Spermatogenesis in Animals as Revealed by Electron Microscopy

VII. Spermatid Differentiation in the Crab, *Eriocheir japonicus*

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PLATES 22 TO 26

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ABSTRACT

Testes of *Eriocheir japonicus* were fixed in buffered (pH 7.2) 1 per cent osmium tetroxide and thin sections of the methacrylate-embedded tissue were studied with the electron microscope. Spermatozoa from the vasa deferentia and spermatids from the testes were examined in smear preparations and sections. The most useful stainings proved to be the Feulgen, Unna-Pappenheim, and PAS reactions.

The present paper covers one of the late stages of spermatogenesis. At the late stage of differentiation of the spermatid, the nucleus shows a concave disc-like contour in longitudinal sections and the karyoplasm is finely alveolar in appearance. A vesicle with a fibrillar or granular content appears situated between the cytoplasm and the nucleus. In an advanced spermatid, a large vacuole develops between the nucleus and this vesicle. As development of the spermatid proceeds, the cytoplasm is sloughed off. Thus, the advanced spermatid consists of an ovoid vesicle surrounded by a flattened nuclear disc. The former corresponds to the "capsule" or the "head," and the latter to the "pseudopodia." Concurrently with the sloughing off of the cytoplasm, a lamellar structure appears at the periphery of the head. It is composed of thin plates imbricated like the leaves of an onion. Each plate consists of triple layers, two dense layers separated by a lighter space. Each layer measures about 7 mμ in diameter.

At the distal pole of the head, a depression develops into a tubule which reaches the proximal pole of this structure. The lumen of the tubule is occupied by a dense substance part of which arises from the limiting membrane of the vacuole. The dense material begins to fill the tubule from the distal towards the proximal end. It finds its way till the middle of the tubule, leaving its proximal half unfiled. Thus, the proximal portion of the mature sperm head has a straight tubule whlich opens on its surface. The latter is covered by a dense membrane about 60 mμ in width.

Cytochemical analysis reveals that the pseudopodia contain DNA, and the head a carbohydrate component as well as PNA. The classical concept that the head becomes the male pronucleus after fertilization can no longer be accepted on the basis of the cytochemical analysis. The present study came to no definite conclusions concerning the functional significance of the head.

INTRODUCTION

Spermatogenesis in decapods has been extensively studied (1–18) mainly because the spermatozoa of this group of Crustacea are so different in shape from those in other animals. They belong to the type commonly known as radial spermatozoon. Light microscope observations are summarized here to provide a background for the description of the electron microscope findings.

In the cytoplasm of the spermatid, a vesicle makes its appearance opposite the nucleus, increases in size, and soon presses against the latter, which then becomes smaller and uniform in density. The shape assumed by the vesicle varies with

the species; in some it is spherical, in others cup-like, cylindrical, or irregular. The vesicle is usually called the "capsule" or the "head," "*Schwanzkapsel*" or "*Kapselkörper*" in the German literature. The origin of the decapod sperm head has been variously interpreted. Some have thought the head develops directly from the nucleus (2, 6, 8, 10–12, 14, 18), while others have considered it a vesicle derived from the nucleus of the spermatid (1, 3, 9). Nath (16) has come to the conclusion that in *Paratelphusa spinigera* the head arises from the fusion of vesicular mitochondria. In *Palaemon lamarrei*, he believes the vesicle to be produced by the general liquefaction of the cytoplasm including the Golgi material and the mitochondria. In *Potamobius astacus*, Grabowska (15) has described a possible relationship between the Golgi material and the vesicle. McCroan (18) has concluded that the vesicle is very probably of mitochondrial origin.

The flagellum-like arms or pseudopodia of the spermatozoa are assumed to arise from a mitochondrial mass, or the nucleus, or the cytoplasm proper by various authors (2, 5, 6, 9, 18). A central body, appearing in the center of the vesicle supposedly arises from the centrosome (1, 6, 7, 15).

It has been assumed (9) on the one hand, that the nuclear material is transferred by diffusion from the nucleus to the vesicle before maturation. When the spermatozoon comes in contact with the egg, the vesicle is usually applied to the shell of the egg and sinks into the ooplasm, where it increases in size and transforms into the male pronucleus, which gives rise to the chromosomes. On the other hand, it has been considered that the vesicle of the radial sperm corresponds to the acrosome of the typical flagellate sperm (13).

Although decapod spermatogenesis has been studied by classical methods as mentioned, many details of the complex differentiation process involved have remained obscure because of the limited resolving power of the light microscope and of the insufficient cytochemical analysis. Recently Moses (19) and Ruthmann (20) have sectioned crayfish testes, and obtained noteworthy electron micrographs of the primary spermatocyte chromosomes as well as the transformation of the nuclear envelope of the spermatid during spermatogenesis (19), and of the basophilic lamellar system in the spermatocyte (20). Spermatozoa and spermatids in late stages of differentiation have not previously been studied by electron microscopy. The present paper is restricted to the electron microscopy and cytochemical analysis of the spermatids of *Eriocheir japonicus* in their late stages of development. The findings which have already been reported in brief (21) are given here in detail.

