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SPERMATOGENESIS AND EGG INVESTMENTS IN *LEPTOSYNAPTA CLARKI*, *CUCUMARIA LUBRICA* AND *CUCUMARIA PSEUDOCURATA* (ECHINODERMATA: HOLOTHUROIDEA), WITH A NOTE ON ACROSOMAL REACTIONS

by

DAVID GRATTAN ATWOOD

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

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EDMONTON, ALBERTA

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FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled SPERMATOGENESIS AND EGG INVESTMENTS IN *LEPTOSYNAPTA CLARKI*, *CUCUMARIA LUBRICA* AND *CUCUMARIA PSEUDOCURATA* (ECHINODERMATA: HOLOTHUROIDEA), WITH A NOTE ON ACROSOMAL REACTIONS, submitted by DAVID GRATTAN ATWOOD in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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Supervisor

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External Examiner

Date May 9th, 1975

ABSTRACT

This research has included studies on spermatogenesis, egg investments and acrosomal reactions in the sea cucumbers: *Leptosynapta clarki*, *Cucumaria lubrica* and *Cucumaria pseudocurata*. The most significant findings are listed below.

1) Spermatogonia, spermatocytes and spermatids of *Leptosynapta clarki* and *Cucumaria lubrica*:

Spermatogonia are joined by dense junctions. Proacrosomal granules are evident in late spermatogonia. A tubular body exists in spermatogonia of *C. lubrica*. Spermatocytes are characterized by flagellar formation, centriolar satellites, striated rootlets and a chromatoid body in *L. clarki*. Spermatids are joined by cytoplasmic bridges. The nucleus of the *C. lubrica* spermatid elongates without the aid of microtubules.

2) Spermatozoon of *Leptosynapta clarki*:

The sperm has a circular head measuring 3.0 μ, a midpiece containing a mitochondrion and a tail 45 μ long. The acrosomal region is at the anterior tip and contains a granule measuring 0.7 μ in diameter which consists of dense concentric lamellae. Typical proximal and distal centrioles lie posterior to the nucleus. The distal centriole gives rise to nine satellite projections.

3) Spermatozoon of *Cucumaria lubrica*:

The sperm consists of a cylindrical head, 1.5 μ in diameter and 5.2 μ in length, a mitochondrial midpiece 1.9 μ in length and a tail 65 μ long. An acrosomal granule, containing a dense sphere, is located at the anterior tip of the cell. The nucleus is 6.8 μ long and tapers to a diameter of 0.5 μ at the posterior end. The mitochondrion surrounds the

posterior 1.6 μ of the nucleus. Typical proximal and distal centrioles lie posterior to the nucleus.

4) Spermatogonia, spermatocytes and spermatids of *Cucumaria pseudocurata*:

Six stages are evident during spermatogenesis: primary spermatogonia, secondary spermatogonia, early spermatocytes, late spermatocytes, spermatids and spermatozoa. Morphogenesis of the acrosome and striated rootlet is initiated in the early spermatocyte. A morphogenic polarity exists in the developing acrosomal granule. Cell shape is altered in the intermediate spermatid with dorso-ventral compression and anterior-posterior elongation resulting. No microtubules are evident.

5) Spermatozoon of *Cucumaria pseudocurata*:

The tabloid sperm consists of a dorsal surface containing a striated rootlet and a ventral surface containing an acrosome. The head is 5.5 μ in length, 1.2 μ in width and 0.8 μ in depth. A mitochondrion lies at the base of the nucleus. The flagellum, 70 μ long, has a 9+3 tubular arrangement in the midtail region. The proximal and distal centrioles contain satellite projections and lie posterior to the nucleus.

6) Acrosomal reaction and egg investments in *Leptosynapta clarki*, *Cucumaria lubrica* and *Cucumaria pseudocurata*:

The sperm of *L. clarki* and *C. lubrica* artificially undergo a typical echinoderm acrosomal reaction, whereas sperm of *C. pseudocurata* do not. Sperm of *C. pseudocurata* attach to the egg surface on their sides rather than head on, where they undergo an atypical acrosomal reaction. The *L. clarki* egg is surrounded by an outer particulate-fibrous layer, follicular cell layer, dense laminate fibrous layer, dense

particulate layer and a lucent particulate layer. In *C. lubnica* a follicular cell layer, dense laminate fibrous layer and dense particulate layer are present. The egg of *C. pseudocurata* is surrounded by a single dense laminate layer.

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Chapter I

GENERAL INTRODUCTION

GENERAL INTRODUCTION

Fertilization: Historical and Present Perspectives

The fertilization process involves the union of male and female gametes and the combination of genetic material from maternal and paternal sources. Sexual reproduction, through the sorting and shuffling of genes into new and different combinations, provides a constant source of phenotypes for testing against the environment. Even though at any designated time there will be a smaller proportion of well-adapted individuals than would arise from a well-adapted asexual population, a sexually reproducing population has the advantage in its ability to adjust to changing environmental demands.

Echinoderms have been important in fertilization studies due to the fact that they are easily accessible and relatively simple to spawn and fertilize under laboratory conditions. Echinoderm species, besides being tools for reproductive research, are key members of the ecosystems they inhabit owing to their vast numbers and varied interactions with species of other phyla.

Fol (1877), a pioneer in the field of fertilization, was one of the first to describe the penetration of the animal oocyte by a spermatozoon. As a result of studies on fertilization in *Asterias*, Fol (1879) concluded that the directive movement of the sperm through egg investment layers and eventually into the oocyte was the result of an attraction exerted by a cone-like projection put forth from the surface of the egg. Fol suggested that a tenuous filament arose from the egg surface cone, came into contact with approaching spermatozoa and drew the sperm into the

main body of the egg. Similar egg-sperm connecting filaments were observed in holothurians (Iwanzoff, 1898; Hörstadius, 1939b) and hemichordates (Colwin and Colwin, 1949, 1954). Fol (1879) noted that the spermatozoon advanced as the filament progressively shortened until the sperm head reached the cone into which it then entered. The theory that the filament was an outgrowth of the sperm rather than the egg was ruled out at that time since no reduction in volume of the head was observed (Fol, 1879). Subsequent work by Chambers (1923, 1930) on *Asterias* and Hörstadius (1939a) on *Astropecten* substantiated Fol's results. Just (1929) argued that the fertilizing filaments he observed in certain asteroids originated in the head of the spermatozoon rather than on the surface of the egg. It was not until the work of Dan (1952, 1954) on echinoids and Colwin and Colwin (1955) on *Holothuria* and *Asterias* that it was conclusively demonstrated that the sperm head gave rise to an "acrosomal filament" (Dan, 1952) or process which preceded the advancing spermatozoon into the entry cone of the egg at the time of fertilization.

The acrosome was initially termed the "perforatorium" by Waldeyer (1870). Brenda (1887) later determined that this organelle originated from a granule located in a vacuole within the Golgi complex and Lenhossek in 1898 proposed that the term "acrosome" was a more appropriate label. Field in his 1893 study of echinoderm sperm referred to the acrosome as the "centrosome." Because of the close association between the acrosome and the nucleus and the fact that it was situated in a slight nuclear indentation, Field obviously mistook this organelle for the region containing the centrioles.

The elongated acrosomal process consists of a single membrane

surrounding a fibrous (filamentous) shaft. Colwin and Colwin (1963) and Dan et al. (1964) have postulated that the filaments originate through the polymerization of precursor materials present within periacrosomal substances of the acrosome. Tilney et al. (1973) and Jessen et al. (1973) have demonstrated that in *Asterias* (Asteroidea) and *Echinocardium* (Echinoidea) G-actin is evident within the periacrosomal material and that its polymerization into linear filaments, F-actin, is responsible for elongation of the acrosomal process. They propose that these filaments may play a dual role in the fertilization process: 1) extension of the acrosomal apparatus through the egg investment layers and 2) incorporation of the sperm nucleus into the egg.

Popa (1927), working with echinoids, was one of the first to report on the acrosomal process. Since that time much research employing light and electron microscopy has been published on echinoderm species (Dan, 1952, 1954, 1956, 1960, 1967, 1970; Colwin and Colwin, 1955, 1956; Rothschild and Tyler, 1955; Afzelius, 1956; Afzelius and Murray, 1957; Collier, 1959; Haino and Dan, 1961; Bernstein, 1962; Dan et al., 1962, 1964, 1972; Dan and Hagiwara, 1967; Franklin, 1970; Summers and Hylander, 1974).

Prior to electron microscopy there was considerable confusion regarding sperm entry into the egg. Loeb (1917) suggested phagocytosis as a possible mechanism in fertilization, whereby the sperm was engulfed or captured by the oocyte. Lillie (1912) stated that following sperm attachment to the egg membrane in *Nereis*, the cortical cytoplasm of the egg became denser at the point of attachment. Subsequent active streaming of the surrounding cytoplasm carried the sperm into the egg by a centripetal movement. Austin (1951) observed sperm entry into the rat egg with

phase contrast microscopy and concluded that penetration was due to some membrane activity by which the sperm head sank into the vitellus. Following studies on reactive systems of fertilizin-antifertilizin and other surface agents, the concept emerged that sperm attachment was due to specific surface reactions followed by a phagocytotic process which was responsible for the swallowing of the sperm. Tyler (1959, 1960, 1962) elaborated this concept in a hypothesis suggesting "Specific Pinocytosis" or phagocytosis, "as a possible Sperm-Engulfing Process." This implied that the entire sperm cell with its plasma membrane intact was surrounded by a vesicle of egg plasma membrane and transported into the cytoplasm of the oocyte. It was thought that the acrosomal filament or the cytoplasm in which it was anchored played the active role in sperm penetration while the egg plasma membrane was mechanically broken.

Even though these concepts of fertilization were appealing there was no direct evidence to support them. In an electron micrograph of a penetrating sea urchin spermatozoon, Rothschild (1957) showed that the sperm plasma membrane was missing. If the sperm head had penetrated through a process of phagocytosis, there should have been at least one membrane surrounding the sperm, namely that of the phagocytotic vesicle. It later became evident from work on *Hydroides* (Annelida) (Colwin and Colwin, 1961) and the rat (Szollosi and Ris, 1961) that sperm penetration actually occurred through a membrane fusion process. Colwin and Colwin (1961) stated: "No matter how the fusion is accomplished, it is clear that by a rather early stage the egg plasma membrane and the sperm plasma membrane become one continuous mosaic membrane, and that the two formerly separate cells then constitute a single cell." Extensive reviews on

fertilization have recently appeared which discuss comparative aspects of this phenomenon throughout the plant and animal kingdoms (Monroy, 1965; Colwin and Colwin, 1967; Austin, 1968; Metz and Monroy, 1969; Longo, 1973).

Summers and Hylander (1974) suggested that gamete contact (at least in sea urchins) is a two-step process consisting of a binding between extracellular materials on the acrosomal process and the egg plasmalemma and then a membrane fusion between the acrosomal process tip and the oolemma. Aketa (1973) has postulated that a species-specific component is present on the apical end of the echinoid sperm which is complementary to a sperm-binding protein of the egg surface. These two molecules are possibly responsible for both initial species recognition and bonding of the gametes. It has been suggested that such specific sperm molecules are contained within the acrosomal vesicle and are made available to the egg surface following the acrosomal reaction and that these molecules form a structural bond with the vitelline envelope before membrane fusion (Summers and Hylander, 1974).

Spermatogenesis in Echinoderms: Historical and Present Perspectives

To gain a firm knowledge of the features that enable the egg and sperm to unite, it is essential to understand the structure and development of the spermatozoon. Relatively few studies have been published on spermatogenesis in the phylum Echinodermata. Initial fragmentary reports appeared in the late 1800's on all five classes: holothurians (Jensen, 1883; Field, 1893), crinoids, echinoids, ophiuroids and asteroids (Field, 1893). Since that time the majority of studies have dealt with asteroids (Delavault, 1961; Cognetti and Delavault, 1962; Delavault and Bruslé,

1968; Bruslé, 1968; Smith, 1971) and echinoids (Fuji, 1960; Longo and Anderson, 1969). To date, there has been no detailed fine structural investigation of spermatogonia, spermatocytes and spermatids for any species of the class Holothuroidea.

Field (1893) examined spermatogenesis in 19 species representative of all five echinoderm classes.

> Throughout all those different species I have found a very general similarity, though in minor details there is considerable variation. The present preliminary account deals mainly with those general facts, which we have reason to believe obtain throughout the entire class, in those species which have retained the general and typical ontogeny.
> . . . by use of new apochromatic homogeneous immersion objectives I have been able to overcome many of the obstacles which have hitherto prevented an exclusive study of the spermatogenesis of the Echinoderms as a group (Field, 1893).

Field found that a general testicular section displayed distinct zones characterized by definite cellular stages: spermatogonia with resting nuclei laid peripherally, next was an area of spermatogonia with nuclei in active mitosis, internal to the spermatogonia was a zone of spermatocytes, then spermatids followed by immature spermatozoa, and finally, in the center of the lumen, mature spermatozoa.

In the asteroid *Leptasterias*, developing male germinal cells form fingers which reach out from the germinal epithelium into the testicular lumen (Smith, 1971). The cells of the fingers are layered into zones according to maturity from spermatogonia to mature spermatozoa. In the echinoids *Arbacia* and *Strongylocentrotus* (Longo and Anderson, 1969) the germinal cells are arranged in a series of cell types progressing from spermatogonia (normally in contact with the testicular wall) to spermatocytes (located among and internal to the spermatogonia) to spermatids and spermatozoa (occurring centrally in the lumen). Developing germinal

cells are not divided into distinct zones but are generally more mature as they proceed from the gonadal wall toward the lumen.

Jensen (1883) noted several basic characteristics of the spermatogonia, spermatocytes and maturing spermatozoa in the holothurian *Cucumaria frondosa*. The spermatogonia contain a large nucleus in relation to the surrounding homogeneous cytoplasm. Lipid droplets of varying sizes are observed surrounding the homogeneous nucleus. The nucleolus, not visible in unstained preparations, is very distinctive when properly stained. Spermatogonia give rise to spermatocytes which contain lipid droplets and consist of a smaller volume than noted in spermatogonia. Following formation of the tail, in maturing sperm, cytoplasm condenses and becomes progressively smaller. The tail does not appear to develop from a predetermined point within the cellular protoplasm. At this stage, lipid droplets are more numerous, the nucleus decreases in size and the nucleolus is no longer visible.

Field (1893) observed in various echinoderm species that a great number of darkly stained granules appeared in the spermatid stage and gradually fused into larger and larger refringent bodies, which in the mature sperm existed as a single large Nebenkern (mitochondrion). In the late spermatid the Nebenkern could take any position in the cytoplasm with reference to the nucleus, but in the mature sperm was always posterior to the nucleus. It was suggested that this change in position was due to mechanical causes, that is, the Nebenkern was drawn into its final location by the changes of the cytoplasm of the spermatid into the tail of the spermatozoon, and the pressure from the cell membrane of the spermatid which became tightly drawn over the head of the mature sperm.

Longo and Anderson (1969) have demonstrated many of the finer points of spermiogenesis in the echinoids *Arbacia* and *Strongylocentrotus*. Echinoid spermatids are connected by short intercellular bridges which are maintained until the sperm cells are nearly mature. Morphogenesis of the membrane-bound acrosomal granule is initiated in the spermatid stage and is associated with the Golgi complex. Early spermatids, which are irregularly circular, elongate during the later stages of spermiogenesis as nuclear chromatin material condenses. The spermatid develops an anterior depression which houses the acrosomal granule and a posterior indentation termed the centriolar fossa. They assume that the single large mitochondrion, which lies posterior to the nucleus, develops through a fusion of smaller ovoid mitochondria noted in earlier stages. Proximal and distal centrioles occur in the midpiece region posterior to the centriolar fossa.

Spermatozoan Structure in Echinoderms: Historical and Present Perspectives

The spermatozoon is a highly specialized cell with a few very precise functions, namely, to transport the genetic material to the oocyte and to introduce the material into the oocyte. Comparative spermatologists have suggested that sperm structure is better correlated with the nature of the environment in which fertilization occurs than with phylogeny (Franzén, 1970; Afzelius, 1972). Spermatozoa which are released freely into the water and fertilize externally have been termed "primitive" (Franzén, 1970). The primitive spermatozoon normally contains a rounded or conical nucleus posterior to a small apical acrosome, a short midpiece consisting of one or a few mitochondria arranged around

a proximal centriole and basal body, and a single flagellum displaying a typical 9+2 tubular pattern (Franzén, 1970). Such sperm are found throughout the animal kingdom occurring in the following phyla: Porifera, Cnidaria, Ctenophora, Nemertini, Aschelminthes, Annelida, Mollusca, Arthropoda, Brachiopoda, Sipunculida, Echiurida, Echinodermata and Chordata (Franzén, 1970; Afzelius, 1972).

Since the late 19th century, numerous light microscopic observations have dealt with sperm morphology in the phylum Echinodermata (Field, 1893, 1895; Retzius, 1905, 1910; Dan, 1950, 1952, 1954; Rothschild, 1951; Colwin and Colwin, 1955, 1956; Chia and Buchanan, 1969). More recently, various detailed anatomical accounts employing electron microscopy have appeared (Afzelius, 1955, 1959, 1972; Dan, 1960, 1967, 1970; Bernstein, 1962; Dan et al., 1964; Franklin, 1965, 1970; Hagiwara et al., 1967; Anderson, 1968; Austin, 1968; Longo and Anderson, 1969; Fawcett, 1970; Inoue et al., 1970; Dan and Sirakami, 1971; Summers, 1972; Longo, 1973; Marshall and Luykx, 1973; Summers and Hylander, 1974; Chia et al., in press; Fontaine and Lambert, unpublished manuscript). The majority of these fine structural studies (usually concerned with the morphology and reactivity of the acrosomal region) have dealt with the classes Echinoidea and Asteroidea. Fragmentary studies have been reported in the classes Ophiuroidea (Dan, 1967, 1970), Crinoidea (Dan, 1967, 1970) and Holothuroidea (Dan, 1967; Summers, 1972). To date, no detailed published account is available concerning the ultrastructure of the spermatozoon from any species of the class Holothuroidea.

Field (1893) studied spermatozoa from the five echinoderm classes and reported the following rather accurate observations:

> The spermatozoon must be studied alive. It is so extremely delicate that the greatest care must be exercised in technique. Satisfactory permanent preparations are scarcely to be hoped for. No one method should be relied upon. The results which I have obtained from living cells stained upon the slide, the changes being watched under the microscope, have been confirmed by killed and hardened material. For the latter, I have teased the testes, in various conditions of advancement towards maturity, in a very small quantity of sea water: then Flemming's Chrom-osm-acetic, strong formula; or Platinum chloride, 0,3% for 24 hours, or more: wash in water for 24 hours; stain either in safranin, gentian violet (decolorize in water slightly acidulated), or in Delafield's Haematoxylin; mount in glycerine; dissociate cells by tapping coverglass gently (Field, 1893).

The nucleus varies greatly in size and shape in the different groups; however, in crinoids and echinoids it is generally conical and small, whereas in holothurians, asteroids and ophiuroids it is spherical and generally larger than in the echinoids. At the apical end of the nucleus a cup-shaped depression containing a highly refringent spherical body, the "centrosome" is noted. The size of the centrosome varies greatly in the different groups, being relatively small in echinoids (0.3—0.66 μ) and comparatively large in holothurians, asteroids and ophiuroids (1.3 μ). This spherical body fits tightly into the anterior depression and can be mechanically separated from the nucleus. In the asteroids investigated, the centrosome appears to consist of two parts: a clearer, slightly staining refringent material, spherical in shape, surrounding a deeply staining dumbbell-shaped body. "This latter reminds one strongly of the figures given by various investigators for the first stage of the division of the centrosome" (Field, 1893). Posterior to the nucleus is a Nebenkern, next in size to the nucleus and flattened in the anterior-posterior plane. This structure varies in size in different species and contains granules of various sizes. The tail of the sperm forms from the cytoplasm of the spermatid and is united with the

cell membrane rather than attached to the nucleus (Field, 1893).

There have been at least 101 echinoderm species for which basic sperm morphology has been reported. Of this total number, 37 are echinoids, 23 asteroids, 23 holothurians, 11 ophiuroids and 7 crinoids (Chia et al., in press). All of these sperm typify the primitive type sperm and contain either a spherical (Asteroidea, Crinoidea, Ophiuroidea, Holothuroidea) or conical (Echinoidea) shaped head positioned anteriorly to a short midpiece consisting of a single uniformly shaped mitochondrion surrounding the centriolar region. A prominent acrosome always occurs at the apex of the nucleus. Except for slight modifications, all sperm of a particular class of echinoderms are morphologically similar and, therefore, the general theory has arisen that sperm structure remains the same within each of the five echinoderm classes.

The outstanding variation in sperm structure among the majority of primitive sperm is in the size and organization of the acrosomal region (Fawcett, 1970; Franklin, 1970). With few exceptions the basic structural plan is retained in all primitive, external fertilizing spermatozoa investigated. In situations where unique environmental demands in sperm transport from the male to the egg have arisen, structural modifications have evolved (Franzén, 1970; Afzelius, 1972). The extent that a spermatozoon deviates from the primitive type is determined by the extensiveness of the external demands placed upon the biology of propagation of that species. Spermatogenesis, likewise, can be correlated with the biology of propagation. By studying the various stages of cytogenesis of the sperm it can be determined at which point, if any, the cell begins to deviate from its primitive features. Franzén (1956)

argues that obvious differences in spermiogenesis between two organisms or groups of organisms may have phylogenetic as well as biological consequences.

Thesis Research Problem

Holothurian species inhabit all seas at all depths with many ecological and anatomical questions being unanswered.

> Doubtless there yet remain many undiscovered species of Holothuriadae in the British seas. Of Starfishes we must not expect to find many more kinds, though Goniaster miliaris, and some few others which have been seen on the Norwegian shores, may be looked for. Of Sea-Urchins there are probably still fewer unnoticed; but of the Sea-Cucumbers many. Their comparatively unattractive aspect, the difficulty of preserving them (they must always be kept in spirits), their habitat in the sea, and the little attention that has hitherto been paid to them by native zoologists, all lead me to believe that many species have been passed over. Much yet remains to be done towards a full investigation of the anatomy of the Sea-Cucumbers, more especially with a view to a comparison of the structure of the Molluscan with the Annelidous forms of Holothuriadae (Forbes, 1841).

