To the Graduate Council:

I am submitting herewith a dissertation written by Rene E. Sotomayor entitled "Analysis of Action of a Male-Sterile Mutation of the Mouse, Blind-Sterile, bs." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Life Sciences.

Mary Ann Handel

Mary Ann Handel, Major Professor

We have read this dissertation
and recommend its acceptance:

Accepted for the Council:

Vice Provost
and Dean of the Graduate School

ANALYSIS OF ACTION OF A MALE-STERILE MUTATION OF THE MOUSE, BLIND-STERILE, *bs*

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

Rene E. Sotomayor

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ABSTRACT

Blind-sterile (bs) is a new autosomal recessive mutation of the mouse that causes sterility in males and bilenticular cataracts in both sexes. The cellular and hormonal effects of the bs mutation on spermatogenesis were investigated by light and electron microscopy, radioimmunoassay and cytochemical techniques.

Sterile bs/bs male mice exhibited normal copulatory behavior, reduced testis weight and few or no epididymal sperm. All sperm present were morphologically abnormal with aberrant head shapes. Testes from adult bs/bs mice were characterized by germ cell deficiency that results in profound alterations of the typical germ cell associations. Only 30% of the tubule cross sections contained relatively normal germ cell associations. The rest showed various degrees of germ cell deficiency, the most deficient tubules containing only Sertoli cells. The most striking effect of the bs mutation was the failure of acrosome formation during spermiogenesis. Disorganized proacrosomic granules were detected up to step 3 of spermiogenesis by both periodic acid-Schiff staining and ultrastructural analysis. Not a single acrosomal cap or fully developed acrosome was detected in over 3500 spermatids scored past steps 3-4 of spermiogenesis. Electron microscopy revealed a thickening of the nuclear envelope in the region where the acrosome should have been located; however, no acrosome was present. The thickening of the envelope spread in a caudal direction over the surface of the nucleus through subsequent spermiogenic stages. The acrosomeless nuclei of mutant spermatids

underwent elongation and chromatin condensation, processes temporally associated with the presence of a well developed manchette in these spermatids. This observation led to the conclusion that nuclear elongation and condensation can be initiated in the absence of acrosome development; therefore, these processes are not mechanistically dependent on the acrosome. Since the final shape attained by bs spermatids was abnormal, either the bs gene affects head shaping as well as acrosome formation or the acrosome itself plays an important role in nuclear shaping.

In order to assess whether these effects of the mutant bs gene on spermiogenesis might be due to steroidogenic and/or gonadotropic disfunctions, circulating levels of testosterone, FSH and LH were determined. Ultrastructural studies of Sertoli and Leydig cells were also performed to investigate possible correlates with the endocrinological profiles. Circulating testosterone levels varied extensively in both mutant and normal mice. Mutant mice showed lower mean testosterone values than the normal littermates, but differences were not statistically significant. Circulating FSH levels were significantly elevated in mutant mice. LH levels were similar in both groups of mice. These hormonal changes may well be related to apparent morphological and quantitative changes of Sertoli and Leydig cells. Estimates of the relative numbers of these cells indicated that they were reduced in mutant testes. Some Sertoli cells contained morphologically abnormal nuclei, characterized by atypical nucleoli and abundant heterochromatin clumps associated with the nuclear envelope. Leydig cells had numerous whorls of SER and lipid droplets

in the cytoplasm. Since similar hormonal and structural changes have been demonstrated in other pathological conditions affecting the testes, it is suggested that the effects of the bs mutation on reproductive hormones is an indirect one, probably triggered by the induction of germ cell depletion.

One of the goals of this research was to determine whether germ cell deficiency was a consequence of the abnormal spermatid differentiation or was an independent effect of the bs mutation. A postnatal developmental study was undertaken to investigate the onset of germ cell depletion in mutant testes. Testes from mice 5 to 40 days of age were cytologically examined. Results showed that germ cell deficiency occurred before 5 days of age in mutant testes, thus demonstrating that germ cell deficiency is independent of spermiogenic effects. The number of gonocytes, type A spermatogonia and the mitotic index were estimated in the 5 day old testes. A reduction in the number of gonocytes and type A spermatogonia per tubule cross section was demonstrated in the mutant testes. However, the mitotic index was similar in mutant and normal testes. These results suggest that germ cell deficiency may occur as a result of insufficient numbers of primordial germ cells populating the testis.

Finally, an investigation was undertaken of the role of the Golgi apparatus in the failure of acrosome assembly. Since acrosomes are derived from the Golgi apparatus, it was possible that the Golgi was structurally and functionally defective in mutant spermatids. Golgi enzyme markers, cytidine monophosphatase (CMPase), thiamine pyrophosphatase (TPPase) and nicotinamide adenine dinucleotide

phosphatase (NADPase) were cytochemically analyzed in spermatocytes and spermatids. CMPase activity was limited to 1 or 2 saccules of the trans side of the Golgi. In early spermatid stages, CMPase was also detected in proacrosomic vesicles and proacrosomic granules in both control and mutant testes. No CMPase activity was observed in the Golgi of spermatids past steps 6-8 of spermiogenesis. TPPase activity was demonstrated in the mid-to-trans saccules of the Golgi of both control and mutant spermatogenic cells. Unlike CMPase, TPPase continue to be present in the Golgi of spermatids past-steps 6-8 of spermiogenesis. No activity was detected in the fully formed acrosomic granules. No NADPase activity was detected in mutant or normal germ cells. These results indicate that the Golgi apparatus of mutant germ cells was structurally and functionally normal and that it contributed to the formation of the proacrosomic vesicles.

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GENERAL INTRODUCTION

Mammalian spermiogenesis is a complex well ordered sequence of events that results in the transformation of a relatively undifferentiated cell, the round spermatid, into a highly differentiated one, the spermatozoon, capable of efficiently transmitting the parental genome to the egg. The differentiative process has been amply described by light microscopy (Leblond and Clermont, 1952; Clermont and Leblond, 1952; Clermont, 1972; Oakberg, 1956a, 1956b), ultrastructural observations (Fawcett and Phillips, 1970; Fawcett et al., 1971; Fawcett, 1975; Sandoz, 1970; Susi et al., 1971; Hermo et al., 1980; Clermont et al., 1981; Pelletier and Friend, 1983) and biochemical studies (see Bellve, 1979; Bellve and O'Brien, 1983, for reviews).

During the course of spermiogenesis haploid spermatids undergo profound morphological and molecular changes. These include: formation of the acrosome by the Golgi apparatus (Burgos and Fawcett 1955; Sandoz, 1970; Susi et al., 1971; and Fawcett, 1975); condensation and elongation of the spermatid nucleus (Fawcett et al., 1971; Dooher and Bennett, 1973); assembly of the flagellum (Fawcett and Phillips, 1969, 1970); formation of a transient microtubular structure, the manchette, which may have a role in redistributing the cytoplasm (Fawcett et al., 1971; elimination of most of the RER and the Golgi apparatus (Dietert, 1966), and the obvious acquisition of a hydrodynamic shape by the differentiated sperm.

Description of Mouse Spermiogenesis

During the Golgi phase of spermiogenesis, stages 1 to 3 of Oakberg's classification (Oakberg, 1956), two concurrent events are taking place. The proacrosomic vesicles derived from the Golgi apparatus appear in the spermatid cytoplasm and fuse to form a single, larger proacrosomic vesicle, containing the proacrosomic granule. Almost simultaneously one member of a pair of centrioles, the distal one, begins to elaborate the flagellum (Dooher and Bennett, 1973; Fawcett, 1975).

At the beginning of stage 3 the large proacrosomic vesicle, containing the proacrosomic granule, attaches to a specific site on the anterior pole of the nucleus and an intimate association between the respective membranes develops. By freeze-fracture and transmission EM analysis it has been possible to determine that the nuclear envelope, at the implantation site, develops extensive modifications. These include a thickening of the nuclear envelope by close contact of the two adjacent membranes, caudal migration of the nuclear pores and the formation of a depression on the nuclear envelope opposite to the acrosomal membrane (Dooher and Bennett, 1973; Pelletier and Friend, 1983).

During the "cap phase" of spermiogenesis, which includes stages 4 to 7 of Oakberg's classification, the acrosome increases in size and spreads caudally over the anterior one-third of the spermatid nucleus.

The next step of mouse spermiogenesis is the acrosomic phase, which according to Oakberg (1956) begins at stage 8 and lasts until stage 11. Several morphological changes are manifest during the

acrosomic phase. Nuclear elongation is pronounced during these stages. Within the acrosome the centrally located granule, which is very prominent during early stages, disappears abruptly and the acrosome content acquires a more homogeneous appearance. The anterior pole of the spermatid becomes closely invested in a cytoplasmatic differentiation of the Sertoli cell, the mantle. No nucleoli are visible during the acrosomic phase (Dooher and Bennett, 1973).

At the beginning of stage 8 a conspicuous, transitory microtubular structure, the manchette becomes established (Fawcett et al., 1971; Dooher and Bennett, 1973). The microtubules of the manchette take up a position tangential to the caudal half of the spermatid nucleus. Simultaneously near the posterior margin of the acrosome and close to the plasma membrane a unique fibrillar specialization occurs, encircling the nucleus. This structure has been called the nuclear ring (Fawcett et al., 1971). The orientation of the microtubules changes as spermiogenesis proceeds. At the beginning the microtubules are oriented obliquely, closely applied to the nuclear envelope in the postacrosomal region, as if they were flattening the round contour of the nucleus. Actually the nucleus acquires a truncated pyramidal shape.

Since the manchette with its nuclear ring is intimately associated with the caudal margin of the acrosome it would be important to determine whether the two events are interdependent and the possible effects of acrosome abnormalities on the development of the manchette. In turn these factors may influence the final shape of the spermatid head. It is interesting to note that when the manchette

microtubules depolymerize by stage 15, mitochondria are able to gather around the flagellum in the typical helical fashion, as if they had been prevented to do so by the manchette (Fawcett et al., 1971).

The maturation phase includes spermatid stages 12 to 16. The most striking event during these stages is the initiation of chromatin condensation at stage 12 (Dooher and Bennett, 1973). The maturation phase lasts about 5 days in the mouse.

Condensation of the spermatid nucleus is manifest by a series of morphological and molecular changes which have been demonstrated by electron microscopic and biochemical techniques. One of the first signs of nuclear condensation in the mouse spermatids is the appearance of a dense core of fibrillar chromatin, centrally located in the nucleus. From detailed EM pictures published by Dooher and Bennett (1973), it can be seen that by stage 13 about one-third of the anterior region of the nucleus has become uniformly condensed while the caudal region is somewhat delayed in condensing. Chromatin fibers and granules are recognizable in the posterior nuclear region up until stage 14. By stage 15, however, chromatin is totally condensed, resembling the general appearance of a mature sperm nucleus.

A precise sequence of molecular events has been correlated with the condensation of chromatin. Histones, which are basic proteins associated with DNA during mitotic stages, are gradually replaced by more basic proteins, first by testis protein (TP) and then by protamines (Bellve, 1979). TP appears during the initiation of chromatin condensation and last until stages 14-15 (Mayer et al., 1981). These proteins are then replaced by protamines which are rich

in cysteine and arginine and are retained until completion of spermiogenesis and sperm maturation in the epididymis (Grimes et al., 1977; O'Brien and Bellve, 1980). The loss of DNA repair capabilities by the mature sperm appears to be also related to the replacement of histones for protamines (Sega, 1974; Sotomayor et al., 1978; Sotomayor et al., 1979; Sega and Sotomayor, 1982).

Many problems of sperm differentiation remain unsolved. For instance, it is tempting to hypothesize that nuclear elongation and condensation depend on the formation of the acrosome, given the close temporal relationship between the two events. Does the attachment of the proacrosomic vesicle to the nuclear envelope represent a signal to start nuclear elongation? Is the presence of an acrosome a prerequisite for the condensation of the spermatid nucleus?

The Genetic Approach

An approach that is proving very informative in trying to elucidate problems of male gamete differentiation is the use of mutations in mice that perturb the normal course of spermatogenesis. The strengths of mutant analysis have been well demonstrated in other fields of biological research where it has been instrumental in the elucidation of mechanisms of gene action and gene control. The effects of these gene mutations on spermatogenesis have been reviewed by Searle (1982).

Of particular interest are various pleiotropic gene mutations which affect structural components of the male germ cells. Some of these mutations include: blind-sterile, bs; Purkinje cell

degeneration, pcd; quaking, qk; pink-eyed sterile, p^s and other alleles of the locus.

The effects of pcd on mouse spermatogenesis have been studied by Handel and Dawson (1981). These authors found that elongating spermatids and maturing spermatids contained structural abnormalities characterized by abnormally shaped heads and tails with extraneous ectopic outer dense fibers. The sperm counts were reduced and the sperm were non-motile. The authors concluded that the presence of extraneous outer dense fibers in the pcd spermatids is probably due to a defect in the assembly process early during spermiogenesis.

Bennett et al. (1971) have studied the effects of qk on spermatogenesis. Two principal morphogenetic effects were found. These included disorganization of the axoneme and abnormal spermatid nuclei.

Bryan (1977) has shown that males homozygous for p^s are sterile because spermiogenesis is abnormal from the Golgi phase on (the initial phase). The acrosome develops abnormally showing multiple implantation fossa on the nucleus and irregular distribution of acrosomal material during the caudal spreading of the acrosome cap. These and other pertubations give rise to a spectrum of sperm abnormalities, ranging from highly aberrant to nearly normal sperm heads. Flagellar ultrastructure appear normal even in those sperm lacking heads.

One observation derived from these studies is that the development of the sperm head appears to be influenced by a number of factors, incluidng genes whose primary effects are not related to

sperm head shaping. For instance, gk and pcd which cause defects of the axoneme and disorganization of the tail outer dense fibers, respectively, also cause sperm head abnormalities. Since all these mutations are pleiotropic, it is likely that effects such as those of pcd and gk on sperm head differentiation are indirect, secondary to the main effect of the gene.

Other gene mutations appear to act upon specific germ cell structures more selectively. Thus, p^s and, as we will see later, also bs, affect sperm head differentiation but have no apparent effects on tail organelles. The "blind-sterile mutation" (bs) is a recessive, pleiotropic mutation, located on chromosome 2 (Varnum, 1983). In the homozygous state it causes sterility in males but not in females and bilenticular cataracts in both sexes. This mutation appears to be an excellent model for studies of the genetic control of specific events during spermiogenesis.

Specific Objectives

The focus of the present research is an analysis of effects of the bs mutation on male gametogenic differentiation. In the first part of this research, the cellular effects of the bs mutation on spermatogenesis were studied. Results indicate that this mutation causes a failure of acrosome assembly in spermatids and extensive deficiency of germ cells. The autonomy of the nuclear elongation and chromatin condensation processes with regard to the formation of the acrosome was established.

In the second part, the objective was to determine reproductive hormonal effects and their possible association with structural abnormalities of the Sertoli and Leydig cells. Results show that mutant mice had lower testosterone levels than normal mice although great variability in the testosterone levels was observed. FSH levels were significantly higher in mutants than in normal mice. No differences were found with regard to LH levels.

The third part was aimed at the problem of germ cell deficiency in the mutant testes. Is deficiency of germ cells an effect independent from the onset of spermiogenesis or is it triggered by the lack of acrosome formation and subsequent elimination of spermatid stages? Results indicate that germ cell deficiency occurs early during postnatal development of the testis. Therefore it is not related to the formation of defective spermatids. It is suggested that insufficient numbers of gonocytes populating the testis may be responsible for this effect.

The final part of this research deals with the Golgi apparatus in mutant germ cells. Is the Golgi apparatus structurally and functionally normal in the mutant spermatids and other germ cells? The presence and distribution of 3 enzyme markers of the Golgi are studied using EM cytochemical techniques. The results suggest that the mutant Golgi is functionally and structurally competent for the enzymes studied and that contributes to the formation of the proacrosomic vesicles during early spermatid stages. However, these results do not exclude the possibility that the Golgi could secrete

other defective proteins or enzymes that might be directly involved in the failure of assembly of the acrosome.

Significance

The present research is important for a number of reasons. The finding that a recessive gene mutation can cause complete failure of acrosome assembly permits an analysis of the specialization of a specific organelle from a genetic perspective. This is the first case reported in mammals of a well documented mutational event which causes the lack of assembly of a unique spermatogenic cell organelle, namely the acrosome. Our research on this mutant has provided information about the autonomy of a major differentiation processes during spermiogenesis: acrosome formation-nuclear elongation-chromatin condensation. Such information has not been hitherto available because of the inability to separate nuclear shaping from acrosome formation, as this mutant permits. The mouse mutant systems provide models for unraveling the causes of human male sterility and, more importantly, for understanding the basic mechanisms of male germ-cell differentiation in mammals.

INTRODUCTION

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PART I

FAILURE OF ACROSOME ASSEMBLY IN A MALE STERILE MOUSE MUTANT

FAILURE OF ACROSOME ASSEMBLY IN A MALE STERILE MOUSE MUTANT

Abstract

Blind-sterile (bs), is a new autosomal recessive mutation of the mouse which causes sterility in males and bilenticular cataracts in both sexes. Sterile bs/bs males exhibited normal copulatory behavior, reduced testis weights, and few or no epididymal sperm. The effects of the bs mutation on spermatogenesis were examined by light and electron microscopy. All sperm present were morphologically abnormal with aberrant head shape. Adult bs/bs testes were characterized by germ cell depletion which resulted in profound alterations of the typical germ cell associations. Only 30% of the tubules contained relatively normal germ cell associations while 39% were extensively depleted, showing only Sertoli cells or Sertoli cells and spermatogonia. The most striking effect of the bs mutation on spermiogenesis was the failure of acrosome formation. Disorganized proacrosomic granules were detected up to step 3 of spermiogenesis by both periodic acid-Schiff staining and ultrastructural analysis. In over 3500 spermatids scored past steps 3-4 of spermiogenesis not a single acrosomal cap or fully developed acrosome was detected. Electron microscopy revealed a thickening of the nuclear envelope of elongating spermatids in the region where the acrosome should have been located, however no acrosome was present. Chromatin condensation and nuclear elongation did occur in these acrosome-less spermatids, suggesting that caudal growth of the acrosome is not a mechanistic factor in these events.

Introduction

Mutations affecting either the process of spermatogenesis or the structure of sperm cells can be useful for elucidating mechanisms of spermatogenic differentiation. A number of such mutations have been identified in the mouse (Searle, 1982). All of these mutations are pleiotropic, affecting processes other than spermatogenesis; and all cause a number of sperm structural abnormalities, head shape abnormalities being predominant. A new mutation, blind-sterile, bs, described here, has potential for elucidating the relationship between acrosome development and sperm nucleus morphogenesis.

The bs mutation, when homozygous, causes sterility in males and bilenticular cataracts in both sexes (Varnum, 1983). The gene is inherited as an autosomal recessive and has been mapped to chromosome 2, adjacent to the agouti locus (Varnum, 1983). We report here an analysis of the cellular effects of this mutation on spermatogenesis, documenting both germ cell depletion and abnormal spermiogenesis. The most striking finding is that bs spermatids fail to develop acrosomes; nonetheless, the nucleus undergoes elongation and condensation, thus suggesting that the acrosome does not play a mechanistic or determinative role in these processes.

Materials and Methods

Mice

Male mice homozygous for the blind-sterile, bs, mutation and their normal littermates (bs/+ or +/?) were used in this study. The bs/bs mice were recognized by prominent cataracts observable soon

after birth. The mice were derived from the F2 of (AKR-bs X 129) nybrids originally received from the Jackson Laboratory (Bar Harbor, ME) and subsequently maintained in the University of Tennessee Animal Facility by brother-sister mating of bs/bs or bs/+ females with bs/+ males. All mice were provided with food and water ad libitum and were maintained on a 14 hr L:10 hr D light schedule.

Mating

To determine whether mutant mice mate, 7 adult bs/bs males (3 to 5 months old) were paired with mature HA/ICR females (procured from Cumberland View Farms, Clinton, TN). Matings were set up with one pair of mice per cage. Females were inspected daily for the presence of vaginal plugs, signs of pregnancy or litters. Two of the males were given another female after a month of mating without a pregnancy.

Light and Electron Microscopy

Sperm preparations for light microscopy were made from animals killed by cervical dislocation or, more frequently, anesthetized with an injection of Avertin. The caudae epididymis and vasa deferentia were removed and sperm expressed into 1 ml of phosphate buffered saline (pH 7.0) with 0.1% glucose. After checking for sperm motility using phase contrast optics, the sperm suspension was made 10% in formaldehyde. The number of sperm in the sample was determined using a hemocytometer. Air dried sperm smears were prepared from the same suspension and stained 24 hours later with Bryan's stain (Bryan, 1970). Preparations were dehydrated in ethanol, and coverslips mounted with Permount (Fisher). Preparations were scored for sperm

abnormalities with a Zeiss microscope at 400X. Criteria for scoring were according to Wyrobeck and Bruce (1975, 1978).

For scanning electron microscopy sperm samples were fixed in 3% glutaraldehyde, 2% paraformaldehyde, in 0.1M sodium cacodylate buffer (pH 7.1) with 0.001M Ca++ and Mg++ at room temperature. Drops of the sperm suspension were placed on round coverslips, presoaked in 0.1% poly-D-Lysine (Sigma) and dehydrated through ethanols and acetone. The preparations were dried in a Sorvall critical-point dryer and coated with gold in a Denton Vacuum DV-515 automatic evaporator. The sperm specimens were examined and photographed with an ETEC Autoscan scanning electron microscope.

Testes for light microscope analysis were weighed and immediately fixed in Zenker-formol, embedded in paraffin, sectioned at 7μm, stained with periodic acid-Schiff (PAS), and counterstained with Ehrlich's hematoxylin (Leblond and Clermont, 1952). The stages of spermatogenesis in 7 bs/bs adult mice were compared to those of three normal littermates. For each animal, more than 200 seminiferous tubule cross sections were analyzed using criteria of the morphology of PAS-stained spermatids (Oakberg, 1956; Leblond and Clermont, 1952). Because bs/bs testes showed extensive germ cell depletion and no development of the acrosome past the granule stage, the classical 12 stages of the seminiferous epithelium could not be recognized in these testes. It was necessary to develop an arbitrary staging for these abnormal testes. Spermatid stages 1-7, 8-12, and 13-16 of bs/bs testes were classified according to presence of proacrosomal granules (for the early stages), nuclear morphology, and germ cell

associations. In order to quantitate the effect of the bs mutation on the acrosome, round and elongating spermatids were scored for the presence of proacrosomic granules, acrosomic caps and acrosomes, respectively, in the paraffin-embedded, 7µm testicular sections.

Testes for ultrastructural analysis were removed from anesthetized mice and immersed in a fixative of 3% glutaraldehyde, 2% paraformaldehyde in 0.1M sodium cacodylate buffer (pH 7.1) with 0.001M Ca++ and Mg++ at room temperature. Tissue pieces were rinsed in cold buffer and post-fixed in 1% OsO4 in the same buffer at 4oC, dehydrated in ethanol, infiltrated with propylene oxide, and embedded in Epon. Thin sections were cut with a Reichert OM U-3 ultratome, stained with uranyl acetate and lead citrate, examined and photographed using a Hitachi H600 or Zeiss 9S electron microscope. Thin sections from 15 bs/bs males and 11 +/? males were analyzed.

Some testes were embedded in Epon-araldite and were sectioned at 1 µm using a Sorvall JB-4 microtome. These preparations were stained with PAS-Ehrlich's hematoxylin for light microphotography.

Results

Mating

All 7 bs/bs males tested mated when provided with females. Vaginal plugs were found at regular intervals in all 9 of the females paired with each of the 7 males used in this study. This evidence suggested that the sterility of bs/bs male mice was not due to failure to mate and was therefore due to other abnormalities of male reproduction.

Sperm Counts, Sperm Morphology and Testis Weight

The majority of the bs/bs mice (61%) had no detectable numbers of sperm in the epididymis or the vas deferens (Table I-1*). The rest of the mutant mice had reduced sperm numbers ($0.22 \pm 0.35 \times 10^6$ sperm), and these sperm were non-motile. In comparison, normal littermates had a mean sperm count of $14.4 \pm 3.5 \times 10^6$ sperm. In both aspermic and oligospermic mutant mice, large numbers of degenerating immature germ cells were observed in the epididymides.

Testis weights in the bs/bs mice averaged about 1/3 to 1/2 that of control littermates, reflecting both germ cell depletion and failure of normal spermatogenesis (Table I-1). The mean testis weight of normal mice was 101.8 ± 16.7 mg.

The mutant sperm when produced were all morphologically abnormal (Fig. I-1,* Table I-2). Three broad classes of sperm abnormalities were detected. The most frequent one was characterized by severely distorted sperm heads. Typically the sperm head which contained an abnormally shaped nucleus was folded back on the middle piece (Fig. I-1). Another class of sperm abnormality was characterized by the presence of mitochondrial clusters and no apparent nuclei. A third class which occurred rarely was distinguished by the presence of highly looped tails and abnormally shaped nuclei. Table I-2 shows the relative frequencies of these classes of sperm abnormalities.

Testicular Histology

Analysis of PAS-stained, paraffin-embedded testicular

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*All tables and figures may be found in the Appendix.

preparations revealed drastic alterations of the structure of the seminiferous epithelium in bs/bs testes, indicative of both germ cell deficiency and degeneration (Fig. I-2). In order to determine the extent of germ cell depletion, the types and frequencies of various germ cell associations were studied in tubule cross-sections. Five types were found in bs/bs testes and were arbitrarily classified as types I, II, III, IV and V, respectively (Table I-3). The most depleted tubules contained only Sertoli cells or Sertoli cells and spermatogonia (type I). This type was found in 39 percent of the tubule cross-sections. Other tubule cross-sections contained Sertoli cells, spermatogonia and few round or elongating spermatids (type II). These occurred at a low frequency. Moderately depleted tubules (type III) represented about 28 percent of the total and were characterized by the presence of Sertoli cells, spermatogonia, meiotic spermatocytes and round spermatids in steps 1-7 of spermiogenesis. In many cases the round spermatids were aggregated into a large multinucleated cell located close to the lumen of the seminiferous tubule (Fig. I-2). Some tubule cross-sections showed relatively normal germ cell associations, comparable to those of the control testes (types IV and V). Type IV contained Sertoli cells, spermatogonia, meiotic spermatocytes and early elongating spermatids, presumably in steps 8 to 12 though definitive staging was not possible given the absence of acrosomes. Type V had Sertoli cells, spermatogonia, meiotic spermatocytes, round spermatids and maturing spermatids, presumably in steps 13 to 16. On the average, 11 and 19 percent of the tubule cross-sections showed these types of germ cell associations,

respectively. In comparison, normal testes showed about 40 and 60 percent of types IV and V.

The most striking finding from the analysis of PAS-stained testicular preparations was the failure of acrosomal development in the spermatids of bs/bs mice (Fig. I-3). In over 3500 spermatids scored past stage 3 of spermiogenesis from 6 bs/bs mice not a single acrosome or acrosomal cap was found (Table I-4). However, round spermatids in stage 2 showed PAS-positive proacrosomic granules (Fig. I-3, Table I-4). The proacrosomic granules appeared disorganized and intensely stained with the PAS technique. In 61% of the early spermatid stages, multiple proacrosomic granules were observed close to the nucleus or lying on the nuclear membrane. This material never formed an acrosomal cap in subsequent spermatid stages (Fig. I-3). In contrast spermatids from control littermates showed normal PAS-staining acrosomal granules and fully formed acrosomes (Fig. I-3, Table I-4).

Ultrastructure of Spermatogenesis in bs/bs Mice

Analysis of the ultrastructure of bs/bs testes revealed abnormal spermiogenesis. Early round spermatids were characterized by abundant profiles of the Golgi apparatus at the apical end of the nucleus with dense material within the cisternae of the Golgi vesicles (Fig. I-4). Numerous proacrosomic granules appeared during stages 1 and 2 between the Golgi saccules and the nucleus. In some cases vesicles fused to form one or more proacrosomal vesicles; and in some round spermatids more than one proacrosomal vesicle attached to the nuclear envelope (Fig. I-4). Subjacent to the proacrosomal vesicle

the nuclear envelope assumed a thickened appearance, resulting from the deposition of fine fibrillar material on the inner aspect of the inner membrane and the apparent fusion of the inner and outer membranes of the nuclear envelope (Fig. I-4 and I-5). No further development of the proacrosome was detected but the thickening of the nuclear envelope continued to extend caudally independently of an acrosome, which was absent in all these spermatids (Fig. I-5). The spermatid nuclei entered a pseudo-cap phase without acrosomes. Nuclei of this stage of differentiation were characterized by finely granular chromatin and the presence of a nucleolus. The chromatoid body was present in these pseudo-cap phase spermatids.

