SPERMIOGENESIS IN *CANCER* CRABS

SUSAN G. LANGRETH

From the Whitman Laboratory, The University of Chicago, Chicago, Illinois 60637. Dr. Langreth's present address is the Department of Anatomy and Cell Biology, School of Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania 15213

ABSTRACT

Spermiogenesis in *Cancer* crabs was studied by light and electron microscopy. The sperm are aflagellate, and when mature consist primarily of a spherical acrosome surrounded by the nucleus with its short radiating arms. The acrosome forms by a coalescence of periodic acid-Schiff-positive (PAS-positive) vesicles. During spermiogenesis one edge of the acrosomal vesicle invaginates to form a PAS-negative central core. The inner region of the acrosome bounding the core contains basic proteins which are not complexed to nucleic acid. The formation of an elaborate lattice-like complex of fused membranes, principally from membranes of the endoplasmic reticulum, is described. These membranes are later taken into the nucleus and subsequently degenerate. In late spermatids, when most of the cytoplasm is sloughed, the nuclear envelope and the cell membrane apparently fuse to become the limiting boundary over most of the sperm cell. In the mature sperm the chromatin of the nucleus and arms, which is Feulgen-positive, contains no detectable protein. The chromatin filaments appear clumped, branched, and anastomosed; morphologically, they resemble the DNA of bacterial nuclei. Mitochondria are absent or degenerate in mature sperm of *Cancer* crabs, but the centrioles persist in the nucleoplasm at the base of the acrosome.

INTRODUCTION

Spermatogenesis in decapods has long been a subject of interest to cytologists mainly because this order of crustaceans has unusual, nonflagellate sperm. In general, these sperm have a spherical, conical, or cylindrical body from which radiate a number of processes or arms. Although an extensive classical literature has developed since the first detailed description of aflagellate sperm by Grobben (1878), the contributions made by early investigators to our knowledge of aflagellate spermatogenesis have been limited for several reasons. Most of these authors attempted to homologize the bizarre structures observed in these cells with the typical structures of flagellate sperm, without the assistance of specific cytochemical methods for localizing the nucleus, acrosome, mitochondria, Golgi material, centrioles or their derivatives. Only in 1940, when McCroan used the Feulgen reaction for DNA on sperm of the crayfish *Cambarus virilis*, was the nucleus first localized unequivocally in one of these forms. However, not until the advent of the electron microscope, particularly when it is used in conjunction with light-microscope cytochemistry (Moses, 1956), was significant progress made in our understanding of spermatogenesis in decapods.

Yasuzumi (1960) studied late stages of spermiogenesis in the crab *Eriocheir japonicus*. He not only identified correctly the major components with the electron microscope, but also demonstrated that in the mature sperm the arms contain DNA and are continuous with the body of the nucleus. The acrosome or "vesicle" of the classical literature was found to be PAS positive and to contain RNA in this species.

Fairly detailed studies of spermiogenesis have been made on two species of crayfish. Moses (1956, 1961a, b) examined *Procambarus clarkii* while

Yasuzumi et al. (1961) and Kaye et al. (1961) looked at *Cambaroides japonicus*. These investigations are in agreement on principal points: continuity of nucleus and arms, loss or degeneration of mitochondria, Golgi elements, centrioles in the mature sperm, and unusual elaborations and associations of major membrane systems (nuclear envelope and endoplasmic reticulum) of the cell.

A preliminary study of the sperm of the crab *Cancer borealis* revealed a number of differences between this brachyuran and the macruran crayfish (Langreth, 1965). The centrioles persisted, the internal structure of the acrosome was distinctive, and the apparent origin of the membrane elaborations from endoplasmic reticulum and their subsequent degeneration followed different patterns. Although decapod sperm have been studied in the past primarily because of their unusual aflagellate form, the recent discoveries of deoxyadenylate-deoxythymidylate copolymer satellite DNA's in the testis and other tissues of several species of crabs (Sueoka, 1961; Sueoka and Cheng, 1962; Smith, 1964; Skinner, 1967) have stimulated a more general interest. For these reasons a detailed investigation of spermiogenesis in *Cancer* crabs was undertaken.

Recently, Pochon-Masson (1968) published an account of spermiogenesis in the crab *Carcinus maenas* and in the anomuran *Eupagurus bernhardus*. While the general scheme of differentiation appears to be similar, there are numerous differences in details and in interpretation between this contribution and the material presented here.

MATERIALS AND METHODS

Animals

North Atlantic crabs, *Cancer borealis* and *Cancer irroratus*, were obtained from the Boston Lobster Company, the Marine Biological Laboratory, Woods Hole, Mass., and the Bayview Fish Market of Manomet Point, Mass. Pacific crabs, *Cancer magister* and *Cancer productus*, were obtained from Dr. Michael Smith, Fisheries Research Board, Vancouver, B. C. The crabs were packed in wet seaweed over ice during shipment by air freight. In Chicago, the animals were maintained in Instant Ocean synthetic sea water (Ward's Natural Science Est., Inc.) in 25 gallon reinforced fiberglass aquaria in a 12.5°C cold room. Charcoal filtration and aeration were continuous. About once a week crabs were fed thawed fresh-frozen shrimp obtained from the supermarket. Fresh crabs were received in all seasons except from December to March.

Light Microscopy

Small pieces of testis, vas deferens, and seminal receptacle were fixed in ethyl alcohol-acetic acid 3:1 or in 10% neutral buffered formalin appropriate for histone staining (Alfert and Geschwind, 1953). Tissue was embedded in paraffin, sectioned at 4 μ, and mounted on albuminized or gelatin-subbed glass slides. Smears were fixed in 10% neutral formalin followed by water rinses, 100% ethanol, or acetic alcohol followed by 100% ethanol and then air dried.

Cytochemical analysis on formalin and acetic-alcohol fixed material was performed by use of the following stains:

(1) Feulgen reaction for DNA according to the method of Swift (1955). Control slides, treated in 60°C water instead of 1 N HCl, were always included.

(2) Azure B for DNA and RNA (Flax and Himes, 1952; Swift, 1955). For RNA alone, slides were treated before staining in 0.2 mg/ml DNase (Worthington) in 0.003 M $MgSO_4$ adjusted to pH 6.5 with 0.01 N NaOH. The DNase control was 0.003 M $MgSO_4$ at pH 6.5. For DNA alone, slides were pretreated in 1 mg/ml RNase (Worthington) in pH 6.5 water adjusted with 0.01 N NaOH. The nuclease control was pH 6.5 water. Other controls to remove both nuclei acids were 5% trichloroacetic acid (TCA) at 90°C for 15 min or a double nuclease. All nuclease pretreatments were for 1 hr at room temperature (25°C).

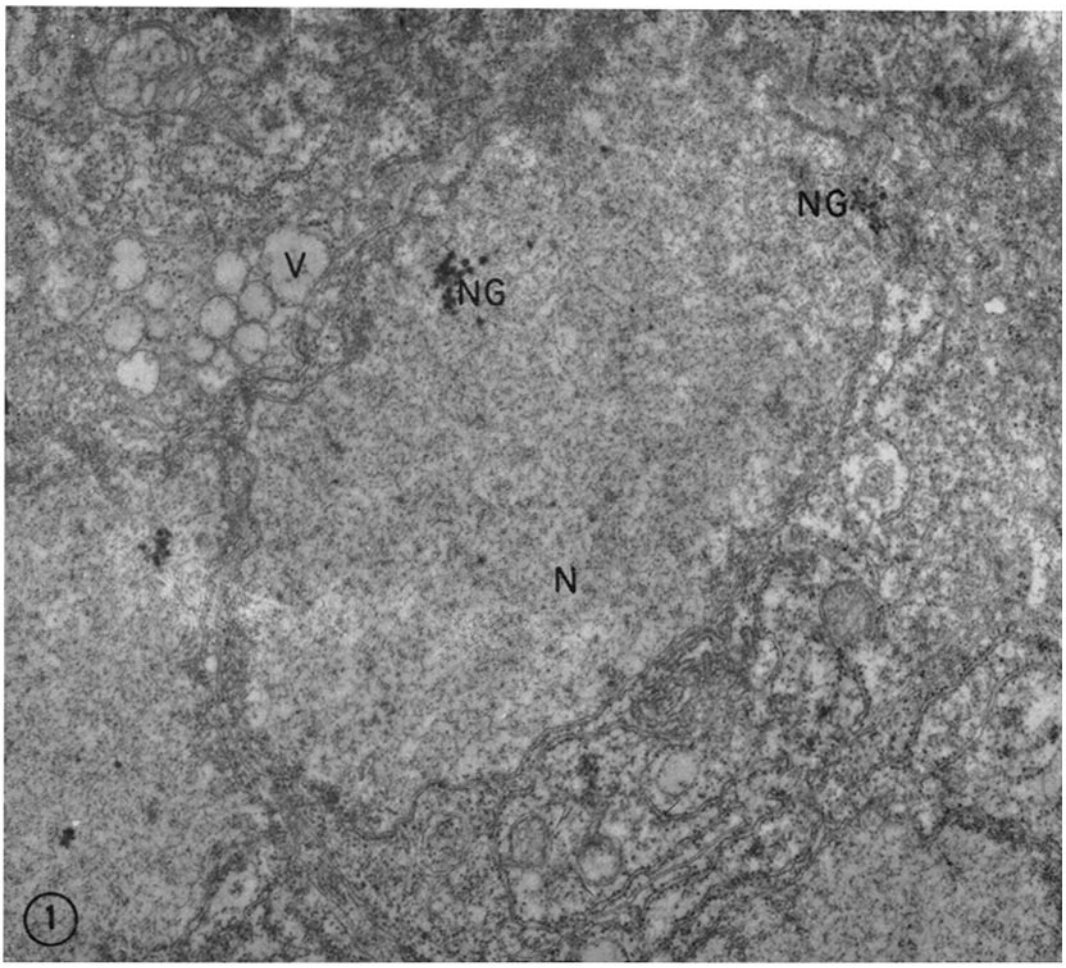
(3) For total protein slides were stained for 1 hr in 0.01% fast green, adjusted to pH 2 with HCl. After staining, slides were rinsed in two 15 min changes of pH 2 distilled water, dehydrated, cleared, and mounted.

(4) The periodic acid-Schiff (PAS) reaction for polysaccharides (1, 2 glycol groups) was performed according to the method of Hotchkiss (1948). A nonperiodic, acid-treated control was included.

(5) The pH 8.1 fast green staining method of Alfert and Geschwind (1953) was employed to detect histone-type basic proteins with one slight modification. After staining, the water rinse was at pH 8.1 nstead of at an unspecified pH. Controls on the hot TCA hydrolysis for nucleic acid removal were hot water, DNase, RNase, and their controls. The effectiveness of the nucleases was checked by azure B istaining on duplicate slides. Only formalin-fixed slides were used.

(6) The bromphenol blue method of Bloch and Hew (1960) to detect histones and protamines also was used only on formalin-fixed slides.

Light micrographs were made with a Zeiss 90X objective (numerical aperture 1.4) and 25X ocular on Kodak M plates. Appropriate filters were used to enhance contrast. Magnifications were calibrated by use of a Zeiss object micrometer (1 mm in hundredths).



All figures are of *Cancer borealis*, except for Fig. 18, which is of *Cancer irroratus*.

FIGURE 1 Spermatid I stage. The nucleus (*N*) is of rather homogeneous consistency, except for a few clumps of granules (*NG*). Typical Golgi material is absent, although many vesicles (*V*) are present in the cytoplasm. × 18,000.

Electron Microscopy

Small pieces of testis tubules, vas deferens, and seminal receptacle were fixed in 1% osmium tetroxide in 0.1 M Sorenson's phosphate buffer at pH 7.6 or in 6% glutaraldehyde in 0.1 M phosphate buffer at pH 7.0 followed by buffer rinses and 1% osmium tetroxide in 0.1 M phosphate at pH 7.6 (Sabatini, Bensch, and Barrnett, 1963) or in 10% formalin in 0.2 M phosphate buffer at pH 7.6. All fixations were done in the cold for 1–4 hr. DNase extractions and controls on formalin-fixed tissues were performed as described by Swift, Adams, and Larsen (1964). Tissues were dehydrated in a cold graded series of alcohols, cleared in propylene oxide, and then embedded in Epon 812 (Luft, 1961). Sections were mounted on carbon-coated copper mesh grids, stained in saturated aqueous uranyl acetate usually followed by lead citrate (Venable and Coggeshall, 1965), and then examined in an RCA EMU 3C electron microscope operated at 50 kv. 1 μ Epon sections adjacent to those used for electron microscopy were cut in order to check staging of spermatogenesis and to compare details with the light microscope. These sections were picked up with plastic loops, mounted on gelatinized slides, and then stained with 0.25% azure B.

RESULTS

Spermiogenesis is a continuous process. Its division into discrete stages is somewhat arbitrary and has

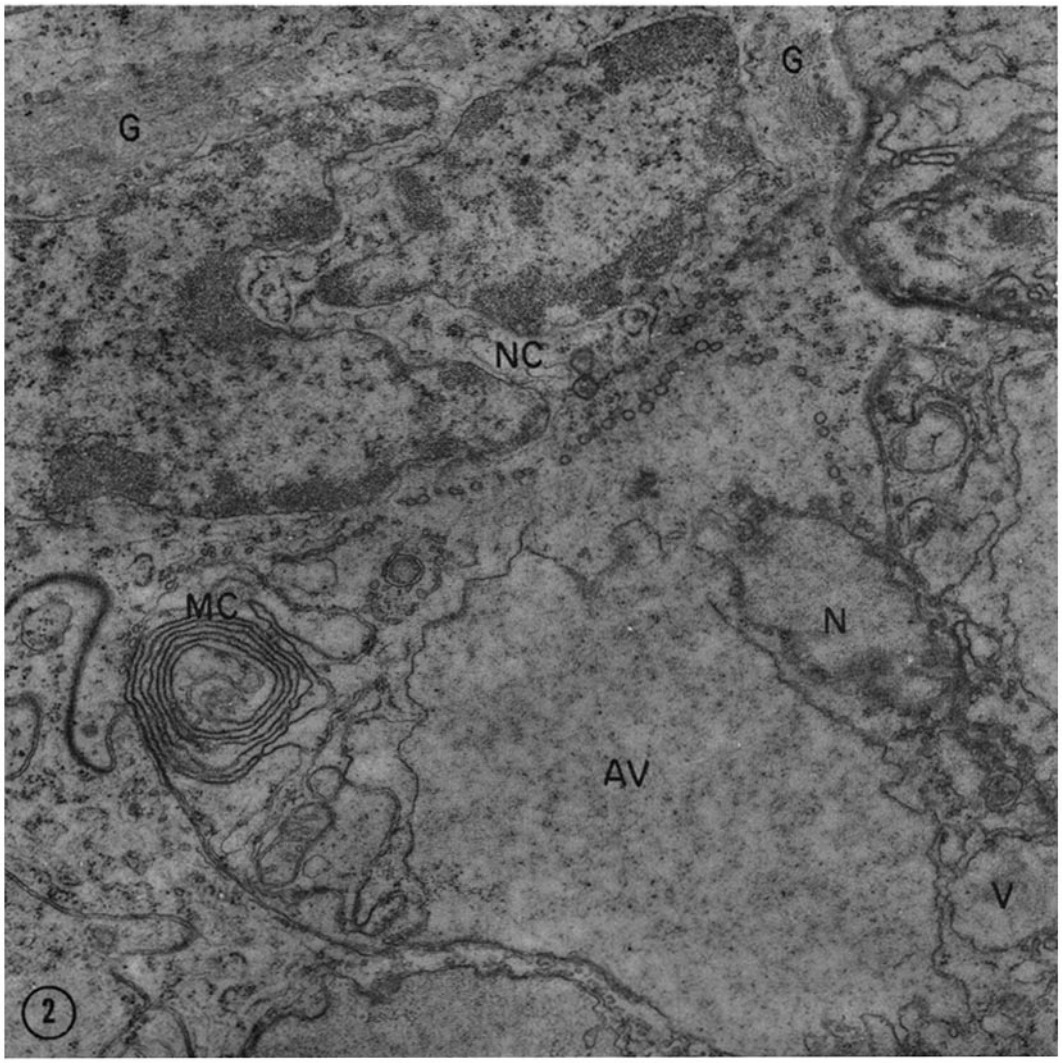


FIGURE 2 Spermatid II stage. Small vesicles (*V*) coalesce to form the acrosomal vesicle (*AV*). The forming membrane complex (*MC*) is closely associated with endoplasmic reticulum but not the nucleus (*N*) at this time. At the upper left is a nurse cell (*NC*), which has prominent Golgi material (*G*). Osmium tetroxide fixation. × 19,000.

been made here only to facilitate description. It should be kept in mind that gradual changes also occur within the stages herein delineated. Although in this study four species of *Cancer* crabs were examined, the following description applies primarily to *C. borealis*. Interspecies differences were comparatively slight.

