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# Spermiogenesis in the Japanese Greater Horseshoe Bat, *Rhinolophus ferrumequinum nippon**

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In the Japanese greater horseshoe bat, ***Rhinolophus ferrumequinum*** *nippon*, the nuclear ring formation in spermiogenesis is characterized by an early appearance of a dense subplasmalemmal matrix which originates from the inner leaflet of the plasma membrane near the caudal end of the acrosome and by the subsequent formation of microtubules from the dense matrix. The Sertoli cell-ectoplasmic specializations consisting of two elements, i.e. the circumferentially arranged filamentous structures around the spermatid heads and the more deeply placed flattened sER cisternae: the specialization seems to play a dual role ; grasping or shaping the spermatid heads by the former and causing the spermiation by the disappearance of both elements. A large amount of the longitudinally lamellated dictyosomes persist in the cytoplasmic droplet until just after spermiation and transform into one- or multi-layered spherical vesicles in the epididymal sperm. The extensive existence of the satellite fibrils seems to be an inherent property of this bat, considering the situation from the phylogenic point of view. The inferiority of sperm mitochondria in size (diam. -0.1 $\mu$m) appears to be compensated by the numerical superiority (160) for prolonged sperm storage in the female reproductive tract during hibernation.

## INTRODUCTION

Since an early electron microscopic analysis on mammalian spermatogenesis (Burgos and Fawcett, 1955), investigations have been extensive. For bats, however, there have been few such studies, except for the report of Uchida and Mōri (1972) on spermiogenesis in the Japanese long-fingered bat, *Miniopterus schreibersi fuliginosus* belonging to the Miniopterinae (Vespertilionidae) and the "delayed implantation" type.

The present study was therefore carried out to examine with the electron microscope the spermiogenesis in the Japanese greater horseshoe bat, *Rhinolophus* ***ferrumequinum*** *nippon*, belonging to the Rhinolophinae (Rhinolophidae) and the "prolonged sperm storage" type (Mōri ***et al., 1982).*** The spermiogenesis in this bat was fundamentally similar to those in other mammals as expected; accordingly, this study clarified details of the nuclear ring formation, relationship of the spermatids to the Sertoli cells, some characteristic structures (dic-

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tyosomes and satellite fibrils) and the mitochondrial property.

## MATERIALS AND METHODS

Five adult males (*R. f. nippon*) were collected at caves in the Yamaguchi and Fukuoka Prefectures from late July to early November. The testes and epididymides were removed and then the tissues were promptly placed in cold 3 % glutaraldehyde in 0.2 M-phosphate buffer (pH 7.4) for 4 h. After being thoroughly rinsed with the same buffer, the tissues were post-fixed with 1.3 % osmium tetroxide in the same buffer, dehydrated with acetone and embedded in Epon 812. Thin sections (-60 nm) were doubly stained with uranyl and lead acetate and examined with an Hitachi HS-9 electron microscope (75 kV).

## RESULTS

Spermatogenic activity in this bat was vigorous from early August to late September. Spermiogenesis was described according to the following successive phases--Golgi, cap, acrosomal and maturation phase--, and the subsequent spermiation.

### Golgi and cap phases

At the Golgi phase, a cytocentrum containing two centrioles and a wide Golgi field was found in the juxtanuclear cytoplasm. A single large acrosomal vesicle was fixed to a deep recess of the nucleus, within which a crescent and dense acrosomal granule was enclosed. Approximately the posterior half of the vesicle was applied to the indented and thickened nuclear membrane devoid of the perinuclear cisternae (Pl. I, Fig. 1). At the late cap phase, the acrosomal vesicle spread outward from the anterior pole of the nucleus and flattened over almost the anterior one-third of the nucleus, and the acrosomal material was not yet completely distributed into the fold of the acrosomal cap. A considerable amount of the spermatid cytoplasm still remained in front of the developing acrosome, and many smooth endoplasmic reticula (sER) with expanded cisternae and a few annulate lamellae were present in the cytoplasm (Pl. I, Fig. 2).

### Acrosomal phase

At the early acrosomal phase, most of the spermatid cytoplasm which had been located between the outer acrosomal and the plasma membranes was removed backward, and consequently both membranes came in contact. A shallow recess of the nucleus was found in the part facing the posterior margin of the acrosomal cap through the subacrosomal space, consequently a small swelling of the nucleus was made just behind the recess. The nuclear ring had not yet formed (PI. I, Fig. 3).

At the slightly advanced stage, ectoplasmic specializations began to occur in the Sertoli cells, which were characterized by the appearance of circumferential filamentous structures attaching to the inner surface of its plasma

membrane adjoined to the spermatid plasma membrane, and of more deeply placed and disconnected flattened sER cisternae (Pl. II, Fig. 4 and inset a). Also in the spermatid, a ring-like plasmalemmal specialization, i.e. an anlage of the nuclear ring with a slightly dense matrix, began to appear beneath the plasma membrane near the above nuclear swelling, from which microtubles arise, and the caudal sheath (manchette) consisting of the numerous microtubules with an inverted truncated cone-like arrangement extended caudally; the nuclear envelope locating along the microtubules became parallel to them (Pl. II, Fig. 4 and inset b).

