FINE STRUCTURAL STUDIES ON THE REPRODUCTIVE BIOLOGY OF THE ANTHOZOAN *BUNODOSOMA CAVERNATA*

A Dissertation

Presented to

The Faculty of the Department of Biology

University of Houston

In Partial Fulfillment

of the Requirements for the Degree

Doctor of Philosophy

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by

WILLIAM CORNELIUS DEWEL

August 1972

ACKNOWLEDGEMENTS

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Abstract

This investigation is concerned with certain aspects of the reproductive biology of the anthozoan Bunodosoma cavernata.

The first portion of the study is a fine structural examination of spermiogenesis and the mature spermatozoon. The young spermatid is located in the peripheral region of the testicular cyst. It possesses a large uncondensed nucleus. The cytoplasm contains multivesicular-like bodies, mitochondria, lipid-like inclusions and a Golgi complex. The flagellum is already present in this early stage of spermiogenesis and is surrounded by two separate centriolar specializations, centriolar satellites and a pericentriolar branching complex. The centriolar satellites, presumably centers for microtubule assembly during maturation, do not persist during spermiogenesis. The pericentriolar branching complex, however, persists and is present in the mature spawned spermatozoon and possibly functions in support. During spermiogenesis the nucleus condenses to form the compact nucleus of the mature spermatozoon. The Golgi complex produces "donut" shaped vesicles which become localized near the nucleus. No distinct acrosome is formed.

The second portion of this study is a fine structural analysis of the surface of the oocyte, unfertilized egg and fertilized egg of B. cavernata. The cortex of the oocyte

contains numerous electron dense cortical granules. Very prominent surface specializations, which resemble microvilli, project from the surface of the egg. As a result of fertilization the cortex of the egg undergoes an extensive reorganization. Fusion and vesiculation of cortical granule membranes and the egg plasma membrane lead to the discharge of the cortical material into the spaces between the microvillus-like surface specializations. The flocculent material forms a coat over the egg surface at the bases of the microvillus-like structures. The egg of B. cavernata is not surrounded by accessory investments which must be traversed by the spermatozoon prior to fertilization. The spermatozoon does not possess a well-defined acrosome but small "donut" shaped vesicles are present in the head region. These vesicles cannot act as an acrosome in the penetration of egg investments since none are present. Such vesicles, however, may be involved in gamete membrane fusion.

TABLE OF CONTENTS

LIST OF FIGURES

I.

INTRODUCTION

Numerous investigations directed to understanding the complex series of events associated with fertilization are present in the literature (review, 8). Contributions from several subdisciplines including biochemistry, physiology, immunology and morphology have provided valuable insights on the problem and have generated continued interest and research toward extending our knowledge regarding this dynamic process.

One problem which has received extensive attention has concerned the interaction of gametes from the time the spermatozoon encounters the outermost investment of the egg to the time it is incorporated into the egg cytoplasm. The fine structural investigations of Colwin and Colwin (4,5,6,7) provided valuable information concerning this interaction. In the annelid Hydroides and the hemichordate Saccoglossus the spermatozoa bear distinct acrosomes at their apices. The acrosome is a complex membrane bound structure derived from the Golgi complex during spermiogenesis which contains hydrolytic enzymes. Upon encountering the outer egg investments the outer acrosomal and sperm plasma membrane dehisce and then fuse releasing the substance of the acrosomal vesicle. These hydrolytic enzymes act as lysins and locally dissolve away the egg envelope. The inner acrosomal membrane becomes extended as a filament(s) passing through the region of local envelope dissolution. The acrosomal filament interdigitates with and then fuses with the egg plasma membrane.

As a result the zygote is bounded by a mosaic of sperm plasma membrane, acrosomal membrane and egg plasma membrane. Since these studies, numerous other investigations on sperm morphology, acrosomal reactions and gamete interactions have indicated that the Hydroides-Saccoglossus pattern of fertilization, as it is now called, is characteristic for several invertebrate and vertebrate groups. The egg also undergoes morphological changes as a consequence of fertilization. These changes characteristically involve a breakdown of granules located in the cortex of the egg (cortical granules) and, concommitantly, an elevation of a "fertilization membrane".

In view of these results it is interesting that recent fine structural examinations of the spermatozoa of lower acoelomate invertebrates reveal that there is no well defined acrosome present (1,2,3,9,10,11,13,14,15,16). Instead in the head region of the sperm there are a few small moderately electron dense vesicles.

Furthermore, unlike the eggs of the invertebrates mentioned above it is reported that the eggs of the Porifera and the Cnidaria are not endowed with accessory investments (12).

This investigation was undertaken to examine the fine structure of developing and mature gametes and gamete interaction in the anthozoan Bunodosoma cavernata with the intent of gaining new information regarding fertilization in lower acoelomates. Specifically, what is the developmental origin

of the vesicles in the spermatozoon? What changes in the morphology of the vesicles, if any, occur during sperm approach to or contact with the egg? Does the egg, in fact, possess investments; and if investments are present, are they elevated during fertilization? What is the response of the egg, if any, to fertilization? It is submitted that answers to these questions will significantly add to our knowledge regarding fertilization in acoelomate invertebrates. Further, an investigation of an apparently simpler system (no acrosome; no investments) may be expected to provide new insights regarding fertilization in general.

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II.

SPERMIOGENESIS AND THE MATURE SPERMATOZOON

Introduction

The ultrasturcture of the spermatozoa of many invertebrate species has been described in the last decade (12). These investigations have demonstrated the presence of a well-defined acrosome which is involved in the local dissolution of egg investments and in the fusion of egg and sperm membranes during fertilization. However, among the acoelomate invertebrates, the existence of an acrosome is in question. Tuzet (23) has reported that the spermatozoan of the sponge Aplysilla rosea does not possess an acrosome. Among the cnidaria, spermatozoa which do not have well defined acrosomes have been observed in the hydrozoans Hydra littoralis (24), H. attenuata (19), Eudendrium racemosum (13), Pennaria tiarella (20), Hydractinia echinata and Tubularia crocea (14), and Campanularia fexuosa (15); and in the scyphozoans Aurelia aurita and Cyanea capillata (14), and Nausithoe sp. (1). Although typical acrosomes have not been seen in the spermatozoa of these cnidaria, small moderately electron dense vesicles of suggested Golgi origin are characteristically found in the head region of the sperm.

Another interesting morphological feature of cnidarian spermatozoa is the presence of a branching system of pericentriolar processes associated with the distal centriole in the hydrozoans P. tiarella (20), H. echinata and T. corcea

(14), and C. flexuosa (15); and in the scyphozoans Philalidium gregarium (21), A. aurita and C. capillita (14), E. racemosum (13) and Nausithoe sp. (1). Several of these authors (1,15,21) have suggested that this system may provide structural support for the axial filament.

Little is known concerning the ultrastructure of developing and mature spermatozoa among the anthozoans. In preliminary reports Hinsch and Clark (14) and Dewel and Clark (6) have observed several interesting characteristics of the spermatozoa of Metridium senile and Bunodosoma cavernata, respectively. First, the spermatozoa of both species show a degree of asymmetry not commonly observed in the spermatozoa of other invertebrates. This asymmetry is more extreme in B. cavernata. Second, the sperm of both species posses a single, large, complex mitochondrion associated with lipid-like inclusions. Third, centriolar specializations similar to those initially described by Szollosi in Phialidium are present in the spermatozoa of these anthozoans. Finally, neither spermatozoan displays a typical acrosome, although both have small vesicles located in the head region.

This investigation of spermiogenesis and mature sperm in anthozoans is preliminary to an understanding of reproduction in this important group of metazoans.

Materials and Methods

Adult animals were collected near Port Bolivar on the Texas Gulf Coast. Preparations of testis were obtained by dissecting the animal and transferring portions of the mesentery containing testicular cysts to fixative. Fixation was carried out in cold (0-4°C) 4.0% glutaraldehyde in 0.2 M s-Collidine buffer containing 8.6% sucrose. Following glutaraldehyde fixation the preparations were washed in buffer and post-fixed in cold 1.0% osmium tetroxide in 0.1 M phosphate buffer for one hour. The tissue was then dehydrated in a graded acetone series, embedded in a low viscosity epoxy resin (18) and sectioned on a Sorvall MT-2 ultramicrotome according to standard techniques. Specimens were mounted on uncoated grids, stained with uranyl acetate and lead citrate (17) and observed with an AEI EM-6B or Hitachi HS-8 electron microscope. Plastic embedded material for light microscopy was stained with 1.0% aqueous toluidine blue.

Mature sperm were obtained in one case by pipetting extracts obtained from macerated testis into shallow aquaria containing adult sea anemones. Spawning occurred in less than 20 minutes. On other occasions spawning occurred naturally within 18 hours after collection. Sperm suspensions were prepared for electron microscopy using the techniques previously described.

Results

The spermatogenic tissue of B. cavernata is organized into testicular cysts bounded by a thin mesogleal layer (Fig. 1). The cysts are located within the tissues of the incomplete septa just radial to the free margins of the septal filaments. The gastrodermal surface of the septa surrounding the testicular cyst has a flagellated epithelium composed of cells possessing numerous secretory accumulations (unpublished observations).

The mesogleal boundary of the testicular cysts is a tripartite structure with a central fibrous layer surrounded by two finely filamentous layers (Figs. 2 and 3). In animals possessing testicular cysts with numerous mature spermatozoa, axonemal arrays resembling sperm tails are present in the central fibrous layer of the mesoglea (Fig. 2).

Located just within the mesogleal boundary of the testicular cyst is a zone of cells in spermatogenic and early spermiogenic stages (Figs. 2 and 4). The cytoplasm of the early spermiogenic cells contains a Golgi complex, multivesicular-like bodies, lipid-like inclusions, mitochondria, ribosome-like particles but no obvious endoplasmic reticulum (Figs. 2 and 4).

Cytoplasmic bridges connecting developing spermatids are noted (Fig. 4). There are dense plaques on the cytoplasmic

surface of the plasma membrane which outline these bridges (Fig. 4). The bulk of the central region of the testicular cyst contains spermatids and spermatozoa which appear mature when compared to spawned sperm (Figs. 1 and 2). The young spermatid contains a prominent tail, centrioles and associated structures (Fig. 4). It is interesting to note that we have observed these structures in spermatocytes also. Spermatogenesis will be the subject of a paper now in preparation.

In the tail the axoneme emanates from the triplets of the distal centriole (Figs. 4 and 7). A cytoplasmic collar surrounding the most anterior region of the tail is present but poorly developed until later spermiogenic stages. Nine electron dense pericentriolar processes extend from an electron dense matrix in which the centriolar triplets lie (Figs. 5-8). The thickened tip of each primary process has a connecting extension (interprimary process) which terminates on an adjacent primary process (Figs. 5 and 8). The tip of each process also gives rise to secondary processes which appear to end blindly in the cytoplasm. This pericentriolar complex (arrows) lies close to the anterior margin of the collar, and the thickened tips appear to attach to the collar (Figs. 6 and 7). The pericentriolar processes are distinct from centriolar satellites (pericentriolar bodies) which are also noted at this stage (Figs. 5, 7 and 9).

Slightly oblique sections through the region where the triplet tubules join the doublets at the most anterior region of the sperm tail reveal Y-shaped fibrillar structures extending between the doublets and tail plasma membrane as well as portions of the pericentriolar processes (Fig. 6). Both the pericentriolar processes and the Y-shaped fibers persist throughout the development of the spermatid and are found in the mature sperm.

In the early spermatid there is a general polarization of cytoplasm. The mitochondria and the Golgi complex are observed near the centrioles in a region of the cell possessing the bulk of the cytoplasm (Figs. 9 and 10). Numerous concave or "donut" shaped vesicles are closely associated with the Golgi cisternae (Figs. 11 and 12). These vesicles possess a fibrillar substructure (Fig. 12). At this stage the opposite end of the spermatid, that destined to be the anterior end of the mature spermatozoon, has only a very thin cytoplasmic layer.