While examining spermatozoa from various holothurian species with light microscopy, it was noted that extensive morphological variation exists in the class. The basic holothurian sperm shape (spherical) is typified by *Leptosynapta clarki*, whereas the shape in *Cucumaria lubrica* is cylindrical (torpedo-shaped) and that in *Cucumaria pseudocurata* is tabloid (elongated and compressed). Closer examination with the light microscope revealed that the *C. pseudocurata* spermatozoon had definite ventral and dorsal surfaces, an extensive groove on the dorsal side and an acrosome-like structure on the ventral side. It is logical to assume that the process of fertilization in *L. clarki* and *C. lubrica* is very similar to that described for other echinoderm species. Likewise,

it can be theorized that the extensively modified sperm morphology in *C. pseudocurata* corresponds to a modified process of fertilization. To understand completely the process of fertilization in a particular species it is first necessary to investigate the process of spermatogenesis, biology of propagation (packaging of sperm for transport to the egg, medium in which sperm must locomote to fertilize the egg, location of egg when fertilized), acrosomal reaction and egg investment substructure.

The purpose of the present study is to investigate spermatogenesis, to a limited extent the biology of propagation, acrosomal reaction and egg investment substructure in three species of holothurians which live under different environmental conditions and exhibit different reproductive habits. Hopefully, new principles will come to light which will add to the existing knowledge of the process of fertilization and spermatozoan structure in marine invertebrates. *Leptosynapta clarki* (Heding, 1928) is an ovarian brooder with internal fertilization which lives in an intertidal sandy habitat. *Cucumaria lubrica* (Clark, 1901) is an external ventral surface brooder with external fertilization which occurs on subtidal rock surfaces in swift water currents. *Cucumaria pseudocurata* (Deichmann, 1938), also an external ventral surface brooder, occurs intertidally within mussel beds attached to rock surfaces.

The thesis research was directed towards answering the following specific questions:

1) How is spermatogenesis in the class Holothuroidea comparable to that reported in other echinoderm classes; and is there variation in the spermatogenic process within the holothurian class?

2) Do holothurian spermatozoa conform to the primitive sperm type exhibited by other echinoderm species; and if not, then at which stages during spermatogenesis are modifications introduced and how are these alterations reflected in the spermatozoa?

3) Does spermatozoan morphology differ significantly within the holothurians; and if so, is it possible to correlate changes in spermatozoan morphology with an altered biology of propagation (sperm transport from the male to the egg) or an alternative factor such as egg investments?

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SPERMATOGONIA, SPERMATOCYTES AND SPERMATIDS OF *CUCUMARIA PSEUDOCURATA*

Introduction

During a recent comparative morphological study of echinoderm sperm it was observed that *Cucumaria pseudocurata* (Holothuroidea) produces a spermatozoon unique to the class, as well as to the phylum. Sperm shapes of two types have previously been reported in the class Holothuroidea: spherical (Atwood, 1974a; Krishnan and Dale, 1975; Fontaine and Lambert, unpublished manuscript) and cylindrical (Atwood and Chia, 1974). The spermatozoon of *C. pseudocurata* represents a third type: tabloid, i.e., elongated and dorso-ventrally compressed (Atwood, 1975). The ventral surface contains a medially situated acrosome with substructure differing from previously reported echinoderm species. The dorsal surface contains an extensive groove containing a striated rootlet-like structure extending from the centriolar region to slightly posterior to the acrosomal region. At the base of the elongated nucleus is a mitochondrion and a posteriorly projecting flagellum displaying a 9+3 tubular arrangement in the midtail region. The proximal and distal centrioles with their satellites lie perpendicular to one another within the mitochondrial mass. Finger-like cytoplasmic extensions project posteriorly from the midpiece (region containing mitochondrion, centrioles, satellites and striated rootlet), partially encompassing the basal region of the flagellum.

Subsequent examination of the spermatogenic process also revealed structural uniqueness, primarily in the morphogenesis of the acrosome and midpiece. To date, only one detailed published account is available on holothurian germinal cells less mature than sperm (Atwood, 1974b).

Accordingly, the present study describes the fine structure of spermatogonia, spermatocytes and spermatids in the sea cucumber, *Cucumaria pseudocurata*.

Materials and Methods

Cucumaria pseudocurata were collected subtidally from September, 1973 through June, 1974 at Eagle Point, San Juan Island, Washington. Testes were removed from mature males and fixed in a glutaraldehyde-H_2O_2 mixture prepared as follows. Twenty-five per cent glutaraldehyde (Fisher Scientific Company) was diluted to a 2.5% solution buffered to pH 7.6 with 0.34 M sodium chloride and 0.4 M phosphate buffer at room temperature. Thirty per cent H_2O_2 (Fisher Scientific Company) was added with continuous stirring in the amount of ten drops per 50 ml of fixative. Fixation was for 2 1/2 hr at room temperature. A similar glutaraldehyde-H_2O_2 technique has been reported by Peracchia and Mittler (1972). Tissues were then passed into 2.5% glutaraldehyde (with sodium chloride and phosphate buffer at pH 7.6) for 1 1/2 hr at room temperature, washed for 1 hr in phosphate buffer and post-fixed in 2% osmium tetroxide (in phosphate buffer) for 2 hr at room temperature.

Testicular tissues were then rinsed in 0.05 M maleic acid (pH 5.2) for 30 min with three changes and stained *en bloc* in saturated aqueous uranyl acetate for 30 min. Specimens were again rinsed in 0.05 M maleic acid, dehydrated, embedded in Araldite 502, and sectioned with a Porter-Blum-MT-2 ultramicrotome. Sections were stained with saturated aqueous uranyl acetate and 0.2% lead citrate and observed with a Philips EM 200. For light microscopy, Araldite sections were cut at 1 μ and stained

according to Richardson et al. (1960).

Observations

General Description

The germinal cells of the testes are arranged in a series of cell stages progressing from spermatogonia (normally in contact with the gonadal wall) to spermatocytes (located among and lumenally to the spermatogonia) to spermatids and spermatozoa (occurring centrally in the testicular lumen). Developing germinal cells are not separated into definite zones; however, they are generally more mature as they proceed from the gonadal wall toward the lumen. Six basic cell stages, determined by cell position, nuclear morphology and previous spermatogenic studies, are recognizable: (1) primary spermatogonia, (2) secondary spermatogonia, (3) early spermatocytes (preleptotene and leptotene stages), (4) late spermatocytes (zygotene, pachytene and diplotene stages), (5) spermatids and (6) spermatozoa.

The primary spermatogonium, roughly spherical in shape (Fig. 1), has a diameter of approximately 8.7 μ and contains a roughly circular nucleus about 5.5 μ in diameter containing one or two nucleoli, each having a diameter of 1.8 μ. The nucleoplasm consists of a fine homogeneous matrix in which is suspended condensed chromatin distributed around the periphery of the nucleus and widely scattered throughout central regions. Connection-like junctions connect the spermatogonia. Cytoplasmic organelles include a Golgi complex, numerous ovoid mitochondria, limited rough endoplasmic reticulum, numerous free ribosomes, scattered polysomes and occasional microtubules. Membrane-bound

osmiophilic granules, lipid droplets and clear vesicles are normally present in the peripheral cytoplasm. Two centrioles, slightly angular to each other, lie in close proximity to the Golgi complex and nuclear membrane. In rare favorable sections the proximal centriole appears atypical in consisting of nine rows of five tubules each, instead of the usual three tubules. This is described further in the section on morphogenesis of the midpiece. Microvilli arise from spermatogonia, spermatocytes and spermatids with the greatest concentration being in the late spermatocyte stage.

Secondary spermatogonia (Fig. 2) presumably arise through mitotic divisions of the primary spermatogonia. This cell has an average diameter of 7.0 μ with a nucleus of 5.0 μ in diameter containing a single nucleolus with a diameter of 1.1 μ. Nuclear changes include a smaller diameter, thickening of peripheral chromatin adjacent to the nuclear envelope and larger aggregations of centrally positioned chromatin. Cytoplasmic organelles and inclusions are the same as noted in the primary spermatogonia. Secondary spermatogonia are connected by connection-like junctions with no evidence of cytoplasmic bridges. While flagellar morphogenesis is occasionally initiated in this secondary stage (Fig. 2), this is infrequently encountered until the early spermatocyte.

Secondary spermatogonia differentiate into primary spermatocytes progressing through the preleptotene, leptotene, zygotene, pachytene and diplotene stages of the first meiotic prophase. Diplotene spermatocytes presumably give rise to secondary spermatocytes at the first meiotic division. Distinction between various spermatocyte stages was

very difficult in *C. pseudocurata*. For convenience, spermatocytes have been designated as early (preleptotene and leptotene stages) and late (zygotene, pachytene and diplotene stages). Secondary spermatocytes could not be conclusively identified.

Early spermatocytes (Fig. 3) have an average diameter of 5.4 μ with a nucleus measuring 3.7 μ in diameter containing a single nucleolus with a diameter of 0.6 μ. Chromatin condensation is greatly accelerated at this stage. Cytoplasmic organelles are the same as noted in the spermatogonia. The single Golgi complex is more extensive with an indication that proacrosomal granule formation has been initiated. The proximal and distal centrioles, in association with peripheral electron dense materials (presumably developing satellites), lie perpendicular to each other in the basal cytoplasm of the cell. The first signs of morphogenesis of the striated rootlet-like structure extending from the centriolar region are noted at this stage.

The late spermatocytes (Fig. 4) have an average diameter of 5.2 μ with a nucleus measuring 4.1 μ in diameter. No nucleolus is present. Condensed chromatin material is associated with distinct synaptonemal complexes. Cytoplasmic organelles are the same as described above. Microvilli arising from the cell surface appear to be more extensive at this stage. Proacrosomal granules have begun to coalesce to form an irregularly shaped acrosomal granule.

Early spermatids are irregularly circular (Fig. 5), measure about 3.4 μ and contain a nucleus with a diameter of 2.5 μ. No nucleolus or synaptonemal complexes are evident. Nuclear condensation has progressed from a particulate-fibrous state to a stage of coarse chromatin

granules interconnected by fine fibrous materials with extensive interchromatin spaces (Figs. 5, 6). Condensation proceeds at a faster rate in peripheral areas of the nucleus and gradually moves to the center. Spermatids contain microvilli and are joined together by intercellular cytoplasmic bridges. Several ovoid mitochondria (Fig. 5), a Golgi complex (Fig. 5), a basally derived flagellum (Fig. 5), and a densely staining chromatoid body (Fig. 12) are present in the cytoplasm. Dense satellite materials have accumulated in the vicinity of the distal (basal body) centriole (Fig. 5). In the basal cellular region, developing cytoplasmic extensions (folds) reach posteriorly encompassing the base of the flagellum (Fig. 5). An immature irregularly circular acrosomal granule is located in a slight nuclear depression on the future ventral surface of the cell (Fig. 5).

As the spermatid matures, nuclear interchromatin spaces are gradually obliterated by massive condensation of the dense chromatin granules (Figs. 9, 10, 11). The cell simultaneously elongates (no microtubules evident in peripheral cytoplasm) and compresses dorso-ventrally. The dorsal surface contains the developing striated rootlet-like structure located within a dorsal groove (morphogenesis described in detail below) and the ventral surface, a maturing acrosome situated in a nuclear depression (described below). Mitochondrial elements have transformed from several ovoid forms to a large single mass encompassing the centriolar-rootlet region. Cytoplasm is confined to the posterior cellular extensions and a narrow area surrounding the developing striated rootlet.

Morphogenesis of Acrosome

Small proacrosomal vesicles are formed in the basal cytoplasm by the Golgi complex in the early spermatocyte stage. The majority of vesicles contain a fine reticular substance with a low affinity for stains with several vesicles being void of contents. In the early spermatid a large irregularly circular acrosomal granule measuring about 0.6 μ x 0.8 μ has formed, presumably from the coalescence of the smaller proacrosomal vesicles (Fig. 6). The contents which are of a highly sparse fine reticular nature are evenly distributed and not localized into definite zones as in *Leptosynapta clarki* (Holothuroidea) (Atwood, 1974a,b) and *Asterina pectinifera* (Asteroidea) (Dan and Sirakami, 1971). The surface of the granule facing away from the nucleus and Golgi complex is overlaid with a thin layer of dense material (Fig. 6). Small vesicles released from Golgi cisternae lie in close proximity to the adnuclear surface of the granule and possibly add to the contents of the granule (Fig. 6).

In a later stage, the granule is still somewhat irregularly circular, but has become reduced in size (0.5 μ x 0.6 μ) (Fig. 7). Granule contents have condensed from a sparse reticular state to a more electron dense particulate-fibrous nature (Fig. 7). The layer of dense material overlying the granule membrane on the surface opposite the Golgi cisternae has become increasingly more osmiophilic, with deposition of material being slightly greater on the inner surface of the membrane. As morphogenesis continues, granule materials become more condensed (Fig. 8). The dense material of the membrane is highly osmiophilic and obviously more concentrated on the inner surface (Fig. 8).

The granule has maintained its same relative position in relationship to the nucleus; i.e. the surface of the granule containing dense material is opposite to the nucleus.

As the spermatid matures, the acrosomal granule (presumably by migration) comes to lie between the plasma membrane and the nuclear envelope (Figs. 5, 9). The slight cup-shaped nuclear depression which houses the granule occurs on the side of the nucleus opposite the remaining cytoplasmic components (Golgi, mitochondria and flagellum). This side is destined to become the ventral surface of the mature spermatozoon. At this stage the granule appears to rotate within the nuclear depression ending up with the surface containing dense material being in an adnuclear position (Fig. 9). The granule is relatively circular, measuring about 0.6 μ in diameter, and contains a homogeneous particulate material (Fig. 9). The dense material of the granule adnuclear membrane has become less osmiophilic, localized to the inside of the membrane and is of a fine particulate consistency (Fig. 9). Completely surrounding the granule is a periacrosomal layer of a homogeneous particulate-fibrous material more electron dense than that of the granule (Fig. 9). This layer, which does not appear to be a Golgi derivative, apparently arises from the cytoplasm which was sandwiched between the granule and the nuclear envelope at the time of granule implantation. A continuity between this material and the narrow zone of cytoplasm surrounding the spermatid is still evident immediately following implantation (Figs. 5, 9).

At a slightly later stage of morphogenesis (spermatid is elongating and becoming dorso-ventrally compressed) the anterior-posterior

surfaces of the nuclear depression flare out forming pockets in the surrounding nucleus. The anterior-posterior granule surfaces simultaneously bulge out as the granule sinks deeper into the nucleus (Fig. 10). The dense particulate material interior to the adnuclear surface of the granule membrane has now appeared to transform into an incomplete membrane-like structure (Fig. 10). The material of the periacrosomal layer remains homogeneous in nature but is becoming less osmiophilic.

In the late spermatid (nuclear elongation and chromatin condensation nearly completed) the acrosomal region is reaching maturity (Fig. 11). The anterior-posterior surfaces of the nuclear depression and granule continue to bulge out into the nucleus. The incomplete membrane-like structure is very distinct and extends from the anterior-posterior inner surfaces around the dorsal face of the granule. A space, consisting of sparse fibrous materials, has become evident between the granule material and the outer granule limiting membrane and extends ventrally from the positions where the inner incomplete membrane terminates (Fig. 11). The internal (dorsal) surface of the granule has correspondingly become slightly indented forming an inward depression (Fig. 11). In the completely mature acrosomal granule a small area of particulate-fibrous osmiophilic material occurs ventro-medial to this depression (Atwood, 1975). Two distinct regions of the previously homogeneous periacrosomal layer are becoming evident. First, dorso-medial to the granule is a particulate-fibrous material lodged within the granule depression. This material, surrounded by a space void of osmiophilic substances, is presumably the precursor of the acrosomal filament

(Fig. 11). The other region occurs ventral to the pockets on either side of the granule (between the granule and nuclear membranes) and is slightly more dense and granular than the remaining periacrosomal material. This area corresponds to the region within the granule where the inner incomplete membrane is lacking (Fig. 11).

Morphogenesis of Midpiece

The region of the *C. pseudocurata* spermatozoon designated as the midpiece includes the proximal and distal centrioles, centriolar satellites, striated rootlet and dorsal rootlet groove. These components are encompassed by a large mitochondrion.

In the early spermatocyte the proximal centriole lies off center and perpendicular to the distal centriole (basal body) in the basal cytoplasm (Fig. 13). The distal centriole is typical in having nine rows of three tubules each, arranged in a pinwheel manner, whereas the proximal centriole is atypical displaying nine rows of what appears to be five tubules each. This configuration which is only occasionally discernible in spermatogonia and spermatocytes, is commonly noted in spermatids (Figs. 16, 19). In the mature spermatozoon, the number of tubules in the proximal centriole is difficult to determine due to the heavy deposition of dense materials associated with the striated rootlet and surrounding satellite. The first indication of development of the striated rootlet and associated structures is the equal deposition of dense materials between all nine rows of tubules in the wall of the proximal and distal centrioles (Fig. 13). Simultaneously, fine filamentous structures are forming at the proximal end of the distal centriole. They extend toward the center of the cell making contact with the

outer surface of the adjacent side of the proximal centriole. Small aggregations of osmiophilic substances are associated with these structures (Fig. 13). Projecting laterally from the wall of the distal centriole is a single club-shaped dense mass of material connected to the centriole by two dense regions (Fig. 13). This is the formative stage of the basal foot-like structure observed in late spermatocytes (Fig. 15). Posterior to this club-shaped mass, dense satellite materials are forming around the base of the distal centriole. The satellite material forms in close association to the developing basal foot and extends away from the centriole toward the plasma membrane (Fig. 13).

In the slightly later spermatocyte there is an increase in dense materials between the tubules in both centrioles with deposition occurring at a much greater rate in the proximal centriole (Fig. 14). At this stage, deposition in the proximal centriole is unequal with greater quantities of materials being formed in the vicinity of the tubules of the anterior (distal to the basal body) centriolar surface. This heavily osmiophilic material reaches away from the centriole in the form of a dense particulate arm (Fig. 14). In sections coinciding with the cross sectional axis of the proximal centriole there is a row of from three to five ill-defined circular densities parallel to the particulate arm. Presumably, the formation of these densities is initiated either directly by the proximal centriole or indirectly by the proximal centriole via the particulate arm (Fig. 14). The filamentous structures emanating from the end of the distal centriole have become more prominent and now display an ill-defined striated pattern

(Figs. 14, 15). The basal foot of the distal centriole, becoming more distinct in the later spermatocyte, clearly consists of a club-shaped cap connected to the centriolar wall by two dense regions. Fine dense fibers radiate from all surfaces of the foot into the surrounding cytoplasm with an exceptionally concentrated zone extending to the wall of the proximal centriole (Fig. 14). Satellite materials of the distal centriole have become more extensive and osmiophilic (Fig. 14).

In the late spermatocyte the dense projecting arm of the proximal centriole has transformed from a particulate to a fibrous state. The circular densities observed lying parallel to the arm are no longer present and evidently have been incorporated into the mass of the arm which has become more extensive (Fig. 15). The basal foot of the distal centriole has now reached maturity and consists of a dense cap separated from a stalk by a less osmiophilic space. The stalk is separated by another less osmiophilic space from the two dense connecting regions attached to the centriolar wall (Fig. 15). Fibers radiating from the basal foot are now restricted to the cap region and lie in a tight zone existing between the foot and the proximal centriole (Fig. 15).

By the early spermatid stage the basal foot and filamentous structures of the distal centriole have degenerated. The only remaining connection between the two centrioles is a narrow region of fibrous material existing from the end of the distal centriole to the basal surface of the proximal centriole (Fig. 16). This material increases in concentration by the intermediate spermatid and persists as a connection point between the centrioles throughout spermiogenesis (Figs. 17, 19, 20). In the early spermatid the proximal centriole has shifted slightly

laterally to lie directly above the distal centriole (Fig. 16). The fibrous arm of the proximal centriole (referred to as striated rootlet from this stage) has developed an ill-defined striation pattern (Fig. 16).

In the intermediate spermatid the striated rootlet has become very extensive, reaching deep into the cytoplasm (Figs. 17, 18). The rootlet consists of dense fibers, measuring 12 mμ in diameter, cross-striated by smaller fibers measuring about 9 mμ. The axial periodicity is about 39 mμ (Fig. 17). The rootlet originates at the proximal centriole as a single entity but bifurcates into several planes as it proceeds toward the nucleus (Fig. 17). As the rootlet elongates, a slight depression, which will become the dorsal rootlet groove in the late spermatid, develops in the posterior surface of the nucleus. Fine fibrous elements radiate from the projecting end of the rootlet into the nuclear depression (Fig. 17). Nuclear chromatin material is heavily condensed in this region and a conspicuous perinuclear space is evident between the outer and inner nuclear membranes (Figs. 17, 18). The nucleus is simultaneously compressing (Fig. 18, from top to bottom of micrograph) and elongating (Fig. 18, from left to right). A cross sectional view at a slightly later stage reveals that the rootlet elements contact only five of the nine rows of tubules of the proximal centriole (Fig. 20). Evidently, the tubules of the centriole have differential potentials for the formation of rootlet elements. The axial periodicity at this stage has increased to approximately 55 mμ (Fig. 20).

In the late spermatid the rootlet extends almost half the length of the cell with the axial periodicity being 55 mμ at the level of the

proximal centriole and 50 mμ further anteriorly (same as observed in mature sperm) (Fig. 21). The terminal anterior end of the rootlet consists of 20—25 dense fibers tightly situated within the dorsal groove between the plasma and outer nuclear membranes. Satellite materials surrounding the proximal centriole are first evident in the late spermatid (Fig. 22). These materials encompass the base of the rootlet and are continuous with the satellite materials of the distal centriole (Fig. 22).

Basic cell shape first becomes altered in the intermediate spermatid where there is a corresponding compression and elongation of the nucleus (Figs. 5, 18). Compression takes place in the dorso-ventral plane, whereas elongation is along the anterior-posterior axis (Fig. 25A,B). In the late spermatid there is a gradual shift of remaining cytoplasm to the posterior dorsal region of the cell (Figs. 23, 24, 25C,D). This cytoplasmic shift is accompanied by an increase in nuclear compression and elongation. The rootlet groove which originated on the dorso-medial surface of the nucleus in the intermediate spermatid (Fig. 25B) elongates posteriorly as the cell matures (Fig. 25C,D). Through the cytoplasmic shifting process, the striated rootlet comes to lie parallel with and tightly situated within the groove (Fig. 25D).

Discussion

The formation of acrosomal substructures in *C. pesudocurata* is very intriguing. Functional significance of these structures will remain obscure until fertilization experiments are completed. It is likewise interesting that a morphogenic polarity exists in the developing acrosome,

as evidenced by the facts: (1) the region of the acrosomal vesicle membrane surrounding the surface opposite the nucleus and Golgi complex always becomes overlaid with dense materials (precursor of the inner incomplete membrane of the mature acrosome), and (2) the acrosomal granule, following migration, appears to rotate within the nuclear depression ending up with the original complete surface lying adnuclear.

In the asteriod, *Asterina pectinifera*, the developing acrosomal granule maintains no specific orientation with respect to the nucleus; however, the surface on which dense materials are deposited is always at the side farther from the Golgi complex as in *C. pseudocurata* (Dan and Sirakami, 1971). In the holothurian, *Leptosynapta clarki*, the dense surface of the granule appears to remain in close association with the nuclear envelope throughout development with the granule undergoing no final rotation as in the present species (Atwood, 1974b).

