ELECTRON MICROSCOPIC STUDIES ON THE SPERM OF FRESHWATER SNAIL, RADIX JAPONICA JAY I. STRUCTURAL CHARACTERIZATION

ELECTRON MICROSCOPIC STUDIES ON THE SPERM OF FRESHWATER SNAIL, *RADIX JAPONICA JAY*

I. STRUCTURAL CHARACTERIZATION

By

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Abstract

Mature sperms of *Radix japonica* have been examined with the light and electron microscope. Sperms obtained from the seminiferous tubules are found tightly clumping in bundles of up to several hundred per bundle. When observed in isotonic saline solution, they are actively motile, extremely elongate and threadlike, approximately 700–850μ in length. The sperm head is 2.5–3μ in length, the middle piece 600–700μ, and the tail 50–70μ. Nucleus is twisted and tightly packed with fibrillar nuclear substance 70 A thick which is arranged in parallel and spiral to the long axis. The basal surface of the nucleus is invaginated and an electron dense disc (proximal centriole ?) is engulfed here. Nine electron dense spherical bodies occur encircling the disc. From such topographical arrangement, these bodies give the impression to be the satellites of the disc. Nine coarse fibers emerge from these bodies, and nine pairs of peripheral filaments of the flagellum originate there also. Central double filaments of the flagellum originate from bottom of the cup (distal centriole). Coarse fiber shows the uniform cross band structures 750 A in periodicity, each bands consisting of five subunits. Coarse fibers have been so far reported only from vertebrate sperm. At the proximal end of the middle piece, the Nebenkern (a mitochondrial ring) lies surrounding the axis. Two long mitochondrial tubes (major whorls) arise from the Nebenkern. They are composed of concentric parallel membranes of high density and granular masses of moderate density. Nebenkern sometimes contains crystalline bodies. Transverse sections of anterior part of the middle piece exhibits radially projecting seven ridges. Two of them are mitochondrial whorls and the rest, undulating ridges. These ridges diminish their height posteriorly, and disappear at the posterior part of the middle piece. A few layers of the endoplasmic reticulum wrap the middle piece. From its outermost layer, the endoplasmic reticulum extrudes into each ridges. This membrane system has never been reported from any kinds of sperms. The crystalloid array of filaments is observed in interspaces between the coarse fiber and the endoplasmic reticulum and outside the latter. Each filaments are 150 A in diameter and composed of two helical subunits. The width of subunits is about 80–

100 A. At the posterior end of the middle piece, there exists the endpiece. Tail loses its coarse fiber. Tail axis is surrounded with a thick layer of PAS positive granules. These are interpreted to be glycogen granules.

Introduction

So called "flagellated sperms" show wide variation in their form according to the kind of animals, but their basic components are common; acrosome, nucleus, neck region, middle piece and tail. Certain structural modifications of these basic elements have been reported in various species of animals with electron microscopy. Concerning the fine structure of the gastropod sperms, however, there are only a few reports; Yasuzumi 1958 (1), Gall 1961 (2) and André 1963 (3). The present paper describes the fine structure of the sperm of pulmonate freshwater snail, *Radix japonica* Jay (mono-ara-gai), widely distributed in Japan.

Materials and Methods

For light microscopic observation: Testis and seminiferous tubules of the snail were dissected in isotonic saline solution (4), and sperms were fixed in buffered formalin. They were treated with PAS reaction, Feulgen reaction and other staining techniques.

For electron microscopic observation: Same samples were cut in isotonic saline into small pieces, fixed for 3 hrs in cold 3% glutaraldehyde buffered to pH 7.4 in 0.2 M s-collidine buffer (5). After glutaraldehyde prefixation specimens were rinsed in multiple changes of the same buffer. Postfixation was carried out with cold 1% osmium tetroxide in 0.2 M s-collidine buffer at pH 7.4. Sucrose (6) was added to these fixatives. The samples were dehydrated with concentrating series of acetone, then transferred to propyrene oxide and embedded in Epon 812 (7). Ultrathin sections ranging from gold to gray in color were cut with glass knives on a Porter-Blum MT-1 ultramicrotome. The sections were mounted on formvar coated copper grids, double stained with lead citrate (8, 9) and uranyl acetate.

Chromium shadow casting preparation was also employed for intact and trypsin treated sperms to clarify the fine structure. Used methods for preparation were as follows: (1) Seminiferous tubules were dissected in isotonic saline solution, and the bundle of sperms were released. Sperms were exposed in osmium vapour. After rinse in distilled water by using centrifugation, the samples were air dried on formvar coated grids. (2) Fresh sperms were incubated in 0.25% trypsin at 27°C for 6-12 hrs. Trypsin treated specimens were transferred to fixatives, rinsed in distilled water and air dried on formvar coated grids. Both samples were shadowed with chromium at an angle of 30-45 degrees in the atmosphere of 10^{-5} mm Hg. This sections and shadowed samples were examined in Hitachi HS-7 electron microscope at 50 KV.

Observation and Discussion

Light microscopic observation: Under the phase contrast microscope, the sperm of *Radix* are found abundantly in seminiferous tubules arranged tightly clumping each other into a bundle. The sperm has a very long flagellum. Results of the measurement obtained from light microscopic observation showed the sperm length of 650–900μ. This is conspiquous in comparison with that of the other animal sperms which usually varies from 50 to 75μ. When transferred to the physiological saline solution, it carries on wavy undulating movement for long periods. The nucleus conical in shape gives a Feulgen positive reaction. Being treated with 0.01% Fast green (pH 2) staining, it is observed that the anterior extremity of the head and the basal part of the flagellum are positively stained. They are acrosome and some neck component (Pl. Fig. 1). PAS positive reaction can be seen prominent only in the tail, which is 1/10 of the whole length of the sperm. After PAS treatment the tail tip was curled and looped, and it was observed as a round thick end embracing a clear spot (Pl. Fig. 2).

Electron microscopic observation: According to the electron microscopic observation, the sperm has the following component parts; acrosome, nucleus, centriole complex, Nebenkern and its derivatives, coarse fibers, filamentous components, agranular endoplasmic reticulum and glycogen granule mantle in the tail.

Among these structures, endoplasmic reticulum in the middle piece was the most striking. Usually it is said that when the spermatid growth is completed, the ergastoplasmic activity disappears and the useless organelle is thrown off together with cytoplasm.

Acrosome (Text Fig. 1, A): Electron microscope studies of some invertebrate sperms showed that the acrosome is a complex organelle, having two or more distinct components (10). In the case of the present snail, the acrosome is an cylindrical protrusion of the head, covered with plasma membrane. It is divided into two parts; the anterior is of spherical shape and is filled with granules light density, while the posterior is of cylindrical and is filled with granules of moderate density (Pl. Fig. 4 and 5). From the tip of the nucleus, a homogeneous protrusion of moderate density goes anteriorly and attains the clear region (Pl. Fig. 4 inset, arrow; Fig. 5a, arrow). The fact observed in the present study that the acrosome has two components may correspond to following observations. The acrosomes of some molluscan and echinoderm sperms may possess two distinct activities (11, 12); they extrude a filament which attaches the sperm to the egg surface, and they appear to contain lysins which act to break down egg membrane.