At the middle acrosomal phase, a nuclear ring-groove was formed between the anlage with the more dense subplasmalemmal matrix originated from the inner leaflet of the plasma membrane and the posterior margin of the acrosomal cap, and the enlarged intercellular space between the Sertoli cell and the spermatid joined the above groove (Pl. III, Fig. 5).

At the late acrosomal phase, an elongation of the nucleus and a condensation of the nucleoplasm had advanced considerably, but the nuclear ring still persisted in its original locality and exhibited a dome-like structure protruding into the more expanded cellular space (Pl. III, Figs. 6 and 7).

**Maturation phase**

At the maturation phase, the nucleus and acrosome of spermatids which were still embedded in recesses of the Sertoli cells began to take on the shape and structures characteristic of the species; the elongation of the apical segment of the acrosome and the condensation of the nucleoplasm were almost completed, and the nuclear ring descended from the original position to the final one (Pl. III, Fig. 8). The Sertoli cell-ectoplasmic specializations also advanced more; the filamentous structures surrounded the apical segment, and sER vesicles were coalesced to form long and flattened sER cisternae all round the acrosome (Pl. III, Fig. 9). The formation of the postacrosomal sheath caused by the complete descent of the nuclear ring resulted in an appearance of the membranous scroll consisting of the redundant nuclear envelope (Pl. IV, Fig. 10).

Just before spermiation, the close associations between the late spermatids and Sertoli cells became loose; the acrosomal region, especially the apical segment of the acrosome was in contact with the Sertoli cells only by their cytoplasmic protrusions, and the Sertoli cell-ectoplasmic specializations had almost disappeared even from there (Pl. IV, Fig. 11). At this stage, the plasma membranes of the Sertoli cell decreased in electron-density, and thus the spematid plasma membranes became prominent (Pl. IV, Fig. 12).

**Spermiation and epididymal immature sperm**

All the immature spermatozoa just released from the Sertoli cells had a cytoplasmic droplet in the upper part of the middle piece, which contained numerous longitudinally lamellated dictyosomes arranged in parallel to the axial filament complex (Pl. V, Fig. 13). In the epididymal spermatozoa, the cytoplasmic droplet became located in the lower part of the middle piece, and

most of the dictyosomes transformed from lamellated structures into one- or multi-layered spherical ones (Pl. V, Fig. 14). Another characteristic finding was the presence of numerous satellite fibrils in a punctate profile between 9 outer dense fibers and between them and the axial filament complex, the no. 9 of which was thick as well as the nos. 1, 5 and 6; some satellite fibrils appeared connected with a cortical substance of each outer dense fiber (Pl. V, Fig. 15). The mitochondrial sheath consisted of 160 mitochondria (i.e. 1 longitudinal and 79 circumferential pairs) arranged in a helical pattern, the size of which was -0.1 $\mu$m in diameter (Pl. V, Fig. 15).

## DISCUSSION

Although the presence of a well developed sER in the Sertoli cytoplasm has been interpreted as evidence for its possible secretion of steroid hormones and possible function of the "nurse cell" (Fawcett, 1975), the mechanical role of the Sertoli cell-ectoplasmic specialization consisting of the filamentous and membranous elements can be categorized into two devices, i.e. a grasping or shaping device for the developing spermatid heads harboring in the recesses of the Sertoli cell, and a releasing device which causes their spermiation to be apt to occur between the late spermatid heads and the Sertoli cells. The former device involves "zipper up" in the rat (Dym and Fawcett, 1970) or firm adhesion in the mouse and rat (Ross and Dobler, 1975; Russell, 1977) : the microfilaments with actin-like property may give rise to a gripping force on the pig and mouse spermatid heads, holding it in place until spermiation (Toyama, 1976) ; also, the network of fibers disposed circumferentially around mammalian spermatid heads could play a role in shaping the acrosome or nucleus of the spermatid (Phillips, 1980). The latter device is closely related to the coalescence or widening of the endoplasmic reticulum in the Sertoli cell of the rat, hamster and/or musk shrew, *Suncus murinus* (Brokelmann, 1963; Vitale-Calpe and Burgos, 1970 a, b; Burgos ***et al.,*** 1973; Cooper and Bedford, 1976), although direct evidence for the mechanism of spermiation has not yet been demonstrated. The results in the present study also suggest that the specialization plays a dual role at least in the process of spermiation; the filamentous structure and the flattened sER cisternae may function as a grasping and/or shaping device from the acrosomal phase to just before spermiation and as a releasing device at the time of spermiation, respectively.

It has been speculated that the nuclear ring has been derived from the cytoplasm or the plasma membrane (Burgos and Fawcett, 1955). The nuclear ring and manchette retain their shape and association in several mammalian spematids in spite of the mechanical dissociation and lysis with detergent, suggesting that both structures are tightly bound together ***in vivo*** (Phillips, 1980). In this connection, the findings in the present study adduced ultrastructural evidence that the dense subplasmalemmal matrix originates from the inner leaflet of the plasma membrane near the caudal end of the acrosome, and subsequently the microtubules develop secondarily from the dense matrix, as indicated by Fawcett (1981).