Subsequent to this early stage in spermatid differentiation, several maturational events occur more or less simultaneously. The aggregation and fusion of mitochondria takes place in the posterior region of the spermatid (Figs. 8, 9 and 10). Closely associated with the aggregating mitochondria is a large lipid-like inclusion (Fig. 14). Nuclear condensation begins (Figs. 11, 13, 14).

At the apical and basal end of the nucleus the membranes of the nuclear envelope become tightly apposed and just within the karyoplasm there are electron dense plaques of granular material (Figs. 13, 14). The basal surface of the nucleus indents and forms a fossa in which the proximal centriole lies.

As nuclear condensation proceeds, areas of uncondensed nucleoplasm form in the posterior region of the nucleus (Fig. 13). In still later stages of nuclear condensation these uncondensed regions form unusual spherical pockets in a rather precise radial array surrounding the fossa in the posterior ridge of the nucleus (Figs. 14, 15). The cytoplasmic collar becomes more highly developed and eventually surrounds the uppermost part of the spermatid axoneme (Fig. 14).

The structure of the mature spermatozoon is observed in spawned spermatozoa or in testicular spermatozoa indistinguishable from those spawned (Figs. 16, 17 and 18).

The anterior region of the spermatozoon is characterized by the presence of an electron dense nucleus surrounded by a double membrane. This nucleus commonly possesses a small, central, electron lucent area. No vesicles are present between the nuclear and sperm plasma membranes at the apex of the sperm (Figs. 16, 17 and 18). However, several biconcave or "donut" shaped vesicles are present lateral and posterior to the head (Figs. 17 and 18). As mentioned earlier in

maturing spermatids similar vesicles are observed in close association with Golgi cisternae.

The midpiece contains a single, complex mitochondrion derived from the fusion of smaller mitochondria during spermiogenesis and consisting of a central, spherical region partly surrounded by a cup-shaped extension (Figs. 18, 19). The mitochondrion is asymmetric to the midline of the sperm. Located in close association with the mitochondrion is a lipid-like inclusion (Fig. 17).

A proximal centriole lying at an approximate 45^{o} angle relative to the midline of the sperm is located in the cup-shaped fossa in the caudal pole of the nucleus. The sperm axoneme arises from the distal centriole and is surrounded by a cytoplasmic collar. In mature sperm the collar is interrupted locally by membrane fusion. By comparing longitudinal sections (Fig. 18) and cross sections (Figs. 19 and 21) the various regions of fusion and regions of uninterrupted collar are observed.

The tubules of the sperm tail display the characteristic 9+2 pattern with the two central tubules arising slightly posterior to the distal centriole (compare Figures 18, 19 and 21).

Discussion

This investigation of spermiogenesis and the mature sperm of the anthozoan B. cavernata provides new information which clarifies several important points concerning the reproductive biology of the cnidarians.

A pattern of organization based on gamete maturity is recognizable in the testicular cyst. The peripheral region contains immature gametes while the central region contains late spermatids often indistinguishable from spawned sperm. A more detailed discussion of the organization of the testicular cyst and early spermatogenesis will be the topic of a forthcoming paper.

The development of the spermatozoa of B. cavernata is interesting in several respects. The flagellum, destined to be the tail of the mature sperm, is already present in the spermatocyte. Hanisch (13) has reported a similar precocious differentiation of the sperm flagellum for the hydrozoan Eudendrium racemosum.

As is typical of invertebrate sperm, the flagellum of B. cavernata arises from the distal centriole. Associated with this centriole are two distinct centriolar specializations which deserve careful attention. First, there are one or more lateral club-shaped appendages which have been termed satellites (pericentriolar bodies) by numerous authors. They appear to function as one of several

nucleating sites for mitotic or cytoplasmic microtubules (2,5,10,16,21,22). In B. cavernata satellites are present in spermatocytes and spermatids but not in mature spermatozoa.

Second, there are other centriolar specializations, distal to the satellites, which in this paper are termed pericentriolar processes. Several authors (1,13,14,15,20, and 21) have described similar structures in male gametes of the cnidaria. In 1964 Szollosi discussed in detail pericentriolar processes found in the epithelial cells and spermatids of P. gregarium (21). However, he did not distinguish these from the satellites mentioned earlier. Although we are in essential agreement with Szollosi concerning the basic structure of them and concur with him that they may function as an anchoring device, we believe that the nucleating sites for microtubules (satellites) and the pericentriolar processes are separate structures. Flock and Duvall (11) showed that these structures were separate in their investigation of the kinocilia of the sensory cells of the inner ear and lateral line organs in a teleost. Since Szollosi's investigation several other authors have described these elaborations. Fallon and Austin (8) called them spokes or ridges in the spermatozoa of Nereis limbata. Summers (20) described their structure in the sperm of the cnidarian P. tiarella and in agreement with Szollosi also called them satellites. However, Hanisch (13) distinguished these

centriolar specializations from satellites and termed them centriolar processes (Centriolenfortsatz). More recently, Afzelius and Franzen (1) observed pericentriolar processes in Nausithoe and termed the combined processes an anchoring fiber apparatus.

Colwin and Colwin (4) described in the spermatozoa of Hydroides Y-shaped connections between the outer doublets of the flagellum and the most anterior region of the tail plasma membrane. Afzelius and Franzen (1) also described similar connections in Nausithoe. Since slightly oblique sections through this region in Bunodosoma reveal both the pericentriolar processes emanating from the triplets and Y-shaped connections associated with doublets of the tail we feel that the latter may be further arborizations of the pericentriolar branching complex. Finally, the term transitional fibers has been used by other authors to describe pericentriolar arborizations similar to those discussed in this paper (3). Since these processes are not transitional in a temporal sense (they are present in mature sperm) or in a positional sense (they are not confined to the junction between the centriole and the tail) the term 'transitional fiber' is inappropriate.

The small "donut" shaped vesicles found lateral to the head in B. cavernata are also of great interest. They appear to be of Golgi origin. As mentioned in the introduction of

this paper, similar vesicles are frequently present in the sperm of other cnidarians. Unlike the vesicles of apparent Golgi body origin in invertebrates of higher taxonomic rank, they do not coalesce to form characteristic acrosomal complexes. They may, however, represent the evolutionary forerunner of such structures as suggested by Summers (20) and Afzelius and Franzen (1).

Cytochemical characterization of the vesicles and fine structural examinations of fertilization now being attempted should extend our knowledge regarding the function of these structures during fertilization.

Similar to spermiogenesis in other invertebrates there is general polarization of spermatid cytoplasm, fusion of mitochondria and condensation of nuclear material. However, there are two features of additional interest. The first is the presence of a lipid-like inclusion which becomes associated with the developing complex mitochondrion. This association probably represents energy storage and utilization. The second is the temporary existence of precisely arranged pockets of uncondensed nucleoplasm at the caudal pole of the nucleus. The significance of this is unclear.

Finally, it is interesting that during this stage the maturing spermatids are connected by intercellular bridges similar to those described by Dym and Fawcett (7) in the rat. Since those spermatids which are interconnected are in the

same differentiation stage, the hypothesis of Fawcett et al. (9), as well as numerous other investigators (review, Dym and Fawcett, 1971) that the bridges are involved in maintaining cell synchrony is indirectly supported.

Legend

Fig. 1. A light micrograph showing several testicular cysts located within the incomplete septa of Bunodosoma. g, gastrodermal surface of septum: sg, region of cyst containing cells undergoing maturation: sp, mature spermatozoa: arrow, mesogleal boundry of the testicular cyst. X 500.

Fig. 2. An electron micrograph showing two adjacent testicular cysts separated by mesoglea. mg, mesoglea: sp, mature spermatozoa: s, spermatid. X 10,500.

Fig. 3. A higher magnification of the mesoglea showing two filamentous layers surrounding a fibrous component. X 30,000.

Fig. 4. An electron micrograph showing a region of the testicular cyst containing cells in early spermiogenic stages. Intercellular bridges (i) are present between spermatids. A cytoplasmic collar (c) is forming around the anterior region of the tail. Spermatid organelles become localized in the posterior region of the cell near the centrioles and tail (arrow). "Donut" shaped vesicles (v) are located near the Golgi cisternae. Mitochondrial fusion is taking place and lipid-like inclusions (l) are present in the spermatid cytoplasm. X 13,000.

Fig. 5. An electron micrograph showing spermatid centrioles. Two pericentriolar specializations are present, a satellite (s) and pericentriolar processes (p). X 45,900.

Fig. 6. An oblique section of the region where the distal centriole is giving rise to the flagellum. Note the thickened tips of the pericentriolar processes (p) and the more posterior Y fibrils (arrow). c, cytoplasmic collar. X 45,600.

Fig. 7. A longitudinal section of the spermatid centrioles and flagellum. pc, proximal centriole: dc, distal centriole: s, satellite: (arrow) pericentriolar processes: mg, mesoglea. X 53,700.

Fig. 8. An electron micrograph showing several spermatid organelles. Note the small moderately electron dense vesicles (v) associated with the Golgi cisternae. arrow, centriole and associated pericentriolar processes. X 31,400.

Fig. 9. An electron micrograph of an early spermatid. The nucleus (n) is just beginning to condense. Spermatid organelles are localized in the posterior region of the cell. g, Golgi complex: m, mitochondria: dc, distal centriole: arrow, satellite. X 22,100.

Fig. 10. An electron micrograph showing the localization and fusion of mitochondria during spermiogenesis. Note the cytoplasmic collar (arrow) surrounding the flagellum. X 17,300.

Fig. 11. An electron micrograph showing two spermatids with "donut" shaped vesicles (arrows) closely associated with Golgi complexes. X 13,300.

Fig. 12. A higher magnification of a "donut" shaped vesicle showing its fibrillar substructure. X 118,000.

Fig. 13. An electron micrograph showing a later stage in nuclear condensation. Spherical pockets of uncondensed nucleoplasm are forming in the posterior region of the nucleus (arrows). Electron dense plaques (p) of granular material are present at the apical and basal surfaces of nuclear mass. X 19,000.

Fig. 14. A later stage in nuclear condensation showing one of the spherical pockets of uncondensed nucleoplasm located in the posterior ridge of the nucleus (arrow). A lipid-like inclusion (l) is associated with the mitochondrion. At this stage the Golgi complex is occasionally noted lateral to the nucleus. X 24,000.

Fig. 15. A still later stage in nuclear condensation showing the radial array of pockets of uncondensed nucleoplasm (arrows) in the posterior ridge of the nucleus. X 30,600.

Fig. 16. A nearly mature spermatid showing a longitudinal section of a portion of the pericentriolar process (arrow) of the distal centriole attached to the anterior margin of the collar (c). X 37,500.

