns that were packed using the same solvent. The following fractions were collected: 2.0 to 6.0 ml (progesterone), 8.0 to 16.0 ml (5 $\alpha$ -dihydrotestosterone), 16.0 to 26.0 ml (testosterone), and 26.0 to 40.0 ml (17 $\alpha$ -hydroxyprogesterone). This chromatographic system completely separates 5 $\alpha$ -dihydrotestosterone and testosterone. The chromatographies were run in a series of seventeen samples, each set

including control samples representing low, medium, and high steroid concentrations. The steroid radioimmunoassays were performed as described previously (Apter et al, 1976; Hammond et al, 1977b). The results were expressed in pg of steroid per 1 cm of seminiferous tubule. For statistical analyses, Student's paired *t*-test was used.

## Results

#### Microdissection Procedure

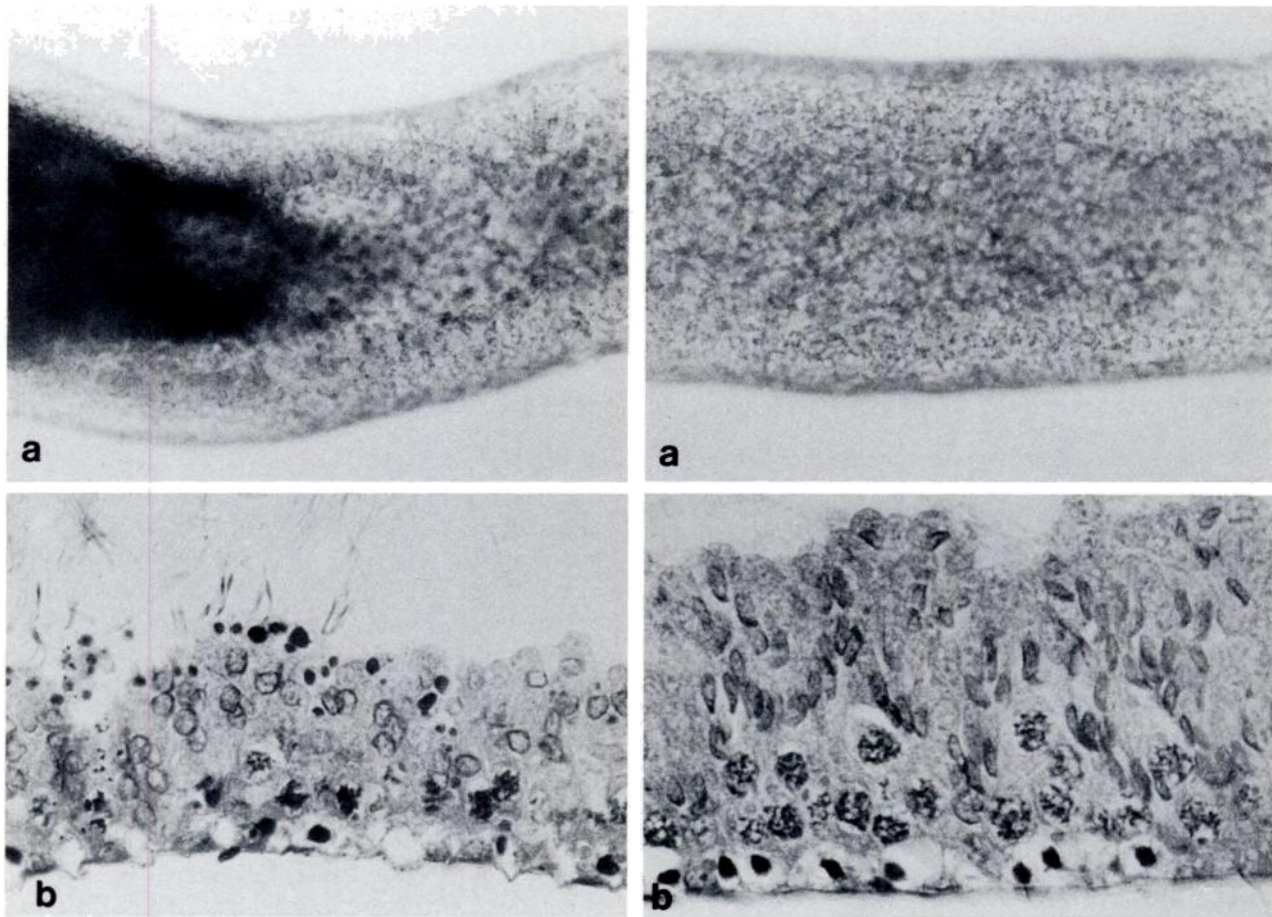
Transillumination patterns characteristic of tubular segments collected and classified by the present procedure are depicted in Figs. 2 to 11. The abrupt stop of dark absorption is a good marker for stage VIII, where spermiation occurs (Fig. 2). During stages IX–XI (Fig. 3), the nuclei of spermatids elongate but are not arranged in groups, and these stages are characterized by pale absorption. At stage XII, the elongating spermatids begin to assemble in bundles (Fig. 4). At the end of this stage, the nuclear condensation of the spermatids results in absorption that can be recognized as a weak spotty configuration in the transilluminated tubules. This configuration is definite in stages XIII (Fig. 5) and I (Fig. 6), when spermatids form tight bundles in association with the Sertoli cells. During stages II–III (Fig. 7), the bundles of spermatids increase in density and move toward the periphery of the seminiferous tubule. This, together with the increasing thickness of the flagellum, causes a strong spot configuration in the transilluminated seminiferous tubules, with peripherally oriented faint stripes. This pattern is even more prominent during stages IV–V, when the bundles of maturation-phase spermatids assume their most peripheral location (Fig. 8). During stage VI, the maturing spermatids are released from the bundles and move toward

