Cytology and Cytochemistry of Spermatogenesis in Two Millipedes *Phyllogonostreptus nigrolabiatus* and *Gonoplectus malayus*

Natwar Raj Kalla¹

Department of Zoology, Panjab University, Chandigarh-14, India

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Introduction

The cytology of spermatogenesis of millipedes has been worked out by a number of investigators Gilson (1886) in *Glomeris marginata*, *Polydesmus complanatus* and *Julus sabulosus*; Oettinger (1909) in *Pachyiulus varius*; Sokoloff (1918) in various species of *Polyxenus*; Warren (1934) in *Odontopyge* sp.; Tuzet and Bessière (1941 and 1943) in *Julus hospitelli* and *Schizophyllum albolineatum* respectively; Bessière (1948) in a number of species belonging to the families Julidae, Glomeridae and Polydesmidae; Nath and Sharma (1952) in *Thyroglutus malayus*; Tuzet *et al.* (1954) in *Polyxenus lucidus*; Grassé *et al.* (1956) in *Spirostreptus castaneus*, etc.

The millipede sperm is usually described as non-flagellate except in *Polyxenus lucidus* where, according to Tuzet and Manier (1955), a long axial filament is given off from the centriole only when the sperm reaches the seminal receptacle of the female. But in *Pachyiulus varius* (Oettinger 1909) the sperm is flagellate even in the male genital tract. Tuzet and Bessière (1943) also found flagellate sperm in the male genital tract of *Schizophyllum albolineatum* though the flagellum is only transitory.

Grassé *et al.* (1956) are the only workers who have described the ultrastructure of the non-flagellate sperm in *Spirostreptus castaneus*.

But, to the best of the author's knowledge, no worker has so far attempted the cytochemistry of the millipede sperm.

In this communication an account of cytology and cytochemistry of spermatogenesis of two millipede species (*Phyllogonostreptus nigrolabiatus* and *Gonoplectus malayus*) have been recorded.

Material and methods

Specimens of the millipedes used were collected from several localities situated at the foot of the Shivaliks in the neighbourhood of Chandigarh. The testes were dissected out in physiological saline and transferred to the following fixatives: Lewitsky saline, Hermann, Bouin, Zenker, Carnoy, buffered osmic acid, 10% neutral formalin, formal calcium and formalin vapour. Paraffin sections used were 5-7 μ , gelatin sections 10 μ thick.

For morphological studies of spermatogenesis the Lewitsky saline-iron haematoxylin and Hermann techniques were employed. Localization of nucleic acid was determined with methyl green-pyronin G (Jordan and Baker 1955) and Feulgen light green (Swift 1950). Changes in the composition of nucleoproteins during spermatogenesis were determined according to Alfert and Geschwind (1953) and Ringertz and Zetterberg (1967) techniques. Sakaguchi reaction for arginine, tetrazolium reaction for SH-groups, coupled tetrazolium (CTZ) reaction with various controls for specific amino-acids and mercuric bromphenol blue (Hg-BPB) for general proteins were employed. Polysaccharides (1:2 glycol groups) were identified by the periodic acid-Schiff reaction (PAS) followed by acetylation control and KOH

¹ Present address: Department of Biophysics, Panjab University, Chandigarh-14, India.

reversal. Methanol-chloroform mixture was employed to extract lipids to exclude the possibility of the lipids giving a positive reaction in PAS. Diastase and hyaluronidase digestion methods for the removal of such polysaccharides as glycogen and hyaluronic acid respectively were employed as additional control for the PAS method. Alcian blue reaction and Bauer's Feulgen technique were employed for the detection of acid mucopolysaccharides and glycogen respectively. Silver-Schiff (Baryan 1964) and Giemsa (Hancock 1956) were employed to stain acrosome. Sulphation technique for metachromasia was also employed. Lipids were studied by the employment of Sudan black B (SBB); Berenbaum's technique (1958) for bound lipids and acid haematein for phospholipids were employed.

For the localization of succinic dehydrogenase and acid and alkaline phosphatases techniques of Hrudka (1965) and Gomori (Burston 1964) were employed respectively. Living material was studied under a Carl Zeiss phase contrast microscope.

Results

The process of spermatogenesis is identical in *Phyllogonostreptus nigrolabiatus* and *Gonoplectus malayus* except that in the latter species the sperm and the sperm-forming cells are smaller.

