EFFECTS OF THE ANTI-ANDROGEN

FLUTAMIDE ON THE DESCENT OF THE

TESTIS

BY

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December, 1996.

ACKNOWLEDGEMENTS

ABSTRACT

Exposure of male Albino Swiss rats to the non-steroidal anti-androgen flutamide during the period from gestational day 10 to birth resulted in feminization of the external genitalia and the suppression of growth of the male reproductive tract. In adulthood, testes were found to be located in diverse positions. True cryptorchidism occurred in 12% of cases, while 44% of testes descended to the scrotum and 44% were located in a suprainguinal ectopic region. Varying degrees of tubule abnormality were seen in the testes of flutamide-treated animals, ranging from completely normal tubules with full spermatogenesis (and the expected frequency of the stages of spermatogenesis) to severely abnormal tubules lined with Sertoli cells only. For each individual testis, the overall severity of tubule damage was strongly correlated with its adult location, with intra-abdominal testes worst affected and scrotally-located testes least; only the latter contained normal tubules. Similarly, intra-abdominal testes were the smallest in weight and contained the least testosterone. By contrast, postnatal treatment of male rats with flutamide from birth to postnatal day 14 did not impair development of the external genitalia, the process of testicular descent or adult spermatogenesis. These findings confirm that androgen blockade during embryonic development interferes with testicular descent but also demonstrate that i) prenatal flutamide treatment *per se* has a detrimental effect on adult testis morphology but ii) the degree of abnormality of the testes is strongly influenced by location, and iii) the varying degrees of seminiferous tubule damage are probably due to the stage-specific effects of reduced testicular testosterone on spermatogenesis. The findings of this study

also demonstrate that outgrowth of the gubernacular cone is not affected by flutamide; however, the regression phase (which occurs postnatally) was delayed. Furthermore, shortening of the gubernacular cord was inhibited in males treated prenatally with flutamide. Exposure of males rats to anti-androgen flutamide resulted in the persistence of the cranial suspensory ligament. This persistence however, was irrespective of the position of the testes in the adult. Nor was there any difference in the structure of the cranial suspensory ligaments from these varying testis locations. This suggests that retention of the cranial suspensory ligament is not an important factor in testicular maldescent. Prenatal flutamide treatment to rats also interfered with Wolffian duct development since most of the offspring of flutamide-treated mothers exhibited varying degrees of inhibition/absence of Wolffian duct derivatives. Moreover, impaired development of the epididymis and vas deferens was not necessarily associated with testicular maldescent since (despite partial to complete absence of epididymis and vas deferens) 44% of testes descended normally into the scrotum in males treated prenatally with flutamide. In contrast to the rat, prenatal flutamide treatment to hamsters did not interfere with testicular descent although there was clear evidence of flutamide action during development including inhibition of development of the Wolffian duct and retention of cranial suspensory ligament.

INTRODUCTION

The process of testicular descent in mammals has been a classic problem of developmental biology since the time of **John Hunter (1762)** - cited by **Backhouse (1964)** who first described the gubernaculum testis and considered its involvement in testicular descent. Despite a great deal of research carried out on a variety of species by many workers, the mechanism of testes descent and its controlling factors remain matters of debate.

This thesis reports on the effects of prenatal exposure to the anti-androgen flutamide on:-

1. the development of the male reproductive tract in two rodent species, the rat and hamster with particular reference to

   a) the position of testes during adulthood,

   b) the morphology of these testes,

   c) the capacity of these testes to produce testosterone, and

   d) the spermatogenic cycle in the seminiferous tubules.

2. the process of testis descent during the prenatal and neonatal period in the rat with particular reference to the developmental morphology of the gubernaculum during the outgrowth phase and the regression phase.

3. the persistence of the cranial suspensory ligament.

1. DEVELOPMENT OF MALE REPRODUCTIVE ORGANS:

Normal sexual development consists of three sequential processes: Firstly, it involves the establishment of chromosomal sex at the time of fertilization, the heterogametic complement (XY) being male and the homogametic state (XX) female. Secondly, it involves the development of gonadal sex. The Y chromosome carries genetic determinants (SRY) in human **(Sinclair, 1990)** and (Sry) in mouse **(Gubbay et al, 1990)** that induce the indifferent gonad to differentiate into a testis. In the absence of this positive influence, as in the normal female and in some forms of gonadal dysgenesis, the indifferent gonad becomes an ovary. Thirdly, it involves the translation of gonadal sex into phenotypic sex; this is normally the direct consequence of the type of gonad formed and its secretions (for review see **Hunter, 1995**).

Development of the sexual phenotype normally conforms to chromosomal and gonadal sex. In the absence of the testis, as in the female or in the male embryo castrated before phenotypic differentiation, phenotypic development is female **(Jost et al, 1973).** In mammals, development of the female pattern does not require the presence of gonad, whereas male development is induced by hormones from the fetal testis.

The internal accessory organs of reproduction in the two sexes are derived from separate anlagen, the Müllerian and Wolffian ducts, which are present in early embryos of both sexes. In normal development, the Wolffian ducts in the male give rise to the epididymis, vas deferens and seminal vesicles,

while the Müllerian ducts disappear. In the female, the Müllerian ducts give rise to the fallopian tubes, uterus and upper vagina, while the Wollfian ducts regress. In contrast, the external genitalia and the urethra in the two sexes develop from a common anlagen - the urogenital sinus. In the male, the urogenital sinus gives rise to the prostate and the prostatic urethra and, in the female, to the urethra and the lower portion of the vagina. The genital tubercle is the progenitor of the glans penis in the male and the clitoris in the female. The genital swellings become the scrotum or the labia majora, and the genital folds develop into the shaft of the penis or the labia minora.

Development of the male phenotype is the result of the action of three hormones: a) Müllerian inhibiting substance (MIS), b) testosterone and c) dihydrotestosterone (DHT). MIS supresses the Müllerian ducts and thereby prevents development of the uterus and fallopian tubes **(Josso _et al_, 1993a)**. Testosterone promotes male development in two ways: 1) by acting directly on the Wolffian ducts to stimulate their conversion into epididymis, vas deferens and seminal vesicles; 2) by serving as a prohormone for the third fetal hormone dihydrotestosterone which induces both formation of prostate from the urogenital sinus and formation of the external genitalia **(Wilson _et al_, 1981)**. Thus, androgens induce the formation of the entire male genital tract during fetal life. Finally, there is migration of the gonad to the position it will occupy in adult life. In the case of the testis this involves descent into the scrotum which will be dealt with in detail below.

a) Development of testis:

Differentiation of the gonads is the first phenotypic evidence of sexual dimorphism. Gonadal development involves the migration, organisation and differentiation of both germinal and somatic cells, and numerous reviews are available on the processes involved in the formation of the gonads in mammals (**Clermont and Huckins, 1961; Clark and Eddy, 1975; Jost et al, 1973; Eddy et al, 1981; Wartenberg, 1989; Jost and Magre, 1993; Hunter, 1995**).

The presence of the testis-determining gene, SRY (human) and Sry (mouse) on the Y chromosome acts to programme the formation of the testis and thus the differentiation of the supporting cell lineage to form Sertoli cells. All other aspects of seminiferous tubule organisation would then be triggered and directed by the Sertoli cells without further Y chromosome involvement (for review see **Hunter, 1995**).

The somatic (i.e. non germ) cells of the developing gonads may involve contributions from 1) the coelomic epithelium and adjacent mesenchymal cells and 2) cells of the mesonephros. There is some controversy on the source of somatic cells in the developing gonad. Some authors believe that somatic cells are derived from the coelomic epithelium (**Yoshinaga et al, 1988; Growney, 1994**), while others believe that these cells arise from the mesonephros (**Upadhyay et al, 1981a; 1981b; Zamboni and Upadhyay, 1982; Satoh, 1985; 1991; Mackay et al, 1989; Smith and Mackay, 1990; Buehr et al, 1993**). A dual

origin for somatic components from both coelomic epithelium and mesonephros is proposed by **Pelliniemi (1975)** and **Wartenberg (1981; 1982).**

In the human embryo, PGCs are first identified in the dorsal endoderm of the yolk sac membrane **(Figure 1a)** at 4 weeks post-conception (~14-somite embryo), in the hindgut epithelium and mesentery at 5 weeks (~22-somite embryo) and then in the genital ridge **(Figure 1b-c)** at 6 weeks **(Fujimoto et al, 1977)**. The latter authors found that germ cells migration is by active amoeboid movement and cells can be readily tracked by plasma membrane alkaline phosphatase activity. The presence of abundant extracellular fibronectin (FN) in the migratory pathway suggests that FN plays a significant role in the migration of PGCs **(Fujimoto et al, 1985)**. However, **Jeon and Kennedy (1973)** who studied PGCs in mouse embryo suggested that PGCs may migrate passively following the morphogenetic movement of the yolk-sac endoderm to form the gut; thus PGCs are enclosed in the gut epithelium in the 4-week embryo as a result of invagination of the the yolk-sac endoderm into the embryo proper to form the gut.

The initial location of the earliest germ cells remains unclear; they have been identified in mouse embryo at 8-9 days post-coitum (d.p.c.) within the mesoderm at the base of the allantois, in the caudal end of the primitive streak **(Eddy et al, 1981; Gardner et al, 1985; Ginsburg et al, 1990)**.

FIGURE 1: Diagrams showing

(a) mid-sagittal view through an early embryo prior to primodial germ cell migration showing the origin of primordial germ cells,

(b) oblique view of the mid-gut and its mesentery showing route of migration of the germ cells and

(c) transverse section of an embryo at the beginning of gonadal differentiation.

(Adapted from Human Embryology and Developmental Biology by Bruce M. Carlson (1994).

1a.

Intestinal loop — Mesonephros

Mesentery — Gonad

Primordial germ cells

b.

Aorta

Mesentery — Gonad

Gut — Primordial germ cells
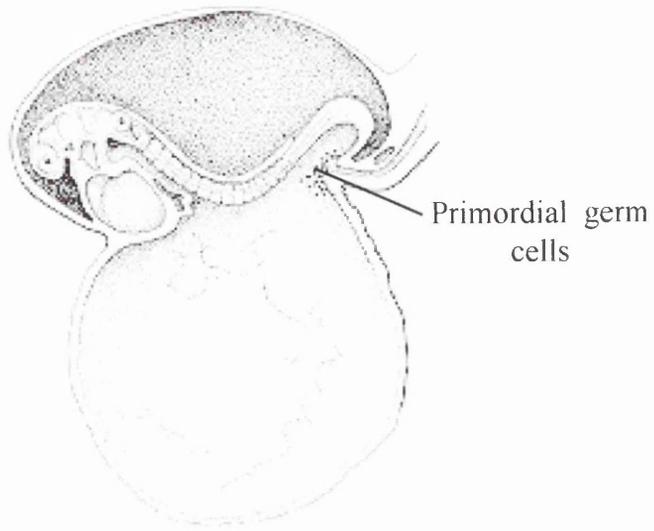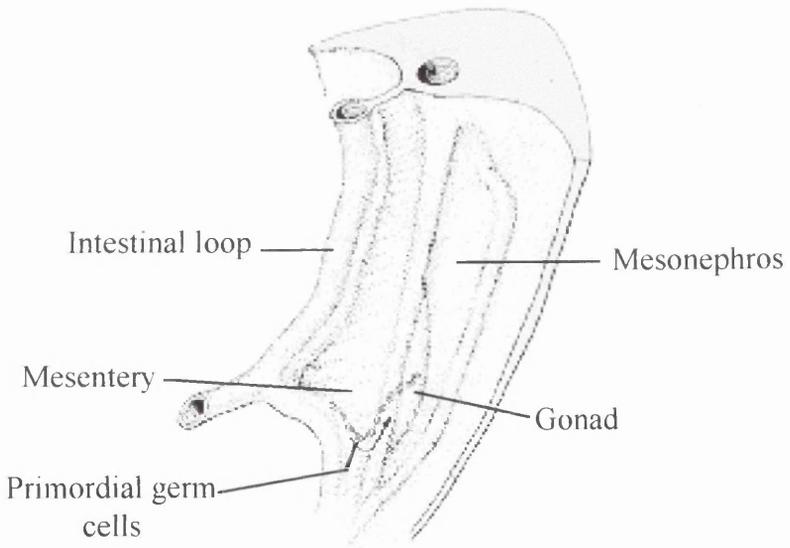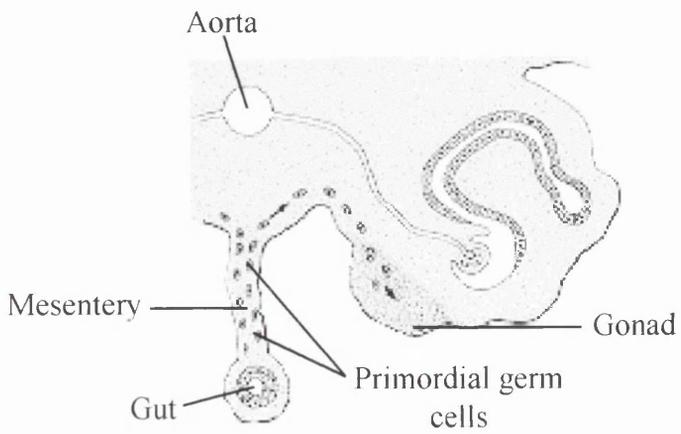
c.

A number of suggestions have been put forward as to what initiates migration of germ cells from the extra-gonadal sites, and what informational cues are required for the successful completion of the process:

1). **Godin et al, (1990)** demonstrated two factors that control migration in mouse embryos in in vitro studies using PGCs from 8.5 d.p.c.: a) the intrinsic capacity of PGCs to spread and move, b) extrinsic (chemotropic) factors which were emitted from the genital ridges and which acted to guide the PGCs. In this study, culture medium conditioned by 10.5 d.p.c. genital ridges, limb buds and hindgut mesenteries showed that PGCs migrate towards the genital ridges in preference to other explanted organs.

2). PGCs express cell-specific and stage-specific molecules on their cell surface. These surface molecules, termed differentiation antigens, are thought to play a role in the events of recognition, attachment and interaction between cells during migration **(Eddy et al, 1981; Thiery et al, 1984)**.

3). An extracellular glycoprotein, FN, may serve to stimulate translocation of the migrating cells since immunocytochemical study of mouse embryo (between 9.5 - 11.5 d.p.c.) showed that the migratory pathway of PGCs (i.e. the hindgut lining and mesentery) is rich in FN **(Fujimoto et al, 1985; De Felici et al, 1992)**.

Primordial germ cells (PGCs) are large cells (15-20 μm in diameter) with a large, round nucleus and prominent nucleolus: the cytoplasm contains a substantial amount of glycogens, numerous lipid droplets, ribosomes and mitochondria **(Fujimoto et al, 1977)**. They increase in number by mitotic

division during migration, but most conspicuously once the germ cells reach the presumptive gonads **(Godin et al, 1990; Tam and Snow, 1981).**

While the process of multiplication of germ cells proceeds in the fetal testis, the initiation of meiosis in the male is delayed until the time of puberty **(Jost and Magre, 1993)**. The XY germ cells in the testis enter a state of mitotic arrest as (pro)spermatogonia at the same time as female germ cells enter meiosis (at 13-15 d.p.c. in the mouse), and they resume mitotic proliferation in the immediate post-natal period **(McLaren, 1983; Jost and Magre, 1993).**

The gonadal anlagen in vertebrates is bipotential, that is it is capable of developing into either an ovary or a testis. The superficial cortical region of the anlagen would differentiate into an ovary whereas the medulla would differentiate into a testis **(Jost et al, 1973)**. **Jost** and his colleagues suggested that there should be an antagonism between the cortex and medulla (i.e. proliferation of one region and regression of the other) if a distinct ovary or testis is to form. Thus, in mammals, the development of the ovary can be viewed as a default pathway although it still requires a specific gene programme, while development of the testis requires definite activation and thus diversion from the default pathway.

Formation of a functional gonad requires the correct differentiation of both somatic and germ cells. The earliest histological indication of sexual dimorphism in the pattern of gonadal differentiation is the appearance of primordial Sertoli cells in genetic males and their organisation as presumptive seminiferous cords separated from the true epithelium by a basal lamina **(Jost**

and Magre, 1993). Development of a testis is reflected in rapid growth of the organ and differentiation of cell types occurs earlier in the testis than in the ovary (**Jost et al, 1973; Jost and Magre, 1988**). The probable reason that male gonads grow sooner and faster is that testicular products such as androgens and MIS are necessary to prevent phenotypic feminization as the 'default' pattern. It is not surprising that the undifferentiated phase before an ovary is distinguishable in genetic females is prolonged compared with the relatively rapid development of a testis in genetic males (**Jost et al, 1973**) since there are no comparable pressures in the developing female fetus.

Testis differentiation begins in the centre of the genital ridge close to the mesonephric tubules. The PGCs are withdrawn towards the medulla and the sex cords become a prominent feature (about 12.5 d.p.c. in the mouse and 13.5 d.p.c. in the rat). Formation of sex cords is accompanied by mitotic multiplication of all cell types: somatic cells and PGCs. However, there is not a complete separation between the mesothelium and the underlying tissue since a basal lamina is only formed 24 hours later (i.e. at 14.5 d.p.c. in the rat) (**Jost and Magre, 1993**).

The primordial Sertoli cells are specialized somatic cells with clear abundant cytoplasm which surround the germ cells and achieve intimate contact by means of long cytoplasmic processes. Differentiation and alignment of Sertoli cells are the first steps in testicular cord formation in genetic males, simultaneous with a rapid proliferation and aggregation of PGCs (**Jost et al, 1981; Magre and Jost, 1980; 1983**).

Normal testis cord formation requires peritubular myoid cells, which migrate from the mesonephric region. The evidence in support for this is when 11.5 d.p.c. testes were grafted to mesonephric regions from mice carrying a marker, the marker was found in some of the peritubular myoid cells and interstitial cells (**Buehr et al, 1993**). These authors concluded that cells can migrate from the mesonephric region into the differentiating testis and contribute to the interstitial cell population that is necessary for the establishment of normal cord structure. The cords become the future seminiferous tubules once a central lumen develops and a basement membrane has formed. Further growth of the testis is then mainly due to proliferation of somatic and germ cells already within it rather than further invasions of mesonephric cells.

The periphery of the developing gonad becomes occupied by the membranous tunica albuginea, and a prominent blood supply is characteristic of the developing testis (**Mackay et al, 1993**). However, although the tunica albuginea and the interstitial tissue become extensively vascularised, the germ cells become isolated from direct contact with blood capillaries by a more or less complex basement membrane and junctional complexes between the Sertoli cells, the so-called blood-testis barrier (**Dym and Fawcett, 1970**).

After waves of mitotic divisions the male germ cell line remains relatively undifferentiated on the basement membrane as prospermatogonia or spermatogonia until shortly before puberty (**Jost and Magre, 1993**). Male germ cells do not usually start meiosis until this time, thus DNA replication and cell

division are not usually resumed until after birth. The nature of the factor(s) causing such prolonged inhibition of meiosis remains uncertain, although it is thought to originate from the surrounding Sertoli cells since germ cells cannot proceed through differentiation unless in contact with somatic cells **(Jost and Magre, 1988).**

Morphological differentiation of Leydig cells within the interstitium occurs later, and is correlated with the onset of steroidogenesis. Leydig cell precursors migrate from the mesonephric region into the genital ridge before 12.5 d.p.c and recognisable Leydig cells occur at 13.5 d.p.c. in the mouse (at 15.5 d.p.c. in the rat). This correlates with activation of the gene for 3β-hydroxysteroid dehydrogenase, which is expressed in the mouse testis by 13.5 d.p.c but not by 12.5 d.p.c. **(Jost and Magre, 1993)**. Differentiation of Leydig cells from mesenchymal precursors is said to be under the influence of Sertoli cells **(Jost and Magre, 1988)**

By 14.5 d.p.c., the seminiferous cords of the fetal rat testis are well organised and made up of the primordial Sertoli cells and PGCs. These cords are disposed in double arcades in planes perpendicular to the length of the gonad **(Clermont and Huckins, 1961; Magre and Jost, 1983)**. The germ cells have a round nucleus and one or more distinct nucleoli; the mitochondria are round and swollen. The tissue between the cords is scarce. A basal lamina forms as flattened mesenchymal cells around the seminiferous cords.

Further development of the seminiferous cords has been detailed in the rat by **Clermont and Huckins (1961).** Briefly, by 15.5 d.p.c., the seminiferous cords have become very large and obvious and are arranged in double arcades. The interstitial cells are packed together. By 16.5 d.p.c., the tissue surrounding the cords becomes less packed and the Leydig cells more conspicuous. The Sertoli cells migrate to the outer surface of the sex cords. The testis appears as an elongated and slightly cresentric organ running along the medial aspect of the mesonephros. The seminiferous cords remain in double arcades with their two extremities connected with the rete testis. By 19 d.p.c., the testis has become spherical, and the seminiferous cords begin to show some convolutions.

In neonates, the seminiferous cords maintain their circular pathway around the testis, though they become increasingly folded into tiny convolutions. There is a 4-fold lengthening of each cord between E19 and birth. In pups at 12 days after birth, the outer seminiferous tubules follow a circular pathway around the testis, but the tubules undergo extensive elongation. In the adult, the basic plan of the seminiferous tubules is maintained; the tubules show a large number of regular convolutions. Although many morphological features are highly modified during the extensive growth of the sex cords into adult seminiferous tubules, the architectural plan of tubules in the adult testis reflects the distribution of sex cords in the embryo.

b). Differentiation of the male genital ducts:

The embryonic duct system may also be viewed as bipotential in that it is duplicated during the initial stages of organogenesis. In the very young embryo, there are two sets of primitive reproductive tracts: the male (Wolffian) and female (Müllerian) duct system respectively **(Figure 2a)**. These ducts can be distinguished alongside each other for a period of time during embryogenesis irrespective of the sex chromosome complement of the individual. Such duplication would imply that there is still the potential to promote formation of either a male or female duct system. The Wolffian (mesonephric) ducts appear first as the excretory ducts for the mesonephros. The Müllerian (paramesonephric) ducts develop later in the proximity of the Wolffian ducts **(Jost et al, 1973).**

During normal development of the embryo, only one of the bilaterally paired duct systems develops whilst the other undergoes regression **(Figure 2b)**. However, this may not be the situation in cases of chromosomal anomalies or hormonal disturbance. With the onset of sexual differentiation, the testis-determining gene (SRY) on the Y chromosome initiates testicular development **(Sinclair, 1994)** and the production of testicular hormones - Müllerian inhibiting substance (MIS) and testosterone **(Lee and Donahoe, 1993; Josso et al, 1993a)**. Under the influence of MIS, the Müllerian duct begins to regress so that the male is not born with fallopian tube, uterus and vagina **(Lee and Donahoe, 1993; Behringer, 1995)**. Testosterone is responsible for stimulating the Wolffian

FIGURE 2:   Diagrams showing

(a) the paired genital ducts at the indifferent stage; both the Müllerian and the Wolffian ducts are present

(b)   the Müllerian ducts persist in the female but regress in the male while the Wolffian ducts persist in the male but regress in the female.

(Adapted from Jost and Magre, 1993).

Adrenal

Ureter

Wolffian duct

Mesonephros

Kidney

Gonad

Mullerian duct

Gut

Gubernaculum

Bladder

2a. Indifferent stage

Mullerian duct

Rete ovarii

Wolffian duct

Seminiferous cord

Mullerian duct

Rete testis

Mesonephros

Wolffian duct

Ovarian cortex

Uterus

Urogenital sinus

Seminal vesicle

Prostate

Urogenital sinus
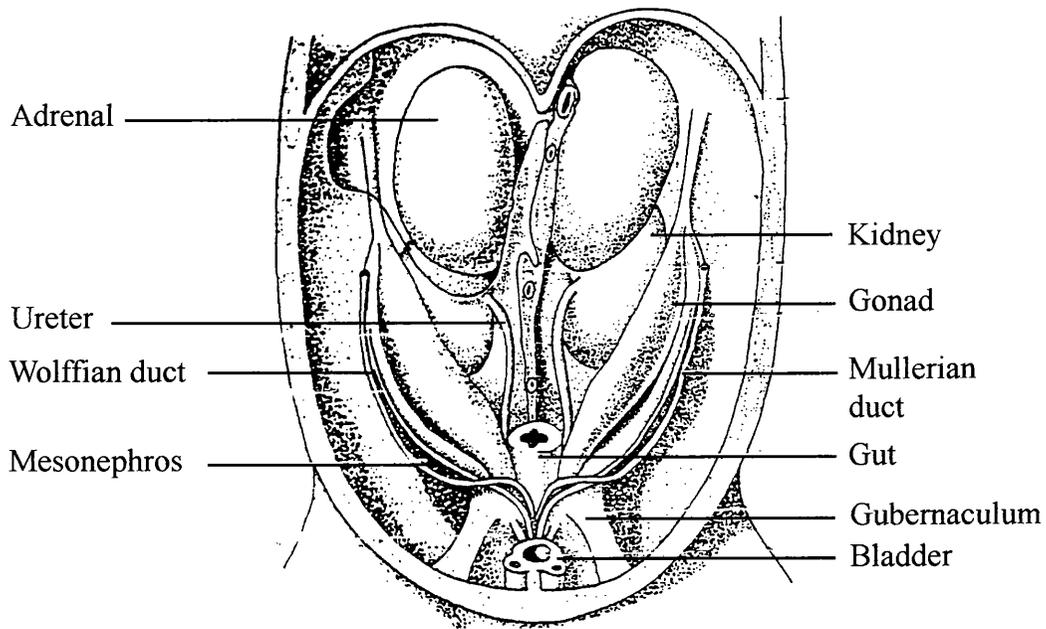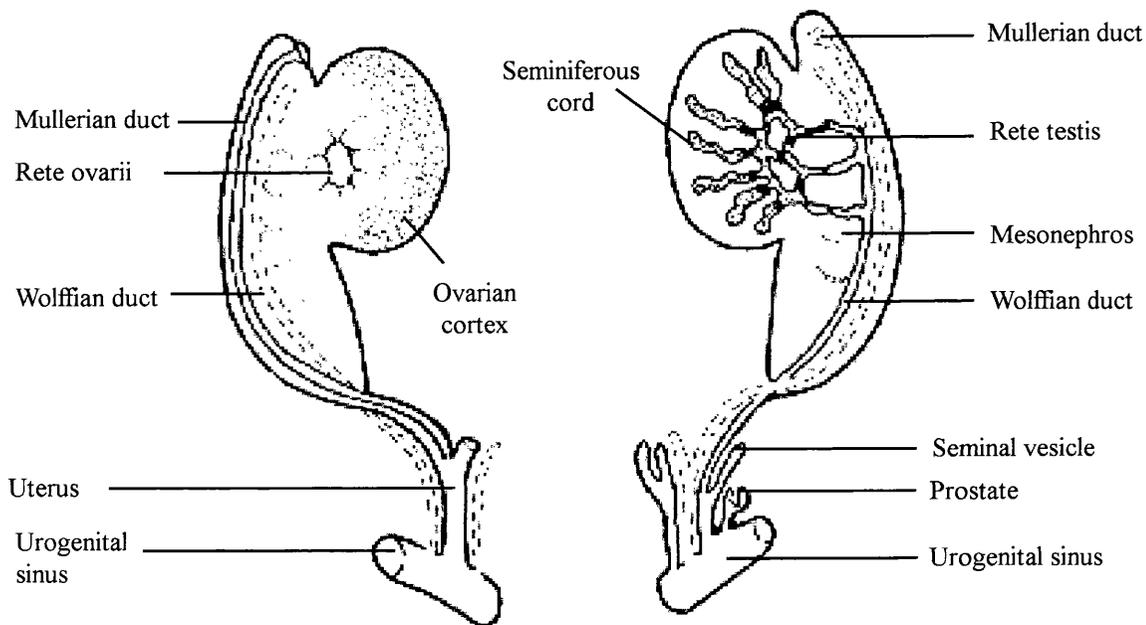
b.   **Female**   **Male**

ducts to continue their development to form the epididymis, vas deferens and seminal vesicles (**Wilson et al, 1981**).

MIS is the first known molecule produced by fetal Sertoli cells (**Tran, 1977; Tran and Josso, 1982**). Its synthesis begins before the appearance of Leydig cells. Furthermore, the Müllerian ducts of the developing male embryo are only sensitive to MIS during a limited early period after differentiation of the gonad. The critical period of responsiveness of Müllerian ducts to MIS in rats is 13.5 -15 d.p.c. and in man is about weeks 7-8 of gestation; exposure to MIS before or after this critical period is ineffective (for review see **Josso et al, 1993a**).

The differentiation of the Wolffian duct to form the paired and convoluted epididymis, the muscular vas deferens and the seminal vesicles begins shortly after the formation of testicular cords and is under the influence of testosterone (**Wilson, 1992**). The paired Wolffian ducts do not contain 5α-reductase (**Griffin and Wilson, 1980; Wilson, 1992**). By contrast, virilisation of the urogenital sinus (to become the prostate and the membranous urethra) and external genitalia during embryogenesis do require dihydrotestosterone since the urogenital sinus and phallus contain high activity of 5α-reductase (the enzyme that converts testosterone to dihydrotestosterone) during the period of differentiation of the genitalia (**Renfree et al, 1992**).

Dihydrotestosterone has some 10x the affinity for androgen receptors than testosterone (**Grino et al, 1990**), enabling the external genitalia to

be masculinised by very low concentrations of testosterone. The action of dihydrotestosterone stimulates the urogenital sinus to form the prostate and the membranous urethra, the urogenital tubercle to form the penis and penile urethra and urogenital swelling to form the scrotum **(Wilson et al, 1981)**. However, the precise cellular mechanisms whereby testosterone acts to promote male duct differentiation and development remains uncertain.

2. NORMAL TESTICULAR DESCENT:

Descent of the testis is one of the normal events during development of the male reproductive system in most (but not all) species of mammals (Backhouse, 1966; 1982; Wensing, 1986; Wensing and Colenbrander, 1986; Heyns, 1987; Hutson and Beasley, 1992). The process of testicular descent has received a great deal of attention because anomalies of testicular descent are frequently encountered in man (Backhouse, 1966; 1982; Scorer and Farrington, 1971; Heyns, 1987) and in other mammalian species (Spencer Barthold et al, 1994).

In man (Backhouse, 1982; Heyns, 1987) and in animals such as pig, calf and dog (Backhouse and Butler, 1960; Hullinger and Wensing, 1985; Heyns et al, 1986; Wensing, 1986; Fujikake et al, 1989) the gubernaculum is a structure extending from the caudal pole of the testis via the epididymis to the region of the future inguinal canal in the anterior abdominal wall and into the scrotal swelling (Figure 3a). A gutter forms around the junction between the gubernaculum and the anterior abdominal wall; this is the beginning of the processus vaginalis. The processus vaginalis thus divides the gubernaculum into three parts: a) the gubernaculum proper, consisting of an intra-abdominal part suspended in a peritoneal fold and an extra-abdominal part suspended by the visceral layer of the processus vaginalis, b) the vaginal part which receives the termination of the cremaster muscle, and c) the infra-vaginal part (Figure 3b). It is generally agreed that during testicular descent, there is never an organized

FIGURE 3: Semi-diagramatic representation of the gubernaculum in species with strip-like cremaster muscles

(a) The appearance of the gubernaculum during the outgrowth phase prior to testicular descent.

(b) The appearance of the gubernaculum at the beginning of testicular descent. Note: the processus vaginalis is formed and thus, at this stage, the gubernaculum may be divided into gubernaculum proper, a vaginal part and an infra-vaginal part.

Testis

Epididymis

Gubernaculum

3a.

Testis

Epididymis

Gubernaculum proper

Vaginal part of gubernaculum

Cremaster muscle

Infra-vaginal part of gubernaculum

Processus vaginalis
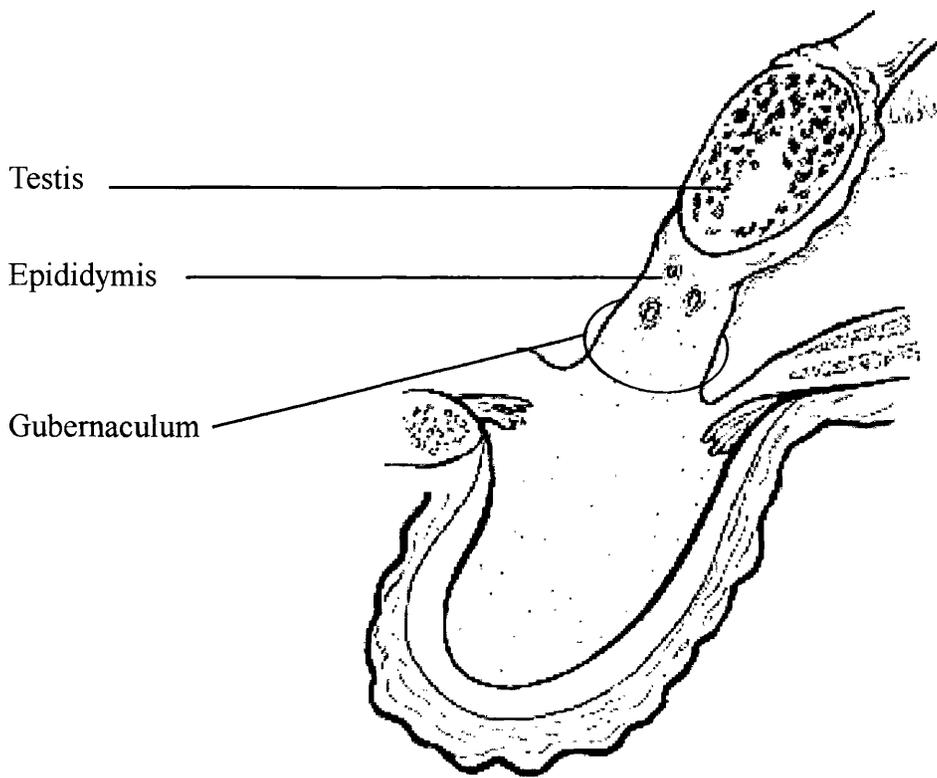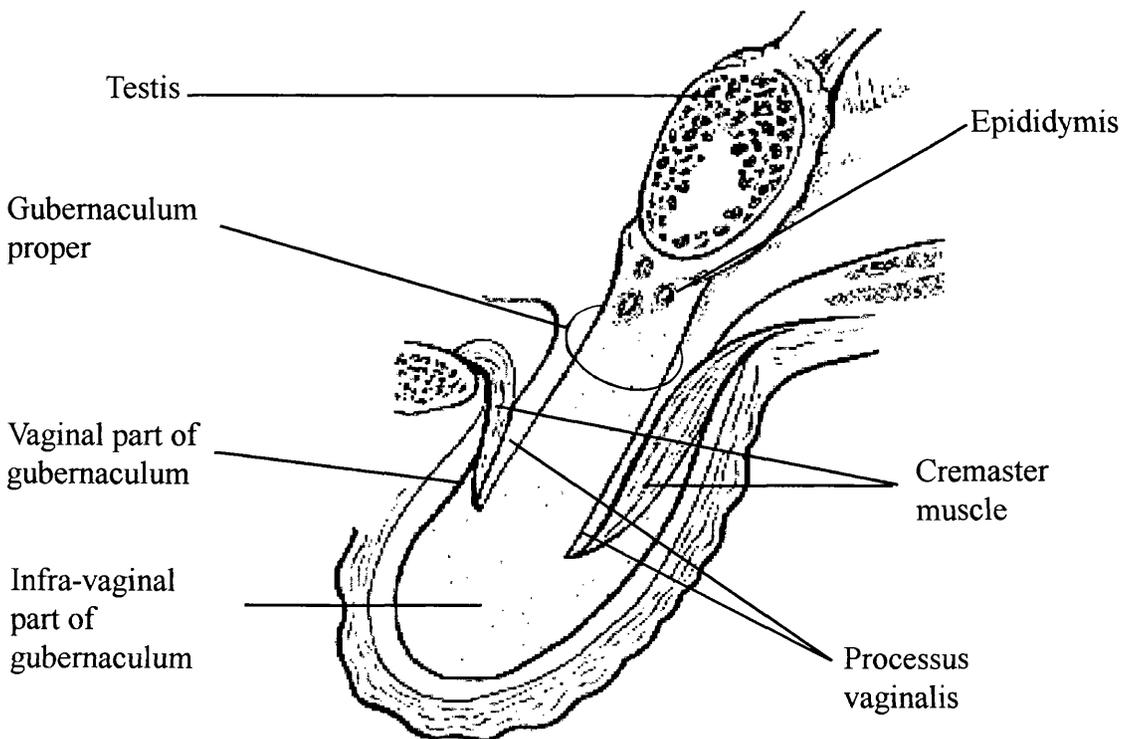
b.

FIGURE 4: Semi-diagramatic representation of the gubernaculum in species with sac-like cremaster muscles

(a) The gubernaculum during the outgrowth phase (at 18 d.p.c. in rat); it may be divided into an intra-abdominal (i.e. the gubernacular cord and cone) and an extra-abdominal parts

(b) The gubernaculum (at birth) at the beginning of gubernacular regression and eversion to form the cremaster sac with the formation of the processus vaginalis.

Testis ─────────

Epididymis ─────────

Gubernacular cord ─────────

Gubernacular cone ─────────

Extra-abdominal part
of gubernaculum ─────

4a. 18 d.p.c.

Testis ─────────

Epididymis ─────────

Gubernacular cord ─────────

Gubernacular cone ─────────

Processus vaginalis ─────────

Cremaster
muscle ─────────

Extra-abdominal part
of gubernaculum ─────
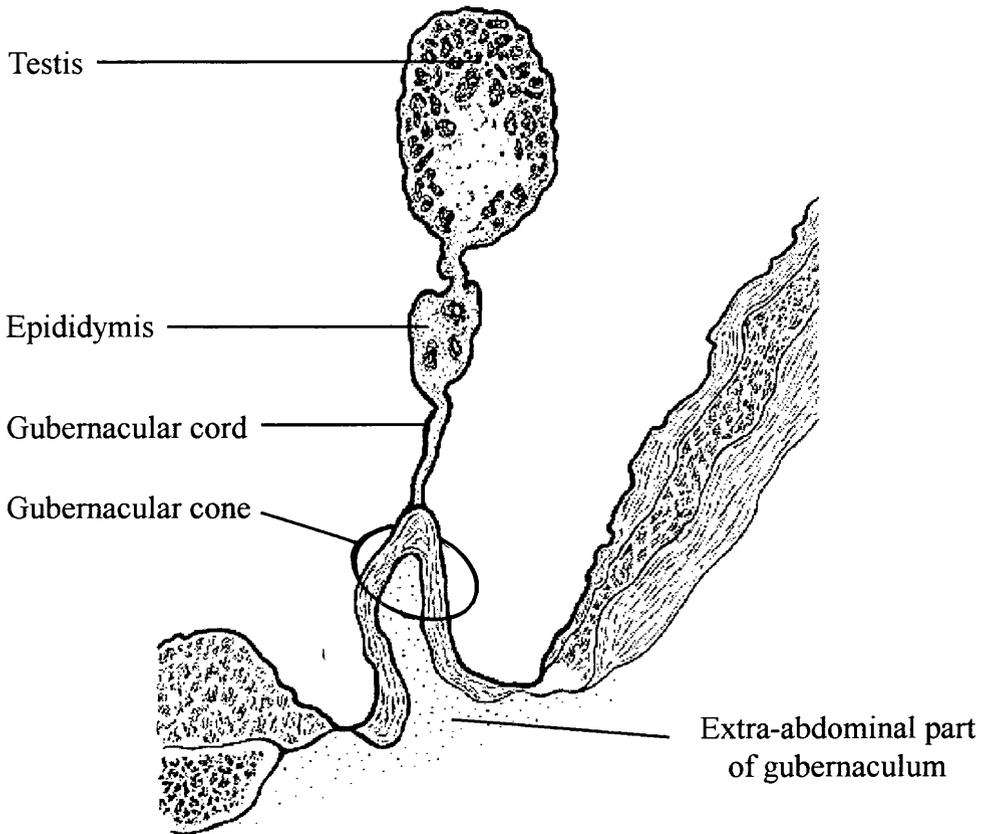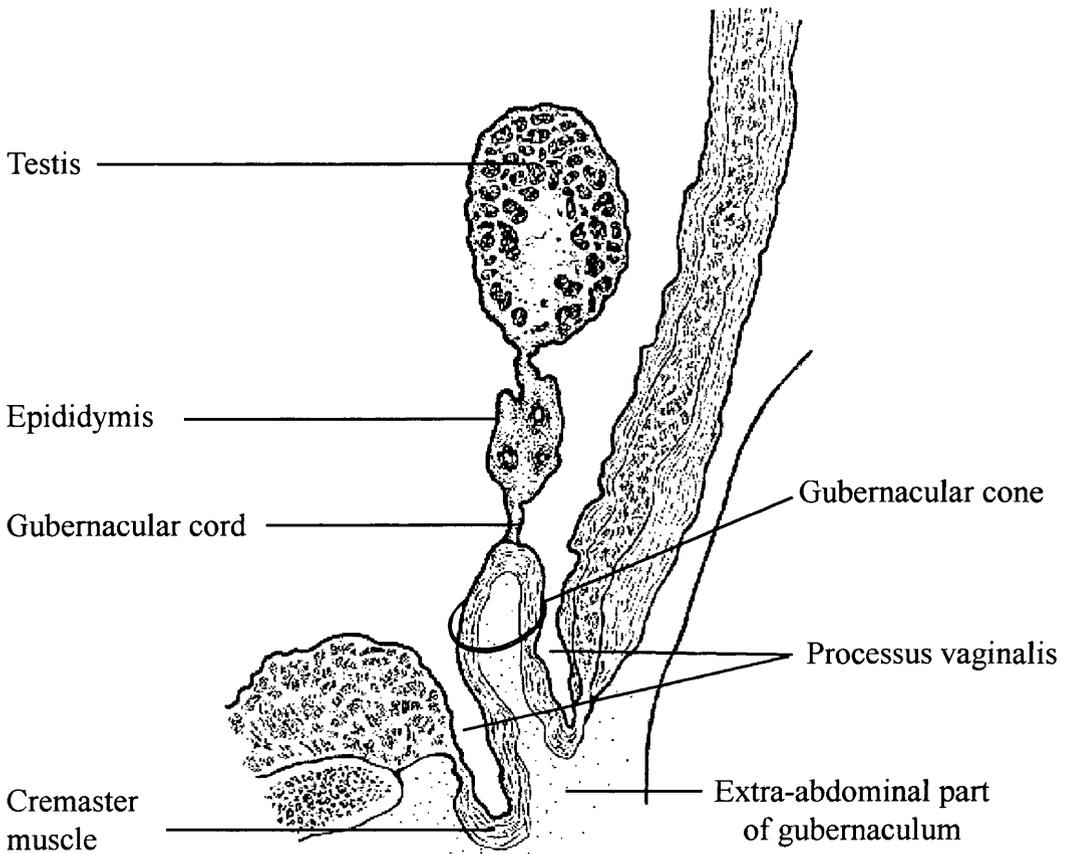
b. at birth

connection between the infra-vaginal part and the scrotum. In the gubernaculum proper, the mesenchymal cells are orientated in a longitudinal direction while, in the scrotal swelling, such orientation is lost and cells lie in a random fashion. However, towards the surface, the cells become orientated parallel to the skin **(Backhouse, 1982).**

In rodents such as the rat **(Wensing, 1986)** the gubernaculum may be divided into a) the cranial part (also called the gubernacular cord) which is an inconspicuous strand of mesenchyme, and b) the caudal part which is subdivided into i) intra-abdominal and ii) extra-abdominal parts **(Figure 4a).** The intra-abdominal part (also called the gubernacular cone) is composed of a mesenchymal core surrounded by several layers of myoblasts which are continuous with myoblasts in the abdominal wall: they form the basis of the future cremaster muscle. The extra-abdominal part (not surrounded by myoblasts) is composed of relatively dense mesenchyme which extends from the inguinal area to the future scrotum. The mesenchyme and myoblasts of the intra-abdominal segment are very conspicuous. Both the gubernacular cord and the cone are contained within the free margin of a peritoneal fold which extends from the testis to the inguinal region.

