SPERMATOZOA AND SPERMATELEOSIS IN THE SALAMANDRIDAE WITH ELECTRON MICROSCOPY OF DIEMICTYLUS[1]

C. L. BAKER

Southwestern College, Memphis, Tennessee

INTRODUCTION

This is the third of a series of investigations dealing with sperm morphology, spermateleosis and motility of the urodelean sperm, observations by light microscopy having been reported on Amphiuma (Baker, 1962), Cryptobranchus and Necturus (Baker, 1963). There is a uniformity in the transformation of the spermatid into the mature sperm in the Amphiumidae, Salamandridae, Cryptobranchidae, Proteidae, and Plethodontidae as all agree in having a prominent granule and ring in the spermatid with a tail complex originating in the granule as a filament that passes through the ring. This ring elongates down the tail of all except the Cryptobranchidae and Proteidae (Necturus), and the granule transforms into a prominent neck piece in all except the Cryptobranchidae. There is no apparent separate middle piece in the Cryptobranchidae and Proteidae.

There is evidence that a diagnostic feature of each family of the Urodela may be the structure of the axial filament as revealed in cross sections; that of the Amphiumidae being dumb-bell shaped, that of the Cryptobranchidae is round while the Salamandridae exhibit a trifoliate appearance (Fawcett, 1961) especially in the principal piece.

Within the Salamandridae the outstanding generic characteristic is the length of the neck piece with minor differences in the flagellar wave length and the total length of the sperm. The differences in the morphology of the sperm of the several families of urodeles may serve to indicate phylogenetic relationships and evolution.

Spermateleosis and sperm morphology were studied in three genera of the Salamandridae, Diemictylus (Triturus) viridescens viridescens, the eastern red-spotted newt, Taricha granulosa, the western rough-skinned newt, and Cynops pyrrhogaster, the Japanese red-bellied newt, by light microscopy, while rather intensive yet preliminary observations were made on the early stages of spermatid elongation and mature sperm morphology of Diemictylus with the electron microscope.[2]

Electron micrographs clarify and substantiate previous hypotheses, disprove some conclusions and open new vistas for the interpretation of ultrastructure along with the creation of new problems that should be investigated.

The various cytoplasmic organelles of the spermatid and a review of their history and their transformation have been discussed (Baker, 1962) and need not be repeated here. A granule and ring are present in each spermatid but their origin is uncertain and there is some disagreement as to their fate. The granule is considered to be the oblique proximal centriole that gives rise to the material of the undulating membrane while the axial distal centriole forms the flagellum (Burgos and Fawcett, 1956), or the oblique centriole is called a "granular juxtanuclear body" that forms the neck piece and axial filament with the other centriole disappearing soon after the beginning of flagellar growth (Sotelo and Trujullo-Cenoz, 1958). The granule is considered to be the proximal centriole and is reported to increase rapidly in size while approaching the nucleus inside of which it becomes wedged (Gatenby, 1913). The ring is considered to be the distal centriole that lacks fibrils and originates from matrix alone (Gatenby, 1961). It is believed to elongate and become the edge of the undulating membrane and the axial filament, while the proximal centriole forms the flagellum.

The neck piece is not recognized as a component of the sperm of Desmognathus fusca (Gatenby, 1913; 1961) although it is clearly designated in the text figures.

It is apparent that some confusion exists regarding urodelean spermateleosis. Two centrioles seem to be present in the spermatid along with the meiotic centriole (Baker, 1962). These two centrioles persist throughout sperm formation and remain in the mature sperm with the proximal one at the base of the flagellum and the distal one at the origin of the axial filament. This does not mean that either of these formed structures should be considered as centrioles. The origin of the granule and ring is unknown and possibly they may arise from centrioles but their composition and subsequent transformation offer no support for considering them as centrioles.

The neck piece is reported to originate from the granule (Meves, 1897; Bowen, 1922). In the Amphiumidae it appears to arise from the nucleus (Baker, 1962) but it must be admitted here that such origin is doubtful. There is considerable uniformity in the process of spermateleosis in the several families of urodeles and observations of the process in Diemictylus, from both light and electron microscopy, tend to confirm these similarities.

These studies began with light microscopy and the results are reported here. Generic differences are evident in the relative size of the nucleus, the neck piece and end piece along with minor variations in the overall length and in the wave length of the flagellum. There is an overall uniformity of structure of the sperm in the three genera, especially in the process of transformation of the spermatid into the mature sperm.

Electron micrographs made from pieces of the testes of *Diemictylus* clarify the structure of the axial filament, the undulating membrane, and the flagellum of the mature sperm, and also present some evidence of their relationships to each other in their early formation. This study is far from complete and many pieces of the puzzle are missing, yet sufficient observations have been made to warrant a presentation of the results with the hope that investigations from other sources and continued studies on testes of representatives of other urodelean families will eventually present a complete and comprehensive description of all aspects of spermateleosis and mature sperm morphology.

MATERIALS AND METHODS

Diemictylus (Triturus) viridescens viridescens were obtained in quantity near Murphy, N. C., *Taricha granulosa* were collected near Gladestone, Oregon, while the *Cynops pyrrhogaster* were purchased from local tropical fish dealers who import them from Japan. All stages of spermateleosis are found in September and October with the vas deferens dilated with mature sperm and by December mature sperm predominate. Spermateleosis was observed by dark phase contrast microscopy in the living state. Permanent preparations had excellent detail after iron-alum hemotoxylin. The periodic acid-Schiff method stains the acroblast and acrosome, and tetrazolium chlorides were used for mitochondria in the living sperm. These methods have been discussed in detail in a previous paper (Baker, 1962).

Tissue was prepared for electron microscopy by opening an animal under isotonic salt solution and dissecting away extraneous organs to expose the prominent testes. Small pieces (1 mm. square or less) were excised and placed in equal parts of 5% glutaraldehyde and Sorenson's buffer (pH 7.3). Immediately a similar piece was examined with the dark phase contrast microscope. If the stages of development observed were those desired they were kept for one hour in the glutaraldehyde fixative. Tissue was then washed for ten minutes in each of three changes of Sorenson's buffer then cut into smaller blocks when necessary. Osmium tetroxide, 2% in distilled water at 4 degrees C., completed the fixation in two hours. The blocks were washed 10 minutes in several changes of distilled water and left in a .5% uranyl acetate solution overnight for staining. The tissue was then washed, dehydrated to 100% ethanol in 30 minutes, followed by three changes of 100% ethanol in 30 minutes, then put into propylene oxide for three changes in 30 minutes. A plastic mixture, composed of 70% DDSA, 20% Araldite, and 10% Epon 812, with 2% DMP30 as a catalyst, was used. Tissue was placed in equal parts propylene oxide and plastic mixture for one hour at 45 degrees C. in a capped container, followed by pure plastic plus accelerator for one hour at 37 degrees C. in an open bottle (Lockwood, 1964). Beem capsules were filled about two-thirds with a fresh plastic mixture and accelerator and one piece of tissue was lifted with fine forceps and placed on the top of each capsule. A faucet vacuum connected to a bell jar degassed the plastic and tissue and removed any of the remaining volatile propylene oxide. When air bubbles ceased to appear the capsules were removed from the vacuum and filled to the top with plastic and placed in an incubator at 45 degrees C. for 24 hours, then at 60 degrees C. for 24 hours for complete polymerization. Sections of grey to silver appearance were cut with a Porter-Blum MT-1 (Sorvall) thin sectioning microtome, spread with toluene vapor while floating on water and then placed on a grid. Reynold's lead stain for three minutes followed by several rinses completed the process. Electron micrographs were made with a Siemens Elmiskop I or Zeiss EM9 electron microscope at original magnifications of 2,000 to 30,000 diameters and were enlarged photographically to the desired size. It is difficult to determine which of these electron microscopes had the better resolution at these lower magnifications.

OBSERVATIONS

In general appearance the sperm of *Diemictylus, Taricha* and *Cynops* are similar in having a needle-shaped nucleus tipped by a pointed perforatorium which is capped by the acrosome. Confluent with the nucleus at its distal end is the prominent neck piece to which is attached a tail (Fig. 35) composed of an axial filament with a marginal flagellum; both held together by an undulating membrane. In each the middle piece composes approximately two-thirds of the tail, with the distal third being the principal piece in which the axial filament is of lesser diameter and a different shape in cross section. Beyond the end of the axial filament the flagellum extends a short distance as an end piece (Figs. 21, 32, 34).

Although of sufficient similarity to indicate close taxonomic and phylogenetic relationships as members of the family Salamandridae, the discrete differences that are readily apparent justify the three as being of separate genera. Measurement of the various components is difficult. A motile sperm may have a different length from a motionless one. A smear is unreliable for tail measurements as considerable stretching seems to occur. More accurate measurements appear to result from sperm undergoing slight flagellar movement. Comparative measurements, in micra, are as follows:

	Diemictylus	*Taricha*	*Cynops*
Perforatorium	8	8	15
Nucleus	142	64	70
Neck Piece	37	21	8
Middle Piece	270	118	220
Principal Piece	105	70	110
End Piece	28	13	8
Total Length	590	294	431

The above measurements are averages and are significant only as they are indicative of differences. The flagellum of *Taricha* sperm is the more prominent as in the principal piece of the tail the wave length tends to increase and become irregular (Fig. 32) with the flagellum appearing to wind around the axial filament (Fig. 38). The sperm of *Diemictylus* is double the length of that of *Taricha* with that of *Cynops* being intermediate.

The pattern of spermateleosis is quite uniform in the three genera. In each a ring and granule appear in the cytoplasm of the spermatid (Figs. 1-3, 33), both enlarge and become oriented in relation to the nucleus with the granule almost touching the nuclear membrane. Before the nucleus elongates a vibratile filament or tail complex extends from the granule through the ring and across the cell membrane, or perhaps is accompanied by an extension of the cell membrane (Fig. 23). Nuclear elongation is accompanied by a further enlargement of the granule and ring with the granule soon being embedded in the distal end of the nucleus (Figs. 4-6, 24-26). The vibratile filament elongates rapidly (Figs. 6-8, 25-28), the proximal half being larger and less vibratile than the very active and filamentous distal portion. The larger proximal part soon reaches a length equivalent to that of the axial filament of the mature sperm with the smaller distal portion extending 200-250 micra beyond (Figs. 8, 28). Alongside the proximal part of this tail filament the smaller process, the flagellum, soon separates from the axial filament and can be traced to its origin on the neck piece. During this stage the flagellum is parallel to the axial filament and has no waves yet it exhibits active motility. It is also quite obvious that the axial filament and flagellum are held together by a membrane that is referred to as the undulating membrane. An acrosome forms within the acroblast and elongates to form the very thin needle-pointed cone over the perforatorium. In the mature sperm the acrosome cannot be seen except after the periodic acid-Schiff (PAS) reaction. The very finely attenuated point observed with iron-alum hemotoxylin is the perforatorium, an anterior extension of the nuclear membrane. A droplet of material is noted frequently on the tip of the acrosome giving the appearance of a hook or barb but there is no regularity or uniformity in structure. Thus it cannot be said that the acrosome has a hook or barb. The cell membrane invests the head of the sperm and is observed frequently, on those that are massed in bundles in the testes, as a globular extension to a sertoli cell. This cell membrane may be present on sperm removed from the vas deferens but it is not evident, perhaps due to being appressed closely to the nucleus.

The prominent ring begins to elongate down the tail as the nucleus reaches its maximum length and its chromatin condenses. This ring elongation is evidently proportional to the length of the middle piece and in *Diemictylus*, with a middle piece length of 270 micra this ring fades from view after it becomes 150 micra long (Fig. 9).

Mitochondria can be observed in the middle piece of the mature sperm after a tetrazolium chloride reaction (Baker, 1962). No centrioles are evident with any degree of certainty in any stage of spermateleosis with the light microscope.

Sperm motility is uniform in the three genera and the normal pattern is for the wave to arise at the base of the neck piece and pass distally with the sperm progressing forward. However when observed by dark field contrast microscopy a wave may originate at any part of the flagellum and pass either proximally or distally or, in some cases, in both directions at once. Waves also may arise simultaneously at opposite ends of the flagellum and cease movement at the point of union. These movements have been recorded by cinematography. When reverse motility is viewed in film reversal the sperm appears to move forward in a normal manner. Critics have accused the author of film reversal in attempting to exhibit reverse motility. Since reverse motility is "impossible" a group of sperm that exhibits such motility is being influenced by "diffusion currents and stroboscopic effects."

The tail of the mature sperm has an elongate coil that contributes to the rotation of the cell as it progresses through the medium. The flagellum is always on the outer convex border of the axial filament. The waves of the flagellum appear two dimensional by light microscopy and are so indicated in sketches (Figs. 21, 32, 34), yet the coiling is definitely three dimensional (Fig. 63).

EXPLANATION OF FIGURES
(Pages 5, 6)

Fig. 1. A spermatid with typical acroblast, acb, and mitochondria. A small granule, perhaps the meiotic centriole, is close to the acroblast.

Figs. 2-3. The earliest appearance of the granule and ring.

Figs. 4-5. The nucleus of the spermatid is elongating. The acroblast contains a dark acrosome. The granule and ring have migrated to the distal end of the nucleus and the granule is seated against the nucleus. A tail complex emerges from the granule, passes through the ring and across the cell membrane. The distal end of the tail filament is smaller and quite vibratile.

Fig. 6. A later stage of spermatid transformation into a sperm. The nucleus continues to elongate, the acroblast-acrosome, acr, is affixed to the proximal end of the nucleus, the granule is enlarging and invading the nucleus and the tail filament is lengthening.

Figs. 7-8. The nuclear material is condensing into a chromophilic filament, the neck piece is elongating and the tail filament has separated into a heavy axial filament and a smaller parallel flagellum that is vibratile. The acrosome is conical and pointed.

Fig. 9. An almost mature sperm showing the greatest extent of the elongating ring (at arrow) possibly marking the end of the middle piece. The flagellum has retracted alongside the axial filament into regular conical waves except for the end piece.

Figs. 10-14. Various stages of ring elongation down the axial filament and parallel flagellum.

Figs. 15-20. Various views of the axial filament, ring, neck piece and flagellum.

Fig 21. A mature sperm of **Diemictylus viridescens.** Note the very elongate neck piece, np, the perforatorium, p, at the proximal end of the nucleus, the heavy axial filament, af, bordered by the flagellum, fl, both extending through the extensive middle piece, mp, (to arrow) and the principal piece, pp, to the end piece, ep.

Fig. 22. An abnormal stage of development. The bulbous neck piece apparently results from fixation at a critical stage of development.

Figs. 23-32. The transformation of the spermatid of **Taricha granulosa** into a mature sperm similar to that which occurs in both **Diemictylus and Cynops.** The neck piece is shorter and the wave lengths of the flagellum are irregular and longer in the principal piece, pp.

Fig. 33. Earliest appearance of the acroblast and ring in the spermatid of **Cynops pyrrhogaster.**

Fig. 34. A mature sperm of **Cynops.** Note the very short neck piece, np, and end piece, ep, in comparison to the two other genera.

An explanation of the electron micrographs will be found near the proper photographs. A Siemens Elmiskop I was used for making the electron micrographs for Figs. 40, 41, 42, 43, 44, 49, 50, 52, 53, 57, and 63, while a Carl Zeiss Electron Microscope EM9 was used for Figs. 48, 51, 54, 55, 56, 58, 59, 60, 61 and 62.