TABLE 1. Diffusion of Testosterone from Testicular Preparations into the Dissection Medium (PBS) during 2½ hours\*

Type of Preparation	Dissection Conditions	Testosterone Concentrations	
		absolute (pg/mg)	relative (%)
Dissociated whole testis	Control (not kept in PBS)	99	100
" " "	1.5 C	86	87
" " "	22 C	66	67
Isolated seminiferous tubules (stages VII-VIII)	Control (dissected without PBS)	44†	100
" " "	1.5 C	33	75
" " "	22 C	20	45

\* n = 2.

† Possible contamination with Leydig cells.



**Fig. 2A** (upper left). Spermiation at stage VIII causes an abrupt disappearance of the dark absorption in transilluminated rat seminiferous tubule (magnification 200). **Fig. 2B** (lower left). Same specimen as shown in Fig. 2A, fixed and processed for histology. The point of spermiation is seen (magnification 850). **Fig. 3A** (upper right). Transillumination pattern in stages IX-XI is homogeneously pale. The large lipid droplets in the Sertoli cells can be seen near the periphery (magnification 200). **Fig. 3B** (lower right). The elongating spermatids at stage X are not arranged in definite bundles, and their nuclear condensation has not progressed to the point of affecting light absorption (magnification 850).

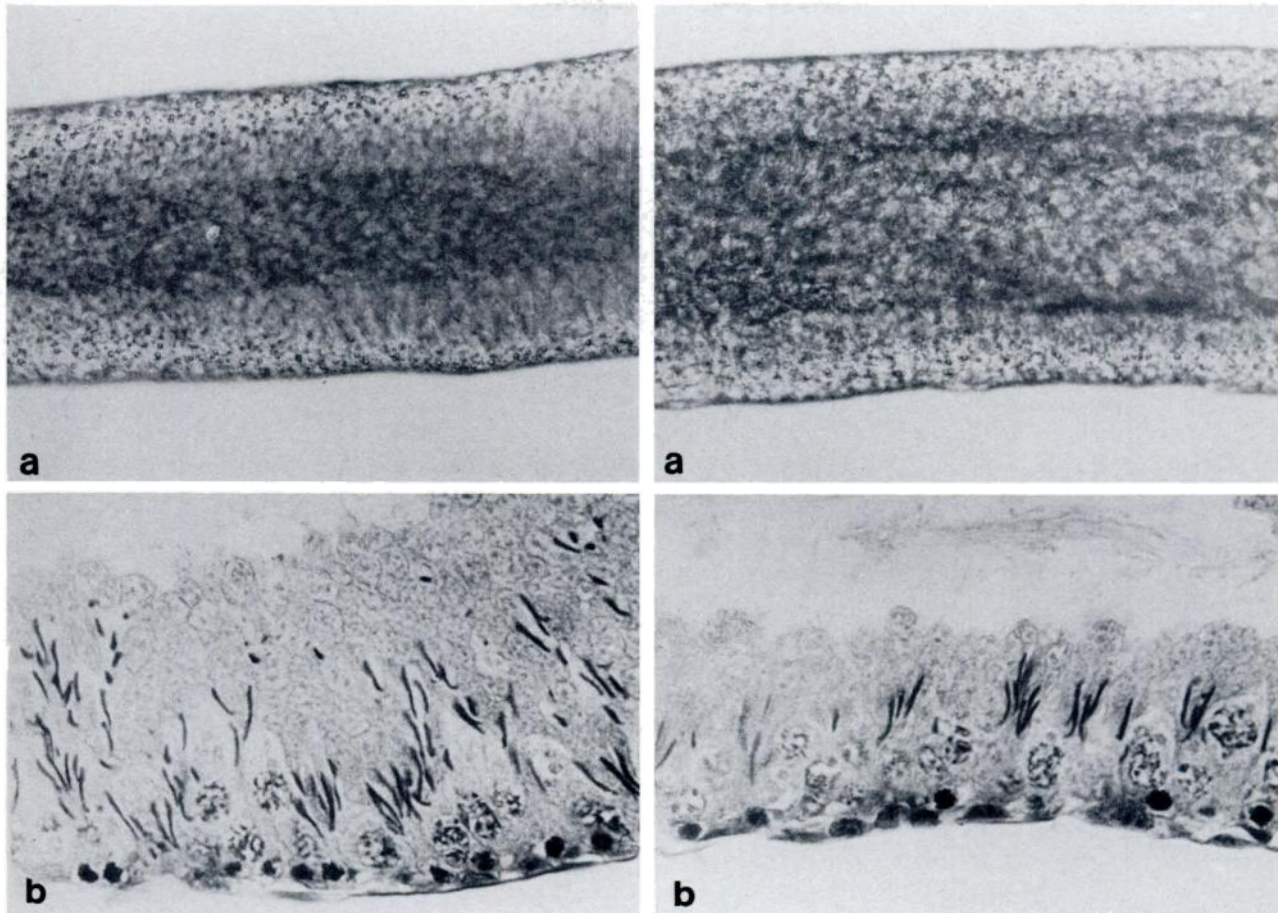
the tubular lumen. In transillumination, this is reflected in transition from the strong spot to the dark zone (Fig. 9). The long stage VII can often be divided in two parts, the beginning representing sub-stages VIIa and VIIb (Fig. 10) and the remainder, sub-stages VIIc and VIId (Fig. 11). There are no obvious differences in the transillumination pattern, but there is a change in the orientation of the residual bodies in relation to the nuclei of late spermatids.

The accuracy of the microdissection procedure is evaluated in Table 2. Stages IV-V, VI, VII, VIII and IX-XI, having the most characteristic transillumination patterns, can be separated most accurately. However, during stages XII-III, the alter-

ation of the spotty configuration of the seminiferous tubule is gradual and the error becomes maximal for the relatively short segments of the tubules in stage XIII. The appearance of the weak spots is often difficult to recognize and requires a stereomicroscope of very good quality. The onset of strong spot zone at stage II is easier to recognize, obviously due to the increasing thickness of the flagella of step 16 spermatids.

#### *Diffusion of the Endogenous Steroids*

Results of a pilot study indicate that cooling slows down the diffusion of endogenous testosterone from testicular tissue and from isolated



