The ultrastructure of spermatogenesis and epididymal spermatozoa of the tuatara *Sphenodon punctatus* (Sphenodontida, Amniota)

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SUMMARY

By using transmission electron microscopy (TEM) the events of spermatogenesis are described for the first time in the tuatara *Sphenodon punctatus punctatus* (Gray), a representative of the 'reptilian' order Sphenodontida. Secondary spermatocytes contain two greatly elongate (8.0 μm), rod-shaped centrioles which lie parallel to one another and are each associated with a small deposit of dense material and a short centriole. Spermatids contain only one rod-shaped centriole (associated with a short centriole) which gives rise to the flagellar axoneme thereby becoming the distal centriole. Four stages of spermatid development can be distinguished: (i) the early stage (nucleus round; nuclear contents granular with a thin, condensed periphery; mitochondria scattered; acrosomal vesicle spheroidal, slightly depressed onto nuclear surface); (ii) the middle stage (nucleus pyriform with two endonuclear canals formed; nuclear contents fibro-granular with thick periphery; mitochondria chiefly posterior; acrosomal vesicle flattened; centriolar complex attached to nucleus); (iii) the advanced stage (nucleus elongate and rod shaped; nuclear contents coarsely granular; mitochondria (containing linear cristae) clustered around the distal centriole; acrosomal vesicle conical; centriolar complex attached to posterior fossa of nucleus); (iv) the late stage (nucleus very elongate and associated with a longitudinally arranged microtubular sheath; nuclear contents very condensed; midpiece fully formed and featuring mitochondria with concentric cristae and a dense intramitochondrial body; centrioles associated with a dense, lateral body). Testicular sperm have a conical acrosomal vesicle (length 4 μm) and subacrosomal cone, an elongate (length 54–56 μm) helical nucleus, a midpiece (length 8 μm, featuring spheroidal mitochondria containing concentric cristae and a dense body), an annulus, an elongate principal piece (length 74–78 μm, featuring a dense, fibrous sheath) and a short end piece (length 2–4 μm). Epididymal sperm differ from those in the testis by having a more developed lateral body in the midpiece and a sheath of flocculent material surrounding the fibrous sheath in the principal piece. The relatively large number of epididymal sperm still associated with a cytoplasmic droplet suggests that sperm spend a significant period maturing within the epididymis. The features of spermatogenesis and mature sperm suggest that the Sphenodontida are primitive amniotes, with only chelonians having fewer spermatozoal apomorphies while the crocodilians are little more advanced.

1. INTRODUCTION

The reptilian order Sphenodontida is represented by only two living species of the genus *Sphenodon* (*S. punctatus*, *S. guntheri*), popularly known as the New Zealand tuatara. Anatomically, *Sphenodon* spp. share numerous features with other amniote groups as well as possessing their own unique characteristics. Many recent workers have regarded the Sphenodontida as the sister group of the Squamata (snakes, lizards) (Fraser 1986; Evans 1984, 1988; Gauthier *et al.* 1988*a*,*b*) or as advanced squamates (Whiteside 1986), while cladistic analysis of sperm morphology (Jamieson & Healy 1992) suggests that sphenodontids are truly basal amniotes, only the turtles having fewer spermatozoal apomorphies.

To date the fine structure of spermatogenesis and spermiogenesis is known for all major groups of the 'Reptilia' with the exception of the Sphenodontida. (The term Reptilia is used in this account as a convenient name for the paraphyletic assemblage, within the Amniota, which includes sphenodontids, turtles, crocodilians and squamates but excludes birds and mammals.) Hogben (1921) studied changes in chromosome morphology in spermatogonia and spermatocytes of *Sphenodon punctatus* but, because of fixation limitations, did not include observations on spermiogenic stages or spermatozoa. The basic histology and seasonal activity of the testis of *S. punctatus* has recently been investigated (Saint Girons & Newman 1987), including the effects of varying sex hormone levels on gametogenesis (Cree *et al.* 1992). In

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a previous paper (Healy & Jamieson 1992) we detailed the ultrastructure of mature testicular spermatozoa and also presented some relevant observations on developing cells in order to determine the origins and therefore the homology of certain key features subsequently used in cladistic analysis (Jamieson & Healy 1992). In the present account we describe for the first time the ultrastructure of spermatogenesis in *S. punctatus*, and conclude with a discussion of comparative spermatogenesis and sperm morphology within the Amniota. We also take the opportunity to demonstrate the ultrastructure of mature spermatozoa taken from the epididymis of *Sphenodon punctatus* and contrast these cells with testicular spermatozoa described by us previously (see Healy & Jamieson 1992).

2. MATERIAL AND METHODS

All testis and epididymal tissues of *Sphenodon punctatus punctatus* (Gray) processed for TEM originate from material supplied by Dr Alison Cree (University of Otago) (material was previously studied at light microscopical level for histology of the testis; see Cree *et al.* 1992). The samples used were fixed and stored in neutral buffered 10% formalin and collected from recently dead or injured males at Stephens Island, New Zealand between July 1987 and February 1989. Our CITES number permitting research on *S. punctatus punctatus* is AU 005.

The following testis tissues were used: sample 1, July 1987, collected by A. Cree and L. J. Guillette (primary and secondary spermatocytogenesis only); sample 2, February 1989, collected by M. McIntyre, M. Brown, R. Ainsworth (full spermiogenesis and mature sperm); sample 3, March 1988, collected by B. Firth and M. B. Thompson (spermiogenesis near end, some mature spermatozoa). All spermatogenic observations presented here were made on cells in sample 2. The epididymal sample (ducts containing mature sperm) derives from the same individual as sample 2 spermatogenic material. For transmission electron microscopy (TEM) small portions (1–2 mm^3) of testis and clumped epididymal spermatozoa were rinsed thoroughly in 0.1 M phosphate buffer (3 × 10 min), osmicated (2 h, 1% osmium tetroxide in buffer), dehydrated in ethanol (ascending from 20%, 40%, etc. to absolute, 30 min each step) then infiltrated and embedded in Spurr's epoxy resin (50%, 75%, 100%). All stages from the initial buffer rinse to 70% ethanol were conducted at 0–4°C and thereafter at room temperature. A LYNX EL Tissue Processor was used to prepare all samples. Semithin and ultrathin sections were cut using an LKB IV 2120 Ultrotome and glass or diamond knives. Ultrathin sections were stained with uranyl acetate and lead citrate according to the method of Daddow (1986, a contrast enhancing double stain method). Grids were examined with a Hitachi 300 TEM operated at 75 kV. Light microscopic observations of spermatozoa, from tissue squashes, were made using an Wild M20 microscope adjusted for phase-contrast microscopy.

3. RESULTS

(*a*) *Spermatogonia and Sertoli cells*

Spermatogonia could not be positively identified in the testicular material examined. Sertoli cells, however, were commonly observed, and could be identified by presence of a polygonal, oblong nucleus (14.0 × 10.0 μm) and an extensive cytoplasm featuring numerous spherical, dense vesicles (figure 1*a*). Typically the Sertoli cells were positioned among the spermatocytes but not always in close proximity to the basal epithelium of the seminiferous tubules. Aside from the dense vesicles, other cytoplasmic components of the Sertoli cells and the plasma membrane were not well preserved. This prevented detailed investigation of the relationship of the Sertoli cells with developing spermatocytes or spermatids.

(*b*) *Spermatocytes*

In spermatocytes, the nucleus is ovate to spheroidal, approximately 9.0–11.0 μm in diameter and, depending on the division stage reached, may exhibit irregular patches of dense chromatin or chromosomal cores (sometimes observed associated with synaptinemal complexes) (figure 1*a*,*b*,*d* and *e*). Small, round to oblong mitochondria and scattered endoplasmic reticular cisternae are scattered throughout the cytoplasm (figure 1*d*). Presumably a Golgi complex is also present in these cells but this organelle could not be identified with certainty. Of particular interest is the consistent presence of very elongate (8.0 μm long), paired, rod-shaped centrioles within the spermatocyte cytoplasm (figure 1*b*–*g*). Each centriole shows triplet microtubular substructure although this is often not clearly visible because of the enveloping, granular matrix (figure 1*f*). The two centrioles lie parallel to one another and each is associated, near one end, with a spheroidal deposit of dense material (figure 1*b*,*d*,*e*–*g*) and a short centriole, the latter arranged approximately perpendicular to the elongate centriole (figure 1*c*).

(*c*) *Spermiogenesis*

(i) *Early spermatids*

In early spermatids the nucleus is spheroidal, with dense chromatin distributed as irregular patches and a thin peripheral layer (figure 1*i*). Only one elongate centriole (and associated short centriole and dense deposit) is present in the cytoplasm of early spermatids, suggesting that elongate centriolar pairs observed in spermatocytes (see above) are partitioned between the two spermatids during the final meiotic division of the spermatocyte. Generally the triplet microtubular substructure of centrioles is more readily apparent in spermatids (see figure 1*h*) than in spermatocytes (figure 1*f*). Mitochondria, endoplasmic reticular cisternae and numerous ribosomal granules are observed throughout the cytoplasm, the mitochondria exhibiting typical (i.e. linear) cristae. Cytoplasmic bridges, reinforced by dense collars, connect spermatids (figure 1*i*). The Golgi complex appears to be poorly developed throughout spermiogenesis in *Sphenodon* and is

not well preserved in the tissues available for study. Nevertheless the acrosomal vesicle, a presumed Golgi product, is clearly visible even at the early spermatid stage (figure 2*a*). On first contacting the nucleus the acrosomal vesicle is spheroidal and its contents sparse, finely granular and only slightly electron dense. A thin, curved, subacrosomal layer lies between the nuclear surface (similarly curved in this region) and the base of the acrosomal vesicle (figure 2*a*).

(ii) *Middle stage spermatids*

In middle stage spermatids, the nucleus is initially oval and its contents very dense peripherally and granular internally (figure 2*b*). The anterior and posterior poles of the spermatid nucleus become identifiable by the attached acrosomal complex (anteriorly) and newly attached centriolar apparatus (posteriorly).

As development proceeds, the nucleus becomes pyriform and the acrosomal complex drapes over the nuclear apex (figure 2*c*). Small, highly electron-dense granules cover the outside of the acrosomal vesicle, particularly on the anterior surface (figure 2*e–h*). Vesicles containing such granules are usually seen within the acrosomal vesicle, and appear to arise by budding off internally from the acrosomal vesicle membrane (figure 2*h* and inset). Contents of the acrosomal vesicle are fibro-granular and sparse (figure 2*e–i*). The subacrosomal material, like the acrosomal vesicle gradually adopts a conical shape (figure 2*g,h*), and eventually will be transformed into the subacrosomal cone of late spermatids and mature spermatozoa (see figures 3*f* and 4*a*).

Commencing anteriorly and progressing posteriorly, the inner granular contents of the nucleus are gradually converted to a fibro-granular consistency (figure 2*b,c,e–h*). During this phase two narrow invaginations into the nucleus develop immediately beneath the attached acrosomal complex (figure 2*e,f*). Both invaginations or 'endonuclear canals' as they are termed in mature sperm (Furieri 1970; Healy & Jamieson 1992), contain a dense perforatorial deposit and are eccentrically positioned in transverse sections (figure 2*i,j*). The developing endonuclear canals were exceptionally difficult to trace in longitudinal section (see figure 2*f*) because of their narrow diameter and helical arrangement (see § 3*d*). However, as figure 2*f* shows, these canals extend posteriorly past the acrosomal region, deeper into the nucleus in middle stage spermatids.

A loose network, composed of moderately electron-dense material and a less dense background matrix, gradually develops around the centriolar region (figure 2*b–d*). The elongate, rod-shaped centriole, which now can be termed the distal centriole or basal body, gives rise to the flagellar (9 + 2 substructure) axoneme (figure 2*c*). At this stage the distal centriole becomes penetrated by the pair of singlet microtubules from the axoneme. Mitochondria throughout the middle spermatid phase remain small with unmodified cristae and begin to collect posteriorly around the distal centriole in preparation for midpiece development (figure 2*c,d*).

(iii) *Advanced spermatids*

Advanced (elongating) spermatids can be recognized by their narrow (1.0 μm) cylindrical nucleus, the contents of which have been converted to coarse granules which sometimes interconnect (figure 3*a,b*). Microtubules are arranged in a low angle helical sheath around the nucleus but were often difficult to discern because of the background granularity of the cytoplasm.

The distal and proximal centrioles are now closely attached to and partly inserted within the nuclear base. As figures 3*a* (inset) and 3*d* show, the C tubules of the proximal and distal centriole are open (i.e. incomplete). The distal centriole also exhibits a central pair of singlet microtubules (continuous with those of the axoneme), nine coarse fibres and a lateral body (figure 3*a–d*); the latter feature almost certainly is derived from condensation of the loose network of pericentriolar material first observed in mid-spermatids (see figure 3*c*). In some sections the lateral body shows traces of periodic striations (see Healy & Jamieson 1992) and sometimes, electron-lucent lacunae.

In advanced spermatids, mitochondrial cristae are arranged parallel to each other, while within the matrix an eccentrically positioned intramitochondrial granule is usually observed (figure 3*c*).

(iv) *Late spermatids*

On reaching the late spermatid stage, the acrosome has become attenuate and conical although still associated with traces of cytoplasm. A sheath of longitudinally aligned microtubules now surrounds the nucleus (figure 3*g*). The nucleus itself exhibits highly condensed contents as well as two rod-filled endonuclear canals, and, like the acrosomal complex, has essentially reached its final form (figure 3*f*). Mitochondria are spherical and are arranged around the elongate distal centriole to form the midpiece. A poorly defined sheath of microtubules, probably continuous with that surrounding the condensing nucleus, is associated with midpiece development. Mitochondrial cristae are transformed into multiple concentric membranes which surround a dense, centrally positioned, intramitochondrial body (figure 3*e,j*). Although a well developed annulus is present at the posterior limit of the mitochondria, the fibrous sheath of the principal piece has only just begun to form (figure 3*h*).

(*d*) *Spermatozoa*

(i) *Testicular spermatozoa*

The fine structure of testicular spermatozoa from *Sphenodon punctatus* has been described in a previous account (Healy & Jamieson 1992). For the purposes of comparison and completeness, however, it is appropriate to restate the basic features of these cells.

Testicular spermatozoa consist of an acrosomal complex (length 4 μm), elongate, helical nucleus (length 54–56 μm), a midpiece (length 7–8 μm), an elongate principal piece (length 74–78 μm) and short end piece (length 2–4 μm).

