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Studies of the Spermatogenesis in the Firebug, *Pyrrhocoris apterus* (Heteroptera). II. Transformation of Spermatid Chondriome*

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Synopsis

The process of formation of mitochondrial derivates (MDs), during the spermatogenesis in firebug, described in this paper, resembles that of other Heteroptera. Nevertheless, new facts are described which had not been previously observed during insect spermiogenesis: 1) the formation of two separate aggregations of mitochondria within immature spermatids, which are probably precursors of the mitochondrial bodies that form the nebenkern (NK); 2) the presence in some spermatids, independently from MDs, of few "additional" mitochondria in which paracrystalline material (PM) is formed, like that in MDs. The appearance and distribution of this material differ from those in the matrix of MDs. Thus it is possible to suggest that the PM is of mitochondrial origin and that the normal course of its formation depends on the immediate proximity of MDs to the axonemal sheath; 3) the occurence in MDs of rows of equally spaced dense particles up to 7.9 nm in diameter, attached to the inner mitochondrial membrane. It is postulated that the appearance of these particles indicates alternations in structure of the inner mitochondrial membrane, caused by its reorganization.

Introduction

The transformation of entire mitochondrial sets of the spermatids into two

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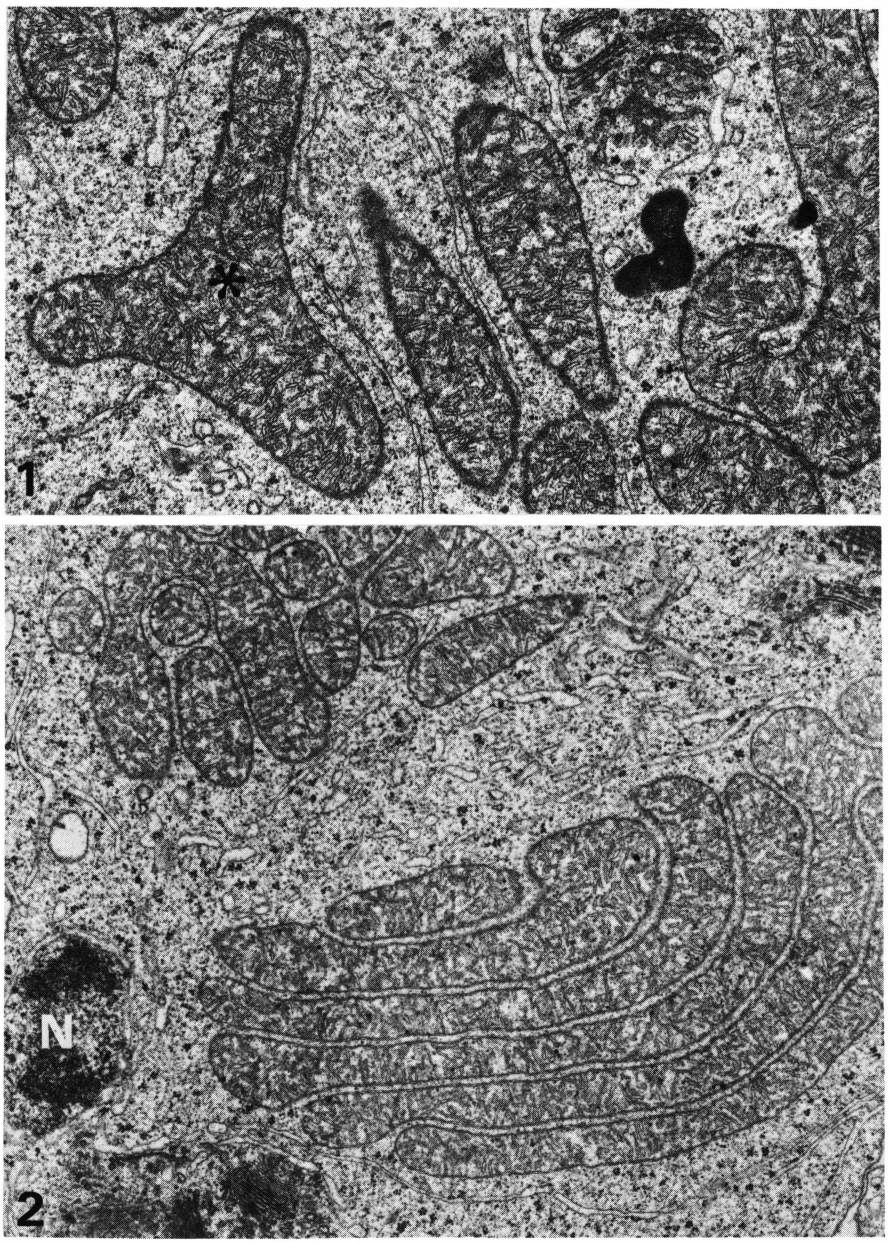

Fig. 1. Fragment of spermatid cytoplasm in telophase of the second meiotic division. The branched mitochondrion is marked by an asterisk. × 20,500.

Fig. 2. Fragment of a spermatid in telophase of the second meiotic division. Mitochondria form two large aggregates. N, nucleus. × 14,500.

mitochondrial derivatives (MDs) which in mature spermatozoa form elongated bodies positioned symmetrically on either side of the flagellar axoneme, is an interesting process of insect spermiogenesis (Stanley *et al.*, 1972; Tokuyasu, 1975; Szöllösi, 1975; for a review see Phillips, 1970; Baccetti, 1972). Several steps can be distinguished during this process: a) a coalescence of all the mitochondria of a young spermatid into a round body called a nebenkern (NK); b) structural transformation of the NK, followed by its division into two separate bodies, called NK derivatives (NKDs), which flank the flagellar axoneme; c) subsequent conversion of NKDs into MDs which are characterized by a specific arrangement of cristae and the presence of intramatrical paracrystalline material (PM).

In the studies on the spermiogenesis in Heteroptera much attention has been paid to the transformation of mitochondria. This process, however, has been presented either as a general outline (Barker and Riess, 1966; Payne, 1966; Trandaburu, 1971; Trandaburu and Trandaburu, 1971; Itaya *et al.*, 1980) or its description has been limited to some of its stages only (Pratt, 1968, 1970; Afzelius *et al.*, 1976; Rosati *et al.*, 1976; Baccetti *et al.*, 1977; Dallai and Afzelius, 1980). No study summing up the information available on the entire process of chondriome transformation in differentiating heteropteran spermatids has been published so far. To bridge this gap, a study on the mechanism of MDs formation in the course of spermiogenesis in the firebug (*Pyrrhocoris apterus*) has been attempted. The results of these investigations, presented in this paper, indicate that the process resembles that in other insect species, yet demonstrating some peculiarities which have not been previously reported.