In considering the gubernaculum and its possible role in testes descent, several factors must be borne in mind

i)   The gubernaculum undergoes 2 distinct phases of development during testicular descent; firstly, the outgrowth phase (also called the swelling reaction) and, secondly, the regression phase **(Wensing, 1986)**.

ii)   **Wensing and Colenbrander (1986)** described 2 forms of testicular descent in mammals a) typical of those species with a strip-like cremaster muscle (i.e. ungulates, carnivores and man), and b) typical of those species with a sac-like cremaster muscle (i.e. rodents and lagomorphs).

iii)   there are two phases of testicular descent:

The first or the **transabdominal phase** refers to migration of the testis from the caudal pole of the ipsilateral kidney to the inguinal region, while the second **inguinoscrotal phase** refers to migration of the testis through the inguinal ring down to the scrotum. Both phases of testicular descent occur prenatally in man **(Backhouse, 1982; Heyns, 1987)**; in the rat, the transabdominal testicular descent occurs during the gubernacular outgrowth phase in the prenatal period with the inguinoscrotal phase completed by the end of the third week of life **(Wensing and Colenbrander, 1986).**

The transabdominal phase of testicular descent occurs between 10 - 15 weeks of gestation in the human **(Jirasek, 1971)** and between 16 - 20 d.p.c. in the rat **(Wensing and Colenbrander, 1986)**. The gubernaculum enlarges in the male, thereby anchoring the embryonic testis near the inguinal region as the

embryo enlarges. By contrast, the gubernaculum remains small and thin in the female so that the ovary undergoes relative ascent with embryonic enlargement. In the human fetus, the ovary remains closely related to the uterus and fallopian tube, whereas, in the rat, it remains closely associated with the kidney **(Backhouse, 1982; Wensing, 1986; Wensing and Colenbrander, 1986).**

a). Morphology of testicular descent in mammals with strip-like cremaster muscle:

During early embryonic development of the human fetus (6 weeks), the gonad develops in the genital ridge between the mesonephros and the dorsal gut mesentery **(Figure 1c)**. The excretory system of the testis is derived from the adjacent mesonephros and the mesonephric duct. The head and body of the epididymis are formed from the mesonephros and subsequently bear a constant postero-lateral relationship to the testis. Initially, numerous mesonephric tubules pass to the primitive testis. Most of these atrophy, leaving between 5-12 definitive vasa efferentia which drain from the rete testis to the head of the epididymis. The cephalic part of the mesonephric duct forms the tail of the epididymis, the caudal portion becoming the vas deferens **(Scorer and Farrington, 1971).**

The gonad and the mesonephros together form the uro-genital ridge, which has connections above and below. These are:-

i) a superior 'plica vascularis' contains the testicular artery and vein. With the involution of the cranial part of the mesonephros, the mesonephric ridge linking the mesonephros to the posterior body wall remains, thus linking the gonad with the developing diaphragm **(Backhouse, 1982)**. This is the basis of the cranial suspensory ligament of the gonad.

ii) an inferior 'plica inguinalis', a mesodermal thickening which forms an elongated structure, and extends caudally from the lower end of the testis and mesonephros into the groin. This is the gubernaculum of the testis. Thus, the gubernaculum is established as a mesenchymatous column extending from the gonad through a preformed inguinal canal in the anterior abdominal wall into the scrotal swelling **(Backhouse, 1982).**

While the above changes are taking place, the anterior abdominal wall develops a trilaminar musculature. The body wall muscles and fascia differentiate around this mesenchymal core; hence, at the site where the gubernaculum meets (or passes through) the anterior abdominal wall, there is a mesenchymal gap in the musculature, which will become the future inguinal canal. In this mesenchyme the genital branch of the genitofemoral nerve also passes through the abdominal wall **(Backhouse, 1982)**. By 8 weeks after conception, the human cremaster muscle develops as medial and lateral slips in the mesenchyme of the inguinal

canal. Similarly, the gubernaculum of the domestic pig (*Sus scrofa*) is also a mesenchymal structure which first runs in a peritoneal fold extending from the testis to the anterior abdominal wall. This gubernacular mesenchyme is continuous with a core of mesenchyme which demarcates the future inguinal canal within the differentiating abdominal wall muscles; This core is, in turn, continuous externally with the mesenchyme of the scrotal swellings **(Backhouse and Butler, 1960).**

With further development of the fetus, the processus vaginalis invades deeper into the gubernacular mesenchyme in the inguinal canal region, and divides the gubernaculum into three parts as above **(Figure 3b)**. There is no organized connection between the caudal tip of the gubernaculum and the area of the future scrotum during the early stages of testicular descent **(Wensing, 1968).**

During the first phase of testicular descent, the swelling reaction (outgrowth phase) of the gubernaculum is confined mainly to the extra-abdominal part of the gubernaculum proper. Gubernacular swelling is partly caused by active cell division **(Wensing, 1968)** but particularly by an increase of extra-cellular substances, glycosaminoglycans (GAG) which may be responsible for the increase in water content in the gubernaculum **(Backhouse, 1982; Heyns et al, 1990)**. Thus, during the outgrowth phase, the gubernacular cell density decreases, especially in that part located in the inguinal canal. The increase in weight and volume of the gubernaculum continues for some time after the passage of the testis through the inguinal canal and remains maximal for some

time before reduction commences (**Backhouse, 1982; Hullinger and Wensing, 1985**).

In pig and calf fetuses, the ratio of the intra- and extra-abdominal parts of the gubernaculum changes substantially during testicular migration. Concurrent with the outgrowth of the extra-abdominal part of the gubernaculum proper, the intra-abdominal part becomes shorter, thus bringing the testis and epididymis closer to the internal inguinal ring (**Wensing, 1968; Hullinger and Wensing, 1985**). The extra-abdominal part forms a progressively greater proportion of the whole gubernaculum as the testis approaches the internal inguinal ring. Shortly before the passage of the testis through the inguinal canal, the cranial part of the gubernaculum proper adjacent to the testis-epididymis reaches a diameter as wide or wider than the testis itself and in this way dilates the inguinal canal (**Wensing, 1968**). Growth of the extra-abdominal part apparently exerts traction upon the abdominal part of the gubernaculum proper, and, as it shortens, it gradually carries the testis beyond the external inguinal ring into the future scrotum.

In the dog, intra-abdominal testicular migration during the outgrowth phase of the gubernaculum is far less pronounced (**Baumans et al, 1981**); however, the outgrowth of the extra-abdominal part is clear, albeit less spectacular than in ungulates. In the male human fetus the changes in the dimensions of the gubernaculum, together with the transabdominal migration of the testis, more or less resemble the situation in ungulates and carnivores (**Backhouse, 1982**). Growth of the processus vaginalis is due to direct invasion

of the gubernacular mesenchyme by the coelomic epithelium to form an annular cavity dividing the gubernaculum into a central mesenchymal column and an outer parietal layer **(Figure 3b)**, while growth of the cremaster muscle is by differentiation of gubernacular mesenchyme **(Hutson and Beasley, 1992)**.

With subsequent regression of the gubernaculum proper and a shift in the relative positions of the superficial and deep inguinal rings, the testis descends within the peritoneal fold that carries the gubernaculum proper **(Wensing and Colenbrander, 1986)**. The scrotum and the inguinal canal become dilated in advance of testicular descent and passage of testis through the inguinal canal is preceded by lengthening of the vas deferens and testicular vessels **(Backhouse, 1964)**. Subsequent descent of the testis to the floor of the scrotum is permitted by lengthening of the structures of the testicular cord and their coverings. Following complete descent of the testis, the central gubernaculum involutes by dissolution of the extra-cellular matrix. The residual parts of the column form the fibrous attachment of the testis to the scrotum **(Hutson and Beasley, 1992)**.

In the human fetus, 75% of testes pass through the inguinal canal between 24-28 weeks of gestation **(Heyns, 1987)** and 93% are through by 32 weeks **(Birnholz, 1983)**. In the dog, passage of the testis through the inguinal canal takes place between 3-4 days after birth with a further 35-40 days before the final scrotal position is reached **(Baumans et al, 1981)**. In normal descent, the inner, visceral layer of the processus vaginalis covers the testis, epididymis, and

spermatic cord. At the inguinal ring, this layer becomes continuous with the peritoneal fold that carries the vas deferens. Caudally, it is reflected from the testis and epididymis as the mesorchium and mesoepididymis respectively to become the parietal layer which is continuous at the internal inguinal ring with the parietal peritoneum. The gubernaculum proper (together with the infra-vaginal parts) are converted into the ligament of the testis and the caudal ligament of the epididymis **(Backhouse and Butler, 1960; Wensing, 1968)**.

In fetal pig and calf, and in the neonatal dog, it is possible to apply gentle traction to the abdominal portion of the gubernaculum and to partially evert the vaginal process, thus returning the extra-abdominal portion of the gubernaculum into the abdomen **(Wensing and Colenbrander, 1986)**. In the pig (with a 28 weeks gestation period), the transformation of the early mucoid gubernaculum into a small fibrous structure takes place between 16 -17 weeks of gestation **(Wensing, 1968)**; in the dog the process occurs in the neonatal period **(Baumans et al, 1981)**.

Backhouse and Butler (1960) sought to explain the first phase of testicular descent from a position ventro-medial to the mesonephros to the deep inguinal ring by degeneration of the mesonephros and growth of the testis which expands to fill the space vacated by it. There are difficulties with this explanation especially the time difference between degeneration of the mesonephros and subsequent testicular migration in several species such as dog and horse **(Wensing, 1968)**. In the dog, there is only a little increase in testicular size and

the testis remains within the abdomen even though the mesonephros is largely degenerated. In the horse on the other hand, the testis undergoes an impressive enlargement during the period of migration. **Wensing (1968)** also noted the correlation between the outgrowth of the extra-abdominal part of the gubernaculum and the intra-abdominal migration. He suggested that intra-abdominal migration is brought about by the outgrowth of the extra-abdominal part of the gubernaculum. However, it was suggested that the separation between the testis and kidney is caused by growth of the lumbar spine and pelvis since the position of the testis relative to the acetabulum remains constant in the early fetus **(Backhouse, 1964).**

The passage of the testis through the inguinal canal is believed to be produced by an increase in intra-abdominal pressure, facilitated by the generous dimensions of the inguinal canal which is further dilated by the gubernaculum **(Wensing and Colenbrander, 1986; Backhouse, 1964).** The most important factor in the last phase of testicular descent is the regression of the gubernaculum proper and its infra-vaginal part together with the rapid growth in length of the vas deferens and the testicular vessels, thus allowing further migration of the testis within the peritoneal fold that formerly carried the gubernaculum proper.

b). Morphology of testicular descent in mammals with sac-like cremaster muscle:

The morphology of testicular descent in the ungulates (with a strip-like cremaster muscle) and rodents (with a sac-like one) are comparable in the following aspects:

i)   The gubernaculum connects the testis and the epididymis to the inguinal area before the process of testicular descent starts. During the outgrowth phase (i.e. at 18 d.p.c.), the gubernaculum of the rat may be divided into two parts as described above **(Figure 4a) (Wensing, 1986; Wensing and Colenbrander, 1986)**.

ii)   The gubernacular cord in the rat is essentially homologous with the intra-abdominal part of the gubernaculum in the pig. During the first phase of testicular descent, when gubernacular swelling occurs, shortening of the cord takes place in both species, bringing the testis and epididymis closer to the enlarged gubernacular mesenchyme.

iii)   The mesenchymal core of the gubernacular cone in the rat can be compared with the extra-abdominal part of the gubernaculum proper and the vaginal part of the gubernaculum in the pig. The swelling reaction takes place mainly in these parts.

iv)   The myoblasts of the gubernacular cone in the rat are essentially homologous to the strip-like cremaster muscles terminating in the vaginal part of the gubernaculum in the pig. However, the muscular component of the cone in

the rat is larger, more elaborate and voluminous than the comparable structure in the pig (Wensing, 1986).

v)   The extra-abdominal segment of the gubernaculum in the rat can be compared with the infra-vaginal part in the pig. These parts in both species extend towards the scrotal region, but have no organised connection with surrounding structures.

The differences between the two species are:

i)   Formation of the processus vaginalis in the rat results from the eversion of the cremaster sac during the regression of the gubernacular mesenchyme. The muscular wall of the gubernacular cone has everted into the space created by this regression (Figure 4b). Eversion begins around birth at the base of the gubernacular cone and gradually deepens (Habenicht and Neumann, 1983; Wensing, 1986) and the processus vaginalis is formed as a consequence of this eversion, while in the pig it is produced by an active peritoneal invasion into the mesenchyme of the extra-abdominal part of the gubernaculum proper during outgrowth phase of the gubernaculum. Once eversion of the gubernacular cone has been completed (at postnatal day 4 in the rat), the muscular coverings form the extra-abdominal cremaster sac. However, despite the difference in formation, the end results are remarkably similar, the main difference being the larger relative diameter of the inguinal canal in the rat. Because of this, movements of the testis from the scrotum to the abdominal cavity remain theoretically possible. However, in practice, when the testis is located scrotally, the epididymal fat pad

blocks the inguinal canal, preventing the herniation of abdominal viscera **(Wensing, 1986)**; this may also prevent the descended testis from returning into the abdominal cavity.

ii)   In man, the bulk of the caudal enlargement of the gubernaculum is resorbed after the completion of testicular migration **(Backhouse, 1966)** while, in rodents, resorption begins prior to the commencement of migration **(Fallat et al, 1992a).**

iii)   The strip-like cremaster muscle of ungulates, carnivores and man is not very conspicuous during the process of testicular descent. In contrast, the sac-like cremaster muscle of rodents and lagomorphs increases in size, and continues to grow even after regression of the gubernacular mesenchyme **(Wensing, 1986)**.

Intra-abdominal pressure seems to play a role in the completion of testicular descent into the scrotum in both species **(Frey et al, 1983; Frey and Rajfer, 1984).**

3. FACTORS CONTROLLING TESTICULAR DESCENT:

Theories of testicular descent:

A number of diverse and often conflicting theories have been proposed to explain the mechanism by which the testis descends from the abdomen into the scrotum. In general, these theories have held that the testis is either pulled (traction) or pushed (propulsion) from the abdomen into the scrotum, or that it reaches its destination by a combination of growth and involution.

The traction theories propose that the contractions of muscle fibres of the gubernaculum act to pull the testis down **(Wyndham, 1943).** These theories rest upon the assumption that the gubernaculum has firm attachments both cranially and caudally. However, **Backhouse (1966)** found that the gubernaculum of a pig fetus lay free in the scrotum but attached to the abdominal wall around the inguinal canal and having the processus vaginalis extending from the abdomen into it. Another related theory is that swelling of the gubernaculum distal to the external inguinal ring exerts traction on the proximal part of the gubernaculum; This has been likened to the inflation of a balloon in a restricted passage exerting traction on the intra-abdominal testis **(Wensing, 1968).**

The epididymal theory of testicular descent is based on the fact that the gubernaculum is adherent to the cauda epididymis and the lower pole of the testis, thus the epididymis accompanies the testis during descent. **Hadziselimovic (1983)** proposed that it was actually the epididymis which descended carrying the testis with it as a result of changes in the centre of gravity due to i) the

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craniocaudal direction of differentiation of the epididymis, ii) peristaltic and secretory activity within the differentiating epididymis.

Theories of propulsion suggest that an increase in intra-abdominal pressure is the primary force that causes the testis to leave the abdomen and enter the inguinal ring. The increase in intra-abdominal pressure may be the result of growth of other abdominal viscera **(Wells, 1943)**, closure of the physiological umbilical hernia **(Rajfer and Walsh, 1977)**, contraction of the abdominal muscles, or respiratory effort by the fetus **(Heyns, 1987)**. These pressures are supposed to cause herniation of the gubernaculum and the testis through the 'weak' part of the abdominal wall, that is, the inguinal canal.

Recently, clinical conditions such as prune belly syndrome or abdominal wall defects which are associated with undescended testis (such as umbilical hernia, omphalocele) **(Pagon et al, 1979; Moerman et al, 1984; Hutson and Beasley, 1987; Kaplan et al, 1986; Hadziselimovic et al, 1987)**, together with experimental evidence such as the descent of prostheses into the scrotum, have appeared to support this concept **(Frey et al, 1983; Frey and Rajfer, 1984)**. After closure of the internal ring, enlargement of the testis forces it to move down the funnel-shaped inguinal canal **(Engle, 1932)**. However, **Heyns (1987)** disagreed with this idea since although the absolute size of the testis and epididymis increase, the size of the testis relative to that the fetus remains constant.

The growth theory states that the differential growth of the lumbar vertebral column, pelvis and abdominal wall are responsible for the apparent transabdominal descent of the testis while the gubernaculum passively anchors it at the internal ring **(Heyns, 1987).** The relative movement of the gonads in male and female fetuses has produced debate, especially as it is difficult to know the 'fixed' point it should be measured against. The absolute distance between the testis and the inguinal region in the fetal rat remained constant during this phase, suggesting that the testis did not descend but that, paradoxically, the ovary did ascend in the female fetus. **Shono et al, (1994)** also proposed that the ovary ascends and the testis never moves downward in the mouse, the distance between the testis and the bladder neck remaining constant during the transabdominal phase of descent. **Van der Schoot (1993a)** suggested that ovarian ascent is caused by persistence of the cranial suspensory ligament while ascent of the testis is prevented by its absence. In flutamide-treated male rats, the cranial suspensory ligament persists, but this is not necessarily associated with undescended testis **(van der Schoot and Elger, 1992).**

Furthermore, persistence of these ligaments in androgen-resistant mice was not associated with failure of the transabdominal phase of descent **(Hutson, 1986).** Paradoxically, if impaired movement of the ovary is passive, then lack of movement of the testis must still be an active process **(Hutson et al, 1996)** because the testis would occupy the same position as the ovary if descent did not occur. **Hutson et al (1996)** have taken the ovary itself as the reference point for

descent, rather than the inguinal region. Thus, with the ovarian position as the starting point for descent, the testis descends relative to the inguinal region with fetal growth.

Whatever the mechanical factors at work, a large number of clinical and experimental observations suggest that the process of testicular descent is under hormonal control **(Elder et al, 1982; Backhouse, 1982; Habenicht and Neumann, 1983; Fentener van Vlissingen et al, 1988; Heyns and Pape, 1991; Heyns et al, 1993; Hutson and Donahoe, 1986; Hutson et al, 1990, 1996).**

Hormonal control of testicular descent

Hormonal control of testicular descent has been a subject of controversy since **Engle (1932)** originally noted premature descent of testis in the monkey after injection of pregnant monkey urine or anterior pituitary extracts. However, the mechanisms by which these hormones act remains controversial. Testicular descent involves two separate phases (see p. 18) which may occur under different hormonal controls.

In previous studies on the hormonal regulations of testicular descent, evidence has been presented that the first phase (that is, the outgrowth of the gubernacular mesenchyme) is androgen-independent **(Wensing, 1973; Colenbrander et al, 1979; Habenicht and Neumann, 1983; Baumans et al, 1983).** However, the second (the regression of this mesenchyme), and possibly

the third (the development and growth of the cremaster muscle), may be androgen-dependent **(Elder et al , 1982; Baumans et al, 1983; Wensing, 1988).**

The embryonic testis produces both testosterone and MIS **(Lee and Donahoe, 1993; Josso et al, 1993a).** Early evidence supported a role for testosterone and 5α-dihydrotestosterone (DHT) in promoting testicular descent. Treatment of immature rhesus monkeys, (which exhibit a cryptorchid condition until the onset of puberty) with testosterone induces testicular descent within 3 weeks **(Hamilton, 1938);** Similarly, administration of DHT to bilaterally-orchidectomised rats (on day 14) induces descent of silicone prostheses (on day 28) **(Frey et al, 1983)**. Moreover, a high activity of the enzyme 5α-reductase (which converts testosterone to 5α-DHT) was found in day 18 fetal rat gubernaculum **(George, 1989)**.

Investigations into the role of androgens on testicular descent in the rat have produced conflicting results. Evidence in support are the findings that premature testicular descent (in rat) can be induced by DHT, but not by testosterone **(Rajfer and Walsh, 1977).** Furthermore, daily injections of estradiol to rat pups from birth to postnatal day 21 inhibits testicular descent, and this inhibition by estradiol can be reversed by DHT, but not by testosterone **(Rajfer and Walsh, 1977).**

In attempting to clarify the role of androgens on descent of the testis, recent studies using the non-steroidal anti-androgen flutamide also have produced

divergent results. When flutamide was administered prenatally, it inhibited testicular descent in rats (Spencer et al, 1991; van der Schoot, 1992b). The maximum effect was found with exposure between 16-17 d.p.c. (Husmann and McPhaul, 1991b) while administration of flutamide to fetal rats from 10 d.p.c. (i.e. prior to differentiation of the Wolffian duct), also prevented regression of the cranial suspensory ligament (van der Schoot and Elger, 1992). While Husmann and McPhaul (1991b) found that the testes were either located at the bladder neck or in the inguinal region, van der Schoot and Elger (1992) found that testicular descent was completely inhibited, with the testes located in the ovarian position near the lower pole of the kidneys, despite normal growth of the gubernaculum and its postnatal eversion to form the cremaster sac. Similar observations have been made in perinatal rabbits (van der Schoot and Elger, 1993).

Hutson et al (1996) have suggested that the difference between these studies may be due to the timing of prenatal flutamide treatment; Spencer et al (1991) and Husmann and McPhaul (1991b) gave flutamide after the Wolffian duct had been exposed to endogenous androgens while van der Schoot and Elger (1992) gave flutamide prior to Wolffian duct stabilization. Hutson et al (1996) agreed that regression of the Wolffian duct, and hence absence of the epididymis, may cause the gubernacular cord to be unusually long, so that the testis is not held near the inguinal canal. By contrast, development of the gubernacular cone is independently controlled and occurs normally despite

flutamide. However, the subsequent migration of the everted cremaster muscle to form the cremaster sac is abnormal, and the sac fails to reach the scrotum in most animals **(van der Schoot, 1992b)**.

Because of these contradictory conclusions, a number of authors **(Wensing, 1973; Hutson and Donahoe, 1986)** have suggested that testicular descent may be controlled, at least in its initial phases, by MIS. This is based on the observations that the completion of regression of the Müllerian duct (which is under MIS control) coincides with the onset of the rapid growth phase of gubernacular development, and almost all patients with persistent Müllerian duct syndrome (PMDS) have one or both testes undescended (for reviews see **Hutson et al, 1990; 1996).** In PMDS, the testes are located in the abdominal cavity in a position analogous to the ovary **(Sloan and Walsh, 1976)**. However, in some patients with this anomaly, only one testis may remain within the abdomen and, in about 10% of patients, both testes were found in the groin on the same side. **Josso et al, (1993b)** found that the testes in patients with PMDS are tightly attached to the fallopian tubes, and the position of the gonads depends on the mobility of the Müllerian duct remnant, thus concluding that the failure of testicular descent is caused by mechanical constraint by the Müllerian ducts. Furthermore, the role of MIS in transabdominal descent has been doubted because testes may be located in the inguinal region in some patient with PMDS.

Controversy over the role of MIS in transabdominal descent of the testis remains. Contradictory evidence includes the failure of antibodies against MIS

administered to pregnant rabbits to prevent testicular descent in male offspring, despite persistence of the Mullerian ducts **(Tran et al, 1986)**. By contrast, pregnant mice exposed to estrogens had male offspring with Mullerian ducts, high intra-abdominal testes and absence of the normal male gubernacular enlargement **(Hutson et al, 1990)**.

i) The role of genitofemoral nerve (GFN) in descent of testis

Inguinoscrotal testicular descent requires migration of the gubernaculum from the inguinal region to the scrotum **(Heyns, 1987; Fallat et al, 1992a)**. This phase may be androgen dependent, since maldescent occurs in animals with complete androgen resistance **(Hutson, 1986)**. The gubernaculum is innervated by the genitofemoral nerve (GFN). In human, the GFN arises from the ventral roots of segments L1-L2 of the spinal cord. It innervates the gubernaculum by the genital branch which leaves the main trunk cranial to the inguinal ligament. The genital branch of GFN descends through the inguinal canal and enters the gubernaculum proper through its caudal **(Figure 4a-b)** to supply both it and the cremaster muscle **(Tayakkanonta, 1963)**. In the rat, the course of the genital branch of the GFN as confirmed by anterograde fluorescent and immunohistochemical tracing was similar to that in the human **(Larkins and Hutson, 1991)**.

The GFN spinal nucleus has been shown to be sexually dimorphic because the GFN spinal nucleus in adult male mice contained more cells than in female

mice **(Larkins et al, 1991)**. Furthermore, immunohistochemical studies have shown that a neurotransmitter, calcitonin gene-related peptide (CGRP) is present within the cell bodies of GFN neurons in the lumbar spinal cord **(Larkins et al, 1991)** and its fibres in the scrotum and pelvis **(Larkins and Hutson, 1991)**. Since division of the genitofemoral nerve not only prevents inguinoscrotal testicular descent but also prevents gubernacular migration **(Beasley and Hutson, 1987; 1988; Fallat et al, 1992a)**, it has been suggested that androgens act indirectly on the GFN to cause the release of the specific neurotransmitter CGRP and the level of CGRP immunoreactivity in the GFN nucleus of flutamide-treated rats is significantly reduced compared to controls **(Goh et al, 1993)**.

Calcitonin gene-related peptide causes rhythmic contractions in the gubernaculum of the neonatal mouse and rat **(Momose et al, 1992)**: the role of these contractions remains uncertain, but is believed to be linked to normal inguinoscrotal descent. Since the developing cremaster muscle fibres in the gubernaculum contain specific binding sites for CGRP **(Yamanaka et al, 1992)**, it was concluded that CGRP plays an important role as a neurotransmitter for migration of the gubernaculum and subsequent testicular descent.

By injecting exogenous CGRP into the suprapubic region of flutamide-treated rat pups, **Abe and Hutson (1994)** readily diverted the migrating gubernaculum leading to 77% of testes becoming located in the superficial inguinal pouch. They concluded that CGRP could modify the process of testicular descent postnatally.

ii). The role of cranial suspensory ligament (CSL) in testicular descent:

The embryonic gonads have both cranial and caudal attachments (**Wydham, 1943; Backhouse, 1982**) derived from peritoneal folds associated with the mesonephric ridge. The caudal attachment connects the gonad to the anterior abdominal wall at the region of the internal inguinal ring and has been described in detail above. Cranially, the gonad is connected to the posterior body wall by the cranial suspensory ligament inferior to the diaphragm at the junction of the middle and the lateral third of the last rib (for the rat; see **Hebel and Stromberg, 1976;** for the hamster; see **Hoffman et al, 1968).**

In normal development of the rat, the CSL persists in the adult female as a prominent structure containing smooth muscle and elastic fibres (**Hebel and Stromberg, 1976)**, whereas in the adult male it disappears under the influence of androgens (**van der Schoot and Elger, 1992).** Thus, when pregnant female rats were treated with testosterone, the female offspring had no CSL. Conversely, male rats exposed prenatally to flutamide (**van der Schoot and Elger, 1992)** or Tfm rats (**Spencer Barthold et al, 1994)** have persistent CSLs and undescended testes. **Van der Schoot and Elger (1992)** proposed that persistence of the CSL in the female leads to ovarian ascent, while loss of the CSL in the normal male allows the testis to descend.

Thus, the final position of the testis/ovary depends on both a) the gubernaculum, and b) the CSL. These two parts of the mesonephric ridge are

envisaged as working together either for the testis to descend or for the ovary to ascend; that is, in the male, testicular descent occurs where there is a gubernacular swelling reaction and migration towards the scrotum together with disappearance of the CSL while, in the female, ovarian ascent occurs as a result of CSL retention coupled with a long and poorly developed gubernaculum. Thus, the CSL in female 'holds' the ovary near the pelvic brim (in human) or at the lower pole of the kidney (in rodents) **(van der Schoot, 1993b)**

4. CRYPTORCHIDISM AND ITS IMPLICATIONS:

Not all mammals exhibit testicular descent: these animals are called testicondia. Examples of these animals are elephants **(Schulte, 1937)**, cetaceans (dolphin) **(van der Schoot, 1995)** and hyrax (bear) **(van der Schoot, 1996)**. Nevertheless, in the majority of mammals the testes are scrotal and maldescent is not without adverse consequences.

Cryptorchidism (that is, the failure of one or both testes to descend into the scrotum) is a common anomaly in man **(Backhouse, 1982; Frey and Rajfer, 1982; Heyns, 1987)** and in some domestic animals **(Wensing, 1986).** In man the incidence is 3.4% at birth at term and 30.3% in premature births. Many of these testes subsequently descend spontaneously and the incidence has reduced to 0.8% by one year of life and during adulthood **(Scorer and Farrington, 1971).** This means that if by the end of the first year the testis has not descended, it will never descend.

Cryptorchidism can occur as an isolated anomaly or associated with other defects **(Griffin and Wilson, 1980; Elder, 1987; Fallat et al, 1992b).** However, the causes of cryptorchidism remain essentially unknown. Most cryptorchid testes are located in the groin, so that transabdominal descent must be relatively normal and the defect occurs chiefly in the inguinoscrotal phase, that is, when the testis should move from the groin to the scrotum **(Hutson et al, 1996).** Thus, the likely causes are defects in gubernacular migration either due to i) primary anomalies of the migratory mechanism itself such as connective tissue

anomalies interrupting migration through the inguinal mesenchyme **(Backhouse, 1964)** or prune belly syndrome **(Moerman et al, 1984; Hutson and Beasley, 1987)** or abdominal wall defects **(Hadziselimovic et al, 1987)** or ii) a consequence of androgen deficiency <u>in utero</u> **(Hutson et al, 1996).**

Not all cryptorchidisms are congenital (i.e. present from birth), some are acquired late in childhood. The latter anomaly may be due to failure of the spermatic cord to elongate in proportion to body growth **(Hutson and Beasley, 1992)**, or to a spastic cremaster muscle associated with cerebral palsy where there is progressive spasticity of muscles with increasing age **(Smith et al, 1989)**. Retractile or ascending testes may be caused by persistence of a patent processus vaginalis **(Atwell, 1985)**.

The postnatal effects of cryptorchidism

Abnormalities and pathological conditions often occur in cryptorchid testes. It is unclear whether the testis is primarily abnormal (and this leads to maldescent) or, becomes secondarily abnormal (with degeneration of seminiferous tubules leading to infertility and malignancy in young adult life) because it is undescended **(Giwercman et al, 1988)**.

The degeneration seen in undescended testes is believed to be caused by the increased temperature compared to the scrotum **(Steinberger, 1991)**. The scrotum is a low-temperature environment with heat exchange mechanisms such as the pampiniform plexus, pigmentation of the scrotal skin, absence of

subcutaneous fat, and regulation by the temperature sensitive cremaster and dartos muscles **(Hutson and Beasley, 1992).** Most enzymes and cellular mechanisms within the testis appear to be well adapted to this lower body temperature **(Steinberger, 1991).** It is not surprising, therefore, that in undescended testes the increase in ambient temperature is often associated with degeneration and dysfunction, for example, normal differentiation of spermatogenic cells requires a temperature lower than the body temperature. In the human, the normal scrotal temperature is 33°C and the intra-abdominal is 37°C **(Hutson and Beasley, 1992).**

Relocation of rat scrotal testes into the abdomen (artificial cryptorchidism) **(Clegg, 1963; Blackshaw and Massey, 1972);** or elevation of the temperature of a normal rat scrotal testis by heating in water at 42°C for 30 minutes **(Blackshaw and Massey, 1972)** resulted in regression of the seminiferous epithelium. On the other hand, continuous cooling of abdominal testes by implanting a cooling device into the tunica albuginea of naturally cryptorchid pig testes for 45 days resulted in induction of spermatogenesis in most seminiferous tubules **(Frankenhuis and Wensing, 1979).** These authors subsequently suggested that spermatogenic arrest in an abdominally located testis is not due to an inborn defect of the testis, but is caused by the maintenance of the testis at abdominal temperature.

The effects of cryptorchidism on sperm and hormone production

Immediately after birth, the undescended human testis is physiologically and morphologically normal. Abnormality of hormone secretion can be detected within a few months. In normal infants, plasma luteinizing hormone (LH) and testosterone levels rise between 1 and 3 months postnatally **(Gendrel et al, 1978; 1980)**, while MIS is normally high between 4 and 12 months after birth **(Baker et al, 1990).** In contrast, plasma levels of testosterone are significantly decreased and the postnatal peak of MIS is inhibited in cryptorchid infants **(Yamanaka et al, 1991).**

Morphological changes become evident at a slightly later age but this may be complicated by late diagnosis (e.g. not until children go to school - at which time the undescended testis appears small and soft at operation or by palpation **(Hutson et al, 1996).** Histologically, this is correlated with severe degeneration, loss of germ cells, atrophy of the seminiferous tubules, thickening of the basement membrane and increase in peritubular fibrosis **(Mengel et al, 1974; 1982).** Where diagnosis is made at birth, histological changes can be seen during the second year of life, when there is a significant fall in the number of spermatogonia per tubule. **Mengel et al (1974)** proposed that orchidopexy should be performed in the second year of life with the aim of preventing degeneration.

Studies on postnatal development of human germ cells at 4 to 8 months of age reveal that the gonocytes which are initially located in the centre of the

testicular cord migrate to the periphery adjacent to the basement membrane where they are transformed into A-spermatogonia (**Hadziselimovic et al, 1984**). Biopsies from undescended testes (**Huff et al, 1989; 1993**) reveal that decreased numbers of germ cells and Leydig cells together with delayed and defective maturation of germ cells are detectable from the first year of life onwards; these features undoubtedly underlie the increased infertility associated with cryptorchidism.

Similar findings were reported by **Hadziselimovic et al (1986)** who postulated that a postnatal surge of gonadotrophins may be responsible for priming the testes for subsequent development and fertility. **Huff** and colleagues **(1989)** proposed that lack of androgens was the reason for these deficiencies because the testes showed features of hypogonadotrophic hypogonadism (that is, failure of gonadal development secondary to deficiency in gonadotrophic hormones such as LH and FSH).

It has also been suggested that there is an association between a postnatal surge of MIS secretion (which occurs between 4 - 12 months of age) and the normal development of germ cells (**Baker et al, 1990**). There is no deficiency in MIS secretion in cryptorchid testes between 1-3 months. However, the subsequent postnatal peak is inhibited in cryptorchid infants (**Yamanaka et al, 1991**). These authors therefore suggested that the inhibition must be secondary rather than reflecting a primary abnormality (**Hutson and Beasley, 1992**).

The association between postnatal MIS secretion and the development of germ cells as suggested by Baker and his co-workers was investigated in mouse testes of the same development stages as seen in humans **(Zhou et al, 1993)**, with transformation of gonocytes to Type A spermatogonia occuring during the first postnatal week. In *in vitro* studies of type A spermatogonia taken from neonatal mouse testis, the germ cells were found to develop normally as long as MIS was added: addition of antibodies to MIS (or failure to add exogenous MIS) blocked germ cell development. By contrast, hCG failed to stimulate transformation of neonatal gonocytes to type A spermatogonia in neonatal organ culture **(Zhou and Hutson, 1995)**. However, when hCG was given *in vivo* to immature mice at 12 days post-partum, precocious spermatogenesis was produced. Thus, **Hutson et al (1996)** suggested that germ cell maturation in the mouse is likely to be controlled by MIS rather than by hCG or androgens. It is not known whether human germ cells are under similar regulation, but the postnatal peaking of MIS secretion in human is consistent with this possibility.

Pathological changes leading to infertility and increase risk of malignancy

It is a common finding in experimental animals that undescended testes exhibit degeneration **(van der Schoot, 1992b; Blackshaw and Massey, 1972)**. In man, a high incidence of infertility is common in patients with unilateral or bilateral cryptorchidism **(Toth et al, 1987; Hutson and Beasley, 1992; Hutson et al, 1996)**. In a retrospective study of patients who had undergone orchidopexy

for a unilateral cryptorchid testis, the mean sperm density (26.8 million per ml) was significantly lower than that of a normal control group (73.6 million per ml) **(Lipshultz et al, 1976).**

Of more concern, the risk of malignancy in an adult testis which was cryptorchid in childhood is 5-10 times greater than normal population **(Hutson and Beasley, 1992).** The histological changes of progressive dysplasia in undescended testes are the putative cause of this increased risk of malignancy **(Krabbe et al, 1979; Giwercman et al, 1988; 1989).** These tumours tend to occur between 20 - 40 years of age and the incidence of malignancy in patients with maldescended testes is around 8% **(Skakkebaek et al, 1982).** Furthermore, secondary histological abnormalities have been documented in the contralateral descended testis in males with unilateral undescended testis. Whether orchidopexy can prevent dysplasia is not known in humans, but is demonstrable in animal models **(Hutson and Beasley, 1992).**

The aims of the present study

Since maldescent of the testis is a common human congenital abnormality and undescended testes are at risk of developing morphological changes leading to sterility and malignancy, it is of great importance to understand the process of testicular descent and its controlling mechanisms in great detail. Although much data regarding testicular descent in mammals including man are available, the mechanism and the factors involved are still poorly understood. There are indications that androgens play an role in testicular descent, at least in the inguinoscrotal phase. However, the exact mechanisms of action and the target tissue involved are still a subject of debate. Therefore, the present study is intended to add to the understanding of this subject by examining the effects of pre- and postnatal administration of the non-steroidal anti-androgen flutamide (α-α-α-trifluoro-2-methyl-4'-nitro-m-propionotoluidide) on:

a) the process of testicular descent in the rats and hamsters,

b) the morphology of the adult testes in both species,

c) the level of testosterone in plasma and within testes,

d) growth and development of the gubernaculum in rats,

e) growth and regression of the cranial suspensory ligaments in rats.

MATERIALS AND METHODS

AND

RESULTS

EXPERIMENT 1

The aims of this experiment were to examine:

a). the effects of pre- and postnatal exposure to flutamide on testis position, weight and structure in Albino Swiss rats and golden hamsters **(Experiment 1a)**

b). the effects of prenatal exposure to flutamide on adult androgen production in Albino Swiss rats and golden hamsters **(Experiment 1b)**

c). the onset of tubular damage in rats* exposed pre-natally to flutamide **(Experiment 1c)**

*Initially, these experiments were planned for two species - rats and hamsters. However, it became clear after the initial parts of Experimental 1 that flutamide did not prevent testicular descent in the hamster, so this species was dropped from subsequent parts of the investigation.

Animals:

The following groups of animals were examined:

A. Prenatal flutamide administration

1) Experimental animals for the prenatal treatment group (54 adult male Albino Swiss rats; 12 adult male hamsters) were obtained by giving subcutaneous injections of the non-steroidal anti-androgen flutamide to time-mated pregnant female rats (10 mg/day in 0.1 ml propylene glycol from E10 to birth), and to time-mated pregnant female hamsters (10 mg/day from E8 to birth). These starting points for flutamide treatment were chosen to ensure exposure of the fetuses to flutamide throughout the period of androgen-dependent male sexual

differentiation which starts after day 13 of gestation in rat **(Magre and Jost, 1980)**, and the dose was chosen as a maximum one not visibly impairing the health of the pregnant females (eg. **van der Schoot, 1992b** for the rat).

2) Control animals (32 adult male Albino Swiss rats; 12 adult male hamsters) whose mothers received 0.1 ml propylene glycol vehicle only.

B. Postnatal flutamide administration

3) The postnatal treatment group received subcutaneous injections of flutamide (5 mg/day in 0.05 ml propylene glycol) from the day of birth to P14.

4) Control animals for the postnatal group were injected with 0.05 ml propylene glycol vehicle only.

From the outset, it became clear that there was no impairment of testicular descent in rats exposed postnatally to flutamide, so numbers were limited to 6 experimental and 6 control rat pups; furthermore the study was not carried out on hamsters where even pre-natal flutamide exposure did not result in failure of descent and subsequent experiments dealt with the rat only. The efficacy of the pre-natal flutamide treatment was verified by examining the degree of feminization of the genitalia of the male offspring.

EXPERIMENT 1(a)

To determine the effects of prenatal exposure to flutamide on the position, weight and structure of testes in adult rats and hamsters

Methods:

As adults (16-20 weeks) the males of both species were killed by intraperitoneal injection of a lethal dose of pentobarbitone sodium B.P. (VET) (Sagatal 60 mg/ml). The animals were then transcardially perfused with mammalian Ringer containing 0.2% of the vasodilator xylocaine before perfusion fixation with a mixture of 3% glutaraldehyde and 1% paraformaldehyde in 0.1M phosphate buffer (pH 7.4) **(Appendix 1)**. The position of the testes was determined in these animals and photographs were taken for a permanent record. The testes were then removed, weighed individually (because testes from different positions in the same animal have different weights) and immersed in the same fixative for 24 hours before processing for light or transmission electron microscopy.

For each testis, two 1 mm slices were removed from the mid-transverse region, cut into blocks approximately 2mm x 3mm x 1mm and each slice processed separately to give an osmicated and unosmicated set of blocks. One set was rinsed in 0.1M phosphate buffer (pH 7.4) for 2 hours (changing the solution every 1/2 hour) before post-fixing with 1% buffered OsO_4 solution for 1 hour; the other set was left in buffer. After rinsing the osmicated set with buffer solution for 1 hour (changing the solution every 1/2 hour), both sets were dehydrated in

ascending concentrations of ethanol, followed by three changes of propylene oxide and a descending ratio of propylene oxide to araldite (1:1, 1:3 and three changes of araldite), before embedding in araldite **(Appendix 2)**.

The degree of tubular abnormality was assessed on at least 200 tubule profiles per testis using semithin sections cut at a thickness of 1 μm from the unosmicated blocks and stained with toluidine blue. Light micrographs were taken at low magnification (x25) and enlarged photographic prints (x50) were made. Each tubular profile was assigned to one of 3 categories:- i) normal tubules with Sertoli cells and all stages of spermatogenic cells up to and including mature spermatozoa, ii) tubules with spermatogenic cells but with no (or only damaged) maturing spermatids, and iii) tubules lined either only with Sertoli cells or by Sertoli cells with occasional spermatogenic cells limited to the spermatogonium stage. Tubules were assessed as appropriate for inclusion in the study provided they possessed a circularity shape factor of at least 0.8 (1.0 = a perfect circle) as determined using a Kontron Vidas Image analyser. This was to eliminate longitudinal profiles which might exhibit different degrees of damage along their length.

Those tubule profiles assessed as normal were further analysed for the 14 stages of the spermatogenic cycle as reported by **Leblond and Clermont (1952).** Again, the use of circular profiles is essential since different stages might occur in neighbouring regions of the same tubule. Staging of the spermatogenic cycle was

carried out on adjacent semithin sections cut at a thickness of 2 μm and stained with PAS-Haematoxylin (**Appendix 3**).

Detailed morphological studies of each testis were carried out using ultra-thin sections (60-80 nm) cut from the osmicated blocks and stained with uranyl acetate followed by lead citrate (**Reynolds, 1963**). A Phillips CM100.BIO transmission electron microscope was used to examine and photograph the prepared material.
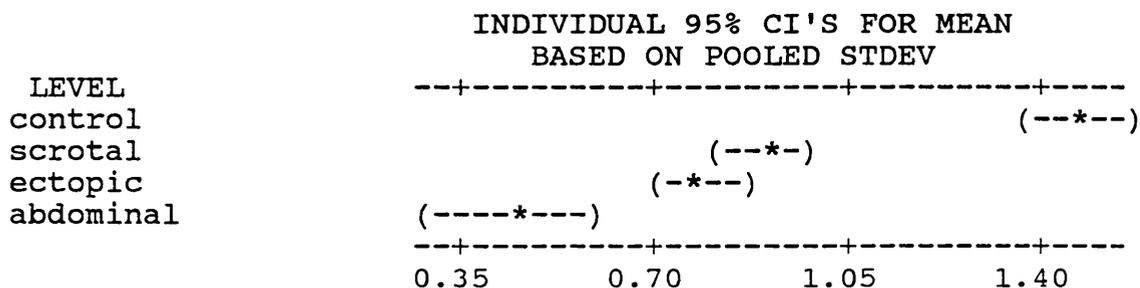
RESULTS

(i). Development of male rats after prenatal exposure to flutamide

Male rats exposed prenatally to flutamide from gestational day 10 to birth had feminized external genitalia as adults, including vaginae and nipples (**Figures 5a-d** and **6a-d**). Moreover, in these adults males, 44% (48/108) of testes occupied a normal scrotal position (25 right, 23 left), while 44% (47/108) were ectopic suprainguinal (19 right, 28 left) and 12% (13/108) remained intra-abdominal (10 right, 3 left) (**Figure 7a-d**). All intra-abdominal testes were located close to the neck of the bladder except one which was located close to the caudal pole of the right kidney. The epididymis and vas deferens were absent in most animals although isolated portions of both were sometimes present. All the testes from this experimental group were smaller than those of controls, particularly testes which remained intra-abdominal (**Table 1**). If the 3 groups of testes from flutamide-treated animals are analysed by a one-way analysis of

ANALYSIS OF VARIANCE

SOURCE	DF	SS	MS	F	p
FACTOR	3	14.3078	4.7693	59.52	<0.001
ERROR	123	9.8566	0.0801		
TOTAL	126	24.1644			


```
                              INDIVIDUAL 95% CI'S FOR MEAN
                                  BASED ON POOLED STDEV
     LEVEL            --+---------+---------+---------+----
control                                                   (--*--)
scrotal                                       (--*-)
ectopic                                      (-*--)
abdominal           (----*---)
                     --+---------+---------+---------+----
                    0.35      0.70      1.05      1.40
```

Analysis of variance for testicular weights (g) in control males (n=38) and males treated prenatally with flutamide where testes in the latter were located scrotally (n=48), in an ectopic suprainguinal position (n=47) or retained within the abdomen (n=12). Individual inter-group comparisons were made using 95% Confidence Intervals.

variance, this is highly significant, with abdominal testes much smaller than those in the scrotal or ectopic suprainguinal positions.

Testes	Mean weight (g ± SEM)	Testosterone (nmol/testis)
Control	1.47 ± 0.02 (n = 32)	0.47 ± 0.03 (n = 10)
Flutamide		
scrotal	0.87 ± 0.05 (n = 48)	0.49 ± 0.09 (n = 6)
suprainguinal	0.78 ± 0.06 (n = 47)	0.32 ± 0.06 (n = 6)
intra-abdominal	0.44 ± 0.07 (n = 12)	0.15 ± 0.04 (n = 6)

Table 1 : Weight of testes and their testosterone content in control adult male Albino Swiss rats and adult males treated pre-natally with flutamide.

(ii). Effects of prenatal exposure of flutamide on the structure of the adult rat testis

Testes from rats exposed pre-natally to flutamide exhibited varying degrees of tubular damage. As described in the Materials and Methods section above, damage was divided into three categories:

- Category 1 - normal tubules with all the expected cell types and normal spermatogenesis;

- Category 2 - moderately damaged tubules ranging from tubules with most of the cell types (but without normal spermatogenesis) to tubules with minimal numbers of germ cells in the spermatocyte stage; and

- Category 3 - severely damaged tubules, with the tubular epithelium consisting mainly (or solely) of Sertoli cells with minimal numbers of germ cells restricted to the spermatogonium stage.