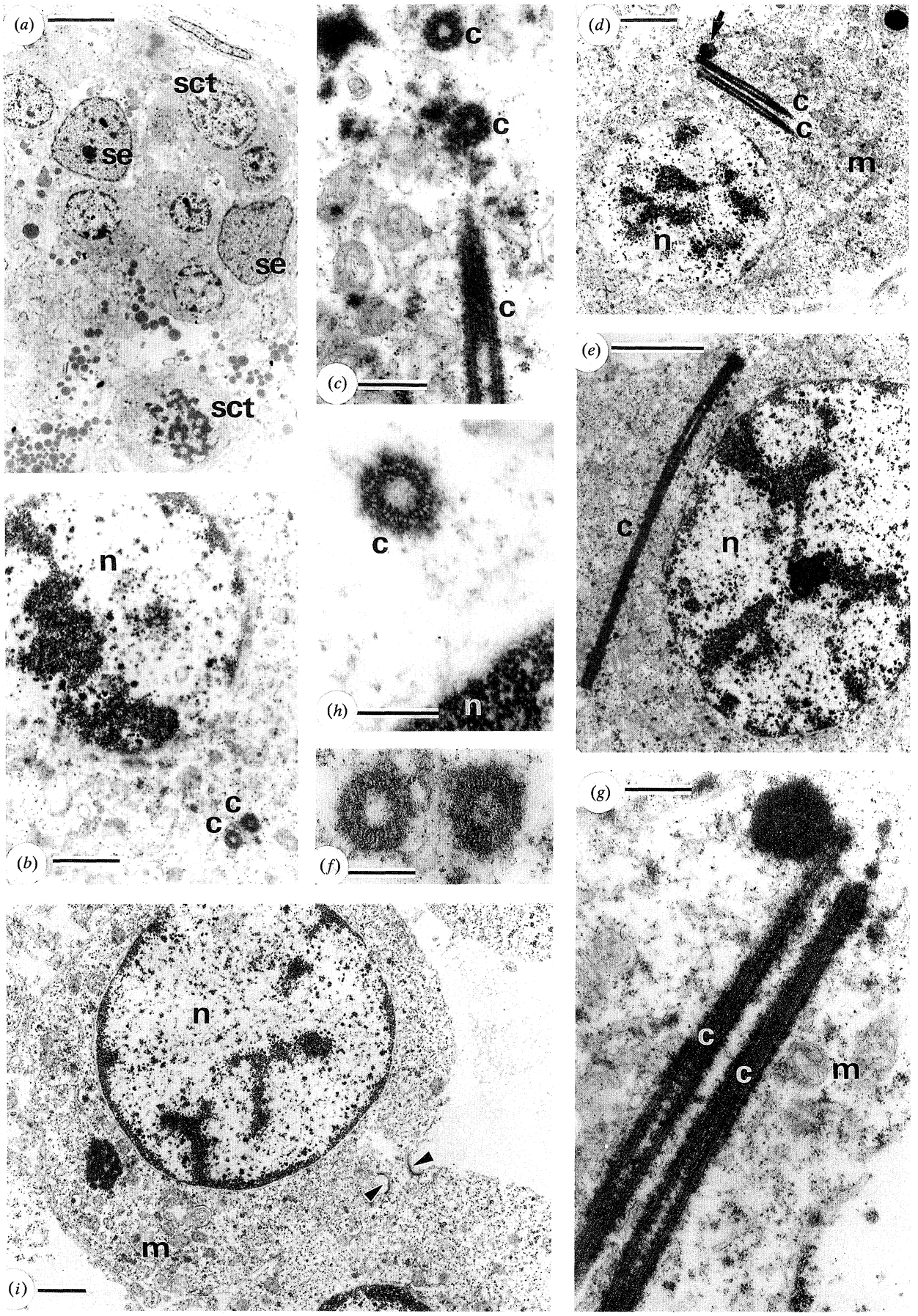

Figure 1. For description see opposite.

The acrosomal vesicle and underlying subacrosomal (inner core) material form a double, curved sheath around the nucleus anteriorly (see figure 3*f*). Two parallel, helically twisted, endonuclear canals (diameter 45 nm) each containing perforatorial material, extend posteriorly from the apex of the nucleus to at least 2.5 μm below the acrosomal complex (figure 3*f* inset). A shoulder of the nucleus marks not only the posterior limit of the acrosomal complex but also the point at which anterior nuclear tapering begins (figure 2*k*). Longitudinal sections of late spermatids show the presence of a nuclear fossa, proximal centriole (triplet substructure, with open C tubules) and an extensive deposit of pericentriolar material,the lateral body, which projects slightly into the nuclear fossa and posteriorly lies in contact with the elongate distal centriole.

The lateral body of testicular sperm is collar-shaped anteriorly and cylindrical distally (within the midpiece) with occasional electron-lucent lacunae. Spherical mitochondria (diameter 0.7–0.9 μm), characterized by concentric cristae and a centrally positioned, often very dense, intramitochondrial body, are grouped around the elongate distal centriole (and posterior portion of the lateral body) to form the midpiece (figure 3*i*,*j*). Eight or nine mitochondria are present anteriorly decreasing to five or six posteriorly (figure 3*j*). Proximal and distal centrioles, despite their marked difference in length, both show an open C tubule within the triplets. A well-developed annulus, attached to the inner surface of the plasma membrane defines the posterior extremity of the midpiece (figure 3*i*). Transverse sections also show that the transition from distal centriole to axoneme occurs within the region of the annulus and that peripheral fibres associated with doublets 3 and 8 are thicker than the other seven fibres (see figure 3*k*). These thicker fibres possibly are continuous with the dense rods which connect doublets 3 and 8 with the fibrous sheath in the principal piece.

The principal piece consists of a 9+2 axoneme surrounded by a highly electron-dense, fibrous sheath (composed of helically coiled and anastomosing fibres) and the plasma membrane (figure 3*i*,*k*). Initially the axoneme emerges from the midpiece accompanied by all nine peripheral fibres (figure 3*k*) but these rapidly decrease in diameter and with the exception of fibres 3 and 8 terminate within the anterior region of the principal piece. Diameter of the principal piece decreases from 0.73 μm anteriorly to 0.38 μm near the end piece. Rod-shaped structures connect doublets 3 and 8 to the periaxonemal surface of the fibrous sheath.

The end piece (length 2–4 μm) of the spermatozoon was not observed ultrastructurally.

(ii) *Epididymal spermatozoa*

Epididymal spermatozoa closely resemble mature testicular spermatozoa in most aspects including the morphology of the acrosome, helical nucleus (including paired endonuclear canals, figure 4*a*,*b*), arrangement of the centrioles and midpiece (centrioles with open C tubules, figure 4*e–g*; elongate distal central centriole with central pair of microtubules, figure 4*g*; prominent lateral body, figure 4*c–e*; mitochondria with concentric cristae and dense intramitochondrial body, figure 4*c*,*e–g*) and presence of a prominent annulus and extensive principal piece (with helical fibrous sheath, figure 4*h–j*). However, we have noted some significant differences between epididymal and testicular sperm. First, in epididymal sperm, the lateral body completely ensheaths the distal centriole anteriorly (figure 4*c*,*d*; versus lateral body of lesser extent in testicular sperm) and becomes a half-sheath posteriorly (figure 4*e*). Second, the anterior extremity of the fibrous sheath projects just inside the annulus (figure 4*f*,*h*; versus annulus and fibrous sheath well separated in testicular sperm, see figure 3*i*). Third, the anterior region of the principal piece not only possesses a fibrous sheath but also has an outer layer of granulo-fibrous material (figure 4*h–j*; such material is absent in testicular sperm, figure 3*j*). A significant proportion of the epididymal sperm observed were still associated with a cytoplasmic droplet, indicating that the epididymis acts not only as a sperm storage organ but also as a site where maturation is completed.

4. DISCUSSION

(*a*) *Sertoli cells and spermatocytes*

Insufficient information on the ultrastructure of spermatocytes or Sertoli cells of reptiles exists to present any useful comparison with *Sphenodon*. From what we can ascertain, the basic morphology of these cells in all reptiles is probably similar to that of *Sphenodon* (e.g. Sertoli cells of turtles; see Sprando & Russell 1988) but this requires confirmation by further work on a range of taxa.

(*b*) *Spermatids and spermatozoa*

(i) *Acrosomal complex*

Acrosomal development in *Sphenodon* resembles most closely the pattern reported in crocodiles (Saita *et al.* 1987), turtles (Sprando & Russell 1988) and non-

Figure 1. *Sphenodon punctatus* : Sertoli cells, spermatocytes, early spermatids. (*a*) Sertoli cells (note dense vesicles) among spermatocytes. (*b*) Spermatocyte with pair of elongate centrioles shown in transverse section. (*c*) Two short centrioles in spermatocyte shown associated with one of two rod-shaped centrioles. (*d*) Rod-shaped centriole pair in spermatocyte. Note deposit of dense material (arrow). (*e*) One of the pair of rod-shaped centrioles shown full length in cytoplasm of spermatocyte. (*f*) Pair of rod-shaped centrioles (in spermatocyte) shown in transverse section. (*g*) Dense material associated with rod-shaped centrioles (in spermatocyte). (*h*) Triplet substructure of spermatid centriole. (*i*) Early spermatid showing round nucleus. Note also cytoplasmic bridge (arrow heads). Scale bars: (*a*) 10.0 μm; (*d*,*e*) 2.0 μm; (*b*,*i*) 1.0 μm; (*c*,*g*) 0.5 μm; (*f*,*h*) 0.25 μm. Abbreviations: c, centriole(s); m, mitochondria; n, nucleus; sct, spermatocyte; sc, Sertoli cell.

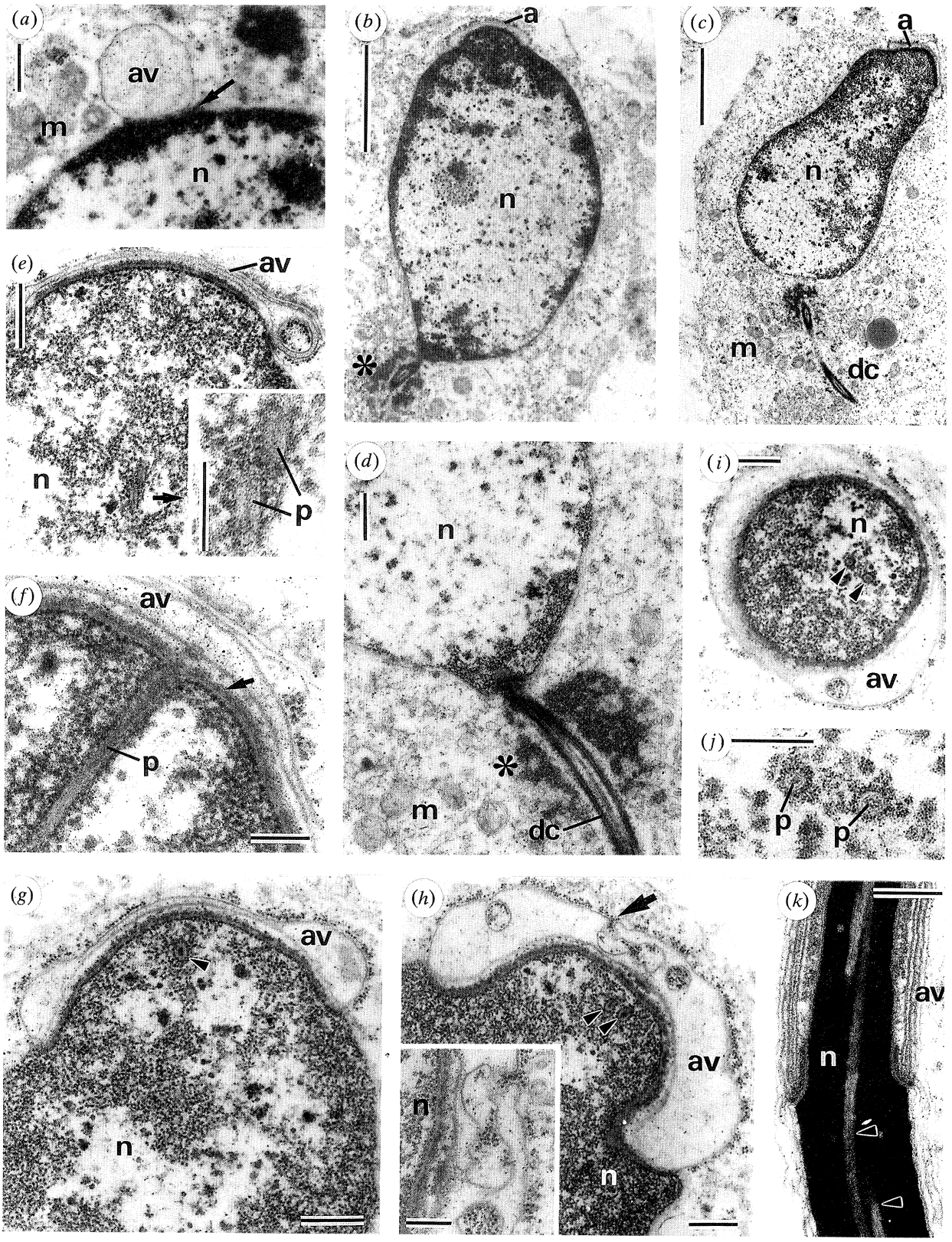

Figure 2. For description see opposite.

passerine birds (for example, the domestic fowl *Gallus domesticus* (Sprando & Russell 1988); and quail *Coturnix coturnix* (Saita *et al.* 1980)). In contrast, during spermiogenesis in squamates the acrosomal vesicle exhibits an obvious basal granule (within the vesicle). In addition, endonuclear (perforatorial) canals, such as those observed in *Sphenodon*, turtles, crocodiles and non-passerine birds, are never formed in squamates (Healy & Jamieson 1992).

(ii) *Nucleus*

In advanced spermatids of *Sphenodon* the contents of the nucleus are converted into closely packed, dense granules which eventually fuse into a loose network. Elsewhere among the Amniota this type of condensation occurs only in turtles, crocodilians and non-passerine birds (see also Yasuzumi *et al.* 1971; Saita *et al.* 1987; Sprando & Russell 1988). In squamates, nuclear condensation follows a complex, fibrous-lamellar pattern (see Clark 1967; Cruz-Hofling & Cruz-Landim 1978; Butler & Gabri 1984; Al-Hajj *et al.* 1987; Saita *et al.* 1988*a*,*b*; Carcupino *et al.* 1989). It seems likely that the simpler, granular pattern seen in *Sphenodon*, turtles, crocodiles and non-passerine birds represents the primitive mode of nuclear condensation for the Amniota. Recently Saperas *et al.* (1993) have shown that the two patterns of condensation occurring in teleost fish (globular versus fibrillar) can be correlated with differing nuclear proteins. By contrast, major differences in sperm basic proteins do not exist between the crocodilians, turtles and squamates although these groups do show differing percentages of arginine (see Kasinsky *et al.* 1987). Chiva *et al.* (1989) have shown that the amino acid composition of sperm basic proteins in turtles (using the freshwater turtle *Chrysemys picta*) is similar to that found in bird sperm protamines. In the context of this result it would be interesting to determine the type and proportion of nuclear proteins (especially protamine) occurring in spermatozoa of *Sphenodon*. In view of the strong morphological similarities between sperm of *Sphenodon* and those of turtles, we would predict that *Sphenodon* sperm nuclear proteins would not differ substantially from those recorded by Chiva *et al.* (1989) for *Chrysemys picta*.

The changing arrangement of perinuclear microtubules during spermiogenesis in *Sphenodon*, that is, from a low angle helical orientation in advanced spermatids to longitudinal in late spermatids, has been observed in all other groups of amniotes (turtles: Yasuzumi & Yasuda 1968; crocodiles: Saita *et al.* 1987; squamates: Clark 1967; Cruz-Hofling & Cruz-Landim 1978; Al-Hajj *et al.* 1987; Saita *et al.* 1988*a*,*b*; and Dehlawi *et al.* 1992; non-passerine birds: Phillips & Asa 1989; monotreme mammals: Carrick & Hughes 1982). Although most authors postulate that the low-angle helically arranged microtubules simply reorientate longitudinally late in spermiogenesis, Saita *et al.* (1987) have presented evidence to suggest that two distinct generations of microtubules are actually being produced, at least in *Caiman crocodilus* (low-angle helical and longitudinal microtubules of differing diameter coexisting in the cytoplasm of many cells).

Development of the endonuclear canals in *Sphenodon* probably commences at the early spermatid stage, as indicated here and as also occurs in crocodiles (Saita *et al.* 1987), turtles (see figure 1 (micrograph) of Sprando & Russell 1988) and certain non-passerine birds (see figure 18 (micrograph) of Sprando & Russell 1988). Similar canals occur in sperm of sarcopterygian fish (coelacanth: Mattei *et al.* 1988; and lungfish (*Neoceratodus*): Jespersen 1971; B. G. M. Jamieson, unpublished results) and primitive frogs (for example, *Ascaphus*, Jamieson *et al.* 1993). This confirms the endonuclear canals as symplesiomorphies of tetrapods. Almost certainly these canals and their granular contents perform some perforatorial function (for further discussion see Healy & Jamieson 1992), although it has also been suggested that they may be involved with nuclear metabolism (Yasuzumi *et al.* 1971). The homology of endonuclear filaments occurring in lampreys and sturgeon (see Jamieson 1991) with those of sarcopterygians is questionable. Those in the sturgeon are supposedly non-perforatorial (Cherr & Clark 1984).