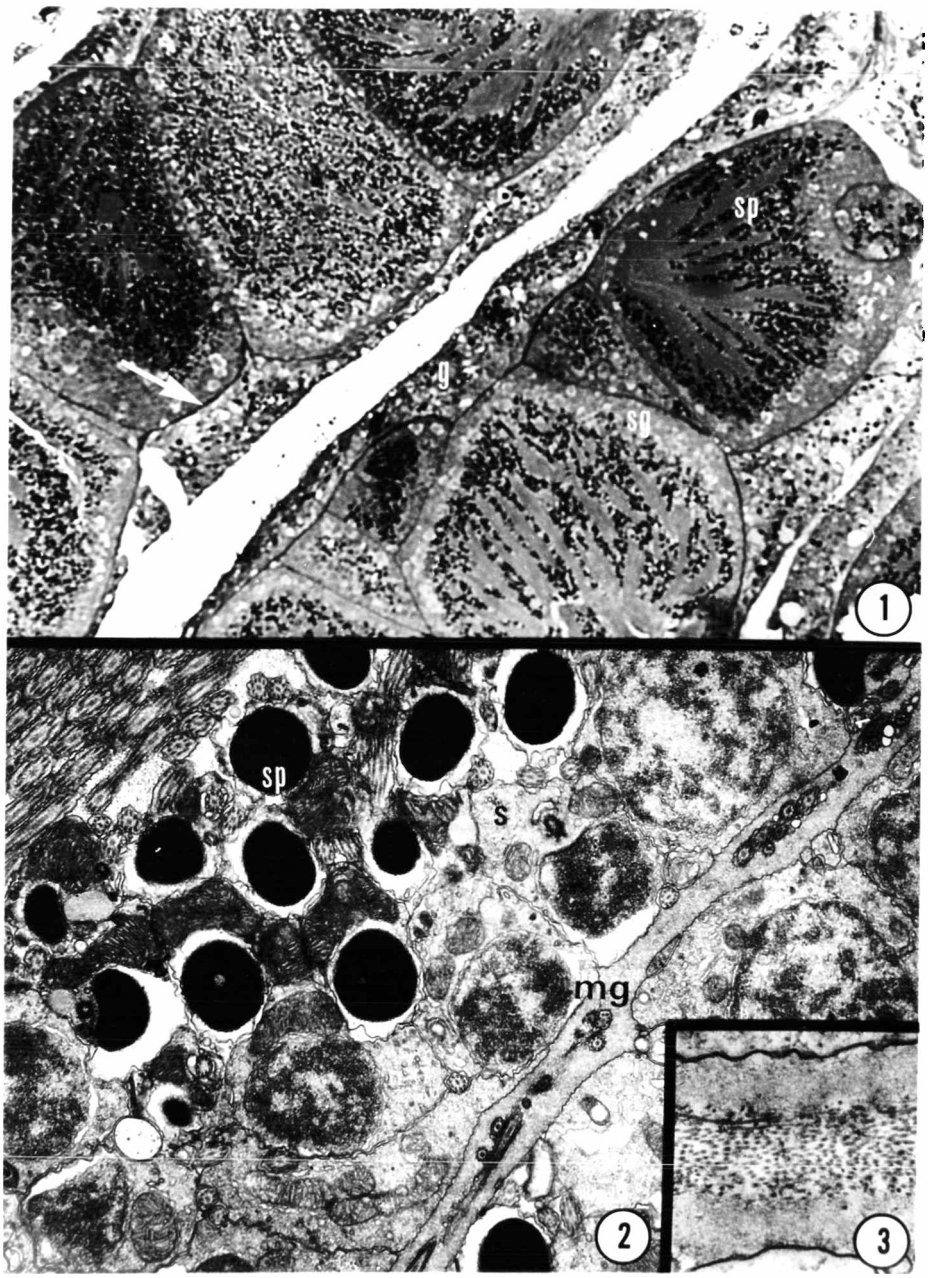
Fig. 17. A mature, spawned spermatozoon showing the highly condensed nucleus (n), the mitochondrial complex (m) and its associated lipid-like inclusion (l). Note also the moderately electron dense vesicles (v) located lateral to the nucleus. X 31,400.

Fig. 18. A longitudinal section of a mature, spawned spermatozoon showing the condensed nucleus (n), moderately electron dense vesicles (v) and mitochondrial complex (m). The membranes of the cytoplasmic collar (c) are locally fused so that the collar is interrupted. X 31,500.

Fig. 19. A cross section through the mitochondrial complex of a spawned spermatozoon showing a region where the collar is interrupted by local membrane fusion. X 50,000.

Fig. 20. Cross sections through two testicular spermatozoa showing regions of uninterrupted collar (c). In the lower cell Y fibrils are present connecting the doublets to the membrane of the flagellum. The upper cell shows a complete collar surrounding the tail in a region where the doublets are present and the central tubules of the axoneme are just forming. X 43,400. Inset: A higher magnification of the region of the flagellum where the Y fibrils are distinct. X 80,300.

