Spermiogenesis in the rainbow trout (*Salmo gairdneri*)

An ultrastructural study

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Summary. In an ultrastructural study on the spermiogenesis of the rainbow trout (*Salmo gairdneri* R.) four spermatogenetic stages were identified. In young round spermatids, the nuclear chromatin was first heterogeneous (euchromatin and heterochromatin). Subsequently, it became more homogeneous and started to condense in the form of coarse granules and fibers and then into fibrils associated in ribbon-like elements which eventually partly fused together. During early spermiogenesis, a juxtanuclear vacuole appeared in the area where the nuclear envelope was specialized due to condensation of material between the two envelopes and a slight accumulation of nuclear material. This area was finally located in the anterior part of spermatids and spermatozoa; it probably plays a role during fertilization. A flagellar rootlet appeared early in spermiogenesis; it may play a role in the attachment of the flagellum to the nucleus since it persisted until the centriolar complex was definitively fixed in the implantation fossa. The flagellum did not display a plasma membrane and was first located in the cytoplasm, but when it was later extruded from the cell, it acquired a membrane. The cytoplasm was rich in ribosomes (free or in small groups) but poor in membranous organelles. The few mitochondria polarized around the centriolar complex were finally organized into an annular mid-piece. The spermatids remained connected by intercellular bridges until the end of spermiogenesis. The complexity of trout spermiogenesis is intermediate between that in poecilids and that in carp and pike, which have very simple spermatozoa. The role of the material from the nucleus and the cytoplasm reaching the Sertoli cell in the control of spermatogenesis has been discussed.

Key words: Spermiogenesis – Rainbow trout – Ultrastructure – Spermatids – Chromatin

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The formation of spermatozoa during the last phase of spermatogenesis, and especially the transformation of nuclear protein (Alfert 1956; Felix 1960; Dixon and Smith 1968; Davies et al. 1976), have been thoroughly studied by biochemical means. Some of the rare, detailed morphological studies that exist, however, indicate that spermiogenesis in salmonids has several particular characteristics.

An ultrastructural and morphometric stucy by Zirkin (1975) has shown that the process of chromatin condensation is complex. Important transformations, such as the temporary presence of flagellar rootlets, also affect the cytoplasmic organelles during the morphogenesis of spermatozoa (Billard 1972). No comprehensive study has been carried out on the entire process of spermiogenesis in one species. The present investigation is an approach to such a study in the rainbow trout (*Salmo gairdneri*).

Materials and methods

Adult male rainbow trout were obtained from various trout farms in the Paris area or from the trout breeding facility at our laboratory in Jouy-en-Josas; these fish had already had at least one reproductive cycle.

They were decapitated between September and February after anesthesia in a 2% solution of MS222. Pieces from the middle of the testis were sampled and fixed for 1 h in a 3.3% glutaraldehyde solution in 0.1 M phosphate buffer at pH 7.25 and then fixed for 1 h in a 2% solution of osmium tetroxide in the same buffer; in some cases, the pieces were fixed directly in the osmium solution. After embedding in Epon, the pieces were cut into sections and stained with uranyl acetate and lead citrate for 5 min before they were covered with a carbon film for observation with a Siemens EM I electron microscope.

Results

Spermiogenesis in trout is divided into four stages which are described below and summarized in Table 1:

Stage 1. Immediately after the last maturation division of secondary spermatocytes, the round spermatids grouped in cysts are interconnected by intercellular bridges. In the nucleus, which remains spherical, granular filamentous chromatin is irregularly distributed (Figs. 1–3), with scattered perichromatin granules (Figs. 1–4) and dense bodies (Fig. 3) which might represent nucleolar remnants. The flagellum is formed in the cytoplasm early in this stage and remains inside the cytoplasm. The centriolar complex is surrounded by small vesicules (Fig. 1) and becomes connected to the nucleus by a flagellar rootlet (Fig. 2, Table 1). The membranous structures (Golgi apparatus and endoplasmic reticulum) of the cytoplasm are poorly developed; ribosomes are usually found free in the cytoplasm. Mitochondria with a dense matrix are polarized around the centriolar complex early in spermiogenesis (Fig. 2).

Another peculiarity of early spermiogenesis is the presence of a small vacuole in the anterior part of the nucleus in close contact with the nuclear envelope which is more dense (Fig. 3). This vacuole expands slightly (Fig. 4)
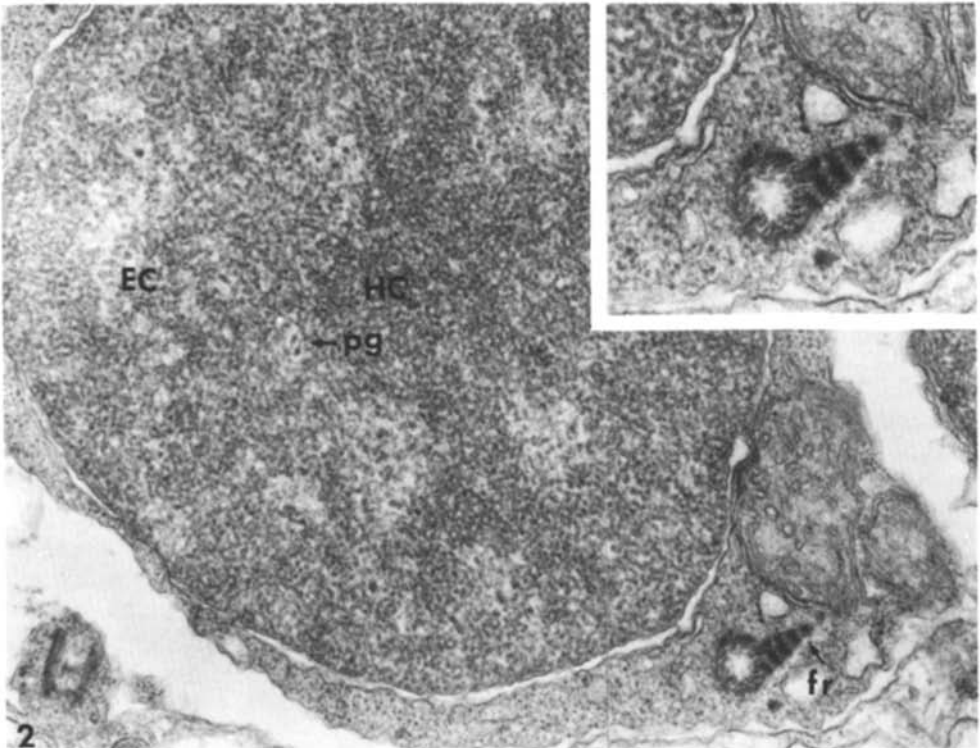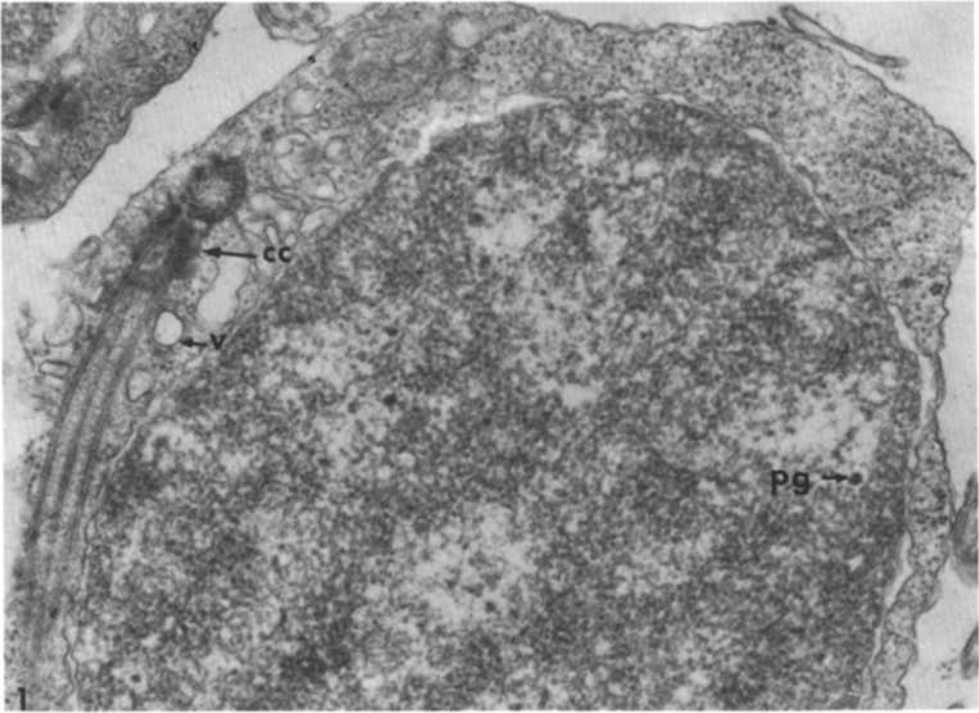
Fig. 1. Young round spermatid (stage 1). The flagellum is still in the cytoplasm. Numerous vesicles (*v*) are seen around the centriolar complex (*cc*); *pg* perichromatin granule. × 30000

