OBSERVATIONS ON SPERMIOGENESIS IN
THE FUNGUS GNAT SCIARA COPROPHILA

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ABSTRACT
Although 9-membered centrioles are found in somatic tissues of Sciara, the centriole which lies at the spindle pole of the second meiotic division in male Sciara is composed of a row of approximately 70 short tubules in an oval array. Shortly after telophase of this unequal division, in the daughter cell destined to undergo spermio genesis, microtubules become confluent with the tubules of the centriole. These tubules have the same density as other cytoplasmic microtubules after glutaraldehyde-OsO₄ fixation and, like them, are not preserved with OsO₄ fixation. As the centriole, now with approximately 70 attached, posteriorly directed, doublet tubules, migrates from the polar to the apolar end of the nucleus to take a final position in an oval groove which forms in the nuclear envelope, the tubules lengthen and become demonstrable after OsO₄ fixation and more electron opaque than other cytoplasmic microtubules following glutaraldehyde-OsO₄ fixation. Later, a singlet tubule appears peripherally to each doublet of the oval and 4 "arms" develop at specific sites on the tubules. Posteriorly, where the oval of tubules becomes discontinuous and forms a spiral, the arrangement of arms is different and the singlet tubules are lacking. Dense solid bodies develop inside this odd flagellum and become enclosed by a smooth double membrane. A single mitochondrial derivative has three components: a central area of homogeneous, moderately electron-opaque, proteinaceous material; a peripheral ring of cristae; and a crystalloid which is specifically oriented with respect to the flagellar tubules.

INTRODUCTION
During the 1920's and 1930's, Metz and his co-workers traced the cytological intricacies of a highly unusual spermatogenesis in the fungus gnat Sciara (references 18–21; for review, see reference 19). They reported that, as a consequence of unequal distribution of chromosomes and cytoplasm in both first and second spermatogenic divisions, only one spermatid is formed for each primary spermatocyte instead of the four products of typical spermatogenesis. This single spermatid contains a set of maternally derived autosomes, two identical, maternally derived X chromosomes, and one or more "limited" chromosomes (heterochromatic chromosomes restricted to the germ tissue). At the same time, Doyle found that spermiationesis in S. coprophila also encompasses some unusual features. The mitochondria accumulate a great deal of proteinaceous material which eventually occupies most of the volume of a single elongate mitochondrion formed during spermiationesis by mitochondrial fusion (7).

A reinvestigation of Sciara testis with the electron microscope has brought to light fine structural features as unexpected and unconventional as those revealed by light microscopy 40 yr ago. The present report deals with spermiationesis with
special reference to the development of an axia filament complex differing markedly from the typical $9 + 2$ tubule pattern in the flagella of other spermatozoa (17, 25). The odd tubule pattern of the flagellum is apparently a reflection of a correspondingly odd tubule pattern of the spermatocyte centriole.

MATERIALS AND METHODS

The spermatogenic divisions last but a few hours in Sciara, and they take place fairly synchronously throughout the testis. Spermiogenesis, which lasts for the entire period of pupation (about 5 days at $16^\circ C$), is also synchronous.

Two methods were employed to determine pupal stage.

1. A group of male pupae which had just undergone the pupal molt were selected, placed in the same environment, and sacrificed at set intervals.

2. Pupal stage was estimated by evaluation of the morphological indices of imaginal eye, leg, and wing development and darkening of the puparium.

Either of these methods gives only an approximation of the stage of spermiogenesis. Animals which undergo their pupal molts simultaneously soon become so developmentally asynchronous that the time of eclosion may span 24 hr. Morphological staging is inexact owing to the gradual nature of observable changes. In addition, stages in spermiogenesis do not correlate well with external morphological characteristics, and individual spermatids in the same testis are somewhat asynchronous. This is especially true for late spermatids.

To stage spermatid development closely it was necessary also to depend upon morphological characteristics of the spermatids themselves. There are many progressive structural changes during spermiogenesis (i.e., cell elongation and sloughing of cytoplasm, nuclear condensation and elongation, mitochondrial fusion and elongation). From many sections through various parts of spermatids of the same testis, we were able to get a general picture of a given stage. By comparing the stage of an animal as determined by pupal development with the stage of a testis as determined by fine structural criteria, we have reconstructed the sequence of changes during spermiogenesis.