Fig. 21. A cross section through the mitochondria just anterior to the section shown in figure 19. The collar (c) is complete but the central tubules of the axoneme are not present at this level. Compare with figure 18. X 42,000.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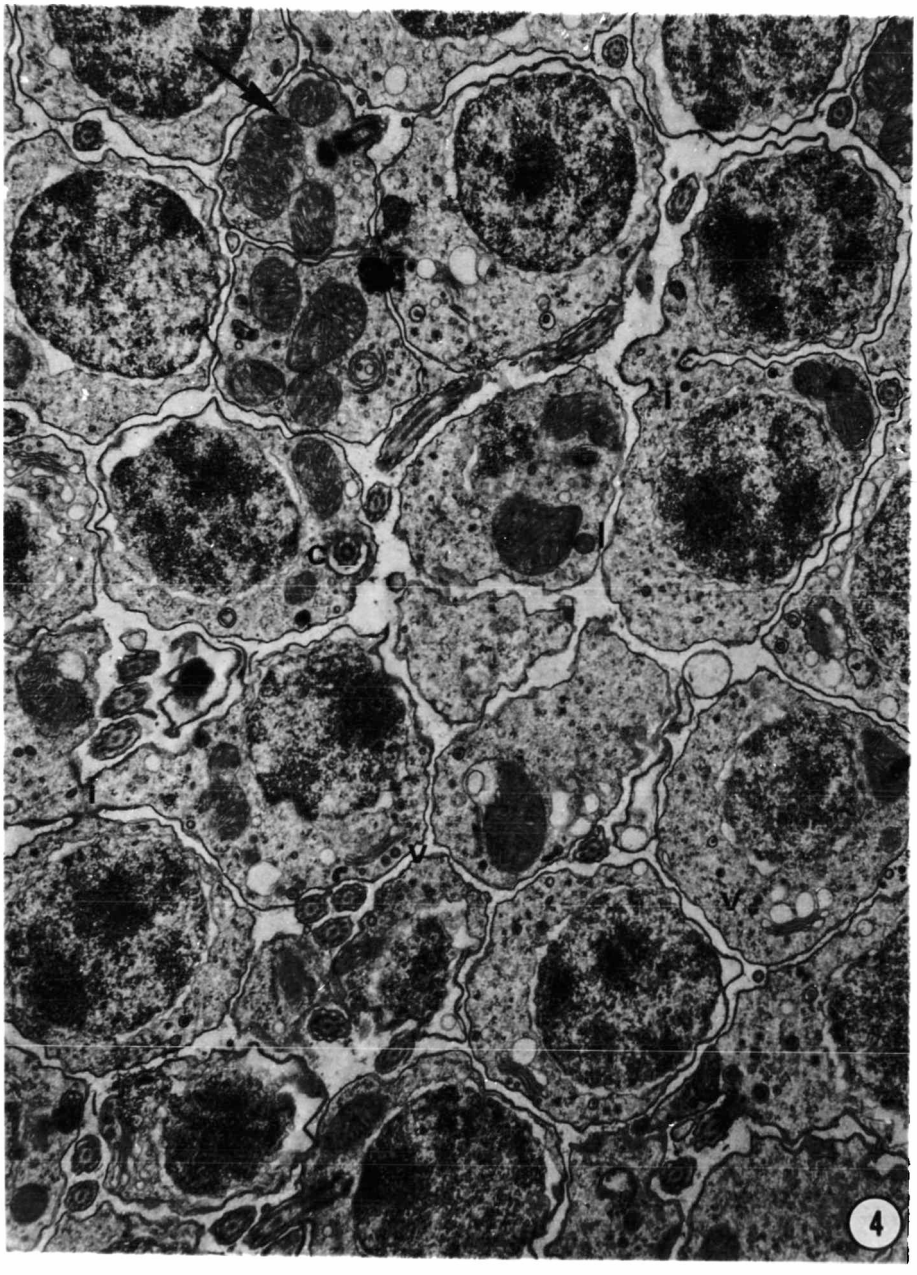
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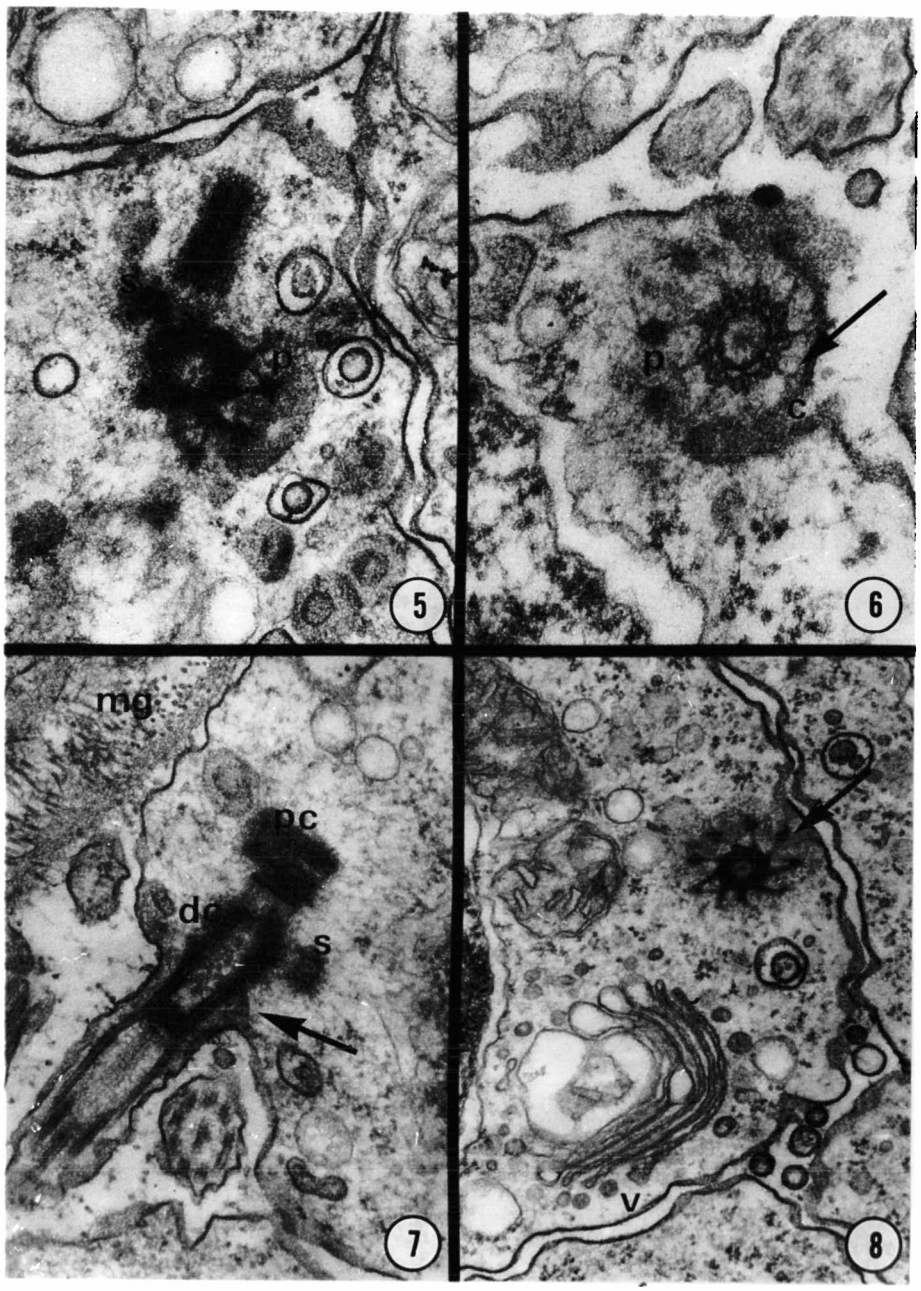
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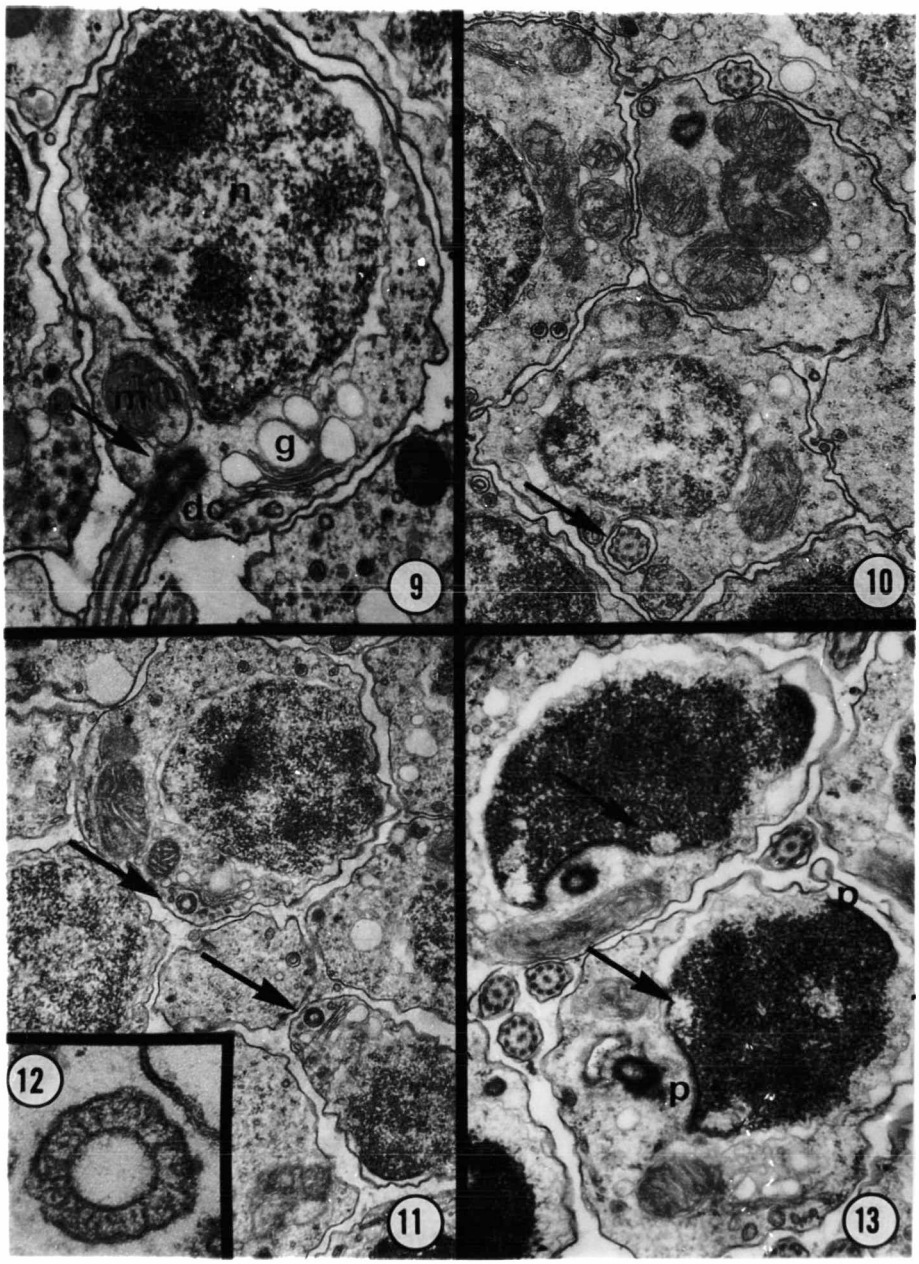
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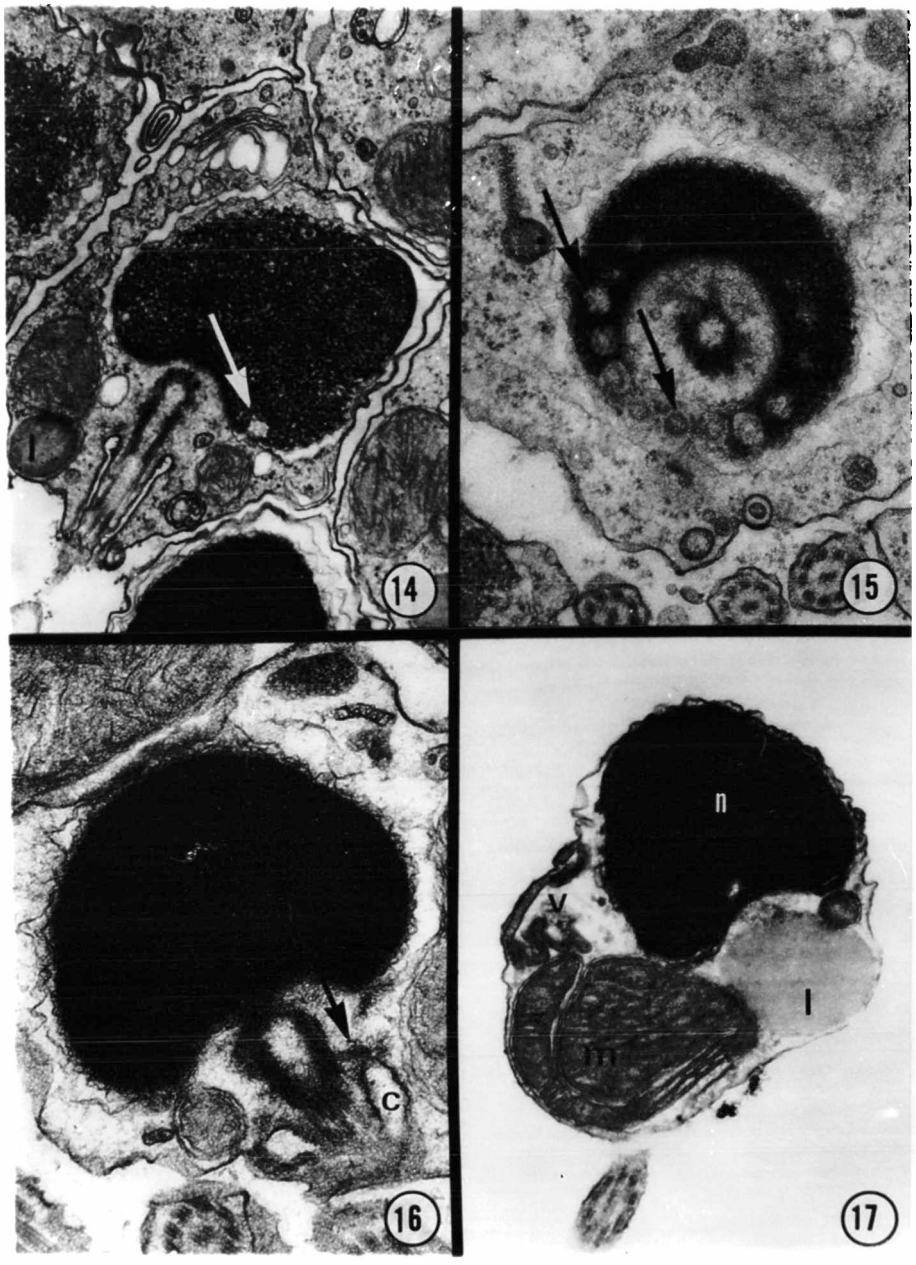
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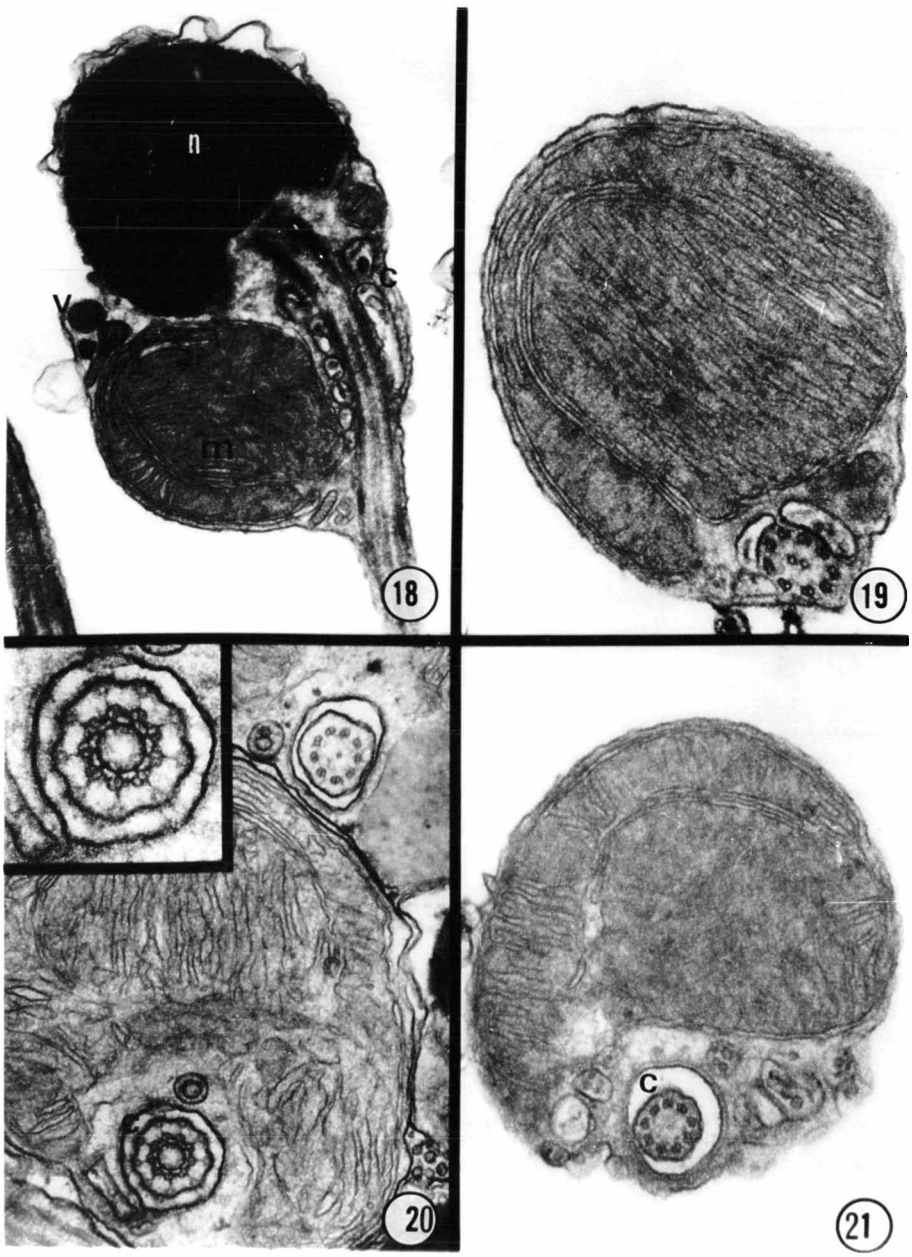
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III.

SURFACE SPECIALIZATIONS AND THE CORTICAL REACTION

Introduction

Many excellent fine structural investigations centered on the reproductive biology of invertebrates have been presented in recent years. Some of these are concerned with the final stages of gamete development and/or with the interaction of gametes during fertilization. With the exception of a few, these investigations are confined to those invertebrate groups in which the spermatozoon possesses a well-defined acrosome and the egg is endowed with an investment(s) that must be traversed by the spermatozoon prior to gametic union (5,13,14,15,16,23,24,31).

In view of this it is interesting that several recent fine structural studies on lower acoelomate invertebrates have revealed that the spermatozoa characteristically lack well-defined acrosomes. These include one poriferan (53) and several members each of the hydrozoans (3,29,30,40,48,52,56), schyphozoans (2,30) and anthozoans (20,30). Although it is reported that the eggs of the poriferans and cnidarians in general lack egg investments other than the egg plasma membrane (43) there have been no fine structural studies on the above representatives to verify the absence of accessory investments.

