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ELECTRON MICROSCOPIC STUDY OF THE BULL SPERMATOZOON IV

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IV STRUCTURE OF THE MIDDLE PIECE

RESULTS

In the current study with suspension materials, inner structures of the middle piece of the bull sperm were generally obscure. This portion was demonstrable only as a rod-like structure with high electron density (fig. 30). By means of repeated washing with distilled water the mitochondrial sheath was collapsed and the axial fibril bundle was revealed.

In sectioned materials, more detail of internal structures were apparent. In cross sections, the entire elements composing the middle piece were detectable: a triple-layered cell membrane, a mitochondrial sheath, 9 peripheral fibrils forming the outer ring of the axial fibril bundle, 9 peripheral fine fibrils forming the inner ring, and a pair of central fine fibrils, thus, totalling 20 fibrils composing the axial fibril bundle. Each of the peripheral fibrils (the outer ring) had very high electron density and the diameters of 4 localizing at both the lateral sides were larger about twice than those of the remaining 5, 3 of which localized at the dorsal side and 2 at the ventral (figs. 36 & 37). The 9 peripheral fine fibrils (the inner ring) and the central pair had lower electron density and smaller diameters as compared with the peripheral fibrils. Each of these inner or central fine fibrils was a tubular structure having no contents or a little contents with very low electron density as similar as that of surrounding matrix (figs. 13 & 37).

In some successful microphotographs of the middle piece which was longitudinally sectioned through the flattened plane of the head, out of the axial fibrils only 2 thicker peripheral fibrils and 3 fine fibrils were shown, running parallelly with each other toward the direction of the tail piece (figs. 13 & 33). The thicker peripheral fibrils originated in the large radices of the neck, but there was so-called mosaic junction between them, as mentioned in the foregoing chapter.

The interspace of these structures was occupied by considerably light matrix. In some microphotographs of the middle piece sectioned longitudinally through the sagittal plane of the head, there appeared 4 fine fibrils (fig. 39). A pair of central fine fibrils did not arrange laterally, but did dorsally and ventrally. The 5 small radices of the neck seemed to become 5 peripheral fibrils of the middle piece. The other findings suggest the peripheral fibrils in longitudinal sections resembled those in the neck. Each of these fibrils of the middle piece

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seemed to run straight through the tail piece and the end piece (figs. 13 & 39).

In the cross section of the middle piece, the arrangement pattern of the fibrils composing the axial fibril bundle was quite symmetrical in a plane through the central pair (figs. 36 & 37). The remaining 9 pairs of fibrils forming the outer ring and the inner ring of the axial fibril bundle were located radially surrounding the central pair. In the outer ring, the 4 thicker peripheral fibrils were located at both lateral sides, taking the nearest position of a provisional straight line between each of the central pair. The remaining 5 (3 & 2) peripheral fibrils were located at the dorsal and ventral sides, respectively. Each of the fine fibrils consisting of the inner ring was disposed at the position of inside of the respectively corresponding peripheral fibrils forming the outer ring. Furthermore, from the findings of many cross sections of the middle piece with the head, it was concluded that the symmetric plane introduced from the arrangement pattern of 20 fibrils of the middle piece was in accord with that of the head.

In longitudinal sections of the middle piece, the mitochondrial helix, the main component of the mitochondrial sheath which originates at the basal granule of the head and loosely covers the neck as a collar, showed a regular pitch of spiral (figs. 33 & 35). The internal structure of the mitochondrial helix had a close resemblance to that of mitochondria of the general somatic cell; a triple-layered mitochondrial membrane (unit-membrane system) contained mitochondrial matrix with moderate electron density, in which the mitochondrial cristae were sometimes noted (figs. 13 & 36). As shown in figures 34 and 35, the mitochondrial helix seemed to be a spiral-like structure surrounding the axial fibril bundle. The number of turns was assumed to be 2 by means of the spiral engineering method (the details are given in discussion of this chapter).

On the basis of the findings of replica preparations, it was clearly shown that the direction of the spiral was regularly counter-clockwise (figs. 31 & 32). The number of times of spiral in this structure was decided as follows; in 43 cases of successful longitudinal sections of this part, the average frequency of spiral at the left side was 64.96 (range: 56~74) while at the right was 65.50 (range: 55~72), thus, in viewpoint of the fact that the mitochondrial sheath consisted of 2 mitochondrial helixes as mentioned above, it can be said that each of the 2 helixes would surround the axial fibril bundle about 32.5 times, respectively ($p < 0.01$).