Mitochondria: In the earliest spermatogonium of *Phyllogonostreptus nigrolabiatus* the mitochondrial granules are uniformly distributed (Fig. 1a); under the phase contrast they present, as usual, a much lower phase than the Golgi bodies. In the spermatocyte the mitochondrial granules are generally circumnuclear in disposition (Figs. 1b and 2). In the spermatid and the ripe sperm the mitochondria again assume a more or less uniform distribution

Figs. 1a-e. Illustrating spermatogenesis in *Phyllogonostreptus nigrolabiatus*. a, spermatogonium showing circumnuclear mitochondrial granules (M) with a few scattered Golgi bodies (G). Nucleolus (N₁). Lewitsky saline/Iron haematoxylin. b, primary spermatocyte showing circumnuclear concentration of mitochondrial granules (M). The Golgi bodies are more and distributed in the cytoplasm. Nucleolus (N₁) attached with the nuclear membrane. Zenker/Hg-BPB. c, secondary spermatocyte showing circumnuclear mitochondria (M). Golgi bodies (G). Zenker/CTZ. d, late spermatid with a few mitochondrial granules, large number of acrosomal vacuoles (AV) with Golgi bodies (G) at their periphery. Clear area of the nucleus (N) touches the centriole (C). Formal calcium/acid haematein. e, mature sperm side view. The acrosome (AC) inbetween the nucleus (N) and centriole (C), cytoplasm (CY) containing mitochondria (M) on either side of the acrosome. Zenker/PAS counterstained with methyl green.

Figs. 2-5. Photomicrographs of living cells taken with phase-contrast microscope. 2, spermatocyte showing circumnuclear mitochondria (M). Golgi bodies (G). The nucleus contains scattered chromatin filaments (CF). $\times 4000$. 3, primary spermatocyte and spermatids. Primary spermatocyte shows distinct and large number of Golgi bodies (G) and acrosomal vacuoles (AV). The spermatids shows prominent acrosomal vacuoles (AV) with Golgi bodies at the periphery. Clear area (arrow) in the nucleus. Centriole (C). Dictyosome (D). $\times 4000$. 4, the spermatids showing acrosomal vacuoles (AV) with Golgi bodies (G) at the periphery. Clear area in the nucleus (arrow) is inbetween chromatin mass and acrosomal vacuoles. $\times 4000$. 5, the spermatid, cytoplasm containing prominent scattered Golgi bodies (G). The clear area (arrow) is inbetween the chromatin mass and the centriole. $\times 4000$.

(Figs. 1d, e, 3, 4 and 5). It may be pointed out that the mitochondria are extremely small in all the sperm-forming cells and the sperm.

Throughout spermatogenesis the mitochondria reveal, more or less, a uniform chemical composition. The presence of general proteins is revealed by the Hg-BPB reaction (Fig. 6). They are negative for basic proteins. They are devoid of SH-groups. Their negative reaction to Sakaguchi establishes the absence of arginine. CTZ reaction with its various controls suggests the presence of tryptophan and tyrosine as the dominant aminoacid components of the mitochondria.

The lipid contents of the mitochondria are revealed by Sudan black B in which they are coloured black. Berenbaum's technique shows that the lipids of the mitochondria are bound with proteins. A positive reaction with acid haematein (negative after pyridine control) suggests phospholipids in them (Fig. 7). All the tests employed to detect carbohydrates, *viz.* PAS, Alcian blue and Bauer's Feulgen gave a negative reaction. At no stage do the mitochondria show any trace of nucleic acids.

The Nitro-BT reaction reveals the presence of succinic dehydrogenase in the mitochondria. They also reveal the presence of alkaline phosphatase in them.

Golgi bodies and acrosome :

The spermatogonia reveal only a few Golgi granules distributed at random in the cytoplasm (Fig. 1a). They are much bigger than the mitochondrial granules. In the primary spermatocyte (Figs. 1b and 4) the Golgi bodies seem to proliferate and also grow in size; they are not distributed in groups. In the early spermatid the Golgi bodies have further grown in size and some of them also appear in the form of dictyosomes (Fig 8); under the phase contrast they reveal a much higher phase than the mitochondria

Figs. 6-7. 6, the spermatocytes showing mitochondria (M), Golgi bodies (G). Nucleus with chromatin filaments. Zenker/Hg-BPB. $\times 4000$. 7, spermatids showing uniformly distributed mitochondria (M) and Golgi bodies (G). Nucleus intensely stained. Formal calcium/Post-chromed/Acid haematein. $\times 4000$.

at all stages of spermatogenesis.