Materials and Methods

Adult male firebugs, used in this study, were collected in Oeiras, Portugal. The testes were dissected in a balanced salt solution, fixed in 3% glutaraldehyde in Millonig's phosphate buffer, pH 7.3, and postfixed in 1% osmium tetroxide in the same buffer. Following dehydration with a graded series of ethanols, the specimens were embedded in Epon 812, sectioned, and stained with uranyl acetate and lead citrate. The sections were observed with a Siemens Elmiskop 101 at 80 kV. For light microscopy, semithin sections were stained with toluidine blue.

Results

Early spermatids at the telophase of the second meiotic division have numerous mitochondria, which do not differ in appearance from mitochondria of mature spermatocytes. Nevertheless, some mitochondria often appeared in branched form, indicating the onset of their fusion (Fig. 1). In more advanced spermatids, the mitochondria group to form two large, independent clusters in which neighbouring mitochondria are separated by a fairly constant space of about 60 nm (Fig. 2). Sometimes, single mitochondria, henceforth called "additional" mitochondria, that did not join the clusters can be observed in cytoplasm. Later, both mitochondrial aggregates clump together into a large body called a nebenkern (NK) situated usually near the posterior

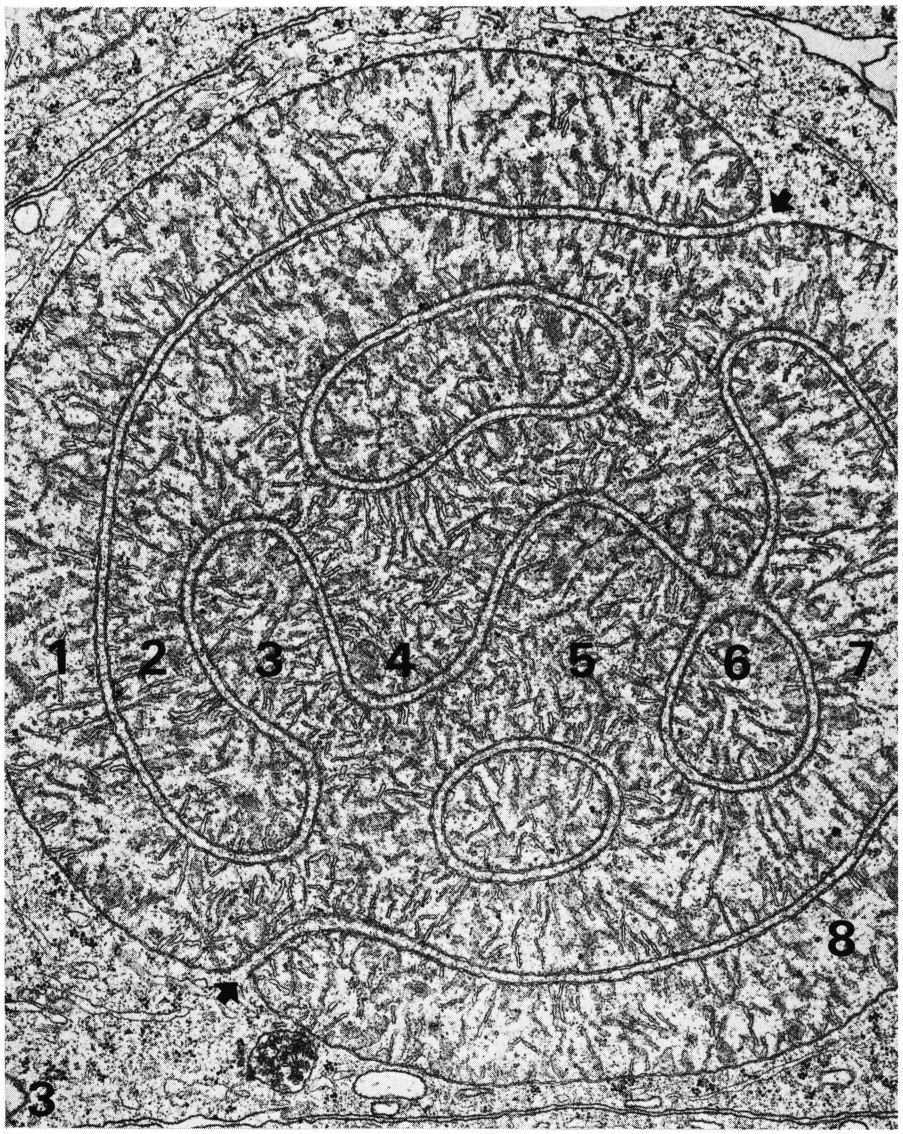

Fig. 3. A "four-layered" NK sectioned in a plane perpendicular to the long axis of spermatid. The narrow fissures visible on its poles (arrows) indicate the presence of a furrow running round the NK. It is clearly visible that the segments marked with even numbers do not merge with those denoted by odd numbers. × 21,000.