Furthermore there is little known about the reaction of the cortex to fertilization in these lower acoelomate invertebrates. In many higher invertebrates it is well established

that the cortical granules of the mature egg respond to fertilization by discharging their contents into the space between the egg surface and outer egg investments (1,4,21, 22). One function ascribed to the cortical reaction is that it is involved in a block to polyspermy (46). However, if there is no accessory egg investment, no enveloping layer would be elevated by a cortical reaction and this element of the reaction, at least, would not participate in a block to polyspermy.

Therefore, the intent of this investigation is: 1) to examine the fine structure of anthozoan oocytes and eggs with special reference to cortical and surface specializations and 2) to gain information regarding the response of an egg not possessing accessory investments to fertilization by a spermatozoon not bearing a distinct acrosome.

Materials and Methods

Adult animals were collected near Port Bolivar on the Texas Gulf Coast and maintained in the laboratory under aeration in shallow aquaria. Preparations of ovary were obtained by dissecting the animal and transferring portions of the mesentery containing ovary to fixative. Fixation was carried out in the paraformaldehyde-glutaraldehyde (pH 7.4) mixture recommended by Karnovsky (35). Subsequently, the preparations were washed with sea water and post-fixed in 1.0% osmium tetroxide in sea water for one hour. The tissue was then rapidly dehydrated in a graded acetone series, embedded in a low viscosity epoxy resin (47) and sectioned on a Sorvall MT-2 ultramicrotome according to standard techniques. Thin sections were mounted on uncoated grids, stained with alcoholic uranyl acetate and lead citrate (54) and observed with an AEI EM-6B or Hitachi HS-8 electron microscope. Thick sections (1u) for general light microscopy were stained with 0.25% aqueous toluidine blue in 0.15% sodium borate.

Unfertilized eggs were obtained from aquaria containing females which released gametes when males were not present or when males failed to exhibit a spawning reaction. Fertilized eggs for this study were obtained from the oral cavity of females kept in the same aquaria with males that had spawned previously. Gametes and zygotes so obtained were fixed according to the techniques already described.

Results

The sexes are separate in B. cavernata. The developing and mature oocytes are located within the mesoglea of the incomplete mesenteries just radial to the septal filaments (Fig. 1). The loose layer of mesoglea that separates the oocytes from the overlying gastrodermis also extends between the developing and mature oocytes throughout the gametic region. Specializations of the surface of the mature oocyte to be discussed later project into the electron lucent mesogleal matrix. Cells with prominent pseudopodial extensions occasionally occupy the mesogleal matrix between oocytes (Fig. 2). These cells possess a large nucleus containing a nucleolus and a granular karyoplasm with local accumulations of heterochromatin. The cytoplasm varies in density but is usually granular and contains numerous, often localized mitochondria, rough endoplasmic reticulum, juxtanuclear Golgi elements and osmiophillic inclusions (Fig. 2).

The nearly mature oocyte has a very large germinal vesicle with a single discrete electron dense nucleolus (Figs. 1, 3). Mitochondria, lipid-like inclusions, annulate lamellae and both membrane and nonmembrane bound fibrous bodies are commonly present (Figs. 3, 4). Yolk bodies fill the cytoplasm occupying all but the cortical region of the oocyte (Figs. 3, 4). In comparison to the cortical granules

the yolk bodies are generally larger, more electron dense and less homogeneous as a result of lipid-like inclusions. The cortical granules are spherical, homogeneous and membrane bound (Fig. 4). Although their relative abundance at the oocyte surface depends on the angle of the section and the degree of maturation they fill the outermost cytoplasm to a depth several times their diameter in the mature oocyte and egg. Regardless of the state of maturity they occasionally discharge their contents to the outside (Fig. 8 and inset). Golgi elements apparently involved in the packaging of the cortical granules (as well as other oocyte constituents such as yolk) are visible (Fig. 7). While the appearance of forming cortical granules is very similar to that of the immature yolk bodies which lack the characteristic lipid-like inclusions, it is distinguishable on the basis of its coarser granularity and lower electron density (Fig. 7).

Finally, the surface of the nearly mature oocyte and egg bear striking extensions which project locally from the surface (Figs. 4, 5, 6, 13). Although these modifications are clearly cytoplasmic extensions of the oocyte surface the term "spines" is used for descriptive purposes since it appears in both early and recent light microscopal studies (10, 25, 26). These spiny, microvillus-like, extensions possess a fibrillar core which extends basally well into the oocyte cytoplasm. The individual filaments of the core are 50-70 A in diameter. While confined in the ovary the spines of neighboring oocytes lie pressed against one another and against

the opposing oocytic gastrodermal or, amebocytic surfaces in groups of 25-50 "spines" (Figs. 1, 2, 4).

Fertilized eggs used in this study could be obtained from the oral cavity of the female during spawning by inserting a Pasteur pipette into the mouth opening. Most of these eggs were free (Fig. 12) but some were still loosely bound by ovarian tissue (Fig. 9). Several were apparently just fertilized since the cortical reaction had occurred over only a portion of the surface (Fig. 9, 15).

The germinal vesicle probably breaks down just prior to or during egg release since it is never present in spawned eggs. Unfertilized eggs later display a small eccentrically located pronucleus (Fig. 14).

Except for the cortical region the cytoplasm of the fertilized egg is similar to that of the mature ovarian oocyte or unfertilized egg with respect to density and the presence and disposition of the cellular organelles and inclusions. In contrast the major portion of the population of cortical granules which before fertilization remain as membrane bound electron dense structures undergo a marked change upon fertilization (Figs. 13, 15, 16). They lose much of their electron opacity and are multiphasic rather than homogeneous in electron density (Figs. 15, 16). The more electron lucent cortical material has a flocculent consistency (Figs. 15, 16, 17, 18, 19, 20). The membranes of those cortical granules at the egg surface fuse with the egg plasma membrane. Subsequent vesiculation of these membranes results in the discharge of cortical material into the spaces between

the spines. However, at the same time more centrally located granules fuse with neighboring granules as well as with those already discharging (Figs. 15, 16). The egg plasma membrane now is largely composed of cortical granule membrane and is more centrally located (compare figures 10 and 11). The formation of an apparently new oolemma from the fusion of the most centrally located cortical granules results in a drastically convoluted egg plasma membrane (Figs. 17, 18). Remnants of the original egg plasma membrane and even membrane enclosed portions of the cortical cytoplasm with characteristic cellular constituents (egg mitochondria, yolk, lipid-like inclusions, ER) are visible together with the cortical material in the extraoocyte spaces between "spines" (Figs. 17, 19). It is important to note at this point that no investments other than the egg plasma membrane were ever seen associated with the ovarian oocytes or with the unfertilized egg mentioned earlier. In addition no membrane was elevated as a consequence of fertilization.

The loose cortical material persists on the egg surface for some time after fertilization. This material together with a corona of unsuccessful sperm is visible with the light microscope. At the fine structural level it is clear that the great majority of the sperm associated with the fertilized eggs are confined to that region outside the area of cortical material (Figs. 9, 19).

Although we have not yet obtained micrographs of many sequential stages of sperm entry into the egg the male pronucleus

is visible in a cone-shaped elevation of the egg surface shortly following fertilization (Fig. 20).

The severity of the cortical reaction almost seems to distrupt the continuity of the microvillus-like spines with the egg surface (Figs. 15, 17, 18, 19, 20). Nevertheless, the spines are never lost to the eggs. Apically they remain in characteristic cone shaped aggregates. As a result of being freed from the confines of the ovary they project radially and form discrete groups of 25-50 spines (Fig. 18). The basal ends of the spines extend into the cortical material and the tips of each of the aggregates remain in tight bundles. Although it is very difficult to observe attachments between the bases of the spines and the egg surface after completion of the reaction these structures remain associated with the developing embryo and are observed with both the light and electron microscope as late as the free swimming planula stage.

Finally, only those eggs which displayed the cortical reaction developed normally. From samples taken prior to fixation we found that normal cleavage occurred in greater than 90% of the eggs. Many of those embryos not fixed continued to develop to the planula stage. In this regard it is important to point out that all attempts at <u>in vitro</u> fertilization have been unsuccessful. The unfertilized eggs, released along with those which were fertilized, remained unfertilized even when exposed to increasing concentrations of sperm from the same males responsible for successful fertilization.

Discussion

It is well known that marine eggs commonly display surface modifications such as microvilli. However, the microvillous-like spines in some anthozoans are exceptional with respect to size and arrangement. Although we know of no fine structural examinations of spines among the anthozoa other than B. cavernata, light microscopal studies are available. Spiny egg surfaces are reported for several anthozoans including Actinia, Anthea, Bolcera Peachia and Tealia (see 9, 10, 25, 26). One can only conclude at this time that these surface modifications are a reflection of animal diversity since the eggs of other anthozoans are not equipped with spiny surfaces (25). The term "spines" is retained here pending fine structural examination of other anthozoan eggs. Following such studies perhaps a more suitable term reflecting the cytoplasmic nature of these structures may be chosen.

Since already fertilized eggs could be obtained prior to release from the oral cavity and since some of these were still loosely bound by ovarian tissue and/or exhibited the cortical reaction over only a portion of their surface (Figs. 9, 15) we conclude that fertilization occurs within the gastrovascular cavity of the female as the eggs are released and that the cortical reaction proceeds from the point of sperm

entry. Similar observations of internal fertilization have been reported for several other anthozoans (9, 10, 26, 33). Many investigators have described cortical granules in the eggs of marine invertebrates. In the polychaete Sabellaria cortical granule release occurs on exposure to sea water (42, 44). However, in some molluscs cortical granules are unresponsive to exposure to sea water or to fertilization (6, 32, 36, 37, 39). In B. cavernata, however, the cortical reaction is in response to fertilization as in the case of sea urchins (5). The reaction is unlike that in sea urchins since no continuous investment acts to contain the product of the cortical granules. The cortical reaction is massive. It involves fusion and vesiculation of cortical granule membranes with each other as well as with the egg plasma membrane. Although the severity of the reaction seems to greatly disrupt the basal portion of the spines they are never lost and are a prominent feature of developing stages, at least to the free swimming planula. It appears that the fibrillar material of the spines, which extends well into the cytoplasm, functions as an anchoring mechanism serving to maintain the integrity of the spines during fertilization. Furthermore, the microvillous-like spines apparently trap the material of the cortical granules near the surface of the egg. Since almost all unsuccessful spermatozoa are confined outside the area of cortical material it is conceivable that the material retained by the spines participates in preventing polyspermy. It will be interesting in this regard to obtain new fine structural analyses of the

cortical region and gamete interaction among those anthozoans not possessing microvillous-like spines.

As a result of the extensive cortical reaction the membrane which bounds the egg, and probably the basal surfaces of the spines, is largely composed of that membrane which enclosed the cortical granules and which was derived originally from Golgi complexes. In contrast, the membrane which covers the more apical portion of the spines after fertilization is composed of the original plasma membrane.

Fertilization in B. cavernata does not fit the Hydroides-Saccoglossus pattern characteristic of many invertebrate forms in which the acrosome is involved in penetration of an egg investment as well as in fusion with egg plasma membrane (see review by Colwin and Colwin, 17). In B. cavernata there is no well defined acrosome present on the sperm. However, several investigators (2, 20, 30, 52) have suggested that the small vesicles characteristically found in the head region of cnidarian sperm (see also 3, 29, 40, 48, 56) may be acrosomal in function. In B. cavernata these vesicles cannot function as an acrosome as far as penetration of egg investments is concerned since there are none present. Nevertheless, we have not obtained micrographs showing sperm and egg membranes actually fused in a mosaic as described by Colwin and Colwin (17) and it is possible that the vesicles may act as an acrosome in the fusion of gametic membranes. Only when such a phenomenon is demonstrated can we ascribe an acrosomal-like function to these vesicles in Bunodosoma spermatozoa.

