SPERMATOGENESIS IN ANIMALS AS REVEALED BY ELECTRON MICROSCOPY

II. Submicroscopic Structure of Developing Spermatid Nuclei of Grasshopper

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Plates 219 to 222

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INTRODUCTION

For a long time the differentiation of the male germ cells has occupied a position of particular interest in cytology and cytogenetics. The nuclear changes taking place during spermatogenesis have been interpreted as a dehydration of the karyoplasm resulting in a reduction in volume without loss of essential genetic substance. Recently, a number of results obtained with the light microscope, polarizing microscope, and x-ray diffraction have suggested that there are remarkable changes in the properties of the chromatin in addition to its concentration. Daoust and Clermont (5) have shown noticeable changes in Feulgen reactivity between early and advanced spermatids treated with desoxyribonuclease. This finding suggests that a physicochemical reorganization takes place in the course of spermatid metamorphosis which renders the chromatin refractory to enzymatic digestion. Measurements of birefringence (18) and ultraviolet dichroism (4) have shown that the nucleic acid molecules in some sperm heads lie parallel to one another. A series of x-ray diffraction studies have further indicated parallelism of the nucleoprotein molecules in sperm heads and suggested a possible crystallinity (15, 22, 23).

More recently, Grassé *et al.* (7–10), Yasuzumi (24, 25), and Yasuzumi *et al.* (26) have demonstrated distinct parallelly arranged and more or less helical fibrils along the long axis of developing sperm heads in *Helix pomatia* L. and *Passer montanus saturatus* Stejnegar. Moreover, it has been suggested that in the fibril the nucleoprotein has the form of helical filaments approximately 10 A in diameter oriented parallel to the fibril axis (25). Rudzinska and Porter (17), and Rudzinska (16) have reported a honeycomb structure in cross-sections and parallel lines in longitudinal sections of the chromatin bodies in macronuclei of overfed organisms of *Tokophrya infusionum.*

In the present paper a peculiar structure, being similar to the fine structure

of the macronucleus chromatin of *Tokophrya infusionum* (16, 17), has been observed in cross-sections through the developing spermatid nuclei of the grasshopper, *Gelastorrhinus bicoler* de Haan, and it has been possible to follow the changes of organization in the spermatid nucleus during its differentiation.

Materials and Methods

Blocks *ca.* 1 mm. in thickness of fresh testis of grasshopper, *Gelastorrhinus bicoler* de Haan, were fixed for 1 hour at 10°C. in 1 per cent osmium tetroxide adjusted with veronal-acetate buffer to pH 7.4 (14). After fixation the specimens were directly (without washing in distilled water) dehydrated in a series of increasing concentrations of ethyl alcohol and embedded in a mixture of 20 per cent methyl methacrylate and 80 per cent *n*-butyl methacrylate (13). Sections were cut at an estimated thickness of 200 A with a Shimadzu microtome with glass knives. The sections were examined, without removing the plastic, with a Hitachi electron microscope, model HU-10. Electron micrographs were taken at calibrated magnifications of 10,000 and 20,000, and photographically enlarged as desired.

OBSERVATIONS

The nuclei of the spermatids are spherical in the early stages of metamorphosis, but later they become conical and then more and more elongated until they are long slender rods, rounded at the base and tapering at the tip to a sharp point. Concurrently with these changes in the nuclei, remarkable changes occur in the fine structure of the karyoplasm. In the early stages of maturation of the spermatid the nebenkern is divided into two parts separated by the developing tail filament. At this stage the content of the spherical nucleus is very dense, so that its internal fine structure is scarcely visible. A pair of centrioles are observed at the base of the nucleus, and a portion of the tail filament is revealed between the halves of the nebenkern (Fig. 1). The nebenkern is enclosed by a thin limiting membrane and contains a large number of round, ellipsoid, or rod-shaped elements which originate from mitochondria (Fig. 1). A slightly oblique cross-section through a more advanced, conical-shaped spermatid shows an ellipsoid nucleus, in which appear fibrillar structures in an irregular arrangement (Fig. 2).

