CHAPTER- III

EVENTS OF SPERMATOGENESIS

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CONTENTS

Introduction

For their easy maintenance in the laboratory and relatively large size of their germ cells have made amphibians very resourceful objects from the very beginning of cytological and genetical investigations and then they have proved to be excellent experimental animal for research in endocrinology, reproductive and developmental biology. A number of important contribution to basic biology have been achieved in the course of investigation of spermatogenesis in amphibians. Long ago in 1876 George described various stages of spermatogenesis in five species of amphibians from observations on testicular tissues, fresh or fixed in osmium tetraoxide and laid the ground work for modern terminology in this field, coining the terms 'spermatocyst', 'spermatogonium' and 'spermatocyte'. Fleming (1887) used the spermatocytes of *Salamandra* in his meticulous study of the meiotic divisions and Witschi (1914) first recognized in *Rana temporaria* that the male sex in frogs is digametic. Yet the literatures lack a single article which might serve as a classic comprehensive description of amphibian testes and spermatogenesis.

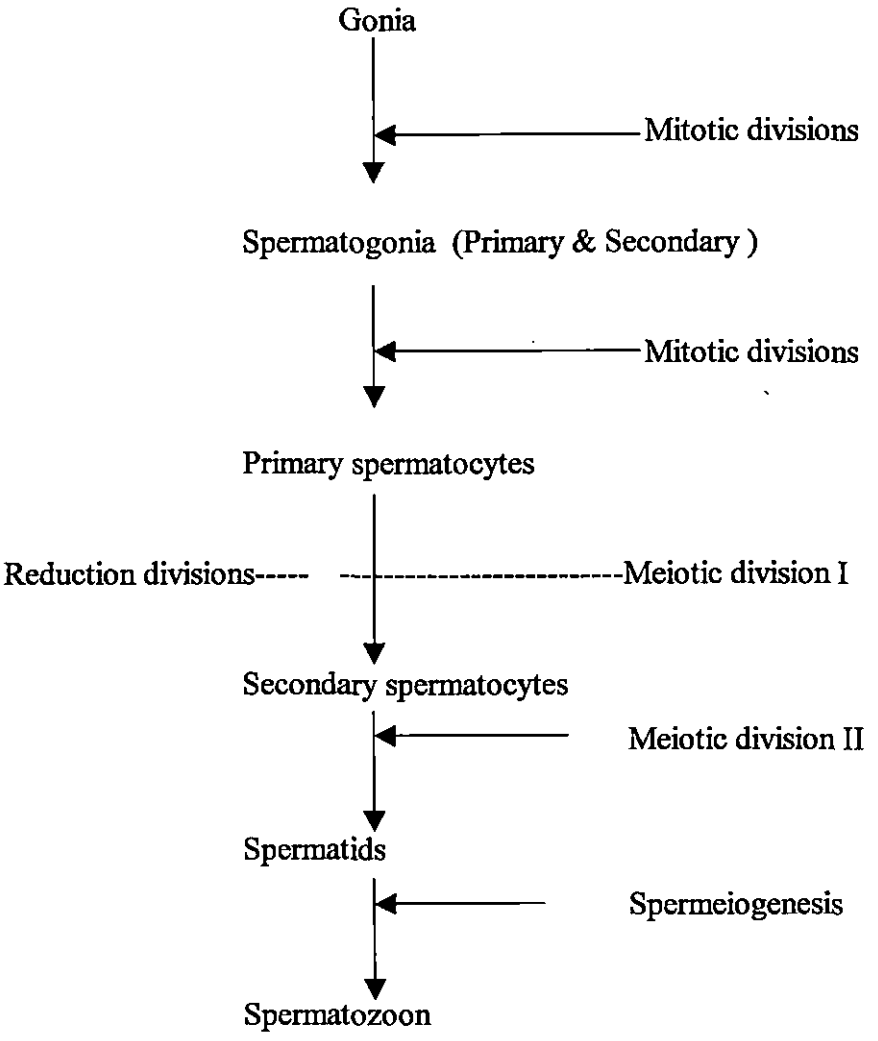
The patterns of cyclical activity and their variations and distribution have been thoroughly reviewed by Van Oordt (1960).

Spermatogenesis is a dynamic process of cellular differentiation (Clermont and Leblond,1953) and is divided into three stages : (i) spermatocytogenesis –which includes development and differentiation of germ cells; (ii) spermatocytic stage- which includes multiplication of primordial germ cells and reduction in chromosome number , and (iii) spermeiogenesis- which includes transformation of spermatids into spermatozoa (Roosen-Runge,1977). The events of spermatogenesis in general found in amphibian species are shown in Table-9.

There are considerable number of notes on investigations on the above parameters in mammals, birds, reptiles and salientian amphibians.. Nuclear polymorphism of anuran species has been observed by (Poska-Teiss,1933). A detailed description of Spermatogenesis in *Rana temporaria* was given by Witschi (1924). Electron Microscopic studies were also made by Brokelmann (1964) in frog. Biochemical studies were made by Van Dongen, Balliex and Geursen (1960).Olivieri (1965); Gould-Somero and Holland (1974); Erickson(1980). Effect of hormones on spermatogenesis and specially on the morphology of the spermiation has been investigated thoroughly by Burgos and Vitale-Calpe (1967); Pisano and Burgos (1971). However, study on sequential events of morphological changes through scanning and transmission electron microscope is meager and obscure (Picheral, 1979). The detailed ultra-structural studies of spermatogenesis in *Bufo himalayanas* were not reported earlier.

Table-9

Events of spermatogenesis



Aims and objectives of the present study

Various authors investigated amphibians and anuran spermatogenesis thoroughly. However, this particular high altitude species, *Bufo himalayanas* lacks proper attention before. So the present study has definite objectives or aims to describe the events of spermatogenesis in this himalayan toad. Specially the ultrastuctural studies of morphology and histology of this particular species may reveal some interesting findings.

The present study has a specific objective to show ultrastructural characteristics and peculiarities, if any, of spermatogenesis which include meiotic stages and the process of spermiogenesis.
The current investigations concentrates on the seasonal cycles of the events of spermatogenesis which is usually evident in the high altitude toad or in the anurans of temperate region (Van Oordt,1960).

In my present study, I want to reveal the basic morphology of the mature spermatozoa as the morphology of the spermatozoa is highly variable in amphibians. Ray et. al.,(1986) and others have reported an unique phenomenon of hypo- and hyperdiploid gonial population in amphibians apart from diploid gonial cells, which may be the reason for the variations in spermatozoan morphology. Thus in the present study an attempt has been made to record variations in sperm morphology as revealed from surface topographies of mature spermatozoa through electron microscopy.