There could be a mixture of different categories of damage within an individual testis.

Normal tubules

Normal tubules (designated here as Category 1) not only contained Sertoli cells, but also exhibited full spermatogenesis with spermatogonia, primary and secondary spermatocytes, round, elongating and maturing spermatids **(Figure 8a-b).** The Sertoli cells of Category 1 tubules appeared normal and extended upward through the full thickness of the epithelium to its luminal surface. They possessed thin processes which surrounded the spermatogenic cells and occupied the interstices between them. They had ovoid nuclei (often with one or more deep infoldings), homogeneous nucleoplasm and a large round or oval nucleolus. The cytoplasm contained numerous slender elongated mitochondria orientated parallel to the long axis of the cell **(Figure 8c)**. Granular and agranular endoplasmic reticulum were sparse, and lipid droplets and inclusion bodies were sometimes present.

The most primitive spermatogonia (Type A) and the later, more differentiated spermatogonia (Type B) appeared normal. The Type A spermatogonium had a spherical or ellipsoid nucleus with very fine chromatin granules and one or more nucleoli attached to the inner aspect of the nuclear envelope. The cytoplasm was homogeneous. Type A spermatogonia were present in all stages of the spermatogenic cycle. The Type B spermatogonium also possessed a spherical nucleus with a single nucleolus which was centrally located and chromatin granules of larger size than in Type A. Type B spermatogonia were present only in stages IV-VI of the spermatogenic cycle. Spermatogonia were usually located adjacent to the basement membrane.

The primary spermatocytes exhibited varying degrees of maturation. Resting (non-dividing) spermatocytes were readily seen in tubules at stage VII and VIII of the cycle. Leptotene and zygotene spermatocytes were seen in tubules during stages IX-X and in stage XIII respectively, together with the older (pachytene) spermatocytes. Pachytene spermatocytes divide by a first maturational meiotic division in stage XIV to give rise to secondary spermatocytes. The latter soon divide again by a second maturational mitotic division to produce young spermatids at the end of stage XIV.

The round (steps 1-3) spermatids were present in tubules in the golgi phase (Stages I - III) of the spermatogenic cycle and (steps 4-7) spermatids in the cap phase (Stages IV-VII). The elongating (steps 8-14) spermatids in the acrosome phase (Stages VIII-XIV) had their head caps orientated towards the

tubular basement membrane and their cytoplasm displaced towards the tubule lumen. The maturing spermatids (also called the immature spermatozoa), which were seen in stages XV-XIX, undergo the terminal changes in shape before release from the seminiferous epithelium as free spermatozoa **(Leblond and Clermont, 1952).**

Tubules with moderate damage

These tubules (designated here as Category 2) exhibited most of the expected cell types, i.e. Sertoli cells, primary and secondary spermatocytes and first generation (steps 1-7) spermatids. Elongating (steps 8-14) spermatids were usually damaged or absent and second generation (steps 15-19) spermatids were usually absent. There was increased intercellular spacing both between germ cells and between germ and Sertoli cells: This appeared to be the result of degenerating germ cells within the tubule wall. Some of the tubules exhibited 'free' germ cells in the lumen while others possessed luminal polynuclear giant cells **(Figure 9a-b)**. These 'free' cells were held by small processes of Sertoli cells as revealed by scanning electron microscopy **(Figure 9d)**. In tubules of this category, the Sertoli cells not only contained mitochondria, but also contained lipid droplets and several inclusion bodies and vacuoles in their cytoplasm **(Figure 9c).**

Tubules with severe damage

These tubules (designated here as Category 3) were lined almost exclusively by cells which electron microscopic examination showed to be Sertoli cells **(Figure 10a-b)**. There were occasional spermatogonia. In these tubules, the Sertoli cells had highly lobulated nuclei with many deep infoldings. The cytoplasm contained minimal numbers of mitochondria or inclusion bodies **(Figure 10c-d)**.

Lamina propria

Tubule profiles designated as moderately or severely damaged (categories 2 and 3) could have dimensions similar to controls, but many were very small and separated from each other by wide interstitial spaces filled with lymphoprotein and increased numbers of interstitial cells. Regarding the latter, mean counts of Leydig cells per mm^2 of interstitium were significantly higher in flutamide-treated animals (849.9 $\pm$ 58) than in controls (683.3 $\pm$ 37) (t = 2.42, df = 8, p < 0.05) based on 5 areas per testis from 5 experimental and 5 control testes.

Tubules in control male testes were surrounded by a smooth lamina propria consisting of a single layer of flattened peritubular myoid cells separated from Sertoli cells by 2 layers of slightly undulating basal lamina **(Figure 11a)**; normal (category 1) tubules from flutamide-treated males were similar. Tubules from moderately (category 2) and severely (category 3) damaged tubules differed

from this pattern, but the nature of the difference depended on the size of the tubule. Thus, tubules which achieved normal size had increased layers of basal laminae (up to 5 - see **Figure 11b**), while shrunken tubules had only 2 highly convoluted layers and their peritubular myoid cells possessed pyramidal nuclei **(Figure 11c).**

In control rats, the Leydig cells were distributed close to the blood vessels of the interstitium. They had lobulated nuclei containing a single nucleolus. The cytoplasm contained numerous mitochondria and agranular endoplasmic reticulum. In contrast, the Leydig cells in the flutamide-treated rats appeared in clusters. Their nuclei were less lobulated and the cytoplasm contained mitochondria but less agranular endoplasmic reticulum. Granular endoplasmic reticulum was minimal in both control and flutamide-treated animals **(Figure 12a-b).**

Tubule damage in relation to testis position

A total of 6,906 tubules was examined from scrotally-located testes, 6,027 from suprainguinal ectopic testes and 1,679 from intra-abdominal testes (the latter being a lower figure as there were fewer examples of these testes). There was a clear gradation of damage according to testis location, with scrotal testes being least affected and intra-abdominal testes the worst **(Figure 13)**.

The abnormality of cryptorchid testes has often been ascribed to their exposure to elevated temperatures. In the case of supra-inguinal ectopic testes, it might be expected that greater damage would be seen in areas close to the

anterior abdominal musculature rather than in the potentially cooler subcutaneous areas. This was not the case. Least damage occurred in the central regions with greater damage observed uniformly around the periphery.

Staging of the spermatogenic cycle

Tubules with the most damage (Category 3) had few or no spermatogenic cells. Those with moderate damage (Category 2) had arrested spermatogenesis at the spermatid stage: Tubules in stages I-VIII of the cycle of the seminiferous epithelium showed apparently normal Steps 1-8 of spermiogenesis but lack second generation maturing (Steps 15-19) spermatids, while tubules in stages IX -XIV showed damaged or absent elongated acrosome-phase (Steps 9-14) spermatids.

For tubular profiles from controls (together with those tubules rated as Category 1 in flutamide-treated male rats), the percentage of profiles at each stage of the cycle of the seminiferous epithelium as defined by **Leblond and Clermont (1952)** was determined (**Figure 14**). There were no major differences in the percentage of occurrence of the different stages of the spermatogenic cycle between the normal tubules of the flutamide-treated males and those from control testes. This demonstrates that tubules which appear normal in terms of possessing all cell types are also normal in terms of stages of the spermatogenic cycle.

60

FIGURE 5:   (a-d) Light micrographs of four consecutive sections (30 μm intervals) of the genito-urinary tract in a male Albino Swiss rat treated prenatally with flutamide. Note:

(i) the urethra (U) opens beneath the prepuce on the ventral aspect of the phallus (P),

(ii) the persistence of a vagina (V).

Magnification: all x 6.3

5a

b

c

d
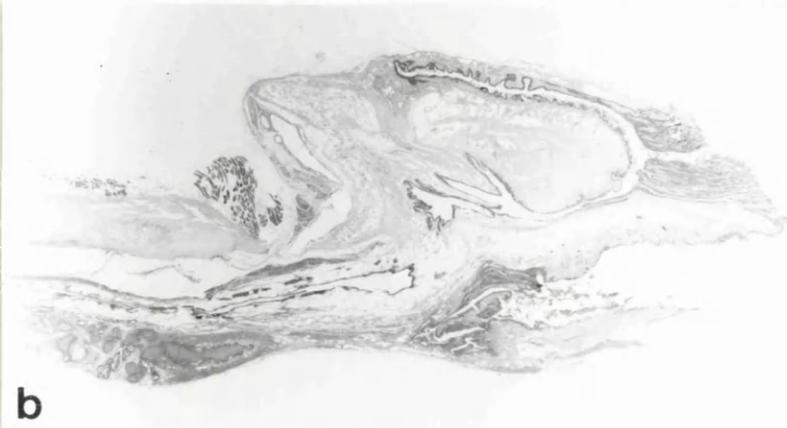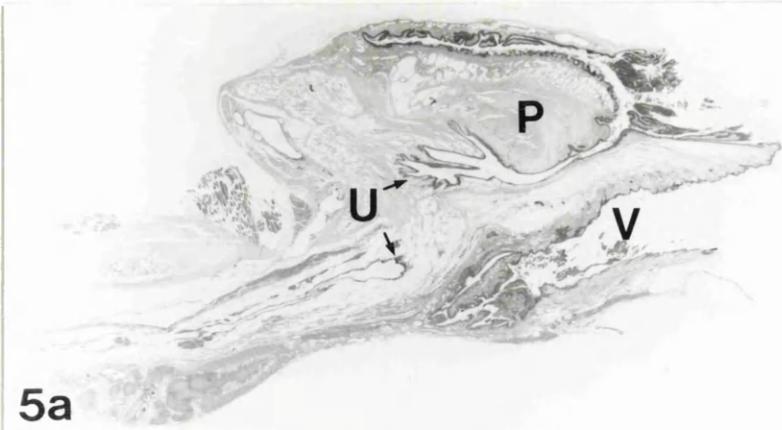
FIGURE 6: Computer reconstruction of sections of the same

specimen as shown in Figure 5:

(a) made up of 27 serial sections across the phallus

Red - urethra and preputial space; Blue - vagina;

Green - urethral glands; Yellow - prostate

(b) made up of 3 consecutive sections (14 - 16) to

show opening of the urethra (U) into the preputial

space below the phallus (P).

6a

b

P

U →

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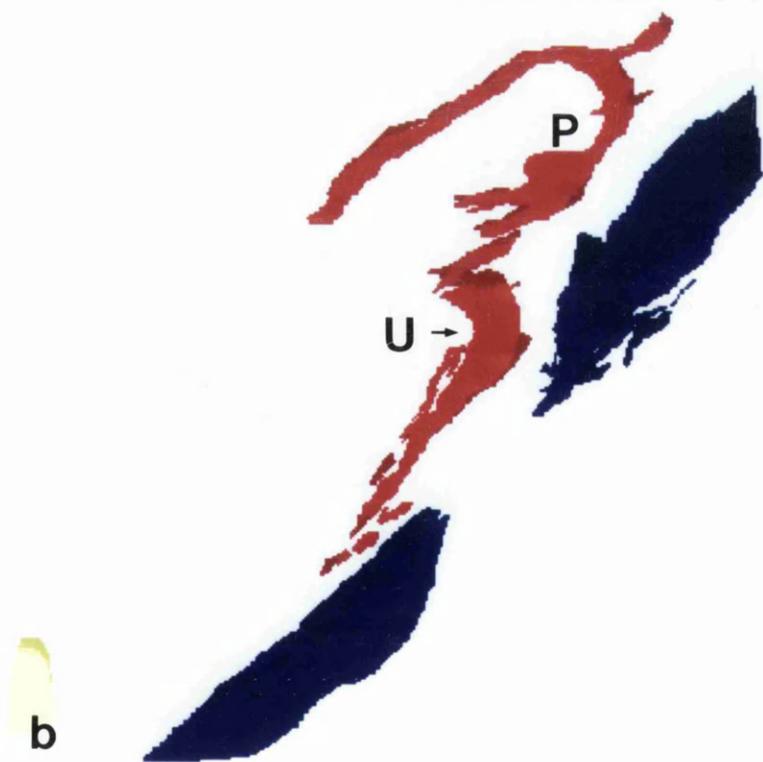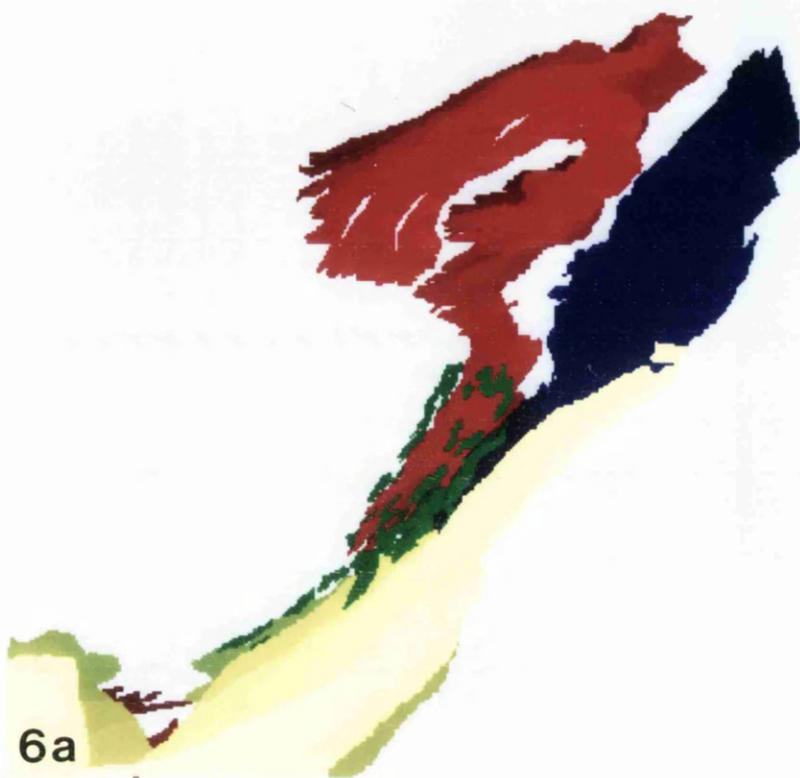
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FIGURE 7: Various positions of the testis in male Albino Swiss rats treated prenatally with flutamide:

a) bilateral descent of testes into the scrotum,

b) unilateral scrotal descent of right testis and descent of left testis to an ectopic suprainguinal position,

c) bilateral descent to ectopic suprainguinal positions, and

d) unilateral descent of left testis to an ectopic suprainguinal position and right undescended testis (arrow).

Magnification: all x 0.8

7a   b

c   d

FIGURE 8: Normal (Category 1) seminiferous tubules from the testes of Albino Swiss rats treated prenatally with flutamide:

a) Light micrograph showing normal tubules with full spermatogenesis. Note that the tails of maturing spermatids occupy the lumen and their nuclei (heads) lie in the apical region of the epithelium. Magnification: x 230

b) Transmission electron micrograph of a Category 1 tubule. Bar = 2 μm.

(c) Transmission electron micrograph of a Sertoli cell within a Category 1 tubule. Bar = 2 μm.

Sn, Sertoli cell nucleus; Pm, peritubular myoid cell; L, Leydig cell; Sp, spermatocyte; S7, step 7 spermatid; Arrows indicate lipid droplets; M, mitochondria.
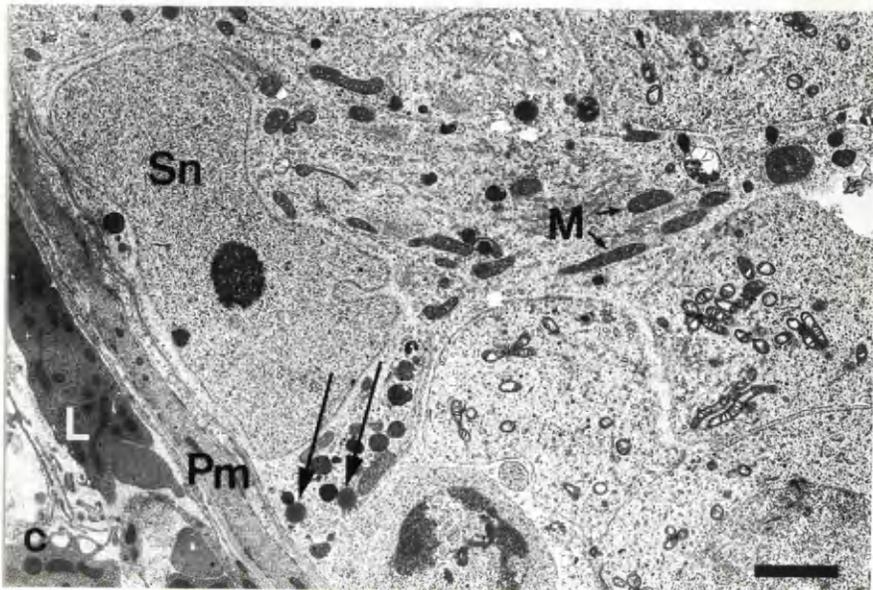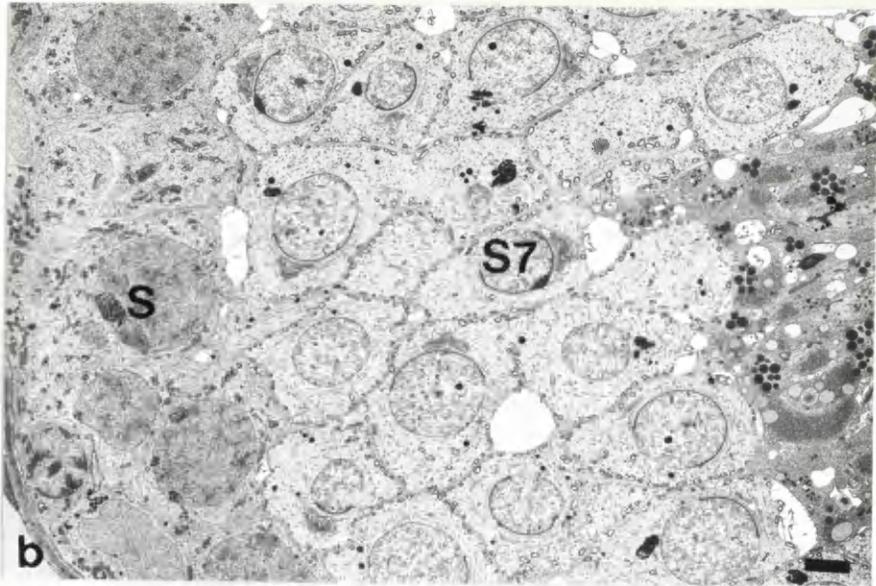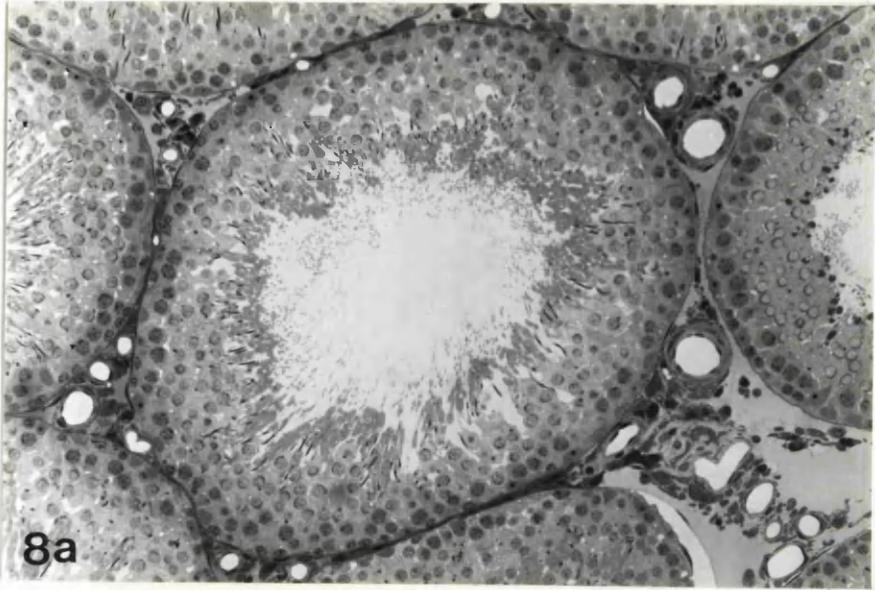
FIGURE 9: Moderately damaged (Category 2) seminiferous tubules from the testes of Albino Swiss rats treated prenatally with flutamide:

a) The lumen of the tubules are lined by round cap-phase spermatids. The maturing spermatids which should lie internal to these have been lost. Instead a few 'free' cells (arrows) occupy the lumen. A multinucleate giant cell is seen in the lumen (arrowhead).

Magnification: x 230.

b) Transmission electron micrograph of a Category 2 tubule. Bar = 2 μm.

(c) Transmission electron micrograph of Sertoli cells in a Category 2 tubule. Bar = 2 μm.

Sn, Sertoli cell nucleus; S8, Step 8 spermatid; Asterisks indicate degenerating cells; V, vacuole; Arrows indicate lipid droplets.
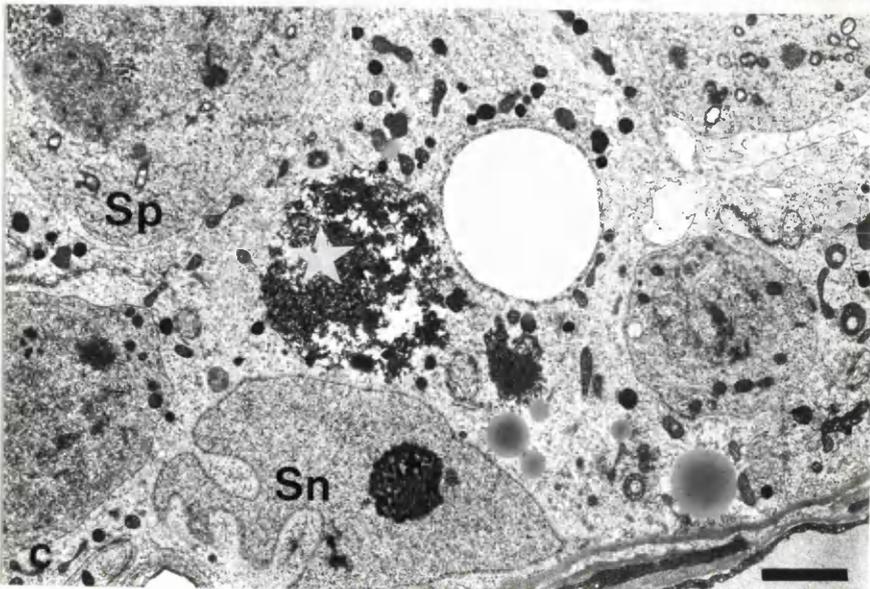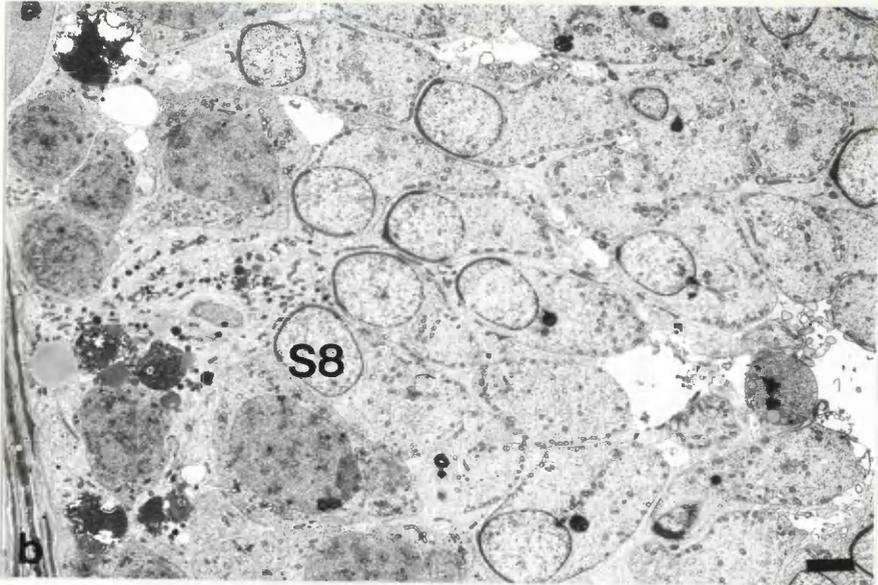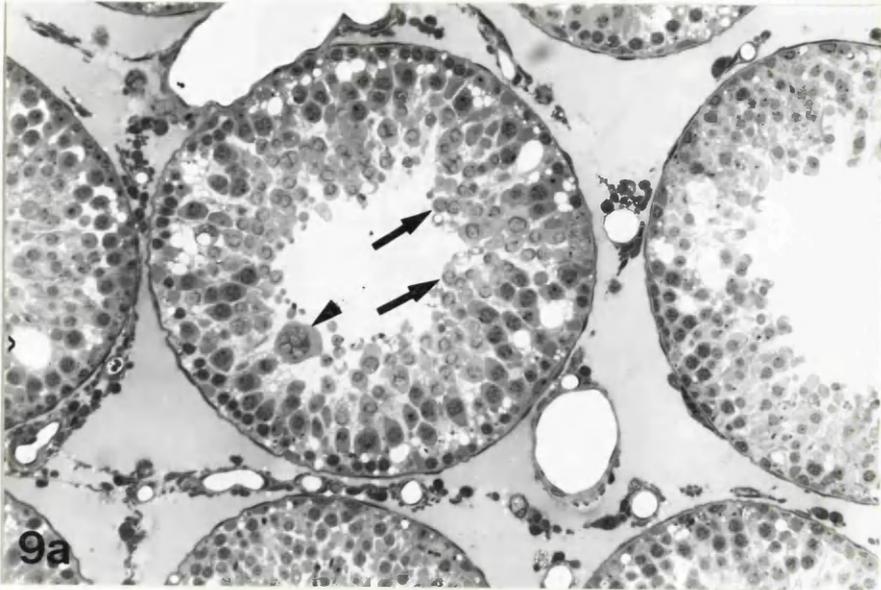
FIGURE 9:   <u>continued</u>:

(d)   Scanning electron micrograph of a Category 2 tubule showing the luminal surface of the tubule. Note: 'free' cells (F) noted on light and transmission electron microsopy are actually held by the apical processes of the Sertoli cells (Sp). Bar = 10 μm
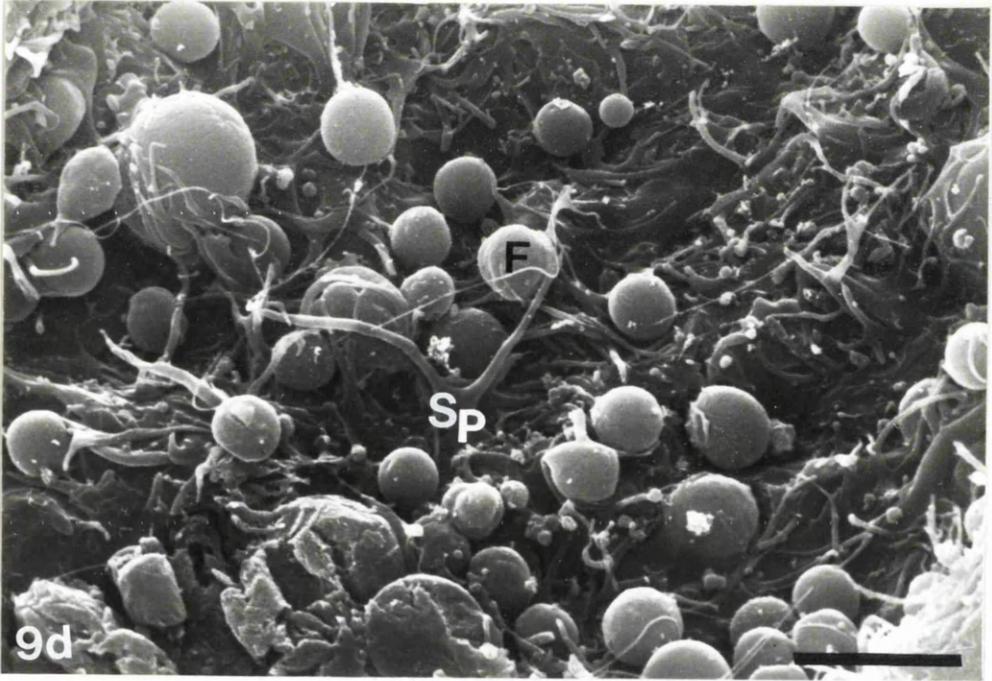
FIGURE 10: Severely damaged (Category 3) seminiferous tubules from the testes of Albino Swiss rats treated prenatally with flutamide:

a) thin walled, and (b) thick walled tubules

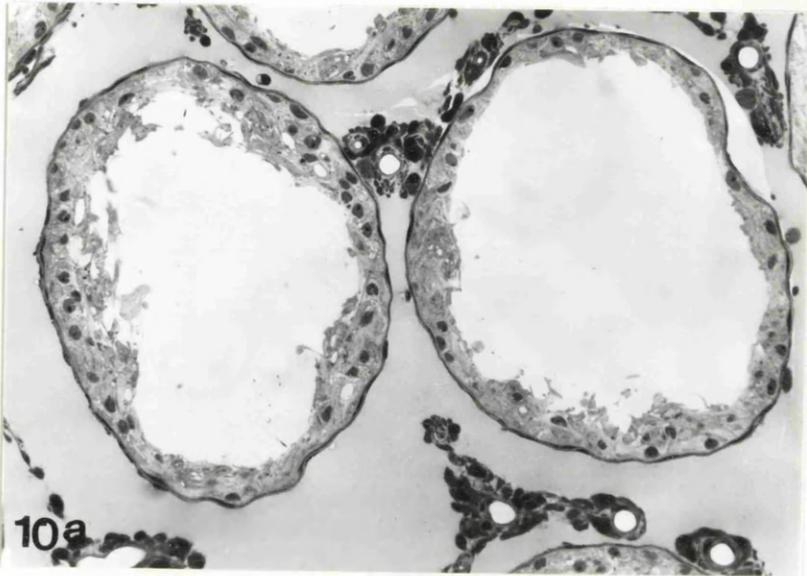These tubules are lined almost exclusively by Sertoli cells.

Magnification: x 230

10a

b
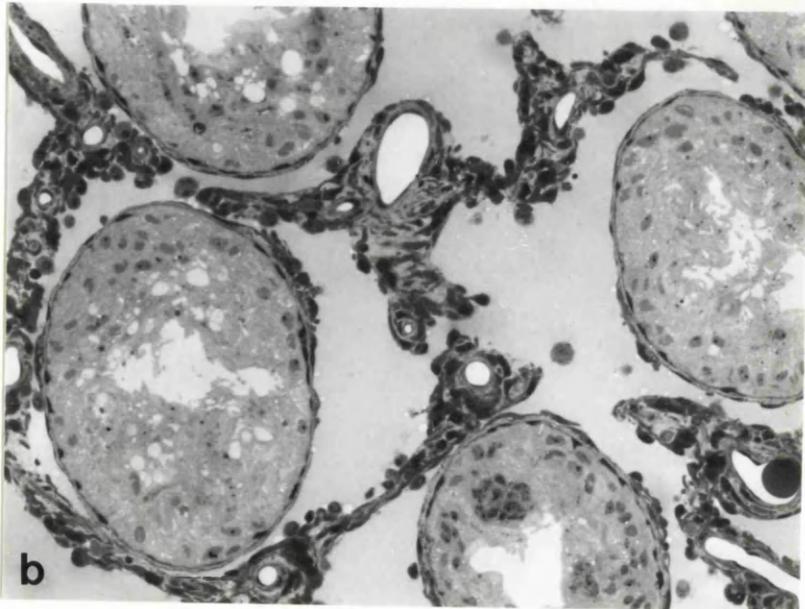
FIGURE 10: continued:

Transmission electron micrographs of Sertoli cells from (c) thin walled, and (d) thick walled tubules in Category 3 tubules.
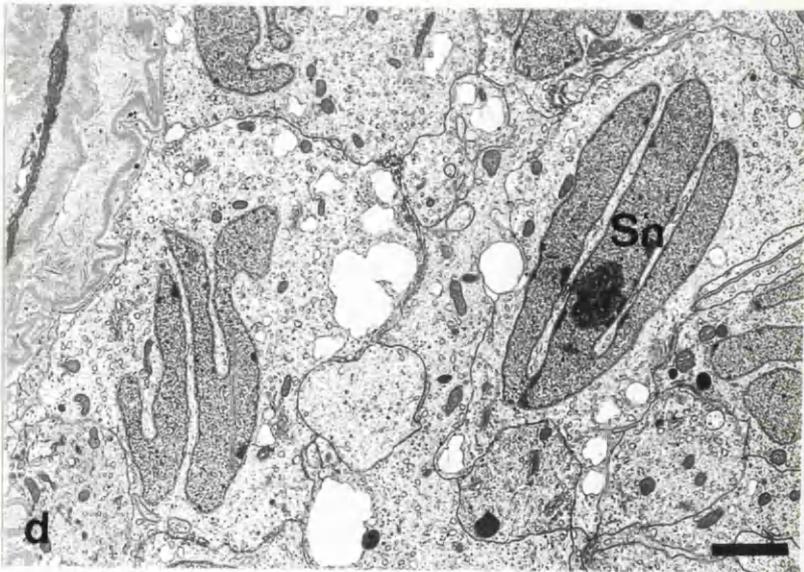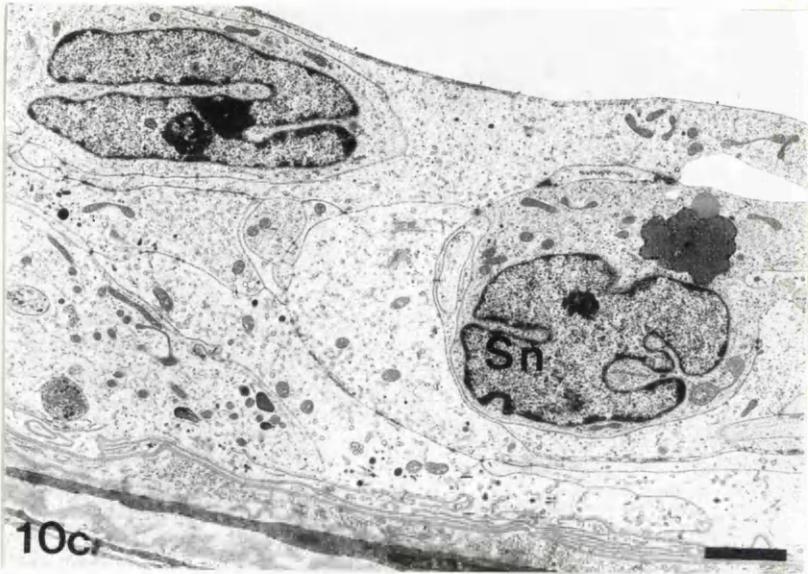
Sn, Sertoli cell nucleus. Bar = 2 μm.

10c

d

FIGURE 11: Transmission electron micrographs of limiting membranes in

a) a normal seminiferous tubule;

b) a severely damaged (Category 3) tubule of normal dimensions;

c) a severely damaged (Category 3) tubule of greatly reduced diameter.

S, Sertoli cell; PM, peritubular myoid cell; E, endothelial cell, Arrows indicate basal lamina; Lc, Leydig cell.
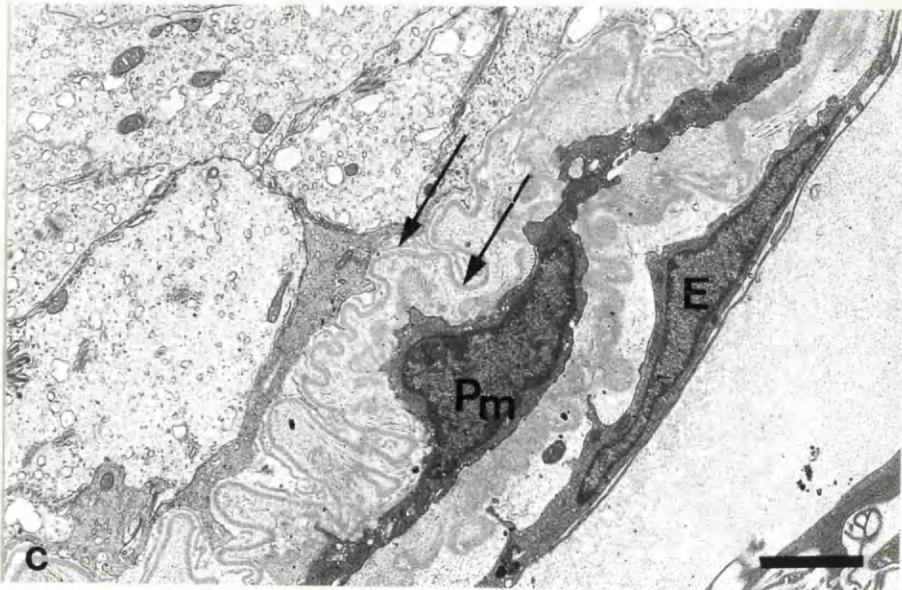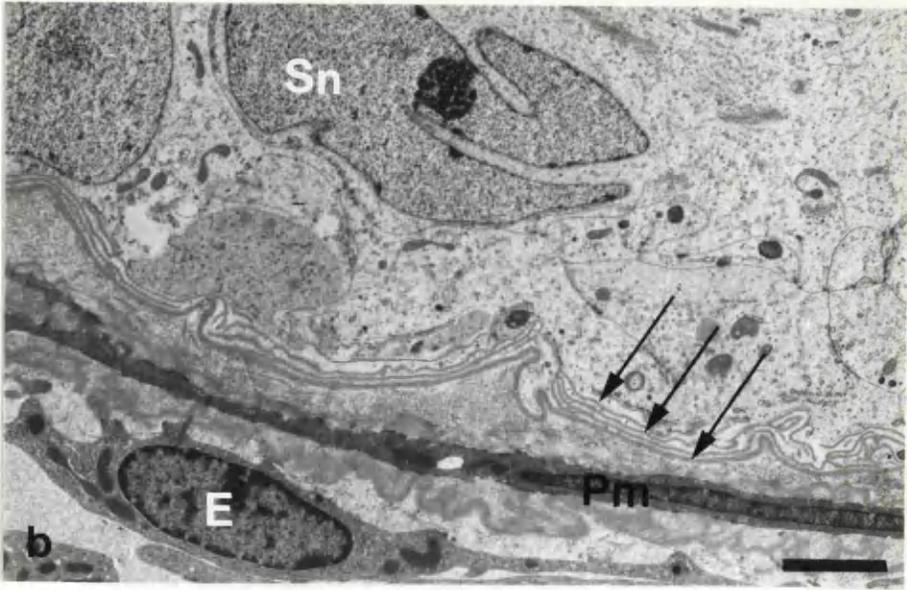
Bar = 2 µm.

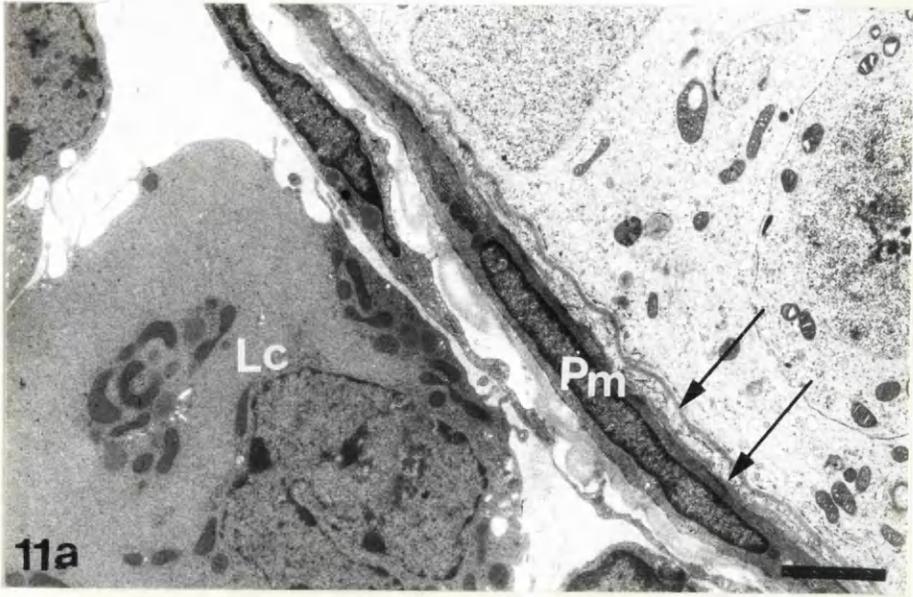**FIGURE 12:** Transmission electron micrographs of the interstitium showing Leydig cells of (a) a normal control male Albino Swiss rats, and (b) a male treated prenatally with flutamide.

Ln, Leydig cell nucleus; M, mitochondria; Q, macrophage.

Bar = 2 μm.
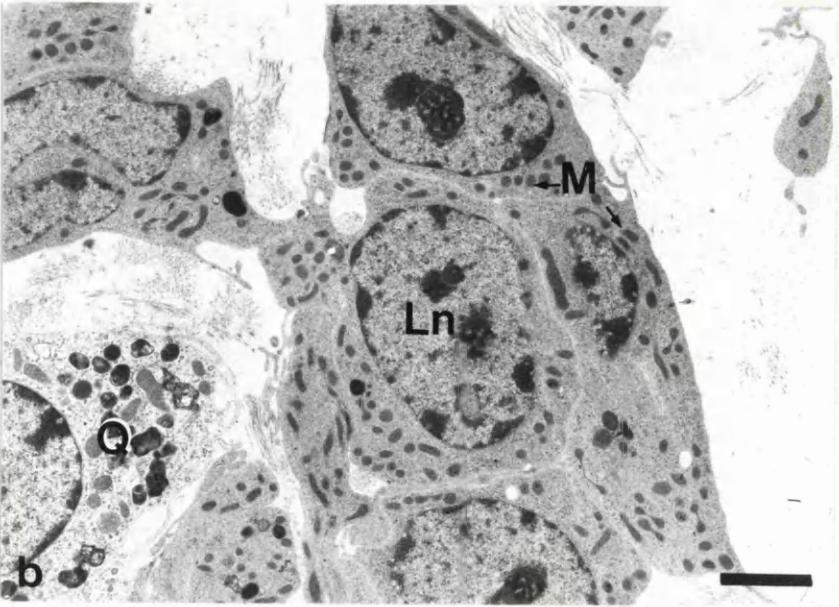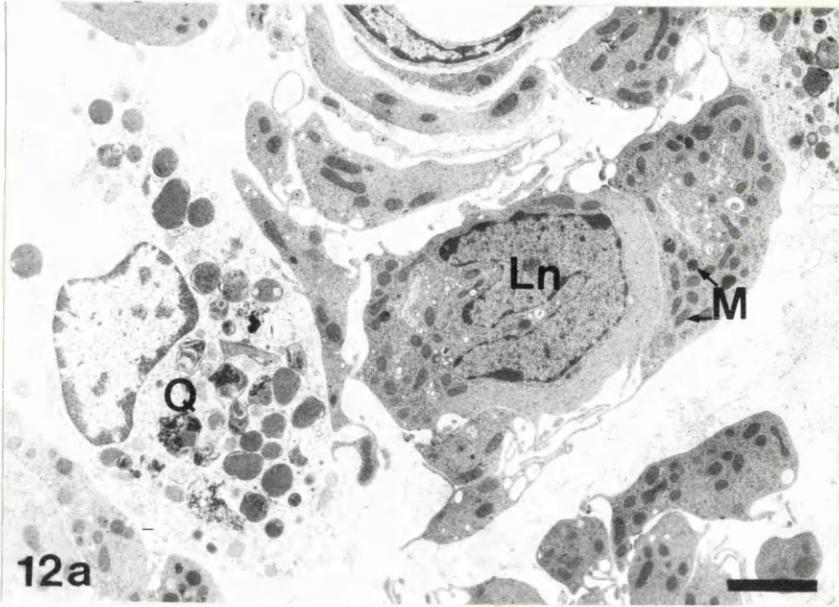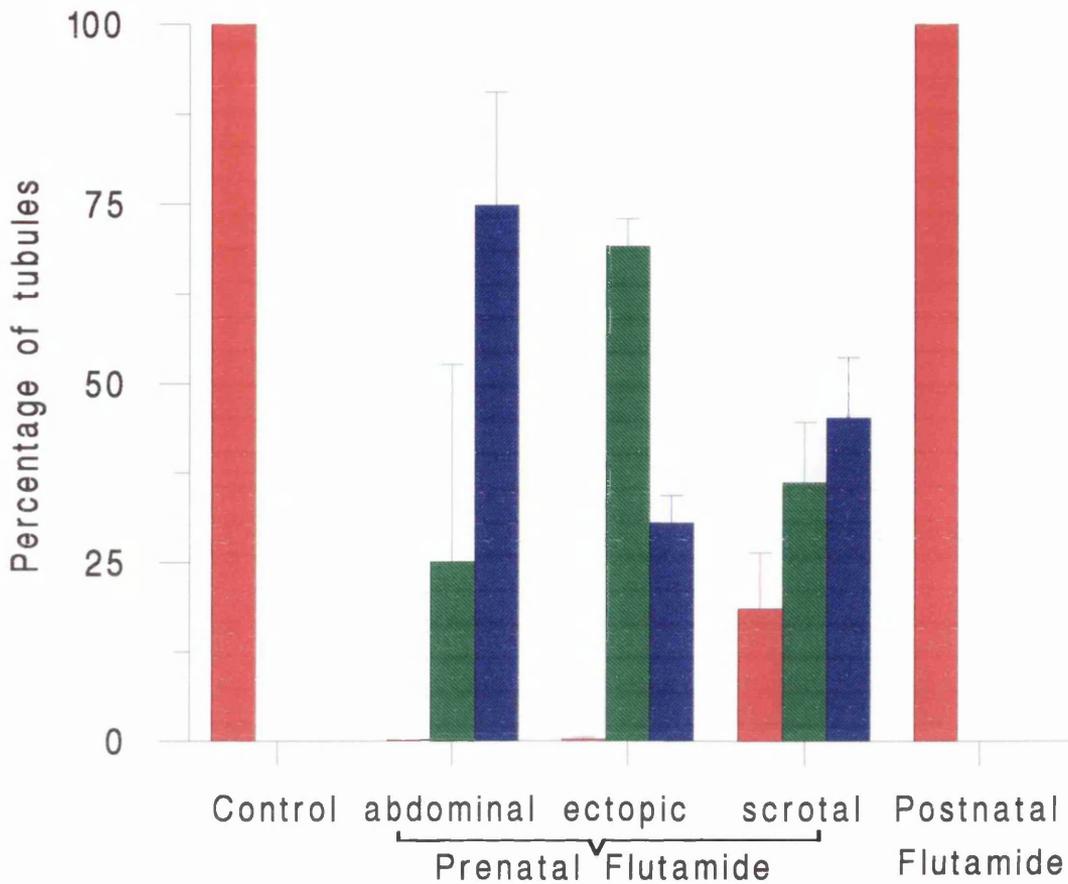
Figure 13: Percentage of seminiferous tubules in different categories of damage in relation to testis position in control male Albino Swiss rats and males treated prenatally with flutamide (percent ± SEM).