Nucleus (Text Fig. 1, N): Nucleus is conical in shape, approximately 2μ long, and is twisted, helical whorls being visible on its surface (Pl. Fig. 4 and 6). The nucleus is of fibrillar structure. Fibrils are 70 A thick, tightly packed and run spiral to the long axis of the nucleus (Pl. Fig. 6, inset). The basal end of the nucleus is invaginated and the disc (considered to be a modification of the proximal centriole) is engulfed here (Pl. Fig. 5b and 6).
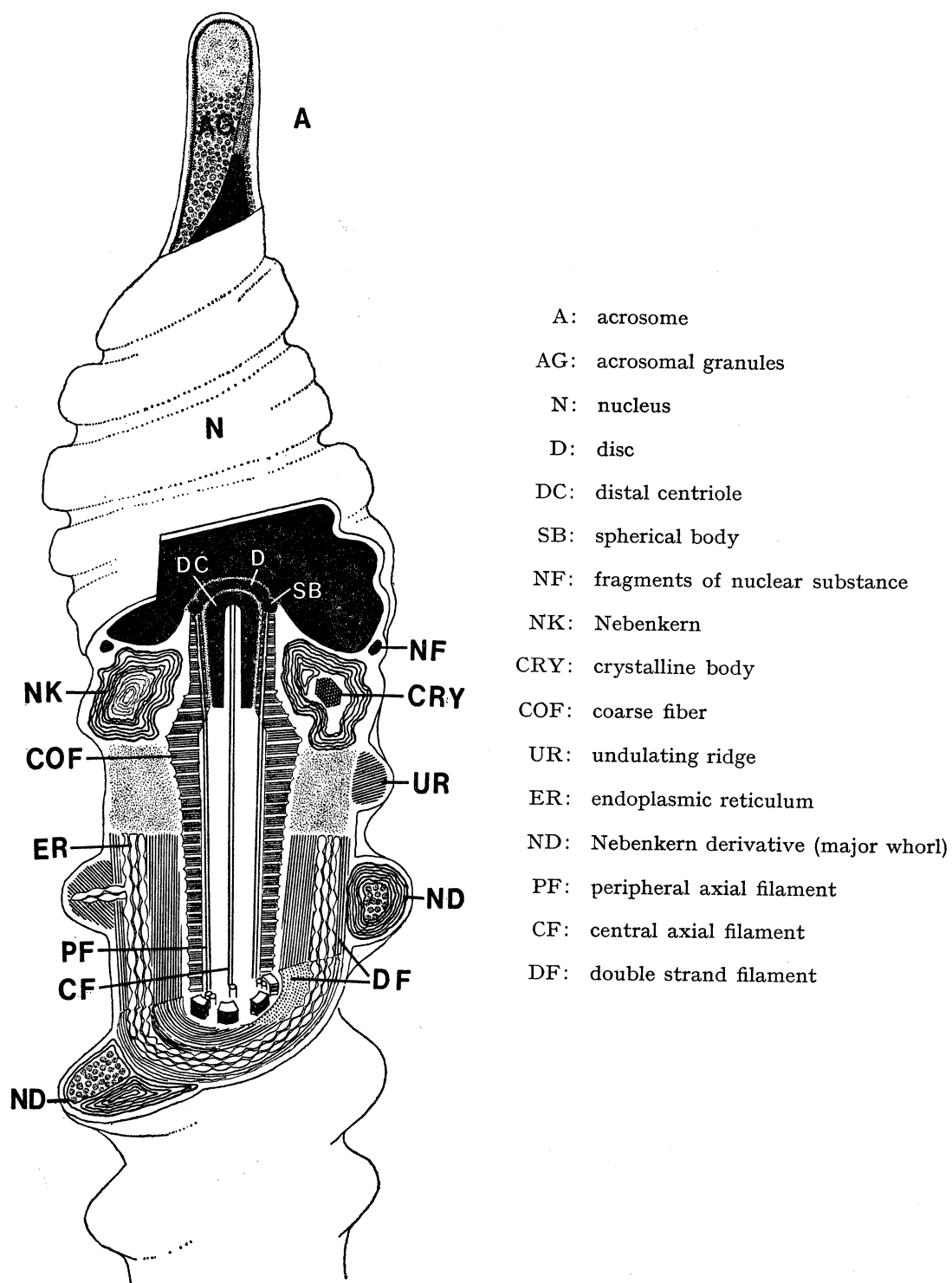
A: acrosome

AG: acrosomal granules

N: nucleus

D: disc

DC: distal centriole

SB: spherical body

NF: fragments of nuclear substance

NK: Nebenkern

CRY: crystalline body

COF: coarse fiber

UR: undulating ridge

ER: endoplasmic reticulum

ND: Nebenkern derivative (major whorl)

PF: peripheral axial filament

CF: central axial filament

DF: double strand filament

Figure 1.   Schematic illustration of *Radix* sperm.

Centriole complex (Text Fig. 1, D, SB and DC): So called "centriole complex" is located in the neck, compactly adhering to the center of the basal end of the nucleus. The centriole complex is composed of the disc, nine electron dense bodies around it, and the cup-like distal centriole (Pl. Fig. 5b). The disc is presumably made to be derived from the proximal centriole. The disc is 400 mμ in diameter and 40 mμ in thickness, the periphery being thicker and being about 65 mμ. It is of the same electron density with the distal centriole. There is cementing substance of moderate density in the space between the disc and the nuclear membrane, and also in the clear narrow space between the former and the distal centriole.

Nine dense bodies are spherical and are about 65 mμ in diameter. They encircle the disc, and attach very closely to the latter (Pl. Fig. 5b, 6, 8 and 11). They are of the same electron density with the disc. These spherical bodies of high density have not been reported in any animal sperms.

The cup-like distal centriole lies posterior to the disc. This centriole is 750 mμ long and 170 mμ thick, the thickness being greater to the bottom, and being 270 mμ thick there. It is of the same electron density with the above mentioned components. The central double filaments arises from its bottom.

Coarse fibers arise from the nine dense bodies and run posteriorly along the cup. There is cementing granular substance of moderate density between the cup and the coarse fiber. Peripheral axial filaments arise also from the dense bodies, go through the basal part of the coarse fiber, go out into the central axial space and take the usual 18 +2 pattern, encircling the double central filament in its center. The peripheral filament root running through the coarse fiber is single and elongated oval in cross section (Pl. Fig. 9, arrow), and becomes a doublet after it goes out into the central space (Pl. Fig. 6 and 10).

The behavior of the two centrioles during spermatogenesis is not yet clarified in invertebrates.