Further information concerning the details of the fibrillar structure of the spermatid nucleus can be obtained at later developmental stages from very thin sections of well fixed material. In micrographs of elongated, slender, rod-shaped spermatid nuclei, it is possible to see a fairly high degree of order. It appears, in cross-sections, as a close packing of light areas enclosed by dense walls. Thus the structure resembles most closely a hexagonal honeycomb (Figs. 3 and 4). The light areas measure 140 to 220 A in diameter. The dimension of the light areas changes from place to place, but this may be due to slight changes in the angle of sectioning or compression during sectioning (Fig. 4). The thickness of the dense walls surrounding the honeycomb is about 70 A (Figs. 3 and 4). Thus the entire structural unit, wall, and enclosed less dense material, measure about 210 to 290 A. The number of the hexagonal light

areas appearing in cross-sections is variable, approximating 450 to 518 in each spermatid nucleus. At the resolution available, no finer structures could be defined in any of the walls of the honeycomb, so it is impossible to say whether each is double and so whether the structure represents a close packing of tiny cylinders, as suggested by Rudzinska and Porter (17).

Fig. 3 demonstrates cross-sectioned, elongated spermatids in which the round profiles of the nuclei appear surrounded by a rim of cytoplasm. The cytoplasm is enclosed by two membranes: a dense inner membrane and a less dense, thicker outer membrane. The cytoplasm is made up of a substance with low density. A discontinuous, thin membrane is visible along the plasma membrane in the homogeneously appearing cytoplasm.

In the longitudinal and oblique-longitudinal sections of elongated spermatid nuclei it is possible to see the parallelly aligned structure (Figs. 5 and 6). Such appearances provide clear evidence that both images (the honeycomb and parallel lines) belong to the same structure seen in different planes of section. The rather variable spacing between the lines 70 A thick is progressively reduced to 100 to 200 A, as the nuclei elongate and their diameter shrinks. In longitudinal sections as well as cross-sections, a discontinuous membrane *ca.* 100 A thick is visible along the plasma membrane in the homogeneously appearing cytoplasm.

The remarkably elongated nucleus of more advanced spermatids contains a material of relatively high density which appears homogeneous in both transverse and longitudinal sections (Figs. 7 and 8). At this time, the nucleus is more sharply defined (especially in longitudinal sections) than in the preceding stages, but the nuclear membrane and the internal structure are no longer visible. The discontinuous membrane noted in the cytoplasm becomes unclear at this stage.

DISCUSSION

The present procedure is characterized by a direct dehydration, without washing of materials fixed with buffered osmium tetroxide, since washing is often accompanied by a loss of cellular substances and drastic distortion. The procedure has enabled us to demonstrate the submicroscopic structure of the spermatid nucleus of a grasshopper in detail.

At the stage in which the nebenkern halves have the shape of hemispheres, the karyoplasm of the spermatid is very dense and this excessive density may interfere with the demonstration of its internal structure. Recently, Beams *et al.* (2), Tahmisian *et al.* (20), and De Robertis and Franco Raffo (6) studied the nebenkern by means of phase contrast and electron microscopy. They agree that the nebenkern is of mitochondrial origin. The present study has shown the nebenkern halves on each side of a portion of the axial filament of the tail. More detailed evidence concerning the nebenkern will be given in another

publication. The nuclei of the spermatids are spherical in the early stages of maturation but later they become ellipsoid, showing fine fibrils distributed at random within them.

In the present study the chief point of interest is the demonstration of clear cut order in the karyoplasm of the elongated spermatids. Cross-sections of the elongated nuclei in developing spermatids demonstrate a hexagonal honeycomb structure, each opening of the honeycomb being surrounded by a dense wall. Since the average thickness of the sections was less than the diameter of the hexagons, the longitudinal sections showed a parallelly arranged fibrillar structure. As far as it is known, Rudzinska and Porter (17) found for the first time such a regular honeycomb structure in bodies of chromosomal character in the macronucleus of *Tokophrya infusionum*.