It is generally believed in echinoderms that the acrosomal granule *migrates* from the basal cytoplasm region of spermatids to its final position at the sperm apex (Longo and Anderson, 1969; Atwood, 1974b). In a recent report on a holothurian, *Cucumaria frondosa*, Krishnan and Dale (1975) state that the acrosomal granule embeds in the nucleus and remains stationary as the remainder of the cytoplasm moves posteriorly beside the nucleus through 180°. In *C. pseudocurata* the granule embeds at a much later stage of development and distant to the region in which it is formed, thus suggesting that the granule migrates through the cytoplasm to its final location as in *Leptosynapta clarki*, *Cucumaria lubrica* (Atwood, 1974b) and echinoids (Longo and Anderson, 1969).

In *L. clarki*, *C. lubrica* as well as *C. pseudocurata*, the peri-acrosomal material appears to originate from the cytoplasm which is sandwiched between the acrosomal granule and nucleus at the time of granule implantation. Krishnan and Dale (1975) report in *C. frondosa* that the material originates directly from the acrosomal granule, since there is no cytoplasm between the nucleus and the acrosomal granule at the time it embeds. Evidently, significant morphogenic variations exist not only within the echinoderm phylum but also within the individual classes.

C. pseudocurata produces a spermatozoon which consists of a midpiece more complex than that observed in previously studied echinoderms. Functional reasons for this complexity remain obscure. In the early and late spermatocyte stages several structures develop which are presumably for anchorage and/or stability during the formative stages of the flagellum. These structures either disappear in later stages (basal foot, dense fibers radiating from basal foot), transform into mature structures (dense arm of proximal centriole, filamentous structures emanating from distal to proximal centriole) or persist throughout spermiogenesis (satellite materials of distal centriole).

An unique feature of the midpiece is the presence of a basal foot projecting from the distal centriole. This structure is almost identical in location and structure to the cup-shaped basal foot described for kinocilia of sensory cells in vertebrate lateral-line and equilibrium organs (Flock, 1965; Flock and Duvall, 1965; Thurm, 1968), retinal rods (Tokuyasu and Yamada, 1959), gill cilia of *Mytilus edulis* (lamellibranch) (Gibbons, 1961), statocyst sensory cells of *Helix pomatia* (Gastropoda) (Laverack, 1968) and anemone epithelial cells

(Peteya, personal communication). It has been postulated that the ciliary basal foot is a possible mechanosensitive structure instrumental in the control of directional movement of cilia (Thurm, 1968; Flock, 1971). Ciliated cells which contain basal feet exhibit a fixed directional ciliary beat which is related to the orientation of the feet. The questions that arise are why should a developing sperm cell be equipped with such a structure and for what reason does it degenerate? Three possibilities exist: (1) the basal foot is strictly an anchorage/stability structure only necessary during the formative stages of the flagellum and striated rootlet, (2) the foot is strictly a mechanosensitive device important in the initial movements of the flagellum or (3) the foot exhibits a combination of the above two functions.

Both the proximal and distal centrioles obviously have the capacity to organize filamentous cross striated materials of the midpiece (arm of proximal centriole and filamentous structures of distal centriole) as reported in mammalian sperm (Fawcett and Phillips, 1969). The filamentous materials observed in early and late spermatocytes passing from the distal to the proximal centriole appear relatively insignificant and degenerate by the early spermatid. It is possible, however, that these structures, under the influence of the distal centriole, act as a template or inducer for the development of the striated rootlet, which is basically a product of the proximal centriole. Striated rootlet-like structures observed in spermatozoa of various other organisms appear to be organized directly through the influence of the distal centriole (Fawcett and Phillips, 1969; Stanley, 1971; Fawcett, 1972; Mattei and Mattei, 1973).

Various views on the role of microtubules in the process of nuclear elongation have recently been published (Fawcett et al., 1971; Ferraguti and Lanzavecchia, 1971; Lanzavecchia and Donin, 1972). As in *Leptosynapta clarki* and *Cucumaria lubrica* (Atwood, 1974b), there is no evidence that microtubules play an active role in nuclear elongation in *C. pseudocurata*. Nuclear elongation in these species is probably due to internal condensation of the chromatin.

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PLATE 1

Figures 1—4. Early stages of spermatogenesis in *Cucumaria pseudocurata*. The primary spermatogonia (fig. 1) normally lie in contact with the inner basal lamina of the haemal sinus and contain one or two large nucleoli. Interior to the primary spermatogonia lie the secondary spermatogonia (fig. 2), early spermatocytes (fig. 3), and late spermatocytes (fig. 4). Refer to text for complete description of cell stages. Figures 1, 2, X 11,600; figure 3, X 17,000; figure 4, X 13,100. Arrows, Flagellum.

Figure 5. Early spermatid. Note the irregularly circular shape, slight acrosomal nuclear depression and coarse nuclear chromatin granules, X 21,600.

A, Acrosomal region
F, Flagellum
G, Golgi complex
M, Mitochondria
X, Cytoplasmic extensions
Arrow, Satellite material of the distal centriole

Figure 6. Initial stage of acrosomal granule formation in early spermatid. Note the dense material overlying the region of the granule membrane opposite to the nucleus and Golgi complex. Granule contents are of a fine reticular nature, X 58,800.

C, Centriole
G, Golgi complex
N, Nucleus
Arrows (small), Limits of dense material overlying complete membrane of acrosomal vesicle
Arrow (large), Small vesicles

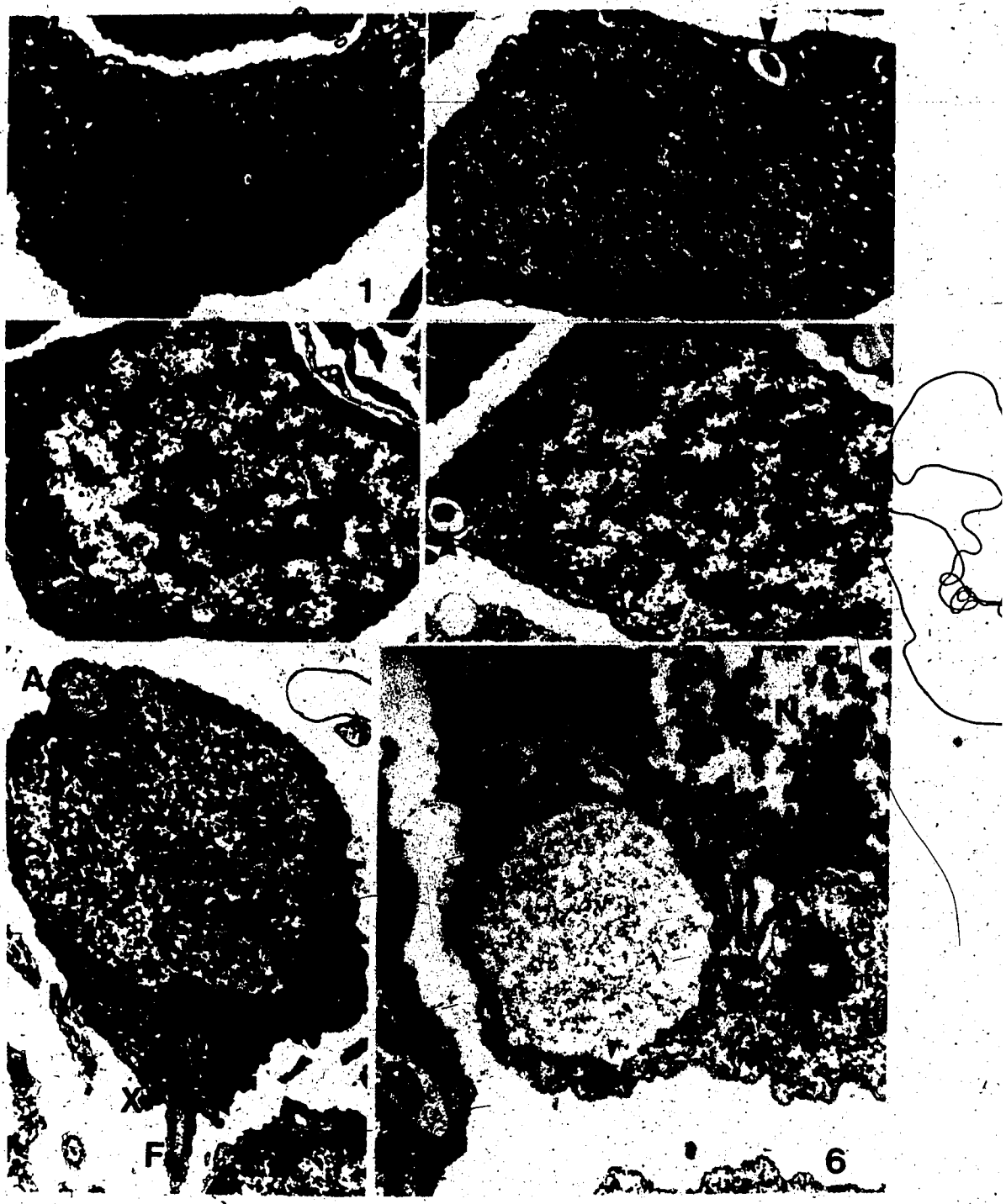

1
A
X
F
N
6

PLATE 2

Figures 7—11. Sequential stages in the morphogenesis of the acrosome. The granule becomes reduced in size and contains a particulate-fibrous material as the layer of dense material overlying the granule membrane becomes more osmiophilic (fig. 7). In a later stage (fig. 8), granule contents are condensing as deposition of dense material is localizing on the inner surface of the granule membrane. As the spermatid matures (figs. 9, 10), the granule comes to lie between the plasma and nuclear membranes in a nuclear depression. The granule rotates within the depression ending up with the surface containing dense material being adnuclear. Surrounding the granule is a periacrosomal layer of a homogeneous particulate-fibrous material. The dense particulate material interior to the granule membrane has transformed into an incomplete membrane-like structure. The anterior-posterior surfaces of the nuclear depression are beginning to flare out forming pockets in the surrounding nucleus. In the late spermatid (fig. 11) the acrosome is reaching maturity. Note the dense material of the periacrosomal layer lodged within the granule depression. Figure 7, X 66,150; figure 8, X 66,150; figure 9, X 61,600; figure 10, X 77,600; figure 11, X 87,300.

G, Golgi complex
GM, Granule membrane
N, Nucleus
NM, Nuclear membrane
PL, Periacrosomal layer
PM, Plasma membrane

Black arrows (small), Limited of dense material (membrane-like structure) interior to granule membrane
Black arrow (large), Particulate-fibrous material of the periacrosomal layer
White arrows (small), Ventral dense, granular areas of the periacrosomal layer

Figure 12. Dense staining chromatoid body in close association with mitochondrion in late spermatocyte and early spermatid stages, X 73,500.

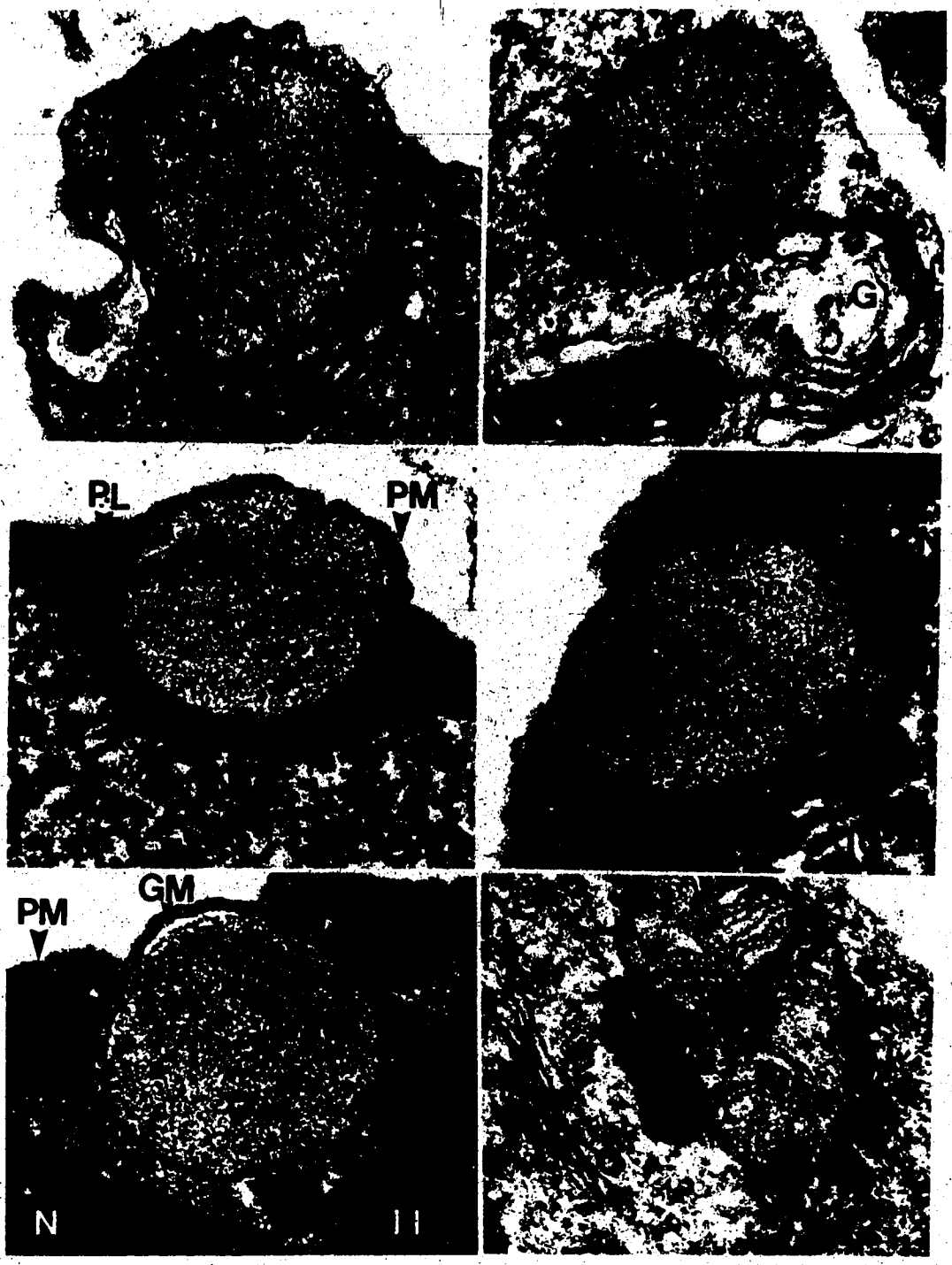

G
PL
PM
PM
GM
N
II

PLATE 3

Figures 13—18. Stages in morphogenesis of the striated rootlet and associated structures of the middle piece. Refer to text for detailed description. Figures 13, 14, early spermatocyte; figure 15, late spermatocyte; figure 16, early spermatid; figures 17, 18, intermediate spermatid. Figure 13, X 67,900; figure 14, X 56,000; figure 15, X 58,800; figure 16, X 66,200; figure 17, X 50,400; figure 18, X 18,900.

A, Arm of proximal centriole
D, Dense fibrous materials radiating from the basal foot to the surface of the proximal centriole
DC, Distal centriole
F, Basal foot of distal centriole
G, Dorsal rootlet groove (formative stage)
N, Nucleus
PC, Proximal centriole
R, Striated rootlet
S, Satellite material of distal centriole
Black arrow (small), Circular densities parallel to arm of proximal centriole
Black arrow (large), Dense connecting material between centrioles
White arrow, Region of bifurcation of striated rootlet.

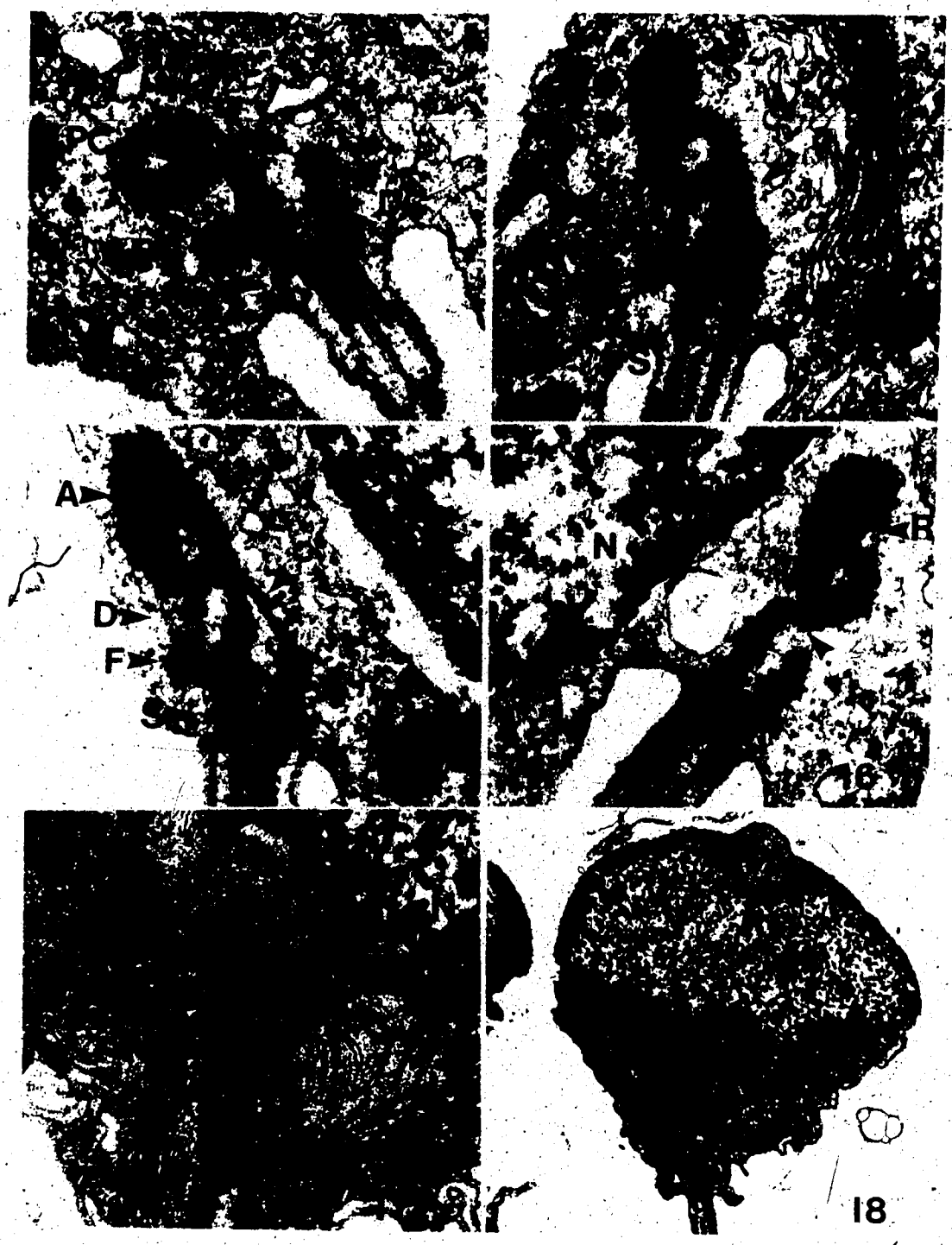

A
D
F
N
18

PLATE 4

Figure 19. Proximal centriole of an early spermatid. Note the nine rows of five tubules, X 147,000.

PC, Proximal centriole
White arrows, Tubules of one of the nine rows

Figure 20. Cross sectional view of the centriolar region of an intermediate spermatid. Note that the rootlet elements contact only five of the nine rows of tubules of the proximal centriole. The axial periodicity at this level of the rootlet is 55 mμ, X 66,200.

Black arrows, Dense connecting material between centrioles.

Figure 21. Section through the longitudinal axis of the centriolar region of a late spermatid. Note the extensiveness of the striated rootlet. Axial periodicity is 55 mμ at the level of the proximal centriole and 50 mμ further anteriorly, X 39,200.

N, Nucleus
PC, Proximal centriole
S, Satellite material of the distal centriole

Figure 22. Cross sectional view of the centriolar region of a late spermatid. Note the satellite materials surrounding the base of the striated rootlet, proximal and distal centrioles, X 56,000.

DC, Distal centriole
PC, Proximal centriole
R, Striated rootlet
Black arrows, Satellite materials

Figures 23, 24. Longitudinal sections through late spermatids. Spermatid in figure 24 is at a slightly later stage demonstrating greater nuclear condensation and elongation. Refer to text and fig. 25 for detailed description of cell orientation and elongation pattern. Figure 23, X 18,900; figure 24, X 15,200.