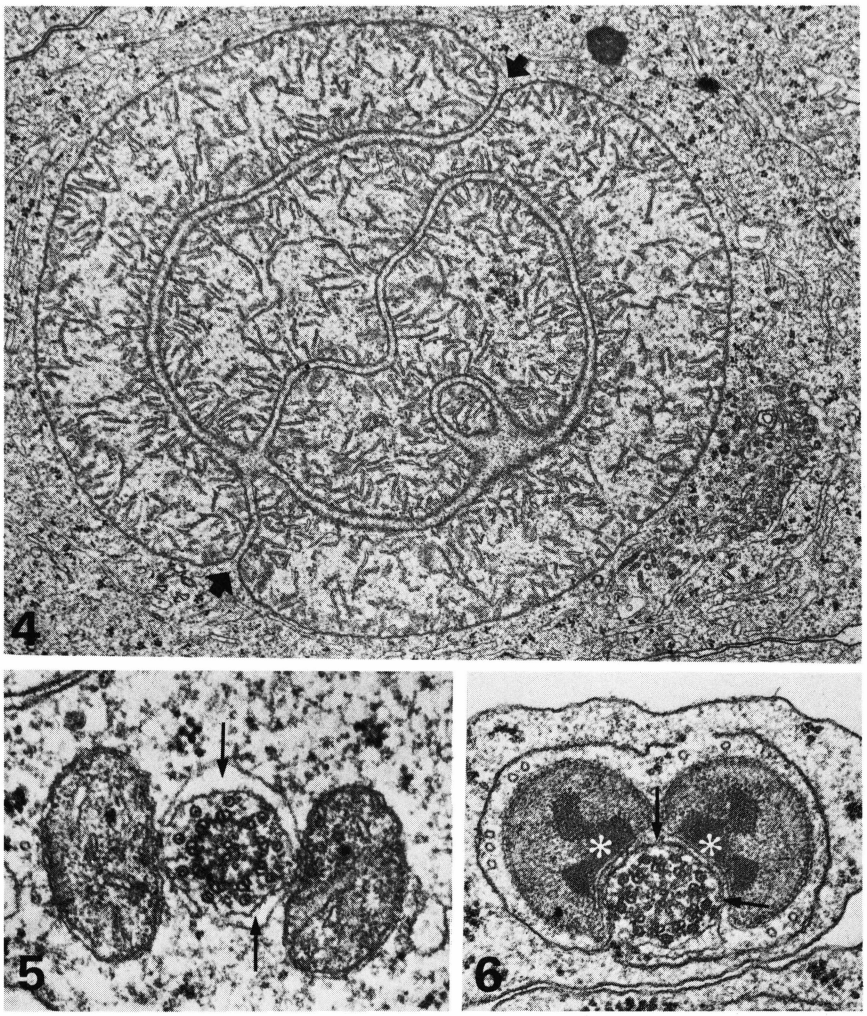

Fig. 4. A "two-layered" NK cut transversely with respect to the axis through the nucleus and the NK. Arrows indicate the presence of a meridionally running furrow on the NK surface. × 20,000.

Fig. 5. Transverse section of the tail region of a spermatid. Note the cisternae of axonemal sheath (arrows) encircling the axoneme. × 62,000.

Fig. 6. Transverse section of a spermatid tail in a later stage than in Fig. 5. The PM (asterisks) lies close to the mitochondrial envelope on the side facing the axoneme. The axonemal sheath (arrows) is in the form of an electron-dense lamella. × 50,000.

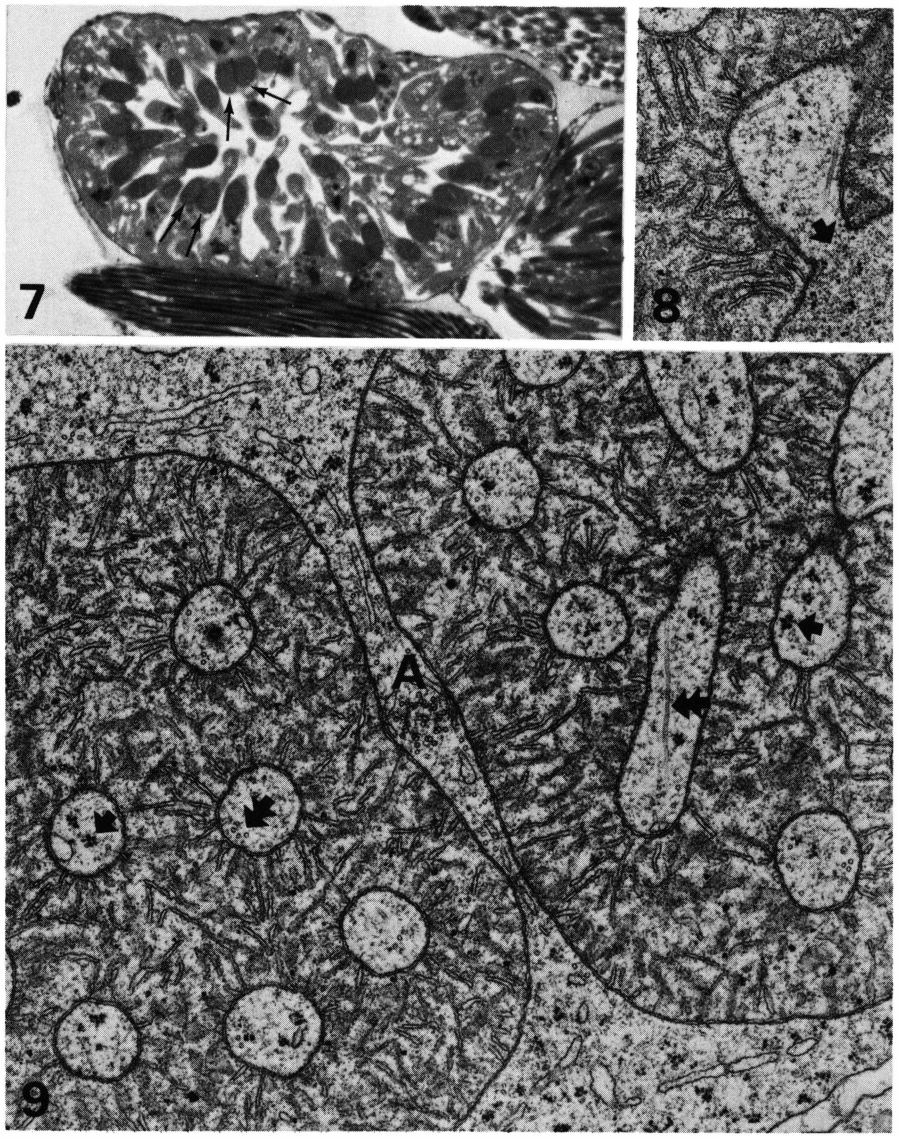

Fig. 7. Cyst with spermatids just after a division of NK into two NKDs (arrows). × 700.
Fig. 8. Fragment of NKD. Continuity of "vacuole" lumen with cytoplasm is indicated by an arrow. × 25,000.
Fig. 9. NKDs sectioned in a plane perpendicular to the axis of the axoneme (A). In "vacuoles" ribosomes (arrows) and microtubules (double arrows) are clearly visible. × 25,000.