Grassé *et al.* (7–10), Yasuzumi (24, 25), and Yasuzumi *et al.* (26) have presented electron micrographs of parallelly arranged and more or less helical fibrils in some animal spermatid neclei. Burgos and Fawcett (3) have demonstrated a characteristic arrangement of coarse chromatin granules in advanced toad spermatids, and have suggested that they are made up of macromolecules in crystalline array. The present study permits a clearer and better three-dimensional understanding of the nuclear structure in the developing spermatids of a grasshopper. The fine structure revealed by our work is similar to that described in a different material by Rudzinska and Porter (17) and Rudzinska (16).

Electron micrographs of sodium desoxyribonucleate have been obtained by Scott (19), Bayley (1), Liquier-Milward (12), and Kahler and Lloyd (11). Kahler and Lloyd (11) have shown that the structural element of desoxyribonucleate is a diameter of 15 $\pm$ 5 A. A model for the molecular structure of desoxyribose nucleic acid has been proposed by Watson and Crick (21); it consists of two helical chains, each coiled round the same axis 10 A in diameter. It is assumed that the structural elements disposed with a high degree of order in the spermatid nucleus of the grasshopper during certain developmental stages contain desoxyribonucleic acid. However, at the resolution available no correlation is possible between the structures described and the DNA fibrils mentioned above.

Schmidt (18) indicated a parallel arrangement of nucleoprotein molecules in certain sperm heads, suggestive of crystallinity. Caspersson (4) has also seen a strong birefringence of negative sign in certain animal sperm heads, and has attributed it to parallel orientation of polymerized thymonucleic acid chains. Rinne (15) has made x-ray diffraction studies of anisotropic sperm heads of *Sepia* and concluded that the nucleoprotein consists of a liquid crystalline arrangement of parallel, rod-shaped molecules. Wilkins and Randall (23) and Wilkins and Battaglia (22) have obtained x-ray diffraction patterns of sperm heads and concluded that each individual head consists of either a single imperfect crystal or an aggregate of parallel microcrystals. The present

electron micrographs have demonstrated the presence of parallelly arranged structural elements of macromolecular dimensions (presumably nucleoprotein macromolecules) in certain stages of development in the grasshopper spermatid nucleus.

Daoust and Clermont (5) have suggested that a physicochemical reorganization occurs in the course of spermatid differentiation which renders the chromatin refractory to enzymatic digestion. The present study has revealed certain remarkable changes in the submicroscopic structure of the nucleus during maturation: when the nucleus is spherical, it appears dense but does not allow a clear visualization of its internal structure; when it is conical, it begins to show a fibrillar structure inside; when it is elongated, it shows a honeycomb structure in cross-sections and parallel lines in longitudinal sections; when it is further elongated and reduced in volume, the spacing between the lines becomes narrower and narrower until it cannot be resolved. This sequence of events is in general agreement with the interpretations of Grassé *et al.* (7–10), who studied the spermatogenesis in *Helix pomatia*.

The discontinuous membrane seen in the cytoplasm of elongated spermatids seems to originate from certain fine granules and vesicles present in the cytoplasm of the spermatids at earlier stages of development; these structures are absent in the cytoplasm of advanced spermatids. A more exhaustive study of the various developmental stages of the spermatid nucleus in the grasshopper and in *Drosophila virilis* will be presented in another publication.

SUMMARY

The submicroscopic structure of the maturing spermatid nucleus of the grasshopper, *Gelastorrhinus bicoler* de Haan, has been studied in thin tissue sections by electron microscopy.

In the early spermatid the nucleus appears dense with no clearly resolvable fine structure. In the advanced spermatid with a conical-shaped nucleus, the karyoplasm begins to show a fibrillar structure. At subsequent stages, the elongated spermatid nucleus displays in cross-sections a hexagonal honeycomb pattern and in longitudinal sections an array of parallel lines, 70 A in diameter and spaced 100 to 220 A apart.

As differentiation of the spermatid proceeds further, the space between the lines becomes narrower and narrower until it can no longer be resolved.