Testes were fixed in either 1% Os$O_4$ (pH 7.6) or 6% glutaraldehyde (pH 6.8) followed by 1% Os$O_4$ (pH 7.6) (31). Fixing solutions were buffered in 0.1 M Sorenson's phosphate buffer (final molarity). The testes were dissected in cold (0 to $4^\circ C$) fixative, and subsequent fixation was carried out in the cold. Tissues were dehydrated in cold ethanol and embedded in Epon 812 according to Luft (16). Sections were cut on a Porter-Blum MT-1 ultramicrotome, stained in 3% aqueous uranyl acetate (6 to 24 hr), poststained with lead citrate using the method of Reynolds (28) or Venable and Coggeshall (35), and examined with a Siemens Elmiskop I.

RESULTS

Spermatogonial Centrioles

Centrioles in somatic tissues of Sciara are similar to the type of centrioles which is apparently almost universally present in the animal kingdom (9). They differ in that they are apparently composed of 9 doublet instead of the usual 9 triplet tubules (Fig. 1). The centrioles of spermatogonia are, however, strikingly different in appearance from any centrioles which, to our knowledge, have previously been described. Spermatogonial centrioles are composed of 50 to 90 (generally about 70) short singlet tubules (Fig. 2). In cross-section the evenly spaced singlet tubules of the centriole describe an oval. Fibrous material occurs on both sides of the oval of singlet tubules and between adjacent singlets. This material is most prevalent on the side of the tubules facing the inside of the oval. Inside this fibrous material is a thin (about 50 A) oval band of fibrous character which is separated from the outer fibrous substance by a narrow region of low electron opacity. A third zone of fibrous substance occupies the center of the spermatogonial centriole.

The spermatogonial centrioles are true centrioles in the classical sense (36) because they lie at the pole of the mitotic spindle (Fig. 3). In addition, these centrioles, like conventional centrioles, are often found in pairs, the two centrioles of the pair being disposed at right angles to each other (Figs. 3 and 4).

Spermatocyte Centrioles

Like the spermatogonial centrioles, the meiotic centrioles in male S. coprophila are composed of many short (0.5 μ) tubules arranged in an oval. We cannot be sure whether the tubules are doublets or singlets because they have very low contrast against the dense material between and on either side of them (Figs. 5 and 6). A region of dense fibrous material is usually present in the immediate vicinity of the centriole (Figs. 5 and 6).

The second meiotic division in male Sciara is an unequal division; the bulk of the cytoplasm accompanies one of the nuclei, and this daughter cell undergoes spermiogenesis; the other nucleus
is pinched off into a small rudimentary process or polar body which degenerates (19, 20). At the spermatid pole of this division a single centriole in the form of a truncated cone is always oriented so that a section bisecting both poles cuts the centriole longitudinally, with the apex of the truncated cone directed towards the opposite pole (Fig. 7). Although numerous microtubules present in the spindle of the second meiotic division are oriented in the direction of the pole, spindle microtubules are not confluent with the doublet tubules which comprise the meiotic centriole (Fig. 7). At telophase, the daughter cell which is destined to differentiate into the spermatozoon is somewhat elongate; the telophase nucleus lies at the end of the cell which is distal to the rudimentary process, and the meiotic centriole is located at the polar end of the telophase nucleus (Fig. 8). This end of the cell will become the anterior end of the spermatid.

Migration of the Centriole

Shortly after telophase of meiosis II, the oriented spindle microtubules are no longer in evidence. At a slightly later stage in spermiogenesis, the microtubules are again oriented towards the centriole.
FIGURE 3  A glancing section of two spermatogonial centrioles (arrows) at the pole of the mitotic spindle. The centrioles are perpendicular to each other and have been cut so that the tubules of the upper one are seen in cross-section and the tubules of the lower one in longitudinal section. Cleavage furrow (cf). Glutaraldehyde-OsO₄. × 25,000.

FIGURE 4 Three spermatogonial centrioles (arrows). The pair of centrioles at the right are disposed at right angles to each other. Glutaraldehyde-OsO₄. × 45,000.
FIGURE 5 Cross-section of a meiotic centriole. Meiotic centrioles consist of ovaly disposed tubules. In this figure a portion of the oval is not in the plane of section. An area of fibrous material extends from 100 to 200 A on either side of and between the tubules of the meiotic centriole. A region of dense fibrous material (d) is usually present in the immediate vicinity of the centriole. Glutaraldehyde-OsO₄, X 49,000.