Materials and methodology

Materials

In the present study the adult and larval stages of himalayan toad, *Bufo himalayanas* have been used as material. In case of adults only males are used.

Methodology

1. Study of the spermatogametic cycle:

Animals, both adults and larval stages were sacrificed and their testes or gonadal masses were collected from the dissected specimens (Plate-14, Figure e, f, g). Gonadal tissues and testicular tissues then preserved in normal saline solution or Bouin's solution(aquous) after removing blood vessels and other undesirable tissue parts. The tissues were prepared for cytological, histological as well as for scanning electron microscopy and transmission electron microscopy as per conventional techniques described in earlier chapter.

After the preparations of suitable sections for both types of microscopies, stained sections were examined and photographed for further examinations and cellular identifications.

2. Cytological preparation of testicular cells:

Permanent squash preparation:

The convenient squash technique for studying meiotic chromosomes (Book and Kjessler, 1964; Ohno,1965; Eicher,1966) was followed in the present study.

The testes were dissected out from the specimens and the blood vessels as well as testicular covering were removed from the adult testes. The gonads thereafter were quickly collected in the saline solution (0.67% Sodium Chloride W/V). Then the tissue was cut into small pieces and treated with 0.5% hypotonic solution for 30 minutes. The tissues were then fixed in freshly prepared aceto-alcohol (Glaciel Acetic acid:Absolute Alcohol, 1:3) for 2-3 hours. Then the fixed tissues were transferred to 45% Glaciel Acetic Acid and placed on a clean grease free albuminised slide, coverslipped and squashed by applying gentle pressure of thumb (Smith's technique, Sharma and Sharma,1975).

3. Identification of cell types:

Spermatogonial cells were divided into two categories for the identification of gonial cells.

(a) *Primary spermatogonia*: Smaller in size and contain chromatin in condensed and dusty appearance.

(b) Secondary spermatogonia: Relatively larger in size and contain chromatin in dispersed condition.

Spermatocytes were classified into leptotene,zygotene,pachytene and metaphase stages according to their chromatin condensation. Diplotene and diakinesis, not being clearly distinguishable in histological sections, were recorded in single category (Dip-Met) along with metaphase-I stage.

On the basis of their gross similarity to the mammalian spermatids, the spermatids were classified into:

a) early or Golgi phase- where PAS positive granules are present in the idiosomes.

b) Mid or cap phase- where PAS positive granules were fused and spread like a cap covering the nucleus and,

c) late spermatids- where definite acrosome formed over the nucleus.

4.Study of spermatogenesis by light, scanning and transmission electron microscopy.

a) **Light microscopic study**: Conventional squashed (Sharma and Sharma, 1975) technique was followed with 2% Lacto-aceto-orcein staining.

b) **Scanning electron microscopic study**: After dissection,the testes lobes were collected in normal saline(0.67% Sodium Chloride W/V) solution and teased longitudinally.These were then stirred in saline. The resulting cloudy solution was centrifused, supernatant discarded and the precipitate was fixed in 2.5% gluteraldehyde with 0.1M Sodium cacodylate buffer (pH 7.4) for four hours. After fixation, the materials were transferred to 2% osmium tetraoxide (OsO4) solution in the same buffer for 90 minutes. Then the material was dehydrated through graded alcohol, treated with a mixture of absolute alcohol and amylacetate (1:1) for 20 minutes and then kept in absolute amylacetate overnight. The preparation was placed on metallic stub and coated with gold and was observed under scanning electron microscope (Hitachi S 530). The selected frames were photographed for further investigations.

c) Transmission electron microscopic study: Methodology of this process was discussed in details in the earlier chapter (Chapter- II.).

Observations

Studying mammalian spermiogenesis by the conventional method on the basis of distribution and concentration of acid mucopolysaccharide in spermatids as detected by PAS reaction (Leblond and Clermont,1952) is not convenient for the study of spermiogenesis in *Bufo himalayanas*. The different stages of spermiogenesis have been studied from smear (air dried) and electron microscopical preparations. Ultra-thin sections prepared for transmission electron microscopy were also studied for the same purpose.

[a] Primary and secondary spermatogonia:

The existence of gonial cells in the developing testes has been observed from transmission electron microscope. The gonial cells are similar to the other anurans and exhibit the characteristic features of gonial cells. According to the size and the nuclear condensation, gonial cells may be categorized into primary and secondary spermatogonia. These gonial cells exhibit irregular surface morphology with myriad of convolutions (Plate 30, Figure a). Transmission electron micrograph shows oval or elliptical shape with oval nucleus and uniformly distributed chromatin granules. The nucleus is prominent, spherical; and electron dense in nature. Cytoplasm is homogeneous and contains oval mitochondria, free ribosomes, granular and agranular endoplasmic reticulum (Plate 28, Figure a).

[b] Spermatocytes:

Light microscopy reveals primary and secondary spermatocytes exist together.Primary spermatocytes, under scanning electron microscope,exhibit spherical and centrally placed nucleus without nucleolus. They have irregular surface morphology. Transmission electron microscopy shows electron dense heterochromatin masses of nucleoplasm that are irregularly distributed and condensed. (Plate 28, Figure a, b). Secondary spermatocytes are more condensed and smooth in appearance (Plate 28, Figure b).

[c] Spermatids

For the sake of easy identification from morphological structures, spermatids undergoing spermiogenesis are categorized into :

(a) early or round shaped spermatids
(b) Mid or oval shaped spermatids
(c) Late or elongated spermatids (elliptical or rod shaped)
(d) Mature spermatozoon.

From the light and electron (scanning and transmission) microscopic observations, the following features of the spermatids undergoing spermiogenesis have been recorded

(i) Each of **round spermatids** has a compact round nucleus and a thin rim of cytoplasm around its nucleus. Under scanning electron microscope these round and early spermatids show very irregular surface texture. Invaginations and buldges are the most prominent features (Plate 30, Figure B). Through TEM, these early spermatids appear as round shaped bodies with prominent nucleus (10μm) with nucleolus having electron dense granules.

(ii) A **mid spermatid** has an ovoid or elongated body with oblong nucleus. Its cytoplasm is condensed in nature. Under scanning electron microscope, apart from its general features, it shows smooth appearance. No invagination or protrusions are found (Plate 30, Figure C, D).Through transmission electron microscope, it has been revealed that acrosome cap spread over the primary surface of the spermatids. So a smooth and continuous texture is superficially observed (Plate 28, Figure c, d).