Diemictylus viridescens
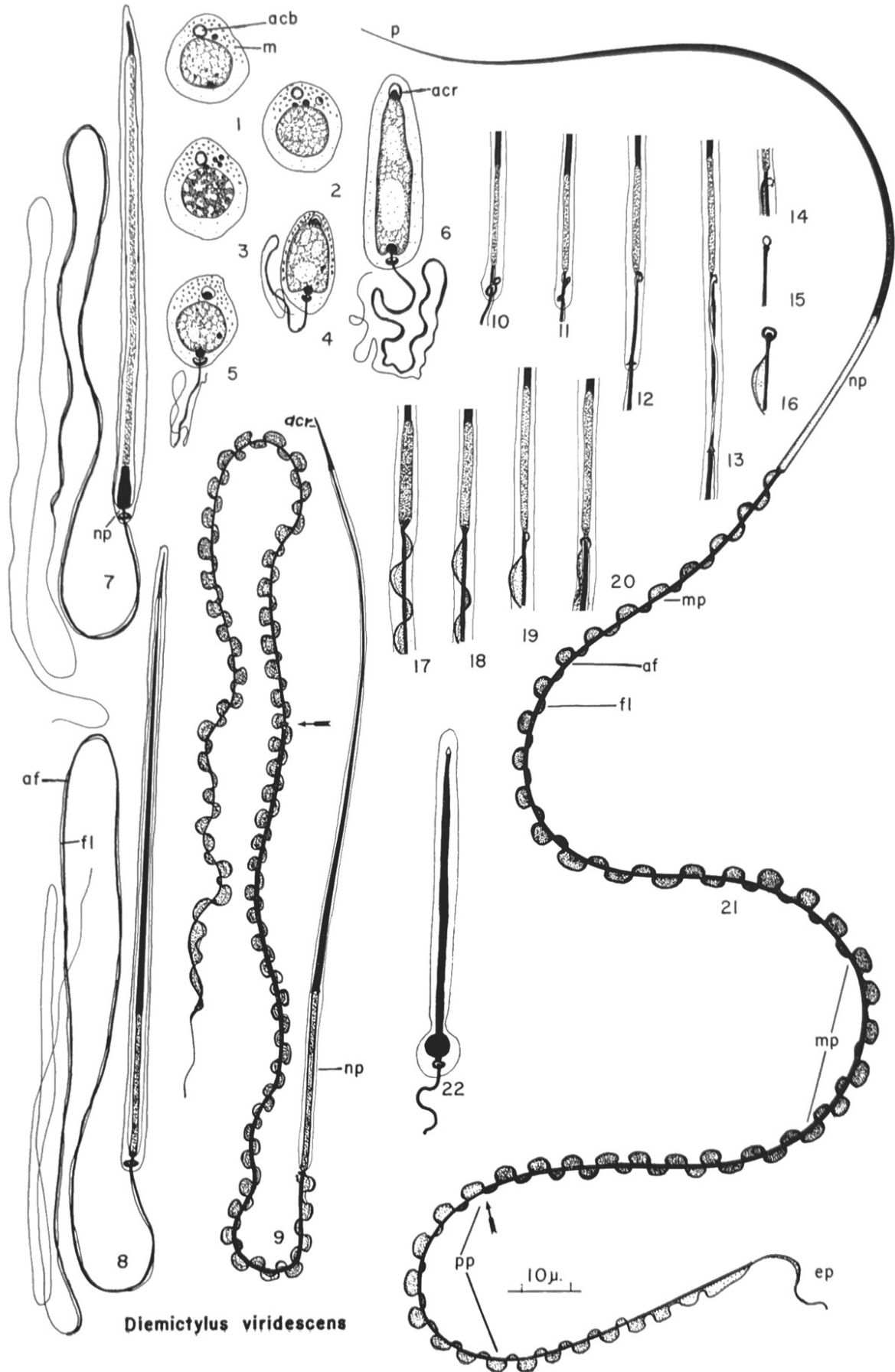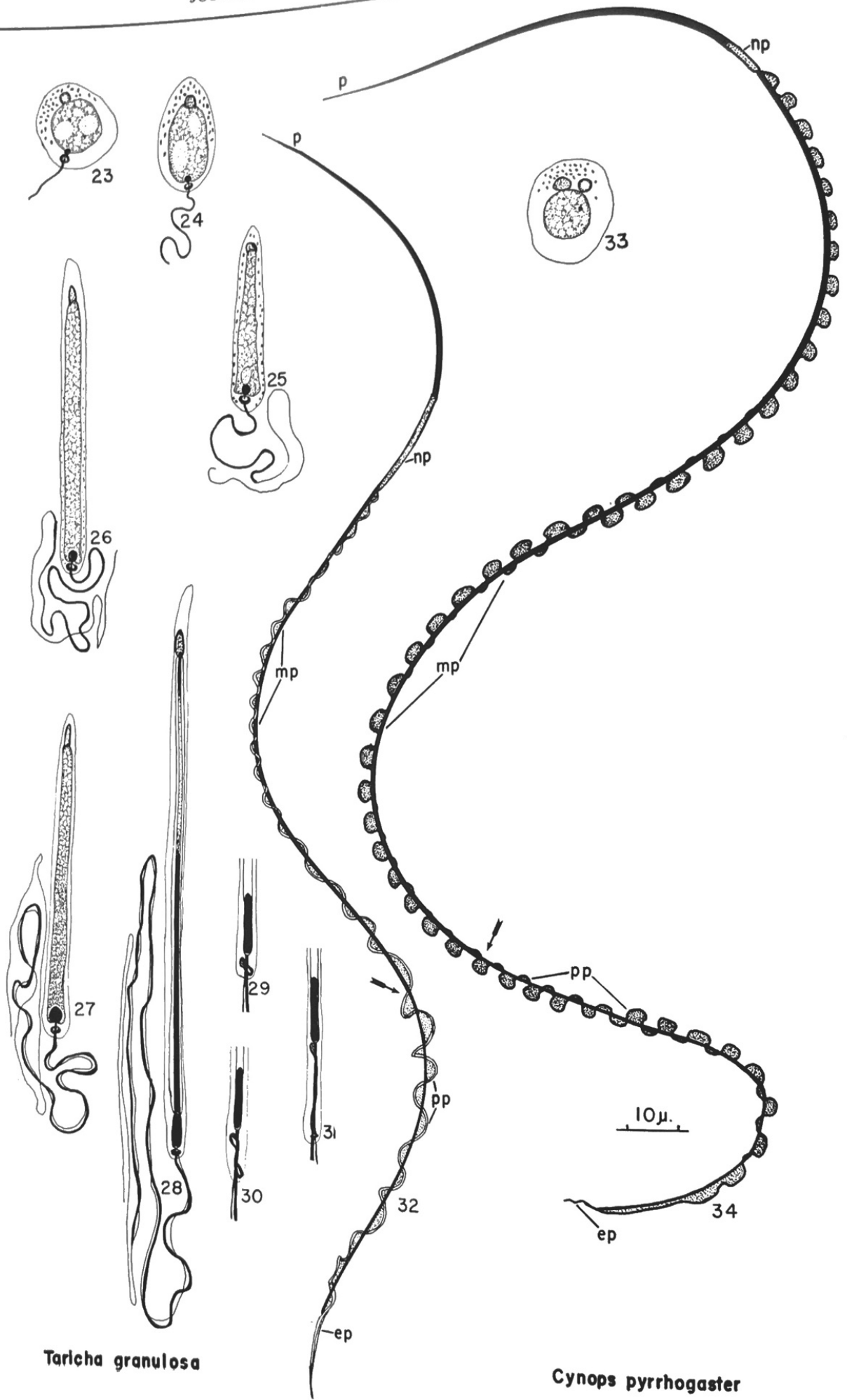
Taricha granulosa

Cynops pyrrhogaster

Groups of sperm are frequently observed in fixed material in abnormal conditions (Fig. 22). It is believed there are critical periods in spermatid transformation when the organelles are more labile and react adversely to fixatives, with the neck piece becoming spherical and the nucleus frequently coiling in an abnormal manner.

ELECTRON MICROSCOPY

The electron micrographs described here are restricted to a few stages of spermateleosis and the mature sperm of *Diemictylus viridescens*. Several small pieces of testes furnished more than 200 sections for observation and it seems incredible that practically all sections were through the middle piece with no evidence of the anterior end of the sperm to show the acrosome.

The axial filament of the mature sperm is a rather homogeneous rod-like process with no evidence of granulation or fibers (Figs. 40-42). The portion extend-
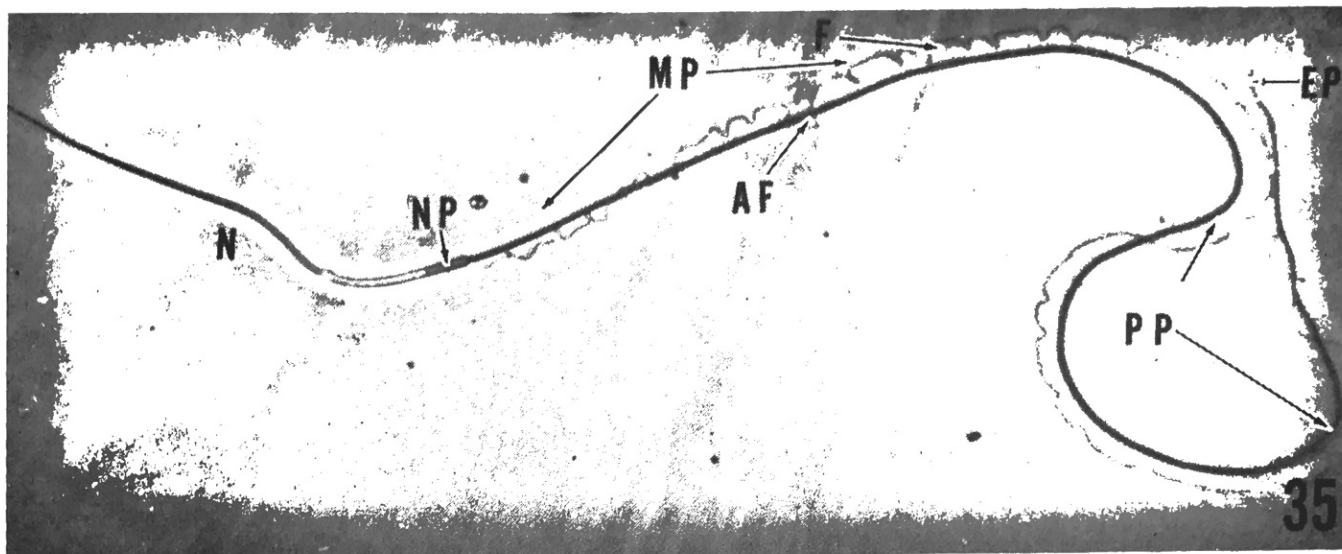
Fig. 35. The sperm of **Cynops pyrrhogaster.** The nucleus, N, is typical of the Salamandridae. This genus has the shortest neck piece, NP. The axial filament, AF, extends throughout the middle piece, MP, and principal piece, PP, and is bordered by the marginal flagellum, F, that terminates in the short end piece, EP. Photo made by dark phase contrast microscopy. X 700.
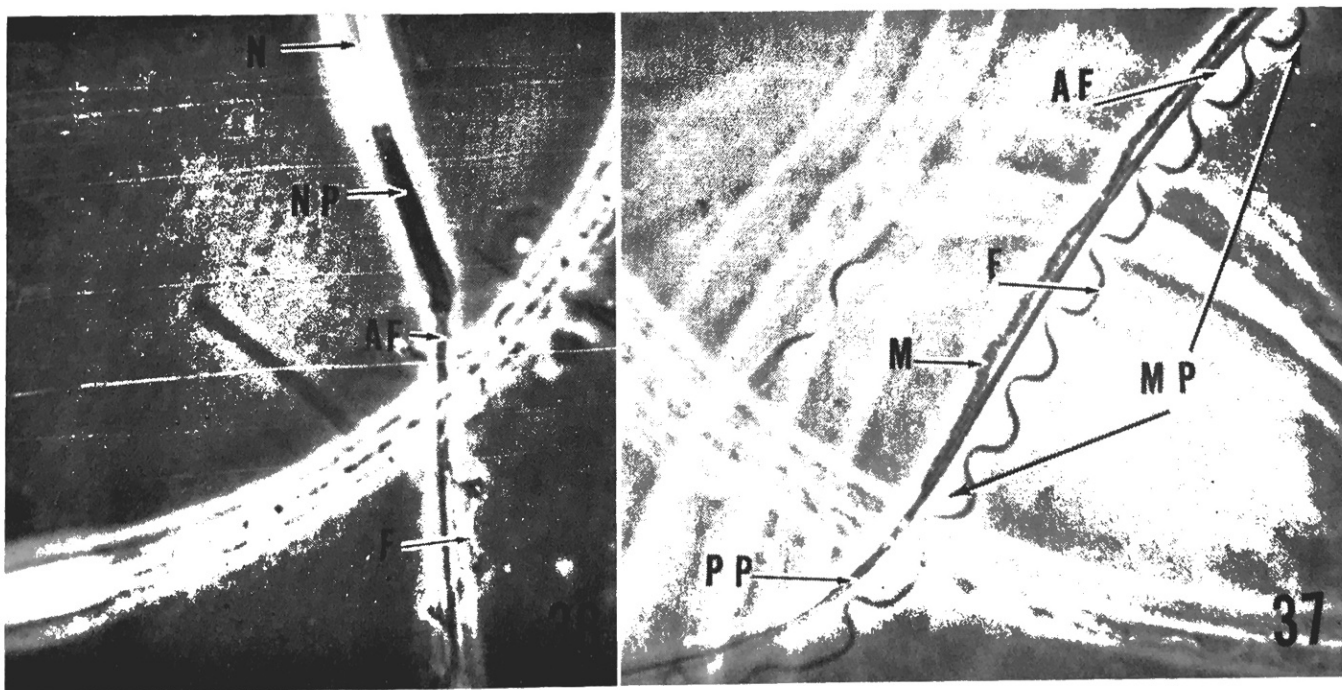

Fig. 36. A portion of the sperm of **Taricha granulosa** showing the neck piece, NP, distal to the nucleus, N, and uniting with the axial filament, AF, and flagellum, F. Dark phase contrast microscopy. X 1400.

Fig. 37. A portion of the sperm tail of **Diemictylus viridescens** with the mitochondria, M, of the middle piece, MP, darkened by the tetrazolium chloride reaction. The flagellum, F, is always on the side of the axial filament, AF, opposite to the mitochondria. (Compare with Fig. 40.) The principal piece, PP, is free of mitochondria (see Fig. 41). Dark phase contrast microscopy. X 1400.

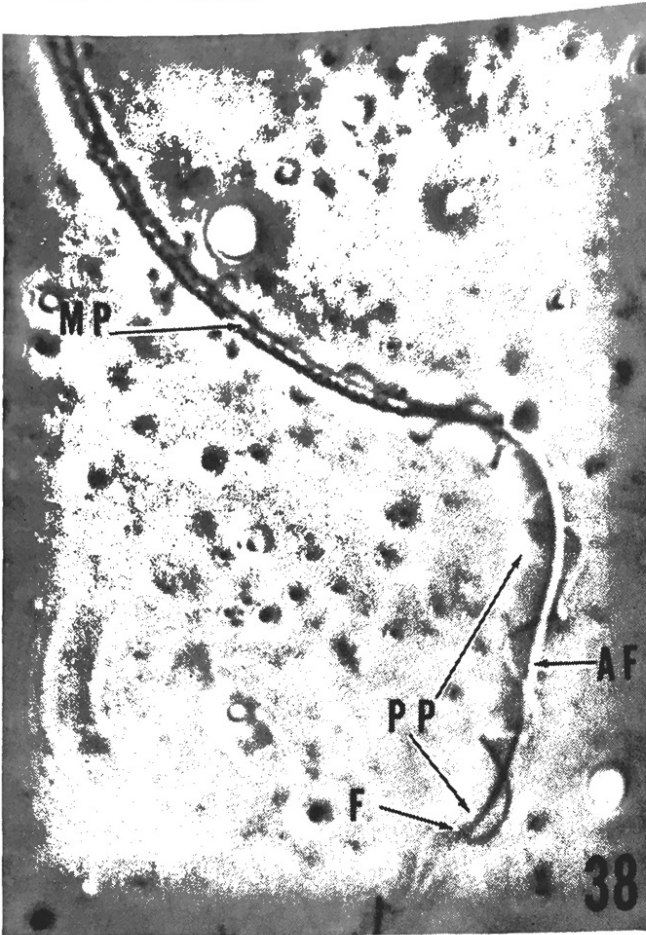EXPLANATION OF FIGURES 38-43
(Pages 8-10)

Fig. 38. A portion of the tail of **Taricha granulosa** sperm showing the juncture of the middle piece, MP, with the principal piece, PP. The mitochondria have been darkened by the tetrazolium chloride reaction. The prominent flagellum, F, is in irregular waves that are characteristic of this species   Dark phase contrast microscopy. X 1400.

Fig. 39. Representative sections through the tail of the sperm of **Diemictylus viridescens** taken from electron micrographs. The first section, through the middle piece, shows the dark axial filament bordered by the mitochondria and connected to the marginal flagellum by the undulating membrane. Five sections are through the principal piece while the last is the end piece with no axial filament. The axial filament gradually diminishes in size. (Sketches are by courtesy of Don W. Fawcett, Harvard Medical School, Boston.)

Fig. 40. Electron micrograph of cross sections of the middle pieces of mature sperm. The mitochondria, M, bordering the outer side of the axial filament, AF, have lost their cristae which seems to be typical of urodele sperm.   The undulating membrane, UM, holds the marginal flagellum, F, in place. A supporting rod, SR, borders the flagellum on its outer edge and these two components are enclosed in the common membrane that also includes the axial filament and mitochondria. X 15,040.