Spermatid I: Nuclear Granule Stage

Newly formed spermatid nuclei are small, about 5 μ in diameter, and nearly spherical. The condensed chromatin of the spermatocyte divisions rapidly disperses, giving the nucleoplasm the appearance of homogeneous fine filaments, except for one or two clumps of granules, which frequently persist through this stage (Fig. 1). Golgi complexes have not been discerned in any spermatid stage. This observation is particularly striking because of the well-characterized role of the Golgi in the formation of the acrosome in the sperm of other animals, such as the cat (Burgos and Fawcett, 1955), the toad (Burgos and Fawcett, 1956), and

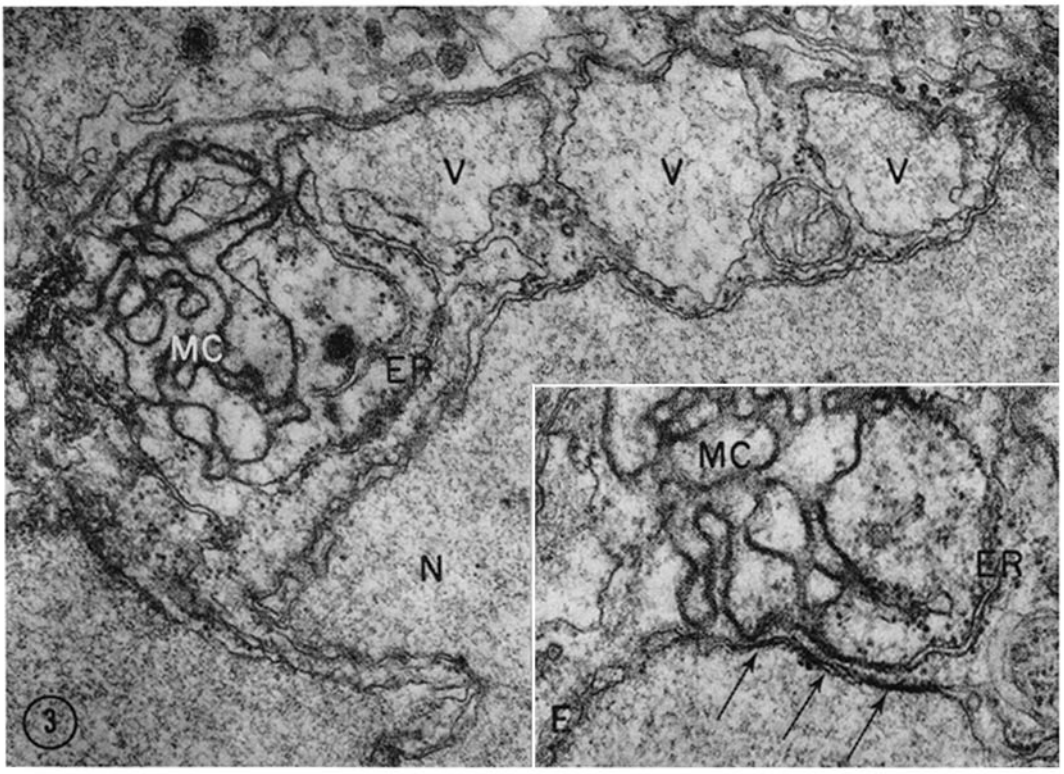


FIGURE 3 Interrelationship of endoplasmic reticulum (*ER*), membrane complex (*MC*), and acrosomal precursor vesicles (*V*) at spermatid II stage. × 40,000. The arrows in the *inset* refer to the fusion of the two layers and apparent closing of the nuclear envelope (*E*) when it is adjacent to the membrane complex (*MC*). × 60,000.

the cricket (Kaye, 1962). However, typical Golgi material appears to be absent in other decapods: the macrurans *Procambarus clarkii* (Moses, 1961b) and *Cambaroides japonicus* (Yasuzumi et al., 1961), the anomuran *Eupagurus bernhardus* (Pochon-Masson, 1963), and the brachyuran *Carcinus maenas* (Pochon-Masson, 1968). In the earliest spermatids, vesicles similar to those seen in secondary spermatocytes are present. Some have smooth membranes and some are lined with ribosomes. A number of these vesicles contain faintly electron-opaque filamentous material and are presumed to be acrosome precursors because of their subsequent fusion and their PAS stainability.

Spermatid II: Acrosomal Vesicle Formation

The vesicles of the previous stage coalesce gradually forming the acrosomal vesicle, a process which can be followed with the light microscope because of the PAS-positive nature of these vesicles (Fig. 5). The interior of the acrosome at this stage is undifferentiated, except for a row of granules along the inner surface of the membrane on one side of the vesicle, which becomes more prominent later (Fig. 4, GB). Figure 2 demonstrates the relationship between a spermatid at stage II and a nurse cell. Nurse cells are recognized easily by their large, irregularly-shaped nuclei which have prominent clumps of peripheral chromatin. The cell membranes of a nurse cell closely appose those of the spermatids, and eventually the spermatids are completely surrounded by nurse cell cytoplasm. The boundary between nurse cell and spermatid sometimes takes the form of a row of small vesicles. It is possible that this is a fixation artefact (Tormey, 1964), but it has been observed under a variety of fixation conditions and has also been seen in the crayfish (Moses, 1961b). Nurse cells

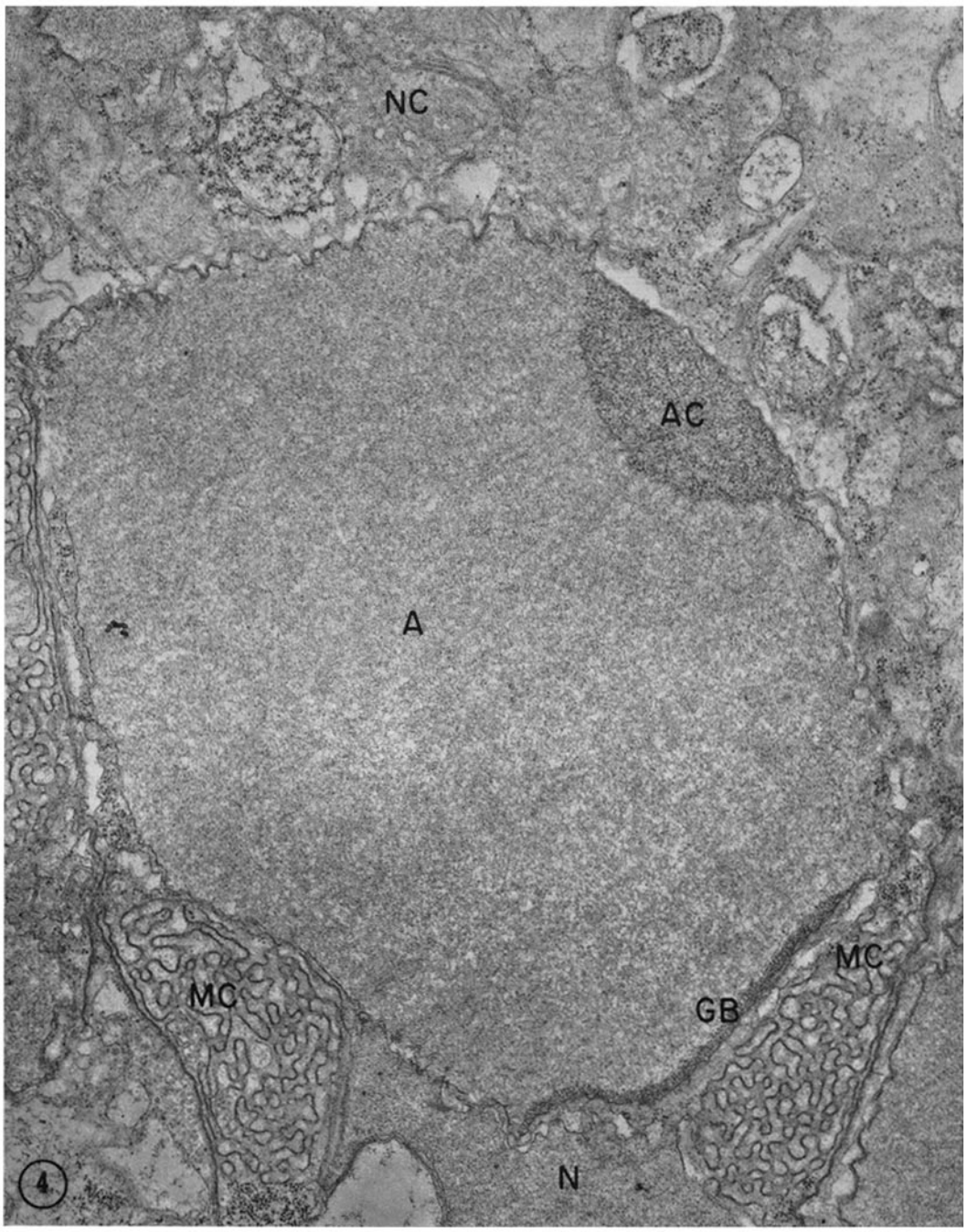


FIGURE 4 Differentiation of structures of the acrosome (*A*) at spermatid III stage include the acrosomal cap (*AC*) and granular belt (*GB*). The nucleus (*N*) is located peripherally; the membrane complex (*MC*) forms a ring between it and the acrosome. Nurse cell (*NC*) cytoplasm surrounds the spermatid. × 20,000.

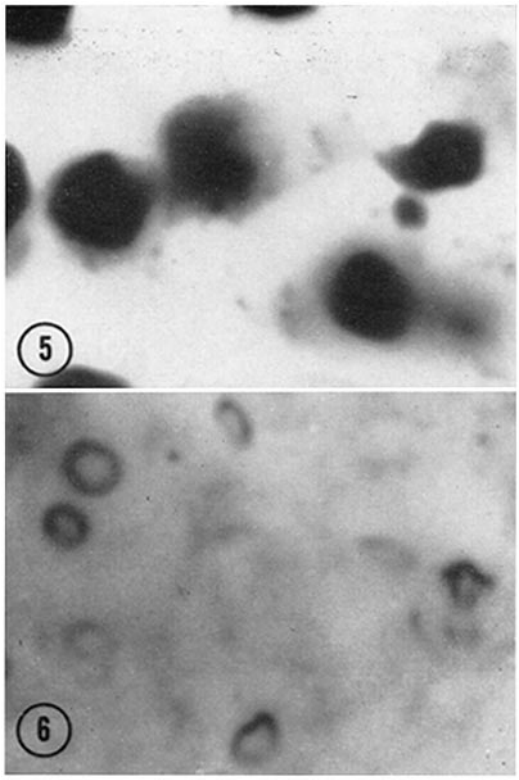


FIGURE 5 PAS staining of spermatid II-III stage showing coalescence of vesicles to form the large acrosomal vesicles. × 4500.

FIGURE 6 DNase treatment followed by azure B staining to detect RNA. Spermatid III–IV stage. The acrosomal cap and granular belt take up the dye. × 4500.

have prominent Golgi complexes and may contribute material to the spermatids.

At this time an intensely osmiophilic complex of membranes arises in association with the endoplasmic reticulum (Fig. 2, *MC*). Presumably, the walls of the cisternae fuse and the ribosomes are shed. Typical, rough endoplasmic reticulum sometimes connects membrane complexes and vesicles which contribute to the formation of the acrosome (Fig. 3). The complex develops a characteristic lattice whose spaces contain microtubules. This structure is not usually formed in conjunction with the nucleus, but it is sometimes observed next to it even at this early stage. Under these circumstances the inner and outer membranes of the nuclear envelope may fuse in the region adjoining the complex and appear identical to the osmiophilic ER membrane derivatives (Fig. 3, *inset*).

During this stage the nucleoplasm is completely homogeneous, consisting of fine filaments and granules of low electron opacity. For light microscopy, both spermatid and nurse cell nuclei stain equally well with pH 8.1 fast green, which indicates that the histone transformation or loss has not yet occurred. The position of the nucleus in the cell is altered slowly as the acrosomal vesicles coalesce, moving it to a more peripheral location (Figs. 2 and 4).

Spermatid III: Acrosomal Cap Stage

The material within the acrosomal vesicle condenses gradually and becomes more electron opaque. Light microscope PAS stainability also becomes more intense. The developing acrosome begins to assume a more spherical shape and internal differentiations become apparent. The single row of granules mentioned earlier elaborates into a thicker complex several rows wide (Fig. 4, *GB*). This belt of granules assumes a position next to the nucleus. In this region the nucleus and acrosome come into close contact. Most cytoplasmic elements appear to be excluded except the centrioles, whose

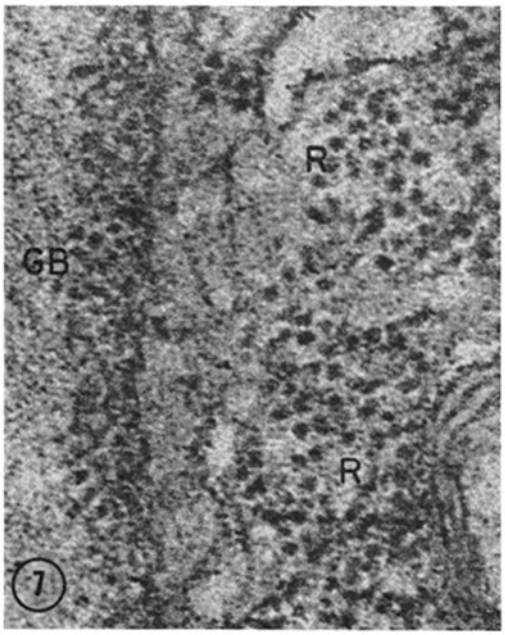


FIGURE 7 Granular belt particles (*GB*) appear to be slightly smaller than ribosomes (*R*). × 115,000.