(iii) *Midpiece: mitochondria and centrioles*

The transformation of unmodified spermatid mitochondria (cristae linear) into the specialized mitochondria of late spermatids and mature sperm (concentric cristae surrounding a dense intramitochondrial body), has elsewhere been observed only in turtles (Yasuzumi & Yasuda 1968), crocodilians (Saita *et al.* 1987) and in a single species of mammal (the woolly opossum *Caluromys philander* (Phillips 1970); it should, however, be noted that woolly opossum mitochondria are

Figure 2. *Sphenodon punctatus*: spermiogenesis. (*a*) Early spermatid with newly attached acrosomal vesicle and (arrow) curved layer of subacrosomal material. (*b*) Middle stage spermatid showing ovate nucleus, flattening acrosomal vesicle (anteriorly), attached centriolar complex (posteriorly) and pericentriolar network (*). (*c*) Middle stage spermatid (slightly more advanced than shown in figure 2*b*). (*d*) Middle stage spermatid. Centriolar complex attached to base of nucleus and network of pericentriolar material (*). (*e*) Middle stage spermatid showing flattening acrosomal vesicle and oblique section of endonuclear canals; (*e*, inset) detail of endonuclear canals and perforatoria. (*f*) Apical region of an endonuclear canal (containing perforatorium) showing confluence with subacrosomal material (arrow). (*g*,*h*) Acrosomal vesicle of middle stage spermatid; arrow heads identifying endonuclear canals, arrow indicating budding-off of vesicles of dense granules; (*h*, inset) detail of budding vesicle. (*i*) Transverse section through acrosome and nuclear apex. Note endonuclear canals (arrow heads). (*j*) Detail of endonuclear canals shown in figure 2*i*. (*k*) Junction of acrosome and nucleus showing helically twisting in endonuclear canals (arrow heads) in testicular spermatozoon. Scale bars: (*a*,*d*,*e*,*g*–*i*) 0.5 μm; (*e* inset,*f*,*h* inset,*j*,*k*) 0.25 μm; (*b*,*c*) 2.5 μm. Abbreviations: a, acrosomal complex; av, acrosomal vesicle; dc, distal centriole; m, mitochondria; n, nucleus; p, perforatoria (within endonuclear canals).

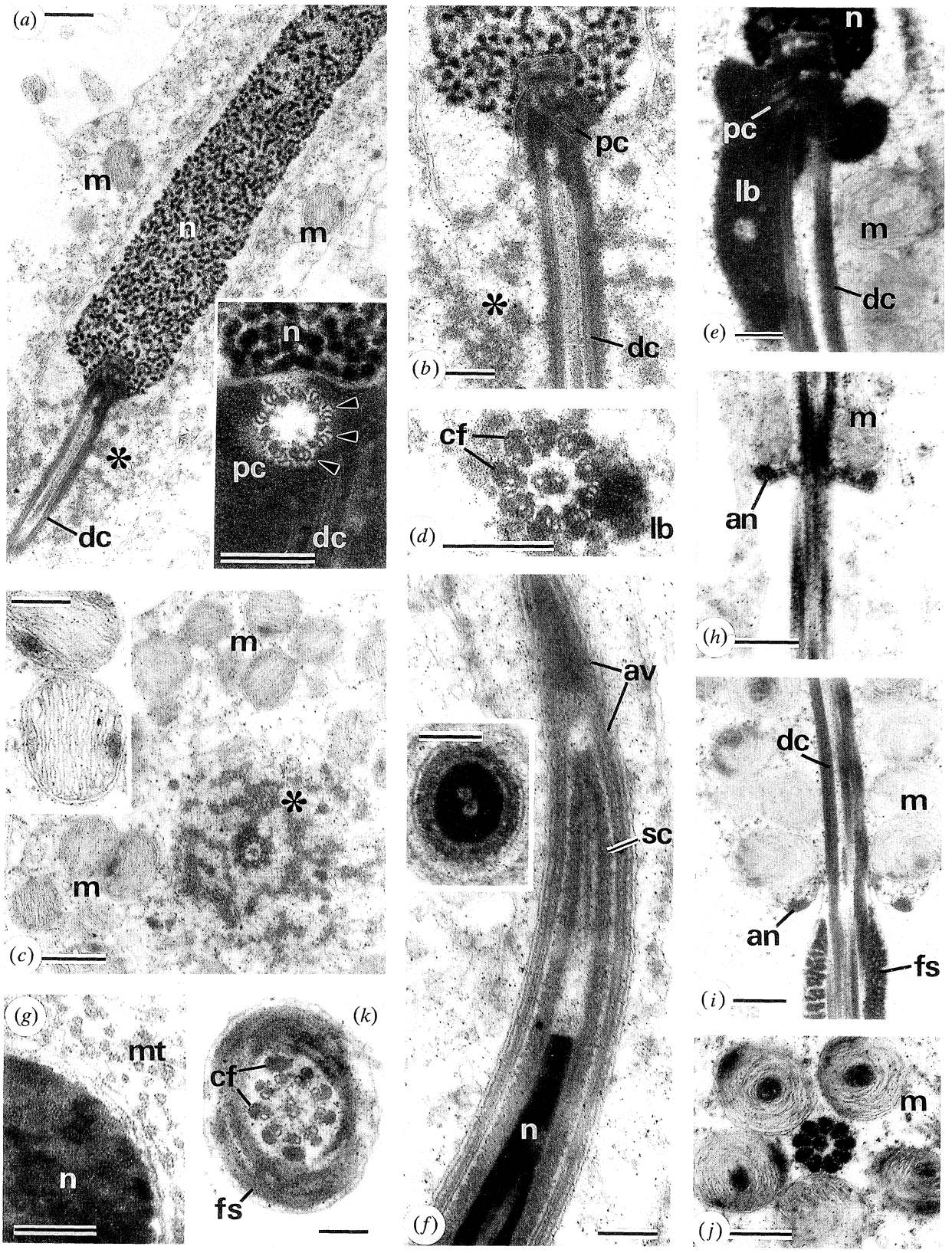

Figure 3. For description see opposite.

flattened, and helically arranged around the axoneme in contrast with *Sphenodon*, turtles and crocodiles in which mitochondria rounded, stacked longitudinally around the distal centriole.). Although the mitochondria of squamates also undergo substantial structural changes during spermiogenesis, these involve an increase in mitochondrial length, helical coiling of mitochondria around the axoneme and the production of dense intra-mitochondrial rings or bodies (Jamieson & Healy 1992; Jamieson & Scheltinga 1993). The exact significance of the substructural changes in mitochondrial cristae occurring in *Sphenodon* (Healy & Jamieson 1992, this paper), turtles (Yasuzumi & Yasuda 1968), crocodilians (Saita *et al.* 1987) and the woolly opossum *Caluromys* (Fawcett 1970) is still unclear, although Yasuzumi & Yasusuda (1968) have related these changes to a possible need for increased mitochondrial efficiency (and therefore cristal surface area) during sperm storage.

The presence of a central pair of microtubules within the lumen of the distal centriole in turtles and crocodiles has led some authors (for example, Furieri 1970; Saita *et al.* 1987) to interpret this elongate structure as the anterior portion of the axoneme. The triplet substructure of this centriole has now been convincingly demonstrated for turtles and *Sphenodon* (see Hess *et al.* 1991; Healy & Jamieson 1992). In addition, Phillips & Asa (1991) present a series of micrographs which establish the presence of a rod-shaped centriole in spermatids of the American alligator (*Alligator mississipiensis*). Aside from crocodilians, turtles and *Sphenodon*, penetration of the elongate distal centriole by paired singlet microtubules late in spermiogenesis has also been recorded in palaeognath birds (Phillips & Asa 1989; Baccetti *et al.* 1991). Presumably these axonemal singlets within the centriolar lumen strengthen the centriole-axoneme connection. The presence of a rod-shaped distal centriole running the full length of the sperm midpiece must be considered an amniote plesiomorphy and not, as suggested by Baccetti *et al.* (1991), an autapomorphy of palaeognath (ratite) birds. Phillips & Asa (1991) correlate the occurrence of this elongate centriole with the first of two modes of midpiece formation in reptiles (1, annulus always posterior to centriole (for example alligator); 2, annulus sliding past centriole during spermiogenesis in squamates). Although Phillips & Asa (1991, p. 1000) state that the first mode of midpiece formation 'will not work if the midpiece is longer than 4 or 5 microns' our study has shown that a midpiece of 8.0 μm can be produced using the same mode. This finding redefines the upper limit for midpiece length in amniote sperm which exhibit a rod-shaped, distal centriole (crocodilians, chelonians, *Sphenodon*, palaeognath birds).

One of the more remarkable features of spermatogenesis in *Sphenodon* is the presence of parallel, highly elongate (8.0 μm long) centrioles in spermatocytes. In spermatids and spermatozoa only one of these rod-shaped centrioles persists. Although no ultrastructural data exist on centrioles in turtle spermatocytes, a detailed light microscopic study by Risley (1936) clearly demonstrates that centrioles are paired and rod-shaped in spermatogonia, spermatocytes and spermatozoa of the freshwater turtles *Sternotherus odoratus* (the musk turtle) and *Malaclemmys centrata* (the diamond-back terrapin). Risley notes that the two rod-shaped centrioles of *Stenotherus* and *Malaclemmys* most commonly are observed as separate entities or as temporary V-shaped arrangements in spermatogonia and most secondary spermatocytes (there are two pairs of elongate centrioles in primary spermatocytes), but during 'interkinesis' of secondary spermatocytes, the centrioles lie parallel to each other and tangential to the nuclear surface; the arrangement described here for *Sphenodon*. Further, Risley established that only one rod-shaped centriole of the pair passes to each newly formed spermatid in *Stenotherus* and *Malaclemmys*; again as observed by us for *Sphenodon*, although here, by TEM, shown to be accompanied by a short centriole.

Surrounding the attached centrioles of the developing midpiece in *Sphenodon* is a loose network of dense material. Although we were unable to determine with certainty the origin of this material, its close association with the centrioles suggests that it may be derived through centriolar influence on precursor materials occurring in the cytoplasm. It remains to be determined whether the dense spheroidal deposits attached to one end of the rod-shaped centrioles in spermatocytes of *Sphenodon* are chemically similar to the more extensive pericentriolar material (probable lateral body precursor) of spermatids. It seems likely

Figure 3. *Sphenodon punctatus*: spermiogenesis. (*a*) Advanced spermatid. Basal portion of nucleus (note granular condensation pattern), attached centriolar complex and pericentriolar network of dense material (*); (*a*, inset) proximal and distal centrioles embedded in lateral body. Note open C tubules (arrow heads). (*b*) Detail of attached centrioles and nuclear invagination of advanced spermatid. Note pericentriolar network (*). (*c*) Transverse section through distal centriole and surrounding network of dense material in advanced spermatid (*); (*c*, inset) parallel cristae of immature mitochondria and dense intramitochondrial body. (*d*) Detail of distal centriole in transverse section showing coarse fibres, developing lateral body and open C tubules of triplets (advanced spermatid). (*e*) Late spermatid in region of nuclear fossa showing lateral body and attached centrioles. Note concentric membranes of mature mitochondria. (*f*) Acrosomal complex of almost mature testicular spermatozoon; (*f*, inset) transverse section showing acrosome, nuclear apex and endonuclear canals. (*g*) Perinuclear microtubules of late spermatid. (*h*) Junction of midpiece and principal piece of late spermatid (annulus present but fibrous sheath not yet formed). (*i*) Junction of midpiece and principal piece of testicular spermatozoon (note presence of fibrous sheath). (*j*) Transverse section of midpiece of testicular spermatozoon (note dense intramitochondrial bodies). (*k*) Transverse section of principal piece of testicular spermatozoon (note absence of outer sheath). Scale bars: (*a*,*c*,*h*–*j*) 0.5 μm; (*a* inset,*b*,*c* inset,*d*–*g*,*k*) 0.25 μm. Abbreviations: an, annulus; av, acrosomal vesicle; cf, coarse fibres; dc, distal centriole; fs, fibrous sheath; lb, lateral body; m, mitochondria; mt, microtubules; n, nucleus; pc, proximal centriole; sc, subacrosomal cone.

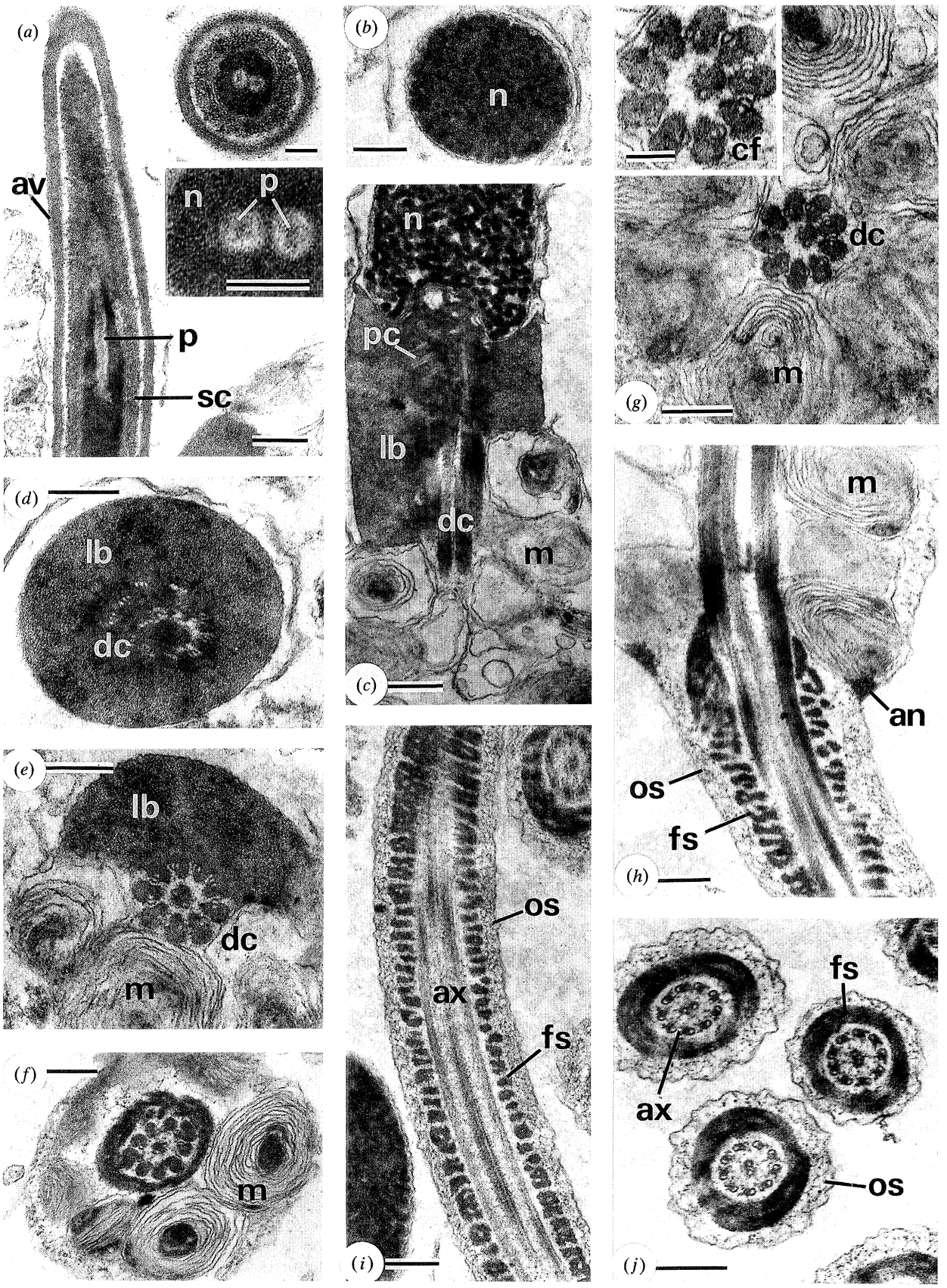

Figure 4. For description see opposite.

that the lateral body of *Sphenodon* is homologous with the 'connecting collar' surrounding the base of the nucleus in turtles (Hess *et al.* 1991). Ultrastructural observations on mid- and advanced stage spermatids of turtles will be necessary to confirm this suggestion.

