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Taenia hydatigena

III. Light and Electron Microscope Study of Spermatogenesis

D. W. FEATHERSTON

Hydatid Research Unit, Medical Research Council of New Zealand, University of Otago Medical School, Dunedin, New Zealand

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Summary. The process of cell division during spermatogenesis in *Taenia hydatigena* followed the general pattern reported by Rybicka (1966) for other cestodes. Sixty-four spermatozoa were formed from each primary spermatogonium after a series of five nuclear divisions. During spermiogenesis, changes in the organisation of sperm tail cytoplasm were evident with four distinct regions along the length of the spermatozoon being distinguishable. The axoneme which was normally single in each sperm tail, had the typical 9 plus 1 structure found in platyhelminths. Nuclear material in the mature spermatozoon was arranged spirally around the axoneme.

Introduction

Recently Rybicka (1966) reviewed the present state of knowledge on cestode spermatogenesis and showed that in the small number of reports available a basic pattern was beginning to emerge. Since then Pashchenko (1965) has reported on the later stages of spermatogenesis in *Taeniarhyncus saginatus* and Morseth (1969) has described the ultrastructure of the sperm tails of *Echinococcus granulosus* and the trematode *Dicrocoelium dendriticum*.

The present study, which is part of a detailed examination of *Taenia hydatigena* (Featherston, 1969, 1971), examined the process of spermatogenesis and the ultrastructure of some of the associated structures.

Materials and Methods

Worms were collected from dogs at autopsy 15, 20, 25, 30 and 40 days after the administration of cysticerci by methods previously described (Featherston, 1969). Fixation of this material was carried out as soon as possible after removal from the host.

Material for examination by the light microscope was fixed in Carnoy's fluid (6:3:1), dehydrated with graded ethanols, cleared with methyl benzoate and embedded in paraffin wax *via* benzene. Facial longitudinal sections 5 μ thick were cut, stained by the Feulgen technique without Fast Green and examined with a Riechert phase contrast microscope.

To determine the external morphology of the developing spermatozoa, a piece of fresh strobila made up of from 3 to 4 proglottids each containing testes was

placed on a microscope slide with a small quantity of Hanks' BSS and gently teased with needles. The excess strobila tissue was removed while the remaining cells were examined under the phase contrast microscope.

The following procedures were used to prepare material for examination in the electron microscope.

Small portions of the terminal and penultimate proglottids were initially fixed in either a formaldehyde-glutaraldehyde mixture (Karnovsky, 1965), or a glutaraldehyde-osmium tetroxide mixture (Franke *et al.*, 1969). After washing in buffer the material was post-fixed in osmium tetroxide, dehydrated with graded ethanols and embedded *via* 1,3-epoxypropane in araldite. Sections were cut with glass knives on an LKB Ultrotome, collected on grids covered with a carbon coated film of collodion, and stained with 1% uranyl acetate overnight followed by lead citrate (Reynolds, 1963) for 30 seconds.

Fresh spermatozoa, obtained by teasing mature proglottids as for light microscopy, were placed on coated grids and stained with 1% phosphotungstic acid (PTA).

All material was examined on a Hitachi HU 11A microscope.

Observations

Light Microscopy

Testes developed in the parenchyma of the terminal proglottid of worms between 15 and 20 days old. There was no evidence that the testes were present in worms 15 days old, but worms aged 20 days showed the early stages of spermatogonia. At 25 days the testes were mature and all stages of development could be identified in the terminal proglottid.

The testes arose by a series of divisions of the parenchyma cells which produced a number of primary spermatogonia within a common envelope (Fig. 1 a). Between these divisions, the spermatogonia were approximately 5 μ in diameter, but when spermatogenesis was about to be initiated the cell enlarged to a diameter of approximately 11 μ (Fig. 1 d). At this stage the nucleus divided and the two halves concentrated at the opposite ends of the original cell before dividing again. The cell wall of the primary spermatogonium did not divide at this stage. At the end of the second division, a tetrad of nuclei was formed within a common cell membrane (Fig. 1 b). Two further divisions of the nuclear components were then observed giving rise to 8 quaternary spermatogonia. These 8 nuclei arranged themselves around the surface of the cytoplasmic mass forming a multinucleate syncytial mass. While the onset of spermatogenesis in all the primary spermatogonia of any one testis was not synchronous, 3 or 4 were generally found in different stages of spermatogenesis at any one time.

Sixteen primary spermatocytes were formed from the division of the 8 quaternary spermatogonia (Fig. 1 c) and developed very clear rosette formations.

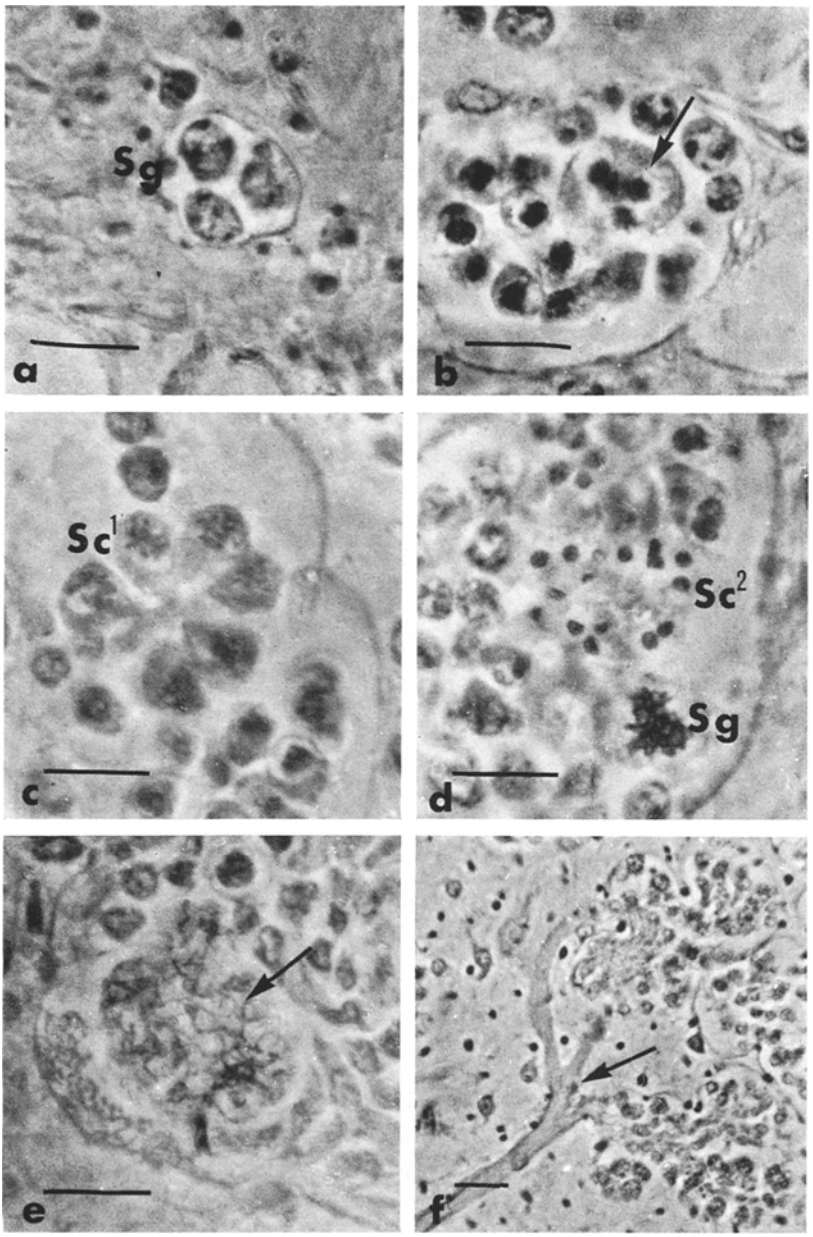

Fig. 1a–f

A further division of the nuclei produced the 32 secondary spermatocyte stage (Fig. 1d) where the nuclei were crowded around the outer edge of the syncytial mass. In fresh teased material this stage gave the appearance of a number of spheres packed around the central mass. This packing was even more evident after the final division when the 64 spermatids were produced.