A characteristic feature of the spermatogenesis is the presence in the cytoplasm of a number of clear hyaline vacuoles (Figs. 1d, 4, 5, 8 and 9) with dense contents. At first only one or two vacuoles appear in the early spermatid but soon with its maturation a larger number of them are observed. In several cases the Golgi bodies are arranged at the periphery of the vacuoles (Figs. 1d, 4, 8 and 9). The conclusion is inescapable that such Golgi bodies are the acroblasts secreting the vacuoles, the acrosomal vacuoles. Soon the acrosomal vacuoles gradually fuse into one larger vacuole, the proacrosome, with the

Figs. 8-10. Photomicrographs of living cells taken with phase-contrast microscope. 8, two spermatids enclosed in a common cytoplasmic mass with remanent Golgi bodies (G) and mitochondria (M). Early spermatids with acrosomal vacuoles (AV), dictyosome (D) and mitochondria (M). $\times 4000$. 9, spermatid showing proacrosome (P.AC) with Golgi bodies (G) clustered around its periphery. The clear area (arrow) is inbetween the centriole (C) and proacrosome. $\times 4000$. 10, mature sperm in side view, the acrosome (AC) is inbetween the centriole (C) and nucleus (N). The cytoplasm is on the either side of the acrosome (arrow). $\times 4000$.

Golgi bodies clustered at its periphery (Fig. 9). The proacrosome is a circular and hyaline sphere which at later stages places itself between the nucleus and the centriole (Figs. 1e and 10). The ripe sperm always contains some granular Golgi remnants in the cytoplasm.

The presence of lipids in the Golgi bodies is confirmed by their deep colouration in SBB. Acid haematein reaction with its pyridine control indicates the presence of phospholipids in them. The presence of proteins in the Golgi bodies is indicated by their positive reaction in Hg-BPB (Figs. 11) and CTZ. The latter technique with its controls establishes tryptophan and tyrosine as their dominant aminoacid constituents as in the case of mitochondria.

The presence of carbohydrates, particularly 1:2 Glycol groups, in the Golgi bodies has been confirmed by their positive reaction in PAS followed by acetylation control and KOH reversal (Fig. 12). The Golgi bodies give negative reaction to Alcian blue (for acid mucopolysaccharides) and Bauer's Feulgen technique (for glycogen). Acid phosphatase is present in the Golgi bodies as revealed by Gomori technique.

The acrosomal vacuoles, the proacrosome and the acrosome itself appear to be chemically inert, never showing any positive reaction to all the above mentioned techniques. They are also negative to Giemsa staining and silver-

Figs. 11-13. 11, the spermatids showing small acrosomal vacuoles (AC) with Golgi bodies (G) and the mitochondria granules. The clear area (arrow) between the chromatin mass and the centriole (C). Zenker/Hg-BPB. $\times 4000$. 12, the spermatocytes showing Golgi bodies (G), nucleus with chromatin filaments. Bouin PAS after acetylation and KOH reversal. Counterstained with methyl green. $\times 4000$. 13, mature sperm side view. The acrosome lies inbetween the centriole (C) and plano-convex nucleus (N). Cytoplasm on either side of the acrosome (arrow). Prepared as in Fig. 12. $\times 4000$.

Schiff technique. Nor do they show any metachromasia.

Nucleus and centriole: The spermatogonial nuclei contain each a prominent nucleolus. A nucleolus can be traced in the nuclei of the primary spermatocytes also but it completely disappears in later stages of spermatogenesis. It stains intense red with methyl green pyronin G technique, revealing an abundance of RNA in it. The nucleus of the earliest spermatid is vesicular in which DNA rich chromatin lumps are distributed at random.

As the process of spermateleosis advances the vesicular nucleus of the spermatid undergoes a complicated series of changes in the arrangement of its

DNA contents. The nucleus now shows a very clear area completely devoid of DNA which, to begin with, is situated between the proacrosome and the DNA containing part of the nucleus (Fig. 9). A little later the nucleus rotates through an angle of 180° with the result that the clear area comes to lie next to the centriole (Figs. 5 and 11). Still later the clear area of the nucleus completely disappears when the nucleus is finally condensed into a plano-convex lens-shaped structure with the mature acrosome now lying between the nucleus and the centriole (Figs. 10 and 13). In a side view of the sperm under the phase contrast a certain amount of cytoplasm, which has escaped sloughing off, can always be detected on each side of the acrosome (Fig. 10).

The ripe sperm has a plano-convex nucleus (Fig. 10) in which the DNA rich chromatin substance is confined to the peripheral regions just under the nuclear membrane so that in a top view the nuclear substance appears in the form of two circular areas, an outer thicker and an inner thinner (Figs. 14

Figs. 14 and 15. Mature sperms top view. Centriole (C) with circular area and two chromatin regions (arrows). 14, *Gonoplectus*. 15, *Phyllogonostreptus*. Phase-contrast. $\times 4000$.

and 15). In a side view of the sperm, however, the plano-convex nucleus generally appears as a thick band (Figs. 1e and 13).