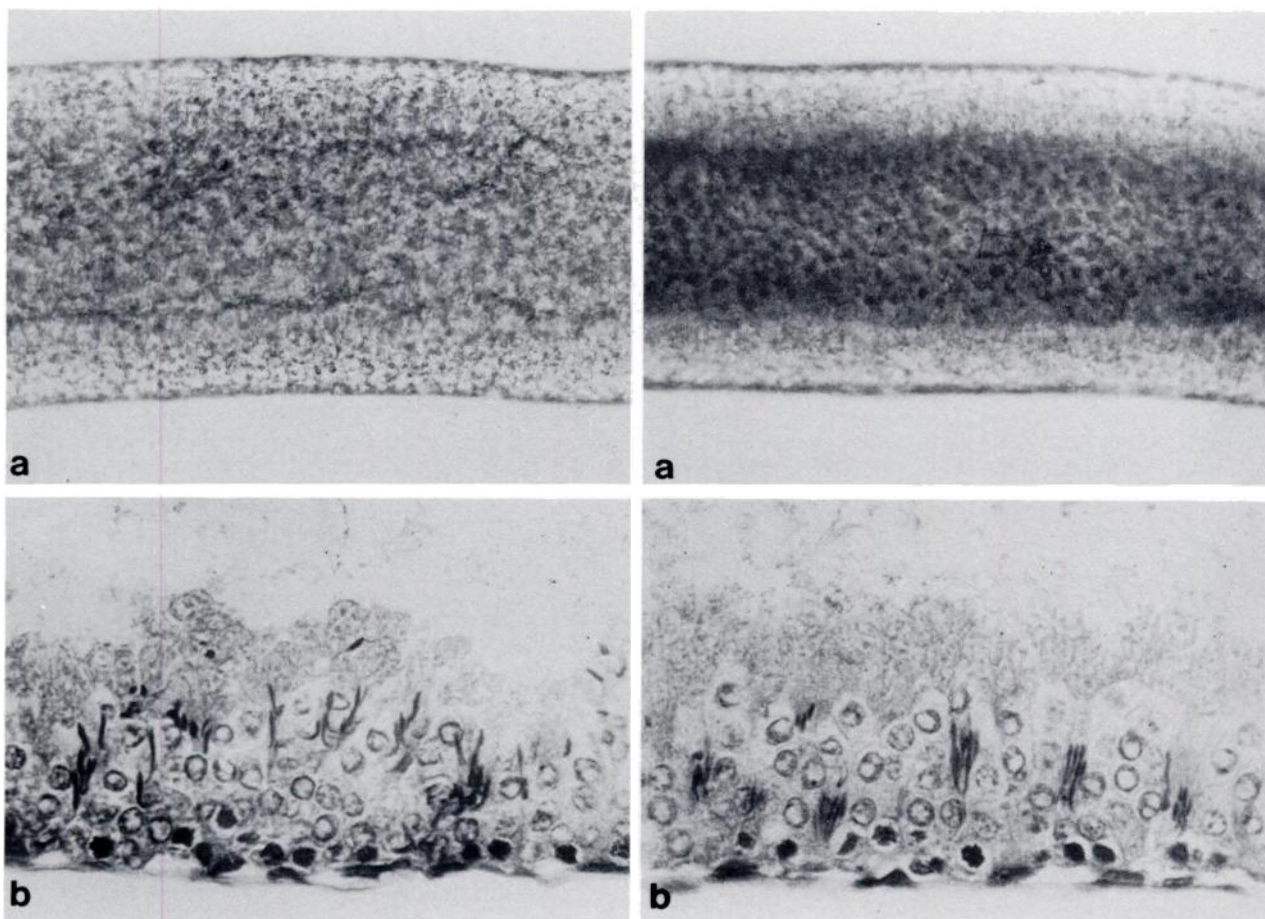
**Fig. 4A** (upper left). In stage XII, the transillumination is still homogeneously pale, but at the end of this stage, faint spots appear as can be seen in the right half of the picture (magnification 200). **Fig. 4B** (lower left). Spermatids become arranged in definite bundles during stage XII. At the end of this stage, the nuclear condensation, together with the increasing density of the bundles, causes the appearance of the weak, spotty transillumination pattern (magnification 850). **Fig. 5A** (upper right). Definite features of the weak, spotty configuration can be seen at stage XIII of the cycle of the rat seminiferous epithelium (magnification 200). **Fig. 5B** (lower right). The spotty configuration is caused by the condensed spermatid nuclei that are arranged in bundles and closely associated with the Sertoli cells (magnification 850).

seminiferous tubules into the dissection medium (Table 1). Therefore, all dissections in the main experiment were performed in temperatures between 1 and 2 C.

#### *Distribution of Endogenous Steroids in the Seminiferous Tubule*

The concentration of testosterone at stage VIII of the cycle was significantly higher than at any other stage ( $P < 0.001$ ). Moreover, the concentration of testosterone in stage VII and in stages XI–XI was significantly ( $P < 0.01$  or  $P < 0.05$ ) above the lowest levels that were observed in stages XIII–V. The absolute mean values (pg/cm  $\pm$  SEM) are given in

Table 3. It can be seen that there were considerable testosterone concentration differences between individual animals, eg, at stage VIII the concentrations of testosterone ranged from 12.0 pg/cm to 48.5 pg/cm. However, all animals showed a similar pattern of differences between different stages, and the deviations between the relative values were smaller. In addition to the prominent testosterone peak at stage VIII, 5 $\alpha$ -dihydrotestosterone levels fluctuated with the cycle and in stage VI attained a maximum value that was significantly above the lowest value found in stage VIIc–d ( $P < 0.001$ ). There were no statistically significant differences between the levels of progesterone or