Fig. 41. Electron micrograph of cross sections through the principal piece of the sperm showing the trifoliate or trefoil axial filament, AF, connected to the marginal flagellum, F, by the undulating membrane, UM. The flagellar supporting rod, SR, of unknown origin and function, is always present. X 15,040.

Fig. 42. Electron micrograph of the middle piece of the sperm; cross sections on the left; longitudinal section on the right. The non-granular homogeneous material of the axial filament, AF, indicates an absence of any motility. X 30,080.

Fig. 43. An electron micrograph of the marginal flagellum, F, and a portion of the undulating membrane, UM, that holds the flagellum alongside the axial filament. Note the double membrane and the flagellum with the typical nine peripheral pairs and one central pair of fibers or tubules. Arms can be seen on the sub-fibers (at arrow). X 105,000.
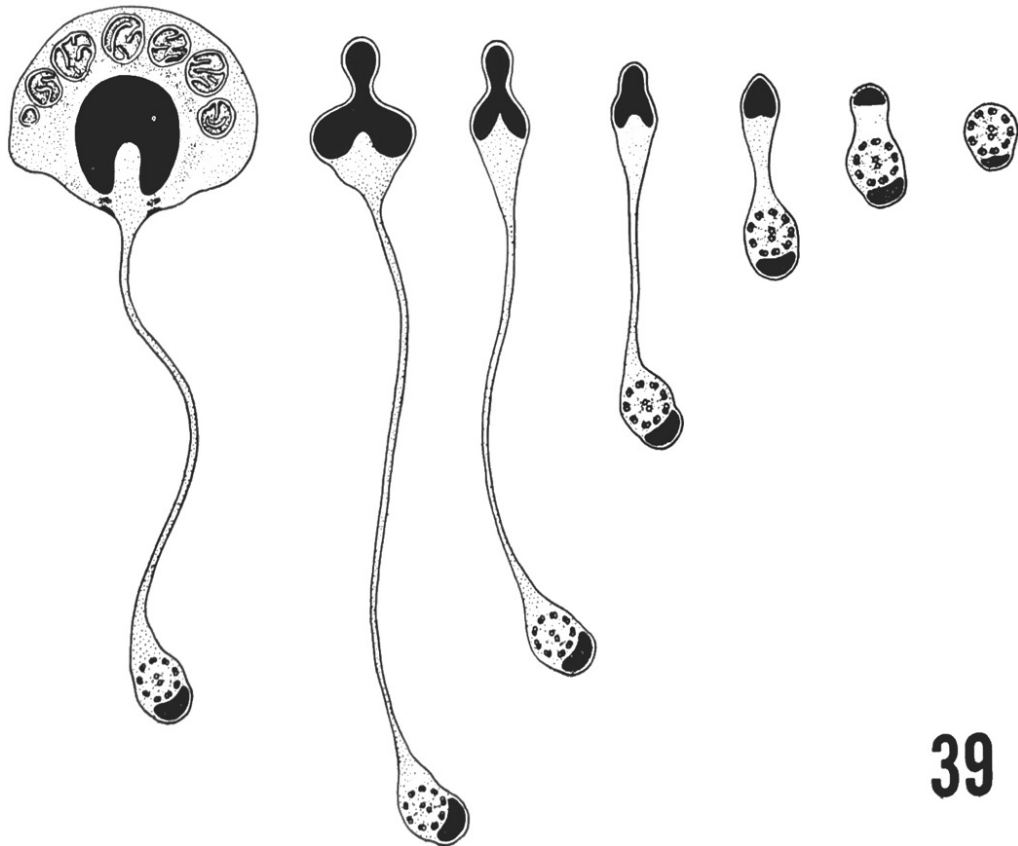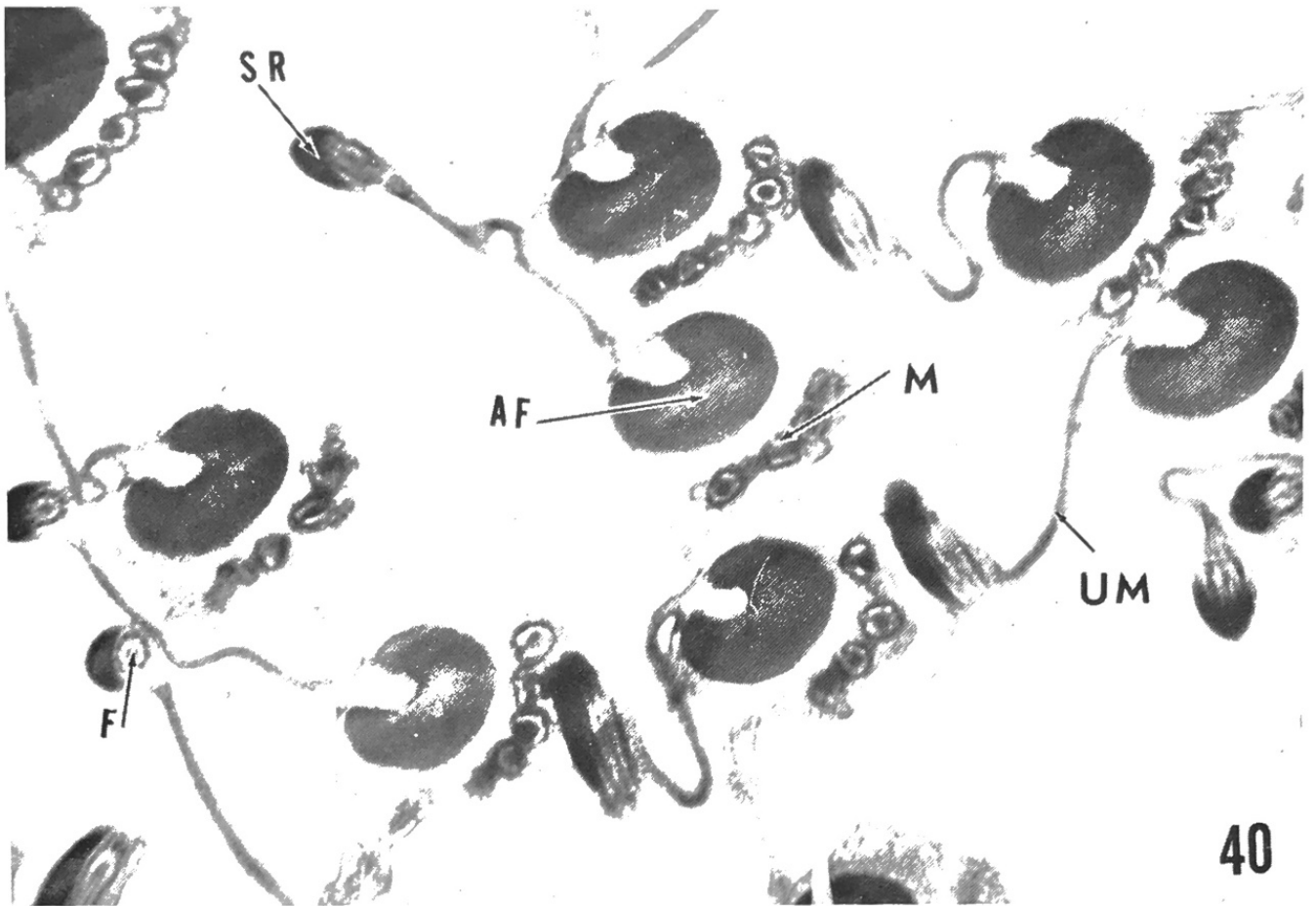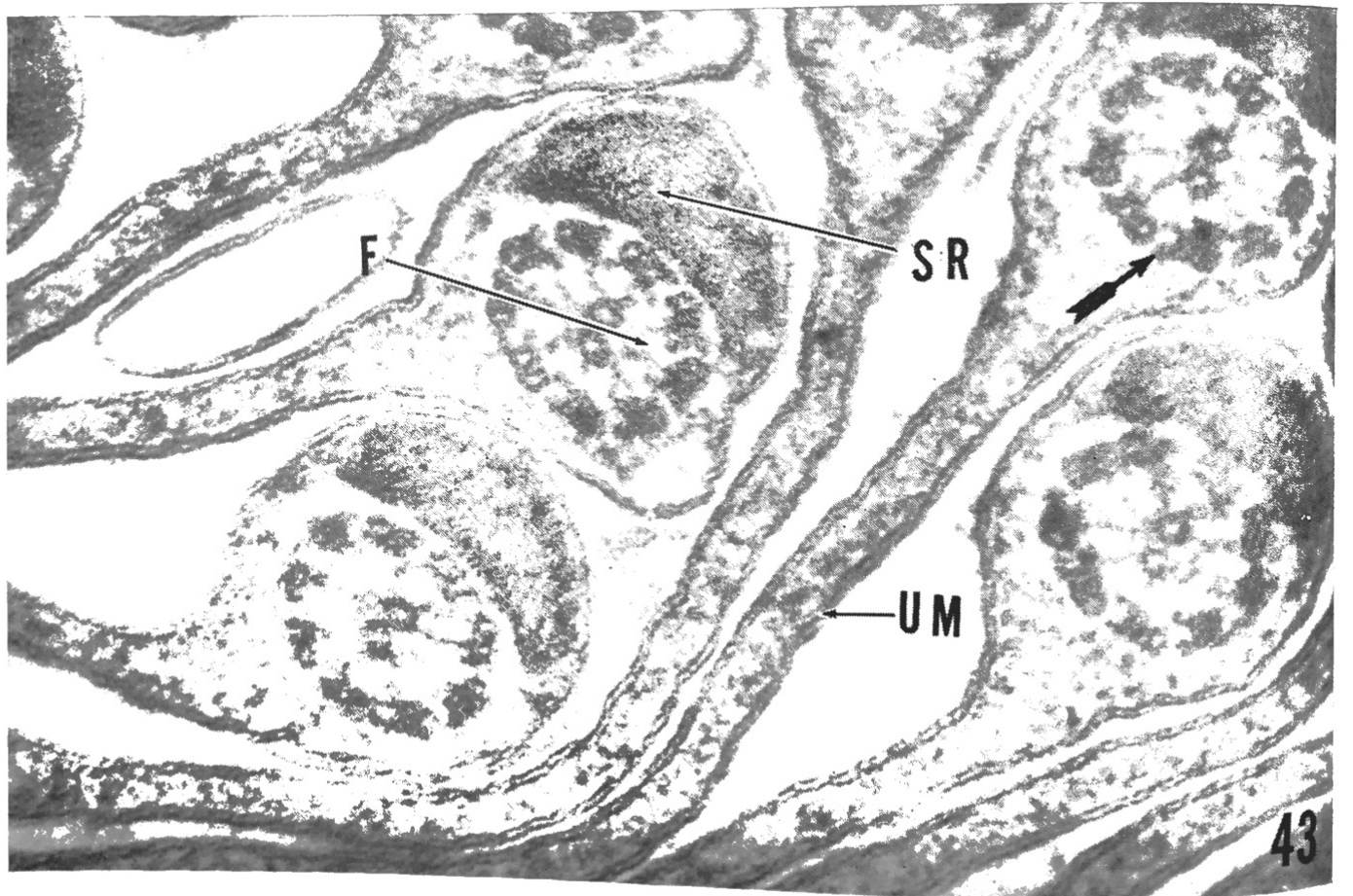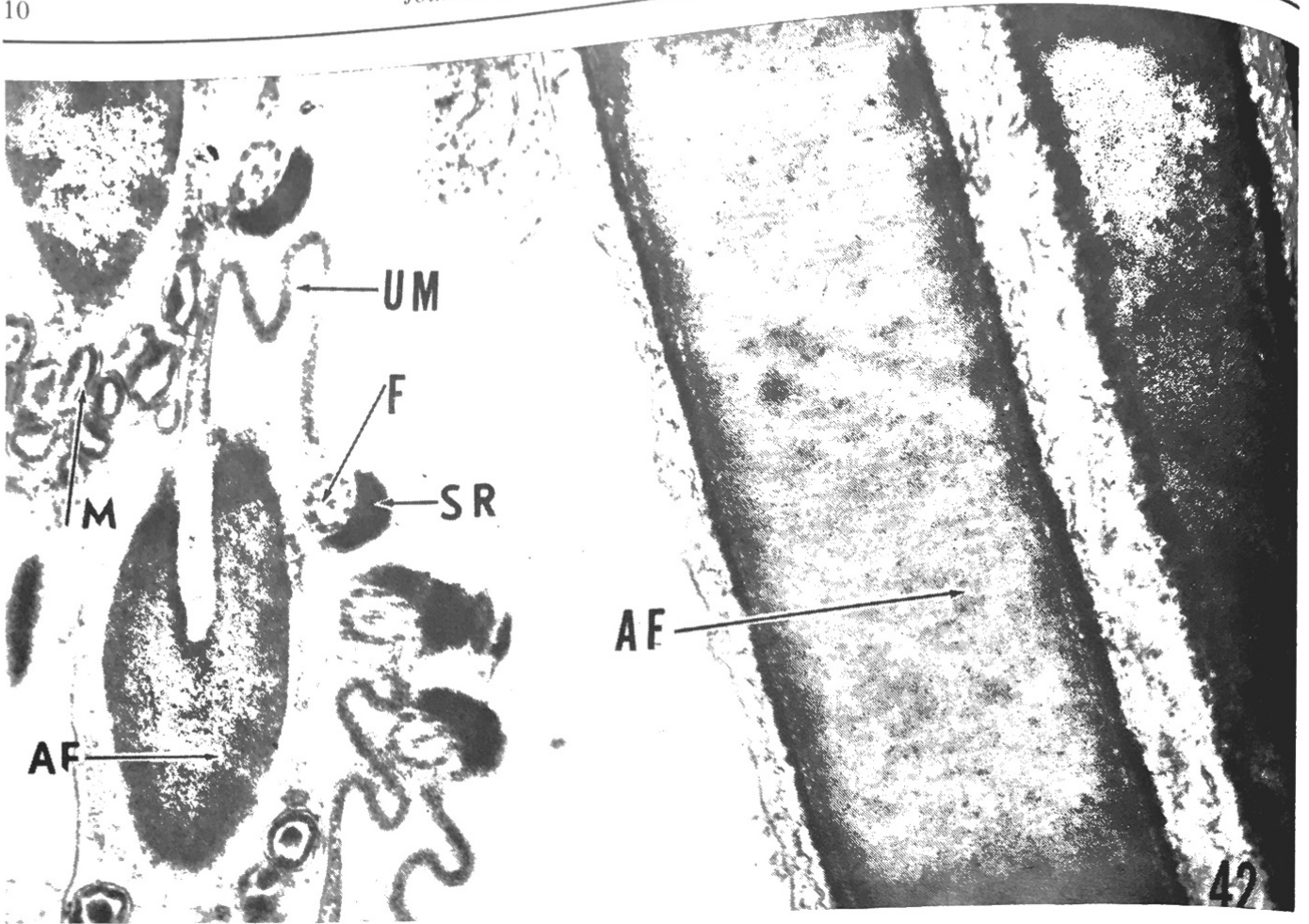
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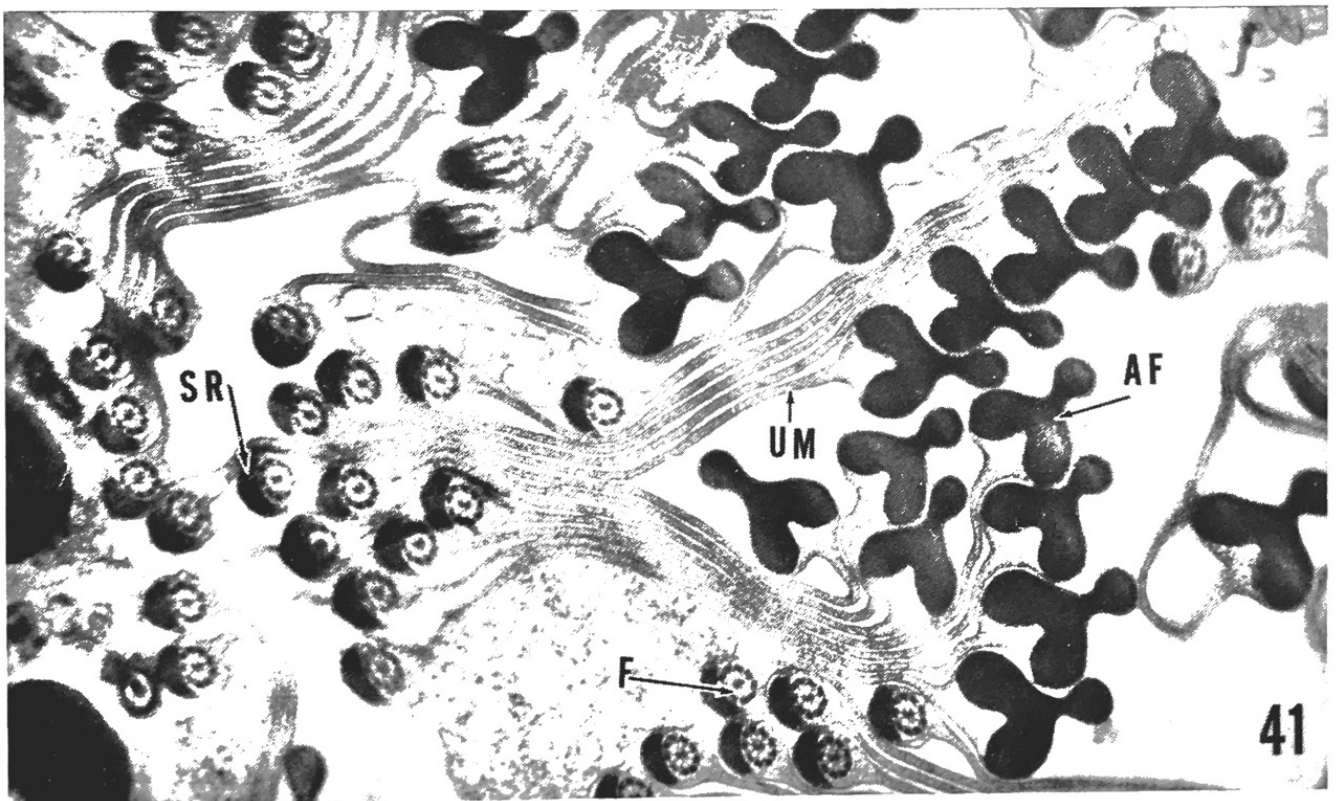
41

ing through the middle piece (Fig. 40) is essentially circular with a U shaped depression on the side facing the flagellum. In the principal piece this axial filament becomes trifoliate (Fig. 41) and towards the distal end of the tail it becomes attenuated and rod shaped (Fig. 39).

