

SPERMIOGENESIS IN THE CRAYFISH

(*PROCAMBARUS CLARKII*)

II. Description of Stages

MONTROSE J. MOSES, Ph.D.

From the Department of Anatomy, Duke University School of Medicine, Durham, North Carolina, and The Rockefeller Institute

ABSTRACT

The sperm of the crayfish, *Procambarus clarkii*, is relatively simple among decapod sperm and was described in the first paper of this series (28). The present paper details the development of this sperm as followed with the light and electron microscopes. The process is divided into six stages for purposes of description. The main points of interest discussed are the absence of mitochondria or mitochondrial derivatives in the mature sperm, the development of a complex acrosome in the absence of highly organized characteristic Golgi apparatus but in the presence of small stacks of annulate lamellae, and the changes in the nucleus. Of the latter, the elaborate convoluted sheets of membrane that are extensions of the nuclear envelope are unique. The nucleus undergoes unusual changes in size and shape that are accompanied by several phases of organization of the chromatin. In the mature sperm the nucleus is empty-appearing and notably lacking in any apparent high degree of order. The entire development of the sperm is consonant with the idea that the fate of the mitochondria and centrioles, structures that figure prominently in the elaborate architecture of flagellate sperm, is associated with the lack of a flagellum.

INTRODUCTION

The differentiation of sperm is, in a sense, a terminal process. With the possible exception of the nucleus, the structural elements of the spermatid undergo profound modifications in the course of sperm development that are apparently irreversible. Such cytoplasmic organelles as are not cast off to the surrounding nutritive cells evolve and become characteristic structures in the mature sperm whose function is primarily concerned with locomotion and penetration into the ovum to accomplish fertilization. Having served their pur-

pose, these structures apparently no longer figure in the essential functioning of the cell, and only the nucleus, containing its structurally and chemically modified complement of chromosomes, undergoes further differentiation to assume a functional role in the zygote. The entire process of spermiogenesis, then, is directed toward transferring the male chromosome complement to the ovum.

There are almost as many variations in the details of spermiogenesis in animals as there are

This paper is dedicated with warmth and appreciation to Professor Franz Schrader on the occasion of his seventieth birthday. In this way I would like to express my gratitude to him for many things, not least among which is the gentle and at the same time strict and critical introduction to cytology that I was fortunate enough to have received from him.

species. The early works of Koltzoff (21), for example, and later of Bowen (6) were outstanding in bringing out common structural denominators in the process. The homologies, especially those proposed by Bowen, rested mainly on the behavior and differentiation of three cytological components: (a) the mitochondria, which in flagellate sperm often aggregate to form a large nebenkern from which develops a tail envelope; (b) the Golgi elements, which differentiate in various ways and serve to produce the acrosome or true penetration apparatus; and (c) the centrioles, which often undergo diverse modifications but appear to function mainly in connection with the flagellum. The nucleus, although it assumes a variety of morphological configurations, seems to be reduced to a passive carrier of the essential genetic material. In the early work, the identification of these cytoplasmic components in their various transformations rested on staining methods of uncertain specificity together with certain recognizable morphological characteristics of the structures concerned (e.g., the Altmann-Champy method for mitochondria and osmium impregnation for the Golgi elements). In widely aberrant forms, however, where the development and appearance of the formed elements diverge widely from the more usual pattern, establishing homologies was more tenuous (see, for example, discussion of this point by Nath, 29).

The morphology and development of the aflagellate sperm in certain nematodes, arachnids, myriapods, and higher crustaceans are so divergent that there is a notable lack of agreement in the literature concerning their precise morphology and homology with flagellate types. In the past few years, electron microscopy has contributed a number of rewarding studies on the structure and development of a variety of flagellate sperm. In addition to providing an insight into more intimate structural interrelationships, the electron microscope permits more positive identification of such minute organelles as mitochondria, dictyosomes, and centrioles, as well as of more recently identified structures that are beyond the resolution of the light microscope. This approach has not yet been applied extensively to the development of the aflagellate sperm.

The present investigation embraces a detailed light and electron microscope study of spermiogenesis in a decapod crustacean, *Procambarus clarkii* (*Cambarus clarkii* Girard; see footnote in 28).

The results of this work have been presented previously elsewhere in brief (24, 25, 27). Although the related form *Cambarus virilis* has figured in at least two light microscope studies (10, 21), spermiogenesis in *Procambarus* has not hitherto been described.

The results of this study are presented in a series of papers. The first, a note describing the mature sperm and briefly outlining the salient steps in its formation, has been published (28). This, the second, will enlarge on the sequence of events by which the spermatid is transformed into a mature sperm. Subsequent papers will deal with the details of certain of these transformations as they affect specific cell organelles.

The mature sperm of crayfish¹ appears to have a simpler morphology than does the sperm of many other decapods (for example, the lobster *Homarus*, the hermit crab *Pagurus*, and the spider crab *Libinia* (40)), and thus offers advantages for an initial study. In order to set the stage for the description and discussion of the events to follow, it will be useful to outline some of the pertinent information about the mature sperm (28).

The mature sperm of *P. clarkii* (25, 28) is an oblate spheroid from which radiate four and occasionally five long processes or arms (Fig. 19). The nucleus, of which the arms appear to be extensions, occupies more than half of the sperm and is indented by a nearly spherical, cup-shaped, complex structure that has been identified as the acrosome (24). A complex membranous integument surrounds both the nucleus and the acrosome and is continuous with occasional trapped masses of convoluted and twisted membranes continuous with the nuclear envelope. As the sperm appears in the testis and in the vasa deferentia, the processes are wrapped around its body and the entire structure is contained in a thin, finely granular encasement which is periodic acid-Schiff-positive. When exposed to fresh water or to physiological solution, the arms are released and extend radially from the body of the sperm. The sperm is not motile. Two degenerating centrioles are seen in final stages of maturation. No recognizable mitochondria or mitochondrial derivatives can be distinguished, which is consistent with the observation that the sperm does not reoxidize and accumulate Janus green. One gains the impression that the absence of accessory structures such as mitochondria, which presumably provide energy for motility, and the atrophy of centrioles are associated with the lack

¹ See discussion of usage of common term "crayfish" and "crayfish" by Penn (33).

of a flagellum. The intracellular transformations that are involved in the maturation of this sperm are as remarkable as the sperm itself.

MATERIALS AND METHODS

Males of the crayfish *Procambarus clarkii* (*Cambarus clarkii* according to the older nomenclature; see reference in 28) were obtained in the spring² and kept in aquaria. They are hardy animals and thrive for several weeks on a diet of raw liver and aquatic plant material. Stages of spermatogenesis are best observed during the months of April through June. Testes were quickly dissected out of living animals and either examined fresh in physiological solution or immediately fixed in chilled fixative for light and electron microscopy.

Light Microscopy: Helly's fixative, followed by paraffin embedding and sectioning at 5 and 10 μ , was used to survey the tissue as a whole. Paraffin sections of testis fixed in Clarke-Carnoy ethyl alcohol-acetic acid 3:1 (3, page 59) or in 4 per cent neutral buffered formaldehyde were used for most cytochemical staining tests. Similar tissue fixed by the Champy-Kull method and embedded either in paraffin or in *n*-butyl methacrylate and sectioned at 5 μ and 2 μ , respectively, was used to relate these morphological observations to older ones in the literature. Close correlation between the light and electron microscope observations was maintained with 1 to 2 μ sections of the methacrylate-embedded osmium tetroxide-fixed material used for electron microscopy. Where appropriate, the thick sections were cut immediately adjacent to the thin ones and alternate sections of the same cells examined in the light and electron microscope (23). Such thick sections, cut on a Porter-Blum (Servall) microtome, were mounted on slides in the following way: Sections were lifted off the surface of the fluid in the microtome knife trough with a fine 00 camel's-hair brush and floated off on the surface of a drop of 10 per cent acetone in water on an albuminized glass slide. The slide was then warmed gently until the section flattened, and then was carefully heated further until the fluid evaporated. Care must be taken not to permit the fluid to boil, which is likely to produce bubbles under the section. When dry, the slide was heated for 5 to 10 minutes on a hotplate at about 100°C. A ring marked around the section on the undersurface of the slide with a diamond glass marking pencil aids in its location. Sections prepared in this way were examined either unstained with the

² From the Carolina Biological Supply Co., Elon College, North Carolina.

phase microscope by mounting the section in refractive index oil $n = 1.46^3$ (31), or following various stains with or without the methacrylate removed (see below).

For purely morphological observation paraffin sections were stained with hematoxylin-eosin, and both paraffin and methacrylate sections with iron hematoxylin. Sections of Champy-Kull fixed material were stained with Altmann's fuchsin to distinguish the "mitochondrial" elements, or impregnated with osmium tetroxide for approximately three days to differentiate "Golgi" elements. Cytochemical staining tests were carried out on paraffin sections of alcohol-acetic acid- and formaldehyde-fixed material as follows: Protein was localized by staining with fast green at pH 1.2 and differentiating overnight in absolute ethanol (39). Basic proteins were identified by the alkaline fast green method (1). Ribonucleic acid (RNA) was distinguished by its metachromasia following staining with azure B at pH 4.0 and differentiation in tertiary butyl alcohol (12). Deoxyribose nucleic acid (DNA), which stains orthochromatically with azure B, was characterized in sections digested with crystalline ribonuclease,⁴ 0.01 per cent at pH 6 for 1 to 3 hours at room temperature, followed by staining with azure B. The residual orthochromatic staining is due to DNA. The localization of DNA was also confirmed by Feulgen staining, which could be applied equally well to osmium-fixed methacrylate-embedded sections (24). Substances containing 1,2 glycol groups (presumably polysaccharides) were localized after oxidation with periodic acid and staining with the Schiff reagent (PAS reaction) (22, 18). This reaction was equally specific in osmium-fixed methacrylate-embedded sections with or without the embedding medium removed. Certain, but not all, proteins, presumably nuclear histones and reactive proteins of certain cytoplasmic organelles, bound appreciable amounts of fast green when such preparations were counterstained at pH 1.2 and differentiated in absolute ethanol. Apart from imparting specific cytochemical information, this combination of stains gives a very handsome preparation in which morphological details are enhanced (24). The most useful stain for both morphological and cytochemical characterization during spermiogenesis is Himes and Moriber's trichome (17), applied to paraffin sections after various fixations. Apart from facilitating distinction between nuclear, acrosomal, and other elements, it permits identification of DNA, polysaccharide, and protein when appropriate light

³ Obtained from Cargille Sons, Little Falls, New Jersey.

⁴ Worthington Biochemical Corp., Freehold, New Jersey.

filters transmitting only wavelengths absorbed by the particular dye complexes are used.

In addition to sectioned material, Feulgen-fast green squashes of alcohol-acetic acid-fixed material were used for quick identification and examination.

Living cells were teased from small pieces of testis kept moist with body fluids. Such cells were mounted in either insect Ringer's or modified Belar's solution (4) and were studied with the phase microscope. Photomicrographs of such living cells were taken with the aid of an electronic flash illuminator which was very kindly made available by Drs. D. W. Fawcett and S. Ito, then in the Department of Anatomy, Cornell University School of Medicine.

Light micrographs were made with Zeiss 1.3 N.A. $\times 90$ apochromat and Zeiss 1.3 N.A. $\times 90$ neofluar objectives, $\times 7$ compens and $\times 8$ projection oculars, on 35 mm microfilm. Appropriate filters were used as described; phase micrographs were made through a Wratten No. 74 green filter.

Electron Microscopy: Small pieces of testis about 1 mm in diameter were fixed in chilled buffered fixatives containing osmium tetroxide. The most commonly used fixative was 1 per cent OsO_4 in veronal acetate buffer adjusted to pH's ranging from 7.0 to

8.0 (32). Only minor variations in fine structure could be observed with changes in pH; the essential morphological features remained constant. However, the pH range 7.4-7.6 was most commonly employed, and fixation was usually from 20 to 30 minutes in the cold. Less distortion, especially shrinkage and agglomeration of particulate and fibrillar elements, was observed if sucrose was added to the fixative (0.045 g/ml according to Caulfield, 7). Fixation in osmium-sucrose was for from 20 to 45 minutes in the cold. The addition of 0.02 per cent CaCl_2 had little effect on the general morphology of germinal epithelium. The same morphological picture was also obtained after fixing overnight in Dalton's chrome osmium (9) fluid. The observations described in these papers were consistent regardless of the fixation used, and hence the various preparative procedures are used interchangeably in illustrating the results. Tissues were embedded in mixtures of *n*-butyl and methyl methacrylate to which Luperco CBD⁵ was added as catalyst. Thin sections for electron microscopy were cut at 100 $\text{m}\mu$ or less on a Porter-Blum microtome

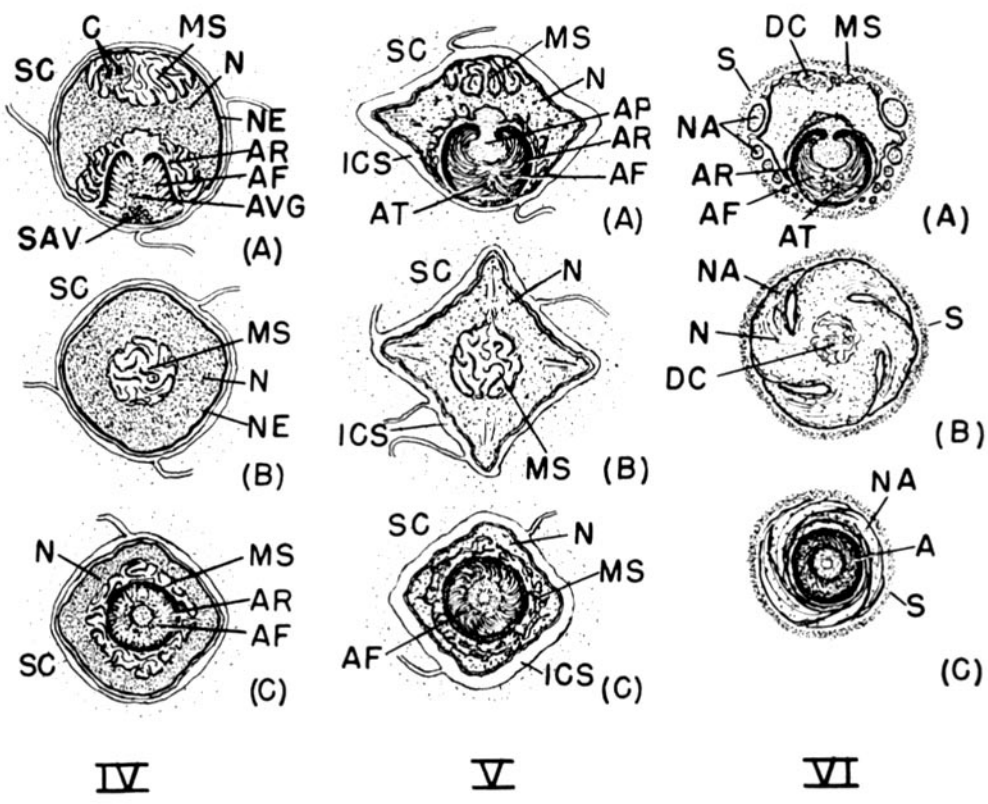
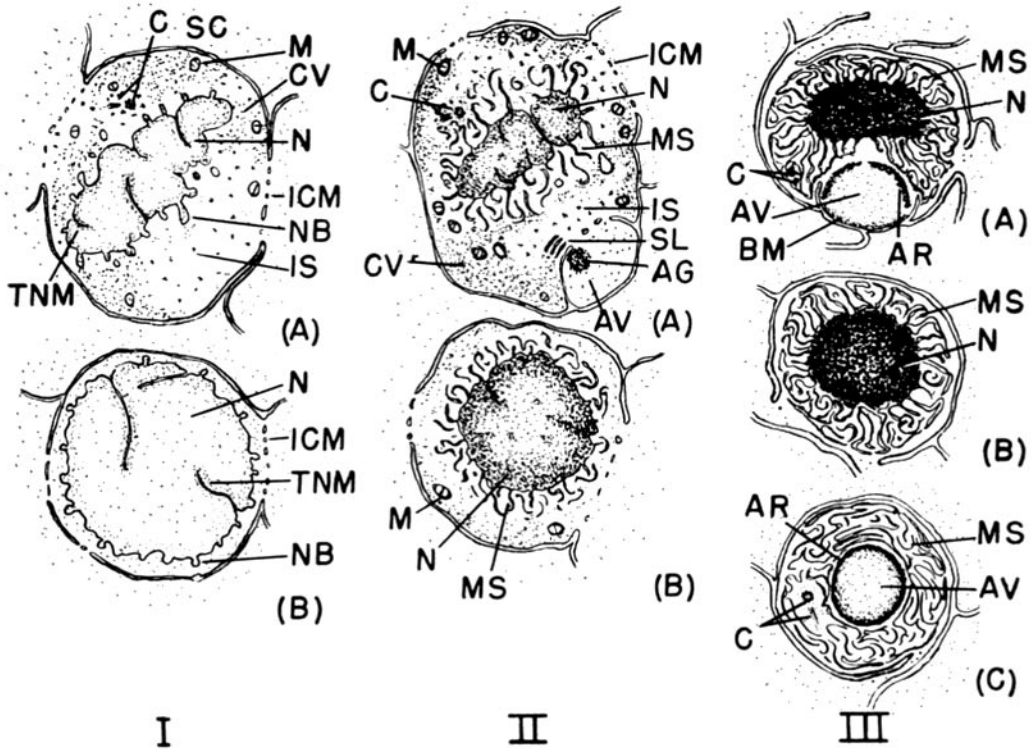
⁵ Obtained from the Lucidol Division of the Wallace and Tiernan Co., Inc., Buffalo, New York.