FIGURE 6 Longitudinal section of a meiotic centriole. The tubules of the centriole are about 0.5 μ in length. Dense fibrous material (d). Glutaraldehyde-OsO₄, X 49,000.

FIGURE 7 A centriole (c) at the pole of the second meiotic division. Spindle tubules (arrows) are oriented in the general direction of the doublet tubules of the centriole. Chromosomes (ch). Glutaraldehyde-OsO₄, X 33,000.

However, unlike the spindle tubules, these microtubules appear to be in direct continuity with the doublet tubules of the centriole (Fig. 9). It is not clear whether the microtubules "grow" from the centriole or whether preformed microtubules attach to the centriolar tubules. Microtubules which extend from the centriole are similar in density and diameter to other cytoplasmic microtubules.
after glutaraldehyde-OsO₄ fixation (Fig. 9). They are also similar in that neither is demonstrable after OsO₄ fixation.

The centriole now migrates caudad (towards the end of the cell which will become the tail). As it does so, the microtubules extending from the centriole elongate, and dense material present in the vicinity of the centriole increases in amount (Fig. 10). During this migration the microtubules extending from the centriole exhibit an orientation parallel to the long axis of the spermatid. Upon reaching the apolar end of the nucleus, the centriole becomes juxtaposed to the nuclear envelope, with the microtubules extending directly caudad.

**Nuclear-Centriolar Contiguity**

At the point of contact between nucleus and centriole an oval indentation which forms in the nuclear envelope contains the tubules of the centriole, and dense material extends laterally from the centriole (Fig. 11). The oval indentation in the nucleus gradually deepens, and material continues to increase in amount laterally from the centriole. Simultaneously, the dense fibrous material gradually disappears from inside the tubules which extend from the centriole (Fig. 12).

**Early Development of the Axial Filament Complex**

Cross-sections of spermatids immediately posterior to the nucleus reveal that the tubules which

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**FIGURE 8** Telophase of the second meiotic division. One daughter nucleus \( n₁ \) is accompanied by almost all the cytoplasm and eventually becomes the sperm nucleus. The other nucleus \( n₂ \) is pinched off into a small polar body which subsequently degenerates. A centriole (arrows) lies at the polar end of the telophase nucleus in the larger daughter cell. Spindle remnant \( s \) and mitochondria \( m \) occur in the cytoplasm of the larger daughter cell. Glutaraldehyde-OsO₄, \( X \) 9000.
FIGURE 9 Shortly after the second meiotic telophase, microtubules (arrows) become confluent with the centriolar doublets. These microtubules are similar in density and diameter to other cytoplasmic microtubules. Glutaraldehyde-OsO₄. × 48,000.

FIGURE 10 The centriole migrates from the polar end of the nucleus (P) to the apolar end (A). Here the migrating centriole (arrows) with its associated microtubules and proliferating dense fibrous material (d) lies laterally to the nucleus. × 21,000.
Upon reaching the apolar end of the nucleus the centriole becomes contiguous with the nuclear membrane. Later an oval indentation forms in the nuclear membrane which accommodates the centriole. Amorphous material proliferates laterally to the centriole (arrows). Microtubules (mt) extend caudad from the centriole. A smooth double membrane appears around these tubules as the dense fibrous material (d) begins to disappear. Glutaraldehyde-OsO₄. X 20,000.

Posterior tapering of spermatids is observable in aceto-orcein-stained squashes. The mitochondrion also tapers posteriorly and terminates considerably anteriorly to the caudal end of the cell. In electron micrographs, cross-sectional profiles of cells with a relatively small diameter possessing small mitochondrial profiles are, therefore, posterior to profiles of cells with a large diameter containing mitochondrial profiles of relatively large diameter.