Fig. 2. Young round spermatid at stage I with a flagellar rootlet (*fr*). The mitochondria start to concentrate around the centriolar complex. Nuclear material is heterogeneous with areas of heterochromatin (*HC*) and euchromatin (*EC*). × 25000

Table 1. Summary of the major changes occurring during spermiogenesis in trout

	Stage 1	Stage 2	Stage 3	Stage 4
Shape of nucleus	round	round	slightly elongated	elongated
Chromatin structure	hetero-geneous	homogeneous	coarse granules and fibers	fibrills and fibers
Fiber diameter (nm), thin (after Zirkin, 1975), thick	17.7 ± 3.4 —	17.5 ± 4 —	17.8 ± 3.7 326 ± 71	– 339 ± 74
Perichromatin granules	+ +	+		
Dense bodies	+	−	−	−
Juxtanuclear vacuole	+	−	−	−
Differentiation of nuclear envelope	+	+	+	(+)
Centriolar complex: position	cytoplasm	→nucleus	implantation fossa	implantation fossa
attachment to nucleus	−	−	+	+
rootlet	+	+	+	+ → −
Position of mitochondria	peri-centriolar	peri-centriolar	peri-flagellar	ring, peri-flagellar
Intercellular bridges	+	+	+	+
Cytoplasm	around spermatid	leave anterior part	pushed backward	latero-posterior
Flagellum	intra-cytoplasmic	extra-cellular	extra-cellular	extra-cellular

and eventually disappears, but the modified nuclear envelope with dense material between the two layers remains.

Stage 2. During this stage the nucleus is spherical and the chromatin is homogeneously distributed. Some perichromatin granules remain (Fig. 5) but there is no trace of the dense body. In the cytoplasm, free ribosomes are still numerous and the mitochondria remain located towards the anterior part of the flagellum where the first signs of organization of the middle piece appear (Fig. 5). The centriolar complex is still connected to the nuclear envelope by a flagellar rootlet (Fig. 5). The flagellum, which appeared earlier in the cytoplasm (Fig. 1), was apparently translocated under the plasma membrane and then seemed to emerge from the cell from this point. The plasma membrane of the flagellum with its lateral expansions also appeared to be formed at that time.

Stage 3. During stage 3, the nucleus elongates slightly and its rotation becomes symmetric due to the implantation of the centriolar complex in the
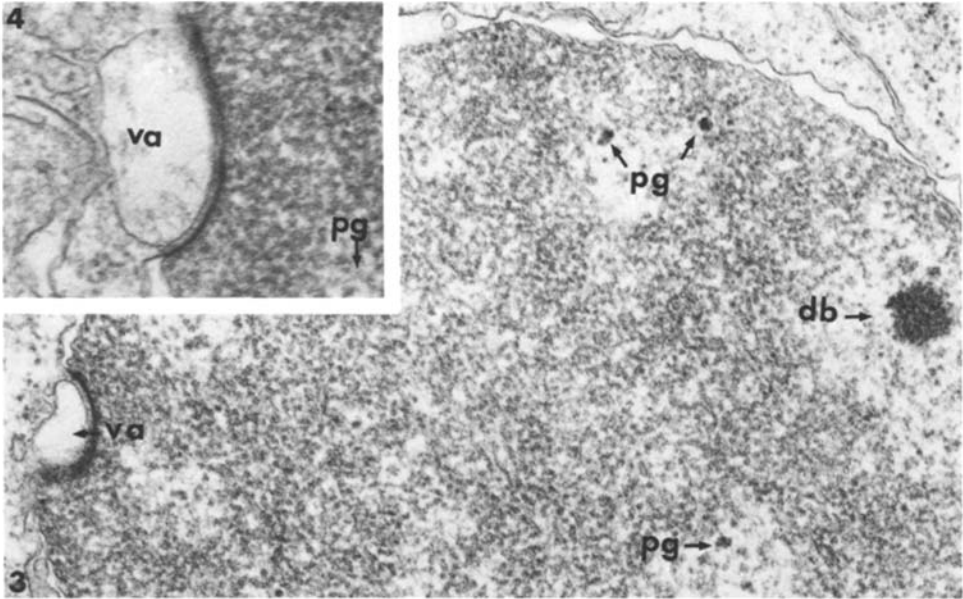
Fig. 3. Round spermatid (stage I) with vacuole (*va*) attached to the nuclear envelope. Perichromatin granules (*pg*) and a dense body (*db*) (nucleolus?) are visible in the nucleus. × 30000