FIGURE 1

Diagrammatic representation of six stages of spermiogenesis in *Procambarus clarkii*. Views marked (A) are lateral, *i.e.* transverse through the nucleus. Those marked (B) are frontal, *i.e.* through the equator of the nucleus. (C) represents frontal sections through the acrosome.

The following list of abbreviations is used in all figures.

A, acrosome	ICM, incomplete cell membrane
AF, acrosomal filaments	ICS, intracellular space
AG, acrosomal granule	IS, interzonal spindle material
AP, cloudy material in acrosomal cup	M, mitochondrion
AR, acrosomal rim, cap, or crescent	MS, membrane sheets
AT, acrosomal tubules	N, nucleus
AV, acrosomal vesicle	NA, nuclear arm or process
AVG, clusters of 200 A granules found in acrosomal vesicle	NB, nuclear blebs
BM, basal membrane of acrosomal vesicle	NE, nuclear envelope
C, centriole	NV, intranuclear vesicle
CG, clusters of 0.05 to 0.1 μ granules around centriole	S, periodic acid-Schiff-positive "shell"
CH, chromatin	SAV, small acrosomal vesicles
CMS, cell membrane of spermatid	SC, sustentacular cell
CM-SC, cell membrane of sustentacular cell	SG, dense (500 A) granules in interzonal spindle area
CV, cytoplasmic vesicles	SI, inclusion in sustentacular cell
DC, degenerating centriole	SL, stacked lamellae
DG, dense granules (150 A)	SM, sperm (spermatid) membrane integument
ERG, granular endoplasmic reticulum	SS, shrinkage space
	TNM, trapped nuclear membrane
	V + B vesicles and dense (150 A) granules



(Servall) and were mounted on carbon-coated grids. Electron micrographs were made with either an RCA EMU 2C (Rockefeller Institute) or a Philips EM 100B (Duke University) electron microscope at 60 kv with compensated objective polepieces and approximately 25 μ objective aperture. Images were registered on plates (2×10 or $3\frac{1}{4} \times 4\frac{1}{4}$ inches) at magnifications of from 5000 to 10,000 and enlarged photographically as needed.

OBSERVATIONS

The transitions through which the spermatid passes during maturation may be divided into six stages. While each of these is somewhat arbitrary in its designation, the progressive alterations are so distinct that they fit easily into the scheme and their description is thus facilitated. Each stage is characterized by at least one sharp morphological change, and although there may be some overlap in timing, the appearance of the stage prototypes among individual crayfish is sufficiently regular to allow generalizations. Stages have been dia-

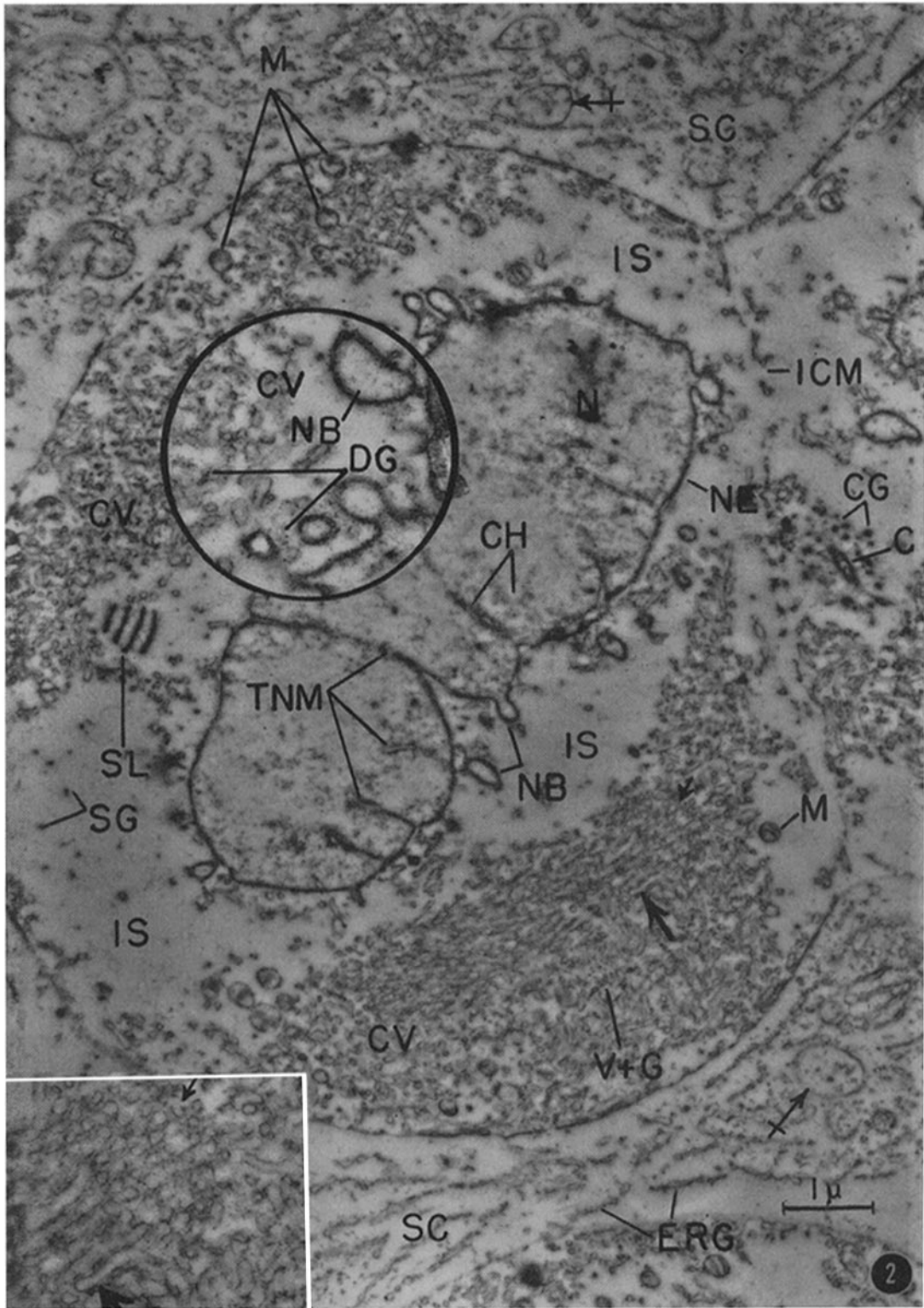
grammed in Fig. 1 and they will be referred to in presenting the evidence.

I. Early Spermatid (Posttelophase of Second Division; Fig. 1, I; Figs. 2 to 5)

The cytoplasm and nucleus of the second meiotic division telophase are superficially quite similar, except for size, to the telophase of first division. The nucleus, occupying roughly one-third of the cell volume, is discoid and presents a sausage-shaped profile in side view (Fig. 1, I, (A)). As has been described previously (26), at late anaphase and telophase large masses of chromosomes form karyomeres partially or entirely surrounded by nuclear envelope. The karyomeres fuse, thereby trapping portions of nuclear membrane between them. These are seen as double or quadruple membranes, often passing from one surface of the nucleus to the other (Fig. 2, *TNM*). The chromatin (Fig. 2, *CH*) is wispy and tends to be peripheralized, adhering to the inner

FIGURE 2

Electron micrograph of an early spermatid (Stage I) fixed in osmium-sucrose. This cell typifies this stage and is characterized by a disc-shaped nucleus (*N*), seen here in side view, with an irregular profile and internum of low density. The spermatid is surrounded by enfolding extensions of the sustentacular cells (*SC*) except where they adjoin their neighboring or sister spermatids. The cell membranes between spermatids are discontinuous (*ICM*) and are represented only by an irregular line of flattened vesicles. The remains of the interzonal spindle material exist as relatively clear areas (*IS*) in which are scattered 500 A granules (*SG*). In a sister spermatid, similar granules (*CG*) surround a centriole (*C*). The major portion of the cytoplasm is made up of small vesicles (*CV*) 50 to 100 m μ in diameter. In most regions, these are mixed with scattered dense 150 A granules (*DG*) which are free and also line the surfaces of the vesicles (*V + G*). Many vesicles are free of adhering granules. In the region marked with the large arrow (see also inset, lower left) free vesicles have fused to give long tube-like structures which, in cross-section, are seen to be hexagonally packed (small arrow). Dense granules do not appear in this region. Mitochondria (*M*) are infrequent and small, and tend to be found toward the periphery of the cell. A small stack of annulate lamellae is indicated at *SL*. The long, thin, double profiles (*TNM*) invading the nucleus from the nuclear envelope (*NE*) are portions of nuclear membrane which have become trapped during karyomere formation at the preceding telophase. Chromatin (*CH*) has a tendency to become condensed along the surfaces of these inclusions and those of the inner nuclear membrane. The entire surface of the nucleus is covered by evaginations or "blebs" (*NB*) most of which can be seen in profile to be continuous with the nuclear surface. Others, however, are unconnected (see circular inset, *NB*) and probably represent sections through the nuclear extensions. The surfaces of the blebs are generally covered with dense granules (*DG*). The content of the blebs is similar to that of the nucleus and has been shown to be Feulgen-positive. The cytoplasm of the sustentacular cells (*SC*) is rich in granular endoplasmic reticulum (*ERG*) and scattered masses of vesicular and granular material enclosed by double-walled membranes (barred arrow). \times approx. 14,000. Insets \times 22,500.



nuclear membrane and to the surfaces of the trapped membranes. The nucleus has an empty appearance in both living and fixed specimens (Figs. 4 and 5) and is intensely Feulgen-positive around the membranes. The entire surface of the nucleus is erupted with extensions or blebs (Fig. 2, *NB*) into which Feulgen-positive material extends (24). In thin sections isolated profiles, whose contents indicate that they are clearly sections of such projections, are seen close to the nuclear surface. In the few cases where serial sections have been followed, these profiles are at some point continuous with the nuclear envelope. While it is possible that they may actually pinch off and float free, there is at present no evidence to prove it, nor is there any reason from the events that follow to assume that they must. The cytoplasmic surface of the blebs, as of most of the nuclear envelope, is studded with dense granules about 150 Å in diameter (Fig. 2, *DG*), similar in appearance to those which are found free and on the surfaces of the granular endoplasmic reticulum of these and other cells (Fig. 2, *ERG*).

At telophase the poleward side of the cell is filled

with large masses of vesicles (Fig. 2, *CV*) ranging roughly from 50 to 100 m μ in diameter, and dense 150 Å particles. Lesser numbers of similar vesicles and granules are seen in the spindle region, often aligned in the direction of the spindle (*cf.* 37). During division, aggregations of mitochondria appear near the poles and as telophase progresses the mitochondria distribute themselves around the periphery of the cell (Fig. 2, *M*) and very seldom penetrate close to the nucleus. The mitochondria are nearly spherical and approximately 0.25 μ in diameter; they usually contain one or two cristae. Transformation from the more common filamentous type of mitochondria to the oblate spheroid found in the spermatid occurs during earlier stages.

Apart from the afore-mentioned small vesicles, aligned and fused along the spindle axis, the spindle region is characterized by a clear area (Fig. 2, *IS*) in which are scattered dense granules (Fig. 2, *SG*) approximately 500 Å in diameter and lacking sharp boundaries. As reconstitution of the nucleus progresses, this region of low density either migrates or is further elaborated around the

FIGURE 3

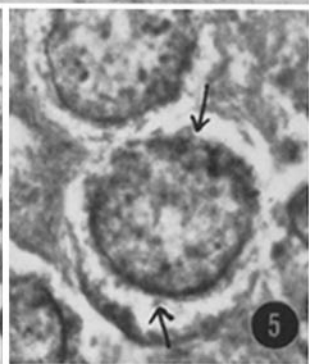
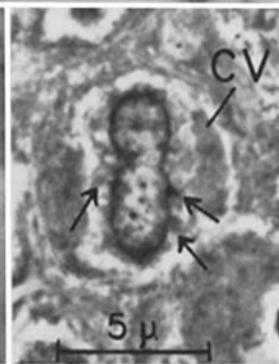
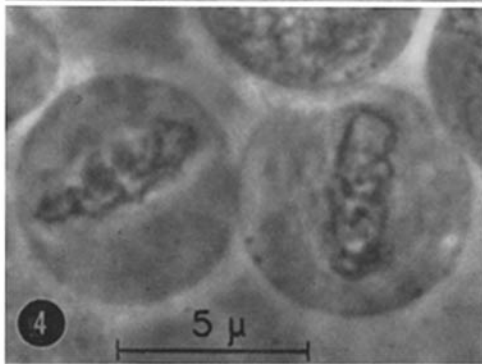
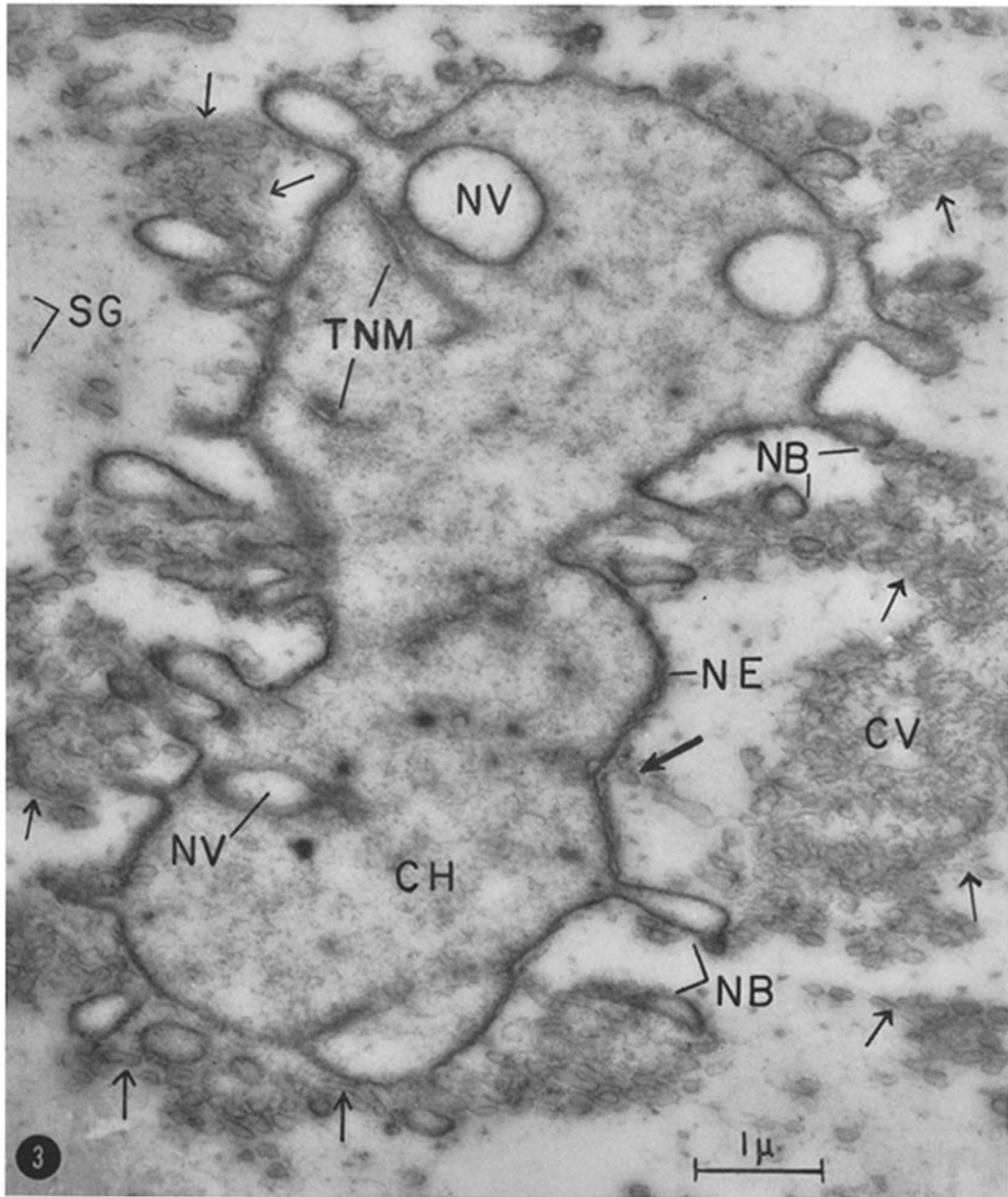
Electron micrograph of a spermatid nucleus (late Stage I) fixed in OsO₄ (Palade) buffered at pH 8.0, showing extensive nuclear evagination. The nuclear envelope (*NE*) consists of two membranes irregularly spaced. At the large arrow, the outer membrane has folded out and is in close contact with an elongate vesicle. All the blebs (*NB*) are in close contact with masses of small vesicles (*CV*) and dense 150 Å granules which often adhere to the outer membrane of the nuclear envelope. Large, double-walled vesicles (*NV*) with sparse contents probably represent trapped nuclear membranes (*TNM*) that have fused and opened out. The blebs also appear to be relatively empty toward their extremities. Chromatin (*CH*) is finely fibrillar. There appears to be a tendency for streams of massed vesicles to be closely associated with the blebs, and often to fuse with each other (small arrows). The 500 Å granules of the interzonal spindle material are seen at *SG*. \times approx. 18,000.