Figure 14: Percentage of seminiferous tubules in different stages of the spermatogenic cycle in normal tubules of control male Albino Swiss rats (▪) and males treated prenatally with flutamide (▪)(percent ± SEM).

(iii). Development of male hamsters after prenatal exposure to flutamide

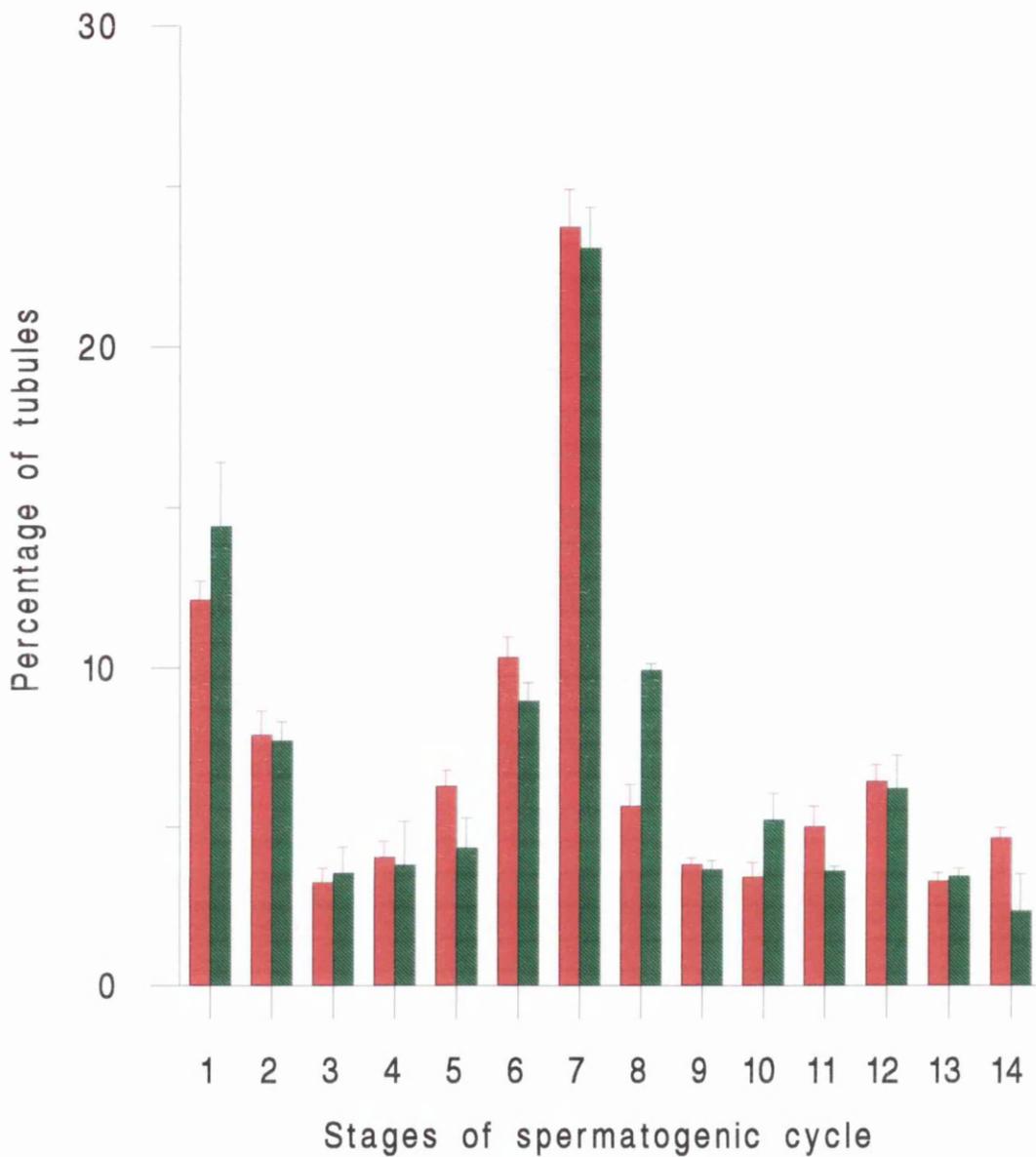In contrast to the rat, male hamsters exposed prenatally to flutamide from gestational day 8 to birth did not have feminized external genitalia; there were no nipples or vaginae as seen in flutamide-treated male rats. However, the development of the epididymis and vas deferens was inhibited to varying degrees in most animals. Furthermore, like the controls, all the testes of flutamide-treated males were located within the scrotum **(Figure 15a-b)**. The testes of the flutamide-treated males were in general smaller than those of the controls: mean weights of testes (g ± SEM) in the control males (n = 24) = 1.33 ± 0.08; and in the flutamide-treated males (n = 24) = 0.80 ± 0.07 (t = 4.84, df = 33, p < 0.005).

(iv). Effects of prenatal exposure to flutamide on the structure of the adult hamster testes:-

Testes from the flutamide-treated hamsters exhibited varying degrees of tubular damage similar to that observed in the rats. A total of 5710 tubular cross-sectional profiles from flutamide-treated testes were examined, and the number of tubular profiles in the three different categories of damage already established for the rat were determined. Percentage of tubules in the different categories were: Category 1 (normal) = 15.69 ± 5.26, Category 2 (moderate damage) = 57.04 ± 6.24, and Category 3 (severe damage) = 27.25 ± 5.57. All the tubular profiles in the control testes (n = 4026) were normal **(Figure 16).**

Normal tubules

The normal (Category 1) tubules were lined by all the expected cell types and exhibited normal spermatogenesis. The Sertoli cells which were located adjacent to the basement membrane had a single nucleus containing a nucleolus. The cytoplasm contained considerable agranular endoplasmic reticulum (SER) and elongated mitochondria which were distributed in the apical cytoplasm. Lipid droplets and small vacuoles were sometimes present. The spermatogonia were located adjacent to the basement membrane together with the Sertoli cells. The primary spermatocytes were in the intermediate layer with the secondary spermatocytes and the round or elongating (i.e. first generation) spermatids in the innermost layer of the tubular epithelium. The maturing (i.e. second generation) spermatids (or immature spermatozoa) lay in rows between the spermatocytes and the first generation spermatids and were surrounded by a network of the apical processes of Sertoli cells **(Figure 17a-c).**

Moderately damaged tubules

Moderately damaged (Category 2) tubules were lined by most of the expected cell types, but spermatogenesis was arrested and the tubular epithelium consisted of Sertoli cells, spermatogonia and spermatocytes; round spermatids were few or absent. Elongating and maturing spermatids were usually absent. The Sertoli cells which were located adjacent to the basement membrane (interspersed

with spermatogonia) often contained increased amount of SER, lipid droplets and inclusion bodies in their cytoplasm **(Figure 18a-c)**.

Severely damaged tubule

Tubules in Category 3 (severely damaged) were lined by Sertoli cells with minimal numbers of germ cells usually limited to the spermatogonium stage. Some Sertoli cells contained increased numbers of mitochondria, and many contained large vacuoles in their cytoplasm **(Figure 19a-b)**.

Lamina propria

Normal (Category 1) seminiferous tubules of the flutamide-treated hamsters and those of the controls were surrounded by a smooth-contoured lamina propria consisting of a) an inner cellular layer of flattened peritubular myoid cells separated from the Sertoli cells by 2 layers of slightly undulating basal lamina, and b) an outer cellular layer of endothelial cells, separated from the peritubular myoid cells by a single layer of basal lamina **(Figure 20a)**. For the moderately or severely damaged (Category 2 and 3) tubules, the lamina propria varied according to their size. In tubules with normal dimensions, the flattened peritubular myoid cells were separated from the Sertoli cells by 3-4 layers of slightly undulating basal lamina but, where tubule size was greatly reduced, tubules were surrounded by a lamina propria consisting of peritubular myoid cells

with "thickened" nuclear profiles and separated from the Sertoli cells by 2-3 layers of highly undulating basal lamina **(Figure 20b)**.

The interstitial spaces were filled with lymphoprotein and interstitial cells, chiefly Leydig cells which were concentrated adjacent to the blood vessels. These Leydig cells were normal with lobulated nuclei, and a cytoplasm containing agranular endoplasmic reticulum and mitochondria. Lipid droplets were sometimes present.

Staging of seminiferous tubules

The stages of the seminiferous tubules of the control testes and the normal (category 1) tubules of flutamide-treated testes were analysed according to the criteria used by **Leblond and Clermont (1952).** The percentages of occurrence of the different stages of the tubular cycle of the flutamide-treated and of the control testes are shown in **Figure 21.** Briefly, 1) some stages occur more often than others in both groups: This is because certain stages of the spermatogenic cycle are of longer duration than others (i.e. stage VII has the longest duration) **(Clermont and Harvey, 1965)**, 2) there were no differences between groups in the frequency of any stage.

FIGURE 15: Position of the testes (T) in (a) a control male hamster, and (b) a male hamster treated prenatally with flutamide.

Note the well-developed epididymis (E) and seminal vesicles (Sv) in the control compared to the flutamide-treated male.

Magnification: all x 1.4

15a

b
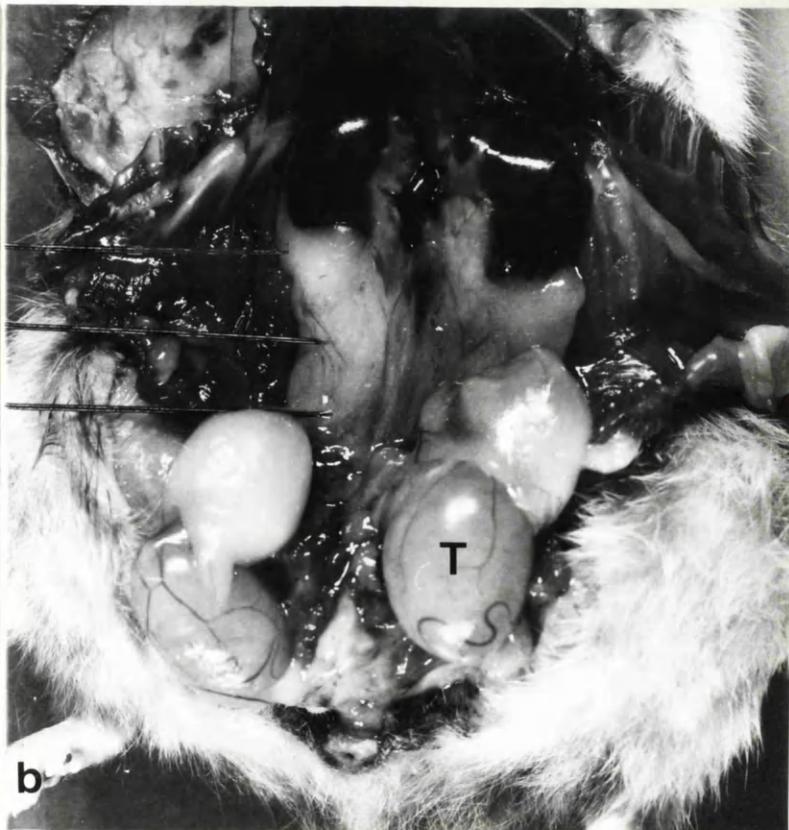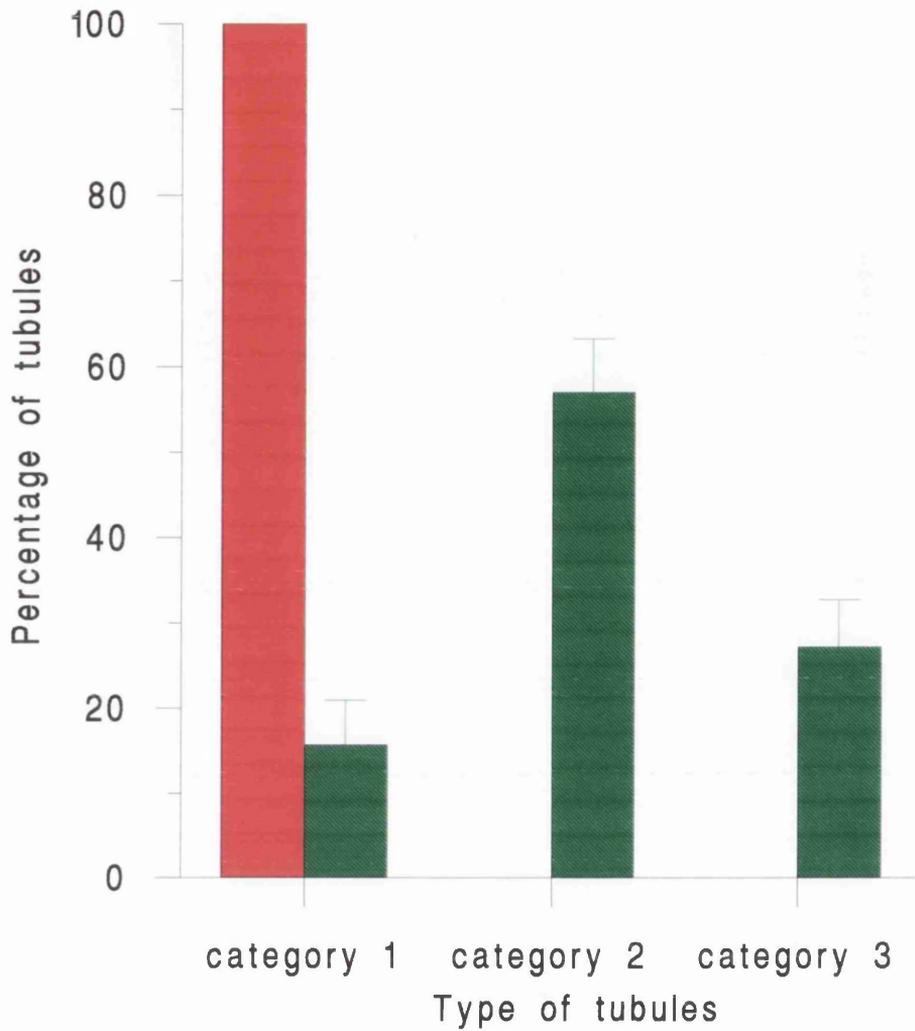
Figure 16: Percentage of seminiferous tubules in different categories of damage in control male golden hamsters (■) and males treated prenatally with flutamide (■) (percent ± SEM).

Category 1 - normal tubules; Category 2 - mild; Category 3 - severe damage. See text for details.

FIGURE 17: Normal (Category 1) seminiferous tubules in male hamsters treated prenatally with flutamide.

a) Light micrograph of normal tubules with full spermatogenesis. Note that the tails of maturing spermatids occupy the lumen and their nuclei (heads) lie in the apical region of the epithelium. Magnification: x 230.

b) Transmission electron micrograph of Category 1 tubule in Stage VII of the spermatogenic cycle. Sn, Sertoli cell nucleus; S7, Step 7 spermatid; Sg, spermatogonium.

Bar = 2 μm.

(c) Transmission electron micrograph of a Sertoli cell from a Category 1 tubule. Sn, Sertoli cell nucleus. Bar = 2 μm.

17a
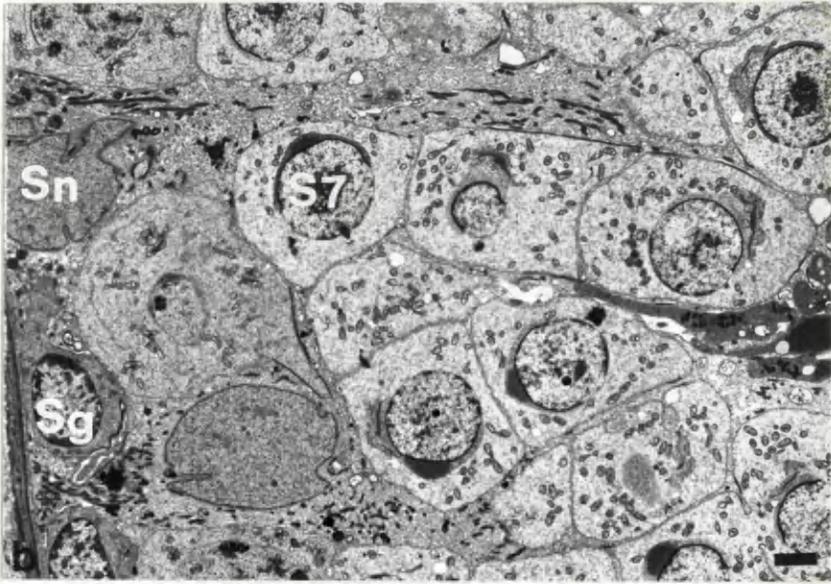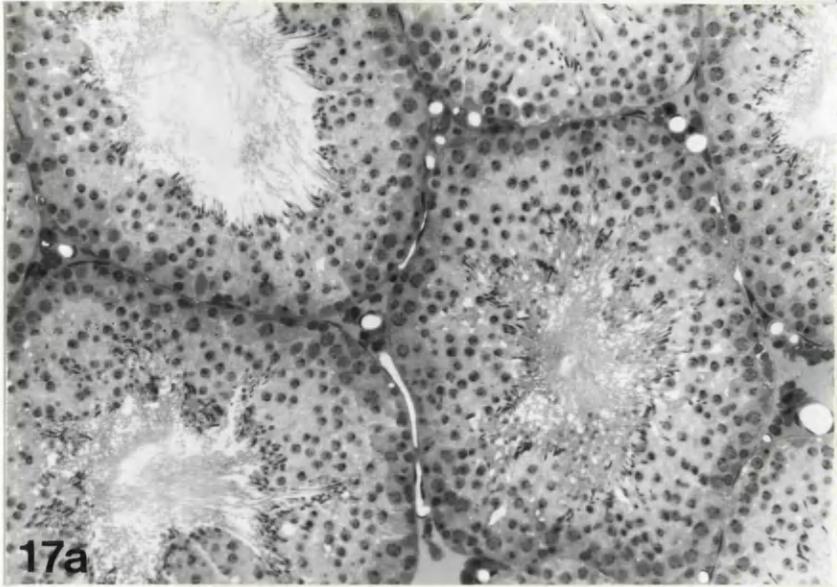
FIGURE 18: Moderately damaged (Category 2) seminiferous tubules from male hamsters treated prenatally with flutamide.

a) The lumen of tubules are lined by round spermatids but mature sperms are absent. Magnification: x 230.

b) Transmission electron micrograph of a Category 2 tubule.

S3, Step 3 spermatid; V, vacuole.

Bar = 2 μm.

(c) Transmission electron micrograph of Sertoli cells from a Category 2 tubule. Sn, Sertoli cell nucleus; Arrows indicate lipid droplets; Thick arrows indicate residual bodies; SER, smooth endoplasmic reticulum; Pm, peritubular myoid cell.

Bar = 2 μm.

18a

b

V

S3

c

Pm

SER

Sn
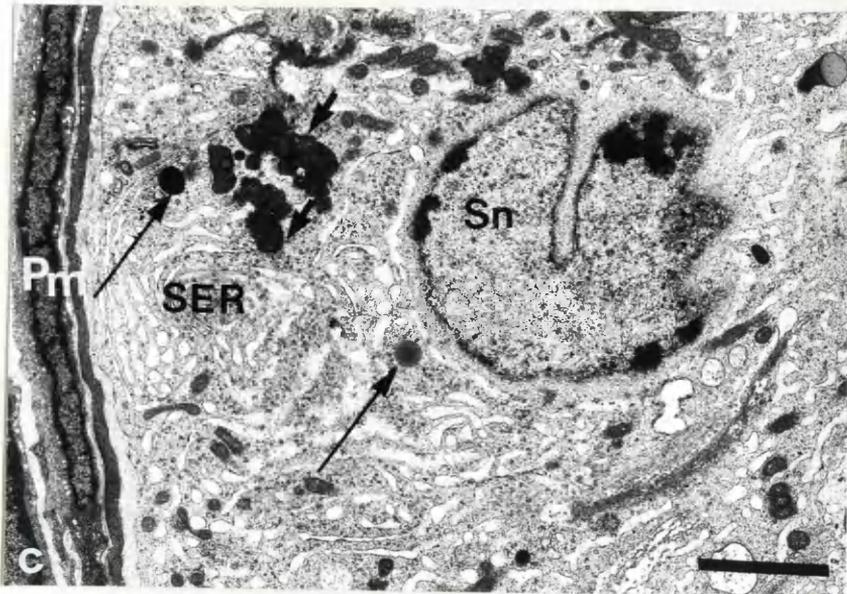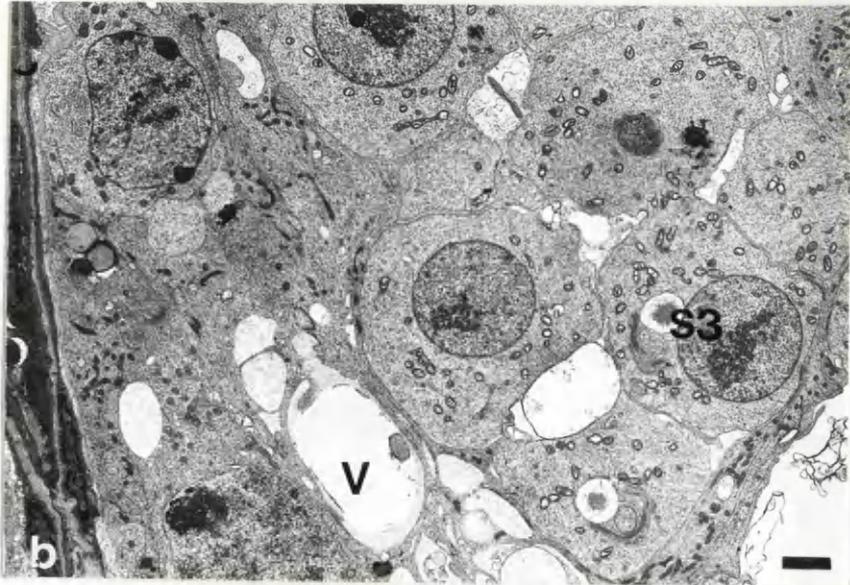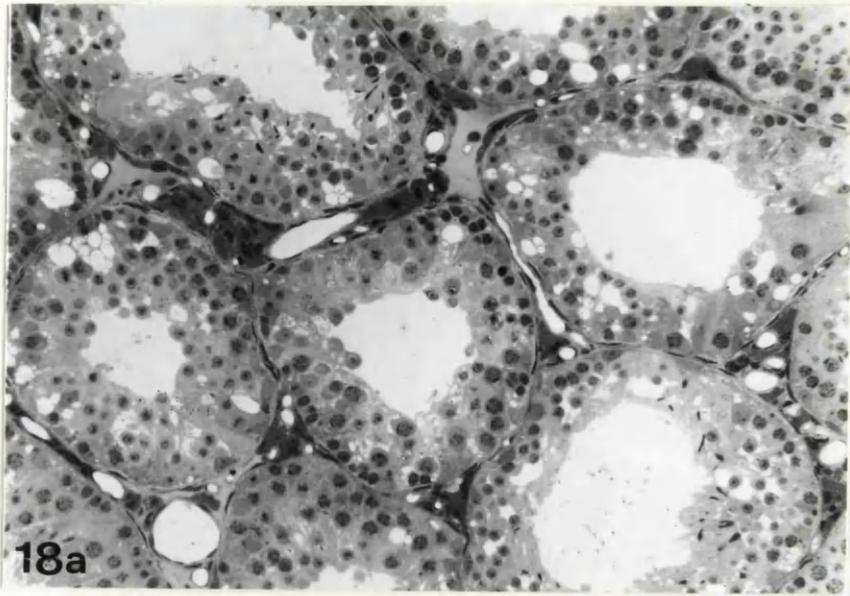
FIGURE 19: Category 3 (severe damage) seminiferous tubules from hamsters treated prenatally with flutamide.

a) Light micrograph of Category 3 tubules, lined almost exclusively by Sertoli cells.

Magnification: x 230.

(b) Transmission electron micrograph of Sertoli cells from a Category 3 tubule. Sn, Sertoli cell nucleus; M, mitochondria. Bar = 2 μm.

19a

b
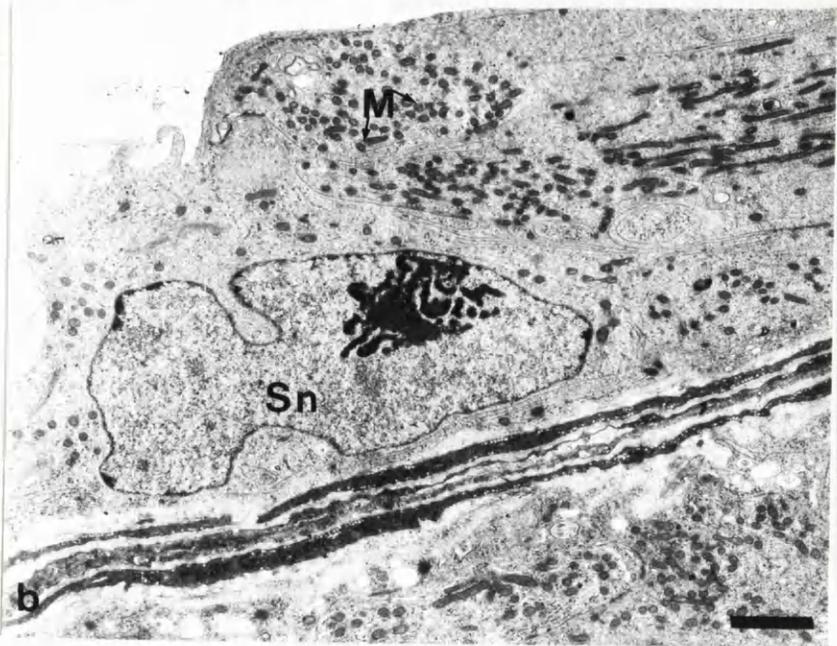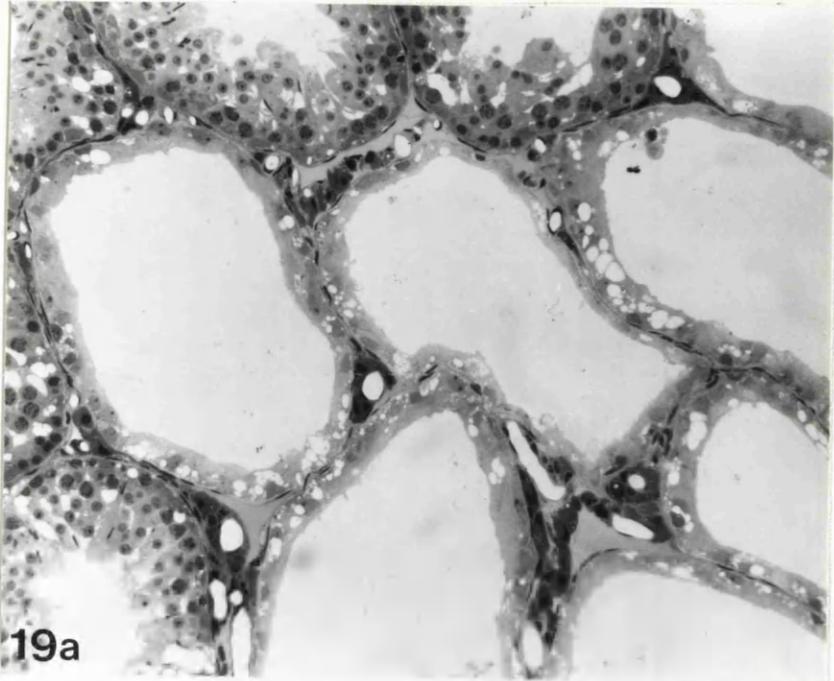
FIGURE 20: Transmission electron micrographs of limiting membranes from the testes of male hamsters treated prenatally with flutamide:

(a) from a normal seminiferous tubule,

(b) from a 'small' severely damaged (Category 3) tubule.

Sn, Sertoli cell; Pm, peritubular myoid cell;

E, endothelial cell, Arrows indicate basal lamina.

Bar = 2 μm.

20a.

b.
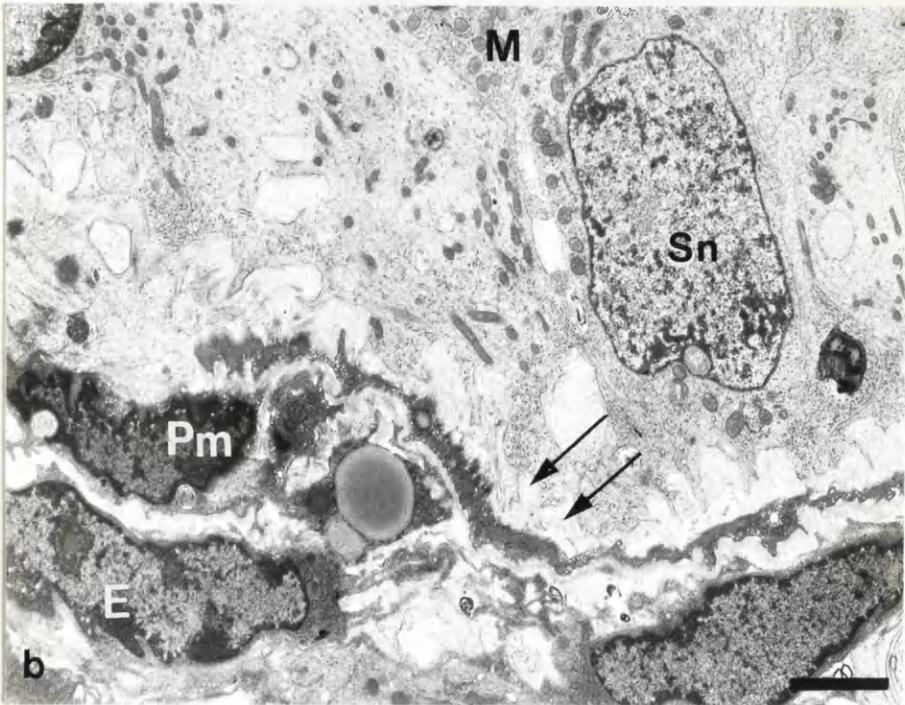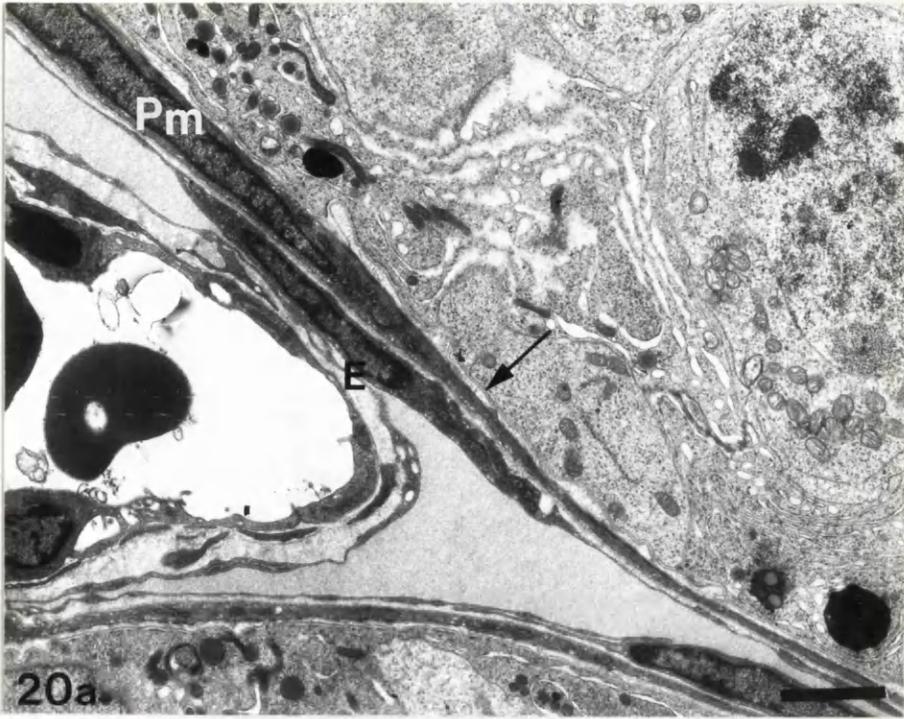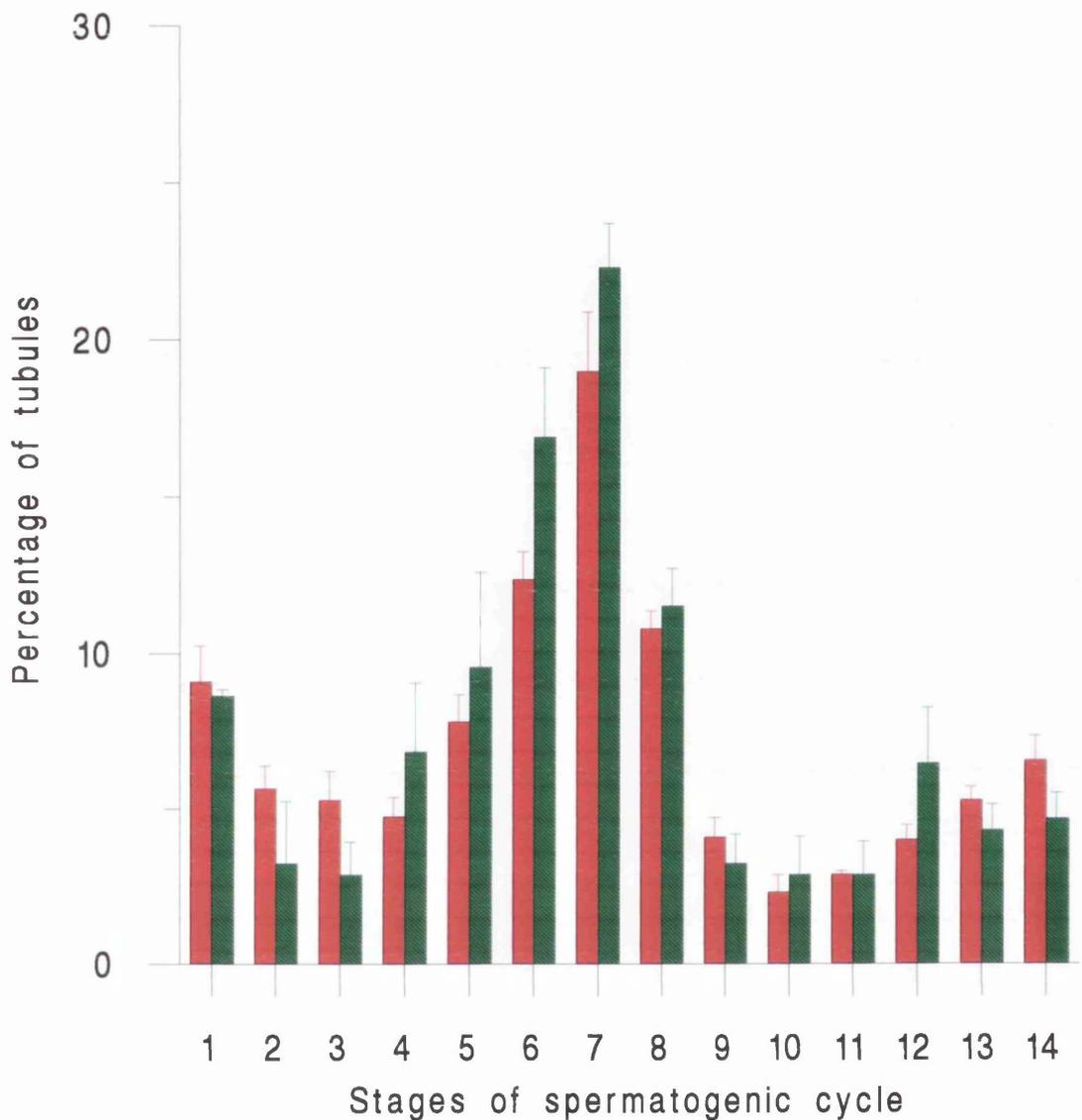
Figure 21: Percentage of seminiferous tubules in different stages of the spermatogenic cycle in control male golden hamsters (■) and males treated prenatally with flutamide (■) (percent ± SEM).

v). Effects of postnatal flutamide treatment in rats:

Postnatal administration of flutamide (birth - P14) to rats did not interfere with the development of the genitalia and all males in this group exhibited normal bilateral descent of testes into the scrotum. The testes were of normal size with mean weight (g ± SEM) : postnatal flutamide-treated males = 1.38 ± 0.02; control males = 1.40 ± 0.03. Morphological examination of these testes revealed that the seminiferous tubule profiles were normal and exhibited normal spermatogenesis. The Sertoli cells and Leydig cells were also normal **(Figure 22a-c).**

FIGURE 22: Category 1 (= normal) seminiferous tubules of male AS rats exposed to flutamide during the early postnatal period:

a) Light micrograph of cross-sections of tubules with normal spermatogenesis. Magnification: x 230

b) Transmission electron micrograph of a Sertoli cell. Sn, Sertoli cell nucleus; Arrows indicate lipid droplets. Bar = 2 μm.

(c) Transmission electron micrograph of Leydig cells. Ln, nucleus; M, mitochondria; SER, smooth endoplasmic reticulum. Bar = 2 μm.