A, Acrosomal region (ventral surface of spermatid)
R, Striated rootlet (dorsal surface of spermatid)
Black arrows, Direction of nuclear elongation (anterior-posterior axis)

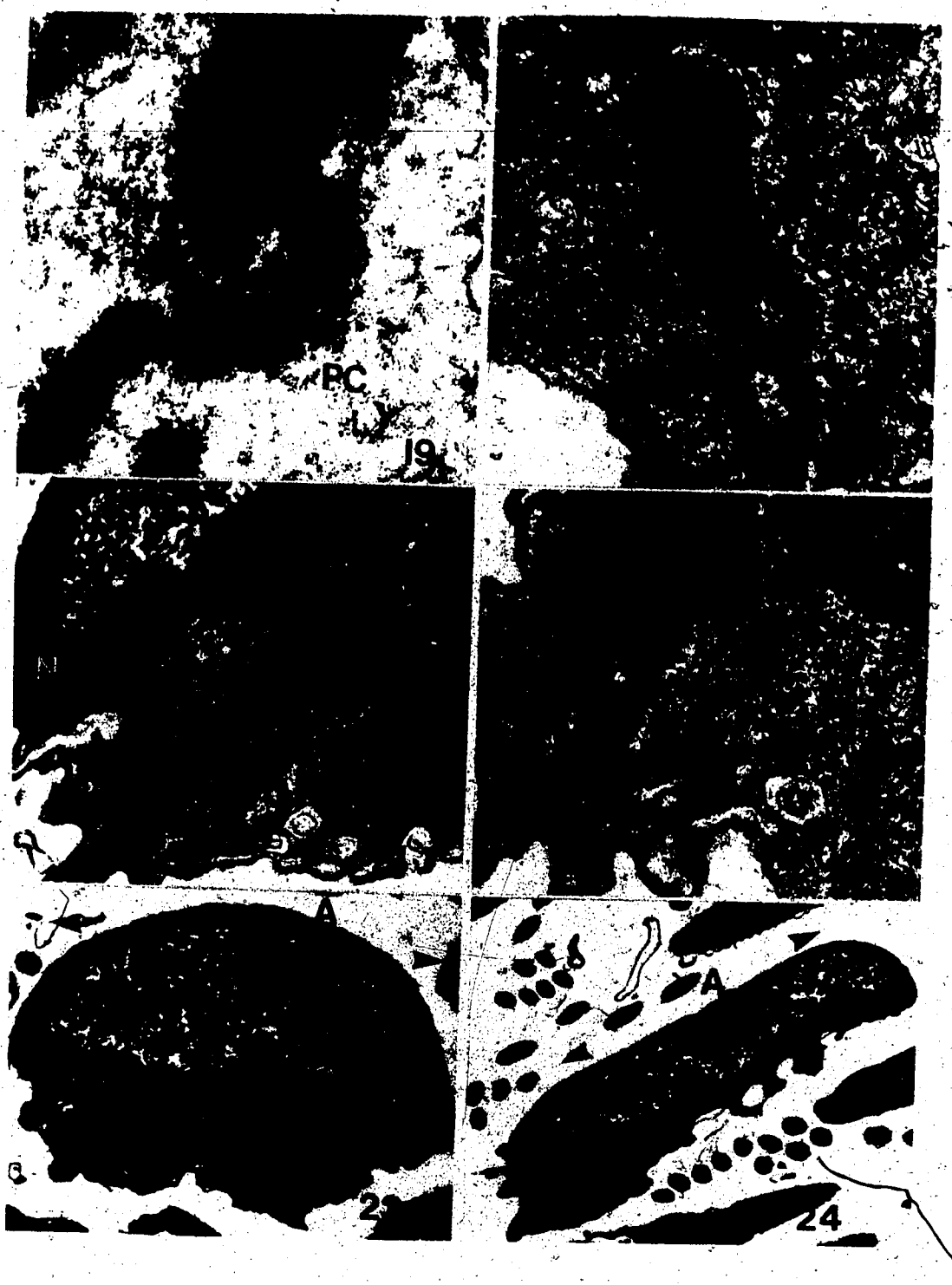
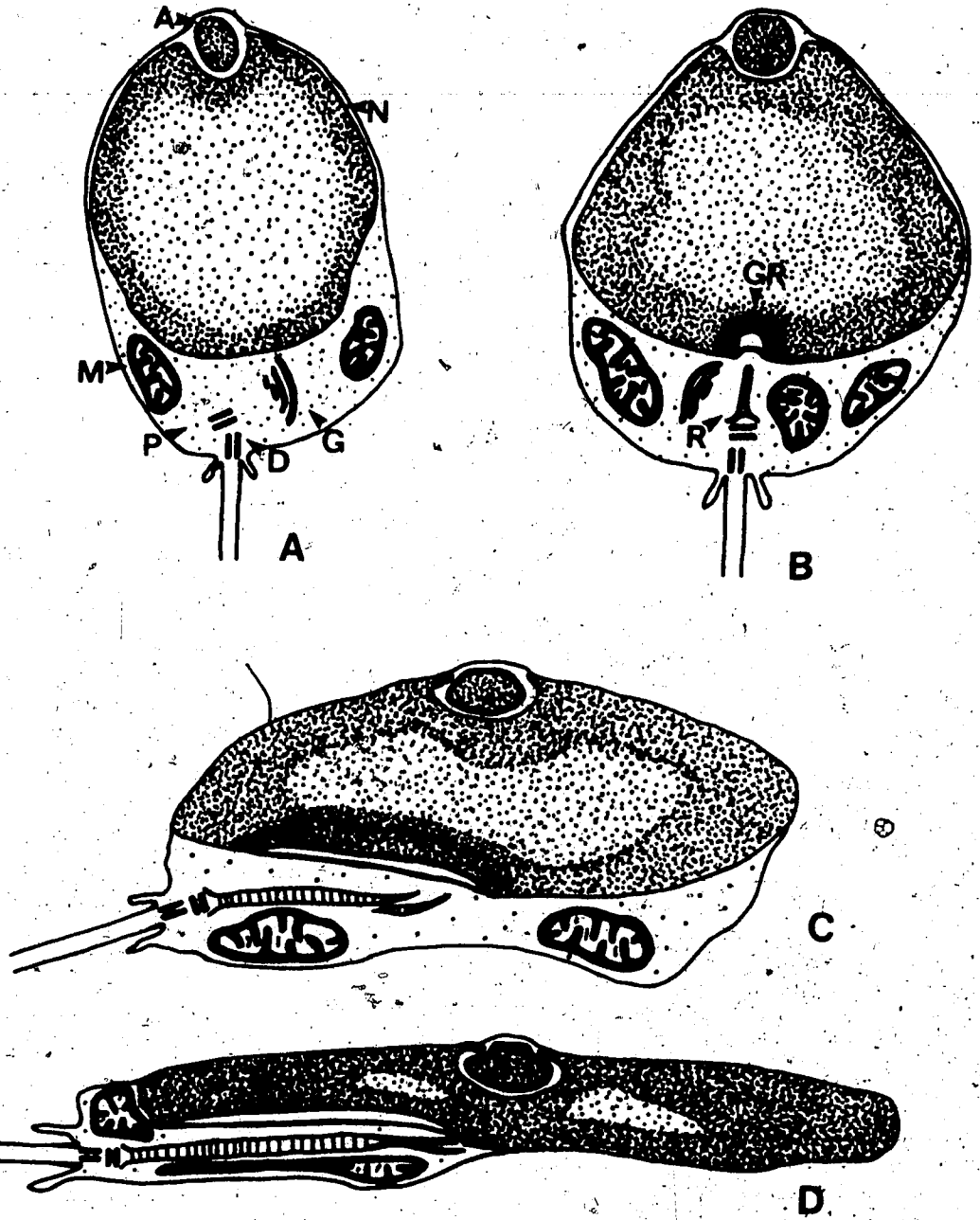

PC
19
N
A
2
A
24

PLATE 5

Figure 25. Diagrammatic representation of nuclear elongation and compression during spermiogenesis in *Cucumaria pseudocurata*. Diagrams are through the longitudinal axis of an early spermatid (fig. 25A), intermediate spermatid (fig. 25B) and two late spermatids at different developmental stages (fig. 25C,D). Orientation is such that the anterior-po or axis runs from right to left on page and the dorso-ventral axis from bottom to top. Note the development of the dorsal striated rootlet groove in the intermediate spermatid stage (fig. 25B). As the nucleus elongates along the anterior-posterior axis, the dorsal groove elongates posteriorly from the level of the acrosome. There is a corresponding shift of remaining cytoplasm to the posterior dorsal region of the cell. The rootlet which develops in a plane perpendicular to the dorsal groove (fig. 25B) ends up in a parallel position in the late spermatid following cytoplasmic shift (fig. 25D).

A, Acrosomal region
D, Distal centriole
G, Golgi complex
GR, Dorsal striated rootlet groove
M, Mitochondrial elements
N, Nucleus
P, Proximal centriole
R, Striated rootlet



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Chapter VII

ACROSOMAL REACTION AND EGG INVESTMENTS IN *LEPTOSYNAPTA CLARKI*, *CUCUMARIA LUBRICA* AND *CUCUMARIA PSEUDOCURATA*

ACROSOMAL REACTION AND EGG INVESTMENTS IN *LEPTOSYNAPTA CLARKI*, *CUCUMARIA LUBRICA* AND *CUCUMARIA PSEUDOCURATA*

Introduction

Fol (1879) reported the existence of a long filament bridging fertilizing sea star sperm with the egg surface. This structure was later interpreted as an extension of the spermatozoon (Just, 1929). This sperm process, "acrosomal filament," and the cytological transformation of the sperm head, "acrosomal reaction," have been extensively studied in many echinoderm species (Dan, 1952, 1954a, 1956, 1960, 1967, 1970; Colwin and Colwin, 1955; Rothschild and Tyler, 1955; Afzelius, 1956a; Colwin and Colwin, 1956; Afzelius and Murray, 1957; Collier, 1959; Haino and Dan, 1961; Bernstein, 1962; Dan et al., 1962, 1964, 1972; Dan and Hagiwara, 1967; Franklin, 1970; Summers and Hylander, 1974). Dan (1952, 1954a, 1954b, 1956) has shown that the acrosomal reaction can be artifically induced by treatment with egg water, calcium-rich sea water, alkaline sea water, albumin sea water or contact with glass and collodion membrane surfaces. Dan (1952) demonstrated that the acrosomal reaction in sea urchins consists of two interrelated processes: 1) breakdown of the acrosomal membrane and the release of substances, possibly lytic enzymes, and 2) formation of a filamentous process from the acrosomal region. The actual dimensions and form of the process varies considerably among echinoderm species. It is generally accepted that in most echinoids the process is short (<0.7 to 6 μ) and broad, while in holothuroids, asteroids, and ophiuroids it is extremely long (10 to 75 μ) and slender (Colwin and Colwin, 1956; Dan, 1956; Tyler and Tyler, 1966).

Ultrastructural studies have shown that all reported echinoderms produce sperm containing an apically located acrosome and, to the author's knowledge, all echinoderm sperm investigated can be artificially induced to discharge an acrosomal process. It has been shown that the sperm of *Cucumaria pseudocurata* (Atwood, 1975) deviates from the typical holothurian sperm model and conceivably deviates from the normal echinoderm acrosomal reaction. Sperm of *Leptosynapta clarki* (Atwood, 1974) and *Cucumaria lubrica* (Atwood and Chia, 1974), on the other hand, possess a typically situated acrosome. It is one aim of the present study to investigate with scanning electron microscopy this cellular phenomenon in these three holothurian species.

Ultrastructural studies of oocyte investment and cortical layers have been published for several echinoderm species (Afzelius, 1956b; Endo, 1961; Mercer and Wolpert, 1962; Franklin, 1965; Monroy, 1965; Tyler and Tyler, 1966; Lönning, 1967; Anderson, 1968; Kessel, 1968; Holland, 1971; Tegner and Epel, 1972; Summers and Hylander, 1974). Except for fragmentary reports in the classes Asteroidea (Monroy, 1965; Lönning, 1967), Ophiuroidea (Kessel, 1968), Holothuroidea (Lönning, 1967) and Crinoidea (Holland, 1971), all studies have dealt with Echinoidea species. No detailed comparable work is available for the class Holothuroidea.

Generally, the echinoderm female gamete is surrounded by a filamentous jelly envelope overlying a thin vitelline envelope which is separated from the egg cortex by a narrow perivitelline space (Austin, 1968). Endo (1961) and Anderson (1968) have shown that it is the vitelline envelope in echinoids which elevates from the oolemna during the cortical reaction to form the

fertilization membrane or activation calyx (Anderson, 1968). Irregularly spaced microvilli arise from the oolemma and extend through the perivitelline space into the vitelline envelope. Immediately interior to the oolemma is a layer of cortical granules (Harvey, 1911; Moser, 1939), sperical, membrane bound vesicles, varying in size and content between species (Tyler and Tyler, 1966; Lönning, 1967).

The second aim of the present research is to investigate the ultrastructure of the egg investment and cortical layers in the holothurian species *L. clarki*, *C. lubrica* and *C. pseudocurata* with scanning and transmission electron microscopy.

Materials and Methods

Leptosynapta clarki were collected in October, 1974 at False Bay; *Cucumaria lubrica* in December, 1974 at Eagle Point; and *Cucumaria pseudocurata* in January, 1975 at Eagle Point, San Juan Island, Washington. Gametes were obtained by dissection of gonads from mature males and females. Female germinal cells of all three species were primary oocytes in the germinal vesicle stage and for convenience were referred to generally as eggs throughout this chapter.

The interaction of sperm and egg was not studied for *L. clarki* (an internal fertilizer) or *C. lubrica* (females not observed spawning). For *C. pseudocurata*, I was fortunate enough to obtain a recently inseminated brood of eggs from a laboratory holding aquarium.

For scanning electron microscopy of acrosomal reactions, 3 ml of dry sperm from each species is diluted in 40 ml of sea water at 8°C. One milliliter aliquots are removed from the diluted sperm solution and centrifuged for 3 min with a hand centrifuge. To artifically induce the acrosomal reaction, alkaline sea water (0.1 N NH_4OH added to sea water) at pH 9.8 is pipetted into the centrifuge tube containing the gently packed sperm pellet. After a period of 2 min, the sample is centrifuged and fixed in a 2.5% glutaraldehyde solution buffered to pH 7.6 with 0.34 M sodium chloride and 0.4 M phosphate buffer for 15 min. Sperm are then washed in buffer (0.4 M phosphate), pelleted by centrifugation and pipetted into Teflon "Flo-Thru Specimen Capsules" (Sargent-Welch Scientific Co.) containing Nuclepore membranes 25 mm in diameter with a 1 μ pore size (Sargent-Welch Scientific Co.). Specimens are post-fixed in 2% osmium tetroxide in 0.4 M phosphate buffer for 15 min at room temperature, dehydrated in ascending concentrations of ethanol and passed through ascending concentrations of amyl acetate to 100%. After critical point drying with carbon dioxide, the capsules are opened and samples on Nuclepore membranes from each capsule mounted on stubs with low-resistance contact cement. The material is coated with carbon then gold to a total thickness of 75 to 125 Å and examined with a Cambridge Stereo-Scan S-4 scanning electron microscope.

For scanning electron microscopy of investment layers, eggs are agitated from dissected ovaries and pipetted directly into a 2.5% glutaraldehyde solution (prepared as above) for 3 hr, followed by a wash in 0.4 M phosphate buffer. Eggs are secondarily fixed in 2% osmium tetroxide (prepared as above) for 1-1/2 hr and pipetted into specimen

capsules as previously described. Subsequent technique is as described for sperm acrosomal reactions. For scanning views of inner egg layers, individual eggs are placed on stubs with double-sided tape, ringed with low-resistant contact cement and fractured with razor blades. Specimens are then coated as usual.

For transmission electron microscopy of egg investment layers, eggs are pipetted into a 2.5% glutaraldehyde solution (prepared as above) for 3 hr at room temperature. Following a 1 hr wash in phosphate buffer with three changes, the eggs are post-fixed in 2% osmium tetroxide (prepared as above) for 2 hr at room temperature. Specimens are then dehydrated, embedded in araldite 502 and sectioned with a Porter-Blum-MT-2 ultramicrotome. Sections are stained with saturated aqueous uranyl acetate and 0.2% lead citrate and observed with a Philips EM 200. For light microscopy araldite sections are cut at 1 μ and stained according to Richardson et al. (1960).

Results

Acrosomal Reaction

The sperm of *Leptosynapta clarki* undergo a typical echinoderm acrosomal reaction, formation and protrusion of an acrosomal process, when treated with alkaline sea water. In the unreacted spermatozoon examined by scanning electron microscopy the acrosomal region normally appears slightly concave (Fig. 1). In many sperm treated for the full 2 min in alkaline sea water, the acrosomal region does not seem to be completely activated. The majority of these sperm are smaller in diameter than the fully reacted ones and instead of a lengthy process,

only a small bleb is apparent at the sperm apex (Fig. 2). The acrosomal process in the fully reacted sperm is relatively short (about 6 μ in length), slightly tapered at its apex and has a maximum width of approximately 0.2 μ (Fig. 3).

The fully reacted sperm of *Cucumaria lubrica* likewise displays a typical acrosomal process. Unlike that in *L. clarki* it does not taper distally and is only about 0.1 μ in width (Fig. 4). The actual length, which exceeds 35 μ, is impossible to determine due to the fragile nature of the process. Many semi-reacted spermatozoa are also evident in this species (Fig. 5). Whereas the apical bleb is similar to that formed in *L. clarki* no dimensional differences are noted between the heads of these sperm and those of the fully reacted sperm.

The sperm of *Cucumaria pseudocurata* differ from sperm of *L. clarki* and *C. lubrica* in that they are packaged in heavy mucous strands and are totally inactive for at least 5 to 7 hr subsequent to release. The sperm are arranged head to tail in long mucous bundles several hundred sperm in diameter. It is necessary for these bundles to be periodically agitated in sea water at 8°C for 10 to 12 hr before all sperm are motile.

Another species difference concerns the reactivity of the acrosomal region. After employing a variety of treatments (alkaline sea water at varying pH, calcium-rich sea water and albumin sea water) it was concluded that a typical acrosomal reaction involving the protrusion of an acrosomal process could not be artificially induced. Scanning electron microscopy revealed no structural changes in the acrosomal region following the above treatments. Subsequent work on naturally inseminated *C. pseudocurata*

eggs showed that the sperm in fact do undergo an acrosomal reaction which is unique to the echinoderms. It is still unclear why this reaction cannot be artifically induced.

A scanning view of the inseminated *C. pseudocurata* egg, reveals that many sperm have attached to the outer egg envelope (detached sperm leave impressions on the egg envelope) (Fig. 6). As shown in figure 6, very few of these spermatozoa remain attached following preparatory procedures. Sperm detachment is possibly either due to technique procedures or it follows the same attachment-detachment sequence described in sea urchin eggs (Tegner and Epel, 1972). The spermatozoon attaches to the egg envelope on its side (lateral) rather than the typical *head-on* position (Figs. 7, 8). Evidently, the surface of the sperm (dorsal or ventral) that first contacts the egg is not critical, since 40% of the sperm observed were ventral side (acrosome containing surface) down and 60% ventral side up (50 eggs containing at least 20 attached sperm per egg were examined). Almost perfect impressions of the entire contacting sperm surface remains on the egg following detachment (Figs. 7, 9, 10, 11). Figure 11 shows the impression of the nuclear, mitochondrial, striated rootlet groove and flagellar regions of a detached sperm which was ventral side up.

Considering the varying configurations of attached sperm flagella (Figs. 7, 10) and the fact that the sperm contacting surface is not constant, it can be assumed that initial sperm orientation to the egg at the time of fertilization is random.

The acrosomal regions of all attached spermatozoa undergo structural modifications (Figs. 7, 8, 9, 10), possibly a modified acrosomal reaction,

in which no acrosomal process is formed. The acrosomal region appears hollowed out, as though the overlying plasma membrane has ruptured (Figs. 7, 9). In the majority of attached sperm, a tubular elevation extends from the center of the acrosomal region posteriorly toward the mitochondrion (Figs. 7, 8, 10). This elevation, which appears to lie under the sperm plasma membrane, cannot be explained until corresponding transmission electron microscopy has been completed.

The egg envelope in the vicinity of the attached sperm also undergoes structural changes, similar to chemical dissolution (Figs. 9, 10, 11). This dissolution is especially noticeable around the sperm body, is also evident along the entire length of the flagellum (Figs. 7, 9) and is obviously responsible for the impressions of detached spermatozoa.

Egg Investment and Cortical Layers

The *Leptosynapta clarki* egg is grayish-yellow in color and measures approximately 0.3 mm in diameter. Envelopes of eggs excised from ovarian tubules consist of the following layers progressing inwardly: 1) outer particulate-fibrous, 2) follicular cell, 3) dense laminate fibrous, 4) dense particulate, and 5) lucent particulate.

The outer particulate-fibrous layer (Figs. 12, 14), which varies in thickness depending on preparatory procedures, consists of a fine particulate material (Figs. 19, 20) and a meshwork of fibers, presumably of a collagenous origin (Figs. 14, 22). Directly beneath this is the layer of scattered follicular cells (Figs. 17, 19, 21) periodically joined together by desmosome-like structures (Fig. 20). These cells are closely applied to the dense laminate layer (Fig. 19) and send out highly branched (Fig. 18), thin (Figs. 19, 20) cytoplasmic processes.

The follicular cell nucleus normally contains one large nucleolus while the cytoplasm contains small ovoid mitochondria, numerous free ribosomes, membrane-bound dense granules and other typical cytoplasmic organelles (Figs. 19, 21).

Underlying the follicular cells is a dense laminate fibrous layer, consisting of fibrous elements in a stacked arrangement (Fig. 19), ranging in thickness from 0.1 to 0.4 μ (Figs. 14, 19, 20). In various regions around the egg this layer appears to bifurcate, forming a relatively large space containing fine dense particles and fibers as noted in the ter particulate-fibrous layer (Fig. 21). This laminate separation normally occurs in the vicinity of a follicular cell body (Fig. 21). Immediately interior to this layer are the two layers, dense particulate (Figs. 15, 19) and lucent particulate (Figs. 19, 20). No fibrous elements are noted in these regions. The two layers vary greatly in thickness ranging from 1.3 to 1.8 μ with the dense particulate normally being the thicker (Figs. 19, 20, 21).

The egg plasma membrane extends through the lucent particulate and into the base of the dense particulate layer as long, thin, microvilli (Figs. 13, 14, 15, 19, 20). The cortical layer directly underlying the plasmalemma contains numerous ribosomes, small ovoid mitochondria, large vesicular structures, endoplasmic reticulum and yolk granules (Figs. 16, 19). No typical echinoderm cortical granules (Harvey, 1911; Lönning, 1967) are apparent in the cortical layer.

The average *Cucumaria lubrica* egg is about 1.1 mm in diameter and green in color. Envelopes of oocytes excised from the ovaries consist of the following layers progressing inwardly:

1) follicular cell, 2) dense laminate fibrous, and 3) dense particulate. The scattered follicular cells contain a large nucleus with a single nucleolus and cytoplasm containing the usual organelles (Fig. 25). The cells send out long, thin cytoplasmic processes over the dense laminate fibrous layer (Fig. 26). The dense laminate fibrous layer (Figs. 23, 24) ranges in thickness from 0.1 to 0.8 μ (Figs. 25, 26) and is continuous, with no obvious separations as noted in *L. clarki*. Beneath this layer is a region of uniformly distributed electron-dense particles, the dense particulate layer (Figs. 24, 25, 26), whose thickness varies from about 4 to 7 μ (Figs. 25, 26).

The plasma membrane underlies this layer and extends slightly into it in the form of rather short, wide microvilli (Figs. 23, 24, 26). Electron-dense particles of the dense particulate layer are scarce at the bases of these cytoplasmic extensions (Fig. 27), possibly due to the physical barrier formed by the folded microvilli (Fig. 24) or fixation shrinkage. The egg cortical layer contains small ovoid mitochondria, few ribosomes, yolk granules, vesicular structures and no apparent cortical granules (Figs. 23, 25, 26).

The *Cucumaria pseudocurata* egg is orange and measures approximately 1 mm in diameter. The large excised eggs, which are tightly packed within the ovarian tubules (Figs. 28, 30), are externally surrounded by an extensively thick, dense laminate layer measuring about 8 μ (Figs. 28, 29, 30). This layer is composed of stacked, non-continuous laminae of relatively low electron-dense materials, each measuring about 0.2 μ in thickness (Figs. 30, 31). Immediately beneath these laminae is a continuous zone of material with the same structure

and density measuring about 0.3 μ in thickness (Figs. 29, 30, 31).