FIGURE 4

Light micrograph (phase) of two living spermatids teased out in modified Ringer's solution. The irregular outlines of the nucleus are visible, indicating that the blebbing seen in sections of fixed material is not an artifact of preparation. \times approx. 2500.

FIGURE 5

Light micrograph (phase) of a 2 μ section of two osmium-sucrose-fixed spermatids, stained with iron hematoxylin. The nucleus on the left is seen in a lateral view similar to that in Fig. 3. Masses of vesicular material (*CV*) lie on either side of the nucleus. The blebs on the nuclear surface, together with masses of associated vesicles, are indicated at the arrows. The periphery of the nucleus stains most heavily. A similar picture is obtained after Feulgen staining. On the right, the nucleus can be seen in top view. \times approx. 2000.



nucleus, which eventually, at spermatid Stage I, is more or less surrounded by it. Since spindle fibers are seldom seen in otherwise well preserved material (*cf.* 37), such elements do not figure in the structure of the spermatid. Centrioles (Fig. 2, *C*) with characteristic organization are found at the poles of the cells, usually surrounded by a cloud of finely granular material in which clusters of dense 500 Å granules similar to those of the spindle region are embedded (Fig. 2, *CG*). No other formed elements are found regularly in the cytoplasm.

Occasionally a spherical body approximately 1 to 2 μ in diameter and appearing to be composed of tightly packed small vesicles (not shown) appears among the masses of the cytoplasmic vesicles. The large masses of stacked annulate lamellae found in primary spermatocytes (36) and secondary spermatocytes are seldom seen at division. Small masses of such lamellae, consisting of from two to five layers, often appear in spermatids (Fig. 2, *SL*), and it is assumed that they are formed after division.

There is nothing in the telophase or Stage I spermatid that resembles the "Golgi membranes" commonly associated with spermiogenesis in flagellate forms. Neither recognizable dictyosomes nor the flattened layers of agranular cisternae associated with the so-called "Golgi apparatus" have been seen. However, within the masses of cytoplasmic vesicles (Fig. 2, *CV*), certain differentiations do occur. Many of the small vesicles are studded with dense 150 Å granules; others are free of granules but are surrounded by unattached granules in the neighborhood (Fig. 2, *V + G*). When numbers of agranular vesicles occur in one area, they often appear to associate with one another in a way that excludes the dense granules. These tight aggregations may involve the fusion of vesicles to form tube-like structures clustered roughly in hexagonal array (Fig. 2, large arrow and lower inset). Although we have found no evidence that this material differentiates any further, in view of its agranular nature it may very well be akin to the Golgi material.

Intercellular bridges, which have been reported in other spermatids (11), are extremely common in this material. They appear to result partly from incomplete cell membrane formation, particularly between sister spermatids (Fig. 2, *ICM*), where chains of vesicles are all that mark the cell boundary. Interruptions in the cell membranes also occur along the boundary of the sustentacular

cells, and cytoplasmic continuity is also evident here. The cytoplasm of the sustentacular cell neighboring the spermatid is rich in organized granular endoplasmic reticulum (Fig. 2, *ERG*). Double-walled vesicles containing dense 150 Å granules, small vesicles, and other structures (Fig. 2, barred arrow) are not uncommon. Mitochondria are also present in these cells.

The most striking characteristic of the Stage I spermatid is the blebbing of the nuclear surface, which becomes more pronounced as Stage I progresses (Fig. 3). The highly irregular nuclear profiles can be seen in the light microscope in both living (Fig. 4) and fixed preparations (Fig. 5), indicating that they are not preparation artifacts. As the blebbing progresses, streamers of small vesicles (Fig. 3, *CV*) and dense 150 Å granules become closely associated with the blebs. Vesicles often appear to fuse (Fig. 3, small arrows). Clusters of vesicles appear to have moved toward the nucleus from the large masses (Fig. 5, *CV*), but it is conceivable that part of them may originate *de novo* within the relatively clear area of spindle material around the nucleus. One frequently gains the impression that the vesicles aligning themselves more or less perpendicularly with the nuclear surface and closely associated with the blebs may actually fuse with the nuclear envelope. Generally, the outer nuclear membrane is involved (Fig. 3, large arrow), though fusion with both membranes may occur. The streamers thus produced are visible in the light microscope (Fig. 5, arrows).

During this stage, a clear area free of any of the structural elements discussed above begins to appear near the periphery of the spermatid and usually on the side of the nucleus away from the pole. Rosettes of 200 Å particles begin to appear in this area, which will become the acrosomal vesicle.

II. Acrosomal Granule Stage (Fig. 1, II; Figs. 6 to 8)

Three structural changes characterize this stage: (*a*) the nucleus shrinks and increases in density, (*b*) the blebs are replaced by sheets of membrane closely associated with the nuclear surface, and (*c*) a large vesicle containing an acrosomal (proacrosomal) granule is set off.

The volume of the spermatid has not changed appreciably although the interruptions in the cell

surface allowing communication between spermatid and sustentacular cell are more numerous. Intercellular connections between sister spermatids persist, but extensions of the sustentacular cells almost completely surround each spermatid. The nucleus undergoes considerable shrinkage and decreases in volume by two-thirds to three-fourths; toward the end of this stage it represents about one-eighth of the total volume of the cell. As a result it is both denser and more homogeneous in the electron image (Fig. 6, *N*) and more densely staining in the light microscope (Fig. 7). Fast green staining, which here parallels Feulgen staining in the nucleus and undoubtedly represents binding to basic protein, is quite intense. Portions of the internalized nuclear membrane persist (Fig. 6, *TNM*). The blebs have receded but the surface of the nucleus is still irregular. A few projections of the nuclear envelope suggest that the outpocketings may have collapsed leaving short, flat sheets of closely apposed envelope membrane. In other areas, the inner nuclear membrane has receded while the outer membrane projects for a short distance, where it is usually associated with chains of vesicles, some of which appear to have fused and collapsed. A few of the 150 A particles which covered the surfaces of the blebs are still attached to the outer membranes, but in greatly reduced numbers.

Closely associated with the irregular envelope are sheets of membrane (Fig. 6, *MS*) with undulating profiles, which may double back on themselves and which can sometimes be followed for several microns in the section. In general, they tend to radiate away from the nucleus, and although numerous vesicles of various sizes are intimately associated with the sheets, the large masses of small vesicles (Fig. 6, *CV*) are excluded from the area of the membrane sheets. The dense, 500 A particles of the spindle material are not, however, excluded. In fact, there is some suggestion that the sheets which develop in this area may do so partially at the expense of this material. At the periphery of the membrane sheets, near the masses of small vesicles, profiles (Fig. 6, arrows) of large vesicles having contents of slightly higher density than the surrounding matrix and with surfaces studded with dense 150 A particles appear to be formed by the fusion of small 0.1 μ vesicles. There is some evidence that these large vesicles collapse, leaving closely apposed membranes. 150 A particles are seen less in association

with the collapsed membranes than with the vesicles. Two centrioles (Fig. 6, *C₁*, *C₂*) are usually found on the poleward side of the nucleus entrapped in the membrane sheets. The centrioles are surrounded by a fine granular material in which are disposed dense 500 A particles (Fig. 6, *CG*) of the sort found in the spindle region.

The masses of small vesicles which appear to have been excluded from the vicinity of the nucleus are packed near the cell periphery, as are the small, nearly spheroidal mitochondria (Fig. 6, *M*). Stacks of from two to six layers of annulate lamellae (Fig. 6, *SL*), from 0.5 to 1 μ in diameter, are generally found associated with the vesicle masses, usually on the side of the nucleus away from the pole and near the clear area which becomes the acrosomal vesicle. The latter has remained free of intrusions of vesicular and granular material from the cytoplasm and is filled with clusters of 200 A particles. The cell membrane of the spermatid (Fig. 6, *CMS*) moves inward around the edges of the material that will become contained by the future vesicle, and presumably by further invagination and fusion with small vesicles begins to form the limiting membranes of the acrosomal vesicle. The part of the spermatid cell membrane lining the base of the acrosomal vesicle is dense and thickened (Fig. 6, *BM*). The entire acrosomal vesicle tends to protrude from the spermatid. This can also be seen in the light micrographs (Fig. 7, *AV*).

In many sections a dense, round, granular structure approximately 1 μ in diameter is visible (Fig. 6, *AG*) in the interior of the forming acrosomal vesicle, often situated near the periphery proximal to the nucleus. This is identified from light micrographs as the proacrosomal granule. There is generally one granule per cell, though occasionally there is a suggestion in electron micrographs that similar, but smaller, aggregations may form in other clear membrane-bound areas, near the cell periphery. The granule is made up of aggregations of the 200 A particles. It often adheres to the invaginating cell membrane or to unattached membrane sheets. In many cases it is associated with the stack of annulate lamellae. When cells at this stage are stained with the periodic acid-Schiff reaction and fast green, both the nucleus and the granule stain intensely with the latter (Fig. 7, *N*, *AG*) while only the granule is PAS-positive (Fig. 8). Although the entire cell, and particularly the membrane area

(Fig. 8, *MS*), is very faintly Schiff-positive, only the granule stains appreciably after oxidation with periodic acid. These observations substantiate identification of the proacrosomal granule, since this is the characteristic reaction of proacrosomal structures in other forms (8, 39).

The sustentacular cells (Fig. 6, *SC*), which nearly surround the spermatid and whose surface membrane is closely applied to the spermatid membrane, contain a number of inclusions in addition to small vesicles, 150 Å granules, organized sheets of rough endoplasmic reticulum, and mitochondria (Fig. 6, *M*). There are also profiles of inclusion bodies surrounded by two membranes and containing spheroidal mitochondria of the sort found in spermatids, together with 500 Å granules of the sort associated with the spindle material, and small vesicles (Fig. 6, large arrows). These, plus other debris, suggest that portions of the spermatid cytoplasm may already have been engulfed. As this stage progresses, large portions of the spermatid cytoplasm outside the membrane sheet (Fig. 6, *MS*) and acrosomal vesicle (Fig. 6, *AV*) areas will be incorporated in the sustentacular cytoplasm by enfoldings of the surface membranes of both cells. Eventually, all the cytoplasmic material including small vesicles, granules, and mitochondria will be lost to the sustentacular cells and only the nucleus, its asso-

ciated membrane sheets, the centrioles, and the acrosomal vesicle will retain their integrity. It is now almost possible to see where this new cell boundary will be formed.

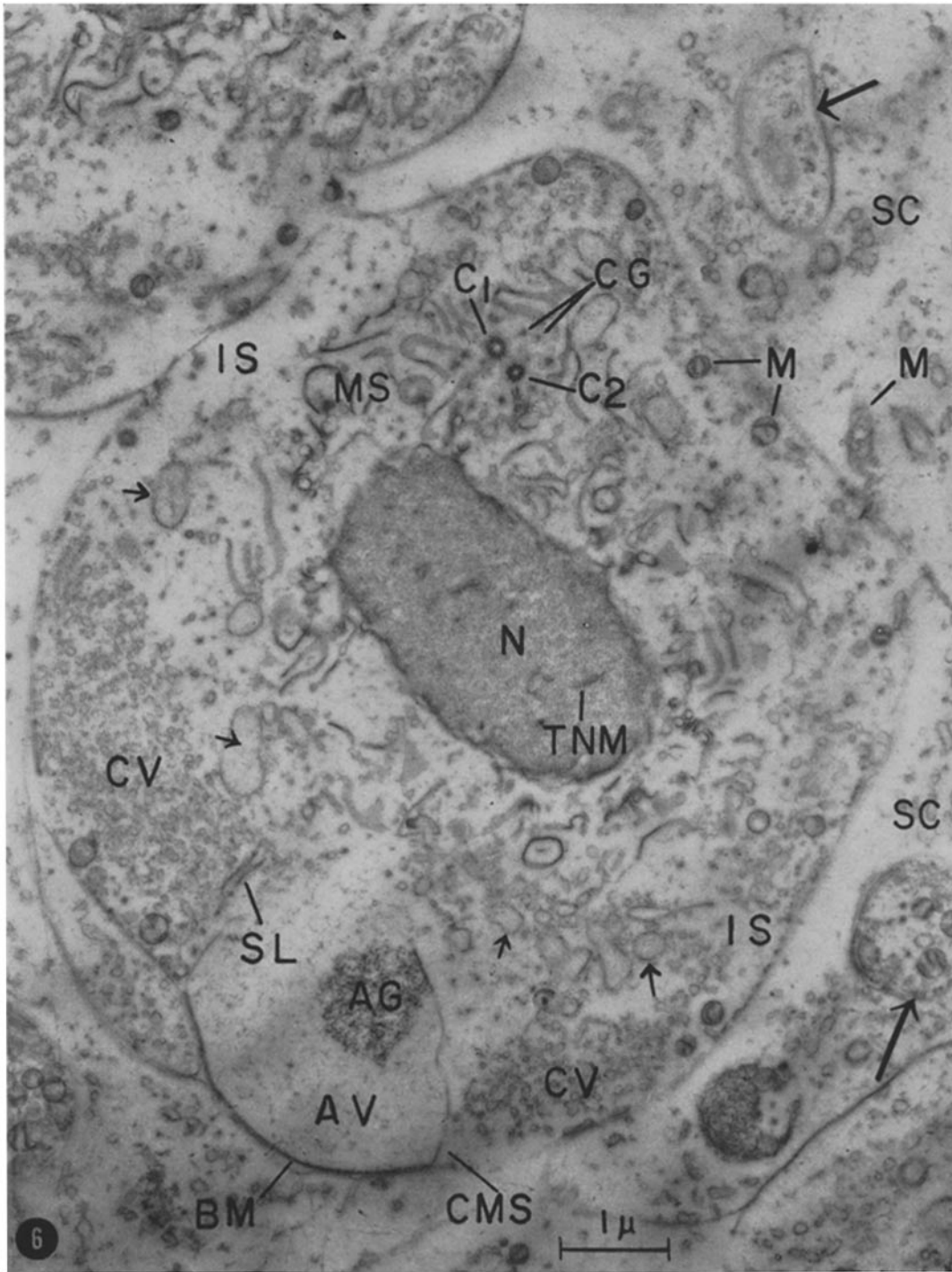
III. Acrosomal Cap Stage (Fig. 1, III; Figs. 9 to 11)

This stage is characterized by (a) further elaboration of membrane sheets around the nucleus, (b) redistribution of the material in the acrosomal granule to form a cap, and (c) exclusion of the remaining cytoplasmic material and delineation of a new cell periphery.