Coarse fibers (Text Fig. 1, COF): Nine coarse fibers originate from nine dense bodies around the disc. They extend posteriorly wrapping the flagellar axis. In longitudinal section, the coarse fibers show the uniform band pattern 750 A in periodicity (Pl. Fig. 6, 7 and 11). At higher magnification, each band is observable to comprise five electron dense laminas (Pl. Fig. 7, thin arrows). The space between these laminas are 20 A wide. Main bands are separated each other by a relatively wide space 150 A. In the median line of this space a thin electron dense lamina is observable (Pl. Fig. 7, thick arrows). These bands are arranged slightly oblique to the plane perpendicular to the long axis of the sperm. In the space between coarse fibers and peripheral axial filaments, minute fibrillar bridges are observable to join these two structures (Pl. Fig. 7, two blank arrows). This fibrillar linkage between coarse fibers and peripheral axial filaments may represent mere mechanical linkage or have some meaning in the conduction of contractile stimulus to each other.

The outer surface of the coarse fibers ae enveloped by a crumpled membrane (Pl.

Fig. 6, 10 and 12). This may give some allowance to contraction and relaxation of the coarse fibers.

The coarse fibers thicken their height at the level near the posterior end of the distal centriole (Pl. Fig. 6, 10, 11 arrow), then diminish their height gradually toward the tail (Pl. Fig. 12, 13 arrow). In the posterior part of the middle piece where the undulating ridges do not exist no coarse fiber is found (Pl. Fig. 13 arrow).

In transverse section at the level of the distal centriole, the coarse fibers are seen as uniform trapezoid units encircling the centriole (Pl. Fig. 9). In each trapezoid unit, along its inner surface, an electron light oval area (Pl. Fig. 9, arrows) is distinguished. This is the root of the double peripheral axial filaments. The doublet pattern is not yet shown in the root.

The coarse fiber of the present species is homologous to the *end knob* and *outer fibers* found in mouse sperm (RHODIN 1963). The *end knob* nominated by RHODIN is the basal part of the coarse fiber, is restricted in the neck ergion, and striated. The *outer fiber* is the extension of the *knob*, non-striated and run along the peripheral axial filament posteriorly in the middle piece and in the tail. In the present species, the coarse fiber is striated even in the middle piece for long part. It gradually tapers, and at last is lost in the posterior part of the middle piece where no undulating ridge exists.

The presence of nine dense bodies around the disc and close topographical relation between the bodies and the coarse fiber in *Radix* sperm are of interest. Coarse fibers and equivalent structures have been reported only in mammalian sperms, and they have never been found accompanied with dense bodies. Many reports have been published on invertebrate sperms, but no structure homologous with the coarse fiber of *Radix* has been described.

Nebenkern and its derivatives, major whorls (Text Fig. 1, NK and ND): A ring-like Nebenkern lies posterior to the nucleus, encircling the sperm axis. It is composed of loosely packed multimembrane (Pl. Fig. 6 and 9). Cross sectioned profile of Nebenkern is represented more or less rugged (Pl. Fig. 6).

The crystalline structure is frequently encountered in the Nebenkern. This is of hexagonal shape. It is not bounded with its own membrane. It is composed of substructures 120 A thick (Pl. Fig. 6). Crystalline inclusions have long been recognized as normal constituents of certain cell types. They were found in nearly all cell organelles by electron microscopy. However, the mode of occurrence and physiological characteristics of these structures are not so clarified.

Two *major whorls* derived from Nebenkern run helically toward tail (Pl. Fig. 4, arrow; 6, 15, 16 and 17). These are filled frequently with electron light 300 A granules and electron dense multimembrane structure (Pl. Fig. 12, 13, 15 and 17).

The middle piece tapers posteriorly as the undulating ridges decrease their height and at last they are lost, lefting the surface smooth, except two slight elevation lines, inside which the major whorls run through and terminate just before they attain the endpiece (Pl. Fig. 13, ND).

Endoplasmic reticulum, double strand filament and other organelles (Text Fig. 1, ER and DF): *Radix* sperm is also characterized by the appearance of agranular endoplasmic reticulum, double strand filament and tail glycogen granules.

The sheath of three to four layers of endoplasmic reticulum begins at the posterior end of the neck region, where the contents are electron light (Pl. Fig. 6). Each layer is composed of regularly cisternated and perforated endoplasmic reticulum. It coalesces with the adjacent as in usual endoplasmic reticulum. The sheath envelopes posteriorly the middle piece. From the outermost layer, projections of endoplasmic reticulum extrude into the undulating ridges (Pl. Fig. 12 and 15). As the middle piece tapers posteriorly, the sheath of endoplasmic reticulum diminishes its thickness, reducing the number of layers (Pl. Fig. 12 and 13). At the posterior end of the middle piece, there can be seen only one layer of endoplasmic reticulum (Pl. Fig. 19). Pl. Fig. 20 represents surface view of the naked endoplasmic reticulum. The specimen was prepared by removal of the cell membrane by trypsin treatment, osmium vapour fixation, and then chromium shadowing. Helical arrangement of the cisternated units of the endoplasmic reticulum is represented here.

The presence of endoplasmic reticulum all through the middle piece in such a large amount may suggest this membrane system has a role in transport of glycolysis product of glycogen involved in the tail.

Double strand filaments exist also in a large amount all through the middle piece. In longitudinal section, these filaments are shown to take the crystalloid array, running in mild spiral around the sperm axis (Pl. Fig. 6 and 16). They fill the space between the coarse fiber and the endoplasmic reticulum sheath and also the space outside the latter. The structure of these filaments is made clear by trypsin treatment for 12 hrs at 27°C, followed by giving mechanical shock of centrifugation. Specimens thus treated represented clear double strand structure (Pl. Fig. 28 and 29). They were composed of two subfilament 80–100 A in thickness and 250 A in pitch. Double strand filaments represented in Pl. Fig. 28 and 29 are still accompanied with some isolated cisternae of endoplasmic reticulum. A key to understand the structure of double strand is seen in Pl. Fig. 28, indicated by an arrow, where the two subfilaments are separated. Further studies are required in order to clarify the exact microstructure of these double strand filaments, employing the negative staining technique and others.

Tremendous amount of double strand filaments wrapping the middle piece suggest two possibilities in their function. First, they may have a close relation with the extraordinarily long length of the middle piece, and play the leading part in retaining its form. Second, they may have some relation with its movement, being contractile. The second possibility is left to future investigations in order to be acknowledged.

Endpiece lies at the end of the middle piece, where the axis limiting membrane terminates (Pl. Fig. 21, arrow). Peripheral filaments which have just passed through the endpiece are shown somewhat undulating. This may due to the lack of the limiting membrane.