A cell membrane encloses the axial filament and flagellum and includes some cytoplasm with numerous mitochondria that always are located on the side of the axial filament opposite the flagellum (Fig. 40). The cell membrane appears to be attached to the axial filament in the middle piece at the edges of the depression by an electron dense material, perhaps to maintain stability of movement. An undulating membrane, composed of both cell membrane and dense cytoplasm, connects the axial filament to the flagellum (Figs. 40, 43).

A supporting rod of electron dense material, of unknown origin and function, extends almost the entire
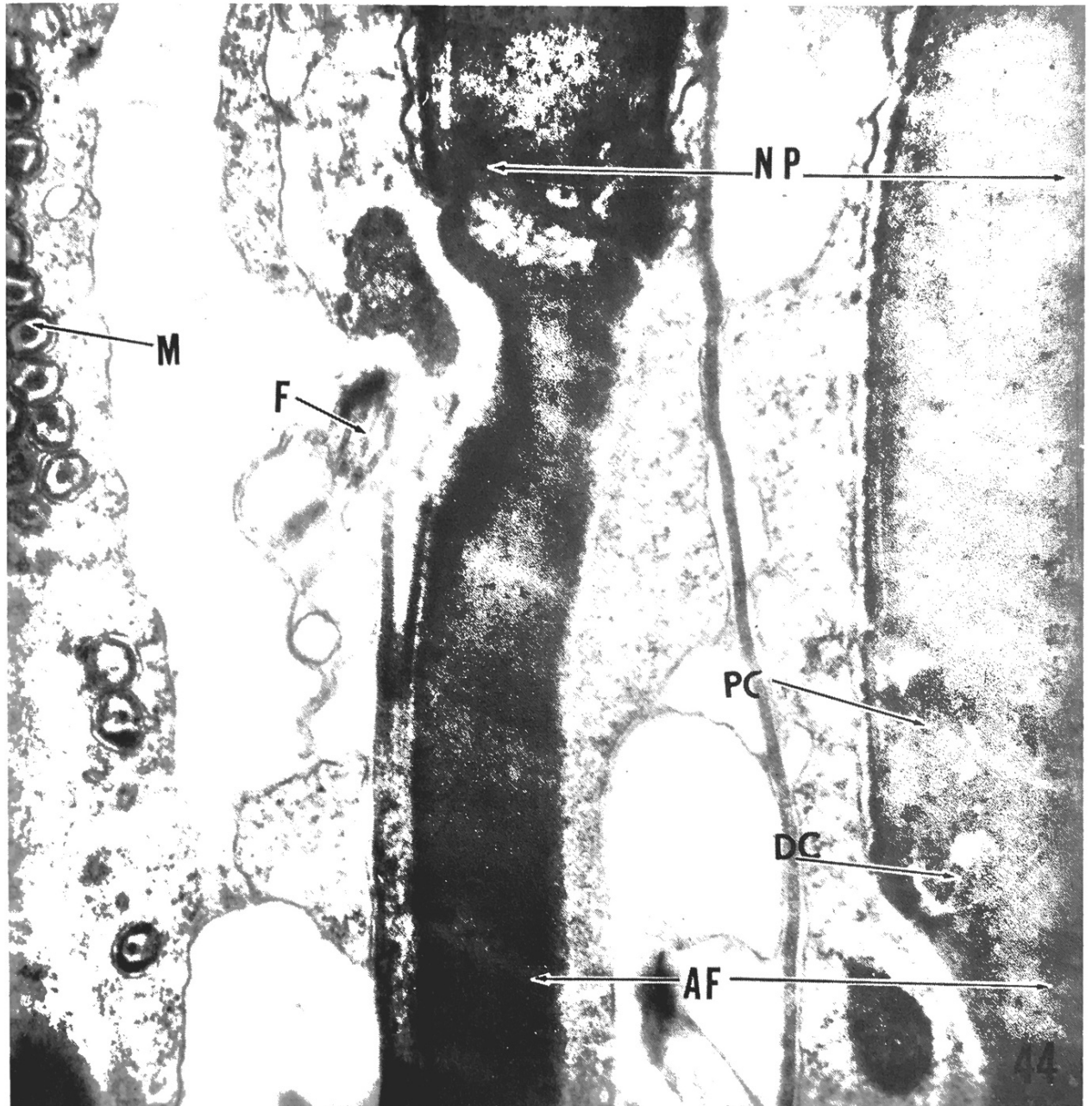

Fig. 44. Electron micrograph of a longitudinal section of two sperm showing the juncture of the neck piece, NP, and axial filament, AF. Above the flagellum, F, is an electron dense mass that may be a portion of the elongating ring. In the distal end of the neck piece on the right are structures that may be the vertical proximal, PC, and horizontal distal centrioles, DC. It is from this region that the flagellum originates. X 60,000.
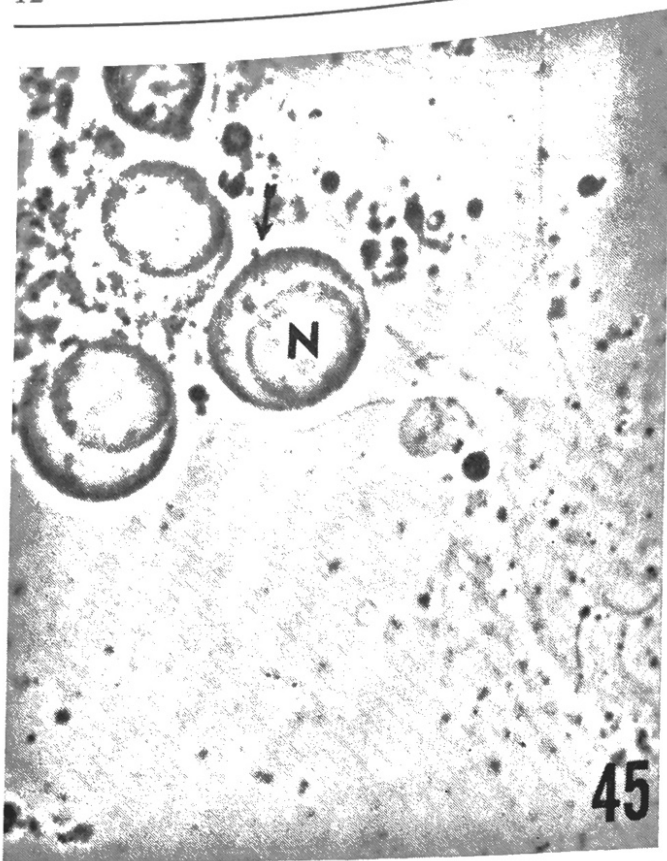
Fig. 45. A dark phase contrast photograph of the spermatids. A granule-ring complex is outside the cell membrane (at arrow), perhaps from a disintegrating spermatid. Note the elongated tail filaments on the right. (Compare with Figs. 47, 48, 51.) X 1400.

Fig. 46. Dark phase contrast photograph of a portion of a developing sperm. The granule has been transformed into the neck piece, NP, at the base of the elongated nucleus, N, and the ring, R, has not yet elongated down the axial filament, AF. X 1400.
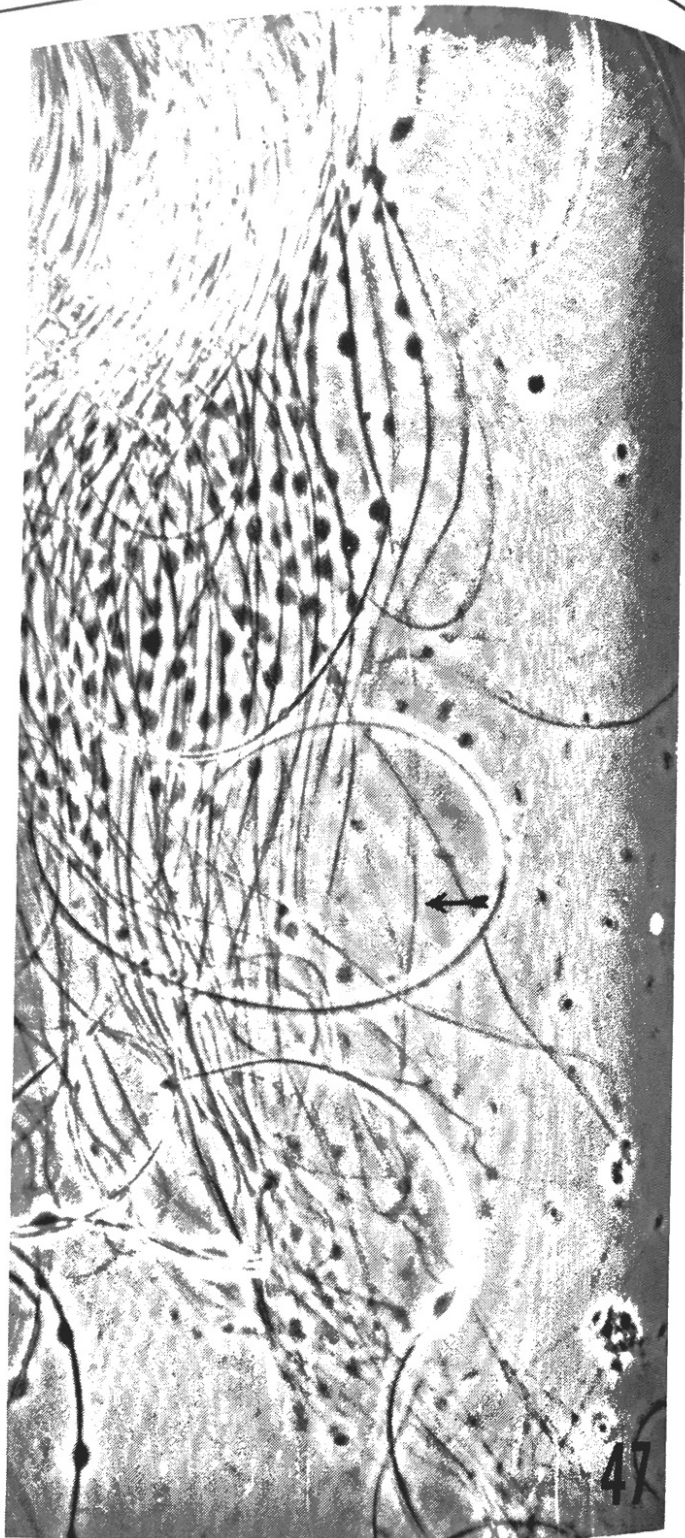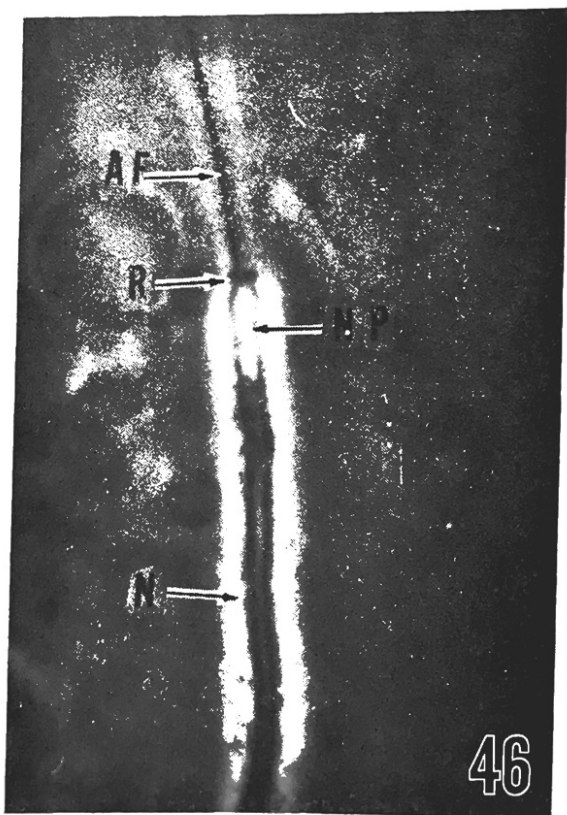

Fig. 47. A mass of developing sperm (same stage as Fig. 46) with the flagellum parallel to the axial filament (at arrow). Two mature sperm are present. Numerous protoplasmic blebs are on the sperm tails. X 700.

length of the flagellum (Figs. 39-43) and is enclosed in the cell membrane.