The nucleus, aside from some possible further shrinkage and resulting increase in stainability, is essentially the same discoid structure as at the previous stage (Fig. 11, *N*). The entire nucleus, however, is now surrounded by a mass of convoluted membrane sheets (Fig. 11, *MS*) which have elaborated further, and which, in many places, can be seen to be continuous with the nuclear envelope (Fig. 11, *I*). Very few small vesicles or dense granules of either the 150 or 500 Å sort are present; they either have been excluded from the new cell or, more probably, have been incorporated in the elaboration of membrane material. Rarely, a mitochondrion, presumably entrapped earlier, is found against

FIGURE 6

Electron micrograph of an osmium-sucrose-fixed spermatid at Stage II. Long streamers of membrane material have been elaborated around the nucleus and tend to radiate from it. A number of these membranes are seen to be continuous with the nuclear envelope. Two centrioles (*C*₁, *C*₂) lie on what is presumably the poleward side of the nucleus (*N*) and are surrounded by a light, cloudy material in which are embedded 500 Å granules (*CG*). Mitochondria (*M*) are found mainly around the margin of the cell near the cell membrane. Scattered outside the region of the membrane sheets are vesicles of various sizes (small arrows) generally studded with 150 Å granules and having a content that is somewhat denser than the surrounding cytoplasm. The relatively vesicle-free interzonal spindle material (*IS*) persists with its 500 Å granules around the equator of the cell. An acrosomal granule (*AG*) has formed and is adhering to the partially complete double membrane (*CMS*) that is continuous with the spermatid cell membrane and that separates the incomplete acrosomal vesicle (*AV*) from the rest of the cell. Dense material lines the membrane (*BM*) at the base of the acrosome. Two layers of annulate lamellae (*SL*) lie close to the acrosomal vesicle. The masses of small vesicles and small granules (*CV*) are excluded from the latter structure and also from the area of membrane elaboration around the nucleus. The nucleus is homogeneous and dense and still contains traces of the trapped nuclear membrane (*TNM*). In the sustentacular cells (*SC*), large double-walled profiles contain mitochondria, vesicles, and granules (large arrows). The cell in the upper left is a sister spermatid. × approx. 16,000.



the nuclear surface. Otherwise, mitochondria are found only at the periphery of the cell and hence are excluded from the region of the membrane elaborations. The two centrioles, little changed in appearance (Fig. 11, *C*), have migrated and may now be found together on either side of the nucleus. Occasional dense structures (Fig. 11, *4*), some of which represent the remains of trapped membranes appear within the nucleus.

The acrosomal vesicle (Figs. 9 to 11, *AV*) is now completely set off from the rest of the cell. The acrosomal granule has been replaced by a dense rim or cap which lines the surface of the vesicle proximal to the nucleus. A thin spot or pore appears centrally (Fig. 11, *5*) in the cap. PAS-

fast green staining (Figs. 9 and 10) shows that the cap material (Figs. 9 and 10, *AR*) has the same staining properties as the granule of the earlier stage. It thus appears that the dense 200 A particles are redistributed along the surface of the invaginated membrane that sets the acrosomal vesicle off from the convoluted membrane mass. The dense material that lines the distal surface of the acrosomal vesicle (Fig. 11, *BM*) joins the acrosomal cap (Fig. 11, *2*). However, it is a different substance in that it stains neither with fast green nor with periodic acid-Schiff treatment. Scattered clusters of 200 A granules are also still found free in the lumen of the vesicle.

The new cell boundary appears to be formed

FIGURES 7 THROUGH 10

Figures 7, 8, 9, and 10 provide a cytochemical comparison between spermatids at Stage II (acrosomal granule) and those at Stage III (acrosomal crescent). The substance of the granule (Figs. 15 and 16) is the same material that is subsequently redistributed to form a crescent on the nuclear side of the acrosomal vesicle (Figs. 17 and 18). The sections were stained with the periodic acid-Schiff reaction followed by fast green. All figures \times approx. 2200.

FIGURE 7

Photomicrograph taken with a Wratten No. 29 (red) filter. The image is primarily one showing fast green staining. The nucleus (*N*) and the acrosomal granule (*AG*) are the only structures binding appreciable amounts of dye. The background image is due to light scatter. The outlines of the acrosomal vesicle (*AV*) and masses of membrane elaborations (*MS*) can be seen. The staining of these nuclei reflects the distribution of basic protein groups.

FIGURE 8

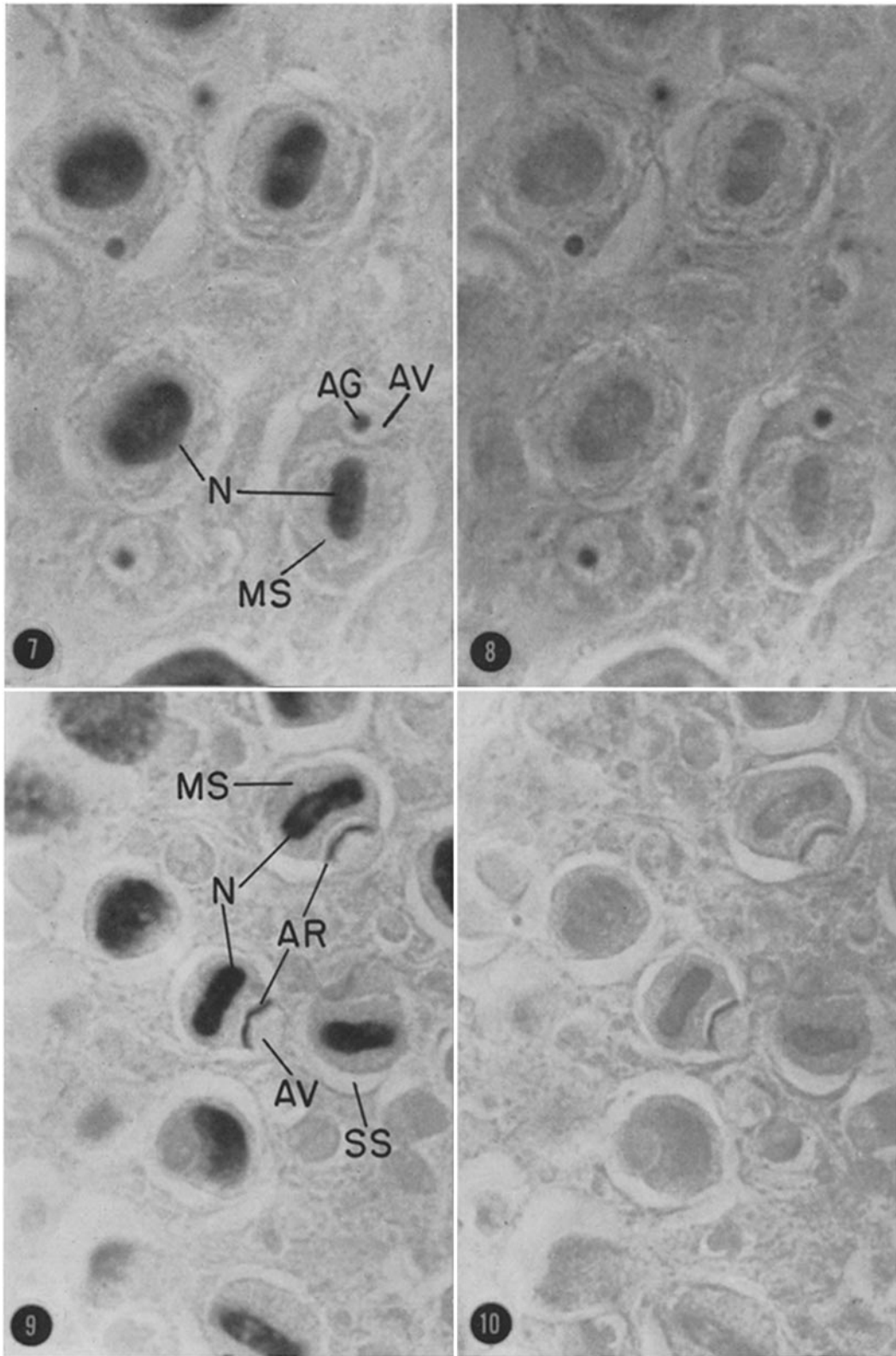
The same area as in Fig. 7 but photographed through a Wratten No. 74 (green) filter. The picture is essentially one of periodic acid-Schiff reactivity. The acrosomal granule is the only structure binding an appreciable amount of dye. There is a faint and non-specific background stain presumably due to non-specific oxidation by the osmium tetroxide during fixation. Light scatter is also greater at this wavelength.

FIGURE 9

(Compare with Fig. 7). In spermatids at Stage III, the mass of membrane elaborations (*MS*) has completely separated itself from the surrounding cytoplasm, leaving a space (*SS*) that is probably produced by shrinkage during preparation. The base of the acrosomal vesicle (*AV*) still adheres to the surrounding cell. The nucleus (*N*) is slightly smaller and the concentration of dye found is consequently somewhat higher. The acrosomal crescent (*AR*) stains exactly as did the acrosomal granule (*AG*) in Fig. 7.

FIGURE 10

(Compare with Fig. 8). This is essentially a periodic acid-Schiff picture showing the only appreciable stain to be in the acrosomal crescent, as it was in the acrosomal granule of Fig. 8.



from three sources: (a) The spermatid is nearly surrounded either by an infolding of the spermatid and adjacent sustentacular surfaces or by an invagination of the spermatid cell membrane only. The original spermatid membrane (Fig. 11, *CM-S*) comes in direct contact with the contents of the new cell. Immediately next to it lies the membrane of the sustentacular cell (Fig. 11, *CM-SC*). (b) The convoluted membrane sheets tend to fuse at the periphery (Fig. 11, *SM*) and thus form a complex membrane barrier. (c) Small vesicles in the sustentacular cell (Fig. 11, 3) may contribute to extensions of the sustentacular and spermatid membranes by fusing with them. Cells at this stage appear to be extremely sensitive to changes in tonicity, and shrinkage spaces (Fig. 11, *SS*) are not uncommon, so that the possibility of attendant membrane damage makes interpretation of the behavior of cell membranes difficult.

IV. Biconcave Nucleus Stage (Fig. 1, IV; Fig. 12)

This stage is characterized by the following three structural differentiations: (a) The nucleus

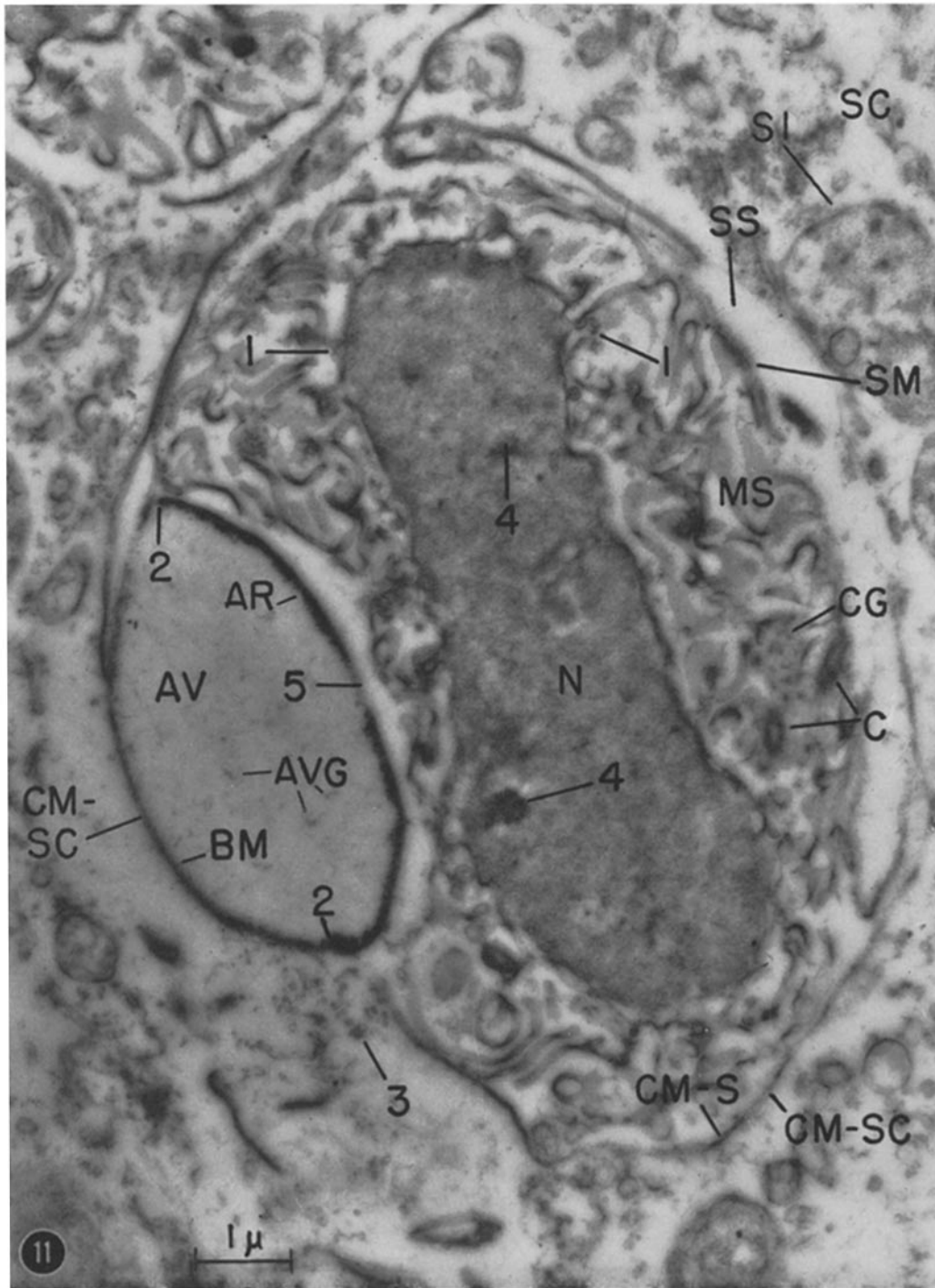
is a biconcave disc; (b) the membrane elaborations have consolidated into larger sheets which are clearly extensions of the entire nuclear envelope; (c) slender filaments extend into the acrosomal vesicle from the dense acrosomal cap, which has begun to invaginate.

The shape of the spermatid at this stage has changed slightly from the preceding one: whereas the Stage III cell tended to be a flattened sphere, with elliptical profile perpendicular to the equator, the Stage IV cell is almost circular in profile in both equatorial and polar planes. From estimations based on the electron micrographs, the nucleus, which is now a biconcave disc, has not changed appreciably in volume. A significant change has occurred, however, in the appearance of its contents, which are homogeneous and less dense than in the preceding stage.

The nucleoplasm is composed almost entirely of fine microfibrils, and the clumps of dense material and trapped nuclear membrane have disappeared. The cells described previously as sperm (24) are actually at this stage, and, as reported, the nuclei stain homogeneously and intensely with the

FIGURE 11

Electron micrograph of an osmium-sucrose-fixed spermatid, Stage III. This is exactly comparable with the stained cells shown in the preceding light micrographs (Figs. 9 and 10). A large fraction of the cell volume is occupied by convolutions of the sheets of membrane elaborations (*MS*). A new limiting membrane is being formed around the spermatid (*SM*). It is partly a highly folded and branching double membrane, the inner membrane of which is an extension of the old spermatid membrane (*CM-S*) while the outer membrane is the cell membrane of the sustentacular cell (*CM-SC*). Fusion of the membrane sheets at the periphery (*SM*) also contributes to the new tegument. Most of the spermatid cytoplasm excluded by this process has now become a part of the sustentacular cell (*SC*), in which inclusion bodies (*SI*) can be found containing recognizable bits of membrane and granular debris. Practically no vesicular, granular, or mitochondrial elements are retained *per se* within the spermatid. The membrane elaborations persist in continuity with the nuclear envelope in many places (e.g., 1). In the acrosomal vesicle (*AV*) the crescent, (*AR*) composed of dense aggregations of 200 Å particles, has replaced the acrosomal granule. The cap thus formed is of uniform thickness. A discontinuity at its apex (5) suggests that the dense material does not bar access between the acrosomal vesicle and the space outside it. Isolated clusters of 200 Å particles (*AVG*) persist in the vesicle. The dense crescent (*AR*) extends only to a point (2) that does not exceed the new cell boundary. Here it meets the basal material (*BM*) which forms a thin, granular layer along the inner surface of the membrane adjacent to the sustentacular cell membrane (*CM-SC*). Clusters of small (0.1 μ) vesicles lie close to the acrosomal vesicle but in the cytoplasm of the sustentacular cell (3). The nucleus (*N*) is homogeneous and dense except for occasional dense areas (4) which may be either nucleoli or local concentrations of chromatin around the trapped nuclear membranes. Two centrioles (*C*) lie on the side of the nucleus away from the acrosome and are associated with dense 500 Å granules (*CG*). The large empty spaces (*SS*) are probably the result of shrinkage during preparation. × approx. 14,300.



