LIGHT AND ELECTRON MICROSCOPE STUDIES OF MORPHOLOGICAL CHANGES OF MITOCHONDRIA DURING SPERMATOGENESIS IN THE GRASSHOPPER*

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PLATES 102 TO 108

It is generally accepted that mitochondria are distributed to the daughter cells during cell division. It is also known that in grasshopper spermatogenesis the mitochondria align themselves on the meiotic spindle and are allotted approximately equally to the spermatocytes or spermatids. However, it is not so generally known that the mitochondria on the spindle divide so that about half of each mitochondrion is received by each newly formed cell.

Furthermore, many investigators have examined insect (especially grasshopper) nebenkern with the aid of the light microscope: Bowen (1), Gatenby (2), Johnson (3), Lewis and Robertson (4), Meves (5), Payne (6), Pollister (7), Poisson (8), and von la Valette St. George (9), and recently Beams et al. (10) studied this body by means of phase contrast and electron microscopy. They agree that the nebenkern is of mitochondrial origin. Gatenby (2) describes a series of changes (from a "chromophilic" to a "chromophobic" state during its development) which warrant further description.

We will present evidence that during these changes the mitochondria become filamentous and entwine to form the early nebenkern. This becomes chromophobic or vacuolated by internal dissolution of the filaments. The evidence is presented as a comparative study of photomicrographs obtained through phase contrast, light, and electron microscopy, employing living and fixed material.

Material and Methods

Testis tissue from adult and 5th instar grasshoppers (Melanoplus differentialis differentialis Thomas) was used for this study. After the testes were removed from the insect and placed in physiological saline adjusted to pH 6.8, adipose tissue layers, connective tissue, and tracheoles were removed from the follicles. A single follicle was then placed with physiological saline on a roto-compressorium, and, under a X 40 dissecting microscope, the large or distal end of the follicular theca was severed and the contents were allowed to spill into the surrounding saline. While the coverslide of the roto-compressorium was lowered with a screwing device, the cells were observed microscopically in order to insure that they were not subjected to undue pressure. These living cells were observed with the aid of a Zeiss-Winkel phase contrast microscope and an oil immersion lens of N.A. 1.30 at a magnification of 1125.

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Testicular follicles to be studied by light microscopy were fixed in Champy's solution for 48 hours, washed, dehydrated in graded alcohols, cleared in newly distilled aniline oil, and embedded in paraffin. Sections were cut at 5 μ and placed serially on slides. Some sections were studied with the osmium left unbleached. Other sections were bleached in 1 per cent KMnO₄ and 10 per cent oxalic acid, then stained with Heidenhain's iron hematoxylin.

For electron microscopy, preparations were made from follicles fixed for 20 minutes either in Palade (11) solution at pH 7.4 or in 1 per cent osmium tetroxide in physiological saline at pH 6.8. The material fixed in Palade solution was transferred directly to 70 per cent alcohol, dehydrated, and embedded in methacrylate. The material fixed in the 1 per cent osmium tetroxide-saline solution was treated in the same manner except that it was washed in physiological saline before dehydration. In a few cases some follicles were fixed in isosomotic HgCl₂ (0.9 per cent NaCl equivalent) solution, saturated with osmium tetroxide at room temperature. These were then treated with Lugol's iodine solution, dehydrated in alcohol, and embedded in methacrylate. The generally poorer appearance of this preparation led to abandonment of the method. The material was sectioned with an International microtome and ultrathin sections (25 to 30 μ) were placed on grids with formvar film and studied with an RCA, EMU-2 electron microscope.

**RESULTS**

Figs. 1, 16, and 23 (lm) represent large mitochondria adjacent to the spindle in phase contrast (living), light (fixed and stained), and electron microscope studies, respectively. Figs. 1 through 6 represent the same living daughter cells at various intervals over a period of 4½ hours, illustrating the progressive changes that the mitochondria undergo, from an extended rod-like (Fig. 1) to a filamentous condition (Fig. 6, f). Figs. 7 through 15 are of various living cells showing the expansion of the filamentous mitochondria (Fig. 8, f), their subsequent contraction (Fig. 10), and the formation of the dense (Fig. 11, nc) and later the vacuolated (Fig. 11, nϕ) nebenkern. The mitochondria on the spindle are very large (Fig. 23, lm). Fig. 16 (m) illustrates a bundle of these long mitochondria that measure 24 μ from tip to tip. In Fig. 23 (lm) the central constriction of the mitochondrion suggests that it is divided rather than distributed at random to a daughter cell. The mitochondrion (Fig. 23, lm), which measures 9.3 μ, is very probably only a small central segment of a much larger body.