As we had determined from the PAS-stained sections, elongating and condensing spermatids were infrequent in bs/bs testes. Those which were found were illustrative, for none of them had an acrosome. Nonetheless, many of the normal features of nuclear elongation and chromatin condensation occurred in these spermatids in spite of the absence of acrosomes (Fig. I-6). Microtubules of the manchette were present in these cells, correlated spatially and temporally with nuclear elongation and the caudal shift of the cytoplasm (Fig. I-6). Anterior to the manchette, the nuclear envelope maintained its thickened appearance in the region where the acrosome is normally present. In spite of the fact that nuclear elongation and chromatin condensation occurred in these spermatids, the nuclear shape assumed was abnormal. This is particularly illustrated in Fig. I-6c, which shows that nuclear elongation is occurring, but that the shape of

the nucleus in the region anterior to the microtubules of the manchette is quite abnormal. These aberrant spermatids appeared to develop a structurally normal relationship with the surrounding Sertoli cell, since mantle specializations of the Sertoli cell were frequently observed closely apposed to the anterior end of the nucleus. Chromatin subjacent to the mantle was condensed in focal clusters close to the nuclear envelope (Fig. I-6). Spermatids advanced to the stages of extensive chromatin condensation (steps 12-16) were occasionally found in bs/bs testes. Though extensive chromatin condensation had occurred in these spermatids, nuclear shape was always abnormal. In many cases, invaginations of the cytoplasm into the nucleus of elongating spermatids and intranuclear vacuoles were observed. Many of these advanced spermatids were phagocytosed and degenerating within Sertoli cell cytoplasm.

Development of the sperm tail structures appeared to occur normally in the spermatids of bs/bs mice (Fig. I-7). The proximal centriole, implantation fossa, striated column, and axonemal complex were found in round and elongating spermatids (Fig. I-7a). There were no apparent abnormalities of the axonemal complex of these sperm tails; mitochondria became associated with these tails; and outer dense fibers and the fibrous sheath were formed (Fig. I-7b, c, d). In the very few epididymal sperm observed, the internal structures of the tail were normal; however the midpiece was frequently wrapped in an aberrant fashion around the acrosome-less sperm head.

Discussion

The most dramatic effect of the bs mutation on spermiogenesis was the complete failure of acrosome assembly in spermatids. Round spermatids were characterized by a well developed Golgi apparatus at the presumptive apical region of the nucleus. This Golgi apparatus apparently packaged secretory products, for vesicles enclosing dense substance (proacrosomal granules) were observed. This material was presumably identical to the PAS-positive granules observed in the cytoplasm of round spermatids. However, the granules failed to assemble into acrosomes; in no spermatids from bs/bs mice were acrosomes observed. To our knowledge this is the first reported case of a mutation which causes the failure of assembly of a specific and unique organelle, namely the acrosome. Although other mutations in mice like the pink-eyed sterile mutation, ps, and some of its alleles are known to affect the acrosome (Hunt and Johnson, 1971; Bryan, 1977), they differ from the bs mutation in that structurally defective acrosomes are produced.

In spite of the absence of a developing acrosome, the nuclear envelope underwent its characteristic differentiation in the region subjacent to the presumptive site of acrosome attachment. This was observed as a deposition of dense material resulting in the apparent fusion of the inner and outer membranes of the nuclear envelope, and the accumulation of a fibrillar lamina on the inner aspect of the envelope. Furthermore, the thickening of the envelope spread in a caudal direction over the surface of the nucleus through subsequent developmental stages just as though the acrosome were accomplishing

its normal caudal expansion. These observations suggest the independence of the nuclear envelope differentiation, characteristically associated with acrosomal development, from the process of acrosome differentiation. The stimulus signal for these events may well emanate from the Golgi, or from the attachment of a proacrosomal granule (which occurs in these spermatids) but the continuation of the process of nuclear envelope modification is apparently not dependent upon acrosome assembly.

The acrosome-less nuclei of spermatids in bs/bs testes underwent elongation and chromatin condensation, processes temporally associated with the presence of a well developed manchette in these spermatids. This observation leads to the important conclusion that nuclear elongation and chromatin condensation can be initiated in the absence of acrosome development and therefore these processes are not mechanistically dependent on the acrosome.

Much has been documented and even more speculated about the processes involved in the morphogenesis of sperm heads (see Fawcett et al., 1971 for review). What has not always been clearly distinguished is that the typical morphology of the sperm nucleus is the result of at least three processes: nuclear elongation, chromatin condensation, and nuclear shaping. There is no a priori reason to assume identical regulatory control or mechanisms for all of the processes. The processes of elongation and condensation do occur in these spermatids. This observation suggests the separation of the processes of nuclear elongation and chromatin condensation, on the one hand, from the process of nuclear shaping, on the other hand. Furthermore, our

observations lead to the conclusion that the presence of a developing acrosome is not a necessary prerequisite to elongation or condensation. It may be that the process of elongation is initiated primarily by the activity of the nuclear envelope since we have observed in these spermatids the apparently autonomous caudal extension of the nuclear envelope thickening associated with the process of nuclear elongation. An active role of the nuclear lamina in this process should be considered as a possible mechanism.

Since the final nuclear shape attained by bs spermatids was abnormal, either the bs gene affects head shaping as well as acrosome formation or the acrosome itself plays a role in nuclear shaping. In this context, we have shown (Fig. I-6) that the anterior (acrosomeless) end of the nucleus in particular is misshapen, suggesting an active role of the acrosome in determining the shape. Though the absence of an acrosome may be a causative factor in the production of abnormal sperm heads in this mutant, the presence of an acrosome is not solely sufficient for accomplishing nuclear shaping since a number of other mutations in the mouse lead to the production of abnormal sperm heads in spite of the presence of acrosomes (see for ex. Bennett et al., 1971 on quaking; Hunt and Johnson, 1971 and Bryan, 1977 on pink-eyed sterile; Dooher and Bennett, 1977 on lethal t-naplotypes; and Handel and Dawson, 1981 on Purkinje cell degeneration).

In addition to the cellular abnormalities of spermiogenesis, the structure of the seminiferous epithelium with its typical germ cell associations was profoundly altered in bs/bs testes. This was

manifested by germ cell depletion and testicular hypoplasia. However in 30% of the tubules complete spermatogenesis was observed. Preliminary results from a developmental study with bs/bs mice of various ages indicate that germ cell depletion occurs long before early spermatid stages appear in the testis (Sotomayor and Handel, unpublished). This suggests that germ cell depletion is not a consequence of the failure of acrosome assembly but a separate effect of the bs mutation itself. It is not known at this time whether the bs mutation might affect the number of primordial germ cells populating the testis or their proliferation capacity once in the testis.

In summary, the present study has documented the following characteristics of spermatogenesis in bs/bs mice: germ cell depletion, limited extent of development of those germ cells which do initiate spermiogenesis, failure of acrosome formation and abnormal head shape of spermatids and spermatozoa.

PART I

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PART I

APPENDIX

Table I-1. Sperm counts, testis weights, and age at the time of sacrifice of mutant (bs/bs) and normal (+/bs; +/?) mice. Values are given as means ± standard deviations.

		Sperm counts X 106	Testis weight mg	Age mos.	Number of Mice
Mutant	A*	N.D.	27.8 ± 7.5	5.4 ± 1.8	25
	B	0.22 ± 0.34	41.1 ± 8.0	5.8 ± 2.2	16
Normal		14.4 ± 3.5**	101.8 ± 16.7**	6.2 ± 1.8	17

*No detectable sperm. These mice placed in separate category from rest of bs/bs mice which showed sperm.

**Significantly different from the mutants at the 99% confidence interval, F-test.

Table I-2. Percentage of sperm abnormalities in mutant and normal mice. Values are given as means ± standard deviations.

	Abnormal			Normal	Number Scored	Number of Mice
	I[a]	II[b]	III[c]			
Mutant	55 ± 3	38 ± 3	7 ± 2	0	2500	10
Normal	0.5 ± 0.4	0.2 ± 0.3	0.2 ± 0.1	99 ± 1	10000	9

[a]Abnormally shaped nucleus folded back on middle piece

[b]No apparent nucleus, mitochondrial cluster

[c]Highly looped tail wrapped around abnormally shaped nucleus

Table I-3. Percentage of tubule cross-sections showing various types of germ cell associations in mutant and normal testes.

	Germcell Association Types*					Tubule Cross-Sections	Number of Mice
	I	II	III	IV	V		
Mutant	39	3	28	11	19	2434	7
Normal	0	0	0	40	60	908	3

*Type I: Sertoli cells and spermatogonia
Type II: Sertoli cells, spermatogonia and few round or elongating spermatids
Type III: Sertoli cells, spermatogonia, meiotic spermatocytes and round spermatids (1-7)
Type IV: Sertoli cells, spermatogonia, meiotic spermatocytes, and elongating spermatids (8-12)
Type V: Sertoli cells, spermatogonia, meiotic spermatocytes, round spermatids and maturing spermatids (13-16)

Table I-4. Percentage of spermatids containing proacrosomic granules (PAG), acrosomal cap (AC), and acrosome (A) in 7μm sections of mutant and normal testes. The number of spermatids scored is in parenthesis.

Testes	PAG	AC	A	Number of Mice
Mutant	91*(2104)	0 (1688)	0 (1881)	6
Normal	90**(1158)	95 (1076)	97 (1004)	3

*61 percent contained 2 or more granules.

**92 percent contained a single granule.

Fig. I-1. Scanning electron microscope views of spermatozoa from mutant and normal mice. X4400. (a,b) Type 1 sperm head abnormalities. c) Normal sperm head. The inset shows a light micrograph of a misshapen mutant sperm head. X1600.

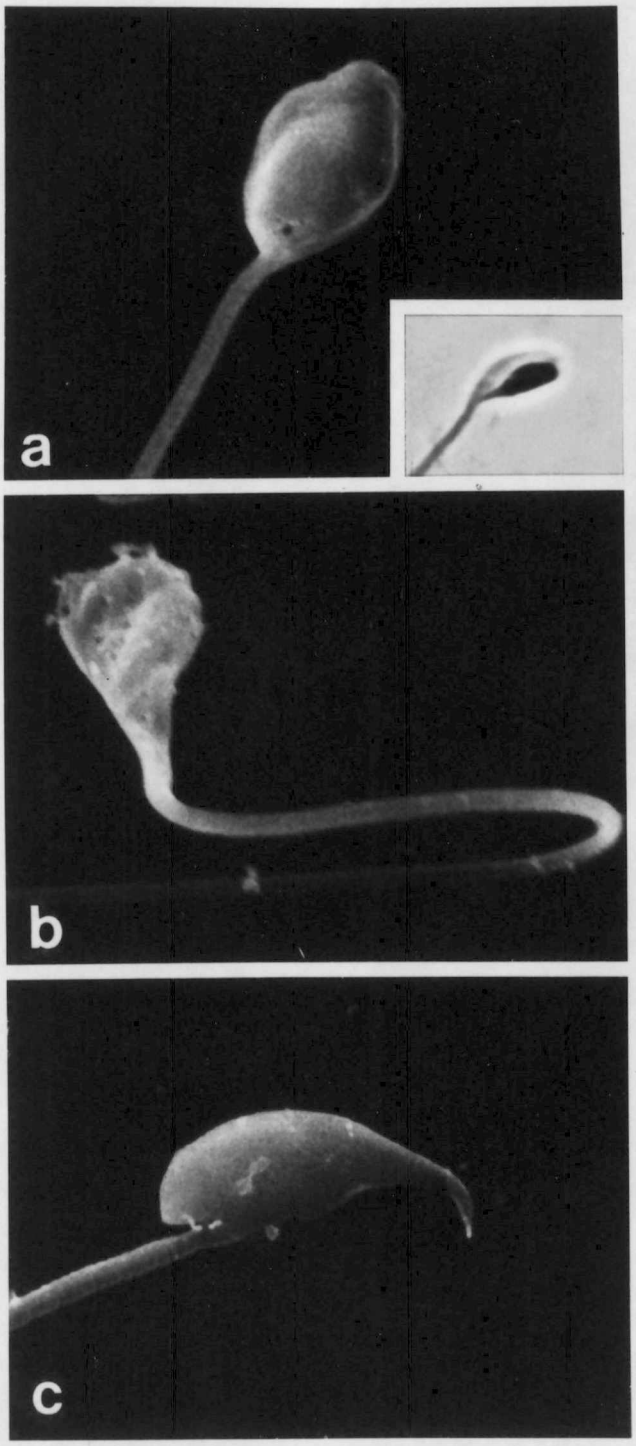

a
b
c

Fig. I-2. Paraffin embedded, PAS-hematoxylin stained sections of normal (a) and mutant testes(b). Note the occurrence of germ cell deficiencies and the presence of giant multinucleated spermatids (arrow) in the mutant testis. X320.

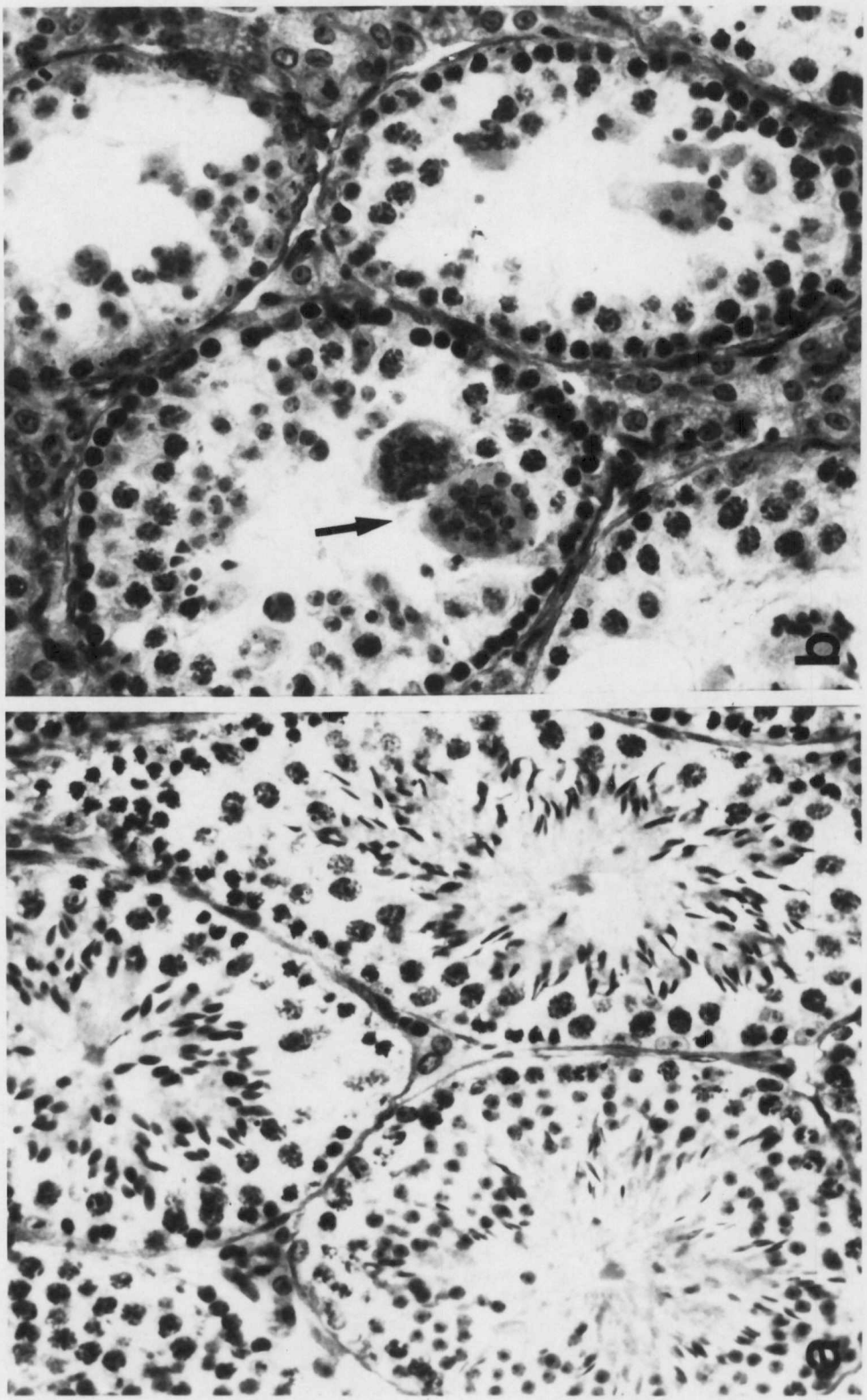

b
a

Fig. I-3. Epon-araldite embedded 1 μm sections of normal (a,c,e) and mutant (b,d,f) testes, stained with PAS-hematoxylin. X1200. a) Normal spermatids in stage 2. b) Mutant spermatids in stages 2-3. The arrow indicates the presence of at least 2 proacrosomic granules. c) Stage 6-7 normal spermatids with intensely PAS-stained acrosomal caps. d) Mutant round spermatids without acrosomal caps. e) Normal elongating spermatids in stages 10-11 showing fully formed acrosomes. f) Mutant spermatids presumably in stages 10-11 showing no acrosomes.

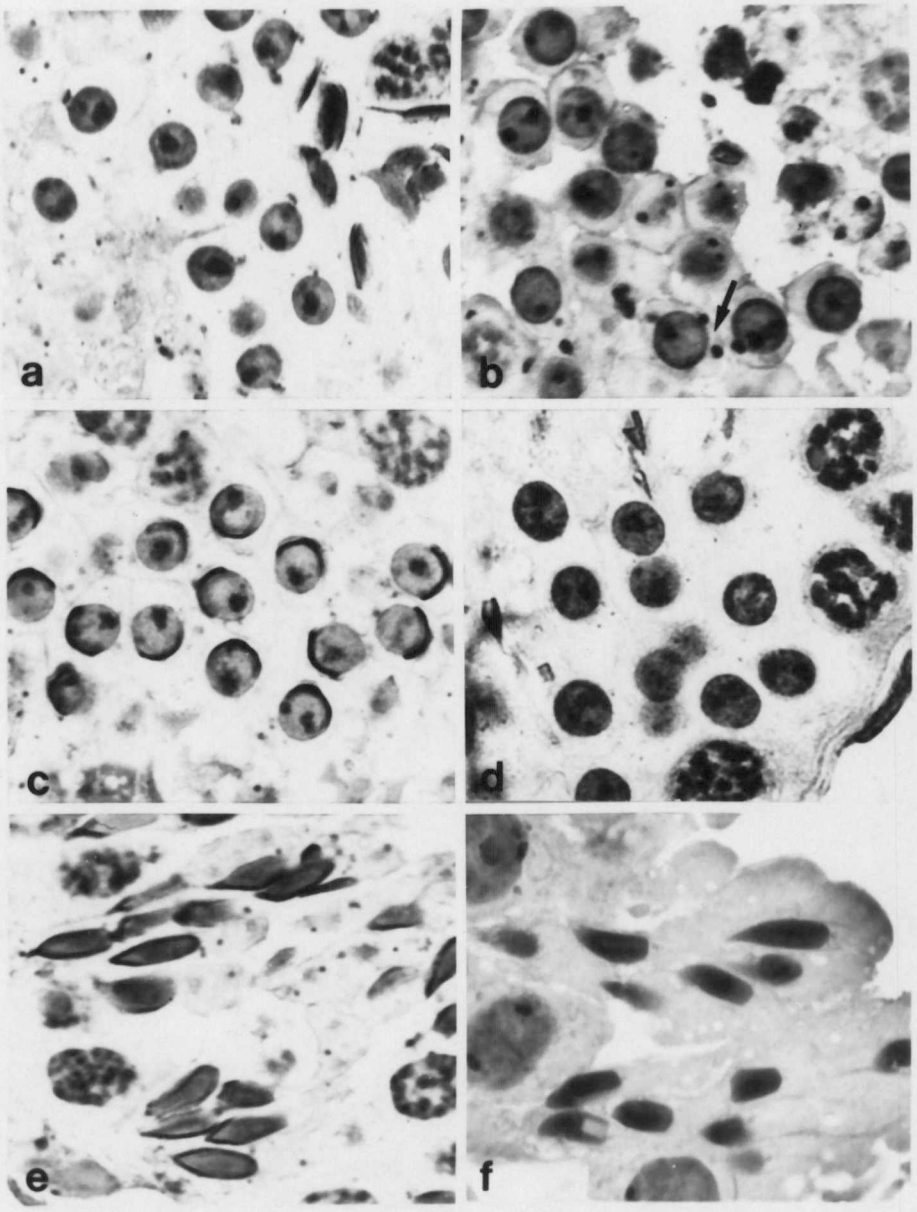
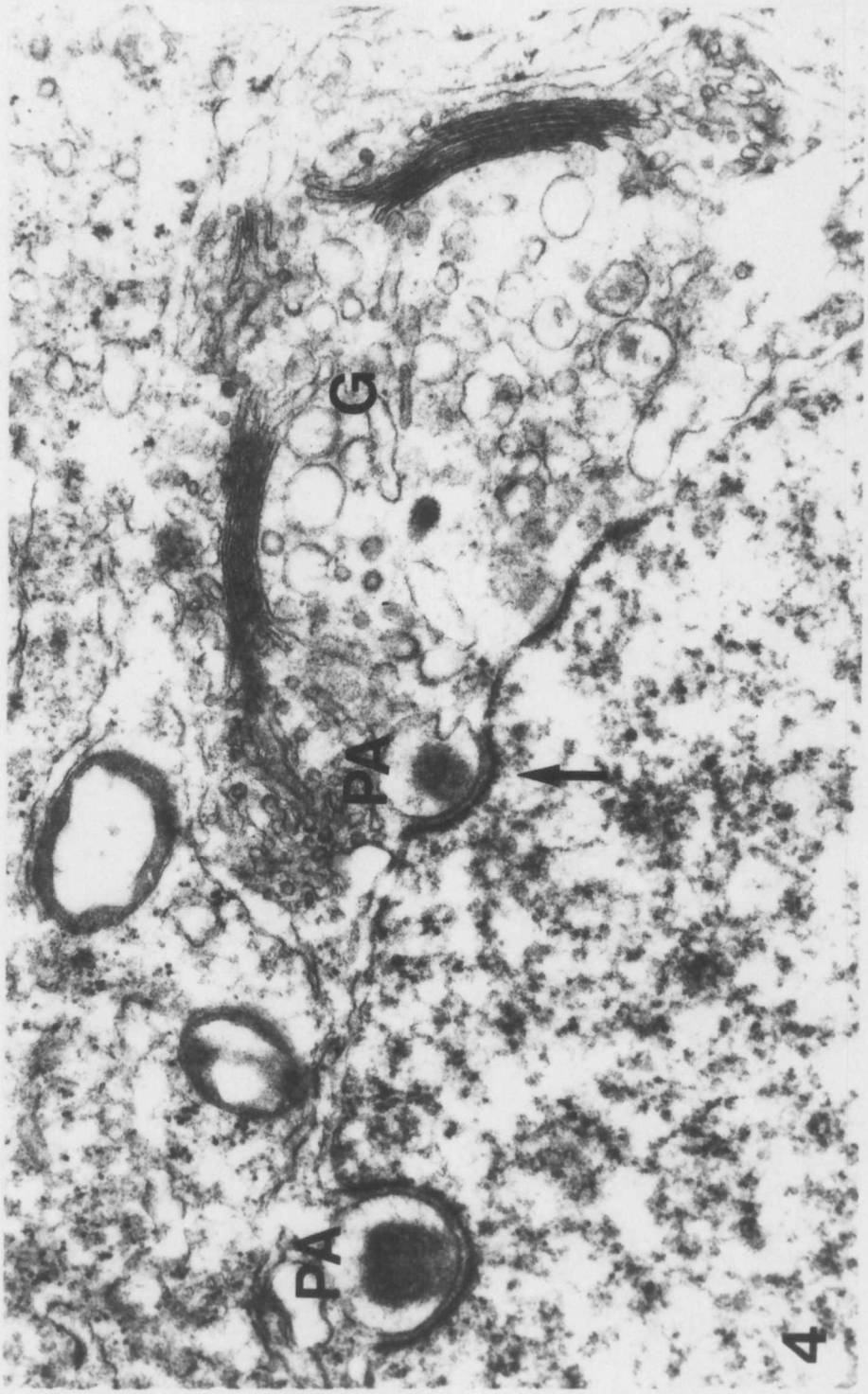

a
b
c
d
e
f

Fig. I-4. Golgi region of bs/bs early round spermatid. Illustrated are the Golgi apparatus (G), the presence of 2 proacrosomes (PA), and the thickening of the nuclear envelope underlying the proacrosome (arrow). X27,000.



4

Fig. I-5. Round spermatids of bs/bs mice showing the absence of acrosomic caps and the thickening of the nuclear envelope. a) A low magnification view. X5600. b) Detail of the caudal extent (arrows) of the nuclear envelope thickening. X28,000. Inset: higher magnification illustrating the deposition of dense fibrillar material on the inner aspect of the nuclear envelope. X63,000.