22a

b   Sn

c   Ln   SER   M
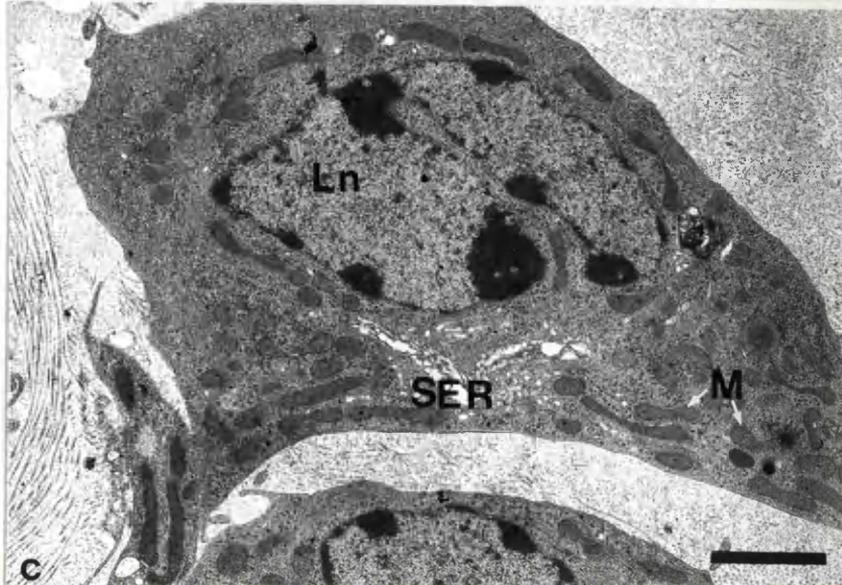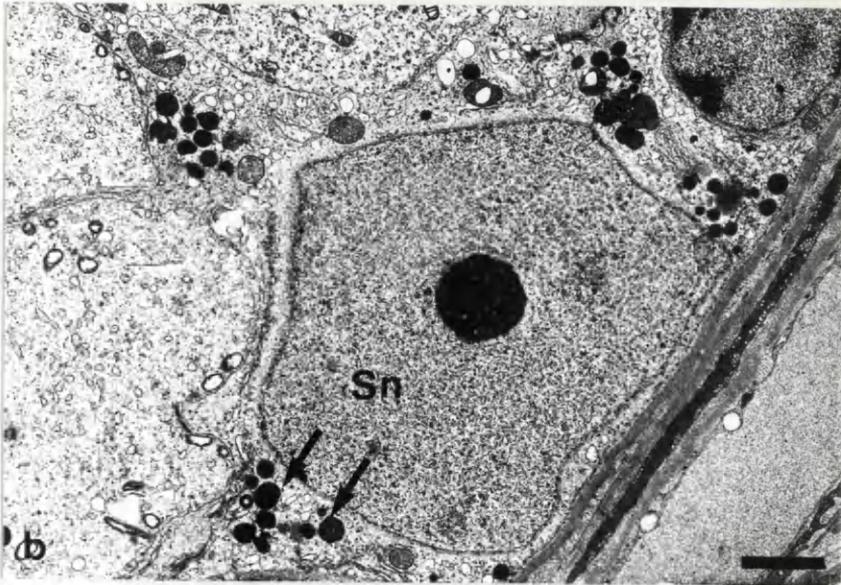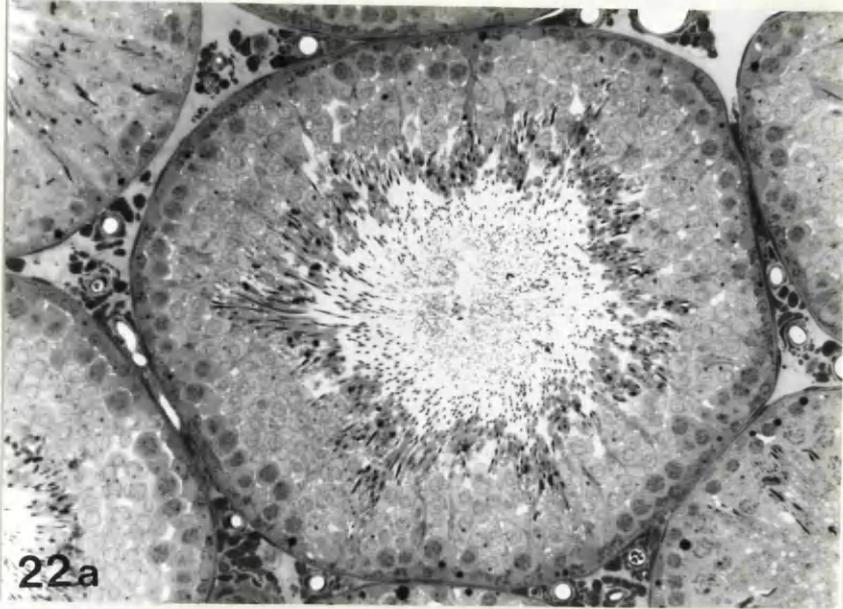
EXPERIMENT 1(b)

Plasma and intra-testicular testosterone levels in the rat and hamster:

Methods:

i). Blood collection

Blood samples were obtained by cardiac puncture from pre-natal flutamide-treated males (20 rats; 6 hamsters) and from control males (16 rats; 6 hamsters). The blood samples were subsequently centrifuged at 3,000 rpm for 5 minutes at 4°C. The separated plasma was then stored at -20°C until assayed for testosterone.

ii). Testis collection

Testes from control males (10 rats; 6 hamsters) and from pre-natal flutamide-treated males (18 rats; 6 hamsters) were removed immediately after blood collection and weighed. The larger number of rats in the latter group was to ensure that sufficient testes were available from all three possible locations. Each testis was then decapsulated and a testicular sample (weighing 0.1 - 0.2 g) was obtained and homogenized in 1.0 ml 0.9% saline in a Jencons Uniform Homogenizer. After centrifugation at 3,000 rpm for 5 minutes at 4°C, the supernatant fraction was stored at -20°C until assayed for testosterone.

iii). Radioimmunoassay (RIA) of testosterone

Plasma and testicular concentrations of testosterone were determined in ether extracts of serum and testicular homogenate respectively, using a double antibody radioimmunoassay **(Appendix 4)** based on a rabbit anti-testosterone-3-0-