Tail and glycogen mantle: Thick accumulation of glycogen granules is observed around the peripheral axial filaments of the tail. Terminal part of the tail lacks axial filaments (Pl. Fig. 24, arrow). Flattened tail ends observed in chromium shadowed specimens (Pl. Fig. 26) and curled ones observed in PAS treated ones (Pl. Fig. 2) may due to this lack of axial filaments. Glycogen granules in sperm tail has been reported on annelida [Anderson on earthworm, 1967 (17); Hirata, Ohsako and Hamasaki on leech, under preparation]. In these cases, the glycogen granules were found dispersed in a layer as large particles in the space between peripheral axial filaments and cell membrane. No case has been reported where glycogen granules are accumulated in such a large amount as observed in *Radix* sperm tail, making a very long mantle around the axis.

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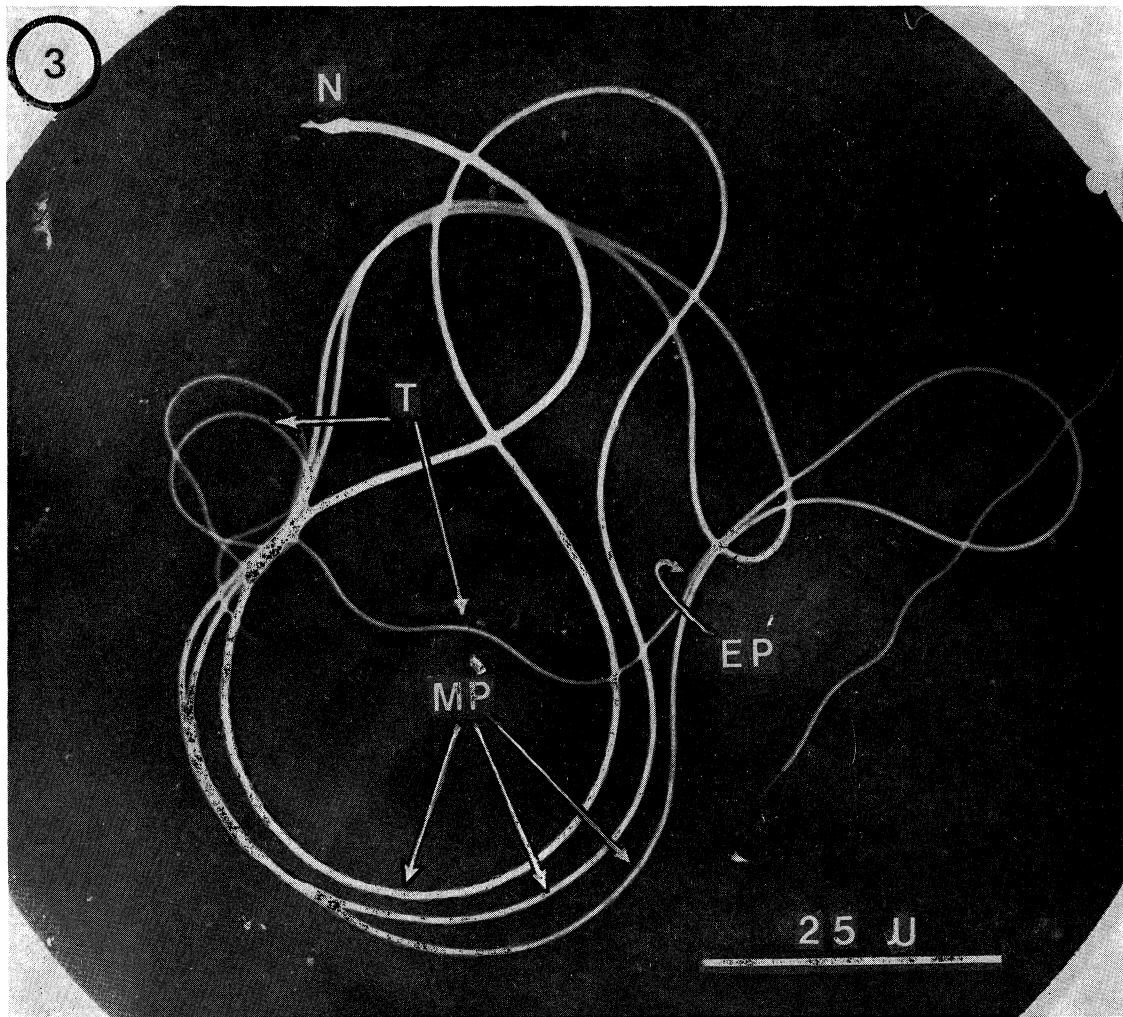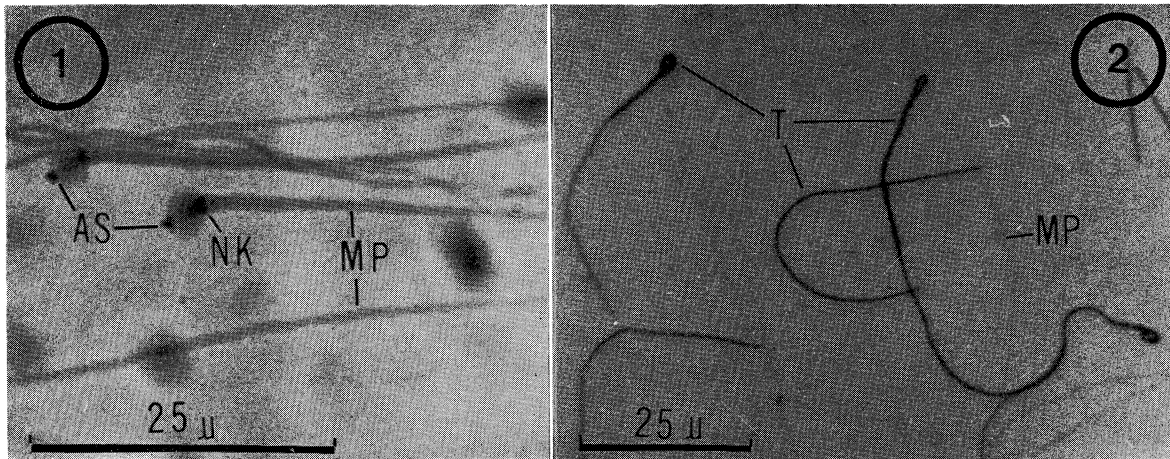EXPLANATION OF PLATE FIGURES

Explanation of Plate Figures

Key to Abbreviation

A:	acrosome	AG:	acrosomal granules
CF:	central filament	COF:	coarse fiber
CRY:	crystalline body	D:	disc
DC:	distal centriole	DF:	double strand filaments
EP:	endpiece	ER:	endoplasmic reticulum
GLY:	glycogen granules	MP:	middle piece
N:	nucleus	MD:	Nebenkern derivative (mitochondrial whorl)
NF:	fragments of nucelar substance		
PF:	peripheral filament	NK:	Nebenkern
T:	tail	SB:	spherical body