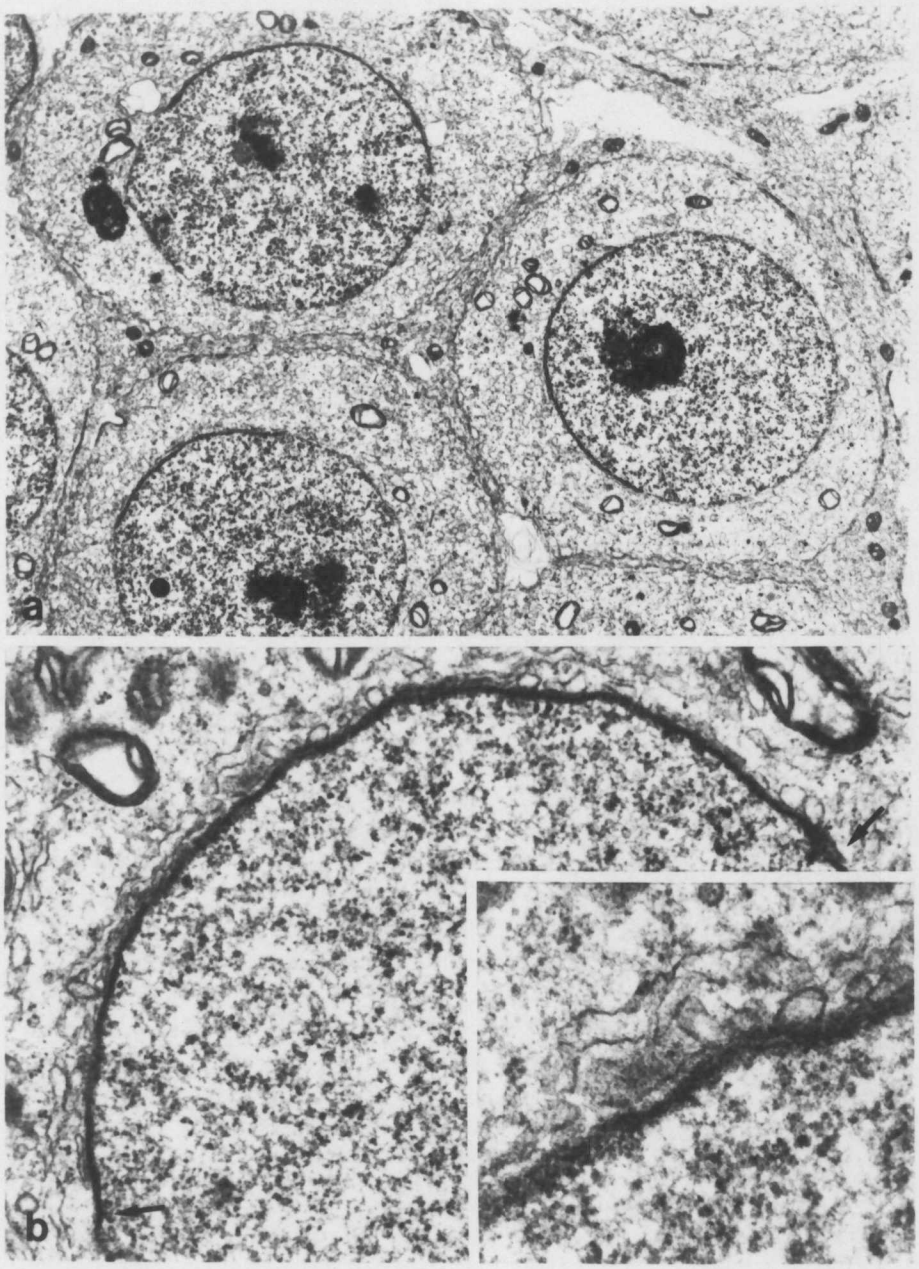

a
b

Fig. I-6. Nuclei of bs/bs elongating spermatids. a) Note the thickening of the nuclear envelope (arrow), the presence of the manchette (M), and the absence of acrosome. X8400. b) Cross section showing manchette microtubules (single arrows) and the surrounding mantle of Sertoli cell (double arrows). Note the focal clusters of condensing chromatin immediately beneath the nuclear envelope (*). X14,600. c) Highly aberrant shape of the nucleus in the acrosomal region. X7400. d) Another view of a misshapen nucleus. X8300

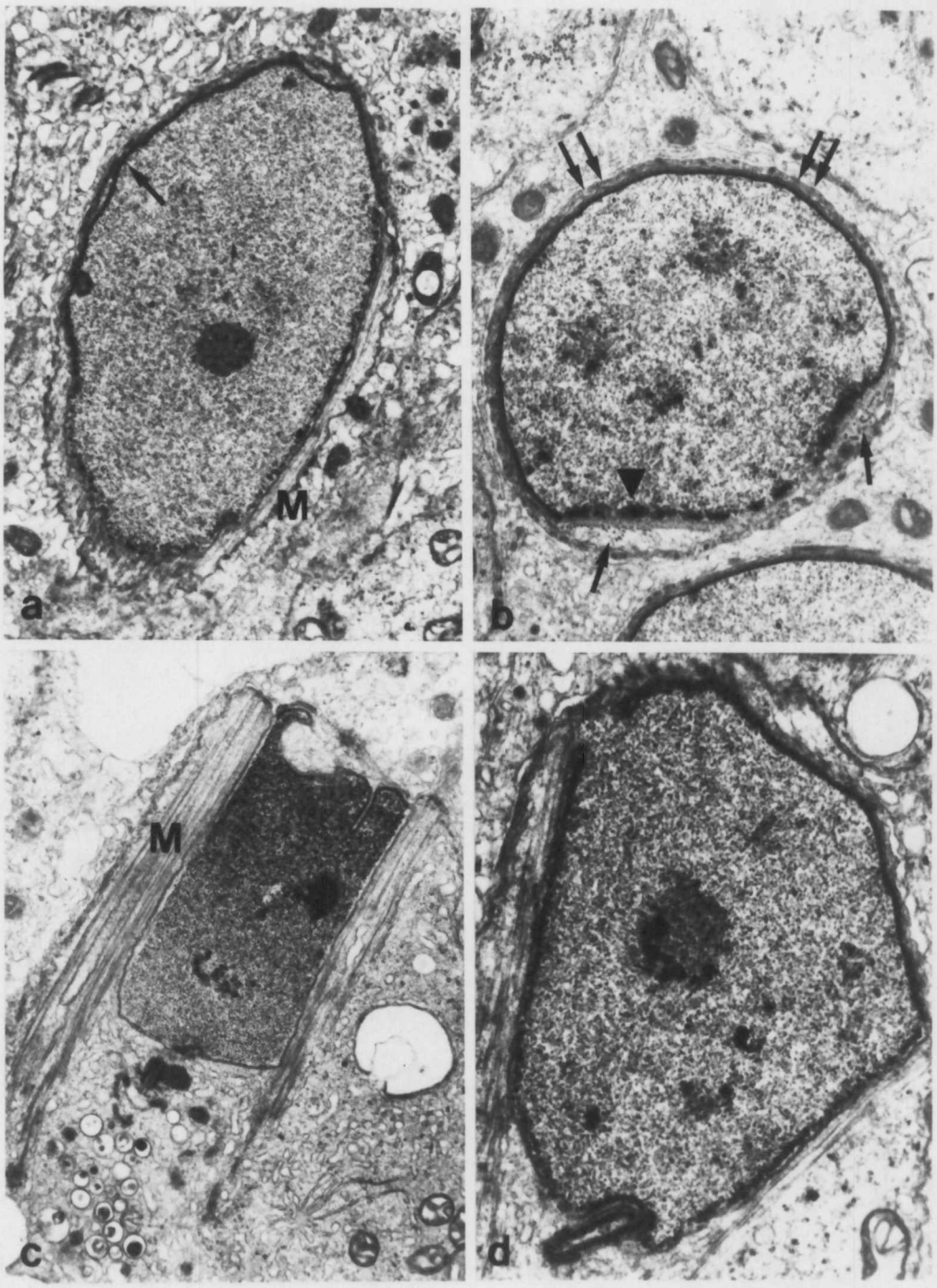
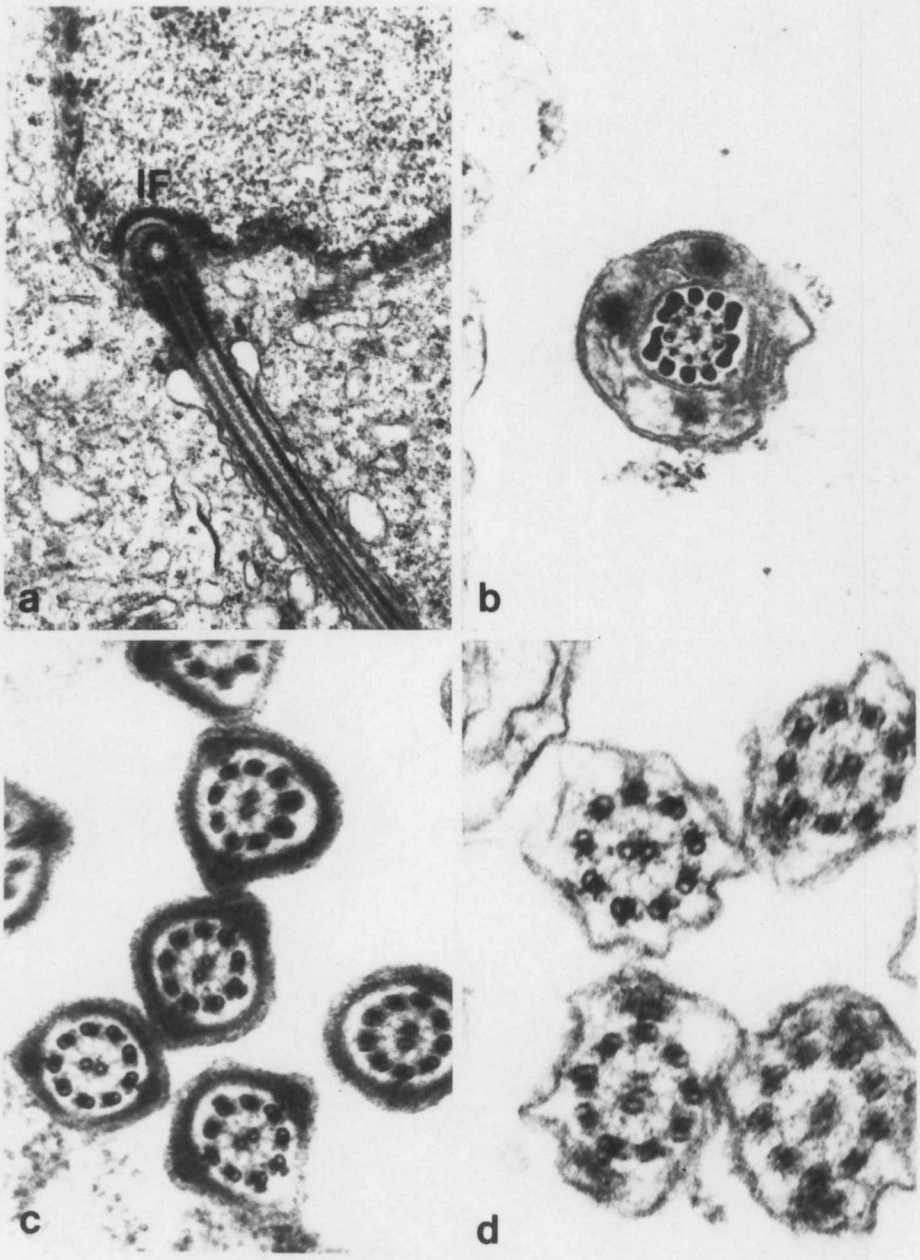

M
a
b
M
c
d

Fig. I-7. Views of tails of bs/bs spermatids. a) Implantation fossa (IF) and proximal centriole. X22,000. b) Distal part of the middle piece showing the outer dense fibers. X43,000. c) Distal segments of the principal piece showing normal axoneme structure, lateral columns and ribs of the fibrous sheath. X64000. d) Section through the proximal part of the end piece showing normal axoneme structure. X92,000.


IF
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PART II

REPRODUCTIVE ENDOCRINE PROFILE IN A MALE STERILE MUTANT MOUSE

REPRODUCTIVE ENDOCRINE PROFILE IN A MALE STERILE MUTANT MOUSE

Abstract

Male mice homozygous for the pleiotropic mutation "blind-sterile" (bs) are characterized by abnormal spermatogenesis with both failure of acrosome assembly and extensive germ-cell deficiency. In order to assess whether these effects may be due to steroidogenic and/or gonadotropic disfunctions, the circulating levels of testosterone, FSH and LH were determined in the homozygous mutants and in their normal littermates. Cytological studies of the Sertoli and Leydig cells were also performed to investigate possible correlates with the endocrinological profiles. Circulating testosterone levels varied extensively in both mutant and normal mice with the mutants showing lower mean testosterone levels than the controls. Nonetheless mutant mice exhibited normal copulatory behavior and normal LH levels. Circulating FSH levels were significantly elevated in mutant mice. The hormonal changes were associated with morphological and quantitative changes in the Sertoli and Leydig cells. In turn, the Sertoli and Leydig cell abnormalities appeared to be a consequence of the profound alterations induced by the bs gene on spermatogenesis, specially the induction of germ cell deficiency.

Introduction

Several gene mutations of the mouse are known to cause structural spermatogenic defects resulting in sterility. Most of these mutations are pleiotropic, affecting various organ systems (see Searle, 1982, for review).

Despite the wealth of information on these pleiotropic gene mutations, little is known about their possible effects on the reproductive endocrine system. Hunt and Johnson (1971) suggested that a pituitary disfunction could account for all the effects of the "pink-eye sterile" mutation, $\underline{p}^{s}$, but no direct determinations of steroids or gonadotropins were made. However, evidence of degenerating nerve axons in the mutant pituitary, reduced binding of estradiol-17B by the hypothalamus and reduced binding of iodine by the thyroid were found in the homozygous mutants. Other hormonal studies have been concentrated on gene mutations with known pituitary effects such as dwarf (dw) (Bartke et al., 1977; Howe et al., 1981) and hypogonadal (hpg) (Cattanach et al., 1977; Iddon et al., 1980; see Lyon et al., 1981 for review).

The "blind-sterile" mutation (bs) has been reported to cause sterility in male mice and bilenticular cataracts in both sexes (Varnum, 1983). We have recently demonstrated the occurrence of profound alterations in the seminiferous epithelium of bs/bs mutant mice (Sotomayor and Handel, 1986). These were characterized by the lack of acrosome assembly in early spermatid stages and extensive germ-cell deficiency. Sperm when formed were all morphologically abnormal.

The extent of physiological and morphological dependence of the Sertoli and Leydig cells on the integrity of the seminiferous epithelium is not well understood. In pathological conditions affecting the testis, such as cryptorchidism (Kerr et al., 1979; Kerr et al., 1979), germinal aplasia in humans (Chemes et al., 1977) and

the XX-sex reversed condition in mice (Chung, 1974; Chung et al., 1977), also resulting in germ cell deficiency, morphological alterations of the Sertoli and Leydig cells have been shown to occur in association with steroidogenic and gonadotropic changes. The present study was conducted to examine hormone levels and structure of Sertoli and Leydig cells in bs/bs germ cell deficient mice. Determinations of circulating testosterone, FSH and LH were made in bs/bs male mice and their respective normal littermates. In addition cytological studies on the Sertoli and Leydig cells were performed to determine correlations with the endocrinological profiles.

Materials and Methods

Animals

Mutant (bs/bs) mice and littermate normal (+/bs or +/?) mice were used in this study. The mice were progeny of (AKR-bs x 129) hybrid mice originally obtained from the Jackson Laboratory (Bar Harbor, ME) and subsequently maintained in our animal facility by brother-sister matings. The mice were provided with food and water ad libitum and were kept on a 14 hr L 10 hr D daily lighting schedule.

Light and Electron Microscopy

The testes, freed of fat, were weighed and were fixed in either Zenker-formol for light microscopy or in 3% glutaraldehyde/2% paraformaldehyde in 0.1M sodium cacodylate buffer (pH 7.1) containing 0.001M Ca^{2+} and Mg^{2+}, for electron microscopy. Testes for light microscopy were embedded in Paraplast, sectioned at 7um, stained with

periodic acid-Schiff (PAS) and counterstained with Ehrlich's hematoxylin (Leblond and Clermont, 1952). Some testes were embedded in JB-4 resin (Polysciences, Inc., Warrington, PA) and were sectioned at 3um using a Sorvall JB-4 microtome. These preparations were PA5 stained as indicated above. Testes for electron microscopy were postfixed in 1% OsO4 in 0.1 M sodium cacodylate buffer at 4 C, dehydrated in ethanol, infiltrated with propylene oxide and embedded in Epon. Thin sections were cut with a Reichert OM U-3 ultratome, strained with uranyl acetate and lead citrate, examined and photographed with a Zeiss 9S or a Hitachi H600 electron microscope.

Morphometry

The number of Sertoli cells per tubule cross-section was determined in 5 bs/bs mice and 3 normal controls. Round tubules were chosen for scoring. Due to the various degrees of germ-cell deficiency in the mutant testes (Sotomayor and Handel, 1986), the tubule cross-sections were classified into 3 types: A-type tubules contained only Sertoli cells; B-type tubules contained Sertoli cells and spermatogonia and C-type tubules contained Sertoli cells, spermatogonia, spermatocytes and various spermatide stages. The tubule perimeters and diameters were determined by digitizing morphometry using a Bioquant II system with an Apple II microcomputer. Leydig cells numbers were estimated by counting the number of nuclei with an occular grid at 1000X in 40 randomly selected fields and multiplying this number by the weight of the testis (McKinney and Desjardins, 1973). The cell counts were not corrected for the

Abercrombie's factor (Abercrombie, 1946) because only relative comparisons were made.

Testosterone Radioimmunoassay

The testosterone radioimmunoassay (T-RIA) was adapted from several sources (Auletta et al., 1979; Selmanoff et al., 1977; Howe et al., 1980). Blood was obtained from anesthetized mice by heart puncture, transferred to 1.5 ml plastic centrifuge tubes without heparin and kept on ice until separating plasma from the cells by centrifugation at 3000 rpm for 15 min at 4°C in a Beckman TJ-6 centrifuge. The plasma samples were then stored at -20°C until use for RIA.

Plasma samples were extracted four times with diethylether (Mallinkrodt Chemical Works, St. Louis, MO). The ether-extracted samples were air-dried and resuspended in 1 ml of phosphate buffered saline (PBS) containing 0.1% bovine serum albumin (BSA, Fraction V, Sigma, St. Louis, MO). Recovery efficiencies were monitored by the addition of approximately 1000 cpm of tritiated-testosterone (95 Ci/mmol (1,2,6,7-^{3}H) testosterone, Amersham, Arlington Heights, IL) to samples prior to extraction. Recovery efficiencies ranged from 80% to 100%. To perform the RIA an additional 10,000 cpm ^{3}H-testosterone was added to the samples. Extracted samples were assayed in duplicate.

The testosterone antibody (GDN #250, anti-testosterone-11-BSA serum) was kindly provided by G. D. Niswender (Colorado State University, Ft. Collins, CO). The antiserum did not cross-react significantly with any other abundant steroid. Antibody dilutions of

1/8000 to 1/12000 were required to produce 36% to 53% binding of the total counts.

Bound testosterone was separated from free testosterone by the addition of 0.5 ml of dextran-coated charcoal (0.025% dextran T-70 plus 0.25% activated Norit A) and subsequent centrifugation of samples at 3000 rpm for 15 min at 4°C. Radioactivity of the samples was determined using a Beckman LS8100 liquid scintillation counter. The data were analyzed using the logit-log analysis of RIA of Faden et al. (1980), programmed in an IBM 370.3031 computer.

LH and FSH Radioimmunoassays

Plasma samples from mutant and normal mice were assayed for LH and FSH using the double antibody assay (Niswender et al., 1968; Kovacic and Parlow, 1972; Beamer et al., 1972). The reagents were supplied by the National Institute of Arthritis, Diabetes and Digestion and Kidney Diseases (NIADDK), Baltimore, MD. Results were expressed in terms of rat FSH (NIADDK-rFSH-RP-2) or rat LH (NIADDK-rLH-RP-2) reference preparations, in ng/ml plasma. Plasma samples were assayed in duplicates. However, plasma volumes from each mouse were not sufficient to test for both LH and FSH from the same individual.

Highly purified preparations of LH (NIADDK-rLH-I-6) and FSH (NIADDK-rFSH-I-6) were iodinated with ^{125}I (Amersham Co., IL), using the Chloramine-T technique (Greenwood et al., 1963). The radioactivity was measured with a Beckmann 4000 Gamma counter. Results were analyzed using the logit-log analysis of Faden et al., (1980) programmed in an IBM computer.

Results

Testosterone Levels

Mutant and normal mice varied considerably with regard to their individual plasma testosterone levels. Values ranged from 40 to over 10,000 pg/ml (Fig. II-1*). The distribution of the individual testosterone concentrations did not follow a normal curve. The transformation to their log10 functions (McCormak and Huseby, 1979) resulted in a fairly normal curve and permitted the use of parametric statistical analysis instead of the less sensitive non-parametric methods.

Mutant mice showed lower mean testosterone levels than the normal littermates (Table II-1*). The difference was statistically significant at the 0.05 level. However, variability was so extensive that significance did not hold at the 0.01 level.

Lower testosterone levels of the mutant mice were associated with low testis weights. Mutant testis weights also showed extensive variation, ranging from 6 to 56 mg with a mean of 31.7 $\pm$ 9.4 mg (Fig. II-2). In comparison the normal testis weights ranged from 67 to 131 mg with a mean of 102.2 $\pm$ 15.1 mg (Fig. II-2). Not surprisingly, a positive linear correlation ($r = 0.3830$; $P < 0.05$) was found between the mutant testis weights and the log testosterone values (Fig. II-3). A negative correlation ($r = -0.0961$) was found in the case of the normal testes but this was not significantly different from a no-correlation hypothesis ($P > 0.2$).

*All tables and figures may be found in the Appendix.

The possible influence of age on the testosterone levels was also investigated in view of the fact that the animals used in our study had a wide range of ages. No significant correlation was detected with age (Fig. II-4).

FSH and LH Levels

Unlike the extreme variation observed in the testosterone levels, FSH and LH levels did not vary extensively. In two different determinations, the mutant mice showed significantly elevated FSH levels compared to the normal mice (Table II-2). No significant differences were found in the LH levels (Table II-2).

Morphometry of Sertoli and Leydig Cells

The structural effects of the bs mutation on spermatogenesis have been previously reported (Sotomayor and Handel, 1986). In the present study we report our findings on the morphology and quantitation of the Sertoli and Leydig cells.

At the light microscope level, the interstitial tissue of mutant testes appeared hyperplastic, with large masses of Leydig cells separating the seminiferous tubules. However, an estimate of the number of Leydig cells per testis indicated that the bs/bs testes contained fewer Leydig cells than the normal testes (Table II-3). The Leydig cells exhibited the typical features described by Christensen and Gillim (1969) for the mouse, with well developed SER, whorls of smooth cisternae, elongated large mitochondria and relatively abundant lipid droplets (Figs. II-5 and II-6). When compared to normal Leydig cells (Fig. II-5) there was an apparent increase in lipid droplets and numerous whorls of SER in the Leydig cells of mutant testes (Fig. II-7).

As mentioned in Material and Methods the tubule cross-sections of mutant testes were classified into three types depending on the extent of germ cell deficiency. The number of Sertoli cells per tubule cross-section increased with the increasing extent of germ cell deficiency reaching a maximum in the most deficient tubules which contained only Sertoli cells (Table II-4). In conjunction with the apparent increase in the number of Sertoli cells per tubule cross section, the diameter and perimeter of the seminiferous tubule decreased considerably (Table II-5). This indicated that the shrinkage of the tubules produced closer packing of the Sertoli cells resulting in an apparent increase of the number per cross section. Further support for this observation comes from the finding that no mitotic Sertoli cells were detected in the mutant testes, despite the apparent increase in the number of Sertoli cells per tubule cross-section (Table II-4).

Light microscopy revealed two types of morphologically different Sertoli cells. One type appeared randomly distributed along the basal lamina with its typical tripartite nucleolar structure. At the ultrastructural level these cells showed the typical features of convoluted nuclear profile, tripartite nucleolus, long, slender mitochondria and specialized intercellular junctions (Fawcett, 1975) (Fig. II-8a,b). The other type of Sertoli cell was found exclusively in the most germ-cell deficient tubules. At the light microscopic level they appeared as few clusters of cells protruding towards the lumen of the seminiferous tubule. At the ultrastructural level, they exhibited extremely convoluted nuclear profiles, atypical nucleoli and numerous

clusters of heterochromatin associated with the nuclear membrane (Figs. II-9 and II-10).

Discussion

Individual testosterone levels varied extensively in both mutant and normal mice, confirming reports by others (Bartke and Dalterio, 1975; McCormack and Huseby, 1979). This variability has been attributed to the occurrence of a pulsatile pattern of secretion by the testis (Bartke and Dalterio, 1975; Mann and Lutwak-Mann, 1981). However, the mutant mice showed significantly lower mean testosterone levels than the normal littermates. The levels of testosterone, although reduced, appeared to be sufficient to maintain a normal copulatory behavior (Sotomayor and Handel, 1986) and a normal feedback mechanism with the pituitary-hypothalamus centers, as shown by the normal levels of LH found in this study.

Age of animals was also considered as a possible factor since researchers have found significant reductions of testosterone levels in mice over 100 days of age (McKinney and Desjardins, 1971). We did not detect any significant change in testosterone levels with age in either mutant or normal mice, despite the wide range of ages used in this study (i.e., from 2.5 to 11 months). Our results are in agreement with those of Eleftheriou and Lucas (1974), who investigated mice up to 28 months of age.

The blood testosterone levels of mutant mice were positively correlated with the testis-body weight ratios. This correlation may reflect the dependency of total testosterone output on the number of

Leydig cells present in the testis. It is logical to assume that the number of Leydig cells will be proportional to the testis mass, up to a certain limit. Since the mutant testis weights varied extensively, ranging from 6 to 56 mg, it is not surprising that the testosterone levels increased as a function of the testis weights (Fig. II-3). No such correlation was found in the case of normal mice probably because there was little variability in testis weights. A gross estimate of the number of Leydig cells per testis showed that mutant testes contained about half the number of Leydig cells compared to the normal testes. A similar finding has been reported by Chung et al. (1977) for the XX sex-reverse condition in mice. As in our case an apparent hyperplasia of the interstitial tissue was observed and the relative number of Leydig cells per unit area was elevated. However, the number of Leydig cells per testis was reduced in comparison to the normal ones.

The Leydig cells of bs/bs mice appeared to be in a functional compensatory state as indicated by our ultrastructural observations. The presence of abundant cytoplasmic lipid droplets and numerous whorls of SER suggested the occurrence of intense steroidogenesis as if the cells were compensating for the reduction in cell number. Almost identical observations have been reported by Chung et al. (1977) in the case of the Sxr testes. Similar findings have also been reported in cryptorchid rats (Kerr et al., 1979) except that lipid droplets are not normally present in rat Leydig cells (Christensen and Gillim, 1969).

Plasma FSH levels were significantly elevated in bs/bs mice. In conjunction with the increased FSH levels, morphological and quantitative changes were detected in the Sertoli cells. The number of Sertoli cells per tubule cross-section appeared to increase depending on the extent of germ cell deficiency. However, the diameter and perimeter of the seminiferous tubules were, on the average, significantly reduced compared to the normal tubules. We have interpreted these results as indicating that shrinkage of the seminiferous tubules due to germ-cell deficiency results in a close packing of the Sertoli cells, altering the intercellular relationships of these cells. In addition, morphologically abnormal Sertoli cells were detected in those tubule cross-sections most deficient of germ cells. These cells resembled immature Sertoli cells (Ramos and Dym, 1979). The elevated FSH levels, spermatogenic deficiency and Sertoli cell abnormalities found in bs/bs mice, have also been observed in other pathological conditions of the testis. These include the XX sex-reversed condition of the mouse (Chung, 1974; Chung et al., 1977), germinal aplasia in humans (Chemes et al., 1977), cryptorchidism in rats (Kerr et al., 1979), and in various other forms of spermatogenic disruption (Setchell et al., 1977). The possible cause for the elevated FSH levels may be a lack of sufficient germ cell stimulus to the Sertoli cells for production of inhibin because of germ cell deficiency. Inhibin is known to control FSH and is produced by the Sertoli cells in response to stimulus from germ cells (Steinberger and Steinberger, 1976; Setchell et al., 1977).

In conclusion the present study has documented the occurrence of abnormal levels of testosterone and FSH in conjunction with morphological and quantitative changes of the Sertoli and Leydig cells in the bs/bs testes. These changes appear to be related to the extensive germ-cell deficiency occurring in the mutant testis rather than to a direct effect of the bs gene on the endocrinological system. This conclusion is supported by other studies indicating that similar hormonal alterations occur in different pathological conditions of the testis, all of which result in germ cell deficiency.

PART II

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PART II

APPENDIX

Table II-1. Mean plasma testosterone concentration in bs/bs and control mice.

	pg Testosterone/ml plasma		Number Mice
	Mean ± SD	Mean Log 10 ± SD	
bs/bs	1073 ± 1769	2.7549 ± 0.4778*	56
Controls	2506 ± 4126	2.9665 ± 0.6062*	31

*P = 0.05

Table II-2. FSH and LH concentrations (mean ± SD) in the plasma of mutant and normal mice.

	Exp.	FSH (ng/ml)	Number of mice	LH (ng/ml)	Number of mice
Mutants	I	58 ± 10*	10	11 ± 2	5
	II	92 ± 55#	9		
Normals	I	40 ± 7 *	12	10 ± 2	7
	II	33 ± 3 #	4		

*P < 0.01

#P < 0.01

Table II-3. Relative number of Leydig cells per testis in mutant and normal mice. Leydig cells were counted in 40 randomly selected fields per testis and the average number multiplied by the testis weight (N x T.W.). Each value represents the mean ± S.D.

	Leydig Cell Index (N x T.W.)	Testis Weights (mg)	Number of mice
Mutants	130 ± 37	40 ± 9	3
Normals	280 ± 13	113 ± 1	3

Table II-4. Average number of Sertoli cells per tubule cross-section in mutant and normal mice. Each value represents the mean ± S.D. The number of tubule cross-sections scored is shown in parentheses. Type A tubules contained Sertoli cells only; Type B tubules contained Sertoli cells and spermatogonia; Type C tubules contained various normal germ cell associations.

	Sertoli Cells/ Tubule Cross-Section				
	Class of Tubules			Mitotic Index	Number of Mice
	A	B	C		
Mutants	29 ± 5 (88)	21 ± 3 (52)	17 ± 3 (82)	0/4974	5
Normals	-	-	12 ± 2 (60)	0/744	3

Table II-5. Perimeters and diameters of the seminiferous tubules in mutant and normal mice. Each value represents the mean ± S.D.