Feulgen reaction. In such preparations the contours of the nucleus are smooth, indicating that no chromatin extends outside the nucleus. This is remarkable in that the nuclear envelope along the faces of the nucleus opposite the membrane masses (Fig. 12, *MS*) does not form a smooth, continuous boundary. At various points along the surfaces (Fig. 12, small arrows) both membranes of the nuclear envelope flare outward and become continuous with the membrane sheets. Where this occurs there is no evidence that the nuclear microfibrils extend into cavities between the sheets. When a favorable profile can be found, the double-membrane nuclear envelope can sometimes be followed all the way out to the cell periphery.

Occasionally, along the nuclear surface, small masses of tangled membrane appear partly or completely surrounded by extensions of the nuclear envelope and folded sheets of membrane (Fig. 12, large arrow). Such knots of membrane material are an inconstant feature of these sperm; they appear to be more numerous in some individuals than in others, although they are generally rare in occurrence in this species. In other species, however, they appear to be fairly common. There is no evidence that they are obligatory or associated with any particular structure or function in these cells. It is possible that they represent disorganized myelin figures, or conceivably even regions where trapped mitochondria are degenerating. No recognizable mitochondria are

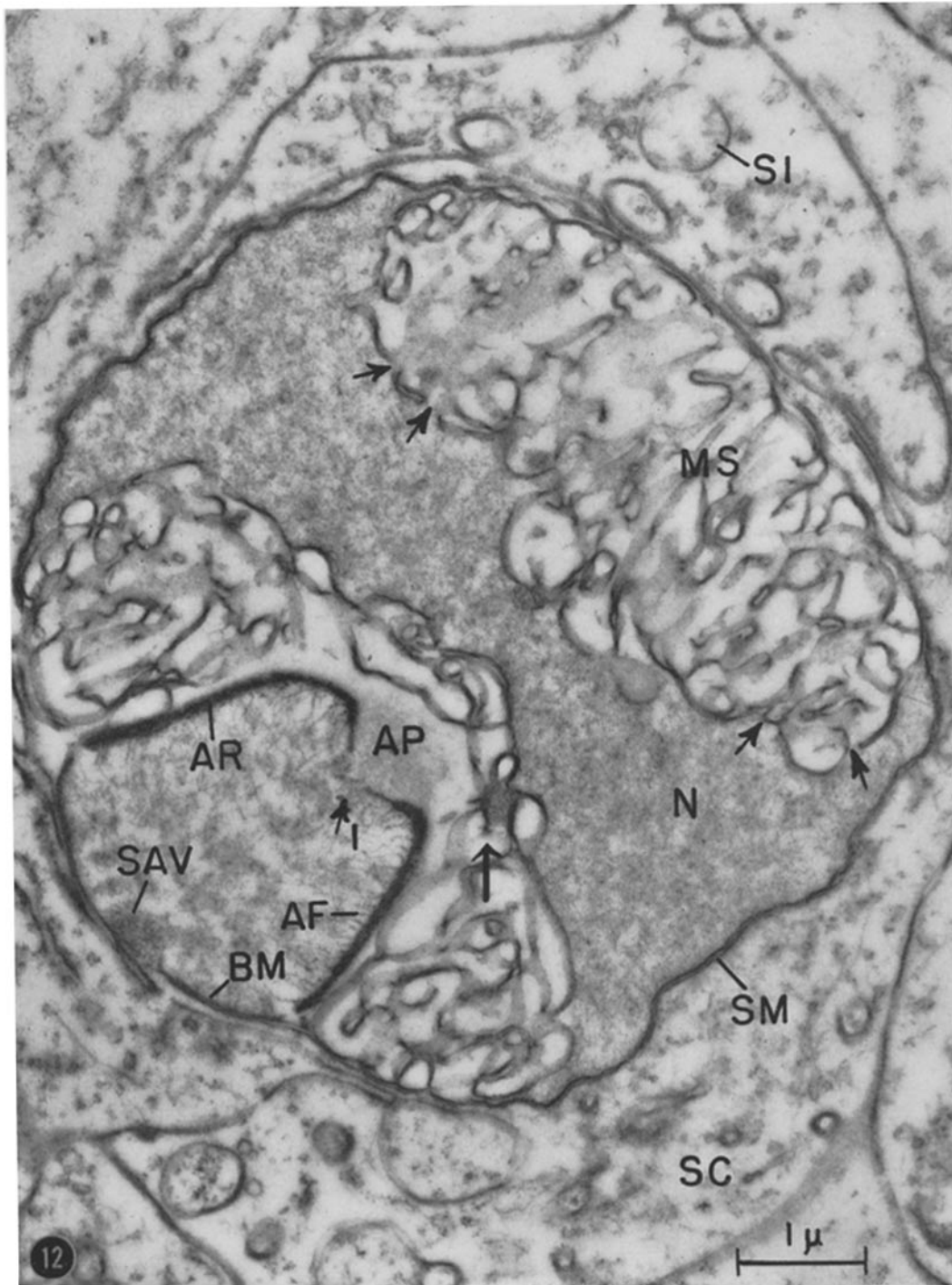
present in the spermatid, and even the occasional one that has been trapped near the nuclear surface is not easily identifiable.

The membrane sheets themselves have become consolidated in that they can be followed for longer distances in thin sections, and tend to be arrayed in a more orderly fashion perpendicular to the surface of the nucleus. Where they reach the periphery of the cell, they tend to turn and lie against the cell membrane and fuse with one another to form an inner sheet which certainly, in places, is an extension of the nuclear envelope. The sheets also form a barrier between the acrosome and the nucleus; it has not been possible to trace clear access between membranes from the acrosome into the nucleoplasm. The membrane sheets are restricted to the concavities of the nucleus; around the equator, where the nucleus lies against the cell membranes, its contours are relatively smooth and free of extensions (Fig. 12, region marked *SM*).

Within the acrosome, several notable changes have taken place. Long and slender filaments (Fig. 12, *AF*) roughly 200 Å thick extend inward from the dense acrosomal cap (Fig. 12, *AR*). The latter still stains intensely with both PAS and fast green. As mentioned above, the central part of the cap never completely closes over, and at this point an invagination of the cap begins (Fig. 12, *I*). The invagination actually appears to begin at some time after the nucleus has become biconcave; hence, the two phenomena are not well syn-

FIGURE 12

Electron micrograph of an osmium-fixed spermatid at Stage IV. The entire cell is now almost completely enclosed by a complex integument consisting of at least four membranes (*SM*). The elaborate mass of convoluted membranes (*MS*) is continuous with the nuclear envelope in many places (small arrows). These sheets obviously contribute to the integument making up the inner two (or more) membranes. The nucleus (*N*) is now biconcave; its chromatin is distributed homogeneously, and it is less dense than that in Fig. 11. The sides of the acrosomal crescent (*AR*) extend away from the nucleus but do not exceed the limits of the cell. The contents of the acrosomal vesicle have become denser and are cloudy. Short filaments (*AF*) extend into the vesicle from the crescent (*AR*). The crescent is beginning to invaginate (*I*) and an amorphous substance (*AP*) is accumulating in the depression thus formed. In the region of the basal material (*BM*) is an aggregation of small vesicles (*SAV*). In the sustentacular cells (*SC*), membrane-bound inclusion bodies (*SI*) presumably containing cytoplasmic debris from the spermatid are numerous. The convoluted sheets of membrane that extended from the sides of the nucleus in the earlier stage are now missing. The contours of the nucleus in this region have become relatively smooth and the periphery lies close against the retaining membranes. A tangle of membrane suggesting the beginning of a myelin figure is shown at the large arrow. \times approx. 19,000.



chronized. The cavity of the acrosomal vesicle has become filled with clouds of moderately dense, finely granular material in which are found scattered a few of the characteristic 200 Å granules. The entire internum is now palely PAS-positive and fast green-negative, in contrast to the acrosomal cap. On the inner face of the acrosomal vesicle, distal to the nucleus, there has been a further accumulation of dense material along the membrane bounding the cell (and the acrosome) at this point (Fig. 12, *BM*). This material is distinct from the adjacent acrosomal cap. In the center of this region where the dense accumulation is thinnest, a small cluster of vesicles, roughly 500 Å in diameter, accumulates. On the opposite side of the acrosome, proximal to the nucleus, an ill defined mass of cloudy material collects in the indentation formed by the invaginating acrosomal cap (Fig. 12, *AP*).

The new cell boundary (Fig. 12, *SM*) is now quite distinct and smooth in contour. The spermatid is virtually completely surrounded by a continuous cell membrane derived from the original spermatid cell membrane. The sustentacular cells entirely enfold the spermatid, and their membranes lie against the spermatid membrane. These two membranes in turn are appressed against the two membranes of the nuclear envelope and its extensions around the cell, except in the region of the acrosome, which is bounded only by the spermatid and sustentacular cell membranes.

The contents of the sustentacular cell (Fig. 12, *SC*) in the vicinity of the spermatid are similar to those in earlier stages. Dense, 150 Å particles and small vesicles as well as more organized sheets of endoplasmic reticulum are abundant. Mitochondria are present, as are the double-walled inclusion bodies (Fig. 12, *SI*) containing structures that are presumably residua of the discarded spermatid cytoplasm.

V. Immature Sperm (Fig. 1, *V*; Figs. 13 to 16)

This stage has been so named mainly because the final morphological changes leading to the mature sperm have begun to take place: (a) The entire cell is surrounded by a complex, membranous integument; (b) the nucleus has begun to extend itself radially in four directions to initiate the long processes that characterize the mature sperm (28), and the organization of the nuclear contents has changed markedly; (c) the invagi-

nated acrosomal complex has undergone further structural differentiation.

The nucleus is still essentially biconcave. Its contours are highly irregular, largely owing to the fact that the continuity of the nuclear envelope and the membrane sheets (Figs. 13 and 16, *MS*) have become very pronounced. Continuity between the nuclear envelope and the periphery of the cell can be seen in numerous places. In the equatorial region, the nucleus has begun to extend itself to form arms (Fig. 16, upper left). The significance of this is most clearly demonstrated in living cells (Figs. 14 and 15). The resemblance between the living cell in lateral view as seen in the light microscope (Fig. 14) and a sperm of the same stage in the electron microscope (Fig. 16) is striking. In equatorial view (Fig. 15) the cell (nucleus) has a nearly square or rhomboidal profile. The straight edges are slightly scalloped, while the corners are pointed away from the center of the cell. These radial extensions of the nucleus will continue to elongate. Fig. 13 is a transverse section through the acrosomal region of a cell at very early Stage V. The rhomboidal profile of the part of the nucleus that extends over the acrosome is evident. The membrane masses (Figs. 14 and 15, *MS*) appear in the phase microscope as areas of considerably higher density than the nucleus. The pairs of lines extending from the membrane masses into the radial extensions of the nucleus (Fig. 15, arrows) probably represent folds of membrane that are pulled out of the membrane masses as the extensions are formed.

A loss in nuclear density, apparent in both the light and electron micrographs, is particularly striking. In the phase microscope a low image density represents low mass concentration. In the electron image the low density could represent either low mass or decrease in propensity for binding osmium. In this case, the empty appearance of the nucleus reflects the former and is in part due to a redistribution of its fine structural elements. Instead of being a homogeneous mass of microfibrils as in the preceding stage, the nucleus (Fig. 16, *N*) is filled with sparsely distributed, dense clumps of material, from which radiate dense fibrils varying in width up to 250 Å. Many of these clumps and fibrils adhere to the inner surface of the nucleus. These structures lie within a homogeneous matrix of low density. Feulgen squashes of this and subsequent stages show the nucleus to be more or less homogeneously Feulgen-

positive (28). Seen in 1 μ and 2 μ sections with the light microscope, Feulgen-positive material is distributed throughout the nucleus but is concentrated around the nuclear periphery and in a few clumps within the nucleus. In the electron microscope, the contours of the Feulgen-positive region are irregular because of the pronounced and erratic extensions of the nuclear envelope in the region of the membrane sheets. There are now numerous channels where continuity from the interior of the nucleus can be followed between the sheets out to the periphery of the cell (Figs. 13 and 16, arrows).

Characteristic centrioles, still surrounded by the finely granular cloudy material, are, as before, to be found together on either side of the nucleus and in no particular relation to other cell structures. There is no evidence that they are in any way connected with the extensions of the nuclear envelope.

Continued invagination of the acrosome has produced a double-walled cup, the outermost wall of which is formed by the dense acrosomal cup material, from which long, fine filaments (Figs. 13 and 16, *AF*) about 200 A wide at their base extend into the inner space. Closely associated with these filaments are tubules approximately 350 to 400 A in diameter which have replaced the vesicular material found in the earlier stage. Distortions that are presumably the result of preparation (Fig. 16, *SS*) suggest that the dense material along the outer membrane of the acrosome (Fig. 16, *BM*) is not integral with the fibrillar and tubular material. The fine cloudy material (Fig. 16, *AP*) has increased in quantity and fills the cup resulting from the invagination. It is faintly acidophilic. The morphology of the acrosomal complex can be seen clearly in lateral and frontal views in micrographs of living cells (Figs. 14 and 15, *A*). The material filling the acrosomal cup is of low density (Figs. 14 and 15, *AP*) while the cap is of very high density, corresponding to the relative densities in the electron image.

The sperm is surrounded at this point by a continuous membrane integument (Figs. 13 and 16, *SM*) which in favorable views can be seen to be composed of four membranes: the two nuclear membranes or extensions thereof, the spermatid membrane, and the sustentacular cell membrane. That the integrity of the cell is maintained by the continuity of its membrane tegument is indicated by the fact that entire cells can be teased out free

from surrounding sustentacular cells, without damage or destruction (Fig. 14).

VI. Mature Sperm (Fig. 1, VI; Figs. 17 to 19)

This term is applied to the most advanced sperm appearing in the testis and in the ducts. It may not describe the final stages in maturation, since further changes may conceivably occur outside the testis and prior to fertilization. The changes that have occurred are, for the most part, continuations of events already underway in the previous stage. The over-all morphology of this stage has been adequately described in detail in a previous publication (28) and will not be repeated here, but a few further observations should be noted.

The nuclear processes or arms (Fig. 17, *NA*) in extending themselves have wrapped themselves around the body of the sperm and in lateral sections are cut transversely. In carefully teased-out living cells at this stage, the coiled arms can be seen clearly (Fig. 18, arrows). Uncoiling occurs after the cells have stood in physiological solution for a short time (Fig. 19), and eventually the arms extend straight out, as is seen to be occurring in one arm in Fig. 19. The arms are about 30 μ in length, enough to encircle the cell two or three times. This accounts for the eight to twelve progressively small cross-sections of arms seen flanking the acrosome in electron micrographs such as Fig. 17 (*NA*).

The nuclear contents appear about as they did in the earlier stage. The membrane extensions of the nuclear envelope have diminished considerably and presumably have been used up, increasing the surface area of the nucleus as a result of growth of the processes. Centrioles no longer have the characteristic appearance of nine concentrically arranged tubes or filaments, but appear to deteriorate and become ill defined masses of fine granular material (Fig. 17, *C*); intermediate stages have been observed. Again, these structures do not appear to be implicated in any morphological events and may be found together on either side of the nucleus (*e.g.* as in Fig. 17).

The one distinct change is the appearance of an amorphous layer of material which invests the entire sperm. It is laid down between the sustentacular cell membrane and the sperm, and hence is extracellular. It gives a positive PAS reaction and may be deposited by the sustentacular

cell at the expense of PAS-positive material in its cytoplasm. Apparently it is the rupture of this investment, either by breaking or by dissolution, that sets the nuclear arms free in fresh water or saline (Fig. 19).