There is no flagellar membrane other than the common membrane enclosing the tail complex and there is no enclosing sheath as suggested (Wilson, 1928). The flagellum always has a uniform structure consisting of two central filaments and nine peripheral doubtlets (Fig. 43). There are radial spokes or fibers extending from the central pair to the peripheral ring and small arms of the peripheral doublets are characteristic. The double nature of the cell membrane is apparent with a thickness
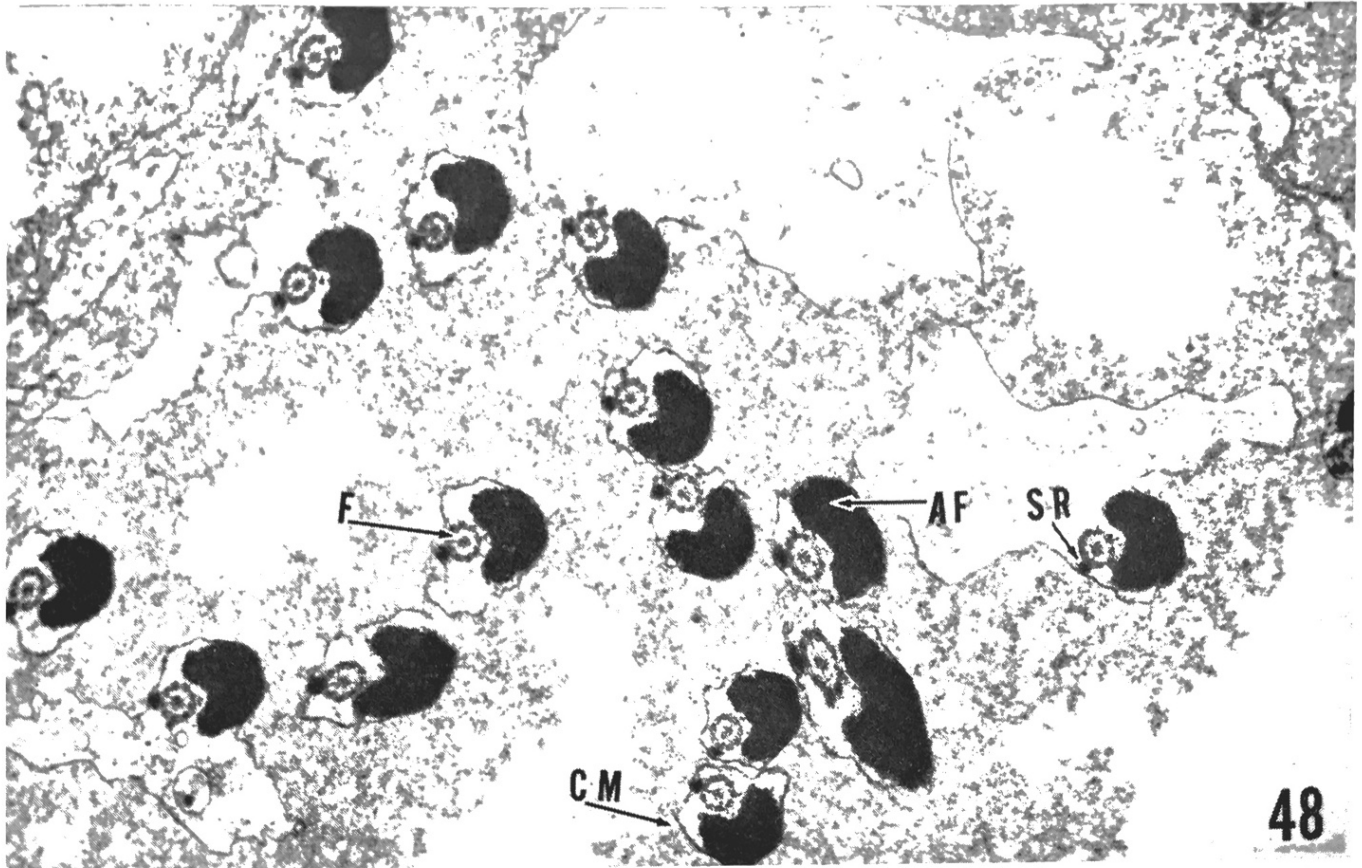
Fig. 48. Electron micrograph of cross sections of an early stage of sperm transformation. These sections are through the proximal part of the elongated tail filament and show the axial filament, AF, and flagellum, F, enclosed in a cell membrane, CM, with no evidence of an undulating membrane. Note the absence of mitochondria. X 30,600.
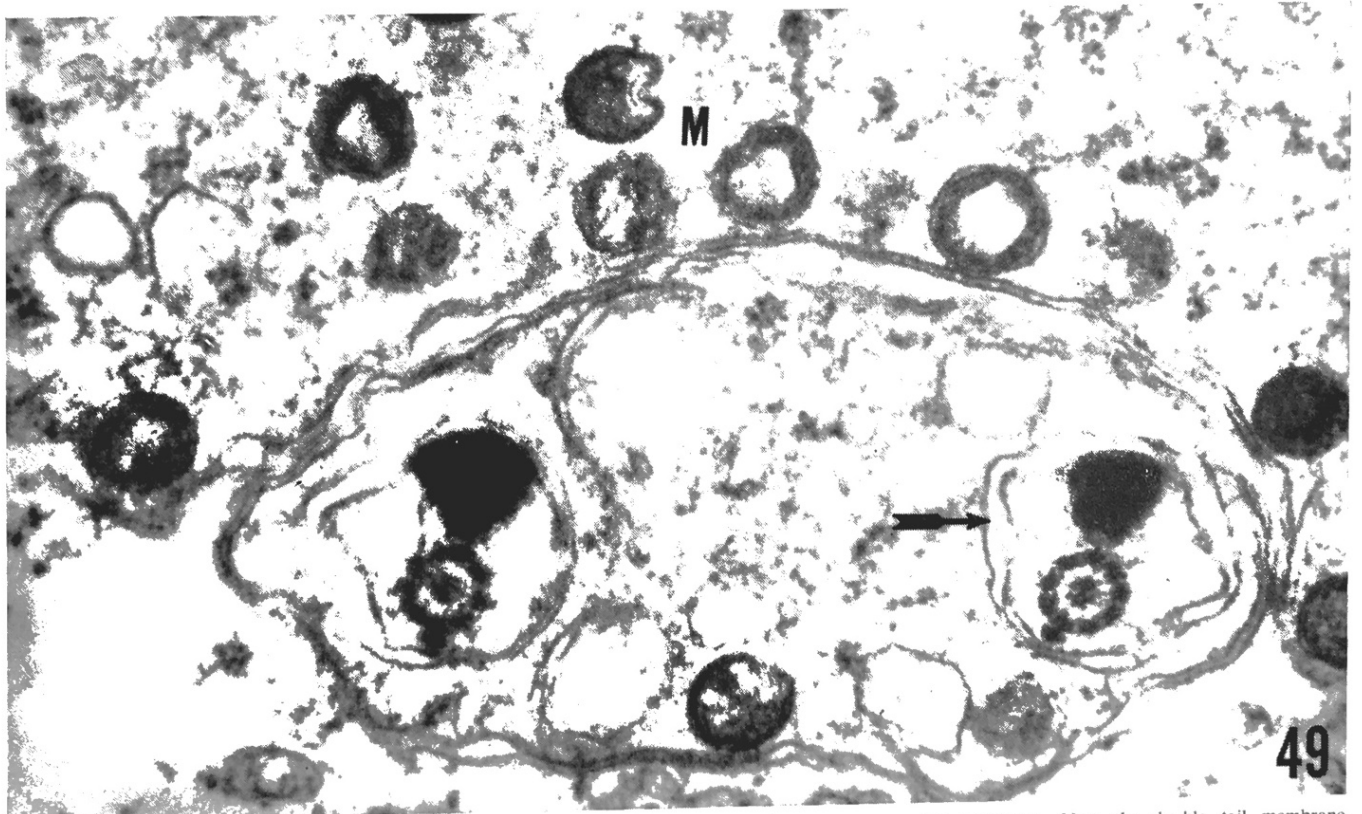

Fig. 49. An electron micrograph of two tail filaments of a developing sperm in a common outer membrane. Note the double tail membrane (at arrow). There is no explanation for this section. The mitochondria of an adjoining spermatid show the loss of cristae. X 60,160.

Fig. 50. Electron micrograph of an unusual section of a tail filament. Several of these flagellar quartets have been observed and there is no expanation presently available X 105,000.
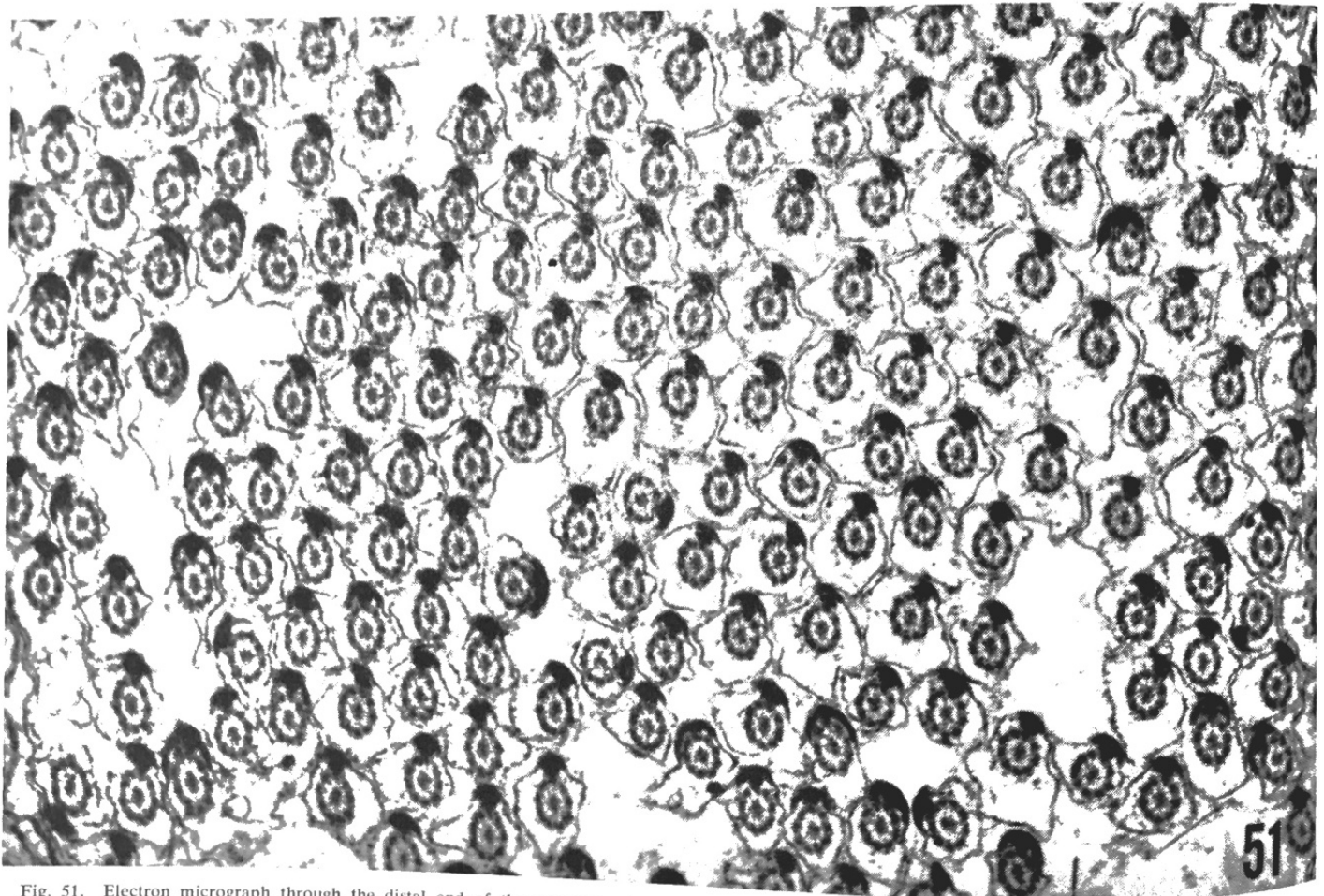

Fig. 51. Electron micrograph through the distal end of the tail filament of developing sperm. The flagellum and its supporting rod are enclosed in a cell membrane but no axial filaments or undulating membranes are present. X 30,600.
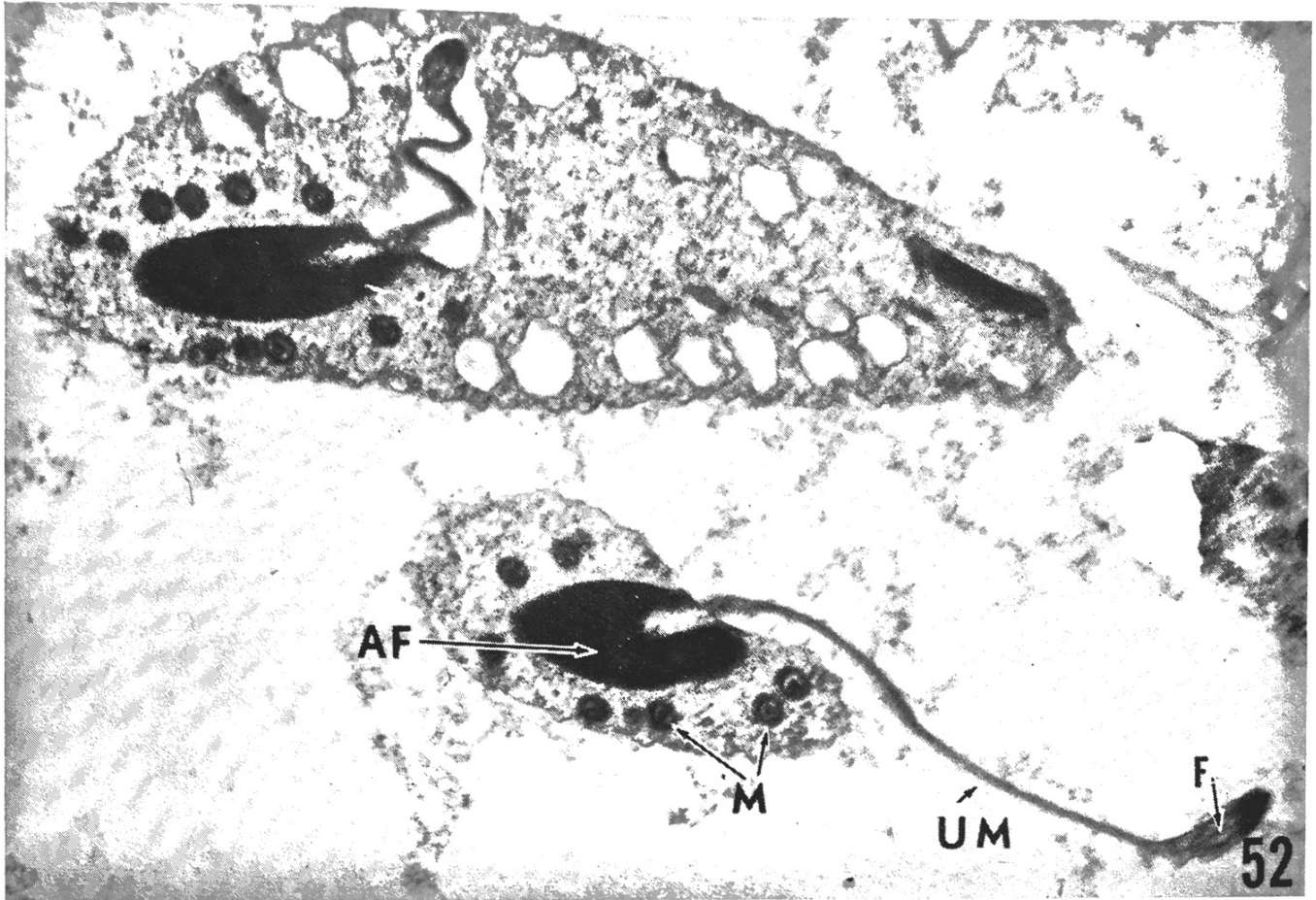
Fig. 52. Electron micrograph of a section through the middle piece of developing sperm. The axial filament, AF, has increased in size and attained its characteristic size and shape, and some cytoplasm with mitochondria surrounds the tail complex. In the upper section an undulating membrane connects the axial filament and flagellum and these components are enclosed in a cell membrane. In the lower figure, which is a later stage of development or possibly more distal, the flagellum has been released and has assumed the condition found in the mature sperm. The origin of the undulating membrane is unknown. (Compare with Figs. 5-7, 25-28.) X 19,550.

Fig. 53. Electron micrographs of a longitudinal section through an early stage of sperm development. The flagellum, F, and the axial filament, AF, are close together (compare with Fig. 48) and pass through the ring, R. The flagellum originates in the base of the neck piece, NP. Centrioles may be present in the neck piece but they cannot be determined from this section. X 30,000.
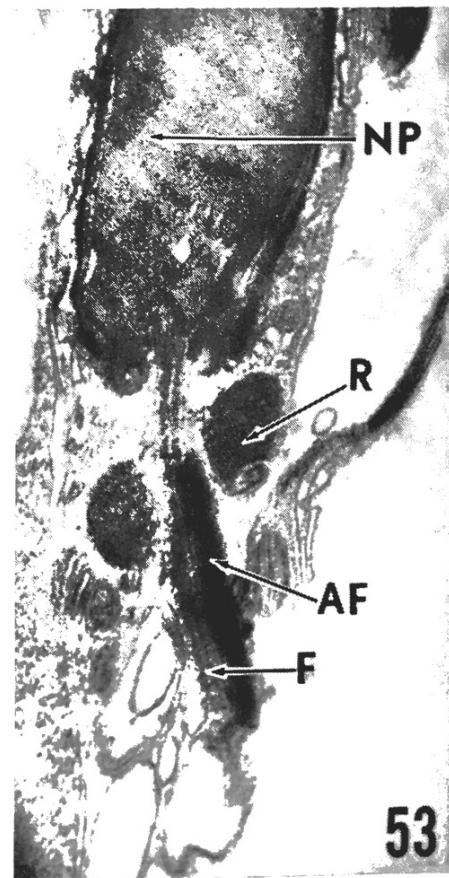
of approximately 100 A.

The axial filament is united with the neck piece with no intervening membrane (Figs. 44, 53). A depression on the side of the proximal end of the axial filament is prominent. A dark mass appears in longitudinal sections that may be the proximal portion of the elongated ring. There may be two centrioles present in the neck piece but their relation to the axial filament and flagellum cannot be determined in these studies.