Explanation of Plate I

1. Light micrograph of sperms obtained from seminiferous tubule, fixed in buffered formalin, and stained with 0.01% of Fast green at pH 2. Acrosome (A) and some organelle in the neck region are stained positively, while middle piece (MP) is negative. ×1.500

2. PAS positive sperm tail (T). The tail tip is curled and looped. Middle piece is PAS negative. ×800

3. Reversed print from chromium shadowed specimen isolated from seminiferous tubule. This low power electron micrograph shows the whole size of the sperm. Total length is measured 780μ. Head (N) occupies 1/400 of the whole length. Middle piece (MP) takes the major length of the cell. Its end piece (EP) and tail (T) are also distinguished. ×1.500
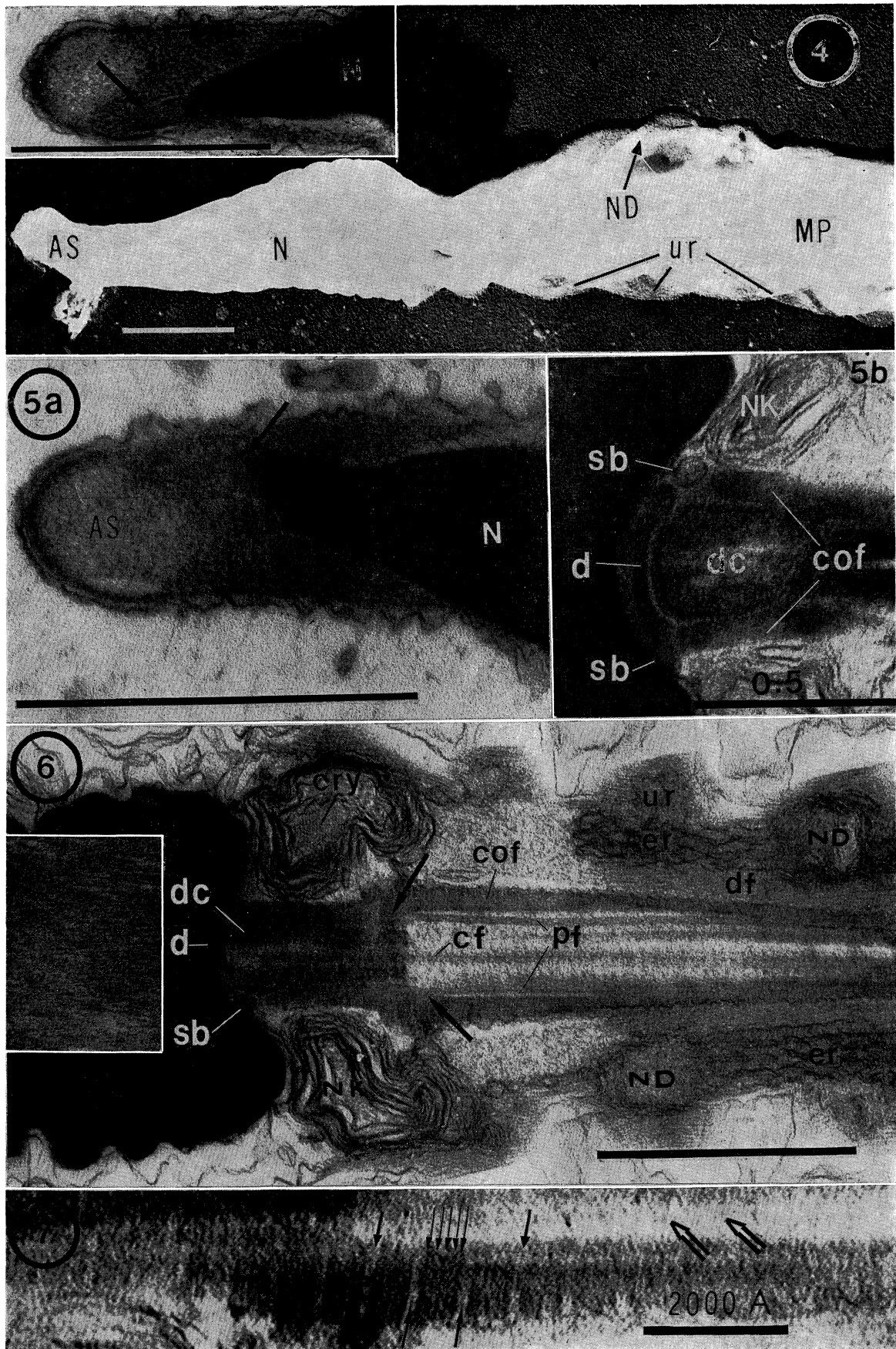
Radix sperm: Plate Figure I

Explanation of Plate II

4. Chromium shadowed sperm. The outline of the sperm head and middle piece (MP) is clearly visible; acrosome (A), head (N), helical whorl (ND) and undulating ridges (UR) are represented in wavy profile. $\times 22.000$
Inset: Longitudinal section of the acrosome. Moderately electron dense protrusion from nucleus apex into acrosome is visible (arrow). $\times 39.000$

5a. Logitudinal section of the apical part of the nucleus. Acrosome (A) is shown as a cylinder-like protrusion that contain two types of granules, and is covered with characteristic electron dense membrane except its dorsal surface. From the tip of the nucleus projects a moderately electron dense protrusion (arrow, same to Fig. 4, inset). $\times 60.000$

5b. Longitudinal section slightly oblique to the long axis, through centriole complex: disc (d), spherical body (sb), distal centriole (dc) and coarse fiber (cof) are represented. $\times 60.000$

6. Longitudinal section of the sperm neck and the basal part of middle piece. Axial complex and helical outlines are encountered. Spherical dense bodies (sb) and disc (d) are not so clear in this figure. Crystalline structure (cry) is involved in Nebenkern (NK). $\times 39.000$
Inset: Enlarged view of the nucleus. Nucleus is composed of 70 A fibrils, tightly packed and run spiral to the long axis.