(iv) *Principal piece*

The fibrous sheath of the principal piece is a synapomorphy of the Amniota (Healy & Jamieson 1992). Absence of this sheath in the sperm of all passerine and some non-passerine birds (Asa & Phillips 1987) appears secondary and therefore apomorphic relative to the plesiomorphic retention in palaeognath birds. According to Fawcett & Phillips (1970) the sheath appears to arise through the accretion of materials around the axonemal complex late in spermiogenesis. In the present study it was found that epididymal sperm of *Sphenodon* differ from those in the testis in having a flocculent outer sheath in addition to the dense fibrous sheath. A similar layer has been observed in sperm of the tinamou (Asa *et al.* 1986; Asa & Phillips 1987; Phillips & Asa 1989) and the woolly opossum (Phillips 1970) but is absent in sperm of most palaeognath birds (Baccetti *et al.* 1991), most mammals (Fawcett 1970), turtles (Furieri 1970; Hess *et al.* 1991; Healy & Jamieson 1992), squamates (Furieri 1970) and probably crocodilians (Saita *et al.* 1987). Asa & Phillips (1987) showed this outer layer within the principal piece of the crested tinamou was structurally similar to glycogen. The corresponding (?homologous) layer in *Sphenodon*, however, shows no morphological resemblance to glycogen granules.

With the exception of the Squamata, the fibrous sheath of amniote sperm occurs posterior to the midpiece. In squamates this sheath extends anteriorly to penetrate almost the full length of the midpiece. The claim by Cruz-Landim & Cruz-Hofling (1977) that the fibrous sheath of the lizard *Tropidurus torquatus* occurs posterior to the midpiece is based on observations on late spermatids in which the midpiece is far from mature (see figure 6a of Cruz-Landim & Cruz-Hofling 1977). We predict that an investigation of fully mature *Tropidurus* sperm would reveal that the fibrous sheath penetrates the midpiece, as observed in all other investigated squamates.

(*c*) *Systematic considerations*

In previous papers (Healy & Jamieson 1992; Jamieson & Healy 1992) we have drawn attention to the remarkable similarities between spermatozoa of *Sphenodon* and those of turtles (Furieri 1970; Hess *et al.* 1991; Healy & Jamieson 1992), the crocodiles (Saita *et al.* 1987) and to a lesser extent, non-passerine birds (Asa *et al.* 1986; Asa & Phillips 1987; Phillips & Asa 1989; Baccetti *et al.* 1991). All of these groups share the following features: (i) an acrosomal complex associated with one or more narrow endonuclear canals (absence of an endonuclear canal in the emu (*Dromaius novaehollandiae*) (Baccetti *et al.* 1991) is almost certainly due to secondary loss because all other examined palaeognath birds exhibit a canal); (ii) a midpiece containing an elongate distal centriole; (iii) non-intrusion of the fibrous sheath into the midpiece. Among the Reptilia, only *Sphenodon*, turtles and crocodiles possess rounded sperm mitochondria characterized by concentric cristae and a prominent intramitochondrial body (see Furieri 1970; Yasuzumi & Yasuda 1968; Saita *et al.* 1987; Hess *et al.