	Testis Weights (mg)	Tubule Perimeters (um)	Tubule Diameters (um)	N
Mutant #				
1	51	481 ± 48	115 ± 15	30
2	50	489 ± 50	156 ± 16	51
3	35	401 ± 35	128 ± 11	31
4	34	401 ± 56	128 ± 18	40
5	32	401 ± 27	128 ± 9	21
6	14	377 ± 17	120 ± 5	22
7	6	296 ± 24	94 ± 7	25
Normal #				
1	113	607 ± 37	193 ± 12	32
2	113	602 ± 79	192 ± 25	23
3	114	585 ± 50	186 ± 16	49

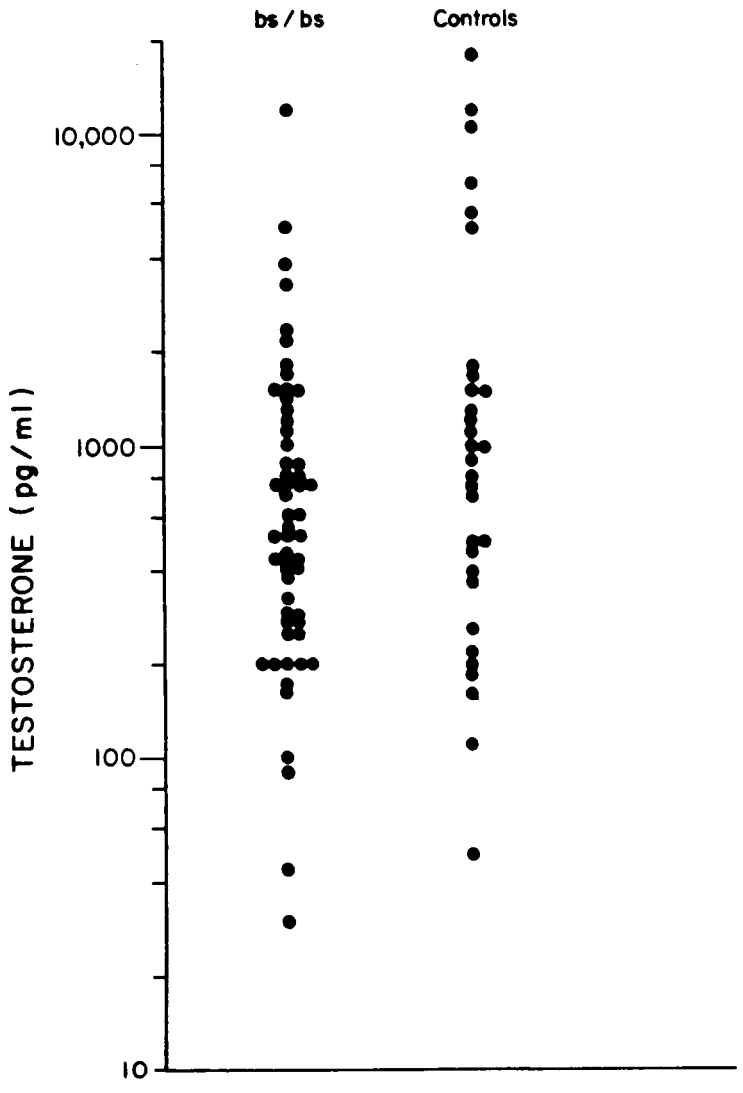


Fig. II-1. Individual testosterone concentrations in mutant (bs/bs) and normal (controls) mice. Each dot represents one mouse.

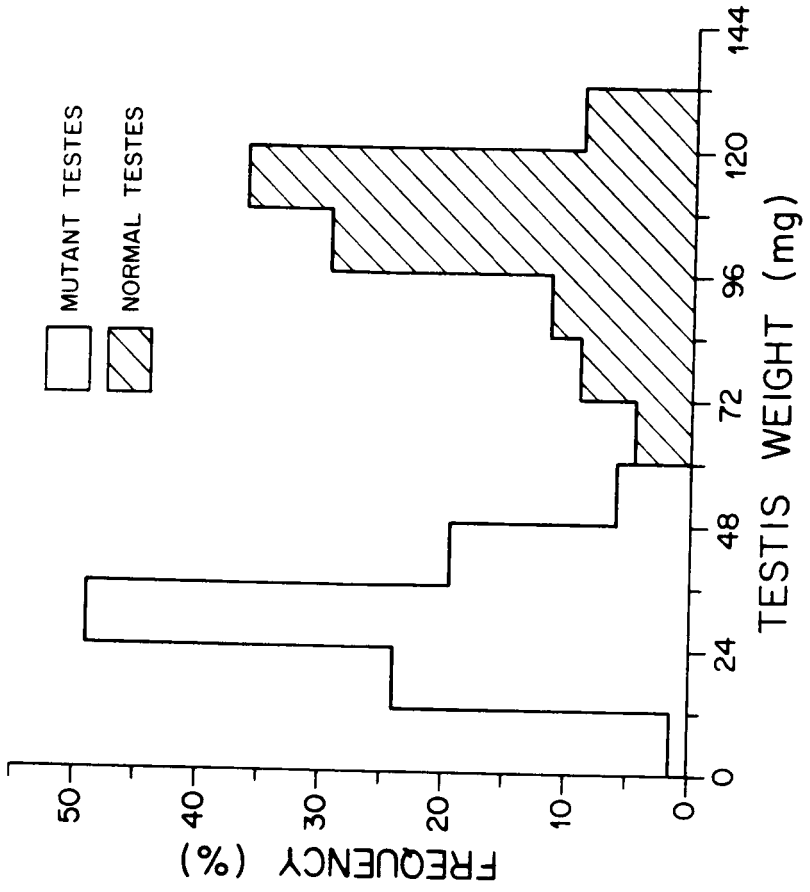


Fig. II-2. Distribution of the testis weights (mg) in mutant and normal mice.

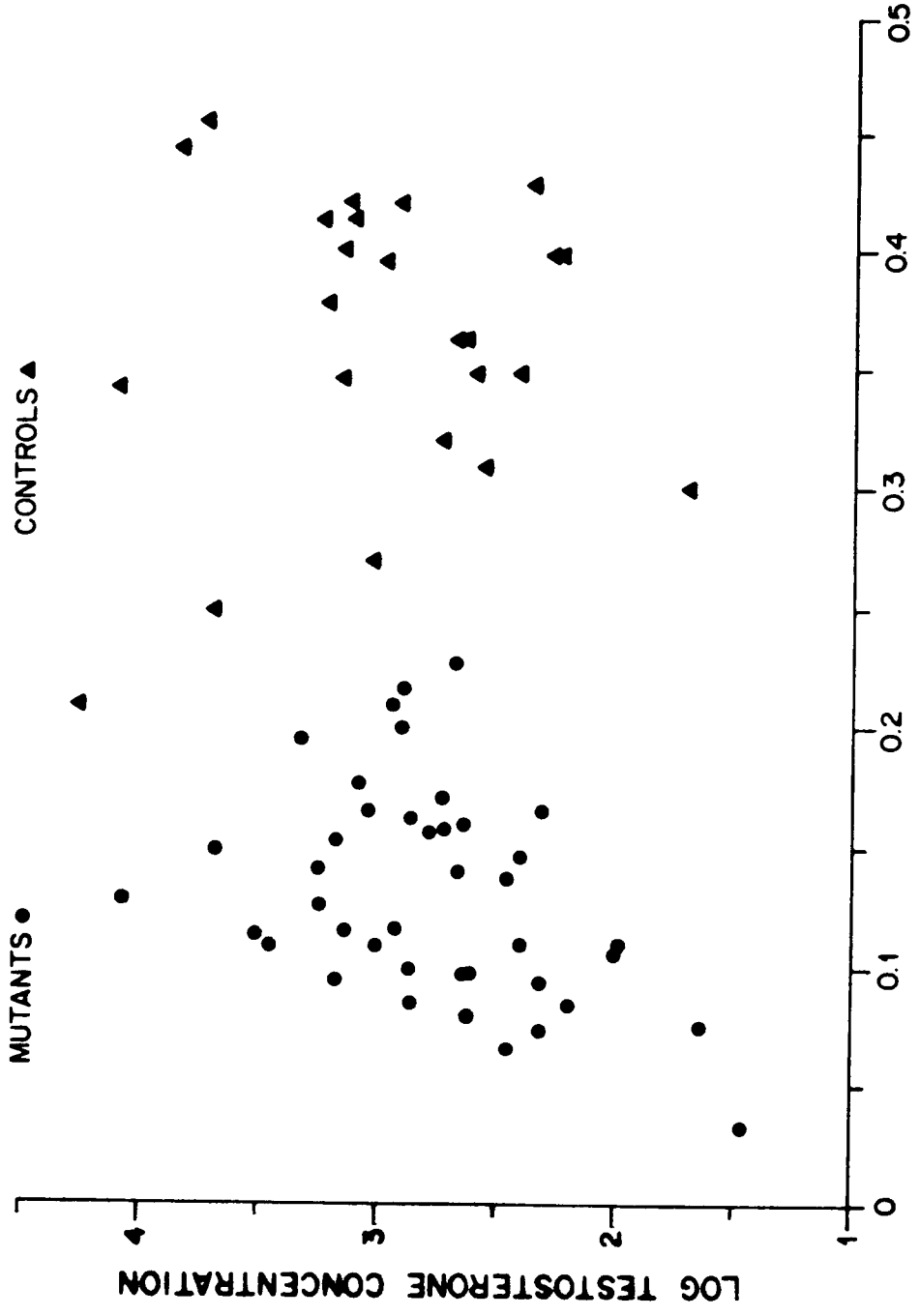


Fig. II-3. The log 10 testosterone concentrations plotted as a function of the ratio of the testis weights (TW) over body weights (BW). Regression analysis shows linear correlation for the mutant data ($r = 0.3830$; $P < 0.05$) and no correlation for the control data ($r = -0.0961$; $P > 0.2$).

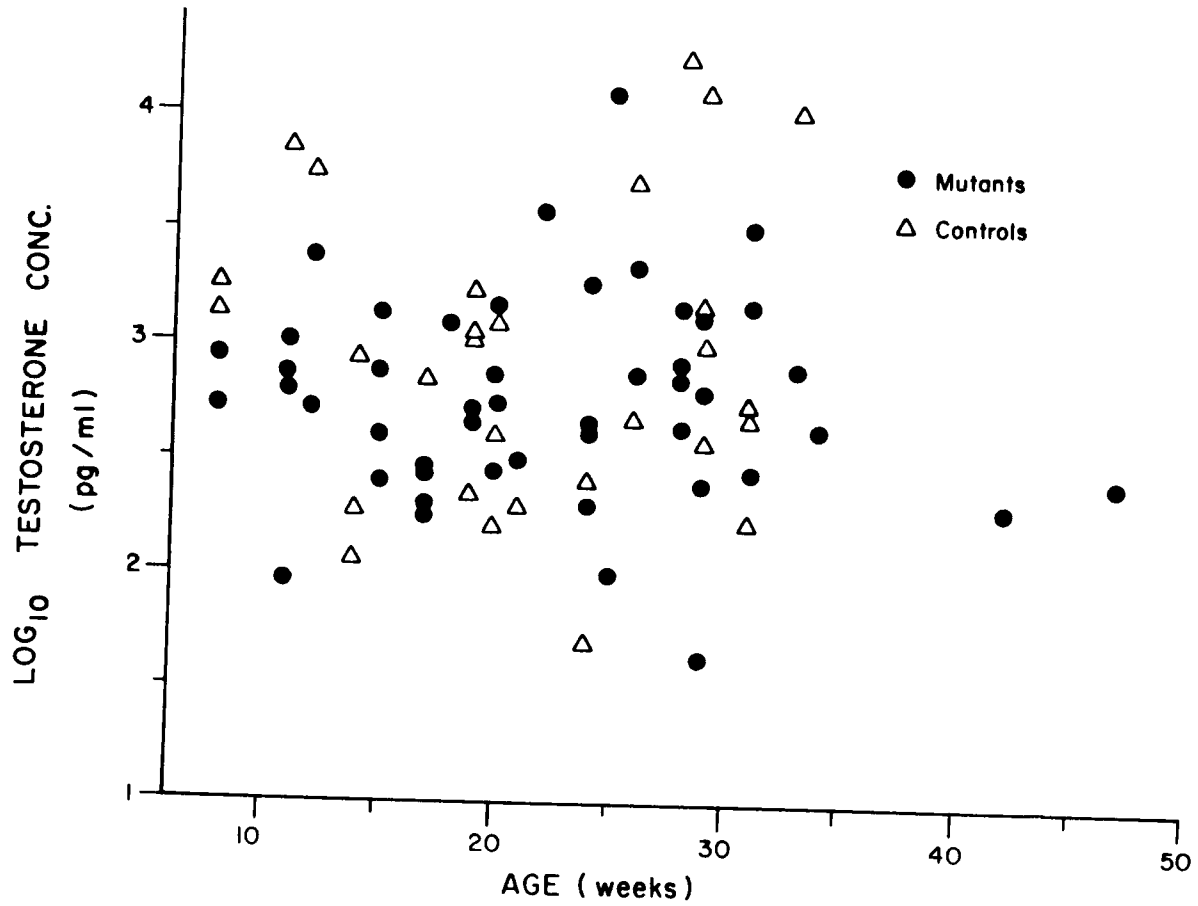
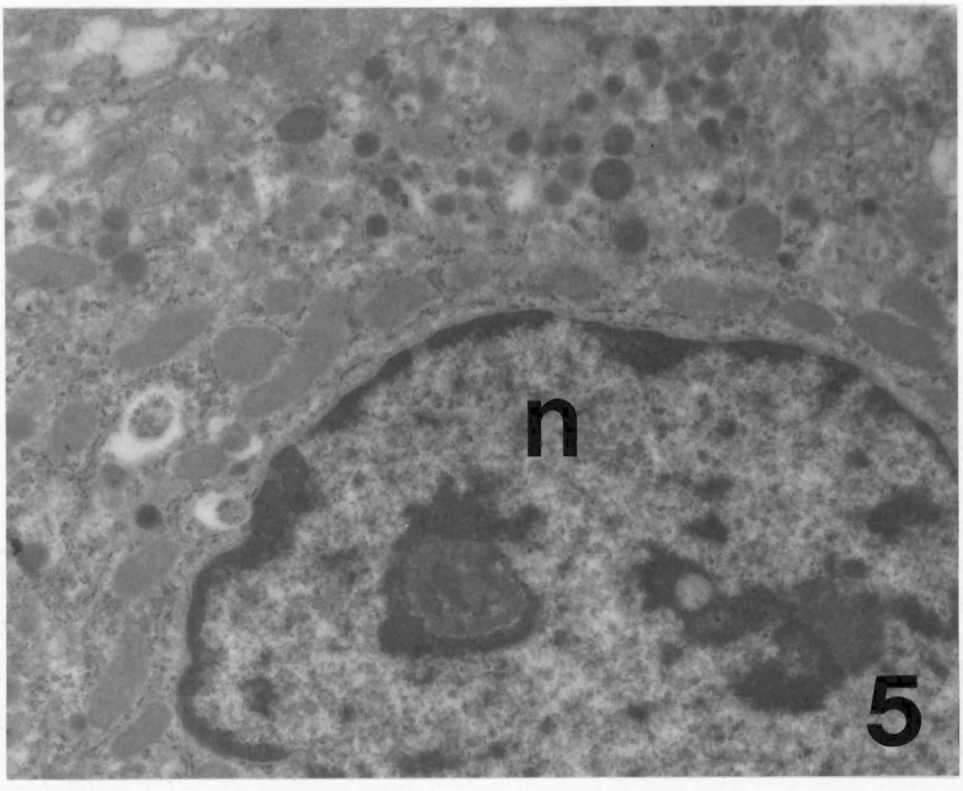


Fig. II-4. The log 10 testosterone concentrations plotted as a function of age. Regression analysis shows no significant linear correlations for the mutant data ($r = -0.0344$; $P > 0.2$) or for the control data ($r = 0.0158$; $P > 0.2$).

Fig. II-5. Electron micrograph of a normal Leydig cell, (n) nucleus. X12,000.

Fig. II-6. Electron micrograph of a mutant (bs/bs) Leydig cell. Note the abundance of lipid droplets (arrow) in the cytoplasm, (n) nucleus. X12,000.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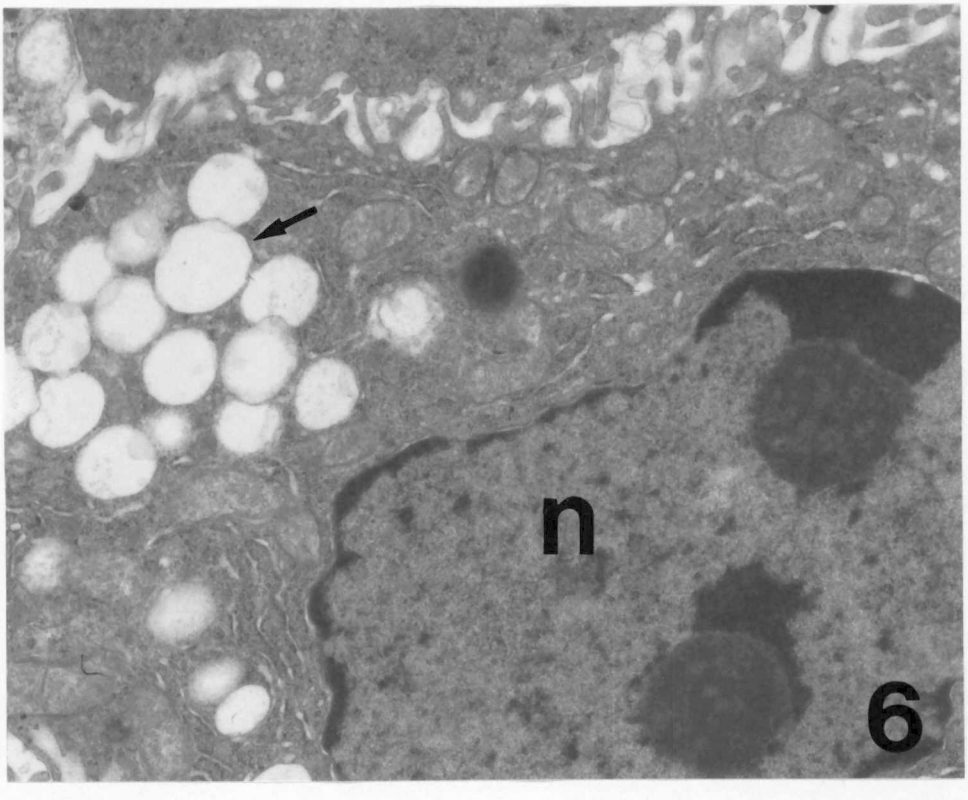
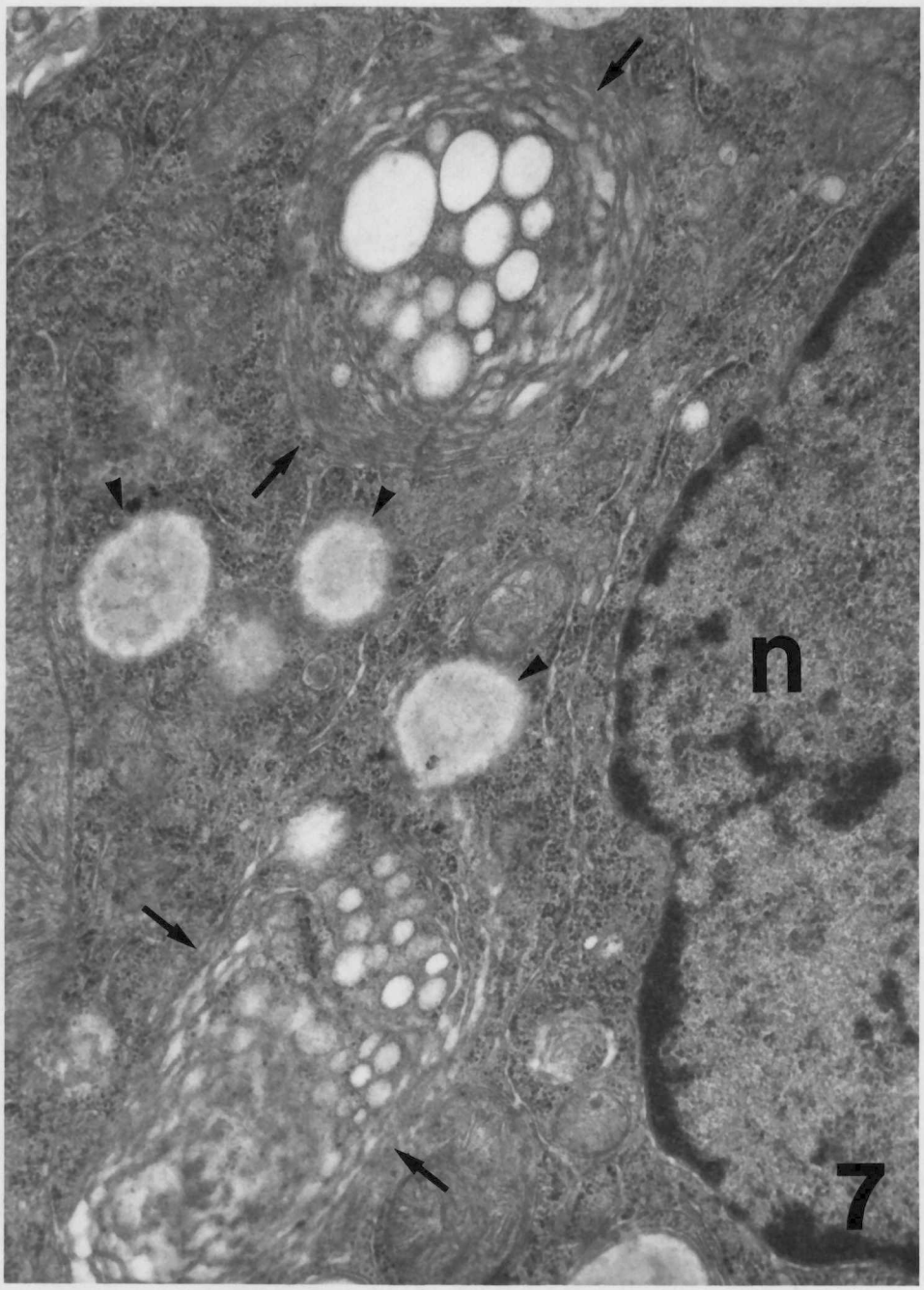

n
6

Fig. II-7. Part of the cytoplasm of a mutant Leydig cell showing large whorls of smooth endoplasmic reticulum (arrows) and lipid droplets (arrowheads); (n) nucleus. X23,000.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7

Fig. II-8. Electron micrograph of a normal Sertoli cell showing the typical tripartite nucleolus (a) and characteristic intercellular junctions (b); (nu), nucleolus. (a) X100,000; (b)X30,000.

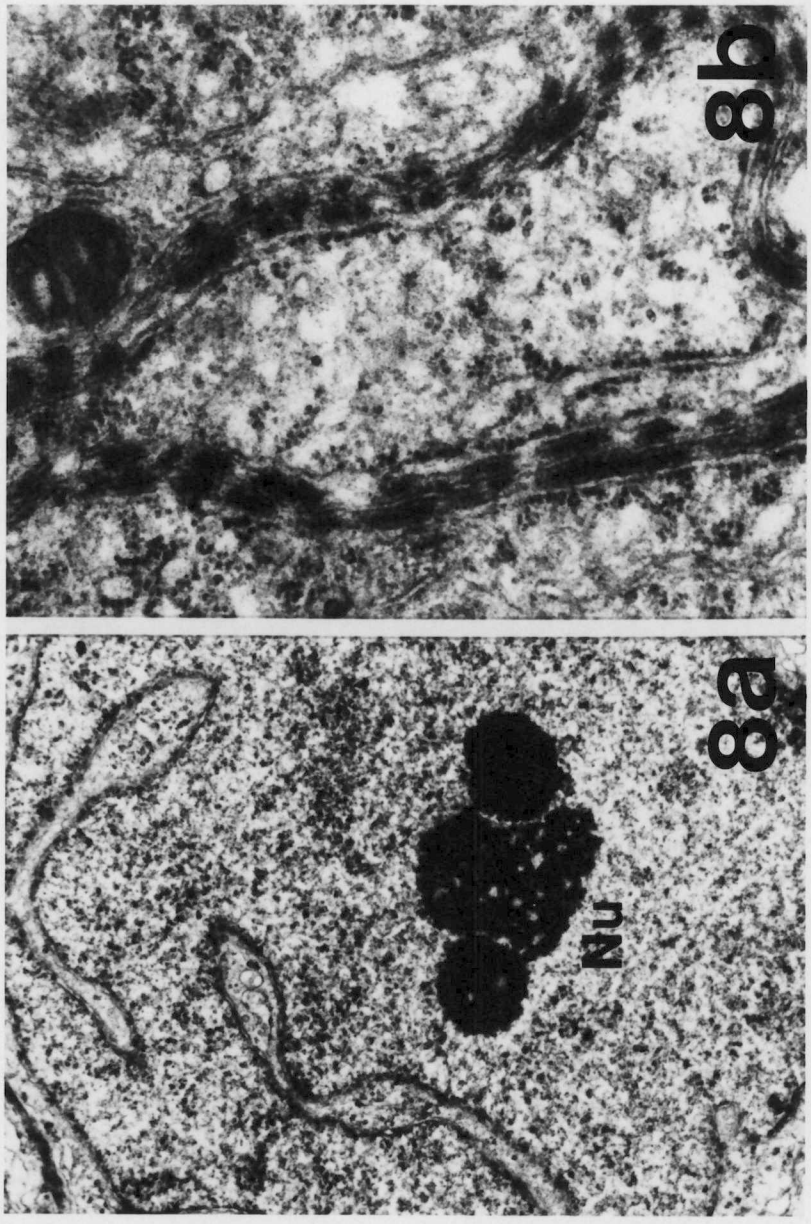
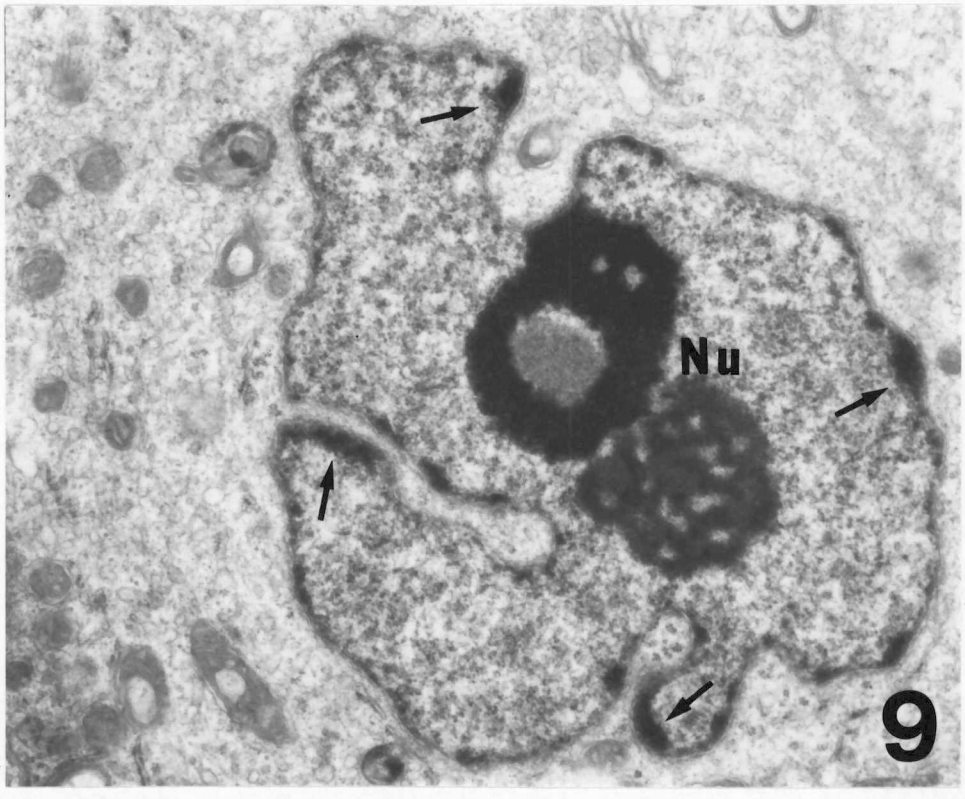
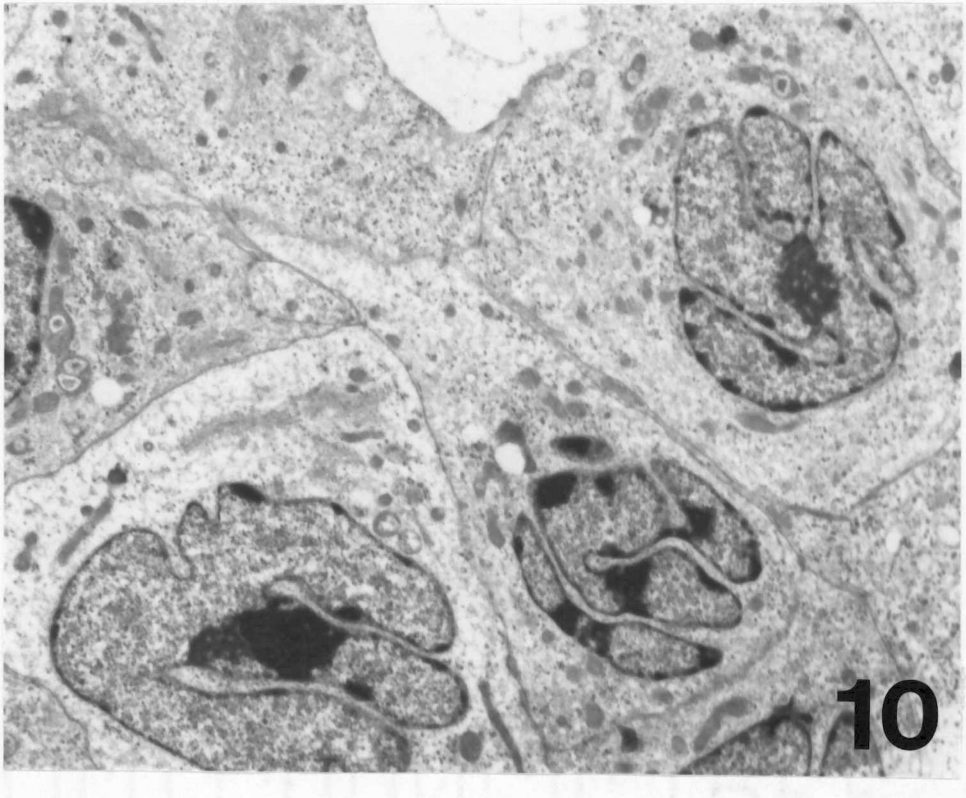

8b
8a
Nu

Fig. II-9. Nuclear morphology of an abnormal Sertoli cell from mutant mice. Note the heterochromatin clumps associated with the nuclear envelope (arrows) and the atypical nucleolus (Nu). X12,000.