In certain other groups the spermatozoa also may gain apparently direct access to the egg. According to the literature the chorion surrounding the egg in cephalopods (7, 38), insects (18) and certain fishes (28, 45) is perforated with micropylar canals through which the spermatozoon passes during fertilization. However, Longo and Anderson (38) have shown that the spermatozoon of the cephalopod Octopus bimaculatus possesses the positional and structural equivalent of an acrosome. Insects also generally are thought to have spermatozoa which bear acrosomes (8, 18, 55, 57). Among the fishes acrosomes are said to be present on the more primitive acipenserids (19, 27, 28), elasmobranchs (50, 51) and lungfishes (34) but are absent on spermatozoa of higher teleosts (28, 45, 49).

Nevertheless, although the chorion is perforated by a micropyle, access to the egg plasma membrane is not necessarily direct. Davey (18) states that the membrane surrounding many insect eggs is covered by a waxy layer or by a vitelline envelope which is continuous even in the region of the micropyle. Furthermore, the micropyle may be filled with maternal secretions. Although we have found no fine structural studies of the egg surface in the region of the micropyle in elasmobranchs, acipenserids, lungfishes or higher teleosts, the light micrographs of Dettlaff (19) suggest to us that there may be investments other than the egg plasma membrane of sturgeon eggs (acipenserid) that must be traversed by the spermatozoon

during fertilization. As a consequence of fertilization in this species a "fertilization membrane" is elevated during a cortical reaction.

Although the relationship between the absence of continuous egg investments and the absence of a distinct acrosome is unclear the concept of a positive relationship should not be discarded until there is sufficient data. This data should demonstrate 1) that if there is direct access to the egg there are no, as yet unresolved, investments present, or 2) that an acrosome or acrosome-like structure is not involved exclusively in fusion with egg plasma membrane. In this regard it is interesting that the higher acoelomate invertebrate Cerebratulus lacteus possesses a spermatozoon with an acrosomal-like vesicle whose morphology remains unchanged during passage through the vitelline envelope (11) but is absent after fusion of egg and sperm plasma membranes (12). Furthermore, no characteristic acrosomal lysins are associated with the spermatozoon of C. lacteus (41).

Legend

Fig. 1. A light micrograph showing oocytes lying within the gastrodermis of the incomplete mesenteries of the gastrovascular cavity. Mesoglea separates the oocytes from one another and from the overlying gastrodermis. g, gastrodermis; gv, germinal vesicle; m, mesoglea; arrow, nucleolus. X 1250.

Fig. 2. An electron micrograph of an ameboid-like cell (A) commonly found in the mesoglea between oocytes. This cell contains a centrally located nucleus (n), large perinuclear Golgi complexes (g), osmophilic inclusion ($_{O}$), and mitochondria (m). Note the tightly packed surface specializations of the oocytes (OC, arrows). cg, cortical granules; y, yolk. X 9000.

Fig. 3. An electron micrograph of the nuclear region of an oocyte. gv, germinal vesicle; nc, nucleolus; y, yolk; l, lipid-like body; m, mitochondrion; f, fibrous inclusion. X 10,000.

Fig. 4. An electron micrograph showing the cortical region of a maturing oocyte. y, yolk; cg, cortical granuale; m, mitochondrion; g, Golgi; l, lipid-like inclusion; mv, microvillous-like "spines"; al, annulate lamellae; large arrows, membrane bound fibrous bodies; small arrows, mesoglea. X 9000.

Fig. 5. An electron micrograph showing the prominent microvillous-like extensions of the oocyte surface. s, "spines"; cg, cortical granules. X 8800.

Fig. 6. A higher magnification of the surface specializations of the oocyte surface. Note the fibrillar core of these specializations and the root-like extensions of the fibrillar material extending into the ooplasm. fc, fibrous core; r, rootlets. X 24,000.

Fig. 7. An electron micrograph showing Golgi elements associated with a developing cortical granule (arrow). cg, cortical granule; m, mesoglea; l, lipid-like inclusion. X 19,500.

Fig. 8. An electron micrograph of the oocyte surface illustrating cortical granule fusion. The cortical granule at the surface has fused with the oocyte membrane leading to the discharge of these cortical granules to the outside. cg, cortical granule; m, mesoglea. X 28,000.

Fig. 9. A light micrograph showing a cluster of lightly adhering fertilized eggs. The cortical granules in one of the eggs have not completed the reaction. Compare this light micrograph with the unfertilized egg in figure 14. cg, undischarged cortical granules; arrows, spermatozoa outside cortical material. X 550.

Figs. 10 and 11. Light micrographs of unfertilized (10) and fertilized (11) whole mount eggs for comparative purposes. Note the extensive cortical reaction in the fertilized egg. s, "spines"; cm, cortical material. X 550.

Fig. 12. A light section of a fertilized egg obtained from the oral cavity of the female. Compare with the unfertilized egg in Fig. 14. s, "spines"; cm, cortical material. X 550.

Fig. 13. An electron micrograph of the surface of an unfertilized egg. Note the thick layer of undischarged cortical granules. s, "spines"; cg, cortical granules; l, lipid-like inclusions; y, yolk; g, Golgi; m, mitochondria. X 8300.

Fig. 14. A light micrograph of an unfertilized egg. Note presence of cortical granules and compare with figure 12. cg, cortical granules; s, "spines". X 530.

Fig. 15. An electron micrograph showing the massive cortical reaction as it is spreading over the egg surface. The arrow points to the transition zone between discharged and largely undischarged cortical granules. dcg, discharging cortical granules; s, "spines", fl, flocculent cortical material. X 9200.

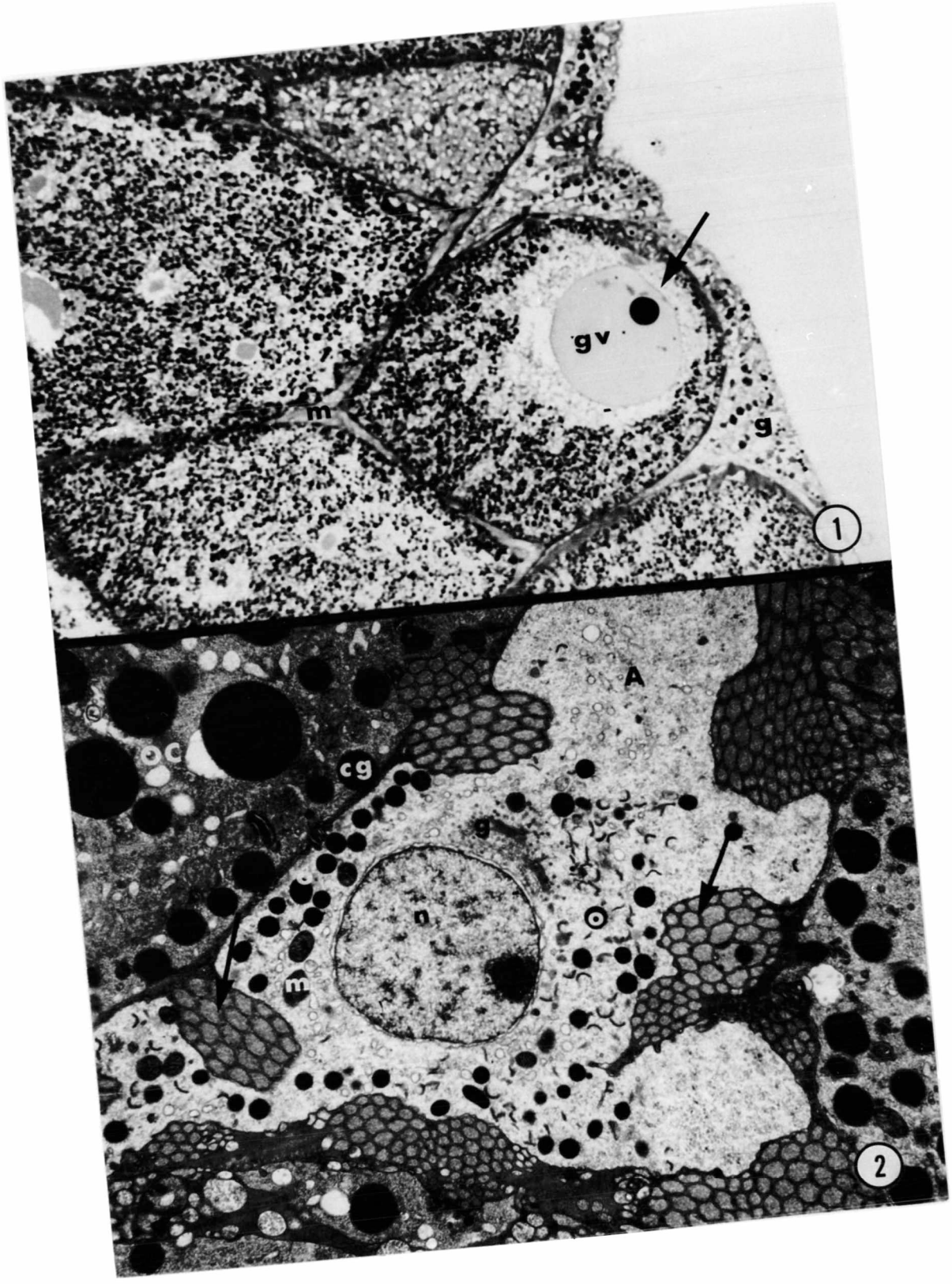
Fig. 16. An electron micrograph showing the cortical reaction taking place. Note the fusion of the cortical granules which occurs sometimes even before the change to the multiphasic appearance (arrows). y, yolk. X 6600.

Fig. 17. An electron micrograph showing the completed cortical reaction. Note the convoluted egg membrane, flocculent cortical material (fl) and membrane-bound regions of cortical cytoplasm (cc) apparently lost to the egg as a result of the reaction. y, yolk. X 7700.

Fig. 18. An electron micrograph showing the fertilized egg surface after completion of the cortical reaction. s, "spines"; y, yolk; arrows, remnants of cortical cytoplasm. X 8700.

Fig. 19. An electron micrograph showing a spermatozoon in the flocculent material near the egg surface. m, mitochondrion; l, lipid-like inclusion; n, nucleus; f, flocculent cortical material; arrows, remnants of cortical cytoplasm. X 9100.