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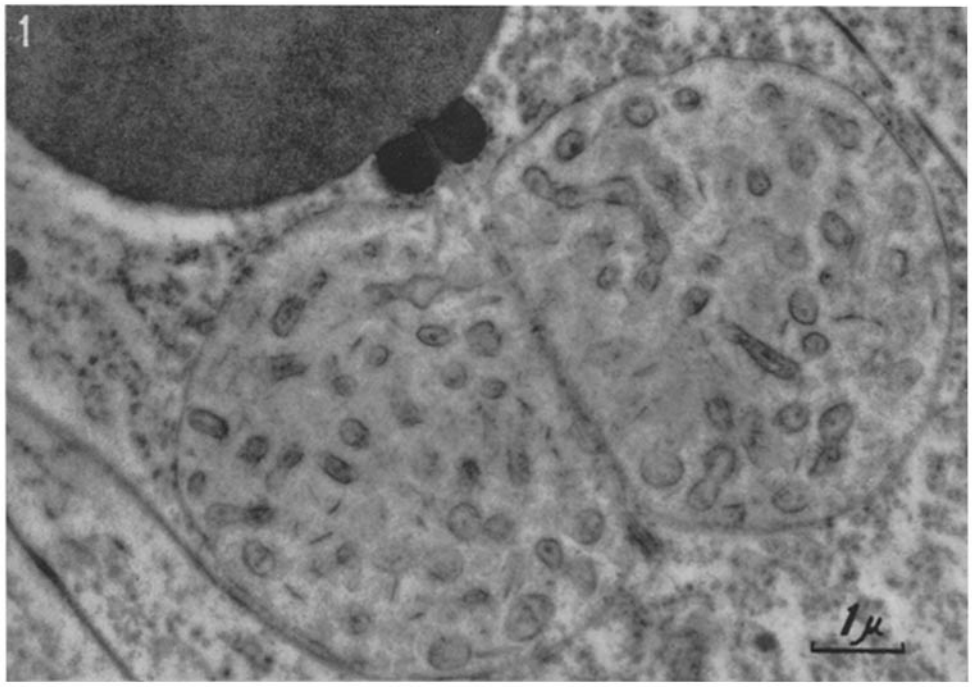
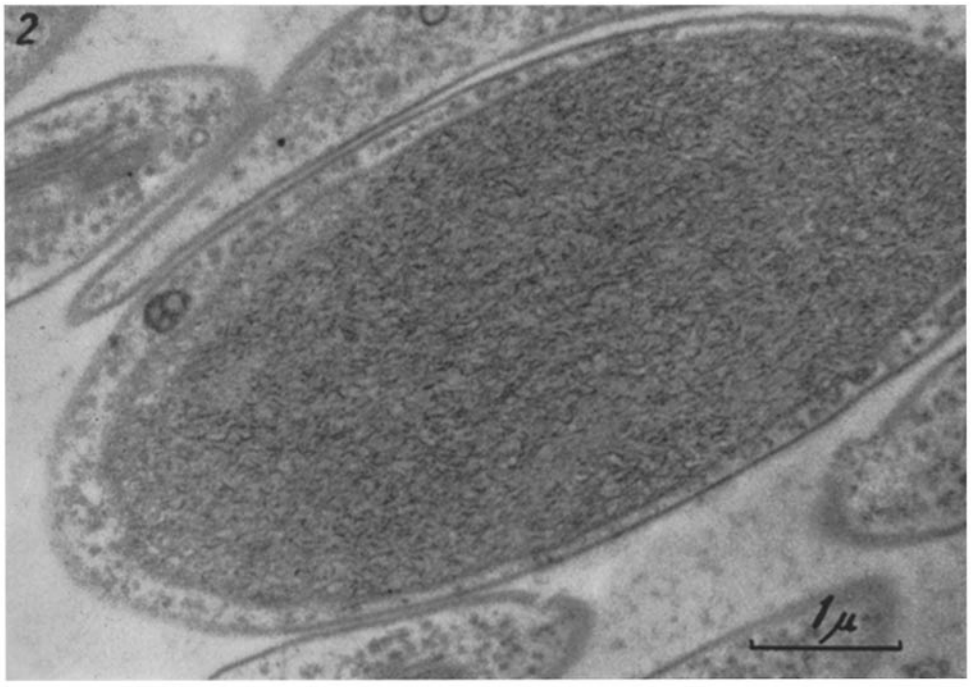
EXPLANATION OF PLATES