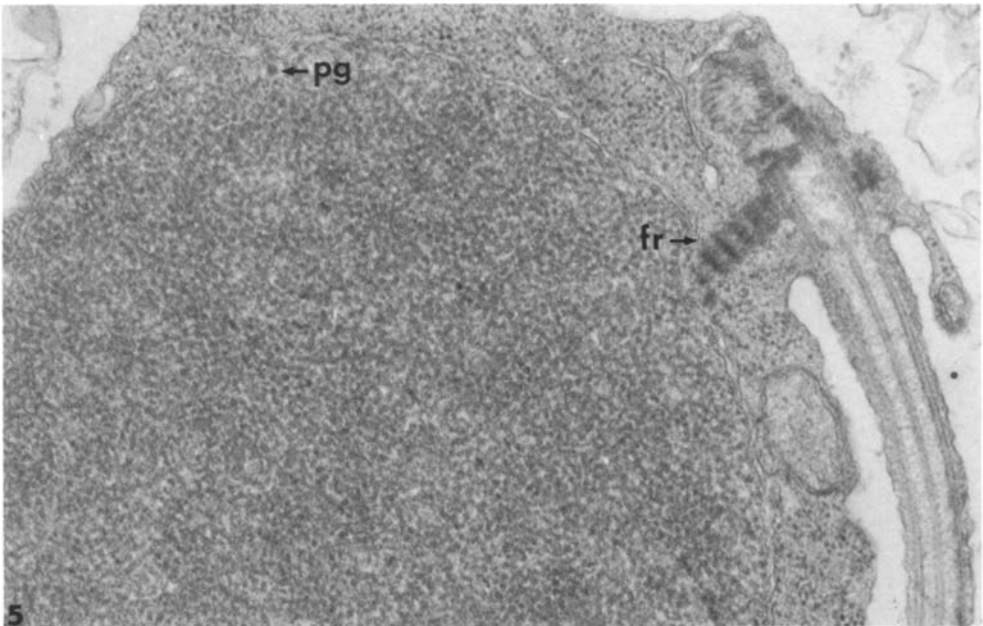**Fig. 4.** Juxtanuclear vacuole (*va*) showing a later stage of development than in Fig. 3. × 47000

Fig. 5. Nucleus at stage II. The chromatin is becoming homogeneous. A flagellar rootlet (*fr*) links the nuclear envelope with the distal centriole. × 30000
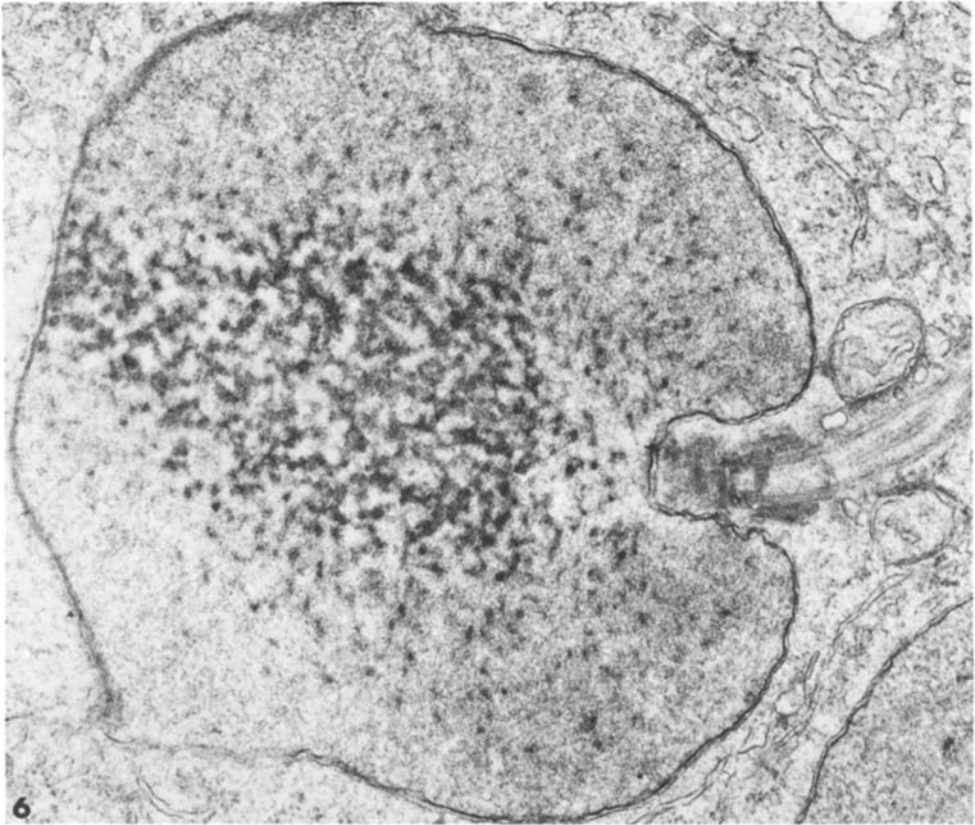
Fig. 6. Spermatid (stage III). Note the beginning of chromatin condensation in the form of coarse granules in the central part. Fixation in OsO$_4$. × 32000

implantation fossa (Figs. 6–9). Two types of material are identified in the nucleus: (i) very fine fibers at the periphery and coarse, fused granules, and (ii) much thicker fibers in the center (Fig. 6). Eventually, these thick fibers occupy the entire center of the nucleus and appear to show a definite orientation (Figs. 7–9). The thick fibers finally appear to condense, starting from the central axis of the nucleus and leaving some clear areas with a limited amount of material (Figs. 7–9). The cytoplasm is progressively pushed towards the posterior part of the spermatid (Figs. 7–9). In the anterior part of the spermatid, the modified nuclear envelope, identified during stage 1, is clearly visible (Figs. 6–9). The final, rough morphology of the sperm head is then acquired with the mitochondria located in the posterior part of the spermatid (Figs. 7, 9) and the centriolar complex inserted in the implantation fossa of the nucleus (Figs. 6–9). The flagellar rootlét associated with the distal centriole (Figs. 2, 5) appears to be involved in the migration of this complex towards the nucleus (Figs. 8, 4, 10). The membranous structures are still poorly developed in the cytoplasm but the ribosomes are numerous.