Fig. 20. An electron micrograph of the surface of a recently fertilized egg showing the male pronucleus in an elevated cone-shaped projection of the egg surface. pn, pronucleus; f, flocculent cortical material; y, yolk. X 5000.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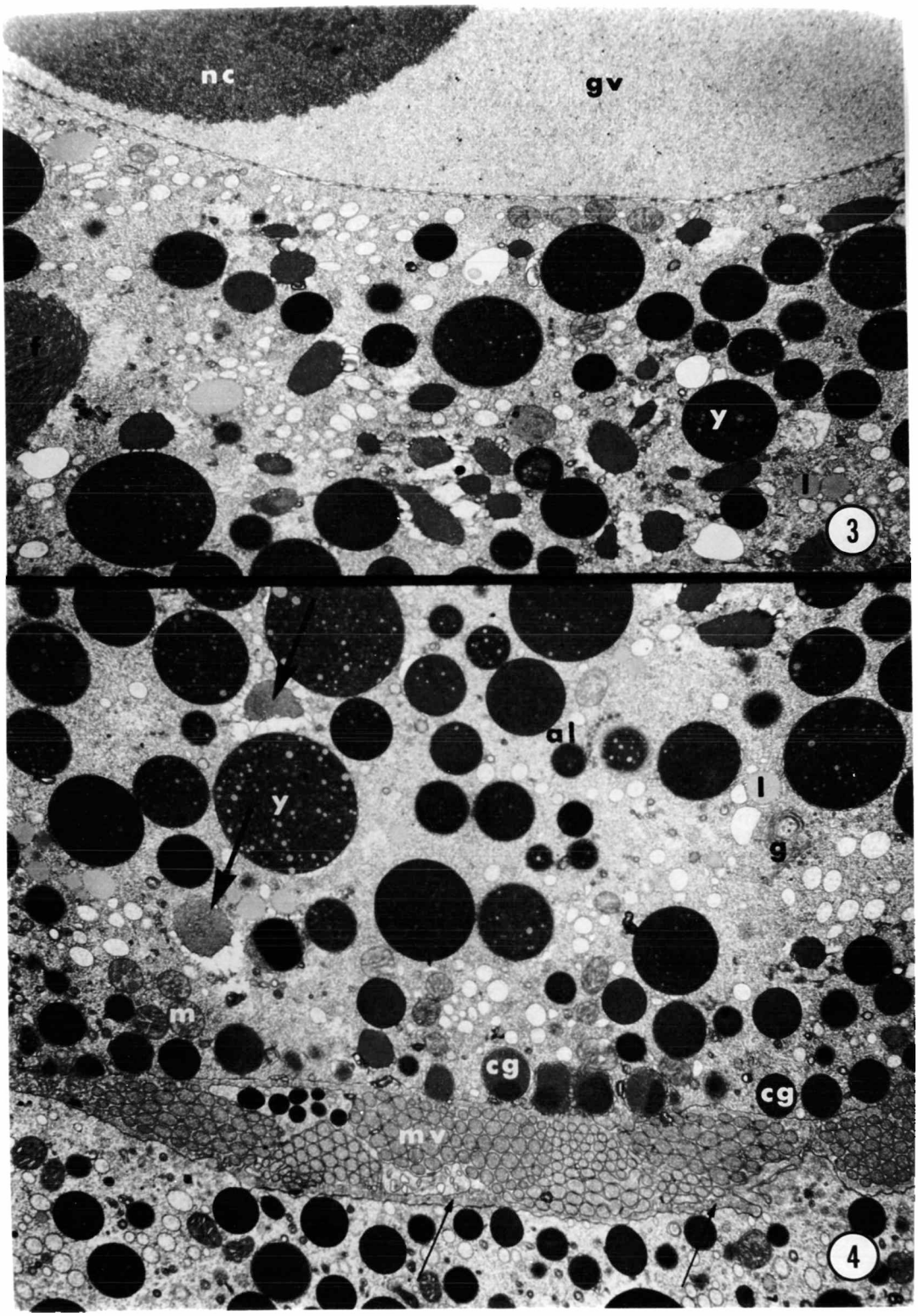
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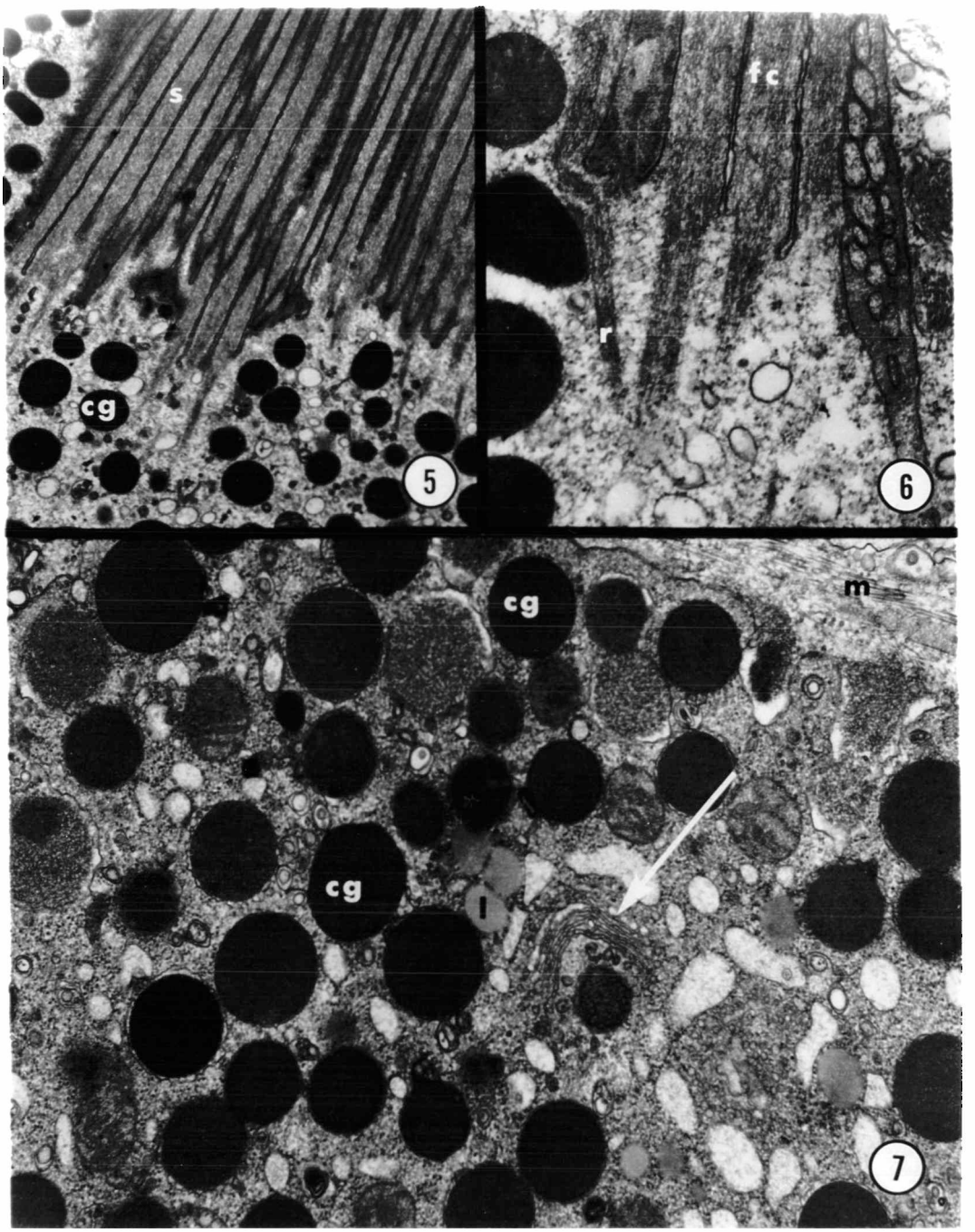
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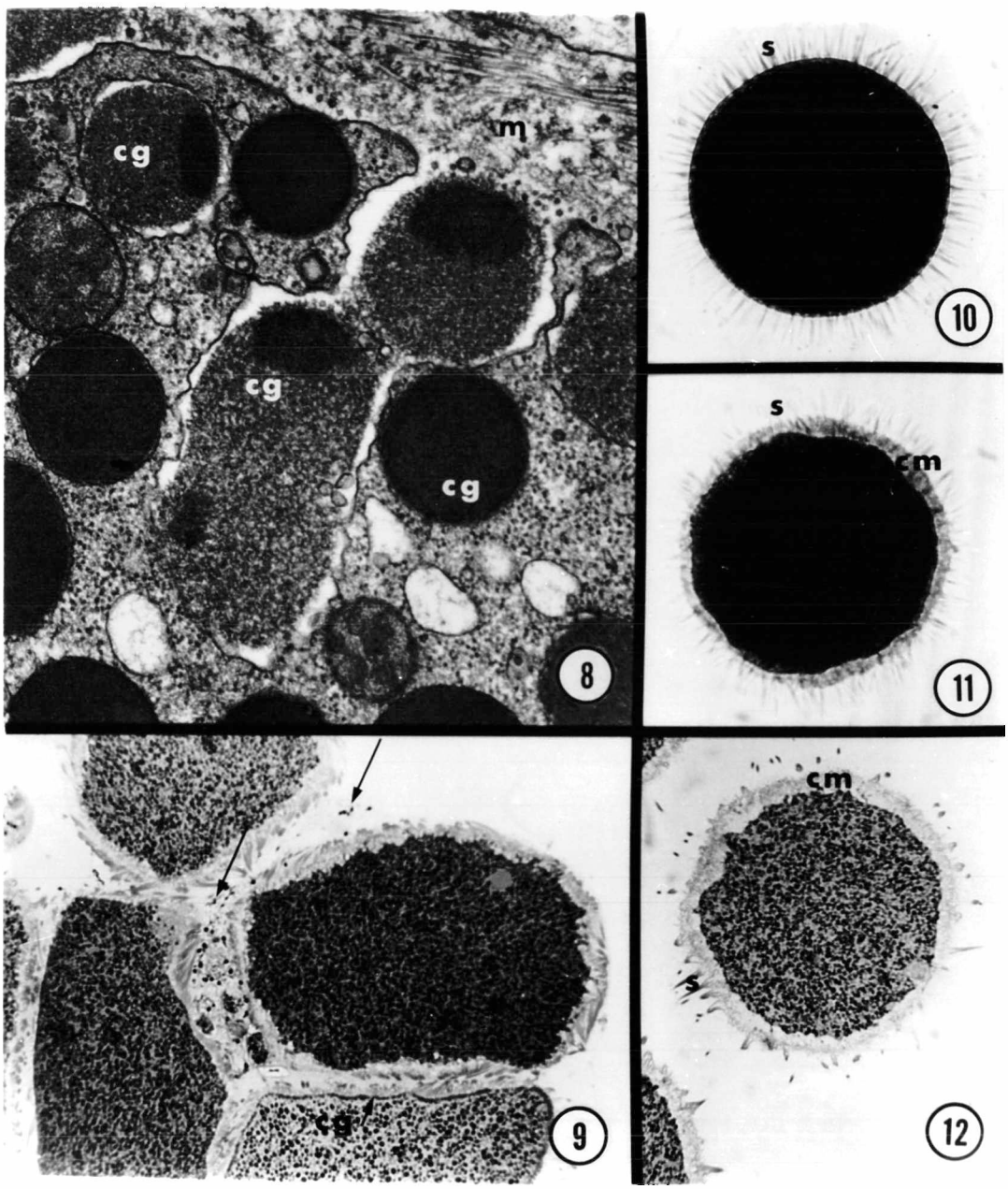
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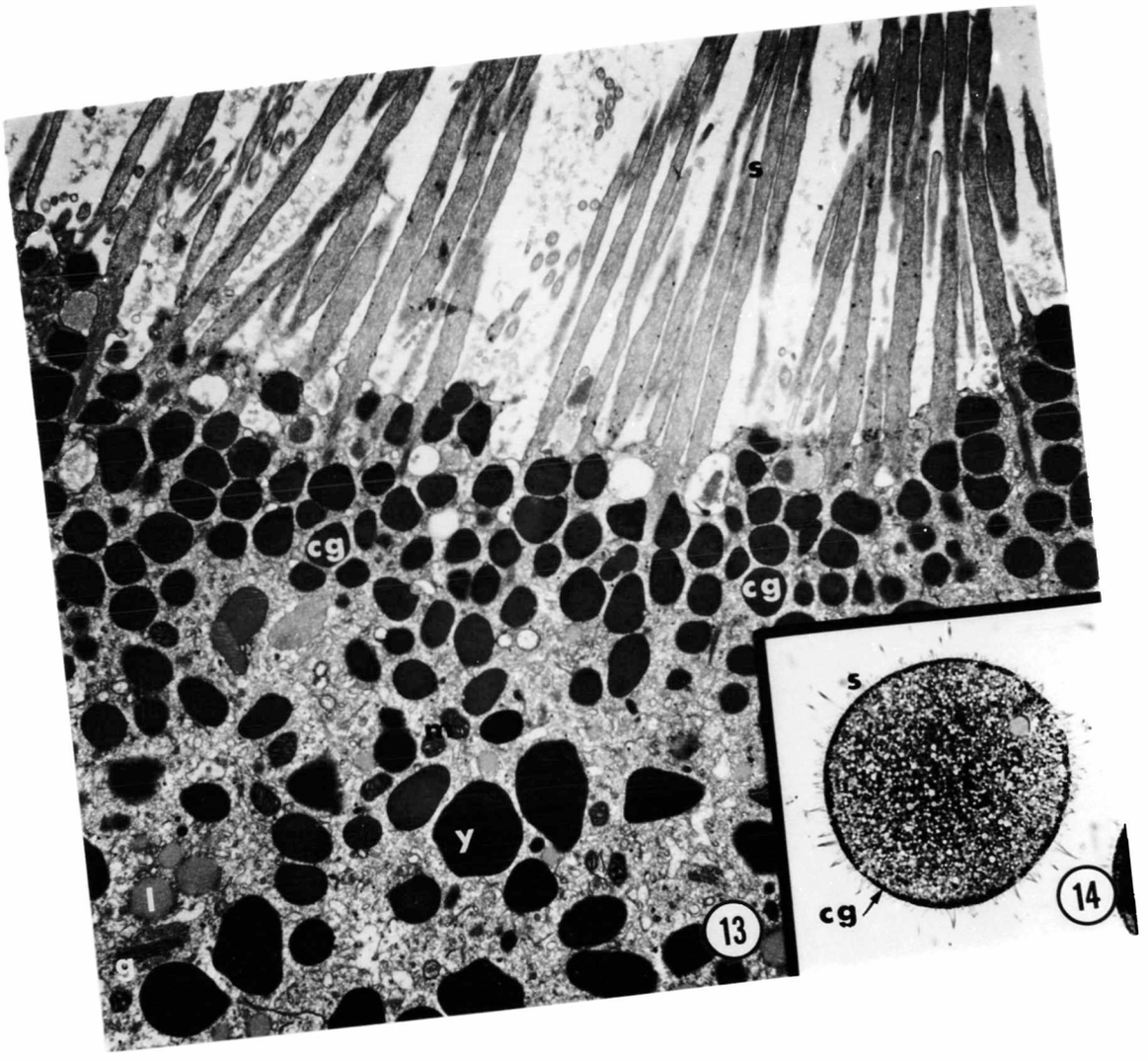
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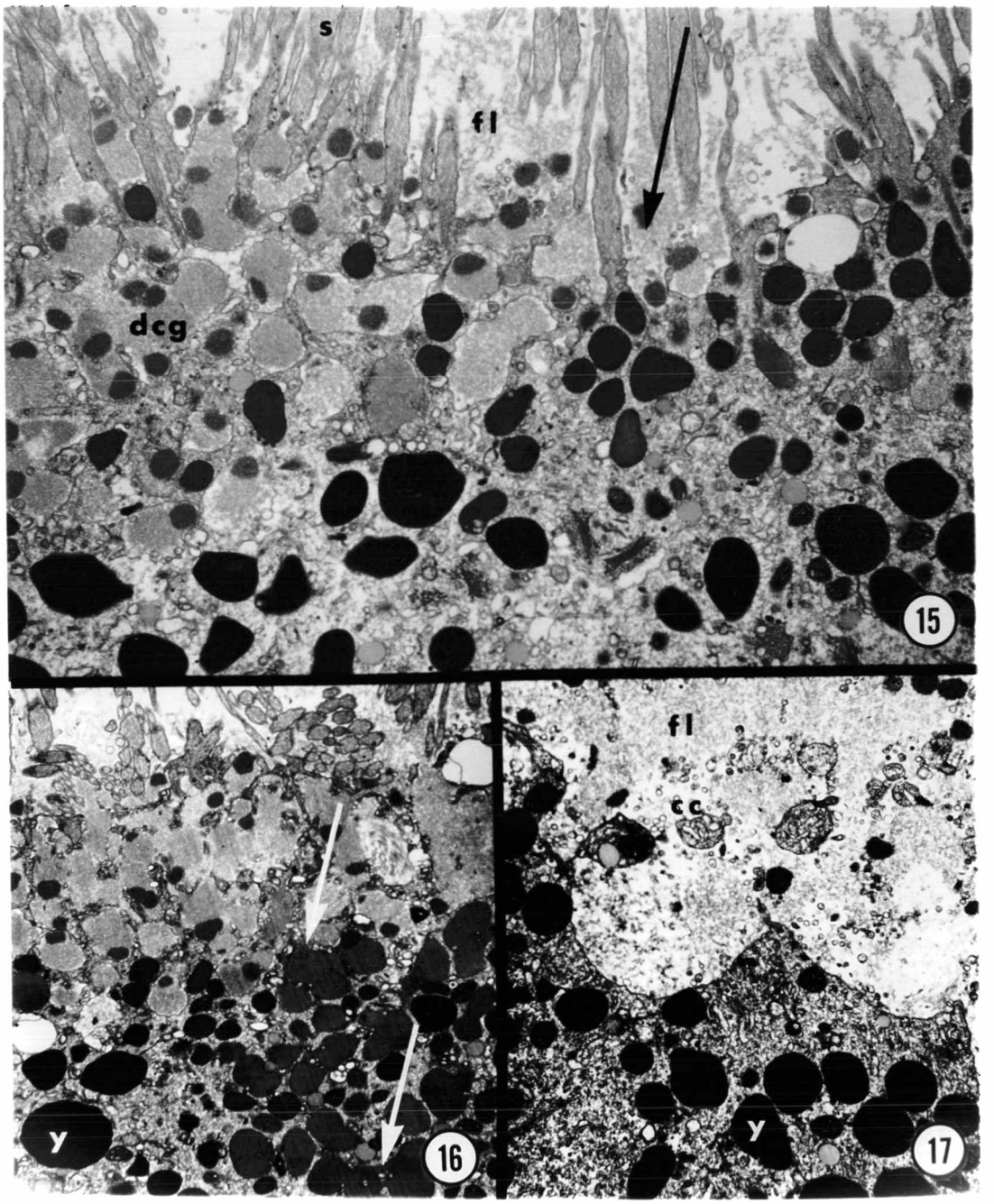
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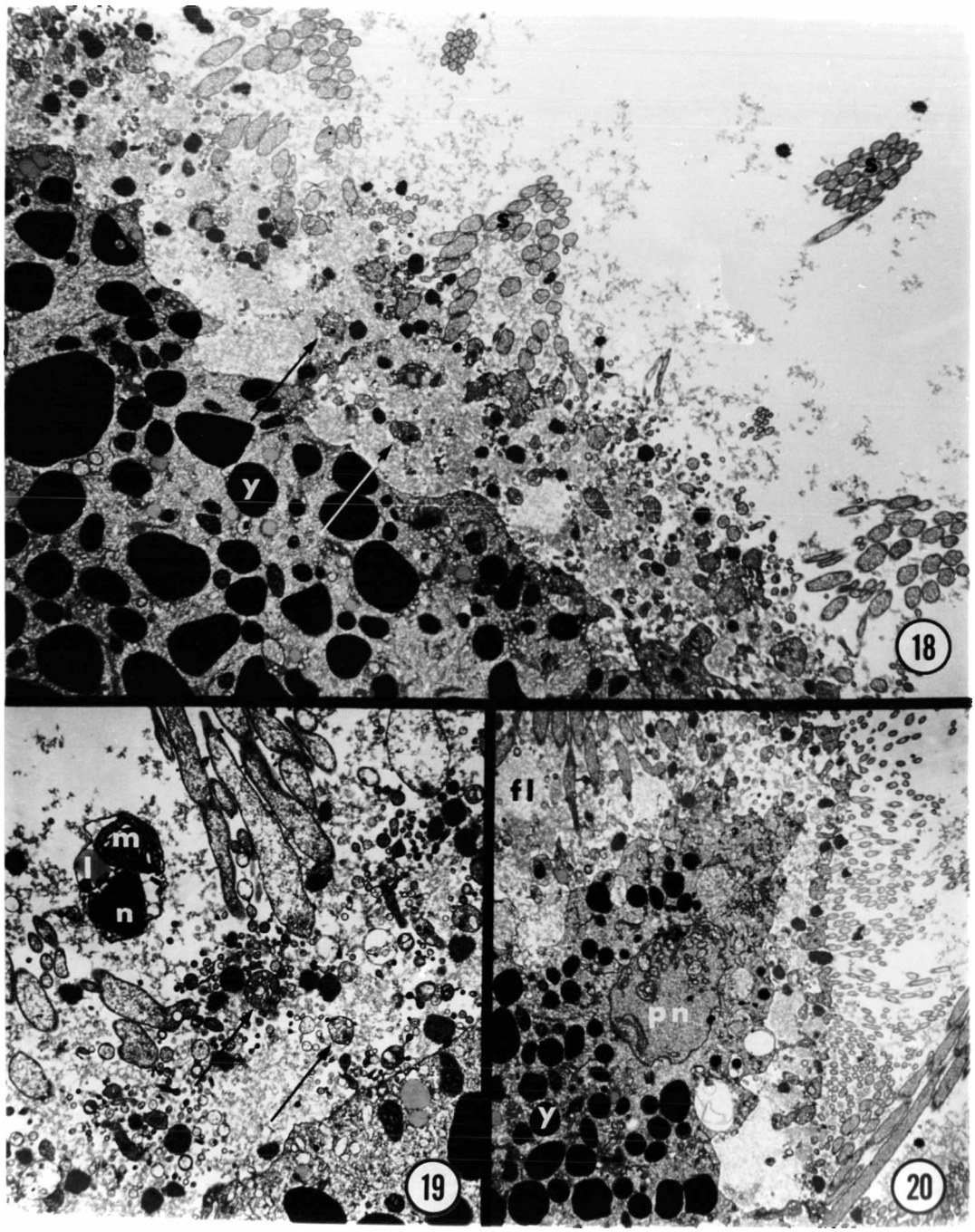
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IV.