The acrosome has undergone very little further change. All the components present in the previous stage are recognizable. The lips of the invaginated cup have become compressed somewhat to form a pore, and the cloudy, granular material (Fig. 17, *AP*), which appears from the light micrographs (Figs. 18 and 19) to have low mass, fills the cup

and plugs the pore. The staining characteristics of the acrosomal complex are essentially unchanged.

DISCUSSION

This paper presents the first detailed electron micrograph study of spermiogenesis in the decapod Crustacea, and it is not our intent to extract generalizations about the process from our present observations. From what we have seen in other decapod species, the events depicted here represent the simplest we have so far encountered, but such

FIGURE 13

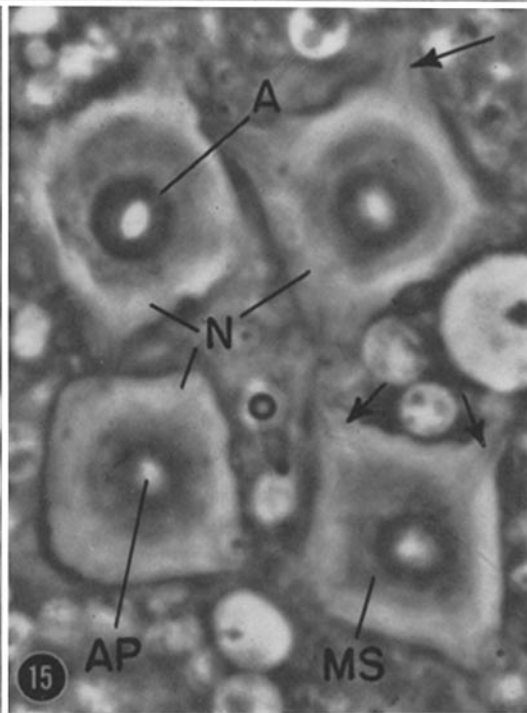
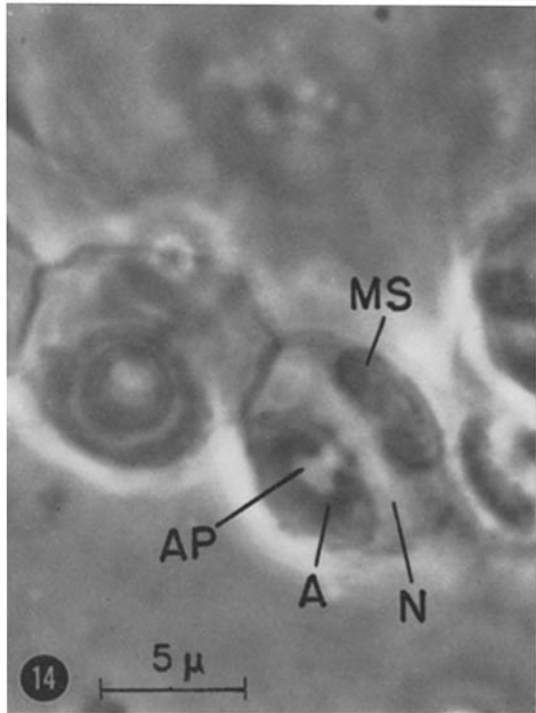
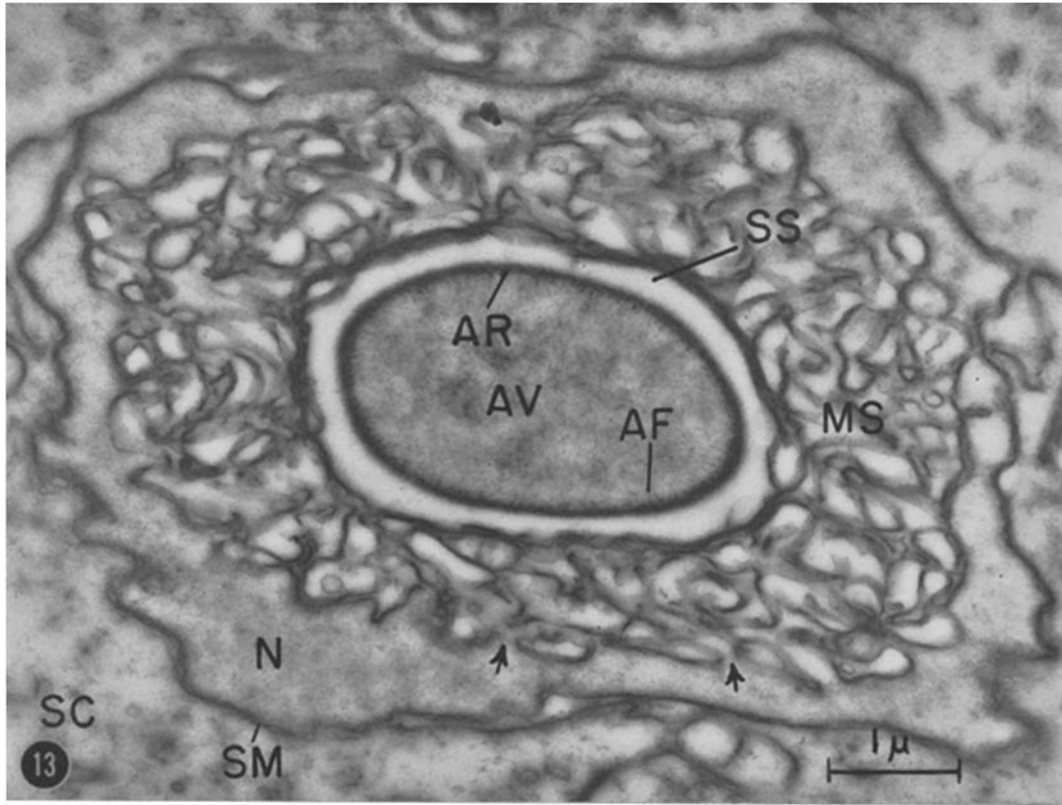
Electron micrograph of an osmium-sucrose-fixed spermatid approaching Stage V. This is a frontal section, cut in a plane perpendicular to that shown in Fig. 12 and passing through the acrosomal vesicle (*AV*). The profile of the cell is roughly square (rectangular, in this case, because of compression during sectioning). Because the nucleus is concave, a rim of nuclear material (*N*) is included in this section at the level of the acrosome. On the inner face of the nuclear profile, elaborations of convoluted membrane (*MS*) are continuous with the nuclear envelope (arrows). At the surface adjacent to the acrosome, the membranes fuse to form a relatively smooth boundary in which the acrosome lies. The space separating them (*SS*) is probably due to shrinkage during preparation. Acrosomal filaments (*AF*) extend from the acrosomal rim or cap (*AR*). The vesicle (*AV*) itself is filled with dense, cloudy material. The boundary (*SM*) between the nucleus and the sustentacular cell (*SC*) is relatively smooth and free of membrane elaborations. \times approx. 17,700.

FIGURE 14

Light micrograph (phase) of a living spermatid at approximately the same stage (V) as that shown in Fig. 16. The spermatid on the right is seen in side view and in the same orientation as in Fig. 16. The regions of the membrane elaborations (*MS*) and acrosome (*A*) are ones of high mass (high refractive index), while the nucleus (*N*) and the material filling the acrosomal cup (*AP*) are of relatively low mass. The low density of these latter structures, then, in the electron image is due to the low concentration of solid material present. This is in marked contrast to the higher density of the nucleus in earlier stages. (Compare with Fig. 11). Note also that the spermatids at this stage are easily dissected out free from adhering sustentacular cell material, indicating that the cell is contained in an intact tegument. \times approx. 3700.

FIGURE 15

Light micrograph (phase) of spermatids similar to those in Fig. 14 but seen in frontal view, as in Fig. 13. The square outline of the cells when they are teased out in physiological solution is characteristic of the stage (V). The four corners of the cell represent the extensions of the nucleus which will grow out into long arms. The membrane masses (*MS*) that lie above and below the nucleus (*N*) can be seen as gray areas. In several places striae extend from the phase-dense masses into the points of the outgoing arms (arrows). These probably represent folds of the convoluted membranes, which are pulled out as the arms extend and contribute the membrane material with which they are surrounded. The region of highest density is the acrosome (*A*), in the center of which is the low-density aperture of the invaginated cup (*AP*). \times approx. 3700.



striking and perhaps fundamentally important differences occur even between closely related species that a careful, comparative study among the more widely divergent types is necessary before meaningful generalizations can be made.

The transformations undergone by various cell organelles as the sperm matures in *Procambarus* are in themselves sufficiently unique to deserve further consideration. It seems important to attempt to derive the functional significance of these special differentiations. In a sense they are probably expressions of potentialities resident in related structures that are common to most cells. However, such considerations will be left for subsequent papers in this series, and the discussion here will be confined to the events taking place during spermiogenesis in this species.

A detailed review of the older literature on decapod spermiogenesis would not be entirely relevant at this point, since most of the observations were made on forms which are not closely related to the subject of the present investigation. Sperm development in *Astacus fluviatilis*, the European crayfish, particularly as described by Grobben (15), Herrmann (16), who incidentally appears to be the first to have reported thoroughly on spermiogenesis in this genus, Sabatier (38), Keppen (20), Retzius (35), Nichols (30), Reinhard (34), Gilson (13), and Grabowska (14), probably comes closest to that of *Procambarus*. In fact, the description by Herrmann, though incomplete with respect to the earlier spermatid stages, fits very well with the present observations, and his drawings, with a few minor modifications pre-

sumably necessitated by species differences, could be used to illustrate certain of our observations. His observations of the development of the acrosome, the extension of processes or arms from the nucleus, and the non-acrosomal cytoplasmic masses in the depressions of the biconcave nucleus are straightforward and accurate and are thus unique. Possibly because later workers were more strongly influenced by emerging schools of thought on mitochondria, Golgi material, acrosome, central body, etc., and at the same time were struggling to homologize with flagellate forms, their descriptions are less easily related to our observations. In any case, until *Astacus* has been examined with the electron microscope and a comparison with *Procambarus* can be made, our observations must stand by themselves. Even the descriptions of spermiogenesis in *Cambarus virilis* by Fasten (10) and McCroan (22) contain anomalies which suggest marked differences in some of the stages when contrasted with *Procambarus*.

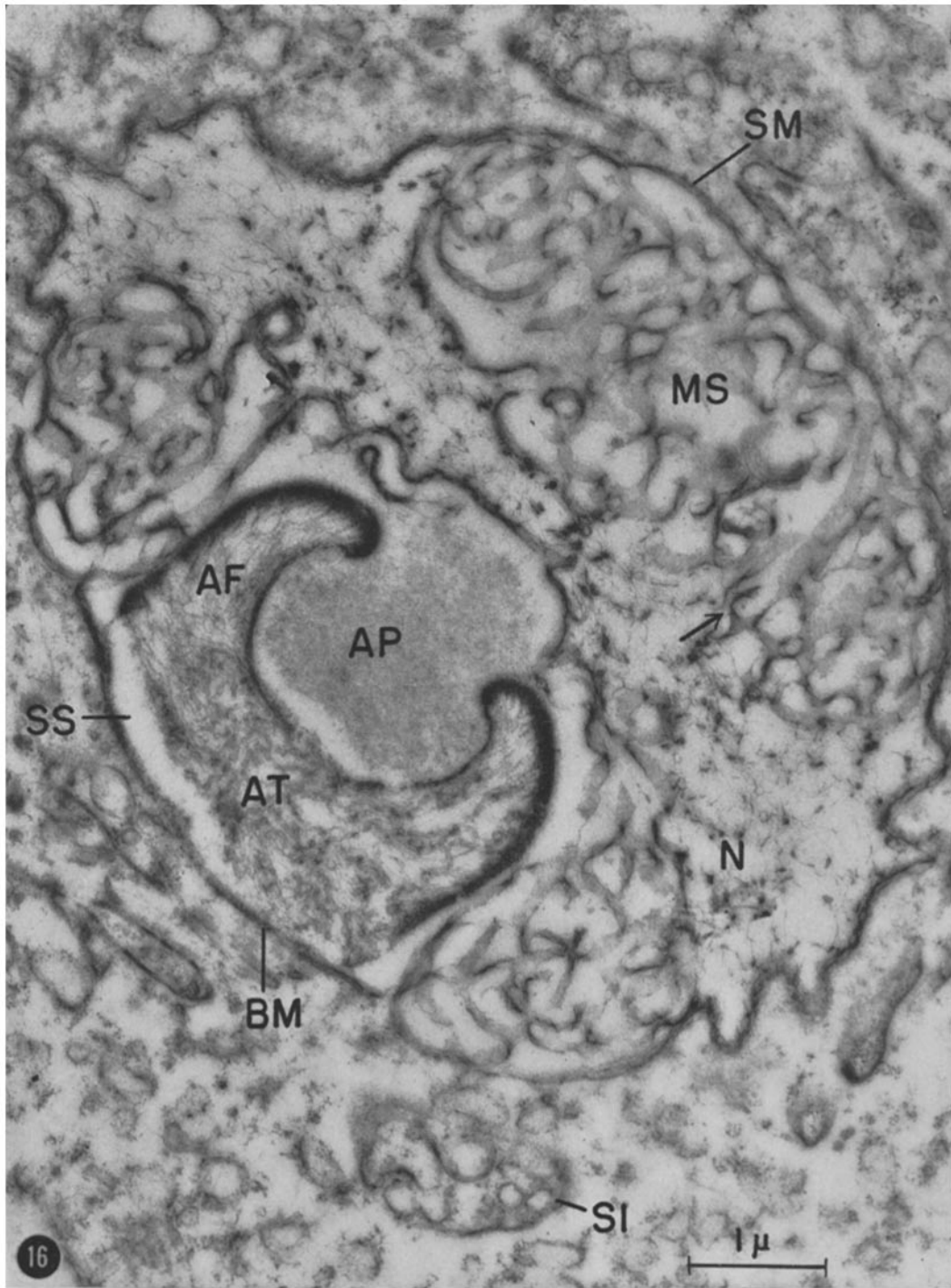
There are, however, several points within the scope of this paper that incidentally bear on questions raised in the literature and hence are worthy of emphasis.

Mitochondria

Mitochondria appear to play a relatively minor role in the spermiogenic transformations. They are sparse and small, with only a few cristae, and are relegated to the periphery of the early spermatid. While occasionally one is seen near the

FIGURE 16

Electron micrograph of a portion of an osmium-fixed (Palade, pH 7.6) spermatid (immature sperm) at Stage V. The sperm cell is completely separated from the surrounding sustentacular cells by a complex membranous tegument (*SM*) that is made up, at least in part, by the confluence of sheets of the convoluted membrane masses (*MS*). The latter can be seen in many places to be extensions of the nuclear surface which can be followed to the periphery of the cell. The contents of the nucleus (*N*) are markedly different from those in Fig. 12: the nucleus is of lower density and is filled with dense, irregular clumps connected with the network of anastomosing microfibrils. The profile of the nucleus is highly irregular. The invagination of the acrosome has produced a double-walled cup, the outer concavity of which is filled with a homogeneous finely granular material (*AP*). The inner space is filled with long fibrillae (*AF*) and tubules (*AT*). The material lining the basal surface of the acrosome (*BM*) is still separate from the dense substance of the wall. Spaces such as that at *SS* are probably due to shrinkage in preparation. The surrounding sustentacular cells are filled with membrane-bound structures containing the remains of the old spermatid cytoplasm (*SI*). \times approx. 20,200.



nucleus, the impression is that it is accidentally trapped there. Certainly, no obligatory relationship appears to exist. Moreover, while the mitochondria may play a subsidiary role in the formation of the membrane elaborations that associate with the nuclear envelope, there is no evidence that they are directly involved with the process. In the same way, they may take a subsidiary part in the formation of the acrosomal vesicle, since occasionally they may be seen disposed in the region where the limiting membrane is extending and being formed. But certainly if they contribute themselves and are actually incorporated at all in the formation of this structure (as postulated by Grabowska, 14; McCroan, 22; and Nath, 29), it must be only to a very small extent, since their total volume represents only a fraction of that of the vesicle. Mitochondria can have very little influence on any of the succeeding transformations, since by Stage III the cell membranes have estab-

lished the new cell boundary, and all mitochondria, with the possible exception of an occasional trapped one, have been excluded. Unless a few mitochondria have become incorporated in either the membrane sheets of the nucleus or the acrosome in some way that has escaped us, any mitochondrial activity necessary to the cell must derive from the structures in the surrounding sustentacular cells.