Fig. 7. Spermatid at stage III. Note the gradual extension of chromatin condensation to the periphery of the nucleus. *Arrow* indicates a zone of uncondensed chromatin. × 33 000
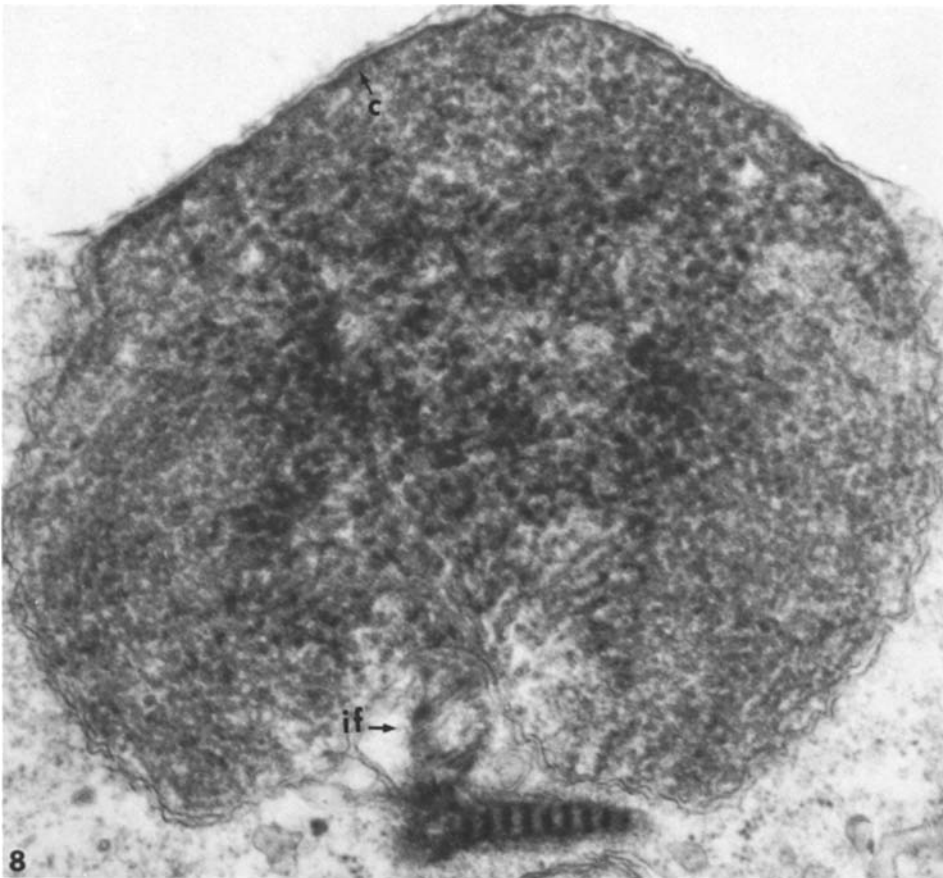
Fig. 8. The proximal centriole is located in the implantation fossa (*if*). First signs of chromatin condensation (stage III); *c* chromatin condensation along the modified nuclear envelope. × 36 000

Stage 4. The nucleus maintains its elongated shape during this stage (Figs. 10, 12, 14). The chromatin becomes more condensed, starting with the formation of thick fibers (the ribbon-like element of Zirkin), composed of smooth fibrillar elements resulting from this condensation (Figs. 10, 12). The ribbon-like structure, but not the fibrillar elements, remain visible in late spermatids (Fig. 13) and in spermatozoa (Fig. 14). However later, at the end of the period of spermiation in February, the fibrillar elements are seen again in the degenerating sperm head in the lobule (Fig. 15). In spermatozoa in the testis or in ejaculated milt, the degree of chromatin condensation is quite variable, some sperm heads being entirely electron-dense while others show numerous clear areas. The modified appearance of the nuclear envelope in the anterior part of the spermatid head, identified during the previous stages, is not visible in this stage but it is still present
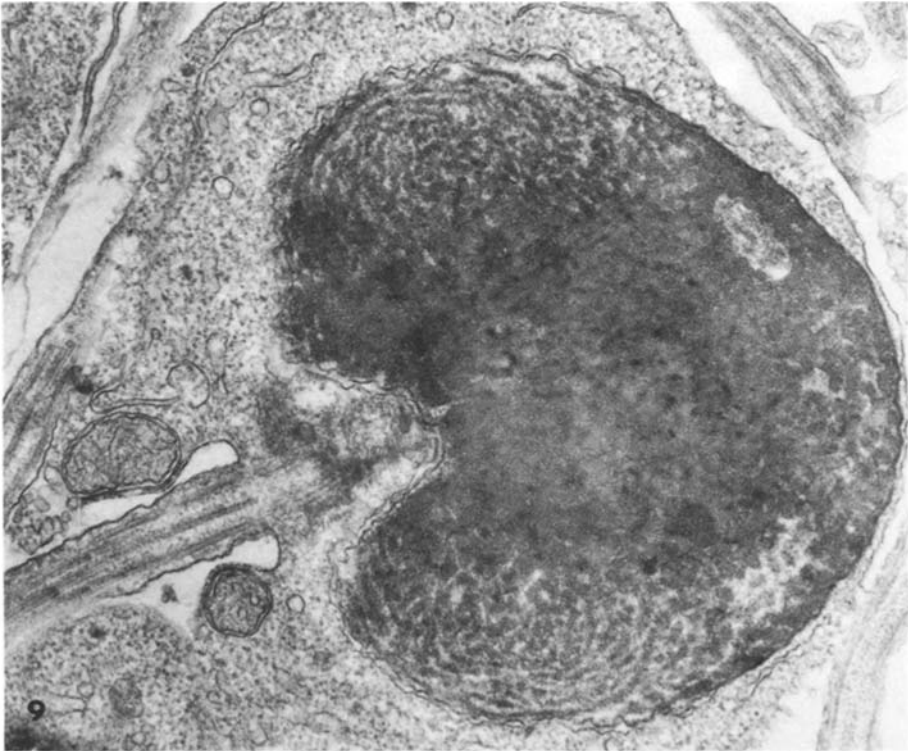
Fig. 9. Spermatid at stage III. The process of chromatin condensation has reached the periphery of the nucleus; the coarse granules and fibers fuse in the central part. × 30000