SUMMARY

This examination of the gametes and certain aspects of gamete interaction in the anthozoan B. cavernata has provided new information regarding the reproductive biology of acoelomate invertebrates.

The first portion of this investigation is concerned with the fine structure of differentiation of the spermatozoon with particular reference to the fate of the Golgi body derivatives and pericentriolar specializations is presented. Like the acrosome of other species the "donut" shaped vesicles of the spermatozoon are derived from the Golgi apparatus during spermiogenesis. Unlike the acrosome the vesicles play no role in penetration of accessory egg investments since there are no investments present before or after fertilization. Nevertheless, the intriguing possibility remains that the vesicles may be involved in gamete membrane fusion, the second well documented function of the acrosome.

Two pericentriolar specializations which have often been confused in the literature are present in spermatids of B. cavernata. They are the satellites, presumably centers for microtubule assembly during maturational divisions, and the pericentriolar branching complex, which may be supportive in function. The pericentriolar branching complex persists during the development of the spermatozoon, the satellites do not.

The second portion of this study is concerned with the oocytes and eggs of B. cavernata with particular reference to morphological changes of the surface during fertilization. Although there are no investing layers surrounding the oocytes and eggs they do possess striking membrane specializations ("spines") which resemble microvilli. Furthermore, the cortex of the mature oocytes and unfertilized eggs contains an abundant supply of cortical granules. As a result of fertilization the cortical granules undergo fusion and vesiculation leading to the discharge of cortical material into the spaces between the "spines". The reaction is so massive that membrane bound remanents of cortical cytoplasm are apparently lost to the egg. The aforementioned "spines", however, are not lost but persist during development at least to the free swimming planula stage. The flocculent cortical material discharged during the reaction seems to be trapped for a period of time in the basal region of the "spines". Since spermatozoa are largely restricted outside the cortical material it is conceivable that the material retained by the "spines" participates in preventing polyspermy.

The preceding study is a fine structural examination of spermiogenesis, the mature spermatozoon, the maturing oocyte and the unfertilized and fertilized egg of the anthozoan Bunodosoma cavernata. It represents a significant addition to our knowledge of the reproductive biology of acoelomate invertebrates. Furthermore, this investigation of

fertilization in a simpler system than those generally studied may prove of great value in increasing our understanding of the essentials of fertilization.