The dictyosomes in the cytoplasmic droplets have been consistently found as dispersed lamellae in the form of flattened sacs in the rat (Dietert, 1966) and as round or semi-circular vesicles in the Japanese long-fingered bat, *Miniopterus schreibersi* ***fuliginosus*** (Uchida and Mōri, 1972), but in this bat, almost all of a large amount of dictyosomes which had been lamellar in shape until the period of testicular immature sperm transformed into one- or multi-layered spherical vesicles in the epididymal immature sperm.

The satellite fibrils have been demonstrated to arise by exfoliation of the cortical substance of the outer dense fiber in the Chinese hamster (Fawcett and Phillips, 1970), which coincided with our observations. As for their functional significance, an extensive development of the satellite fibrils has been explained to be an adaptive modification for increasing tensility of the thick outer dense fibers in the golden-mantled ground squirrel, *Citellus* ***lateralis*** (Fawcet and Phillips, 1970) as well as in the bandicoot, *Parameles* ***nasuta*** (Cleland and Rothschild, 1959). According to our unpublished observations of insectivores (shrews and moles) and a primitive primate (tupai) sperm tails, a successive gradual decline in quantity of the satellite fibrils was recognized as animals evolve. On the basis of the phylogenetic conception, the predominant existence of the satellite fibrils in this bat is considered to inherit the nature of shrews, contrasting with the poor appearance of those in other bats examined, i.e. *Myotis macrodactylus*, ***M. nattereri***, ***M. s. fuliginosus*** and *Pipistrellus abramus*.

The number and size of mitochondria in bat spermatozoa seem to be profoundly concerned with their prolonged survival in the female reproductive tract during hibernation (Uchida and Mōri, 1972); the mitochondria of ***M. nattereri*** (135 in number and 0.3-0.8 $\mu$m in diameter), *M. macrodactylus* ***(117*** and 0.3-O. 6 $\mu$m) and ***P. abramus (138*** and 0.2-O. 8 $\mu$m), belonging to the "prolonged sperm storage" type, are relatively more numerous and larger than those of ***M. s. fuliginosus (78*** and 0.1 $\mu$m) belonging to the "delayed implantation" type. As to ***R. f. nippon***, in spite of belonging to the former type, the mitochondria (160 and -0.1 $\mu$m) are large in number but far small in size compared with those of other bats belonging to the same type. Accordingly, the inferiority of the mitochondria in size seems to be compensated by their superiority in number.

## ACKNOWLEDGEMENTS

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## Explanation of Plates I-V

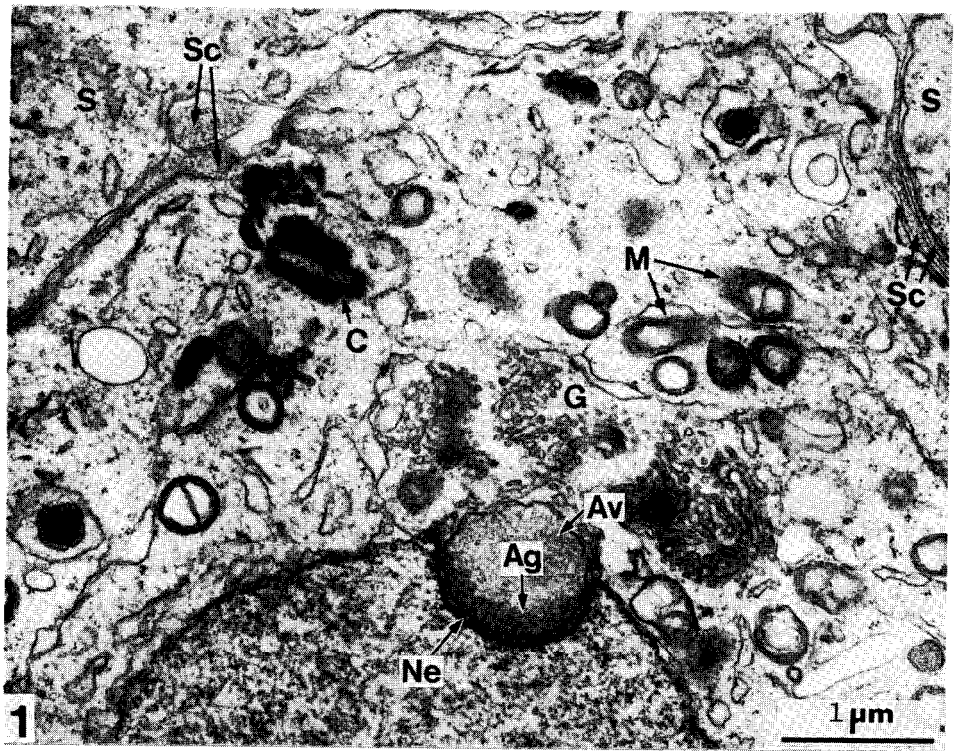
**Abbreviations: A,** acrosome; **Ag,** acrosomal granule; **Al,** annulate lamellae; An, anlage of nuclear ring; Au, annulus; Av, acrosomal vesicle; C. centriole; Cd, cytoplasmic droplet; Ch, Chromatin ; **D.** dictyosome; Es, equatorial segment; **F,** filamentous structure; Fe, flattened sER; G, Golgi complex; I, inner acrosomal membrane ; Is, intercellular space ; L, lumen of seminiferous tubule ; **M,** mitochondrion ; Ms, membranous scroll: Mt, microtubule; N, nucleus; Ne, nuclear envelope; No, nucleolus; Nr, nuclear ring; 0, outer acrosomal membrane; Od, outer dense fiber; P, postacrosomal sheath; Ps, plasma membrane of Sertoli cell; R, recess of nucleus ; S, spermatid ; Sc, Sertoli cell ; Se, smooth endoplasmic reticulum ; Sf, satellite fibrils; Sn, swelling of nucleus ; Sp, spermatid plasma membrane ; Ss, subacrosomal space; *, nuclear ring-groove.