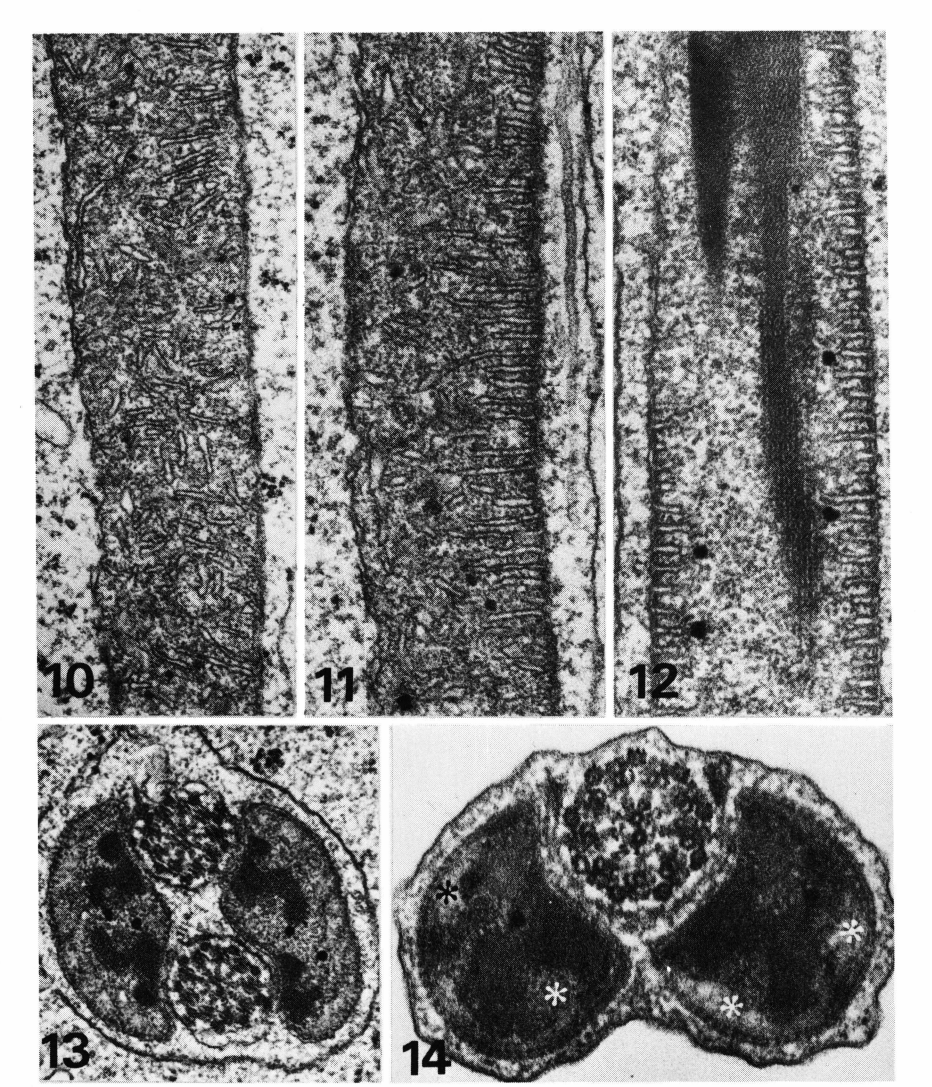

Fig. 10. Fragment of NKDs on a longitudinal section. Mitochondrial cristae are randomly distributed. × 50,000.
Fig. 11. Longitudinal section through MD showing periodic alignment of mitochondrial cristae perpendicular to the mitochondrial long axis. × 56,000.
Fig. 12. Fragment of MD with a developing PM. × 65,000.
Fig. 13. Cross section through the biaxonemal spermatid. In MDs two paracrystals independently of each other are formed. × 50,000.
Fig. 14. Cross section through the sperm tail. PM occupies most of the mitochondrial matrix. Asterisks indicate areas free of this material. × 80,000.

pole of the spermatid nucleus. Next, the NK undergoes complex structural transformations. Fig. 3 shows one of the stages of NK transformation, resembling the stage termed by Pratt (1968) as a "four-layered nebenkern" . Numbering the segments of NK layers (terminology applied after Pratt, 1968), in the manner shown in Fig. 3, indicates that the segments marked with even numbers do not merge with those denoted by uneven numbers. This implies that the "even" and "uneven" segments belong to two separate, but interlocked mitochondrial bodies forming the NK. In the course of further development the number of layers in the NK gradually decreases. A "two-layered nebenkern" is shown in Fig. 4. Two fissures which appear on the poles of NK (Figs. 3, 4) are, as shown by Pratt (1968), the sections of a meridional furrow on the NK surface. The furrow lies approximately in the plane running through the centres of the spermatid nucleus and the NK, *i. e.* in the plane of the future NK division into two NKDs. NK transformation entails its division into two NKDs which then take positions symmetrically on either side of the axoneme (Figs. 7, 9). The NKDs are not solid bodies; they contain many "vacuoles" within which ribosomes and microtubules are often observed (Fig. 9). It has been found that in some cases the lumen of the "vacuole" communicates with the cytoplasm (Fig. 8).

Initially, the NKDs assume an ellipsoidal form, and as the spermatids elongate their diameter progressively diminishes. Eventually they transform into two cylindrical structures oriented alongside the axoneme. Previously observed "vacuoles" disappear completely during this process (Figs. 10, 11).

As NKDs elongate, membranous cisternae penetrate between them and the axoneme, thus forming a ring-like structure called an axonemal sheath (Fig. 5). At later stages, the membranes of this sheath merge and, as a result, give the axonemal sheath an appearance of a thickened lamella (Fig. 6).

In the course of the elongation of the NKDs, their internal structure undergoes extensive reorganization leading to the transformation of NKDs into MDs. At first, randomly arranged cristae begin to arrange themselves on one side of NKDs, gradually assuming the form of evenly spaced shelf-like invaginations oriented perpendicularly to the NKD long axis (compare Figs. 10, 11). During the later stages of spermiogenesis arrangement of the cristae becomes more regular (Figs. 12, 15). As can be seen on Fig. 17, the arrangement of the cristae in MDs of the mature spermatozoa shows a striking regularity. The width of the cristae and the distance between them are almost constant at 22 and 20 nm, respectively. Concurrently to these changes, patches of electron-dense PM appear within the matrix of MDs adjacent to the axonemal sheath (Fig. 6). Such material emerges simultaneously in all the spermatids of a given cyst, always assuming the same, definite shape and size. It is worth noting, that in sporadically observed biaxonemal spermatids two identical patches of such material appear independently in the matrix of MDs, close to each axoneme (Fig. 13). When viewed in longitudinal sections, the PM appears as a complex of closely packed sinusoidal filaments running parallel to each other and to the long axis of MDs (Figs. 12, 15). The center-to-center spacing of the filaments is about 9 nm and the distance from peak-to-peak in sinusoids in 48 nm. On some of the longitudinal sections of PM periodic cross-striations with a 48 nm period can be seen (Fig. 16). These striations result from the sinusoidal shape of filaments. This probably occurs when the filaments become more closely packed; then the sinusoidal shape becomes indistinct until only 48 nm transverse periods remain