because it can be seen again in degenerating spermatozoa found later in the lumen of the lobule (Fig. 15). During final development of the spermatid and the slight elongation of the nucleus, the flagellar rootlet (Fig. 11) remains associated with the distal centriole and in close contact with the nuclear envelope (Fig. 10). The rootlet disappears when the sperm head is fully formed (Fig. 14). The mitochondria are still found lining the cytoplasmic canal in the anterior part of the flagellum (Figs. 10, 12, 13); they tend to fuse and a single mitochondrion is usually found surrounding the cytoplasmic canal (Fig. 16). Some of the mitochondria not jncorporated into the mid-piece are eliminated and occur isolated or associated with other organelles in residual bodies in the extracellular space (Fig. 17). The cytoplasm, which has been gradually pushed backwards during spermiogenesis, is finally detached from the spermatid (Fig. 14) and left in the cyst, from which cellular debris is taken and phagocytized by the Sertoli cells (Fig. 17); some debris remains in the lumen of the lobule. At no time during spermiogenesis does the head of the spermatid enter into close contact with the Sertoli cell cytoplasm. At the end of spermatogenesis, the cyst wall facing the central lumen of the lobule ruptures, and the spermatozoa are
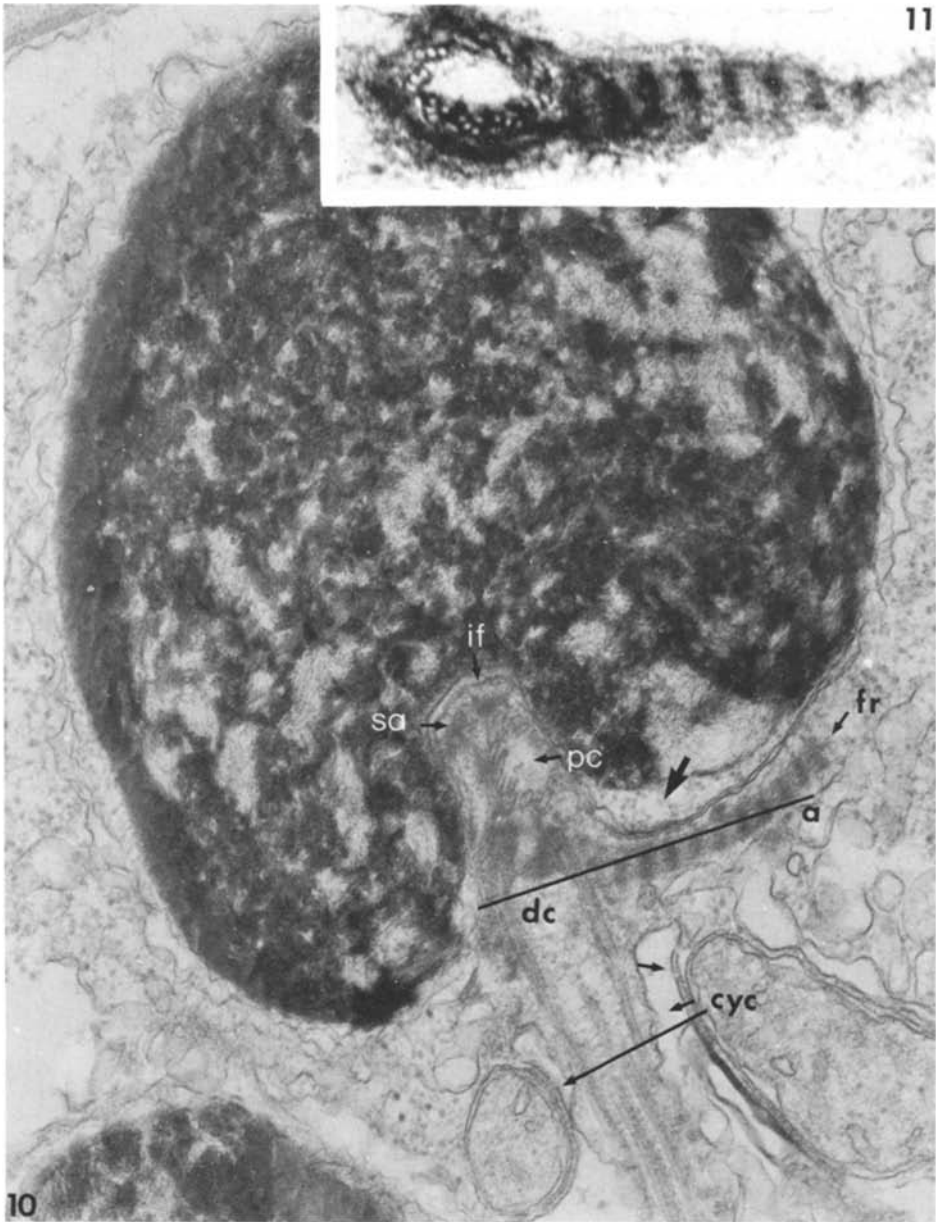
Fig. 10. Anterior-posterior section of a spermatid at stage IV. The flagellar rootlet (*fr*) is still attached to the distal centriole (*dc*) deeply embedded in the implantation fossa (*if*). The system of centriolar complex attachment to the nucleus is now formed. An additional membrane (*arrow*) can be seen between the plasma membrane of the cytoplasmic canal (*cyc*) and the mitochondrial envelope. *Large arrow* indicates a zone of uncondensed chromatin. × 45000

Fig. 11. Detail showing the flagellar rootlet sectioned along the line shown in Fig. 10. × 60000
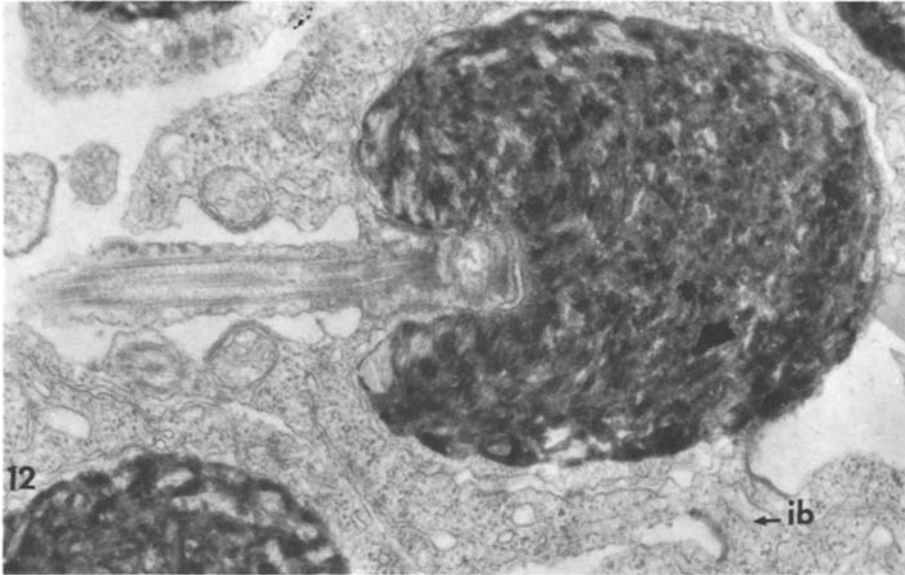
Fig. 12. Spermatid at stage IV. Ribbon-like elements appear in the nucleus. The spermatids remain attached by intercellular bridges (*ib*). The cytoplasm is shifted to the posterior part of the spermatid. × 28 000

freed into the lumen. The Sertoli cells remain in a monolayer at the internal periphery of the lobule.

Spermatids at different stages are sometimes found side by side, showing that spermiogenesis is not always synchronous. Also, some cells do not appear to go through the entire process of spermiogenesis, remaining in the spermatid stage in the lumen of the lobule. Sometimes, the cyst opens prematurely, releasing the spermatids into the lumen of the lobule.