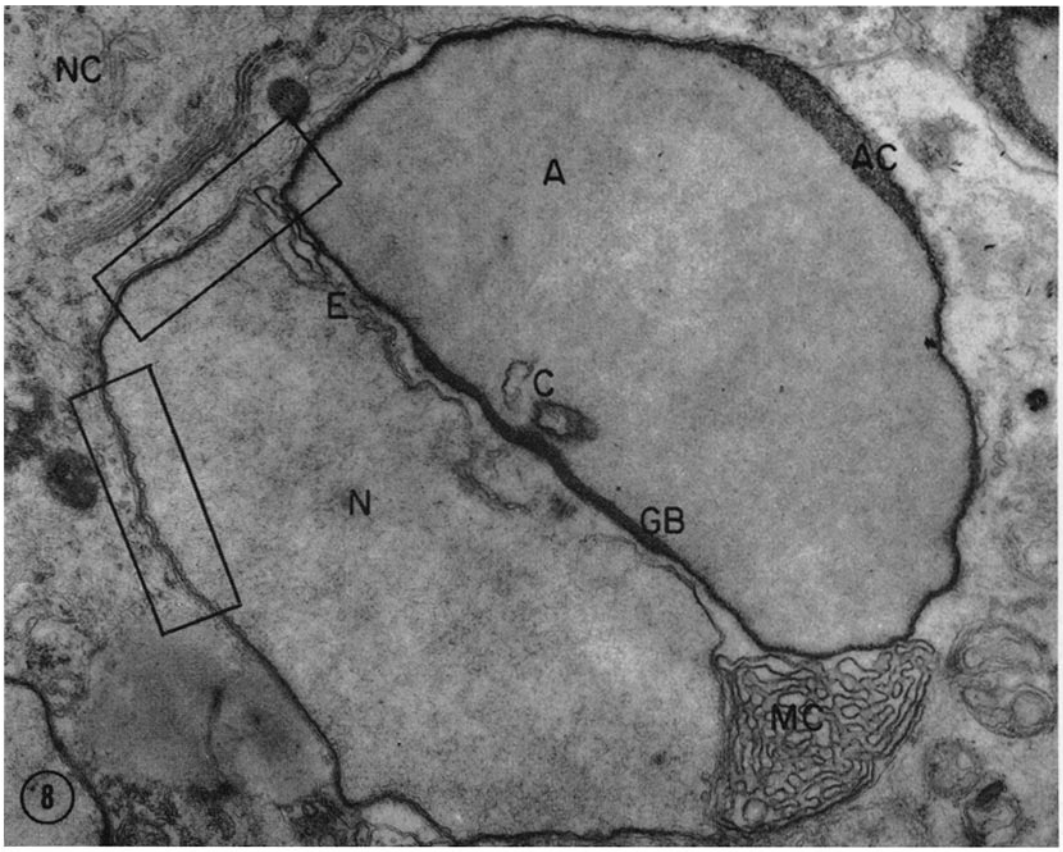


FIGURE 8 Spermatid stage IV. Perforations at the base of the acrosome (*A*) next to the granular belt (*GB*) indicate the beginning of acrosomal core (*C*) formation. Sloughing of cytoplasm is completed, and the nuclear envelope lies against the cell membrane. × 21,000.

characteristic position in late spermatids is between nucleus and acrosome (Fig. 19).

The acrosomal granules seem to be related to ribosomes, but examination at high magnification indicates that they are consistently somewhat smaller than the ribosomes in the cytoplasm (Fig. 7). They probably contain RNA, for there is an RNA-containing structure in this region of the acrosome at this and at the next stage in spermatid development (Fig. 6). The size, position, and particulate nature of the granules (and the cap, to be described shortly) make them the most likely candidates for the components responsible for RNA stainability.

A condensation of other granular and fibrillar material at an edge of the acrosome some distance from the belt of granules occurs next and is the earliest detectable differentiation of the acrosomal cap (Fig. 4). As the acrosome becomes more spherical, the cap positions itself directly opposite the nucleus and belt of granules, as is seen in all subsequent stages.

As the membrane complex becomes larger and more elaborate, it takes the shape of a ring and is located around the nucleus and the acrosome in the region of their juncture (Figs. 4 and 10). The nuclear envelope is observed frequently to be continuous with the complex at this and later stages. The centrioles come to lie at the center of the membrane complex ring, between nucleus and acrosome (Fig. 10). Mitochondria are often caught up in whorls of this complex, but their inclusion appears to be fortuitous. Most mitochondria apparently are lost from the spermatid as its cytoplasm is sloughed to the nurse cells, a process which begins at this stage. Elaborate in-

foldings and vesiculation of the cell membranes of both the spermatid and the surrounding nurse cell appear to be involved in this cutting off of all but the nucleus, acrosome, centrioles, and membrane complex.

Spermatid IV: Core Formation

The further differentiation of the acrosome can be followed in Figures 8 and 10. As the cap becomes larger, its consistency becomes more homogeneous, electron opaque, and very finely particulate. The particles of the granular belt, however, retain their near-ribosomal size. Adjacent to the center of the belt are the first indications of acrosomal core formation. An invagination of the acrosomal vesicle occurs in this region, and the inpocketing elongates until it reaches the acrosomal cap. The belt of granules is perforated at its center and turns in at the base of the developing core (Figs. 8 and 20). Acrosomal cap material moves down along the internal membrane of the core until it reaches the granular belt. At the base of the core (Fig. 10) is some irregularly fibrous material which may be the precursor of the tubules which form within the core at a later stage.

Core formation can also be followed with the light microscope because of the cytochemical changes which accompany the process. As the core forms, the PAS reaction demonstrates a change from a solid sphere to a structure resembling a thick doughnut. As mentioned earlier, both the acrosomal cap and the granular belt stain for RNA at this stage (Fig. 6). The RNA staining, which is RNase removable, changes from a ring (granular belt) and a disc (acrosomal cap) to an hourglass configuration as the belt and cap materials move along the edges of the developing core and meet one another. At this time the cap and belt stain with pH 8.1 fast green in a pattern identical to that of the RNA. Since this basic protein stains without nucleic acid removal by hot TCA hydrolysis, it does not fit the traditional definition of histone as DNA-associated basic protein (Bloch, 1966). Crab basic proteins will be described in more detail at a later stage. It should be noted now, however, that the nucleus still stains faintly for histone and does not appear to contribute basic protein to the acrosome as has been suggested by Chevaillier (1966a) and Vaughn (1968).

Sloughing of cytoplasm is completed at this stage. Although the spermatids are surrounded by elaborately enfolded nurse cell cytoplasm, their own cell membranes are intact (Figs. 8–10). However, because of the sloughing of most of the cytoplasm, the cell membrane comes to lie against the nuclear envelope. Along this region of contact no nuclear pores are evident. In fact, the two membranes of the nuclear envelope and the cell membrane apparently fuse, forming a highly electron-opaque structure. Only in fortuitous sections can the tri-membrane nature of the resulting cell boundary be resolved after this stage.

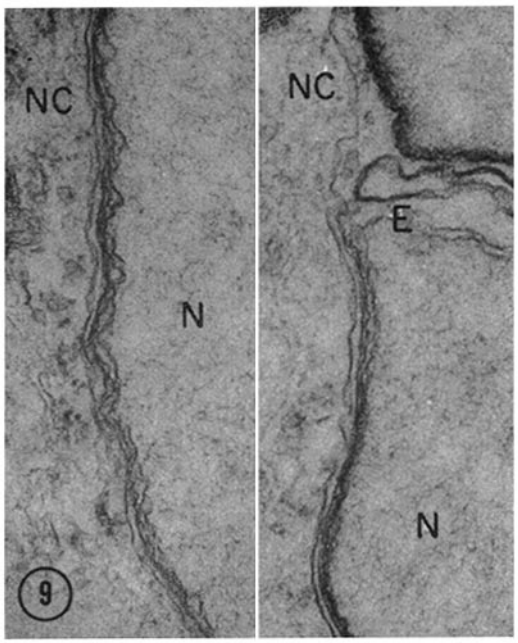


FIGURE 9 Higher magnification of the two areas enclosed by rectangles in Fig. 8, showing apposition of nurse cell membrane, spermatid cell membrane, and the spermatid nuclear envelope. × 50,000.

The part of the nuclear envelope next to the membrane complex also undergoes unusual modifications. Nuclear membranes and membranes from the complex become continuous with one another, and nucleoplasm begins to surround the membrane complex (Figs. 10 and 11). Together they invest the acrosome up to the level of the cap. The spermatid cell membrane adheres tightly to the outer edge of the nuclear envelope-membrane complex and then continues separately over the top of the acrosome.

The changes in the limiting membranes of the spermatid can now be summarized as follows:

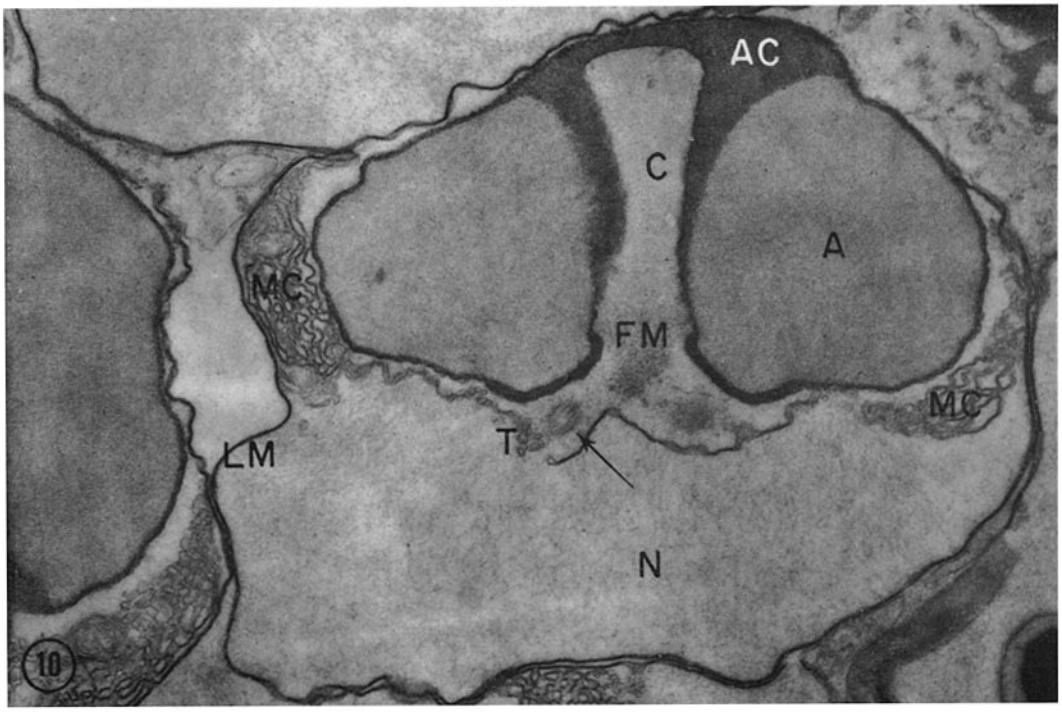


FIGURE 10 Late stage IV spermatid. The cell membrane and nuclear envelope have fused to form an extremely electron-dense limiting membrane (*LM*). Acrosomal cap material (*AC*) is now distributed along the edge of the fully-invaginated acrosomal core (*C*). At the base of the core is some irregularly fibrous material (*FM*), which is separate from the centriole (arrow). Apparent tubulation (*T*) of the nuclear envelope is evident in this region. The membrane complex (*MC*) still forms a peripheral ring between nucleus (*N*) and acrosome (*A*). × 21,000.

over the acrosomal cap are one acrosomal vesicle membrane and the unmodified cell membrane, while the boundary of the rest of the cell is an extremely electron-opaque tripartite membrane consisting of the original cell membrane and the two layers of the nuclear envelope. Also surrounding the spermatid are the cell membranes of the nurse cell. These are often folded extensively, possibly a result of their involvement in phagocytosis of the sloughing spermatid cytoplasm.

Changes also occur in the part of the nuclear envelope next to the acrosome, where small vesicular or tubular structures of about 300 A frequently appear (Fig. 10). At the base of the acrosomal core discontinuities appear in the nuclear envelope, which always breaks down in this specialized region and in no other. The centrioles, located between nucleus and acrosome, then become surrounded by nucleoplasm and chromatin filaments (Figs. 11, 20, and 21).

Immature Sperm

As the nucleus and membrane complex surround the acrosome, both the entire sperm and the acrosome itself become more spherical. The acrosome increases in density as evidenced by its enhanced electron opacity. The top part of the acrosomal cap is now so dense that its former granular nature can no longer be resolved (Fig. 11). RNA staining of the cap material or any other structure can no longer be detected by light microscope cytochemical techniques, a further indication of organizational changes.

Each nurse cell surrounds several sperm cells (Fig. 11). A striking feature of the nurse cells at this stage is the degenerating material in their cytoplasm. This is undoubtedly the remnant of the sloughed cytoplasm of the spermatids. Phagocytosis and degradation of residual spermatid cytoplasm has been studied in Sertoli cells of the

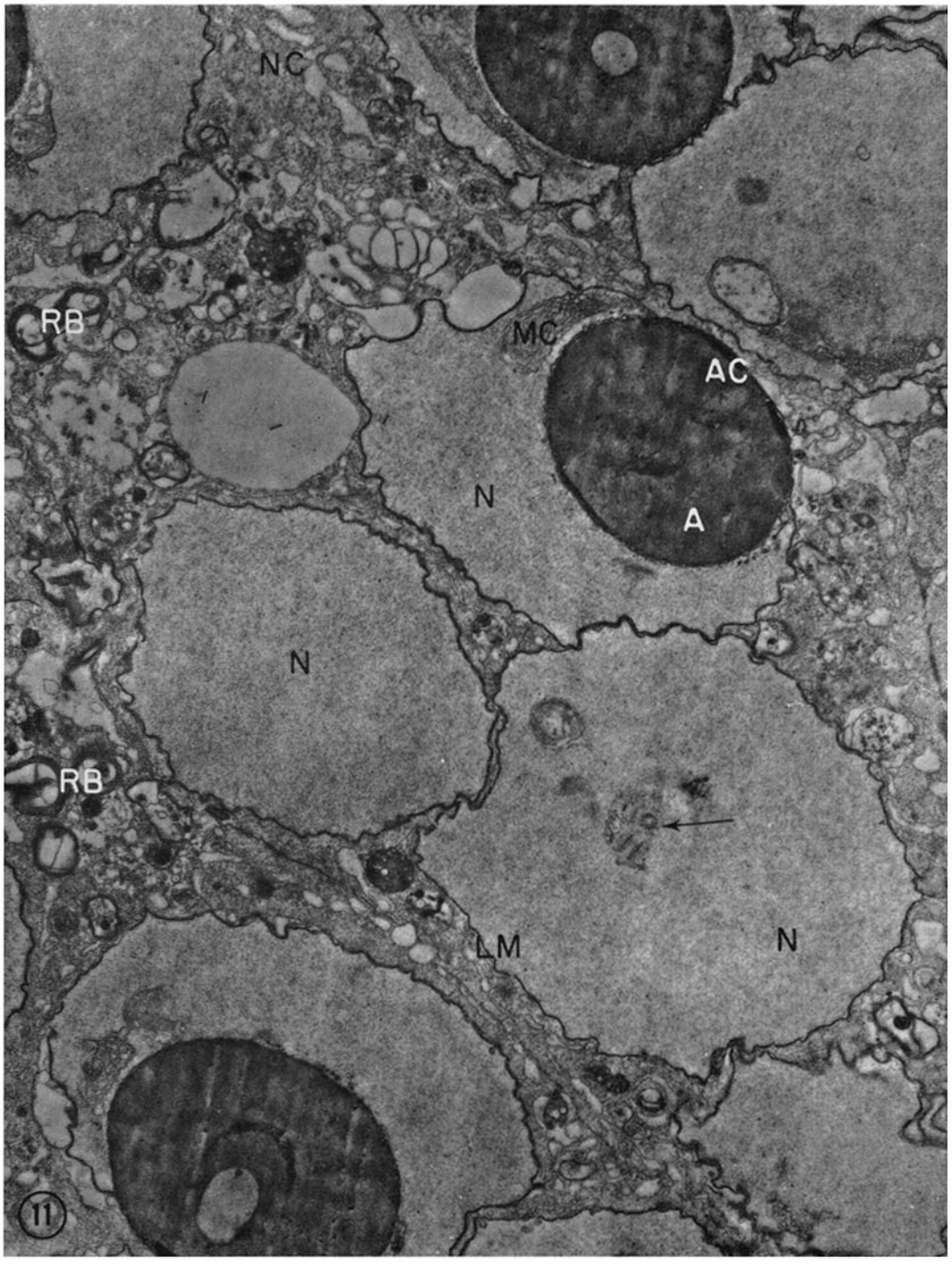
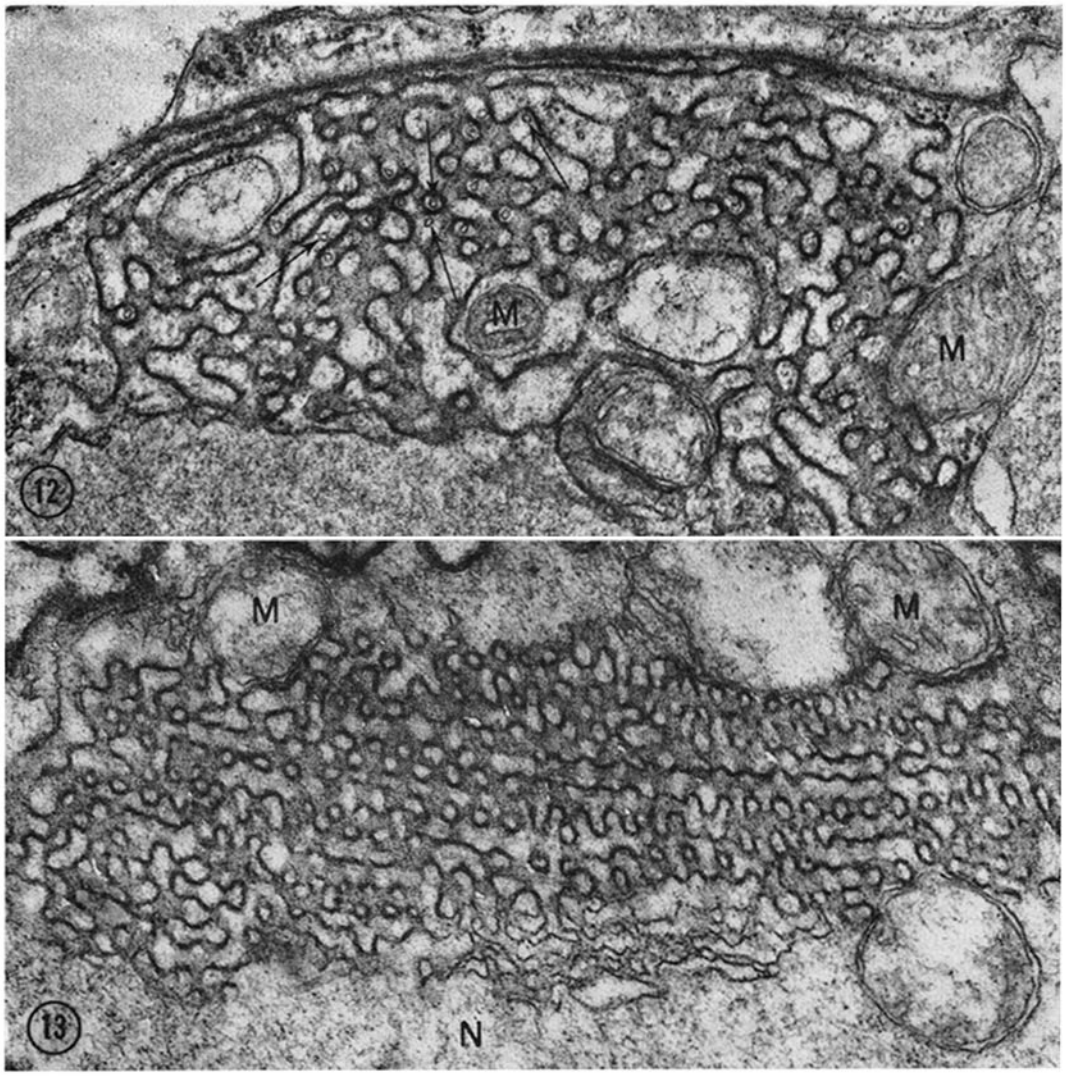