The first stage of spermiogenesis showed the nuclei of the spermatids elongating and forming thread-like structures which were Feulgen positive (Fig. 1e). These thread-like nuclei appeared to be randomly scattered throughout the cytoplasmic mass in the early stages, but later were again concentrated around the periphery of the cytoplasmic mass. Normally at this stage the vasa efferentia began to make their appearance and could be seen as small tubules making contact with each testis (Fig. 1f).

In fresh material the initiation of spermiogenesis was heralded by the appearance of sperm tails (Fig. 2a) projecting from the young spermatids. These sperm tails continued to elongate (Fig. 2b), while the cytoplasmic mass which made up the young spermatid began to diminish. In a number of cases small cytoplasmic enlargements were seen to develop on the sperm tail at irregular intervals along its length. Fig. 2c shows a single such sperm tail with these enlargements. Eventually these disappeared and the spermatozoon so formed then assumed a filamentous shape with a corkscrew segment at one end (Fig. 2d). These fine filamentous spermatozoa were up to 300 μ long.

Whereas Feulgen positive areas were easily distinguished in the cytoplasmic mass at the onset of spermiogenesis, no such areas were detectable in the mature spermatozoa of fixed material.

Electron Microscopy

Within mature testes, which were easily identifiable by the presence of sperm tails with their characteristic axoneme, all stages of development could be distinguished. Spermatogonia and spermatocytes were identified by the size of the former and the characteristic arrangement of cells in the

Fig. 1a–f. *Taenia hydatigena*. Stages in spermatogenesis. a Primary spermatogonia cells (*Sg*) in an early testis. b A tetrad of tertiary spermatogonia nuclei (arrow). c Primary spermatocytes (Sc^1) showing rosette formation. d Secondary spermatocytes (Sc^2) and a primary spermatogonium (*Sg*) during division. e The elongated nuclei of the spermatids during early spermiogenesis. f Branches of the vasa efferentia (arrow) seen leaving the testes. (The bar in each micrograph represents 10 μ. Feulgen technique without Fast Green. Phase contrast)

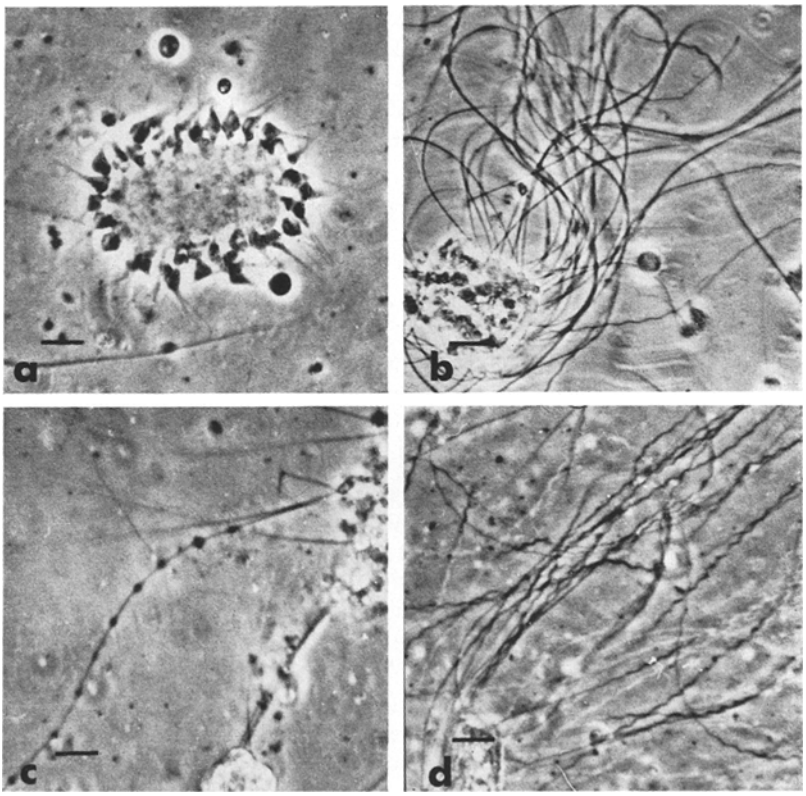

Fig. 2a–d. *Taenia hydatigena*. Stages in spermiogenesis. a The early stages of sperm tail development with the spermatids arranged around the residual cytoplasm. b A later stage showing the elongation of the sperm tails. c Cytoplasmic enlargements along the length of a sperm tail. d A cluster of mature spermatozoa showing the "corkscrew" shaped area. (The bar in each micrograph represents 10 μ. Fresh teased material. Phase contrast)

latter. Spermatogonia in the resting stage, shown in Fig. 3a, had a high concentration of mitochondria, a sign that division was about to occur. This high concentration of mitochondria was also evident in those cells dividing to produce the more advanced stages of spermatogonia or primary spermatocytes.

As observed with the light microscope, cell wall division was not necessarily complete during the early stages of cell division. This situation is shown in Fig. 3b where there is an area of continuity between adjacent cells at what was probably the primary spermatocyte stage with the