ANALYSIS OF VARIANCE

SOURCE	DF	SS	MS	F	p
FACTOR	3	5068	1689	8.43	<0.001
ERROR	24	4811	200		
TOTAL	27	9879			

```
                         INDIVIDUAL 95% CI'S FOR MEAN
                             BASED ON POOLED STDEV
Group           ---------+---------+---------+-------
control                                   (-----*----)
scrotal                                   (-------*------)
ectopic                         (-------*------)
abdominal           (------*-------)
                ---------+---------+---------+-------
                        16        32        48
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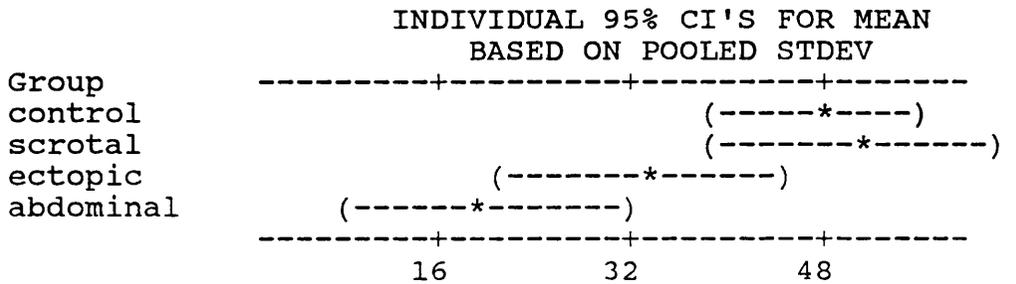
Figure 24: Analysis of variance for testicular testos-
terone levels (nmol/testis) in control males (n=10) and
males treated prenatally with flutamide where testes in
the latter were located scrotally (n=6), in an ectopic
suprainguinal position (n=6) or retained within the
abdomen (n=6). Individual inter-group comparisons were
made using 95% Confidence Intervals.

carboxymethyl oxime-BSA serum and a ^{125}I radioligand prepared from the histamine derivative of testosterone-3-0-carboxymethyl oxime **(Semple et al, 1988).**

RESULTS

Plasma and intra-testicular concentrations of testosterone

Plasma concentrations of testosterone in the male rats treated with flutamide were not significantly different from those in the controls (t = 1.23, df = 31, p > 0.05). Mean plasma concentrations: for controls = 6.9 ± 0.6, and flutamide-treated males = 5.9 ± 0.5 (nmol/l ± SEM).

The mean intra-testicular testosterone concentrations in control male rats were not significantly higher than the mean concentrations of the scrotally located testes in flutamide-treated animals. However, the mean concentrations in testes located in the scrotal and ectopic suprainguinal position of the flutamide-treated males was significantly higher than those testes which remained in the abdomen (see **Table 1**). If the 3 groups of testes from flutamide-treated animals are analysed by a one-way analysis of variance, the intratesticular testosterone concentrations in the abdominal testes is significantly lower than those in the scrotal or ectopic suprainguinal positions.

Similarly, there was no difference between the mean plasma testosterone concentrations in the control and the flutamide-treated hamsters (t = 0.78, df = 5, p > 0.05). The mean plasma testosterone concentrations: for controls

= 9.37 ± 0.32; for flutamide-treated males = 8.15 ± 0.15 (nmol/l ± SEM). There was also no difference between the mean testosterone concentrations per testis in the control males and those of the flutamide-treated groups (t = 0.38, df = 7, p > 0.5). Mean testosterone concentration per testis in controls hamsters = 0.23 ± 0.05, and flutamide-treated males = 0.21 ± 0.03 (nmol/testis ± SEM).

EXPERIMENT 1(c)

The onset of testicular abnormality in experimental male rats exposed prenatally to flutamide:

Since the testes in adult flutamide-treated rats showed varying degree of tubular damage, a limited study was conducted to determine the onset of tubular damage in these experimental animals.

Methods:

Male rats were killed at 4 and 8 weeks post-partum (n = 2 in each age group for controls and for flutamide-treated males) and processed as in Experiment 1(a) for light and electron microscopy. The first evidence of spermiation in the rat is normally seen in the seventh week of postnatal life **(Clermont and Perey, 1957)**. Thus the chosen times are before and just after complete spermiogenesis is established.

RESULTS

As already noted, male rats exposed prenatally to flutamide had feminized genitalia. All control males exhibited normal bilateral descent of testes into the scrotum while flutamide-treated males had their testes located in various positions (see below). Testes of flutamide-treated males at 4 weeks (2 scrotal; 2 intra-abdominal) were about the same size as those of the controls ($t = 2.39$, $df = 5$, $p > 0.05$); however, the testes at 8 weeks (1 scrotal; 2 supra-inguinal ectopic; 1 intra-abdominal) appeared much smaller than those of the controls ($t = 3.34$, $df = 3$, $p < 0.05$) **(Table 2).**

Age group	position (n = 4)	weight (g ± SEM)
4 weeks - Control	4 - S	0.17 ± 0.01
- Flutamide	2-A, 0-E, 2-S	0.13 ± 0.01
8 weeks - Control	4 - S	1.29 ± 0.03
- Flutamide	1-A, 2-E, 1-S	0.64 ± 0.20

Table 2: Shows the position of testes and their weights in control male Albino Swiss rats and males treated prenatally with flutamide at 4 and 8 weeks after birth. **Note:-** S, scrotal; E, suprainguinal ectopic; A, intra-abdominal

Morphology of testes at 4 weeks:

The tubules were close together, separated by narrow interstitial spaces; most tubules had acquired a lumen **(Figure 23a** and **24a)**. The tubular epithelium was composed of Sertoli cells, spermatogonia (Type A and B) and spermatocytes; some tubules had early spermatids. The seminiferous tubules of the flutamide-treated testes were at approximately the same stage of development as in controls. However, there were increased amounts of lipid droplets in the epithelium of most seminiferous tubules of flutamide-treated males, together with increased numbers of degenerating cells and enlarged intercellular spaces **(Figure 23b** and **24b)**. There were no badly damaged tubules (which could be designated as Category 3) at this stage of development. The Sertoli cells of both control and flutamide-treated males had numerous round to elongated mitochondria and SER distributed throughout the cytoplasm.

Each tubule was surrounded by a lamina propria similar to that seen in the adult. There was no apparent difference between the lamina propria of the controls and flutamide-treated males, and those of the normal adult males. The interstitial cells (mainly Leydig cells) of control and flutamide-treated males were grouped together in clusters; in both groups they possessed spherical to ovoid nuclei, and their cytoplasm contained numerous SER and mitochondria. However, lipid inclusions were prominent features in the flutamide-treated males **(Figure 25a-b)**.

FIGURE 23: Seminiferous tubules of 4 week old male control Albino Swiss rats:

:

(a) Light micrograph. Magnification (x 230), and

(b) Transmission electron micrograph.

Sn, Sertoli cell nucleus; Sg, spermatogonia; Sp, primary spermatocyte; Arrows indicate lipid droplets.

Bar = 10 μm.

23a

b

Sn

Sp

Sg
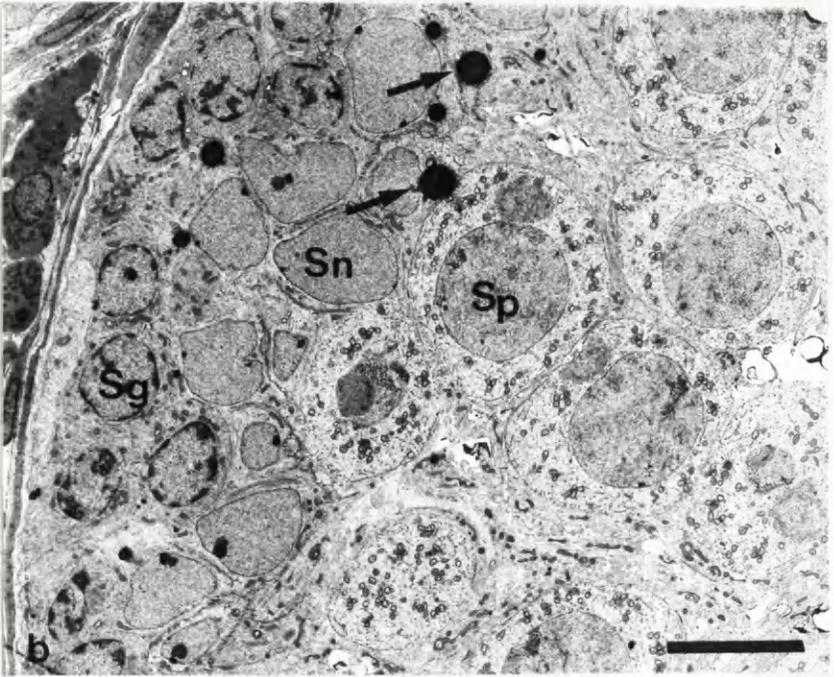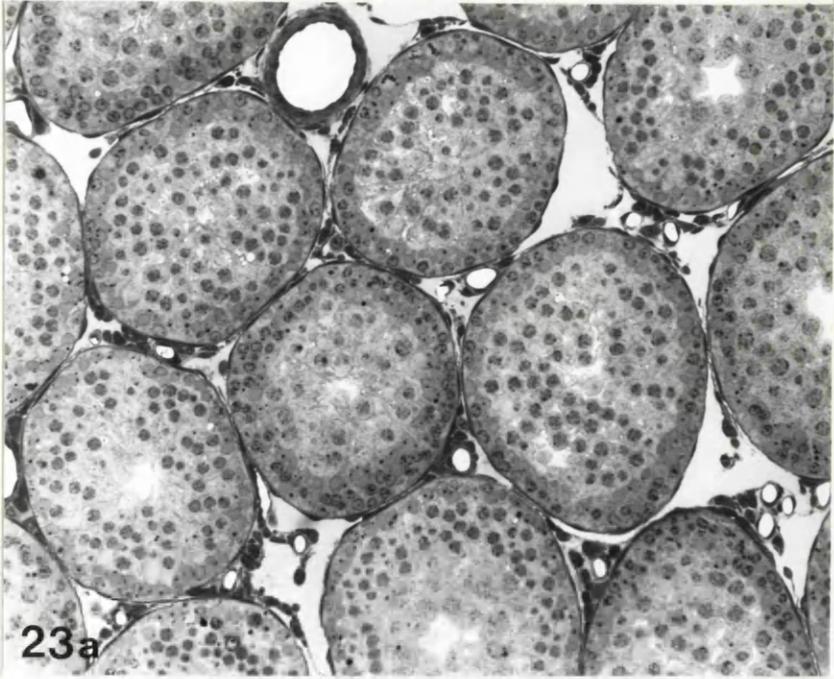
FIGURE 24: Seminiferous tubules of flutamide-treated 4 week old male Albino Swiss rats:

(a) Light micrograph. Magnification (x 230), and

(b) Transmission electron micrograph

Sn, Sertoli cell nucleus; Sg, spermatogonia; Sp, primary spermatocyte; Asterisks indicate degenerating cells; Arrows indicate lipid droplets. Bar = 10 μm.
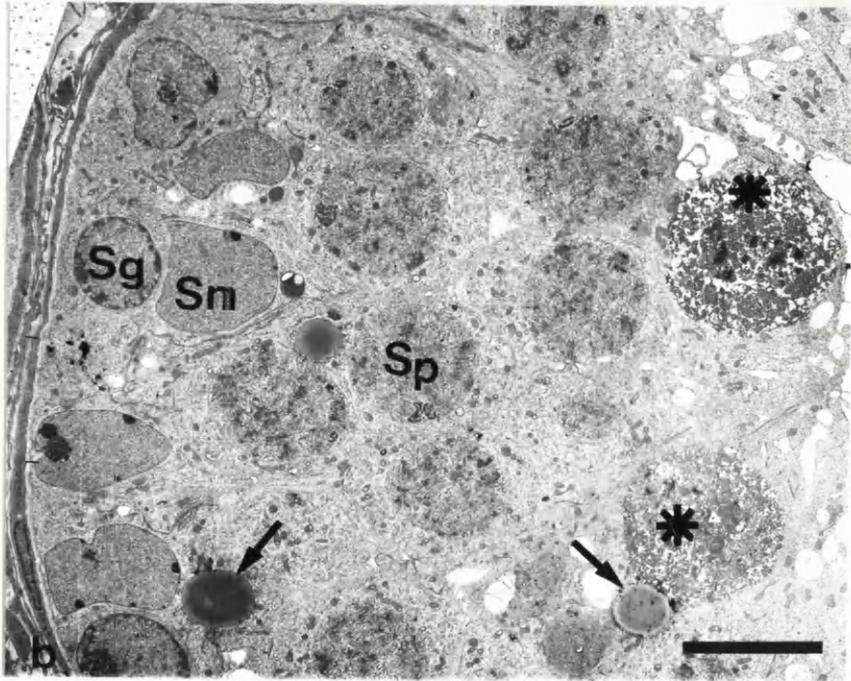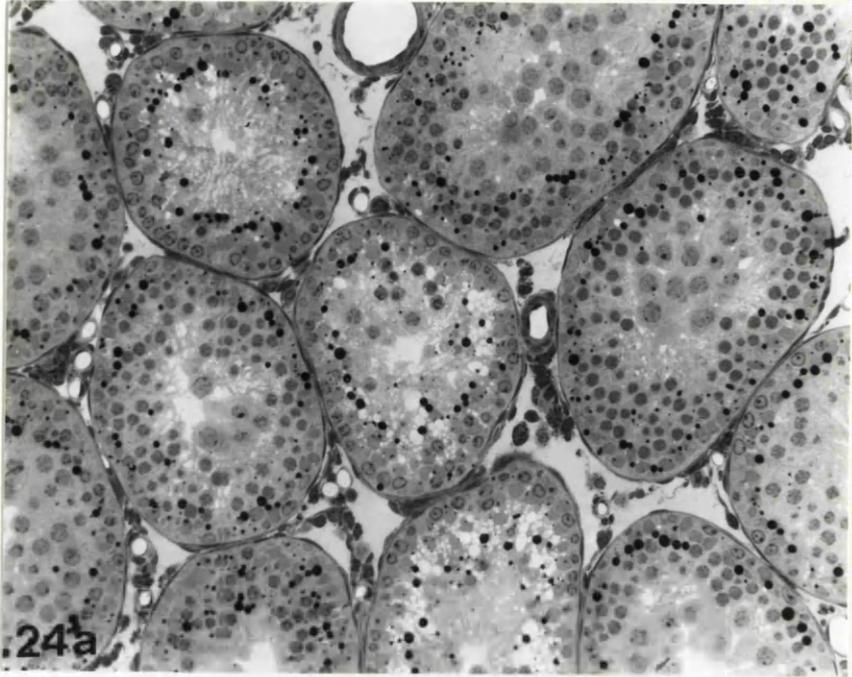
FIGURE 25: Transmission electron micrographs of Leydig cells

of 4 week old male Albino Swiss rats:

(a) a control male, and (b) a male treated prenatally

with flutamide.

Ln, Leydig cell nuclei; M, mitochondria;

Arrows indicate lipid droplets.

Bar = 1 μm.

25a

b
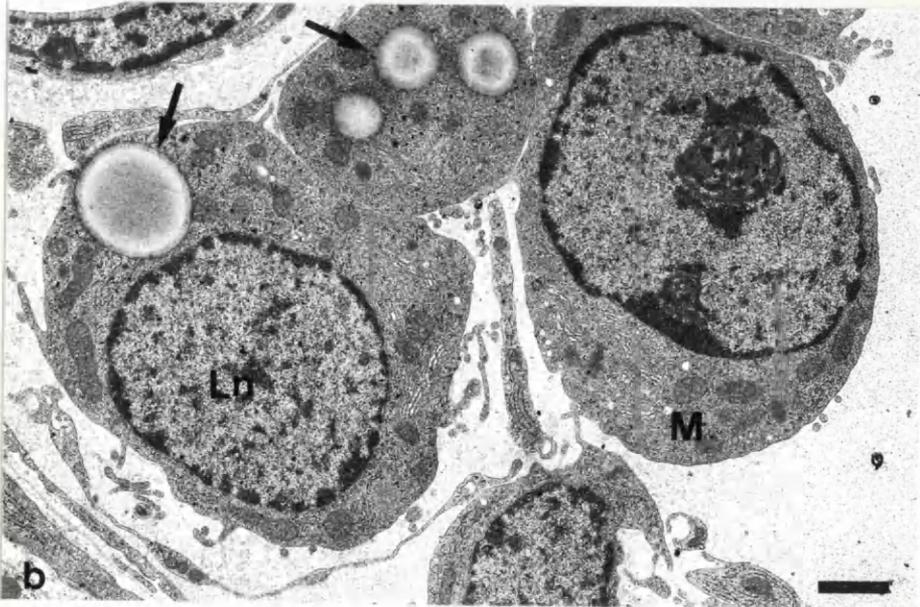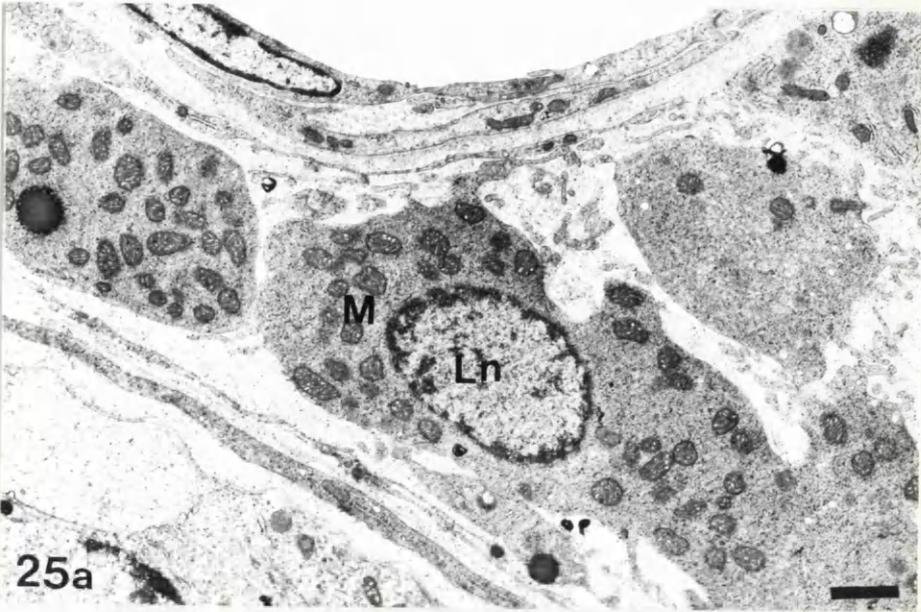
Morphology of testes at 8 weeks:

At 8 weeks the seminiferous tubules of control males appeared similar to those of the adult **(Figure 26a)**. They were lined by all the expected cell types including spermatids at different steps of maturation which corresponded to the stages of the spermatogenic cycle as described by **Leblond and Clermont (1952).** In contrast, most of the seminiferous tubule profiles of flutamide-treated males appeared smaller and these tubules were surrounded by wide interstitial spaces **(Figure 26b)**. Spermatogenesis was disrupted. Most tubules only contained Sertoli cells, spermatogonia and spermatocytes in their tubular epithelium, together with numerous degenerating cells.

The Sertoli cells of the control males were similar to those of the adult in that their nuclei (with their prominent nucleoli) were located close to the basement membrane, and their cytoplasm contained mitochondria, lipid inclusions and several small vacuoles. In contrast, the nuclei of the Sertoli cells of the flutamide-treated males appeared in different layers of the tubule epithelium and the cytoplasm contained lipid inclusions and large vacuoles.

The lamina propria of the seminiferous tubules in the 8 week control and flutamide-treated testes were similar to those of the adults as described earlier. Leydig cells in the control males appeared singly and possessed nuclei which were slightly lobulated. In contrast, the Leydig cells of the flutamide-treated males appeared in clusters, had spherical to ovoid nuclei. Other cell organelles including numerous mitochondria, SER and RER appeared similar in both

groups, There was an increased amount of collagen fibres in the interstitium of

the flutamide-treated males.

FIGURE 26: Light micrographs of seminiferous tubules of 8 week

old male Albino Swiss rats:

(a) a control, and (b) a male treated prenatally with

flutamide.

Magnification: all x 230.

26a
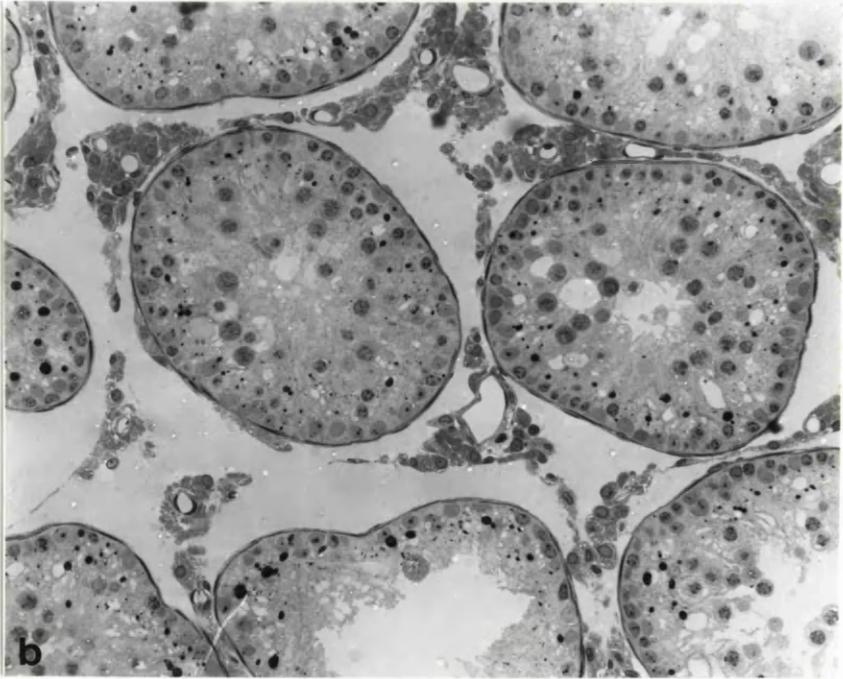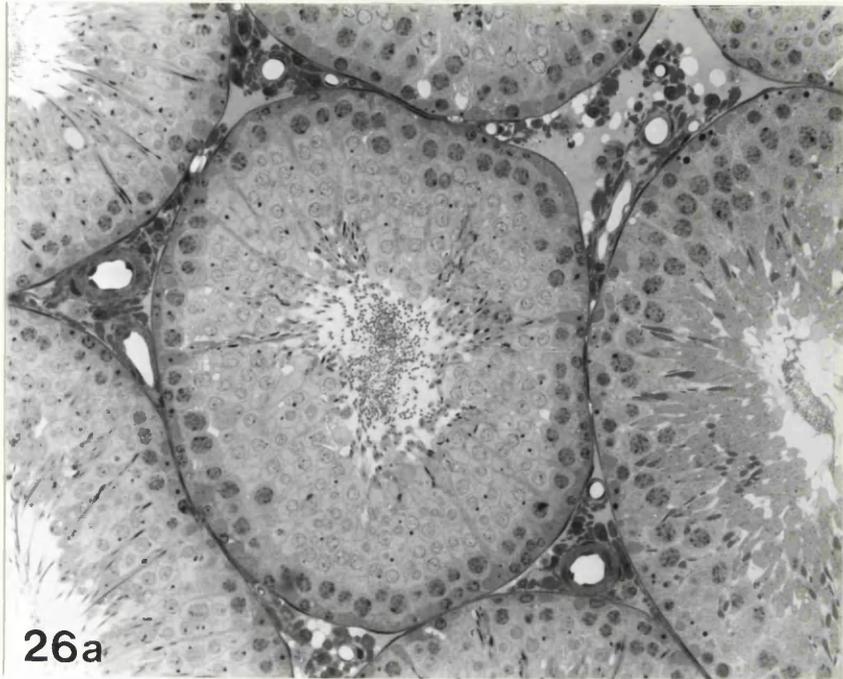
EXPERIMENT 2

The development and regression of the gubernaculum in rat fetuses and neonates.

Results from Experiment 1 showed that prenatal (but not postnatal) exposure to the non-steroidal anti-androgen flutamide affected testis descent in male Albino Swiss rats, with testes occupying a variety of positions in adulthood (intra-abdominal, ectopic supra-inguinal, scrotal). Because the gubernaculum has often been considered an important structure in testis descent, Experiment 2 was undertaken to examine the effects of flutamide on gubernacular development and subsequent regression.

Animals:

A total of 40 experimental and 40 control male fetuses and neonates were used for this experiment. The experimental males were born to mothers which received injections of the non-steroidal anti-androgen flutamide (10mg/day in 0.1 ml propylene glycol from E10 to birth) while control pregnant females received 0.1 ml propylene glycol vehicle only. Day 10 post-coitum was chosen to start treatment to ensure exposure of the fetuses to flutamide before the period of testis-dependent male sexual differentiation which begins after day 13 of gestation in the rat **(Magre and Jost, 1980).**

Five control and experimental males were examined at each of the following ages: i) 16 days of gestation, ii) 18 days of gestation, iii) 20 days of

gestation, iv) the day of birth, v) 2 days post-partum, vi) 4 days post-partum, vii)

8 days post-partum, and viii) 12 days post-partum.

Methods:

Pregnant females were killed by an overdose of carbon dioxide at 2-day

intervals from day 16 to day 20 of gestation. The fetuses were removed,

decapitated and the lower half of the body was immersed in fixative. Animals

intended for paraffin sectioning were immersed in 10% buffered formalin fixitive

for 3 days, while animals intended for scanning and transmission electron

microscopy were immersed in a mixture of 3% glutaraldehyde and 1%

paraformaldehyde in 0.1M phosphate buffer for 3 days (**Appendix 1**).

Neonates were killed by a lethal i.p. dose of pentobarbitone sodium B.P.

VET (Sagatal 60mg/ml) and subsequently perfusion-fixed using the appropriate

fixative (see above) by transcardiac infusion. The lower half of the body was

again immersed in the same fixative overnight before further processing when

these animals were initially rinsed in 0.1M phosphate buffer for 2 hours before

being dissected.

Animals intended for LM serial sectioning were subsequently immersed in

10% buffered EDTA for 1-2 weeks (changing the EDTA daily) to decalcify any

bony parts. The specimens were then gradually dehydrated in a Reichert-Jung

Histokinette 2000 automatic tissue processor (**Appendix 5**), and subsequently

vacuum-embedded in paraffin wax and sectioned serially at 6 μm. The sections

were mounted as a 1 in 5 series on glass slides (i.e. 30 μm intervals between two adjacent sections) and stained with Hematoxylin and Eosin (**Appendix 6**). Light micrographs were taken from representative areas. Measurement of the gubernaculum was carried out on the corresponding sections using a Wild microscope with appropriate attachments.

The animals for scanning electron microscopy were processed accordingly (**Appendix 7**). Specimens were viewed using a Jeol JSM-T300 scanning electron microscope and micrographs were taken from representative areas.

RESULTS

Development of the gubernaculum in the male rat fetus

At day 16 post-coitum

At 16 day post-coitum (d.p.c.), the male fetus possessed elongated testes located antero-lateral to the kidney **(Figure 27).** Superiorly, the testis and Wolffian duct were attached to the posterior abdominal wall behind (i.e. cranio-lateral to) the kidney by means of the cranial suspensory ligament. Inferiorly, the testis and epididymis were attached to the inguinal region of the anterior abdominal wall by means of the gubernaculum. There was no obvious difference between the gubernacula of flutamide-treated males and controls at this stage of fetal development. The gubernaculum appeared as a long slender structure (the cord) with a small distal swelling (the cone) which was not as prominent as in later stages of development **(Figure 28)**. The gubernaculum (both the cone and cord) was composed largely of mesenchymal cells and numerous fibroblast-like

cells. There were no myoblasts in either the control or the flutamide-treated gubernaculum at this stage.

The testis contained a few testicular cords, each surrounded by a distinct basement membrane. The testicular cords contained two types of cells: the large cells (i.e. the gonocytes) possessed a deep-staining eosinophilic cytoplasm (from H&E stained sections), and the smaller pale staining cells were the supporting or pre-Sertoli cells.

At day 18 - 20 post-coitum

At 18 d.p.c., the gubernacular cone had become more prominent and the gubernacular cord had elongated **(Figure 28)**. However, by 20 d.p.c. **(Figure 29)**, while the gubernacular cone became increasingly prominent, the length of the gubernacular cord remained relatively constant in both control and flutamide-treated males. With the continued growth of the body, the testes thus appear to approach the apex of the gubernacular cone about half-way between the caudal pole of the ipsilateral kidney and the inguinal ring in the control animals. The gubernaculum had become more cellular and distinct layers of myoblasts were clearly seen. These cells proliferated at the periphery of the gubernaculum while the core still had the loose organization of mesenchymal cells **(Figure 30a-d)**. These mesenchymal cells were oriented parallel to the long axis of the gubernaculum.

By 20 d.p.c., the testis became more ovoid in shape and contained increased numbers of testicular cords. There was a variable degree of inhibition of Wolffian duct development in flutamide-treated males.

At birth and in the neonatal period

Control male and female rats were easily distinguishable at birth by the difference in the ano-genital distance. Normal males did not have nipples at birth while normal females had 2-3 pairs. Both male and female offspring of flutamide-treated mothers appeared identical to normal females; they had feminized genitalia, their ano-genital distance was similar to normal females (i.e. circa 1 mm) and they had 2-3 pairs of nipples.

The testes of the normal male neonates were located in the lower abdomen, held close to the neck of the bladder by the gubernaculum. The gubernacular cones started to undergo marked structural changes at birth: a) the myoblasts differentiated resulting in distinct layers of muscle cells surrounding a mesenchymal core, b) cavitations were formed within the mesenchymal core and base of the gubernacular cone, and c) formation of the tunica vaginalis began **(Figure 31a)**. Examination of semithin sections showed that the testicular cords in flutamide-treated males appeared similar to those of the controls. Within the testicular cords, the supporting cells were located peripherally adjacent to the basement membrane while the primordial germ cells with large spherical nuclei were located centrally. The limiting membrane was composed of distinct layers of

elongated cells (presumably the precursors of peritubular myoid cells and endothelial cells) surrounding each cord **(Figure 31b).**

By post-natal day two, the loose mesenchymatous core of the gubernaculum had become the mesenchymatous outer layer to the everting (cremaster) muscle layers. It was separated from the remaining connective tissue of the scrotum by tissue spaces (the cavitations formed earlier at the begining of gubernacular eversion). Gubernacular eversion (and progressive descent of the testes) continued in the control and in most of the flutamide-treated males. In the control males and some flutamide-treated males, eversion was completed by day 4 post-partum **(Figure 32a-b)**, while in some of the flutamide-treated males eversion was not completed until day 8 post-partum. The organization of cells in the testicular cords at day 4 post-partum appeared similar in both groups. While some of the primordial germ cells remained unchanged, others showed changes suggestive of degeneration; their nuclei appeared swollen and poorly stained, and the nuclear membranes were wrinkled.

While the gubernacular cone was everting, the gubernacular cord in some flutamide-treated males had become excessively long, stretching from the caudal end of the testis to the base of the cremaster sac **(Figure 33)**. By day 12 post-partum, eversion of the gubernaculum was completed in all animals of both groups. Testis descent was accompanied by descent of the epididymis which, in control males, was located within the inguinal canal attached to the scrotal floor by a short gubernacular cord while the testis itself was located close to the

inguinal ring. Thus, the epididymis reaches the scrotum in advance of the testis. At this stage, the testicular cords contained both supporting cells and spermatogonia (Type A and Type B), and some tubules contained resting spermatocytes. There was little change in the appearance of the supporting cells.

Length of gubernacular cone and cord in control and flutamide-treated males

In the early stages of gubernacular development, the gubernacular cones of flutamide-exposed fetuses appeared indistinguishable from those of the controls. As development proceeds (i.e. up to 20 d.p.c.), the gubernacular cones in both control and flutamide-treated males became bigger and longer. While the length of the gubernacular cord in control males remained relatively constant after 18 d.p.c. in both groups, the gubernacular cords in some of the flutamide-treated males were significantly longer than those of controls. In particular, the gubernacular cords in some of the flutamide-treated males became excessively long structures connecting testes which remained in the abdomen to the base of the scrotal (cremaster) sac **(Figure 33)**.

The length of the gubernacular cones and cords of the flutamide-exposed males were measured and compared to those of controls. Measurement of the cord was made from its epididymal attachment to the apex of the cone while the cone was measured from the apex to its base. The base corresponds to the anterior abdominal wall in pre-natal males and the lowest point of the tunica

vaginalis in post-natal males, i.e. when eversion had started. It was found that the mean length of the gubernacular cone in flutamide-treated males was not different from that of controls at least up to the day of birth. However, the eversion process in flutamide-treated males was delayed; eversion was completed by post-natal day 4 in control males, but not until post-natal day 8 in the flutamide-treated males.

The mean length of the gubernacular cord increased slightly in both groups between 16 - 20 d.p.c. and remained relatively constant thereafter until the day of birth. The mean cord length prior to birth was similar in both the flutamide-treated and control males. However after birth, while the mean cord length of control males remained constant, the cord of flutamide-treated males became longer at post-natal day 2 and remained constant thereafter at least up to day 8 post-partum. However, the cords in both groups appeared to become longer at day 12 post-partum **(Figure 34a-c).** While there was no difference between the mean cord length of both groups prior to birth, the mean cord length of flutamide-treated males became significantly longer than that of controls after post-natal day 2 (t = -2.51, df = 11, p < 0.05).

FIGURE 27: Scanning electron micrograph of a fetal rat at 16 d.p.c. showing the testis (T), kidney (K), adrenal (A), Wolffian duct (W), cranial suspensory ligament (L), gubernacular cord (D), gubernacular cone (N) and urinary bladder (B).

Bar = 100 μm.

27
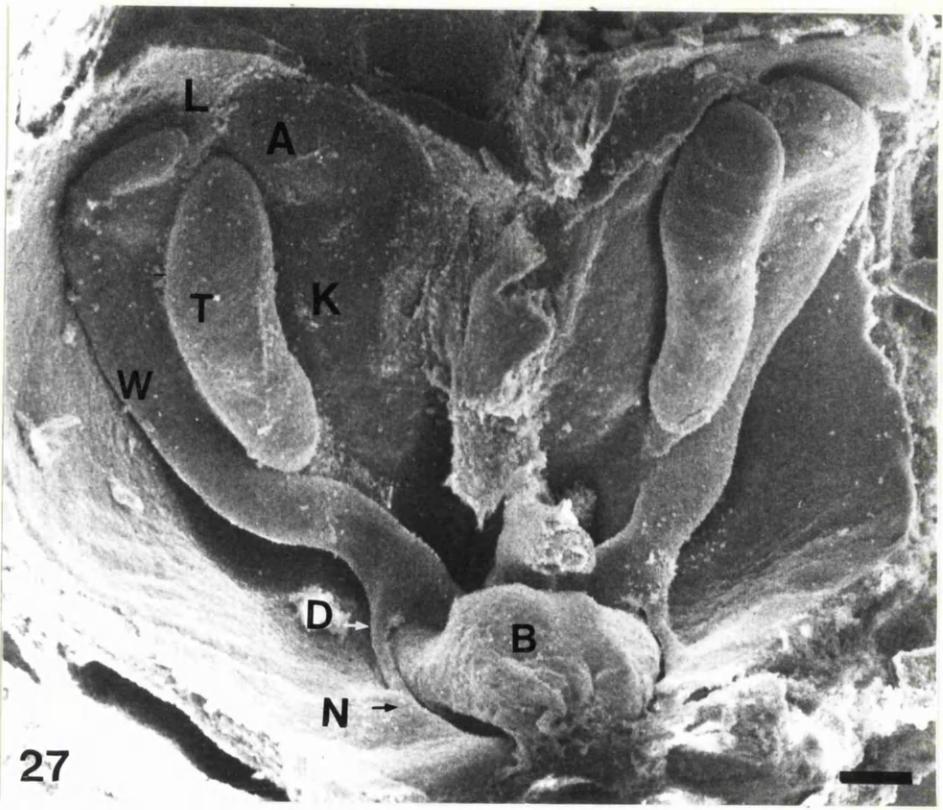
FIGURE 28: Scanning electron micrograph of a fetal rat at 18 d.p.c. showing the testis (T), kidney (K), cranial suspensory ligament (L), gubernacular cord (D), gubernacular cone (N) and bladder neck (B). Bar = 100 μm.
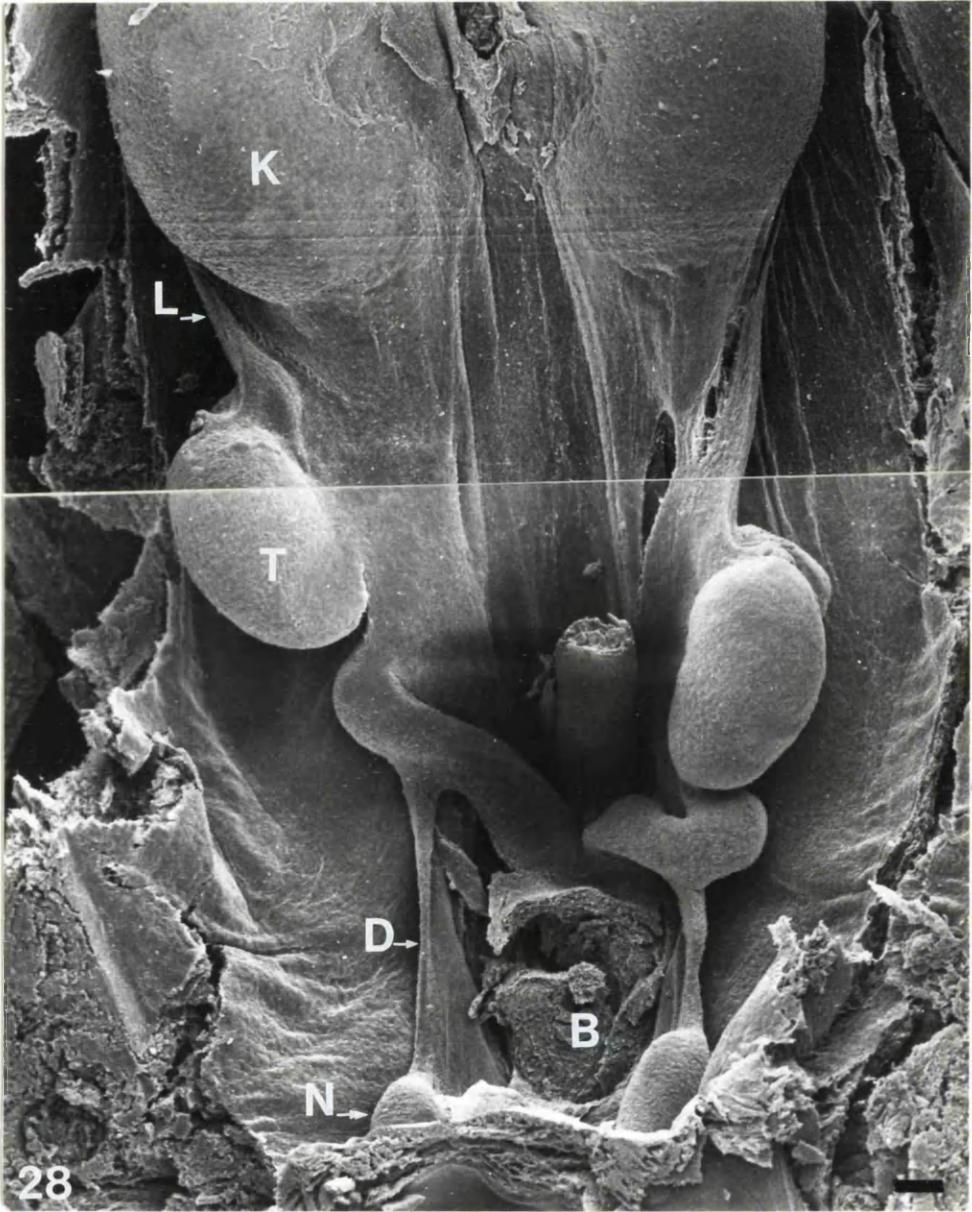
FIGURE 29: Scanning electron micrograph of a fetal rat at 20 d.p.c. showing the testis (T), epididymis (E), kidney (K), cranial suspensory ligament (L), gubernacular cord (D), gubernacular cone (N) and bladder neck (B).

Bar = 100 µm.

29
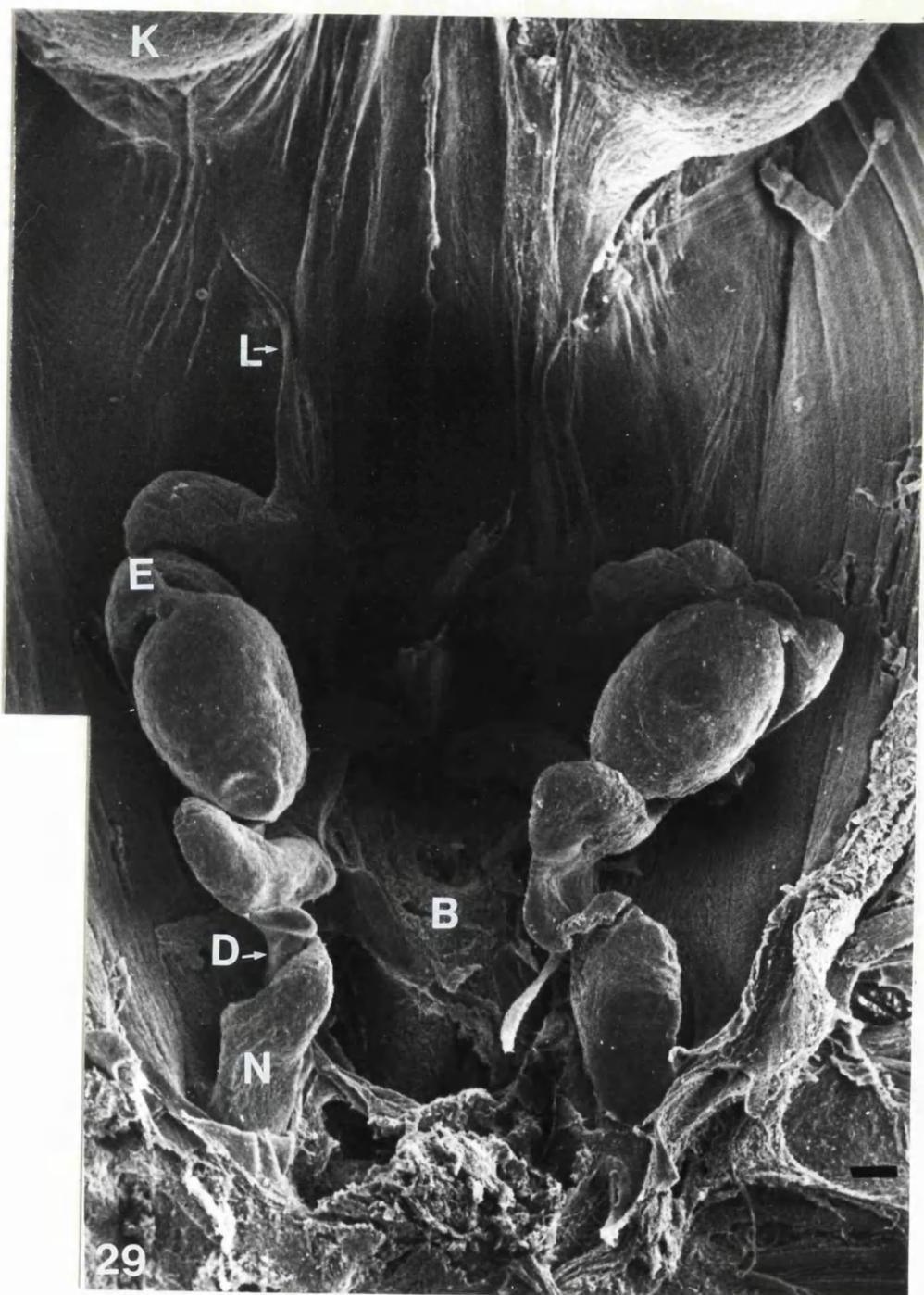
FIGURE 30: The gubernacular cone during the outgrowth phase:

(a) and (b) Light micrographs of gubernacular cone at 18 and 20 d.p.c. respectively, showing myoblasts (which are more developed in the latter stage) at the periphery of the cone.

Magnification: x 170

(c) and (d) Transmission electron micrographs of gubernacular cone at the corresponding stages as above. Sm, myofibrils; Mn, nucleus of myoblast.

Bar = 1 μm.

30a

b

c

d
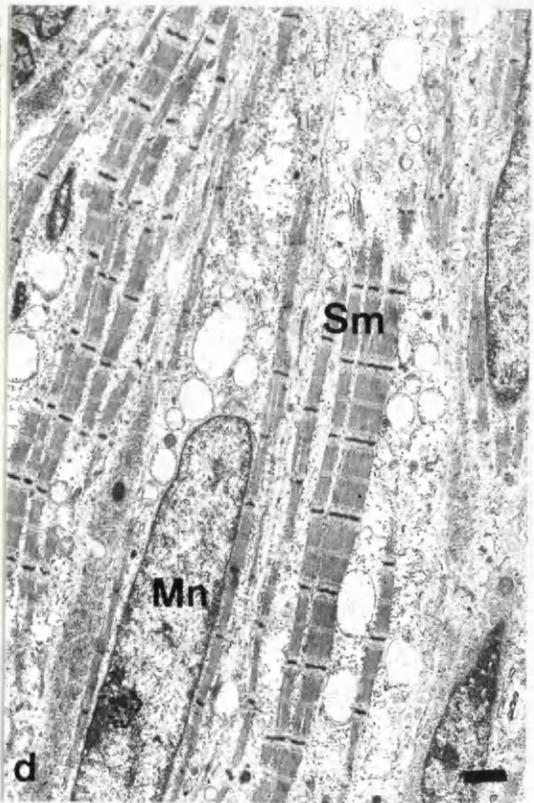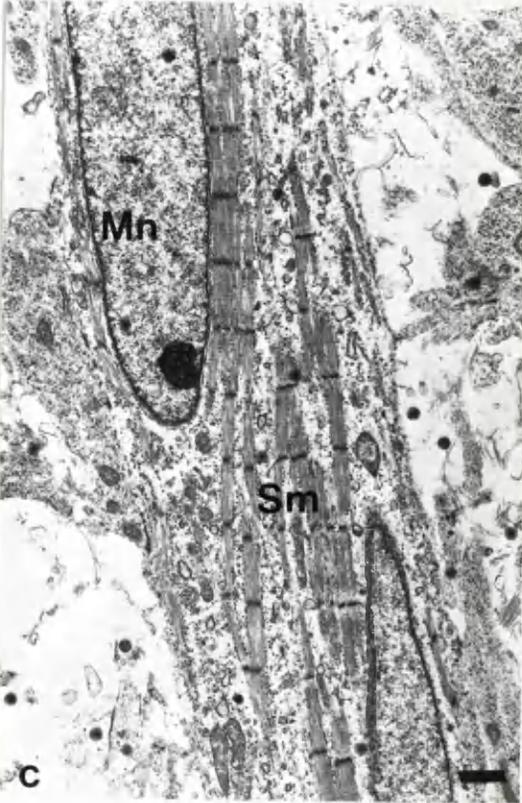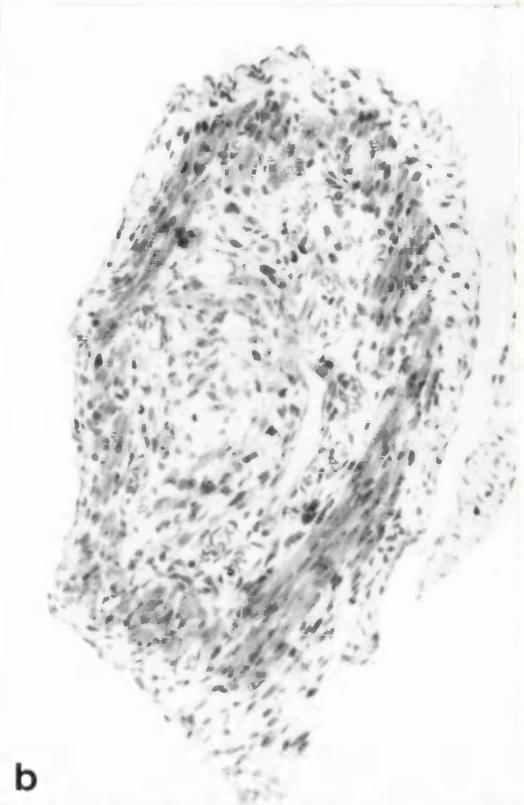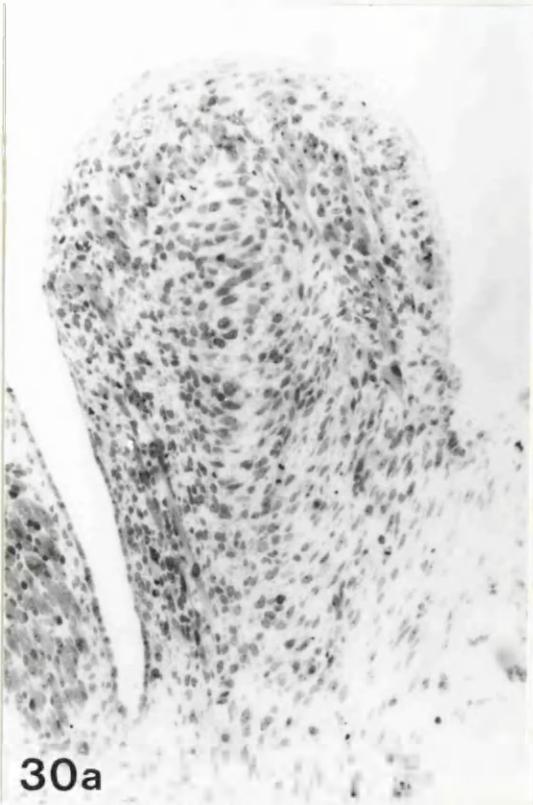
FIGURE 31: Light micrograph of a newborn Albino Swiss rat showing

(a) right gubernaculum cone (N) and cord (D), testis (T) and epididymis (E) at the beginning of testicular descent. Note that the testis is located close to the bladder neck (B); Arrows indicate 'cavitations' in the mesenchymal core of gubernaculum. Magnification: x 36

(b) testicular cords with primordial germ cells (G) and pre-Sertoli cells (S). Mitotic figures (M) are commonly seen. Magnification: x 580

31a

b
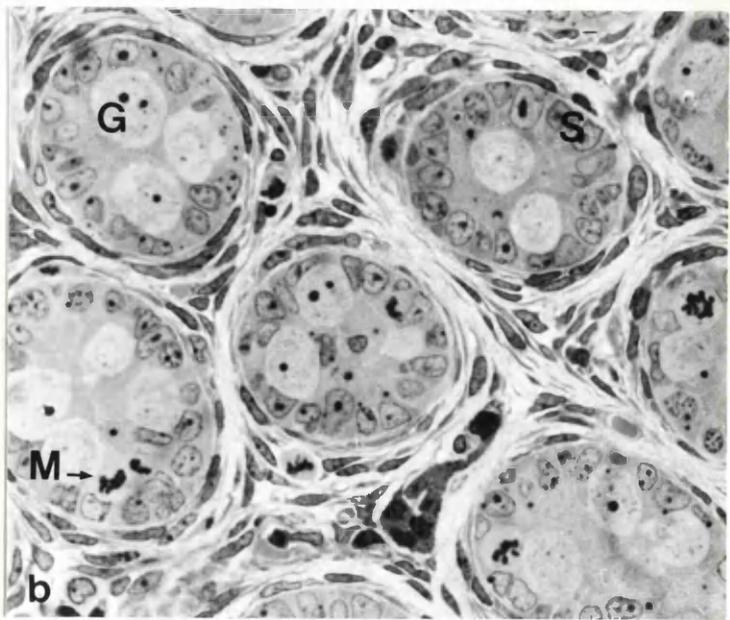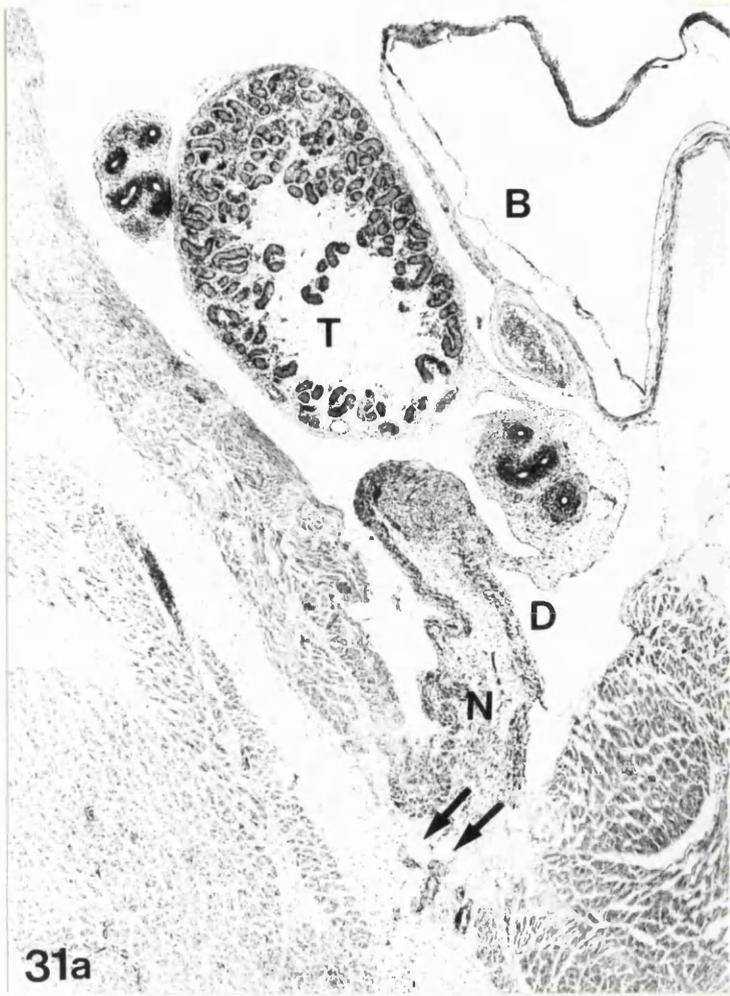
FIGURE 32: Light micrographs of the gubernaculum of 4 day-old

male Albino Swiss rats showing

(a) control male in which the gubernaculum is

completely everted with a short gubernacular cord

(D) and descended epididymis (E), but testis (T) still

at the inguinal ring, and

(b) flutamide-treated male with a long gubernacular

cord and testis close to bladder neck (B).

Magnification: x 30

32a

b
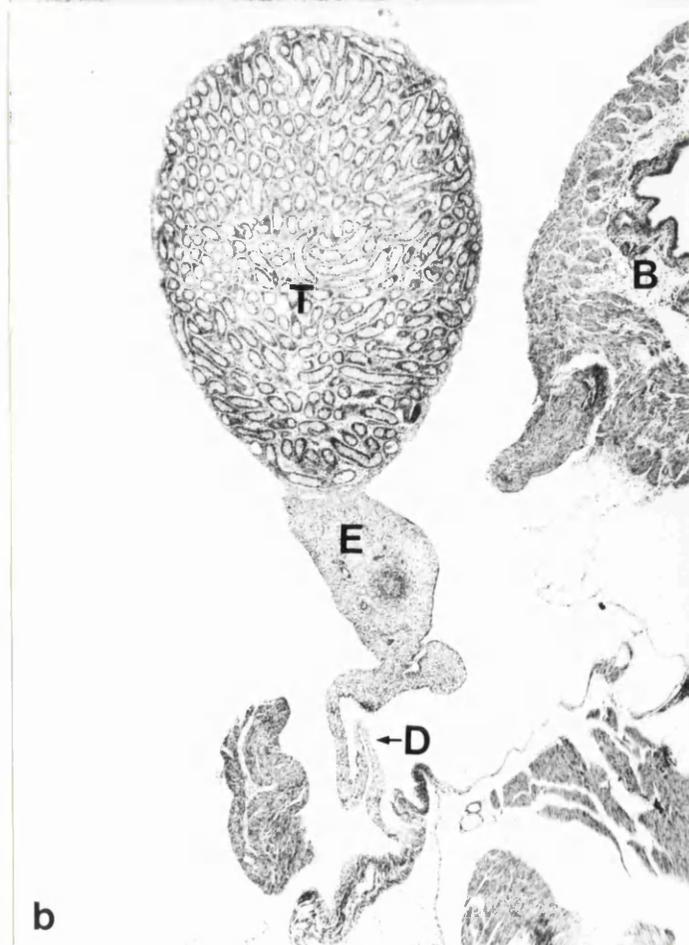
FIGURE 33: Scanning electron micrograph of an 8 day-old flutamide-treated male showing a long gubernacular cord (D) and testis (T) located close to the caudal pole of the kidney (K).

Bar = 200 μm.

K

T

D

33
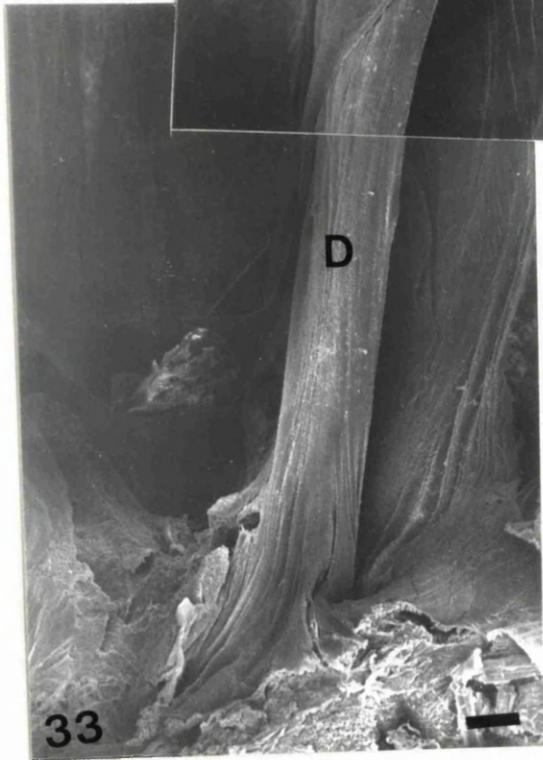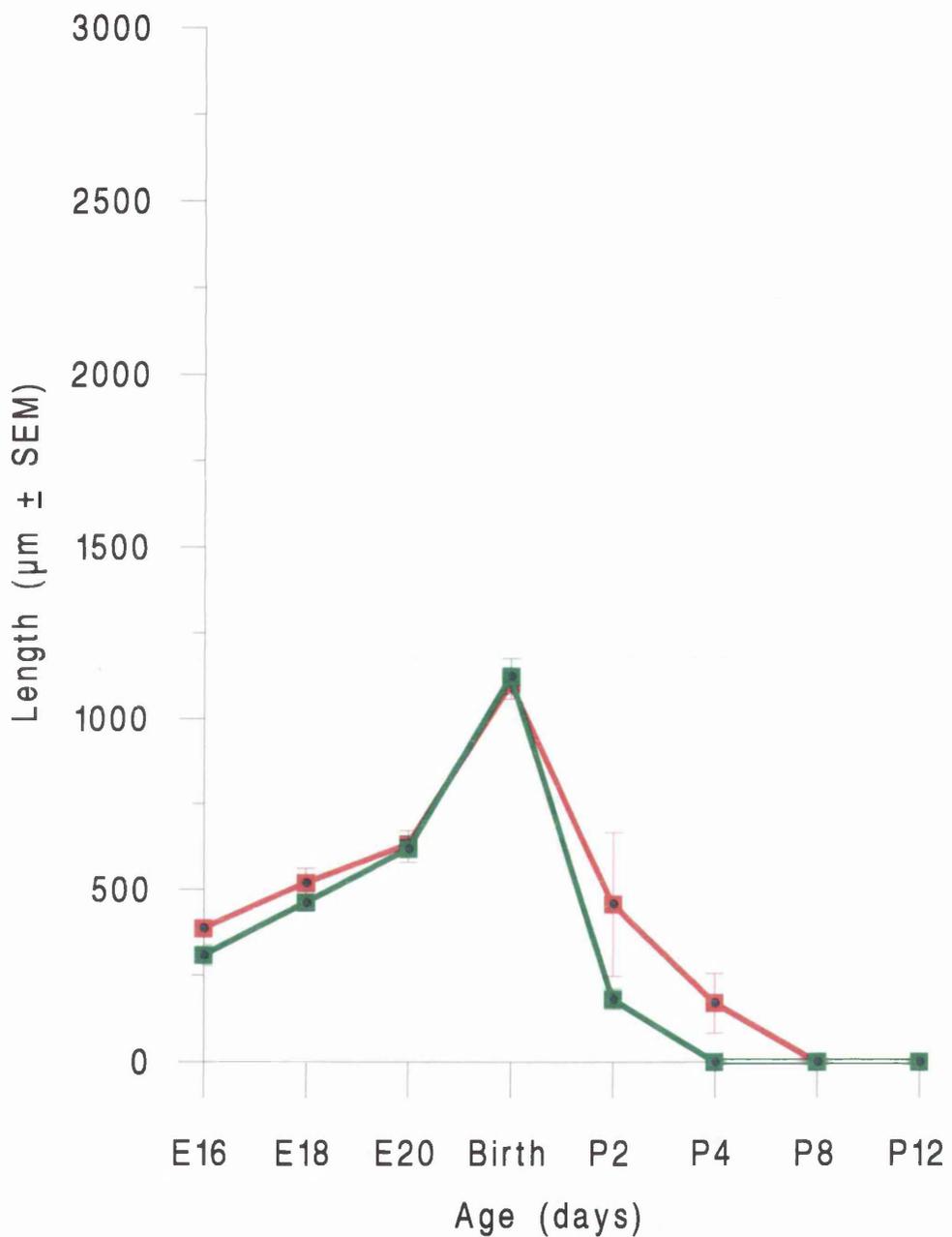
Figure 34a: Graph showing the length of the gubernacular cone of control male Albino Swiss rats (——■——) and males treated prenatally with flutamide (——■——) (mean length ± SEM)
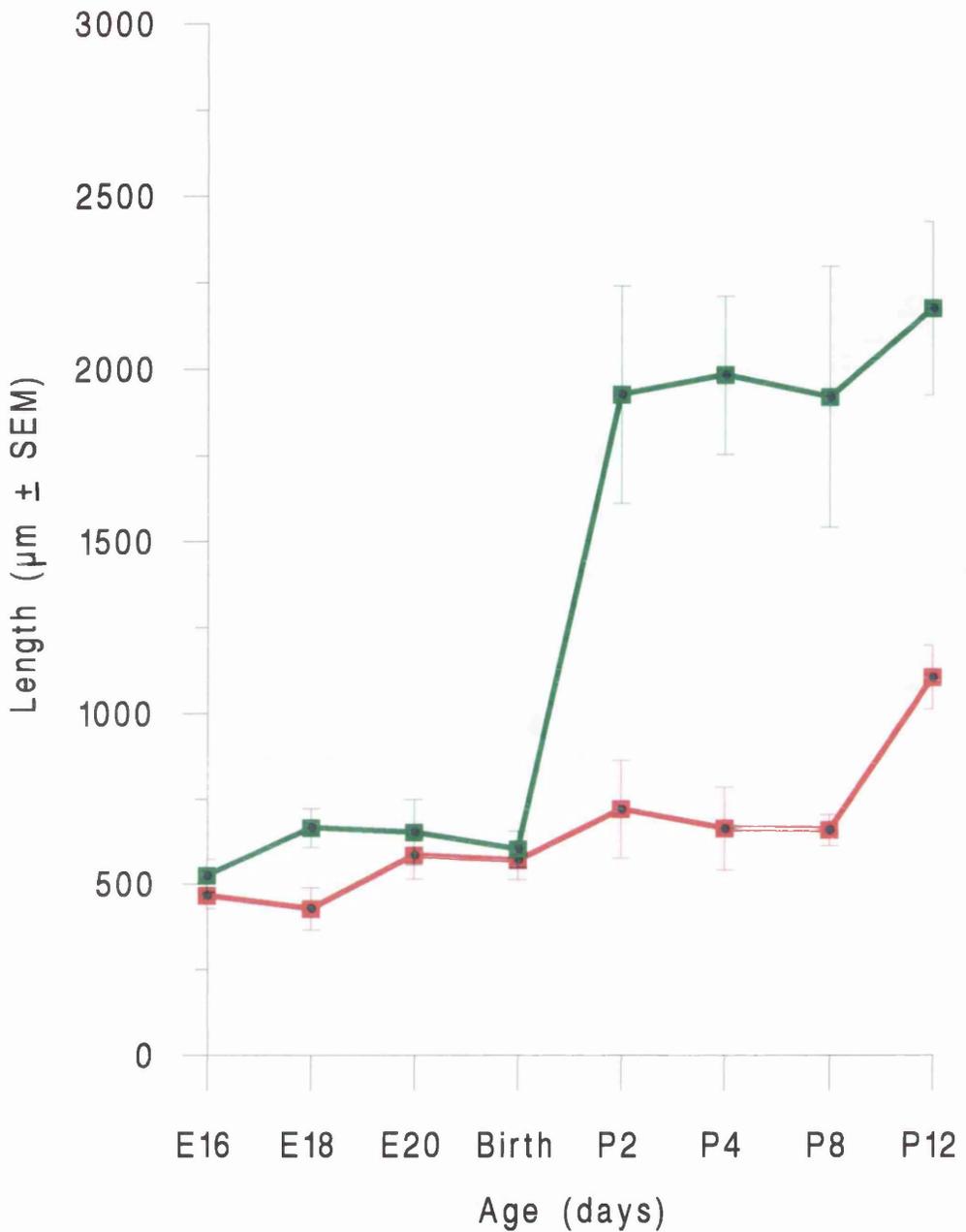
FIGURE 34b:Graph showing the length of the
gubernacular cord of control male Albino Swiss
rats (—■—) and males treated prenatally with
flutamide (—■—) (mean length ± SEM).
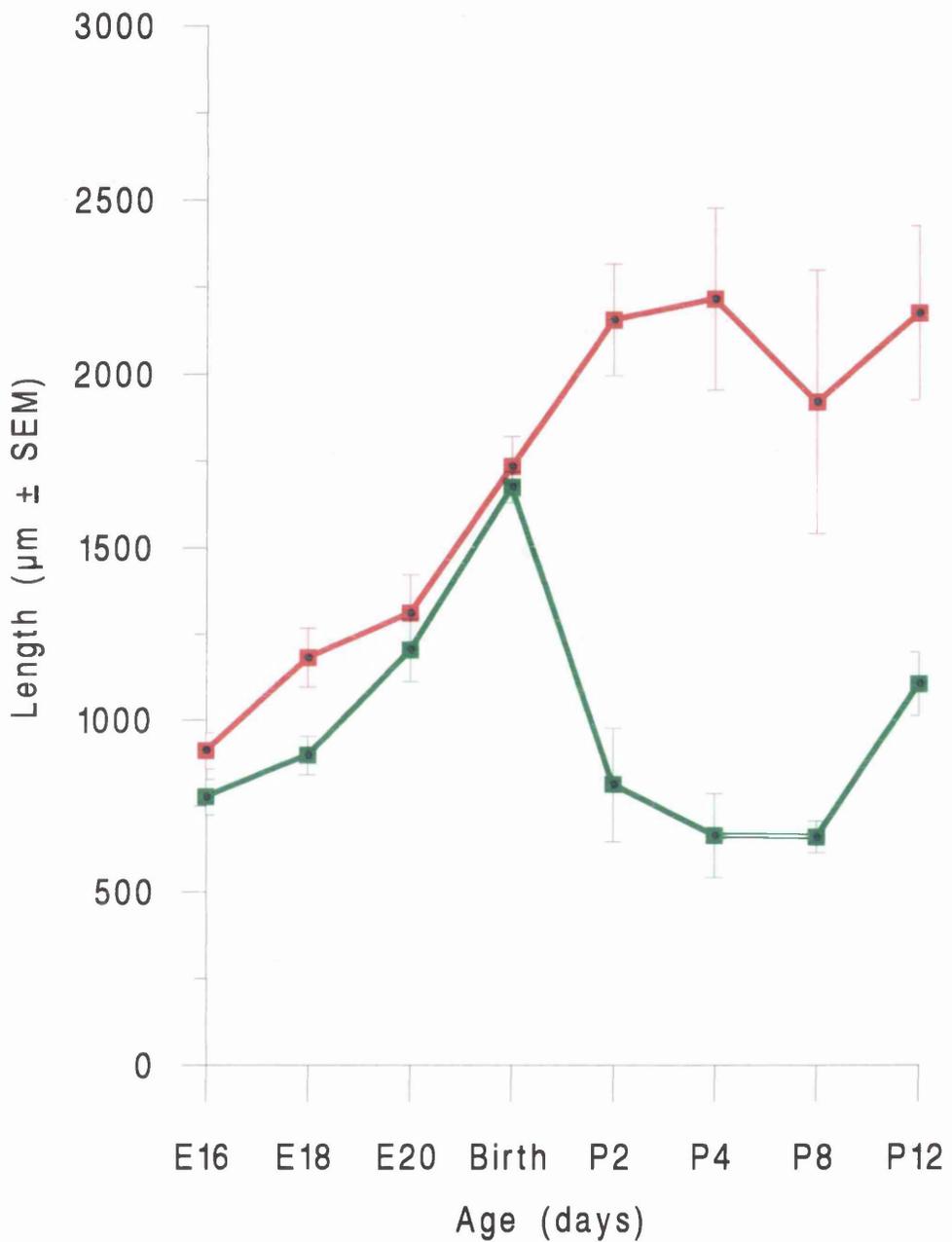
Figure 34c: Graph showing the length of the gubernaculum (cone + cord) of control male Albino Swiss rats (———) and males treated prenatally with flutamide (———) (mean length ± SEM)

EXPERIMENT 3

The effects of prenatal exposure to flutamide on the persistence of the cranial suspensory ligament

A cranial suspensory ligament (CSL) of the testis was found in all adult male rats and hamsters exposed prenatally to flutamide, but in none of the control males. This experiment aimed to study: a) the development of the CSL in the male rat and b) the composition of this ligament in fetal, neonatal and adult males.

Methods:

1)   Fetal and neonatal CSL were studied from the same animals used in Experiment 2 using serial sections and scanning electron microscopy.

2)   Specimens of CSL were also obtained from a variety of adult flutamide-treated males (with testes in the three different locations) fixed with a mixture of glutaraldehyde and paraformaldehyde and processed for general histology and electron microscopy.

RESULTS

In animals at 16 d.p.c., the CSL was a short, thick structure connecting the cranial part of testis to the diaphragm at the posterior abdominal wall **(Figure 35a).** As the testis moved caudally with growth of the body, the CSL became longer and thinner **(Figure 35b)**. From parasagittal sections of the fetus at 18 d.p.c. **(Figure 36a)**, the CSL at this stage was composed of mesenchymal cells, fibroblast-like cells (readily seen at higher magnification) and numerous blood vessels. By 20 d.p.c., the CSL in control males had become much thinner than those in flutamide-treated males **(Figure 36b-c)**, and disappeared thereafter. By contrast, the CSL in the flutamide-treated males persisted through to adult life.

All flutamide-treated adult males used in this study possessed CSL which were fine structures extending from the testes (no matter where the testes were located) to the posterior abdominal wall cranial to the kidney **(Figure 37a-b)**. The CSLs were located retroperitoneally in the dorsolateral fat pad of the posterior abdominal wall and contained smooth muscle fibres, fibroblasts, blood vessels and nerves **(Figure 38a-c** and **Figure 39a-c).** No difference could be found between the morphology of the CSL associated with testes in different adult locations.

FIGURE 35: Scanning electron micrographs of cranial suspensory ligaments (L) of (a) 16 d.p.c., and (b) 18 d.p.c. male Albino Swiss rats. K, kidney; A, adrenal; W, Wolffian duct; T, testis.

Bar = 100 μm.

35a

b
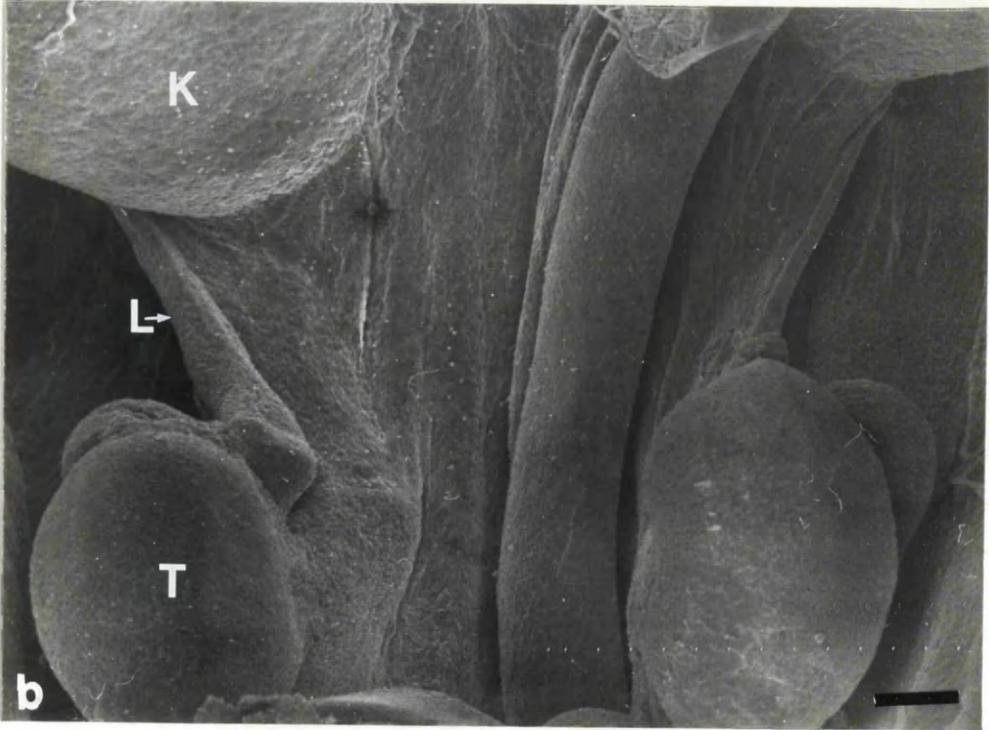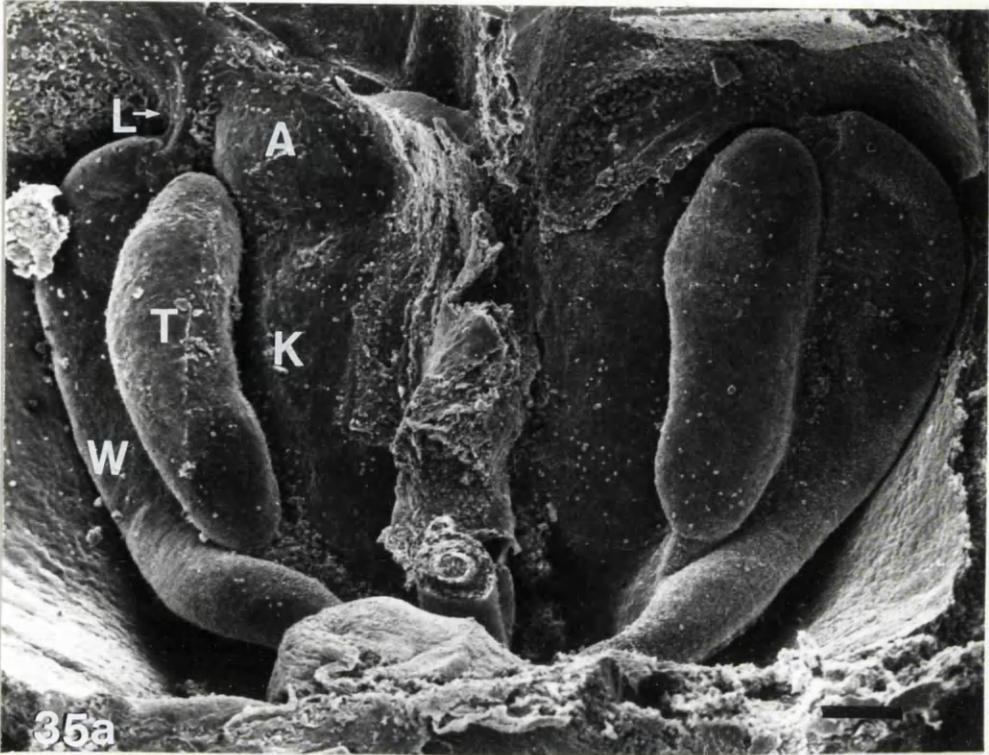
FIGURE 36: Cranial suspensory ligaments of male Albino Swiss

rats:

(a) light micrograph of a sagittal section of cranial

suspensory ligament of 18 d.p.c. male AS rats.

Cranial suspensory ligament (L), testis (T),

gubernacular cone (N).

Magnification: x 50

(b) and (c) Scanning electron micrographs of cranial

suspensory ligament of 20 d.p.c. control and

flutamide-treated males respectively. Kidney (K),

cranial suspensory ligament (L), epididymis (E).

Bar = 100 μm.

36a
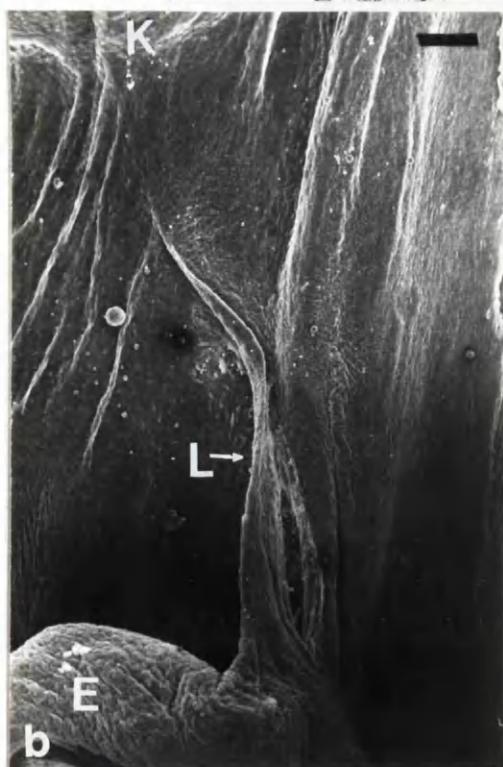
FIGURE 37: A dissection to show the cranial suspensory ligament (L) of (a) an adult male AS rat treated prenatally with flutamide.

K - kidney; T - testis, B - urinary bladder.

Magnification: x 0.9

37a
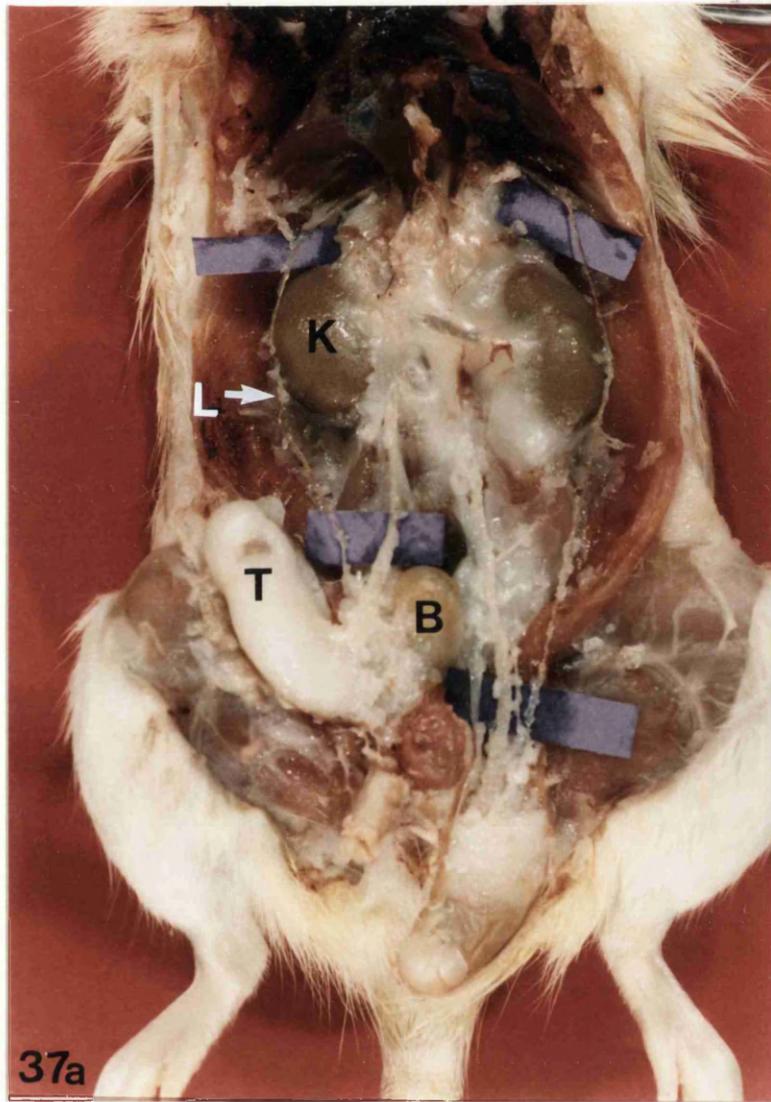
FIGURE 37: <u>continued</u>:

(b) an adult golden hamster treated prenatally with flutamide. Magnification: x 1.4

K - kidney; T - testis, B - urinary bladder, L - cranial suspensory ligament.
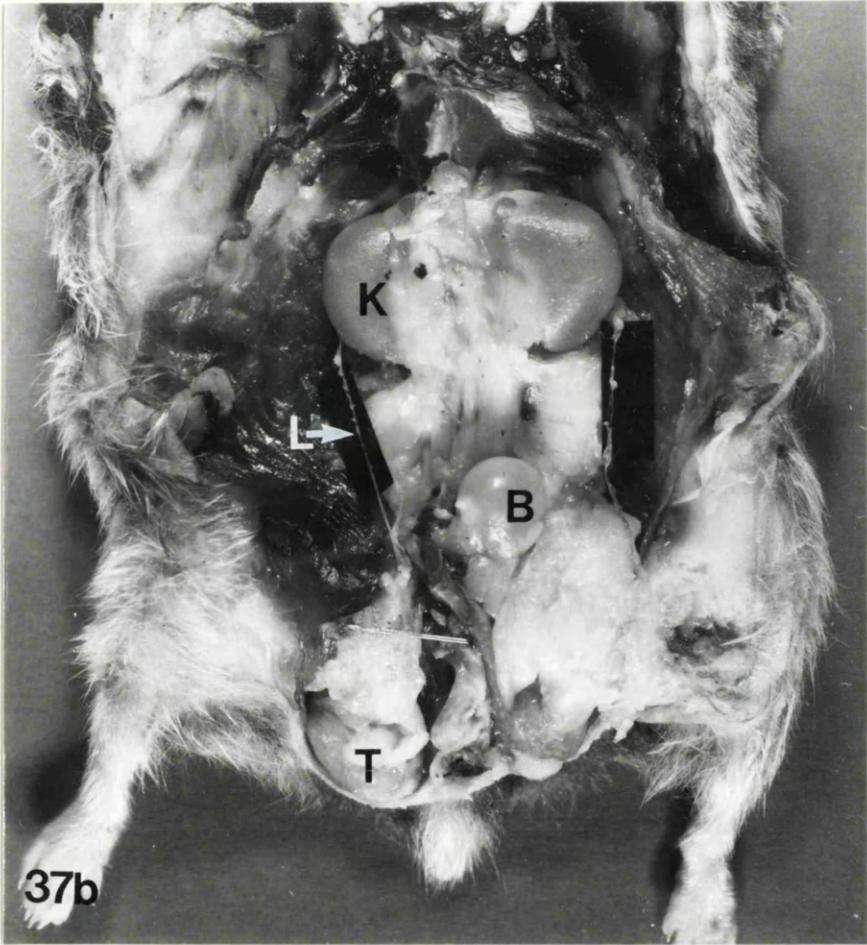
37b

FIGURE 38: Light micrographs of sections of the cranial suspensory ligaments from adult male Albino swiss rats:

(a) the cranial part,

(b) the middle part, and

(c) the caudal part near the testis.

L - abdominal cavity, V - blood vessels.

Magnification: x 160

38a

L

V

•

L

V

b

L

V

c
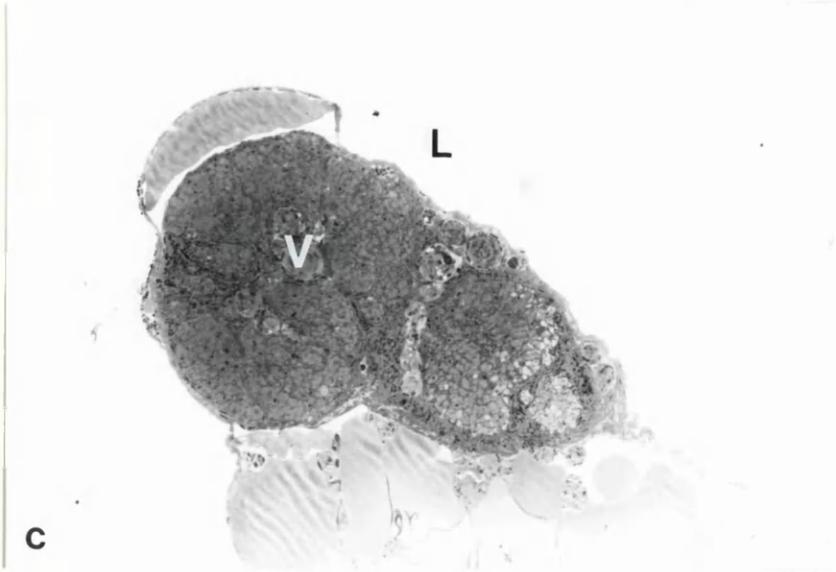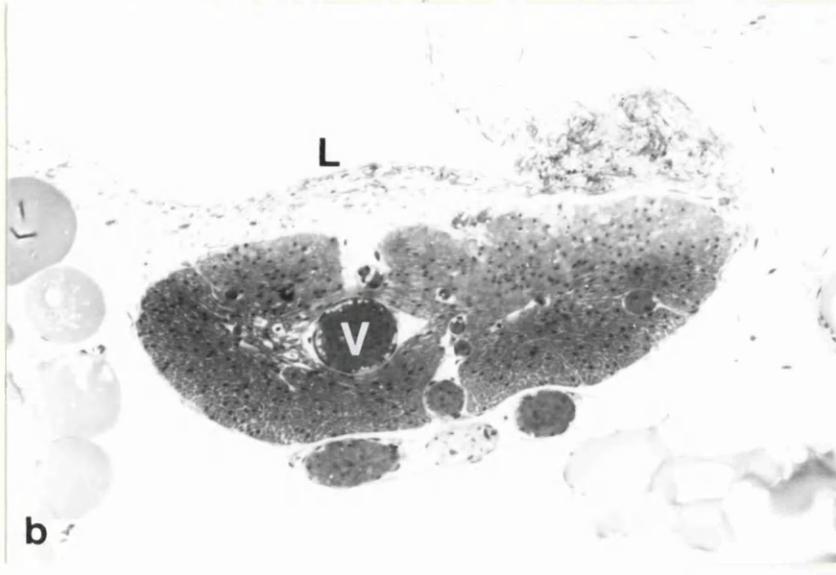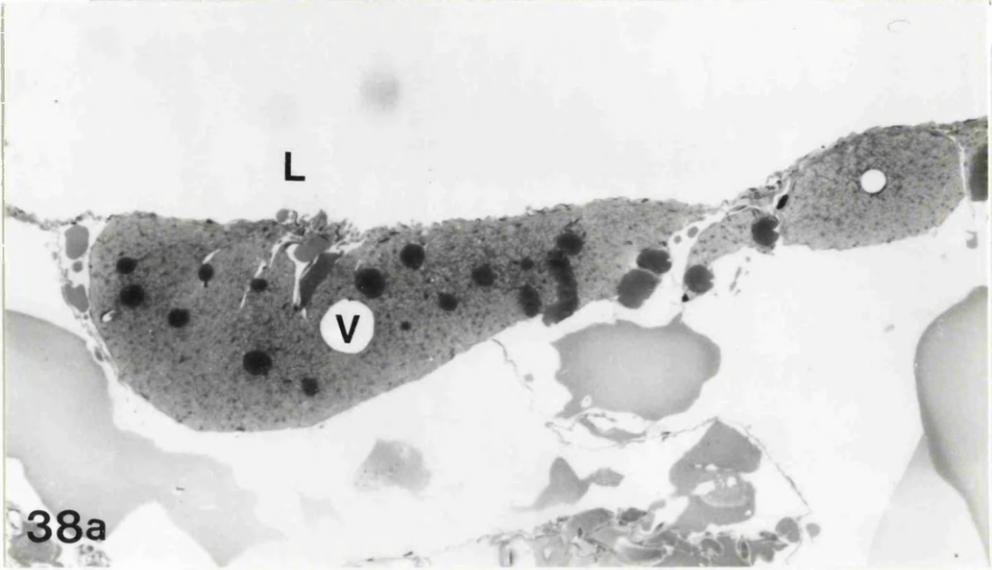
FIGURE 39: Transmission electron micrographs of cranial

suspensory ligaments:

(a) showing 'septation' in the cranial suspensory

ligament. Bar = 1 µm.

(b) showing smooth muscle. Bar = 0.5 µm.

(c) showing microtubules. Bar = 0.1 µm.

Sm, smooth muscle; N, nucleus of smooth muscle;

M, mitochondria; Arrows indicate microtubules.
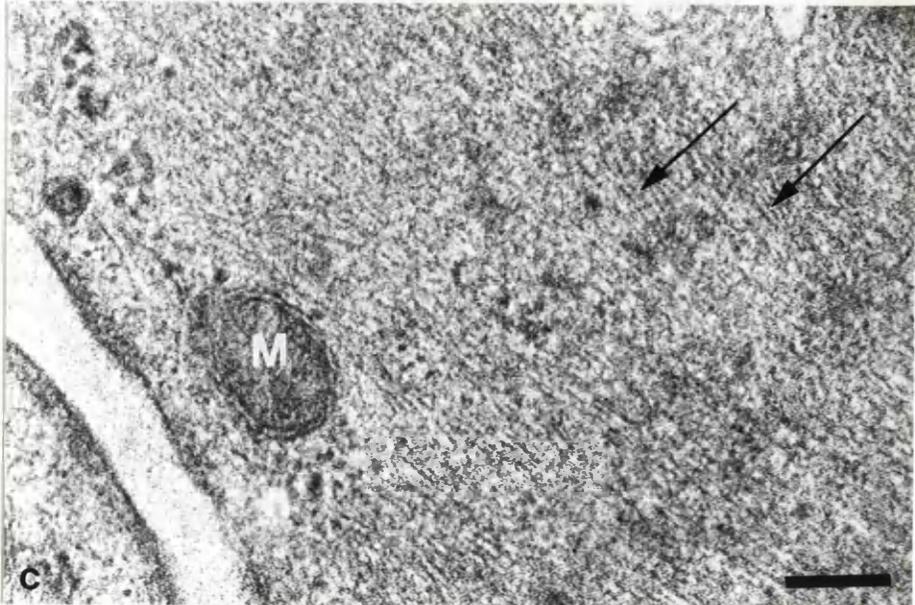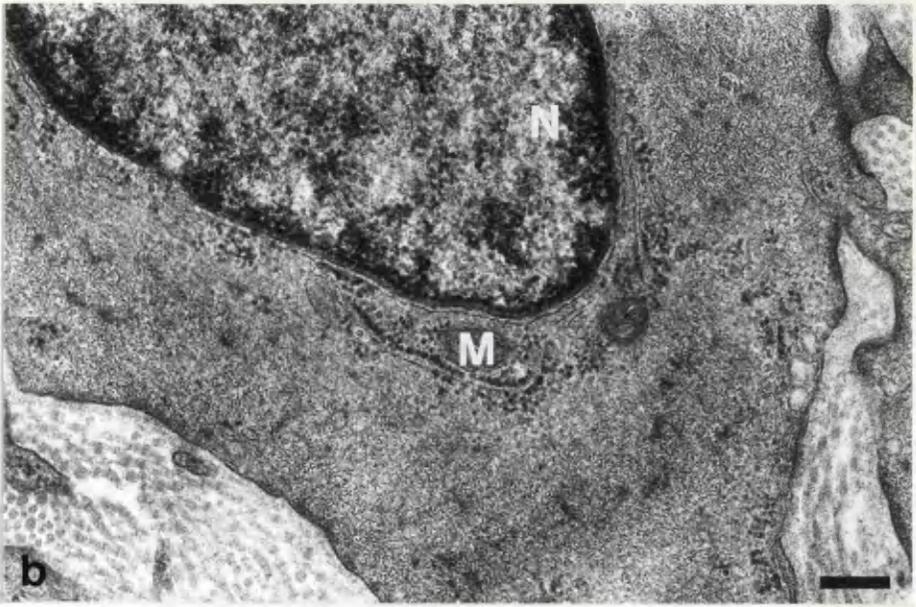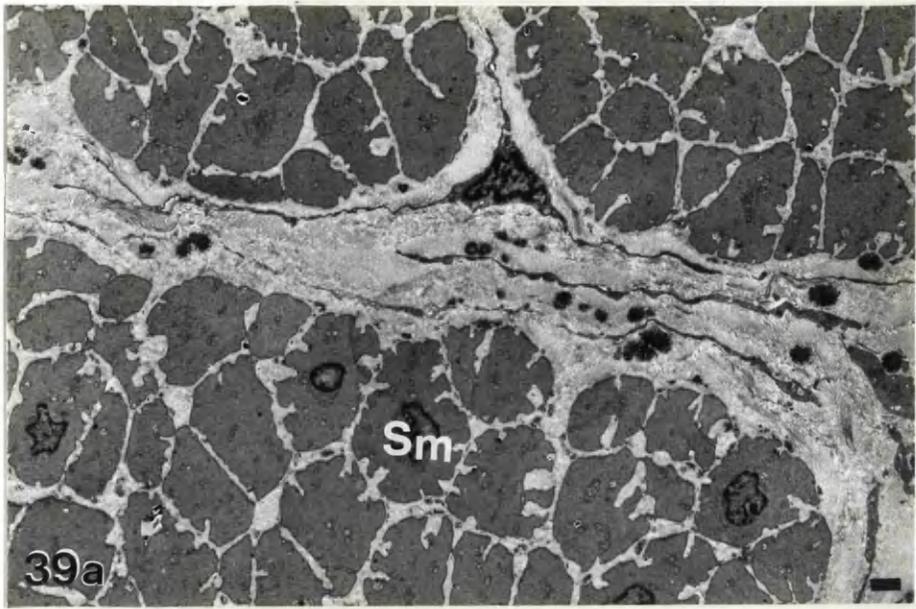
EXPERIMENT 4

Immunostaining for androgen receptors

The aim of this study was to confirm that androgen receptor is present in

a) the gubernaculum of fetal and neonatal rats,

b) the cranial suspensory ligament of fetal and adult rats,

c) the testes of fetal and adult rats.

Methods

i)   Fetal and neonatal specimens were obtained from the serial sections of

Experiment 2 (see **p. 74**).

ii)   Adult specimens were obtained by perfusion of adult males with 10%

buffered formalin after flushing out the circulation with Mammalian ringer

containing the vasodilator 0.2 % xylocaine. The animals were dissected and

samples were taken and immersed in the same fixative overnight and rinsed in

buffer (x 3), before dehydrating in a Reichert-Jung Histokinette 2000 (see

Appendix 5). The specimens were embedded in paraffin wax and sectioned at

6 µm.

<u>**Staining for androgen receptor**</u>

Immunostaining for androgen receptors was carried out according to the methods devised in the ICC laboratory in the Laboratory of Human Anatomy, Glasgow University. The primary antibody used in this study was a polyclonal androgen receptor antibody raised in rabbit (NCL-AR70 from NovoCastra) and was diluted at 1:30 with the antibody diluent 1% NGS (normal goat serum) in 0.3% Triton **(Appendix 8).**

Briefly, the paraffin sections were deparaffinised, hydrated to water and washed in 3 x 5 minute changes with 0.1 M phosphate buffer saline (PBS). The sections were then incubated in 1% NGS for 1 hour at room temperature prior to incubation in the primary antibody for 24 hours. The control sections (i.e. adjacent sections) were incubated in 1% NGS for the same duration. Subsequently, the excess 1° antibody was washed off, followed by incubation in horse anti-rabbit IgG coupled to biotin (2° antibody) for 1 hour. After the excess 2° antibody was washed off, the sections were incubated in avidin-biotin horseradish-peroxidase complex (ABC complex) for 1 hour. The horseradish peroxidase was revealed by the diaminobenzidine (DAB) reaction.

<u>RESULTS</u>

Staining sections with this polyclonal androgen receptor antibody resulted in dark brown staining (positive reaction) and light golden brown background

staining. The sections from the controls which were not incubated in the primary antibody were stained light golden brown.

Gubernaculum, testes and epididymis

Staining of androgen receptor was demonstrated in the nuclei of the mesenchymal cells of the gubernaculum (both cone and cord) **(Figure 40a-b),** testicular cords and in the epididymal epithelium in both control and flutamide-treated fetuses and neonates. In adult testes, staining was demonstrated in the seminiferous tubule epithelium and in some cells of the interstitium. The positive staining in the tubular epithelium was limited to the acrosome (i.e. the head cap on the nuclei) of round and elongating spermatids and the heads of the maturing spermatids **(Figure 41a-d)**.

Cranial suspensory ligament

While the fetal cranial suspensory ligament (at 18 d.p.c.) showed positive staining for androgen receptor **(Figure 42a-b)**, the cranial suspensory ligament of adult flutamide-treated males showed negative staining.

FIGURE 40: Androgen receptor staining of the gubernaculum of

20 d.p.c. male Albino Swiss rats

(a) positive androgen receptor staining of the nuclei

of cells (arrows) in the mesenchymal core of the

gubernaculum, and

(b) negative staining of an adjacent section incubated

in the absence of primary antibody.

Magnification: all x 120

40a

b
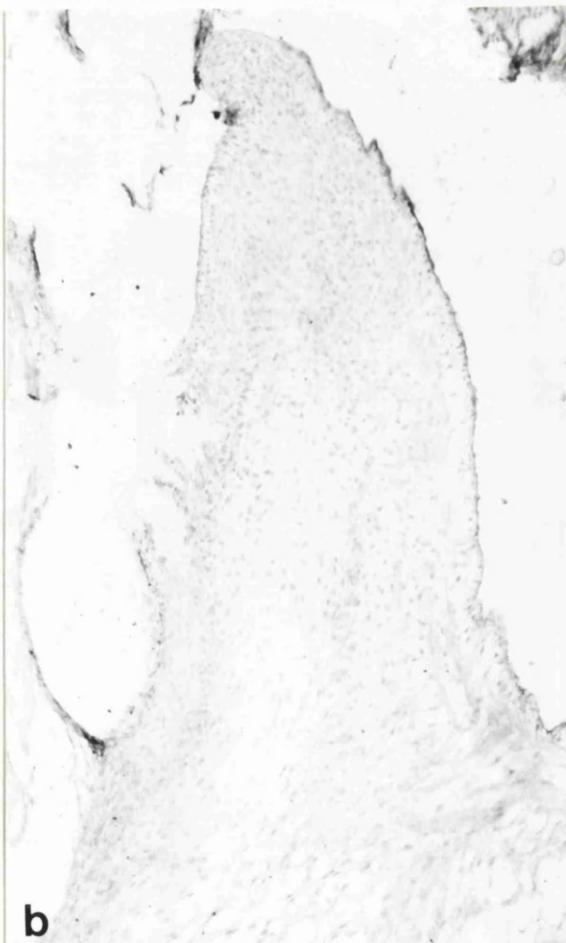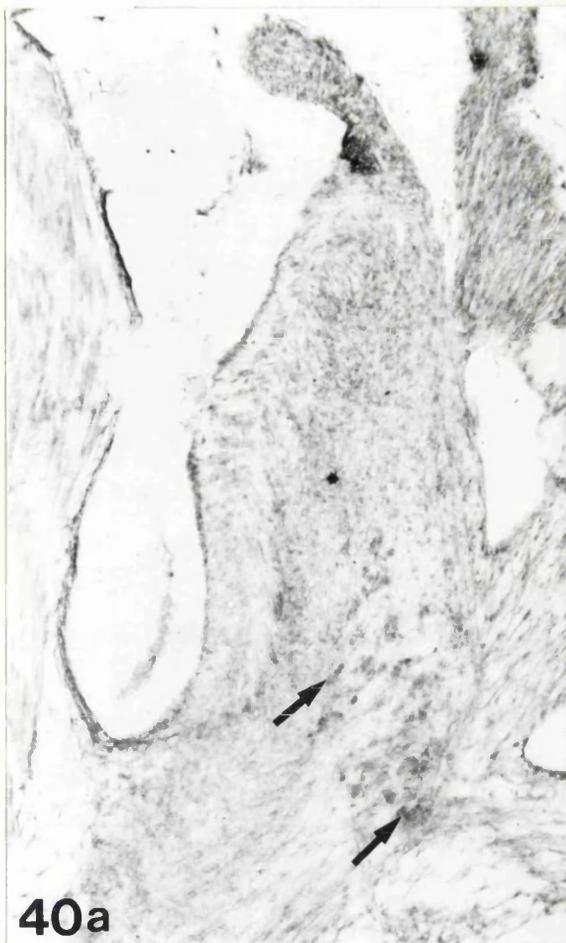
FIGURE 41: Androgen receptor staining of testes in

(a) a control adult Albino Swiss rat, and (c) a male

treated prenatally with flutamide.

Note positive staining of the acrosomal cap of round

spermatids (arrows) and heads of maturing

spermatids (arrowheads) and some cells in the

interstitium. Sections incubated in the absence of the

primary antibody are also shown - (b) and (d).

Magnification: all x 200

41a
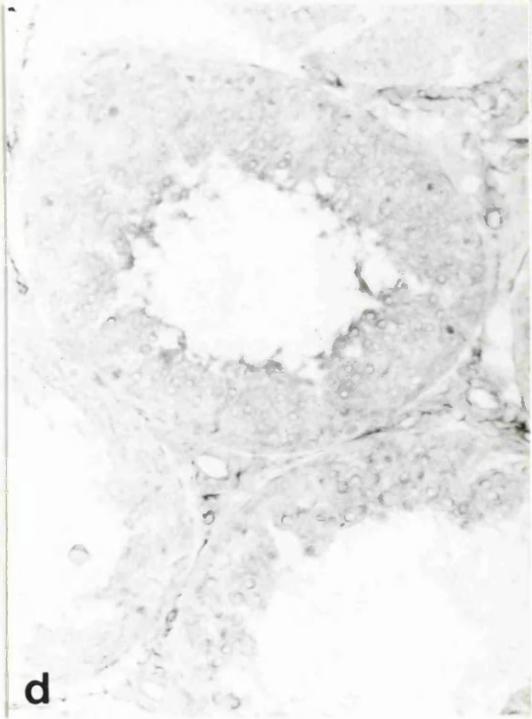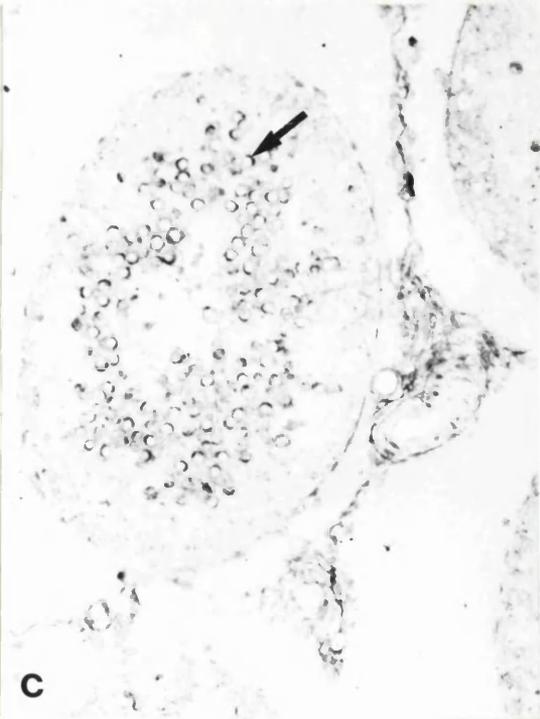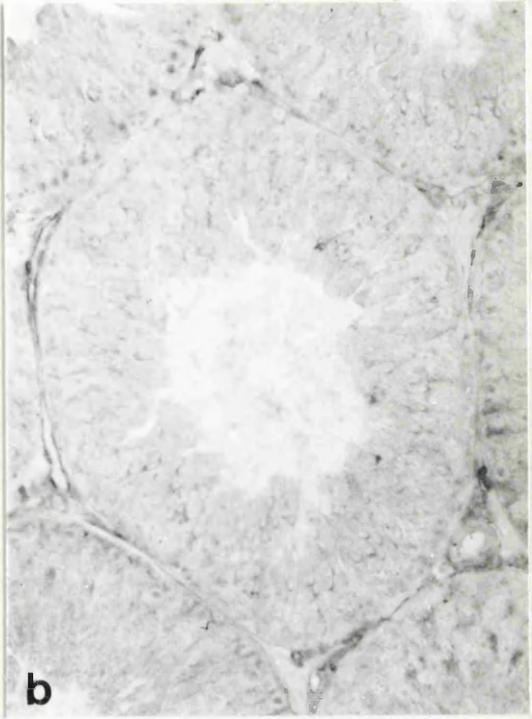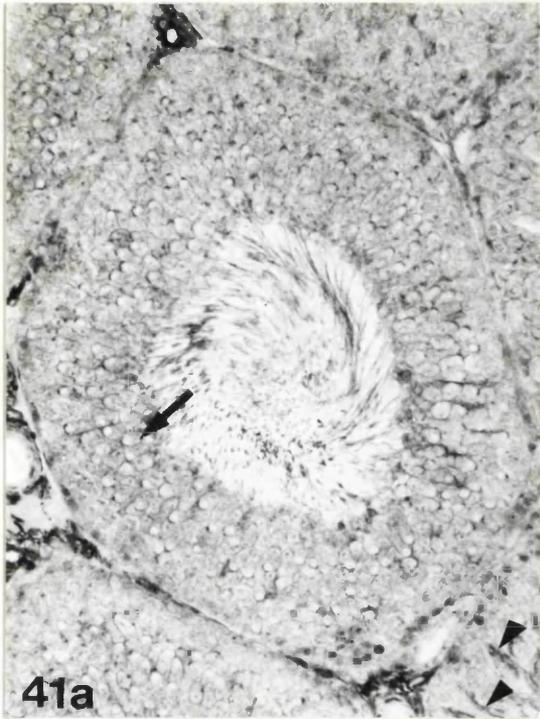
FIGURE 42: Androgen receptor staining of the cranial suspensory

ligament of Albino Swiss rats at 18 d.p.c

(a) stained for androgen receptor, and

(b) negative control incubated in the absence of the

primary antibody.

Note positive androgen receptor staining of nuclei of

cells in the cranial suspensory ligament (arrows) and

testicular cords (T) in (a).

Magnification: all x 80

42a

T

b

T
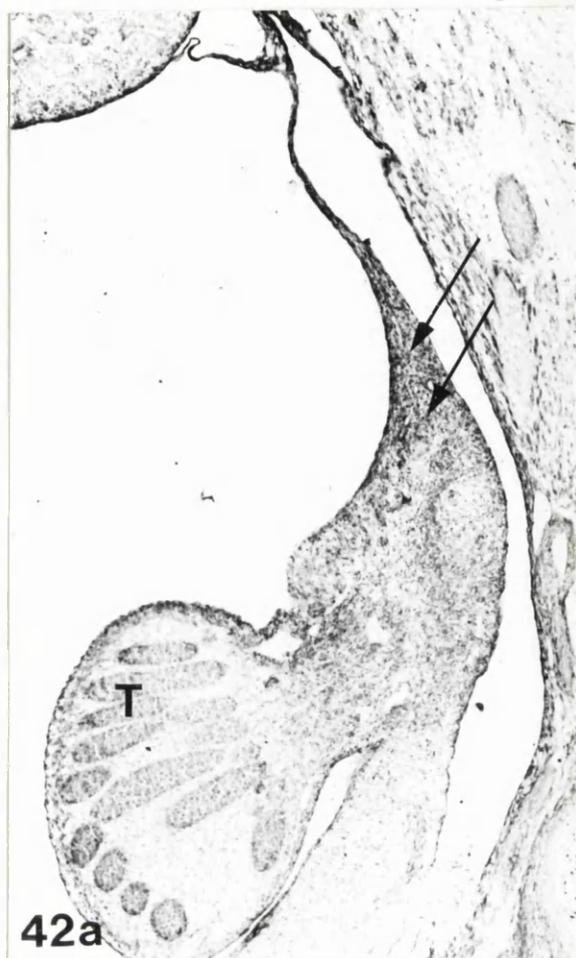
DISCUSSION

The aim of the present study was to examine the effects of exposure to the anti-androgen flutamide on testis descent in view of the continuing uncertainty over whether androgens are involved in this process. The study examined the effects of flutamide on the location and viability of testes in two species (the rat and the hamster) and examined both gubernacular growth and regression and persistence of the cranial suspensory ligament, two fetal structures which may influence descent.

1. Is flutamide an appropriate choice of anti-androgen ?

Anti-androgens are drugs that inhibit the biological activity of androgens (**Lerner, 1964**). They may be steroidal or non-steroidal compounds. Their mechanism(s) of action is to compete for androgen receptor sites in target tissues (**McLeod, 1993**). Steroidal anti-androgenic drugs include cyproterone, cyproterone acetate and megestrol acetate while non-steroidal anti-androgenic drugs include flutamide, casedox and nilutamide (**McLeod, 1993**). Both steroidal and non-steroidal anti-androgenic drugs compete with naturally occurring androgens such as testosterone and DHT for an androgen receptor which is predominantly a nuclear protein (**Husmann et al, 1990**). These drugs suppress the uptake and retention of androgens by target tissues, and inhibit the formation of the nuclear binding of androgens (**Peets et al, 1974**). However, while steroidal anti-androgens also have progestational activity which inhibits testosterone production by Leydig cells as a result of suppression of pituitary luteinizing hormone, non-steroidal anti-androgens block cellular binding of androgens only

(Neumann **et al**, 1977; **McLeod, 1993**). Thus, with non-steroidal anti-androgens, there is no primary reduction of testosterone production, there may even be an increase of LH resulting in increased plasma testosterone levels **(McLeod, 1993)**. The non-steroidal anti-androgen flutamide is metabolised to form its active metabolite (flutamide-OH) in the target cells before it is able to compete effectively for androgen binding sites **(Katchen and Buxbaum, 1975).**

Other anti-androgens have been used in the past to examine many aspects of reproductive tract development. For example, cyproterone and cyproterone acetate developed by Schering (for review see **Neumann et al, 1970)** have been used to study