* 1991; Healy & Jamieson 1992; Jamieson & Healy 1992; B. G. M. Jamieson & A. Georges, unpublished data). Further, of these three groups, only *Sphenodon* and turtles possess multiple (usually paired), elongate, helically twisted nuclear canals. The fact that morphological differences between sperm of *Sphenodon* and turtles are relatively minor (turtle sperm lacking the outer fibrous layer in the principal piece and occasionally showing three rather than two endonuclear canals) is highly significant because it does not support the widely held view that sphenodontids are closely allied to the Squamata. Many ultrastructural studies have now been done on spermatozoa and spermiogenesis of squamate reptiles (Clark 1967; Hamilton & Fawcett 1968; Furieri 1970; Del Conte 1976; Cruz-Landim & Cruz-Hofling 1977; Cruz-Hofling & Cruz-Landim 1978; Butler & Gabri 1978; Saita *et al.* 1988*a*,*b*; Carcupino *et al.* 1989; Newton & Trauth 1992). Squamate sperm possess the following features not observed in *Sphenodon* (or in turtles or crocodilians): (i) presence of a paracrystalline inner core in acrosomal complex; (ii) absence of endonuclear canals, the perforatorium where present, always being pre-nuclear; (iii) midpiece with the axoneme, not a centriole, always forming the major axial component of this region; (iv) fibrous sheath penetrating the midpiece, almost to the base of the nucleus; (v) presence of dense inter-mitochondrial rings (or equivalent, smaller bodies (Jamieson & Scheltinga 1993). All of these squamate features are here interpreted as apomorphic (see also Jamieson & Healy 1992);

Figure 4. *Sphenodon punctatus* : epididymal spermatozoa. (*a*) Apical region of acrosomal complex and nucleus; (*a*, inset top) transverse section of acrosomal complex, nucleus and paired endonuclear canals; (*a*, inset bottom) detail of endonuclear canals. (*b*) Transverse section of nucleus below level of endonuclear canals. (*c*) Junction of nucleus and midpiece showing lateral body (around centrioles). (*d*) Transverse section through anterior region of lateral body (sheathing distal centriole). (*e*) Transverse section through posterior region of lateral body (partly ensheathing distal centriole) within midpiece. Note coarse fibres associated with triplets. (*f*) Transverse section of midpiece close to annulus. Initial portion of fibrous sheath surrounding axoneme and coarse fibres. Note concentric cristae and dense intramitochondrial body. (*g*) Transverse section of midpiece; (*g*, inset) detail of distal centriole. (*h*) Junction of midpiece and principal piece. Note annulus, fibrous sheath and outer sheath. Unlike squamates, fibrous sheath does not extend throughout midpiece. (*i*) Principal piece showing fibrous and fibro-granular sheaths. (*j*) Transverse section through principal piece region. Scale bars: (*a* inset, *g* inset) 0.1 μm; all others 0.25 μm. Abbreviations: an, annulus; av, acrosomal vesicle; ax, axoneme; cf, coarse fibres; dc, distal centriole; fs, fibrous sheath; lb, lateral body; m, mitochondria; n, nucleus; os, outer sheath; p, perforatoria; pc, proximal centriole; sc, subacrosomal cone.

features (i), (iv) and (v) being here considered as autapomorphic for the Squamata. Our cladistic analysis of sperm characters within the Amniota (Jamieson & Healy 1992), although partly hampered by incomplete data for some groups, clearly showed that *Sphenodon* cannot be regarded as an aberrant squamate, but is in fact is a very primitive amniote, only turtles having fewer spermatozoal apomorphies.

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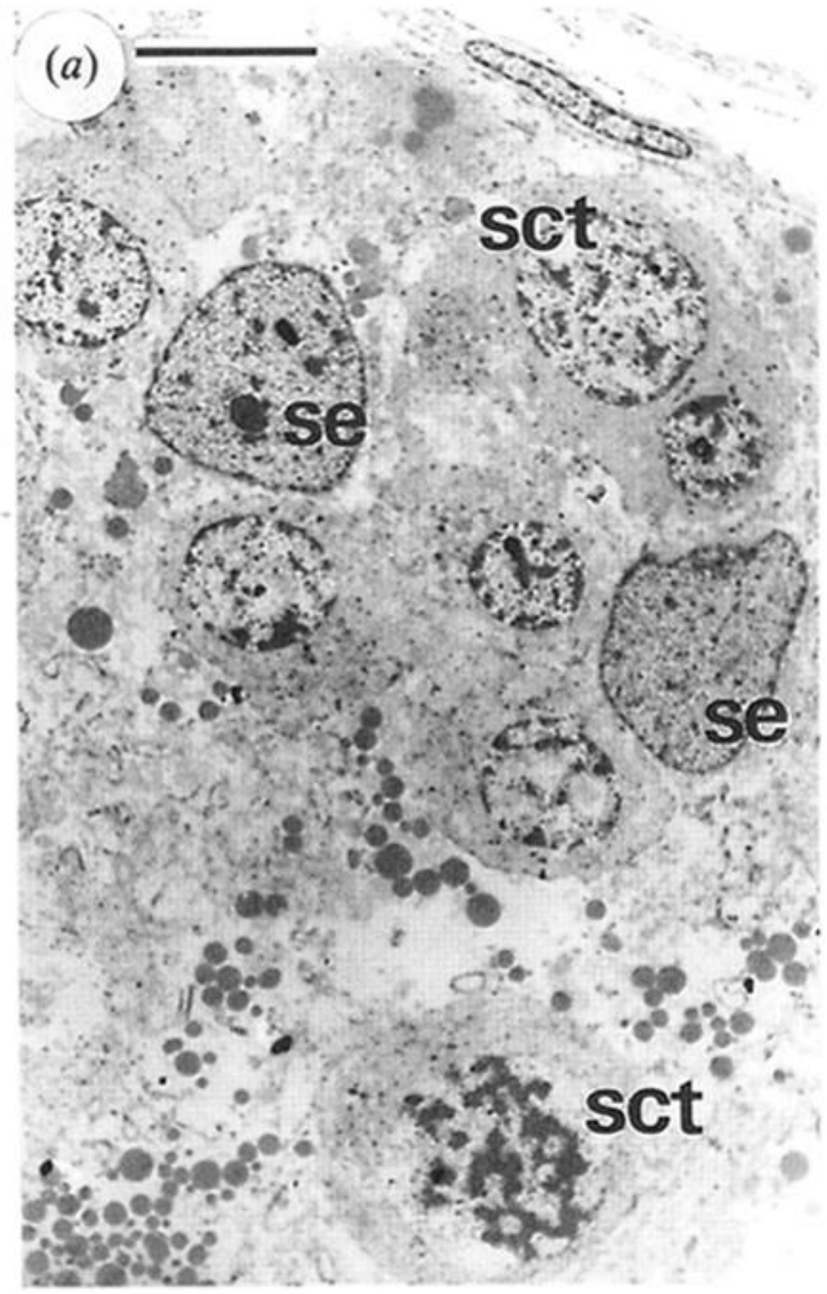
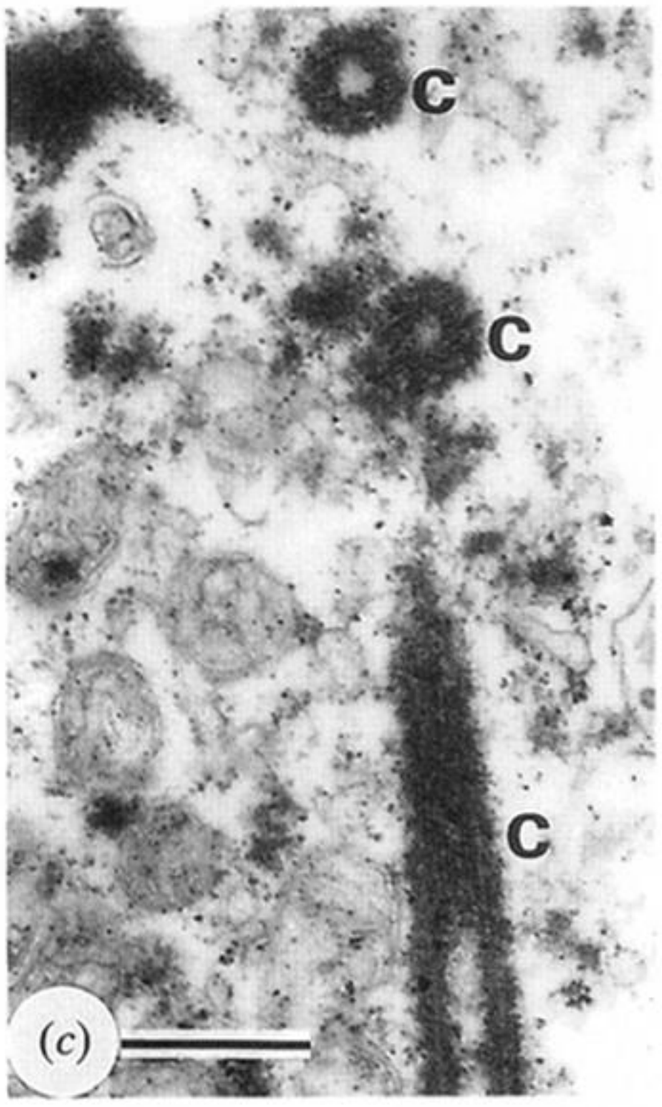
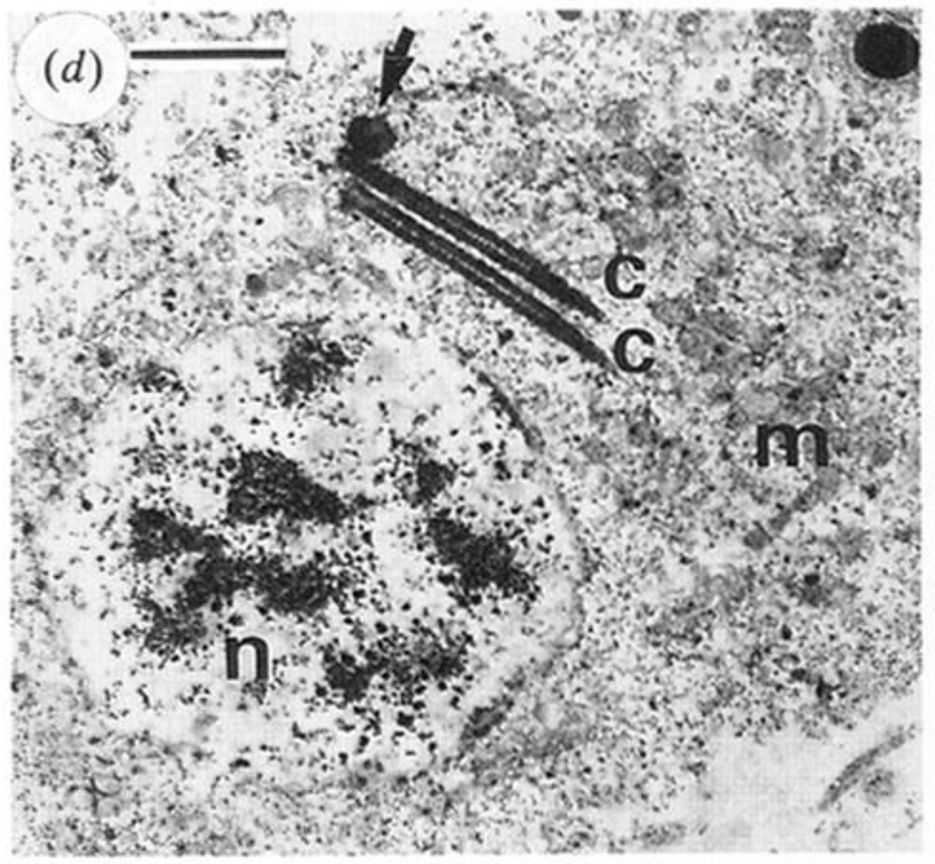
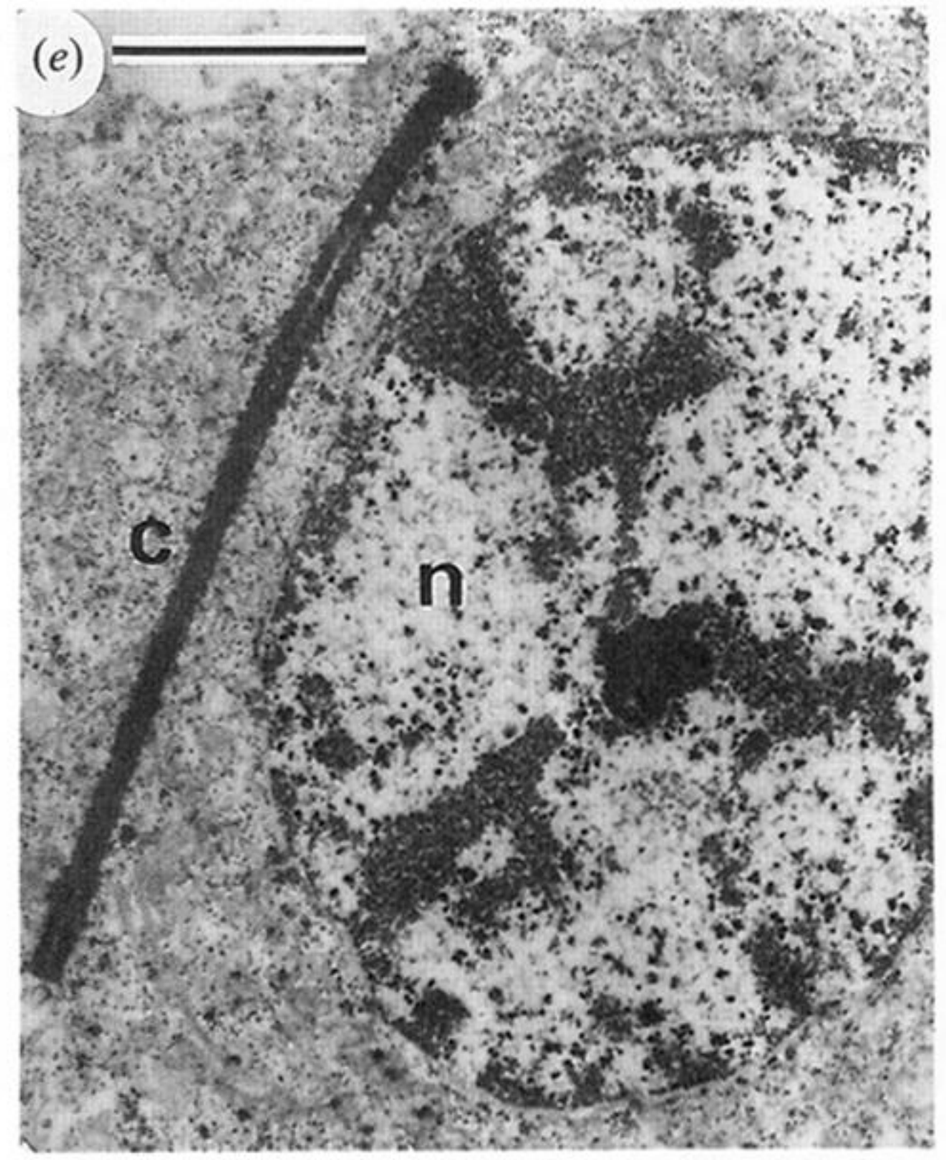
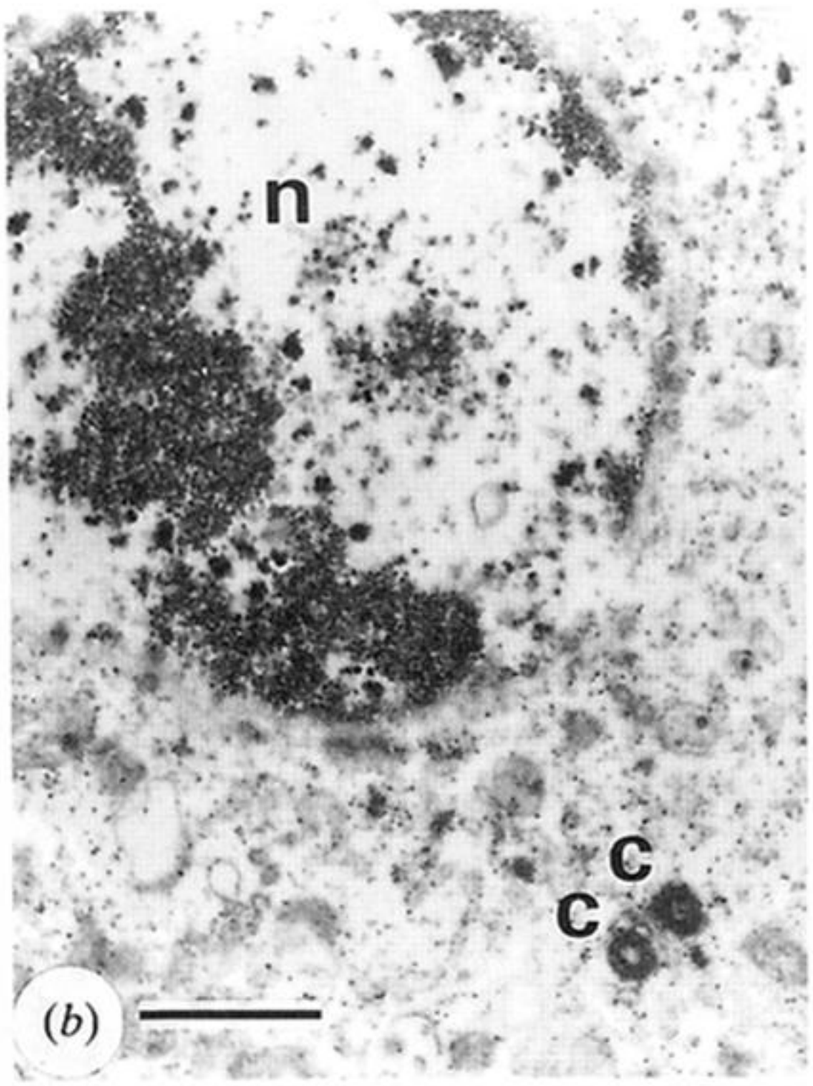
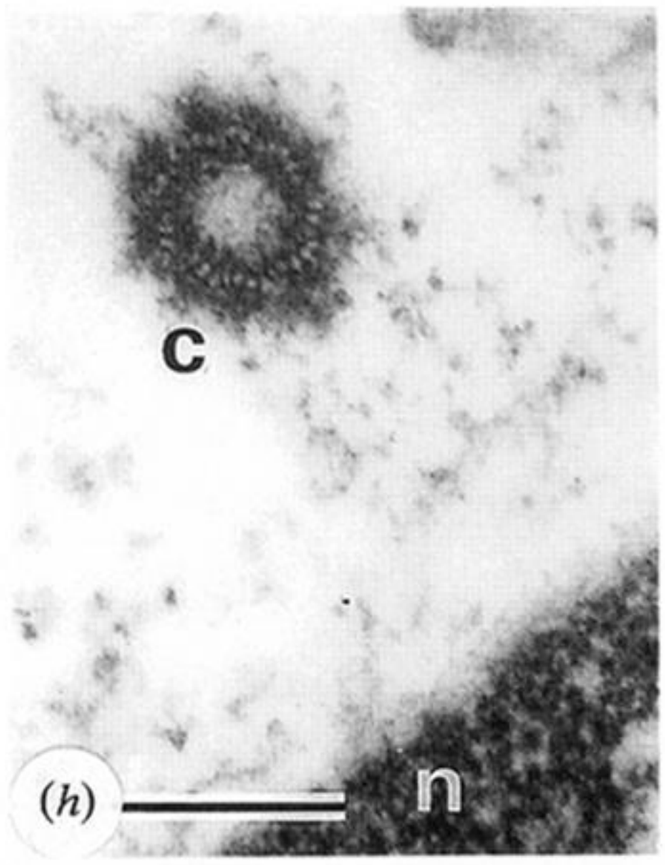