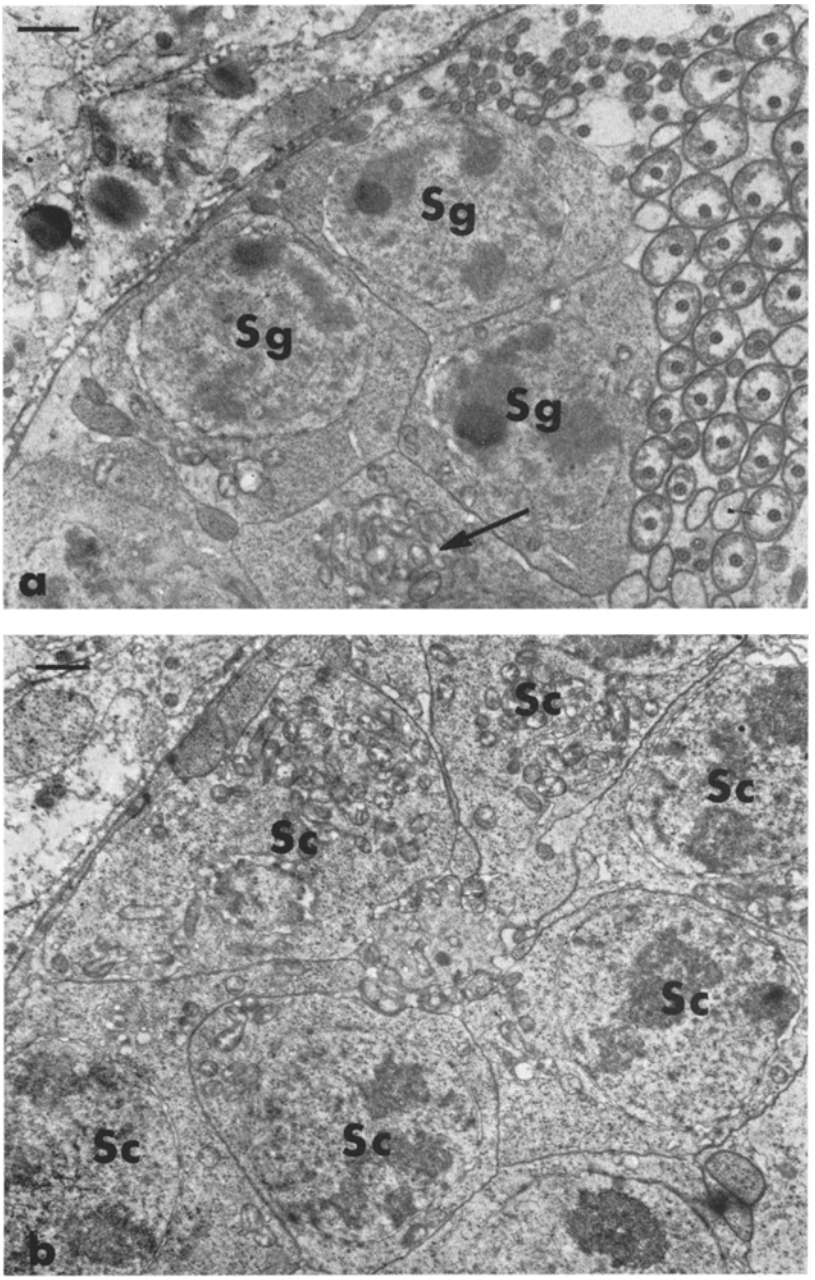

Fig. 3a and b. *Taenia hydatigena*. a Primary spermatogonia (*Sg*) prior to the onset of nuclear division. Note the high concentration of mitochondria (arrow). b Early spermatocytes (*Sc*) showing rosette formation. (The bar in each micrograph represents 1.0 μ. Formaldehyde-glutaraldehyde, osmium fixation)

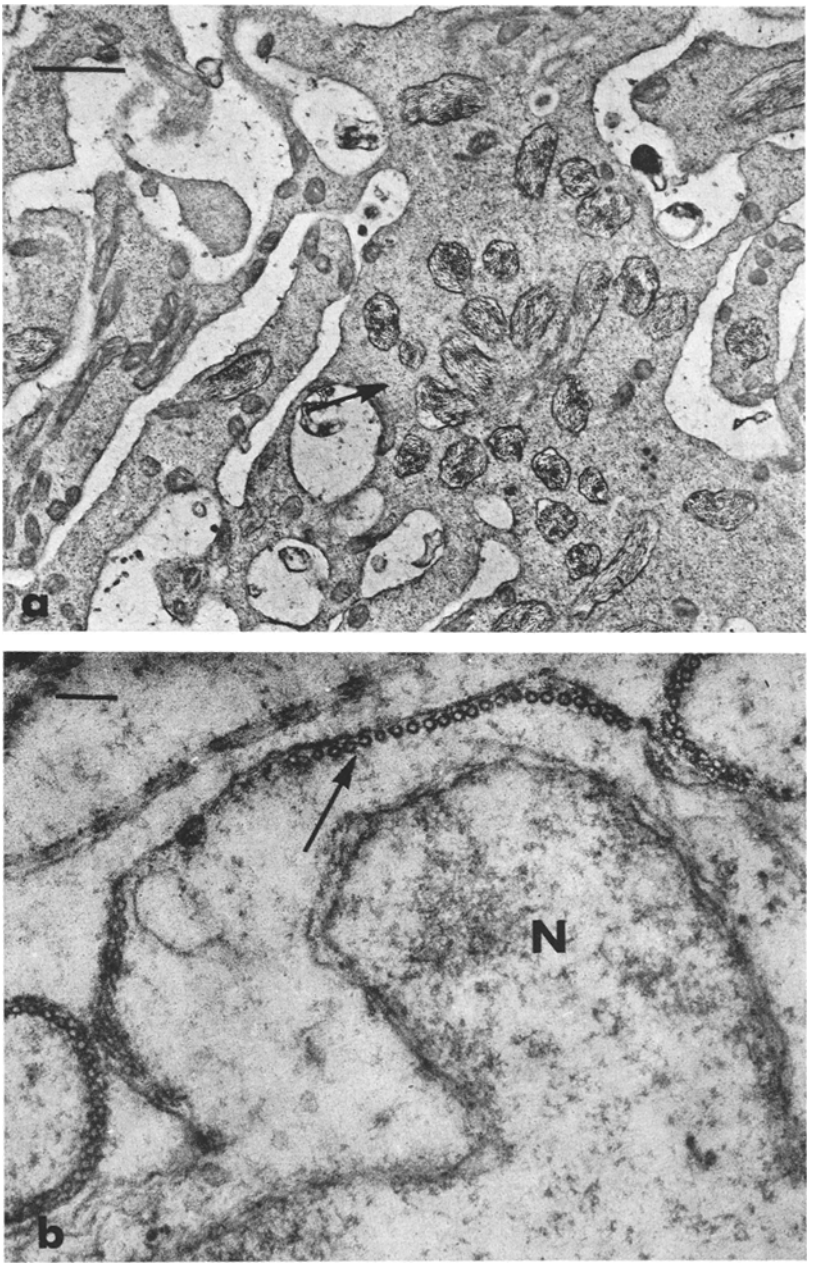

Fig. 4a and b. *Taenia hydatigena.* a Condensed nuclear material (arrow) scattered throughout the cytoplasm of the spermatid mass during the early stages of spermiogenesis. (The bar represents 1.0 μ. Formaldehyde-glutaraldehyde, osmium fixation). b Migration of the nucleus (*N*) towards the periphery of the spermatid cell. Note the development of the microtubules (arrow) directly beneath the cell membrane. (The bar represents 0.1 μ. Formaldehyde-glutaraldehyde, osmium fixation)