Discussion

Spermiogenesis in rainbow trout appears to be a relatively complex process. Zirkin (1975) divided this process into four stages based only on the degree of transformation of nuclear material. These stages also correspond to changes in other cellular components (Table 1).

With regard to changes in the nuclear material, the present findings are in agreement with the observations of Zirkin (1975) on *Oncorhynchus tshawytscha*; the main difference is that the so-called fibrils, identified at the beginning of stage 3, seem to compose the ribbon-like elements of the trout. In this species, as well as in other salmonids, nucleohistones (very homogeneous in trout: Seyedin and Cole 1981) are transformed directly into nucleo-protamine due to the replacement of histones by protamine during spermiogenesis (Marushige and Dixon 1971; Louie and Dixon 1972; Dixon and Smith 1968). This transformation is said to occur at an advanced stage
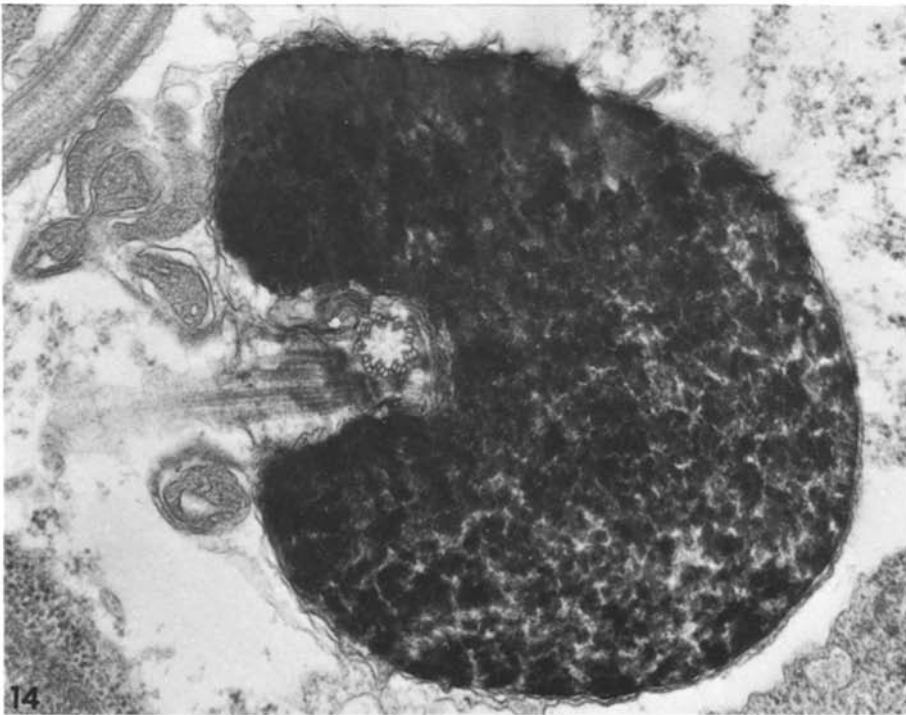
Fig. 13. Late stage-IV spermatid. × 30000

Fig. 14. A spermatozoon is shorter after the cytoplasm has finally been eliminated. × 35000
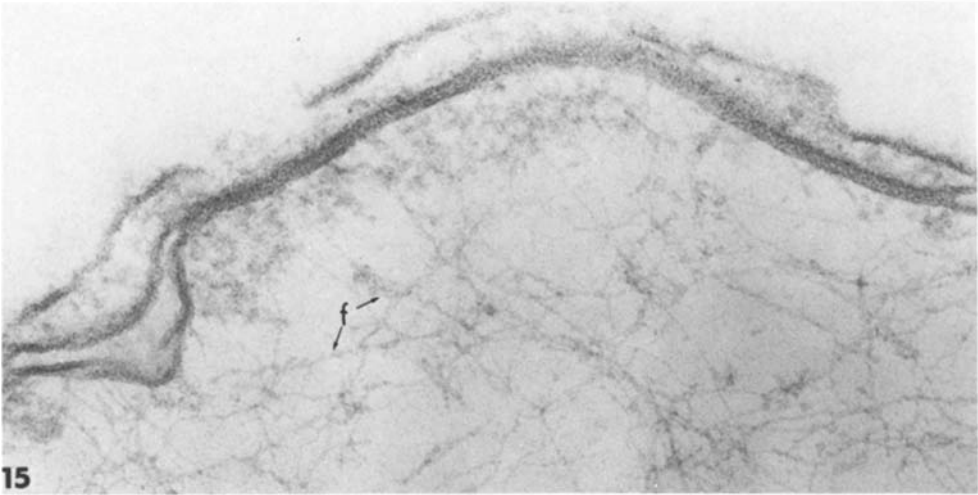
Fig. 15. Detail showing differentiation of the nuclear envelope in a degenerating sperm head in the lobular lumen. Dense material is condensed between the two layers of the envelope; *f* chromatin fibrils. × 96000

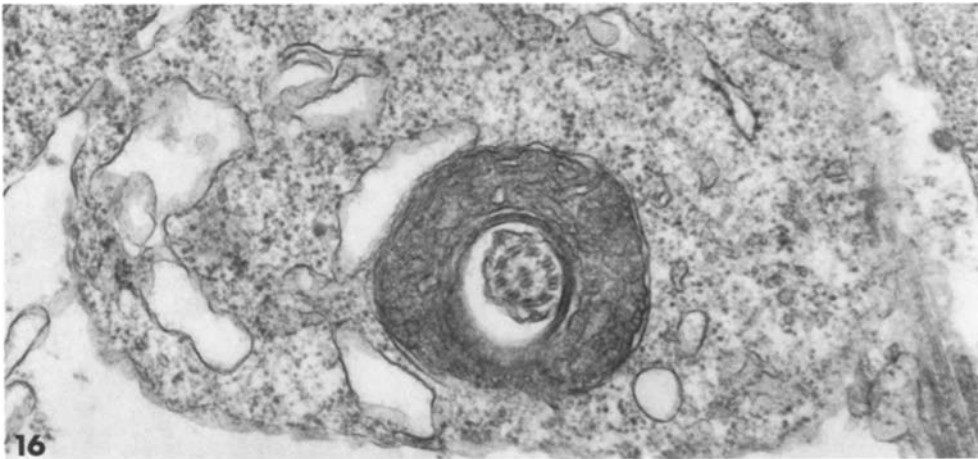**Fig. 16.** Cross-section of the posterior part of a late spermatid showing annular mitochondria. × 30000