(iii) At the **late stage**, neck part originates from the more broad posterior end as irregular bands. Under scanning electron microscope, this irregular bands appear distinctly and under higher resolution neck part is found to be featured by transversely arranged tubules in a zig-zag fashion (Plate 30, Figure E, F, G). This is probably due to spirally arranged multiple cords at the neck region. Further condensation of the apical region along with the elaboration of the neck region is found under the transmission electron microscope. Cytoplasmic organelles were also elaborated at this stage (Plate 31, Figure c, D, E).

[d] Spermatozoon

The **mature spermatozoa** are found at the end of spermiogenesis . The spermatozoa show appearance of two axial filaments coiled upon one another. The head part becomes more elongated to assume a rod like shape, tapering at the apex area (Plate 30A, Figure A, B). Under scanning electron microscope a barb like perforatorium appears from the pointed anterior end (Plate 32, Figure b). The head measures about 18 micromillimeter in length, a short neck and a long filamentous and twin tail of about 25 micromillimeter in length. Under transmission electron microscope, these features revealed some more structural details and also about cytoplasmic inclusions which were seen in the ultra-thin section (Plate 33, Figure A, B, C, D).

The mature spermatozoa shows polymorphism in sperm morphology as revealed from surface topographies specially from scanning electron microscopy. Under scanning electron microscope, it was evident that sperms have different shapes and morphologies. Apart from normal spermatozoon, I have recorded at least four different types of spermatozoon as followings:

a) Spermatozoon with ovoid head, without perforatorium and ill-recognizable neck region (Plate 30A, Figure C).

b) Spermatozoon with sickle shaped head, without neck and tail (Plate 30A, Figure E).

c) Spermatozoon with round and highly conspicuous head and a long tail (Plate 34, Figure A).

d) Spermatozoon with a tail consisting of a single axial filament (Plate 30A, Figure D).

PLATE 30

Legends

Scanning electron micrographs of different developmental stages of spermatogenesis

A. Primary spermatogonia with irregular surface

B. Round spermatids with myriad convolutions

C. Mid-spermatids with smooth surface

D. A mid spermatid (ovoid) with smooth surface

E. Spermatid with a bulging protrusion

F. Late spermatid with growing neck region

G. Later spermatid with developing neck and tail region

PLATE-30

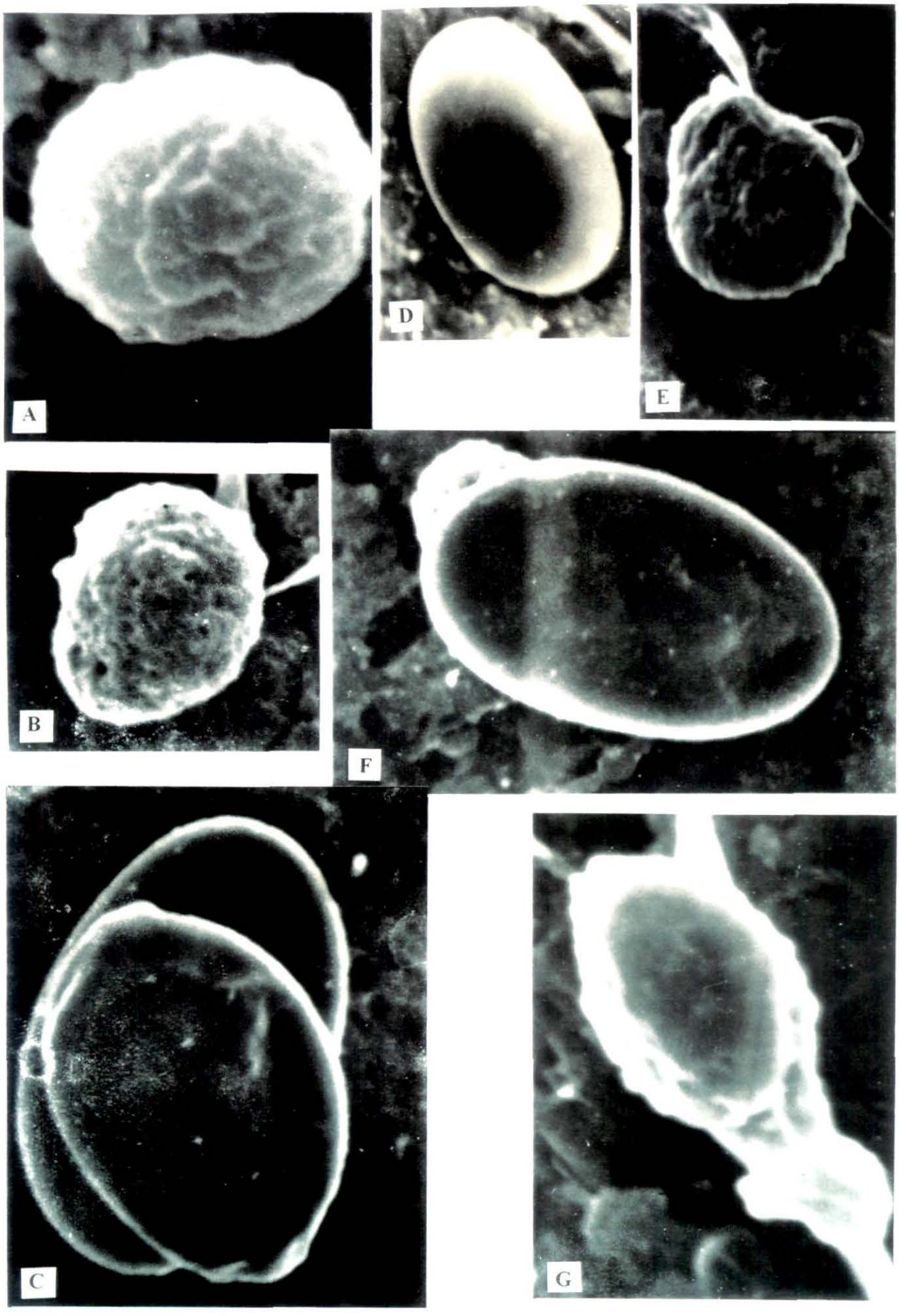


PLATE 30A

Legends

Scanning electron micrographs of mature spermatozoa and different atypical spermatozoa