FIGURE 11 Immature sperm surrounded by nurse cell cytoplasm (*NC*). Membrane complex material (*MC*) and centrioles (arrow) are within sperm nuclei (*N*). The acrosome (*A*) and cap (*AC*) are highly electron dense. Residual bodies (*RB*) of degenerating material are prominent in the nurse cells. × 12,000.



FIGURES 12 and 13 Membrane complexes of immature sperm. Within the lattice framework are microtubules (arrows). A few degenerate mitochondria (*M*) are associated with the complex. × 42,000.

rat by Kingsley Smith and Lacy (1959) and Lacy (1960, 1962). The phagocytic inclusions in crab nurse cells are quite similar in morphology to those seen in Sertoli cells, and also to sites of cytoplasmic resorption in many other cell systems.

In the immature sperm the membrane complex still retains its lattice-like structure (Figs. 12 and 13). Microtubules run for distances of up to 3 μ through the complex, possibly contributing to its rigidity as well as its organizational framework. A few mitochondria are still associated with the complex, although most were lost when spermatid cytoplasm was sloughed. Filaments like those characterized by Nass, Nass, and Afzelius (1965) as mitochondrial DNA have been observed within them (Fig. 14).

At this stage the previously homogeneous contents of the nucleus change to fine anastomosing fibrils and dense clumps (Fig. 14). This morphology is identical to that of bacterial chromatin that has not been treated with uranyl acetate prior to alcohol dehydration (Ryter and Kellenberger, 1958; Kellenberger, Ryter, and Séchaud, 1958). Coincident with this change in chromatin mor-

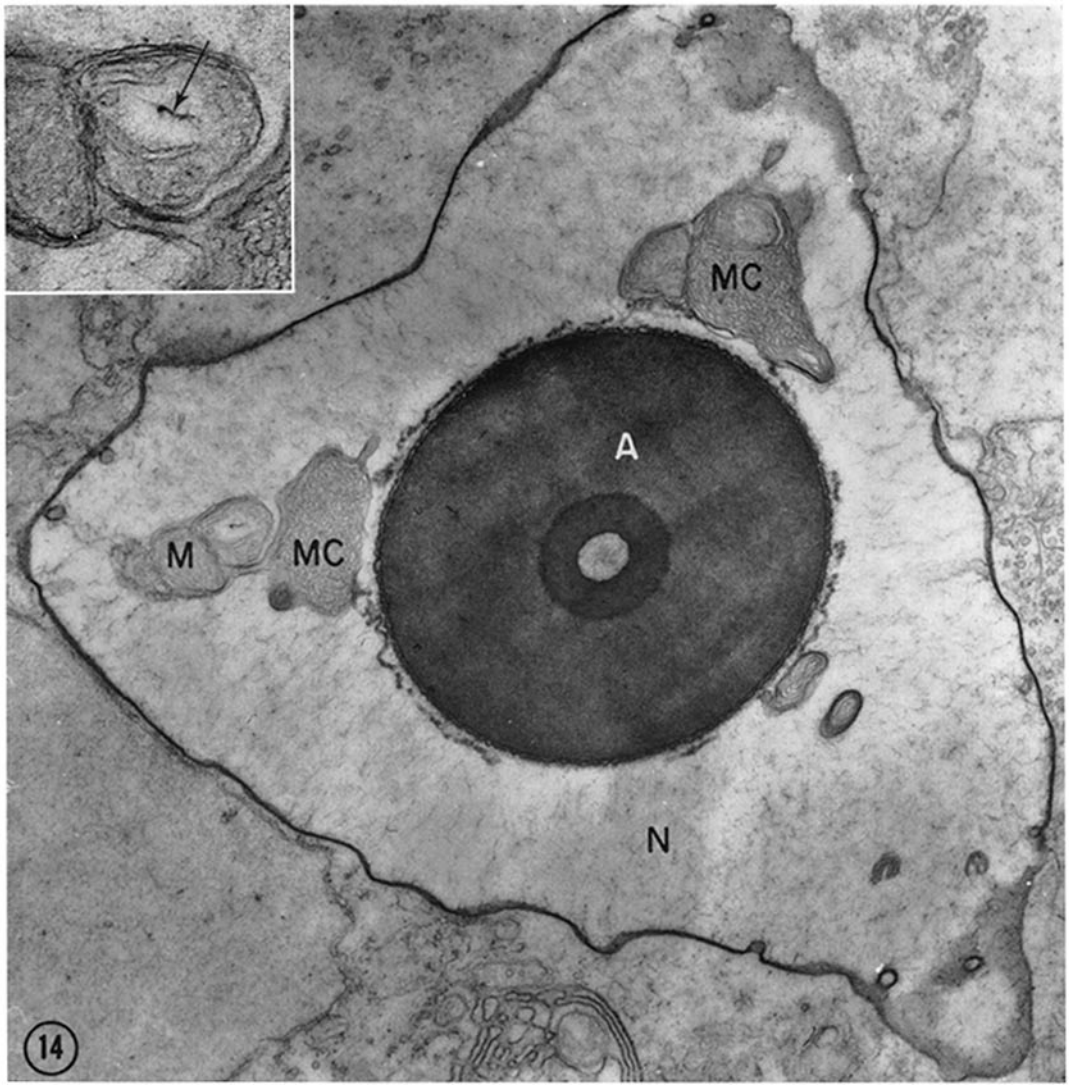


FIGURE 14 Immature sperm in cross section through the acrosomal core. Fine branching filaments characterize the nucleus (*N*). × 18,000. The *inset* shows a DNA filament (arrow) within a mitochondrion (*M*). × 42,000.

phology is the loss of histone stainability; there is no detectable stain with fast green at pH 8.1 under the light microscope. The result of brom phenol blue staining was also negative, which indicates that there is no detectable histone, protamine, or other basic protein in the nucleus at this time.

Mature Sperm

ULTRASTRUCTURE: The changes from the previous stage which result in a mature sperm occur mostly in the acrosome. The inpocketing which forms the acrosomal core now perforates the cap region so that two acrosomal vesicle membranes appose one another (Fig. 15). The limiting boundary of the cell continues to be a highly electron-opaque complex of membranes. The nuclear envelope, which is scalloped where it is adjacent to the acrosome, does not extend beyond the acrosomal lip. Three types of components are differentiated within the acrosomal core. The upper two-thirds of the core (towards the

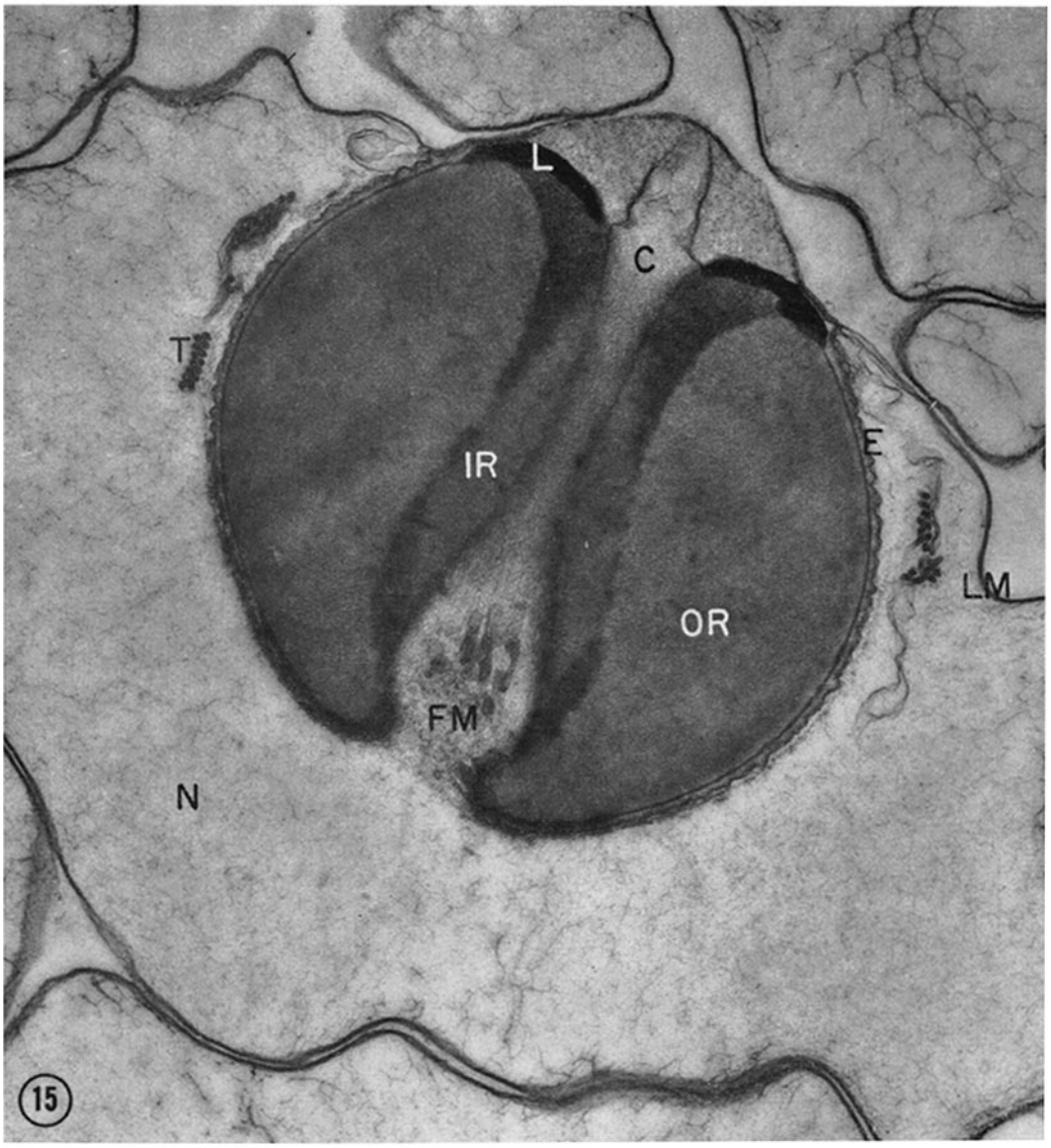
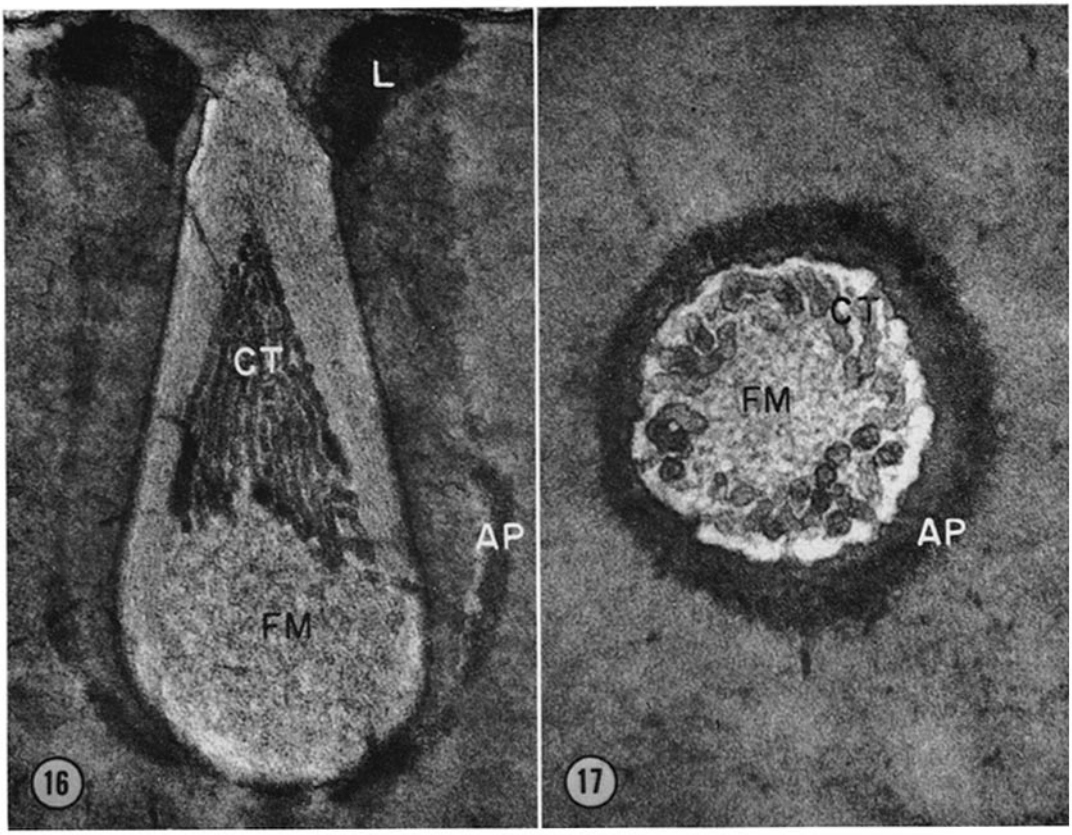


FIGURE 15 Mature sperm in longitudinal section through the acrosomal core (*C*). Outer (*OR*) and inner regions (*IR*) of the acrosome and the acrosomal lip (*L*) are fully differentiated. The nuclear envelope (*E*) is intact except in the region of the fibrous material (*FM*) at the base of the core. Tubules (*T*) in the nucleus (*N*) are probably remains of the membrane complex. × 33,000.

cap) is composed of fine filaments oriented along its long axis. At the base is an irregularly fibrous material. Between these components and at the widest part of the core, are a group of thick tubules 500–600 A in diameter, which have a dense center and are arranged into a conical shape, the apex of which is situated toward the top of the acrosome (Fig. 16). The interior of this cone of tubules is filled by the irregularly fibrous material (Fig. 17). Contrary to the opinions of Fasten (1918) and Pochon-Masson (1965b, 1968), no evidence was found to indicate the involvement of the centrioles in the differentiation of these structures.