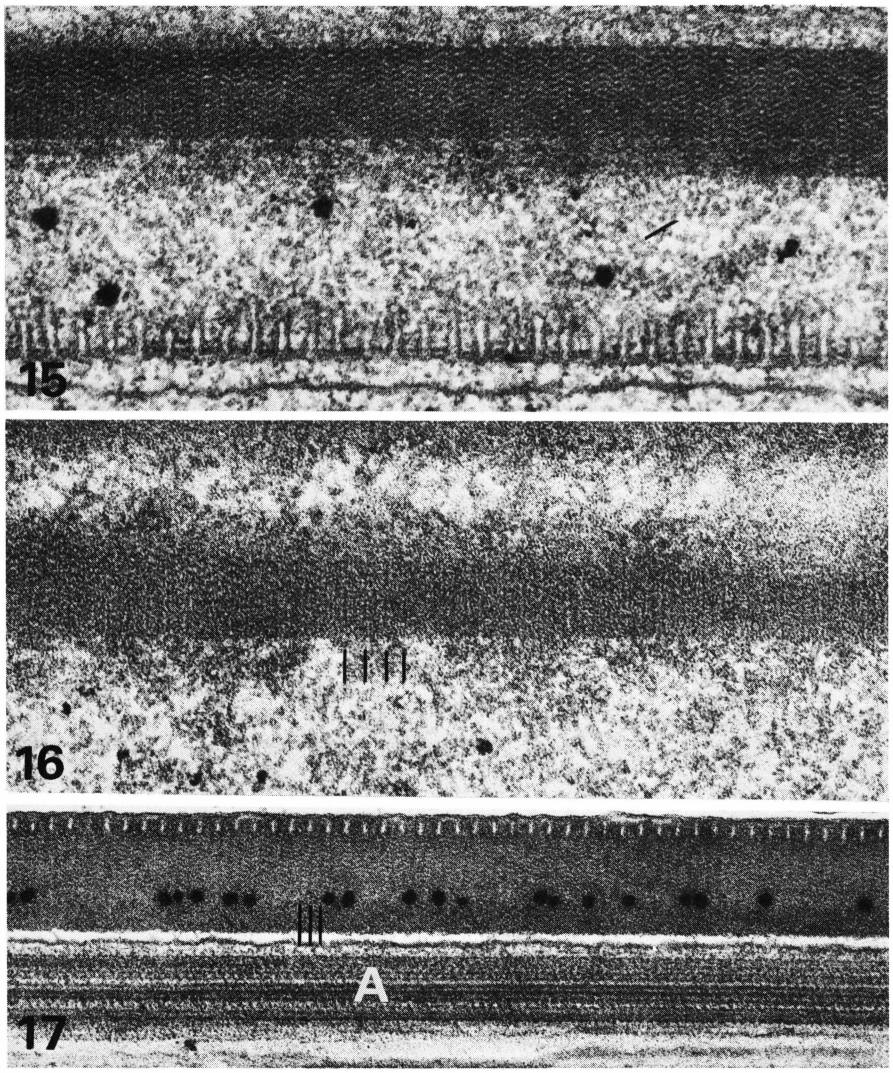

Fig. 15. Longitudinal section showing a fragment of MD in the late spermatid. PM is composed of sinusoidal filaments running parallel to each other. Note regularly spaced parallel cristae. × 87,000.

Fig. 16. On some longitudinal sections the PM shows periodic cross-striations. × 123,000.

Fig. 17. Longitudinal section of the mature spermatozoon. Note periodic alignment of cristae, sinusoidal filaments and regular cross-striations. A, axoneme. × 52,000.