PLATE 219

FIG. 1. Electron micrograph of a longitudinal section through a spermatid at an early stage of development, showing a portion of a dense nucleus, a pair of highly dense centrioles situated at the base of the nucleus, and a nebenkern divided into hemisphere-shaped halves. The nebenkern is enveloped by a distinct limiting membrane and contains a large number of spherical, oval, and rod-shaped bodies of mitochondrial origin. Some of them show an internal dense region, surrounded by a pale zone which is covered up by a well defined membrane. The cytoplasm shows a granular appearance, which in part may be an artifact resulting from fixation and dehydration. Magnification, 12,000.

FIG. 2. Electron micrograph of slightly oblique cross-sections through a more advanced, conical-shaped spermatid, showing an ellipsoid nucleus surrounded by a ring of cytoplasm in the center of the figure and at least six elongated spermatids at the level of developing sperm tail flagellum in the peripheral part of the figure. The ellipsoid nucleus shows a fibrillar structure of irregular arrangement. Numerous fine granules of various shapes and fine vesicles are in the cytoplasm. A dense body with light spots is found in the ring of cytoplasm, suggesting a chromidial body. Magnification, 20,000.





(Yasuzumi and Ishida: Spermatogenesis in animals. II)

PLATE 220

FIG. 3. Electron micrograph of cross-sections of elongated spermatids, showing the peculiar appearance of the nuclei. The cells have become greatly elongated and simultaneously their cross-sectional diameter has considerably diminished. Each of the spermatids appears to be surrounded by a double membrane, an outer membrane of low density, and an inner, thinner, and denser membrane. A discontinuous thin membrane (*DM*) is visible along the plasma membrane in the agranular cytoplasm. The round nuclei are enclosed by a dense nuclear membrane and their content shows a honeycomb structure. Magnification, 50,000.

FIG. 4. High resolution electron micrograph of a cross-section of an elongated spermatid, showing at a higher magnification the light areas surrounded by the dense walls which characterize the honeycomb structure of the karyoplasm. The openings of the honeycomb are quite regular in size and disposition; larger openings are marked by the arrows. The nuclear membrane is well defined and the double cell membrane is clearly visible. Magnification, 110,000.