Materials and Methods

Testes of *Eriocheir japonicus* were fixed for 1 to 2 hours in 1 per cent osmium tetroxide buffered to pH 7.2 with the Michaelis veronal-acetate buffer (22). Without washing in distilled water (23), the specimens were dehydrated in a series of increasing concentrations of ethyl alcohol, impregnated with a mixture of 20 per cent methyl methacrylate and 80 per cent *n*-butyl methacrylate, and finally embedded in the same resin polymerized by 2,6-dichlorobenzoyl peroxide at 46°C. Sections were cut on a Shimadzu microtome with glass knives. They were mounted on collodion-coated copper grids and examined, without removal of the embedding plastic, either in an Akashi electron microscope, model TRS-50, or in a Siemens Elmiskop I.

For light microscopy, spermatozoa from the vasa deferentia were examined in smear preparations which were fixed with methanol or sublimate alcohol. Light microscope observations on the cytology of spermatids were carried out on paraffin or plastic sections, 1 to 5 μ in thickness. The testes were fixed in methanol, 1 per cent osmium tetroxide (pH 7.2), or 10 per cent formaldehyde. Both smear preparations and sections were stained by the Feulgen nuclear technique, methyl green–pyronine or PAS reagent before and after treatment with ribonuclease or trichloroacetic acid. Standard cytological methods were also used to demonstrate general cell structure.

RESULTS

At the late stage of differentiation of the spermatid, the nucleus shows a concave thick disc-like contour in longitudinal section, and the karyoplasm is finely alveolar. The vesicle which contains finely fibrillar or granular masses appears between the cytoplasm and the nucleus. At this stage a dense membrane irregular in outline makes its appearance between the vesicle and the deformed nucleus, and apparently adheres to the nuclear membrane on one side, and to the wall of the vesicle on the other (Fig. 1). Numerous vacuoles of irregular shape and different sizes appear between the dense membrane and the deformed nucleus and finally coalesce to form a single sizable vacuole which, in life, is probably occupied by a fluid. The concave disc-like contour of the nucleus transforms gradually in an arch-like profile bent around the growing vacuole, as if the fluid contents of the latter were under sufficient tension to deform the underlying nucleus (Figs. 1 and 2).

With further expansion of the vacuole and exten-

sion of its area of adherence to the nuclear membrane, the deformation of the nucleus progresses until it is flattened into a concave, thin disc. At this stage a major change in the distribution of the cytoplasm takes place, resulting in the displacement of the cytoplasmic body to the opposite side of the nucleus. A pair of dense projections appears upon the vesicle and separates it from the cytoplasm. As development of the spermatid proceeds, the cytoplasm is sloughed off. Thus the advanced spermatid consists of an ovoid vesicle surrounded by the irregularly shaped vacuole and a flattened nuclear disc. Concurrently with the sloughing off of the cytoplasm, a lamellar structure appears at the periphery of the vesicle. The vesicular content becomes more and more dense, as the vesicle develops into the head of the mature sperm, while the flattened nuclear disc gives rise gradually to the pseudopodia (Figs. 2 and 3).

The dense membrane develops to surround the whole vacuole. At the distal pole of the head, a depression develops into a tubule which finally reaches the proximal pole of this structure. The lumen of the tubule is occupied by a dense substance part of which apparently arises from the dense membrane which covers the vacuole (Figs. 3 and 4). The dense material begins to fill the tubule from the distal end upwards. It finds its way to the middle of the tubule, leaving its upper half unfilled (Fig. 5). It must be noted that in the peripheral portion of the head there is evidence of lamellar structure parallel to the envelope of the head, and comprising about eight layers 19 to 27 mμ thick, separated by lighter spaces 16 to 24 mμ in width (Figs. 4 and 5).

In favorably oriented sections, it can be seen that in the apical part of the head the tubule is surrounded by a dense wall 150 to 260 mμ thick, and the proximal pole of the head is covered by a remarkably dense band 60 mμ thick (Fig. 6).

Fig. 7 shows a slightly oblique longitudinal section of the tubule judged to be in a stage similar to that of Fig. 6. It will be noticed that the tubular wall contains fibrous elements which are arranged parallel to its major axis. The lamellar structure at the periphery of the head is more clearly visualized than in earlier stages.

Fig. 8 shows a longitudinal section including the tubule within the mature sperm head. It shows a lamellar structure consisting of four to twelve layers which are arranged in parallel along the envelope of the head. Each layer measures about 21 mμ in diameter and is separated from the next layer by a light space about 21 mμ in diameter. Arrows point to places where the triple-layered structure of these lamellae can be seen with clarity. It is seen to consist of two dense layers about 7 mμ thick separated by a space with similar dimensions. The layers bend back to form a U-shaped or V-shaped profile at each terminal. Some of the innermost layers do not show complete lines, but become vague and intermix with the internal fibrillar components. The proximal portion is a straight tubule which opens on the head surface. The latter is covered by a dense band about 60 mμ thick. The content of the tubule is inhomogeneous and shows ill defined structures which are surrounded by a dense wall 0.35 μ in width at the widest (Fig. 8 and Text-fig. 1).