A. A normal mature spermatozoon showing head, neck and typical bi-flagellate tail
B. Enlarged view of a spermatozoon's head, mid-piece and tail
C. An atypical spermatozoon with ovoid head, ill-developed neck and without perforatorium
D. An atypical spermatozoon's head, ill-developed neck and mono-flagellate tail
E. An atypical spermatozoon with sickle shaped head, without neck and tail
F. A normal spermatozoon with a spermatid

PLATE-30 A

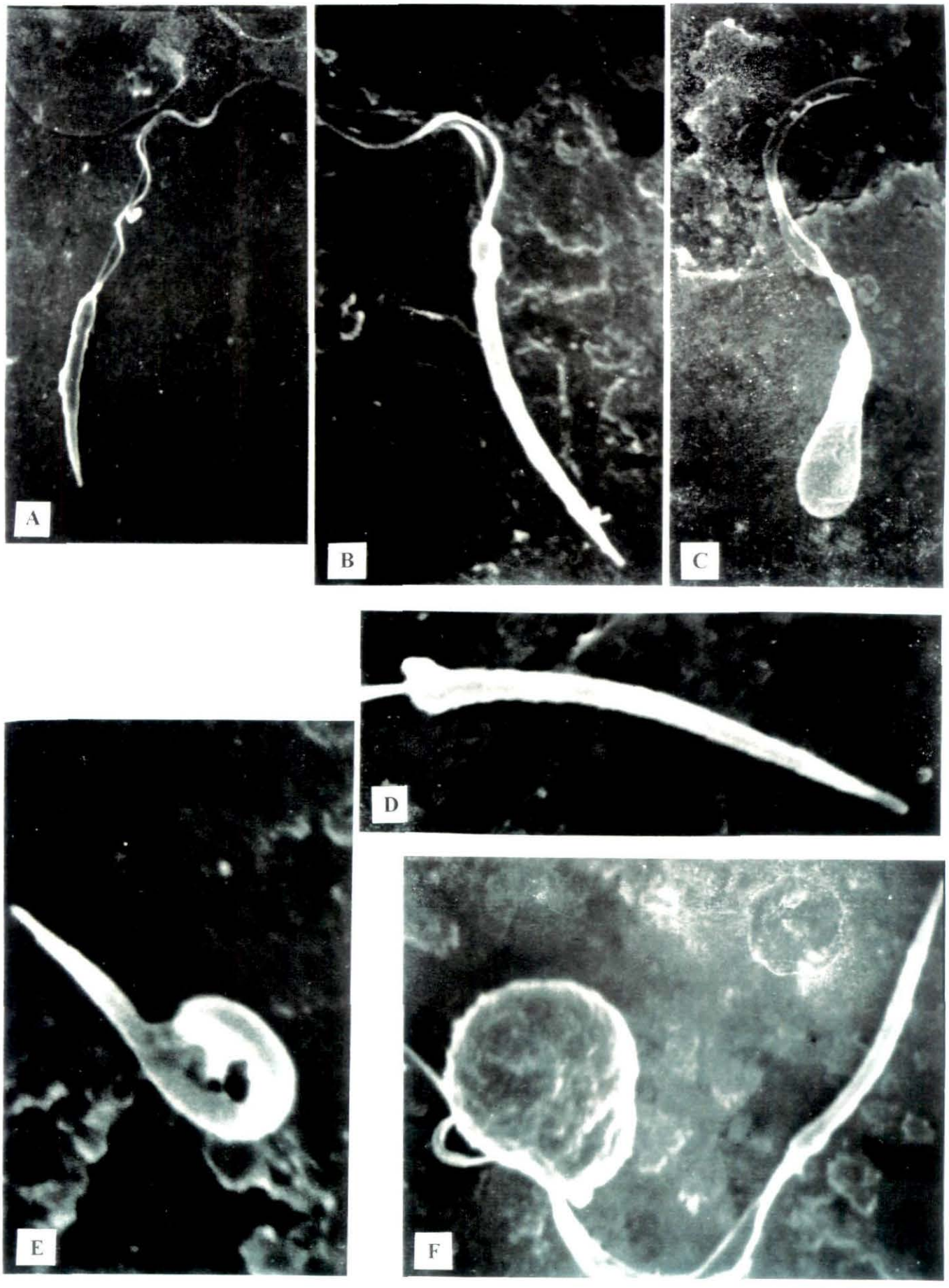


PLATE 31

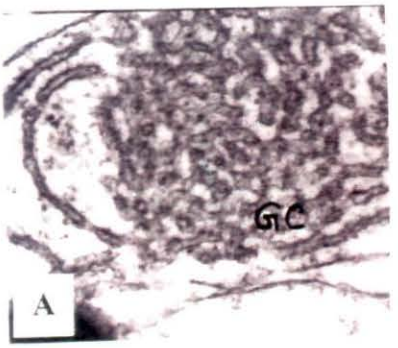
Legends

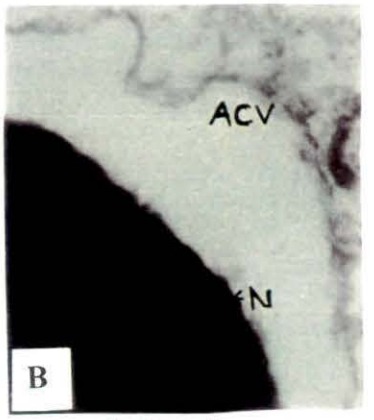
Electron micrographs of different parts of maturing spermatids and mature spermatozoa

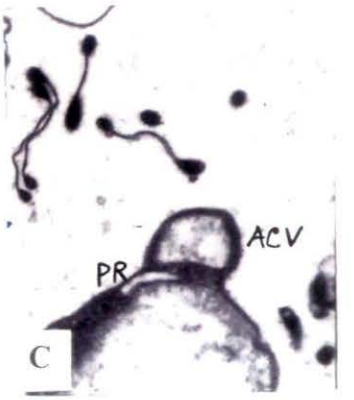
A. Golgi complex of an early spermatid
B. Acrosomal complex developing in late spermatid
C. Origin of acrosomal cap and perforatorium of a spermatozoon
D. Elongated and late spermatid with developing neck region
E. Late spermatids
F. Pre-mature spermatozoon
G. Maturing spermatozoon (posterior part)
H. Maturing spermatozoon (anterior part)