FIGURES 16 and 17 Structures of the acrosome in mature sperm in longitudinal and cross section: core tubules (CT), irregularly fibrous material (*FM*) of the basal region of the acrosomal core, acrosomal particles (*AP*) separating inner and outer regions, and the electron-dense acrosomal lip (*L*). × 50,000.

The acrosomal cap material is now differentiated into several structures. As noted earlier, the top part of the cap becomes so electron-opaque that its internal structure cannot be resolved. The lip of the cap curls inward along the core so that it is no longer in contact with the membrane surface of the acrosome except at its outer edges (Fig. 15). Most of the rest of the cap material is finely granular and of slightly higher density than the acrosome outer region. In the area of the core tubules is another probable cap derivative, i.e., a region of particles which separates the core and other components of the inner region from the outer region of the acrosome. These particles are smaller and more closely packed than those of the granular belt (Figs. 15, 17, and 28).

The breakdown of the nuclear envelope at the base of the acrosomal core is complete so that the contents of the nucleus seemingly have free access to the core. However, the irregularly fibrous material of the core and the chromatin filaments of the nucleus maintain their distinct separation; there is no detectable mixing (Figs. 15, 28, and 29). In the nucleus around the rest of the acrosome are tubular structures. These are remnants of the membrane complex and of the earlier tubular derivatives of the nuclear envelope.

The membrane complex and associated structures, now completely embedded in nucleoplasm and chromatin filaments, appear to degenerate in the mature sperm (Fig. 18). The regular lattice framework disappears, and is replaced by membrane whorls resembling myelin figures. Microtubules can no longer be detected within the membrane complex. Entrapped mitochondria appear to lose their few cristae and degenerate further. No coloration by Janus green B was

observed, and thus no evidence was obtained for functional mitochondria.

The distinguishing feature of the mature sperm in the male is its packaging into a spermatophore. Spermatophore material is produced by the secretions of the innermost layer of the proximal end of the vas deferens. This tissue is columnar epithelium, rich in ergastoplasm and filled with vacuoles containing inclusions resembling spermatophore material under the electron microscope. Both stain for polysaccharide by the PAS reaction as viewed with the light microscope. The secretory role of this epithelium has also been studied by Fasten (1917). A few to several hundred sperm cells are packaged into a spermatophore by a condensation of the secretion around them (Figs. 18 and 28). The sperm stay in spermatophores throughout the time of their passage through the vas deferens and until they are transferred to the female seminal receptacle. The sperm are nonmotile. Their arms, which are very short and stubby in all *Cancer* species observed, are interdigitated among adjacent cells in the spermatophore.

The structure of individual sperm cells in the seminal receptacle of the female is essentially the same as that of mature sperm in spermatophores of the male. The membrane complex is also degenerated, indicating that the sperm in the male exhibiting this degeneration were not abnormal. The sperm are no longer packaged in spermatophores, however, a fact noted by Pearson (1908). Among the masses of sperm one finds some material with spermatophore-like properties as viewed with the electron microscope, but it contributes to no organized structure.

Centrioles have been found in the mature sperm in both spermatophores and seminal receptacles in all *Cancer* species examined. Contrary to the case of the crayfish (Moses, 1961a, b; Yasuzumi et al., 1961), the centrioles do not become degenerate. The structure of crab centrioles is typical (a ring of nine triplet microtubules arranged in the usual "pinwheel" configuration), but their orientation with respect to one another is not typical. From spermatid stage IV (core formation) onward, the centrioles lose their perpendicular arrangement and are disposed in an apparently random fashion (Figs. 20–27). Pochon-Masson (1968) has also observed this change in centriole orientation in *Carcinus maenas*.

CYTOCHEMISTRY: DNA localization in the mature sperm has been investigated by both electron and light microscope cytochemistry (Figs. 28–30). DNA is found only in the nucleus and in the arms, which are of the same morphology as the nucleus. Although the nucleus is not separated by any visible structure from the acrosomal core, no detectable DNA staining was observed within any acrosomal structure. No RNA could be found in the mature sperm by the DNase-azure B method.

The outer region of the acrosome is intensely PAS positive (Fig. 31). All of the acrosome including the core stains for total protein with fast green at pH 2, but the cap derivatives, particularly the lip, stain most intensely (Fig. 32). When the pH 8.1 fast green stain for basic protein is used only the cap derivatives and the granular belt take up the dye. Although stainability is enhanced with TCA treatment, the dye distribution is the same with or without removal of nuclei acids with hot TCA (Figs. 34 and 35). The consequence of digestion with DNase or RNase prior to fast green treatment, however, is the staining of only the lip of the acrosome (Figs. 36 and 37). Since the use of either nuclease results in the loss of dye binding from the same region, as compared with the non-TCA-treated control, it is most likely that the nucleases are acting as protein anions which, by binding to basic proteins, block their affinity for pH 8.1 fast green. Artefacts of this kind produced by nuclease extractions are discussed by Swift (1966). The dye distribution with brom phenol blue is similar to that with basic fast green in respect to acrosomal structures (Fig. 33). Slight differences between the two methods were evident, however. Picric acid-extracted cells were slightly smaller, and structures at the base of the acrosome stained less strongly.

No protein can be detected in the nucleus of mature sperm. Staining with pH 2 fast green, pH 8.1 fast green, and brom phenol blue all give negative results (Figs. 32–37). As far as the present cytochemical methods can determine, the histones, lost at an earlier stage, do not appear to be replaced by protamines or other proteins.

DISCUSSION

Membrane Complex

The membrane complex, as described in this study, is composed of three elements: an elaborate lattice framework of fused double membranes,

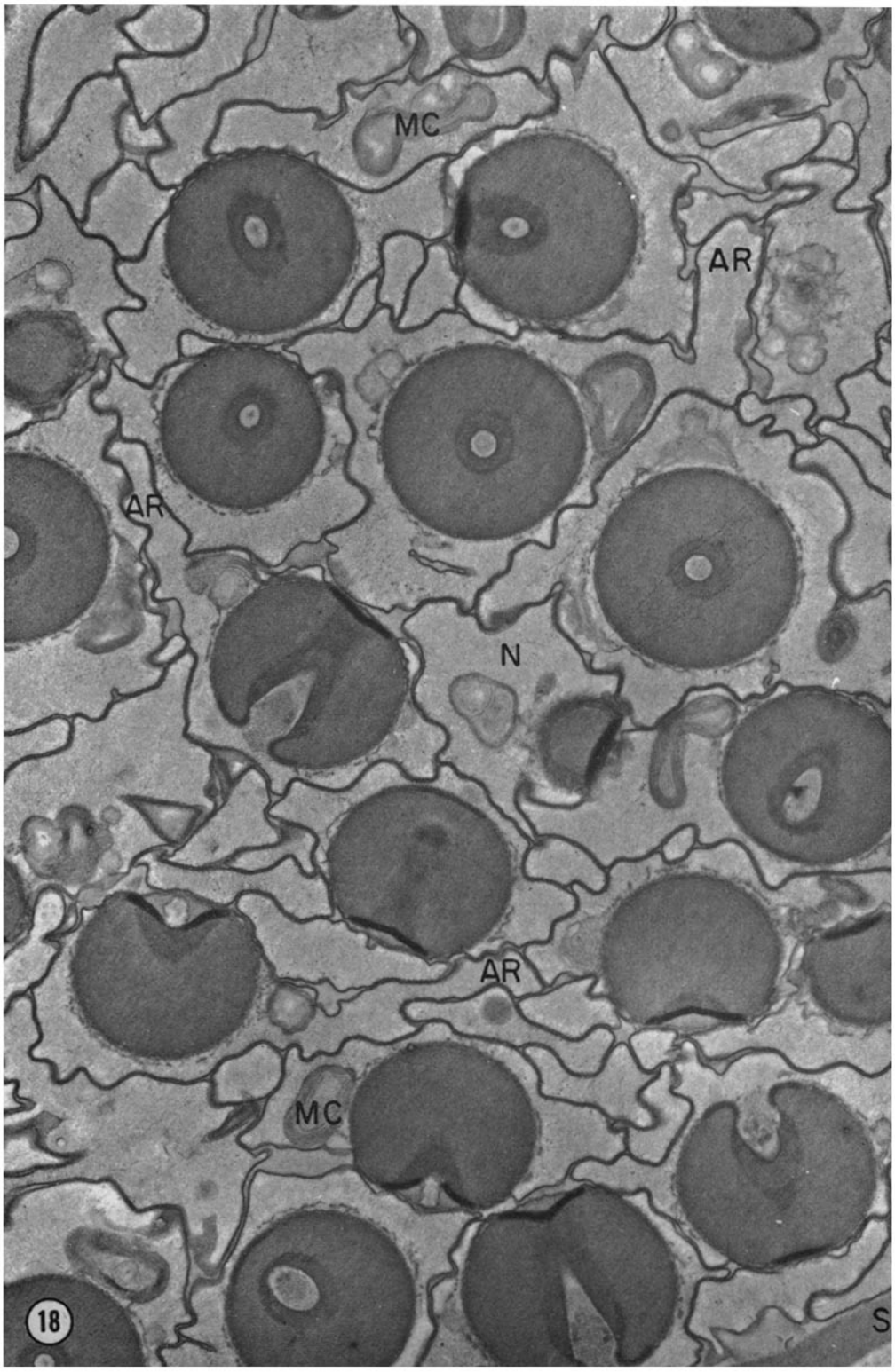
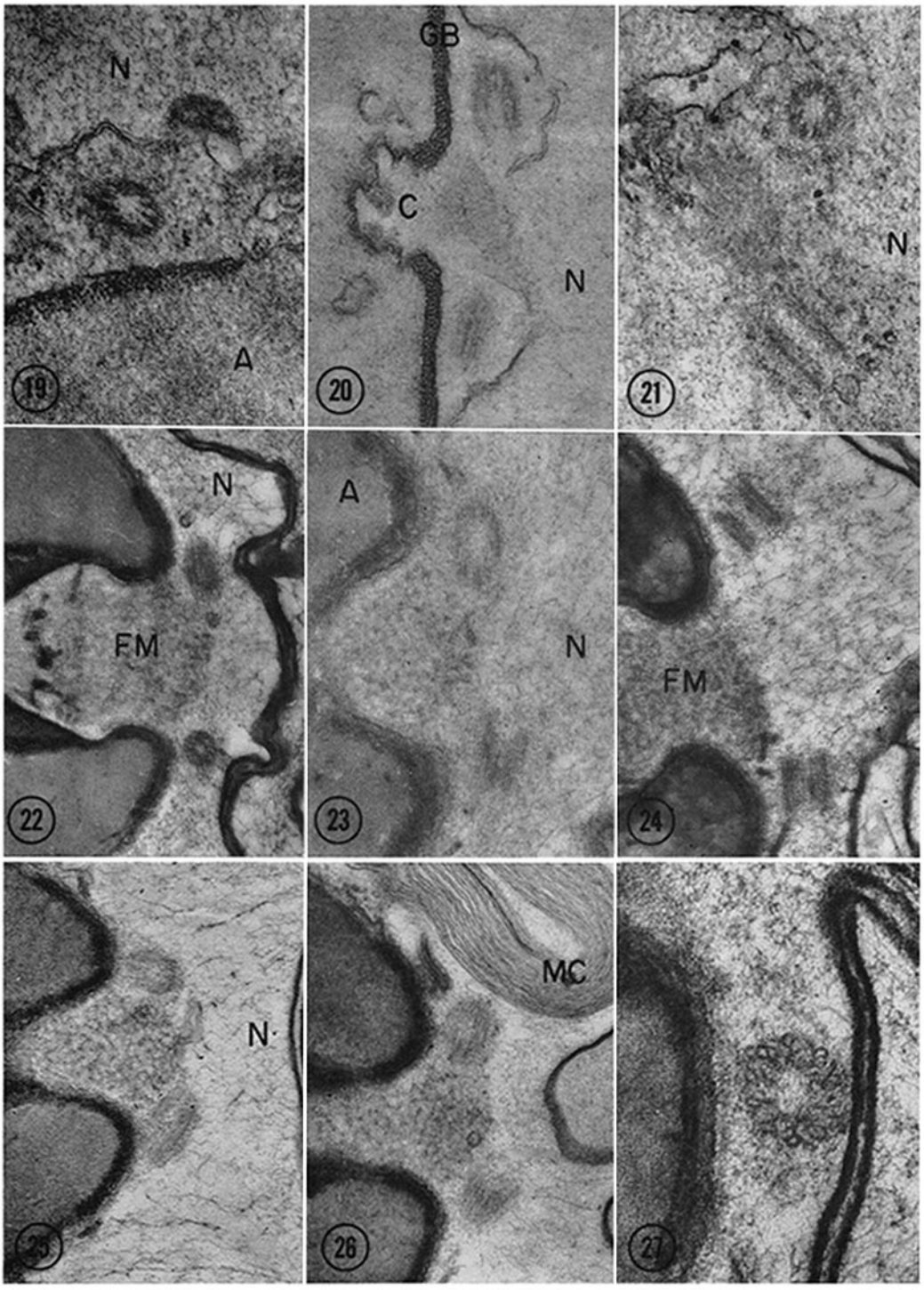
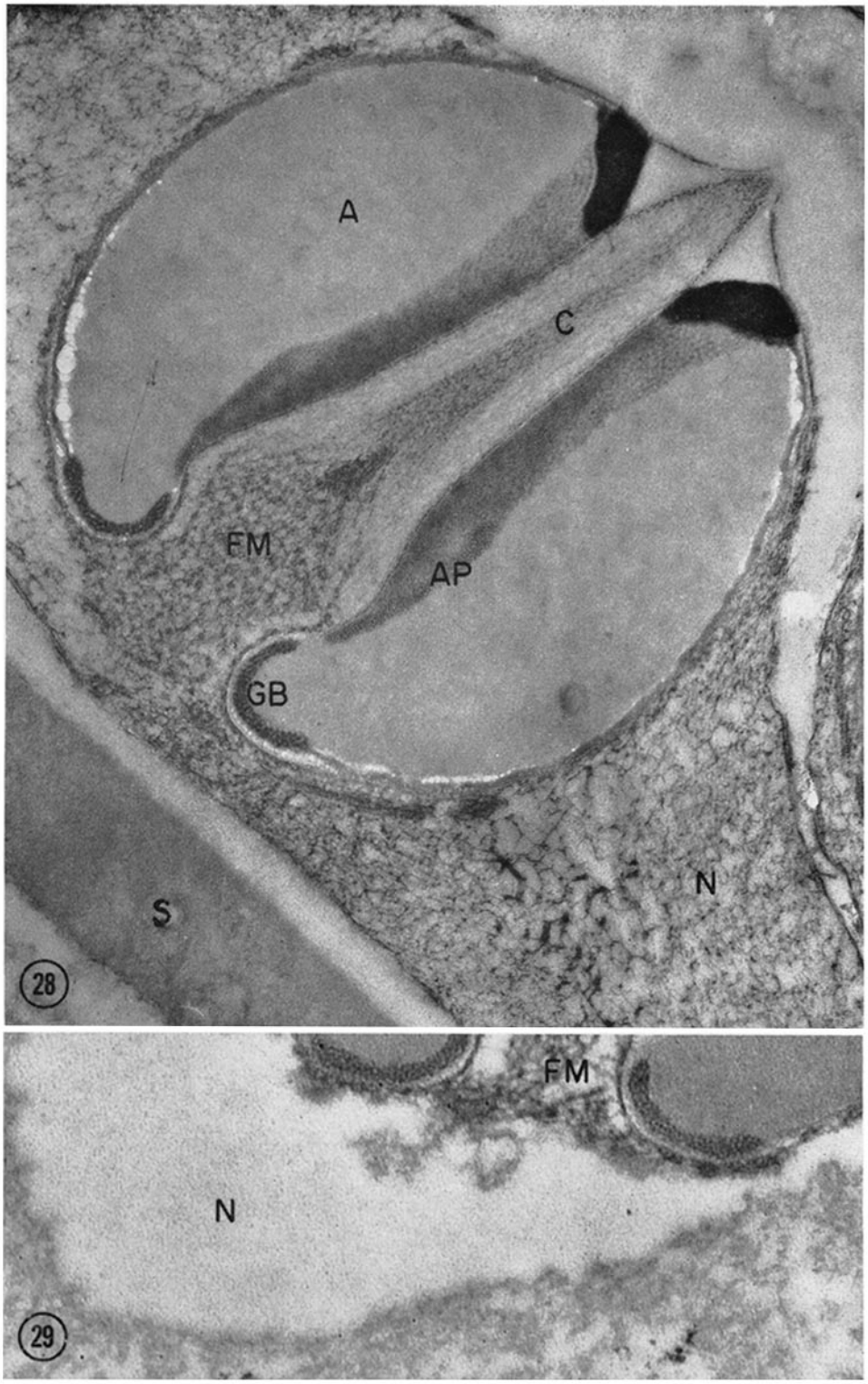