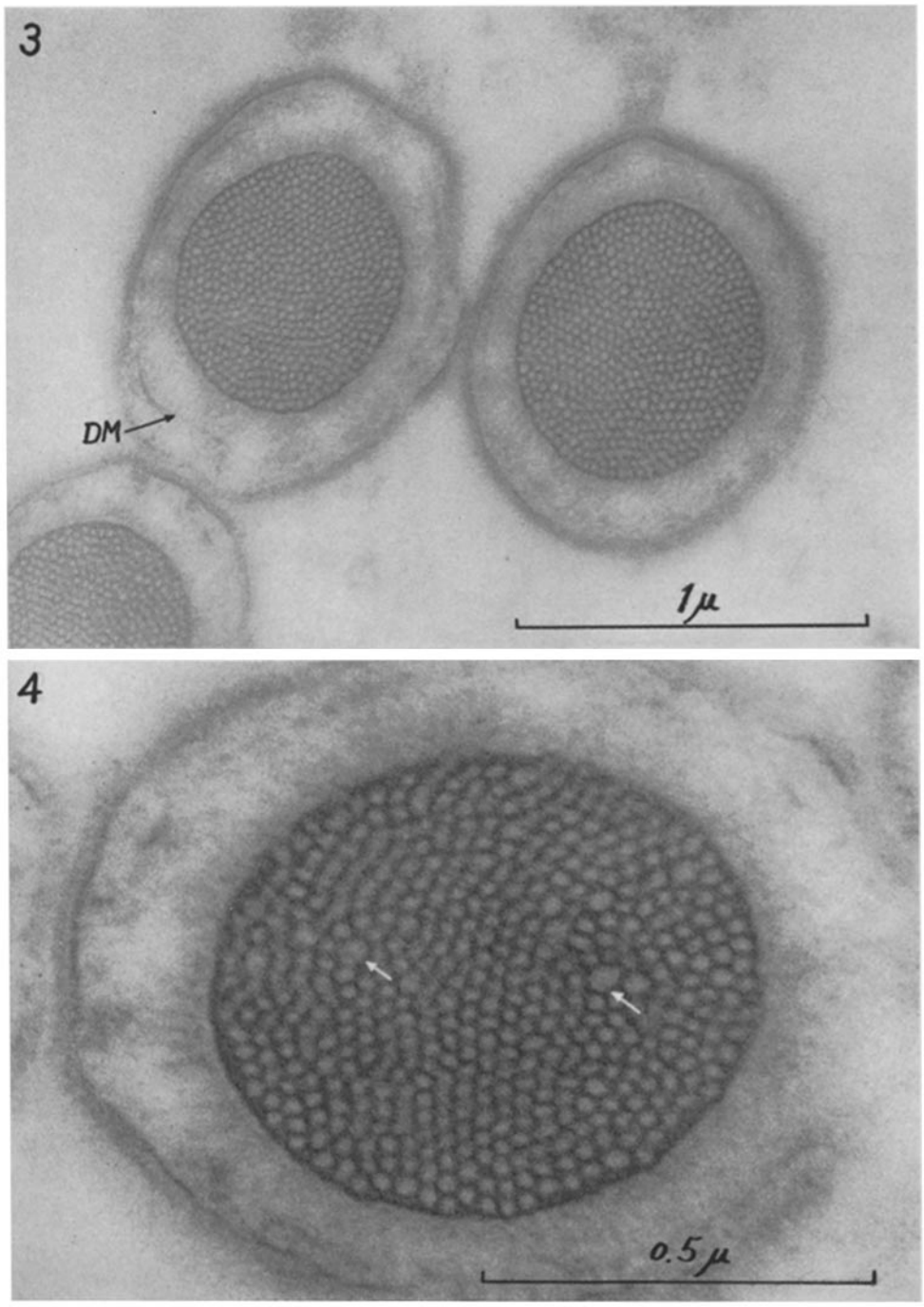


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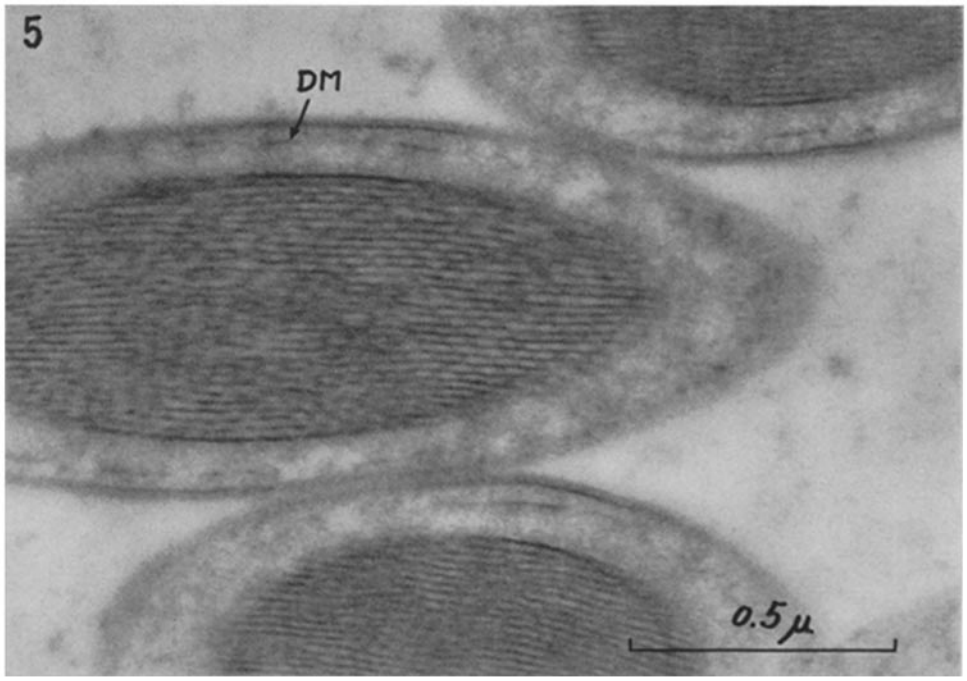
PLATE 221

FIG. 5. Oblique sections through elongated spermatids, showing a considerable number of parallely arranged lines in the nucleoplasm. The spaces in between the lines appear to contain a material of a higher density than the background. A discontinuous membrane (*DM*) is also visible in the cytoplasm along the plasma membrane. Magnification, 70,000.