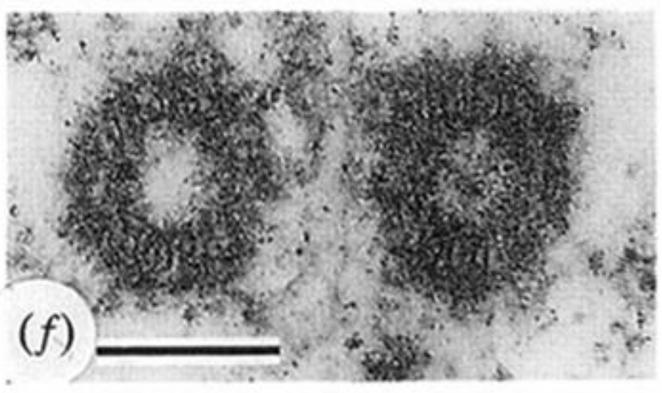


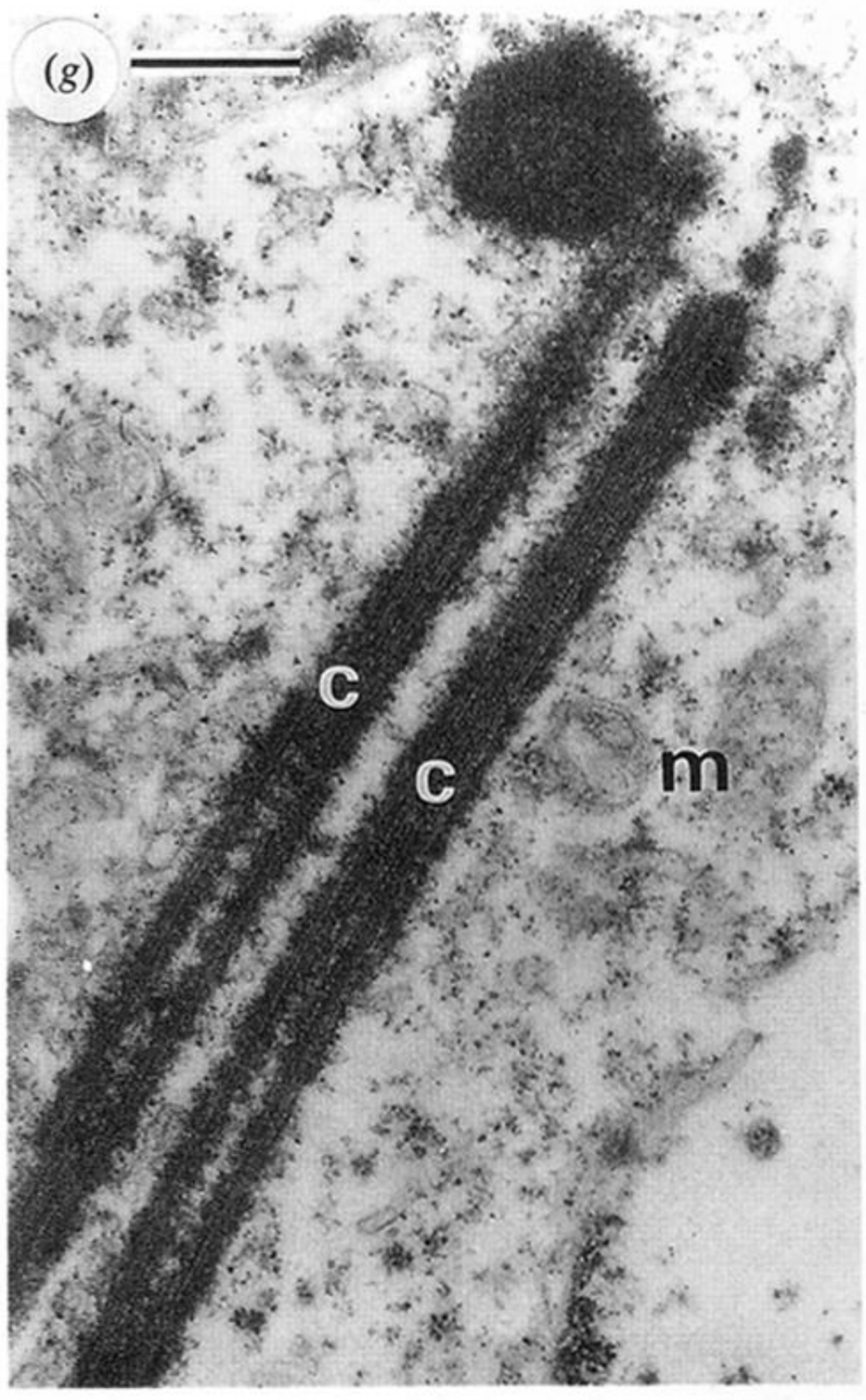












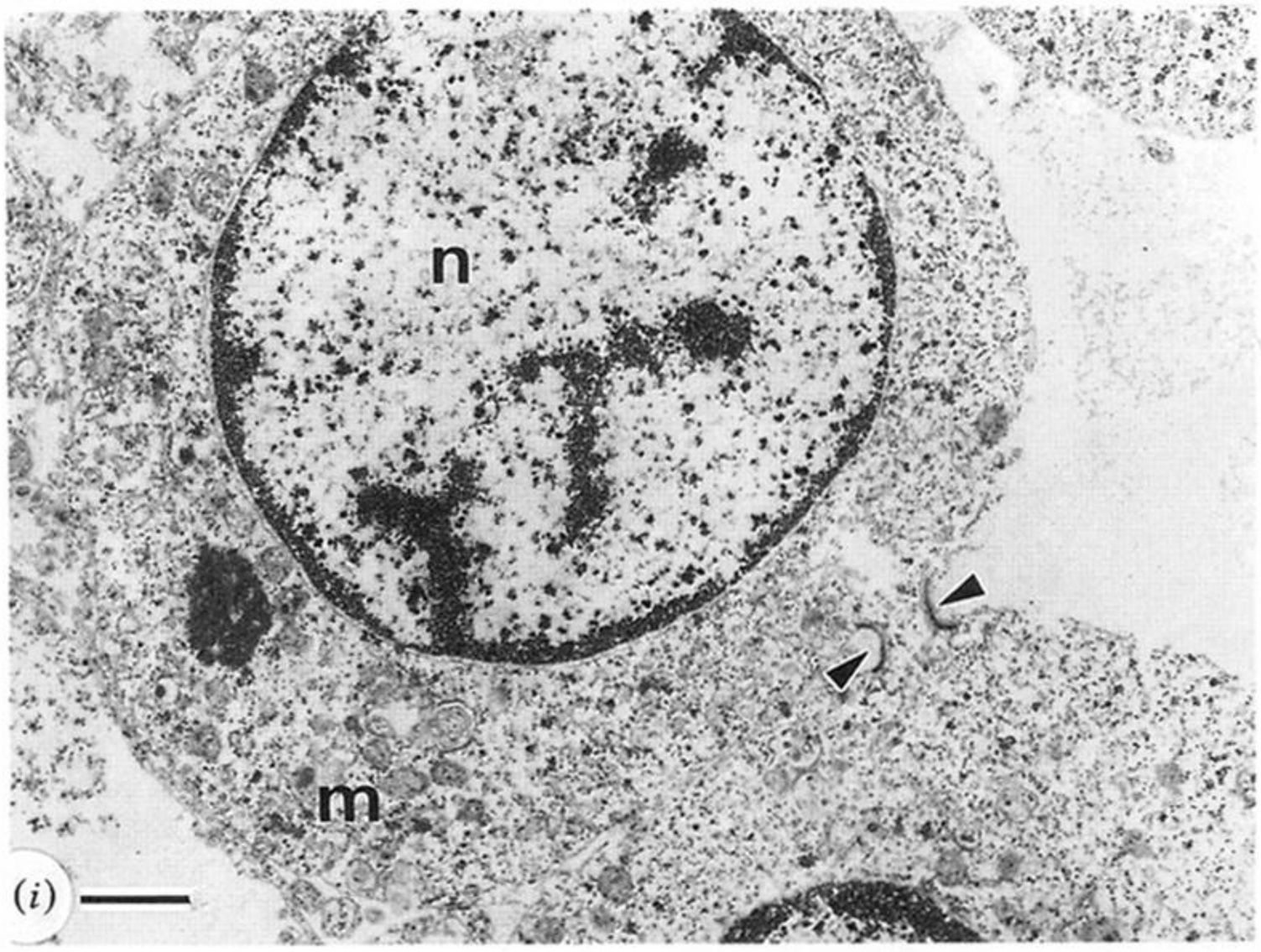
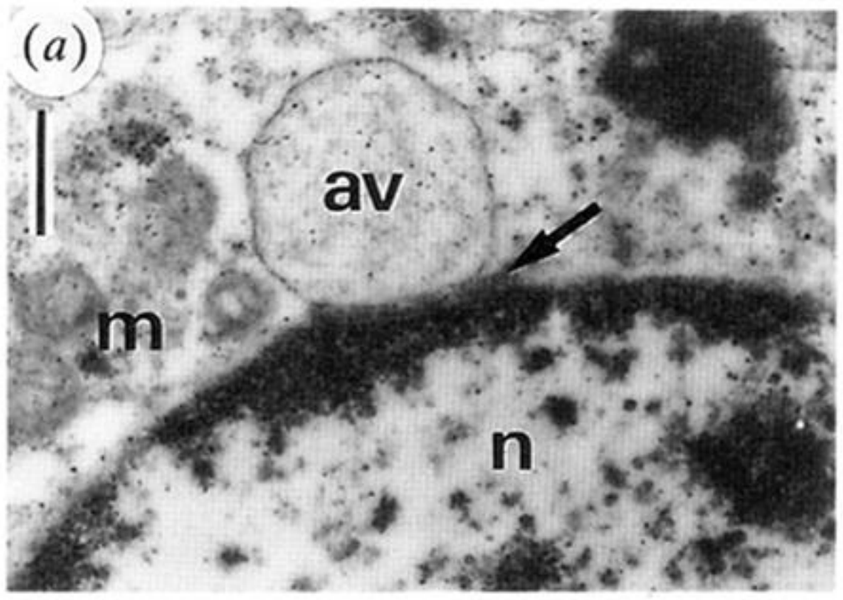
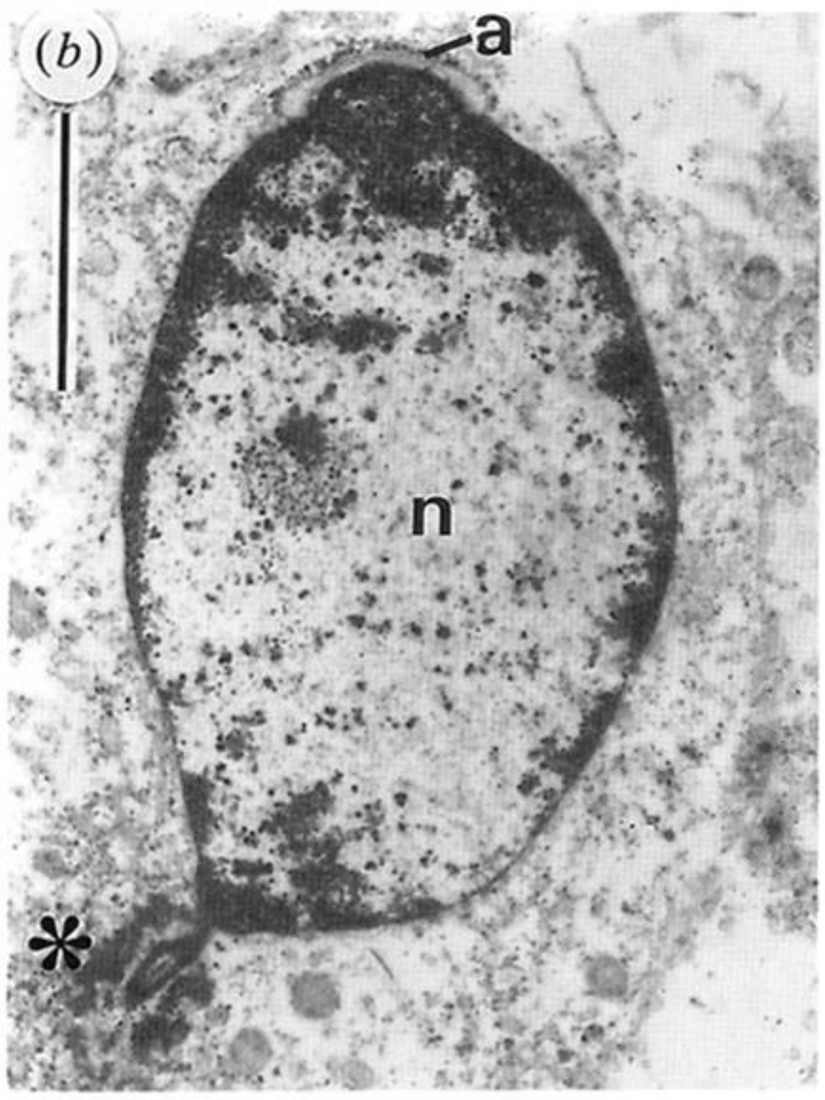
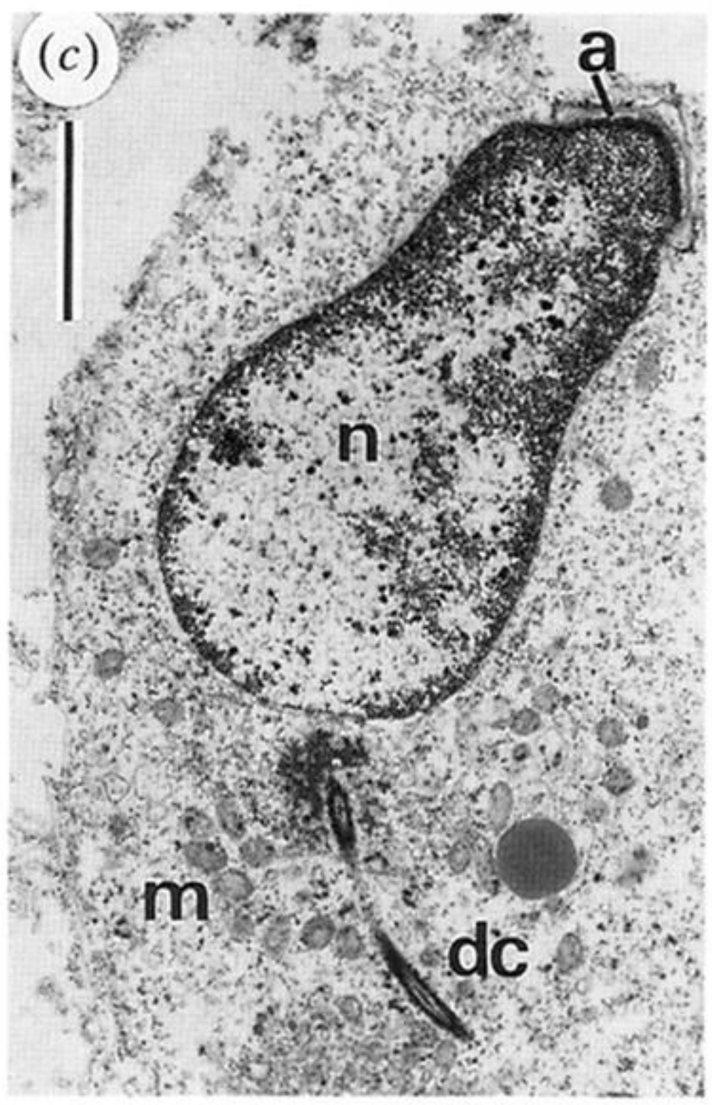
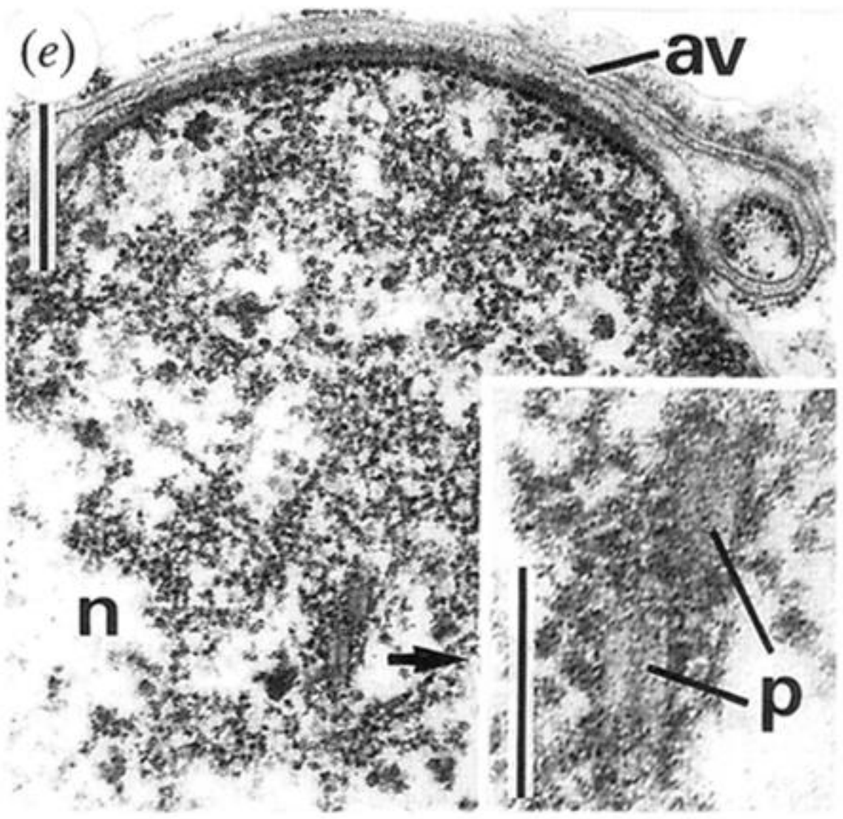


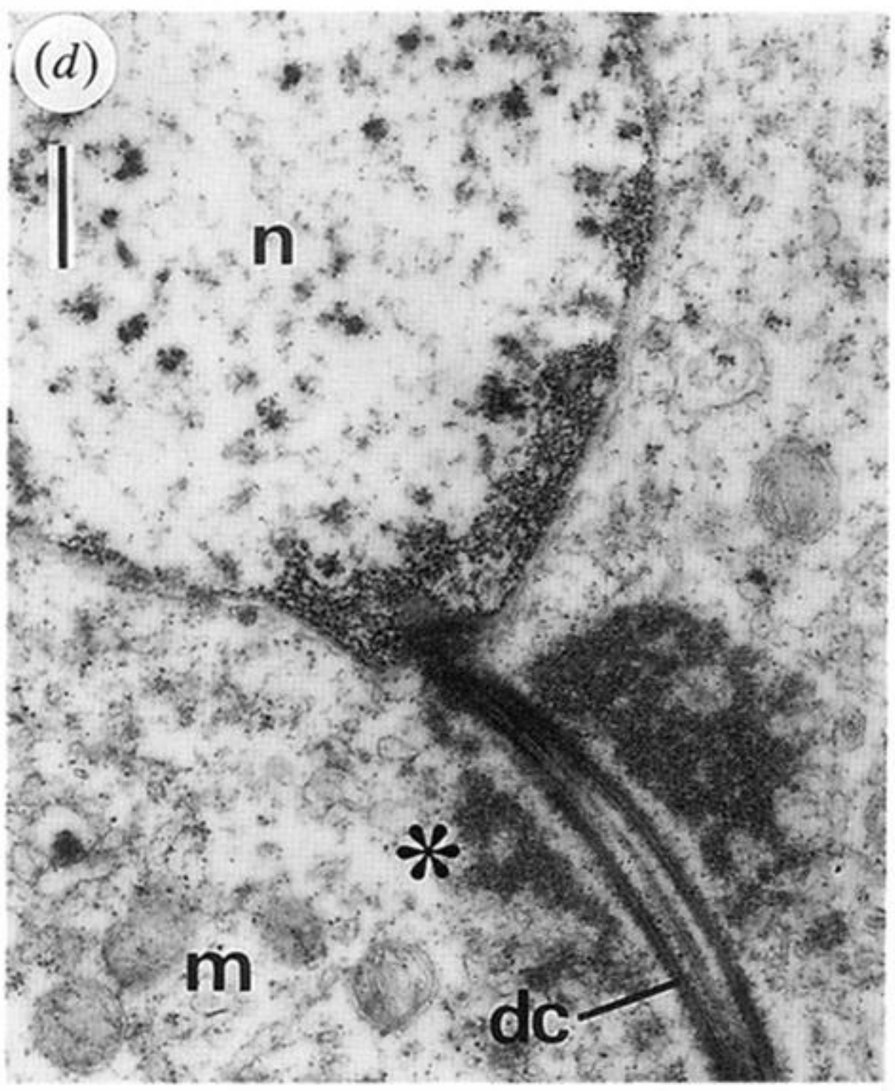
Figure 1. For description see opposite.