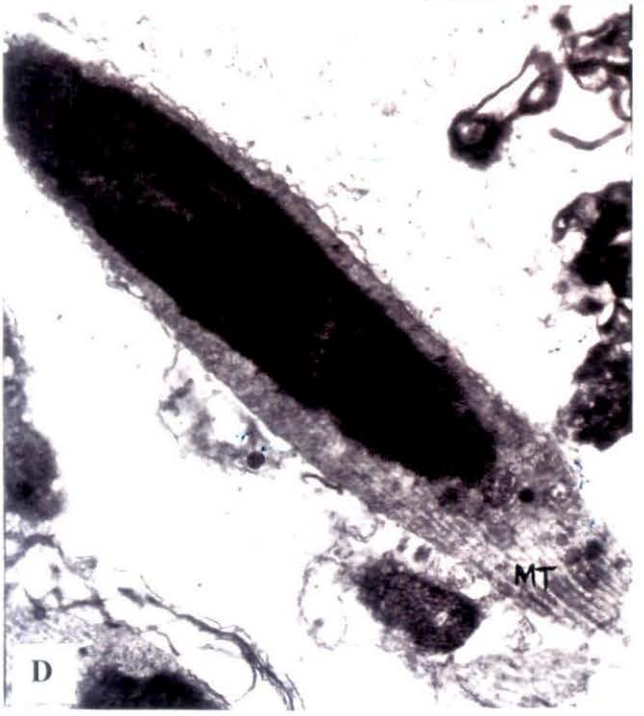
N- Nucleus GC- Golgi complex ACV- Acrosomal vesicle
PR- Perforatorium MT- Microtubules TL- Tail AC- Acrosome
M- Mitochondria DN- Developing neck

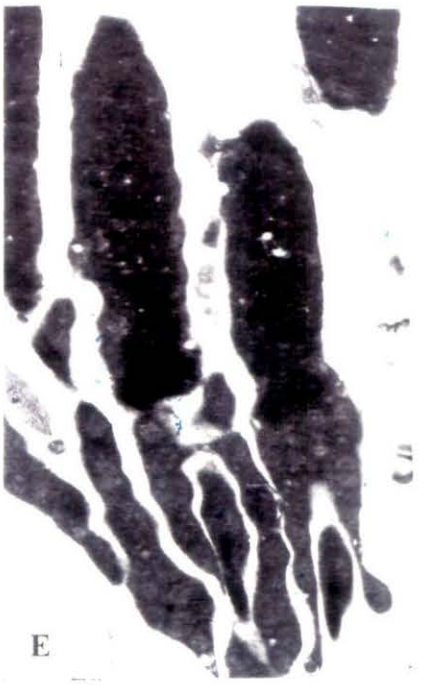
PLATE-31

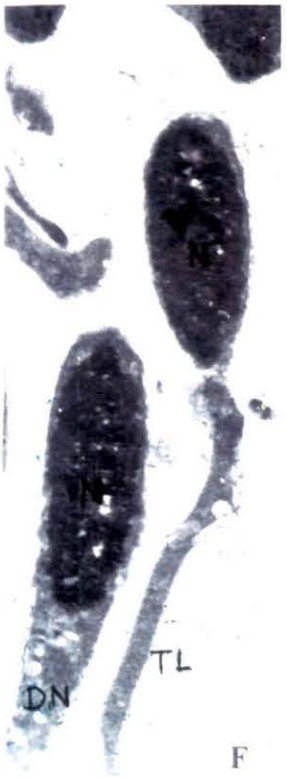












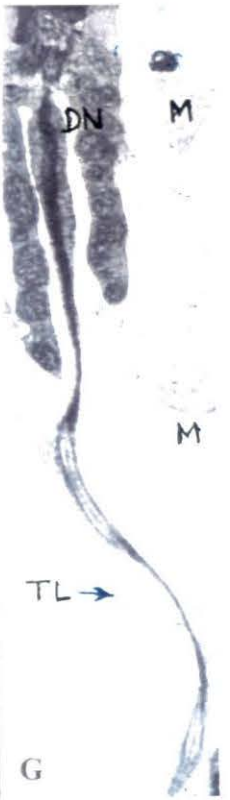


PLATE 32

Legends

Light and scanning electron micrographs showing different parts of mature and normal spermatozoa

Fig.a. Light micrograph of a normal, mature spermatozoon showing typical features of this species

Fig.b. Enlarged view of the anteriormost part of the spermatozoon showing a barb-like perforatorium

Fig.c. Scanning electron micrograph of a typical spermatozoon

Fig.d. Part of the tail highly enlarged to show bi-flagellate tail connected by a membrane medially

Fig.e. End portion of the tail highly enlarged to show the discontinuation of the membrane towards the end