The absence of mitochondria is unusual and implies a decrease in the demand for the products of mitochondrial metabolism, specifically of ATP. This may also be inferred from the small size of spermatid mitochondria and the small number of cristae in them, which together suggest low metabolic involvement. This is not surprising, however, if it is assumed that the main function of mitochondria in sperm is to supply energy for flagellar movement, as is certainly suggested by the morphology of the sperm tail (*e.g.*, 19). It would

FIGURE 17

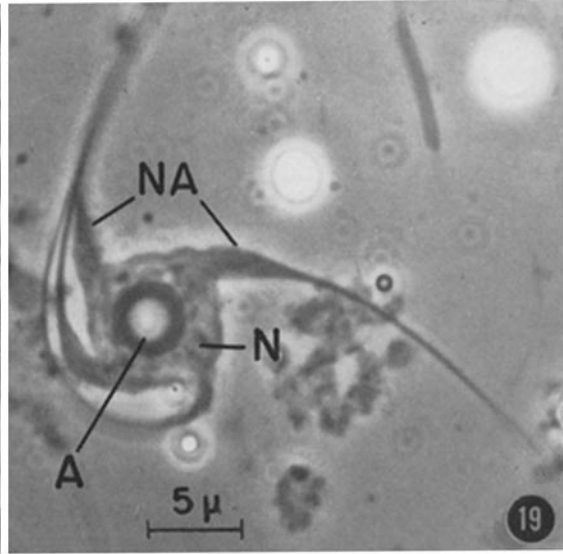
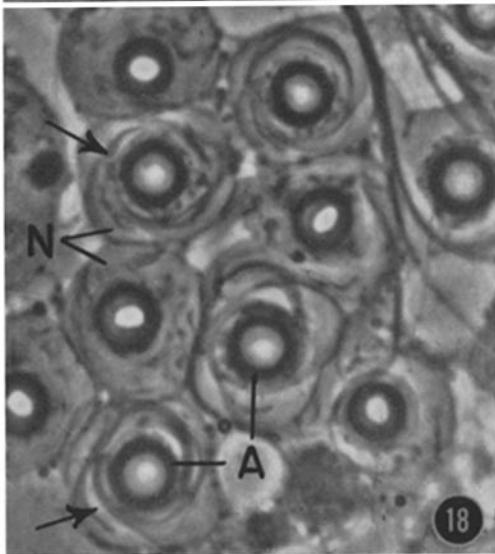
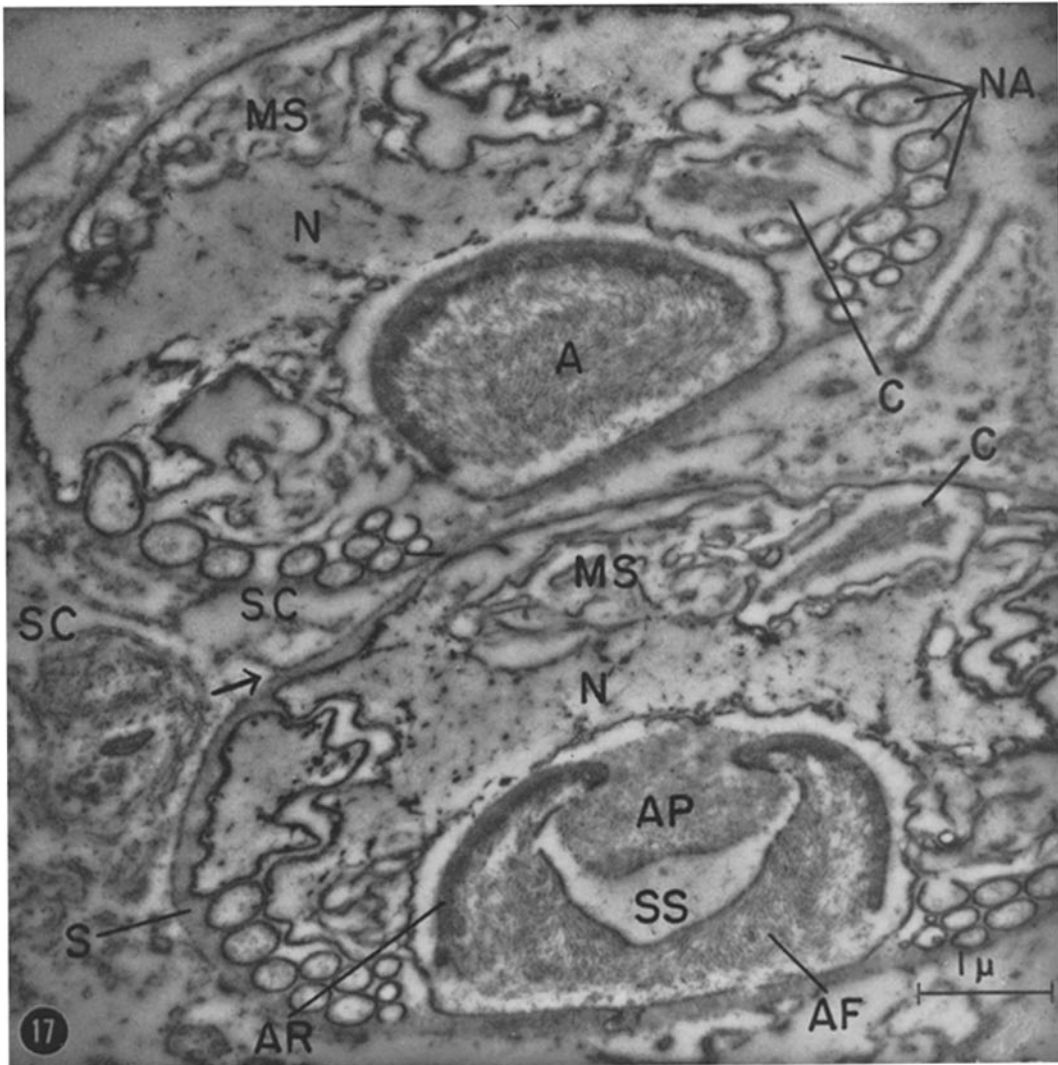
Electron micrograph (longitudinal section) of two nearly mature sperm (early Stage VI) fixed in chrome-osmium (Dalton). These two cells are still *in situ* and surrounded by sustentacular cells (*SC*). They have become invested with a shell of homogeneous material of intermediate density (*S*). The arrow points to a junction between two sustentacular cells. The nucleus (*N*) has a highly tortuous outline and contains clumps of dense material, microfibrils, and a structureless matrix of intermediate density. The nuclear arms or processes have extended and wrapped themselves around the body of the sperm several times; in these sections they are cut transversely (*NA*). Their content is similar to that of the nucleus. The convoluted membranes are considerably reduced in quantity (*MS*). In both cells, masses of granular material (*C*) represent the residua of the centrioles. In the upper cell, the mass is on the side of the nucleus near the acrosome (*A*) while in the lower cell it is on the side of the nucleus away from the acrosome, indicating that there is no specific relation between the two. The acrosomal complex consists of a dense outer shell (*AR*) which is independent of the base of the acrosome, masses of microfibrils and tubules (*AF*), and the homogeneous finely granular material filling the invaginated cup (*AP*). The cells are extremely sensitive to changes in tonicity, etc., and shrinkage spaces (*SS*) are common in such preparations. \times approx. 18,000.

FIGURE 18

Light micrograph (phase) of living early Stage VI sperm teased out in modified Ringer's solution. The cells are seen in frontal view and show the nuclear arms (*N*) wrapped around the body of the sperm particularly at the arrows. The acrosomes (*A*) appear in frontal view as annuli. \times approx. 2500.

FIGURE 19

Light micrograph (phase) of a preparation similar to that in Fig. 18. A single sperm is shown after standing in physiological solution. The arms (*NA*), which are extensions of the nucleus (*N*), have begun to unfurl. The acrosome (*A*) appears as an annulus in this frontal view. \times approx. 2500.



follow, then, that the sperm is otherwise a metabolically inert cell, whose role is simply that of passive carrier of nucleus and penetration apparatus. On the other hand, failure to find morphological evidence of mitochondrial structure in *Procambarus* sperm does not exclude the possibility that mitochondrial activity may be taken over by other cell structures. It is true that no Janus green accumulation could be observed in mature sperm (28), but this is by no means a conclusive test for mitochondrial activity. This question, then, must also remain open until other observations can be applied toward an answer.

Acrosome

The acrosome of *Procambarus* (corresponding to the "Schwanzkapsel", "capsule," or "vesicle" of earlier workers; see also 40) is far more complex than the acrosome of flagellate sperm. Since the only periodic acid-Schiff-positive structure so far described in sperm of any sort is the acrosome (8, 39), the homology between this structure in *Procambarus* and that in flagellate forms rests mainly on the presence of a PAS-positive protein component and on its evolution from a vesicle-contained dense granule in the spermatid. The apparently causal relation between the acrosomal anlage and a characteristic Golgi or idiosomal complex, as discussed by Bowen (6), is not strictly upheld in this form, since the packed configurations of agranular, flattened vesicles that have come to be recognized as organized "Golgi" structures or dictyosomes are not present. Instead, the organelles associated with the acrosome are for the most part strikingly lacking in elaborate organization, and take the form of fairly uniform small vesicles, both with and without attached 150 Å dense particles.

The structures described in this study that do appear to be associated with the developing acrosome in *Procambarus* were unknown to the older microscopists. These are the small stacks of punctate or "annulate" lamellae, which differ from the Golgi structures in that they are not of the "smooth" membrane variety, and which, in crayfish spermatocytes, are basophilic (37). We have had equivocal results in trying to determine the basophilia of these small objects in spermatids. Although they have been described in a number of forms (see references in 37), their function is unknown. Their frequent appearance in sections

is characteristic enough to warrant the supposition that one or more such bodies are involved in some way with the organization of the acrosomal vesicle and granule in *Procambarus*. In any case it would be an innovation at the moment to propose that these structures correspond to the Golgi material. It is more likely that the agranular vesicles that occasionally fuse, elongate, and demonstrate a slight amount of organization (Fig. 2, lower inset) carry on the functions of the Golgi material. If this is the case, however, the situation in Stages I and II represents another example of the close relationship between the vesicular components of the granular and agranular type, which are presumably differentiations of the endoplasmic reticulum. Again, whatever the role of this material may be, it seems to be finished upon formation of the acrosomal granule, since by Stage III the entire vesicular and granular mass is relegated to the sustentacular cell, where, if it has any further effect, it must be outside the spermatid proper.

Within the acrosome itself, the synthesis of the protein and (presumably) carbohydrate moieties and their subsequent arrangement into fibrils, tubules, and regions of varying organization provide an exceedingly interesting case of cellular differentiation. These aspects will be dealt with in further detail elsewhere.

The Nucleus

The morphological changes undergone by the nucleus are dominant in the evolution of this sperm. The full significance of these transformations cannot be deduced from morphological observations alone and must await the results of further studies which are in progress. A number of the observations made here have no recorded counterpart in flagellate sperm. How much of this is common to all aflagellate sperm cannot, at the moment, be decided; but there is a strong possibility that it may be causally related to the absence of a flagellar apparatus.

The posttelophase spermatid nucleus is unusual from its beginning. During re-formation of the nuclear envelope and reconstitution of the nucleus during the second division, a new envelope forms in close association with the chromatin. Karyomeres are formed, often appearing in profiles to be entirely surrounded by membranes. Karyomeres fuse, eventually forming a single nucleus, while

sizable segments of the envelope become trapped in the nucleoplasm (26). The newly formed nucleus is "vesiculate" in that the Feulgen-positive material (chromatin) is localized peripherally and around trapped membranes, while the remaining nuclear space is only palely Feulgen-positive and otherwise gives the impression of containing relatively little material (Figs. 2 to 5). Almost as soon as the spermatid nucleus is formed, its surface erupts in multiple evaginations involving both membranes. They occur all over the surface of the flat disc-like nucleus and are not necessarily associated with particular areas such as the trapped membranes, for example. These evaginations or "blebs" are lined with chromatin. Neither the inner nor the outer membrane is especially unique, though the latter is likely to be coated with dense particles of the order of 150 Å (Fig. 2, circular inset). Although nuclear pores are in evidence, they are not common, particularly in areas of blebbing. Some hint of membrane activity is suggested by the occurrence of bulges and separations of the two membranes (Fig. 3, large arrow). The blebs themselves become intimately associated with the small vesicles of the cytoplasm. As the blebs disappear, they are replaced by membrane sheets which fuse with similar sheets apparently formed in the cytoplasm by transformation of the small vesicles.

Two other nuclear alterations then occur (Stage II): first, the nucleus becomes less vesiculate and more homogeneous; second, it loses roughly two-thirds of its volume. It cannot be said at the moment whether the loss is of water, or of nuclear dry mass, but it is apparent that the clear interchromatin space diminishes while the Feulgen-positive, fast green-staining chromatin becomes dense and evenly distributed. Finally, by Stage III, when the formation of the tightly packed convoluted membrane sheets continuous with the nuclear envelope is complete and the nucleus is completely surrounded by this membrane mass, its volume is one-third to one-quarter of the original volume. At this stage also the chromatin is homogeneously distributed throughout the nucleus as a dense, heavily staining mass (Figs. 9 and 11). Occasional small nucleolus-like structures (Fig. 11, 4) and shreds of trapped membrane are also present. To summarize, concomitant with the formation of membrane sheets continuous with the nuclear envelope, and with the condensation of chromatin

within the nucleus, is a loss of from two-thirds to three-quarters of the nuclear volume.

Following Stage III the nucleus undergoes further alterations. Elaborations of the nuclear envelope are no longer being proliferated, and masses of the membrane material lie above and below the nucleus (Fig. 12). The acrosome undergoes considerable modification and growth at this point, forming a concavity in the nucleus as it does so. Presumably the nucleus would be pushed into a crescent against the opposite wall of the cell, were it not for the membrane mass on the other side away from the acrosome. It appears that this mass is responsible for the second depression which gives the nucleus its biconcave configuration.

During this phase, the appearance of the nucleus changes. Although it is still Feulgen-positive as it was in Stage III, there is a loss of density in both the phase microscope and the electron microscope, as well as a change in texture of the chromatin. There thus appears to be a loss of some other material, or at least a transformation of the material already there. It is possible that this is a manifestation of a change in basic protein type (histone to protamine-like, 5). Although it is difficult to measure accurately, the nuclear volume appears to be approximately the same or possibly slightly less than that at Stage III.

The fine, even texture of the chromatin at Stage IV is replaced by clots and strands of dense fibrous material scattered throughout an otherwise empty-appearing matrix (Fig. 16), much of it adhering to the irregular nuclear surface. This appearance of the interior of the nucleus is the same regardless of fixation procedure or embedding methods. For example, cells fixed in buffered OsO_4 and embedded in Araldite have the same internal structure. Although the nuclei look homogeneous and empty in the phase microscope, Feulgen staining shows the chromatin to be more concentrated at the nuclear border. Concurrent with these changes is a further decrease in volume which leaves the nucleus about half the size it was at Stage III. However, the accuracy of such an estimate is dubious because of the irregular contours (Fig. 16). In any case, in this phase of development such decrease in volume as there is, is accompanied by a *decrease* in density of nuclear material.

At this point, at four equally spaced loci around the periphery of the nucleus, projections begin to

be formed. The forces governing this are not now understood. The result is to give the nucleus a square appearance when seen from above (Figs. 13 and 15). The corners eventually grow out to become the arms which, as they extend, wrap themselves around the sperm in the region of the acrosome. As the arms extend, the membrane material associated with the main body of the nucleus appears to diminish, giving the impression that it serves as surface covering for these projections.

The number of radiating arms appears to be a species characteristic of varying constancy. Four processes are regularly observed in *P. clarkii*; a few exceptions of five have been found, and three have never been seen. In a species of *Cambarus* collected locally, four is again the common number, but five and six are not infrequent. Andrews (2) describes mostly four, but also five, six, and seven arms in *Cambarus affinis*, and Fasten (10) reports six arms in *C. virilis*, with five and seven also common. He also reports five, six, seven, and rarely eight arms in *C. immunitis*, with six and seven frequent. McCroan (22) also shows six and seven arms in *C. virilis*. According to Andrews (2), more than twenty arms are present in *Astacus lenisculus*. It appears, then, that both the modal number and the variance are characteristic for a given species; it is possible that these parameters could serve as taxonomic criteria. For some reason, of all the numbers observed, the number three seems to be forbidden. It is not at all clear morphologically how the number is dictated.