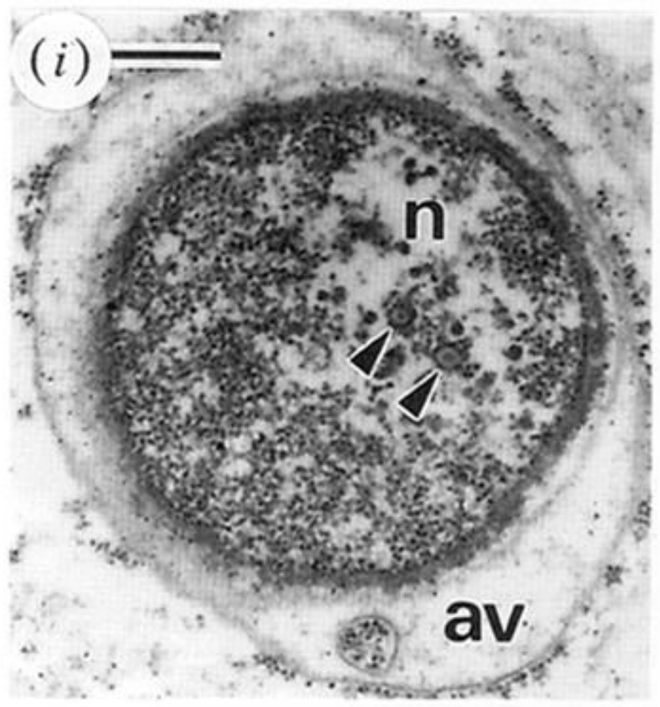


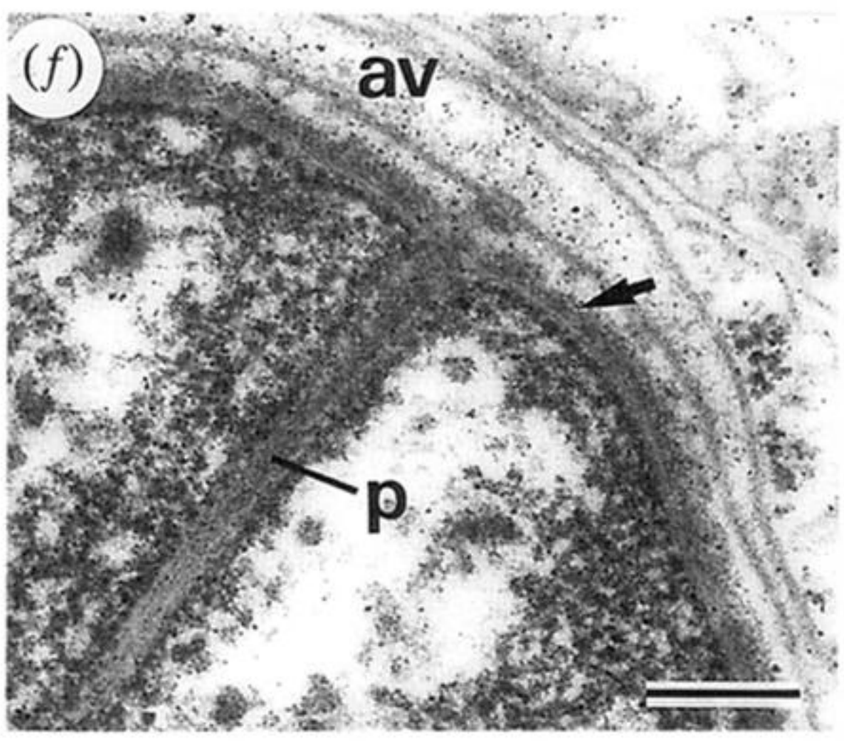


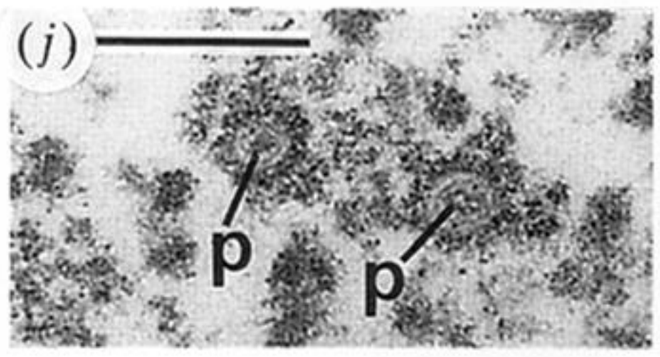


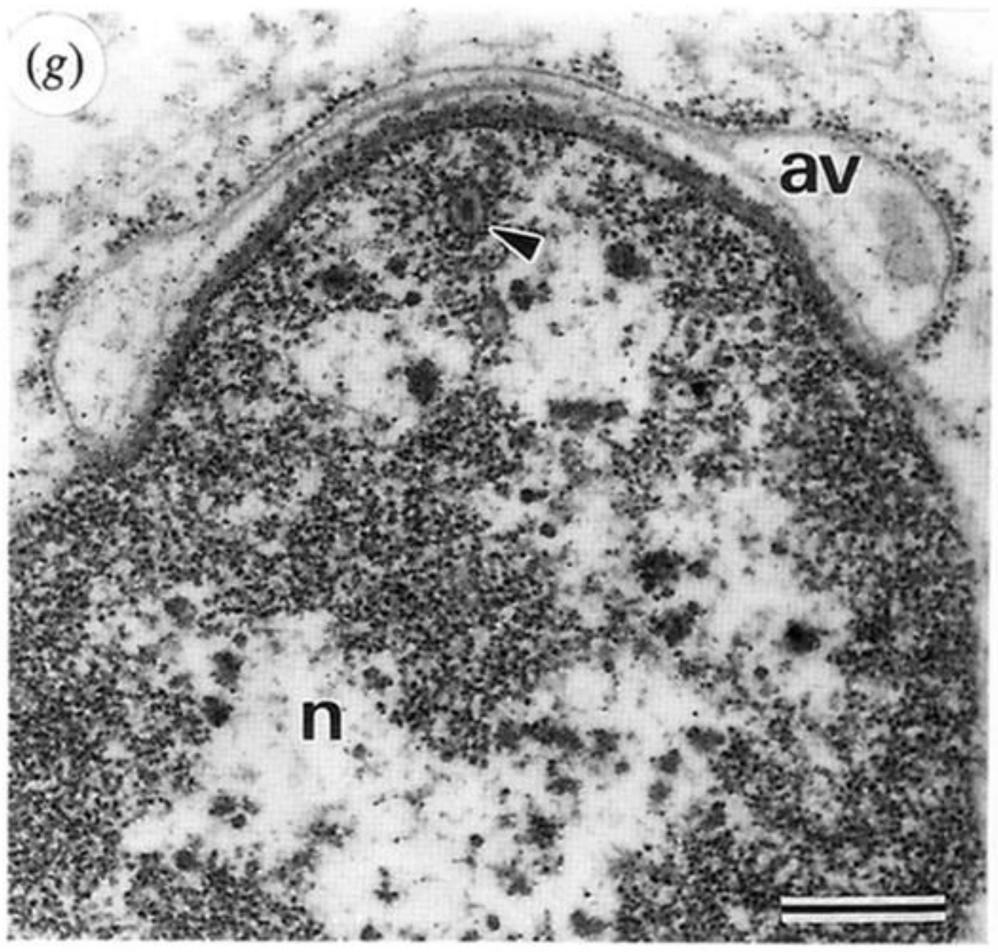


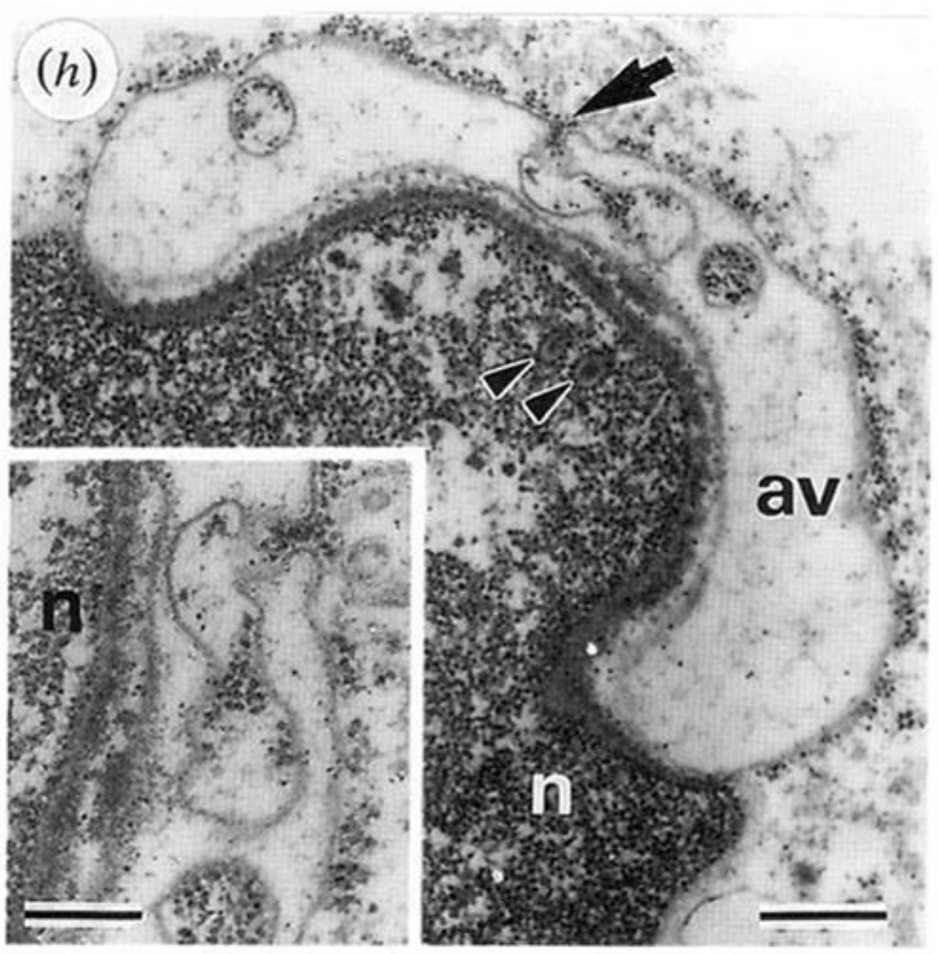












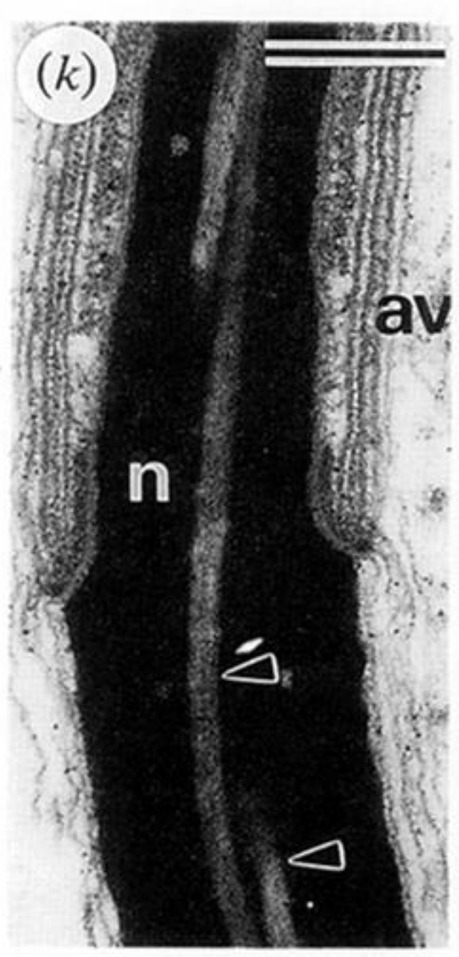
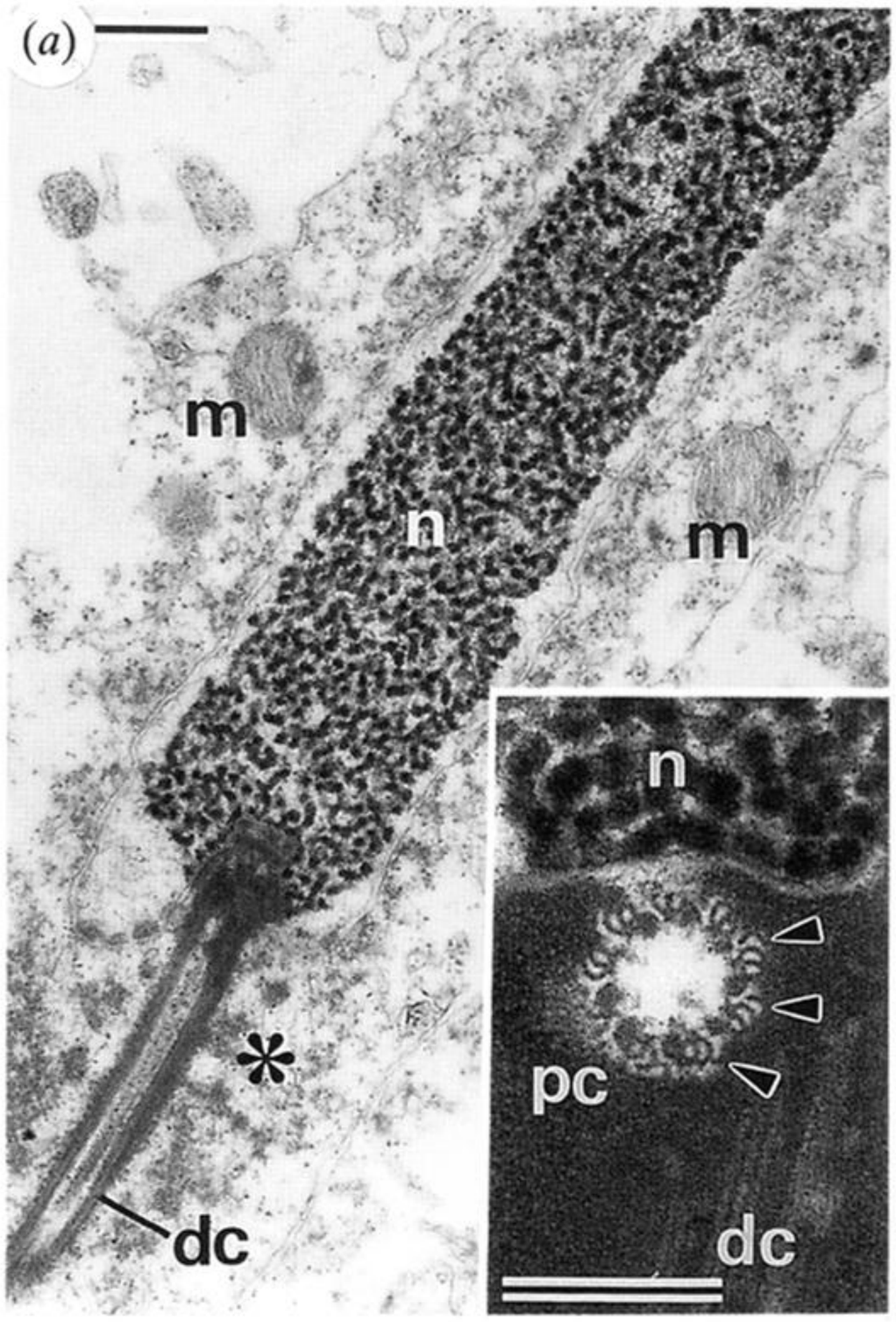
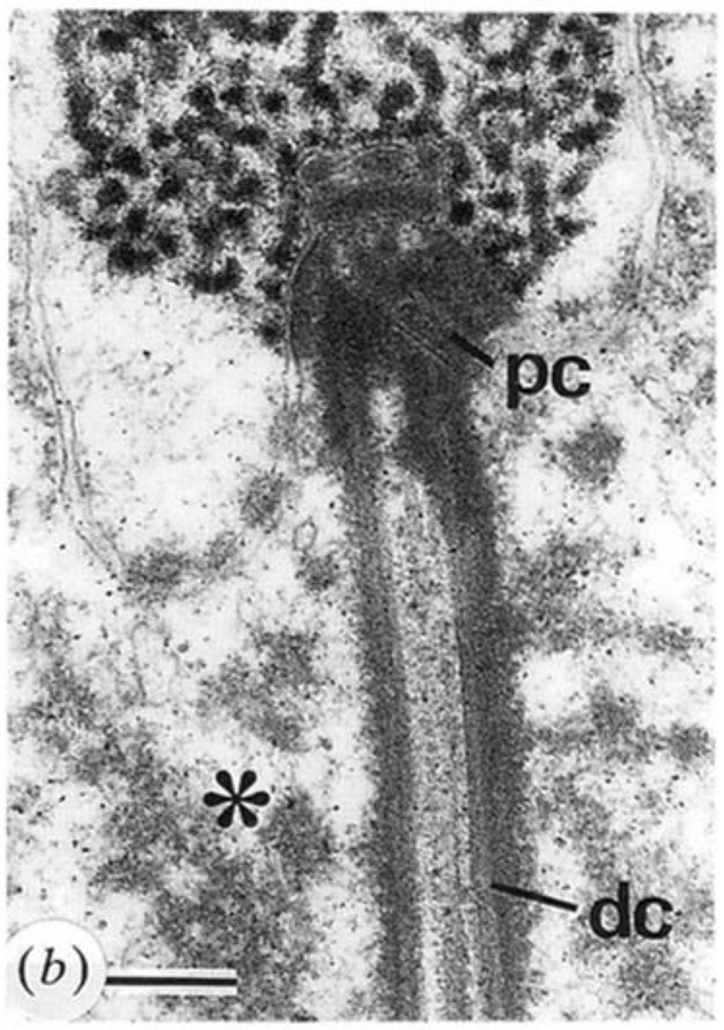
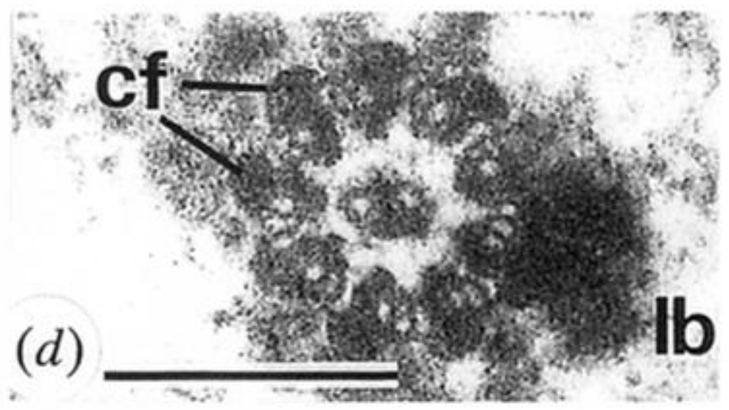
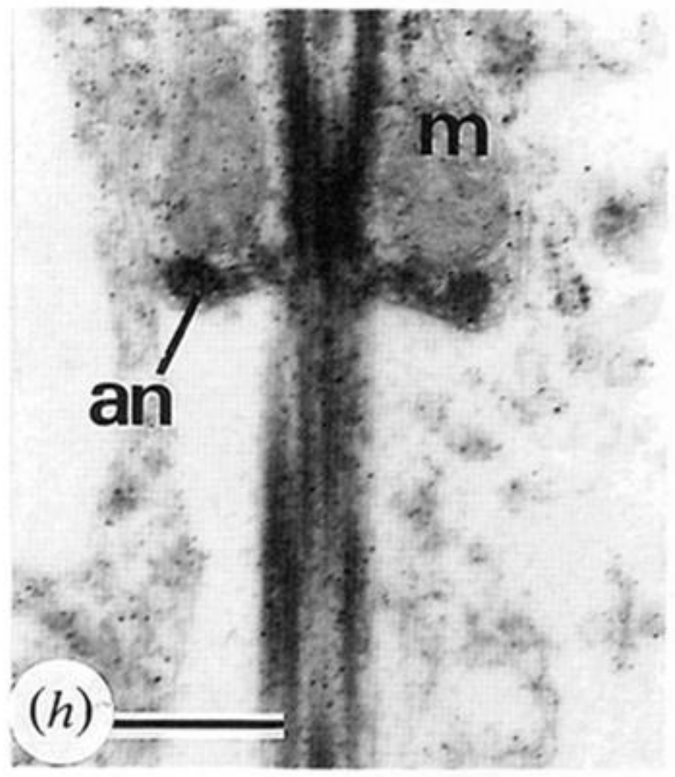