PLATE-32

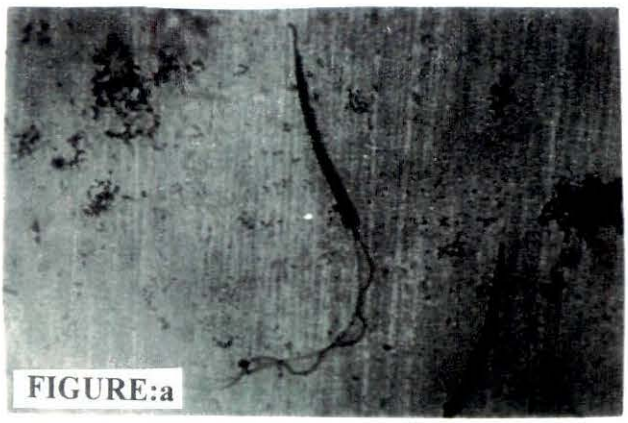


FIGURE:a

FIGURE: b

FIGURE: c

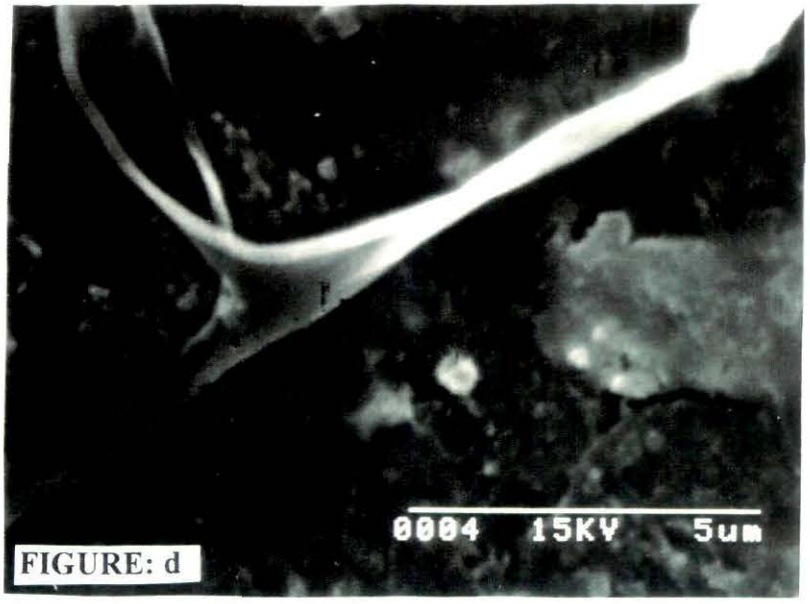


FIGURE: d

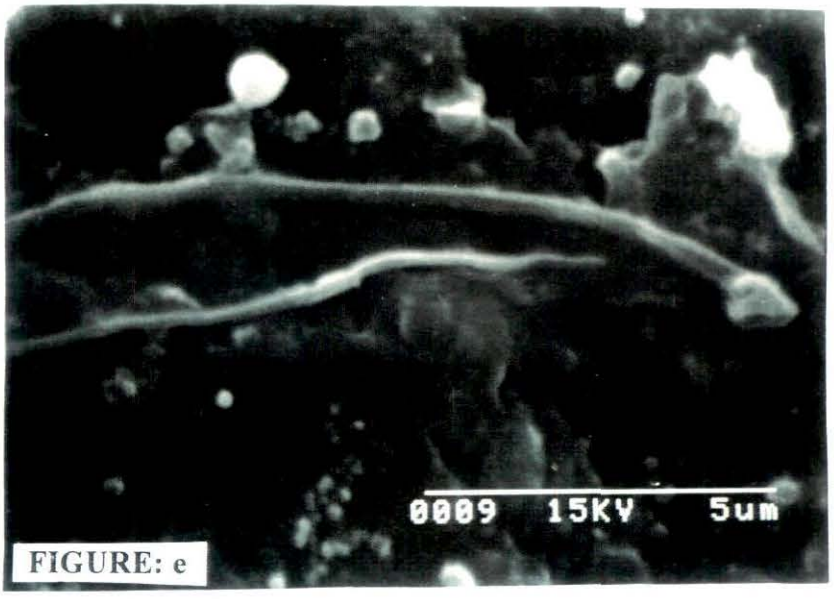


FIGURE: e

PLATE 33

Legends

Electron micrographs showing different parts of mature spermatozoa

A. Enlarged head of a spermatozoon showing perforatorium, acrosome and elongated nucleus
B. Numerous sperm heads in sperm bundle in cross section
C. Section through middle pieces of spermatozoa
D. Section through tail portion showing microtubules in cross section