TEXT-FIG. 1. Three dimensional drawing of the mature spermatozoon from the testis of a crab. The spherical head is enveloped by the irregularly shaped vacuoles and the pseudopodia, but its proximal part is covered by a dense band. The tubule situated at the upper half of the head opens at its proximal pole. The tubule is enveloped by a thick, dense wall. A lamellar structure appears at the periphery of the head, and consists of thin plates imbricated like the leaves of an onion.

Fig. 9 shows a slightly oblique cross-section through the head at the level of the lower part of the tubule, which is judged to be in a stage similar to that of Fig. 8. A highly dense material surrounding the right side of the tubule is assumed to correspond to part of its dense wall. A lamellar structure consisting of four to eight layers appears at the periphery of the round profile of the head, not in its inner region which shows a finely alveolar structure. Irregularly shaped vacuoles can be seen between the head and the pseudopodia. In some places the limiting membranes at both outer and

inner surfaces of the pseudopodia are well defined but in others they are ill defined. The pseudopodia have a zigzag outline and contain inhomogeneous masses.

After appropriate staining the head and the pseudopodia appeared intensively basophilic in the light microscope. It is particularly interesting that the head gave a Feulgen-negative reaction but the pseudopodia a Feulgen-positive reaction. The head, except the tubule, was stained in red by the Unna-Pappenheim technique but failed to stain so when the preparation was pretreated with ribonuclease or trichloroacetic acid. Subsequent experiments demonstrated that the head was stained homogeneously in red by the Schiff reagent for PAS reaction, but the pseudopodia and the tubule failed to stain. The PAS reaction for the head remained positive, of course, after pretreatment with ribonuclease or trichloroacetic acid.

DISCUSSION

Although a full evaluation of the functional significance of the structures described in this paper must await further biological analysis, some of the relations they suggest deserve consideration. The primary point of modern interest in the decapod sperm is the contents of its head. Hermann (3) suggested that when one follows in parallel the differentiation process of the head and of the nucleus, one gets the impression that there is a sort of migration of the chromatic substance from the nucleus to the head. Brandes (4) found that there are two substances, one basophilic and the other acidophilic, in the nucleus of the spermatid, and that the latter settles to one side of the nucleus and then passes out into the cytoplasm. Binford (9) noted a decrease in size of the nucleus and in its affinity for chromatic dyes; the head, on the other hand, showed an increasing affinity for iron-hematoxylin and safranine. The facts mentioned above suggested a transfer of nuclear material to the head. According to Binford (9) the contents of the head are probably oxychromatin which concentrates after fertilization and gives rise to the chromosomes of the male pronucleus. The present study has revealed that the head contains a carbohydrate component as well as PNA. Thus the assumption that the head becomes the male pronucleus after fertilization and the pseudopodia remains on the outside of the egg (9) can no longer be accepted and should be reinvestigated.

Another point of interest has been the question of the validity of Bowen's suggestion that the head represents the acrosome. The acrosome is known to contain a carbohydrate component (24). The present study has revealed a carbohydrate component in the head. Hence it may be identified as an acrosomal material. But the pyronine reaction for PNA has shown the head to be positive. One body appearing in the late spermatid in the crayfish, *Cambaroides japonicus*, which seems to correspond to the head of *Eriocheir japonicus* in terms of size, form, and site, contains only a carbohydrate component and possesses no PNA (25). This fact means that the chemical constituents of certain cellular elements in different species are not always the same although the structures are apparently similar. Therefore, in the light of these findings, it would appear that the sperm head of *Eriocheir japonicus* can be considered neither a nucleus nor an acrosome completely homologous with the corresponding component of a typical flagellate sperm.

Au unusual structure has been demonstrated in section through the sperm head of the crab. The contents of the sperm head consist of tightly coiled fibrous elements of moderate density in the central portion and lamellae oriented parallel to the envelope at the periphery. The lamellae consist of four to twelve layers about 21 mμ in diameter separated from one another by a lighter space about 21 mμ in diameter. Some of the innermost layers do not show complete circular lines, but are vaguely confluent, apparently intermixing with the fibrous elements. It is characteristic that every layer appears to recur to form a U-shaped or V-shaped profile at each terminal in longitudinal sections.

The lines appearing in the electron micrographs of sections through the sperm head of the crab are considered to be profiles of thin plates imbricated like the leaves of an onion (Text-fig. 1). The present author (21) has previously reported that each linear profile is composed of triple layers; two dense lines separated by a lighter space, measuring about 4 mμ thick on an average in each layer. The present electron micrographs have shown the same structure as that reported previously, but each layer measures about 7 mμ in width. It seems plausible to ascribe the present differences to the variables of the developing stage of the spermatid.

The differentiation of the pseudopodia of the decapod spermatozoon was described by many light microscopists and considered by them to be an outgrowth of the cytoplasm or mitochondria (2, 6). Binford (9) confessed to be unable to determine whether the pseudopodia arise from the mitochon-

drial substance or from the nucleus. Andrews (5) and McCroan (18) asserted that an examination of the stages of spermatogenesis in the crayfish leads to the provisional acceptance of the view that the pseudopodia of the sperm are made from the nucleus of the spermatid. The present cytochemical analysis on *Eriocheir japonicus* has shown that the pseudopodia arise neither from the cytoplasm, nor the mitochondria, but from the nucleus. Electron microscopic observations on differentiation of the pseudopodia will be reported in another publication.