At the terminal portion of the mitochondrial sheath, there appeared a Jensen's ring being a triangular structure in sectioned material with high electron density in longitudinal or oblique sections (fig. 38). In suspension (fig. 30) or replica preparations (fig. 32), this structure was only detected as a slight swelling at the posterior end of the middle piece. In oblique sections of this region it was clearly shown that the helix surrounded the axial fibril bundle with consistent pitch from the neck to the middle piece and reduced in thickness posteriorly, finally terminating at Jensen's ring.

The triple-layered cell membrane enveloped the outside of the mitochondrial sheath, in general, with a short distance, and sometimes with close contact, but it adhered tightly to the Jensen's ring (figs. 13, 33, & 38).

DISCUSSION

It has pointed out by RETZIUS⁸⁵⁾ that there are morphological differences

between the middle piece and the tail piece of the sperm. As the most important characteristic of the middle piece, the existence of the mitochondrial sheath has been reported in most text books^{56,102,108}, also this structure has its origin in mitochondria of the spermatid has been ascertained by many optical^{10,106} or electron microscopic studies^{3,8,28,32,64,92,118}.

The internal structures of the mitochondrial sheath are, in general, considered as similar to those of general somatic cells^{76,97}. There are some lamellar structures in the mitochondrial matrix, but their three dimensional structure is not yet well known³⁸. In this work, the morphological composition of the mitochondrial sheath of the bull sperm showed a close resemblance to that of somatic cells; there appeared a triple-layered mitochondrial membrane containing a matrix with considerably low electron density and some mitochondrial cristae (figs. 13 & 36). Some findings with vacuoles inside of the mitochondrial membrane, instead of the matrix, seem to be an artifact during the course of making preparation.

There are not always in accord with each other in many authors' opinions in viewpoint of that the mitochondrial sheath envelops the axial fibril bundle^{11,38}. Most of electron microscopists believe that the mitochondrial sheath is limited in the middle piece^{3,17,26,37,38,53,93}, while some of them have the opinion that the structure is extended up to the neck portion²⁹. SCHULTZ-LARSEN (in human sperm) and BLOM & BIRCH-ANDERSEN¹⁴ (in bull sperm) reported respectively in the study with sectioned preparations that the mitochondrial sheath was continued from the neck portion, but they could not reveal its originating part. The present author noticed that the mitochondrial helixes were originated from the basal granules of the head, irregularly surrounding the neck as a collar and spiraling down to the middle piece with a regular pitch. It is generally accepted that the mitochondrial sheath in mammalian mature sperms takes a continuous spiral form^{17,24,26~29,37,53,63,81}, as well as shown in the spermatocytes by YASUZUMI¹¹³ (in rat) and CHALLICE (in mouse and hamster). In the study of section preparations of the human sperm, ÅNBERG and FAWCETT reported that each helix of the mitochondrial sheath showed an arrangement similar to that of somatic cells, while SCHULTZ-LARSEN stated that the 2 mitochondrial helixes spiraled surrounding the axial fibril bundle. The present author is, in general, agreeable with the later opinion from the findings of replica (fig. 31) and sectioned preparations (fig. 35), although in a few exceptional cases there appeared discontinuous profile or adhesions (fig. 34).

It has been illustrated in most reports that the direction of the spiral of the mitochondrial helix is counter-clockwise^{55,106}. In the electron microscopic studies in the human^{3,95} and farm animals' sperms^{26,81}, this also has been considered to be true, without any distinct proof. The first worker who has undoubtedly demon-

strated the direction of spiral in farm animals' sperm by means of replica method is KESSLER. Furthermore, it is very interesting that FAWCETT illustrated the structure having a clockwise direction, on the contrary to the reports of ÅNBERG and SCHULTZ-LARSEN studying the same species (human).

The determination of the spiral direction is very difficult using the optical or electron microscope with suspended materials or with sectioned materials; with the latter method it is necessary to make a three dimensional reconstruction from numerous serial sections. In the present author's work, the replica method was used to study the problem of spiral direction (figs. 31 & 32). Each replica of this region showed a regular oblique angle; thus the spiral direction was decided to be counter-clockwise.