Fig. II-10. Low power electron micrograph showing a cluster of abnormal Sertoli cells from mutant mice. X5000.


Nu
9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PART III

GERM CELL DEFICIENCY IN THE POSTNATAL DEVELOPMENT OF A MALE STERILE MOUSE MUTANT

GERM CELL DEFICIENCY IN THE POSTNATAL DEVELOPMENT OF A MALE STERILE MOUSE MUTANT

Abstract

The phenomenon of germ cell deficiency during postnatal development of male mice homozygous for the blind-sterile mutation (bs) was studied. Spermatogenesis is defective (in adults) and is characterized by extensive germ cell deficiency and failure of acrosome assembly. It was not known if germ cell deficiency was a consequence of abnormal differentiation of spermatids or was an independent effect of the bs mutation. The present study shows that germ cell deficiency is present as early as 5 days of age and continues throughout the postnatal development, therefore, germ cell deficiency is independent from the appearance of defective early spermatids. The number of gonocytes, type A spermatogonia and the mitotic index were estimated in the 5 day old testes. In all cases a reduction in the number of germ cells per tubule cross-section was demonstrated. However mitotic indices were similar in mutant and normal testes. These results suggested that germ cell deficiency may occur as a result of insufficient numbers of primordial germ cells populating the gonads. A marked delay in the progression of the spermatogenic wave was observed in those tubules containing relatively normal germ cell associations at all ages up to 40 days. Sertoli cell maturation was also delayed.

Introduction

Testis hypoplasia, germinal aplasia and germ-cell depletion or deficiency are all descriptive terms for a generalized pathological condition characterized by extensive loss of germ cells from the seminiferous tubules (Bishop, 1972). Low levels of germ-cell deficiency can also occur in normal testes (Roosen-Runge, 1973). Various pleiotropic gene mutations of the mouse which produce sterility in the homozygous state are known to cause extensive germ-cell depletion in the testis. Alleles of the T-gene complex (Bryson, 1944), of the W-locus (Mintz, 1957; Mintz and Russell, 1957; Coulumbre and Russell, 1954), the "blind-sterile" mutation, bs, (Sotomayor and Handel, 1986), and male sex reversal (Cattanach, 1971; Chung, 1974) are all known to cause profound germinal deficiencies.

We have investigated the occurrence of germ cell deficiency during postnatal development of bs/bs mice. The bs gene mutation in the homozygous state causes failure of acrosome assembly and extensive deficiency of spermatogenic cells (Sotomayor and Handel, 1986). It was not known whether germ cell deficiency occured as a consequence of the abnormal early spermatid stages or independently of this abnormality.

Materials and Methods

Mice

Male mice homozygous for the blind-sterile (bs) mutation were used in this study. Normal littermates (bs/+ or +/?) were used as controls. The mice were derived from the F2 of (AKR-bs X 129) hybrids

originally received from the Jackson Laboratory (Bar Harbor, ME). They were maintained in the University of Tennessee Animal Facility by brother-sister matings of bs/bs or +/bs females with +/bs males. All mice were provided with food and water ad libitum and were maintained on a 14L : 10D schedule.

Cytological Analysis

Thirty-one normal mice and 26 mutant mice were sacrificed at 5, 10, 15, 20, 30, 40, 75, and 120 days post-birth, respectively. The bs/bs mice were recognized by the presence of prominent bilateral cataracts within 5 days of birth. The animals were weighed and their testes removed, weighed in pairs and fixed in Zenker-formaldehyde. After embedding in Paraplast (Lancer, St. Louis, MO) the testes were sectioned at 5u and were stained with the periodic acid-Schiff-hematoxylin technique (Leblond and Clermont, 1952).

Identification of all cell types in the testis was based on previously established criteria (Leblond and Clermont, 1952; Oakberg, 1956; Clermont and Perey, 1957). Preparations were scored with a Zeiss photomicroscope at various magnifications. Estimates of the number of gonocytes, type A spermatogonia and the mitotic index were made in the 5 day old testes only. Quantitative analysis of various germ cell associations per tubule cross-section was performed in all age groups excepting the 75 and 120 day old. In general the quantitative procedures of Clermont and Perey (1957) and Beaumont and Mandl (1963) were followed but no correction for the Abercrombie's factor (Abercrombie, 1946) was made since only relative cell numbers were compared.

Because of the occurrence of extensive germ cell deficiency and lack of acrosome assembly in early spermatids of bs/bs testis, the classical 12 stages of the seminiferous epithelium could not be detected. It was necessary to develop an arbitrary staging in order to compare the mutant testes with the normal ones. Six types of germ-cell associations were developed as described by Sotomayor and Handel (1986). Type I represented the most deficient tubule cross-sections observed, containing no germ cells but only Sertoli cells or Sertoli cell precursors, depending on the age of the mouse. Type II tubules were characterized by the presence of gonocytes, type A spermatogonia and Sertoli cells. Type III tubules included spermatogonia A through B, preleptotene-S spermatocytes, mitotic spermatocytes and Sertoli cells. Type IV tubules contained similar stages to those of type III but the spermatogenic wave had progressed to round spermatids steps 1-7. Type V associations were characterized by the presence of elongating spermatids, steps 8 to 12 in addition to the stages described in type IV. Finally, type VI tubules contained maturing spermatids in steps 13 to 16 and a new wave of early round spermatid stages. For each of these arbitrary types of germ-cell associations, the most advanced germ-cell stage present is indicated.

Results

Table III-1* shows quantitative data on the progression of the different germ-cell association types throughout postnatal development

*All tables may be found in the Appendix.

of normal and mutant testes. Table III-2 shows the qualitative results on the most advanced germ-cell stages present in the various germ-cell associations at different ages post-birth. We will describe in detail these observations for each of the ages studied.

5-Day Old Mice

In normal testes of this age, 99 percent of the tubule cross-sections were characterized by type II germ cell associations (Table III-1). These tubules contained Sertoli cell precursors forming a palisade-like layer along the basement membrane, some gonocytes, type A spermatogonia and few intermediate spermatogonia (Table III-2).

Gonocytes were the largest cells in the tubule with a large spherical nucleus containing light fine chromatin and two or more prominent nucleoli. Type A spermatogonia showed the typical characteristics of adult type A spermatogonia with an oval nucleus containing dust-like chromatin (Fig. III-1*).

Several mitotic figures were observed in the 5-day old testis. These comprised large and small mitosis as has been previously reported by Clermont and Perey (1957). As suggested by these authors the large mitosis appear to come from dividing gonocytes (Fig. III-1) and the small ones from immature Sertoli cells.

In the mutant testes, the majority of the tubule cross-sections (68%) contained type I cell association, i.e., only Sertoli cell precursors (Fig. III-2). This observation documents that germ-cell deficiency had occurred prior to 5 days of age in the mutant testes.

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*All figures may be found in the Appendix.

Gonocytes and type A spermatogonia were found in 32 percent of the tubule cross sections (Table III-1); however, no intermediate spermatogonia were present (Table III-2).

The number of gonocytes per tubule cross-section and the total number of germ cells per tubule cross-section were significantly reduced in the mutant testes (Tables III-3 and III-4). Tubule cross-sections containing only 1 germ cell were equally frequent in mutant and normal testes (Fig. III-3 and Table III-4). However, frequencies of tubule cross-sections containing 2 or more germ cells were reduced in mutant testes. The mitotic index (number of mitotic figures/total number of germ cells X 100) were similar in normal and mutant testes (Table III-3).

10-Day Old Mice

In normal testes a new type of germ cell association classified as Type III was observed by 10 days of postnatal development. Forty-nine percent of the tubule cross-sections were of this type (Table III-1). The most advanced mitotic stage observed was leptotene spermatocyte (Table III-2), but most tubule cross-sections contained preleptone S-spermatocytes (Fig. III-4). Some tubule-cross sections still contained gonocytes. The Sertoli cells showed the typical morphology of immature Sertoli cells.

In contrast, only 1 percent of tubule cross-sections in mutant testes were type III (Table III-1). The most advanced germ-cell stage was preleptotene S-spermatocyte (Table III-2). The majority of the tubule cross-sections (84%) showed only immature Sertoli cells (type I association) and no germ cells (Fig. III-5).

15-Day Old Mice

In the normal testes at 15 days of postnatal development, type III germ cell associations represented 85 percent of the total. Meiotic spermatocytes had advanced to pachytene spermatocyte stages (Table III-2). Gonocytes were no longer present (Fig. III-6). This is the first age at which a morphological change was seen in the Sertoli cell nucleus. Fewer heterochromatic clumps were associated with the nuclear membrane and the typical adult tripartite nucleolus had developed (Fig. III-7 and Table III-2).

In comparison, in mutant testes only 13 percent of the tubule cross-sections contained type III germ cell associations (Table III-1) and the most advanced germ-cell stage was still preleptotene S-spermatocyte (Table III-2). Type I germ cell associations were the most prevalent with 62 percent of the cross-sections (Fig. III-8). Sertoli cells still appeared immature, with abundant heterochromatin clumps associated with the nuclear envelope, and poorly defined nucleoli.

20-Day Old Mice

In the normal testes spermatogenesis by day 20 had progressed to round spermatid stages. Most of these were in steps 1-2. This characterized type IV germ cell associations, which were detected in 10 percent of the tubule cross-sections (Table III-1). Type II associations were no longer detected and type III comprised 90 percent of the total. Sertoli cells were morphologically similar to those of 15 day old testes.

No tubule cross-sections containing type IV germ cell association were found in mutant testes (Table III-1). Type II associations were observed in 11 percent of the tubules indicating spermatogenic arrest or out of phase spermatogenic differentiation. This characteristic of partial progress of the spermatogenic wave persisted throughout the postnatal development of the bs/bs testes. The most depleted tubules were detected in 82 percent of the total. Sertoli cells still appeared immature, similar to those of 10 or 15 day old testes.

30-Day Old Mice

In the normal testes at 30 days of development, spermatids were observed in all tubule cross-sections. The most advanced spermatid stages were maturing spermatids in steps 13 to 16 which characterized type VI germ cell associations. These represented 58 percent of the tubule cross-sections. The typical morphology of the adult Sertoli cells was seen for the first time (Fig. III-9). The nuclei contained no heterochromatin clumps, as seen in the 20-day old testes, and the tripartite nucleolus was present (Table III-2).

In the mutant testes only 5 percent of tubule cross sections had advanced to type IV germ cell associations. The most advanced germ cell stage was round spermatid (Table III-2). Tubule cross-sections showing incomplete germ cell associations (types II and III) were observed in 8 and 13 percent of the tubules, respectively (Table III-1). Extensive germ-cell deficiency, demonstrated by the type I germ cell association, was detected in 74 percent of the tubule cross-sections (Table III-1). Some mutant Sertoli cells were

morphologically similar to those described in younger mutant testes. However, several Sertoli cells showed, for the first time, the tripartite nucleolar structure characteristic of the adult testes (Fig. III-10 and Table III-2).

40-Day Old Mice

The different types of germ cell associations observed in the normal testes at 40 days were similar to those seen in the 30 day old testes. Sixty-three percent of the tubule cross-sections contained type VI germ cell associations (Table III-1).

In contrast, mutant testes showed only 4 percent of the tubule cross-sections with type VI germ cell associations. The percentage of type I associations decreased to 33 percent, while partially depleted germ-cell associations were observed in different frequencies (Table III-1). This is the first developmental stage when the mutant Sertoli cells appeared with the typical nuclear morphology of the adult Sertoli cell, showing no differences with those of the normal mice. However, in some tubules containing only Sertoli cells, a few clusters of immature Sertoli cells were seen.

Body and Testis Weights

Body and testis weights were measured in mutant and normal mice from 5 to 120 days of age (Table III-5). No major differences in body weight were seen between mutant and normal mice up to 20 days of age, however, the slopes of the respective curves (slope bs/bs = 0.2013 and slope +/- = 0.2425), indicate that the normal mice grew slightly faster than the mutant ones (Fig. III-11).

Testis weights of mutant mice were reduced at all ages after birth (Table III-5). When the testis weight/body weight ratios were plotted against age, a linear increase in testis weights was detected from 5 to 30 days of age post-birth (Fig. III-12). No significant increase occurred thereafter, apparently reaching a plateau as shown by the broken lines in Fig. III-12. A linear regression analysis of the data between 5 and 30 days of age confirmed the linear fit for both groups of mice. The correlation coefficients (r) were 0.9877 and 0.9815 for the normal and mutant testes, respectively. The slopes of the curves were 0.0126 for the normal testes and 0.0044 for the mutant ones. Therefore, during the 5-to-30 day period post-birth, the normal testes increased in weight about 3 times faster than the mutant testes.

Discussion

The present results show that extensive germ cell deficiency occurred throughout the entire postnatal development of mutant testes. Tubule cross-sections containing only Sertoli cells or Sertoli cells precursors were found in high frequencies (up to 84%) in testes ranging from 5 to 40 days of age. In contrast, practically no tubule cross-section of normal testes contained only Sertoli cells. The results indicate that germ cell deficiency occurs much earlier than the first appearance of defective spermatids in the bs/bs testes. Spermatids were detected for the first time in the 30 day old mutant testes. In comparison, in the normal testes, early spermatid stages were observed for the first time at 20 days of age. Therefore it can

be concluded that germ cell deficiency does not occur as a consequence of the formation of acrosome-lacking spermatids but is an independent effect of the bs mutation.

A significant delay in the progression of the spermatogenic wave was evident in all the mutant seminiferous tubules otherwise containing relatively normal germ cell associations. This delay was also manifest in the maturation of the Sertoli cells. In the normal testes the typical tripartite nucleolus was detected for the first time at 15 days of age. However, the definitive nuclear morphology of the adult Sertoli cells was not seen until the 30 days of age, in agreement with the observations of Clermont and Perey (1957). In contrast, the tripartite nucleolus of mutant Sertoli cells was not detected until 30 days of age. At this time, some tubule cross-sections contained clusters of apparently immature Sertoli cells. This abnormal characteristic has also been reported in the XX sex-reversed condition of the mouse (Chung, 1974).

The possibility that mutant gonocytes and type A spermatogonia were not as mitotically active as the normal ones was also investigated. In addition, the number of gonocytes and total germ cells per tubule cross-section was estimated. As expected, the number of gonocytes and type A spermatogonia per tubule cross-section was significantly reduced in the mutant testes. Also the percentage of tubule cross-sections containing 2 or more germ cells was much lower than those of the controls. However, the mitotic index for these germ cells was about the same in both groups of mice. Our data on the normal germ cells from the 5 day old testes are in general agreement

with those of Clermont and Perey (12957) and Beaumont and Mandl (1963).

The results suggest that mutant gonocytes and spermatogonia are mitotically competent. The cause for their reduced numbers might, therefore, be related to problems in the migration of the primordial germ cells to the genital ridges of male mice. A comparable situation which also results in extensive germ cell deficiency has been reported for the mutants of the W-locus in mice (Coulombre and Russell, 1954; Mintz, 1957; Mintz and Russell, 1957). In this case it was demonstrated that the mutant primordial germ cells exhibited migratory retardation and mitotic insufficiency resulting in dramatically reduced germ cells at birth. We suggest that bs/bs primordial germ cells fail to populate male gonads in sufficient numbers, thus producing the typical pattern of germ cell deficiency demonstrated in this study. Extensive germ cell deficiency is the common result of a variety of genetic and non-genetic pathological conditions affecting the mammalian testis (Bishop, 1972). Whatever the cause for germ cell deficiency, the trigger mechanism must operate early in the differentiation of the germ cells.

PART III

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REFERENCES

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PART III

APPENDIX

Table III-1. Percentage of tubule cross-sections containing various types of germ cell associations at different ages post-birth. Description of the different types of germ cell associations is given in Material and Methods.

Age In Days	Genotypes	Germ Cell Association Types						Number of Tubule Cross-Sections	Number of Mice
		I	II	III	IV	V	VI		
5	bs/bs	68	32	0	0	0	0	1030	3
	+/-	1	99	0	0	0	0	1002	3
10	bs/bs	84	15	1	0	0	0	1115	3
	+/-	0	51	49	0	0	0	1437	3
15	bs/bs	62	25	13	0	0	0	1231	3
	+/-	0	15	85	0	0	0	1283	3
20	bs/bs	82	11	7	0	0	0	1054	3
	+/-	0	0	90	10	0	0	855	3
30	bs/bs	74	8	13	5	0	0	696	3
	+/-	0	0	0	21	21	58	1342	3
40	bs/bs	33	14	16	27	6	4	716	3
	+/-	0	0	1	7	29	63	656	3

Table III-2. The most advanced germ cell stages present in the testes at various ages post-birth. The number of tubule cross-sections examined are shown in Table III-1.

Age Days	Genotype	Type A Spgonia	Interm. Spgonia	Type B Spgonia	Prelept. S-Sperma.	Lepto.	Pachy. Sperma.	R.S. (1-7)	R.S. (8-12)	Mature Sptds. (13-16)	Sertoli Cell Tripartite Nucleolus
5	bs/bs	+	-	-	-	-	-	-	-	-	-
	+/-	+	+	-	-	-	-	-	-	-	-
10	bs/bs	+	+	+	-	-	-	-	-	-	-
	+/-	+	+	+	+	+	-	-	-	-	-
15	bs/bs	+	+	+	+	-	-	-	-	-	-
	+/-	+	+	+	+	+	+	-	-	-	+
20	bs/bs	+	+	+	+	+	+	-	-	-	-
	+/-	+	+	+	+	+	+	+	-	-	+
30	bs/bs	+	+	+	+	+	+	+	-	-	+
	+/-	+	+	+	+	+	+	+	+	+	+
40	bs/bs	+	+	+	+	+	+	+	+	+	+
	+/-	+	+	+	+	+	+	+	+	+	+

Abbreviations: Spgonia., spermatogonia; Interm., intermediate; Prelept., preleptotene; Sperma., spermatocyte; Lepto, leptotene; Pachy., pachytene; R.S., round spermatids; Sptds., spermatids.

Table III-3. Average number of germ cells per tubule cross-section and their mitotic index in 5 day old testes.

Geno-type	Gono-cytes	Gonocytes + Type A Spgonia	Percent Mitotic Germ	Number Tubule Cross-Sections	Number of Mice
+/-	0.8 ± 0.1*	2.6 ± 0.2*	6 ± 2	300	3
bs/bs	0.2 ± 0.1*	0.8 ± 0.9*	8 ± 1	300	3

*F-test ($P < 0.01$)

Table III-4. Mean percentage of tubule cross-sections containing 0 to 5 gonocytes and/or type A spermatogonia in normal and mutant testes of 5 day old mice.

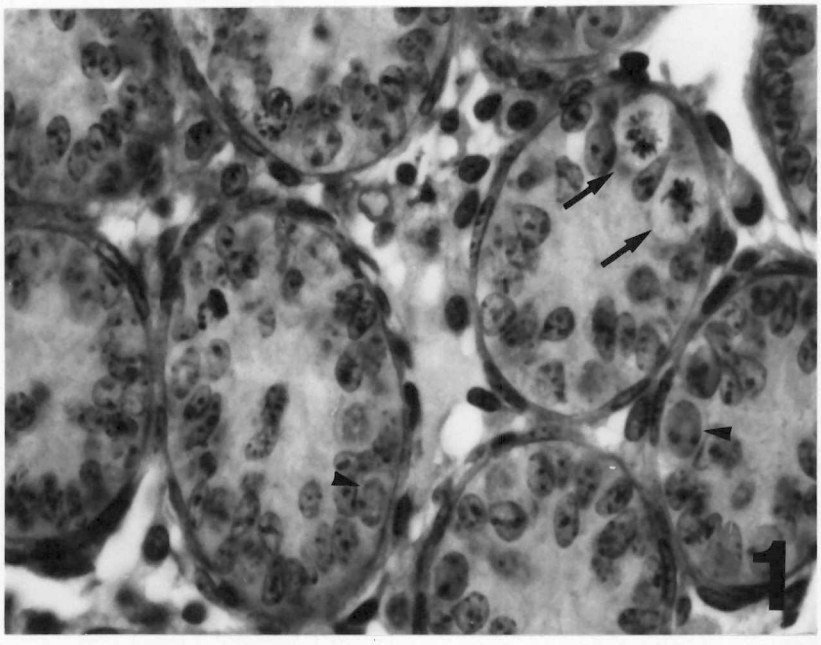
	Mean Percentage ± Standard Deviation						Number Cross-Sections	Number Mice
	0	1	2	3	4	5		
Normal (+/-; +/bs)	1 ± .6	15 ± 3	33 ± 3	32 ± 4	16 ± 2	2 ± 2	300	3
Mutants (bs/bs)	60 ± 33	14 ± 9	14 ± 15	9 ± 3	3 ± 5	.3 ± .6	300	3

Table III-5. Body and testis weights during postnatal development of bs/bs and +/? or +/bs mice. The mean ± S.D. are shown.

Age In Days	+/? or +/bs			bs/bs		
	Body Weight (g)	Testis Weights (mg)	N	Body Weight (g)	Testis Weights (mg)	N
5	4.0 ± 0.6	3.4 ± 0.7	3	4.0 ± 0.2	2.5 ± 0.3	3
10	6.1 ± 0.8	6.0 ± 0.5	8	5.8 ± 1.2	3.6 1 0.4	3
15	5.7 ± 1.4	10.1 ± 3.3	4	8.5 ± 0.4	8.6 ± 2.0	3
20	7.7 ± 1.3	19.5 ± 4.5	3	5.9 ± 1.5	7.1 ± 2.9	3
30	15.4 ± 2.1	58.6 ± 4.6	3	11.6 ± 1.0	19.0 ± 2.0	3
40	17.4 ± 4.3	63.3 ± 23.6	3	13.6 ± 5.0	26.7 ± 14.9	3
75	25.7 ± 1.5	109.0 ± 6.0	3	22.4 ± 1.3	32.8 ± 9.3	5
120	30.5 ± 3.1	113.0 ± 1.4	4	26.3 ± 0.6	42.0 ± 4.6	3

Fig. III-1. Section through a 5 day old normal testes showing gonocytes (arrows) and type A spermatogonia (arrowheads). X800.

Fig. III-2. Section through a 5 day old mutant testis showing germ cell deficient seminiferous tubules. Only Sertoli cell precursors are present (arrows). X800.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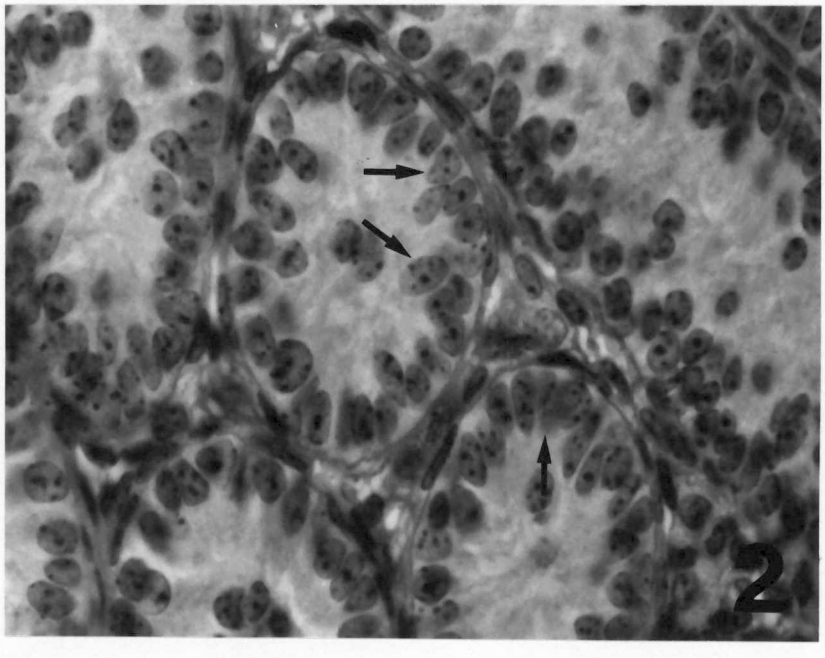
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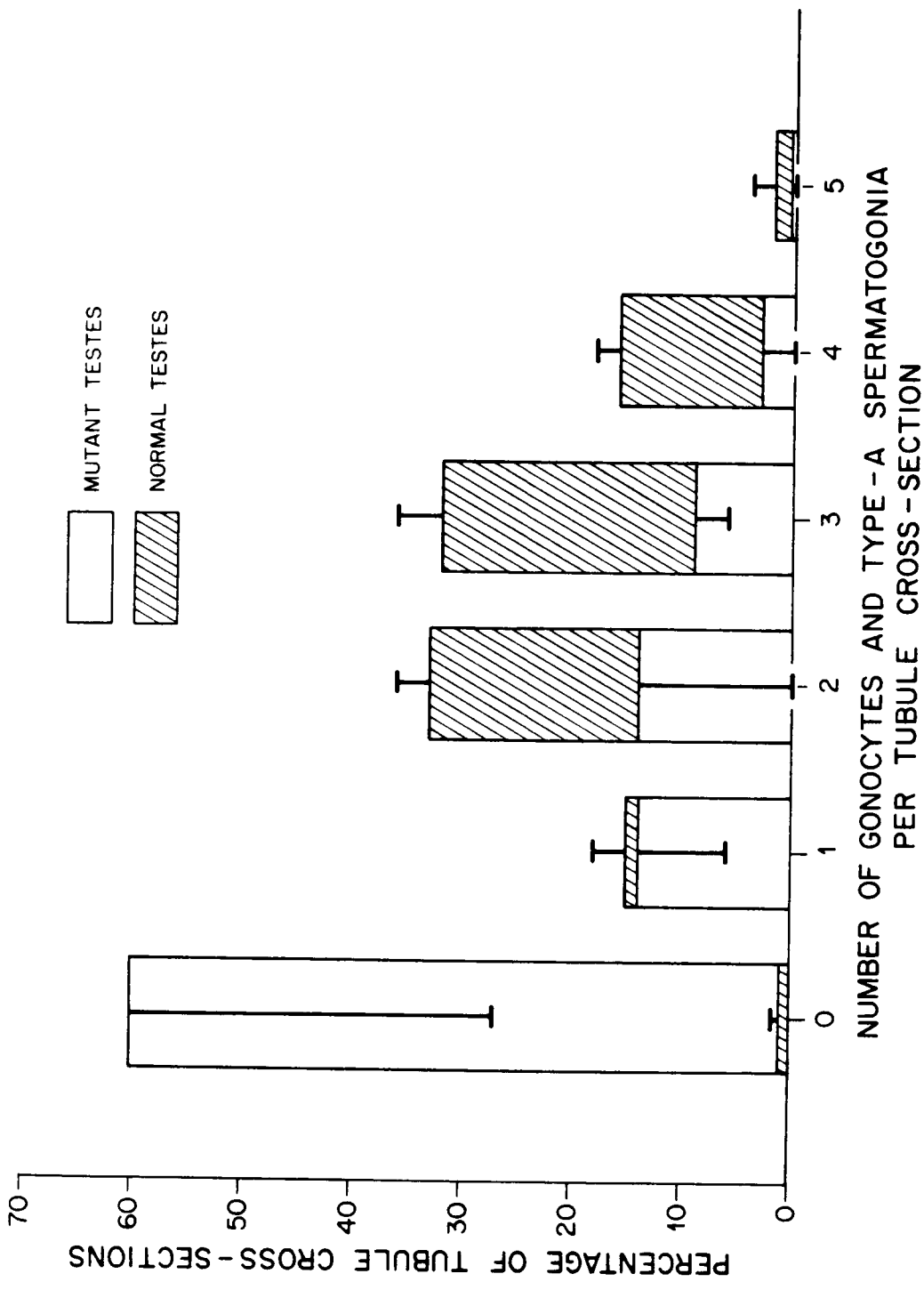
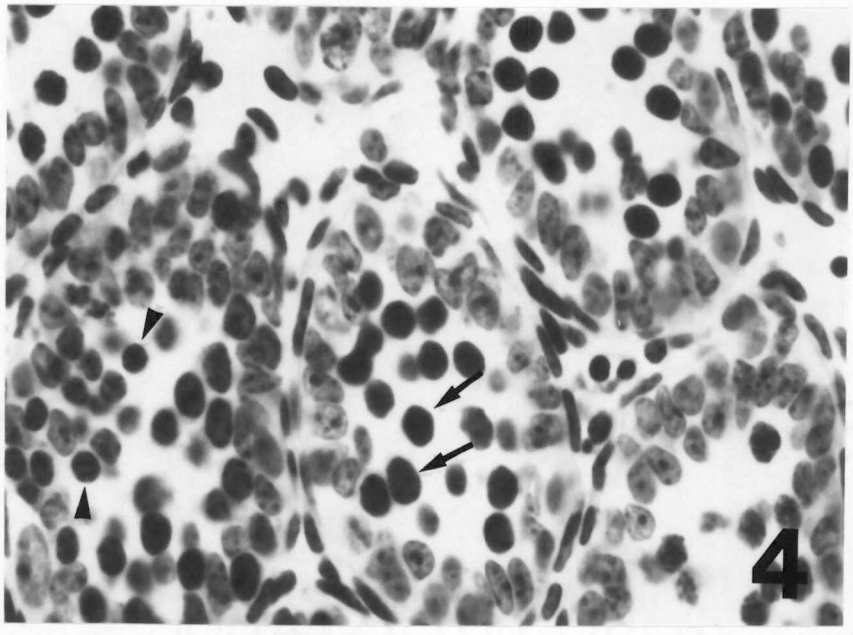


Fig. III-3. Histogram of the distribution of gonocytes and type A spermatogonia per tubule cross-section in mutant and normal testes from 5 day old mice.