Koltzoff (7) and Binford (9) have observed one or two deeply staining granules on the border line between the nucleus and the vesicle. Koltzoff (7) in his researches on the spermatogenesis in *Galathea squamifera*, has identified these granules with the centrosome. In the present study it has not been possible to distinguish any granule which can with any certainty be identified as the centrosome. Binford (9) has called the structure developed from the granule, the "central body" that may correspond to the dense mass derived from the dense membrane covering the lumen of the vacuole.

A dense band of uncertain origin appears on the proximal pole of the sperm head and separates it from the cytoplasm. So far we have not found in the light microscope literature observations concerning the presence of such a band and the process of the sloughing off of the cytoplasm. A homogeneously dense character of the band suggests that it is composed of lipoprotein.

Most electron micrographs for the present study were made with a Siemens electron microscope, model Elmiskop I, at the Anatomical Institute of Kiel University, Germany. The author is deeply grateful to Prof. Dr. W. Bargmann and Dr. A. Knoop for the privilege of using this instrument and other equipment in the Institute.

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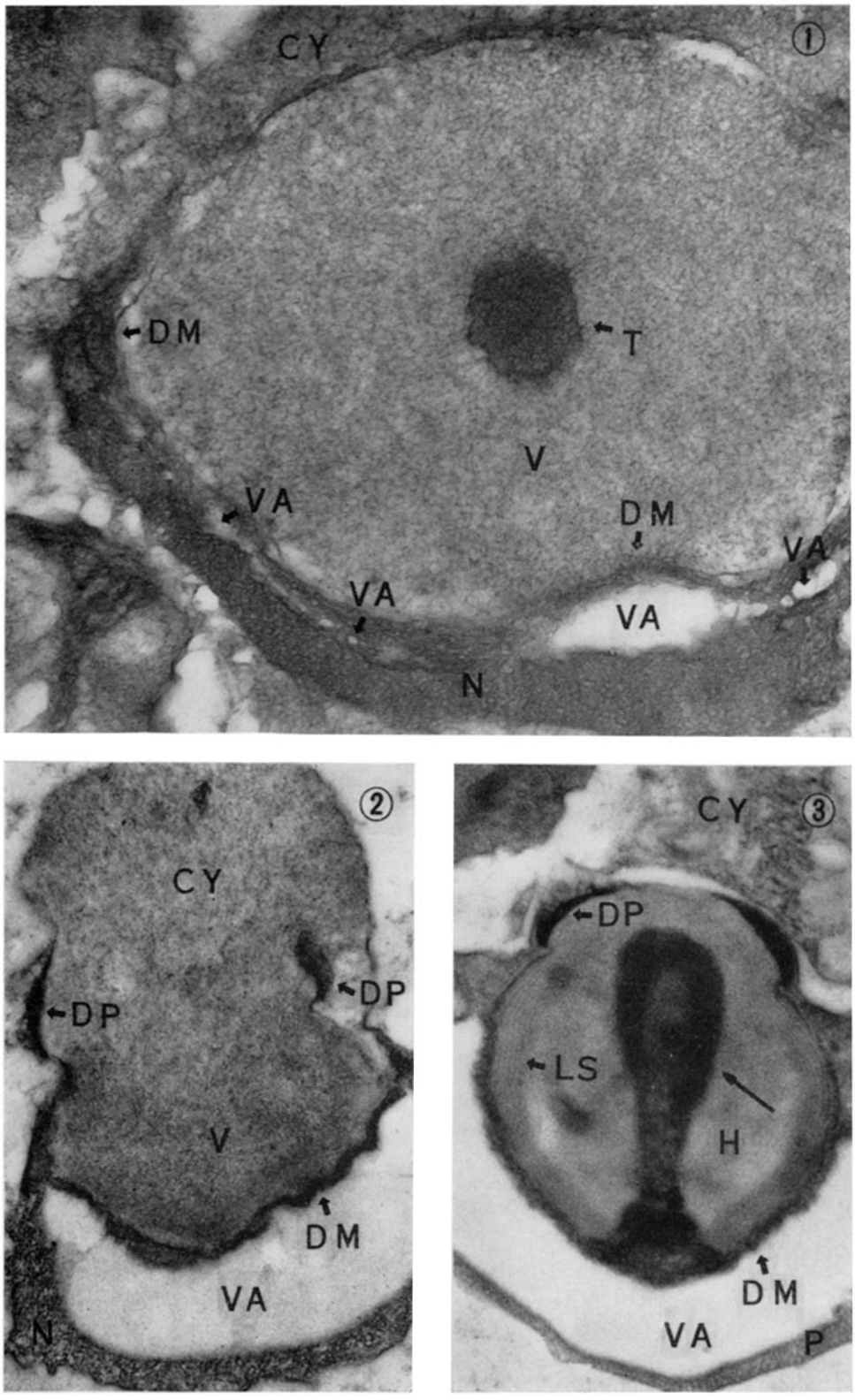
EXPLANATION OF PLATES

PLATE 22

FIG. 1. A slightly oblique longitudinal section through a developing spermatid at the late stage, showing the vesicle (*V*), the deformed nucleus (*N*), and part of the cytoplasm (*CY*). The distal two-thirds of the vesicle (*V*) are enveloped by the dense, concave disc-shaped nucleus (*N*), while the proximal third is covered by the cytoplasm (*CY*). The round profile (*T*) of a dense body can be seen in the vesicle (*V*). A thin, dense membrane (*DM*) is visible along the distal edge of the vesicle (*V*). Vacuoles (*VA*) appear between the dense membrane (*DM*) and the deformed nucleus (*N*). × 25,000.