of spermiogenesis (Alfert 1956) or in the middle phase of spermatid development (Louie and Dixon 1972). In addition, protamine undergoes enzymatic phosphorylation and dephosphorylation (Louie and Dixon 1974). It is supposed that the two phenomena, i.e. the appearance of protamine and phosphorylation-dephosphorylation, are connected with chromatin condensation, but the exact stages of this process are not known. More investigations are necessary to determine if there is a relationship between the two major
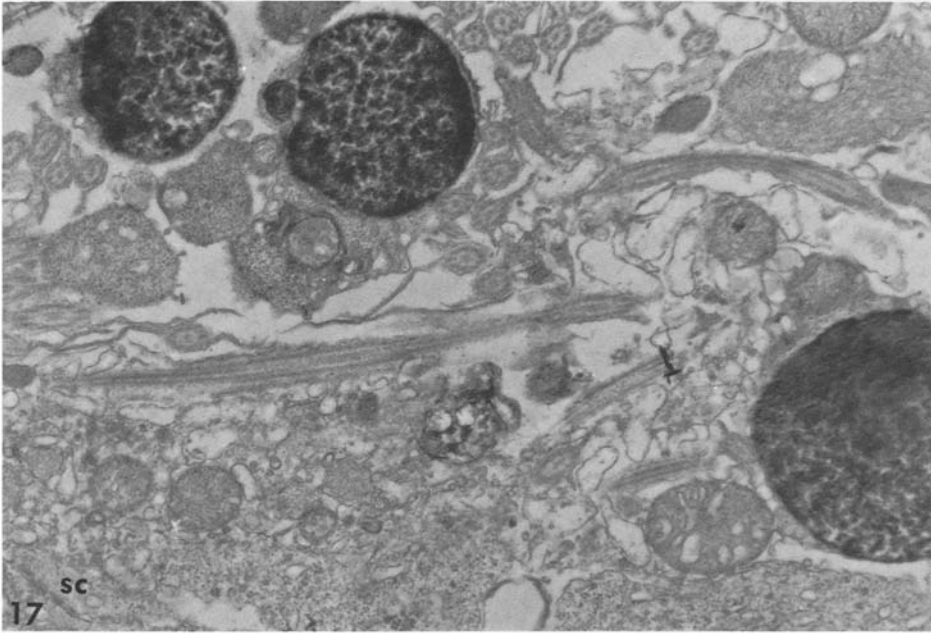
Fig. 17. Lumen of a lobule with free spermatozoa and various residual bodies; *Sc* Sertoli cell. × 15000

events of chromatin alteration in spermatids, i.e. the formation of thick fibers and the appearance of the smooth fibrils associated with the ribbon-like elements.

The presence of nucleoprotamine fibrils has also been reported in mature trout sperm after extraction in ammonium acetate (Inoue and Fuke 1970) and treatment with NaCl (Pollister and Mirsky 1946; Felix 1960). Fibrils can be observed in degenerating sperm nuclei in the testis (Fig. 18) or in the sperm duct (Billard and Takashima 1983) and after deep freezing (Billard 1983), indicating that this fibrillar association is not very tight and can be disrupted easily. The sperm head has been found to change in size with differences in the osmotic pressure of the fixative (Lowman 1953), but not with differences in the osmotic pressure of the saline solution (Billard and Breton 1970). Some morphological changes also occur after dilution in fresh water (Billard 1978).

The cytoplasm has relatively few organelles. The dense material (intermitochondrial 'cement'), very abundant in cyprinids (Clerot 1976), is quite scarce in trout. Possibly as a result of this (Toury et al. (1977) have suggested that the cement is incoporated into the mitochondria), the number of mitochondria is limited and the mid-piece is relatively small, consisting of a single mitochondrion shaped like an incompletely closed ring. The final structure of the mid-piece is gradually acquired while the spermatozoa rest in the lumen of the lobule. A kind of structural maturation then occurs after the spermatozoa are released from the cyst. This may be related to
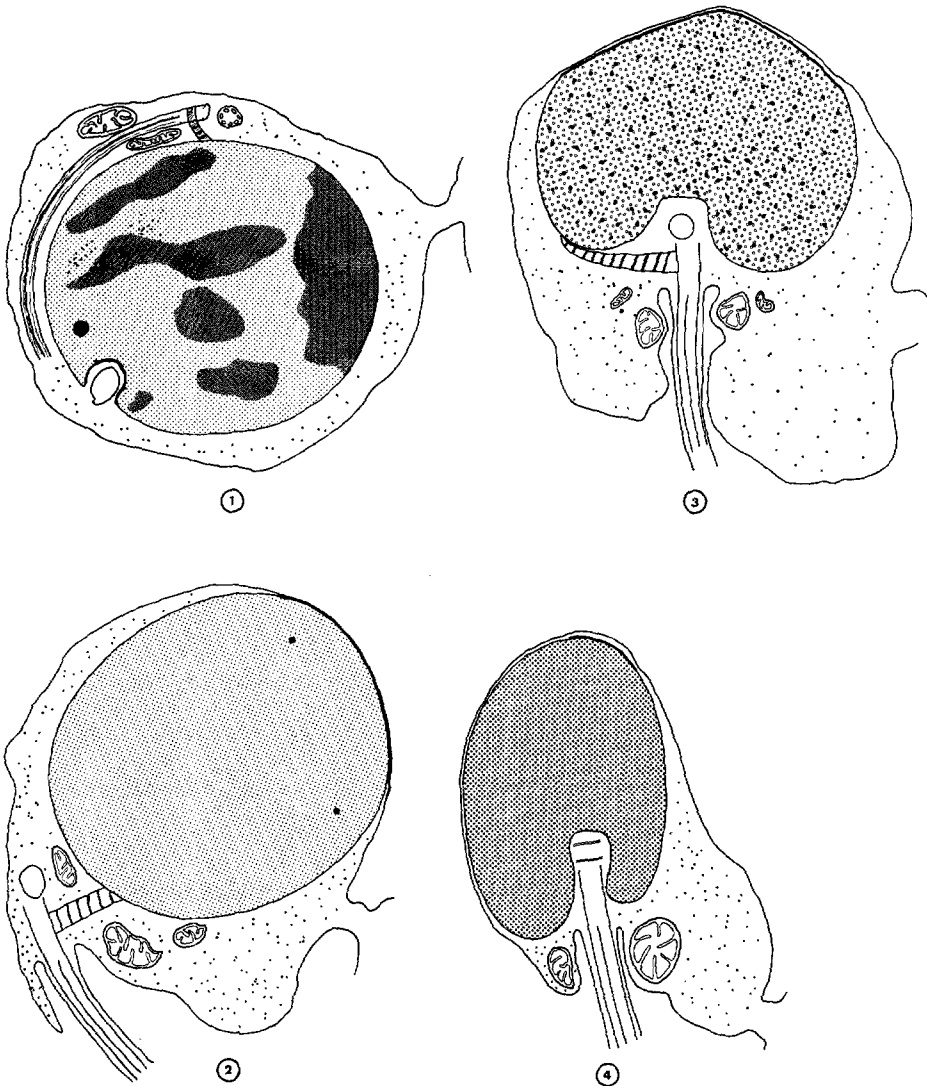
Fig. 18. Summary of the major changes occurring during spermiogenesis in trout

a change in sperm physiology; for instance, the motility of intratesticular spermatozoa is not inhibited by K^+ at the very end of spermatogenesis as it is once spermiation has started (Billard and Mattéi, unpublished).