Directly beneath and in contact with the above layer is the plasma membrane of the egg (Fig. 31). No microvilli are evident. Underlying the plasmalemma is the egg cortical layer which consists of small ovoid mitochondria (Fig. 30), yolk granules (Figs. 28, 29, 30, 31), numerous vesicles occurring either singly or in groups of up to 20 (Figs. 29, 31) and no cortical granules. Also in this area are numerous membrane-bound electron-dense granules considerably smaller in diameter than the yolk granules (Figs. 28, 29, 30, 31). The granules vary in shape from spherical to elliptical and are of the same electron density as the yolk granules (Figs. 30, 31).

Figure 32 shows the outer layers of the inseminated *C. pseudocurata* egg. At the scanning level there appears to be no great structural changes induced by insemination, as observed in various echinoderm species. The small electron-dense granules are still present and are probably not involved in any type of cortical reaction (Fig. 32). Two minor morphological differences between the uninseminated and inseminated eggs are evident: 1) the dense laminate layer is slightly thicker after fertilization and 2) a layer with a fibrous consistency forms between the laminate layer and the cell membrane (Fig. 32).

Discussion

In *Leptosynapta clarki* and *Cucumaria lubrica* two types of acrosomal reactivity are observed. The semi-reacted condition, where a small bleb is formed at the sperm apex, either represents a malformed process or a natural intermediate structural stage occurring prior to complete

acrosomal discharge. The latter is doubtful since the extension of the acrosomal process in other species is complete after a few seconds of exposure to the inductive solution (Dan, 1956). This raises the question of whether or not two types of sperm are produced in *L. clarki* and *C. lubrica*, one of which is non-functional. Dan (1954a) found that in the asteroids, *Asterina* and *Asterias*, the majority of the sperm which failed to react had smaller heads and likewise suggested the possibility of a type of non-functional sperm.

The sperm of *L. clarki* and *C. lubrica* undergo acrosomal reactions typical of echinoderms, whereas the reaction of the *C. pseudocurata* sperm is unique to the phylum. Subsequent sperm passage through egg investment layers is likewise unique to the phylum and tends to be similar to that reported in trematodes (Burton, 1967) and mammals (Austin, 1968; Bedford, 1972) which exhibit lateral fusion of gametes. The dissolution of the underlying egg investments could be postulated to be the result of egg lysins (trypsin-like enzymes) produced by the sperm. Presently, there are conflicting reports concerning the lytic activity of echinoderm sperm (Stambaugh and Buckley, 1972; Vacquier et al., 1972; Longo and Schuel, 1973; Summers and Hylander, 1974) with the majority of evidence disfavoring a trypsin-like sperm component. If in fact such enzymes are formed by the *C. pseudocurata* sperm, it is probable that they are not confined to the acrosomal region as in mammals (Stambaugh and Buckley, 1970), molluscs (Wada et al., 1956) and annelids (Colwin and Colwin, 1961a,b) but present along the entire length of the sperm.

Initial gamete contact in the holothurian *C. pseudocurata*

may be different from that described for other echinoderms. Summers and Hylander (1974) have suggested that echinoid gamete contact is a two-step process consisting of a binding between extracellular materials on the acrosomal process and the oolemma and then a membrane fusion between the acrosomal process tip and the oolemma. Aketa (1973) has postulated that a species-specific component is present on the apical end of the echinoid sperm which is complementary to a sperm-binding vitelline layer protein. These two molecules are possibly responsible for both initial species recognition and bonding of gametes. Summers and Hylander (1974) correspondingly suggest that such specific sperm molecules are contained within the acrosomal vesicle and are made available to the egg surface following the acrosomal reaction and that these molecules form a structural bond with the vitelline envelope prior to membrane fusion. Since observations indicate that no acrosomal process is formed in *C. pseudocurata*, species recognition by the egg surface must be mediated through another structural region on the sperm. Conceivably, the egg-binding molecule, as suggested in echinoids, is localized in the acrosomal region and is discharged over the surface of the sperm at the time of sperm-egg contact. If this is the case, then the entire sperm surface rather than just the apical tip is responsible for species recognition. The mode of initial membrane contact and subsequent fusion is at the present unknown.

The physiological significance of the observed dissimilarity in the acrosomal reactions from spermatozoa of these three species remains to be elucidated. It is noted, however, that in *L. clarki* and *C. lubrica* the egg investment layers are quite similar as are the acrosomal reactions,

whereas, in *C. pseudocurata* both the egg investments and acrosomal reaction are quite different.

Lönning (196[illegible]) reported in *Cucumaria frondosa* that the jelly coat surrounding the oocyte is electron-dense and has an outer border with a layer of adhering follicle cell[illegible]ternal to it and an inner border near the oolemma. The inner border is external to microvilli of the oolemma and is separated from the oolemma by a space void of jelly granular substances. Lönning referred to granule-like structures in the cortical region as cortical granules but, from the single micrograph, it appears as though they differ from cortical granules in asteroids and echinoids in density and substructure. There is no evidence that these structures are involved in the typical cortical reaction noted in echinoids. Substructure of the investment layers in *Cucumaria frondosa* due to poor fixation and resolution cannot be adequately compared to the substructure of the investment layers in this study.

Preliminary observations indicate that the outer particulate-fibrous layer of the *L. clarki* egg comes from the haemal sinus of the ovarian wall. The particulate as well as the collagenous-like fibrous component of this layer are characteristically noted in the ovarian haemal sinus in the earlier stages of oogenesis (Atwood, 1973). It appears as though developing oocytes pass through the germinal epithelium of the ovary into the haemal sinus at an early stage of development, presumably for nutritive reasons. The manner of release of the mature oocyte from the sinus is presently unknown.

Present observations indicate that significant species variation occurs in the substructure of holothurian egg surfaces. The egg investments of *L. clarki* and *C. lubrica* are similar, whereas that of *C. pseudocurata*

is different. The external layers of the *C. pseudocurata* egg are structurally dissimilar to the above two species in that: 1) the follicular cell layer is absent, 2) the laminate layer is very extensive and contains no fibrous component, 3) the dense particulate layer is absent, 4) microvilli of the plasma membrane are not evident, and 5) the egg cortical layer is vesiculated and contains small electron-dense granules different in morphology from the yolk granules. The physiological significance of these structural variations cannot be determined until the fine structure of fertilization has been described.

It is interesting, that the egg envelope in *C. pseudocurata* consists of only one homogeneous, extremely dense layer rather than the several different layers observed in *L. clarki* and *C. fabrica*. This single dense layer could possibly present a greater barrier to advancing sperm than the investments of the other two species. Perhaps a typical echinoderm acrosomal process (one without accompanying enzymatic activity) could not penetrate such an investment, whereas a sperm that does possess such activity could. Until research is completed on the biochemical properties of the acrosomal region, physical properties of acrosomal process and chemical properties of egg investments in many holothurian species, this problem will remain unsolved.

Investment layers of the three species studied in the present research surround pre-spawned primary oocytes in the germinal vesicle stage. It is conceivable that structural changes could occur in the investments following natural spawning. In the case of *L. clarki*, which is an internal fertilizer, mature eggs are retained within ovarian tubules and are not freely spawned to the outside. There is no doubt that the outer follicular cell layer remains intact during fertilization since it per-

sists through the early developmental stages of the embryo! In *C. pseudocurata*, naturally spawned eggs have been examined and no structural differences noted. In the case of *C. lubrica*, mature naturally spawned eggs have not been investigated. It is possible that in this species the outer follicular cell layer is shed prior to spawning.

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PLATE 1

Figure 1. Scanning electron microg[illegible]M) of the unreacted *Leptosynapta clarki* spermatozoon, [illegible],000.

Figure 2. SEM of semi-reacted *L. clarki* sperm. Note: small bleb at sperm apex (arrow), X 27,000.

Figure 3. SEM of reacted *L. clarki* spermatozoon. Note: acrosomal process (arrow), X 18,000.

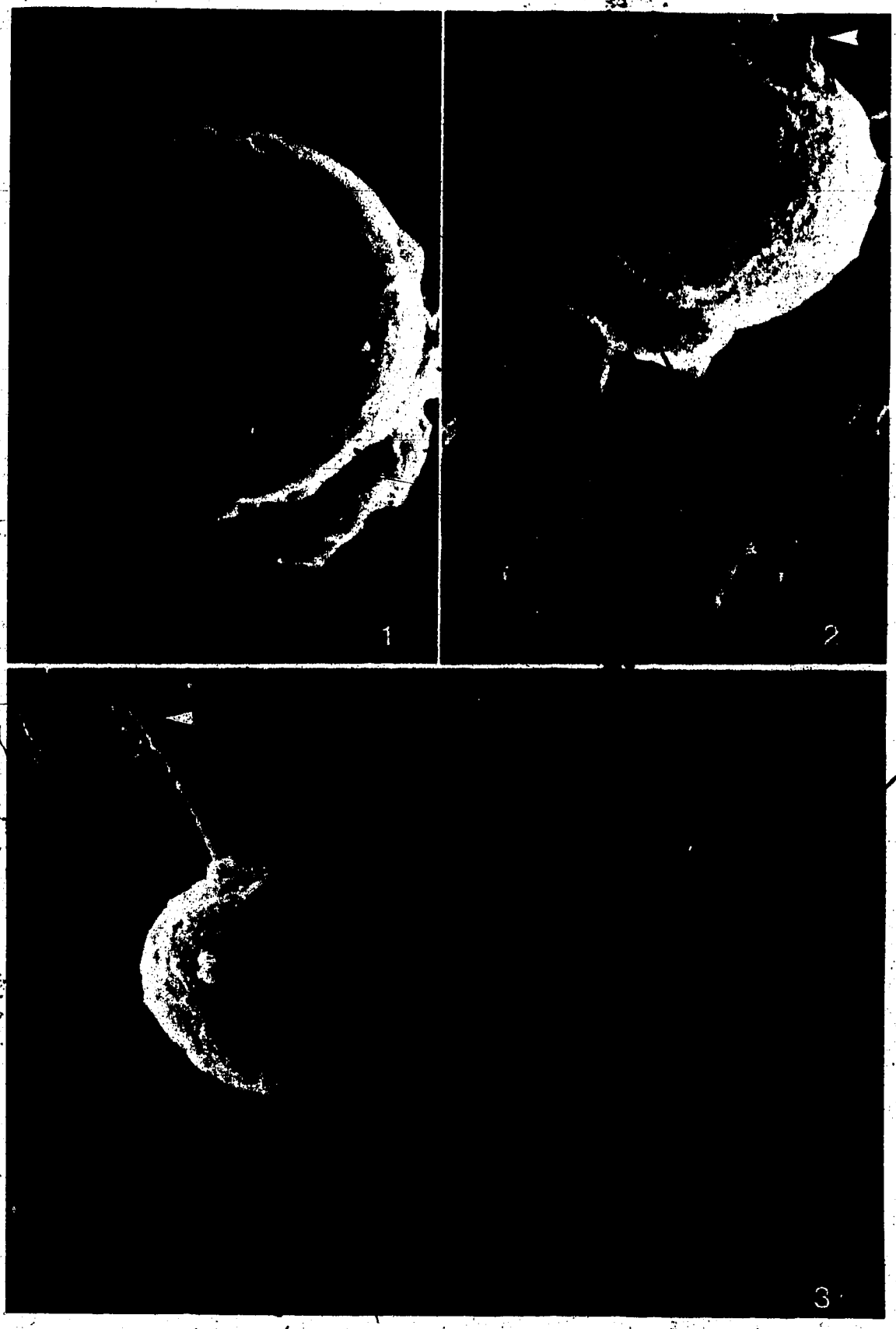
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PLATE 2

Figure 4. SEM of the reacted spermatozoon of *Cucumaria lubrica*. Note: extensive acrosomal process (arrow), X 12,250.

Figure 5. SEM of semi-reacted *C. lubrica* sperm. Note: small bleb at sperm apex (arrow), X 17,500.

4

PLATE 3

Figure 6. SEM of the outer surface of an inseminated *Cucumaria pseudocurata* egg. Note: impressions of detached sperm as well as attached sperm (large arrow) and attached spermatozoon tail (small arrow), X 1,200.

Figure 7. SEM of an attached spermatozoon on the surface of a *C. pseudocurata* egg. Note: impression remaining on egg surface in detached region of the flagellum (arrow); and the hollowed out reacted acrosomal region, X 12,250.

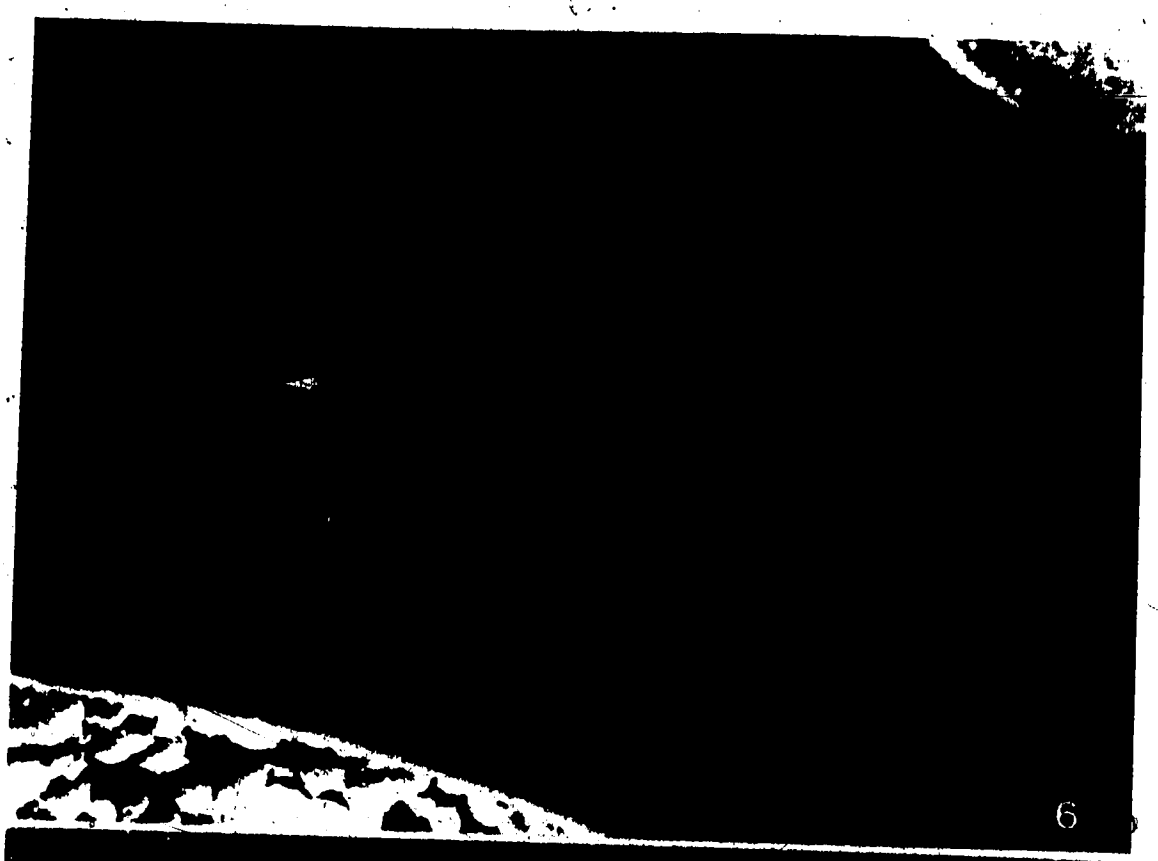

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PLATE 4

Figure 8. Scanning electron micrograph of attached spermatozoon on the surface of an inseminated *C. pseudocurata* egg. Note: dissolution of egg investment layer around nuclear and mitochondrial regions of sperm; and reacted acrosomal area, X 13,800.

Figure 9. Same as figure 8. Note: impressions on egg investment layer of detached flagellar regions (arrows); reacted acrosomes; and extensive dissolution of the egg envelope surrounding attached spermatozoa, X 8,750.

Figure 10. Same as figure 8. Note: extensive dissolution of egg envelope; and tubular elevation extending from the center of the acrosomal region posteriorly toward the mitochondrion (arrow), X 16,250.

Figure 11. SEM showing impression of detached sperm on surface of *C. pseudocurata* egg. Note: nuclear (N), mitochondrial (M), striated rootlet groove (arrow) and flagellar (F) regions; and extensive egg surface dissolution surrounding sperm impression, X 17,500.

8

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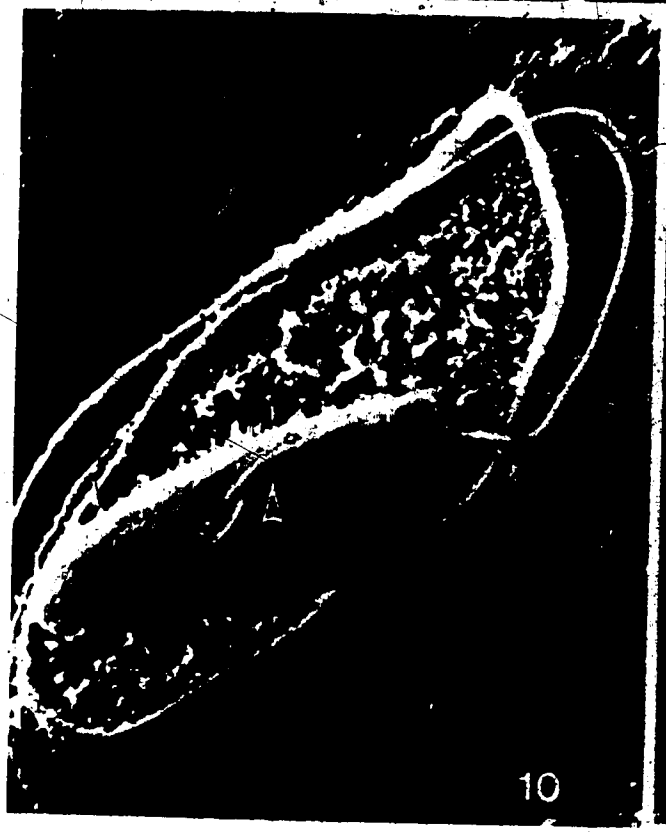

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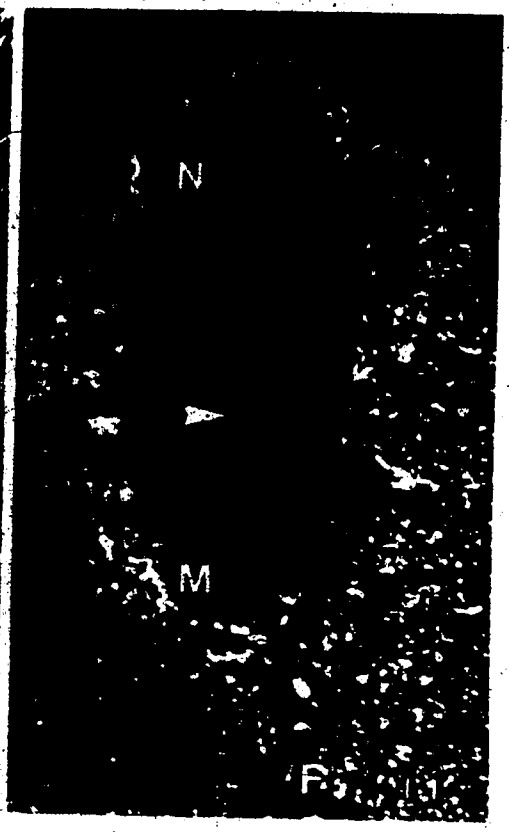

PLATE 5

Figure 12. SEM of *Leptosynapta clarki* egg. Note: outer particulate-fibrous layer (P) and dense laminate fibrous layer (L), X 200.

Figure 13. Same as figure 12. Note: outer investment layers have been removed by heavy agitation thus exposing the underlying microvilli of the plasma membrane (M), X 450.

Figure 14. SEM of *L. clarki* egg showing outer particulate-fibrous layer (P), dense laminate fibrous layer (L) and microvilli of the plasma membrane (M), X 4,200.

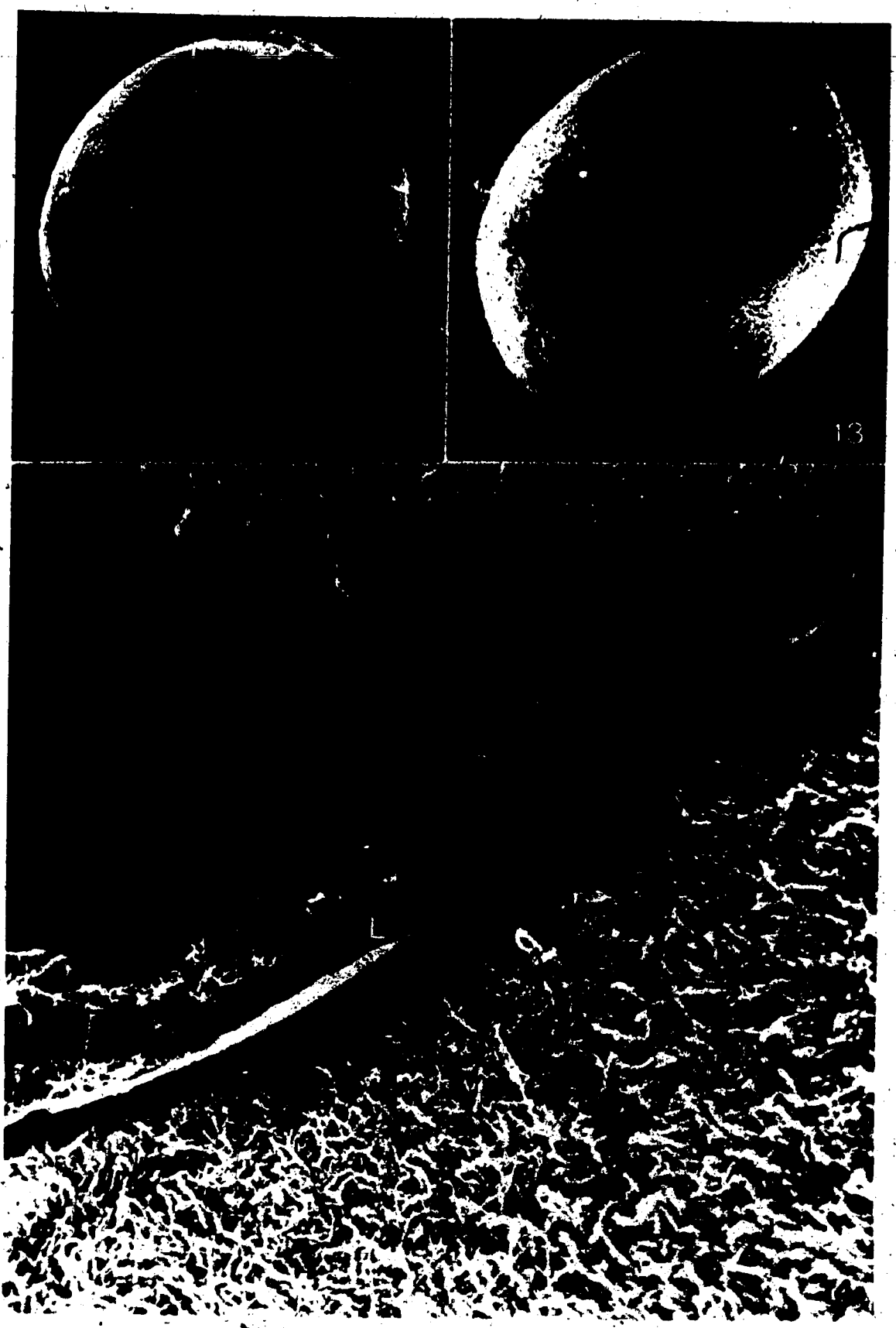
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PLATE 6

Figure 15. Scanning electron micrograph of *L. clarki* egg showing the dense laminate fibrous layer (L), dense particulate layer (D) and microvilli (M), X 10,500.