7. Enlarged view of the coarse fiber. Each periodical band is composed of five electron dense laminas (thin arrows). These laminas are closely set each other with an interspace of 20 A. Between bands there exist a wider space of 150 A, having thin intermediate lamina of high density in it (thick arrows). Fibrous connections are observed in the space between coarse fiber and peripheral axial filaments (two blank arrows). $\times 120.000$
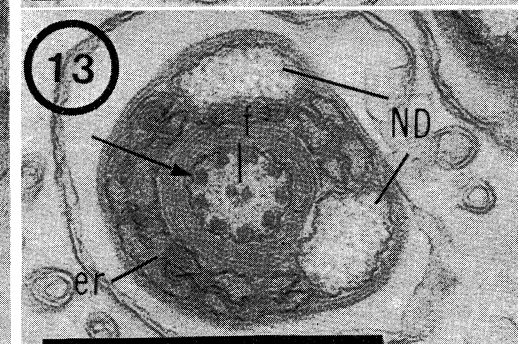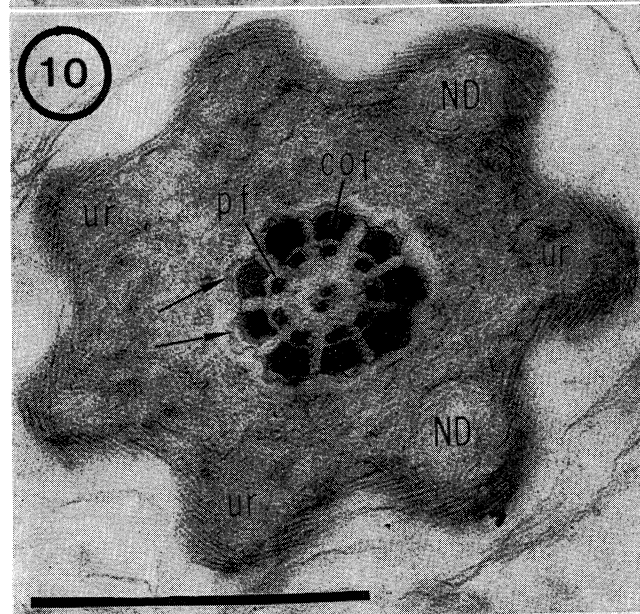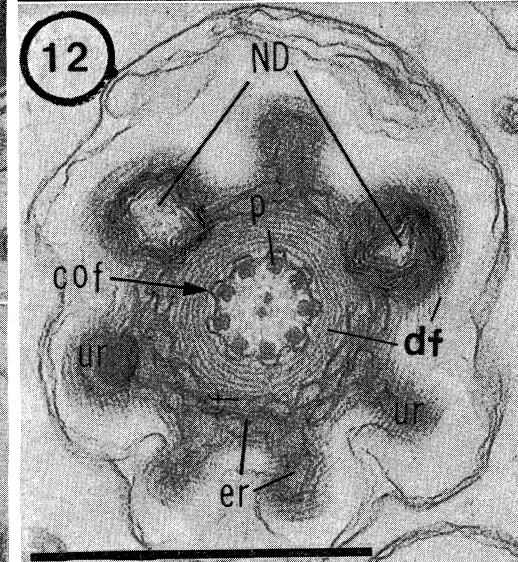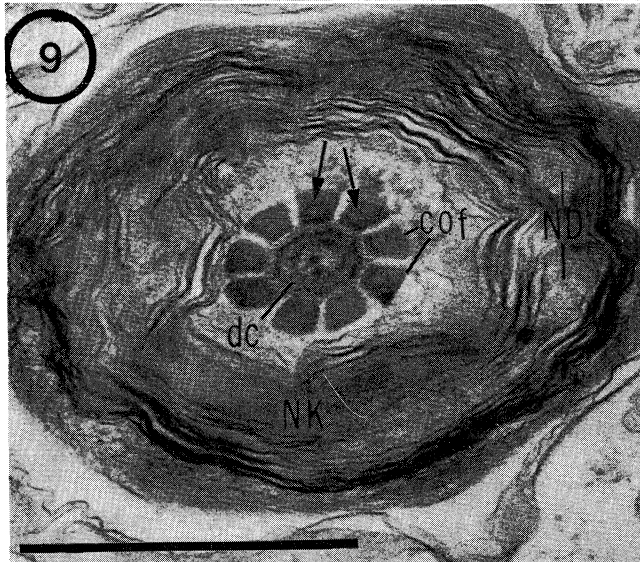
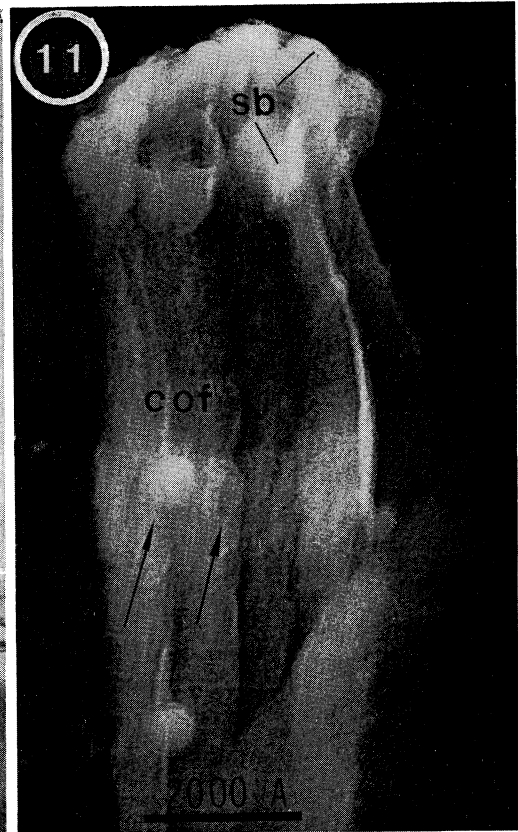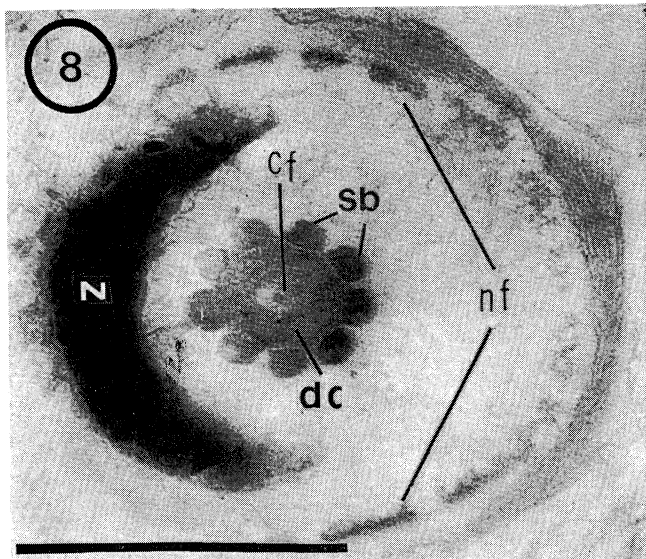## Explanation of Plate III

8. Cross section through the posterior end of the nucleus. Nine electron dense spherical bodies (sb) encircle the distal centriole (dc). Central axial filaments (cf) are located in the center of the distal centriole. Fragments of nuclear substance are encountered in circular arrangement (NF). ×39.000

9. Cross section through Nebenkern, which is composed of loosely packed multimembrane. Granular parts of moderate density (ND) are shown in one side. Nine trapezoid coarse fibers encircle the distal centriole (dc). An electron light oval area (arrows) are distinguished in each trapezoid. ×39.000

10. Transverse section through the clear part, posterior to the Nebenkern. Two mitochondrial worls (ND) derived from Nebenkern and five undulating ridges (ur) are distinguished. Peripheral axial filaments (pf) are seen inside the coarse fibers (cof). Arrows show the crumpled membrane enveloping the tail axis. ×39.000