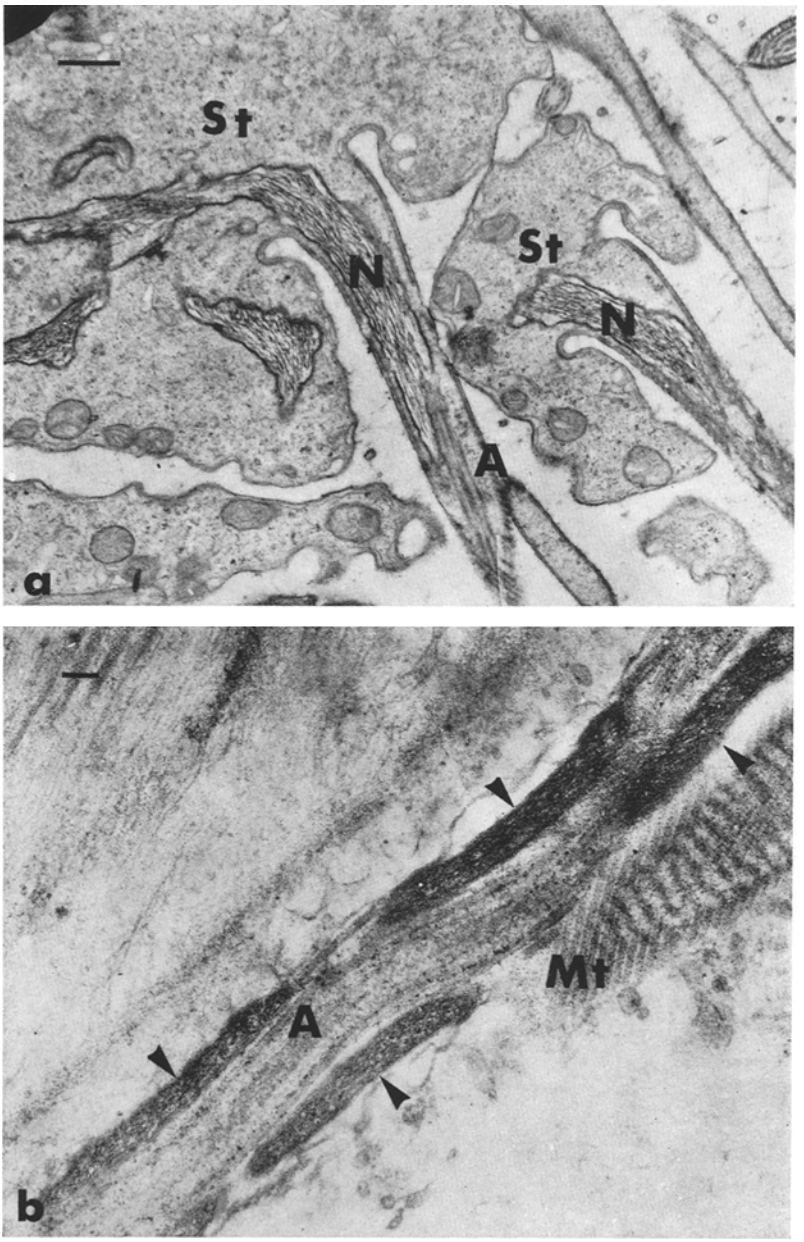

Fig. 5a and b. *Taenia hydatigena*. a Nuclear material migrating into the sperm tail from the spermatid mass. *A* axoneme. *St* spermatid (The bar represents 0.5 μ. Glutaraldehydeosmium fixation). b Longitudinal section through a spermatozoon showing the spiral arrangement of the nuclear material (◀) around the axoneme *A*. Note also the microtubules *Mt* on an adjacent spermatozoon. (The bar represents 0.1 μ. Formaldehyde-glutaraldehyde, osmium fixation)

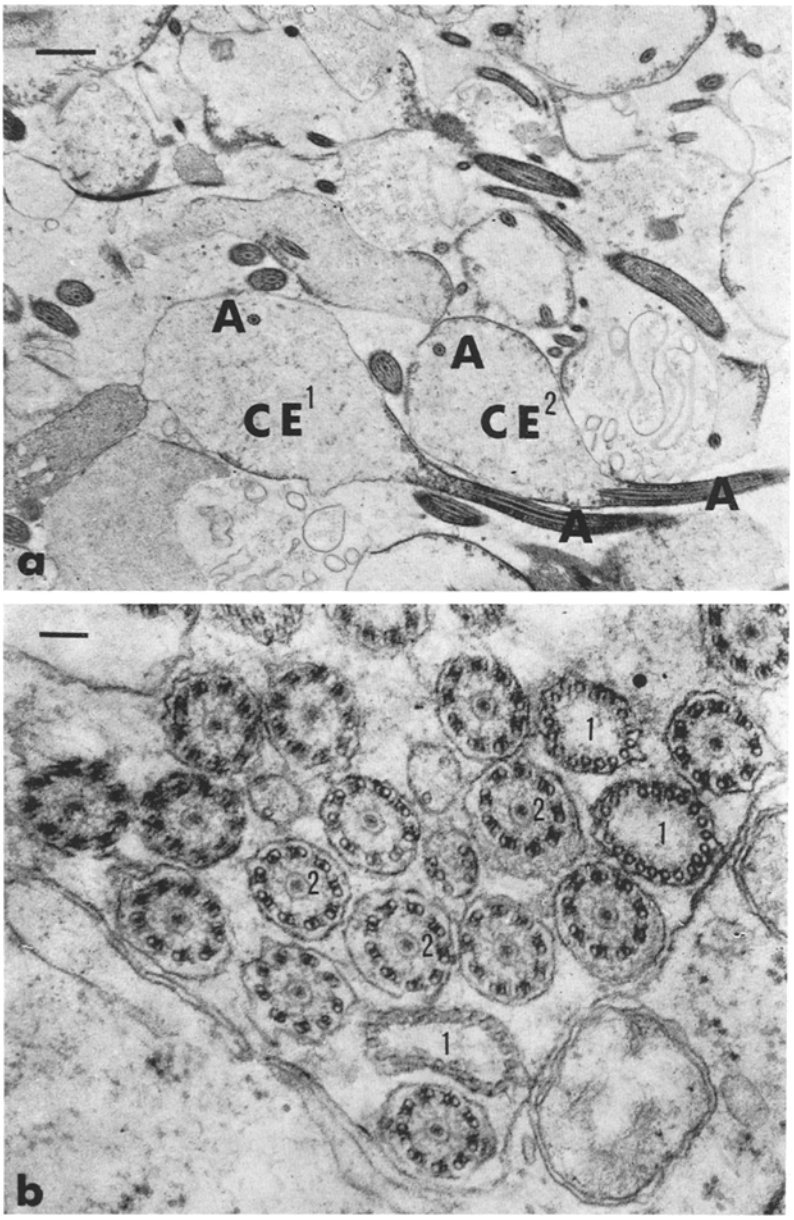

Fig. 6a and b

nuclei at the periphery of the rosette; a stage similar to that shown in Fig. 1c.

The other dominating feature of the mature testis was the spermatid stage which was characterised by an array of nuclei surrounding a common cytoplasmic mass. Because of its size it was not possible to show the complete structure in an electron micrograph. At the completion of this stage spermiogenesis was initiated.

Following the initial differentiation of the spermatids, two different conditions were observed. In the first case the nuclei of the spermatids appeared to elongate and become randomly distributed throughout the cytoplasmic mass as shown in Figs. 1e, 4a. While this condition was common, the more frequent situation found was that shown in Fig. 4b, where the nucleus of the differentiating spermatid was seen to grow out towards the cell wall. At the same time as this was happening microtubules made their appearance just inside the cell wall which then proceeded to elongate and form a tube into which the nucleus migrated (Fig. 5a). Within this tube the axoneme also developed and eventually the nucleus became coiled around it (Fig. 5b). While there was in the early stage of spermiogenesis a head region which contained a nucleus, this area disappeared as the spermatozoon matured, so that the final form of the spermatozoon was a long thin filamentous structure.

The cytoplasmic enlargements seen on the sperm tail of fresh material (Fig. 2c) with the phase contrast microscope were also evident in the thin sectioned material examined in the electron microscope. In Fig. 6a two such enlargements on different sperm tails have been sectioned in a semi-longitudinal plane and are characterised by a lack of cytoplasmic detail. A feature of these areas was the ability of the axoneme to twist around within the cytoplasmic enlargement. It was possible therefore to section this structure in both the longitudinal and transverse planes.