In the posterior part of the cell as determined by the above criteria, the axial filament complex appears in cross-section as a spiral (Fig. 15). This spiraling occurs by a breakage and coiling of the oval of the axial filament complex. This transition is depicted schematically in the diagram of an early spermatid (Fig. 16). The tubules of the spiral, like those of the oval, are doublets and of greater density than singlet tubules of the cytoplasm (Fig. 17). The tubules of the axial filament complex are preserved following fixation with OsO₄ whereas other cytoplasmic microtubules are not. The double membrane which encircled the oval of the axial filament complex more anteriorly is also continuous with the centriole, doublets arranged in an oval. The doublet tubules gradually become more dense than other cytoplasmic microtubules (Figs. 13 and 14). These doublet tubules are henceforth referred to as the axial filament complex (10). A smooth double membrane develops around the axial filament complex, and an "arm" extending towards the outer margin of the adjacent doublet appears on one of the members of the doublet pair (Fig. 14). In accordance with the nomenclature established by Gibbons and Grimstone (13), the tubule bearing the arm will be referred to as subfiber B.
FIOURE 12 Longitudinal section of a spermatid nucleus. Anteriorly an acrosome (a) occupies an indentation in the nuclear membrane; posteriorly the centriole is accommodated by the groove in the nuclear membrane (arrows). Glutaraldehyde-OsO₄. × 19,000.

Late Development of the Axial Filament Complex

After the doublet tubules of the axial filament complex have formed, a singlet tubule is added peripherally to each doublet (Figs. 18 and 19). These singlet tubules, like the doublet tubules of the axial filament complex and unlike other cytoplasmic microtubules, are preserved with OsO₄ fixation, appear dense after glutaraldehyde-OsO₄ fixation, and are destined to become an integral part of the spermatozoon axial filament complex. The singlet tubules extend caudad from the oval groove in the nuclear membrane (Figs. 20 and 21).
FIGURES 13 and 14  Cross-section near the nucleus. (Same stage as Fig. 13). The doublet tubules of the developing axial filament complex appear denser than other cytoplasmic microtubules. A single arm on subfiber B is oriented parallel to the long axis of each doublet. A smooth double membrane surrounds the axial filament complex. The spermatid mitochondrion is composed of a narrow peripheral ring of cristae (Fig. 13, cs) and a central area of homogeneous, moderately electron-opaque material. Glutaraldehyde-OsO₄. Fig. 13, X 44,000; Fig. 14, X 142,000.

and terminate at the point where the oval of the axial filament begins to spiral (see succeeding paper). The arm on subfiber B changes 90° in its orientation so that it is perpendicular instead of parallel to the long axis of the doublet. An additional arm on each singlet tubule and two smaller less obvious arms on subfiber A can now be discerned. The arms on subfiber A resemble in their location the arms which were originally described by Gibbons and Grimstone (13) on subfiber A of protozoan flagella, in that they all point towards subfiber B of the adjacent doublet (Fig. 19). Subfiber B can be distinguished from subfiber A not only by the position and number of its arms, but because it is larger, more clearly defined, and has a less dense center.

At about the same time that singlet microtubules develop peripherally to doublets, another ring of singlets appears in the center of the axial filament oval (Fig. 18). These singlets are similar in electron opacity, after glutaraldehyde-OsO₄ fixation, to cytoplasmic microtubules rather than to the doublets and peripheral singlets of the axial filament complex. Like the former, they are not preserved by OsO₄ fixation. The oval of singlets soon gives way to electron-opaque material which develops in its center and is destined to persist throughout the remainder of spermiogenesis. In longitudinal section this electron-opaque material appears as a row of regularly disposed solid bodies separated by a homogeneous space, in the center of which is a thin band of fibrous material (Fig. 22). In cross-section or in glancing sections the dense bodies usually appear roughly oval (Figs. 19 to 21). A smooth double membrane soon encloses all the bodies (Fig. 22).

The dense material which was first associated with the centriole at the base of the axial filament
complex gradually disappears during spermiogenesis. In late spermatids it is difficult to discern where the centriole ends and tubules begin (Fig. 21).

**Mitochondrial Changes**

The mitochondria of spermatocytes and young spermatids are large, highly refractile spheroids consisting of a peripheral cortex of cristae and a central area of moderately electron-opaque homogeneous material (Fig. 8). Doyle (7) found that the peripheral area stained vitally with neutral red (vital Ehrlich) or Janus green B and that the central portion was Millon positive indicating the presence of protein-bound tyrosine. Using the acid dye fast green (0.01% at pH 2), we found that the central portion of the mitochondria showed typical protein acidophilia throughout spermiogenesis, stained very faintly to not at all with the PAS reaction, and was negative to Sudan IV stain for lipid. Thus no lipids were demonstrable, and polysaccharides were present in very low quantities or not at all.