11. Naked spherical bodies (sb) and correlated coarse fibers (cof). Cross striated structure of 750 A periodicity is observed. Arrows show the partial thickneing of the coarse fibers. Trypsin treated for 12 hrs at 27°C and shadowed. ×90.000

12. Cross section through the main part of the middle piece. Undulating projections (ur) are prominent. Coarse fibers (cof) are here very slender, running just outside the peripheral axial filaments. ×39.000

13. Cross section through the posterior part of the middle piece. Undulating projection has disappeared and two mitochondrial whorls run under mild swollen lines. Arrow indicates the crumpled limiting membrane accompanied with no coarse fiber. ×39.000
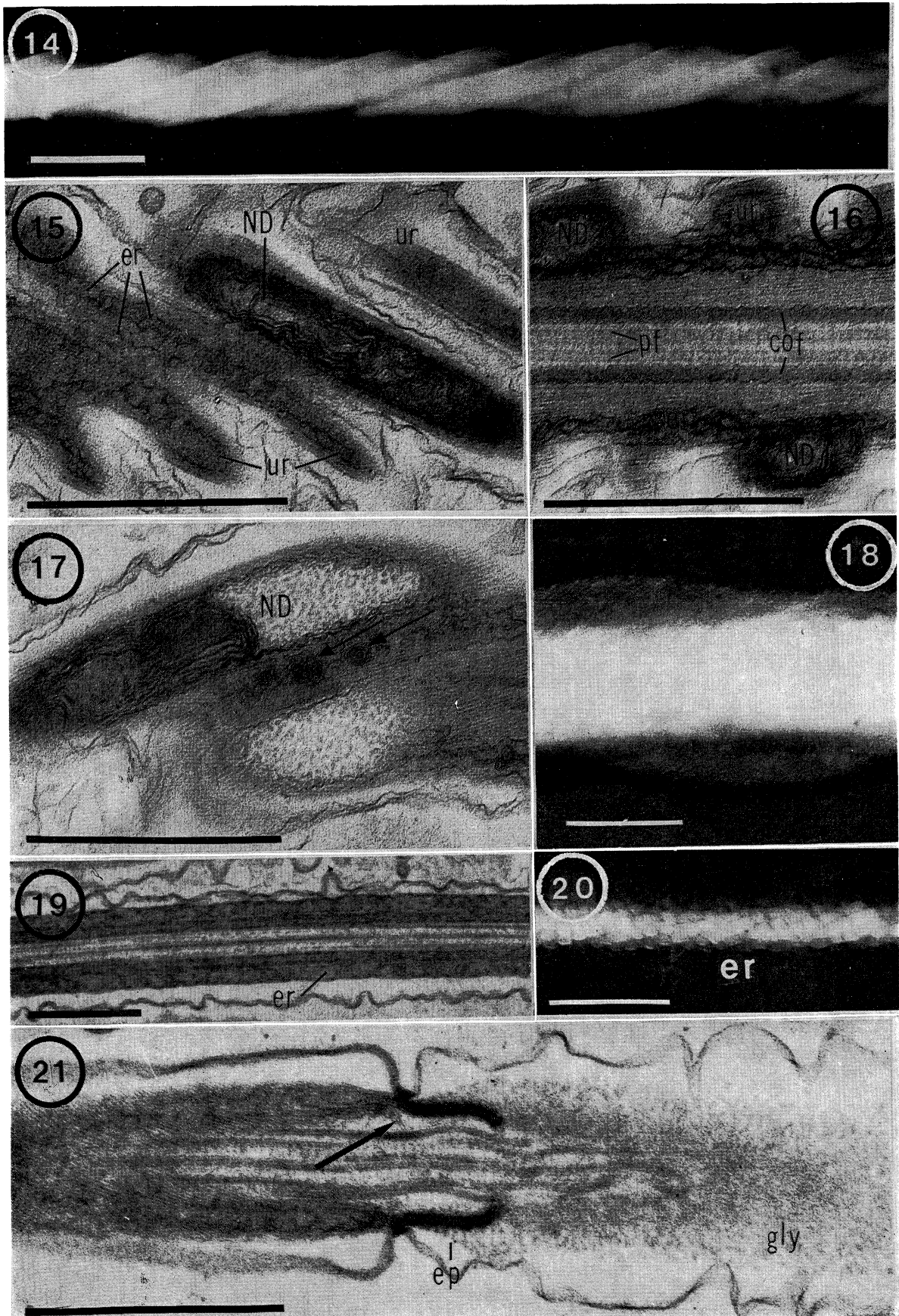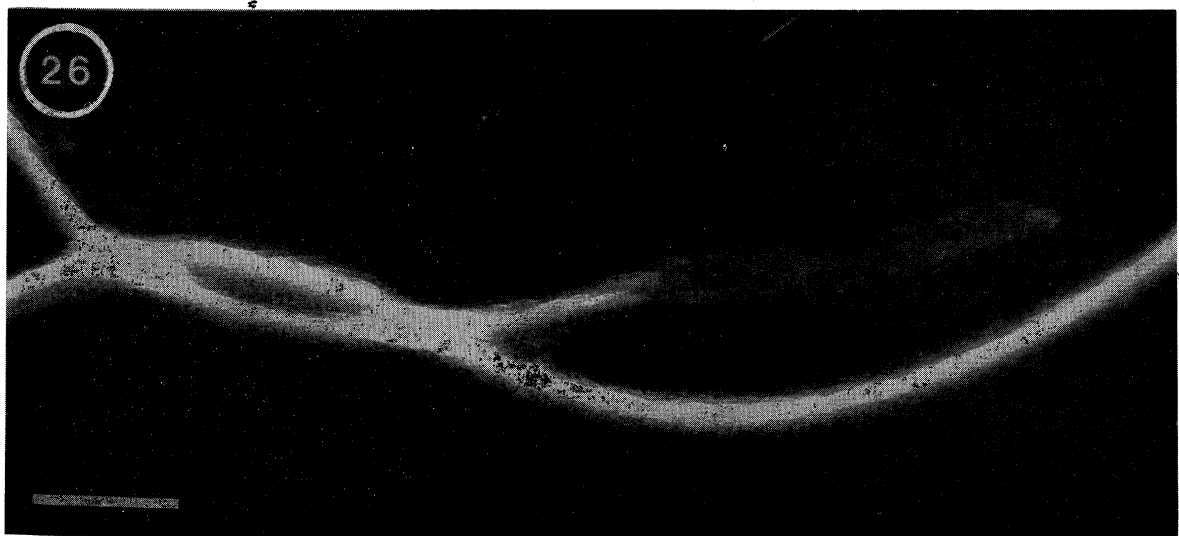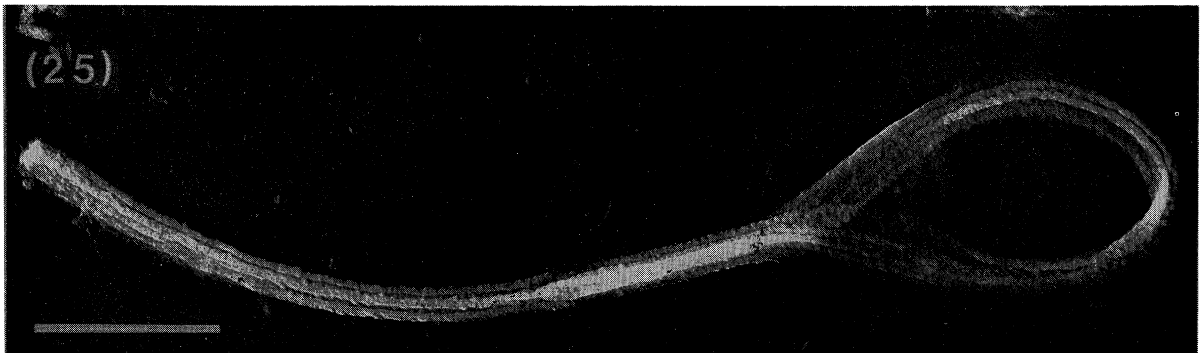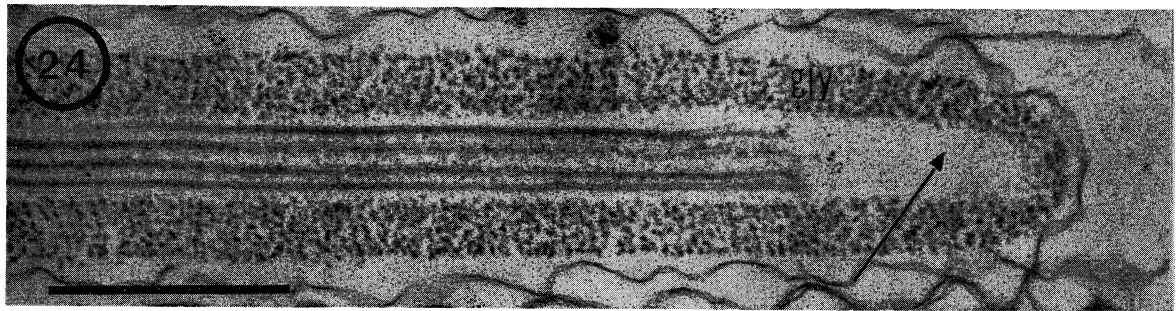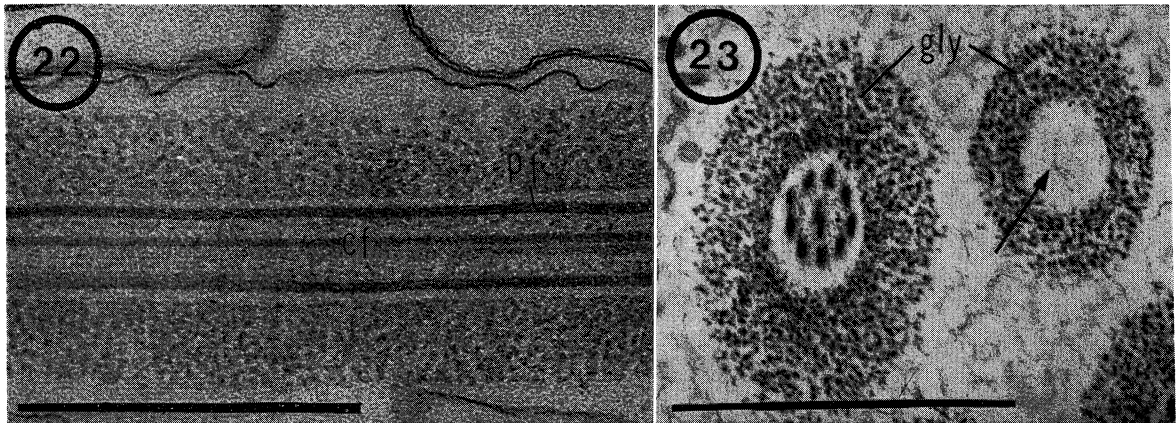
Explanation of Plate IV