Light microscopy reveals a definite ring and granule within each developing spermatid (Figs. 45, 46). In rare instances this granule-ring breaks through the cell membrane when bits of fresh testicular material are macerated (Fig. 45). A fine tail filament extends for a considerable distance from the granule, through the ring and apparently across the cell membrane. Each filament has on it several protoplasmic blebs of unknown significance (Fig. 47). The granule becomes the neck piece with the ring closely adherent (Fig. 46). Electron
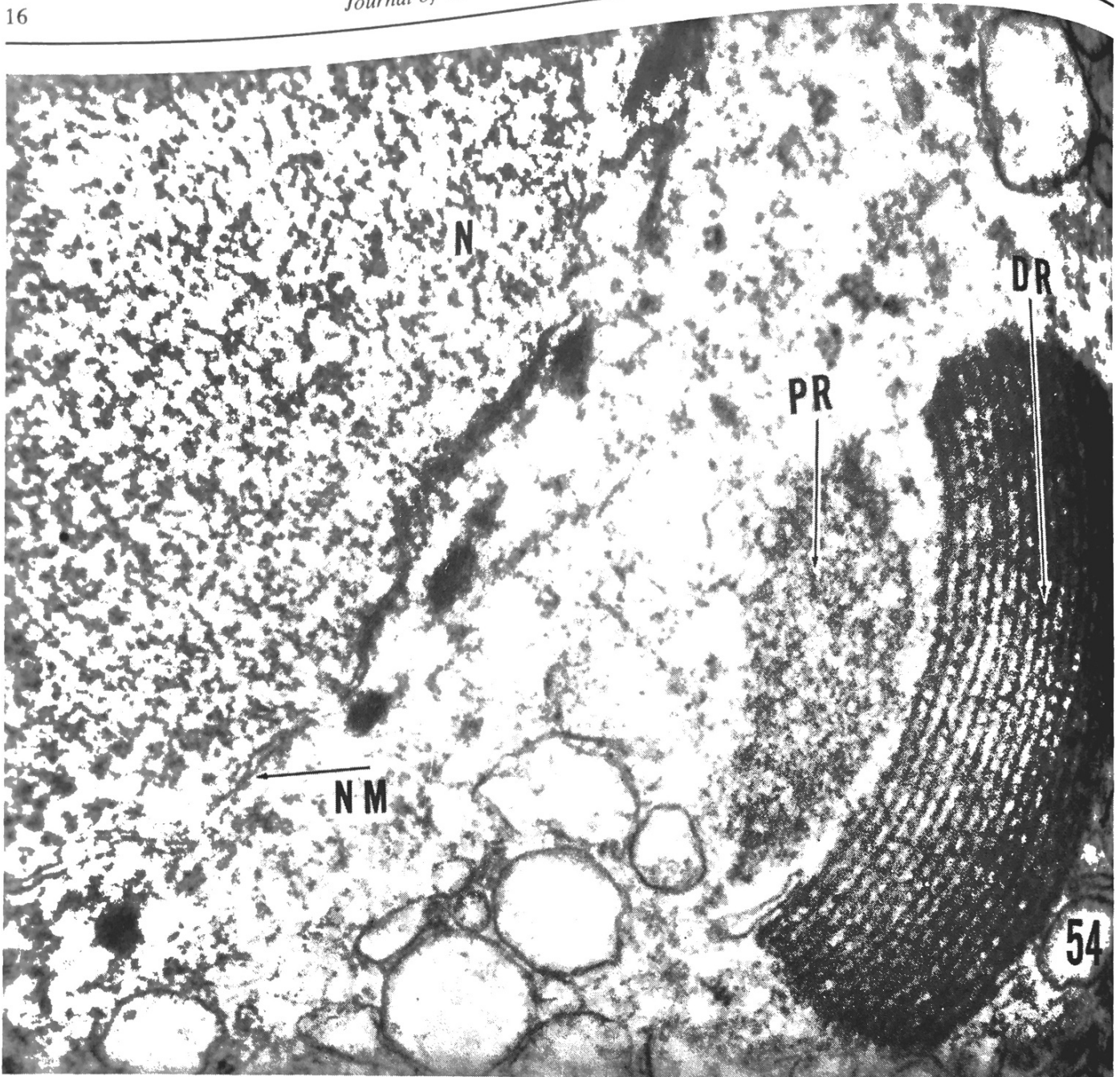
Fig. 54. Electron micrograph of an early stage of sperm development. The double nuclear membrane, NM, is visible. The section cuts through the outer edge of the granular proximal ring, PR, and the lamellar distal ring, DR, which is composed of 16 layers. The dark mass in the nucleus, N, may be associated with the developing neck piece. X 70,000.

micrographs, to be described, will clarify the structure and the association of these components to each other.

The early elongating tail filament of the transforming spermatid contains a definite axial filament, a flagellum and a supporting rod; all within a membrane that is evidently an extension of the cell membrane (Fig. 49). The axial filament in this early stage is of the same general shape as that of the mature sperm but is considerably smaller (compare with Fig. 40). This axial filament obviously increases in size as it lengthens. There is no evidence of an undulating membrane in this early stage. The distal half of this tail filament has a flagellum and a supporting rod, enclosed by a cell membrane, but no axial filament (Fig. 51). In light microscopy one observes sweeping movements of this distal end as compared to the slower and sluggish activity of the proximal

end containing the axial filament.

A bit of cytoplasm encloses the proximal part of this tail complex as development progresses and an undulating membrane develops between the axial filament and flagellum (Fig. 52), and numerous mitochondria are present in this middle piece of the tail. The original membrane still encloses the undulating membrane and flagellum and an additional outer membrane surrounds the entire component. The origin of these membranes is not understood.

Light microscopy reveals the separation of the flagellum from the axial filament or tail complex and electron micrographs confirm that the flagellum is released to the exterior, being held in place by the undulating membrane (Fig. 52). The cytoplasm orients about the axial filament in a manner similar to that

Fig. 55. Electron micrograph of an early stage of development of the sperm This center longitudinal section shows the true bipartite nature of the ring with a proximal granular, PR, and a distal lamellar, DR, composition through which the axial filament, AF, and flagellum, F, pass. The large granule, G, not centriolar in appearance, is affixed to the distal end of the nucleus, N. (Compare with Figs. 4, 23.) X 75,200.

found in the mature sperm. (Compare with Fig. 40.) No comparable sections through the principal piece were observed and therefore it is not determined how the axial filament of this region appears in developmental stages.

Sections made through the neck region of a developing sperm may show two components that logically would be interpreted as a granule and a ring adjoining the nucleus (Fig. 54). The darker and larger portion is lamellar and is composed of 16 layers; the smaller proximal part is granular. Additional sections clearly reveal that the ring is bipartite and the two portions of this ring are referred to as proximal and distal rings (Figs. 55, 56). Lateral sections through the edge of the
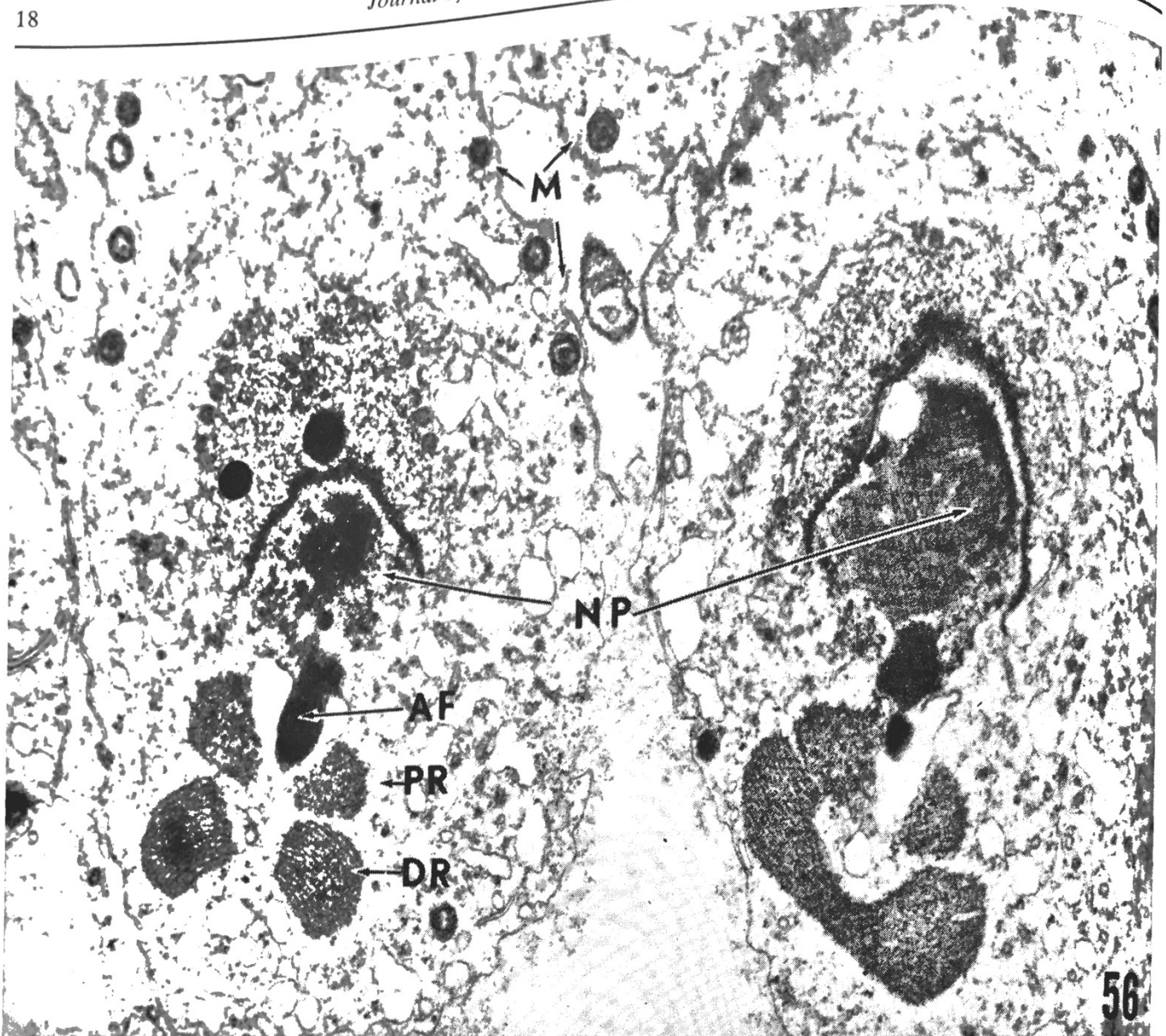
Fig. 56. Electron micrograph showing further development of the sperm. The granule is now embedded in the base of the nucleus and is hereafter designated as the neck piece, NP. (Compare with Figs. 6, 26.) The double nature of the ring. PR, DR, through which the axial filament, AF, passes is quite obvious. X 30,600.

ring may not show the granule at all. The axial filament and flagellum are encircled by this ring and the large granule, proximal to the ring, contacts the nucleus (Fig. 55). No undulating membrane is between the axial filament and the flagellum at this time (Fig. 57). The axial filament seems to originate at the base of the modified granule or neck piece (Figs. 56, 58). This neck piece is embedded in the base of the nucleus and the double nuclear membrane can be observed quite easily as an invagination into the distal end of the nucleus (Figs. 58, 59). The flagellum parallels the axial filament closely and has no waves at this stage (Fig. 60).

Further evidence of centrioles associated with the neck piece is indicated in other sections (Fig. 59) where a proximal centriole appears to be partially embedded in the side of the neck piece with a probable distal centriole at its base.

Little can be determined regarding the nuclear material during the above mentioned stages. It is possible that the sperm that were sectioned were curved sharply at the neck piece and the expected condensed chromatin was not visible. Other sections from this same tubule (Fig. 61) show excellent electron dense granular material in the nucleus surrounded by a layer of finer granules. Further observations on this nuclear material must be made before its composition can be considered.

The mitochondria of certain cells of the testes, probably sertoli cells, are of typical structure with a double peripheral membrane and inner cristae. In the transforming spermatid the mitochondria become modified by the cristae changing from double membrane partitions to condensed electron dense masses and/or a peripheral ring (Figs. 44, 60).

DISCUSSION

The sperm of the three genera of the Salamandridae are quite uniform in general structure, especially when viewed by the light microscope, yet, like the external differences of the adult animals, there are definite
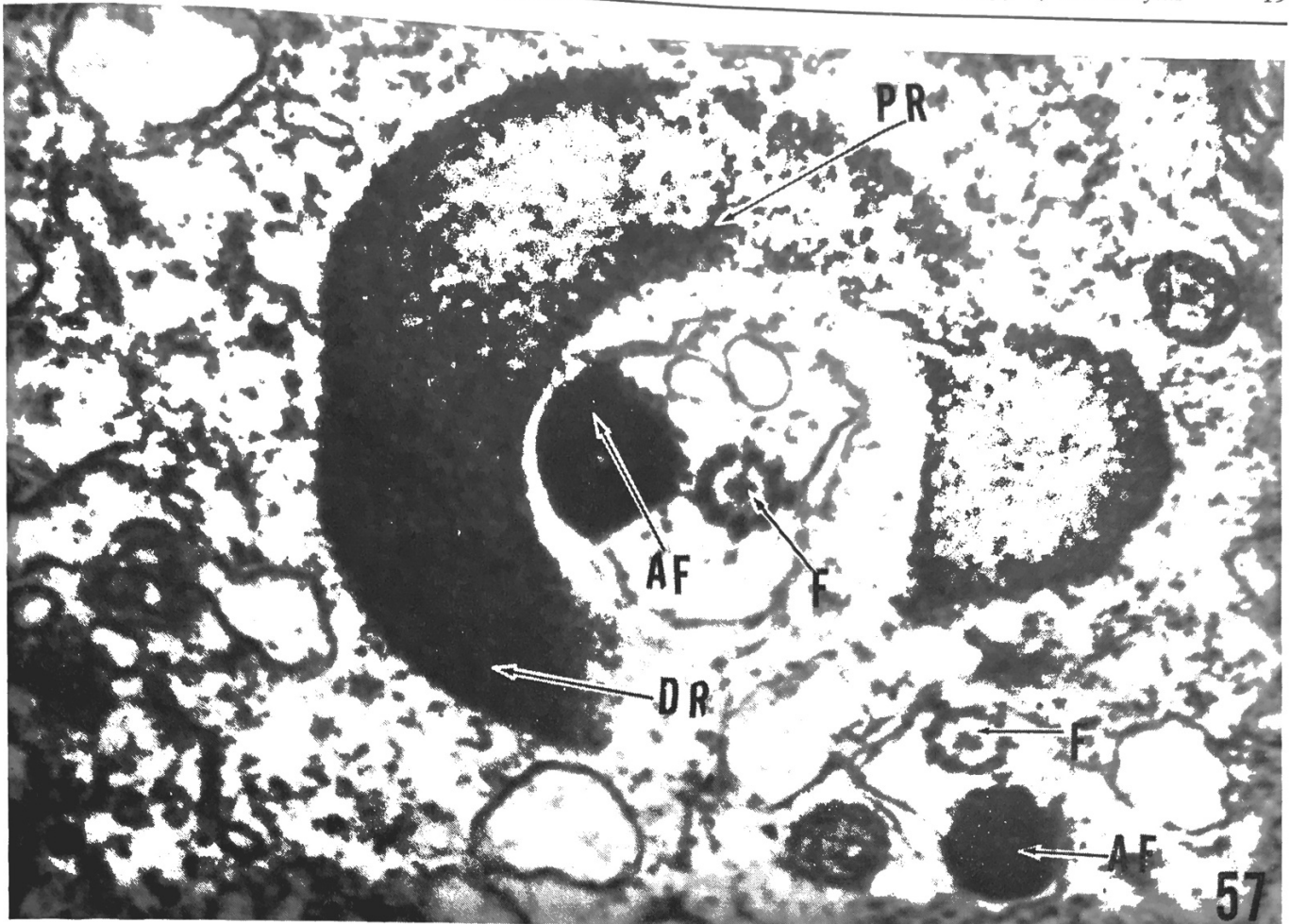
Fig. 57   Electron micrograph of a cross section through the irregular shaped ring of the developing sperm. The ring has both proximal granular, PR, and distal lamellar, DR, material. The axial filament, AF, and flagellum, F, both enclosed in a cell membrane, pass through the ring. An additional axial filament and flagellum of another developing tail are at the lower right. X 60,160.

diagnostic features such as the total length and the length of the neck piece. Perhaps by coincidence the neck piece of *Cynops,* the Japanese newt, is 8 micra long and is nearer to that of the *Taricha,* a west coast newt, of 21 micra than it is to that of *Diemictylus* of 37 micra. The differences in the urogenital systems of the males of these three newts coincide with these neck piece lengths (Baker, 1965).

These three genera of sperm are uniform in having the flagellum on the convex or outer curve of the axial filament, motility is restricted to the flagellum, and a granule and ring appear in the spermatid with the granule giving rise to the tail complex of axial filament and flagellum and later being partially enclosed by the distal end of the nucleus to become the neck piece. The tail complex lengthens rapidly and consists of a proximal portion with both axial filament and flagellum enclosed in a membraneous sheath, and a distal part with the flagellum alone. The entire flagellar component is vibratile as soon as it emerges from the spermatid. When the axial filament reaches its maximum length the flagellum separates from it and parallels the axial filament for its entire length, being held alongside by an undulating membrane. Later the flagellum is drawn into a series of waves with only the end piece remain-

ing free.