As the secondary spermatocyte divides the large mitochondria begin to divide; they become thin centrally, thus assuming a dumb-bell shape. At each distal end the mitochondria elongate parallel to the cleavage furrow (Fig. 22, mf) thus becoming filamentous (Figs. 2, 6, f, and 8). These filaments congregate to form a C or crescent in a limited plane about each daughter nucleus of the spermatids. Figs. 1 through 6 of living cells illustrate bilateral migration of filamentous mitochondria perpendicular to the original mitochondrial orientation for division. The newly divided mitochondria become the filaments that compose the mitochondrial crescent in each spermatid. If we compare the filamentous nature of mitochondria in Fig. 8, f and fixed cells (Figs. 17, f and 18, f) it becomes easy to reconcile these to electron micrographs which show filamentous endoplasmic reticulum-like constituents (Figs. 24 through 29).
At times the crescent almost completely encircles the nucleus (Fig. 17). The long filamentous mitochondria form nodes at each end of the crescent, and this initiates the formation of the nebenkern (Figs. 10, 18–21, 26, n, 21, n, 28, n). As the two nodes proceed toward each other, the filamentous mitochondria are transformed into the dense early nebenkern. The high density of this structure is due to numerous edges of individual filamentous mitochondria, closely opposed, which form layers of refractive surfaces (see Figs. 30, 31). The diameters of the filaments within the electron-dense (chromophilic) nebenkern (nc, Fig. 31) and of portions of the endoplasmic reticulum-like (Palade and Porter, 12) material (Figs. 28 and 29) are the same, namely 70 mµ.

The transition of the nebenkern from the dense to the translucent (chromophobic) type is illustrated (np, Figs. 11 and 32 through 36). The dense nebenkern becomes less dense peripherally by the disappearance of many of the internal filaments, and numerous large, almost empty, pockets still surrounded by double membranes (np, Fig. 32) form. The double membranes separating the individual sacs also disappear until only two sacs remain, one on each side of the early tail filament (Figs. 36 and 37). These sacs are surrounded by a single layer of electron-dense material and contain numerous remnants of the closely apposed single walls of the original mitochondria. These structures, in section, are very similar to the structures called cristae by many authors, even bearing the same relationship to the outside edge of the nebenkern that the “cristae” do to the edge of the mitochondrion. The nebenkern (np) now reaches and touches the now visible centrosome (cs, Fig. 37), and grows downward with the tail filament (Figs. 14, 15, and 38).

The cleavage furrow of the cell is also of interest. An involution of the cell wall is present at the greatest depth of the division plane (c in Figs. 22 and 23). Primary and secondary spermatocytes invariably show a band of material about the mitochondria at the plane of cleavage in fixed and stained cells as observed with the light microscope (mb, Fig. 16). This ring, sometimes referred to as the mid-bodies, is not seen in phase contrast (Figs. 1 to 4) or electron micrographs (Figs. 22 and 23). The structure may be the infolding of the cell membrane at the cleavage furrow (c, Fig. 23).

DISCUSSION

The formation of the nebenkern in insect spermatogenesis has attracted much interest in the past. The mitochondrial origin of nebenkern is well established, but the derivation was thought to occur by coalescence without preliminary morphological changes in mitochondria.

We present evidence here that in the secondary spermatocytes mitochondria become filamentous by elongation, after which they form a narrow band about the nucleus in a crescent that occupies the space between the nucleus and the cell membrane. Two centers of condensation occur at the opposite ends of the
crescent, forming knobs that gradually come together. The two centers form a compact body of filamentous material which is called the early (chromophilic) or dense nebenkern. The “filaments” of the early nebenkern appear to be composed of two edges of individual mitochondria closely apposed, separated by 70 mμ. In the later transforming nebenkern only one of these membranes remains at the limiting surface of the organelle.

The dense nebenkern undergoes transformation into the late, chromophobic nebenkern by the disappearance of many of the numerous twisted double structures representing the adjacent edges of the mitochondria that fused to form the nebenkern in the first place. The double-lined structures of Fig. 35 connected to the outside edge are remnants of these that have become separated from the internal (tail side) portion of the nebenkern.

The mode of nebenkern formation by the elongation of each mitochondrion may serve a physiological purpose. Each mitochondrion is equally divided between the two nebenkern halves on each side of the tail filament. This accomplishes equal distribution of mitochondrial enzymes and constituents to each half of the nebenkern.

SUMMARY

1. Observations on the morphological changes of mitochondria preparatory to the formation of the nebenkern, as well as changes within the nebenkern, are reported.
2. Mitochondria enlarge and divide during the meiotic divisions.
3. The mitochondria of the spermatid elongate, become filamentous, form a crescent, and partially encircle the nucleus.
4. Nodes which develop on either end of the crescent become entwined as they move toward each other.
5. The two nodes coalesce to form a filamentous or early type nebenkern which is described by others as chromophilic.
6. Internal rearrangement and partial dissolution of the filaments result in the development of the late or chromophobic nebenkern which separates into two distinct bodies.
7. The nebenkern moieties send out processes toward the centrosome, and after making contact, elongate and occupy part of the space between the tail filaments and sheath of the spermatozoon.