## Plate I

Fig. 1. Electron micrograph of a cytocentrum of a spermatid at the Golgi phase, showing a single acrosomal vesicle fixed to a deep recess of the nucleus, a centriole and a wide Golgi field.

Fig. 2. Electron micrograph of a spermatid at the late cap phase, showing the acrosome spreading over the anterior one-third of the nucleus, many sER vesicles and a few annulate lamellae.

Fig. 3. Electron micrograph of the part from which an anlage of the nuclear ring develops in the future in a spermatid at the early acrosomal phase, showing a contact between the outer acrosomal and plasma membrane.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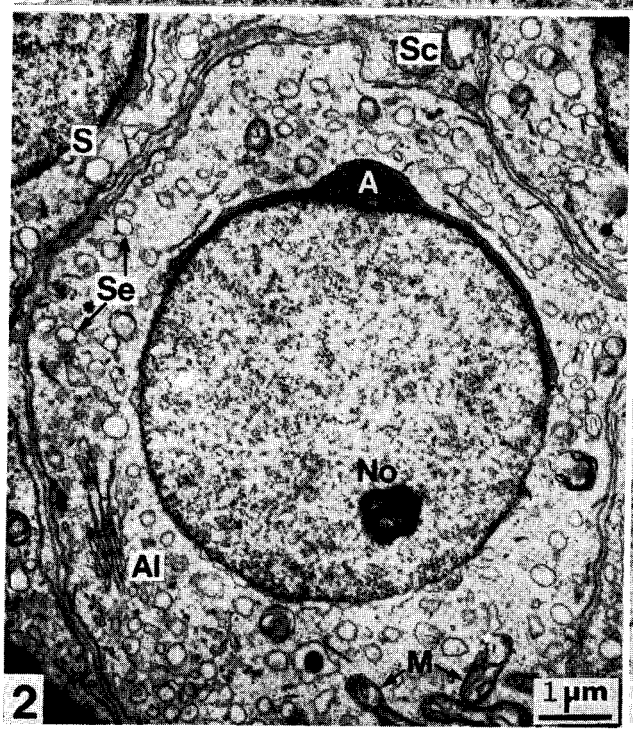
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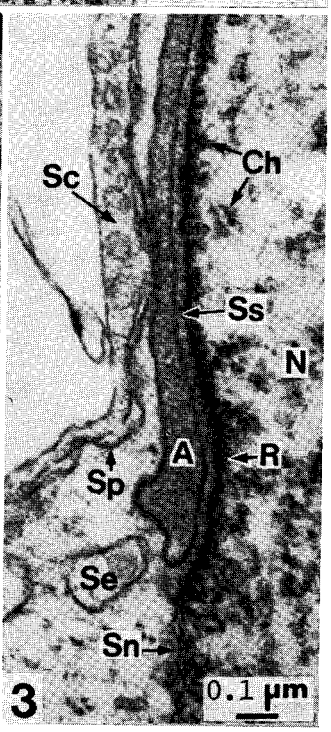
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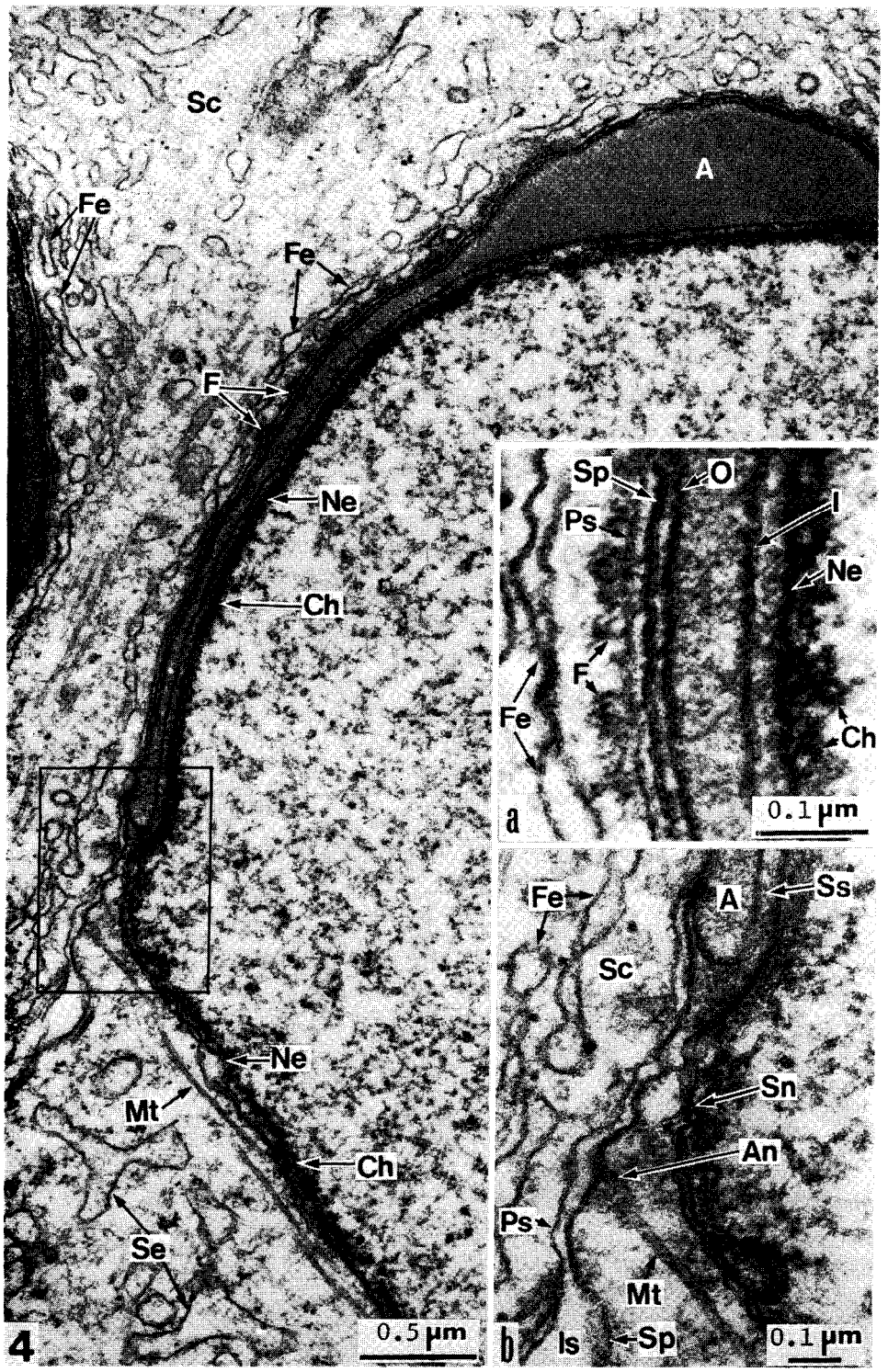
## Plate II