Another constant factor is the direction of coiling of the arms. All are wrapped around the body in the same direction. Viewed with the acrosome up, the direction is clockwise, in agreement with the observation presented by Fasten (10) in *Cambarus*. Andrews (2), however, reports both left- and right-hand spirals in *C. affinis*. Since, at least in some instances, this is not a random affair, the process must be under some kind of control.

Contrary to the belief of certain earlier workers with the light microscope (10, 22), the arms do not develop in connection with the centrioles; neither are they, nor do they contain, extensions of a centriolar apparatus. It was stated above that after Stage III the pair of centrioles may be found on either side of the nucleus and in no particular relation to structures of the spermatid. In all cases the centrioles are separated from the nucleus by at least two membranes of the nuclear envelope.

After the nuclear arms have formed, the centrioles appear to degenerate. In the nearly mature sperm shown in Fig. 17, degenerating centrioles (*C*) are present in both cells. In the uppermost, the degenerating mass lies next to the acrosome. In the lower sperm, the mass lies in the concavity of the nucleus away from the acrosome. In both cases, the centriolar masses are separated from the nucleus by membrane complexes and hence do not contribute any of their material to the nuclear processes.

The arms themselves, as previously reported (28), are continuous with the body of the nucleus. This is in agreement with the early contentions of Andrews (2), Herrmann (16), and later of McCroan (22). When seen in longitudinal section, no evidences of septa have been detected and the fine fibrous material contained within the arms seems to be identical with that of the nucleus. These observations are consistent with the notion that the arms are merely extensions of the nucleus and are not otherwise specialized processes. The fact that they are Feulgen-positive (22, 25) supports this concept. That they cannot be seen to be Feulgen-positive all the way to their extremities is not surprising (*cf.* 41), in view of the fact that in the regions where the arms are very thin (0.2 μ or less) the concentration of Feulgen dye (and of DNA) would have to be extraordinarily high in order for the stain to be visible. While the arms could conceivably be of different composition at their extremities, there is no structural evidence to suggest it. The arms remain furled around the body of the nucleus (Fig. 18) until liberated either by dissolution or by rupture of the PAS-positive shell that invests the sperm (Fig. 17, *S*; see 28). This encasement is probably the "glue"-like material supposed by Fasten (10) to contain the arms until it diffuses away in Ringer's solution, and described by McCroan (22) as staining with Nile blue sulfate. In the latter case, it is not clear whether it is actually this shell that is staining, or the phospholipid of the limiting membrane complex. The arms are, of course, non-motile, as recognized by most previous workers on decapod sperm.

The full significance of the membranal elaborations of the nuclear envelope elude understanding at the moment. That the nuclear envelope is an integral part of the cytoplasmic membrane system has been emphasized by a number of authors (see 26 for discussion) and it is not surprising that

the elaborations are formed in conjunction with cytoplasmic material. But the elaborations themselves are unparalleled in any other cell, and, although they appear eventually to serve as covering for the nuclear arms, the functional reason for their formation cannot be guessed. A key to this question may lie in the nucleus, which is concurrently undergoing marked changes in size and fine structure, and presumably also in chemical composition. The end result of these changes is a nucleus which, at least as seen in this study, does not resemble any nucleus so far reported.

Regardless of the possibility that appearance of the nucleus may in part be due to preparative artifact despite our efforts to rule it out, the lack of order within the nucleus is certainly in marked contrast to that found in certain other forms (for example, see 19) and indicates that regular and periodic organization of chromosomal material is not essential to the functioning of the sperm.

Relationship between Sustentacular Cell and Spermatid

Usually from four to ten large sustentacular cells are visible per testicular lobule per section. They are characterized by their large, irregular nuclei and dense, clumped chromatin. By the time the spermatid begins its differentiation, its cell membrane is in contact with that of the sustentacular cell over a large proportion of its surface, the remaining surfaces being in contact with sister spermatids.

The discontinuities seen in the membranes separating such cells take the form of chains of vesicles (Figs. 2, *ICM*). They may be true intercellular bridges or they may be artifacts resulting from preparative procedures. In any case, they persist regardless of the fixative or embedding method. On the other hand, when such cells are teased living into physiological fluid (Fig. 4) they are generally intact and behave as though they were surrounded by a complete membrane. Thus, either such intercellular connections must heal themselves when the cells are separated or the connections are more highly organized than is visible in these micrographs. However, the cells at Stage II and especially III are almost impossible to tease out intact from the living testis. The nucleus and acrosomal apparatus invariably come free, indicating that at this point the cell is

unbounded or at least surrounded by a membrane that is easily ruptured. Presumably the cell membranes that move in and cut off the nucleus, with its attached membrane sheets, and the acrosome from the residuum of the spermatid cytoplasm are outgrowths from such intercellular discontinuities. The enveloping membranes apparently weave and fuse until the new spermatid is completely surrounded and its cytoplasm has mixed with the cytoplasm of the sustentacular cell. The membrane surface adjacent to the spermatid becomes intimately associated with the convoluted membranes of the nuclear envelope and with the acrosome, and eventually, by Stage IV or V, completely surrounds the spermatid except, of course, at the point of juncture between two sustentacular cells. The spermatid membrane becomes closely associated with the nuclear envelope by Stage V, while the sustentacular cell membrane appears to be separable from it, often by a considerable space. As maturity approaches, the space between the two membranes becomes filled with the fine, PAS-positive material (Fig. 17, *S*). The sustentacular cell membrane becomes almost unrecognizable as a membrane and becomes finely granular around the shell. The PAS-positive material, then, is an extracellular product presumably produced by the sustentacular cells. In Fig. 17 the juncture between two sustentacular cells where they merge to surround the sperm is indicated by the arrow. That the PAS-positive material is not produced by the sperm is indicated by the fact that it appears only around the periphery of the sperm and does not penetrate the interstices between the arms. Finally, when the sperm are free to be discharged, they are surrounded by the PAS-positive extracellular envelope only (28).

General

The spermatozoa of decapods and of a few other orders are unique in that they possess no flagella. Hence they stand in sharp contradistinction to the vast majority of animal spermatozoa, which are constructed with an often very elaborate flagellar apparatus. The aflagellate forms are probably excursions from the main evolutionary stream, since the decapods are not considered primitive types, while those animal forms thought to be closer to primordial Metazoa possess motile gametes.

Despite efforts to homologize, the early light

microscope studies of decapod spermiogenesis (21) demonstrate the process to be as strikingly different from that in flagellate forms as are the mature spermatozoa themselves. Even the more refined observations made possible by the electron microscope, which correct and confirm details of the earlier studies, show the process of sperm maturation in decapods to be distinguished by events so far not encountered in flagellate forms.

Most obvious, of course, is the absence of a flagellum. There is not even a suggestion of an abortive apparatus such as may be the case, for example, in the sperm of certain ticks (29), although centrioles which are probably primary organizers of the flagellum are present throughout most of the period of development. At best it is difficult to answer the question whether the flagellum does not develop because of the absence of necessary accessory structures such as mitochondria, or whether the unusual morphology of this cell during its morphogenesis results largely from the failure of the flagellum to develop. A comparative study of various aflagellate forms carefully chosen with respect to the evolutionary status of the organism may provide some clue.

In any case, at both the light and electron microscope levels it is clear that in decapods in general and in *P. clarkii* in particular, spermiogenesis is a cytomorphogenetic process of unique proportions and consequences, having no obvious counterpart in other forms or cell types; but the specializations observed here must have implications of more general significance.

BIBLIOGRAPHY

1. ALFERT, M., and GESCHWIND, I. I., A selective staining method for the basic proteins of cell nuclei, *Proc. Nat. Acad. Sc.*, 1953, **39**, 991.
2. ANDREWS, E. A., Crayfish spermatozoa, *Anat. Anz.*, 1904, **25**, 456.
3. BAKER, J. R., *Cytological Technique*, New York, J. Wiley and Sons, 4th edition, 1960.
4. BELAR, K., Beiträge zur Kausalanalyse der Mitose. II. Untersuchungen an der Spermatozyten von *Chorthippus* u. s. w., *Arch. Entwickl. mech. Organ.*, 1929, **118**, 359.
5. BLOCH, D. P., and HEW, H. Y. C., Schedule of spermatogenesis in the pulmonate snail *Helix aspersa* with special reference to histone transition, *J. Biophysic. and Biochem. Cytol.*, 1960, **7**, 515.
6. BOWEN, R. H., Further notes on the acrosome of the animal sperm. The homologies of the non-flagellate sperms, *Anat. Rec.*, 1925, **31**, 201.
7. CAULFIELD, J. B., Effects of varying the vehicle for OsO₄ in tissue fixation, *J. Biophysic. and Biochem. Cytol.*, 1956, **3**, 827.
8. CLERMONT, Y., and LEBLOND, C. P., Spermiogenesis of man, monkey, ram and other mammals as shown by the "periodic acid-Schiff" technique, *Am. J. Anat.*, 1955, **96**, 299.
9. DALTON, A. J., A chrome-osmium fixative for electron microscopy, *Anat. Rec.*, 1955, **121**, 281.
10. FASTEN, N., Spermatogenesis of the American crayfish, *Cambarus virilis* and *Cambarus immunitis* (?) with special reference to synapsis and the chromatoid bodies, *J. Morphol.*, 1914, **25**, 587.
11. FAWCETT, D. W., ITO, S., and SLAUTTERBACK, D., The occurrence of intercellular bridges in groups of cells exhibiting synchronous differentiation, *J. Biophysic. and Biochem. Cytol.*, 1959, **5**, 453.

Having established the morphology of the spermatozoa of *Procambarus* (28) and having outlined in this paper the stages in sperm formation and some of the problems that these observations have raised, we shall examine some of the details of the processes involved in sperm formation in *Procambarus* in greater detail in the next paper of this series.

This work was begun at the Rockefeller Institute in the laboratory of Dr. Keith R. Porter. I am grateful to him, to Dr. George Palade, and to their associates for the stimulating climate they provided. I wish also to acknowledge the technical assistance at various times of Mrs. Susan Schwingle Gleason, Mr. Gordon Kaye, Miss Beatrix Yuhasz, and Mr. Martin Clark. Parts of the work described were supported at various times by grants from the American Cancer Society (INSP-85, E-213) and the National Institutes of Health (RG-6753).

Note Added to Proof:

Two papers describing certain aspects of sperm differentiation in the Japanese crayfish *Cambaroides japonicus* have recently appeared (Yasuzumi, G., Kaye, G. I., Pappas, G. D., Yamamoto, H., and Tsubo, I., *Z. Zellforsch. u. mikr. Anat.*, 1961, **53**, 141; and Kaye, G. I., Pappas, G. D., Yasuzumi, G., and Yamamoto, H., *Ibid.*, 1961, **53**, 159.) These electron microscope observations suggest that in general the process resembles that in *P. clarkii*, though differing in detail. The extent of the actual differences, however, depends somewhat on the authors' interpretations, which will be discussed elsewhere.

Received for publication, December 18, 1960.

12. FLAX, M. H., and HIMES, M., Microspectrophotometric analysis of metachromatic staining of nucleic acids, *Physiol. Zool.*, 1952, **25**, 297.
13. GILSON, G., Etude comparée de la spermatogénèse chez les arthropodes, *Cellule*, 1886, **2**, 83.
14. GRABOWSKA, Z., L'appareil de Golgi dans les spermatoïdes des crustacés, *Astacus fluviatilis* et *A. Leptodactylus*, *Compt. rend. Soc. biol.*, 1927, **97**, 3.
15. GROBBEN, C., Beiträge zur Kenntniss der männlichen Geschlechtsorgane der Dekapoden, *Arb. zool. Inst. Univ. Wien u. zool. Stat. Triest*, 1878, **16**, 300.
16. HERRMANN, G., Notes sur la structure et le développement des spermatozoïdes chez les décapodes, *Bull. sc. France et Belg.*, 1890, **22**, 1.
17. HIMES, M., and MORIBER, L., A triple stain for deoxyribonucleic acid, polysaccharides and proteins, *Stain Technol.*, 1956, **31**, 67.
18. HOTCHKISS, R. D., A microchemical reaction resulting in the staining of polysaccharide structures in fixed tissue preparations, *Arch. Biochem.*, 1948, **16**, 131.
19. KAYE, J. S., Changes in the fine structure of mitochondria during spermatogenesis, *J. Morphol.*, 1958, **102**, 347.
20. KEPPEN, N. K., De la spermatogénèse chez *Astacus fluviatilis*, *Mém. Soc. naturalistes Kiev*, 1906, **20**(2), 231.
21. KOLTZOFF, N. K., Studien über die Gestalt der Zella. I. Untersuchungen über die Spermien der Decapoden, *Arch. mikr. Anat.*, 1906, **67**, 364.
22. McCROAN, J. E., Spermatogenesis of the crayfish, *Cambarus virilis*, with special reference to the Golgi material and mitochondria, *Cytologia*, 1940, **11**, 136.
23. McMANUS, J. F. A., Histological demonstration of mucin after periodic acid, *Nature*, 1946, **158**, 202.
24. MOSES, M. J., Studies on nuclei using correlated cytochemical, light and electron microscope techniques, *J. Biophysic. and Biochem. Cytol.*, 1956, **2**, No. 4, suppl., 397.
25. MOSES, M. J., Aflagellate spermiogenesis in the crayfish, *Anat. Rec.*, 1958, **130**, 343.
26. MOSES, M. J., Breakdown and reformation of the nuclear envelope at cell division, in *Proc. IV Internat. Kong. Elektronenmikroskopie*, (W. Bargmann *et al*, editors), Berlin, Springer-Verlag, 1960, **2**, 230.
27. MOSES, M. J., A light and electron microscope study of spermiogenesis in decapod crustacea, *Anat. Rec.*, 1960, **136**, 342.
28. MOSES, M. J., Spermiogenesis in the crayfish (*Procambarus clarkii*). I. Structural characterization of the mature sperm, *J. Biophysic. and Biochem. Cytol.*, 1961, **9**, 222.
29. NATH, V., Cytology of spermatogenesis, *Internat. Rev. Cytol.*, 1956, **5**, 395.
30. NICHOLS, M. L., Comparative studies in crustacean spermatogenesis, *J. Morphol.*, 1909, **20**, 461.
31. ORNSTEIN, L., and POLLISTER, A. W., Applications of phase microscopy in cytology and electron micrography, *Tr. New York Acad. Sc.*, 1952, **14**, 5.
32. PALADE, G. E., A study of fixation for electron microscopy, *J. Exp. Med.*, 1952, **95**, 285.
33. PENN, G. H., A study of the life history of the Louisiana red-crawfish, *Cambarus clarkii* Girard, *Ecology*, 1943, **24**, 1.
34. REINHARD, L., Zum Bau der Spermien and zur Spermatogenese von *Potamobius leptodactylus* (*Astacus leptodactylus*), *Arch. Zellforsch.*, 1913, **10**, 324.
35. RETZIUS, G., Die Spermien der Crustaceen, *Biol. Untersuch.*, 1909, **14**, 1.
36. RUTHMANN, A., Basophilic lamellar systems in the crayfish spermatocyte, *J. Biophysic. and Biochem. Cytol.*, 1957, **4**, 267.
37. RUTHMANN, A., The fine structure of the meiotic spindle of the crayfish, *J. Biophysic. and Biochem. Cytol.*, 1959, **5**, 177.
38. SABATIER, A., Spermatogénèse chez les crustacés décapodes, *Trav. Inst. zool. Montpellier et Sta. mar. Cette*, Series 9, Mem. No. 3, 1893.
39. SCHRADER, F., and LEUCHTENBERGER, C., A cytochemical analysis of the functional interrelationships of various cell structures in *Arvelius albopunctatus* (de Geer), *Exp. Cell Research*, 1950, **1**, 421.
40. WORLEY, E. K., A study of the sperm-forming components in three species of decapoda, *Cellule*, 1939, **48**, 149.
41. YASUZUMI, G., Spermatogenesis in animals as revealed by electron microscopy. VII. Spermatid differentiation in the crab, *Eriocheir japonicus*, *J. Biophysic. and Biochem. Cytol.*, 1960, **7**, 73.