Fig. III-4. Tubule cross-sections from a 10 day old normal testis showing preleptotene S-spermatocytes (arrowheads) and some leptotene spermatocytes (arrows). X600.

Fig. III-5. Tubule cross-sections from a 10 day old mutant testis showing preponderance of immature Sertoli cells (arrows). X500.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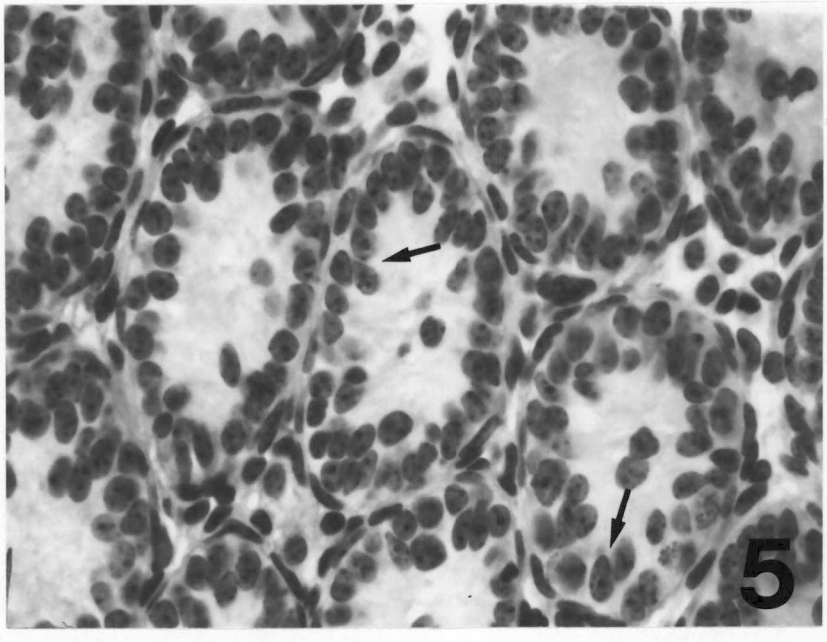
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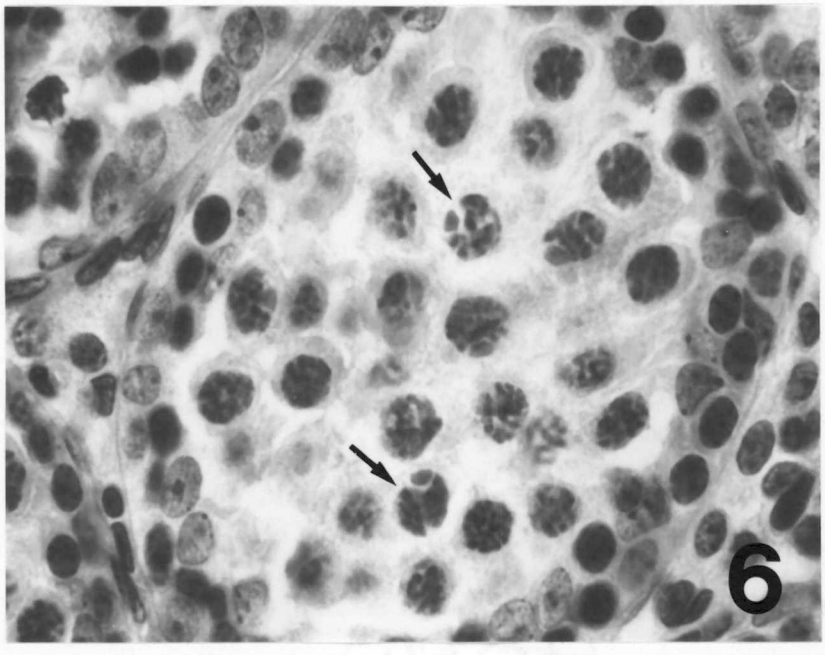
Fig. III-6. Tubule cross-sections from a 15 day old normal testis. Pachytene spermatocytes are the most advanced germ-cell stages present in these testes (arrows). X1200.

Fig. III-7. Sertoli cells from a normal 15 day old testis showing the typical tripartite nucleolar structure (arrows). X1400.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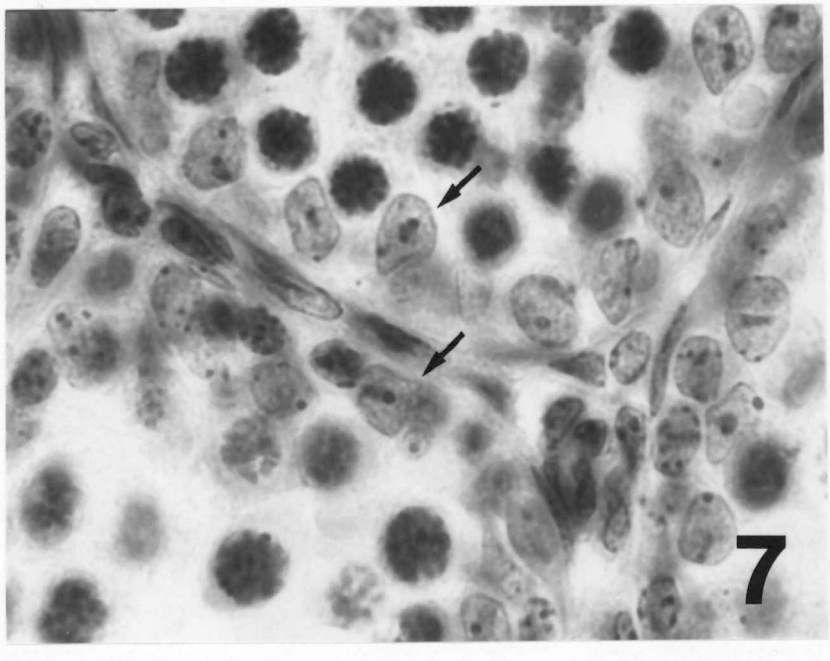
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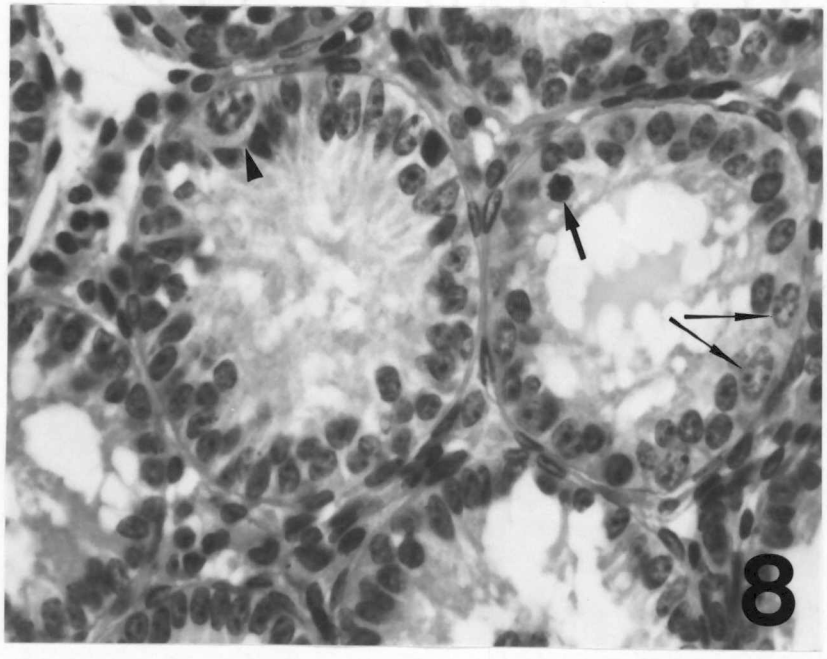
Fig. III-8. Testis cross-section from a 15 day old mutant testis showing mostly germ cell deficient tubules. Some preleptotene S-spermatocytes (arrows), gonocytes (arrowhead) and type A spermatogonia (long arrows) can be seen. X500.

Fig. III-9. Sertoli cells from a normal 30 day old mouse showing the typical tripartite nucleolar structure (arrow). Note the presence of round spermatids in steps 5-6 of spermiogenesis (arrowheads). X800.

Fig. III-10. Sertoli cells from a mutant 30 day old mouse showing the tripartite nucleolar structure (arrow). Some heterochromatin clumps are still associated with the nuclear membrane. X800.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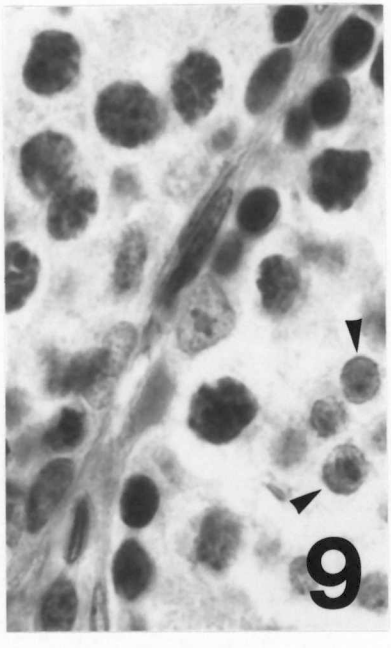
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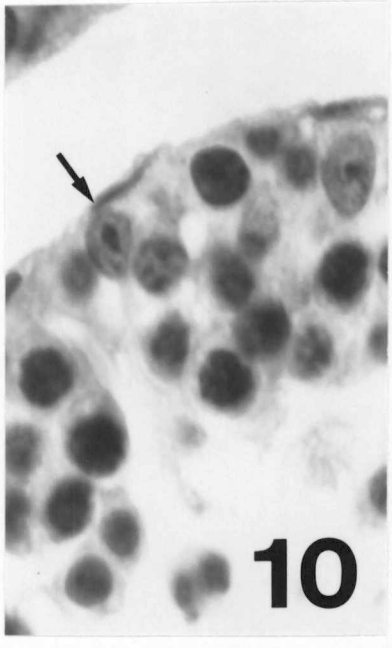
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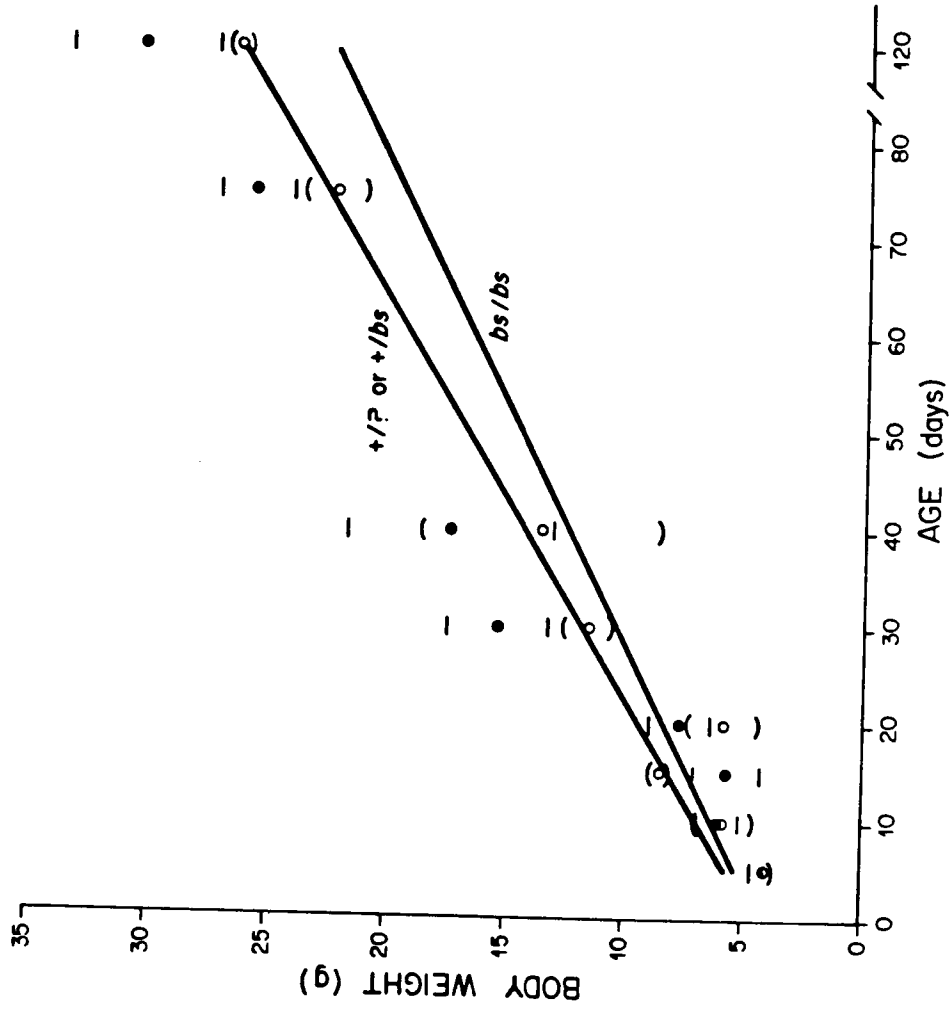

Fig. III-11. Body weight growth in mutant and normal mice from 5 to 120 days of age. The straight lines represent the least squares linear fit to the data; $r_{\underline{bs/bs}} = 0.9717$ and $r_{\underline{+/-}} = 0.9600$.

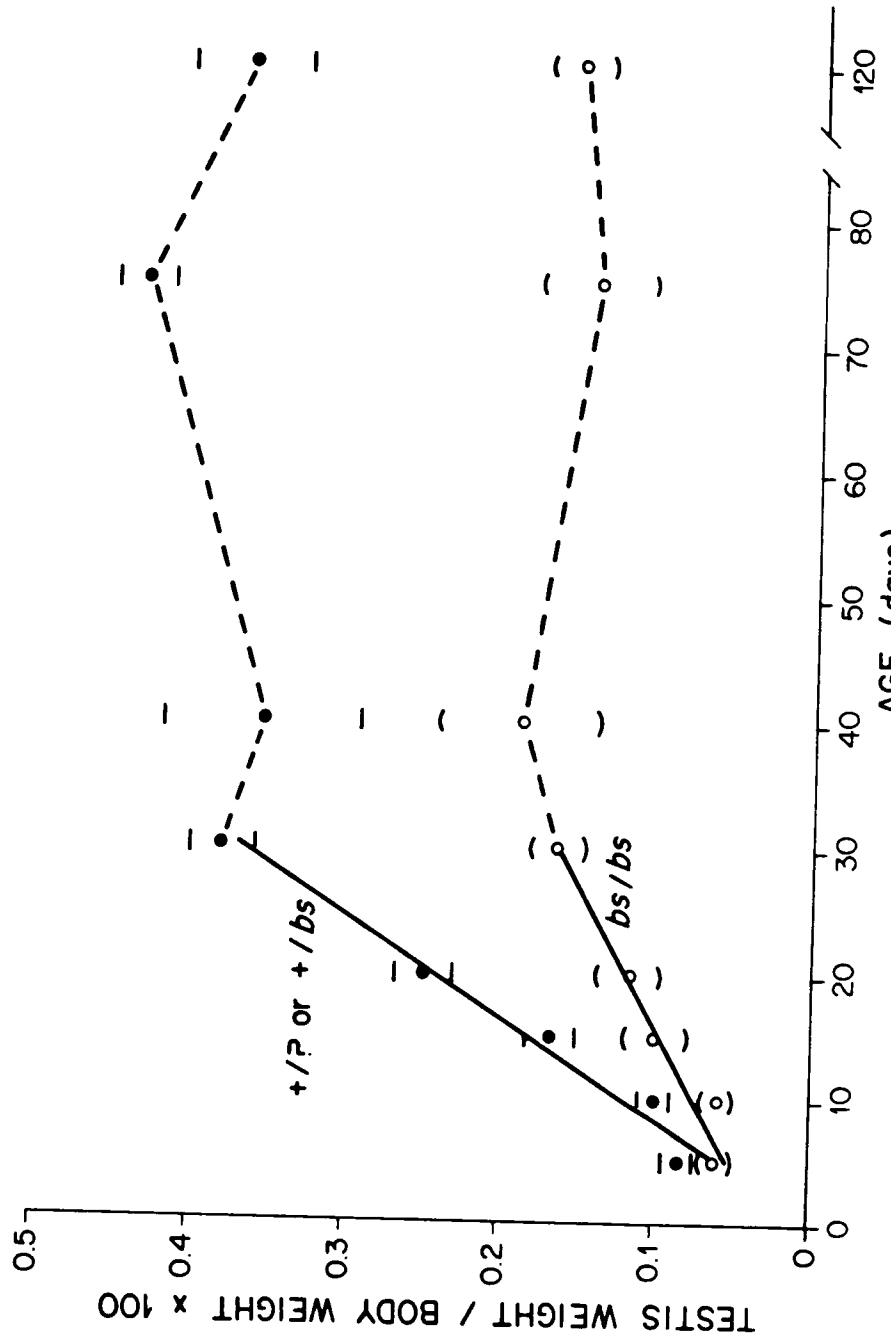

Fig. III-12. Testis weight growth in mutant and normal mice from 5 to 120 days of age. The straight lines represent the least squares linear fit to the data up to 30 days of age; $r_{\underline{bs/bs}} = 0.9815$ and $r_{\underline{+/-}} = 0.9877$.

PART IV

A CYTOCHEMICAL STUDY OF THE GOLGI APPARATUS IN SPERMATOCYTES AND SPERMATIDS OF A MALE STERILE MOUSE MUTANT

A CYTOCHEMICAL STUDY OF THE GOLGI APPARATUS IN SPERMATOCYTES AND SPERMATIDS OF A MALE STERILE MOUSE MUTANT

Abstract

Three Golgi enzyme markers, cytidine monophosphatase (CMPase), thiamine pyrophosphatase (TPPase) and nicotinamide adenine dinucleotide phosphatase (NADPase) were analyzed cytochemically in spermatocytes and spermatids from blind-sterile (bs) mutant and normal mice. No major differences in the reactivity of the Golgi apparatus for CMPase or TPPase was found between normal and mutant germ cells. CMPase activity was limited to 1 or 2 saccules on the trans side of the Golgi of early spermatids and was also detected in the proacrosomic vesicles and proacrosomic granules. No CMPase activity was observed on the Golgi of spermatids past steps 6-8 of spermiogenesis. A weak spotty reaction for CMPase was detected in the acrosome membrane of normal spermatids. Intense TPPase activity was found in mid-to-trans saccules of the Golgi apparatus in both spermatocytes and spermatids. Unlike CMPase, TPPase continue to be present in the Golgi of spermatids past steps 6-8 of spermiogenesis. A light reaction for TPPase was observed in proacrosomic granules but no activity was detected in the fully formed acrosomic granules. Only a spotty reaction was oserved in the anterior aspects of the acrosome membrane. No NADPase activity was detected in the Golgi, proacrosomic vesicles or acrosomes of mutant and normal spermatids. These results suggest that the Golgi apparatus of mutant spermatocytes and spermatids was functionally active for CMPase and TPPase.

Furthermore, the Golgi contributed to the formation of the proacrosomic vesicles in the mutant spermatids.

Introduction

The formation of the acrosome in mammalian spermatids is one of the most critical steps in the differentiation of the male gamete. In the rat and mouse this process has been subdivided into four phases: the Golgi, cap, acrosome and maturation phases (Leblond and Clermont, 1952; Clermont and Leblond, 1955).

Numerous classical ultrastructural studies support the conclusion that the acrosome precursor is secreted by the Golgi apparatus (Burgos and Fawcett, 1955; Fawcett and Hollenberg, 1963; Sandoz, 1970). The Golgi apparatus itself is formed by stacks of six or more flattened membranous sacs with vacuoles or vesicles budding from them. This type of structure is closely conserved in all eukaryotic cells (Novikoff, 1976; Leblond and Bennett, 1977; Rothman, 1981), including highly specialized cells such as Sertoli cells (Rambourg et al., 1979) and mammalian spermatids (Susi et al., 1971; Hermo et al., 1979; Hermo et al., 1980). In most cell types the Golgi apparatus is morphologically and physiologically polarized having a cis or forming face and a trans or maturing face (Rothman, 1981).

One approach to demonstrate the physiological polarization of the Golgi is the use of enzyme markers that can be detected by special cytochemical techniques. Various acid phosphatases have been shown to be localized in discrete compartments of the Golgi in many different cell types (Novikoff and Goldfischer, 1961; Friend, 1969; Goldfischer

et al., 1971; Novikoff, 1976; Novikoff and Novikoff, 1977; Smith, 1980). This approach has been used to study the cytochemical reactivity of the Golgi during spermiogenesis of the rat and has helped clarify the physiological relationships between the Golgi and the acrosome system (Clermont et al., 1981; Tang et al.; 1982).

In a previous study we demonstrated that the blind-sterile mutation (bs) of the mouse causes the failure in complete acromosome assembly. Since the proacrosomic vesicles and hence the acromosome are formed by the Golgi apparatus, implication of a physiologically defective Golgi appeared possible.

In the present study we have tested the functionality of the Golgi in germ cells from bs/bs mutants. We have used three enzyme markers: (1) cytidine monophosphatase (CMPase) which is localized in 1 or 2 saccules of the trans side of the Golgi (Novikoff and Novikoff, 1977; Clermont et al., 1981); (2) thiamine pyrophosphatase (TPPase) associated with the trans aspects of the Golgi apparatus (Novikoff and Goldfischer, 1961; Clermont et al., 1981); (3) nicotinamide adenine dinucleotide phosphatase (NADPase) associated with intermediate saccules of the Golgi (Smith, 1980; Clermont et al., 1981).

Materials and Methods

Animals

Mutant (bs/bs) mice and littermate normal (+/bs or +/?) mice were used in this study. The mice were progeny of (AKR-bs x 129) hybrid mice originally obtained from the Jackson Laboratory (Bar

Harbor, ME) and subsequently maintained in our animal facility by brother-sister matings.

Fixation and Embedding

Techniques followed were essentially those of Clermont et al. (1981). The testes of adult bs/bs and +/- or +/bs mice were fixed by perfusion through the left ventricle with 2.5% glutaraldehyde buffered at pH 7.4 with 0.1M sodium cacodylate containing 0.05% Ca Cl_2. The animals were perfused for 10 minutes at room temperature. Following perfusion the testes were removed, cut into rectangular pieces about 1x 1x 4mm and post-fixed in fresh fixative at $4^{o}C$ for 1 hr. The tissue was washed overnight in at least 3 changes of 0.1M sodium cacodylate containing 4% sucrose and 0.05% Ca Cl_2 at pH 7.2 and 4^{o} C. The tissue pieces were then embedded in a 3% agar solution in small vials and kept in refrigerator at $4^{o}C$. The agar blocks were trimmed into small cubes and were chopped at 100 um with a Lancer-1000 Microvibrotome. The sections were kept in washing buffer until used in the cytochemical studies.

Localization of Enzymes

For cytidine monophosphatase (CMPase) and nicotinamide adenine dinucleotide phosphatase (NADPase) the tissue sections were rinsed several times in 0.1 M sodium acetate buffer, containing 4% sucrose at pH 5. For thiamine pyrophosphatase (TPPase), the tissue sections were washed in 0.1M Tris-maleate buffer containing 4% sucrose at pH 5.

CMPase. Tissue sections were incubated for 2 hr in Novikoff

medium at 37°C and pH 5.0 (Novikoff, 1963; Essner, 1973). This contained 0.005M CMP (Sigma, St. Louis, MO) 0.05M acetate buffer pH 5.0, 0.005 M lead nitrate, 0.005 M $MnCl_2$ and 0.25 M sucrose.

NADPase. Tissue sections were incubated in the medium described by Smith (1980) for 1 hr at 37°C. This consisted of 0.004 M NADP (Sigma, St. Louis, MO), 0.04 M sodium acetate buffer pH 5.0, 0.004 M lead acetate and 5% sucrose.

TPPase. Tissue sections were incubated in the medium described by Novikoff and Goldfischer (1961) for 2 hr at 37°C. This preparation consisted of TPP (Sigma, St. Louis, MO), 0.2 M Tris-maleate buffer pH 7.2, 1% lead nitrate and 0.025 M $MnCl_2$.

Controls. Tissue sections were incubated in the respective media free of the substrates.

Post-Incubation

After incubation the tissue sections were washed in the same incubation buffer. They were then rinsed in sodium cacodylate buffer pH 7.2 containing 4% sucrose and 0.05% CaCl. After the rinse the sections were post fixed in reduced osmium for 3 hr at 4°C (Karnovsky, 1971), dehydrated in ethanols, infiltrated with propylene oxyde and embedded in Epon 812. Thin sections were stained with lead citrate and uranyl acetate, examined and photographed using a Hitachi H600 or a Zeiss 9S electron microscope.

Results

CMPase

A prominent Golgi apparatus containing numerous stacks of cisternae was observed in pachytene spermatocytes. A positive CMPase reaction was detected in the most trans cisternae and buds associated with them (Fig. IV-1*). The Golgi apparatus of normal spermatids steps 1-3 also showed a positive CMPase reaction. This was restricted to 1 or 2 saccules of the trans side of the Golgi (Figs. IV-2 and IV-3). In steps 1 or 2 of early spermatid differentiation large vesicles, interpreted as the proacrosomic vesicles, were strongly CMPase positive (Figs. IV-2 and IV-3). In more advanced spermatid stages (steps 6-8), the Golgi apparatus appeared CMPase negative. The acrosomic system showed a weak positive reaction. This was detected as spotty lead precipitation over the inner membrane of the acrosome and on the acrosome granule (Figs. IV-4 and IV-5).

Early spermatids in steps 1-3 were not found in the sections of mutant testes. Therefore no conclusive evidence for the localization of CMPase in the Golgi and proacromosomic vesicles was obtained. However in earlier stages such as pachytene spermatocytes a prominent Golgi apparatus similar to that found in normal pachytene spermatocytes showed a positive CMPase reaction (Fig. IV-6). A negative CMPase reaction was observed in the Golgi of mutant spermatids in steps 6 to 8. These spermatids lack acrosomes and had to be staged on the basis of the thickening of the nuclear envelope

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*All figures may be found in the Appendix.