The ultrastructure of the sperm tail varied according to where the section was taken, and to the stage of differentiation of the spermatozoon. Because of its length, over 300 μ, it was not possible to obtain a longitudinal section running the complete length of a single sperm tail. From

Fig. 6a and b. *Taenia hydatigena*. a Section through sperm tails showing cytoplasmic enlargements CE^1, CE^2, with the axonemes *A* cut in different planes. Note the lack of cytoplasmic structure in the enlargements. (The bar represents 1.0 μ. Formaldehyde-glutaraldehyde, osmium fixation). b Transverse section through sperm tails showing different arrangements for the axoneme and microtubules. *1* Sperm tails with microtubules lacking an axoneme; *2* Sperm tails with an axoneme lacking microtubules. (The bar represents 0.1 μ. Formaldehyde-glutaraldehyde, osmium fixation)

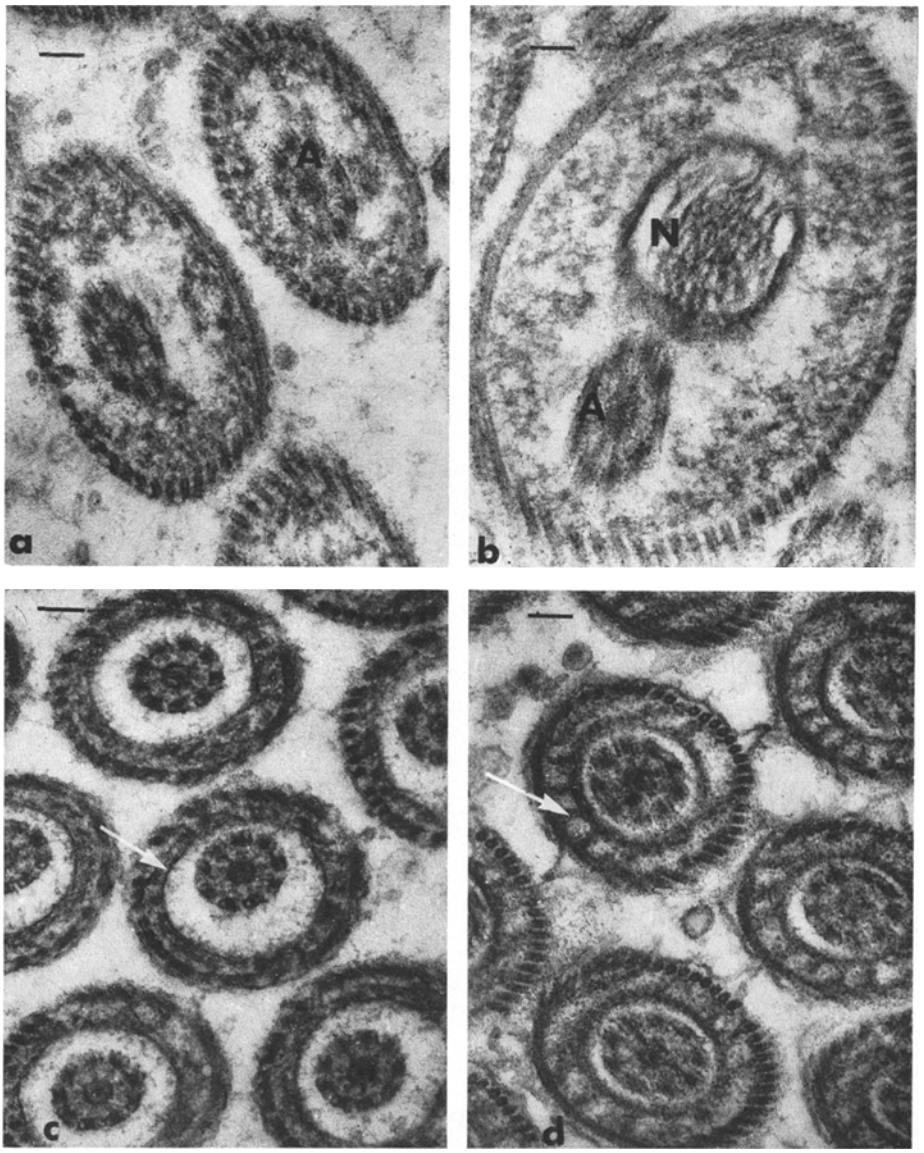

Fig. 7a–d. *Taenia hydatigena*. Transverse sections taken from the four regions of the spermatozoon showing the arrangement of the structures. a Region I. b Region II. c Region III. Arrow indicates possible membrane separating the clear and granular components of this area. d Region IV. Arrow indicates the spiral septum in the outer granular area. (The bar in each micrograph represents 0.1 μ. Formaldehyde-glutaraldehyde, osmium fixation)