Fig. 4. Electron micrograph showing the Sertoli cell-ectoplasmic specializations (circumferential filamentous structures and flattened sER cisternae) and the anlage of the nuclear ring in a spermatid at the slightly advanced acrosomal phase. *Inset a.* Sertoli specializations at higher magnification. Inset *b.* Magnified view of the area enclosed in the rectangle, showing the anlage of the nuclear ring with the slightly dense subplasmalemmal matrix.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a
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An
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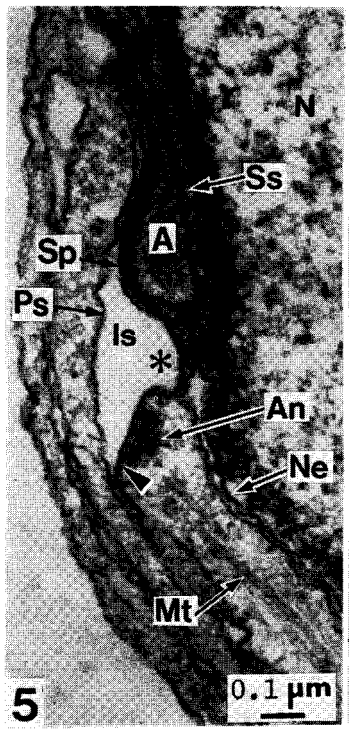
b

## Plate III

Fig. 5. Electron micrograph showing a developing nuclear ring in a spermatid at the middle acrosomal phase. Note the inner leaflet of the plasma membrane (arrowhead) from which the dense subplasmalemmal matrix originates, nuclear ring-groove and intercellular space.

Figs. 6 and 7. Electron micrographs of a spermatid at the late acrosomal phase. Fig. 6. Note an elongating and condensing nucleus, and the nuclear ring still persisting in its original position. Fig. 7. Magnified view of the area enclosed in the rectangle on Fig. 6, showing the developing dome-like nuclear ring.