During spermiogenesis the mitochondria fuse and elongate (Fig. 23); late spermatids possess but a single large mitochondrial derivative which extends caudad from the nucleus for most of the length of the cell. Both cristae and homogeneous
component are retained during spermiogenesis (Figs. 13 and 20).

At about the same time that the axial filament singlets appear, a crystalloid becomes apparent in the fused mitochondrion. The crystalloid is always located in the portion of the mitochondrial derivative immediately adjacent to the axial filament complex (Fig. 22). This consistent disposition is diagrammed in Fig. 16. The crystalline material extends the entire length of the mitochondrial derivative. Anteriorly it projects as a fingerlike process into the nucleus, but remains separated from the nucleoplasm by mitochondrial and nuclear membranes (Fig. 24).

**Nuclear Morphogenesis**

At telophase of the second meiotic division the chromatin is very densely compacted (Fig. 8). The chromatin becomes increasingly more diffuse during early spermiogenesis (Fig. 10). At the time when the contiguity between centriole and posterior nuclear membrane is established, only a few small areas of compacted chromatin remain (Fig. 11).

When areas of dense chromatin are no longer in evidence, the spermatid nucleus begins to condense and elongate (Fig. 12), and the over-all electron opacity of the nucleoplasm subsequently increases (Figs. 15 and 24). The nucleoplasm now appears as a homogeneous meshwork of fibers devoid of clumped chromatin.

The spermatid nucleus gradually assumes a characteristic cross-sectional profile which is symmetric with respect to two axes (Fig. 24). The two prominent indentations on either side of the longer axis will be referred to as the lateral grooves. In glutaraldehyde-OsO₄-fixed material, the nucleus is found to be partially ringed by a row of microtubules whose axis is parallel to the long axis of the cell (Fig. 25). The microtubules do not occur in the lateral grooves.

Later, the spermatid nucleus undergoes a rapid condensation. The first evidence of this is a sloughing of nuclear membrane on both sides of the nucleus at two diagonally opposite points (Fig. 26). A
spiral configuration of membranes occurs simultaneously in each lateral groove (Fig. 26). The nucleus now condenses to less than a quarter of its previous volume (Fig. 27). Nuclear membrane sloughed into the cytoplasm is identifiable by the presence of annuli and by its continuity with the membrane circumscribing the nucleoplasm (Fig. 27). This nuclear condensation is a very rapid one. Nuclei appear either not condensed (Figs. 24 and 26) or strongly condensed (Figs. 21 and 27). Partially condensed nuclei are rarely seen. The microtubules which are present in close proximity to the nuclear membrane disappear soon after condensation.

**Acrosome Formation**

The acrosome in *Sciara*, as in many other species (5, 8, 14, 36), arises as a dense, spherical granule in a Golgi zone at the anterior end of young spermatids (Fig. 28). Later, the Golgi zone migrates caudally, and the dense proacrosome granule remains at the anterior end of the nucleus. The granule becomes lodged in an indentation in the nuclear envelope and subsequently elongates longitudinally (Fig. 12).

**DISCUSSION**

**The Centriole**

Their location at the pole of the spindle during spermatogonial and spermatogenic divisions identifies testicular centrioles of *Sciara* as true centrioles in the classical sense (36). Spermatogonial centrioles, like conventional centrioles (3), often occur in pairs disposed at right angles to each other. Meiotic centrioles are similar to somatic centrioles of *Sciara* in that they are composed of short tubules (probably doublets) and, although the number of tubules is much greater, the tubules are the same length as those which compose the familiar 9-membered centriole (9). In addition, the meiotic centrioles of *Sciara* are surrounded by
FIGURE 18 After the doublet tubules of the axial filament form, a singlet develops peripherally to each doublet. The singlet extends from the groove in the nuclear membrane caudad to the level where the oval becomes a spiral. Dense solids form in association with microtubules within the center portion of the oval near the mitochondrial crystallloid. Glutaraldehyde-OsO₄. X 80,000.