A centriole can be detected in all the spermatogonial and meiotic divisions, one arranged at each pole of the spindle. In spermatids the centriole is situated at one pole of the cell. It contains proteins and lipids as indicated by Hg-BPB and SBB techniques respectively. Its positive reaction to bromphenol blue technique suggests the presence of basic proteins in it. The centriole gives a faint reaction for acid phosphatase.

Quite unexpectedly the centriole gives a strong positive reaction for polysaccharides as suggested by PAS technique followed by acetylation control and KOH reversal (Figs. 13).

Ripe sperm: The sperm of millipedes under discussion becomes finally mature in the testicular tissue of the male, as no change, whatsoever, in its morphology has been observed either in the efferent male duct or the gonopod or the female genital tract.

The morphology of the ripe sperm is characterized by the complete

absence of a flagellum. The ripe sperm is a highly compressed cell with a plano-convex nucleus which stretches across at one end of the cell. At the opposite end lies a centriole which is differentiated into a central granule and an outer ring. Between the nucleus and the centriole lies the cytoplasm containing in its axis a vesicular, hyaline acrosome (Figs. 1e, 10 and 13). The nucleus of the sperm measures across 6.8μ ; in thickness it measures 1.7μ . The sperm measures 3.4μ in its vertical axis.

Discussion

The morphology of the spermatozoa of the two millipedes under discussion viz. *Phyllogonostreptus nigrolabiat* and *Gonoplectus malayus* is characterized by the complete absence of a flagellum. This is in conformity with the findings of Nath and Sharma (1952) in *Gonoplectus* (= *Thyroglutus*) *malayus*, of Gilson (1886) in *Glomeris marginata*, *Polydesmus complanatus* and three species of Julidae, of Warren (1934) in five species of millipedes, of Tuzet and Bessière (1941) in *Julus hospitelli* and of Bessière (1948) in several species of millipedes. More recently Grassé *et al.* (1956) have also described a non-flagellate sperm in *Spirostreptus castaneus* with electron microscopy.

On the contrary, Oettinger (1909) in *Pachyiulus varius* has described a very distinct tail filament in the living spermatozoa which grows to a relatively long fibre measuring approximately three times the size of the whole cell. It is not known with certainty whether the filament is capable of making any movement. Tuzet and Bessière (1943) have described a transitory tail filament in the sperm of *Schizophyllum albolineatum*. Tuzet and Manier (1955) in *Polydesmus lucidus* have shown that the spermatozoa unfold their flagellum only in the genital tract of the female and become spermatozoa of the normal type though it is immobile.

Before proceeding further it must be stated categorically that the nucleus and the centriolar ends of the compressed millipede sperm are the anterior and posterior ends respectively in conformity with the structure of a typical flagellate sperm—not considering some few atypical flagellate sperms in which the centriole occupies a pre-nuclear position (see Nath 1965 for references). The post-nuclear position of the acrosome in the millipede sperm is paralleled, so far as the author is aware, only by *Lepisma* (Nath *et al.* 1960 and 1962, Bowen 1924, and Nath and Bhatia 1953), by *Ctenolepisma* (Nath *et al.* 1962) and by *Thermobia* (Bawa 1964).

Schrader and Leuchtenberger (1951) by employing the technique of periodic acid-Schiff (Hotchkiss 1948) discovered that the acrosome in a variety of sperms gives an intense positive reaction. Subsequent research has shown that the acrosome is invariably PAS positive but in *Phyllogonostreptus* and *Gonoplectus* sperm the acrosome gives a very feeble reaction with PAS which must be regarded as negative. On the contrary, quite surprisingly, the centriole

gives a strong PAS positive reaction. This is paralleled by the unusual granular structural pattern of the centriole in the non-flagellate sperm of *Heteropachyoidellus* (Sotelo and Trujillo-Cenóz 1958).

Summary

Spermatogenesis in two millipedes *Phyllogonostreptus nigrolabiatus* and *Gonoplectus malayus* has been studied with phase contrast and cytochemical techniques. The process is the same in both species. The Golgi bodies of the early spermatid secrete acrosomal vacuoles which in turn unite to form a large proacrosome. This in turn forms the acrosome. The mitochondria react positively for tryptophan, tyrosine, phospholipides, succinic dehydrogenase and alkaline phosphatase. The Golgi bodies contain carbohydrates (1:2 glycol groups), phospholipids, tyrosine, tryptophan and acid phosphatase. The acrosomal vacuoles, the proacrosome, and the acrosome did not show positive reactions in any of the technique employed, including that of PAS.

The centriole contains carbohydrates and acid phosphatase; it is PAS positive. The mature sperm is a compressed cell with a plano-convex nucleus.

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