N-Nucleus PR- Perforatorium AR- Acrosome SPH- Sperm heads MT- Microtubules

PLATE-33

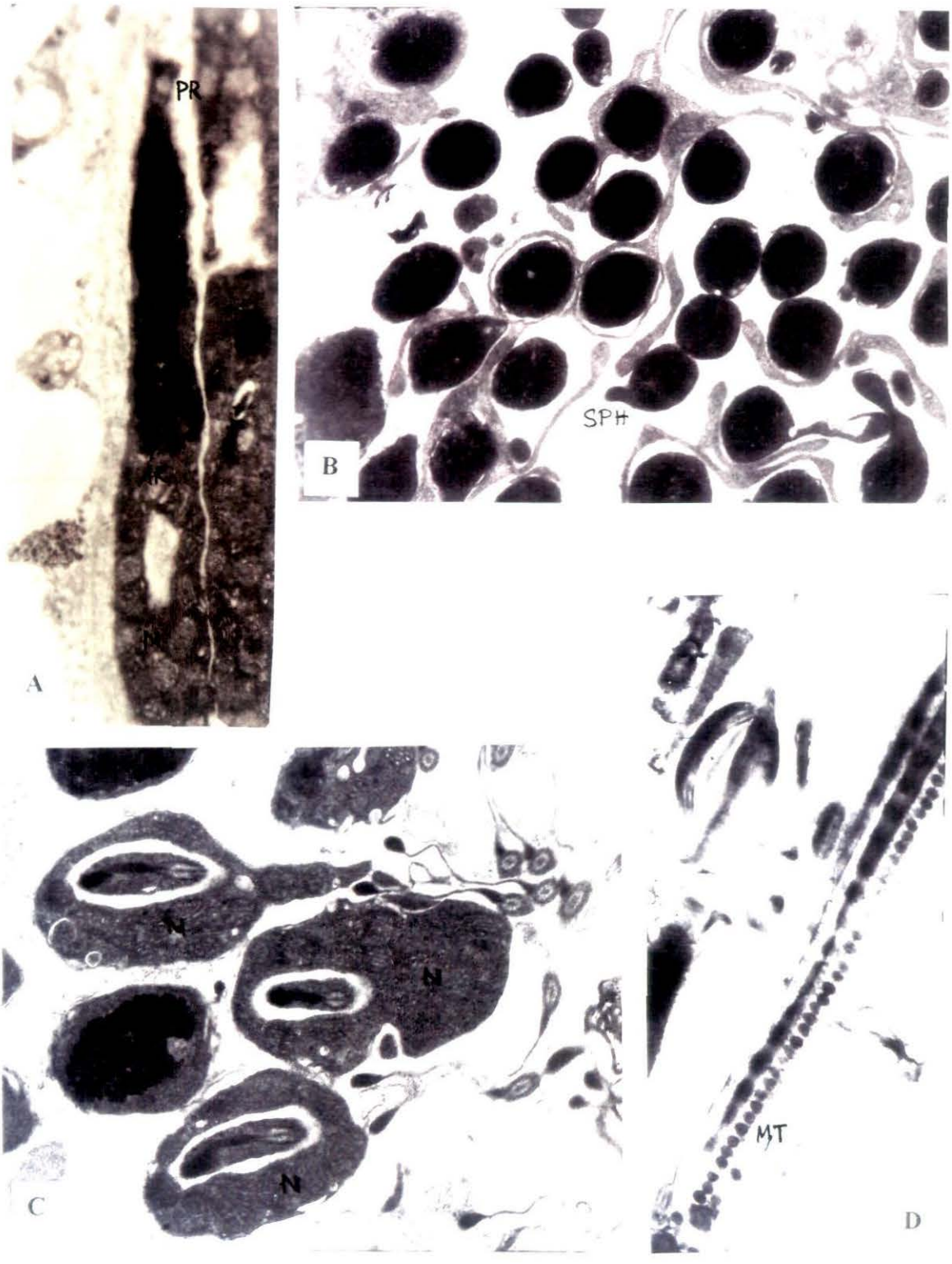


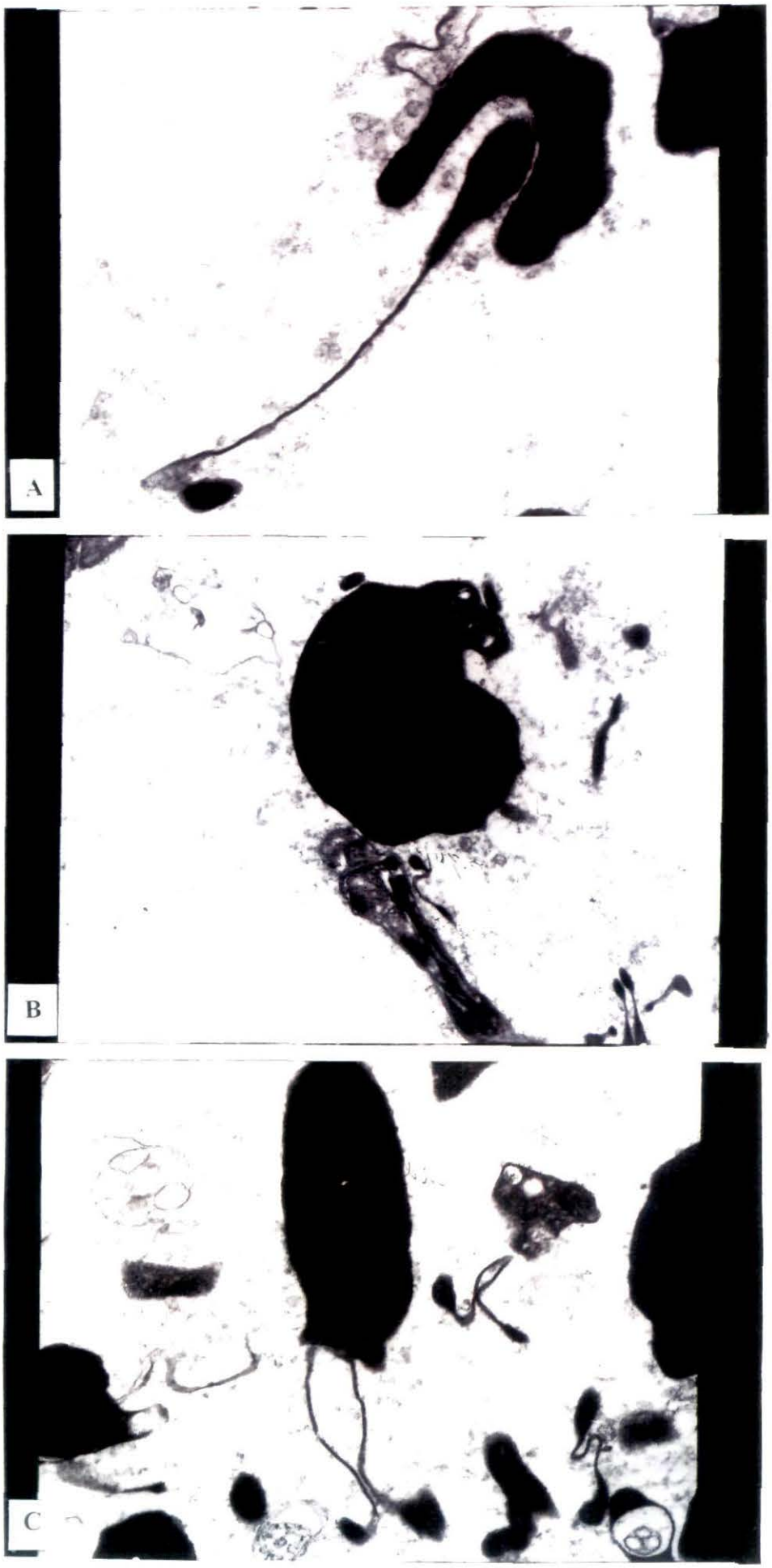
PLATE 34

Legends

Electron micrographs of some atypical spermatozoa

A. Megacephalic sperm with large and conspicuous head
B. Microcephalic sperm with highly condensed head region
C. Globular sperm with large oval head and ill- developed neck and tail region

PLATE-34



Discussion

In amphibians spermatogenesis is of cystic type, i.e. the cells present in a cyst are in the same stage of development and are derived from a single spermatogonium.

The identification of various stages of spermatogenesis in amphibians, particularly anurans, is done following the description of van Oordt (1956). However, in recent years, a modified method of qualitative assessment of spermatogenetic activity is adopted which is as follows:

Stage O: Primary spermatogonia
Stage I: Secondary spermatogonia
Stage II: Primary spermatocytes
Stage III: Secondary spermatocytes
Stage IV: Spermatids
Stage V: Sperm bundles attached to the Sertoli cells.

The primary spermatogonia are the largest germ cells located adjacent to the basement membrane of the seminiferous tubule. Such cells are with voluminous eosiniphilic cytoplasm and with an irregular nucleus. Rastogi et.al. (1985), based on the nuclear characters, recorded two types of primary spermatogonia in the adult testis of *Rana esculenta* and designated them as pale and dark types of primary spermatogonia comparable with A0 to A4 types of primary spermatogonia of mammals (Ray et.al.,1975; Ray et.al., 1978, 1979 and 1980).

The primary spermatogonia divide mitotically to form secondary spermatogonia. **The secondary spermatogonia** are smaller than primary spermatogonia and both cytoplasm and nuclei are basophilic in nature.

The secondary spermatogonia again undergo mitosis to form **primary spermatocytes**. The primary spermatocytes are larger than secondary spermatocytes with eosiniphilic cytoplasm and basophilic nuclei. Meiotic features are noted in the primary spermatocytes. Primary spermatocytes are found in the central part of the cross section of the testis or found attached to the wall of the tubule. The primary spermatocytes usually form a nest and small intracellular vacuoles may be detected in the cell nest (Saidapur,1989).