Figure 16. Same as figure 15. Note: outer particulate-fibrous layer (P) and yolk granules (Y) of the egg cortical layer, X 9,750.

Figure 17. SEM of follicular cells (arrows) on surface of *L. clarki*, X 3,500.

Figure 18. Same as figure 17. Note: highly branched, thin cytoplasmic processes extending from the follicular cell (arrow), X 10,500.


I
D
M


16


17

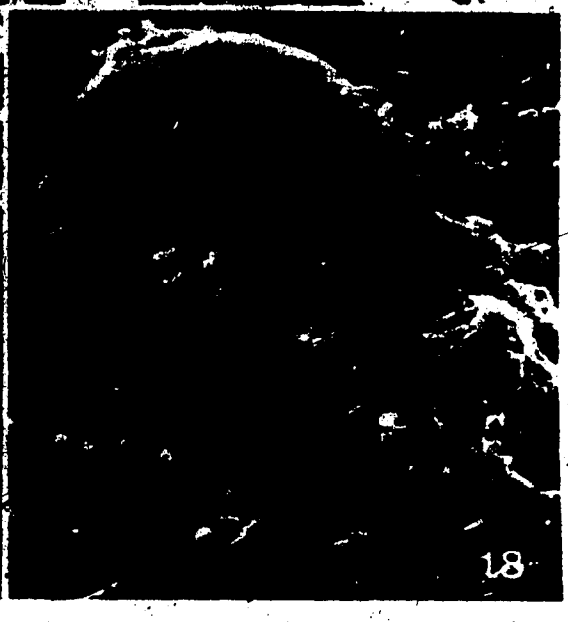
18

PLATE 7

Figure 19. Transmission electron micrograph (TEM) of the investment layers of the *L. clarki* egg. Note: outer particulate-fibrous layer (P), follicular cell process (FC), dense laminate fibrous layer (L), dense particulate layer (D), lucent particulate layer (S), microvilli of the plasmalemma (M) and egg cortical layer (C), X 22,800.

Figure 20. Same as figure 19. Note: desmosome-like structure connecting two follicular cells (arrow); remaining legend is same as for figure 19, X 18,900.

Figure 21. Same as figure 19. Note: separation in the dense laminate fibrous layer (arrows) by space containing electron-dense particles and fibers (area between arrows); and large overlying follicular cell (FC), X 8,050.

Figure 22. TEM of fibers present in the outer particulate-fibrous layer of the *L. clarki* egg, X 66,150.


P
FC
S
19
P
FC
M
S
FC
2

PLATE 8

Figure 23. Scanning electron micrograph of the *Cucumaria lubrica* egg. Note: dense laminate fibrous layer (L), dense particulate layer (D), microvilli of the plasma membrane (M) and yolk granules (Y) of the egg cortical layer, X 2,650.

Figure 24. Same as figure 23. Note: dense laminate fibrous layer (L), dense particulate layer (D) and microvilli (M), X 12,250.

PLATE 9

Figure 25. TEM of investment layers of the *Cucumaria lubrica* egg. Note: follicular cell (FC), underlying dense laminate fibrous layer (L), dense particulate layer (D) and yolk granules (Y), X 9,100.

Figure 26. Same as figure 25. Note: microvilli of the plasma membrane (M); remaining legend is same as for figure 25, X 9,000.

Figure 27. TEM of *C. lubrica* egg showing plasma membrane with microvilli (M) and cortical layer with yolk granules (Y). Note the lack of cortical granules, X 24,300.


FC
25
FC

PLATE 10

Figure 28. Scanning electron micrograph of the outer investment layer of the *Cucumaria pseudocurata* egg within an ovarian tubule. Note: tall columnar epithelial cells of the ovary (O), dense laminate layer of the oocyte (L), small electron-dense granules in egg cortical layer (X) and large yolk granules also in the egg cortical layer (Y), X 4,200.

Figure 29. SEM of the outer layers of the *C. pseudocurata* egg. Note: dense laminate layer (L), basal zone of the dense laminate layer (arrows), vesicles of the egg cortical layer (V), small electron-dense granules (X) and yolk granules (Y) in the egg cortical layer, X 4,200.

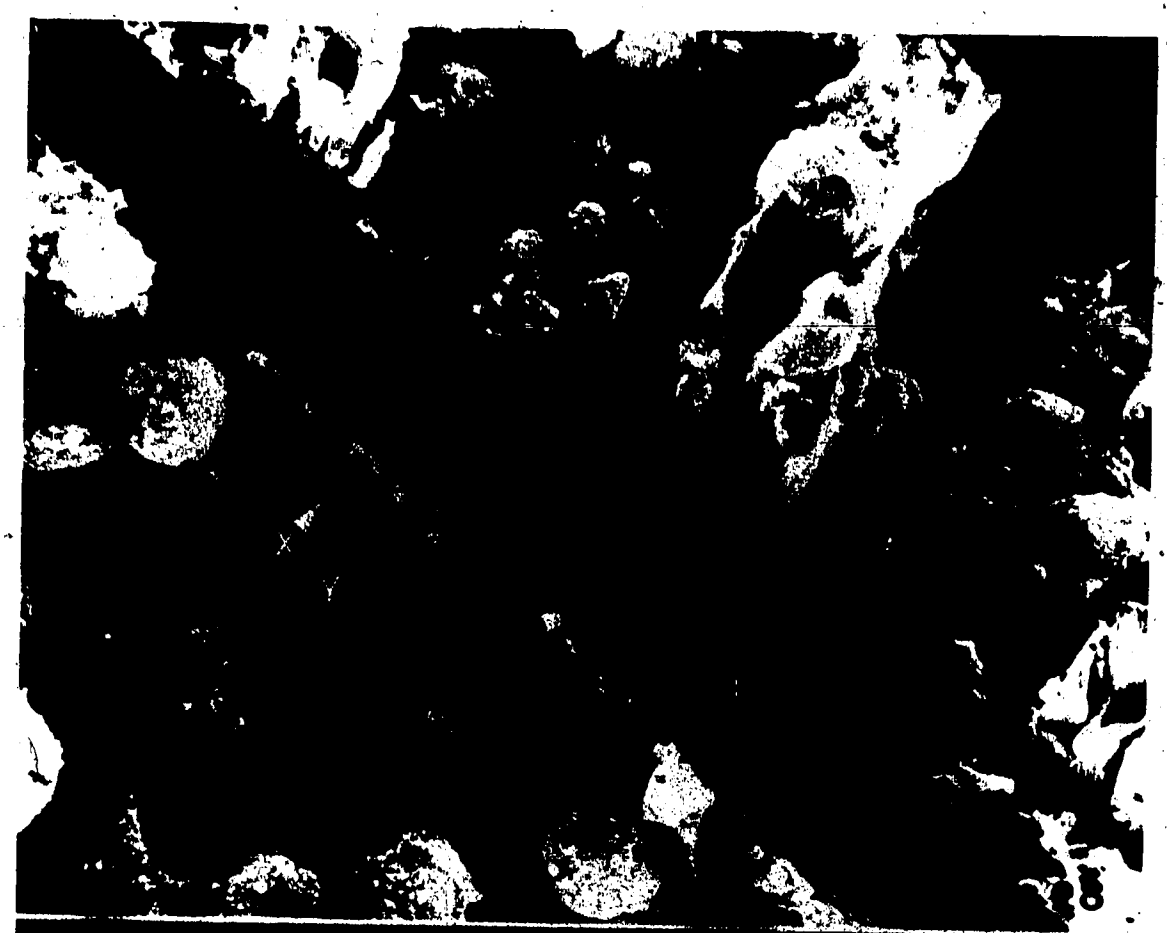

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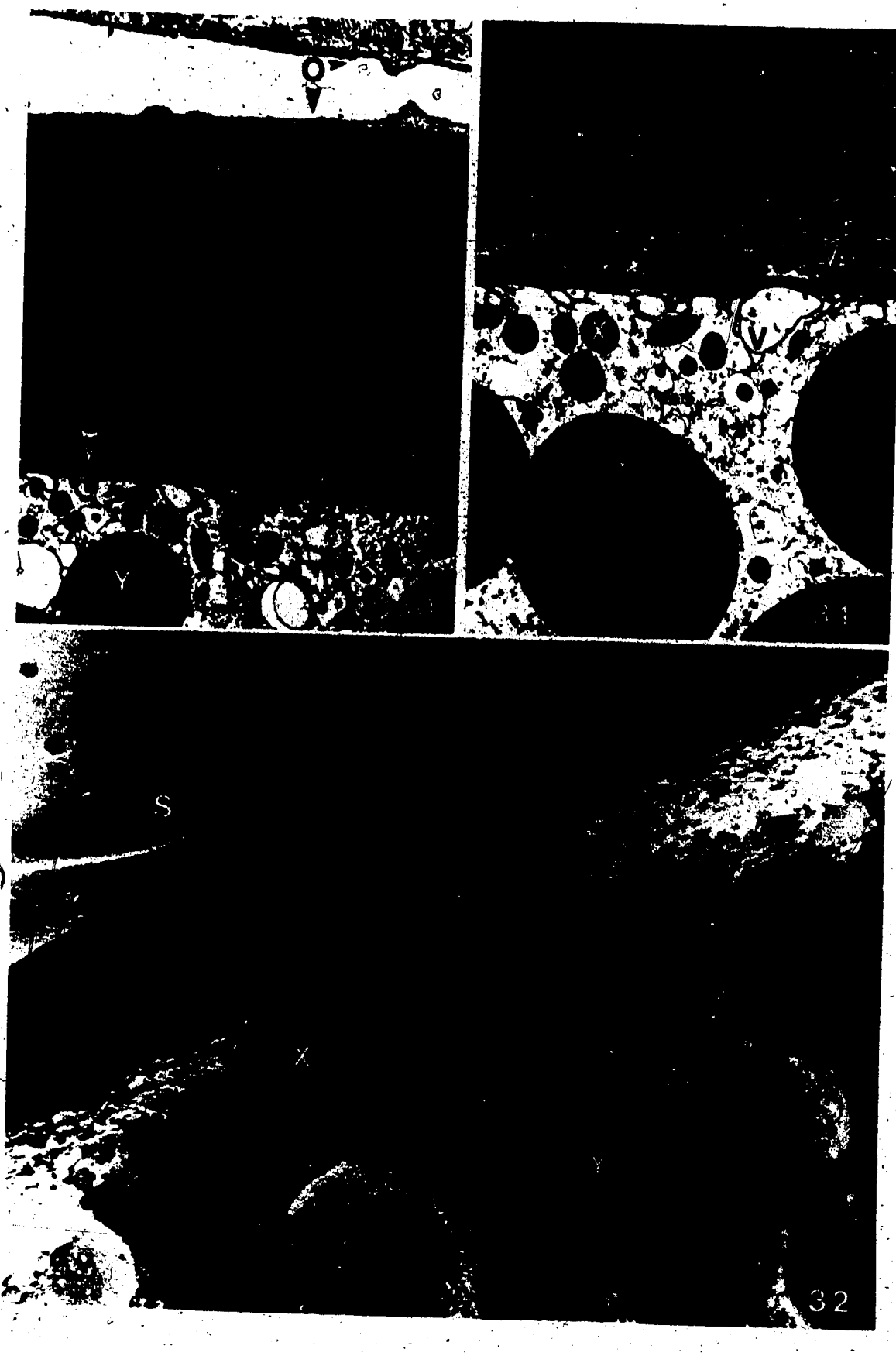
PLATE 11

Figure 30. Transmission electron micrograph of the outer layers of the *C. pseudocurata* egg within an ovarian tubule. Note: ovarian tissue (O), dense laminate layer of the egg (L), basal zone of the dense laminate layer (arrows), small dense granules in egg cortical layer (X) and yolk granules (Y), X 7,700.

Figure 31. TEM of the outer layers of the *C. pseudocurata* egg. Note: vesicles beneath the plasma membrane (V); remaining legend is same as for figure 30, X 10,800.

Figure 32. SEM of the surface of the inseminated *C. pseudocurata* egg. Note; impression of detached sperm (S), dense laminate layer of the egg (L), fibrous layer (F) which forms between laminate layer and cell membrane, small dense granules of egg cortical layer (X) and yolk granules (Y), X 4,550.


S
X
32

Chapter VIII

GENERAL DISCUSSION AND CONCLUSION

GENERAL DISCUSSION AND CONCLUSION

This chapter is an attempt to draw together the discussions from the preceding six chapters in a manner so as to answer the questions set forth in the general introduction. Summaries, conclusions and hypotheses will be presented in the same order as the questions.

1) How is spermatogenesis in the class Holothuroidea comparable to that reported in other echinoderm classes; and is there variation in the spermatogenic process within the holothurian class?

Except in the case of the echinoids *Arbacia punctulata* and *Strongylocentrotus purpuratus* (Longo and Anderson, 1969) there is no detailed comparative work available on the phylum. Nevertheless, general observations are available in the class Asteroidea. In the asteroid *Leptasterias* developing germinal cells form fingers or colonnettes (Cognetti and Delavault, 1962) which bulge out from the germinal epithelium into the lumen of the testes (Smith, 1971). The cells of the colonnettes are layered according to maturity and proceed from spermatogonia at the base to mature spermatozoa at the lumenal tip. In the echinoids (Longo and Anderson, 1969) and holothurians (present study) the germinal cells are arranged in a series of cell stages progressing from spermatogonia (normally in contact with the testicular wall) to spermatocytes (located among and lumenally to the spermatogonia) to spermatids and spermatozoa (occurring centrally in the testicular lumen). Developing cells are not separated into definite zones but are generally more mature as they proceed from the gonadal wall toward the lumen.

Morphogenesis of Acrosomal Region

Information concerning acrosomal granule morphogenesis in the echinoderms is very sparse. It is generally agreed that the Golgi complex is associated with the formation of proacrosomal vesicles. These vesicles probably coalesce to form the initial acrosomal granule during the late spermatocyte-early spermatid stage of spermatogenesis. The initial granule in the asteroid *Asterina* contains a central region of sparse, randomly oriented, dense material surrounded by a region of material in a radial micellar arrangement. The centrally located material condenses as spermiogenesis proceeds and becomes more electron-dense than the peripheral region (Dan and Sirakami, 1971). In the echinoids *Arbacia* and *Strongylocentrotus* the initial granule appears as a membrane-bound vesicle void of electron-dense material. The granule, at a late stage of spermiogenesis, consists of a fine granular homogeneous matrix which contains no characteristic substructure (Longo and Anderson, 1969). In the holothurian *Cucumaria lubrica*, acrosomal formation is similar to that reported in *Asterina*. The initial granule, which is irregularly circular and enclosed by a membrane, consists of a dense homogeneous material with a reticular consistency. The granule material loses the reticular appearance during migration toward the apical sperm surface and becomes less electron-dense. By the time the granule reaches the nuclear region, destined to become the acrosomal depression, it consists of a homogeneous material except for an electron-dense sphere displaced anteriorly.

In the present investigation, it was found that morphogenesis of the acrosomal granule in *Leptosynapta clarki* differs in details from

other echinoderm species. The initial granule is irregularly circular, surrounded by a limiting membrane and consists of a heterogeneous material distinctly segregated into a dense reticular peripheral zone and a less dense vesicular central zone. As the granule matures, the central vesicular structures break down, forming a zone composed of a homogeneous material less electron-dense than that of the peripheral zone, which has become less reticular in nature. Later in morphogenesis the granule becomes relatively circular in shape and develops four or five concentric dense bands in the central zone. Two electron-dense, cup-shaped bands occur in the posterior region of the granules of both *L. clarki* and *C. lubrica*. These bands, which can be equated to similar structures within the asteroid granule, have been shown by Dan and Hagiwara (1967) to be involved in the acrosomal reaction.

Morphogenesis of the acrosomal granule in *Cucumaria pseudocurata* is likewise unique to previously reported echinoderms. The granule begins as a large irregularly circular structure containing a highly sparse, fine reticular material. In the late spermatocyte the granule contents have condensed from a sparse reticulate state to a more electron-dense particulate-fibrous nature. As in *L. clarki*, the granule membrane destined to become the adnuclear surface becomes overlaid with a thin layer of dense materials. As the granule matures, this dense material becomes less osmiophilic and localized to the inside of the adnuclear granule membrane. The granule changes from irregularly circular in shape to a structure with its anterior-posterior surfaces flared out into the surrounding periacrosomal material. The osmiophilic material of the adnuclear surface progresses to the state of an incomplete membrane-like

structure which extends from the anterior-posterior inner surfaces around the dorsal face of the granule. This structure could be correlated to the cup-shaped bands in the posterior granule regions of *L. clarki* and *C. lubrica*.

Encircling the acrosomal granule in *L. clarki*, *C. lubrica* and *C. pseudocurata*, within a nuclear depression, is a periacrosomal layer generally composed of a reticular material. This material appears to arise from the cytoplasm lying in the region of the nuclear depression rather than from other areas of the cell.

It is generally believed in echinoderms that the acrosomal granule is formed in the basal cytoplasm region of the spermatocyte-spermatid stage and migrates to the apical end of the cell by time of maturity. In the asteroid *Asterina*, the migrating granule maintains no specific orientation with respect to the nucleus. However, the surface on which dense materials are deposited is always at the side farther from the Golgi complex (Dan and Sirakami, 1971). In *L. clarki* the dense surface of the granule appears to remain in close association with the nuclear envelope throughout development. In *C. pseudocurata* the dense surface of the granule faces away from the nuclear envelope during migration and rotates within the nuclear depression into an adnuclear position at the spermatid stage. Acrosomal rotation of this type has not been previously reported in echinoderm species.

Morphogenesis of Mitochondrial Region

In all three holothurian species investigated in the present study, mitochondrial elements transform from small ovoid forms in spermatogonia

and spermatocytes to a single large structure in spermatids. Two hypotheses are available to explain this final shape of the mitochondrion. The first possibility is that mitochondrial elements observed in spermatogonia and spermatocytes fuse to form one single organelle, as reported in echinoids (Longo and Anderson, 1969). A second possibility is that only one mitochondrion exists in the cell throughout the entire process of spermatogenesis. The mitochondrion, in the early stages, would be highly branched, consisting of many greatly folded tubular units. This model could explain the many small ovoid mitochondria observed in sections of earlier stages of spermatogenesis. It is then conceivable that as the germinal cell matures, the mitochondrion condenses into a single compact organelle lying at the base of the nucleus. Preliminary data obtained from serial sections of early spermatids of *L. clarki* and *C. pseudocurata* indicate that this model is feasible. Hoffmann and Avers (1973) have shown that a single tubular mitochondrion rather than numerous separate units exists in the yeast cell and have indicated that a similar situation is noted in mammalian cells.

Morphogenesis of Nuclear Region

During the process of spermatogenesis, in all three holothurian species, the nucleus becomes relatively circular. In *D. clarki* the circular shape is maintained throughout spermiogenesis and remains unchanged in the spermatozoon. Spermatids of *C. lubrica* and *C. pseudocurata*, on the other hand, undergo an elongation process which results in a torpedo-shaped (cylindrical) nucleus in the *C. lubrica* sperm and a tabloid-shaped nucleus in *C. pseudocurata*. A similar nuclear elongation

process is observed in the echinoids *Arbacia* and *Strongylocentrotus* (Longo and Anderson, 1969). Nuclear elongation in the holothurians and echinoids does not appear to be accompanied by the presence of microtubules but is probably due to internal condensation of the chromatin material of the nucleus.

In *L. clarki* and *C. lubrica* spermatids, nuclear indentations form at the posterior (centriolar fossa) and anterior (acrosomal depression) regions. Extending from the nuclear surface of the proximal centriole into the posterior centriolar fossa is a dense projection with a fibrous consistency in *L. clarki* and a tubular-fibrous consistency in *C. lubrica*. The formation of the fibrous projections parallel that of the centriolar fossa in the early spermatid. The projections gradually become less prominent during late spermiogenesis but remain in a reduced form in the mature spermatozoon. Longo and Anderson (1969) reported that similar projections occurred in echinoid spermatids but were absent from spermatozoa. It was ventured that they were instrumental in the formation of the centriolar fossa. A dense fibrous structure extending from the proximal centriole and similar to the fibrous arms in *L. clarki*, *C. lubrica* and echinoids, develops in the early spermatocyte stage of *C. pseudocurata*. In this holothurian species the fibrous arm is a transitory structure which is instrumental in the formation of the extensive striated rootlet-like structure observed in the spermatozoon. It is possible that the fibrous arms of *L. clarki* and *C. lubrica* are likewise involved in the morphogenesis of the transitory striated rootlet-like structures noted in the centriolar region of the spermatocytes of these two species.

Tubular and Chromatoid Bodies

Two cytoplasmic organelles not previously reported in echinoderms have now been observed in germinal cells of holothurians. The spermatogonia of *C. lubrica* contain a tubular body which consists of parallel microtubules. This structure has been termed the Crystalloid of Lubarsch (Lubarsch, 1896) and has been observed in human (Nagano, 1969; Rowley et al., 1971) as well as rooster (Nagano, 1969) spermatogonia. The ultimate fate and function of this structure remains obscure. Tubular bodies were never found in *L. clarki* or *C. pseudocurata* germinal cells. In *L. clarki* spermatocytes and *C. pseudocurata* spermatids there occurs a densely staining chromatoid body. The structure is of a honeycomb configuration with a fine granular consistency. The body is frequently surrounded by mitochondria and normally lies closely associated with the nuclear envelope. Similar chromatoid bodies have been reported in various vertebrate and invertebrate germinal cells (Sud, 1961a,b; Fawcett et al., 1970; Comings and Okada, 1972; Fawcett, 1972). Previous instances in invertebrates (Fawcett, 1972) have been limited to the classes Arachnida, Crustacea and Insecta of the phylum Arthropoda. Fawcett (1972) indicates that the chromatoid body is primarily a basic protein which may contain small amounts of RNA at certain times and that there is some experimental evidence that it is essential for germinal cell formation. The initial speculation was that the body functioned as precursor material for the formation of tail components but since its discovery in vertebrate oocytes this theory has been discredited. Its function is presently unknown (Fawcett, 1972).