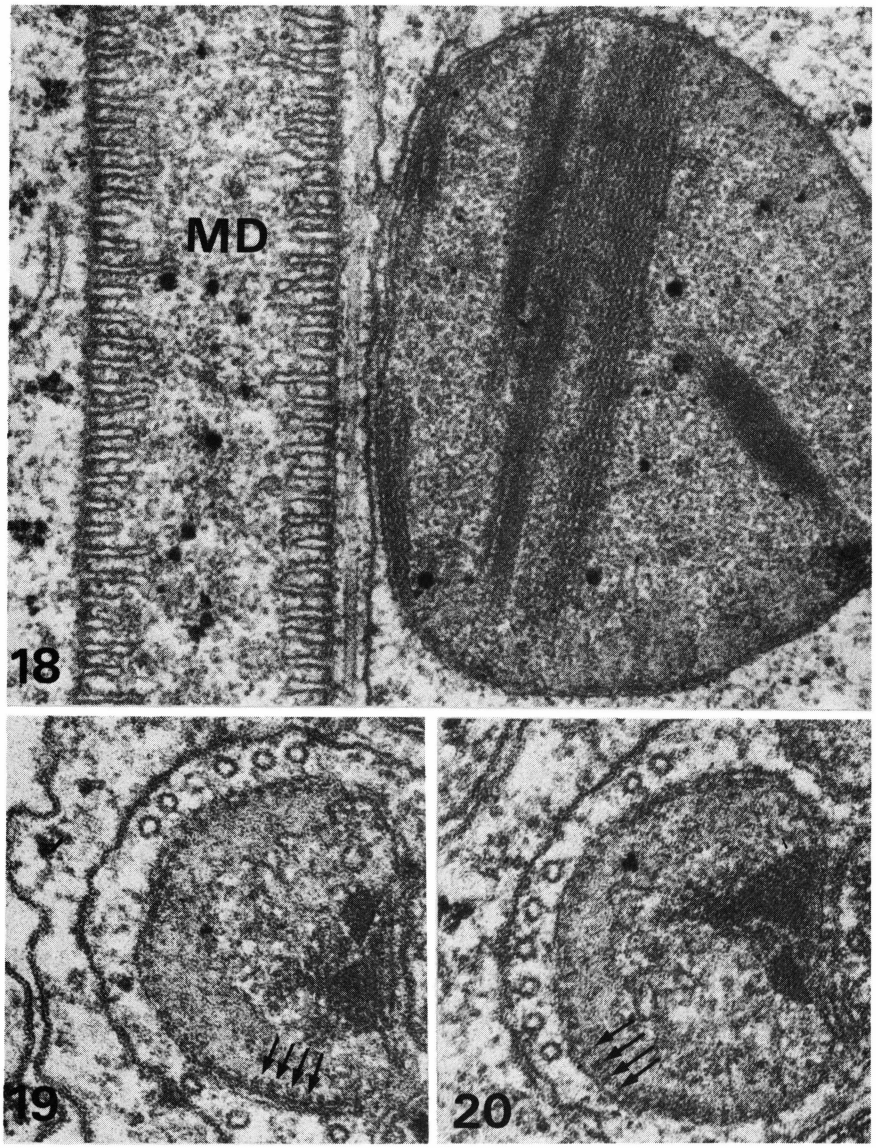

Fig. 18. "Additional" mitochondrion in the late spermatid. Patches of PM are irregularly scattered throughout the matrix. Note the sinusoidal shape of filaments forming the PM. MD, mitochondrial derivative. × 73,000.

Figs. 19, 20. Transverse sections through MDs. Arrows indicate rows of evenly spaced dense particles attached to the matrix facing surface of the inner mitochondrial membrane (Fig. 19) and lying free in MD (Fig. 20). MDs seen on Figs. 19 and 20 were probably sliced in the places which are marked on Fig. 25 as plane A and B, respectively. × 126,000.

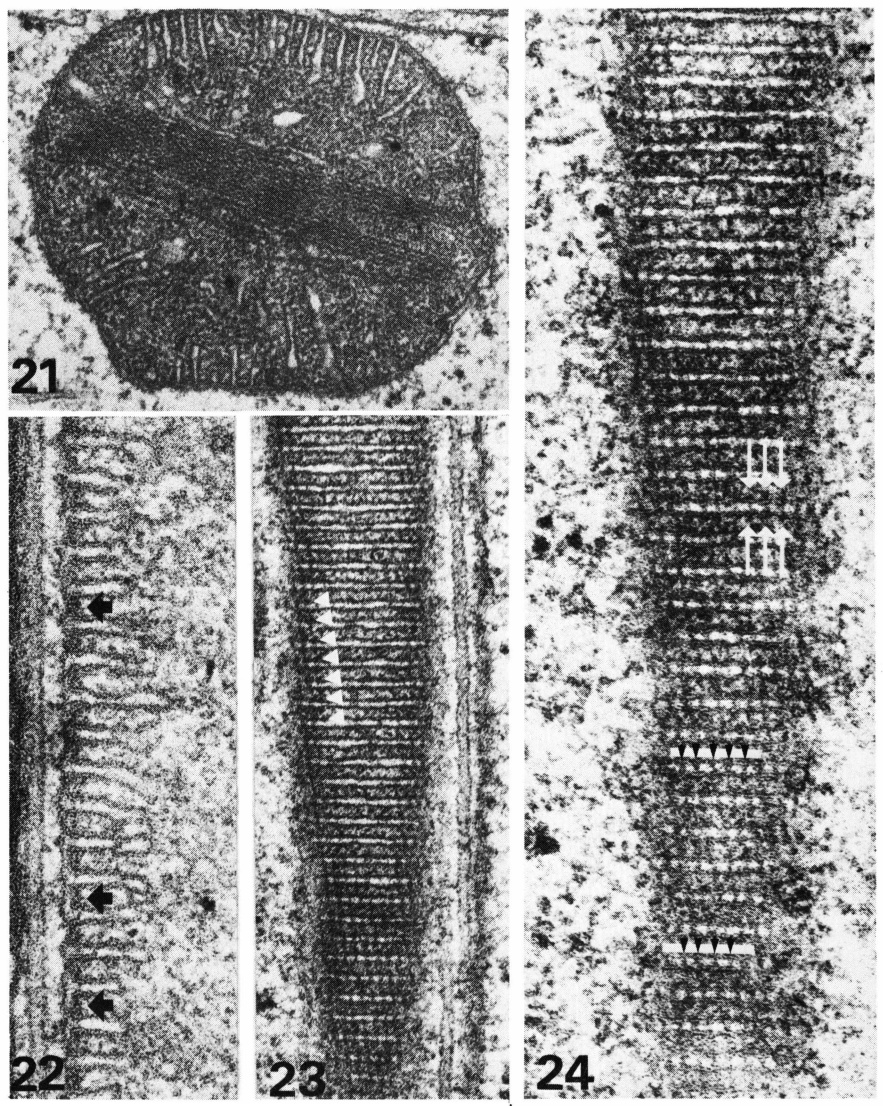

Fig. 21. "Additional" mitochondrion in the late spermatid. Note sinusoidal shape of PM filaments and regular arrangement of cristae. × 50,000.

Fig. 22. Longitudinal section through the MD. Note the dense material (arrows) between cristae attached to the matrix facing surface of the inner mitochondrial membrane. × 106,000.

Figs. 23, 24. Longitudinal sections through the distal parts of MDs. In both cases the planes of section runs tangentially to the inner mitochondrial membrane, crossing the sites of its transition into membranes of cristae. Equidistantly disposed "lines" running parallel to one another and slightly obliquely to the plane of the cristae membranes are clearly visible. In fact, the "lines" are arrays of dense particles connected with the membranes of mitochondrial cristae (white arrows) on Fig. 23. Note on Fig. 24 equidistant-spaced small densities in lumini of cristae (black arrowheads) and crista with dense particles attached to both its sides (white arrows). Fig. 23, × 70,800; Fig. 24, × 110,500.

visible.

Sections of the tail of spermatozoa show that most of the MD matrix is occupied by PM (Fig. 14). As in the earlier stages, a characteristic stratification and sinusoidal shape of filaments can still be seen in its structure (Fig. 17).

A striking feature of the internal structure of MDs in *P. apterus* is the presence of rows of electron-sense particle ~9.7 nm in diameter, with a center-to-center spacing of about 20 nm. On some transverse sections of MDs these particles are attached to the matrix-facing surface of the inner mitochondrial membrane (Fig. 19), while on the other sections they do not seem to have any contact with membranes of the mitochondrial envelope (Fig. 20). It appears that in the first case the plane of section ran just between two neighbouring cristae (Fig. 25, plane A), while in the second case the supposed plane of the MD section is represented by plane B on Fig. 25. Similar rows of particles have been also seen on superficial longitudinal sections of the distal parts of MDs (Figs. 23, 24). These particles are attached to the cristae membranes with center-to-center spacing ~20 nm. Their diameter is about 9.3 nm. These values correspond well with those calculated for particles observed on transverse sections. It seems that in the case of superficial sections the plane of section runs tangentially to the inner mitochondrial membrane, cutting across the sites of its transition into membranes of cristae (the position of such a section is represented by plane C on Fig. 25).