Many early morphologists considered the mitochondrial helix as being single^{55,106,108}). Likewise, some of the earlier workers using the electron microscope agreed to this concept³⁷). On the contrary to these, HERRNLEBEN decided the number of helixes in farm animals' sperm to be 3, on the basis of the nature of the spiral pitch, and also other authors tried to estimate the helix to be more than 3 based upon the degree of the spiral angle. In addition, BRETSCHNEIDER has revised his opinion from single, at first²⁹), to double²⁶) or triple²⁷), in the bull sperm. Nowadays, however most of the morphologists consider the number of the helixes both in the human and farm animals' sperms as 2^{10,32,63,81,92,95}). The present author decided it to be two helixes according to the theory of spiral-engineering. That is, in figure 35, a schematical copy of the mitochondrial sheath, if next formulae would be satisfied geometrically, it may be said that the number of the helix consisting the mitochondrial sheath is two.

$$\angle MOE = \angle R$$

$$\triangle MOE \equiv \triangle MOG$$

The formulae mentioned above should be employed only in the case where the middle piece would have an approximately equal thickness, and where the spiral helixes surrounding it would have a regular pitch. The actual figure in thickness of the middle piece was slightly different between the portion near the neck and that of near the tail piece. The difference measured was, however, only about 2 mm in 15,000 times magnified microphotographs. Such a slight difference seems to be negligible. Furthermore, another theorem in the field of spiral-engineering may be allowed to be introduced into the problem concerned; if two tubes with an equal thickness would surround a cylinder closely attached with each other, the minimum number of cutting surfaces of the tubes which would appear in a correct transverse section across the cylinder is to be two. Actually, the minimum number of cutting surfaces of the spiral helixes appeared in correct transverse sections of

the middle piece was completely in accord with this theoretical value (fig. 37). In addition, the fact that the mitochondrial helixes have their origin in two basal granules which are located on the bilateral sides of the basal portion of the head would strongly support that the number of the mitochondrial helix is two.

It has been said that the number of spiral of the mitochondrial helix is ten times or so in human and farm animals' sperms, on the basis of the earlier electron microscopic studies^{37,81,84,93}. Recently, FUJII reported that the number of the spiral is nearly 30 in the bull sperm and about 20 in the boar sperm by means of the argentine stain method. BRETSCHNEIDER²⁷, using suspension materials of the bull sperm, reported 10~17 turns. On the other hand, BLOM & BIRCH-ANDERSEN¹⁴ reported about 100 turns, using sectioned materials of sperms in the same species. As for the number of the spiral in the human sperm by means of the section technique, 5~7, 9 and 10 were reported by SCHULTZ-LARSEN, ÅNBERG and FAWCETT, respectively. From these reports, the number of the spiral in the human sperm is suggested to be nearly 10. But, in farm animals, it is suspected that the number may considerably vary with species and within species. In the present work of the bull sperm, the number averaged 32 (range: 28~37). In this connection, it is interesting to note RAHLMANN's report in which in Jersey bull sperms the number was approximately 75.

Fine structures of the terminal part of the mitochondrial helix, excepting so-called Jensen's ring, are not yet clearly detailed, as far as the present author is aware. Since JENSEN (1887) had reported a terminal ring structure at this part by the optical microscope with special stained stallion sperm, the structure is called Jensen's ring and has been reported in the sperm of various animals^{17,53,55,92,106,108}, however, some investigators could not find such structure^{3,47,95}. This may be due to the comparatively fragile nature, of the structure as suggested by FRIEDLAENDER. In section preparations in the present study, the structure was clearly shown as a triangular form with a high electron density at the terminal portion of the mitochondrial sheath (fig. 38). Since Jensen's ring was observed in all sections of the terminal portion of the middle piece, it is suggested that this structure is a ring and probably plays a role as a band finishing the mitochondrial helixes. The present study has failed in demonstrating a distinct connection between the Jensen's ring and the fibrillar coil sheath of the tail piece which originates just below Jensen's ring.

Nowadays, the structure of the axial fibril bundle is known as a very complex one. But, in earlier age, it was considered to be rather simple^{82,83,85,86,108}. In 1888, BALLOWITZ at first reported that the tail of the avian sperm sometimes gets loose, becoming into several fine fibrils, but for a long time this finding had not been widely agreeable. In the age of 1940, since introduction of the electron