Figs. 8 and 9. Electron micrographs showing the relationship between a spermatid and the Sertoli cell at the maturation phase. Fig. 8. Note the species-specific shape of the spermatid head and the nuclear ring in its final position. Fig. 9. Note the advanced ectoplasmic specializations of the same Sertoli cell as in Fig. 8, compared with those of the Sertoli cell in Fig. 4.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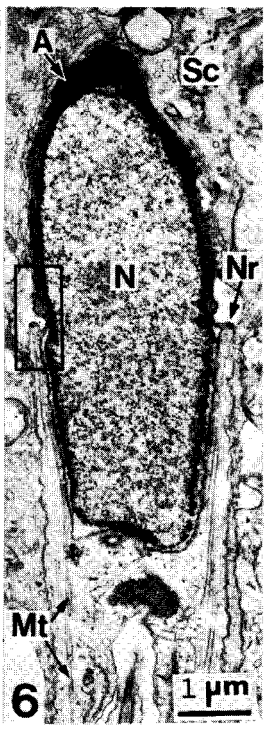
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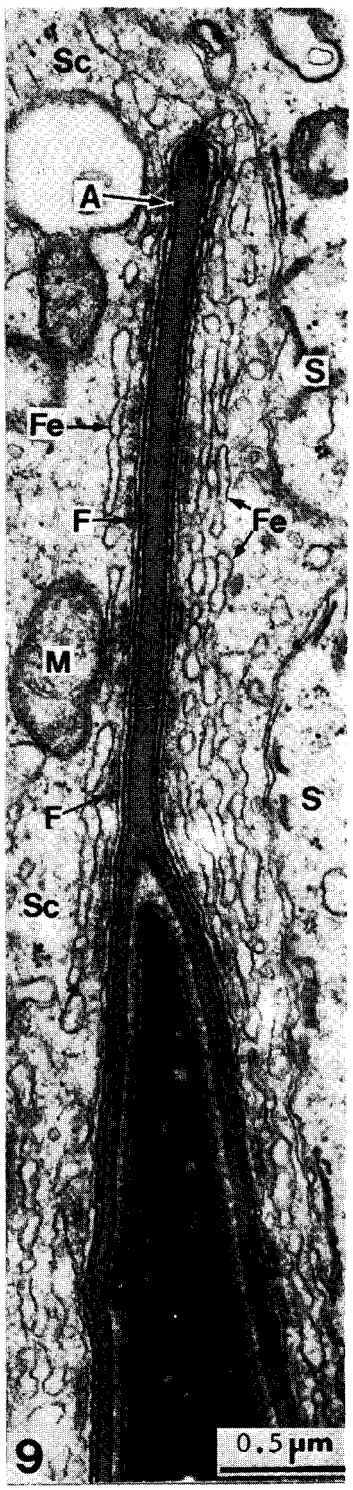
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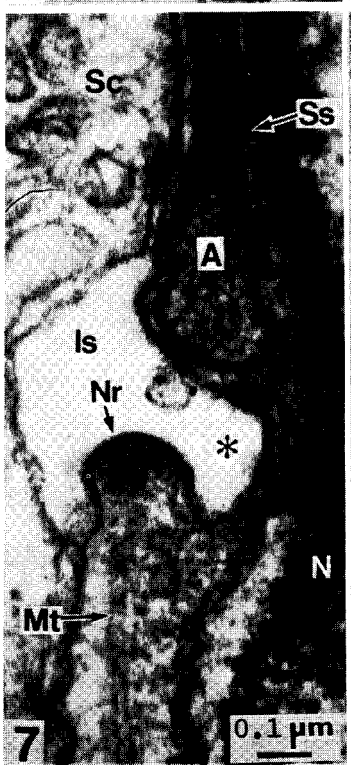
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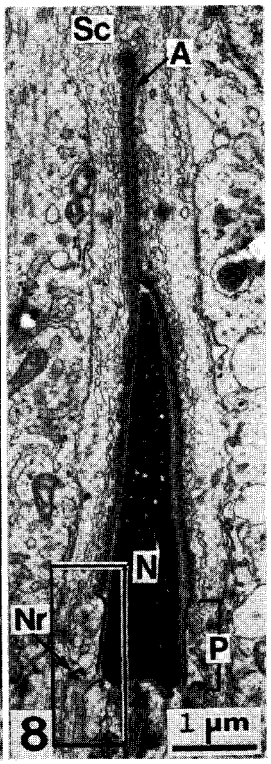
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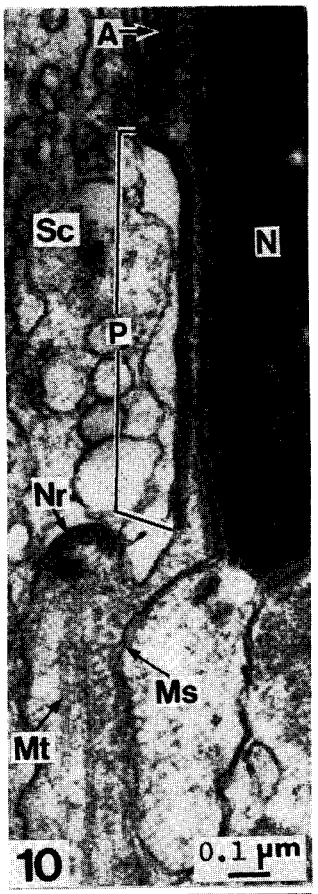
## Plate IV

**Fig. 10.** Magnified view of the area enclosed in the rectangle on Fig. 8, showing the postacrosomal sheath, fully developed nuclear ring and membranous scroll.