(Sotomayor and Handel, 1986). No lead precipitates were seen in the area adjacent to the Golgi apparatus or over the cytoplasmic face of the thickened nuclear envelope, which in normal spermatids of similar stages appeared spottily labeled (Figs. IV-7 and IV-8).

TPPase

TPPase activity was concentrated on several saccules of the Golgi apparatus starting at the mid part of the stack of cisternae to the most trans aspect of the saccules.

The Golgi apparatus of normal pachytene spermatocytes showed an intense reaction along the entire length of the mid-to-trans saccules but showed no reaction in the most cis face of the Golgi. The thick saccules and vesicles associated with the trans aspect of the Golgi were also intensely labeled (Fig. IV-9). The Golgi apparatus of early spermatids (steps 2-4) was characteristically labeled as described for the pachytene spermatocytes (Fig. IV-10). The proacromosomic granules showed less reaction than the Golgi saccules (Fig. IV-11). It was evident that as the differentiation of spermatids progressed the TPPase activity decreased in the Golgi. In mid-spermatid stages (steps 8-9) the TPPase reaction was limited to only one or 2 of the most trans-saccules of the Golgi (Fig. IV-12). The acrosome systems of mid spermatid stages showed no TPPase reaction except for a weak spotty reaction on the anterior aspect of the acrosome membrane (Fig. IV-13).

The Golgi apparatus of mutant pachytene spermatocytes showed no significant differences from normal ones with regard to TPPase reactivity. Mid-to-trans saccules were intensely labeled (Fig. IV-

14). As in the case of normal pachytene spermatocytes, the prominent Golgi was generally found in close proximity to the sex vesicle. No early spermatid stages were found in these sections of mutant testes. Therefore, we do not have direct experimental evidence of the localization of TPPase in the Golgi or proacrosomic vesicles of mutant early spermatids. However, in more advanced mutant spermatids (steps 6-8) the Golgi apparatus reacted positively for TPPase. Lead deposits were seen in 2 or 3 of the saccules located on the trans side of the Golgi (Fig. IV-15). No lead deposits were found on the cytoplasmic face of the thickened nuclear membrane at the place where the acrosome should have been located.

NADPase

In both normal and mutant spermatids and spermatocytes no reaction for NADPase was found. However, lysosomes from Sertoli and Leydig cells showed a positive reaction for NADPase indicating that the negative reaction found in the germ cells was not an artifact of technique (Figs. IV-16 and IV-17).

Controls

Substrate-free controls showed no reactivity in the Golgi apparatus, proacrosomic vesicles or acrosomes.

Discussion

The present investigation was undertaken to determine the functionality of the Golgi apparatus in mutant spermatids. These spermatids lack acrosomes due to the action of the recessive gene

mutation "blind sterile" operating in the homozygous state (Sotomayor and Handel, 1986). Since it is well known that the acrosome originates from vesicles formed by the Golgi apparatus (Burgos and Fawcett, 1955; Fawcett, 1958; Fawcett and Hollenberg, 1963; Sandoz, 1970; Susi et al., 1971) and since we know that the effect caused by the bs mutation occurs immediately subsequent to the Golgi phase of spermiogenesis (Sotomayor and Handel, 1986), it was important to determine whether the Golgi was functionally normal.

We have used three acid phosphatase enzyme markers for this histochemical study. These were cytidine monophosphatase (CMPase), thiamine pyrophosphatase (TPPase) and nicotinamide adenine dinucleotide phosphatase (NADPase). These enzymes have been shown to be localized in discrete compartments of the Golgi apparatus in rat spermatids (Clermont et al., 1981; Tang et al., 1982) and in the Golgi of various other cells types (Novikoff and Goldfischer, 1961; Novikoff, 1963; Goldfischer et al., 1971; Novikoff and Novikoff, 1977; Smith, 1980).

CMPase and TPPase were localized in the Golgi apparatus of pachytene spermatocytes and spermatids in both mutant and normal testes. Furthermore, in agreement with the observations of Clermont et al., (1981) and Tang et al., (1982), the localization of CMPase was limited to about the two most trans cisternae and associated vesicles. The localization of TPPase in mouse spermatids was somewhat different than that observed in rat spermatids by the previously cited authors. In mouse spermatocytes and early spermatids TPPase occupied several of the Golgi cisternae, from the middle to the most trans saccules. In

rat spermatids, this enzyme is localized only in the two most trans saccules of the Golgi apparatus (Clermont et al., 1981; Tang et al., 1982).

Positive reactions for CMPase and TPPase were detected in the proacrosomic vesicles of normal early spermatids (steps 1-4). Unfortunately similar stages could not be found in the mutant samples investigated. Therefore no direct evidence for the presence of these enzymes in proacrosomic vesicles of mutant spermatids was obtained. However the fact that in mutant pachytene spermatocytes and in mid-spermatids stages the respective Golgi's were labeled strongly suggest that the proacrosomic vesicles of mutant spermatids were also positive for these enzymes.

Our observations on the fate of CMPase showed that the Golgi apparatus of both mutant and normal spermatids in steps 6-8 no longer contained this enzyme. CMPase was detected as a weak spotty reaction only in the acrosomal membrane but not on the acrosomal granule of the normal spermatids. No CMPase activity was found in the thickened portion of the nuclear membrane at the acrosome implantation site in mutant spermatids. These findings with CMPase are different from those of Tang et al. (1982). These authors found a CMPase activity in the Golgi of rat spermatids in steps 8 to 17 of spermiogenesis.

Unlike CMPase, TPPase activity was observed in the Golgi of normal and mutant spermatids in steps 8-10 but limited to only 1 or 2 of the most trans cisternae. No TPPase reaction was seen in the acrosome granule of normal spermatids. Only a slight reaction was detected in anterior aspects of the acrosome membrane. These results

suggest that the Golgi apparatus of mutant spermatocytes and spermatids is functionally normal for CMPase and TPPase. Furthermore, the similarity in the localization and fate of these enzymes between normal and mutant germ cells suggests that the Golgi contributes to the formation of the proacrosomic vesicles in the mutant spermatids. The functionality of the Golgi in this process is also indirectly supported by our previous observation that PAS-positive material (presumably glycoprotein) was detected in the proacrosomic vesicles of mutant spermatids (Sotomayor and Handel, 1986).

NADPase activity was not detected in either mutant or normal spermatids or pachytene spermatocytes. Some positive material was detected in the lysosomes of Sertoli and Leydig cells. The lack of NADPase activity in mouse sperm cells is in disagreement with the studies by Clermont et al. (1981) and Tang et al. (1982) for rat spermatids and those of Smith (1980) for rat incisor ameloblasts. In all these cases a strong positive reaction was found in the middle saccules of the Golgi. A possibility for the lack of reaction in our case is that NADPase may not be found in germ cells of male mice but this possibility needs further experimental verification.

PART IV

REFERENCES

REFERENCES

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PART IV

APPENDIX

Fig. IV-1. Golgi apparatus of a normal pachytene spermatocyte showing CMPase activity on the trans saccules (arrow). CMPase activity is also present in buds and vesicles associated with the edges of the saccules (arrowheads). X18,000.

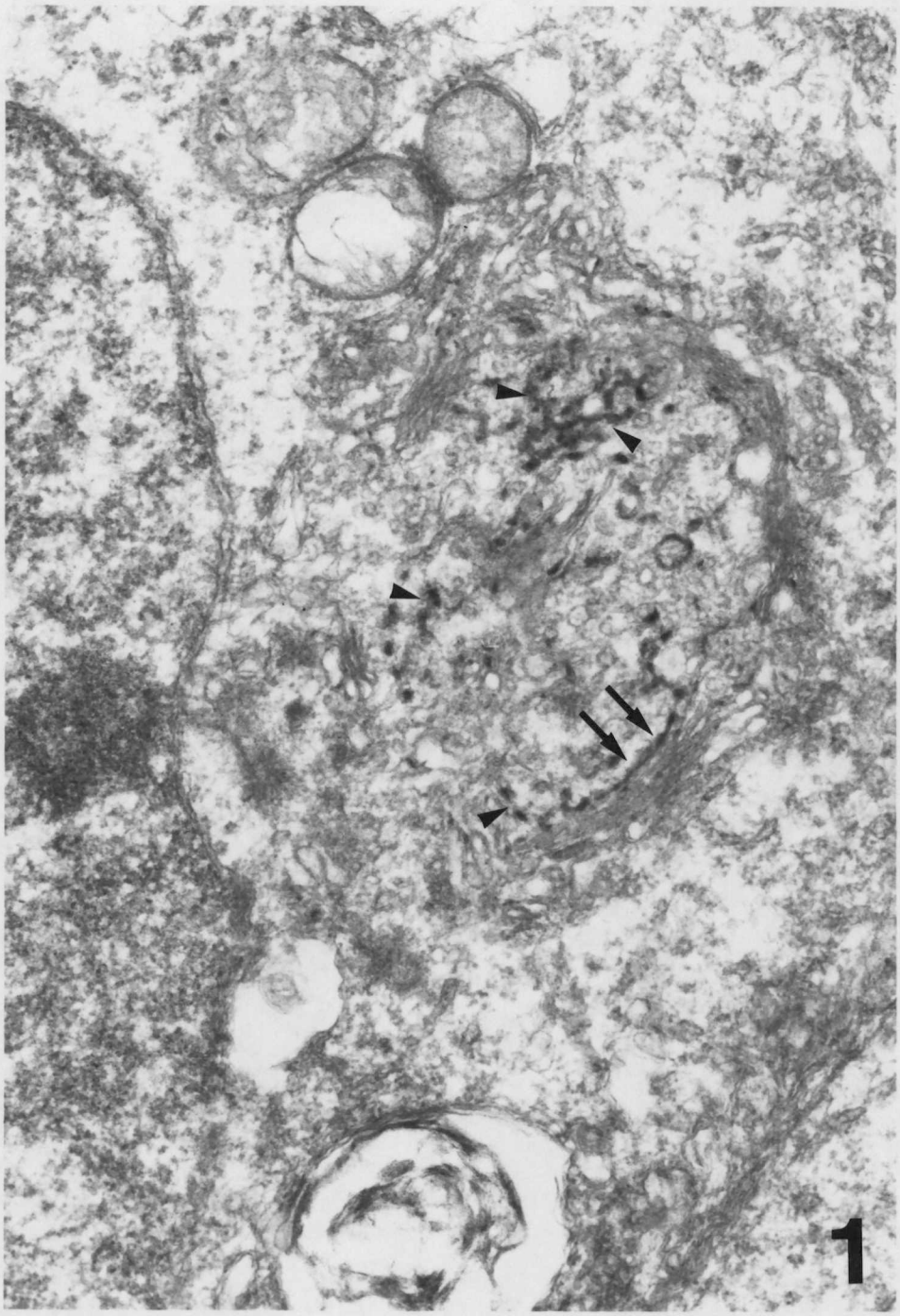
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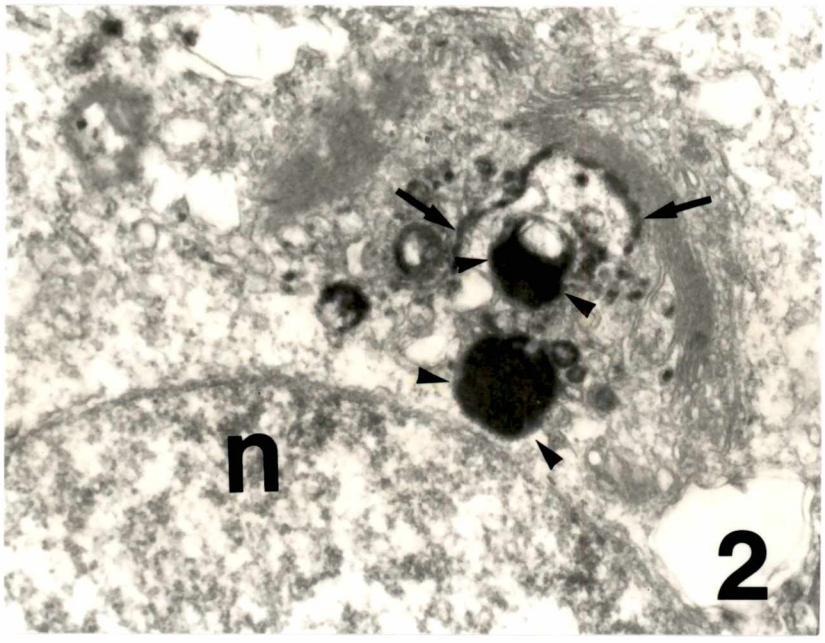
Fig. IV-2. Normal spermatid in steps 1-2 of spermiogenesis showing the Golgi apparatus and proacrosomic vesicles. CMPase activity is present on the saccules and vesicles of the trans aspect of the Golgi (arrows). Proacrosomic vesicles show intense CMPase activity (arrowheads). N, nucleus. X23,000.

Fig. IV-3. Normal spermatid in steps 1-2 of spermiogenesis showing the Golgi apparatus and proacrosomic vesicles at a higher magnification. The photograph illustrates the CMPase reaction on 1 or 2 saccules of the trans side of the Golgi (arrows) and on the proacrosomic vesicle (arrowheads). N, nucleus. X36,000.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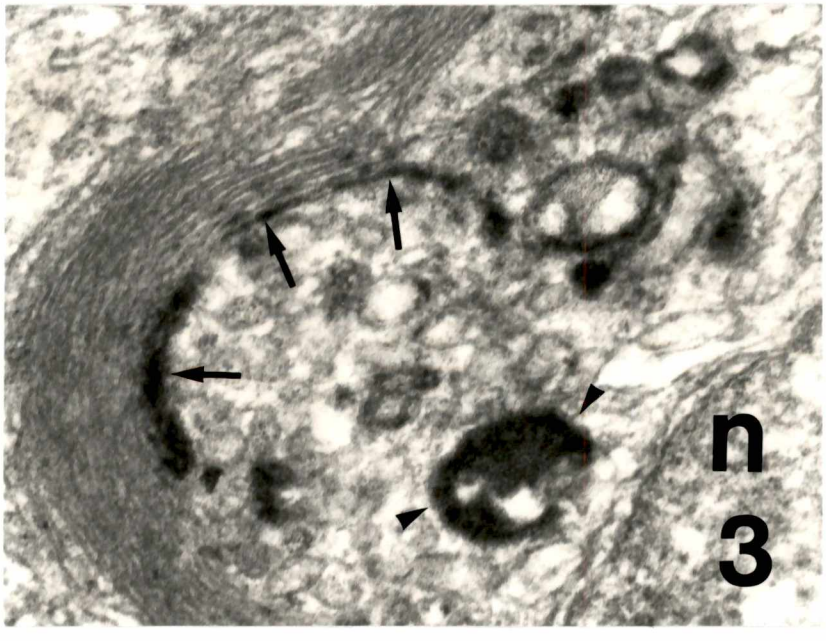
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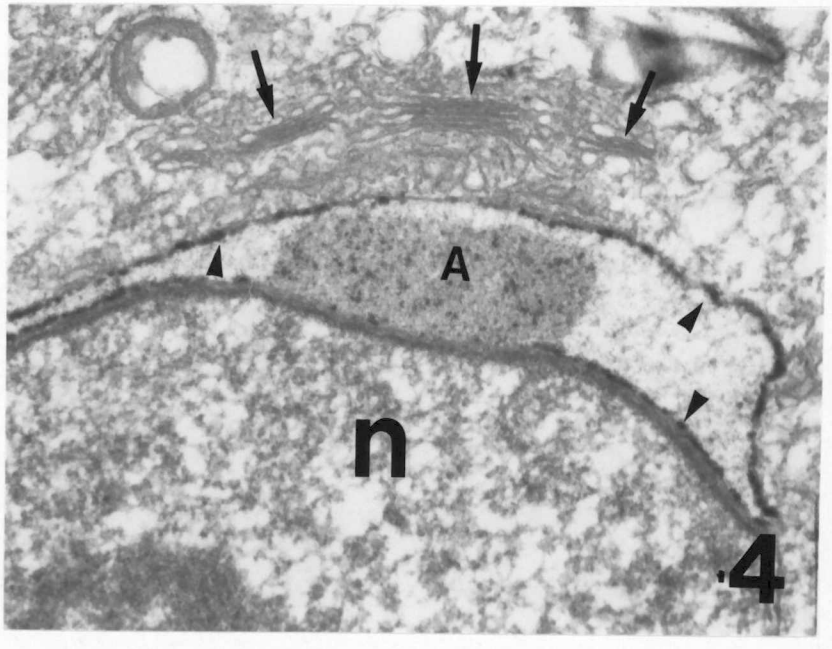
Fig. IV-4. Micrograph of a normal spermatid in steps 6-8 of spermiogenesis showing lack of CMPase activity in the Golgi apparatus (arrows). A light, spotty reaction can be seen on the anterior aspects of the acrosome membrane (arrowheads) and on the acrosome granule (A). N, nucleus. X23,000.

Fig. IV-5. Photograph of a normal spermatid in steps 6-8 of spermiogenesis illustrating the lack of reactivity of the Golgi apparatus (arrows) for CMPase. As shown in Fig IV-4 only a weak, spotty CMPase reaction can be seen on the acrosome membrane (arrowheads) and on the acrosome granule (A). N, nucleus. X23,000.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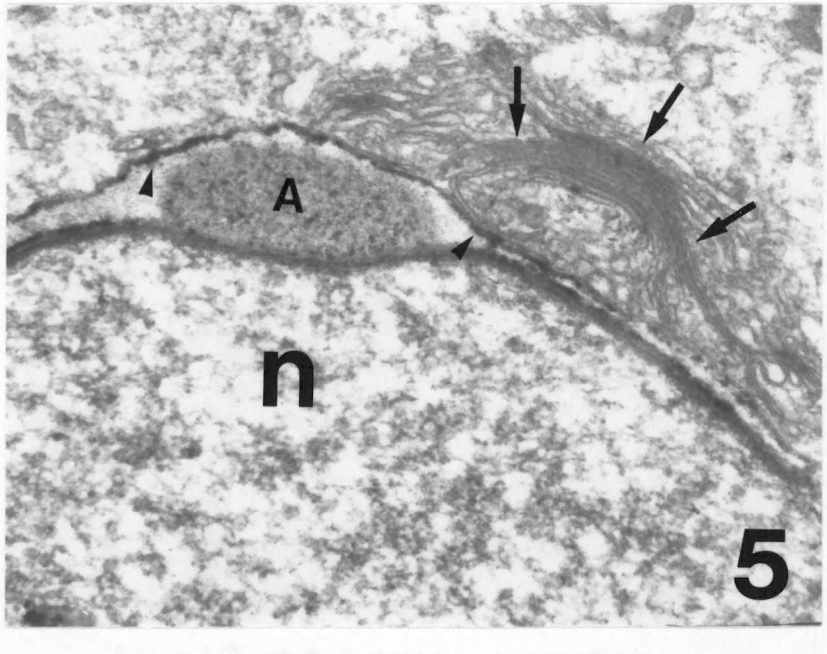
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Fig. IV-6. A prominent Golgi apparatus in a mutant pachytene spermatocyte. CMPase activity is present in one or two saccules of the trans face of the Golgi (arrows). Several vesicles are also CMPase positive (arrowheads). X23,000.

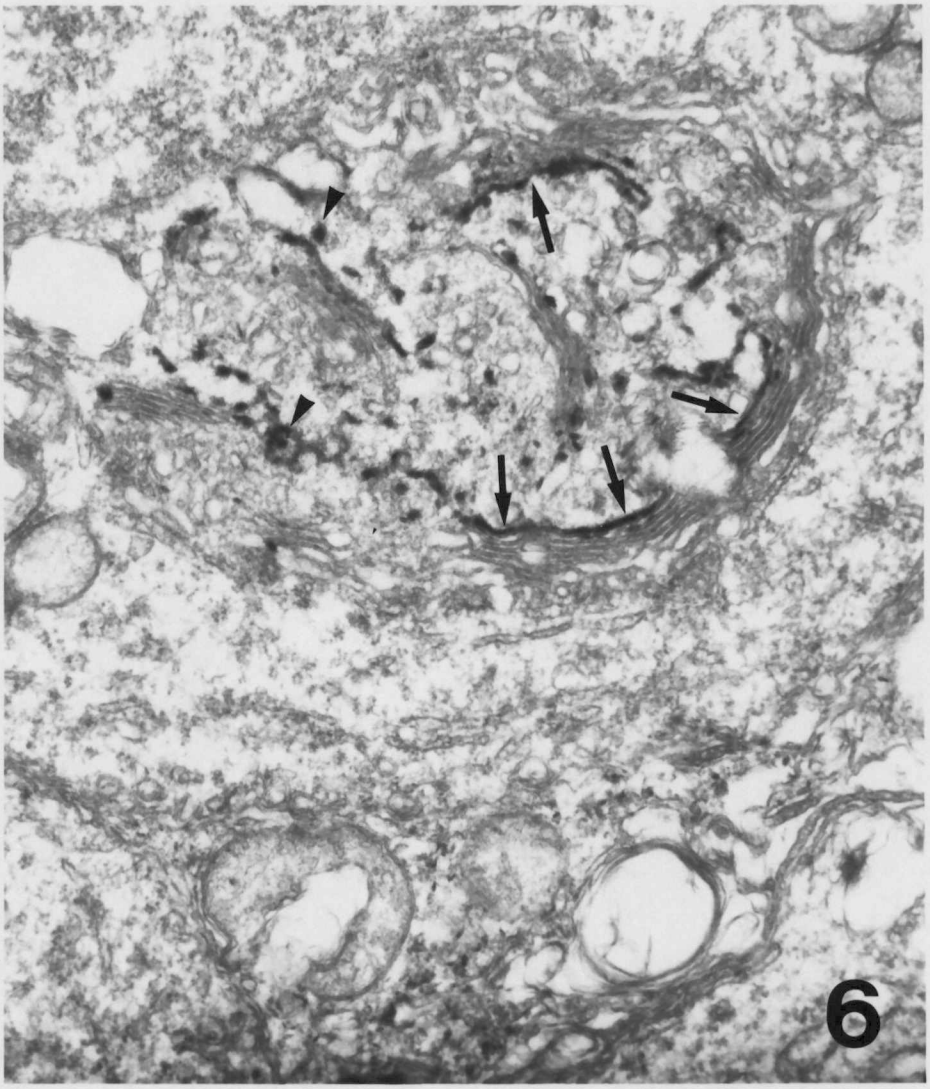
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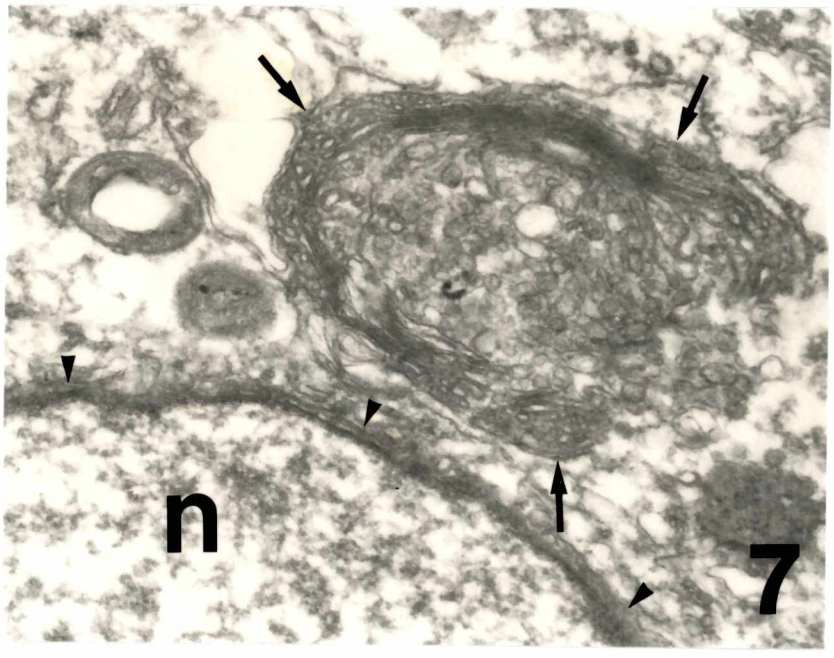
Fig. IV-7. Mutant spermatid presumably in steps 6-8 of spermiogenesis showing lack of CMPase reaction in the Golgi apparatus (arrows). Notice the absence of acrosome and the thickening of the nuclear envelope in the area where the acrosome is normally implanted (arrowheads). N, nucleus. X23,000.

Fig. IV-8. Mutant spermatid similar to that shown in Fig. IV-7 illustrating the lack of CMPase activity in the Golgi apparatus (arrows). The arrowheads indicate the thickening of the nuclear envelope and the absence of the acrosome. No CMPase positive material is present on the area adjacent to the thickened nuclear membrane. N, nucleus. X27,000.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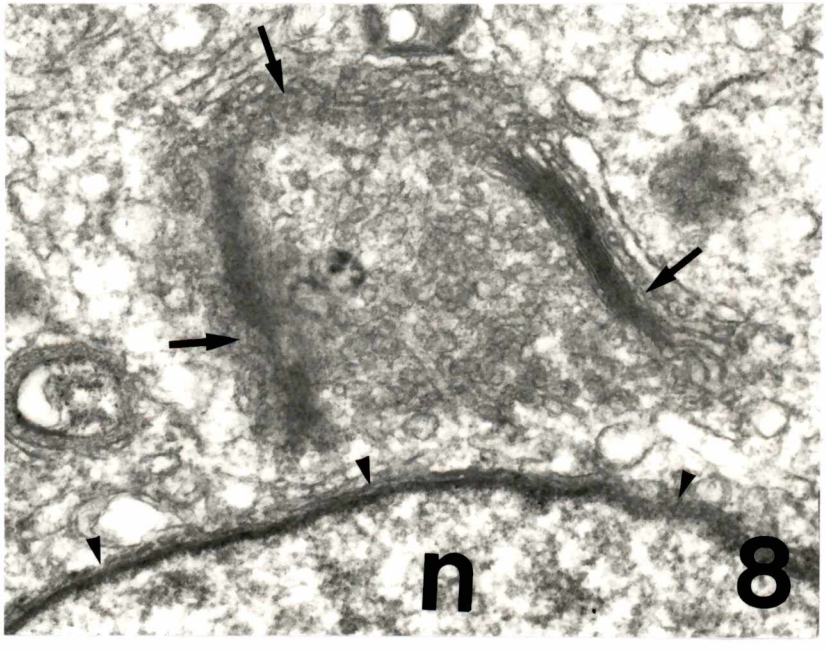
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Fig. IV-9. Intense TPPase reaction in the Golgi apparatus of normal pachytene spermatocytes. TPPase activity is seen along the entire length of the middle-to-trans saccules of the Golgi (arrows). No activity is seen in the cis face of the organelle (long arrows). The GERL elements and vesicles associated with them show TPPase activity (arrowheads). X23,000.