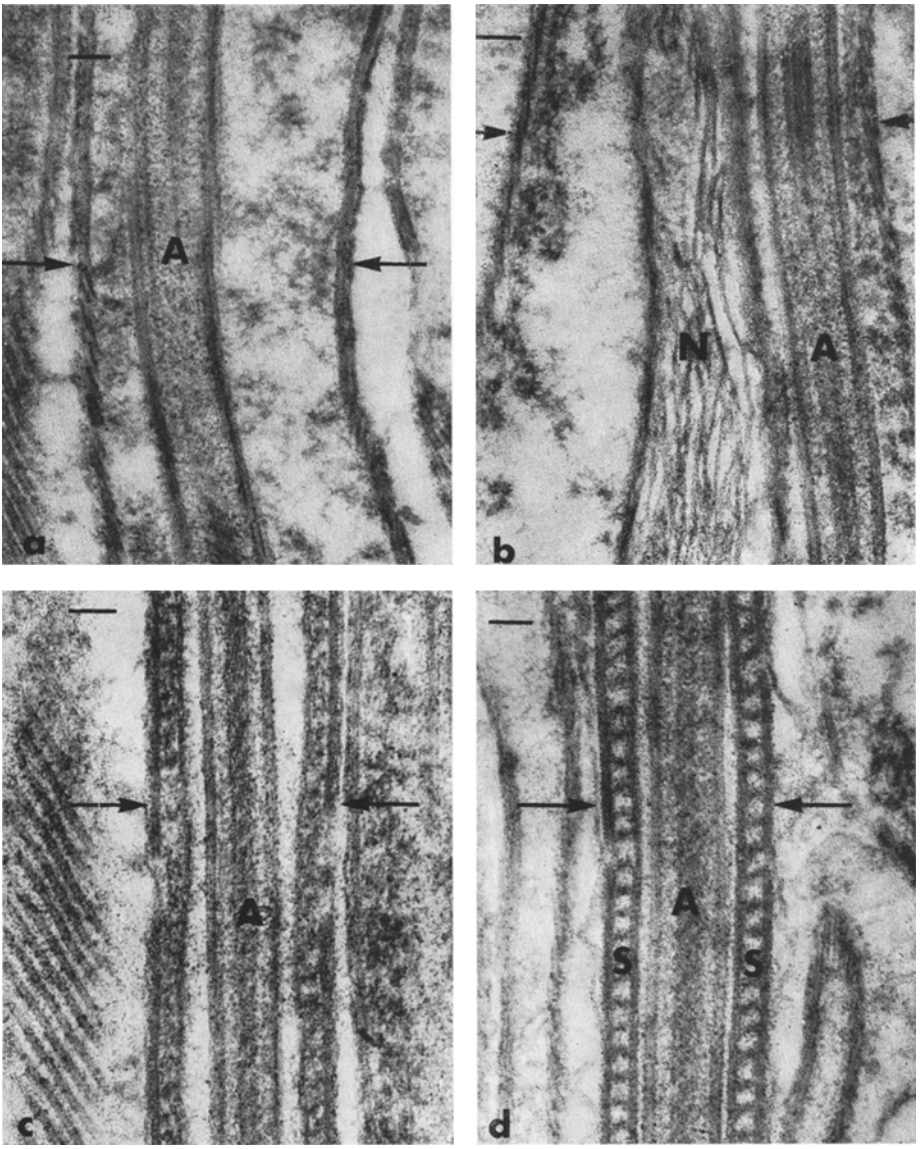

Fig. 8a–d. *Taenia hydatigena*. Longitudinal sections from the four regions of the spermatozoon showing the arrangement of structures comparable to those shown in Fig. 7. In each micrograph the section of the spermatozoon lies between the arrows. a Region I. b Region II. c Region III. d Region IV. *A* axoneme; *N* nucleus; *S* spiral septum. (The bar in each micrograph represents 0.1 μ. Formaldehyde-glutaraldehyde, osmium fixation)

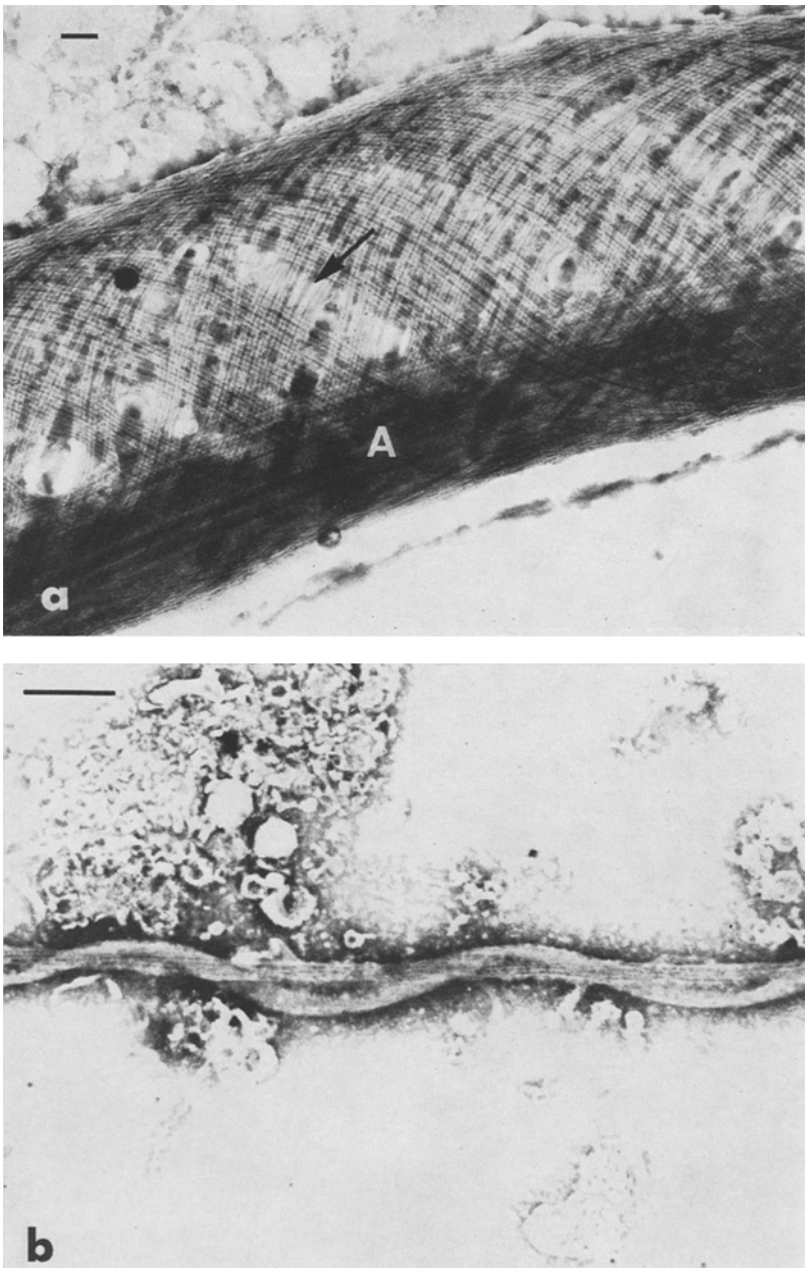

Fig. 9a and b. *Taenia hydatigena*. a Segment of a fresh sperm tail showing the arrangement of the surface microtubules (arrow) and the position of the axoneme *A*. (The bar represents 0.1 μ. PTA). b Segment of a spermatozoon showing the "corkscrew" area where the nucleus is wrapped around the axoneme. (The bar represents 1.0 μ. PTA)