A specific arrangement of particles on cristae membranes also implies a particular arrangement of these particles along the long axes of MDs. As shown by Figs. 23 and 24, they form a system of equidistant parallel "lines" running slightly obliquely (at about 10°) to the plane of the cristae membranes. The "lines" are more obvious if the reader tilts the micrographs and looks obliquely along their planes. The presence of these "lines" is enhanced by the fact that in the electron-lucent intracristal space there are some regularly arrayed small densities that coincide with particles attached to the cristae membranes (Fig. 24). It can be inferred without much hesitation that "free" particles seen on Fig. 20 are cross-sections of these intracristal densities (Fig. 25, plane B).

If the longitudinal sections through MDs are obtained along the plane shown in Fig. 25 (plane D), aggregations of dense material will be observed between cristae (Fig. 22). They are most probably dense particles attached to the inner mitochondrial membrane.

The above observations provide a basis for working out a tentative model which represents the supposed spatial arrangement of particles in MDs (Fig. 25).

In some spermatids at the time of PM formation in MDs a few single "additional" mitochondria can be observed (Figs. 18, 21). Such mitochondria, like MDs, are characterized by the presence of PM with filamentous substructure, and by a regular arrangement of cristae. In marked contrast to MDs, however, the PM in "additional" mitochondria appears in the form of patches scattered irregularly in the matrix (Fig. 18).

Discussion

The process of transformation of mitochondria in the spermiogenesis in *P. apterus*,

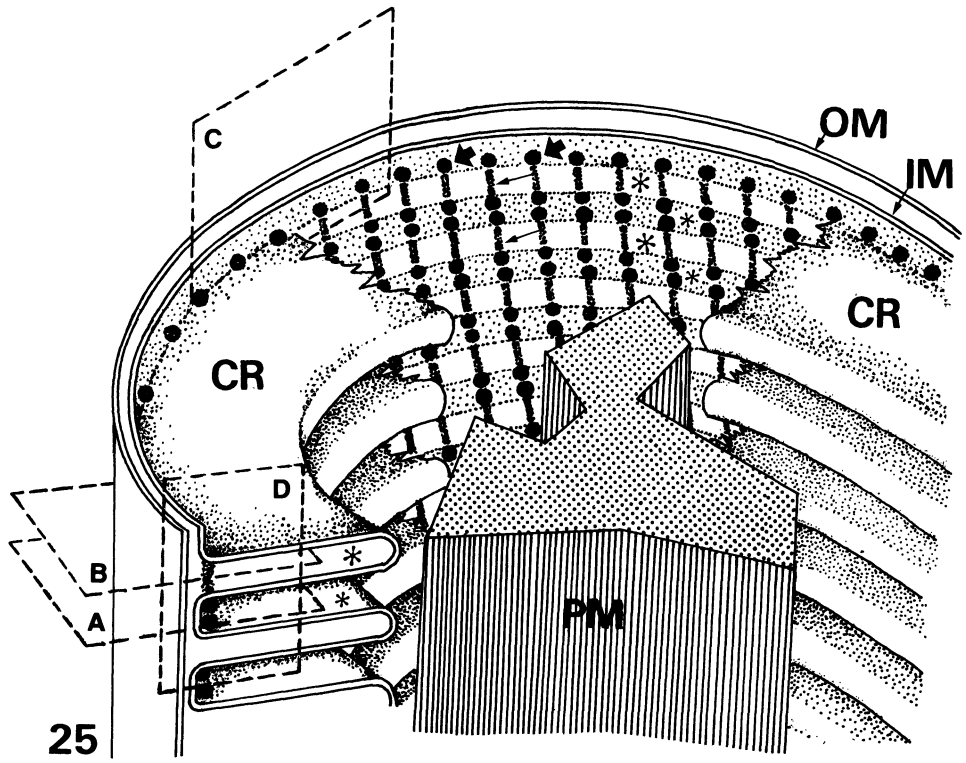

Fig. 25. Tentative model of spatial arrangement of particles in MD. Planes A, B, C and D indicate the places in which the MDs seen in Figs. 19, 20, 22, 23 and 24 were most probably cut (Fig. 19 — plane A; Fig. 20 — plane B; Fig. 22 — plane D; Figs. 23, 24 — plane C). CR, crista; IM, inner mitochondrial membrane; OM, outer mitochondrial membrane; PM, paracrystalline material; boldface arrows, dense particles; large asterisks, intracristal space; small asterisks, intercristal space; thin arrows, intracristal densities.

in result of which two rod-shaped MDs are formed in late spermatids, resembles that in other species of heteropterans studied so far (Bowen, 1922; Payne, 1966; Pratt, 1968, 1970; Phillips, 1970; Itaya *et al.*, 1980). Nevertheless, this study has provided some new information which, in our opinion, is worth commenting on. It should be noted that during the meiotic telophase, mitochondria cluster to form two independent aggregates fusing subsequently into one NK. A question arises whether there is any relation between these two aggregations that precede formation of the NK and the two interlocking mitochondrial bodies which later organize it according to André (1962) and Pratt (1968). The possibility that each mitochondrial aggregate is a precursor of one of the two mitochondrial bodies forming the NK cannot be excluded. However, it may well be that individualization of the two mitochondrial bodies takes place later, inside the NK.

In this paper the complex process of transformation of the NK, and its subsequent

division into two parts has not been analysed. A comparison between sections of NK obtained at various stages of its transformation in *P. apterus* with sections of differentiating NK in *Murgantia histrionica* (Pratt, 1968), seems to indicate that these processes are quite similar in both species.