i)   Differentiation of the reproductive tract and external genitalia in male rabbit and dog fetuses. This led to failure of Wolffian duct maintenance, inhibition of prostate and scrotal development, shortening of the anogenital distance and formation of a vagina.

ii)   Adult testicular functions: Treatment with cyproterone led to inhibition of spermatogenesis in animals and man with stimulation of Leydig cell activity and the secretion of testosterone.

These are not compounds of choice because they are steroidal in nature themselves and therefore have less predictable actions **(Peets et al, 1974; Poyet and Labrie, 1985)**. For example, cyproterone acetate may not only compete with testosterone and DHT for androgen receptors, but also may have progestational

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activity which reduces pituitary luteinizing hormone and subsequently reduces plasma testosterone.

2. What evidence is there that flutamide has successfully crossed the placental barrier in the present study and acted as an anti-androgen ?

Since the results of the present study demonstrate pronounced effects of flutamide on testis descent, gubernacular development and cranial suspensory ligament retention (all of which will be discussed below), it is important to be able to demonstrate that flutamide has acted in its expected manner as an anti-androgen. The most obvious confirmation of this comes from the marked feminization of the external genitalia of prenatally exposed males and the failure of the internal reproductive tract to develop normally. It is well established in the male fetus that

i)   differentiation of the indifferent gonad into the testis is genetic, requiring the presence of Sry (mouse) or SRY (human) gene on the Y chromosome **(Gubbay et al, 1990; Sinclair, 1990)** and that

ii)   inhibition of development of the Müllerian ducts depends on the production of MIS by the pre-Sertoli cells of the testis **(Jost and Magre, 1993; Josso et al, 1993a).**

Naturally, administration of flutamide cannot alter the genetic constitution of rat fetuses so that all treated males possessed testes. Furthermore, flutamide neither interferes with MIS production nor, apparently, blocks MIS receptor sites.

As might be expected, no derivatives of the Müllerian duct were present in treated males. On the other hand,

iii)   virilization of the Wolffian ducts to form the epididymis, vas deferens and seminal vesicles is under the influence of testosterone, while differentiation of the urogenital sinus to form the prostate and prostatic urethra, and of the genital tubercle/swelling to become the penis and penile urethra/scrotum respectively, are under the influence of DHT (the 5α-reduced derivative of testosterone) **(Wilson, 1992)**.

Synthesis of testosterone begins soon after the differentiation of Leydig cells which begins at 15.5 d.p.c. in rat **(Jost and Magre, 1993)**. Testosterone is the principle androgen secreted by the testes and enters target cells by passive diffusion **(Wilson, 1992)**. Within the cells, testosterone may a) combine with the androgen receptor to form a testosterone-receptor complex in the nucleus which influences the expression of certain genes, b) be converted to dihydrotestosterone by the enzyme 5α-reductase; this hormone then combines with the same receptor to form a dihydrotestosterone-receptor complex, which modulate the expression of other genes, or c) be aromatized to estradiol in target tissues which then acts via its own receptor **(Wilson, 1992)**.

Individuals with X-linked androgen insensitivity due to mutation of the androgen receptor have impairment of all aspects of androgen action; both testosterone and DHT are present but these individuals have a phenotype which

varies from wholly feminine (testicular feminization) to men with ambiguous genitalia or with infertility **(Brown et al, 1982; Wilson, 1992).**

The fact that testosterone influences Wolffian duct development whereas DHT influences virilization of the urogenital sinus and genitalia are based on the following observations: a) high activity of the enzyme 5α-reductase in the urogenital sinus and the phallus during the period of sexual differentiation but not in the Wolffian duct **(Wilson, 1973; Renfree et al, 1992),** b) treatment with a 5α-reductase inhibitor inhibits development of rat prostate **(George and Peterson, 1988),** c) human males with 5α-reductase deficiency (an autosomal recessive abnormality) have normal testes, androgen levels and development of Wolffian duct derivatives but impaired phallic development **(Walsh et al, 1974; Wilson, 1992).**

In experimental studies where animals are exposed to anti-androgens, the development of the Wolffian duct and urogenital sinus derivatives are both affected **(Spencer et al, 1991; van der Schoot, 1992b; van der Schoot and Elger, 1993).** Briefly, exposure of male rats to flutamide during prenatal life inhibits virilisation of the Wolffian duct, so that they exhibit poor development (or absence) of the epididymis, vas deferens and seminal vesicle as well as feminized genitalia and hypospadias. Similar results were obtained in this study where the urethra opens onto the ventral aspect of the phallus, the vagina persists and seminal vesicles and prostate (which lie ventral to the urinary bladder) are extremely small In the present study feminization of the genitalia and underdevelopment (or absence) of

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the epididymis and vas deferens are good indicators that flutamide was not only crossing the placental barrier but also acting as an anti-androgen at target tissues **(van der Schoot, 1992b; Husmann and McPhaul, 1991b).**

3. Maldescent

One aim of the present investigation was to examine the effects of flutamide exposure on testicular descent in Albino Swiss rats and on the functional morphology of testes in different final locations. In Experiment 1, 44% of testes descended into the scrotum, while 44% occupied a suprainguinal ectopic position and 12% remained in the abdomen. All control males (exposed to propylene glycol vehicle only) exhibited normal descent of the testes into the scrotum. These findings differ somewhat from those of **van der Schoot (1992b)** who reported that in Sprague-Dawley rats exposed to flutamide from gestational day 11 - birth, 50% of testes descended into the scrotum while 50% of testes remained intra-abdominal. Nevertheless, the finding that 50% of testes occupy a normal scrotal position is common to both studies.

In the present study, cryptorchidism in the rat occurred either unilaterally (nine) or bilaterally (two) and most cases were on the right side (10 right, 3 left). This asymmetry of descent has been documented in human fetuses **(Heyns, 1987)** and in Sprague-Dawley rats **(van der Schoot, 1992b).** There is no agreed reason for this asymmetry. However, **van der Schoot (1992b)** has commented that development of the reproductive organs occurs close to the umbilical vessels.

During the later part of development, there is difference in suppression of the right and left umbilical arteries. While the left umbilical artery atrophies, the right artery hypertrophies. Thus, the two sides of the embryo may receive different amount of exposure to flutamide. This may offer a possible explanation to the asymmetry of undescended testes.

It has been suggested that the process of testicular descent can be divided into two phases which are controlled by separate hormones **(Hutson, 1985)**. The first, transabdominal phase which involves the migration of the testis from the caudal pole of the kidney to the inguinal region about the level of the bladder neck is widely held to be androgen-independent **(Baumans et al, 1983; Hutson and Donahoe, 1986; Fentener van Vlissingen et al, 1988; van der Schoot, 1992a).** The present study supports this view since most of the intra-abdominal testes of flutamide-treated males were located close to the neck of the bladder. **Fentener van Vlissingen et al (1988)** suggested the name "descendin" for a non-androgenic fetal testicular factor (which could be MIS) that controls the first phase of testicular descent. Furthermore, individuals with MIS insufficiency (confirmed by persistence of the Müllerian ducts) have their testes in an ovarian position **(Hutson et al, 1990;1996).**

The second, inguinoscrotal phase (which involves the migration of the testis from the inguinal region through the inguinal canal and down to the scrotum) is believed to be androgen-dependent. Investigations on laboratory species such as rat **(Rajfer and Walsh, 1977)** and rabbit **(Rajfer, 1982)**, revealed that testicular

descent can be induced by administration of human chorionic gonadotrophin (hCG) or DHT, or inhibited by estradiol.

However, the use of anti-androgens has produced conflicting results; cyproterone acetate failed to prevent testicular descent **(Wensing, 1973)** while flutamide did **(Husmann and McPhaul, 1991b; Spencer et al, 1991; van der Schoot, 1992b; McMahon et al, 1995).** Experimental investigations such as the present one (and previous reports such as **Husmann and McPhaul, 1991b; Husmann et al, 1994; van der Schoot 1992b)** support a role for androgens in controlling normal testicular descent.

Although much experimental evidence suggests a role for androgens in testicular descent, the exact mechanism responsible is undetermined, nor is it completely accepted which target tissues are involved. The gubernaculum (which connects the testis and epididymis to the scrotum, and is common to all animals exhibiting testicular descent) has long been the most likely candidate.

If the gubernaculum is indeed the androgen-dependent target tissue responsible for testicular descent, then it must possess androgen receptors. Partial characterisation of an androgen receptor has indeed been made in the gubernacula of the neonatal rat **(George and Peterson, 1988; Husmann and McPhaul, 1991a)** and the fetal and neonatal pig **(Oprins et al, 1988).** In the present study, the attempted demonstration of an androgen receptor in the gubernaculum using a polyclonal androgen receptor antibody raised in rabbit did not yield a wholly convincing result due to relatively strong background staining. Nevertheless, there

was staining of the nuclei of the cells in the mesenchymal core of the gubernaculum at 20 d.p.c. and staining of the nuclei of the cells in the gubernacular cord. The appropriateness of the antibody was confirmed by staining of the nuclei of the epithelium of the epididymis and of the vas deferens.

Not only does the gubernaculum possess androgen receptors, but also the enzyme 5α-reductase necessary for the conversion of testosterone to DHT **(Rajfer and Walsh, 1977; Rajfer, 1982; Renfree et al, 1992)**. George (1989) found a relatively high 5α-reductase activity in 18 d.p.c. rat gubernaculum (when it is composed of mesenchyme); however, the enzyme activity rapidly declines as the gubernaculum becomes predominantly muscular during postnatal life.

4. Consequences of maldescent on the adult testis

All testes from flutamide-treated males were reduced in size compared with controls. Moreover, intra-abdominal testes were even smaller than those which eventually reached scrotal or ectopic suprainguinal positions. In the present study, all males exposed prenatally to flutamide possessed seminiferous tubules exhibiting varying degrees of damage. This is probably due to long term disruption of hypothalamo-pituitary-gonadal axis function where a variety of experimental manipulations adversely affect sperm production **(Russell and Clermont, 1977; Awoniyi et al, 1989; Huang et al, 1991; Sinha-Hikim and Swerdloff, 1993; 1994; Billig et al, 1995)**. In the present study, androgen receptor was demonstrated in the acrosome of round (steps 4-7) and elongating spermatids as

well as the heads of maturing spermatids. Given the positive correlation between low androgen levels and high rates of tubule damage seen in Experiment 1, this is in agreement with the suggestion that the damage seen is due to stage-specific effects of lowered testosterone on spermatogenesis **(Kerr et al, 1993; O'Donnell et al, 1994)** and that in diverse studies, spermatogenesis could be restored by testosterone replacement **(Awoniyi, 1989; Kerr et al, 1993; O'Donnell et al, 1994)** or by cessation of gonadotropin-RH antagonist treatment **(Sinha-Hikim and Swerdloff, 1994)**.

Androgens promote the maturational transition of round to elongated spermatids by maintenance of the Sertoli cell-spermatid binding **(McLachlan et al, 1994)** particularly where Sertoli cells have been rendered "binding competent" by FSH **(O'Donnell et al, 1994; Sinha-Hikim and Swerdloff, 1995)**. This priming effect of FSH on normal spermatogenesis has been specifically associated with restoration of the junction-related Sertoli cell cytoskeleton **(O'Donnell et al, 1994; Muffly et al, 1994)**.

In the most recent studies, the expression of the FSH receptor gene in the rat seminiferous epithelium has been shown to be stage-specific **(Rannikko et al, 1996)** where the level of receptor mRNA in stages XIII-I was sixfold compared with stage VI of the spermatogenic cycle which has the lowest level. In the present study, where testicular androgen levels are reduced, there is marked disruption of the acrosome and maturation phases (Steps 8-19) of spermiogenesis. The cap phases (Steps 4-7) were moderately affected while the Golgi phases (Steps 1-3)

seemed normal. However, where tubules were categorised as "normal" in males exposed prenatally to flutamide, the percentage of occurrence of different stages of spermatogenesis was also normal and, in general corresponded to those recorded in the Albino Swiss rat by **McDonald and Scothorne (1988).**

The results of Experiment 1a suggest that the degree of tubular damage was maximum in cryptorchid testes where no normal tubules were seen at all. This could be due to the increased temperature of the testes **(Clegg, 1963)** which may lead to direct damage of germ cells, or inhibition of mitosis, or may block the action of gonadotrophins on the seminiferous tubules.

It should be appreciated that flutamide does not in itself reduce androgen production. Indeed, flutamide administration to an adult male usually results in increased LH release and elevated levels of circulating testosterone **(Sardanons et al, 1989; Sodersten et al, 1975)** in rat and **(Knuth et al, 1984)** in man.

5. Structural factors in testicular descent: the cranial suspensory ligament and the gubernaculum

The cranial suspensory ligament, an embryonic structure derived from the cranial mesonephric ridge, is initially present in both sexes. It connects the gonad, mesonephros and mesonephric duct to the posterior abdominal wall at the junction between the middle and the lateral thirds of the lowest rib **(Hebel and Stromberg, 1976; van der Schoot and Elger, 1992).** During normal development, while the CSL persists in the female, it disappears in the male exhibiting testicular descent.

Disappearance of the CSL is thought to be influenced by androgens and this is consistent with persistence of the CSL in flutamide-treated male rats (van der Schoot and Elger, 1992) and rabbits (van der Schoot and Elger, 1993) as well as in Tfm mice (Hutson, 1986) and rats (Spencer Barthold et al, 1994). Van der Schoot and Elger, (1993) have suggested that persistence of the CSLs in these cases prevents testicular descent. In studies of "intersexuality" (Greene, 1939) the ovaries descend to a varying degree in female rats exposed prenatally to androgens. Although there is no mention of the CSL in the latter study, it could be that androgens interfered with maintenance of the female CSL.

Kersten et al (1996) reported persistence of the CSLs in a dog associated with cryptorchidism and again suggested that the CSL could be a major factor in preventing testicular descent. However, in the present study, the CSL persisted in all flutamide-treated male rats despite 44% of testes descending normally into the scrotum. Furthermore, it was present in all hamsters, even though they exhibited full descent into the scrotum in all cases. These findings do not support the notion (van der Schoot and Elger, 1992; 1993) that the CSL can hold the testis firmly to the caudal pole of the kidney despite the normal outgrowth of the gubernaculum.

Transabdominal migration of the testis occurs even in mice with complete androgen resistance and in which the CSL persists (Hutson, 1986). In the present study, I have examined the histology of the CSL and found that there was no difference - either in persistence, size or structure - in the CSL from the three

possible locations of the testes. Taken as a whole, these findings suggest that the CSL is <u>not</u> a major factor in preventing testicular descent.

In contrast to the CSL, the gubernaculum is a structure derived from the caudal mesonephric ridge connecting the lower pole of the gonad to the anterior abdominal wall. The gubernaculum is also present in early embryos of both sexes. During normal development, while the gubernaculum in the male begins an outgrowth phase, the gubernaculum in the female regresses **(van der Schoot <u>et al</u>, 1995).**

The gubernaculum is believed to play a major role in the process of testicular descent **(Backhouse, 1982; Wensing and Colenbrander, 1986; Heyns, 1987).** Thus when the gubernaculum of neonatal rats was removed, or it's distal attachments were severed, testicular descent was prevented **(Frey and Rajfer, 1984)**. However, when the proximal attachments to the testis / epididymis was severed, testicular descent occurred normally. In addition, when one testis was removed and was replaced with a prosthesis, and the gubernaculum was left intact, there was normal descent of the prosthesis into the scrotum. Thus, a gubernaculum with intact distal attachments (i.e. with an intact genitofemoral nerve) is a prerequisite for cremaster sac formation and the subsequent testicular descent to occur.

In the male fetus, growth and subsequent regression of the mesenchymatous core of the gubernaculum play a key role in the mechanism for perinatal gubernacular cone eversion and subsequent testicular descent **(Wensing**

and Colenbrander, 1986). During the first, transabdominal phase of testicular descent, the testis migrates from the caudal pole of the kidney to the inguinal region at the level of the bladder neck. This caudal migration of the testis is accompanied by outgrowth of the gubernacular cone and relative shortening of the gubernacular cord.

In the present study, the outgrowth of the gubernacular cone in flutamide-treated males was normal compared to controls, however, gubernacular cone regression was delayed. Furthermore, the gubernacular cords failed to shorten **(Figure 34a-c).** Thus, the gubernaculum in flutamide-treated males is different from that of controls. It is not clear from the present experiments a) How this difference arises, b) What its significance may be. Nevertheless, it suggests that further research should be focussed on the gubernaculum (and not the CSL). Why does eversion takes longer? Why are the cords elongated? Among other factors, attention should be paid to:

i).   Whether there is any difference in the ratio of cells to matrix content in the gubernaculum of control males and males treated prenatally with flutamide.

ii)   Whether there are differences in the rates of cell division or of cell death (necrosis or apoptosis)?

iii)   Whether there are differences in the extra-cellular matrix composition e.g. the expression of extracellular matrix molecules?

iv)   Whether there is any difference in the time of invasion of the gubernaculum by myoblasts, formation of the definitive cremaster muscles or innervation by the genitofemoral nerve?

It is also a frequent observation in man that epididymal abnormalities are associated with testicular maldescent **(Marshall and Shermeta, 1979; Mininberg and Schlossberg, 1983; Heath   et al, 1984; Merksz and Toth, 1987).** In the present study, regression of the Wolffian duct (and hence absence of the epididymis) may have been one of the factors responsible for an unusually long gubernacular cord in some of the flutamide-treated males, so that the testis is not held close to the inguinal canal. However, impaired development of the epididymis and vas deferens was not necessarily associated with testicular maldescent on the same side confirming the suggestion **(Spencer et al, 1991)** that an intact epididymis is not required for descent of the testis. This is an important point because it has occasionally been proposed that, since the gubernaculum is attached to the cauda epididymidis as well as the lower pole of the testis, it is the movements of the epididymis which are responsible for testicular descent **(Mininberg and Schlossberg, 1983; Hadziselimovic, 1983).**

6) Effects of flutamide administered postnatally to rat pups

Postnatal treatment with flutamide did not prevent testicular descent nor had it any effect on the masculization of the accessory sex organs: thus the epididymis and vas deferens developed normally and there was no feminization of

of the external genitalia. These findings support the suggestion **(Spencer et al, 1993)** that androgens act early (i.e. during gubernacular outgrowth): these authors found complete descent of the testes when flutamide was administered during late gubernacular outgrowth and early regression in the rat. The present study also supports the view **(van der Schoot, 1992b; Husmann and McPhaul, 1991b)** that by the time of birth or early in postnatal life the "critical period" of development in which androgens can act on the developing reproductive system has been passed. **Van der Schoot (1992b)** found an increase in testicular weights of rats treated postnatally with flutamide and suggested that the possibility of a failure of the feedback control of gonadotrophin secretion leading to increased gonadotrophin secretion and enhanced testosterone production. However, no evidence to support this was given.

7. Effects of prenatal flutamide on hamsters

Prenatal administration of flutamide to hamsters (10mg/day from E8 to birth) did not affect the process of testicular descent despite various degrees of impairment of masculinization of the male reproductive tract including partial to complete absence of epididymis, vas deferens and seminal vesicles. Treated males also possessed small testes and a small phallus, but no vagina.

Previous studies on prenatal exposure to the anti-androgen cyproterone acetate **(Swanson,** pers comm) resulted in male offspring with a small phallus (with a urethral opening at its base), so that the genitalia were neither completely

male nor female; testicular descent was completed by 3 weeks post-partum. However, in a study examining guinea pigs **(Graf and Neumann, 1972)**, cyproterone acetate induced regression of the Wolffian duct but failed to induce persistence of the vagina or to interfere with testicular descent in this species. These findings lead to the important question: what factors control testicular descent in rodents such as hamsters? One main difference between hamsters and rats is that the hamster is a seasonal breeder which exhibits ascent of the testes during the non-breeding season; It may be that the factors controlling descent are different. Several possibilities must be considered, all leading to further avenues of study

i). <u>Androgens play no part in testis descent</u>.

Although testes clearly descended in hamsters treated with flutamide, it was equally clear that flutamide was getting across the placental barrier and preventing development of the Wolffian duct derivatives. We need to know a) whether the gubernaculum and CSL possessed androgen receptors during development in this species, b) what happens to the hamster gubernaculum and CSL during development in normal and flutamide-treated males.

ii). <u>Androgens play a part, but it is postnatal</u>.

Although I did not try giving flutamide postnatally, there is no reason to think that the period for testis descent is very different in the hamsters compared to the rat. For example, the postnatal administration of estrogens had no effect on phenotypic sex - even though testis weight was reduced in adulthood **(Swanson,**

1966). Secondly, prenatal administration of cyproterone acetate (20 mg/day from E10 to birth) did lead to testicular maldescent **(Swanson,** pers comm)**.** This would seem to established the <u>timing</u> of events which culminate in descent.

iii). <u>Dose was too low</u>.

The dose used in the present study was that which proved effective in the rat. Given the difference in body weight, hamsters would have received a higher dose than rats. While there may be species differences in the ability of flutamide to cross the placental barrier, the absence of Wolffian duct derivatives is itself a guarantee of flutamide exposure.

CONCLUSIONS

The present study has demonstrated

• that administration of the non-steroidal anti-androgen flutamide to pregnant female Albino Swiss rats from gestational day 10 to birth resulted in disturbance of the process of testicular descent in male offspring. These males also exhibited small testes, reduced testosterone levels within the testes (despite higher numbers of Leydig cells) and varying degrees of seminiferous tubule damage probably due to the stage-specific effects of reduced testicular testosterone on spermatogenesis. The overall degree of damage was strongly correlated with the final location of the testis, with scrotal testes being least affected, supra-inguinal ectopic testes intermediate, and intra-abdominal testes severely affected.

• that outgrowth of the gubernacular cone is not affected by flutamide; however, the regression phase (which occurs postnatally) was delayed.

• that shortening of the gubernacular cord was inhibited in males treated prenatally with flutamide.

• that males rats exposed to flutamide exhibit persistence of the cranial suspensory ligament. This persistence however, was irrespective of the position of the testes in the adult. Nor was there any difference in the structure of the cranial suspensory ligaments from these varying testis locations. This suggests that retention of the cranial suspensory ligament is not an important factor in testicular maldescent.

- that prenatal flutamide treatment to rats also interfered with Wolffian duct development since most of the offspring of flutamide-treated mothers exhibited varying degrees of inhibition/absence of Wolffian duct derivatives.

- that impaired development of the epididymis and vas deferens was not necessarily associated with testicular maldescent since (despite partial to complete absence of epididymis and vas deferens) 44% of testes descended normally into the scrotum in males treated prenatally with flutamide.

- that prenatal flutamide treatment to hamsters did not interfere with testicular descent although there was evidence of flutamide action during development (partial/complete inhibition of development of the Wolffian duct) and retention of CSL.

- that postnatal treatment with flutamide did not interfere with testicular descent.

REFERENCES

ABE T AND HUTSON JM (1994): "Calcitonin gene-related peptide injected
ectopically alters gubernacular migration in the flutamide-treated rat with
cryptorchidism." Pediatric Surgery International, 9: 551-554.

ATWELL JD (1985): "Ascent of the testis. Fact or fiction." British Journal of
Urology, 57: 474-477.

AWONIYI CA, SANTULLI R, CHANDRASHEKAR V, SCHANBACHER BD
AND ZIRKIN BR (1989): "Quantitative restoration of advanced
spermatogenic cell in adult male rats made azoospermic by active
immunization against luteinizing hormone or gonadotropin-releasing
hormone." Endocrinology, 125: 1303-1309.

BACKHOUSE KM AND BUTLER H (1960): "The gubernaculum testis of the
pig (Sus scrofa)." Journal of Anatomy, 94: 107-121.

BACKHOUSE KM (1964): "The gubernaculum testis hunteri: testicular descent
and maldescent." Annals of Royal College of Surgery, 35: 15-33.

BACKHOUSE KM (1966): "The natural history of testicular descent and
maldescent." Proceedings of the Royal Society of Medicine, 59: 15-18.

BACKHOUSE KM (1982): "Embryology of testicular descent and maldescent."
Urologic Clinics of North America, 9: 315-325.

BAKER ML, METCALFE SA AND HUTSON JM (1990): "Serum levels of
Müllerian inhibiting substance in boys from birth to 18 years, as determined
by enzyme immunoassay." Journal of Clinical Endocrinology and
Metabolism, 70: 11-15.

BAUMANS V, DIJKSTRA G AND WENSING CJG (1981): "Testicular descent in the dog." Zbl Vet Med, Reihe C10: 97-110.

BAUMANS V, DIJKSTRA G AND WENSING CJG (1983): "The role of a non-androgenic testicular factor in the process of testicular descent in the dog." International Journal of Andrology, 6: 541-552.

BEASLEY SW AND HUTSON JM (1987): "Effect of division of genitofemoral nerve on testicular descent in the rat." Australia New Zealand Journal of Surgery, 57: 49-51.

BEASLEY SW AND HUTSON JM (1988): "The role of the gubernaculum in testicular descent." Journal of Urology, 140: 1191-1193.

BEHRINGER RR (1995): "The Müllerian inhibitor and mammalian sexual development." Philosophical Transaction of Royal Society of London B, 350 : 285-289.

BILLIG H, FURUTA I, RIVIER C, TAPANAINEN J, PARVINEN M AND HSUEH AJW (1995): "Apoptosis in testis germ cells: Developmental changes in gonadotropin dependence and localization to selective tubule stages." Endocrinology, 136: 5-12.

BIRNHOLZ JC (1983): "Determination of fetal sex." New England Journal of Medicine, 309: 942-944.

BLACKSHAW AW AND MASSEY PF (1972): "The recovery of spermatogenesis in the rat testis after heat-induced degeneration." Journal of Reproduction and Fertility, 28:142-143.

BROWN TR, MAES M, ROTHWELL SW AND MIGEON CJ (1982): "Human complete androgen insensitivity with normal dihydrotestosterone receptor binding capacity in cultured genital skin fibroblasts: Evidence for a qualitative abnormality of the receptor." Journal of Clinical Endocrinology and Metabolism, 55: 61-69.

BUEHR M, GU S AND McLAREN A (1993): "Mesonephric contribution to testis differentiation in the fetal mouse." Development, 117: 273-281.

CLARK JM AND EDDY EM (1975): "Fine structural observations on the origin and associations of primordial germ cells of the mouse." Developmental Biology, 47:136-155.

CLEGG EJ (1963): "Studies on artificial cryptorchidism : Degenerative and regenerative changes in the germinal epithelium of the rat testis." Journal of Endocrinology, 27 : 241-251.

CLERMONT Y AND PEREY B (1957): "Quantitative study of the cell population of the seminiferous tubules in immature rats." American Journal of Anatomy, 100: 241-268.

CLERMONT Y AND HUCKINS C (1961): "Microscopic anatomy of the sex cords and seminiferous tubules in growing and adult male Albino rats." American Journal Anatomy, 108: 79-97.

CLERMONT Y AND HARVEY SC (1965): "Duration of the cycle of the seminiferous epithelium of normal, hypophysectomized and hypophysectomized-hormone treated Albino rats." Endocrinology, 76: 80-89.

COLENBRANDER B, VAN ROSSUM-KOK CMJE, VAN STRAATEN HMW AND WENSING CJG (1979): "The effect of fetal decapitation on the testis and other endocrine organs in the pig." Biology of Reproduction, 20: 198 - 204.

DE FELICI M, DOLCI S, AND PESCE M (1992): "Cellular and molecular aspects of mouse primordial germ cell migration and proliferation in culture." International Journal of Developmental Biology, 36: 205-213.

DYM M AND FAWCETT DW (1970): "The blood-testis barrier in the rat and the physiological compartmentation of the seminiferous epithelium." Biology of Reproduction, 3: 308-326.

EDDY EM, CLARK JM, GONG D AND FENDERSON BA (1981): Review article: "Origin and migration of primodial germ cells in mammals." Gamete Research, 4: 333-362.

ELDER JS, ISAACS JT AND WALSH PC (1982): "Androgenic sensitivity of the gubernaculum testis: evidence for hormornal/mechanical interactions in testicular descent." Journal of Urology, 127: 170-176.

ELDER JS (1987): "Cryptorchidism: isolated and associated with other genitourinary defects." Pediatric Clinics of North America, 34: 1033-1053.

ENGLE ET (1932): "Experimentally induced descent of the testis in the Macacus monkey by hormones from the anterior pituitary and pregnancy urine." Endocrinology, 16: 513-520

FALLAT ME, WILLIAMS MPL, FARMER PJ AND HUTSON JM (1992a): "Histologic evaluation of inguinoscrotal migration of the gubernaculum in rodents during testicular descent and its relationship to the genitofemoral nerve." Pediatric Surgery International, 7: 265-270.

FALLAT ME, HERSH JH, AND HUTSON JM (1992b): "Theories on the relationship between cryptorchidism and arthrogryposis." Pediatric Surgery International, 7: 271-273..

FENTENER VAN VLISSINGEN JM, VAN ZOELEN EJJ, URSEM PJF AND WENSING CJG (1988): "*In vitro* model of the first phase of testicular descent: Identification of a low molecular weight factor from fetal testis involved in proliferation of gubernaculum testis cells and distinct from specified polypeptide growth factors and fetal gonadal hormones." Endocrinology, 123: 2868-2877.

FRANKENHUIS MT AND WENSING CJG (1979): "Induction of spermatogenesis in the naturally cryptorchid pig." Fertility and Sterility, 31: 428-433.

FREY HL AND RAJFER J (1982): "Incidence of cryptorchidism." Urologic Clinic of North America, 9: 327-329.

FREY HL, PENG S AND RAJFER J (1983): "Synergy of abdominal pressure and androgens in testicular descent." Biology of Reproduction, 29: 1233-1239.

FREY HL AND RAJFER J (1984): "Role of the gubernaculum and intra-abdominal pressure in the process of testicular descent." Journal of Urology, 131: 574-579.

FUJIKAKE N, TOGASHI H, YAMAMOTO M, ARISHIMA K, UEDA Y AND EGUCHI Y (1989): "Relationship between the gubernaculum and the testicular descent in the rat fetus: macroscopic and light and electron microscopic observation." Japanese Journal of Veterinary Science, 51(2): 416-424.

FUJIMOTO T, MIYAYAMA Y AND FUYUTA M (1977): "The origin, migration and fine morphology of human primordial germ cells." Anatomical Record, 188: 315-330.

FUJIMOTO T, YOSHINAGA K AND KONO I (1985): "Distribution of fibronectin on the migratory pathway of primordial germ cells in mice." Anatomical Record, 211:271-278.

GARDNER RL, LYON MF, EVANS EP AND BURTENSHAW MD (1985): "Clonal analysis of X-chromosome inactivation and the origin of the germ line in the mouse embryo." Journal of Embryology and Experimental Morphology, 88: 349-363.

GENDREL D, JOB JD AND ROGER M (1978): "Reduced post-natal rise of testosterone in plasma of cryptorchid infants." Acta Endocrinologica, 89: 372- 378.

GENDREL D, ROGER M AND JOB JD (1980): "Plasma gonadotropin and testosterone values in infants with cryptorchidism." Journal of Pediatrics, 97: 217-220.

GEORGE FW AND PETERSON KG (1988): "Partial characterization of the androgen receptor of the newborn rat gubernaculum." <u>Biology of Reproduction</u>, 39: 536-539.

GEORGE FW (1989): "Developmental pattern of 5α-reductase activity in the rat gubernaculum." <u>Endocrinology</u>, 124: 727-732.