FIG. 2. A longitudinal section through a spermatid in a late stage of development, showing part of the deformed nucleus (*N*), vesicle (*V*), enlarged vacuole (*VA*), cytoplasm (*CY*), and a pair of dense projections (*DP*) appearing at the border between the cytoplasm (*CY*) and the vesicle (*V*). The dense membrane (*DM*) covers the distal part of the vesicle (*V*). × 16,000.

FIG. 3. As differentiation proceeds, the cytoplasm (*CY*) is isolated from the vesicle by a pair of dense projections (*DP*). The spherical vesicle isolated from the cytoplasm (*CY*) is termed the "head" here. As the vacuole (*VA*) increases in volume, the nucleus decreases gradually in its thickness, and forms the "pseudopodia" (*P*). A dense body (arrow) shaped like a cudgel is continuous with the dense membrane (*DM*). A lamellar structure (*LS*) composed of several layers appears at the periphery of the head (*H*). × 16,000.

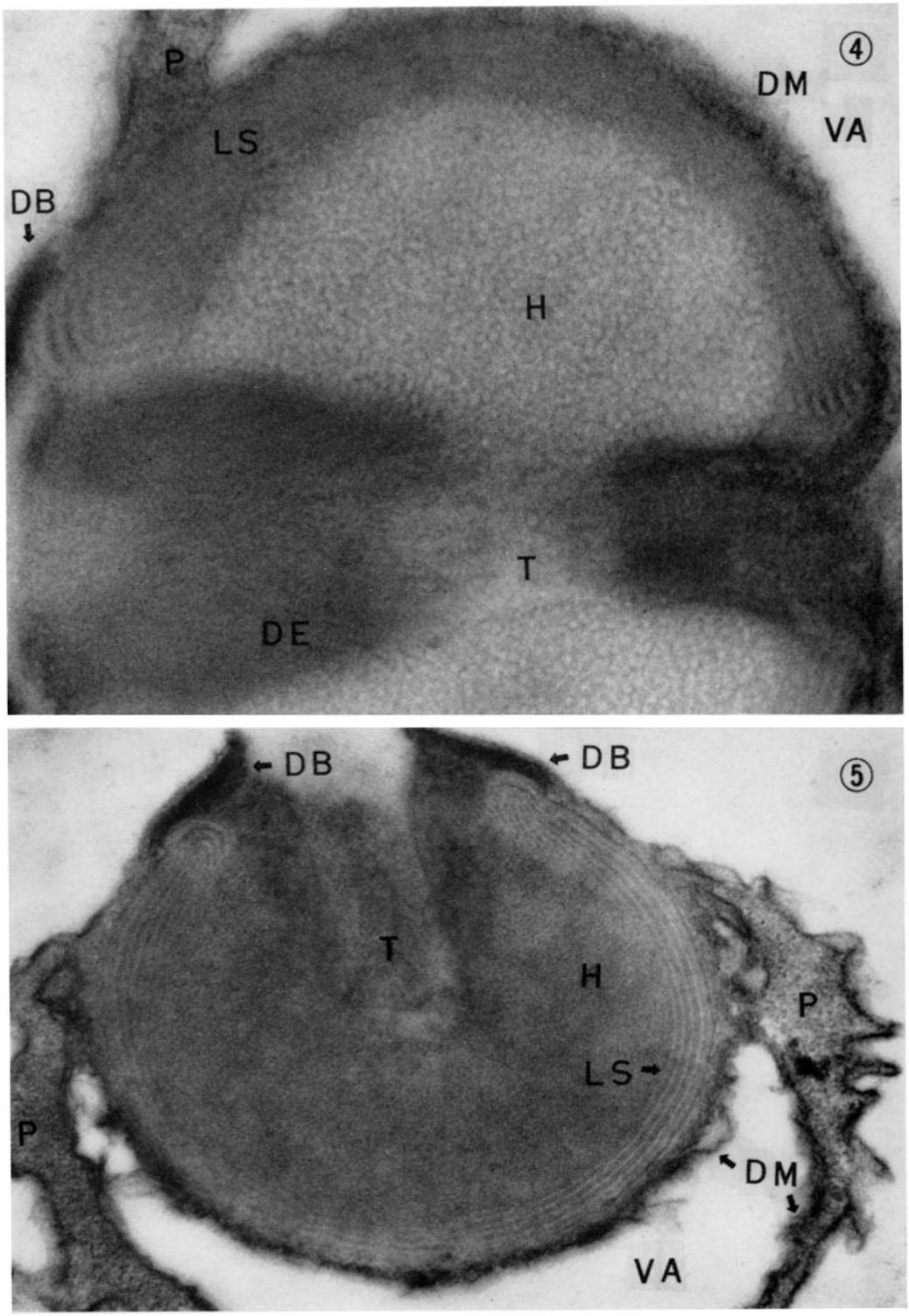


(Yasuzumi: Spermatid differentiation in crab)