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EXPLANATION OF PLATES

PLATE 102

Phase contrast micrographs of living cells from testis follicles of the grasshopper. X 800 except Fig. 13 which is X 1,600.

Figs. 1 to 6. The same cell is shown in each figure illustrating the progressive change of the mitochondria to the filamentous state.

Fig. 7. The mitochondrial bundle has almost completely divided in half, with only a trace connection remaining between the two daughter cells.

Fig. 8. The long strands of mitochondrial filaments (f) have stretched about the nucleus in the typical C-shape.

Figs. 9 and 10. The mitochondrial filaments have begun to contract at opposite ends preliminary to the formation of the nebenkern.

Fig. 11. The upper cell illustrates the complete contraction of the mitochondria into a chromophilic type nebenkern, while the lower cell contains the more advanced chromophobic type nebenkern.

Fig. 12. These cells contain nebenkern which have just begun to undergo a change from the chromophilic to the chromophobic stage.

Figs. 13 to 15. These cells are typical spermatids, with the nebenkern moieties attached to or touching the centriole and elongated about the tract of the tail filament.
PLATE 103

Light microscope micrographs of grasshopper testis cells fixed in Champy's solution for 48 hours, bleached, and stained with Heidenhain's iron hematoxylin. × 2,000.

Fig. 16. The mitochondria have elongated and are arranged on the spindle of the dividing cell showing mid-bodies (mb).

Fig. 17. The filamentous mitochondria (f) have spread out about the nucleus, almost completely encircling it.

Figs. 18 to 21. The filamentous mitochondria have begun to contract. Fig. 20 clearly illustrates the nodes which form at the extreme ends of the mitochondrial crescent as the entwining continues inward in each node and the nodes are brought closer to each other.
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Electron micrographs of testis cells of last instar or young adult grasshoppers.

Figs. 22 to 24, 27, 32 to 35, and 37. Fixed in 1 per cent OsO₄ according to Palade's method, washed in Belar solution, and dehydrated and embedded as usual.

Figs. 25, 26, 28, and 29. Fixed in 1 per cent OsO₄ in Belar solution, washed in Belar, and dehydrated and embedded as usual.

Figs. 30, 31, 36, and 38. Fixed in isosmotic HgCl₂ with saturated OsO₄, treated with Lugol's iodine solution, washed in distilled water, and subsequently dehydrated and embedded.

PLATE 104

Fig. 22. The mitochondria have begun to lengthen into filaments (mf) in a plane parallel to the cleavage furrow. Note the infolding of the cleavage furrow (c). × 12,000.
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PLATE 105

FIG. 23. The elongated mitochondria (im), aggregated along the length of the spindle at the time of meiosis, and gradually pinched in half at the constricting cleavage furrow (c). Note the infolding of cleavage furrow which may appear as a ring in the center of the mitochondria giving the illusion of mid-bodies. × 7,800.

FIG. 24. The filamentous mitochondrial material (mf) has been distributed in a “C” band about the nucleus. × 6,300.

FIG. 25. The filaments appear to be aggregating into a node (n). × 6,400.
PLATE 106

Fig. 26. Filamentous mitochondria are converging into a node (n), cut in cross-section. × 12,000.

Figs. 27 and 28. A portion of the crescent of filamentous mitochondria, with one of the resulting nodes (n) shown in sections of two different cells. × 12,000 and 16,000 respectively.

Fig. 29. Filaments (each 70 mμ in width) aligned in parallel, which are probably a part of the crescent. × 12,000.
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Fig. 30. The early nebenkern (nc) shows the filamentous condition, as well as the reason it is considered a chromophilic body by cytologists. X 12,500.

Fig. 31. The coils of filaments make up an early chromophilic nebenkern (nc). The width of the filaments is 70 mμ. X 14,000.

Fig. 32. An early stage in the transition of chromophilic to chromophobic type nebenkern is shown. Notice that the peripheral limiting membrane is still double. Several pockets (np) have been formed by internal dissolution of fibrillar material. X 16,000.

Figs. 33 to 35. These nebenkern show gradual dissolution of filaments peripherally (np) as well as the coalition of smaller pockets into longer ones. Centrally, the filaments are still present and intact. Notice the transition of the limiting membrane to a single layer. Cristae-like structures attached to the membranes represent remnants of filaments that previously traversed these regions. X 12,000.
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Fig. 36. Internal dissolution has continued to the point at which practically all of the dense core of filamentous material has disappeared. × 13,000.

Fig. 37. The tail filament (t) has emerged and is elongating from the centrosome in the maturing spermatid. The nebenkern moieties, aligned on either side of the tail filament, have also begun to extend. Note the almost complete absence of cristae-like structures in this and the preceding (Fig. 36) nebenkern. × 13,000.

Fig. 38. This section illustrates the lengthening of the mature spermatid cell and the nebenkern (np) which accompanies the elongation of the tail filament (not shown). × 7,500.
(Tahmisian et al.: Mitochondrial changes during spermatogenesis)