**Figs. 11 and 12.** Electron micrographs of late spermatid heads just before spermiation. Fig. 11. Note the loose association of the spermatid plasma membrane with the Sertoli plasma membrane, and almost all the disappearance of the ectoplasmic specializations. Fig. 12. Magnified view of a part of the above loose association site, showing a conspicuous disparity in electron-density between the spermatid and the Sertoli plasma membranes.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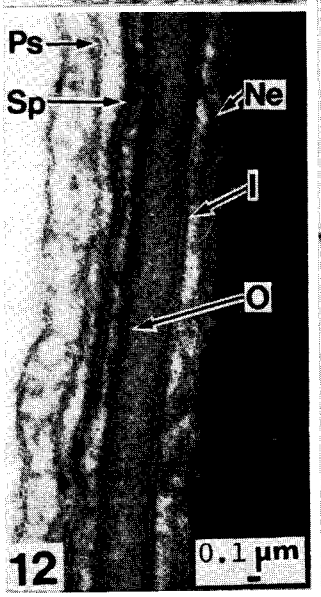
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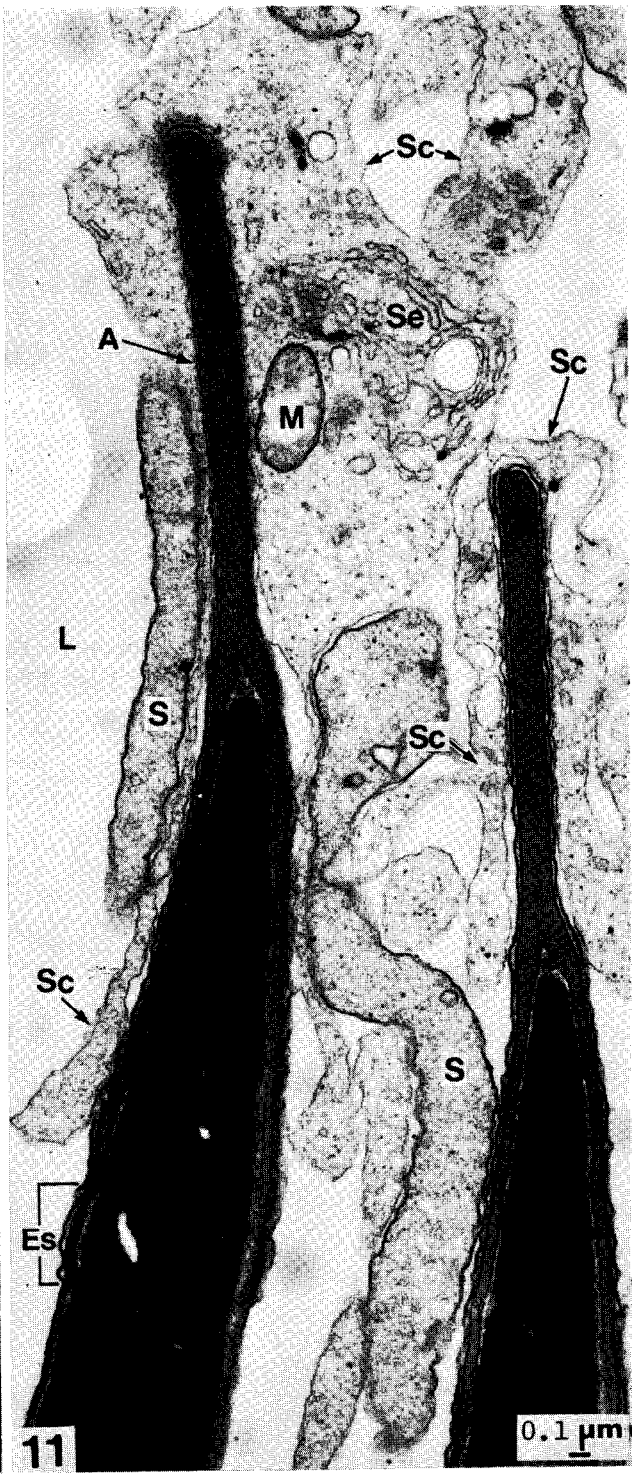
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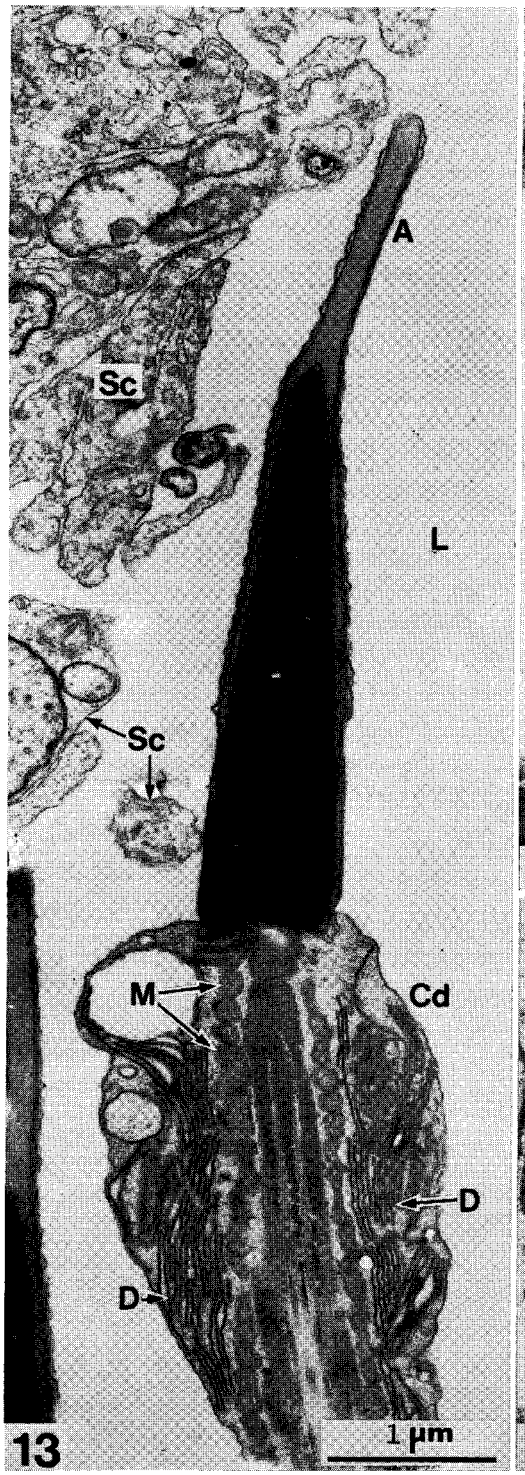
0.1 μm
11