FIGURE 18 Mature sperm of *Cancer irroratus* packaged in a spermatophore (*S*). Membrane complex material (*MC*) appears degenerate. The arms (*AR*) are continuous with and of the same morphology as the body of the nucleus (*N*). × 10,000.



FIGURES 19–27 Centrioles in spermatid and sperm cells. Fig. 19. A centriole in its characteristic position between nucleus (*N*) and acrosome (*A*) at spermatid stage III. Fig. 20. Spermatid IV stage when acrosomal core (*C*) is invaginating. Fig. 21. Centrioles surrounded by nucleoplasm in the immature sperm. Figs. 22–26. Centrioles in mature sperm. Their orientation with respect to one another is variable, but they persist in the nucleus (*N*) near the irregularly fibrous material (*FM*) at the base of the acrosome. × 33,000. Fig. 27. The structure of the crab centriole in mature sperm shows the characteristic pinwheel configuration of nine triplet microtubules. × 65,000.



FIGURES 28 and 29 EM cytochemistry of the mature sperm. Fig. 28. Formalin-fixed control, showing branching and anastomosing DNA filaments in the nucleus (*N*). × 44,000. Fig. 29. DNase treated. The filaments in the nucleus have been removed, but the irregularly fibrous material (*FM*) of the acrosomal core (*C*) remains. × 50,000.

microtubules within the lattice, and some mitochondria or their derivatives caught in the membrane framework.

RELATIONSHIPS TO ER AND NUCLEAR ENVELOPE: In the present investigation the membrane complex first began to form by apparent fusion of the cisternae of the endoplasmic reticulum, concomitant with a loss of ribosomes. Only at later stages did portions of the nuclear envelope become continuous with the complex and then presumably contribute membrane to the growing complex. This viewpoint is contrary to that of Pochon-Masson (1962, 1968), who believes that the complex forms exclusively from the nuclear envelope in *Carcinus maenas*. She notes the frequency of nuclear pores in the region of a forming complex and cautiously suggests the possibility of transfer through the pores of material which participates in complex formation. However, in *Cancer* crabs pores are conspicuously absent in areas in which the membrane complex is in contact with the nuclear envelope.

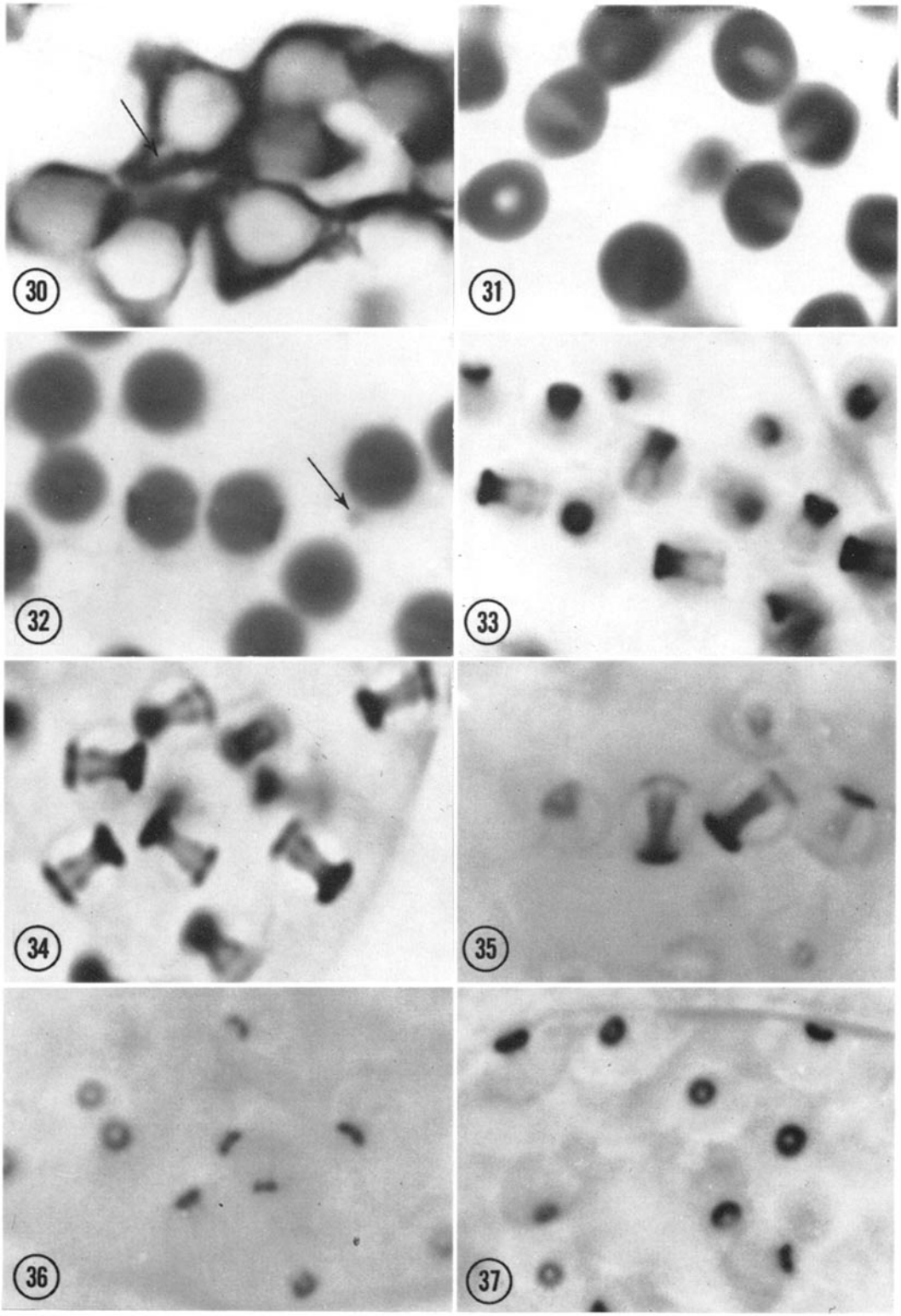
In *Procambarus clarkii* Moses (1956, 1961b) found that the extensive membrane convolutions in the sperm of this crayfish were formed from elaborations of the nuclear envelope and associations between cytoplasmic and nuclear membranes. On the other hand, Kaye et al. (1961) found several different but related types of membrane systems derived from the endoplasmic reticulum in the crayfish *Cambaroides japonicus*. They homologize their "dense filamentous ER" to the membrane convolutions of Moses and note that late in development this ER is continuous with the nuclear envelope, which probably contributes membrane at this time.

The variations in the formation of an ultimately similar membrane complex in the above four species emphasize the interrelationships of endoplasmic reticulum, nuclear envelope, and other membrane systems in the cell. In fact, in *Cancer* the three types of membrane, i.e. ER, membrane complex, and nuclear envelope, have been observed interconnected to one other (Fig. 3). Each element is distinct and presumably has different functions.

MITOCHONDRIA: Several references to what is here called the membrane complex appear in the classical literature. Koltzoff (1906) observed a coalescence of small dark granules, which he believed to be mitochondria, which form a mass adjoining the nucleus. He homologized this mass to the midpiece of flagellated sperm. Retzius (1909) found small masses on the sides of the capsule (acrosome) in *Cancer pagurus* and *Carcinus maenas* which he likened to *Nebenkorper*. According to Binford (1913), in the crab *Menippe mercenaria* a darkly-staining portion of the cytoplasm wedges its way between nucleus and acrosome to form a ring. Like Koltzoff, he believed this portion of cytoplasm to be mitochondrial in nature. Fasten (1918) described a mitochondria-like mass that stains like chromatin in *Cancer magister*, which suggests a possible nuclear contribution to its formation. The mass later becomes a ring between the nucleus and acrosome and ultimately unites with the nucleus, to produce a nucleus-mitochondrial cup which gives rise to the arms of the sperm, according to this investigator.

The above classical descriptions all emphasize the role of mitochondria in the membrane complex. Mitochondrial contribution to the membrane complex in spermatids has been observed under the electron microscope; however, mitochondria are absent, degenerate, or considerably altered in the mature sperm (Yasuzumi, 1960; Moses, 1961a, b; Yasuzumi et al., 1961; Kaye et al., 1961; Pochon-Masson, 1962, 1965b, c, 1968; Langreth, 1965; Chevaillier and Maillet, 1965; Brown, 1966; Anderson and Ellis, 1967). The ques-

FIGURES 30–37 Light microscope cytochemistry of the mature sperm. Fig. 30. Feulgen staining of nucleus and arms. Membrane complex areas (arrow) do not stain. Fig. 31. PAS reaction for polysaccharides. The outer region of the acrosome stains intensely; the core is unstained. Fig. 32. Fast green pH 2 staining for total protein. The arrow refers to a membrane complex area outside the acrosome, which stains faintly. Fig. 33. Picric acid-brom phenol blue reaction for basic protein in the acrosome inner region. Fig. 34. Fast green pH 8.1 stain for basic protein. Fig. 35. Fast green pH 8.1 stain for basic protein. Non-TCA-treated control. Fig. 36. DNase treatment and fast green pH 8.1 staining. Only the acrosomal lip takes up the dye. Fig. 37. RNase treatment and fast green pH 8.1 staining. The dye distribution is the same as with DNase pretreatment. × 4500.


30
31
32
33
34
35
36
37

tion of whether or not these dervativies in the membrane complex retain mitochondrial energy functions is equivocal. Although André (1962) was able to obtain a strong Janus green B coloration in three species of European crayfish (*Astacus spp.*), this test for mitochondrial activity was negative in the crayfish *Procambarus clarkii* (Moses, 1961a, b) and negative in *Cancer borealis* in the present study.

MICROTUBULES: Microtubules have been observed in the membrane complex of several decapods besides *Cancer* crabs. Pochon-Masson (1965c, 1968) claims an unusual mode of origin for the microtubules in *Astacus leptodactylus* and *Carcinus maenas*. According to her, they arise in the nucleoplasm and secondarily become incorporated into the membrane complex. Yasuzumi and Lee (1966) also claim a nuclear origin for the microtubules of *Cambaroides japonicus*. In *Cambarus sp.*, Anderson and Ellis (1967) observed intranuclear and intra-arm tubules of 300 A diameter which seemed to originate during spermiogenesis from pericentriolar densities. In *Cancer* crabs, on the other hand, the microtubules first appear in the lattice framework of the membrane complex as it forms in early spermatids. Only in very late spermatids, when nucleoplasm surrounds the membrane complex, do the microtubules gain a nuclear association.

Acrosome and Related Structures

FORMATION: The classical literature on decapod spermiogenesis is particularly confused in its descriptions of the origin of the "capsule" or acrosome. Its formation is attributed to Golgi elements by Bowen (1925), Grabowska (1927, 1929), and Nath (1937, 1965); to mitochondria by Koltzoff (1906), McCroan (1940), and Nath (1932, 1937, 1942, 1956, 1965); and even to nuclear derivatives by Grobben (1878) and Herrmann (1890). Several authors simply state that vesicles fuse or that a vacuole appears in the cytoplasm (Gilson, 1886; Labbé, 1903; Binford, 1913; Fasten, 1914, 1918, 1924, 1926; Worley, 1939). However, electron microscope studies eliminate direct Golgi, mitochondrial, and nuclear contributions to acrosome formation. Yasuzumi et al. (1961) pinpoint certain dense granules, which first appear in the interzonal spindle region, as the acrosomal precursor. Although Yasuzumi and his associates observed clusters of mitochondria adjacent to the acrosomal vesicle in the spermatid, they found no conclusive evidence that these clusters participate in acrosomal development. Pochon-Masson (1963, 1968) concludes that the acrosome is derived from the fusion of dilated ergastoplasmic cisternae, and she further notes that nuclear envelope derivatives may contribute to the acrosome. The results of the present study agree with those of Moses (1961b) and Chevaillier (1965) in that the acrosome forms from the fusion of cytoplasmic vesicles, some of which have RNP granules on their surfaces, and therefore appear to be derived from rough ER. Other vesicles involved in acrosome formation are agranular; a possible smooth ER contribution to the developing acrosome was also observed by Anderson and Ellis (1967).

The acrosomal core forms by the inpocketing of the acrosomal vesicle in the region closest to the nucleus. The invagination continues until it reaches the acrosomal cap on the opposite side. The formation of this core, "central canal," or "axial rod" in other decapods is similar to that described for *Cancer* (Yasuzumi, 1960; Moses, 1961a, b; Yasuzumi et al., 1961; Chevaillier, 1965; Chevaillier and Maillet, 1965; Pochon-Masson, 1965b, 1968). These investigators also noted fibrous and granular material within the core which resembles the types of material observed in mature *Cancer* sperm. As in the present study, most investigators could not relate these structures to any previously existing body other than some irregularly fibrous material. Pochon-Masson (1968), however, claims that the centrioles give rise to the core structures or "percutor organ," even though she admits finding no continuity between them. The centrioles in *Cancer* are located at the base of the acrosome and bear the same relationship to the core as do those in *Carcinus maenas*, studied by Pochon-Masson. In the present study, proximity was not considered sufficient evidence to claim a precursor-product relationship or inducer role for the centrioles. Perhaps Pochon-Masson was influenced by the classical literature, which depicted the centrioles themselves as transforming into central bodies, rods, tubes, rings, and other bizzare structures (Retzius, 1909; Binford, 1913; Reinhard, 1913; Fasten, 1918, 1924, 1926; Nath, 1932, 1937, 1942, 1956, 1965; Worley, 1939).