PLATE 23

FIG. 4. A longitudinal section of a spermatid in an almost mature stage, showing a tubule (*T*) running from the proximal to the distal pole of the head (*H*), and which is occupied by dense fibrous elements derived from the dense fibrous membrane (*DM*) covering the surface of the vacuole (*VA*). The head (*H*) shows a lamellar structure (*LS*) at the periphery and a finely alveolar structure in the inner region. Part of the dense band (*DB*) and of the pseudopodia (*P*) can be seen at the proximal part of the head (*H*). × 63,000.

FIG. 5. A longitudinal section through a mature spermatozoon, showing a tubule (*T*) opened at the proximal pole of the head (*H*) and ended at its middle portion. A pair of dense bands (*DB*) can be seen at the proximal part of the head (*H*). A lamellar structure (*LS*) is visualized at the periphery of the head (*H*). The dense fibrous membrane (*DM*) develops to cover most of the surface of the vacuole (*VA*). The pseudopodia (*P*) have a zigzag outline. ×37,000.

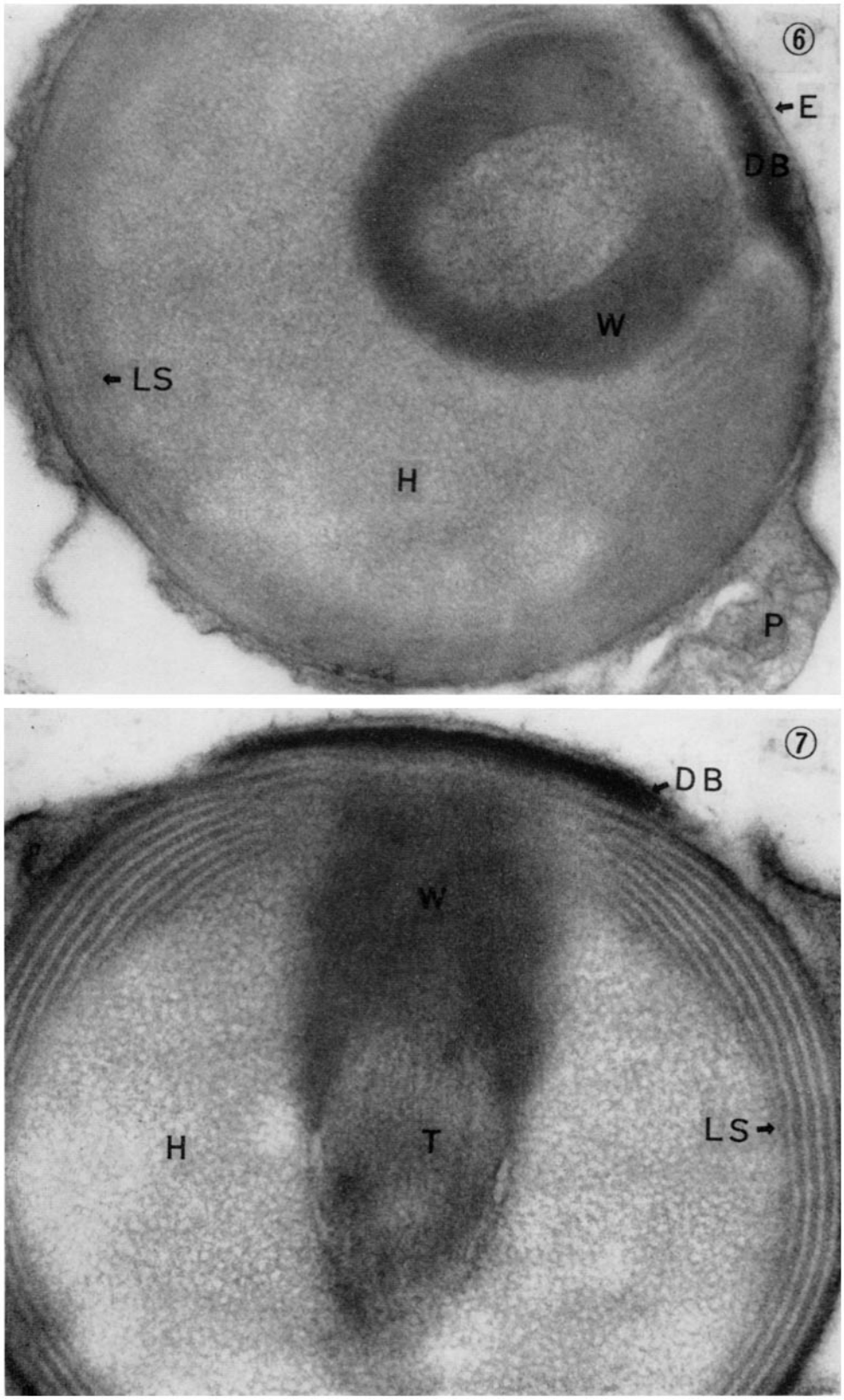


(Yasuzumi: Spermatid differentiation in crab)