It is now well established that teleost fish have no acrosome. However, a vacuole very similar to the acrosome vacuole, visible at the beginning of acrosome formation in mammals, is identified in trout; this may represent an attempt to form the acrosome. Differentiation and densification of the nuclear envelope are observed in the anterior part of spermatids and sperma-

tozoa in trout (Fig. 15; Billard 1983) and in *Lepadogaster lepadogaster* (Mattéi and Mattéi 1978). These authors interpreted this structure as a vestigial acrosome. This differentiated nuclear envelope might play a role in the process of membrane fusion during fertilization. The anterior part of the sperm head first seems to be in contact with the eggs at the bottom of the micropyle in trout (Ginsburg 1963) as well as in *Oryzias latipes* (Iwamatsu and Ohta 1981) and carp (Kuo 1980). Differentiation of the nuclear envelope also seems to prevent the anterior part of the envelope from swelling when the sperm is exposed to fresh water (Billard, unpublished observation).

Contrary to centrioles in the guppy, which cannot be recognized in mature spermatozoa (Billard 1969, 1970), the centriolar complex in trout undergoes only slight changes during spermiogenesis. At the beginning of this process, the axoneme without a plasma membrane is found in the cytoplasm (Fig. 1). The fusion of vesicles near the centriolar complex (Fig. 1), as in guppy (Billard and Flechon 1969) and *Oryzias latipes* (Sakai 1976), seems to be responsible for the formation of the membrane and the anterior part of the cytoplasmic canal. The remainder of the membrane appears to be formed when the flagellum is pushed outside the cell. The formation of the flagellum is different in trout compared to *Trichiurus* and some other teleosts in which the distal centriole remains attached to the plasma membrane (Mattéi and Mattéi 1976; Mattéi 1969).

Another interesting feature of trout spermiogenesis is the presence of a flagellar rootlet connected with the centriolar complex during the major morphological changes (elongation of the nucleus and attachment of the flagellum to the sperm head) in the spermatid. This rootlet, which may be constituted of contractile material, is possibly involved in the attraction and stabilization of the centriolar complex in the implantation fossa while the definitive structure attaching the centriolar complex to the nucleus (bars and filaments), and found in mature trout spermatozoa (Billard 1969, 1983), is progressively established. Data in the literature show that more or less complex associated cytoplasmic structures are present at the time the flagellum is attached to the nucleus; these include rootlets in trout, various structures such as intercentriolar lamellar bodies, electron-dense material and microtubules in *Oryzias latipes* (Grier 1976), *Trichiurus lepturus* (Mattéi and Mattéi 1976) or *Pantolon bucholzi* (Deurs and Lastein 1973), and bundles of microtubules in poecilids (Billard 1970; Russo and Pisano 1973; Grier 1975). In some cases, microtubules are associated with rootlets (eel, *Albula*: Billard and Ginsburg 1973; Mattéi and Mattéi 1974) or with satellites and fibrous material as in *Upeneus prayensis* (Boisson et al. 1969). Such direct means of anchoring the centriolar complex to the nucleus may be completed by additional cytoplasmic structures increasing the strength and rigidity of the system; these structures include thickening of the plasma membrane limiting the cytoplasmic canal in *Trichiurus lepturus* (Mattéi and Mattéi 1976) or the well-developed mid-piece in poecilids and other species with an elongated nucleus (except for *Anguilla anguilla* which has no mid-piece but perinuclear microtubules that remain in the mature spermatozoa).

Rootlets are not observed in species like pike or carp in which the sperm head remains spherical and the flagellar attachment is very simple, i.e. only in contact with the nuclear envelope (Billard 1969; Caloianu-Iordāchel and Sicoe 1976). As complex structures for attaching the flagellum to the nucleus are present only in spermatids that undergo complex spermiogenesis, and as these structures coincide with the elongation of the nucleus and chromatin condensation, they are also involved in these events; this is the case for microtubules. A role in shaping the nucleus has been given to the manchette, but Fawcett et al. (1971) demonstrated that such a role was not obvious and suggested that the shape of the nucleus might be determined by forces resulting from the aggregation of DNA and protein during chromatin condensation. However, Courtens and Loir (1981) proposed that microtubules might play a role in the organization of chromatin and its transformation. This hypothesis would not apply in the case of trout since chromatin condensation occurs without the presence of a manchette or microtubules. Courtens (1982) suggested that the flagellum itself might play a similar role; in the case of salmon, the insertion of the flagellum into the implantation fossa may be responsible for the radial orientation of the chromatin fibers observed by Zirkin (1975).

The mechanism controlling the transformation of nuclear material during spermiogenesis is not clear. The suggestions of Jergil and Dixon (1970) and Marushige and Dixon (1971) regarding endocrine control have not been confirmed, although it has been shown that plasma gonadotropin and androgens rise markedly during spermiogenesis (Billard et al. 1978; Scott et al. 1980; Stuart-Kregor et al. 1981; Hunt et al. 1982) and that steroidogenic activity is detectable in Sertoli cells of spermatid cysts (Hurk et al. 1978a, b). More generally, the fact that most spermatids are not in direct contact with the Sertoli cells is intriguing in a species in which morphological and biochemical changes in the spermatid head are important. In the guppy, all the spermatids are in direct contact with the Sertoli cells at the end of spermatogenesis but the transformations in guppy are more extensive than in trout. However, in trout the spermatids have indirect contact with the Sertoli cells via the intercellular bridges that seem to connect all the cells in a cyst, as shown in other fish (Clerot 1971; Grier 1976) and mammals (Dym and Fawcett 1971). Secretory material found inside the cyst and originating from the Sertoli cells represents another indirect way of contact between the spermatids and the Sertoli cells. In turn, in salmonids some signals reaching the Sertoli cells may be emitted by the spermatids. Spermatogenesis is discontinuous, generally ceasing in autumn, although the endocrine environment (gonadotropin and steroids) still seems favorable. Further spermatogonial division may be inhibited when the testis reaches a given size (sperm release starts only one month after the end of spermatogenesis so that the testis continues to increase in size until spermatogenesis ceases). Compensatory hypertrophy has also been shown in trout (Billard et al. 1982). Therefore, a chalone-type substance may be involved and signals emitted from the spermatids via the release of nuclear or cytoplasmic material. This nuclear material, eliminated during spermiogenesis or taken from

the cyst to be phagocytized (Billard et al. 1972), has informative value and could play a role in the control of spermatogenesis.

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