**Fig. 6A** (upper left). The second part of the weak, spotty absorption pattern is assumed to represent stage I (magnification 200). **Fig. 6B** (lower left). Although transillumination does not show any major differences as compared with stage XIII, the histology is distinctly different; numerous newly formed round spermatids are present in stage I (magnification 850). **Fig. 7A** (upper right). The zone of strong spots begins at stage II. Together with the increasing density of the spots, peripheral faint stripes characterize the transillumination pattern at stages II–III (magnification 200). **Fig. 7B** (lower right). The increasing light absorption of the spots is caused by the increasing condensation of the spermatid nuclei, together with the increasing density of the bundles. Simultaneously, the bundles move towards the tubular periphery, and the flagella increase in thickness (magnification 850).

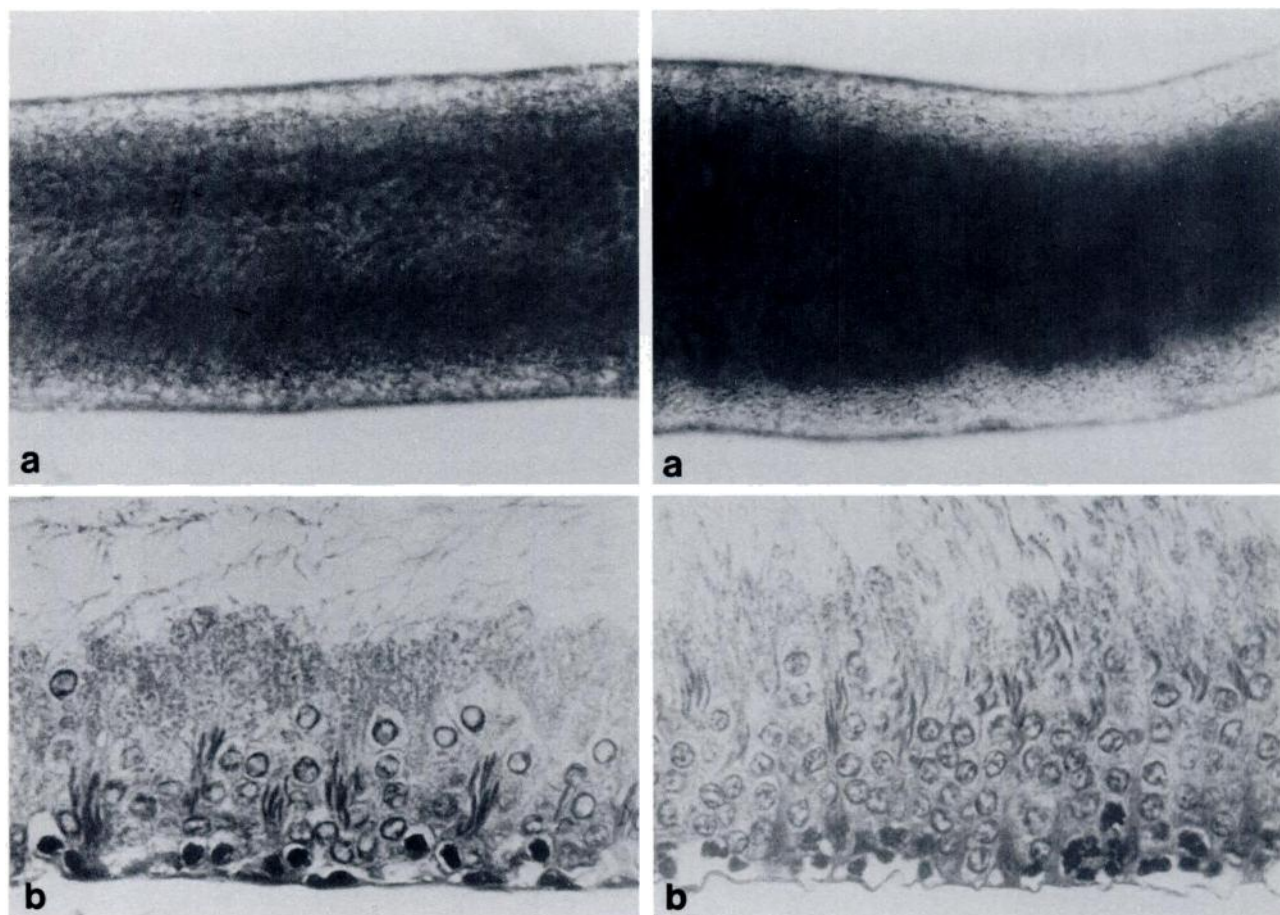
17 $\alpha$ -hydroxyprogesterone in different stages of the cycle of the rat seminiferous epithelium.