After the first meiotic division, primary spermatocytes give rise to **secondary spermatocytes**. They are smaller than primary spermatocytes with eosinophilic cytoplasm, basophilic nuclei and condensed chromatin. The intracellular vacuoles increase in size and become more evenly distributed. The cell nests are usually situated in the central part of the cross section of the testis (Saidapur,1989).

After the second meiotic division, the spermatids are formed. The **spermatids** are small globular cells, distinctly smaller than secondary spermatocytes, with eosinophilic cytoplasm and a spherical basophilic nucleus (Saidapur,1989).

With the initiation of spermiogenesis, the spermatids change morphology from globular to oval to elliptical and ultimately more and more elongated. The intracellular vacuoles first fuse into one big central vacuole and the cells are situated against the wall of the cysts. During spermiogenesis, the heads of the **maturing sperm** cells are found embedded in the Sertoli cells. Such cell nests are often found attached to the wall of the seminiferous tubule (Saidapur,1989).

The various cell nests may or may not be present in the testis round the year depending on the species or pattern of the spermatogenetic activity. In specieses like *Rana cyanophlyctis, Rana hexadactyla, Bufo melanostictus* which exhibit continuous type of spermatogenesis, all the stages are present throughout the year. Whereas *Rana tigerina, Bufo marinus* exhibit potentially continuous spermatogenic activity or discontinuous type as in *Rana temporaria* , the various stages of spermatogenesis undergo distinct changes.

The event of spermiogenesis has been studied in a number of amphibian species following conventional technique (Sharma and Sekhri,1955; Hirschler, 1928; Mc Gregor, 1899; Terni, 1914; Sharma and Dhindsa,1956; Baker,1963; Bandopadhyay,1977; Roy,1978; Midya et.al,,1981; Mallick,1987; Roy,1989).

Sharma and Dhindsa (1955) have provided an excellent account of spermiogenesis in Indian toad, *Bufo stomaticus*. At early spermatid stage deeply stained Golgi granules uniformly spread out throughout the cytoplasm. During the maturation of the spermatid the Golgi granules begin to fuse to form bigger granules which in turn form still bigger granules by fusion. One of these bigger granules now come in close contact with the end of the nucleus which is opposite the axial filament and form proacrosome. To begin with the proacrosoome satins deeply and uniformly. But as it grows it becomes differentiated in an outer chromophilic cortex and inner chromophobic core. As the maturation of the sermatid continues, its nucleus begins to elongate with the proacrosome at its anterior end and the cluster of small mitochondria at the posterior end . The proacrosome form the acrosome; it first becomes triangular and then pointed and needle like. It may be noted that no acrosomal granule is formed in the toad (Diagram 11). The ripe spermatozoon possess a deeply stained head with cylindrical nucleus and pointed acrosomal tip, small middle piece and two axial filaments joined together by a rudimentary undulating membrane.

Burgos and Fawcett (1956) have given a remarkable account of the electron micrographs of the maturing spermatids of *Bufo arenarum* including formation of the acrosome changes in the nucleus, formation of perforatorium, centriole and tail and undulation membrane. Their account confirms the main observations of Sharma and Dhindsa (1955) with light microscopy.

Burgos and Fawcett (1956) described that at the very onset of spermiogenesis, the spermatids lack a conspicuous Golgi complex such as occurs in the mammalian spermatids; instead it possesses one or many very small aggregation of spherical vesicles of varying sizes. According to the authors,no acrosomal granule is differentiated in the acrosomal vesicles, contrary to the mammalian spermiogenesis where one or more acrosomal granules are found. The acrosomal granules gradually increase in area of contact with the nuclear membrane, extending further down over the elongated nucleus at its anterior pole.

Burgos and Fawcett (1956) have described progressive condensation of the nucleus during spermiogenesis. At the early stages, the nucleus is spherical and in the course of spermiogenesis it becomes ovoid, then elongated and finally rod like in the spermatozoon. Burgos and Fawcett (1956) have described some striking changes in the fine structure of the nuclear content. At the early spermatid stage the nuclear contents are " homogeneous" and consists of very fine granules of about 100 to 150 Å in diameter. Later these very fine granules become randomly distributed and form coarse granules. However, the authors have not provided any explanation of such changes and their fate.

Burgos and Fawcett (1956) described for the first time a "perforatorium" in the maturing spermatid of the toad. They described that in the later stages of spermiogenesis, when the nucleus is progressively becoming elongated, a narrow, conical cleft appears between nuclei and head cap as assign of perforatorium appearance. It consists of a number of coarse dense strands which arise around the tapering end of the nucleus to converge in front of it to form a dense pointed structure.

Burgos and Fawcett (1956) described two centrioles of cylindrical shape in the maturing spermatids of toad. In cross section, each centriole appears "as thick walled moderately dense ring with a scalloped outline". About nine scallops are distinguished at the periphery and within each of these one or more minute mass of low density has been described. Centrioles have typical structure as found in others.

Sharma and Dhindsa (1955) first described in *Bufo* the existence of two centrioles in the middle piece which are located side by side. Each gives rise to individual axial filament.

Burgos and Fawcett (1956) described in the axial filament in case of toad of a bundle of 20 submicroscopic longitudinal fibrils. Of these, there is a centrally located fibril and nine pairs of evenly spaced circumferential fibrils. All the 20 fibrils appear tubular in cross section since their core seems to be of low density.

The undulating membrane is made up of a thin ribbon like band of the dense fibrous substance (Burgos and Fawcett,1956)

In the present investigation, all the changes that occur in a normal spermatogenic event have a conformity with the earlier observations made from light and electron microscopy. However, the following changes may be described as 'unique' for the species:

(i) Presence of primary and secondary spermatogonia with rough and irregular surface morphology. Cytoplasm homogeneous with oval mitochondria, free ribosomes and other organelles.

(ii) Spermatogonial cells exhibit hypo- and hyperdiploid gonial cells along with normal diploid gonial cells. These polyploidy may be an important factor for sperm polymorphism in this species.

(iii) Spermatids also exhibit different types of stages according to the degree of development. Anuploidy may also contribute a factor for the variations in the spermatids.

(iv) Mature sperms show polymorphism clearly in their morphology. Few types of morphologically different spermatozoa is an unique characteristic of this species.

(v) A mature normal spermatozoon exhibits an unique biflagellate tail with undulating membrane present in between except the posterior-most end. The flagellum is provided with inner core of axial filament which has submicroscopic microtubules.

(vi) Neck region or middle piece part of a mature spermatozoon is provided with a pair of centrioles. These centrioles are the site of origin of the two axial filaments or flagella.