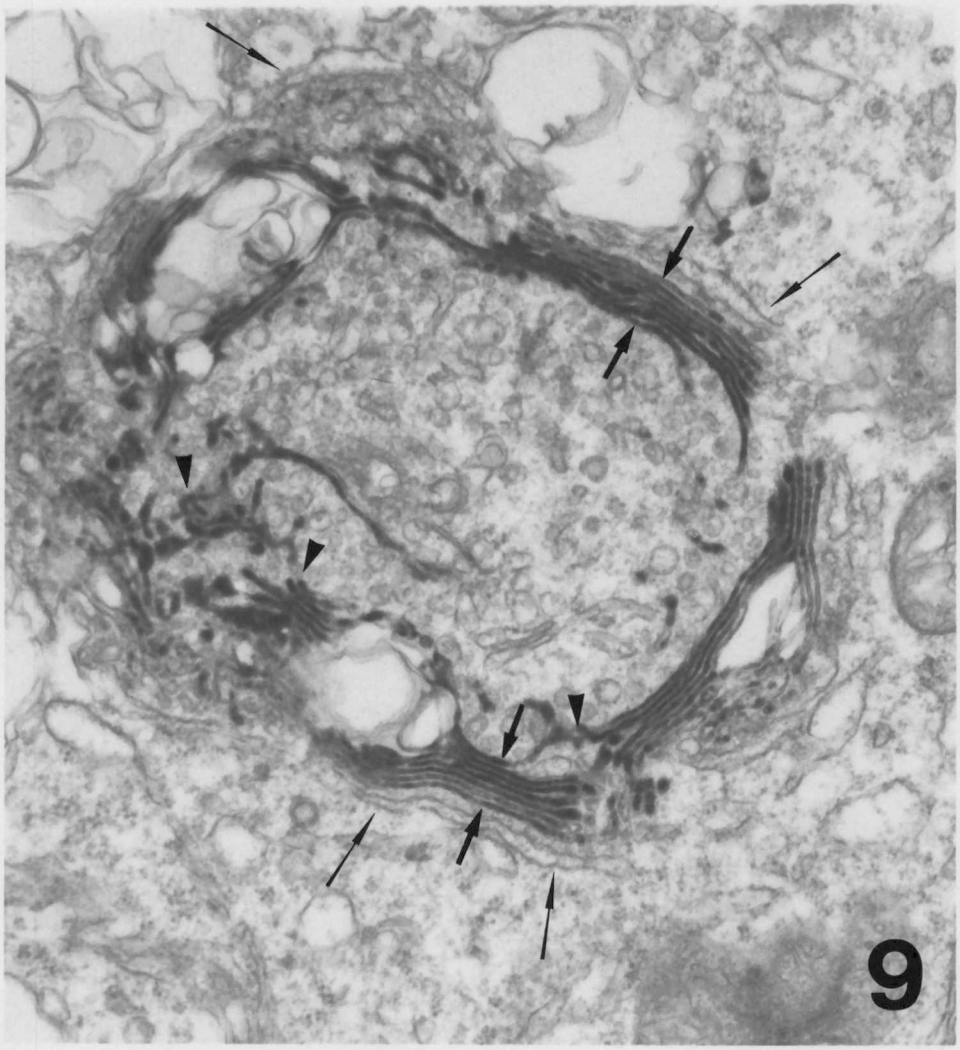
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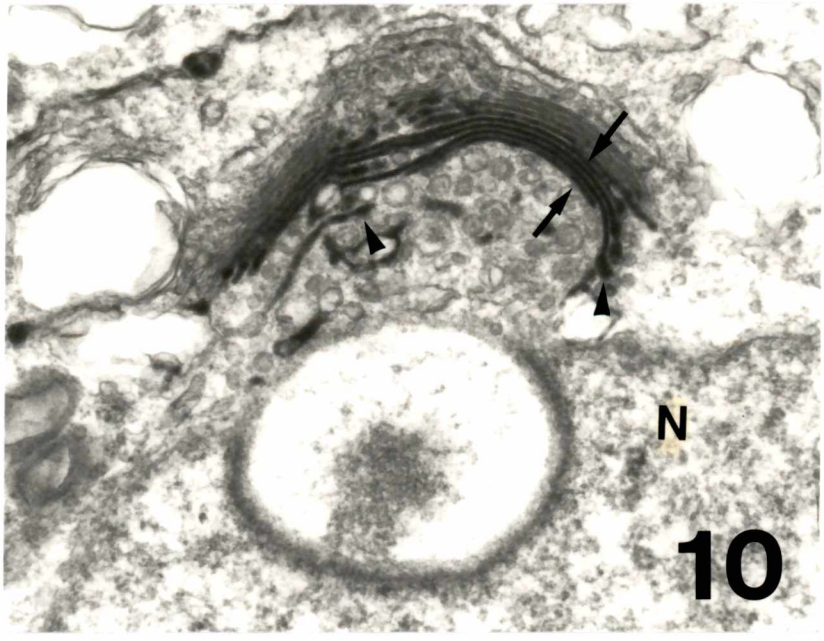
Fig. IV-10. Golgi apparatus of a normal spermatid in steps 3-4 of spermiogenesis showing intense TPPase activity in the mid-to-trans saccules (arrows) and in the vesicles associated with them (arrowheads). N, nucleus. X23,000.

Fig. IV-11. Golgi apparatus and proacrosome of a normal spermatid in steps 3-4 of spermiogenesis. TPPase activity is present in the mid-to-trans saccules of the Golgi apparatus (arrows) and in the vesicles associated with the most trans aspect of the Golgi (arrowheads). Notice the lack of TPPase activity in the proacrosomic granule (PA). N, nucleus. X23,000.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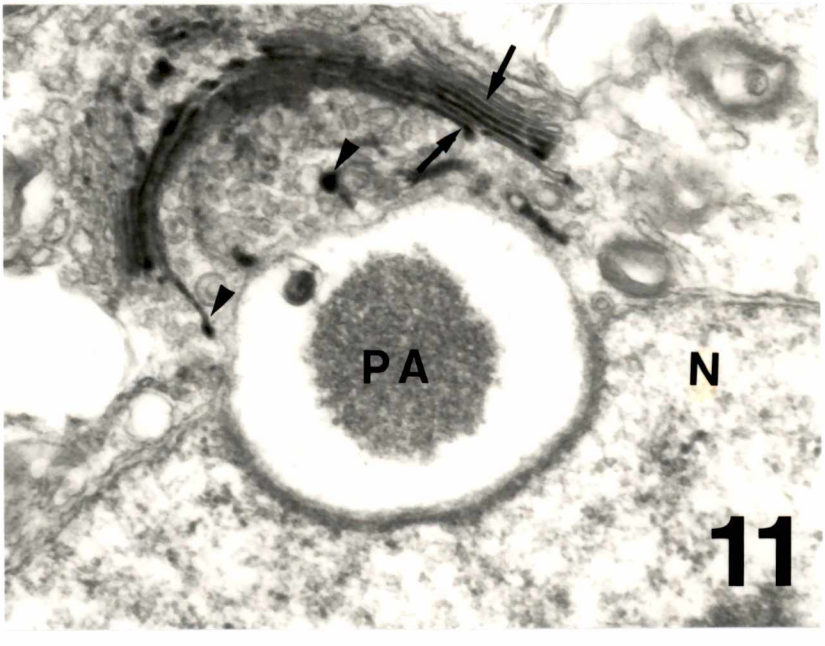
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Fig. IV-12. Normal spermatids in steps 8-9 of spermiogenesis. Notice the caudal position of the Golgi apparatus in these spermatids (G). One or two saccules of the trans side of the Golgi show TPPase activity (short arrows). N, nucleus; acrosome (long arrows). X12,000.

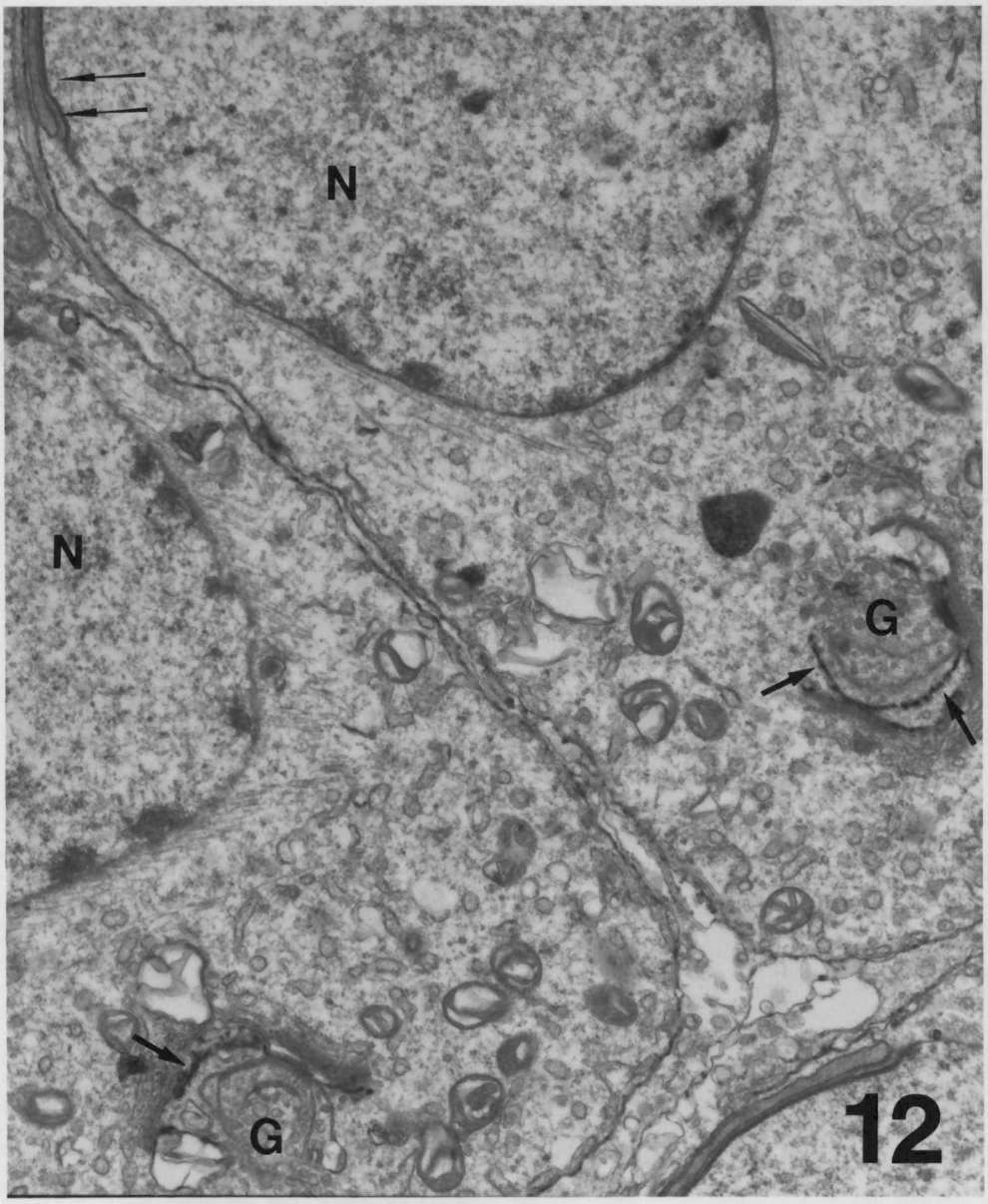
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Fig. IV-13. Normal spermatid in steps 8-9 of spermiogenesis. A weak and spotty TPPase reaction can be seen only on the anterior aspects of the acrosome membrane (arrows). No TPPase activity is observed on the acrosome granule (A) or in any other part of the acrosome. N, nucleus. X23,000.

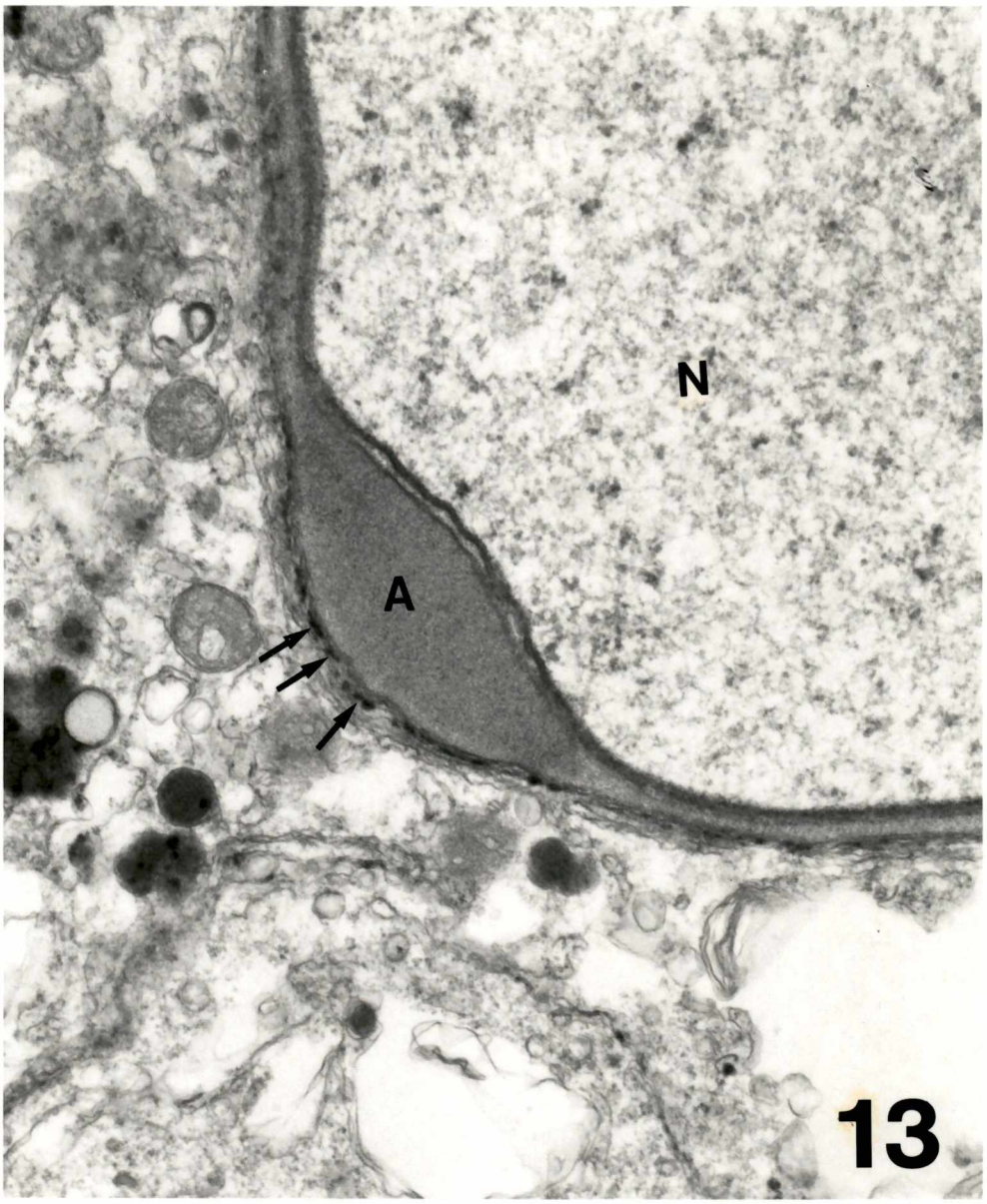
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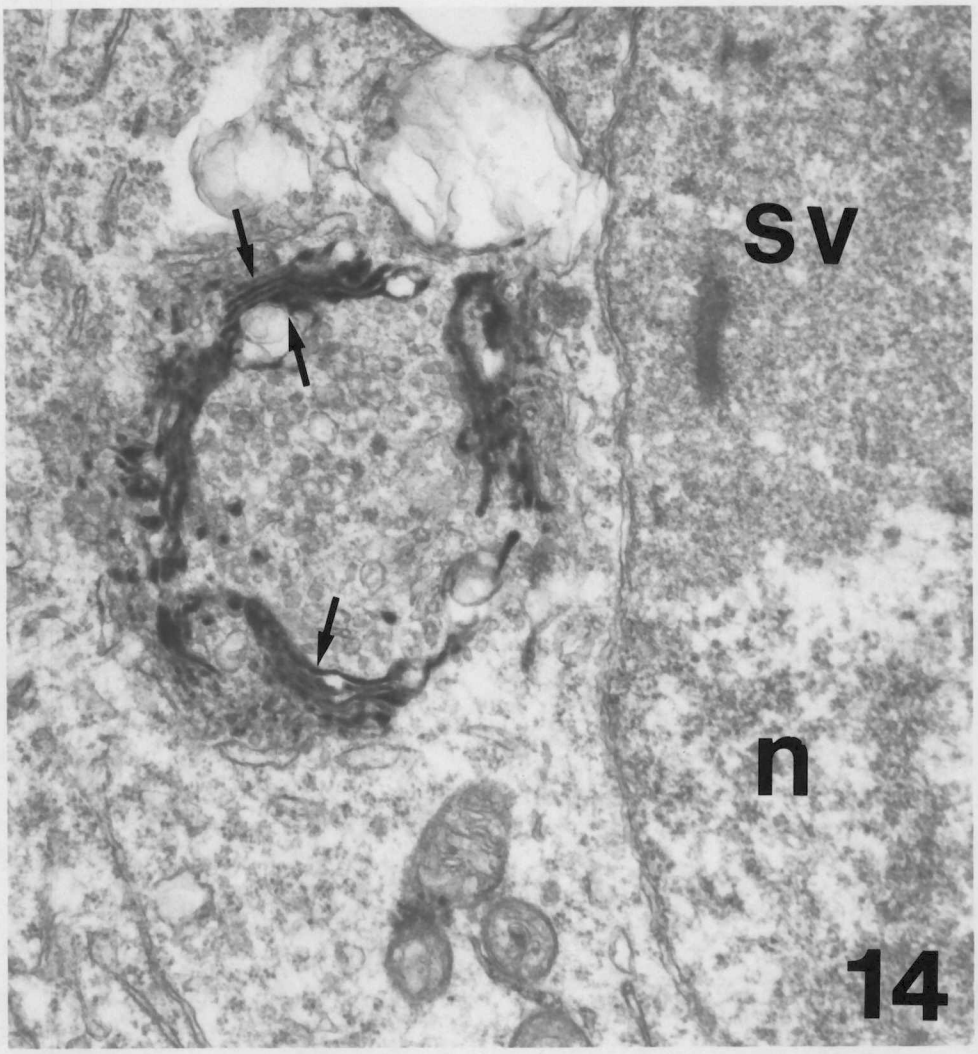
Fig. IV-14. Golgi apparatus of a mutant pachytene spermatocyte. Mid-to-trans saccules of the Golgi are intensely labeled (arrows). N, nucleus; SV, sex vesicle. X23,000.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14

Fig. IV-15. Mutant spermatid presumably in steps 6-8 of spermiogenesis. TPPase activity is seen in two or three saccules of the trans side of the Golgi apparatus (arrows). Note the absence of acrosome and the thickening of the nuclear envelope (arrowheads). N, nucleus. X27,000.

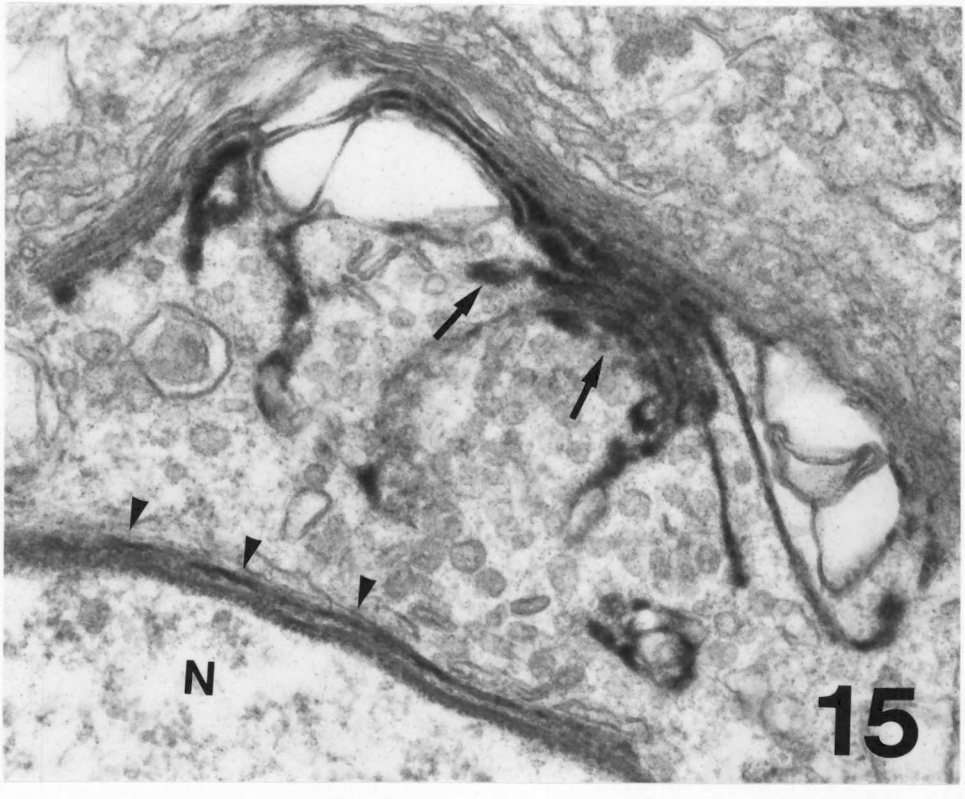
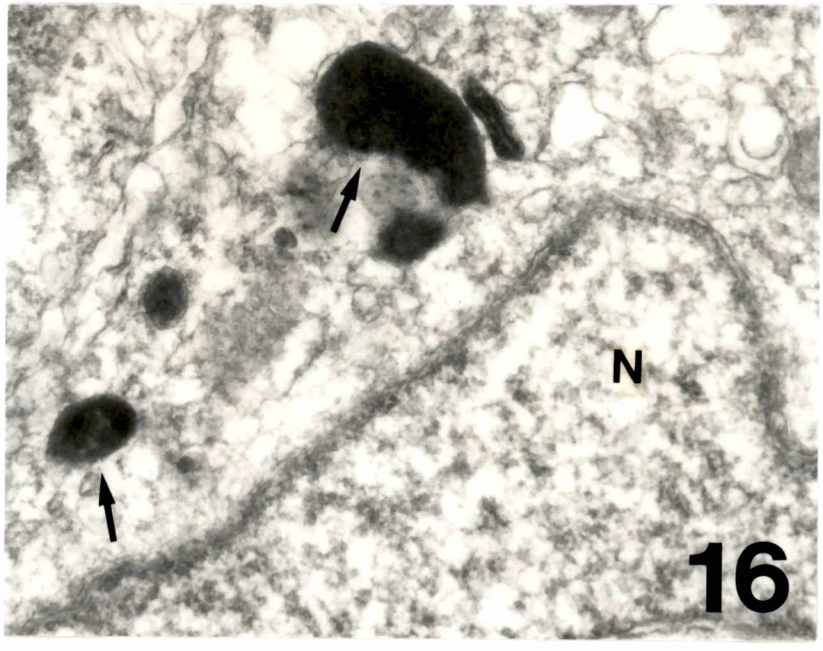

N
15

Fig. IV-16. Normal Sertoli cell showing NADPase-positive material in lysosomes (arrows). N, nucleus. X23,000.

Fig. IV-17. Normal Leydig cell showing NADPase-positive material in lysosomes (arrows). N, nucleus. X23,000.


N
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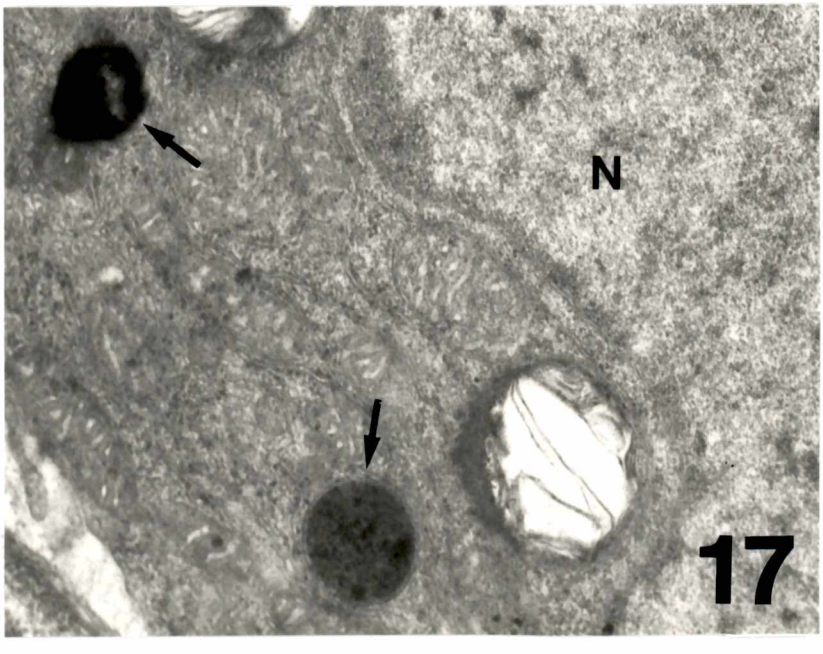
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GENERAL CONCLUSIONS

Most gene mutations causing male sterility in the mouse are pleiotropic, acting upon different tissues and organs. This pleiotropism does not necessarily render these mutations less informative with regard to mechanisms of sperm differentiation. On the contrary, it is likely that their phenotypic expression in various tissues have a similar molecular basis. The elucidation of that molecular basis will contribute greatly to the understanding of pleiotropic gene action in higher organisms. The use of sterile gene mutations has already provided valuable information about factors controlling spermiogenesis. For instance, the findings obtained with qk/qk and hpy/hpy mouse mutants have supported the view that axoneme stability is an essential prerequisite for the proper assembly of other components of the spermatid flagellum such as the outer dense fibers. Investigations with these and other mutants like the pink-eyed sterile (p^s/p^s) have indicated that sperm nuclear shaping is under the control of several genes. Our studies with the pleiotropic recessive gene mutation "blind-sterile" (bs) have established, for the first time, the independence of two phenomena in spermiogenesis. On the one hand, the process of nuclear elongation and chromatin condensation, on the other, the process of acrosome development. It is clear now that nuclear elongation and chromatin condensation can occur in the absence of acrosome development. Thus our finding suggests that acrosome formation is not a mechanistic factor in these processes. Furthermore, our studies with bs/bs mutants indicate that either bs is another genetic factor controlling sperm nuclear shaping

or the acrosome itself has an active role in determining the final shape. Our studies also suggest that reproductive hormonal deficiency is not a primary effect of the bs gene action on spermatogenesis. Thus, it appears that the effects of bs mutation on spermatogenesis are at the cellular level.

We have shown that spermatids of bs/bs mice do synthesize proacrosomic granules but do not form mature acrosomes. This failure might be due to several possible causes: (1) a defective protein of the acrosomal membrane that interacts with the nuclear envelope; (2) defects in glycosylation of acrosomal proteins; (3) defects in lysosomal degradation of oligosaccharides (similar to the effects described for the human lysosomal storage disease); (4) failure of the Golgi apparatus to synthesize acrosomal enzymes; (5) errors in galactose metabolism producing accumulation of toxic products in the proacrosomic vesicles. In any case, the molecular basis for the lack of formation of the acrosome may also explain the formation of bilenticular cataracts in these mutants. It is here, at the molecular level, where one can appreciate the scientific importance of pleiotropic mutations as models for human genetic disease. We have been able to eliminate some possible causes. Both galactokinase and galacto-1-phosphate uridyl transferase, enzymes involved in the initial steps of galactose metabolism, appear to be normal in bs/bs mutants. Mutations affecting these enzymes can result in galactosemia, galactose toxicity, accumulation of galactotitol and cataracts. Our EM cytochemical studies using enzyme markers of the Golgi apparatus have ruled out the possibility of a functional failure

in the Golgi of mutant spermatids. We have limited experimental evidence indicating that glycosylation of testicular proteins is normal in mutant mice. Direct evidence is presently lacking. An attractive hypothesis is the possibility that the failure of acrosome assembly might be a manifestation of lysosomal storage disease. Various mutations are known in humans that cause glycoprotein storage disease. Most of the clinical phenotypes include the formation of cataracts. It is conceivable that accumulation of glycoproteins due to an enzymatic deficiency could produce the breakdown of the proacrosomic vesicles, since these are essentially lysosomes. Unfortunately clinical data on glycoprotein storage disease do not have any reference to sterility problems and therefore the association between cataracts and sterility is missing. The molecular basis for the pleiotropism in bs/bs mice remains to be investigated. If a defective protein or enzyme is found to be responsible for the pleiotropic effects of the bs mutation, the next step is to isolate the specific mRNA and from there to sequence the mutated gene. We are still far from unraveling these possibilities.

VITA

Rene E. Sotomayor was born in Santiago, Chile, on January 3, 1937. He attended elementary schools in that city and in December 1954 he graduated from the Instituto Nacional (High School). In 1956 he obtained the Bachelor of Humanities degree from the University of Chile. He received the Licenciate of Phylosophy degree with a major in Genetics from the University of Chile in 1966. He was appointed as an Instructor and Lecturer at the Chair of General Biology in the School of Veterinary Medicine of the same university. In 1968 he was appointed Assistant Professor at the School of Medicine and at the School of Nursing.

In March 1972 he was awarded a post-graduate fellowship from the Pan American Health Organization to perform studies in mammalian cytogenetics at the Biology Division of the Oak Ridge National Laboratory. In 1975 he was appointed research associate in the genetics Section of the Biology Division where he remained until June 1980. In the fall of 1980 he was invited to join the Institute of Genetics of the Gesellschaft fur Strahlen- und Umweltforschung, Munich, W. Germany, as a visiting scientist. He returned to the U.S. to join The University of Tennessee as a teaching and research associate in the Department of Zoology. He entered the Graduate School in September 1982 and received the Doctor of Phylosophy degree with a major in Life Sciences in June 1986.

The author has been invited to lecture in Mexico, Colombia, Chile and W. Germany. He has presented seminars in several national and international scientific meetings. He is a member of several

scientific societies including the International Federation of Genetics, The Environmental Mutagen Society (USA), the Genetics Society of Chile. He has published several scientific papers and numerous abstracts in national and international journals.