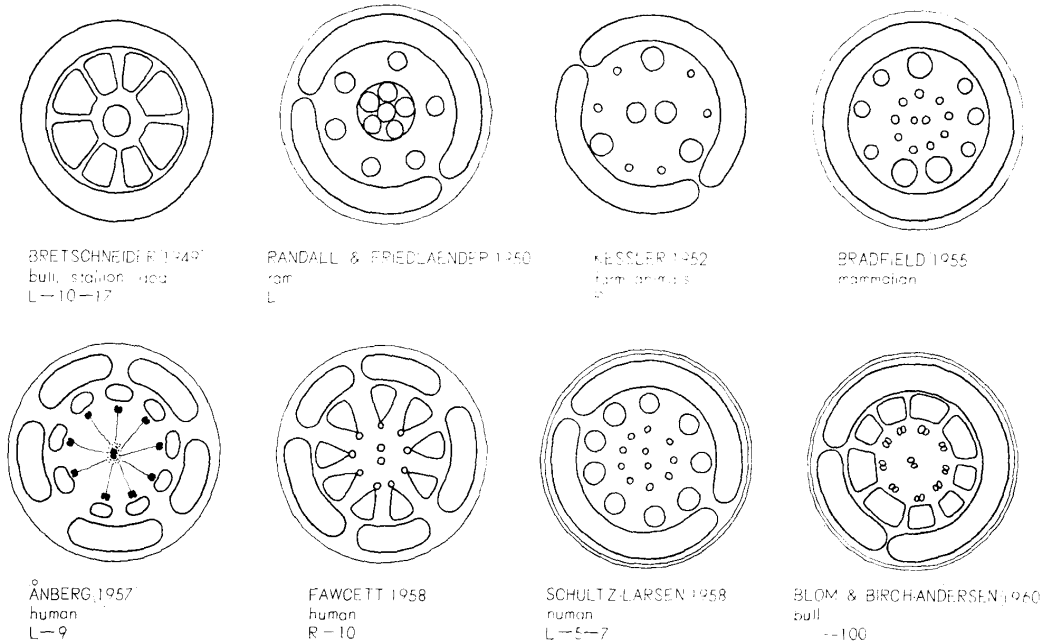
microscope into biology, SEYMOUR & BENMOSCHE ascertained the existence of the fibrils in the human sperm, and then BAYLOR et al. followed them.

In 1943, SCHMITT et al. treated human, bull, rabbit and rat sperms with ultrasonic waves, and revealed electron microscopically that the axial fibril bundle of these sperms was consisted with 9~11 fibrils. Thereafter, many workers with deep interests in the structure treated the sperm with various chemical drugs^{28,37,62,84,110,111,115} or destroyed it by physical methods^{3,63,81}, in order to remove the mitochondrial sheath and to observe naked fibrils. From the results of these wide examinations, the number of fibril consisting the axial fibril bundle in the human and farm animals was concluded as 9~11^{6,27,46,62,91,93,110,111}. Although the arrangement pattern of each fibril in the axial fibril bundle had varied in these reports, with the advances in the ultra-sectioning technique, these earlier interpretations have been rapidly revised, and nowadays a uniformity in the arrangement pattern of the fibrils in the middle piece and the sperm tail piece is recognizable through almost all of the mammalian sperms^{22,38,116}. It is generally accepted that the axial fibril bundle is composed of 9 peripheral fibrils forming the outer ring, 9 peripheral fine fibrils forming the inner ring, and a pair of central fine fibrils, thus total number of fibrils being 20^{11,33,95,113}.

For the convenience of the following descriptions, this pattern of the fibrils in the axial fibril bundle will be abbreviated as [9 : 9 : 2] in this paper. This arrangement pattern has been reported not only in Mammalia, including the human, but also in Aves, Amphibia and some invertebrates^{22,31,38,45,46}. It should be worthy of attention that the flagella of some species of protozoa and the cilia of the general somatic cells also have this pattern^{22,40,89,100}, while the sperm of the sea-urchin and the male gamete of some species of plants are lacking the outer ring, the pattern of these showing [0 : (n×9) : 2]^{3,22,23}. However, some discordances in the pattern are seen in a same species among workers for example, in the human sperm, SCHULTZ-LARSEN reported the [9 : 9 : 2] pattern as mentioned above, whereas ÅNBERG, FAWCETT, and ROTHSCHILD stated the [9 : (2×9) : 2] pattern in which each of the peripheral fine fibrils of the inner ring consists pairs.