Fawcett (1972) has suggested that the chromatoid body originates, at least in mammals, as loosely packed dense materials within mitochondrial clusters of spermatocytes. The mitochondria gradually disperse as the dense materials of the chromatoid body aggregate into a single mass. As the cell matures into a spermatid the body forms a close association with the nuclear envelope and migrates round to the base of the flagellum. At this position the body forms a ring around the flagellum adjacent to the annulus and finally disperses leaving no structural remnants in the mature spermatozoon. The fact that the chromatoid body appears to have a parallel in the process of oogenesis (Eddy and Ito, 1971; Mahowald, 1968, 1971) adds to the curiosity concerning this organelle.

Many of the cellular changes observed during spermiogenesis in *C. pseudocurata* are unparalleled in the class as well as in the phylum. Morphogenesis of the extensive striated rootlet and the alteration of cell shape in late spermiogenesis is very similar to that reported during sperm formation in mammalian species. In the spermatocyte stage several structures develop which are presumably for anchorage and/or stability during the developmental stages of the flagellum. These structures either disappear in later stages (basal foot, dense fibers radiating from basal foot), transform into mature structures (dense arm of proximal centriole, filamentous structures extending from distal to proximal centriole) or persist throughout spermiogenesis (satellite materials of distal centriole).

Basically, therefore, spermatogenesis in holothurians is comparable in several aspects with other echinoderm species, with many differences also being evident. As shown from this research on only three species,

great variation also occurs within the class Holothuroidea.

2) Do holothurian spermatozoa conform to the primitive sperm type exhibited by other echinoderm species; and if not, then at which stages during spermatogenesis are modifications introduced and how are these alterations reflected in the spermatozoa?

Franzén (1967a,b, 1970) has noted a definite relation between sperm morphology, spermiogenesis and the biology of propagation. Spermatozoa which are produced by species that retain the primitive mode of sperm discharge directly into the water and fertilize externally have been termed the primitive type. This type of sperm is generally a small cell with a short rounded or conical nucleus, a midpiece consisting from one to a few mitochondria and a long flagellum. The apical end of the sperm houses the acrosome which has the capabilities of undergoing an acrosomal reaction as a consequence of contact with the egg. The tip of the spermatozoon will be the first region of the cell to contact the oocyte at the time of fertilization. "If the spermatozoa are not discharged freely into the water for the fertilization of the eggs but are transferred directly to the receiving female animal by spermatophores or by organs of copulation, the morphology of the spermatozoon is altered in some way or other" (Franzén, 1970). In those species which have sperm with an altered morphology it is possible to follow the earliest and most conspicuous steps in the spermatozoon's modification by carefully studying the process of spermiogenesis.

Morphologically, the spermatozoon of *L. clarki* is of the primitive type and the events noted during spermatogenesis are typical of the process in those organisms that produce primitive sperm. The sperm of

C. lubrica deviates from the primitive type in that the nucleus is elongate and the mitochondrial elements are rearranged along the posterior portion of the nucleus rather than positioned between the nucleus and flagellum. Nuclear elongation and mitochondrial rearrangement first becomes evident in the intermediate spermatid stage. A tapering of the posterior 1.6 μ of the nucleus accompanies the re-positioning of the mitochondrion.

The spermatozoon of *C. pseudocurata* deviates from the structural pattern observed in the primitive sperm in that it is elongated and dorsal-ventrally compressed, contains an extensive striated rootlet-like structure in the midpiece, has an enlargement and rearrangement of mitochondrial elements of the midpiece, and contains an additional fiber in the flagellum. The stage where it becomes evident that the spermatogenic process of *C. pseudocurata* is deviating from the characteristic pattern observed in formation of the primitive sperm, is in the early spermatocyte. At this point the formative materials for the striated rootlet are being deposited in the regions of the proximal and distal centrioles. Basic cell shape first becomes altered in the intermediate spermatid stage where there is a corresponding compression and elongation of the nucleus. Compression occurs in the dorso-ventral plane, whereas elongation is along the anterior-posterior axis. In the late spermatid there is a gradual shift of remaining cytoplasm to the posterior-dorsal region of the cell. This shift in cytoplasm is accompanied by an increase in nuclear compression and elongation. The dorsal groove which houses the striated rootlet originates on the dorsomedial surface of the nucleus in the intermediate spermatid and elongates posteriorly as the cell

matures. Through this cytoplasmic shifting process the striated rootlet comes to lie parallel with and tightly situated within the groove. In the late spermatid stage the small ovoid mitochondria transform into a single large mitochondrion. Mitochondrial elements surround the centrioles and the posterior region of the striated rootlet as well as extending through the dorsal rootlet groove on the dorsal sperm surface. Mitochondrial elements extend for approximately two-thirds of the length of the groove and terminate posterior to the acrosomal region.

An interesting structure develops during the spermatogenic process in *C. pseudocurata* and completely degenerates by the time the cell reaches maturity. The structure is a basal foot-like projection which extends laterally from the wall of the distal centriole. Formation of the basal foot first becomes evident in the early spermatocyte stage. In the late spermatocyte the basal foot reaches maturity and consists of a dense cap separated from a stalk by a less osmiophilic space. The stalk is separated by another less osmiophilic space from the two dense connecting regions attached to the centriolar wall. Fibers radiate from the cap region of the foot and exist in a tight zone between the foot and the proximal centriole. In the early spermatid stage the basal foot and radiating fibers have totally degenerated. It has been postulated in other cells that the basal foot is a possible mechanosensitive structure instrumental in the control of directional movement of cilia (Thurm, 1968; Flock, 1971). Ciliated cells which contain basal feet show a fixed directional ciliary beat which is related to the orientation of the feet. The questions that arise are why should a developing sperm cell be equipped with such a structure and for what reason does it

degenerate? There are at least three possibilities: 1) the basal foot is strictly an anchorage/stability structure only necessary during the formative stages of the flagellum and striated rootlet, 2) the foot is strictly a mechanosensitive device important in the initial movements of the flagellum, or 3) the foot exhibits a combination of the above two functions.

3) Does spermatozoan morphology differ significantly within the holothurians; and if so, is it possible to correlate changes in spermatozoan morphology with an altered biology of propagation or an alternative factor such as egg investments?

Comparative Aspects of Holothurian Spermatozoan Structure

Basic sperm morphology (based on light and scanning electron microscopy) has been described for at least 20 species of holothurians: *Anapta gracili* , *Chimofota incongna*, *Cucumaria cucumis*, *Cucumaria elongata*, *Cucumaria frondosa*, *Cucumaria miniata*, *Cucumaria piperata*, *Cucumaria planci*, *Eupentacta pseudoquinquesemita*, *Holothuria atra*, *Holothuria poli*, *Holothuria tubulosa*, *Holothurua edulis*, *Mesothuria intestinalis*, *Molpadia oolitica*, *Parastichopus californicus*, *Psolus chitonoides*, *Synapta inhaerens*, *Thyone briareus*, *Trochostoma thompson*. The sperm of these species are reviewed by Chia et al. (in press).

All of these spermatozoa, without exception, contain a relatively small spherical-shaped head measuring 2 to 4 μ in diameter (Chia et al., in press) positioned anteriorly to a small midpiece connected to a relatively long flagellum. The present study has demonstrated that two additional sperm shapes, cylindrical *(Cucumaria lubrica)* and tabloid

(Cucumaria pseudocurata), exist in the class.

The significant structural differences among the three spermatozoa examined in the present study occur in the shape of the head or nuclear region, substructure and location of the acrosome, components of the midpiece and number of tubules in the flagellum.

Nuclear region. The nucleus of the *L. clarki* sperm is irregularly circular in shape and measures approximately 2.9 μ in width. The anterior-posterior axis is shorter due to the anterior acrosomal depression and posterior centriolar fossa. The *C. lubrica* nucleus is elongated measuring about 6.8 μ in length and 1.4 μ at its greatest diameter. The posterior 1.6 μ of the nucleus is tapered to a diameter of 0.5 μ. The nucleus is indented anteriorly by an acrosomal depression and posteriorly by a centriolar fossa. In *C. pseudocurata* the nucleus is dorso-ventrally compressed measuring approximately 5.5 μ in length, 1.2 μ in width and 0.8 μ in depth. An extensive acrosomal depression is noted on the ventral surface. The indentation is medially located along the anterior-posterior axis of the spermatozoon and extends almost completely through the depth of the nucleus. The nucleus is indented posteriorly along the dorsal surface by the striated rootlet groove. The nucleus is tapered at the anterior and posterior ends and reaches its maximum dimensions slightly posterior to the acrosomal depression.

Acrosomal region. In *L. clarki* the acrosomal granule is relatively circular measuring 0.7 μ in diameter and is housed in a nuclear acrosomal depression. The granule is membrane-bound and consists of electron-dense material which in the central region is arranged in concentric lamellae. Five such very osmiophilic areas alternate with areas of much less

electron-dense material. Two osmiophilic bands occasionally occur in the posterior region of the granule. The acrosomal granule in *C. lubrica* measures about 0.6 μ in width and 0.5 μ in length and in the majority of sections is membrane-bound. The limiting membrane is frequently thrown into a number of small folds at the apex of the granule, giving a serrated effect to the anterior end of the acrosome. Granule contents are of a homogeneous nature except for a dense osmiophilic sphere displaced toward the anterior surface. As in *L. clarki*, two osmiophilic, cup-shaped bands occur in the posterior region of the granule and have been termed primary membrane precursors which are involved in the asteroid acrosomal reaction (Dan and Hagiwara, 1967).

In *C. pseudocurata* the acrosomal granule is composed of a dense homogeneous particulate material surrounded by a limiting membrane. The granule is irregular in shape with the width (anterior-posterior axis) measuring 730 mμ and the depth (dorsal-ventral axis) being 425 mμ. The anterior-posterior surfaces flare out forming pockets in the surrounding periacrosomal material. The external (ventral) granule surface bulges out to form a close association with the cell membrane and the internal (dorsal) surface is indented forming an inward depression. Ventral to this depression, within the granule, is a small area containing particulate-fibrous material of a greater osmiophilic density than the surrounding material. To the inside of the granule limiting membrane there is an incomplete membrane-like structure which extends from the anterior-posterior surfaces around the dorsal face. This structure can possibly be equated to the cup-shaped bands noted in the posterior granule region in *L. clarki* and *C. lubrica*.

Completely surrounding the acrosomal granule in the sperm of all three species is a periacrosomal layer of basically homogeneous reticular material less electron-dense than the granule. In *L. clarki* an ill-defined area containing a small amount of fibrous material is located posterior to the granule within the periacrosomal layer. This area forms no obvious subdepression and is infrequently observed. A similar fibrous area is noted in *C. lubrica*. In *C. pseudocurata*, areas of the periacrosomal layer ventral to the pockets (sandwiched between the nuclear envelope and the granule membrane) are more dense and granular than the remaining periacrosomal material. These areas could be correlated with the material composing the fibrous plate precursor of asteroid acrosomes (Dan and Hagiwara, 1967). Dorsomedial to the granule is a distinct particulate-fibrous region lodged within the granule depression. This region corresponds to the periacrosomal fibrous zones noted in *L. clarki* and *C. lubrica* and is presumed to be the precursor of the acrosomal filament.

Midpiece region. The midpiece regions of the *L. clarki* and *C. lubrica* spermatozoa are very similar except for the arrangement of the mitochondrial elements. The major difference is that in *L. clarki* the entire nucleus lies anterior to the mitochondrion whereas, in *C. lubrica*, the tapered posterior 1.6 μ of the nucleus is surrounded by the mitochondrion. Both species contain typical proximal and distal centrioles oriented perpendicular to one another lodged within the mitochondrial mass. A dense fibrous arm projects from the proximal centriole into the centriolar fossa in both species. The distal centriole, which is connected to a series of satellite projections and y-shaped membrane

doublet connectives, gives rise to a typical 9+2 flagellum. The satellite of the distal centriole consists of nine radiating fibers each of which branches into two secondary fibers and in turn branch into fine tertiary fibers which form a network with adjacent tertiary fibers. This network, with which microtubules are closely associated, is very extensive and lies in close proximity to mitochondrial elements.

The midpiece of the *C. pseudocurata* spermatozoon structurally is more complex than that of the other two species investigated. The mitochondrial mass lies immediately posterior to the nucleus and surrounds the proximal and distal centrioles, satellite projections and much of the striated rootlet. The greatest mitochondrial mass occurs on the dorsal side of the sperm where it lies within the rootlet groove and extends for about two-thirds of its length. The centrioles are situated posterior to the nucleus, perpendicular to one another with the distal centriole giving rise to a flagellum. The distal centriole contains the typical nine rows of three tubules, whereas the proximal centriole appears to consist of nine rows of five tubules. Extending anteriorly from the distal centriole to just below the acrosomal region is a striated rootlet. The rootlet originates at the proximal surface of the distal centriole and encompasses the proximal centriole as it projects anteriorly toward the sperm apex. The structure and location of the rootlet suggests that it may function as an anchoring and/or stabilizing device for the distal centriole and flagellum. In *C. pseudocurata* both the proximal and distal centrioles contain satellite projections. The satellite materials of the proximal centriole assume various shapes ranging from slender extensions protruding from the triplets to large dumbbell-

shaped spheres connected to the triplets by thin bridges. The satellite material of the distal centriole, which is a continuation from the proximal centriole, is arranged in more of an ordered manner and is similar in configuration to that observed in *L. clarki* and *C. lubrica*.

Flagellar region. In *L. clarki* and *C. lubrica* the tail flagellum contains the typical 9+2 microtubule arrangement. In *C. pseudocurata* a third central tubule exists in the midtail region between and slightly peripheral to the central pair, thus giving a 9+3 pattern to the flagellum. This third tubule is normally in direct contact with the central pair but is separated by a small space from one or the other in many sections. The 9+3 pattern was never observed in the basal or tip regions of the tail, indicating that the third tubule originates and terminates along the midsection of the tail.

Great variation is noted throughout the animal kingdom in the microtubule pattern of the motile sperm flagellum. The arrangement is a 9+9+0 in mayflies, 9+0 in *Vejovis* and *Hadurus* (scorpions), 9+9+2 in the majority of insects and mammals, 9+9+1 in mosquito species, 9+1 in many flatworms and *Centrorides* (scorpion), and 9+7 in two species of caddis flies (Phillips, 1970, 1974). A 9+3 arrangement has been observed in a fungus gnat and several species of spiders (Phillips, 1970, 1974). In *Pisaurina* (spider) the three central tubules originate at the center base of the distal centriole and run throughout the length of the tail (Reger, 1970). The 9+3 pattern of *C. pseudocurata* differs from the above species in that the additional central tubule originates and terminates in the midsection of the flagellum.

Correlation of Sperm Structure with Biology of Propagation

Franzén (1967a, 1970) and Afzelius (1972) have discussed in length the relationships that exist between spermatozoan morphology and the biology of fertilization. It has been shown that the majority of marine invertebrate species investigated conform to the hypothesis that modified sperm structure can be correlated to a modification in the process of propagation; namely, sperm transport from the male to the egg of the female (packaging of sperm and movement of sperm through the female reproductive tract). An alternative factor that may be considered as a feasible cause for sperm modification is egg investments. It is logical that species with eggs containing complex and specialized investment layers would likewise require a modified spermatozoon for penetration and ultimately fertilization.

Four main factors, therefore, can be considered as possible causes for structural modifications in mature sperm: 1) packaging of sperm for release from the male and transport to the egg (Afzelius, 1972), 2) movement of sperm through media of varied viscosities (Afzelius, 1972), 3) movement of sperm through the female reproductive tract (Afzelius, 1972), and 4) adaptation to a particular egg envelope (present study).

Leptosynapta clarki. *L. clarki* is an internal (ovarian brooder with internal fertilization, which produces a sperm typical of the primitive type. During the spawning season, small groups of individuals lie in close contact with their anterior ends lying in cylindrical depressions in the sand substratum. These sand pockets contain sea water and could act as closed reservoirs in which spermatozoa could move from males to females (Everingham, 1961). The manner of sperm entry into the female

is unknown. Everingham (1961) suggests that the sperm might enter the ovarian lumen through the gonopore and fertilize the eggs or they might enter into the coelomic cavity through the rectum or small perforations in the body wall.

According to Afzelius (1972) several other organisms exhibit internal fertilization and produce a primitive type sperm: *Tealia felina* (sea anemone), *Alcyonium* (coral), *Unio*, *Anodonta*, *Sphaerium*, *Teredo* (mussels) and *Polycarpa* (tunicate). Afzelius proposes that in these species, the internal fertilization habit is not extensively different from that of external fertilization, in that large numbers of sperm are produced and released into the water, which are then drawn into the female by an inhalant current. Water currents are constantly circulating through these animals, presenting an open system directly connected with the external sea water environment. In *L. clarki* the internal fertilization habit *is* different from that of external fertilization in that water currents are not constantly circulated, thus presenting a closed system with respect to the surrounding sea water. Also, in *L. clarki*, the spermatozoa must move through the female reproductive system prior to fertilization, whether sperm entry is through the gonopore or rectum.

L. clarki, therefore, is a typical internal fertilizer with a female tract that sperm must travel through prior to fertilization, which produces a primitive type spermatozoon typical of an external fertilizer. Thus, Franzén's hypothesis on sperm structure is not adequate to explain sperm structure in this particular species.

Cucumaria lubrica. *C. lubrica* is an external ventral surface brooder with external fertilization which produces a sperm that deviates

from the primitive type in that the nucleus is elongate and the mitochondrial elements are rearranged around the posterior region of the nucleus. This species occurs in swift water currents in great abundance ($5000/m^2$) on subtidal rocks. It has been noted in the laboratory that following spawning, the sperm remain together in bundles for an extended length of time before they are suspended. The elongated sperm head may reflect a specific adaptation for the convenience of packaging of sperm bundles. Spermatophore-like structures containing elongate spermatozoa are formed in many organisms: *Littorina* (Prosobranchia) (Buckland-Nicks, 1973), *Hadrurus* (Scorpion) (Jespersen and Hartwick, 1973), *Octopus* (Cephalopod) (Franzén, 1967b; Longo and Anderson, 1970), and *Sibolinum* (Pogonophora) (Franzén, 1973).

Many organisms (such as mammals and gastropods) with internal fertilization produce modified sperm in which the mitochondrial elements extend down the sides of the flagellum. In *C. lubrica* as well as in some turbellarians (Silveira and Porter, 1964), ostracods (Reger and Florendo, 1969) and Nemertines (Afzelius, 1971) the mitochondrial elements spread forward along the nucleus. It is well known that the presence of mitochondria in sperm is related to the aerobic metabolism of the cell. The ATP formed by oxidative phosphorylation is transformed into mechanical energy which permits the spermatozoon to move and penetrate the egg. In the primitive sperm, which are released externally into a low viscosity medium and are normally short-lived, the mitochondrial elements are concentrated in a short midpiece in close association with the centriolar complex and the axoneme. On the other hand, in species with internal fertilization, where the surrounding medium is of a higher viscosity and

sperm life is longer, the midpiece becomes elongated with an increase in the ratio of the volume of the mitochondrion to the volume of remaining regions of the cell. It has been proposed that an increase in mitochondrial mass (Favard and André, 1970) is a modification which has been introduced to cope with a more viscous medium in which the sperm must swim to reach and to fertilize the egg.

As Afzelius (1971) has pointed out, an elongated shape may be a reflection of other factors in the biology of propagation such as the dimension and configuration of the female reproductive tract. *C. lubrica* fertilizes externally, thus eliminating possible modifications to deal with a specialized female tract.

C. lubrica, therefore, is an external fertilizer that produces a modified sperm. It can be argued that the elongated head of the sperm is a modification for packaging but this does not explain the rearrangement of the mitochondrial elements in the midpiece. Since the sperm is modified but fertilizes externally in a medium of a low viscosity and does not travel through a female reproductive tract prior to fertilization, it is obvious that Franzén's hypothesis does not adequately apply to this species.

Cucumaria pseudocurata. *C. pseudocurata* is an external ventral surface brooder with external fertilization which produces a sperm that deviates greatly from the primitive type. This species occurs in great abundance within mussel beds that are attached to intertidal rock surfaces. Massive byssal threads attach the mussels to the substratum and provide additional protection for the cucumbers.

The modified spermatozoon of *C. pseudocurata* has developed many of

the adaptations noted in sperm specialized for internal fertilization: 1) elongate, compressed head, 2) enlargement and rearrangement of mitochondrial elements, 3) striated structure in midpiece, and 4) additional fiber in flagellum.

Since the presence of a globular primitive type head becomes a limiting factor as the viscosity of the medium increases, the internally fertilizing sperm head is usually elongated and/or dorso-ventrally flattened with a filamentous, ovate, falciform or ensiform shape. Structural adaptations in the motor apparatus to deal with this increased viscosity can include an enlargement of the midpiece, formation of a striated midpiece structure, and the addition of fibers in the flagellum (gastropods, cephalopods, cyclostomes, higher vertebrates).

The rootlet-like structure in the *C. pseudocurata* sperm can be compared to the cross-striated elements of the connecting piece in the neck region of internal fertilizing sperm (Fawcett and Phillips, 1969). In mammalian species, the striated columns originate mainly from the polymerization of filamentous material located in interstices in the wall of the distal centriole. This interstitial accumulation of material eventually results in total disruption of the distal centriole in the mature sperm (Fawcett, 1972). These cross-striated elements become continuous with the outer fibers of the flagellum and continue anteriorly past the proximal centriole. Morphogenesis of the cross-striated elements in the *C. pseudocurata* sperm appears to represent a modification of the process observed in mammals, based on the following observations: (1) the distal centriole is intact in the mature sperm, (2) striated elements do not appear to be continuous with flagellar components,

(3) the elements appear to originate at the anterior surface rather than the sides of the distal centriole and (4) the proximal centriole (rather than the distal) appears to be more intimately involved with the cross-striated elements. The structure and location of the rootlet in *C. pseudocurata* suggest that it may function as an anchoring and/or stabilizing device for the distal centriole and flagellum. The presence of striated rootlets in cells with non-motile cilia and on non-ciliated centrioles implies that dissipation of force is not necessarily their only role (Pitelka, 1974).

C. pseudocurata broods its young externally between the ventral body surface and the substratum. The sperm are tightly packaged head to tail and released in dense strands of mucus. Since embryos as young as the two-cell stage have been observed being brooded externally (Chia, personal communication) it is presumed that external fertilization occurs. The question that arises is for what reasons have these structural modifications evolved in a spermatozoon that fertilizes externally? The female *C. pseudocurata* could possibly extrude unfertilized eggs into the sea, transfer them to the ventral surface with the use of tentacles and arrange with podia in superficial surface folds. The brooded egg masses are extremely sticky and surrounded by large quantities of mucus (field observations) similar to that described for *Cucumaria curata* (Smith, 1962). The mucous secretions cover the entire body and evidently aid in the attachment of the eggs. The male being in close proximity to the female could spawn packaged sperm which would be carried into the mucus of the egg mass by water currents and fertilize the eggs externally. Structural modifications of the spermatozoon, therefore, possibly could

be correlated with the convenience of packaging as well as locomotion through a medium of high viscosity.