FIG. 6. A segment of an elongated, rod-shaped spermatid nucleus in a longitudinal section, showing the parallel lines running along the longitudinal axis of the elongated nucleus. In this section a discontinous thin membrane (*DM*) is visible along the plasma membrane in the homogeneously appearing cytoplasm. Magnification, 52,000.



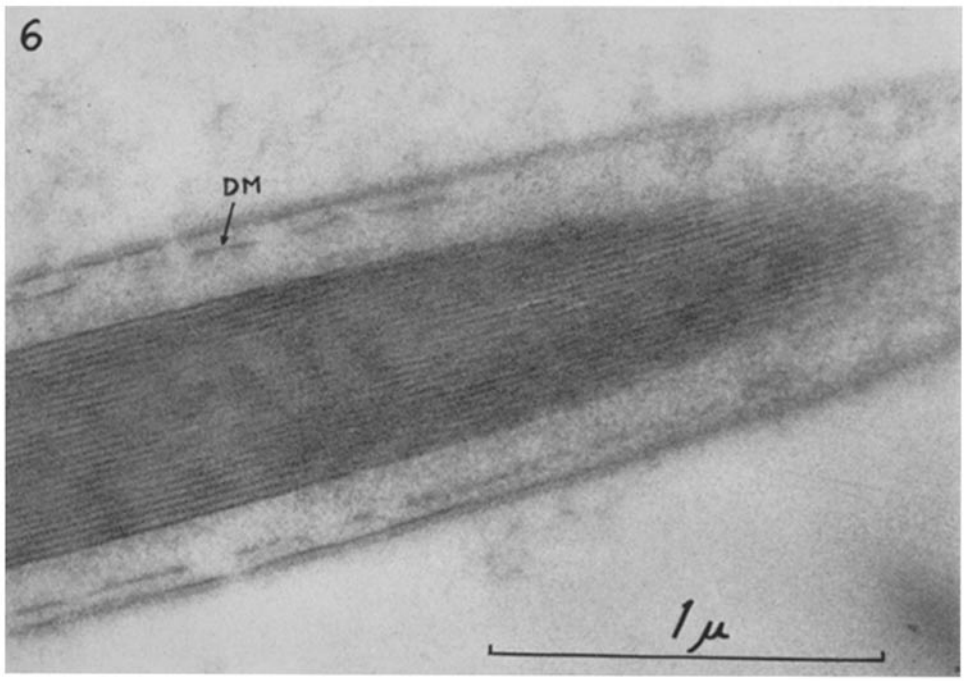


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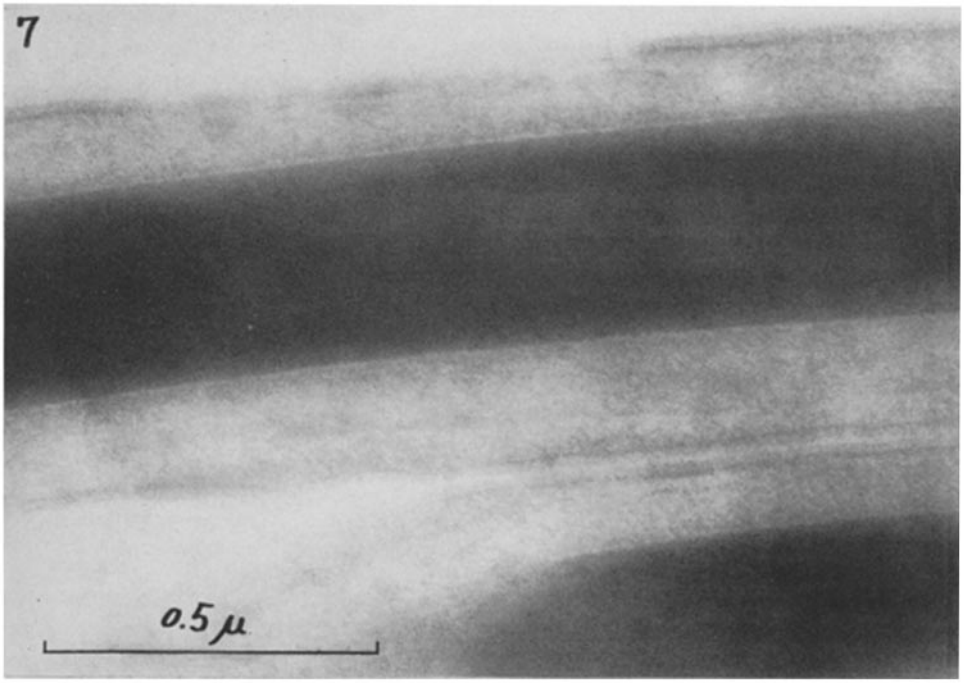
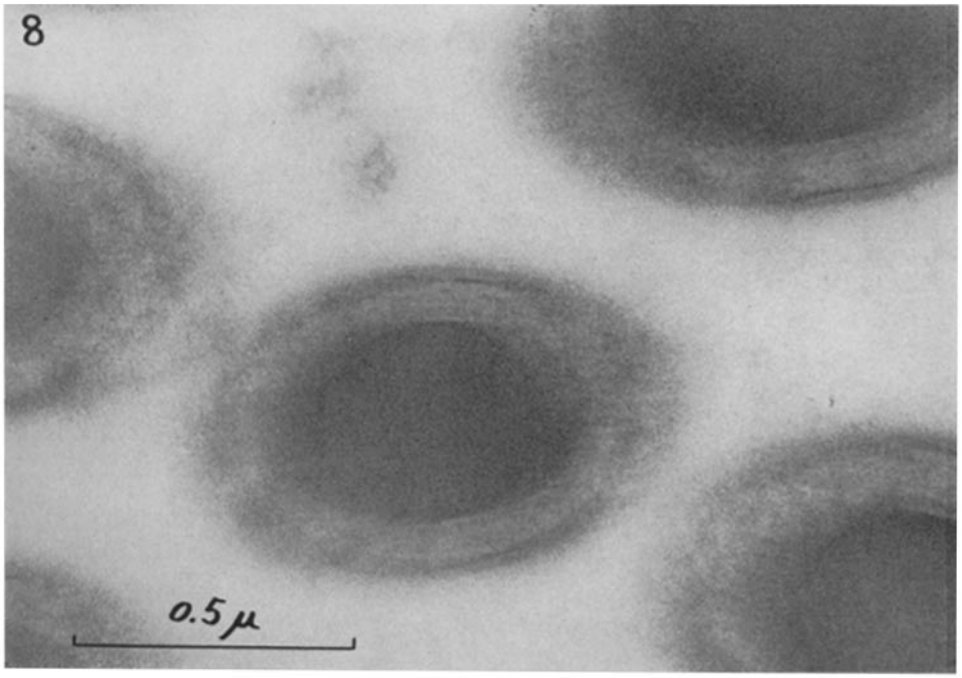
PLATE 222

FIG. 7. Longitudinal sections through long, slender, rod-shaped spermatids, showing the dense homogeneous appearance of the nuclei and the less dense homogeneous cytoplasm surrounded by a double membrane. The discontinuous membrane is difficult to identify. The nucleus is sharply defined and the nuclear membrane is no longer visible. Magnification, 90,000.

FIG. 8. Cross-sections through five long rod-shaped spermatids, showing the dense homogeneous appearance of the nuclei. They demonstrate the disappearance of the honeycomb structure of the nucleoplasm. The discontinuous membrane is scarcely visible. Magnification, 74,000.





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