This flagellar transformation introduces problems that are difficult to explain. When the axial filament reaches its maximum length the distal portion of the tail complex extends 200-250 micra beyond as a free flagellum. The part of the flagellum in the proximal portion of the tail piece parallels the axial filament (Figs. 8, 28) and these two organelles are held together by the undulating membrane. It is apparent and quite definite that when the flagellum appears as a three dimensional coil alongside the axial filament in the mature sperm (Figs. 21, 32, 34) the distal free portion is reduced to a short end piece. Therefore it is obvious that the flagellum has been drawn up beside the axial filament with the total flagellar length remaining approximately the same. A comparable transformation occurs in Cryptobranchus and Necturus (Baker, 1963) and in Amphiuma. No explanation is available regarding this transformation since the flagellum is attached to the axial filament by the undulating membrane for the entire time. Also it is obvious that further observations and interpretations are necessary.

No centrioles can be observed with any degree of certainty by light microscopy and the origin of the granule and ring cannot be determined even though
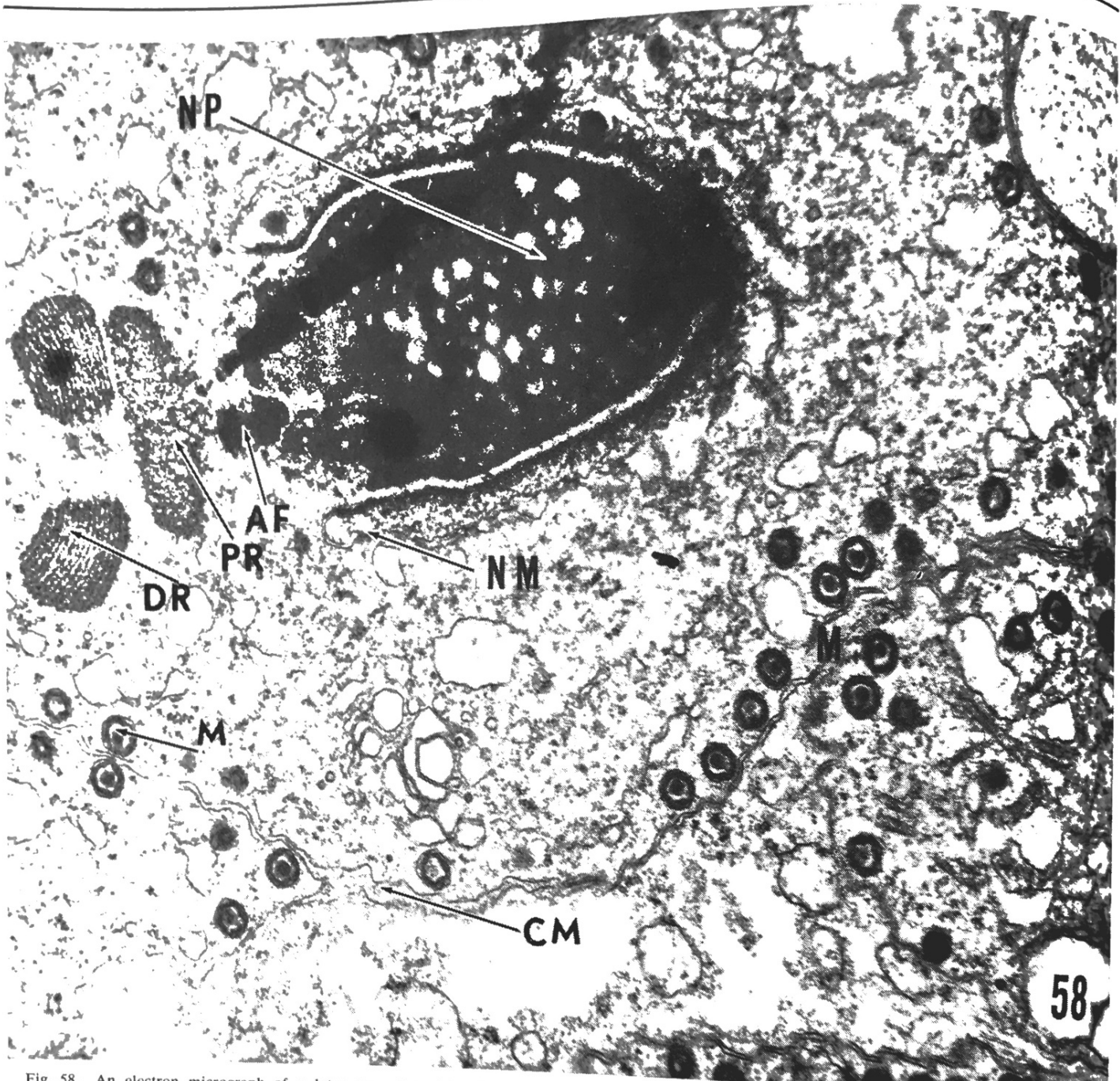
Fig. 58. An electron micrograph of a later stage than Fig. 56 of the developing sperm, with the neck piece, NP, firmly embedded in the base of the nucleus as indicated by the enfolded double nuclear membrane, NM. The double cell membrane. CM, encloses numerous peripheral mitochondria, M, and the bipartite ring, PR, DR. The axial filament, AF, emerges from the base of the neck piece. It is believed these sperm were curved just proximal to the neck piece, which would account for the non-appearance of chromatin granules in the nucleus. X 28,400.

these two components are referred to as centrioles (Bowen, 1922; Wilson, 1928; Burgos and Fawcett, 1956; Gatenby, 1931, 1962). They may be of centriolar origin as is the flagellum and possibly the axial filament but when once formed the flagellum especially is no longer referred to as a centriole and there seems no valid reason for referring to this large granule that forms the neck piece, and the elongated ring, as centrioles.

Electron micrographs indicate quite clearly that the neck piece originates outside the nucleus, from the granule, becomes partially enclosed by the nuclear membrane, and is closely appressed to its distal end in *Diemictylus*. This granule is referred to as a juxtanuclear body in Ambystoma (Sotelo and Trujillo-Cenoz, 1958) that gives rise to the axial filament and is shown

becoming wedged into an increasingly deeper nuclear fossette, while in *Desmognathus fusca* a number of cytoplasmic granular bodies along the nuclear border coalesce to form a distinct region at the base of the nucleus (Gatenby, 1931) which becomes a "region" between the nucleus and the granule.

The ring has a bipartite structure: a proximal portion that is granular and a distal part that is composed of 16 lamellae. A double ring also has been described for Ambystoma and the two components are called "light distal" and "dense distal" rings (Sotelo and Trujillo-Cenoz, 1958).

The elongation of the ring down the axial filament-flagellum tail complex is uniform in the three genera. This ring elongation is accompanied by some cyto-
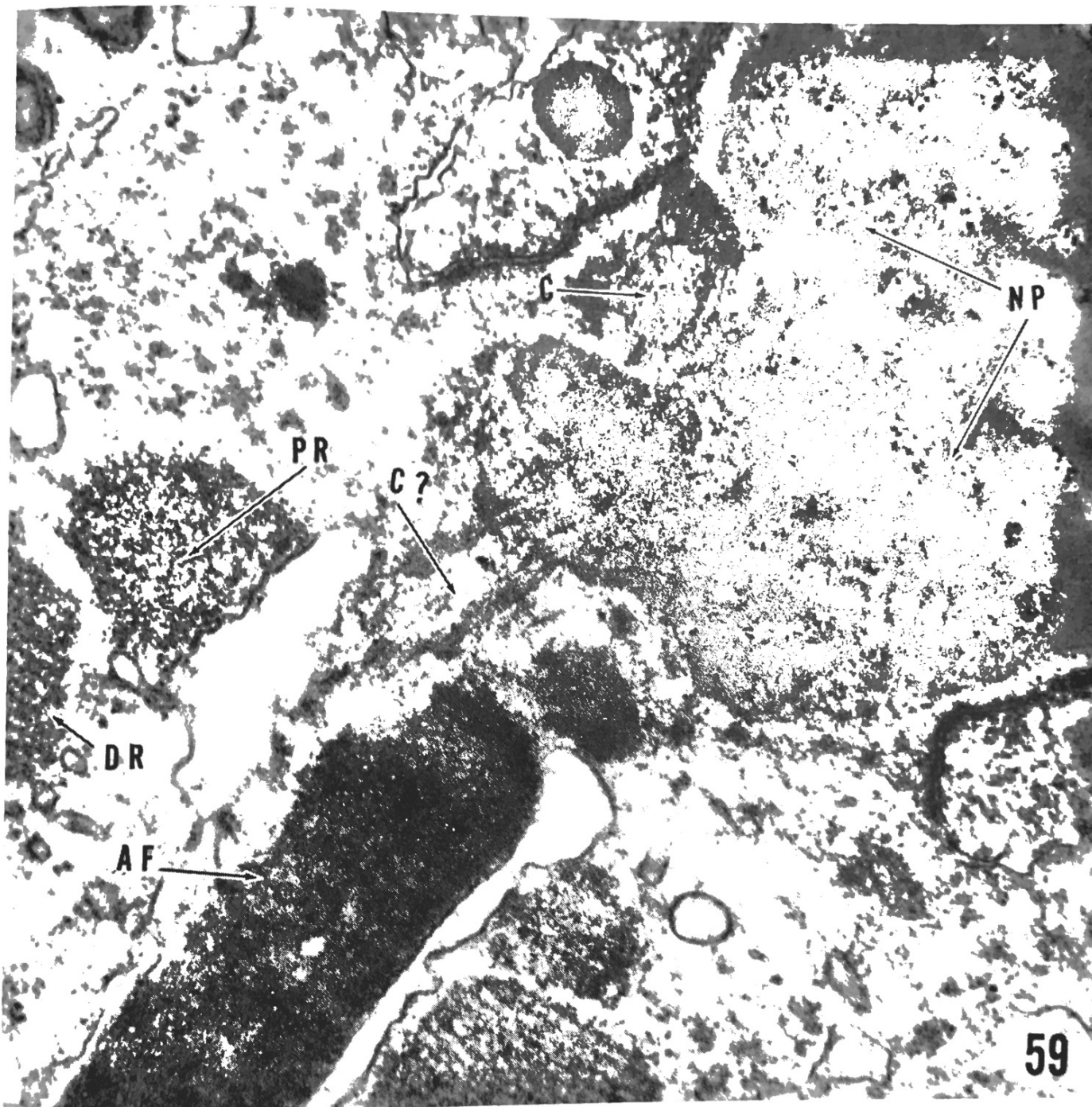
Fig. 59. An electron micrograph of an enlarged section of a stage of sperm development similar to that of Fig. 58. Electron dense clumps of granules of unknown significance are in the neck piece, NP. One centriole, C, embedded in the neck piece, seems typical and another centriole, C?, may be present. The enfolded double nuclear membrane is evident along with the bipartite ring, DR, PR, and axial filament, AF. X 76,800.

plasm with numerous mitochondria and its extent marks the end of the middle piece, but its purpose in elongation and ultimate function remains unknown. It has been reported that the axial filament is a modification of the ring which contains no fibrils (Gatenby, 1931). It is suggested also that the ring is a centriole that turns over to become the elongated edge of an undulating membrane (Gatenby, 1961). The axial filament increases in diameter during this ring elongation and since the ring apparently disappears after its elongation there may be a correlation between the enlargement of the axial filament and this disappearance.

The origin of the undulating membrane is unknown. It is bordered by a double membrane that apparently develops after the axial filament and flagellum are formed but before the flagellum emerges from the common membrane that encloses it and the axial filament.

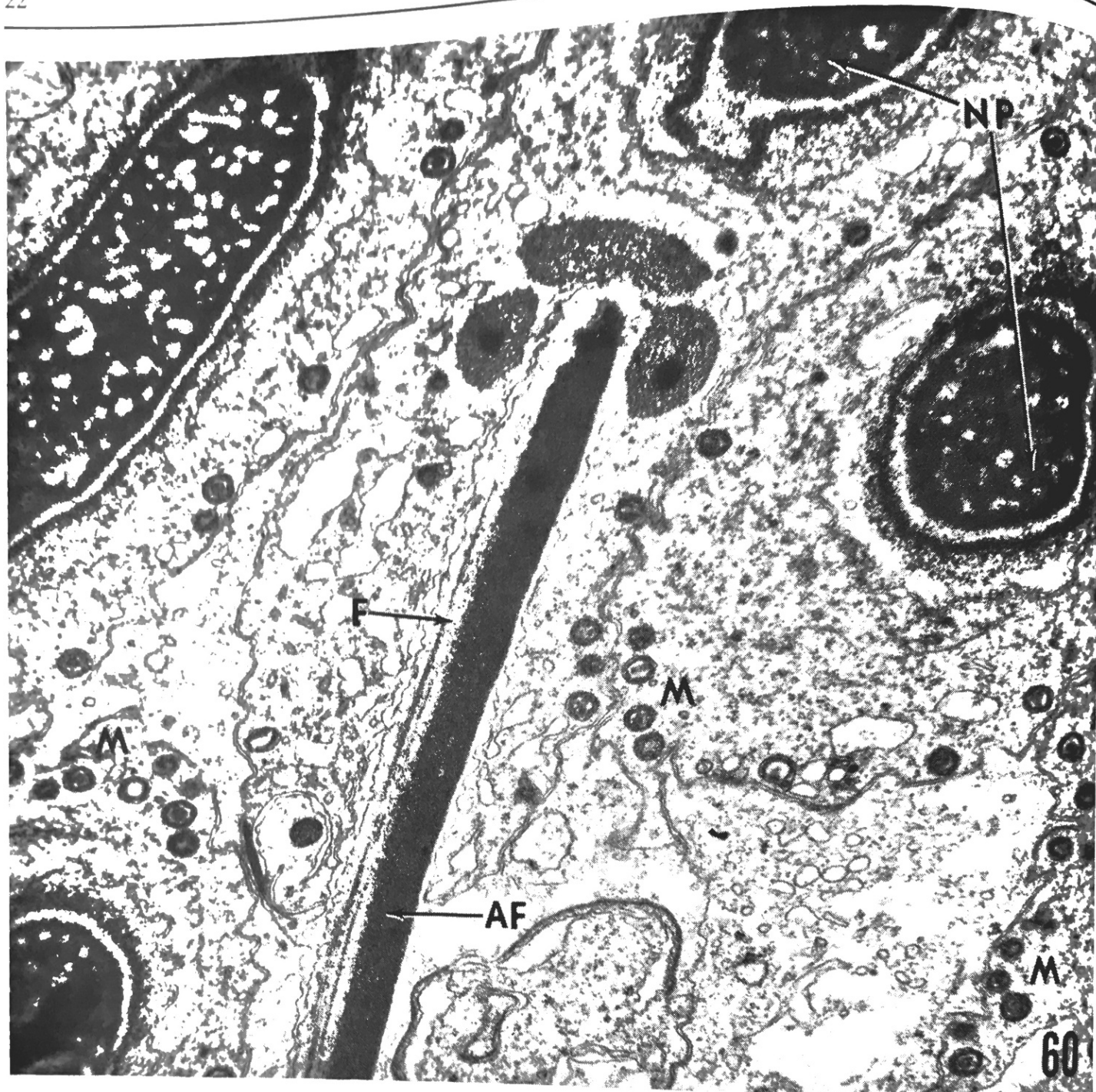The mitochondria do not form a nebenkern body as

Fig. 60. Electron micrograph of a section similar to those of Figs. 58 and 59. The neck piece is surrounded by a heavy nuclear membrane, the flagellum, F, is close to and parallels the axial filament, AF, and the mitochondria, M, tend to be distributed along the cell membrane. X 28,800.

is typical for insect sperm although such a structure has been mentioned for Ambystoma (Sotelo and Trujillo-Cenoz, 1958) as "constituted by many long filamentous mitochondria packed in bundles". This same study, however, shows isolated spherical mitochondria of similar size and appearance as described here. The cristae, present in sertoli cell mitochondria, disappear in the spermatids and seem to be transformed into a prominent dark peripheral ring and a dark central mass. In Squalus sperm they are described as "becoming spherical and develop a denser matrix, and the intramitochondrial membranes become concentrically oriented" (Stanley, 1964). The significance of this mitochondrial transformation will require further study.