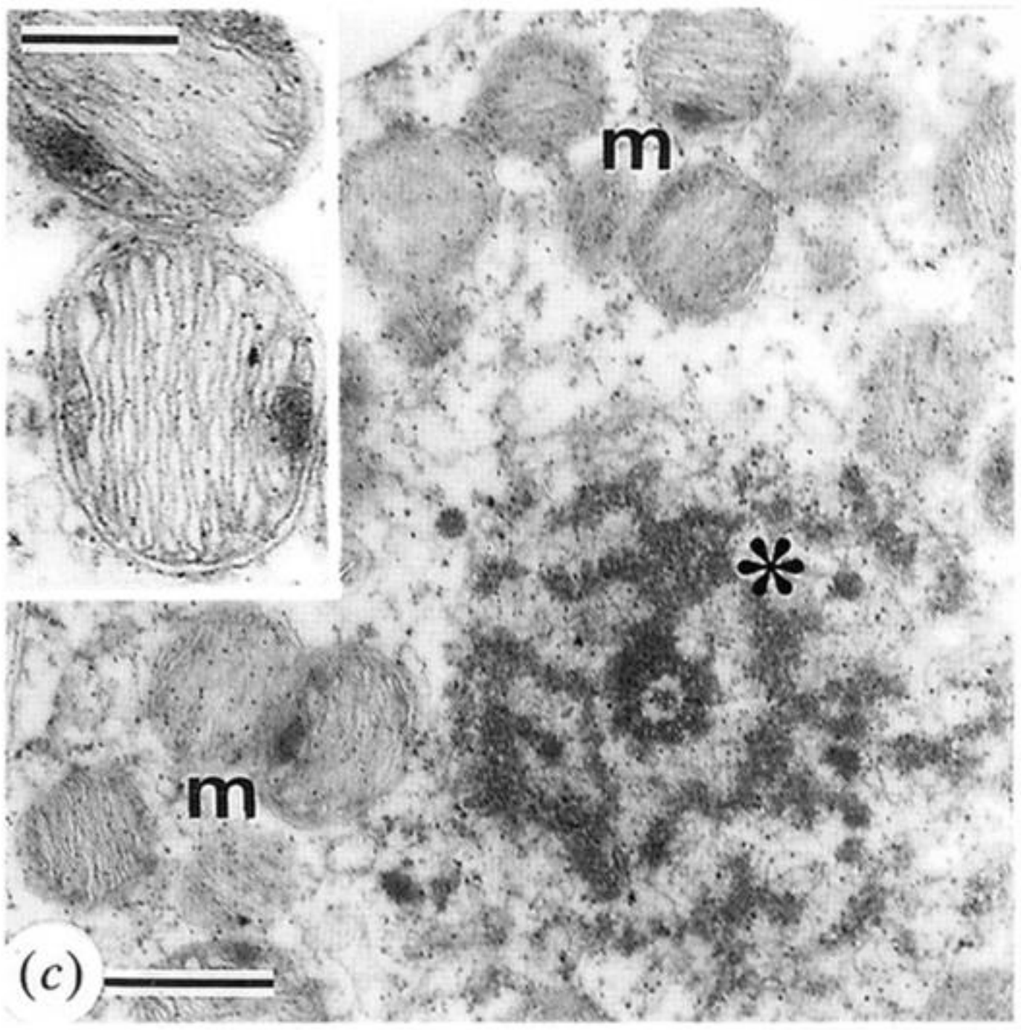
Figure 2. For description see opposite.

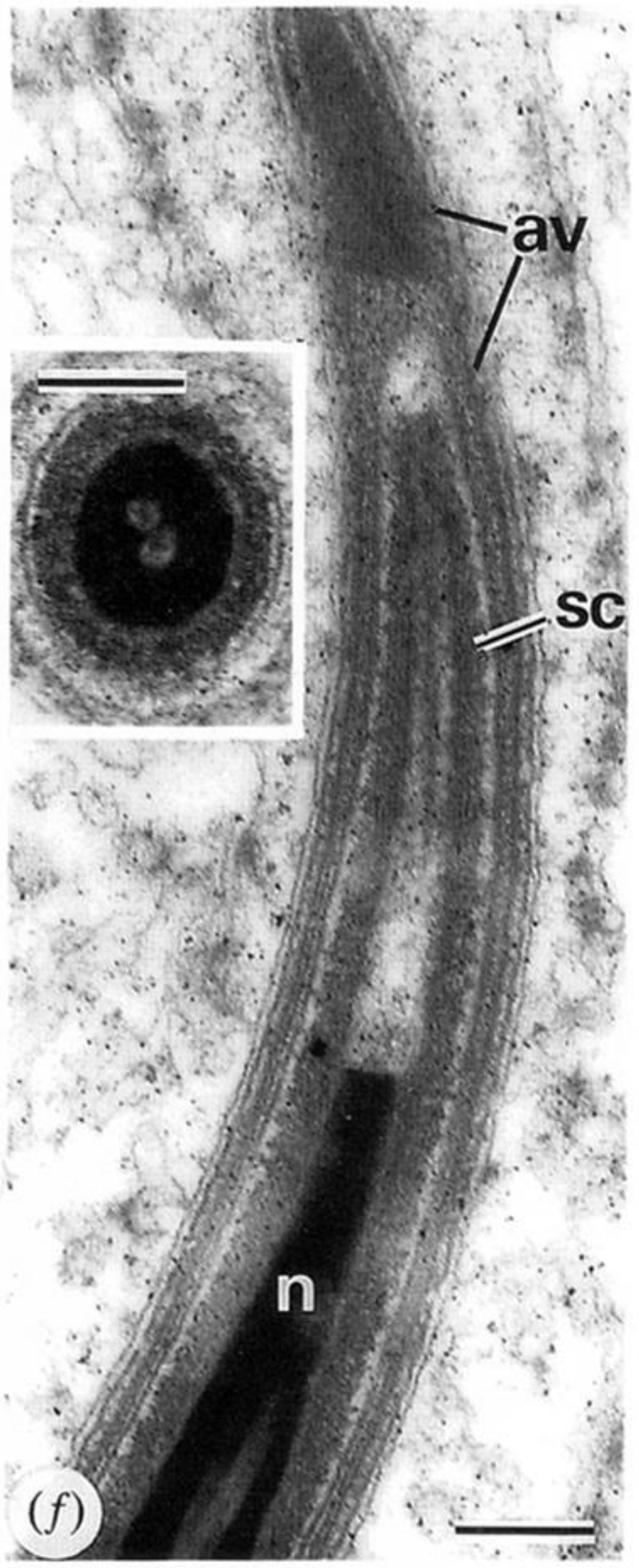


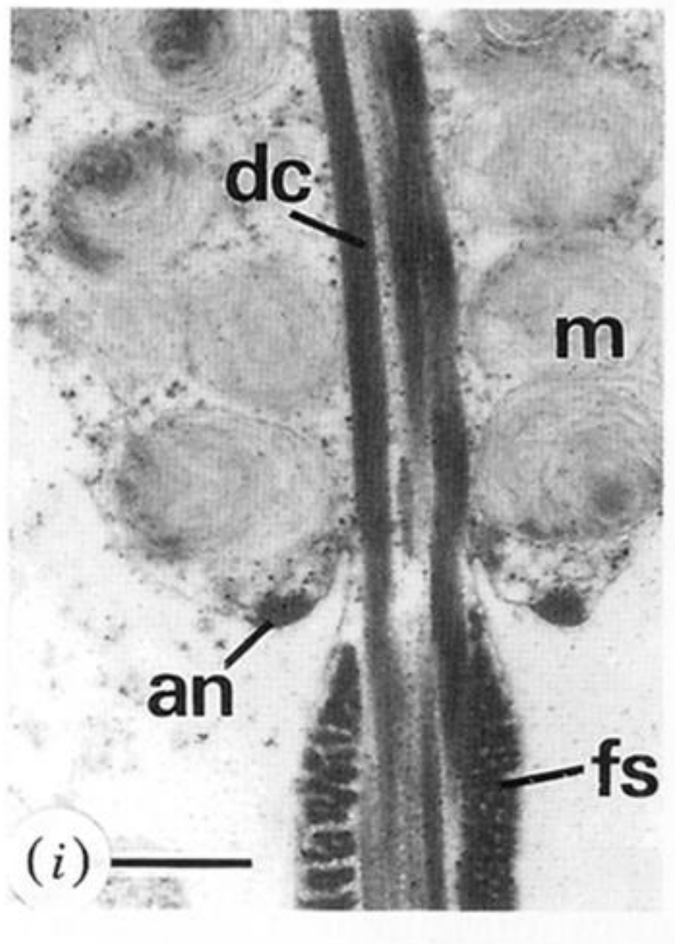


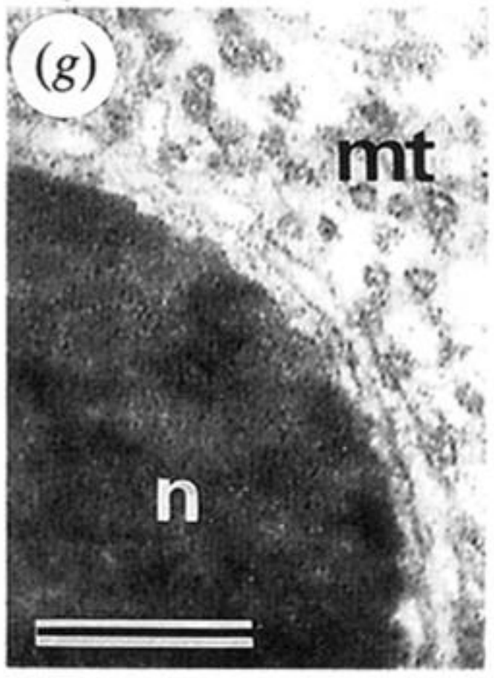


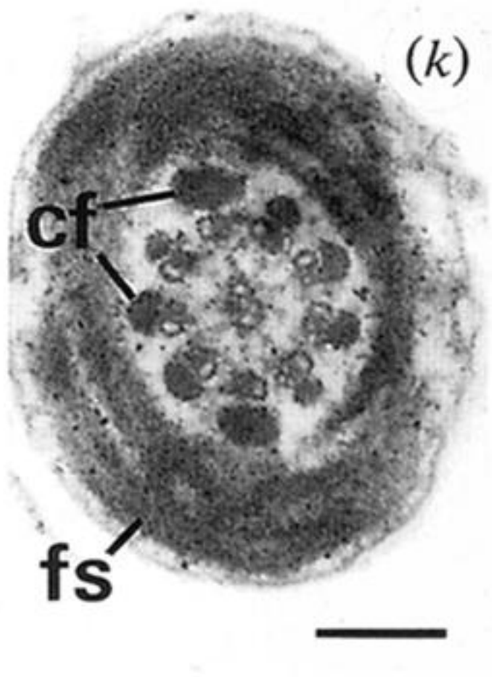












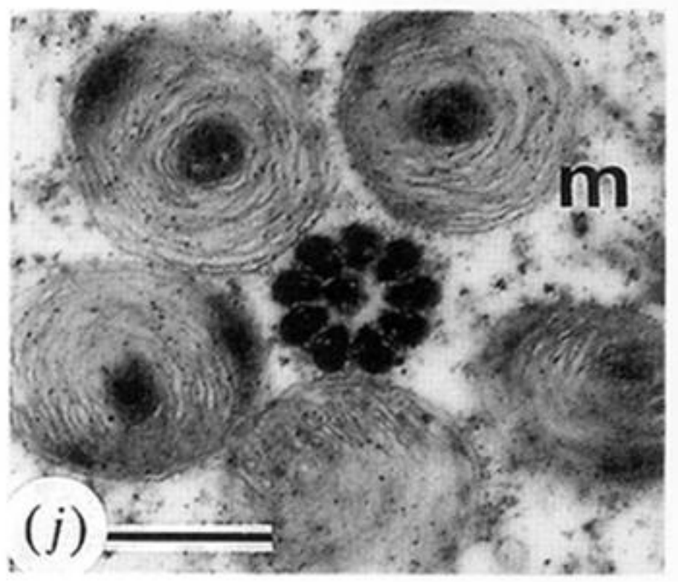


Figure 3. For description see opposite.

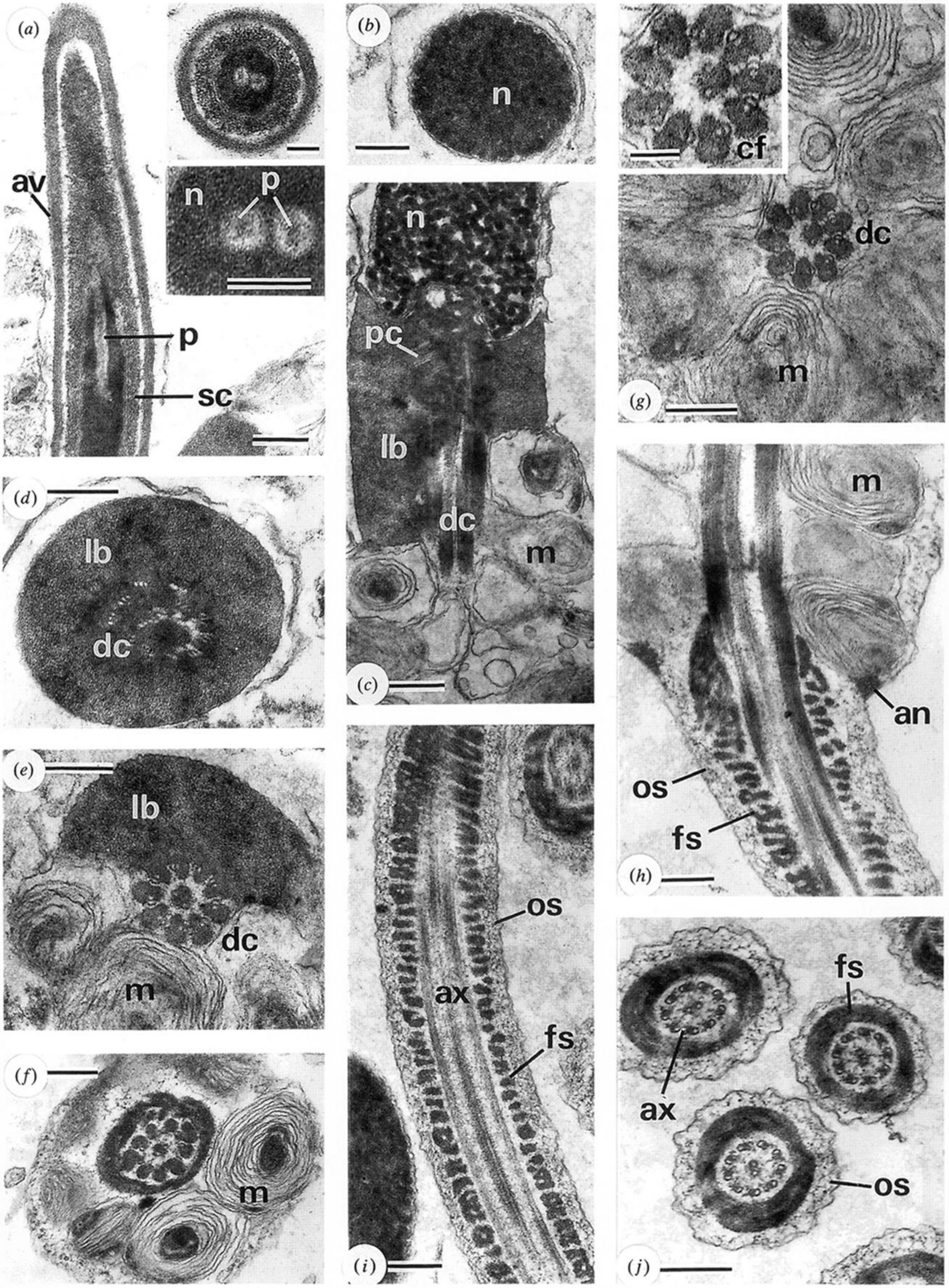


Figure 4. For description see opposite.