Derivatives of the acrosomal cap, as here described, make up the inner portion of the acrosome in the mature sperm of *Cancer* species. Similar structures, under a variety of names, have been observed by most EM investigators. For instance, the

dense lip described here and previously (Langreth, 1965) for *Cancer* appears to be homologous to the dense band of *Eriocheir japonicus* (Yasuzumi, 1960), the acrosomal rim of *Procambarus clarkii* (Moses, 1961a, b), the apical granule of *Nephrops norvegicus* (Chevaillier and Maillet, 1965), the apical cap of *Callinectes sapidus* (Brown, 1966), the horns of *Emerita analoga* (Bloch, 1966), and the opercular sphincter of *Carcinus maenas* and *Eupagurus bernhardus* (Pochon-Masson, 1965b, 1968). However, the structures of the inner region of the acrosome are best distinguished cytochemically, as will be discussed presently.

CYTOCHEMISTRY: The "capsule" structure of decapod sperm has been homologized to the acrosome of flagellated sperm because of its presumed formation from Golgi elements (Bowen, 1925). Although the concept of its direct origin from classical Golgi material is now no longer tenable, the structure can still be called an acrosome on the basis of its eversion upon fertilization and its PAS stainability, which has been noted by a number of investigators (Moses, 1956, 1961a, b; Yasuzumi, 1960; Langreth, 1965; Pochon-Masson, 1965b, 1968; Chevaillier and Maillet, 1965; Chevaillier, 1965, 1966b, 1967; Brown, 1966).

The structures of the acrosome have been subdivided into three regions on the basis of electron microscope observations. These regions are further characterized and distinguished by cytochemical studies. In *Cancer* the relationships are as follows:

(1) The outer region of the acrosome or "capsule", which is homogeneous and of moderate electron opacity, stains positively for polysaccharide (PAS) and total protein (pH 2 fast green).

(2) The inner region of the acrosome, including lip, cap derivatives, and granular belt, is composed of fibrous and granular structures of greater electron opacity and looks like an hourglass when viewed with the light microscope (after staining). It stains positively for basic protein (pH 8.1 fast green, picric acid-brom phenol blue), particularly the acrosomal lip. The total protein staining intensity is considerably greater than that of the outer region. PAS staining is absent. During the development of inner region structures, RNA is localized here, but it is absent in more mature sperm.

(3) The acrosomal core, the region formed by the invagination of the acrosomal vesicle, stains only for total protein.

In *Cancer* the acrosome has been observed to form by the coalescence of PAS-positive vesicles. Then as the acrosomal vesicle matures and differentiates, PAS staining becomes more homogeneous and intense in the outer region. This process differs slightly from acrosome formation in the langouste *Nephrops norvegicus* (Chevaillier, 1965). Until the vesicles coalesce there is no PAS staining. PAS-positive granules first appear at the superior pole and around the periphery of the acrosomal vesicle. They gradually fill in the vesicle as the sperm matures, so that the outer region is ultimately homogeneous for PAS stain. Chevaillier and Maillet (1965) characterized the PAS-positive material in *Nephrops* as a polysaccharide other than glycogen. This polysaccharide is insensitive to salivary or pancreatic amylase and to hyaluronidase, but PAS stainability can be blocked by prior treatment with dimedone.

A puzzling difference between *Cancer* and *Nephrops* is the absence of any protein in the outer region of the acrosome of the langouste (Chevaillier and Maillet, 1965). *Nephrops* appears to be exceptional, however, for the outer acrosomal regions of both *Carcinus maenas* and *Eupagurus bernhardus* stain for total protein (Chevaillier, 1966b, 1967). As in *Cancer* crabs, ordinary proteins are present in the acrosomal core and inner region of the acrosome in all three species. Although basic proteins were variable in amount in these decapods, Chevaillier found them only in the inner regions of the acrosome, which is the same location as in *Cancer* species. The basic proteins appeared to be uncomplexed with nucleic acids, for staining with pH 8.1 fast green occurred without hot TCA pretreatment and was not enhanced after RNase digestion (Chevaillier, 1967). In *Cancer*, neither DNase nor RNase enhanced the staining of the lip region, but staining of the basal regions was inhibited. As mentioned previously, the nucleases probably were behaving like protein anions, thereby blocking fast green binding by the basic proteins. When compared to the non-TCA-treated control, hot TCA treatment increased fast green staining of the basic proteins in *Cancer*. This effect was also observed by Chevaillier and Maillet (1965) in *Nephrops*. This evidence is not convincing proof that acrosomal basic proteins are complexed to any nucleic acid because of the nuclease results, and the fact that TCA hydrolysis of pure histone spots on filter paper is known to increase their stainability with acid dyes (Swift, H., and M. Gorovsky, unpub.).

In *Cancer* species and the decapods studied by Chevaillier (1966b, 1967), only the inner region of the acrosome contained basic protein. In the mole crab *Emerita analoga* the entire capsule as well as the horns (the latter homologous to the acrosomal lip of the present study) stained with pH 8.1 fast green (Bloch, 1966). Bloch further characterized the capsule as lysine-rich and the horns as arginine-rich by deamination, fluorodinitrobenzene (FDNB), Sakaguchi, and fast green-eosin techniques. On the other hand, Chevaillier (1967) found only arginine-rich basic proteins with similar methods. It seems, therefore, that the capsule basic protein of *Emerita* is distinctive from other decapods in both its distribution and its amino acid composition.

For a brief period during spermiogenesis (spermatid stages III and IV) while the inner region of the acrosome is differentiating, RNA is distributed in a pattern like that of the basic protein. However, this RNA is lost by the end of stage IV. Although Yasuzumi (1960) reported finding RNA in the acrosome of nearly mature sperm of *Eriocheir japonicus*, he used methyl green pyronin rather than azure B, which was used here, and did not perform RNase and DNase controls. Why RNA is present only at a specific time in *Cancer* and perhaps in *Eriocheir* and why it is not observed in other decapods which have been examined cytochemically (Moses, 1961b; Chevaillier and Maillet, 1965; Chevaillier, 1967; Pochon-Masson, 1968) are unknown.

Nucleus

CHROMATIN: During spermiogenesis in *Cancer* the appearance of the chromatin changes from homogeneous fine granules and fibrils of fairly low electron opacity to branching and anastomosing filaments and clumps of fibers of varying dimensions in electron-translucent nucleoplasm. This transformation occurs at the time that histone stainability of the nucleus is lost. If fact, in the mature sperm no histone, protamine, or other protein can be detected in the nucleus. The "bare" DNA of the mature sperm resembles the DNA of bacterial chromatin (Ryter and Kellenberger, 1958; Kellenberger, Ryter, and Séchaud, 1958) and the DNA found in mitochondria and chloroplasts of eukaryotes (Nass, Nass, and Afzelius, 1965; Ris and Plaut, 1962; Swift, Rabinowitz, and Getz, 1968).

This unusual nuclear morphology of the mature sperm has been observed in *Procambarus clarkii* (Moses, 1961a, b), *Cambaroides japonicus* (Yasuzumi et al., 1961), *Cancer borealis* (Langreth, 1965), *Callinectes sapidus* (Brown, 1966), and *Eupagurus bernhardus* (Chevaillier, 1966a). Moreover, examination of published electron micrographs of other decapod sperm show that the nuclear morphology also consists of branching fibrils embedded in empty, structureless nucleoplasm. The only exceptions appear to be *Homarus vulgaris* (Pochon-Masson, 1965b, c) and *Nephrops norvegicus* (Chevaillier and Maillet; Chevaillier, 1966a) where the nucleus remains homogeneous, granular, and fairly electron opaque.

Chevaillier (1966a) has correlated appearance of fibrillar chromatin with the loss of all protein staining in the sperm nucleus of *Eupagurus bernhardus*. He has further observed that the chromatin behaves like that of bacteria in respect to EM appearance after Kellenberger's fixation and the versene test (Ryter and Kellenberger, 1958). Loss of protein staining in the nucleus of mole crab *Emerita analoga* sperm has been observed by Bloch (1966) and Vaughn (1968). It is interesting that *Nephrops norvegicus*, the only decapod known to retain histone in its sperm nucleus, does not have chromatin resembling that of bacteria in EM morphology (Chevaillier and Maillet, 1965; Chevaillier, 1966a).

POSSIBLE RELATIONSHIPS BETWEEN NUCLEAR AND ACROSOMAL BASIC PROTEINS: It has been suggested by Chevaillier (1966a) that the histones of the nucleus of the developing spermatid migrate to the cytoplasm and aggregate in the inner region of the acrosome, where basic proteins not complexed to nucleic acids are localized in mature decapod sperm. This hypothesis, however, does not account for the origin of the acrosomal basic proteins of *Nephrops norvegicus* sperm, whose nuclei do not lose their histone. On the other hand, Vaughn (1968) reported that the isolated basic proteins of the sperm of *Emerita analoga*, which has no detectable nuclear protein, show overall similarity to somatic histones as studied by polyacrylamide electrophoresis. He believes that microspectrophotometric evaluation of DNA/protein ratios in the nuclei of developing spermatids also supports Chevaillier's suggestion because cytoplasmic basic proteins accumulated as nuclear histones declined. His preliminary experiments on autoradiography of H^3-arginine and

H^3-lysine incorporation, however, indicate that at least some of the sperm basic protein may be synthesized in the cytoplasm. Bloch (1966), also studying *Emerita analoga*, noted that the basic proteins arose in a vacuole in the early spermatid at a time when the nucleus still stained for histone. He presumed that the acrosomal basic proteins were synthesized in the cytoplasm and suggested that newly-made sperm histones may accumulate and be stored there before entering the nucleus at some later undetermined time.

In *Cancer* crabs basic proteins appear in the inner region of the acrosome in late spermatids at a time when the nucleus still stains for histone. At the EM level, the acrosomal cap and its derivatives (the regions staining for basic protein) show corresponding changes at this time: greater electron opacity and a more homogeneous, finely particulate consistency. The nucleus is still homogeneous, granular, and fairly electron opaque, i.e., its morphology has not yet changed to branching fibrils in empty nucleoplasm, which is the electron image correlated with loss of basic protein. Therefore, in *Cancer* there is no clear evidence for a transfer of the nuclear basic proteins to the inner region of the acrosome. It thus seems likely that these acrosomal basic proteins are synthesized in the cytoplasm and are not of nuclear origin.

ARMS: The radiating projections or arms are clearly extensions of the nucleus in *Cancer* crabs. They are Feulgen-positive, continuous with the body of the nucleus, and exhibit the characteristic chromatin morphology of these species under the electron microscope. As noted earlier, in very late spermatids the membrane complex becomes surrounded by nucleoplasm, and complex membrane systems can be observed in the nucleus of the mature sperm. These membranes, usually located at the bases of the arms, are Feulgen-negative and stain for total protein with pH 2 fast green.

The origin of the arms has long been a subject of controversy in decapod literature. Andrews (1904), studying the crayfish *Cambarus affinis*, appears to be the first to state clearly that the arms derived from the nucleus. But not until McCroan (1940) found the arms of *Cambarus virilis* to be Feulgen-positive was Andrews' view generally accepted. Still, in decapods with long spindling arms, no DNA can be detected in their distal portions (Chevaillier and Maillet, 1965, for *Nephrops norvegicus*; Barker and Austin, 1963, for *Emerita talpoida*; Brown, 1966, for *Callinectes sapidus*; Pochon-Masson, 1965c, for *Homarus vulgaris*; Chevaillier, 1966a, for *Eupagurus bernhardus*). It is possible, as was suggested by Moses (1961b), that in the extremities the DNA concentration is too low to be detectable cytochemically. In several decapods DNA is found throughout the entire length of the arms, as in *Cancer*. These include the crab *Eriocheir japonicus* (Yasuzumi, 1960), the crayfish *Cambaroides japonicus* (Yasuzumi et al., 1961), and the crayfish *Procambarus clarkii* (Moses, 1961a, b).

The membrane systems in the arms of most decapods appear to be derivatives of what is here called the membrane complex. Chevaillier (1966a) has found them to be Feulgen-negative but pH 2 fast green-positive in *Eupagurus bernhardus*, as in *Cancer* crabs. In *Carcinus maenas*, membrane systems are also located as they are in *Cancer*, at the bases of the arms next to the acrosome and surrounded by nucleoplasm (Pochon-Masson, 1968).

One can conclude that in decapods at least part of the arms are nuclear in origin, but that another, originally cytoplasmic component is also present, which appears to be derived from the membrane complex (fused double membranes, altered or degenerate mitochondria, and, in some species, microtubules). In species with longer arms, the cytoplasmic component seems to be more highly developed in the mature sperm than in species with short stubby arms, such as *Cancer* and *Carcinus* (Chevaillier and Maillet, 1965; Pochon-Masson, 1965c, 1968). Possibly the membranes and tubules help to support these long arms, as has been suggested by Anderson and Ellis (1967).

NUCLEAR ENVELOPE RELATIONSHIPS: The outer membrane over the nucleus and arms of mature *Cancer* sperm is a complex structure which has been shown to result from the fusion of the two layers of the nuclear envelope and the cell membrane (Langreth, 1965). Similar complicated cell boundaries have been observed in other decapods. Brown (1966) noted that the nucleus of *Callinectes sapidus* in mature sperm is covered externally by at least three and possibly more membranes. Chevaillier (1966a) has observed a thick, dense, and tight complex, limiting boundary in *Eupagurus bernhardus*, which he presumed to be tripartite like *Cancer*, but he could

not distinguish the membranes. Moses (1961a) was uncertain of the number of layers in the nuclear boundary of *Procambarus clarkii* sperm but reported a close association of the nuclear envelope and the cell membrane by late spermatid stages (1961b). Chromatin fibrils have been observed frequently in close relationship to this multiple membrane wall, where they appear to contribute to its density. This association seems to be a general feature of decapod sperm (see Chevaillier, 1966a, for review).

In late spermiogenesis the nuclear envelope always appears to break down specifically in the region opposite the acrosomal core. Since the appearance of small 300 A vesicular or tubular structures accompanies this breakdown, it seems reasonable to assume that they are at least partly nuclear envelope derivatives. These tubules are unlike the 150–180 A microtubules proliferated at the nuclear envelope of *Astacus fluviatilis* spermatocytes in postmating season individuals (Meek and Moses, 1961). Breakdown of the nuclear envelope next to the acrosomal core may be of functional advantage, since electron microscope studies of the fertilization reaction (Brown, 1966; Pochon-Masson, 1965a, 1968) suggest that the nucleus is injected into the egg through the core after acrosome eversion. Because of technical difficulties, the incorporation of the sperm nucleus into the egg cytoplasm has not been observed. What happens to a nucleus with a tripartite membrane on one side and no apparent membrane on the other side, and by what means an intact nuclear envelope is reformed, if it is, are unanswered questions. Localized areas of membranes can be disrupted by fixation artefact, but it seems unlikely that that is the case here, since loss of a clearly recognizable nuclear envelope where it apposes the acrosomal core has been observed under a variety of fixation conditions and in other crabs (*Callinectes sapidus* by Brown, 1966; *Carcinus maenas* by Pochon-Masson, 1968). Also, the complete surrounding of the centrioles by nucleoplasm, as discussed below, clearly involves controlled localized membrane disruption.