14. Surface view of the main part of the middle piece. Helically arranged undulating projections are well observed. $\times 17.500$

15. Longitudinal, tangential section of the middle piece, illustrating one mitochondrial whorl (ND) and four undulating ridges (ur), in three of which endoplasmic reticulum (er) are represented. The mitochondrion (ND) is here multimembraneous. $\times 39.000$

16. Longitudinal section of the main part of middle piece. Somewhat slender coarse fibers run through. The sheath of endoplasmic reticulum are made of two layers in this part. $\times 39.000$

17. Longitudinal, tangential section of the middle piece. Two components of mitochondrion are represented. Arrows indicate myelin-like structure occurred between two mitochondrial whorls (ND). $\times 39.000$

18. Surface view of the posterior part of the middle piece. Array of minute double strand filaments are observable on the surface of two mitochondrial whorls. Chromium shadowed. $\times 17.500$

19. Longitudinal section of the terminal part of the middle piece. Mitochondrial whorls are not observable. Only one layer of endoplasmic reticulum (er) is reserved. $\times 17.000$

20. Trypsinized and shadowed posterior end of the middle piece. Helical arrangement of the endoplasmic reticulum is exposed. $\times 17.500$

21. Longitudinal section of the terminal portion of the middle piece, representing endpiece (EP). Limiting membrane of the axis terminate just at the junction (arrow). $\times 39.000$

Explanation of Plate V

22. Longitudinal section of the tail. Thick layer of glycogen granules mantle the axis.
$\times$ 39.000

23. Cross sections of the tail. Right hand smaller one shows the tail end where the axial filaments is lost.
$\times$ 39.000

24. Longitudinal section of the tail end. Axial filaments terminate near the tip of the tail, blank area remains (arrows).
$\times$ 28.000

25. Coarse fibers isolated and chromium shadowed. They are segmented and persist their thickness posteriorly for more than 10 microns.
$\times$ 21.000

26. Flattened tail end of unfixed and dried specimen, chromium shadowed.
$\times$ 1.700

Explanation of Plate VI

27. Surface view near the posterior part of the middle piece trypsin treated for 6 hrs. Regular array of the double strand filaments is clearly represented (df). $\times 125.000$
Inset: A section through the mantle of double strand filaments, representing two areas; cut transverse and longitudinal. In the latter area, strand structure is revealed obscurely. $\times 150.000$

28 and 29. Double strand filaments isolated by trypsin treatment for 12 hrs and multiple shock with centrifugation, and then chromium shadowing. Thick arrow shows the single strand. Fragments of endoplasmic reticulum (er) are reserved, being absorbed on the filaments.

er

df

2000 A

27

28

er

2000A

29

er

2000A