PLATE 24

FIG. 6. An oblique cross-section through the tubule (*T*) of a mature sperm head (*H*), showing the dense band (*DB*) at the apical part of the sperm head (*H*), the less dense wall (*W*) surrounding the tubule (*T*) with contents of intermediate density, a lamellar structure (*LS*) at the periphery of the head (*H*), an envelope (*E*) of the head (*H*), and part of the pseudopodia (*P*). × 53,000.

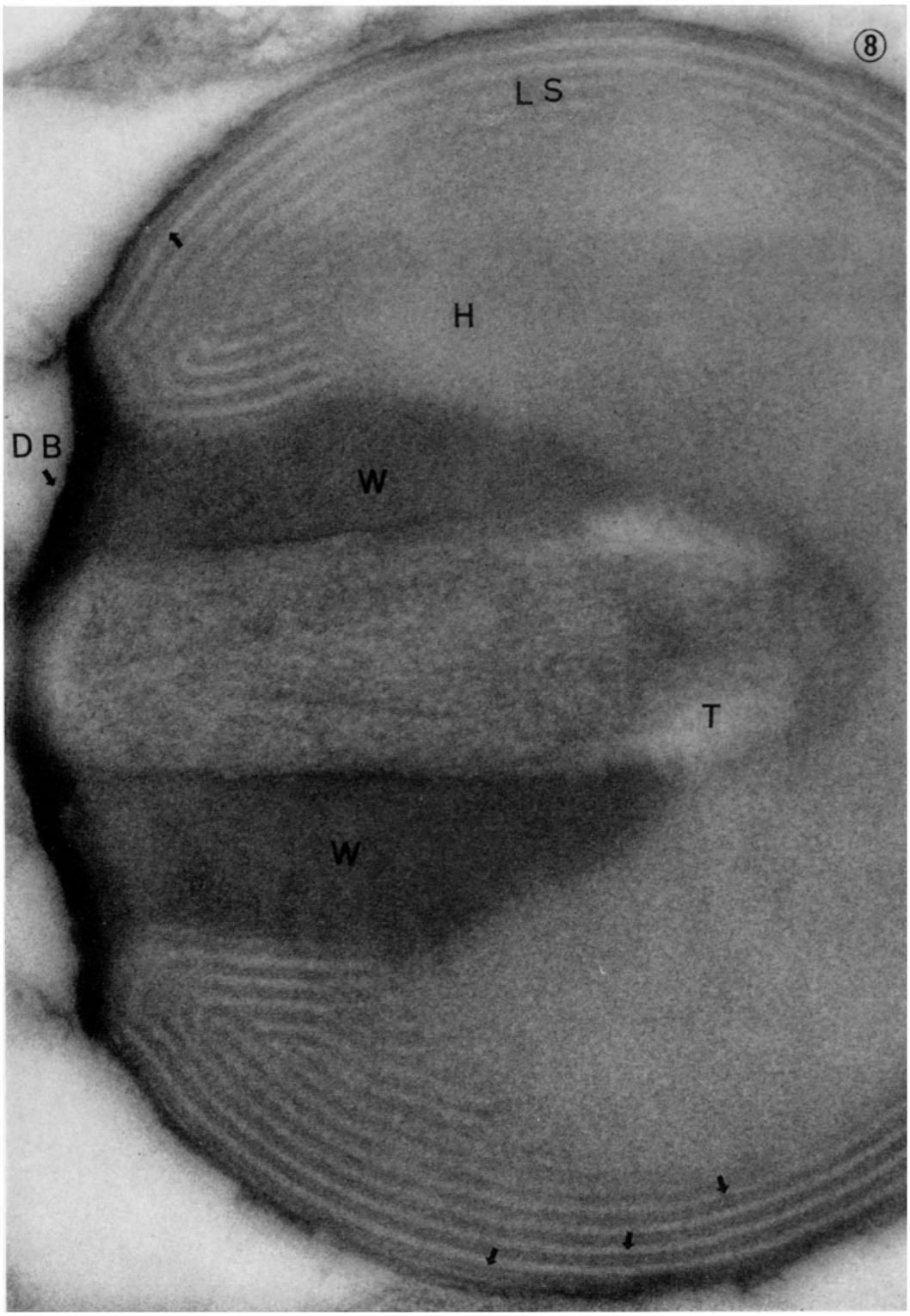
FIG. 7. An oblique longitudinal section through the tubule (*T*) of a mature sperm head (*H*) which is covered by a dense band (*DB*) at the apical part. The dense fibrous elements (*W*) appear to be part of the wall surrounding the tubule (*T*). A lamellar structure (*LS*) is clearly visualized at the periphery of the head (*H*), which shows a finely alveolar structure in its inner region. × 62,000.