## Plate V

**Fig. 13.** Electron micrograph of an immature spermatozoon just after spermiation, showing numerous longitudinally lamellated dictyosomes in the cytoplasmic droplet.

**Fig. 14.** Electron micrograph of the cytoplasmic droplet of an immature epididymal spermatozoon, showing one- or multi-layered spherical dictyosomes.

**Fig. 15.** Transection of the middle piece of an epididymal spermatozoon, showing extensively developed satellite fibrils, 9 outer dense fibers and a pair of mitochondria.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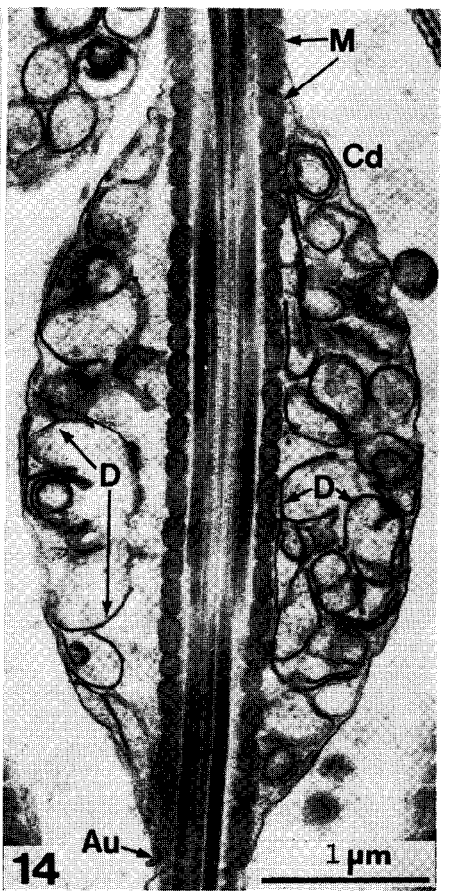
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D
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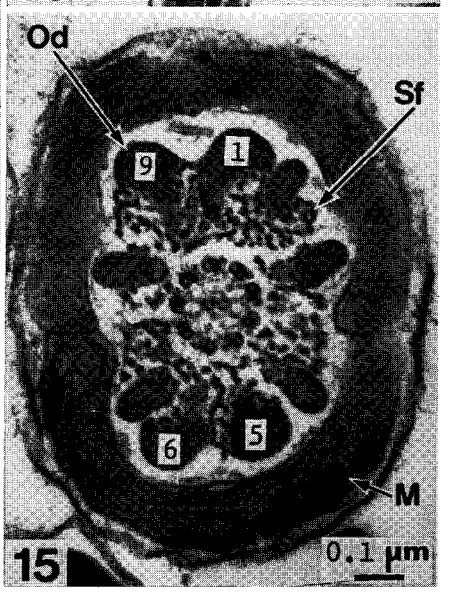
Od
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