C. pseudocurata, therefore, is an external fertilizer that produces a modified sperm. It can be argued that the elongated head is a modification for packaging. The compressed sperm head, striated rootlet, rearrangement and enlargement of the mitochondrion, and the additional tail fiber could possibly be explained by sperm movement through the mucous coat (presumably of a high viscosity) surrounding the eggs. Since the actual viscosity of this medium is not known and since the ventral brood surface is not a closed system with respect to the surrounding sea water, it must be assumed that this modified holothurian sperm, as with *L. clarki* and *C. lubrica*, cannot be adequately explained by Franzén's hypothesis.

Correlation of Sperm Structure with Egg Investments

The egg investments in *L. clarki* and *C. lubrica* resemble each other in that both contain a follicular cell layer, dense laminate fibrous layer, dense particulate layer and microvilli of the plasmalemma. They differ in that *L. clarki* has an outer particulate-fibrous layer and an inner lucent particulate layer lying adjacent to the plasmalemma. The external surface of the *C. pseudocurata* egg is structurally dissimilar to the other two species in that the follicular cell layer is absent, the laminate layer is very extensive and contains no fibrous component, the dense particulate layer is absent, and microvilli of the plasmalemma are absent. In fact, the outer envelope in this species is a single extensive layer.

The physiological significance of these dissimilarities is not

known but it is shown in these species that three different sperm shapes are present as well as three different egg envelopes and that sperm structure (primitive vs. modified) is not correlated with sperm movement from the male to the egg. It is suggested, therefore, that sperm structure in the class Holothuroidea and possibly in other animal groups may be correlated with the structure of egg investment layers.

The *C. pseudocurata* spermatozoon undergoes an acrosomal reaction upon contact with the egg which does not involve the formation and extension of a typical acrosomal process. The sperm attach to the egg surface in a side rather than the typical head-on position. It appears as though the plasma membrane overlying the acrosome ruptures allowing a lytic agent to be released onto the surface of the oocyte. The dissolution of the egg investments is especially noticeable around the sperm body but is also evident along the entire length of the flagellum.

It is intriguing that the spermatozoon of *C. pseudocurata* contains an acrosome that structurally conforms to the typical echinoderm (and most marine invertebrate) acrosome but functionally deviates to a great extent. If in fact, the scanning electron microscope observations have revealed the actual events of the acrosomal reaction in this species, it will be necessary for the definition of an "invertebrate type" acrosome to be reconsidered. The majority of substructures in the *C. pseudocurata* acrosome can be equated to similar substructures in acrosomes of many other organisms, especially echinoids and asteroids. It is interesting that organelles having very similar configurations could function in such different manners.

The eggs of most organisms are surrounded by a varying number of

investment layers which differ considerably in thickness and composition. Spermatozoa must penetrate these investments in order to fertilize the egg. In some species (insects, fish) the sperm are aided in this process by the presence of a micropyle leading directly into the oolemma. In other animals it is necessary for the sperm to either mechanically (echinoids) or chemically (annelids, hemichordates, mammals) penetrate these surrounding layers. Whichever is the case, the spermatozoa are equipped for the event of penetration with the presence of a specialized acrosomal region, which is usually at the anterior tip. The advancing sperm besides dealing with a penetration barrier must also deal with a species-recognition barrier. It is theorized that species recognition (at least in echinoids) is handled by the acrosomal region at the tip of the sperm. Aketa (1973) has postulated that a species-specific component is present on the apical end of the echinoid sperm which is complementary to a sperm-binding protein of the egg surface. These two molecules are possibly responsible for both initial species recognition and bonding of gametes. Summers and Hylander (1974) correspondingly suggest that such specific sperm molecules are contained within the acrosomal granule and are made available to the egg surface following the acrosomal reaction and that these molecules form a structural bond with the vitelline envelope prior to membrane fusion.

Since present observations indicate that no acrosomal process is formed in *C. pseudocurata*, species recognition by the egg surface must be mediated through another structural region on the sperm. Conceivably, the egg-binding molecule is localized in the acrosomal region and is discharged over the entire surface of the sperm at the time of sperm-egg

contact. If this is the case, then the entire sperm surface rather than just the apical tip is responsible for species recognition. The mode of initial membrane contact and subsequent fusion is at the present unknown.

> Modern biologists sometimes adopt a lofty attitude towards morphologists, making unfavorable comparisons between their work and that of biochemists and biophysicists. I hope that this article will help to dispose of the idea that morphology is a dull subject. It is as exciting today as in the seventeenth century, when Leeuwenhoek was studying the morphology of his spermatic animalcules (Rothschild, 1956).

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Appendix

TECHNIQUE FOR PROCESSING SPERMATOZOA FOR SCANNING ELECTRON MICROSCOPY

TECHNIQUE FOR PROCESSING SPERMATOZOA FOR SCANNING ELECTRON MICROSCOPY

Introduction

Scanning electron microscopy has recently been shown to be instrumental in the field of spermatology in determining basic cellular form and shape of surface structures (Brown and Humphreys, 1971; Lung and Bahr, 1972; Baccetti and Burrini, 1973; Buckland-Nicks, 1973; Atwood and Chia, 1974). A technical problem arises in those marine invertebrate species that produce sperm packaged in dense strands of mucus. This mucous covering obscures surface details and enhances clumping when specimens are processed by usual SEM methods. Additional problems encountered when processing spermatozoa with previous techniques are tail breakage (due to mechanical damage incurred during processing and air drying techniques), distortion of surface details (due to air and freeze drying techniques), cellular compression (due to air drying techniques) and loss of sperm (due to processing). This paper introduces a technique for the removal of surface contamination. It also describes a method which facilitates the processing of sperm for SEM, reducing mechanical damage and allowing the material to be dried by the critical point method.

Materials and Methods

Mature males of *Orthasterias koehleri* (Echinodermata: Asteroidea) and *Littorina sitkana* (Mollusca: Gastropoda) were collected in June, 1974 at Friday Harbor Laboratories, Washington. Sperm samples were

obtained either by injection of 2 ml of 1-methyl adenine or by dissection of the gonads. Dry sperm samples were pipetted directly into the following solutions at 10°C for 1 to 2 hr in quantities sufficient to give a milky suspension:

(A) Natural sea water

(B) Natural sea water containing 1500 NF units/ml of ovine hyaluronidase (Sigma Chemical Co.)

(C) Millipored natural sea water containing hyaluronidase (above) (shown to depolymerize protein-polysaccharides)

Material was then processed in the following manner:

(1) Ten ml aliquots were pipetted into 12 ml conical centrifuge tubes and centrifuged for 3 to 4 min at full speed in a clinical centrifuge.

(2) Supernatants were removed and sperm samples (in the form of small pellets) gently re-suspended in a 10 ml wash of millipored sea water for 5 min. Samples were washed two additional times in this manner.

(3) Sperm were fixed (in centrifuge tubes) for 15 min at room temperature in a 2.5% glutaraldehyde solution buffered to pH 7.6 with 0.34 M sodium chloride and 0.4 M Millonig's phosphate buffer.

(4) The sperm were pelleted by centrifugation, washed in 2.5% sodium bicarbonate buffer (pH 7.4), re-pelleted and pipetted into Teflon "Flo-Thru Specimen Capsules" (Sargent-Welch Scientific Co.) containing Nuclepore membranes 25 mm in diameter with a 1.0 μ pore size (Sargent-Welch Scientific Co.).

(5) Specimens were post-fixed in 2% osmium tetroxide in 2.5% sodium bicarbonate buffer for 15 min at room temperature, dehydrated in ascending concentrations of ethanol and passed through ascending concentrations of amyl acetate to 100%.

(6) After critical point drying with carbon dioxide the capsules were opened and samples on Nuclepore filters from each capsule were mounted on stubs for examination. Sperm were also shaken onto stubs covered with double sided tape. The material was then coated with carbon then gold to a total thickness of 7.5 to 12.5 nm and examined with a Cambridge Stereo-Scan S-4 scanning electron microscope.

Observations

Freshly spawned sperm of *Orthasterias* and *Littorina* are heavily coated with a mucous layer which greatly enhances clumping and completely obscures surface details (Figs. 1 and 9). Initial washing with hyaluronidase in natural sea water (1500 NF units/ml) reduces clumping and surface contamination; this, however, leaves behind small contaminating spherules (0.2 to 1.8 μ) adhered to the specimens and Nuclepore membrane (Fig. 2). Superb results were obtained when sperm were initially washed with 1500 NF units/ml hyaluronidase in millipored sea water. The capsules, which are resistant to processing chemicals, allow the sperm to be transferred through the remaining solutions without fear of excessive mechanical damage and simplify the process of critical point drying. The Nuclepore membranes allow efficient chemical filtration and ensure

against loss of materials.

Spermatozoa processed with this technique are not excessively clumped (Fig. 3) and appear amazingly clean (Figs. 4, 5, 6, 7, 8 and 10). Sperm are well preserved and allow three dimensional observations of major surface structures (Fig. 4). The most acceptable sperm were the ones adhering to the membranes rather than those occurring free within the bags. The main regions of the *Orthasterias* sperm: acrosomal (Figs. 5 and 6), nuclear (Figs. 5, 6 and 7), mitochondrial (Figs. 5, 6 and 7), centriolar satellite complex (Figs. 7 and 8) and flagellar (Figs. 5, 6, 7 and 8) are easily distinguishable.

Substructure of the centriolar satellite complex (Summers, 1972; Atwood, 1974; Atwood and Chia, 1974; Fontaine and Lambert, unpublished manuscript) is for the first time shown from a surface view (Fig. 7). The primary and secondary satellite projections as well as the inner ring of dense thickenings and outer marginal ring are quite evident in Fig. 8.

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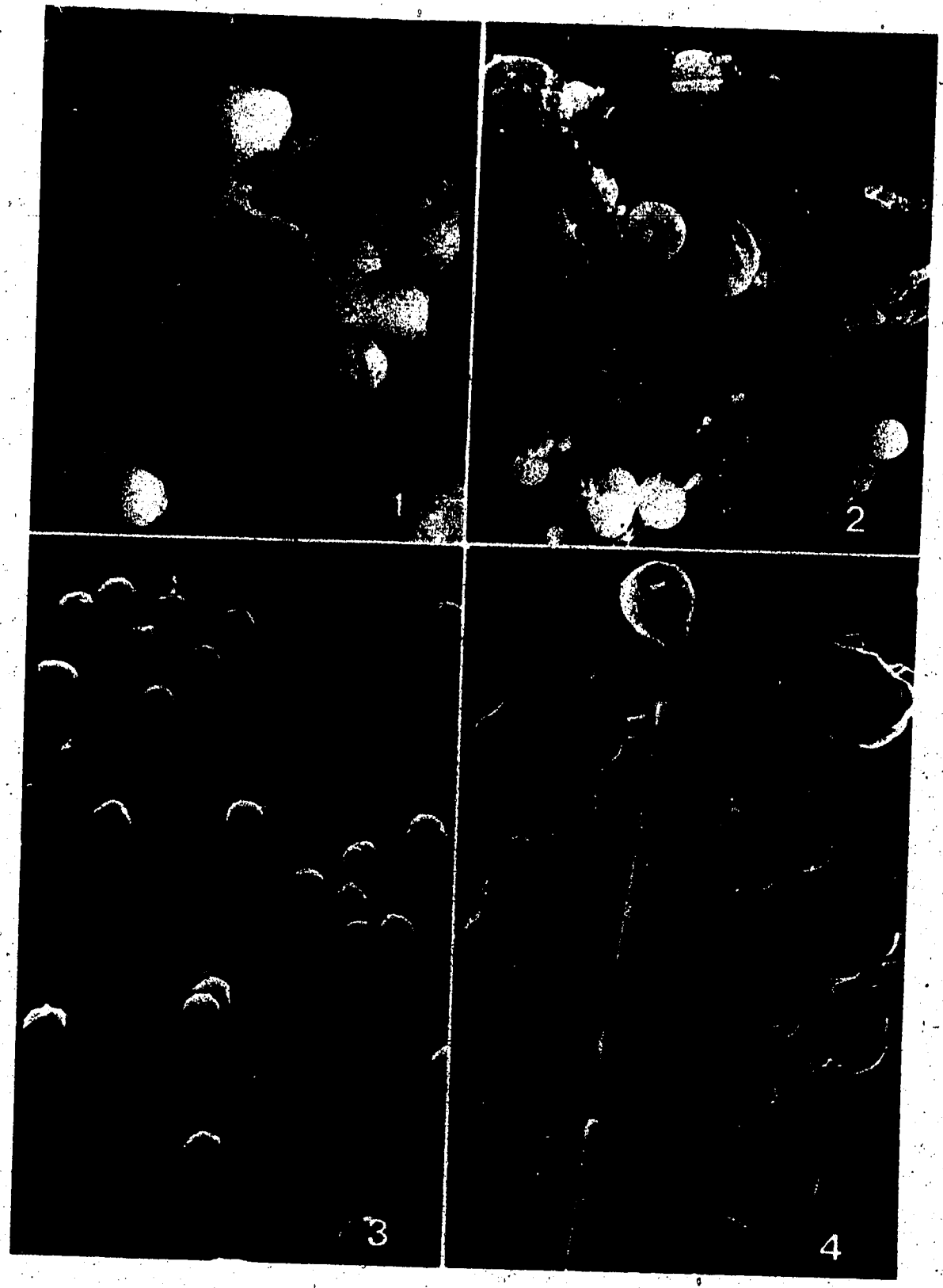
PLATE 1

Figure 1. SEM micrograph of clumped *Orthasterias* spermatozoa. Natural sea water control, X 4,500.

Figure 2. SEM micrograph of *Orthasterias* spermatozoa. Initial wash in 1500 NF units/ml hyaluronidase in natural sea water, X 8,500.

Figure 3. Micrograph of *Orthasterias* sperm. Initial wash in 1500 NF units/ml hyaluronidase in millipored sea water, secondary wash in millipored sea water, fixed in glutaraldehyde, rinsed with sodium bicarbonate buffer, pipetted into specimen capsules and post-fixed in osmium tetroxide in sodium bicarbonate buffer, X 3,900.

Figure 4. Micrograph of an *Orthasterias* spermatozoon. Same procedure as in figure 3, X 7,000.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3
4

PLATE 2

Figure 5—8. *Orthasterias* spermatozoa. Same procedure as in figure 3.

A, Acrosomal region
C, Centriolar satellite complex;
F, Flagellum
I, Inner satellite ring
M, Mitochondrial region
N, Nuclear region
O, Outer satellite ring
P, Primary satellite projections
S, Secondary satellite projections

Figures 5 and 6, X 35,000; figure 7, X 39,000; figure 8 X 70,000.

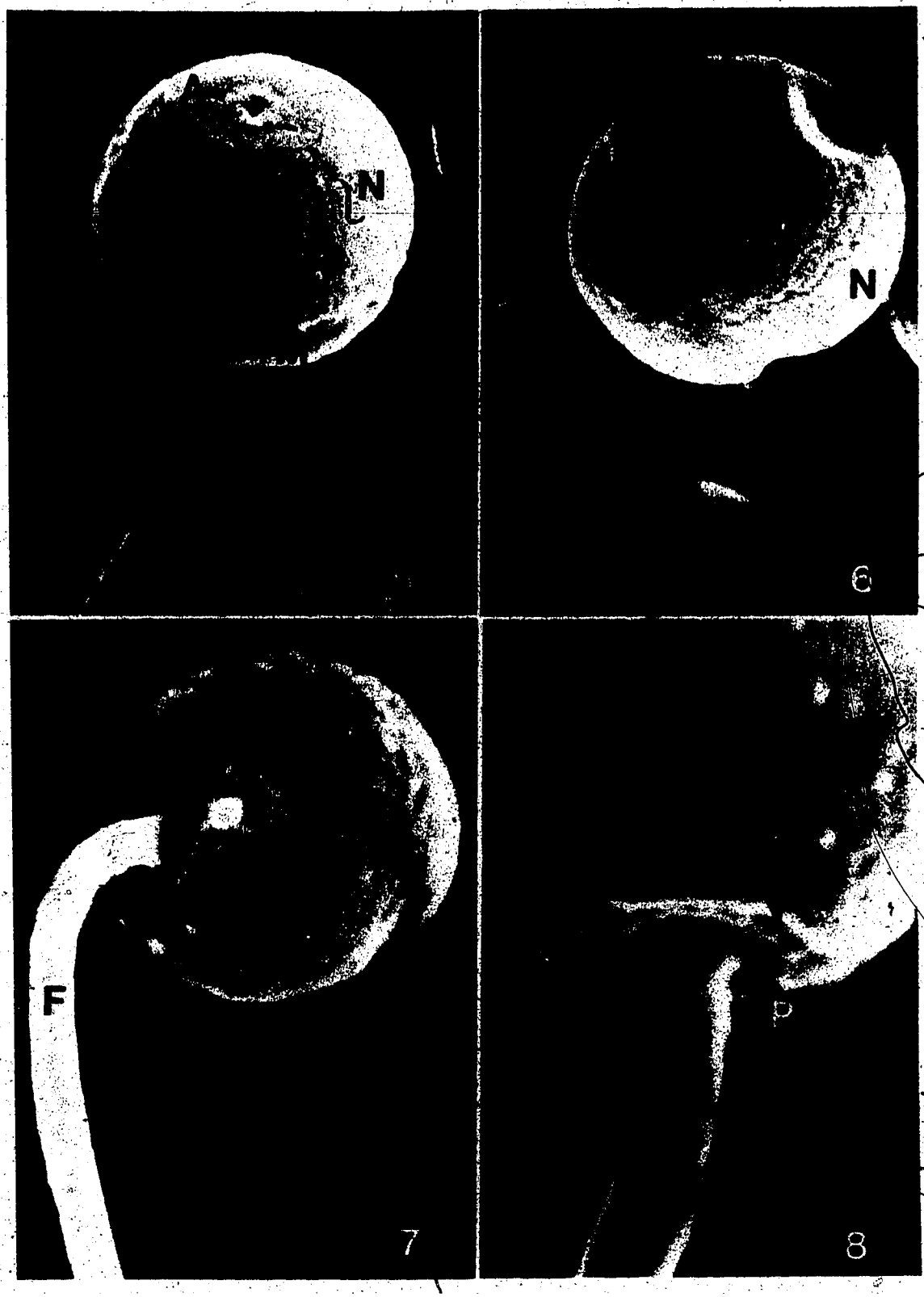
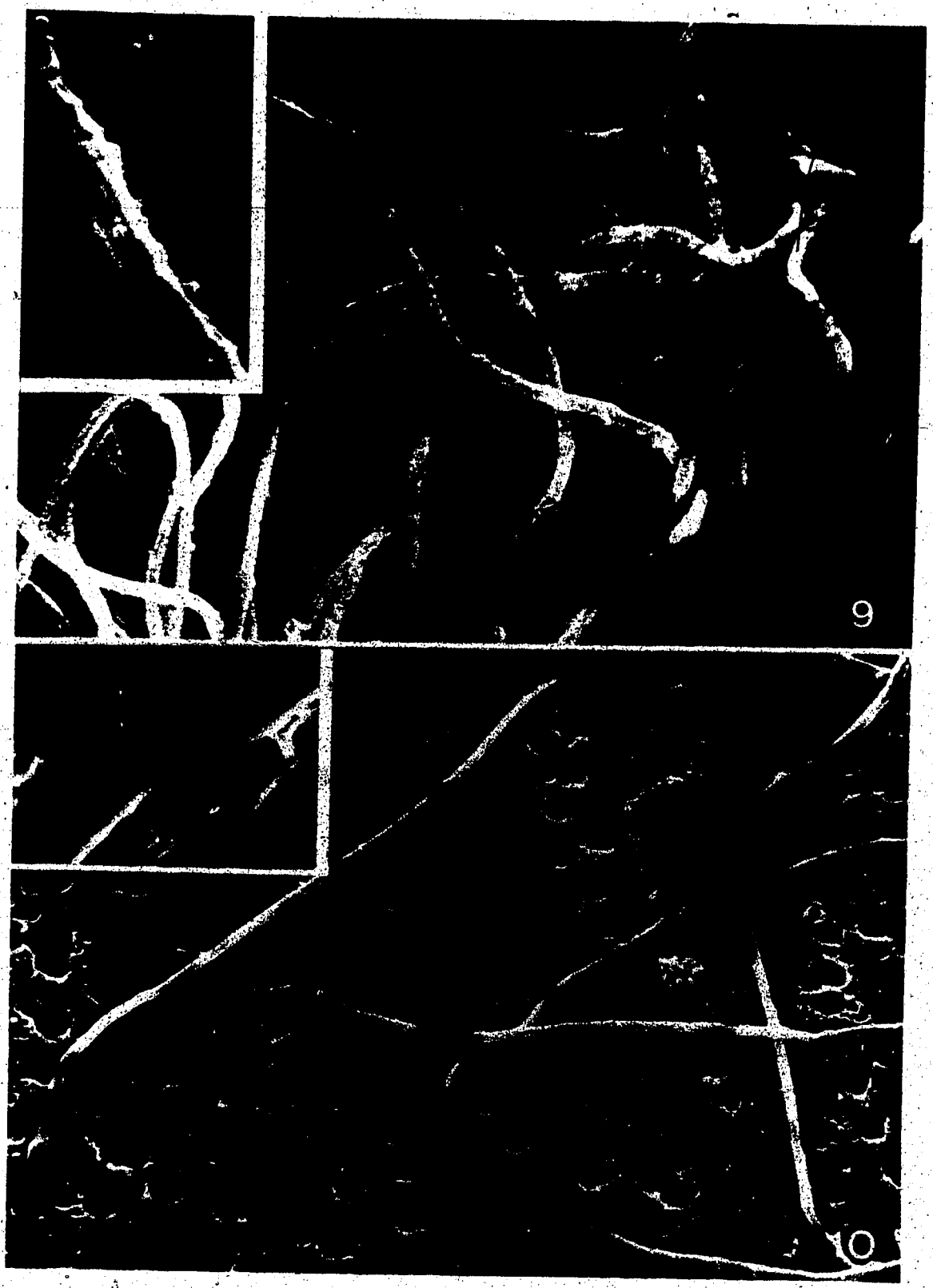

N
N
F
P
6
7
8

PLATE 3

Figure 9. SEM micrograph of clumped *Littorina* spermatozoa. Natural sea water control, X 5,000. Inset, X 21,000 (courtesy Dr. J. Buckland-Nicks).

Figure 10. SEM micrograph of *Littorina* spermatozoa. Same procedure as in figure 3, X 3,000. Inset, X 14,000.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