Centrioles

In *Cancer* crabs the centrioles can be observed in all stages of spermatogenesis. They take up a position between the nucleus and acrosome, inside the ring of membrane complex, midway through spermiogenesis. When the nuclear envelope breaks down at the base of the acrosome core in late spermiogenesis, the centrioles become surrounded by nucleoplasm but remain in the same location with respect to the acrosome. From the time they lose their perpendicular orientation midway through spermiogenesis, their position with regard to one another is variable. The centrioles persist in *Cancer*, for they are found in the sperm in the seminal receptacles of females. Centrioles become degenerate or are absent in mature sperm of several decapods: *Procambarus clarkii* (Moses, 1961a, b), *Eriocheir japonicus* (Yasuzumi, 1960), *Cambaroides japonicus* (Yasuzumi et al., 1961), and *Eupagurus bernhardus* (Chevaillier, 1966a). But in others they persist: *Homarus vulgaris* (Pochon-Masson, 1965a), *Cancer borealis* (Langreth, 1965), *Nephrops norvegicus* (Chevaillier and Maillet, 1965), and *Carcinus maenas* (Pochon-Masson, 1968). Why they become degenerate in some decapods and not in others cannot yet be explained. As noted earlier, they do not appear to be functional in these aflagellate sperm. Whether or not the centrioles, in the species which have them, reach the egg and function there is also unknown.

The author gratefully acknowledges the advice and encouragement of Dr. Hewson Swift throughout the course of this investigation and in the preparation of the manuscript.

This research was supported by a National Science Foundation Predoctoral Fellowship, by National Institutes of Health Training Grant HD 174, and research grants to Dr. Swift. The work was done in partial fulfillment of the requirements for the Ph.D. degree.

Received for publication 18 April 1969, and in revised form 23 July 1969.

REFERENCES

ALFERT, M., and I. I. GESCHWIND. 1952. A selective staining method for the basic proteins of cell nuclei. *Proc. Nat. Acad. Sci. U.S.A.* **39**:991.

ANDERSON, W. A., and R. A. ELLIS. 1967. Cytodifferentiation of the crayfish spermatozoon: acrosome formation, transformation of mitochondria and development of microtubules. *Z. Zellforsch.* **77**:80.

ANDRÉ, J. 1962. Contribution à la connaissance du

chondriome. Etude de ses modifications ultrastructurales pendant la spermatogenèse. *J. Ultrastruct. Res. Suppl.* **3**:1.

ANDREWS, E. A. 1904. Crayfish spermatozoa. *Anat. Anz.* **25**:456.

BARKER, K. R., and C. R. AUSTIN. 1963. Sperm morphology of *Emerita talpoida*. *Biol. Bull.* **125**:361.

BINFORD, R. 1913. The germ-cells and the process of fertilization in the crab, *Menippe mercenaria*. *J. Morphol.* **24**:147.

BLOCH, D. P. 1966. Cytochemistry of the histones. *Protoplasmatol. Handb. Protoplasmaforsch.* **5**(3d):1.

BLOCH, D. P., and H. Y. C. HEW. 1960. Schedule of spermatogenesis in the pulmonate snail *Helix aspersa* with special reference to histone transition. *J. Biophys. Biochem. Cytol.* **7**:515.

BOWEN, R. H. 1925. Further notes on the acrosome of the animal sperm. The homologies of non-flagellate sperms. *Anat. Rec.* **31**:201.

BROWN, G. G. 1966. Ultrastructural studies of sperm morphology and sperm-egg interaction in the decapod *Callinectes sapidus*. *J. Ultrastruct. Res.* **14**: 425.

BURGOS, M. H., and D. W. FAWCETT. 1955. Studies on the fine structure of the mammalian testis. I. Differentiation of the spermatids in the cat (*Felis domestica*). *J. Biophys. Biochem. Cytol.* **1**:287.

BURGOS, M. H., and D. W. FAWCETT. 1956. An electron microscope study of spermatid differentiation in the toad, *Bufo arenarum* Hensel. *J. Biophys. Biochem. Cytol.* **2**:223.

CHEVAILLIER, P. 1965. Formation de la capsule du spermatozöide de la Langoustine *Nephrops norvegicus* L.: évolution des polysaccharides au cours de la spermiogenèse. *C. R. Acad. Sci. Paris.* **261**:3225.

CHEVAILLIER, P. 1966a. Contribution à l'étude du complexe ADN-histone dans le spermatozöide du pagure *Eupagurus bernhardus* L. (Crustacé Décapode). *J. Microsc.* **5**:739.

CHEVAILLIER, P. 1966b. Structure et constitution cytochimique de la capsule du spermatozöide des Crustacés Décapodes. *C. R. Acad. Sci. Paris.* **262**: 1546.

CHEVAILLIER, P. 1967. Mise en évidence et étude cytochimique d'une proteine basique extranucléaire dans les spermatozöides des Crustacés Décapodes. *J. Cell Biol.* **32**:547.

CHEVAILLIER, P., and P. L. MAILLET. 1965. Structure fine et constitution cytochimique du spermatozöide de la Langoustine *Nephrops norvegicus* L. (Crustacé Décapode). *J. Microsc.* **4**:679.

FASTEN, N. 1914. Spermatogenesis of the American crayfish, *Cambarus virilis* and *Cambarus immunis* with special reference to synapsis and the chromatoid bodies. *J. Morphol.* **25**:587.

FASTEN, N. 1917. Male reproductive organs of Decapoda, with special reference to Puget Sound forms. *Puget Sound Marine Sta. Pub.* **1**:285.

FASTEN, N. 1918. Spermatogenesis of the Pacific coast edible crab, *Cancer magister* Dana. *Biol. Bull.* **34**:277.

FASTEN, N. 1924. Comparative stages in the spermatogenesis of various *Cancer* crabs. *J. Morphol.* **39**:47.

FASTEN, N. 1926. Spermatogenesis of the black-clawed crab, *Lophopanopeus bellus* (Stimpson) Rathbun. *Biol. Bull.* **50**:277.

FLAX, M. H., and M. HIMES. 1952. Microspectrophotometric analysis of metachromatic staining of nucleic acids. *Physiol. Zool.* **25**:297.

GILSON, G. 1886. Etude comparée de la spermatogenèse chez les arthropodes. *Cellule.* **2**:83.

GRABOWSKA, Z. 1927. L'appareil de Golgi dans les spermatozöides des crustacés (*Astacus fluviatilis* et *Astacus leptodactylus*). *C. R. Soc. Biol.* **97**:3.

GRABOWSKA, Z. 1929. Über die Plasmakomponenten (Golgi-Apparat u.a.) in dem männlichen Geschlectszellen von *Potamobius astacus* L. *Polska Akad. Umiejętności. Krakow. Wydzial. Mat. Przyrod. Bull. Intern. B. Sci. Nat. II. Zool.* **2**:197.

GROBBEN, C. 1878. Beiträge zur Kenntniss der männlichen Geschlectsorgane der Dekapoden. *Arbeiten aus den Zoologischen Instituten der Universität Wien und der Zoologischen Station in Triest.* **1**:57.

HERRMANN, G. 1890. Notes sur la structure et le développement des spermatozöides chez les décapodes. *Bull. Sci. France et Belgique.* **22**:1.

HOTCHKISS, R. D. 1948. A microchemical reaction resulting in the staining of polysaccharide structures in fixed tissue preparations. *Arch. Biochem.* **16**:131.

KAYE, G. I., G. D. PAPPAS, G. YASUZUMI, and H. YAMAMOTO. 1961. The distribution and form of the endoplasmic reticulum during spermatogenesis in the crayfish, *Cambaroides japonicus*. *Z. Zellforsch.* **53**:159.

KAYE, J. S. 1962. Acrosome formation in the house cricket. *J. Cell Biol.* **12**:411.

KELLENBERGER, E., A. RYTER, and J. SÉCHAUD. 1958. Electron microscope study of DNA-containing plasms. II. Vegetative and mature phage DNA compared with normal bacterial nucleoids in different physiological states. *J. Biophys. Biochem. Cytol.* **4**:671.

KINGSLEY SMITH, B. V., and D. LACY. 1959. Residual bodies of seminiferous tubules of the rat. *Nature (London).* **184**:249.

KOLTZOFF, N. K. 1906. Studien über die Gestalt der Zelle. I. Untersuchungen über die Spermien der Decapoden. *Arch. mikr. Anat.* **67**:364.

LABBÉ, A. 1903. Sur la spermatogenèse des Crustacés Décapodes. *C. R. Acad. Sci. Paris.* **137**:272.

LACY, D. 1960. Light and electron microscopy and its

use in the study of factors influencing spermatogenesis in the rat. *J. Roy. Microsc. Soc.* **79**:209.

LACY, D. 1962. Certain aspects of testis structure and function. *Brit. Med. Bull.* **18**:205.

LANGRETH, S. G. 1965. Ultrastructural observations on the sperm of the crab *Cancer borealis*. *J. Cell Biol.* **27**:56A.

LUFT, J. H. 1961. Improvements in epoxy resin embedding methods. *J. Biophys. Biochem. Cytol.* **9**:409.

MCCROAN, J. E. 1940. Spermatogenesis of the crayfish, *Cambarus virilis*, with special reference to the Golgi material and mitochondria. *Cytologia.* **11**:136.

MEEK, G. A., and M. J. MOSES. 1961. Microtubulation of the inner membrane of the nuclear envelope. *J. Biophys. Biochem. Cytol.* **10**:121.

MOSES, M. J. 1956. Studies on nuclei using correlated cytochemical, light, and electron microscope techniques. *J. Biophys. Biochem. Cytol. Suppl.* **2**:397.

MOSES, M. J. 1961a. Spermiogenesis in the crayfish (*Procambarus clarkii*). I. Structural characterization of the mature sperm. *J. Biophys. Biochem. Cytol.* **9**:222.

MOSES, M. J. 1961b. Spermiogenesis in the crayfish (*Procambarus clarkii*). II. Description of stages. *J. Biophys. Biochem. Cytol.* **10**:301.

NASS, M. M. K., S. NASS, and B. A. AFZELIUS. 1965. The general occurrence of mitochondrial DNA. *Exp. Cell Res.* **37**:516.

NATH, V. 1932. The spermatid and the sperm of the crab, *Paratelphusa spinigera*. *Quart. J. Microsc. Sci.* **75**:543.

NATH, V. 1937. Spermatogenesis of the prawn, *Palaemon lamarrei*. *J. Morphol.* **61**:149.

NATH, V. 1942. The decapod sperm. *Trans. Nat. Inst. Sci. India.* **2**:87.

NATH, V. 1956. Cytology of spermatogenesis. *Int. Rev. Cytol.* G. Bourne and J. Danielli, editors. Academic Press Inc., New York. **5**:395.

NATH, V. 965. Animal Gametes (Male). A Morphological and Cytochemical Account of Spermatogenesis. Asia Publishing House, Bombay. p. 78.

PEARSON, J. 1908. *Cancer*. Liverpool Marine Biological Committee Memoirs, Memoir XVI. W. A. Herdman, editor. Williams & Norgate Ltd., London.

POCHON-MASSON, J. 1962. Le chondriofusome des gamètes mâles du Crustacé Décapode *Carcinus maenas*. *C. R. Acad. Sci. Paris.* **254**:4076.

POCHON-MASSON, J. 1963. Origine et formation de la vésicule du spermatozöide d'*Eupagurus bernhardus* (Décapode Anomoure). *C. R. Acad. Sci. Paris.* **256**:2226.

POCHON-MASSON, J. 1965a. Schéma du spermatozöide dévaginé de *Homarus vulgaris* (Décapode Macroure). *C. R. Acad. Sci. Paris.* **260**:5352.

POCHON-MASSON, J. 1965b. Schéma général du spermatozöide vésiculaire des Décapodes. *C. R. Acad. Sci. Paris.* **260**:5093.

POCHON-MASSON, J. 1965c. L'Ultrastructure des épines du spermatozöide chez les Décapodes (Macroures, Anomoures, Brachyoures). *C. R. Acad. Sci. Paris.* **260**:3762.

POCHON-MASSON, J. 1968. L'Ultrastructure des spermatozöides vésiculaires chez les Crustacés Décapodes avant et au cours de leur dévagination expérimentale. I. Brachyoures et Anomoures. *Ann. Sci. Natur. Zool. Biol. Anim. Sér.* 12, **10**:1.

REINHARD, L. 1913. Zum Bau der Spermien and zur Spermatogenese von *Potamobius leptodactylus* (*Astacus leptodactylus*). *Arch. Zellforsch.* **10**:324.

RETZIUS, F. 1909. Die Spermien der Crustaceen. *Biol. Untersuch.* **14**:1.

RIS, H., and W. PLAUT. 1962. Ultrastructure of DNA-containing areas in the chloroplast of *Chlamydomonas*. *J. Cell Biol.* **13**:383.

RYTER, A., and E. KELLENBERGER. 1958. Etude au microscope électronique de plasmas contenant de l'acide désoxyribonucleique. I. Les nucléoides des bactéries en croissance active. *Z. Naturforsch.* **13b**:597.

SABATINI, D. D., K. BENSCH, and R. J. BARRNETT. 1963. Cytochemistry and electron microscopy. The preservation of cellular ultrastructure and enzymatic activity by aldehyde fixation. *J. Cell Biol.* **17**:19.

SKINNER, D. M. 1967. Satellite DNA's in the crabs *Gecarcinus lateralis* and *Cancer pagurus*. *Proc. Nat. Acad. Sci. U.S.A.* **58**:103.

SMITH, M. 1964. Deoxyribonucleic acids of Crustacea. *J. Mol. Biol.* **9**:17.

SUEOKA, N. 1961. Variation and heterogeneity of base composition of deoxyribonucleic acids: a compilation of old and new data. *J. Mol. Biol.* **3**:31.

SUEOKA, N., and T. CHENG. 1962. Natural occurrence of a DNA resembling the deoxyadenylate-deoxythymidylate polymer. *Proc. Nat. Acad. Sci. U.S.A.* **48**:1851.

SWIFT, H. 1955. Cytochemical techniques for nucleic acids. *In* The Nucleic Acids. E. Chargaff and J. Davidson, editors. Academic Press Inc., New York. Vol. 2. p. 51.

SWIFT, H. 1966. The quantitative cytochemistry of RNA. *In* Introduction to Quantitative Cytochemistry. G. Weid, editor. Academic Press Inc., New York. p. 355.

SWIFT, H., B. J. ADAMS, and K. LARSEN. 1964. Electron microscope cytochemistry of nucleic acids in Drosophila salivary glands and *Tetrahymena*. *J. Roy. Microsc. Soc.* **83**:161.

SWIFT, H., M. RABINOWITZ, and G. GETZ. 1968. Cytochemical studies on mitochondrial nucleic acids. *In* Biochemical Aspects of the Biogenesis of Mitochondria. E. C. Slater, J. M. Tager, S. Papa,

and E. Quagliariello, editors. Adriatica Editrice, Bari, Italy. 3.

TORMEY, J. M. 1964. Differences in membrane configuration between osmium tetroxide-fixed and glutaraldehyde-fixed ciliary epithelium. *J. Cell Biol.* **23**:658.

VAUGHN, J. C. 1968. Histone metabolism in the crab *Emerita analoga*: spermiogenesis and early embryogenesis. *J. Cell Biol.* **39**:138A.

VENABLE, J. H., and R. COGGESHALL. 1965. A simplified lead citrate stain for use in electron microscopy. *J. Cell Biol.* **25**:407.

WORLEY, E. K. 1939. A study of the sperm-forming components in three species of Decapoda. *Cellule.* **48**:149.

YASUZUMI, G. 1960. Spermatogenesis in animals as revealed by electron microscopy. VII. Spermatid differentiation in the crab, *Eriocheir japonicus. J. Biophys. Biochem. Cytol.* **7**:73.

YASUZUMI, G., G. I. KAYE, G. K. PAPPAS, H. YAMAMOTO, and I. TSUBO. 1961. Nuclear and cytoplasmic differentiation in developing sperm of the crayfish, *Cambaroides japonicus. Z. Zellforsch.* **53**:141.

YASUZUMI, G., and K. J. LEE. 1966. Spermatogenesis in animals as revealed by electron microscopy. XVI. The microtubular structure and sites of thiamine pyrophosphatase activity in premature sperm of the Japanese crayfish. *Z. Zellforsch.* **73**:384.