FIGURE 19 When a singlet tubule forms distally to each doublet, the arm on subfiber B reorientates from parallel to perpendicular to the long axis of each doublet. Each singlet bears an arm which is directed toward the arm on subfiber B, and each subfiber A possesses two small arms which are directed towards subfiber B of the adjacent doublet. A double membrane surrounds the dense bodies (b) in the center of the axial filament oval. OsO₄. X 86,000.
an area of amorphous material which extends on either side and between the tubules of the centriole. Morphologically similar material is present in association with the 9-membered centriole of *Sciara* somatic tissues (Fig. 1) and other species (3, 6, 23, 29).

**The Axial Filament Complex**

The pattern of the flagellum of *Sciara* sperm differs from the 9 + 2 pattern so far shown to be present in cilia and flagella of members of practically every plant and animal phylum (8, 11). [The few exceptions to 9 + 2 pattern involve either abnormal cilia (1, 32), mutants (26), or minor differences affecting only the central pair (33).]

Typical cilia and flagella apparently arise from centrioles (22) possessing a tubule pattern only slightly different from that of the flagellum itself (12, 27, 34). The meiotic centriole in *Sciara*, though composed of an unconventional array of about 70 tubules, is analogous to the familiar flagellar type in that it seems to play a role in organizing the axial filament of which it forms the base. It appears, in fact, as though the tubule pattern in the axial filament precisely reflects the tubule pattern of the centriole.

Robison (30) has recently described, in the sperm of an armored scale insect, a motile apparatus consisting of a spiral of singlet microtubules which extend the entire length of the cell. This type of motile system is clearly different from the modified flagellum in *Sciara*, since it probably does not arise from a centriole and bears virtually no resemblance to a typical flagellum. The distinction between a flagellum and a ring of singlet microtubules is most evident in flatworm sperm where both types of motile apparatus are present in each spermatid (33).

The presence of a singlet peripheral to each doublet and an arm on subfiber B is an unusual feature in flagella, but it is not restricted to the *Sciarridae*. Kaye (15) described an arm on subfiber B of the flagellum of early spermatids in the house cricket. This arm is oriented parallel to the long axis of the doublet as in young spermatids of *Sciara*. Later a singlet forms peripherally to each doublet just as in *Sciara*, and the arm on subfiber B apparently changes its orientation. An arm on subfiber B has also been described in the lepidopteran *Macroglossum stellatarum* (2), and a peripheral singlet and arm on subfiber B occur in the sperm of the midge *Chironomus thummi* (author, unpublished).

When the microtubules of the axial filament complex are first laid down, they have the same density as other cytoplasmic microtubules after glutaraldehyde-OsO₄ fixation, and, like other microtubules, are not demonstrable following OsO₄ fixation. They remain so during centriolar migration, but, after the centriole reaches the apolar end of the nucleus, the tubules of the axial filament complex gradually increase in density and in demonstrability after OsO₄ fixation as compared to other microtubules in the same cell. This leads us to believe that the axial filament tubules may at first bear a chemical similarity to microtubules but are later modified so that the two types of tubules react differently to fixation.

**Nuclear Morphogenesis**

The row of microtubules which lie in close association with the nuclear membrane disappears...
In cross-section spermatid nuclei have a characteristic shape which is bilaterally symmetric with respect to two axes. A mitochondrial crystallloid (cr) is seen in one nucleus. Longitudinal groove (arrows). OsO₄. × 22,000.

Many singlet microtubules occur close to the nuclear membrane. The tubules are disposed parallel to the long axis of the cell and are absent from the longitudinal grooves. Glutaraldehyde-OsO₄. × 54,000.

shortly after the rapid condensation of the nucleus of late spermatids. It is possible that the condensation is facilitated by this row of microtubules.

The spermatid nucleus of *Chironomus thummi* has a longitudinal row of microtubules closely apposed to the nuclear membrane and similarly undergoes
FIGURE 26 The first evidence of the rapid condensation of the nucleus is sloughing of nuclear membrane
at two diagonally opposite points (arrows). Whorls of membrane appear simultaneously in the two
longitudinal grooves. OsO₄. × 33,000.

FIGURE 27 Sloughed membrane is continuous with the nuclear envelope in two diagonally opposite
places (arrows). Annuli (an) present in the sloughed double membrane indicate that it is nuclear in origin.
OsO₄. × 33,000.

FIGURE 28 A dense proacrosome granule (gr) forms in a Golgi area of the anterior end of the early
spermatid nucleus (n). Glutaraldehyde-OsO₄. × 40,000.
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