#### Discussion

The molecular mechanisms responsible for the effects of androgens on different steps of germ cell development are not clearly understood. However, there is a considerable amount of information on the hormonal regulation of the Sertoli cell. Sertoli cells contain nuclear and cytosol androgen receptors (Mulder et al, 1975; Sanborn et al, 1977) that bind to Sertoli cell chromatin with high affinity (Tsai et al, 1980). Furthermore, testosterone and 5 $\alpha$ -dihydrotestosterone were reported to stimulate RNA polymerase activity in the Sertoli

cells (Lamb et al, 1981). These observations are consistent with the general scheme of steroid action on their target tissues (Chan and O'Malley, 1976). However, it is a matter of debate whether the germ cells are targets of androgen action. There is some evidence for androgen binding to the nuclei of germ cells (Tsai et al, 1980; Wright and Frankel, 1980), but most investigators agree that Sertoli cells are the principal, if not exclusive, targets of androgens in the seminiferous epithelium (Fritz, 1978; Ritzén et al, 1981b; Grootegoed et al, 1977).

There is a considerable amount of evidence to suggest that steroid levels measured in the present study provide valid information on the concentrations of these hormones in the seminiferous



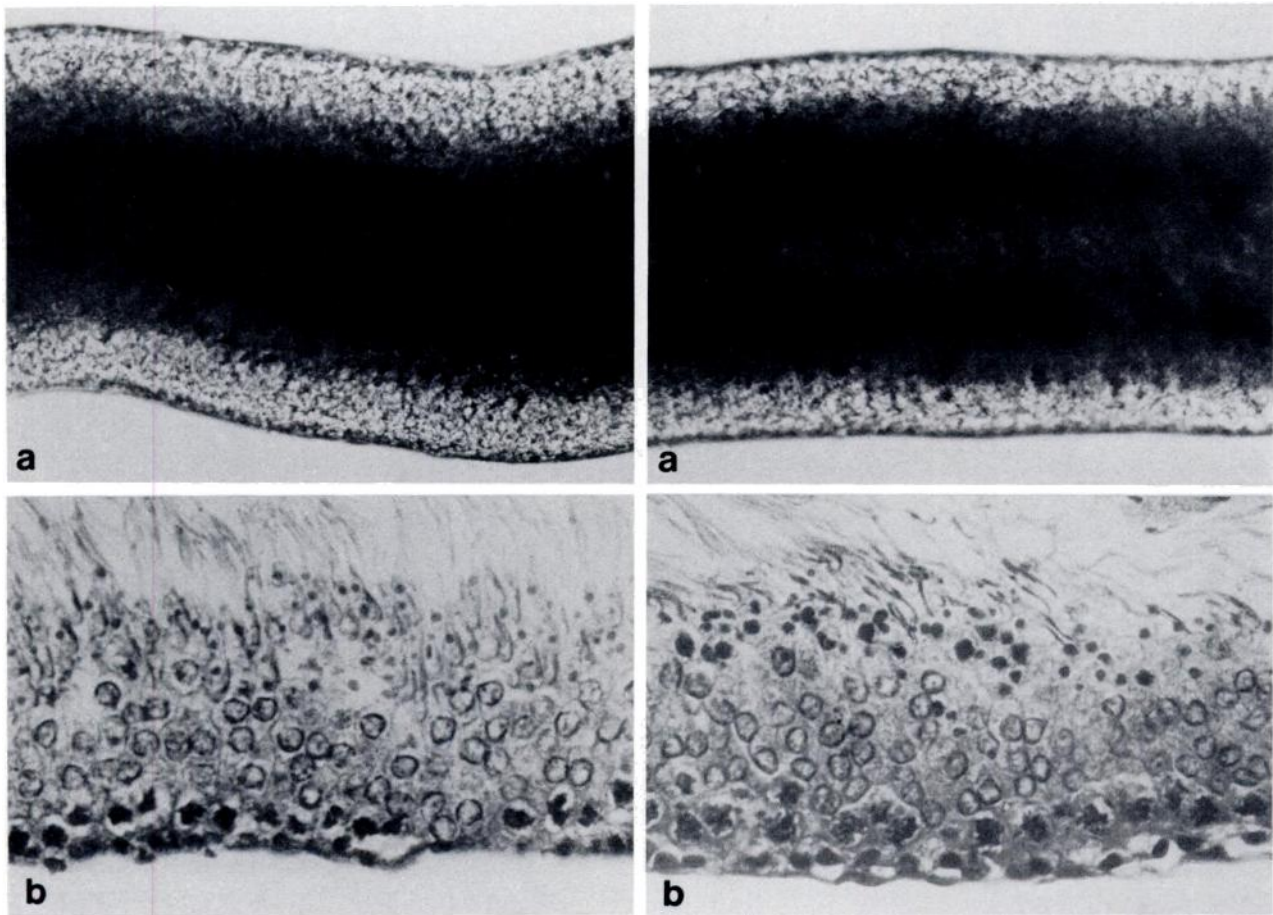
**Fig. 8A** (upper left). At stages IV and V, the strong spot configuration has its most pronounced appearance (magnification 200). **Fig. 8B** (lower left). The peripheral stripes seen in the transillumination pattern are caused by the bundles of spermatids penetrating deeply into the Sertoli cell cytoplasm and reaching their most peripheral location (magnification 850). **Fig. 9A** (upper right). At stage VI, the spots fuse and give rise to the continuous strong absorption pattern in the center of the seminiferous tubule (magnification 200). **Fig. 9B** (lower right). The bundles of spermatids are released from the inner cytoplasm of the Sertoli cells. The spermatids move centripetally and become arranged in a continuous layer at the surface of the seminiferous epithelium at stage VI (magnification 850).