The stimulus for motility appears to be restricted to the flagellum. There is no evidence for independent movement of the axial filament (centriolar edge of the undulating membrane, Gatenby, 1961) or that the undulating membrane can operate while the flagellum remains quiescent (Burgos and Fawcett, 1956). There is evidence for believing that the undulating membrane may appear to move while the flagellum is non-motile. The axial filament becomes smaller toward the end of the tail until it may be smaller than the flagellum, and a stimulus for motility originating in the flagellum may result in more extensive movement of the axial filament than the flagellum. Such stimulus may be the explanation of movement of the distal end of the sperm tail of
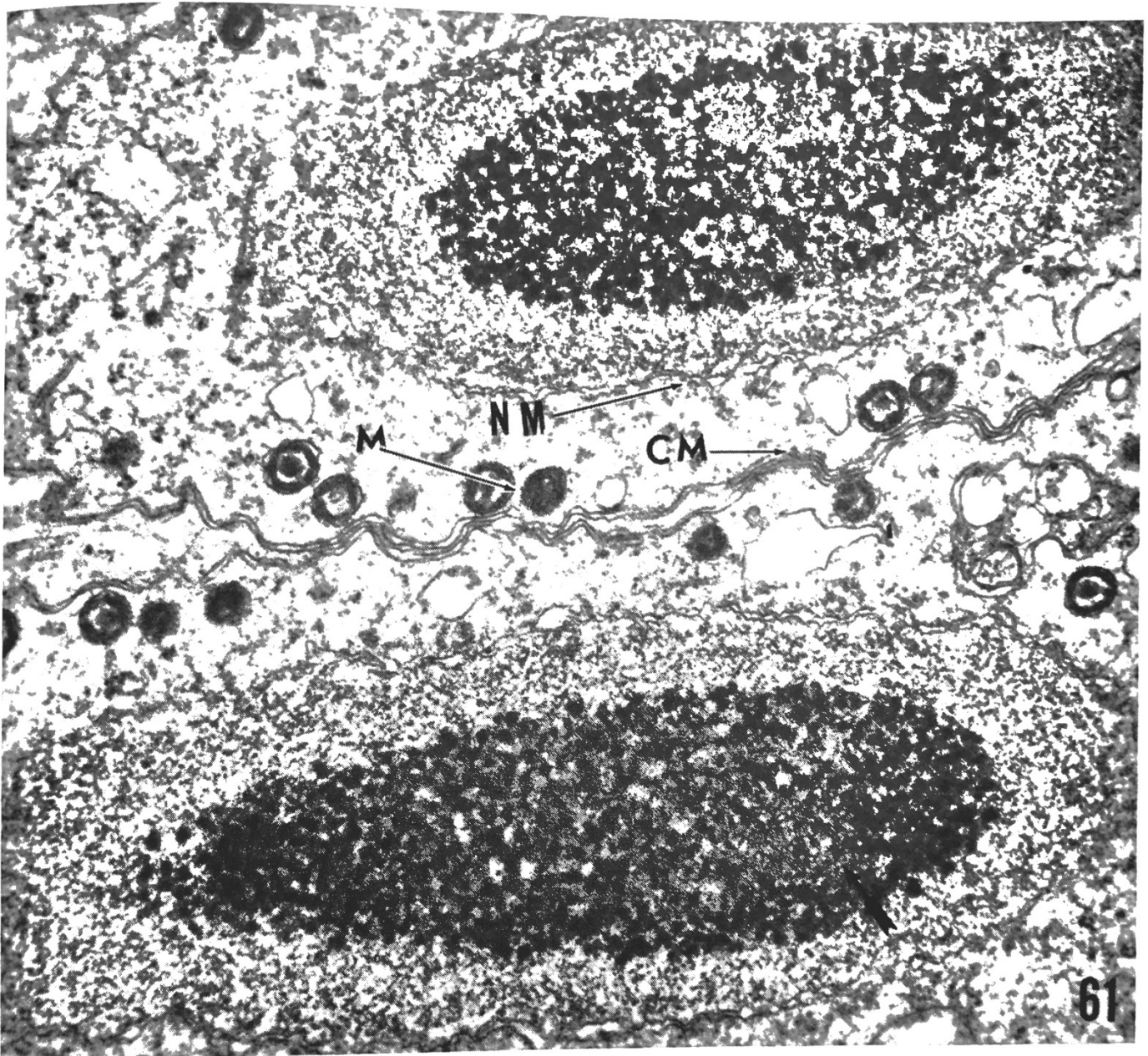
Fig. 61. Electron micrograph of cross sections of the nuclei of developing sperm. These are from the same tubules as those shown in Figs. 58 and 60. The double nuclear membrane, NM, is quite clear as is the double cell membrane, CM. The mitochondria, M, are distributed along the cell membrane. In the lower nucleus a nucleolus may be present (at arrow). X 44,640.

Necturus (Baker, 1963) where the "flagellum dominates the (axial) filament and bends it into a series of small helical coils that rotate with considerable speed."

This study is incomplete in that no observations have been made with the electron microscope on the origin of the granule and ring, the acroblast and acrosome, and no sections have been encountered that show the presumptive principal piece of an incompletely developed sperm. More than 100 sections of the mature sperm and an equal number of developmental stages have been examined but no sections included these stages of development. When one considers that more than 9,000 sections, of approximately 600 A in thickness, are necessary to view an entire sperm of *Diemictylus* by electron microscopy it is easy to understand that one can easily specialize on a study of only one part of a urodele sperm.

CONCLUSION

The sperm of three genera of Salamandridae may be used to indicate phylogenetic relationships within the family.

The neck piece of the sperm of *Diemictylus viridescens* arises from a granule, of unknown origin, and is enclosed partially by the nuclear membrane.

The ring, through which the axial filament and flagellum pass, has a dual structure of a proximal
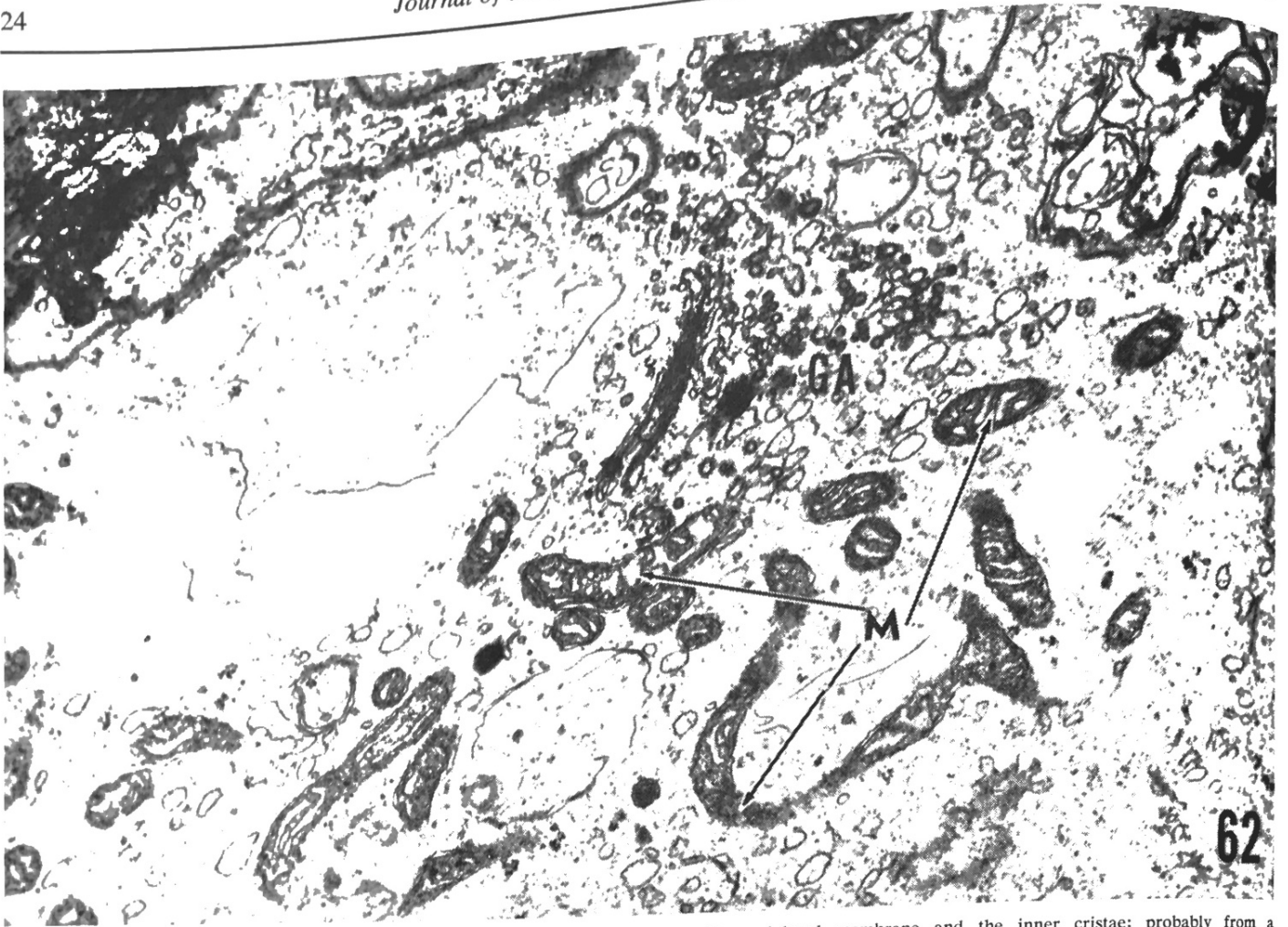
Fig. 62. Electron micrograph of normal mitochondria, M, with the typical double peripheral membrane and the inner cristae; probably from a sertoli cell. Cross sections of the tubules of the Golgi apparatus, GA, may be present. X 30,600.
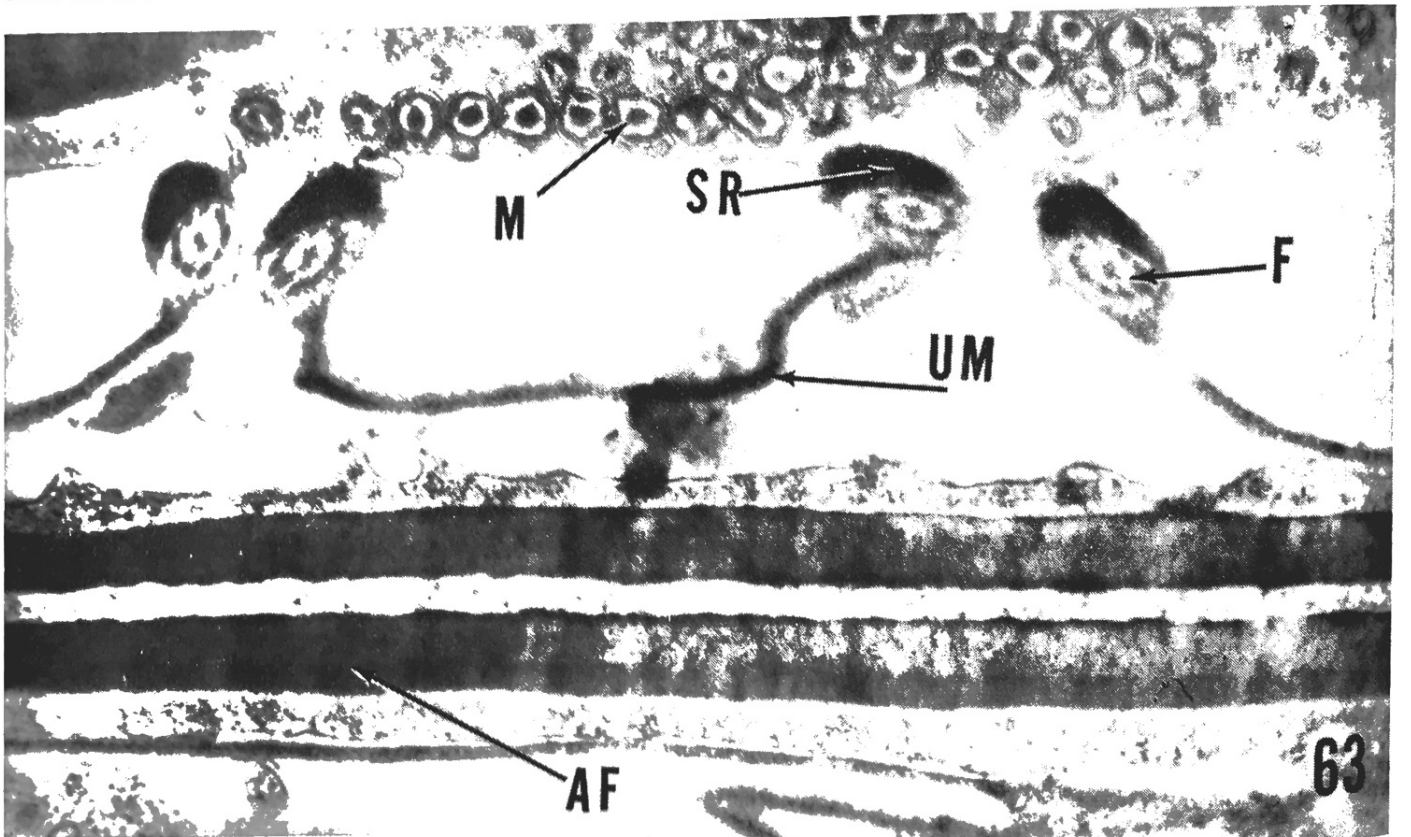

Fig 63. An electron micrograph of a longitudinal section of the middle piece with the U shaped axial filament, AF, flagellum, F, and undulating membrane, UM. The four sections of the same flagellum are evidence that it is arranged in a three dimensional coil somewhat like the top of a "ruffle." The usual flagellar supporting rod, SR, and the modified mitochondria, M, are evident. X 30,080.

granular material and a distal lamellar component of 16 layers.

A vibratile filament extends from the granule of a spermatid through the ring and then exterior to the cell. Its proximal portion is composed of an axial filament and a parallel flagellum while the distal part is of flagellum alone.

When the nucleus has elongated to form the needle or lance-shaped head and the ring has elongated to demark the middle piece, the flagellum, now separated from the axial filament but held in place by an undulating membrane, is drawn up alongside the axial filament into regular waves in a three dimensional coil.

Flagellar motility is reversible in the three genera of Salamandridae and a stimulus for motility may arise at any point along the flagellum and progress in either direction or, in some cases, both directions.

The flagellum is the only source of motility as there is no evidence that the axial filament or the undulating membrane has any independent motility.

There is no evidence of any barbs or hooks on the perforatorium or acrosome.

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NEWS OF TENNESSEE SCIENCE

Oak Ridge, Tenn.—Nearly six million persons viewed showings of the U. S. Atomic Energy Commission's domestic exhibits during 1965—the biggest year in the Commission's continuing program aimed at public understanding of the atom. Increased emphasis on the AEC's high school lecture-demonstration program and fabrication of new traveling exhibits highlight the Commission's 1966 exhibit program. The American Museum of Atomic Energy at Oak Ridge, now in its seventeenth year, had its biggest season to date with 130,445 visitors. The free display, the only museum in the nation devoted exclusively to atomic energy, brought 124,664 visitors to the Atomic City in 1964 and 127,104 in 1963. Museum visitors included more than 400 student-educational groups, 138 various size groups of foreign visitors and 181 miscellaneous groups. The New York World's Fair, which closed last fall, attracted the largest number of visitors. The Commission's two major exhibits at the Fair's Hall of Science, "Radiation and Man," and "Atomsville, U.S.A." were visited by 2,684,000 persons during the Fair's 1965 season. The exhibits are now being viewed elsewhere throughout the country.

A paper delivered by Dr. A. J. Sharp, University of Tennessee botany professor, has been selected for use in two major scientific books. The paper, entitled "The Compleat Botanist," was delivered in 1963 in Cleveland at a meeting of the American Association for the Advancement of Science. Dr. Sharp was vice president of that association at the time of his talk. The paper was first published in the Association's journal, *Science*. The paper was chosen for publication in *The New Treasury of Science*, published by Harper and Row and just recently released. The book covers current developments in nearly all sciences, and Dr. Sharp's article is one of five on plant life. The other book is a leading annual scientific book in Sweden, called *Svensk Naturvetenskap 1965*. Taking the word "compleat" from Izaak Walton's "The Compleat Angler," Dr. Sharp's paper deals with society's "urgent need" for trained botanists with a broad background and a liberal education.

The National Science Foundation has approved a grant of $25,000 to the University of Tennessee for the study of a fern that up to now has received little classification and intensive study. Dr. Murray Evans, of the U T botany department, will direct the research which is aimed at learning the reasons behind the specialization of some ferns to cold climates and others to warm environments. Dr. Evans seeks a better understanding of the genus of ferns named Polypodium, which prefers warm climates. Up to the present time, Polypodium has been largely ignored in its possible relationships to other