GINSBURG M, SNOW MHL AND McLAREN A (1990): "Primordial germ cells in the mouse embryo during gastrulation." <u>Development</u>, 110: 521-528.

GIWERCMAN A, MULLER J AND SKAKKEBAEK NE (1988): "Cryptorchidism and testicular neoplasia." <u>Hormone Research</u>, 30:157-163.

GIWERCMAN A, BRUUN E, FRIMODT-MOLLER C AND SKAKKEBAEK NE (1989): "Prevalence of carcinoma in-situ and other histopathological abnormalities in testes of men with a history of cryptorchidism." <u>Journal of Urology</u>, 142: 998-1002.

GODIN I, WYLIE C AND HEASMAN J (1990): "Genital ridges exert long-range effects on mouse primordial germ cell numbers and direction of migration in culture." <u>Development</u>, 108: 357-363.

GOH DW, MOMOSE Y, MIDDLESWORTH W AND HUTSON JM (1993): "The relationship among calcitonin gene-related peptide, androgens and gubernacular development in 3 animal models of cryptorchidism." <u>Journal of Urology</u>, 150: 574-576.

GRAF KJ AND NEUMANN F (1972) "Influence of cyproterone acetate on sexual differentiation of male guinea pigs." Z Anat Entwicklungsgesch, 137: 200-220.

GREENE RR, BURRILL MW AND IVY AC (1939) "Experimental intersexuality. The effects of antenatal androgens on sexual development of female rats." American Journal of Anatomy 65, 415-469.

GRIFFIN JE AND WILSON JD (1980): "The syndromes of androgen resistance." New England Journal of Medicine, 302 : 198-209.

GRINO FB, GRIFFIN JE AND WILSON JD (1990): "Testosterone at high concentrations interacts with the human androgen receptor similarly to dihydrotestosterone." Endocrinology, 126:1165-1172.

GROWNEY CA (1994): "The role of the coelomic epithelium in the initial formation and further differentiation of the mouse gonad." B.Sc. Thesis. Department of Anatomy, University of Glasgow.

GUBBAY J, COLLIGNON J, KOOPMAN P, CAPEL B, ECONOMOU A, MUNSTERBERG A, VIVIAN N, GOODFELLOW P AND LOVELL-BADGE R (1990): "A gene mapping to the sex-determining region of the mouse Y chromosome is a member of a novel family of embryonically expressed genes." Nature (London), 346: 245-250.

HABENICHT UF AND NEUMANN F (1983): "Hormonal regulation of testicular descent." Advances in Anatomy, Embryology and Cell Biology, 81 : 1-55.

HADZISELIMOVIC F (1983): Cryptorchidism - management and implications. Springer-Verlag, Berlin Heidelberg New York.

HADZISELIMOVIC F, HERZOG B, GIRARD J AND STALDER G (1984): "Cryptorchidism - histology, fertility and treatment." Progress in Reproductive Biology and Medcine, 10:1-15.

HADZISELIMOVIC F, THOMMEN L, GIRARD J AND HERZOG B (1986): "The significance of postnatal gonadotropin surge for testicular development in normal and cryptorchid testes." Journal of Urology, 136: 274-276.

HADZISELIMOVIC F, DUCKETT JW, SNYDER HM III, SCHNAUFER L AND HUFF D (1987): "Omphalocele, cryptorchidism and brain malformations." Journal of Pediatric Surgery, 22: 854-856.

HAMILTON JB (1938): "The effect of male hormone upon the descent of the testes." Anatomical Record, 70:533-541.

HEATH AL, MAN DWK AND ECKSTEIN HB (1984): "Epididymal abnormalities associated with maldescent of the testis." Journal of Pediatric Surgery, 19:47-49.

HEBEL R AND STROMBERG MW (1976): Anatomy of the laboratory rat. The Williams and Wilkins Company, Baltimore.

HEYNS CF, HUMAN HJ AND DE KLERK DP (1986): "Hyperplasia and hypertrophy of the gubernaculum during testicular descent in the fetus." Journal of Urology, 135: 1043-1047.

HEYNS CF (1987): "The gubernaculum during testicular descent in the human fetus." Journal of Anatomy, 153: 93-112.

HEYNS CF, HUMAN HJ, WERELY CJ AND DE KLERK DP (1990): "The glycosaminoglycans of the gubernaculum during testicular descent in the fetus." Journal of Urology, 143: 612-717.

HEYNS CF AND PAPE VC (1991): "Presence of a low capacity androgen receptor in the gubernaculum of the pig fetus." Journal of Urology, 145: 161-167.

HEYNS CF, TATE R, SARGENT NSE, HABIB FK AND CHISHOLM GD (1993): "Absense of 5α-reductase activity in the gubernaculum during descent of the fetal pig testis." Journal of Urology, 150: 510-513.

HOFFMAN RA, ROBINSON PF AND MAGALHAES H (1968): "The golden hamster. Its biology and use in medical research. Iowa State University Press, Ames, Iowa.

HUANG HFS, POGACH LM, NATHAN E, GIGLIO W AND SEFBODE JJ (1991): "Synergistic effects of follicle-stimulating hormone and testosterone on the maintenance of spermatogenesis in hypophysectomized rats: Relationship with the androgen-binding protein status." Endocrinology, 128: 3152-3161.

HUFF DS, HADZISELIMOVIC F, SNYDER HMC, III, DUCKETT JW AND KEATING MA (1989): "Postnatal testicular maldevelopment in unilateral cryptorchidism." Journal of Urology, 142: 546-548.

HUFF DS, HADZISELIMOVIC F, SNYDER III HMC, BLYTHE B AND
    DUCKET JW (1993): "Histologic maldevelopment of unilaterally
    cryptorchid testes and their descended partners." European Journal of
    Pediatric, 152 (Suppl 2): S10-S14.

HULLINGER RL AND WENSING CJG (1985): "Descent of the testis in the
    fetal calf." Acta Anatomica, 121: 63-68.

HUNTER RHF (1995): In Sex Determination, Differentiation and Intersexuality
    in Placental Mammals. Cambridge, University Press.

HUSMANN DA, WILSON CM, McPHAUL MJ, TILLEY WD AND WILSON
    JD (1990): Antipeptide antibodies to two distinct regions of the androgen
    receptor localise the receptor protein to the nuclei of target cells in the rat
    and human prostate." Endocrinology, 126: 2359-2368.

HUSMANN DA AND McPHAUL MJ (1991a): "Localisation of the androgen
    receptor in the developing rat gubernaculum." Endocrinology, 128:
    383-387.

HUSMANN DA AND McPHAUL MJ (1991b): "Time-specific androgen
    blockade with flutamide inhibits testicular descent in the rat."
    Endocrinology, 129: 1409-1416.

HUSMANN DA, BOONE TB AND McPHAUL MJ (1994): "Flutamide-induced
    testicular undescent in the rat is associated with alterations in genitofemoral
    nerve morphology." Journal of Urology, 151: 509-513.

HUTSON JM (1985): "A biphasic model for the hormonal control of testicular
    descent." Lancet, ii: 419-421.

HUTSON JM (1986): "Testicular feminization: a model for testicular descent in mice and men." Journal of Pediatric Surgery, 21: 195-198.

HUTSON JM AND DONAHOE PK (1986): "The hormonal control of testicular descent." Endocrine Reviews, 7:270-283.

HUTSON JM AND BEASLEY SW (1987): "Aetiology of the prune belly syndrome." Austr. Pediatric Journal, 23: 309-310.

HUTSON JM, BEASLEY SW AND BRYAN AD (1988): "Cryptorchidism in spina bifida and spinal cord transection: a clue to the mechanism of transinguinal descent of the testis." Journal of Pediatric Surgery, 23: 275-277.

HUTSON JM, WILLIAMS MPL, FALLAT ME AND ATTAH A (1990): "Testicular descent: new insights into its hormonal control." Oxford Review of Reproductive Biology, 12:1-56.

HUTSON JM AND BEASLEY SW (1992): "Descent of the testis." Arnold. London.

HUTSON JM, TERADA M, ZHOU B AND WILLIAMS MPL (1996): "Normal testicular descent and the aetiology of cryptorchidism." Advances in Anatomy Embryology and Cell Biology, 132:1-56.

JEON KW AND KENNEDY JR (1973): "The primordial germ cells in early mouse embryos: light and electron microscopic studies." Developmental Biology, 31: 275-284.

JIRASEK JE (1971): "Development of the genital system and male pseudohermaphroditism." Edited by Michael Cohen M Jr., Johns Hopkins Press, Baltimore and London.

JOSSO N, CATE RL, PICARD JY, VIGIER B, DI CLEMENTE N, WILSON C, IMBEAUD S, PEPINSKY RB, GUERRIER D, BOUSSIN L, LEGEAL L AND CARRE-EUSEBE D (1993a): "Anti-Mullerian hormone: The Jost factor." Recent progress in Hormone Reseach, 48:1-59.

JOSSO N, PICARD JY, IMBEAUD S, CARRE-EUSEBE D, ZELLER J AND ADAMSBAUM C (1993b): "The persistent Müllerian duct syndrome: a rare cause of cryptorchidism." European Journal of Pediatrics, 152 (Suppl. 2): S76-S78.

JOST A, VIGIER B, PREPIN J, PERCHELLET JP (1973) "Studies on sex differentiation in mammals." Recent Progress in Hormone Research, 29: 1-41.

JOST A, MAGRE S AND AGELOPOULOU R (1981): "Early stages of testicular differentiation in the rat." Human Genetics, 58:59-63.

JOST A AND MAGRE S (1988): "Control mechanisms of testicular differentiation." Philosophical Transactions of the Royal Society of London, Series B, 322: 55-61.

JOST A AND MAGRE S (1993) "Sexual differentiation". Reproduction In Mammals and Man. Edited by Thibault C, Levasseur MC, Hunter RHF. Paris, Ellipses, pp 197-212.

KAPLAN LM, KOYLE MA, KAPLAN GW, FARRER JH AND RAJFER J
(1986): "Association between abdominal wall defects and cryptorchidism."
Journal of Urology, 136: 645-647.

KATCHEN B AND BUXBAUM S (1975): "Disposition of a new, non-steroid,
anti-androgen, α-α-α-trifluoro-2-methyl-4'nitro-m-propionotoluidide
(flutamide), in men following a single oral 200 mg dose." Journal of Clinical
Endocrinology and Metabolism, 41: 373-379.

KERR JB, MILLAR M, MADDOCKS S AND SHARPE RM (1993): "Stage-
dependent changes in spermatogenesis and Sertoli cells in relation to the
onset of spermatogenic failure following withdrawal of testosterone." The
Anatomical Record, 235:547-559.

KERSTEN W, MOLENAAR GJ, EMMEN JMA AND VAN DER SCHOOT P
(1996): "Bilateral cryptorchidism in a dog with persistent cranial testis
suspensory ligaments and inverted gubernacula: report of a case with
implications for understanding normal and aberrant testis descent." Journal
of Anatomy, 189: 171-176.

KNUTH UA, HANO R AND NIESCHLAG E (1984): "Effect of flutamide or
cyproterone acetate on pituitary and testicular hormones in normal men."
Journal of Clinical Endocrinology Metabolism, 59: 963-969.

KRABBE S, BERTHELSEN JG, VOLSTED P, ELDRUP J, SKAKKEBAEK
NE, EYBEN FV, MAURITZEN K AND NIELSEN AH (1979): "High
incidence of undetected neoplasia in maldescended testes." Lancet i:
999-1000.

LARKINS SL AND HUTSON JM (1991): "Fluorescent anterograde labelling of the genitofemoral nerve shows that it supplies the scrotal region before migration of the gubernaculum." Pediatric Surgery International, 6: 167-171.

LARKINS SL, HUTSON JM AND WILLIAMS MPL (1991): "Localisation of calcitonin gene-related peptide immunoreactivity within the spinal nucleus of the genitofemoral nerve." Pediatric Surgery International, 6: 176-179.

LEBLOND CP AND CLERMONT Y (1952): "Spermatogenesis of rat, mouse, hamster and guinea pig by the periodic acid-fuchsin sulfurous acid technique." American Journal of Anatomy, 90: 167-215.

LEE MM AND DONAHOE PK (1993) "Mullerian inhibiting substance: a gonadal hormone with multiple functions." Endocrine reviews, 14: 152-164.

LERNER LJ (1964): "Hormone antagonists: Inhibitors of specific activities of estrogen and androgen." Recent Progress in Hormone Research, 20: 435-490.

LIPSHULTZ LI, CAMINOS-TORRES R, GREENSPAN CS AND SNYDER PJ (1976): "Testicular function after orchidopexy for unilaterally undescended testis." New England Journal of Medicine, 295: 15-18.

MACKAY S, BASHIR AA AND BIRNIE DH (1989): "Primordial germ cells and gonadal development in the golden hamster." Journal of Anatomy, 164: 155-163.

MACKAY S, STRACHAN L AND Mc DONALD SW (1993): "Increased vascularity is the first sign of testicular differentiation in the mouse." Journal of Anatomy, 183(1): 171.

MAGRE S AND JOST A (1980): "The initial phases of testicular organogenesis in the rat." Archives d'Anatomie microscopique, Tome 69: 297-318.

MAGRE S AND JOST A (1983): "Early stages of the differentiation of the rat testis: relations between Sertoli and germ cells". In Current Problems In Germ Cell Differentiation. Editors: Mc Laren, A and Wylie, CC. Cambridge University Press.

MARSHALL FF AND SHERMETA DW (1979): "Epididymal abnormalities associated with undescended testis. Journal of Urology, 121: 341-43.

MCDONALD SW, SCOTHORNE RJ (1988) A quantative study of the effects of vasectomy on spermatogenesis in rats. Journal of Anatomy, 159: 219-225.

MCLACHLAN RI, WREFORD NG, TSONIS C, DE KRETSER DM AND ROBERTSON DM (1994): "Testosterone effects on spermatogenesis in the gonadotropin-releasing hormone-immunized rat." Biology of Reproduction, 50: 271-280.

MCLAREN A (1983): "Studies on mouse germ cells inside and outside the gonad." Journal of Experimental Zoology, 228: 167-171.

MCLEOD DG (1993): Antiandrogenic drugs. Cancer, 71: 1046-1049.

MCMAHON DR, KRAMER SA AND HUSMANN DA (1995): "Anti-androgen induced cryptorchidism in the pig is associated with failed gubernacular regression and epididymal malformations." Journal of Urology, 154: 553-557.

MENGEL W, HIENZ HA, SIPPE WG, HECKER WC. (1974): "Studies on cryptorchidism: a comparison of histological findings in the germinative epithelium before and after the second year of life." Journal of Pediatric Surgery, 9: 445-450.

MENGEL W, WRONECKI K, SCHROEDER J AND ZIMMERMANN FA (1982): "Histopathology of the cryptorchid testis." Urologic Clinics of North America, 9:331-338.

MERKSZ M AND TOTH J (1987): "Testicular-epididymal fusion abnormality in undescended testis." International Urology and Nephrology, 19: 179-187.

MININBERG DT AND SCHLOSSBERG S (1983): "The role of the epididymis in testicular descent." Journal of Urology, 129: 1207- 1208.

MOERMAN P, FRYNS JP, GODDEERIS P AND LAUWERYNS JM (1984): "Pathogenesis of the prune-belly syndrome: a functional urethral obstruction caused by prostatic hypoplasia." Pediatrics, 73: 470-475.

MOMOSE Y, GRIFFITHS AL AND HUTSON JM (1992): Testicular descent III. The neonatal mouse gubernaculum shows rhythmic contraction in organ culture in response to calcitonin gene-related peptide." Endocrinology, 131: 2881-2884.

MUFFLY KE, NAZIAN SJ AND CAMERON DF (1994): "Effects of follicle-stimulating hormone on the junction-related Sertoli cell cytoskeleton and daily sperm production in testosterone-treated hypophysectomized rats." Biology of Reproduction, 51: 158-166.

NEUMANN F, VON BERSWORDT-WALLRABE R, ELGER W, STEINBECK H, HAHN JD AND KRAMER M (1970): "Aspects of androgen-dependent events as studied by anti-androgens." Recent Progress in Hormone Research, 26: 337-410.

NEUMANN F, GRAF KJ, HASSAN SH, SCHENCK B AND STEUNBECK H (1977): "Central actions of anti-androgens." In Androgens and Anti-androgens, edited by Martini L and Motta M. Raven Press, New York.

NUNN IN AND STEPHENS FD (1961): "The triad syndrome: A composite anomaly of the abdominal wall, urinary system and testis." Journal of Urology, 86: 782-794.

O'DONNELL L, McLACHLAN RI, WREFORD NG AND ROBERTSON DM (1994): "Testosterone promotes the conversion of round spermatids between stages VII and VIII of the rat spermatogenic cycle." Endocrinology, 135: 2608-2614.

OPRINS AC, FENTENER VAN VLISSINGEN JM AND BLANKENSTEIN MA (1988): "Testicular descent: Androgen receptors in cultured porcine gubernaculum cells." Journal of Steroid Biochemistry, 31: 387-391.

PAGON RA, SMITH DW AND SHEPARD TH (1979): "Urethral obstruction malformation complex: A cause of abdominal muscle deficiency and the "prune belly". Journal of Pediatrics, 94: 900-906.

PEETS EA, HENSON MF AND NERI R (1974): "On the mechanism of the anti-androgenic action of flutamide in the rat." <u>Endocrinology</u>, 94: 532-540.

PELLINIEMI LJ (1975): "Ultrastructure of the early ovary and testis in pig embryos." <u>American Journal of Anatomy</u>, 144: 89-112.

POYET P AND LABRIE F (1985): "Comparison of the antiandrogenic/androgenic activities of flutamide, cyproterone acetate and megestrol acetate." <u>Molecular and Cellular Endocrinology</u>, 42: 283-288.

RAJFER J AND WALSH PC (1977): "Hormonal regulation of testicular descent: Experimental and clinical observations." <u>Journal of Urology</u>, 118: 985-990.

RAJFER J (1982): "Endocrinological study of testicular descent in the rabbit." <u>Journal of Surgical Research</u>, 33:158-163.

RAJFER J (1987): "Hormonal regulation of testicular descent." <u>European Journal Pediatric</u>, 146 (Supp. 2): S6-S7.

RANNIKKO A, PENTTILA TL, ZHANG FP, TOPPARI J, PARVINEN M AND HUHTANIEMI I (1996): Stage-specific expression of the FSH receptor gene in the prepubertal and adult rat seminiferous epithelium." <u>Journal of Endocrinology</u>, 151: 29-35.

RENFREE MB, WILSON JD, SHORT RV, SHAW G AND GEORGE FW (1992): "Steroid hormone content of the gonads of the Tammar Wallaby during sexual differentiation." <u>Biology of Reproduction</u>, 47: 644-647.

REYNOLDS ES (1963): "The use of lead citrate at high pH as an electron-opaque stain in electron microscopy." Journal of Cell Biology, 17: 208-212.

RUSSELL LD AND CLERMONT Y (1977): "Degeneration of germ cells in normal, hypohysectomized and hormone treated hypophysectomized rats." Anatomical Record, 187 : 347-366.

SARDANONS ML, DE LAS HERAS MA, CALANDRA RS, SOLANO AR AND PODESTA EJ (1989): "Effect of the anti-androgen flutamide on pituitary LH content and release." Neuroendocrinology, 50: 211-216.

SATOH M (1985): "The histogenesis of the gonad in rat embryos." Journal of Anatomy, 143: 17-37.

SATOH M (1991): "Histogenesis and organogenesis of the gonad in human embryos." Journal of Anatomy, 177: 85-107.

SCHULTE TL (1937): "The genito-urinary system of the elephas indicus male." American Journal of Anatomy, 61: 131-157.

SCORER CG AND FARRINGTON GH (1971): "Congenital deformities of the testis and epididymis." New York, Appleton-Century-Crofts.

SEMPLE CG, GRAY CE, AND BEASTALL GH (1988): "Androgen levels in men with diabetes mellitus." Diabetic Medicine, 5: 122-125.

SHONO T, RAMM-ANDERSON S AND HUTSON JM (1994): "Transabdominal testicular descent is really ovarian ascent." Journal of Urology, 152: 781-84.

SINCLAIR AH (1990): "A gene from the human sex-determining region encodes a protein with homology to a conserved DNA-binding motif." Nature (London), 346: 240-244.

SINCLAIR AH (1994): "The cloning of SRY." Molecular Genetics of Sex Determination. Edited by Wachtel SS, Academic, London. pp 23-42.

SINHA-HIKIM AP AND SWERDLOFF RS (1993): "Temporal and stage-specific changes in spermatogenesis of rat after gonadotropin deprivation by a potent gonadotropin-releasing hormone antagonist treatment." Endocrinology, 133: 2161-2170.

SINHA-HIKIM AP AND SWERDLOFF RS (1994): "Time course of recovery of spermatogenesis and Leydig cell function after cessation of gonadotropin-releasing hormone antagonist treatment in the adult rat." Endocrinology, 134: 1627-1634.

SINHA-HIKIM AP AND SWERDLOFF RS (1995): "Temporal and stage-specific effects of recombinant human follicle-stimulating hormone on the maintenance of spermatogenesis in gonadotropin-releasing hormone antagonist-treated rat." Endocrinology, 136: 253-261.

SKAKKEBAEK NE, BERTHELSEN JG, AND MULLER J (1982): "Carcinoma-in-situ of the undescended testis." Urologic Clinic of North America, 9:377-385.

SLOAN WR AND WALSH PC (1976): "Familial persistent Mullerian duct syndrome." Journal of Urology, 115: 459-461.

SMITH C AND MACKAY S (1990): "The development of mesonephric tubules and their possible contribution to gonadal and adrenal cortical anlagen in the CBA mouse." Abstract in Journal of Anatomy, 170: 240.

SMITH JA, HUTSON JM, BEASLEY SW AND REDDIHOUGH DS (1989): "The relationship between cerebral palsy and cryptorchidism." Journal of Pediatric Surgery, 24: 1303-1305.

SODERSTEN P, GRAY G, DAMASSA DA, SMITH ER AND DAVIDSON JM (1975): "Effects of a non-steroidal antiandrogen on sexual behavior and pituitary-gonadal function in the male rat." Endocrinology, 97:1468-1475.

SPENCER JR, RORRADO T, SANCHEZ RS, VAUGHAN ED JR AND IMPERATO-McGINLEY J (1991): "Effects of flutamide and finastride on rat testicular descent." Endocrinology, 129: 741-748.

SPENCER JR, VAUGHAN ED JR AND IMPERATO-McGINLEY J (1993): Studies of the hormonal control of postnatal testicular descent in the rat. Journal of Urology, 149: 618-23.

SPENCER BARTHOLD J, MAHLER HR AND NEWTON BW (1994): "Lack of feminization of the cremaster nucleus in cryporchid androgen insensitive rats." Journal of Urology, 152: 2280-2286.

STEINBERGER A (1991): "Effects of temperature on the biochemistry of the testis." Advances in Experimental Medicine and Biology, 286: 33 - 47.

SWANSON HH: Personal communication.

SWANSON HH (1966): "Modification of the reproductive tract of hamsters of both sexes by neonatal administration of androgen or oestrogen." Journal of Endocrinology, 36: 326-327.

TAM PPL AND SNOW MHL (1981): "Proliferation and migration of primordial germ cells during compensatory growth in mouse embryos." Journal of Embryology Experimental Morphology, 64:133-147.

TAYAKKANONTA K (1963): "The gubernaculum testis and its nerve supply." Australian New Zealand Journal of Surgery, 33: 61-67.

THIERY J-P, DELOUVEE A, GALLIN WJ, CUNNINGHAM BA AND EDELMAN GM (1984): "Ontogenic expression of cell adheision molecules: L-CAM is found in epithelia derived from the three primary germ layers." Developmental Biology, 102: 61-78.

TOTH J, MERKSZ M AND SZONYI P (1987): "States causing infertility in adulthood in children with undescended testis." Acta Chirurgica Hungarica, 28: 243-246.

TRAN D (1977): "Anti-Mullerian hormone is a functional marker of fetal Sertoli cells." Nature, 269: 411-412.

TRAN D AND JOSSO N (1982): "Localization of anti-Mullerian hormone in the rough endoplasmic reticulum of the developing bovine Sertoli cell using immunocytochemistry with a monoclonal antibody." Endocrinology, 111: 1562-1567.

TRAN D, PICARD JY, VIGIER B, BERGER R AND JOSSO N (1986): "Persistence of Mullerian ducts in male rabbits passsively immunized agaist

bovine anti-Mullerian hormone during fetal life." Developmental Biology, 116: 160-167.

UPADHYAY S, LUCIANI J-M AND ZAMBONI L (1981a): "The role of the mesonephros in the development of the mouse testis and its excurrent pathways." In Development and Function of Reproductive Organs. ed. Byskov AG and Reters H, Amsterdam: excerpta Medica. pp. 18-27.

UPADHYAY S, LUCIANI J-M AND ZAMBONI L (1981b): "The role of the mesonephros in the development of the mouse testis and its excurrent pathways." In Development and Function of Reproductive Organs. ed. Byskov AG and Reters H, Amsterdam: excerpta Medica. pp. 31-39.

VAN DER SCHOOT P (1992a): "Androgen in relation to prenatal development and postnatal inversion of of the gubernacula in rats." Journal of Reproduction and Fertility, 95:145-58.

VAN DER SCHOOT P (1992b): "Disturbed testicular descent in the rat after prenatal exposure to the antiandrogen flutamide." Journal of Reproduction and Fertility, 96: 483-496.

VAN DER SCHOOT P AND ELGER W (1992): "Androgen-induced prevention of the outgrowth of cranial gonadal suspensory ligaments in fetal rats." Journal of Andrology, 13:534-542.

VAN DER SCHOOT P (1993a): "Doubts about the 'first phase of testis descent' in the rat as a valid concept." Anatomy and Embryology, 187: 203-208.

VAN DER SCHOOT P (1993b): "The name cranial suspensory ligaments in mammalian anatomy should be used only to indicate the structures derived

from the fetal cranial mesonephric and gonadal ligaments." <u>Anatomical Record</u>, 237:434-438.

VAN DER SCHOOT P AND ELGER W (1993): "Perinatal development of gubernacular cones in rats and rabbits: effect of exposure to anti-androgens." <u>Anatomical Record</u>, 236: 399-407.

VAN DER SCHOOT P (1995): "Studies on the fetal development of the gubernaculum in Cetacea." <u>Anatomical Record</u>, 243: 449-460.

VAN DER SCHOOT P, VIGIER B, PREPIN J, PERCHELLET JP AND GITTENBERGER-DE GROOT A (1995): "Development of the gubernaculum and processus vaginalis in freemartinism: Further evidence in support of a specific fetal testis hormone governing male-specific development." <u>Anatomical Record</u>, 241: 211-24.

VAN DER SCHOOT P (1996): "Fetal genital development in Hyrax capensis, a species with primary testicondia: proposal for the evolution of Hunter's gubernaculum." <u>Anatomical Record</u>, 244: 386-401.

WALSH PC, MADDEN JD, HARROD MJ, GOLSTEIN JL, MACDONALD PC AND WILSON JD (1974): "Familial incomplete male pseudohermaphroditism, Type 2. Decreased dihydrotestosterone formation in pseudovaginal perineoscrotal hypospadias." <u>New England Journal of Medicine</u>, 291: 944-949.

WARTENBERG H (1981): "The influence of the mesonephric blastema on gonadal development and sexual differentiation." In Development and Function of Reproductive Organs, ed. A. G. Byskov and H. Peters, Amsterdam: Excerpta Medica. pp 3-12.

WARTENBERG H (1982): "Development of the early human ovary and role of the mesonephros in the differentiation of the cortex." <u>Anatomy and Embryology</u>, 165: 253-280.

WARTENBERG H (1989): "Differentiation and development of the testes." In The Testis, 2nd edn, ed. Burger H & de Kretser D, pp 67-118. New York: Raven Press.

WELLS LJ (1943): "Descent of the testis: anatomical and hormonal considerations." <u>Recent Advances in Surgery</u>, 14: 436-472.

WENSING CJG (1968): "Testicular descent in some domestic mammals I. Anatomial aspect of testicular descent." <u>Proc Kon Akad Wetensch</u>, C71: 423-434.

WENSING CJG (1973): "Testicular descent in some domestic mammals III. Search for the factors that regulate the gubernacular reaction." <u>Proc Kon Akad Wetensch</u>, C76: 196-202.

WENSING CJG (1986): "Testicular descent in the rat and a comparison of this process in the rat with that in the pig." <u>Anatomical Record</u>, 214: 154-160.

WENSING CJG AND COLENBRANDER B (1986): Normal and abnormal testicular descent. <u>Oxford Reviews of Reproductive Biology</u>, 8: 130-164.

WENSING CJG (1988): "The embryology of testicular descent." <u>Hormone Research</u>, 30: 144-152.

WILSON JD (1973): "Testosterone uptake by the urogenital tract of the rabbit embryo." Endocrinology, 92: 1192-1199.

WILSON JD, GEORGE FW, GRIFFIN JE (1981): "The hormonal control of sexual development." Science, 211: 1278-1284.

WILSON JD (1992): "Syndromes of androgen resistance." Biology of Reproduction, 46: 168-173.

WYNDHAM NR (1943): "A morphological study of testicular descent." Journal of Anatomy, 77: 179-88

YAMANAKA J, BAKER M, METCALFE S AND HUTSON JM (1991): "Serum level of Mullerian Inhibiting Substance in boys with cryptorchidism." Journal of Pediatric Surgery, 26: 621-623.

YAMANAKA J, METCALFE SA AND HUTSON JM (1992): "Demonstration of calcitonin gene-related peptide receptors in the gubernaculum by computerized densitometry." Journal of Pediatric Surgery, 27: 876-78.

YOSHINAGA K, HESS DL, HENDRICKX AG AND ZAMBONI L (1988): "The development of the sexually indifferent gonad in the prosimian, Galago crassicaudatus crassicaudatus." American Journal of Anatomy, 181: 89-105.

ZAMBONI L AND UPADHYAY S (1982): "The contribution of the mesonephros to the development of the sheep fetal testis." American Journal of Anatomy, 165: 339-356.

ZHOU B, WATTS LM AND HUTSON JM (1993): "Germ cell development in neonatal mouse testes *in vitro* requires Mullerian inhibiting substance." Journal of Urology, 150: 613-616.

ZHOU B AND HUTSON JM (1995): "Human chorionic gonadotropin (hCG) fails to stimulate gonocyte differentiation in newborn mouse testes in organ culture." Journal of Urology, 153: 501-05.

APPENDICES

APPENDIX 1

PREPARING GLUTARALDEHYDE FIXATIVE

To prepare 1000 ml of 3% glutaraldehyde and 1% paraformaldehyde in 0.1 M

phosphate buffer:

1) Heat 350 ml distilled water in a conical flask until water temperature

reaches 68 - 70 °C.

2) Measure 10 mg of paraformaldehyde and empty into the above flask and

stir. Add concentrated sodium hydroxide solution drop-by-drop (about 10 - 12

drops) into it until all dissolved.

3) Measure and add 120 ml of 25% glutaraldehyde.

4) Add 4 ml of 0.5% calcium chloride.

5) Add 500 ml of 0.2M phosphate buffer.

6) Make the volume to 1000 ml by adding distilled water.

7) Filter the solution.

APPENDIX 2

PROCESSING TISSUE FOR TRANSMISSION ELECTRON

MICROSCOPY

Specimens must be small (about 1 x 2 x 3 mm)

1) Rinse in 0.1M phosphate buffer	30 min (x 3)
2) Osmicate with 1% osmium tetrahydroxide	1 - $1\frac{1}{2}$ hours
3) Rinse in 0.1M phosphate buffer	30 min (x3)

Dehydrate:

4) 70% ethanol	2 hours
5) 90% ethanol	2 hours
6) Absolute alcohol	2 hours (x 4)
7) Propylene oxide (PO)	15 min (x 3)
8) Mixture of PO : Araldite (1 : 1)	4 hours
9) Mixture of PO : Araldite (1 : 3)	overnight
10) Araldite	48 hours (x 2)

11) Embed in araldite and leave to polymerise in an oven at 60°C for 24 hours

APPENDIX 3

Periodic acid, Schiffs - Haematoxylin (PAS - H) staining

Paraffin sections cut at 5 μm thick, mounted on glass slide and dehydrated in an oven at 37°C overnight. For fragile tissue a further 2 hrs in an oven at 56° C is necessary to prevent sections floating off.

1) Place slides in xylene to deparaffinize   10 min.

OR:

Resin sections cut at 2 μm thick, mounted on glass slides and dehydrated on a hotplate.

1) Deresinate resin sections in ethoxide   30 min.

(saturated sodium hydroxide in ethanol)

Hydrate:

2) 1st Absolute Alcohol   30 sec - 1 min.

3) 2nd Absolute Alcohol   30 sec - 1 min.

4) 90% Alcohol   1 min.

5) 70% Alcohol   2 min.

6) Wash in running water   5 min. (x 2)

7) Periodic Acid   10 min.

8) Wash in running water   5 min.

9) Schiffs (Feulgens) stain   10 - 30 min.

                                               (Until brick red)

10) Wash in running water until clear

11) Celestine Blue   5 min.

12) Haematoxylin (Meyer's)   5 min.

13) Wash in running water until blue

14) Dehydrate to absolute alcohol

15) Clear in xylene   5 min.

16) Mount in histomount

RESULTS

Glycogen, mucin, etc   bright red

Nuclei   blue

General connective tissues   yellowish

APPENDIX 4

RADIOIMMUNOASSAY (RIA) OF PLASMA AND TESTICULAR TESTOSTERONE:

Extraction of testosterone

All tubes are set up in duplicate. For the standard curve, the tubes consisted of total counts, normal bovine serum and standards TO to T9, and the quality control (QC) pools (low, medium, high, normal male and high female).

For the samples, testosterone was extracted from plasma and testicular homogenates: 2 measure of 100 µl of plasma (or 50 µl of testicular homogenates) were placed in two separate tubes. (For testicular homogenates - dilute with an equal amount (50 µl) of distilled water to make the volume to 100 µl). Then, 3 ml of diethyl ether was added before agitating the mixture using a multi-tube vortexer for 3 minutes with a speed set to ensure that the lower aqueous phase is spun off the bottom of the tube.

The tubes were allowed to stand for 5 minutes at room temperature to separate the different phases (i.e. the lower aqueous phase and the upper ether phase); the lower phase was then allowed to freeze in a bath of methanol containing carbon dioxide pellets for about 30 seconds before decanting the solvent phase into another assay tube. The solvent was then allowed to evaporate in the 'Buchler Vortex Evaporator', thus leaving the testosterone as sediment at the bottom of the tube.

Staining the testosterone

300 μl of 0.25% bovine serum albumen (NSB) in buffer pH 7.4 (prepared fresh for each assay) was added to each tube containing the dried testosterone sediment or control (without testosterone) followed by 100 μl of the primary antibody (1° antibody against testosterone raised in rabbit with titre 1:6000) and 100 μl of the radioactive tracer (^{125}I-histamine-testosterone). The mixture was then briefly agitated and incubated for 1-2 hours at room temperature. Afterwards, 500 μl of doubled antibody reagent (Donkey anti-rabbit in rabbit serum) was added and then incubated at 4° C overnight.

Separation:

Finally, the assay tubes were centrifuged at 2,500 rpm for 25 minutes at 4°C. The supernatant fraction was then removed using a finely drawn out glass Pasteur pipette. Assay tubes were counted for sufficient time on the NEN 1600 gamma counter (linked to the Commodore 4032 computer and printer 4022) to accumulate 10,000 counts in the total count tubes. (NB: Where a 50 μl sample has been extracted, the result obtained should be multiplied by 2).

APPENDIX 5

HISTOKINETTE AUTOMATIC TISSUE PROCESSOR

Fixed specimens were rinsed to remove as much fixitive as possible with three changes of 0.1 M phosphate buffer. Any bony components were decalcified with buffered 10% EDTA to facilitate cutting. Specimens were placed in separate histokinette baskets and labelled.

1) 70% ethanol   2 hours

2) 90% ethanol   2 hours

3) Absolute alcohol   2 hours   (x 3)

4) Amyl acetate   2 hours   (x 3)

5) Wax bath   2 hours   (x 2)

Multi-tissue specimens were subjected to vacuum extraction to get rid of air which may be present. After embedding in wax they could then be sectioned once properly set.

APPENDIX 6

HAEMATOXYLIN & EOSIN STAINING

Slides should always be left overnight in an oven at 37° C (for fragile tissue a further 2 hrs in an oven at 56° C) to prevent sections floating off.

1) Place slides in xylene to dewax   10 min.

Hydrate:

2) 1st Absolute Alcohol   30 sec - 1 min.

3) 2nd Absolute Alcohol   30 sec - 1 min.

4) 90% Alcohol   1 min.

5) 70% Alcohol   2 min.

6) Wash in water   5 min. (x 2)

7) Haematoxylin (Mayer's or Harris's)   4 - 5 min.

8) Wash in water   30 sec - 1 min. (x 2)

9) Differentiate in 1% Acid Alcohol   a dip

10) Wash in water   30 sec - 1 min.

11) Blue in Scotts. Microscopic check. If haematoxylin is too heavy further differentiation in acid alcohol is required

12) Wash in water   30 sec -1 min. (x 2)

13) Eosin   1 - 2 min.

14) Wash in water   30 sec -1 min. (x 2)

15) Dehydrate to alcohols

16) Mount in histomount

Staining times are a guide. Microscopic examination is essential

<u>RESULTS</u>

Nuclei   - Blue

Other tissues - Varying shades of pink and red

APPENDIX 7

PROCESSING TISSUE FOR SCANNING ELECTRON MICROSCOPY

1) Specimens rinsed with 0.1M phosphate buffer   30 min. (x 3)

2) Post-fixed with 1.0% buffered OsO_4 30 min. - 1 hours

 (osmium tetrahydroxide)

3) Rinse again in 0.1M phosphate buffer   30 min. (x 3)

Dehydrate:

4) 50 % Acetone   2 hours

5) 70 % Acetone   2 hours

6) Acetone   2 hours (x 3)

7) *HexaMethyl-DiSilazane   1 - $1\frac{1}{2}$ hours

8) Leave to evaporate   overnight (~16 hours)

9) Mount on stub and coat with gold

* For smaller specimens, HexaMethyl-Disilazane is sufficient to dry the

specimens. Bigger specimens require critical point drying using liquid CO_2.

APPENDIX 8

IMMUNOCYTOCHEMICAL STAINING FOR ANDROGEN

RECEPTORS

1) Dewax sections and hydrate to water

2) Ring individual sections with a wax pen

3) PBS rinse	5 min. (x 3)
4) Blocking serum (1% NGS)	60 min.
5) Primary antibody in diluent	overnight
6) PBS rinse	5 min. (x 3)
7) Biotinylated secondary antibody in diluent	60 min.
8) PBS rinse	5 min. (x 3)
9) ABC complex	60 min.
10) PBS rinse	5 min. (x 3)
11) Phosphate buffer 0.1M PB	5 min.
12) DAB + 1% H_2O_2 to conc. of 0.01%	5-10 min.
13) PB rinse	5 min. (x 2)

14) Dehydrate, clear and mount.

0.3% Triton in PBS: 30 µl Triton in 10 ml PBS

Blocking serum: 1% goat serum in PBS with 0.3% Triton

              10 µl goat serum + 990 µl 0.3% Triton/PBS

Antiserum diluent: 1% goat serum in PBS with 0.3% Triton

              10 µl goat serum + 990 µl 0.3% Triton/PBS

Primary antibody: Androgen Receptor antibody

              Dilute in above diluent - dilution used was 1:30

Biotinylated second antibody: (anti-Rabbit Ig)

              5 µl antibody in 1 ml diluent

ABC reagent: 1 ml PBS + 20 µl reagent A + 20 µl reagent B

              Mix well immediately and allow to stand for > 30 min

DAB solution: 1 aliquot DAB + 50 ml PB + 7.5 µl H_2O_2 and filter (used

immediately)

              Dip sections between 5 - 20 min - depends on colour.