tubules *in vivo*. The isolation of the seminiferous tubules from the interstitial tissue by the procedure described by Christensen and Mason (1965) has been shown to produce clean preparations (Murono and Payne, 1976; Cooke et al, 1972), and the absence of interstitial tissue contamination has been suggested by the absence of  $^{125}\text{I}$ -LH binding (Huhtaniemi and Parvinen, unpublished observation). Diffusion of testosterone into the dissection medium was unlikely to affect the present findings (Cooke et al, 1972).

Our observations on the levels of androgens in pooled fragments of the tubules support previous reports that  $5\alpha$ -dihydrotestosterone occurs in the seminiferous tubules and in the testicular venous blood in lower concentrations than does testoster-

one (Folman et al, 1972; Hammond et al, 1977a). It cannot be concluded from the present study whether  $5\alpha$ -dihydrotestosterone plays a specific role at any of the stages of the spermatogenic cycle. Its pattern of distribution is clearly different from that of testosterone, and it does not have any obvious stage-specific accumulation.

The present observations of the high concentration of endogenous testosterone at stage VIII of the rat seminiferous epithelial cycle suggest that this stage of spermatogenesis may be particularly dependent on androgen stimulation. This observation is in agreement with the finding that androgen binding protein is also maximally secreted in this stage (Ritzén et al, 1981a). It is possible that ABP is required for concentrating testos-



**Fig. 10A** (upper left). Stages VII and VIII are characterized by a strong homogenous central region of light absorption. The first part of this zone represents substages VIIa and VIIb (magnification 200). **Fig. 10B** (lower left). The basophilic (residual) bodies have appeared in the cytoplasm of the maturing spermatids. The continuous darkness in the transillumination pattern is caused by the dense spermatid nuclei that form a continuous layer at the surface of the seminiferous epithelium. This picture is from the end of substage VIIb (magnification 850). **Fig. 11A** (upper right). Stage VIIId, showing continuous darkness in the center of the tubule (magnification 200). **Fig. 11B** (lower right). Continuous surface layer of maturing spermatids, residual bodies below the nuclei (magnification 850).