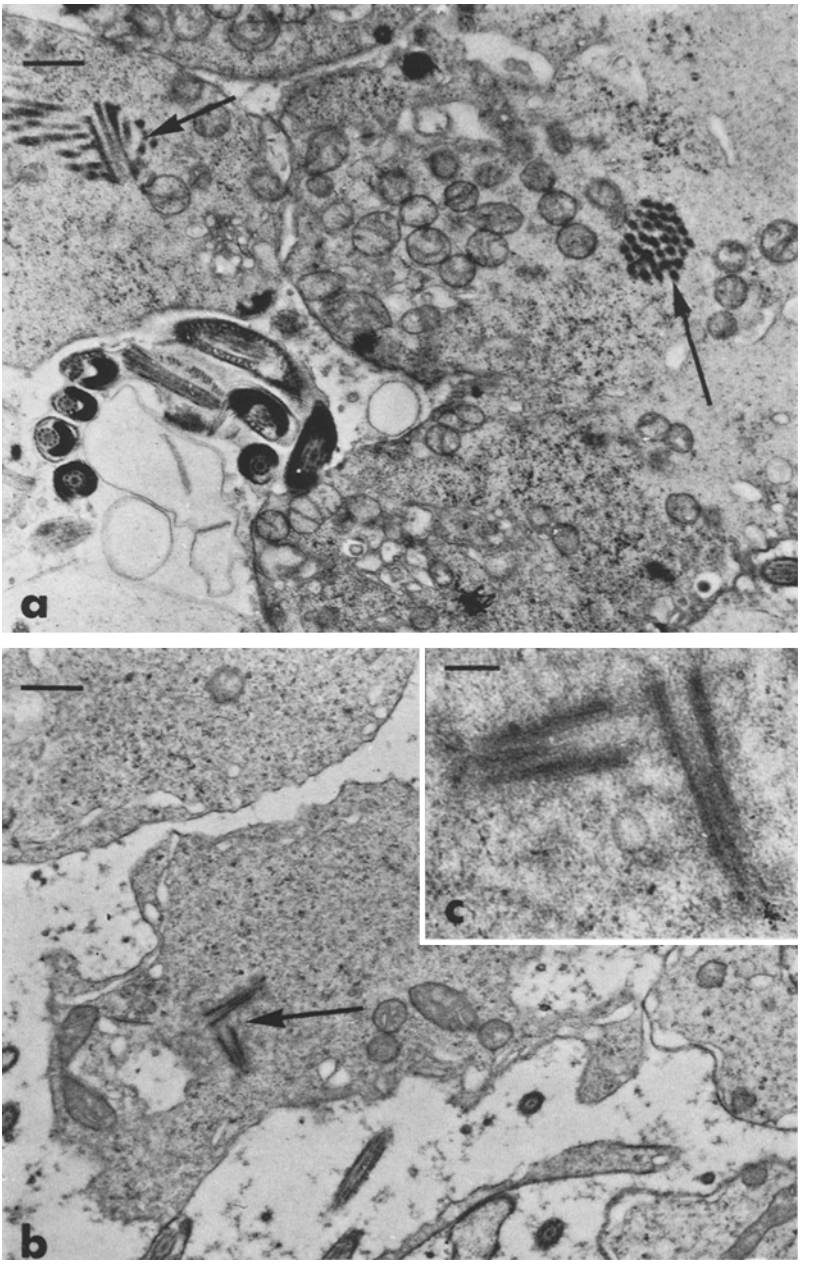

Fig. 10a–c. *Taenia hydatigena*. a Area of spermatid mass showing crystalline-like array of particles (arrows). (The bar represents 1.0 μ. Formaldehyde-glutaraldehyde, osmium fixation). b Centrioles (arrow) in a spermatid showing the typical arrangement of structures. (The bar represents 0.1 μ.) c Higher magnification of centrioles. (The bar represents 0.2 μ. Both b and c glutaraldehyde-osmium fixation)

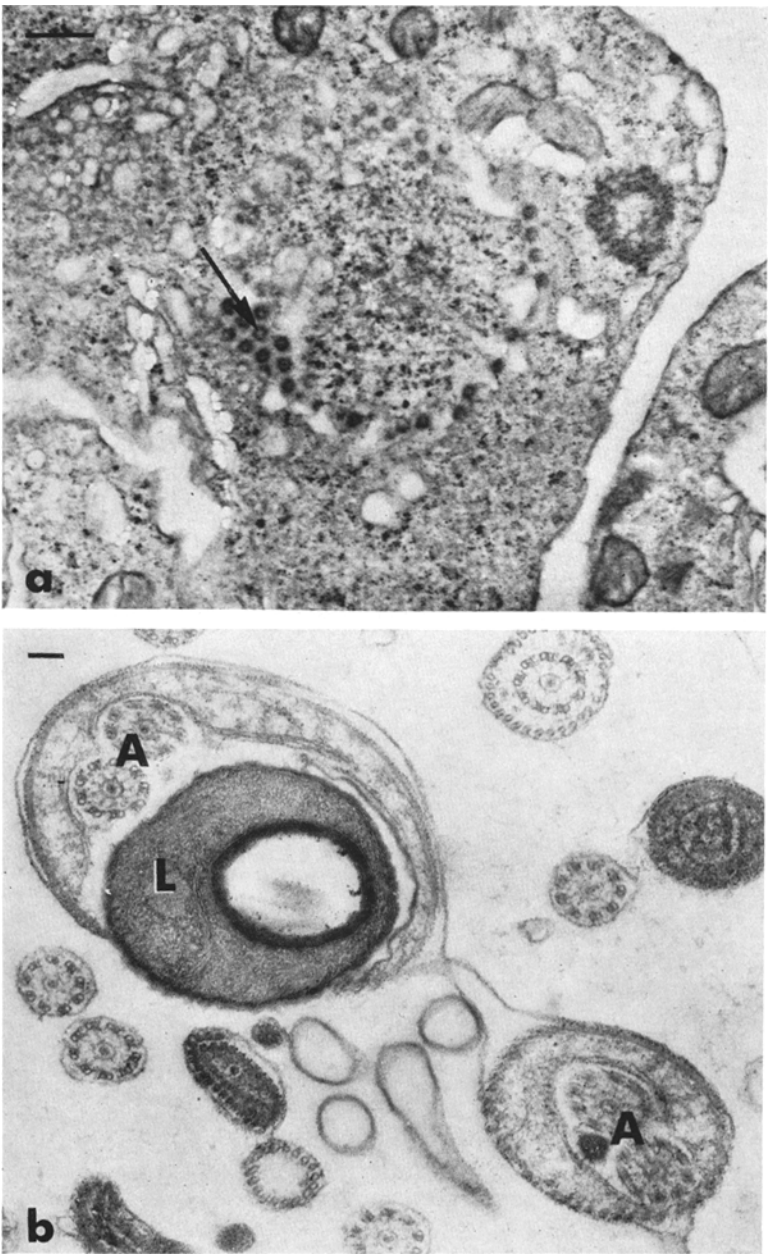

Fig. 11a and b

the transverse and longitudinal sections examined, 4 distinct areas were seen with the possibility of another being differentiated. The four major regions were characterised as follows:

Region I (Figs. 7a; 8a). A limiting membrane on the outside surrounded a layer of microtubules. These microtubules completely surrounded the cytoplasmic contents which were made up of a granular area and an axoneme.

Region II (Figs. 7b; 8b). This region was similar to that of Region I except that in addition to the structure of that area there was nuclear material present.

Region III (Figs. 7c; 8c). This region was distinguished from the others described by having the granular area restricted to a cylinder just beneath the microtubules. There was possibly a membrane separating the granular from the clear areas with the axoneme restricted to the clear area.

Region IV (Figs. 7d; 8d). By far the most complex of all the regions seen, it had in the granular area a further differentiation of a spiral septum.

Further to these 4 regions, sections through other sperm tails showed a lack of axoneme with the presence of microtubules beneath the cell membrane, or the presence of an axoneme but no microtubules beneath the cell membrane (Fig. 6b).

The axoneme was made up of the normal $9+1$ structure commonly found in the sperm tails of cestodes. The doublets surrounding the central complex were joined to it by means of electron dense spoke-like structures. The only other motile cell process examined for comparative purposes was the flame cell, which showed a typical axoneme structure of $9+2$.

The microtubules which formed beneath the cell wall had an external diameter of approximately 270 Å. These tubules completely surrounded the sperm tail and extended in most cases to the tip. They formed an angle of between 40 and 50° with the long axis of the sperm tail. While the arrangement of these microtubules were seen in longitudinal thin sections which skimmed the surface of the sperm tails (Figs. 5a, b; 8a, c) clearer

Fig. 11a and b. *Taenia hydatigena*. a Particles (arrow) found associated with the area where the nuclear membrane would be located in a spermatocyte. (The bar represents 0.5 μ. Glutaraldehyde-osmium fixation). b An atypical spermatozoon with two axonemes *A* and a lamellated structure *L* present. The tail of this particular example was folded on itself and enclosed in a common membrane. (The bar represents 0.1 μ. Formaldehyde-glutaraldehyde, osmium fixation)

detail was obtained by using entire sperm tails treated with PTA. Fig. 9a shows that in some areas, the microtubules were not always regularly spaced along the length of the sperm tail. It further showed that while the axoneme was generally found near the centre of the sperm tail, it could also wander to the edges. Finally the corkscrew effect at one end of the mature spermatozoa seen in Fig. 2d was more clearly seen in PTA prepared material (Fig. 9b). When this micrograph is compared with Fig. 5b, a thin section showing the nucleus twisted around the axoneme, the similarity of arrangement is clear.