A characteristic feature of NKDs in *P. apterus* is that numerous "vacuoles" of low electron density are found in their interior. The presence of ribosomes and microtubules in the "vacuoles" indicates that they represent islands of cytoplasm "trapped" inside the NKDs. Similar "vacuoles" occur also in NKDs in other insects (Pratt, 1968; Phillips, 1970; Baccetti, 1972; Stanley *et al.*, 1972; Szöllösi, 1975; Lai-Fook, 1982). These islands are reported as spaces appearing in result of an incomplete fusion of neighbouring layers in NK (Bertaud and Gatenby, 1960) or an invagination of their walls (Pratt, 1968). No previous studies have given any satisfactory explanation of the way in which the reduction and the subsequent disappearance of the cytoplasmic islands proceed during the transformation of NKDs into MDs. In *P. apterus* and presumably also in other insects, this process may proceed by way of compressing and later squeezing out the cytoplasm contained in "vacuoles" of NKDs, as their diameters decrease. This may happen only if one assumes, that "vacuoles" are not isolated cytoplasmic islands but form within the NKDs a continuous system that communicates with the remaining cytoplasm of the spermatid. The presence in NKDs of *P. apterus* of "vacuoles" whose lumini open to the surrounding cytoplasm, proves beyond any doubt that such communication does take place.

One of the most interesting aspects of insect spermiogenesis are the reorganizational changes in structure of MDs, which comprise partial or complete crystallization of matrix and periodic alignment of cristae (Barker and Riess, 1966; Phillips, 1966; Warner, 1971; Baccetti *et al.*, 1974; Rosati *et al.*, 1976; Dallai and Afzelius, 1980; Itaya *et al.*, 1980; for review see Phillips, 1970; Baccetti, 1972). In *P. apterus* these changes are similar to mitochondrial changes previously described in other heteropterans (Baccetti *et al.*, 1977; Dallai and Afzelius, 1980; Itaya *et al.*, 1980) as well as in other insects (compare: Perotti, 1969; Warner, 1971; Baccetti *et al.*, 1973; Rosati *et al.*, 1976). Some differences in the width of striations and in the size of sinusoidal filaments between *P. apterus* and other insect species may be attributed to different techniques of preparation employed and/or to specificity of the species. According to the classification of MDs proposed by Rosati *et al.* (1976), MDs of *P. apterus*, as in other heteropteran species (Barker and Riess, 1966; Rosati *et al.*, 1976; Itaya *et al.*, 1980; Dallai and Afzelius, 1980, 1982), belong to the category described as partially crystallized MDs.

The formation of the PM in *P. apterus*, as in other insects (Warner, 1971; Tokuyasu, 1974; Baccetti, 1975; Dallai and Afzelius, 1980, 1982), always starts in the part of the MD matrix closest to the axonemal sheath. This may suggest that the axonemal sheath exerts a certain inducing influence upon formation of this material. The fact that in the case of biaxonemal spermatids, observed occassionally in *P. apterus*, identical paracrystals are formed close to each axonemal sheath in each of the two MDs, may be used to support this suggestion.

The exact nature of the close relationship between the axonemal sheath and the site of PM formation in MDs is not known. It may be suggested that the cisternae of the axonemal sheath release a factor into MDs that determines normal arrangement of PM in MDs. Loss of close proximity of the mitochondrial structures and axonemal

sheath causes a disturbance in the crystallization process. "Additional" mitochondria in which PM is randomly scattered in the matrix provide an example of such disturbance.

An interesting hypothesis concerning the origin of the PM in MDs of *Tenebrio molitor* has been put forward by Baccetti (1972, 1975). According to him, the substance forming PM is transferred to MDs from Golgi-derived cisternae which penetrate between MDs and the axoneme and have close contact with MD envelope. Similar cisternae forming the axonemal sheath occur in *P. apterus* and, as our observations (unpublished data) indicate, they originate also from the Golgi apparatus. There is, however, virtually no morphological evidence suggesting an extramitochondrial origin of the PM and its transfer to MDs from the cisternae of the axonemal sheath. It seems that the PM in *P. apterus* is formed within MDs. In our opinion, the chondriome of a spermatid possesses a coded information allowing it to produce PM. Under the influence of as yet unknown factors a mechanism initiating the formation of the PM is released, at a definite stage of spermatid development. This may be supported by the fact that such material appears synchronically in both MDs and the "additional" mitochondria lying free in the cytoplasm with no apparent connection with the axonemal sheath.

The hypotheses concerning the origin of PM and the relationship between PM and axonemal sheath in *P. apterus*, put forward here, may of course be a far-fetched speculation, but the authors let themselves be guided by the words of Porter (1976): "We may be misinterpreting the observations, or overinterpreting them, but in either event, the fault would be greater were we not no attempt an interpretation" .

As was mentioned above, in some spermatids in *P. apterus* a few "additional" mitochondria can be found. In search for the genesis of such mitochondria it is not unreasonable to suppose that these mitochondria have not been incorporated into NK during its formation in immature spermatids. Most probably these mitochondria do not to play any part in the differentiating spermatids and are eliminated from the cell to the lumen of the cyst together with the residual mass of cytoplasm at the end of spermiogenesis. A similar occurrence of single mitochondria that are not incorporated into the NK has been observed by Szöllösi (1976) in spermatids of the locust, *Locusta migratoria*, bred under infra-optimal temperature conditions. Contrary to "additional" mitochondria in *P. apterus*, the extramitochondria in the locust elongate similarly to MDs.

There are many observations of granular inclusions in mitochondria (Munn, 1974), but the particles found in the MDs of *P. apterus* are unlike those described in other organisms in respect to both their form and specific distribution in mitochondria. These particles occur in the form of parallel rows attached to the inner mitochondrial membrane at the sites of its transition into the cristae membranes (Fig. 25). They emerge in MDs when the mitochondrial cristae become regularly arranged along the MD long axis. In our opinion it cannot be ruled out that such spatial rearrangement of the inner mitochondrial membrane may bring about certain changes in its structure and again that the occurrence of these particles is, in some way, a reflection of such alterations. Nevertheless, more research will be needed to explain the nature and role of the particles.

Particles similar to those described in *P. apterus* are also clearly visible in the MDs of transverse-sectioned spermatozoa of some heteropterans (Dallai and Afzelius, 1980;

Itaya *et al.*, 1980); these authors, however, have not commented on their presence.

Recently, the studies of Afzelius *et al.* (1985) on freeze-fractured spermatozoa of saldid bugs have shown the presence of regularly arranged particles of a size of about 9 nm, on the membranes of MDs. The center-to-center space of these particles, as obtained by measurements taken by the authors of this study from Fig. 13 in Afzelius's paper (Afzelius *et al.*, 1985), amounts to about 19 nm. Such close resemblance in the size of these particles, the distance between them, and their arrangement in the MDs of these bugs may suggest that in both cases we were dealing with the same structural element.

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