**TABLE 2. Accuracy of Transillumination Method for Identifying the Stages of Spermatogenesis in Rat Seminiferous Tubules (Comparison of Results Obtained by Transillumination with those Obtained by Histological Study of the Same Tubular Segments)**

Stages Identified by Transillumination	Stages Identified in the Same Tubules by Histologic Examination (Leblond and Clermont, 1952)									Correct (%)	Wrong (%)	Maximal error (No. of stages)		
	n													
VIII a-b	23	VI	4	VIIa	4	VIIb	13	VIIc	1	VIIId	1	74	26	1
VII c-d	24	VIIa	1	VIIb	7	VIIc	11	VIIId	5			67	33	0
VIII	18	VIIId	5	VIII	13							72	28	1
IX-XI	31	IX	13	X	7	XI	8	XII	3			90	10	1
XII	35	IX	1	X	5	XI	7	XII	20	XIII	2	57	43	3
XIII-XIV	21	XII	4	XIII	10	XIV	2	I	5			57	43	1
I	38	XIII	9	XIV	8	I	19	II	2			50	50	2
II-III	23	XIV	1	I	7	II	9	III	6			65	35	2
IV-V	26	III	1	IV	10	V	15					96	4	1
VI	18	V	4	VI	14							78	22	1

TABLE 3. Concentrations of Endogenous Steroids in Rat Seminiferous Tubules at Various Stages of the Spermatogenic Cycle\*

Steroid analyzed	I	II-III	IV-V	VI	VIIa-b	VIIc-d	VIII	IX-XI	XII	XIII-XIV
Testosterone	14.0 ± 2.4	15.0 ± 2.9	13.5 ± 2.5	16.1 ± 3.6	16.8 ± 2.8	17.8 ± 3.8	23.0 ± 5.0	17.8 ± 3.4	14.9 ± 2.8	13.3 ± 2.3
5 $\alpha$ -dihydro-testosterone	2.5 ± 0.26	2.4 ± 0.38	2.7 ± 0.50	2.8 ± 0.22	2.2 ± 0.32	1.8 ± 0.10	2.4 ± 0.31	2.2 ± 0.32	1.9 ± 0.17	2.4 ± 0.32
Progesterone	10.1 ± 1.0	10.7 ± 1.0	10.0 ± 1.1	10.4 ± 0.8	10.2 ± 0.9	10.2 ± 1.2	9.8 ± 1.2	9.1 ± 1.2	10.3 ± 1.0	9.1 ± 0.9
17 $\alpha$ -hydroxy-progesterone	7.1 ± 0.6	7.1 ± 0.6	6.2 ± 0.9	6.4 ± 0.7	7.4 ± 0.6	7.7 ± 0.7	7.7 ± 1.0	7.6 ± 0.4	7.8 ± 0.9	6.9 ± 0.8

\* pg/cm ± SEM; n = 7

terone in the tubule at this particular stage of the cycle. Although detailed immunohistochemical studies have not been conducted, there is some evidence for variations in the concentration of ABP in different stages of the seminiferous epithelial cycle (Pelliniemi et al, 1981). Furthermore, Russell and Clermont (1977) have shown that 5.5 days after hypophysectomy, there is an increase in the number of degenerating mid-pachytene spermatocytes and steps 7 and 19 spermatids in stages VII and VIII, and that this can be prevented by treatment with LH and FSH. The particular androgen dependency at this point of the cycle of the seminiferous epithelium can be suspected from the fact that LH had a more pronounced effect than does FSH.

A number of other observations suggest specific changes in the metabolic activity of Sertoli cells during stages VII and VIII. There is an increase in the amount of the flattened type of endoplasmic reticulum (Assaf, 1980) which is indicative of increased protein synthesis. Furthermore, plasminogen activator is secreted specifically during stages VII and VIII (Fritz et al, 1981) and secretion of a meiosis-inducing substance which is presumably produced by the Sertoli cells is maximal in the same stages (Parvinen et al, 1981). The ectoplasmic specializations of the Sertoli cells that face the germ cells undergo recycling during stage VIII. At this time, pachytene spermatocytes and steps 7 to 8 spermatids receive these structures, presumably from the spermatids released during spermiation (Russell et al, 1980). It is possible that all these morphologic and functional changes during stages VII and VIII are specifically controlled by androgens.

Collection of tubular fragments at stage VIII of the cycle for biochemical studies is convenient and effective because, due to the obvious difference between the dark and pale zones, this stage is the most easily isolated point in the transilluminated seminiferous tubule. It would be of considerable interest to study the possible specific mechanisms of androgen action during this stage, when numerous events occur in the development of the germ cells. The yield of material may prove to be the main difficulty because 1 cm of seminiferous tubulus weighs approximately 1 mg (wet weight) and contains approximately 100,000 cells. However, pooling of the material should allow studies of hormonal regulation of protein synthesis in the

Sertoli cells at various stages of the cycle. This would result in new insights into the mechanisms by which Sertoli cells, under the control of FSH and androgens, create a micro-milieu that is optimal for the germ cells at various stages of their development.

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