Concerning the bull sperm, BRETSCHNEIDER²⁶) illustrated the [8 : 0 : 1] pattern, using suspension materials, whereas, with section materials, BRADFIELD²²) reported the [9 : 9 : 2] pattern, and BLOM & BIRCH-ANDERSEN¹⁴) did the [9 : (2×9) : 2]. In this way, a considerable degree of variation is seen in number of fibrils or in their arrangement, among some species and sometimes even in the same species or by authors. Text figure 4 shows these different schemata of transverse sectioned middle piece of the human and other mammalian sperms by many authors. In the present work, the number of the fibrils coincides with that of BRADFIELD (farm animals) and of FAWCETT (human), while the pattern of arrange-

TEXT FIGURE 4 Schematic diagrams of middle piece in cross section postulated by various authors with some species in Mammalia



(partially revised by the present author)

N. B. L : Counter-clockwise direction of mitochondrial spiral
 R : Clockwise direction
 Number : The spiral number of mitochondrial helix

ment is closely resembled to that of BLOM & BIRCH-ANDERSEN (bull). In the case of the sperm from one Guernsey bull used in the preliminary examination, the results obtained were same as those of Holstein bulls. A few photographs showed the findings as if each of the peripheral fine fibrils makes pairs (fig. 36), however, this was not a constant view. Therefore, the number of the fibrils concerned are 9, in relation to the numbers of the corresponding fibrils in the tail piece and the end piece, as will be described in the latter chapters.

In suspension materials of the human sperm, KASHIWAGI observed a moniliform structure in each fibril. The present author also observed such a structure in suspension materials. With repeated washing, the structure becomes more distinct, and at last it separates into several pieces by severe washing. From these findings, the moniliform seems to appear during collapsing processes of the fibrils. A constructive principle in the striated structure of the general "proto-fibril" may suggest something concerning molecular structure of the sperm fibril. In less frequent microphotographs, each fine fibril appeared like a dotted line in longitudinal sections of the middle piece (fig. 38).

ÅNBERG in the human sperm and CLELAND & ROTHSCHILD in the bandicoot sperm observed "interconnections" or "lamellae" which is a fine thread-like structure connecting between the peripheral fine fibrils and the central ones. This structure, however, undoubtedly discords with the above mentioned dotted line observed by the present author, in viewpoint of its location and direction. If the bull sperm would have such an interconnecting structure in the axial fibril bundle, it should appear anywhere in transverse sections, but no such structure could be demonstrated in this work. Therefore, the "dotted line" seems presumably to be one of artifacts caused by physical or chemical treatments.

The arrangement of the fibrils appeared in cross sections at the presumably anterior portion of the middle piece—sections with comparatively greater diameters—(fig. 36) showed a close resemblance to that of cross sections at the presumably posterior portion of the middle piece—sections with smaller diameters—(fig. 37), both having the [9 : 9 : 2] pattern. In addition, in longitudinal sections of the middle piece, each of the fibrils ran straight from the neck to the tail piece. From these findings, it can be said that the fibrils composing the axial fibril bundle are constantly 20, running throughout the middle piece straight toward the tail piece without changing the arrangement pattern.

There arises an important question how is the situated relation between the sperm head with racket-like form and the pattern of fibril arrangement. The problem of symmetry was formerly discussed by BRADFIELD²²⁾ in the axial fibril bundle of mammalian sperms and in fibrils of cilia of the somatic cells in various animals. He thought that the symmetrizing plane in the middle piece is located between the 2 central fine fibrils of the axial fibril bundle, thus, because of the discordance between the arrangement pattern illustrated by him and that of the present author, BRADFIELD's opinion cannot be applied to interpretation for the result of the present work. CLELAND & ROTHSCHILD tried to resolve this problem with the bandicoot sperm. As the cross section of the middle piece of the bandicoot sperm takes a characteristic ridged rhombic form, it seems difficult to apply the results of these authors to the case of the bull sperm of which the cross section of the middle piece shows approximately round.

The symmetrical plane employed by the present author in this paper is a plane through on the 2 central fine fibrils. Such a plane has ever been considered by BRADFIELD as an inadequate one in the pattern of fibrils in the cilia. Furthermore, this symmetrical plane corresponds to a variation produced by 90 degrees movement from FAWCETT's diagram in the human sperm. In addition, there is another important discord between FAWCETT and the present author; the former illustrates each of the fibrils consisting the outer ring as equal in thickness, while the latter does thicker 4 and thinner 5. This illustration of the present author about the

outer ring has a close resemblance to that of BLOM & BIRCH-ANDERSEN¹⁴⁾, though as for the inner ring, their schema differs from that of the present author, the former showing a paired-fibril pattern (2×9). If an assumption that each of the fibrils composing the axial fibril bundle run straight throughout from the neck portion to the middle piece would be allowed, it is expected that each of the two pairs of 4 thicker peripheral fibrils originating from the medium radices of the neck run along with each of the lateral sides in the middle piece, while 3 and 2 of the remaining 5 peripheral fibrils originating from the small radices of the neck do along with the dorsal (fig. 22) and ventral sides (fig. 21), respectively. This expectation was ascertained to be true without any contradiction in micrographs from longitudinal sections of the middle piece with the flattened (figs. 13 & 33) and sagittal sectioned head (fig. 39). Thus, it can be understood that the symmetrical plane concerned becomes common to that of the head. Accordingly, the symmetric nature of the sperm is recognized throughout every portion covering from the head to the middle piece.