Among the many sections examined during this study, a number of additional structures were occasionally found which had not been reported previously as occurring during cestode spermatogenesis. Fig. 10a shows two groups of electron dense particles resembling crystalline arrays present in the cytoplasm of differentiating spermatids. Similar arrays were also found in other parts of the spermatid mass with no regularity to the composition or arrangement of the structures, some of which were rod-like in appearance. The centrioles were another structure occasionally found associated with the secondary spermatocyte or spermatid stage. Such structures are shown in Figs. 10b, c. A further series of structures which showed as electron dense particles were found associated with the early stages of spermiogenesis (Fig. 11a). While some of these particles had a hollow core, others were seen to have a dense structure in the centre. These structures had an external diameter of 100 mμ and while they appeared to be associated with the line of the nuclear membrane, they were sometimes seen to form clusters.

Within the sperm tail of some atypical spermatozoa, 2 axonemes were occasionally found. In one particular example (Fig. 11b) a type of hollow lamellated body was also present.

Discussion

The results obtained by the use of the Feulgen technique interpreted with the phase contrast microscope showed that *T. hydatigena* followed the same basic pattern of cell division during spermatogenesis as that observed in other cestodes (Rybicka, 1966). A minor but significant difference observed in the sectioned material was the lack of complete separation by the cell walls once the primary spermatogonium had started to divide. This produced a multinucleate syncytium which was retained until the spermatozoa were shed from the residual cytoplasmic mass at the completion of the process. In other platyhelminth species, a fusion process was considered to occur at the spermatocyte stage of development where 8 or 16 primary spermatocyte cells all fused together to

form the typical rosette. Sato *et al.* (1967) showed an electron micrograph of this fusion occurring in the spermatocyte stage of *Paragonimus miyazakii* but omitted to mention when it first occurred.

The size of the nuclei and chromosomes still make it very difficult to determine at what stage meiosis occurs. Until the number of chromosomes have been accurately determined this question must remain unanswered.

The results here have also shown that the process of spermiogenesis was not as simple as that reported for other cestodes. Rosario (1964) suggested for *Hymenolepis nana* and *H. diminuta*, that the spermatozoon was a relatively simple structure derived by a simple process of invagination of the cell wall of the spermatid mass. He further suggested that the sperm nucleus was free to move along the length of the spermatozoon. This situation was seen in a large number of *T. hydatigena* spermatozoa which were still immature, whereas in the more advanced stages of spermiogenesis a quite different picture emerged. Because of these differences the interpretation of the results became more difficult.

A suggested line of development for the spermatozoon was that Region I, consisting of the axoneme and sheath, was restricted to the extreme ends of the spermatozoon and possibly a short length of the sperm tail. It was also the first area which was differentiated from the developing spermatid hence its frequent occurrence in younger testes. Region II was an area which was only transient in the process of spermiogenesis. During this time the nucleus was probably able to move within the sperm tail as suggested by Rosario (1964). This stage could have been followed by reduction in the diameter of the sperm tail and the twisting of the nucleus around the axoneme as shown in Fig. 5b. That this twisted area was restricted to one end of the spermatozoon is supported by the arrangements shown in Figs. 2d, 9b. In the areas of the spermatozoon which did not contain the nucleus, there was a possible condensation of the cytoplasm and an initial separation of material. This was represented by Region III which was either a small area along the sperm tail, or of a transient nature leading to the more complicated arrangements of Region IV represented by Figs. 7d, 8d. The spatial relationships of the various regions to one another still require elucidation.

The problem of cytoplasmic enlargements along the sperm tail has also to be set into the context of the above. It seems likely that it was one which occurred at a very early stage in the process of spermiogenesis and was completed by the time the sperm tail had reached its full length. Since this disappearance of the cytoplasmic enlargements coincided with the elongation of the sperm tails, it would appear that their presence was related to this elongation process.

The other significant feature was the absence of typical mitochondria in the sperm tail. These structures were completely lacking from all stages examined during this study. This feature may be a characteristic of cestodes as Rosario (1964) made no mention of them occurring in the sperm tails of *H. nana* or *H. diminuta*. A similar situation was reported by Bonsdorff and Telkka (1965) in *Diphyllobothrium latum*, and Morseth (1969) for *E. granulosus*. On the other hand Sato *et al.* (1967) found them in the spermatozoa of the trematode *P. miyazakii*. While it appears from this evidence that they are completely absent from the cestodes, there is the alternative that they are present but in a highly modified form. The septate structure seen in Region IV of the mature spermatozoa could be a highly modified form of mitochondrion. Similar structures have been identified in insect spermatozoa where they have been referred to as the "nebenkern" and have been shown to be derived from mitochondria (Andre, 1962). Phillips (1969) also showed an electron micrograph with an arrangement in the sperm tail of the siphonapteron, *Ctenocephalides felis* similar to that found in Region IV of *T. hydatigena*. What was not seen during this study was any sign of the transition stages between the mitochondria of the spermatid and the final structure in the mature spermatozoa. It may however be that the structure seen in Region III where there was a separation of the cytoplasm from the axoneme were the initial stages in the modification of such areas to form the "nebenkern".

The migration of the nucleus along the sperm tail may have been similar to that reported by Hendelberg (1962) as occurring in the trematodes *Dicrocoelium* and *Fasciola*. In both these species the nucleus migrated from the spermatid to the distal end of the sperm tail during spermiogenesis. Such a phenomenon was possible in the case of *T. hydatigena* and the way in which the nucleus first moved into the differentiating sperm tail would support the argument but there was not sufficient evidence available from the light microscope material to show this conclusively.

While the function of the centriole during spermiogenesis was shown to be concerned with the development of the axoneme (Fawcett, 1961; de Kretser, 1969), at no stage were sections found which conclusively showed such a process occurring in *T. hydatigena*. Because of the time of their appearance, it may be considered that the crystalline structures seen in Fig. 10a were in some way connected with the development of the axoneme. On the other hand the fact that these structures were variable in number and arrangement suggests that some other function will have to be sought for them since the centriole shown in Figs. 10a, b appears to be similar to that found in other cells.

It is similarly difficult to interpret the electron dense particles seen in Fig. 11a. These while not being as closely arranged as those in Fig. 10a suggest that they may be connected in some way with the nuclear membrane since they were found in spermatid masses arranged along the line that the nuclear membrane would have taken had it been present. From this evidence it is suggested that the particles may be the dense areas associated with the fusion of the nuclear membrane and formation of the nuclear pore.

As yet insufficient observations have been made on the sperm tails of different cestodes to determine whether there is an overall pattern of structure. The number of axonemes present does vary with the species. The cyclophyllids examined so far have all had 1, whereas 2 have been recorded in the pseudophyllid *D. latum*. There does however seem to be a common pattern to the structure of the axoneme where in all species examined it has been shown to have a $9+1$ arrangement.

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Dr. D.W. Featherston
Hydatid Research Unit
Medical Research Council of New Zealand
University of Otago Medical School
P.O. Box 913
Dunedin, New Zealand