(Yasuzumi: Spermatid differentiation in crab)

PLATE 25

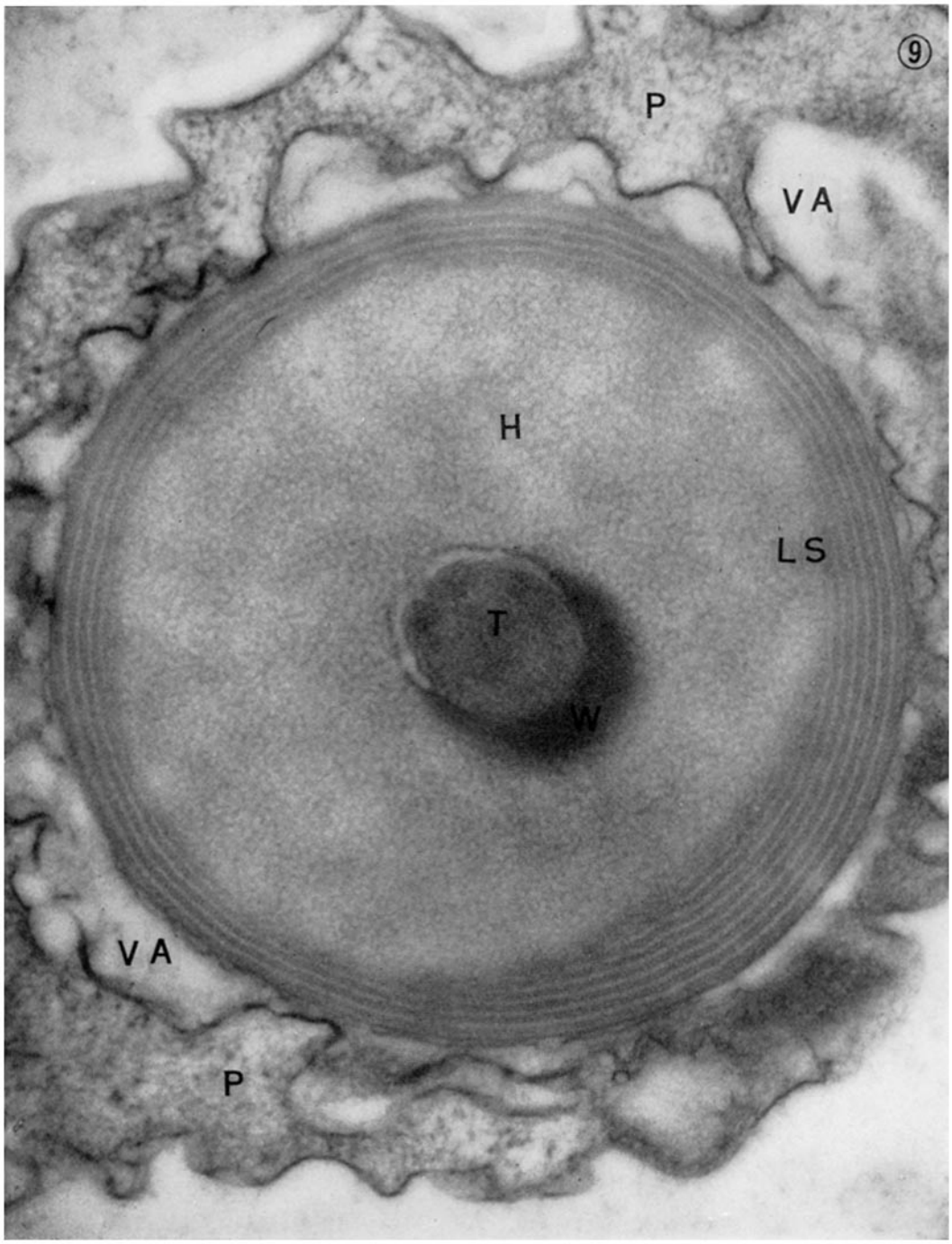
FIG. 8. A longitudinal section through the head (*H*) of a mature spermatozoon which is enveloped by a dense limiting membrane about 30 mμ thick and covered by a denser band (*DB*) at the proximal pole. Within the head (*H*) can be seen the tubule (*T*) with its inhomogeneous contents surrounded by a homogeneous dense mass, and at the periphery a lamellar structure (*LS*) consisting of several layers. Each layer appears to bend back at each terminal. Each layer is composed of three strata which appear as two dense lines separated by a lighter space at the points marked by arrows. × 72,000.



(Yasuzumi: Spermatid differentiation in crab)

PLATE 26

FIG. 9. A slightly oblique cross-section through a mature spermatozoon, showing a round profile of the head (*H*) and the irregularly shaped pseudopodia (*P*). The tubule (*T*) with its contents of moderate density can be seen in the central portion of the head (*H*). A lamellar structure (*LS*) consisting of 4 to 8 layers appears at the periphery of the head (*H*). Vacuoles (*VA*) can be seen between the head (*H*) and the pseudopodia (*P*). The highly dense material surrounding the right side of the tubule (*T*) is the same material as seen in Fig. 8. × 47,000.



(Yasuzumi: Spermatid differentiation in crab)