There are some interesting reports concerning the size of the middle piece and its physiological activity. BRADFIELD²²⁾ offered an opinion that the diameter of the middle piece depends on the phylogenetic level of the species; the more evolution the greater the thickness of the middle piece. FAWCETT thought that the length of the middle piece varies correspondingly with the size or shape of the head, and that it is affected by viscosity of seminal fluids. Other authors^{10,101)} presented the opinion that the main energetic source for the sperm motility is concentrated in the fibrils in the portion below the neck, and it is activated by the enzyme system of the mitochondrial sheath.

Furthermore, several authors^{3,14,33,38,95)} measured the thickness of each fibril and the distances between them. In this work, the average figure was calculated as 13.3μ in the actual length, with a range of 10.7μ to 16.9μ .

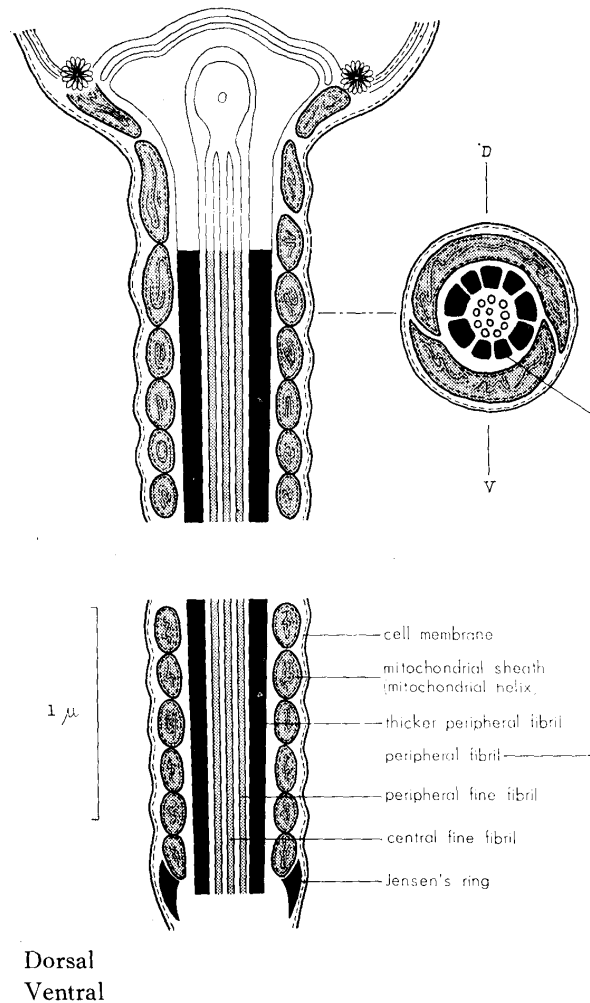
CONCLUSION

As for the middle piece, the following findings were obtained by the present author (text fig. 5).

1) The mitochondrial sheath originating in the basal granule of the head and covering the neck is composed of 2 mitochondrial helixes which twist regularly about 32 times surrounding the axial fibril bundle.

2) The direction of twisting of these helixes is counter-clockwise.

3) The axial fibril bundle is composed of 20 fibrils; 9 peripheral fibrils consisting of the outer ring, 9 peripheral fine fibrils consisting of the inner ring and 2 central fine fibrils. In a transverse section of the middle piece, the pattern of arrangement of these fibrils is shown in a formula as $[9 : 9 : 2]$.

TEXT FIGURE 5 *Schematic diagrams of middle piece*

N. B.: Left shows longitudinal section of flattened view and right shows cross section at anterior portion of middle piece

4) The arrangement of these fibrils is characteristic. A pair of central fine fibrils are located in the central portion with an arrangement of dorsal and ventral direction. Each of the 2 pairs of the 4 peripheral fibrils of the outer ring which are thicker than the remaining 5 (3 in dorsal side and 2 in ventral) run through at each of the lateral sides, respectively.

5) In the cross section of the middle piece, the arrangement pattern is symmetric, and the symmetrical plane is common that of the anterior portions above the middle piece.