THE FINE STRUCTURE OF PRONUCLEAR DEVELOPMENT AND FUSION IN THE SEA URCHIN, *ARBACIA PUNCTULATA*

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ABSTRACT

Fertilization events following coalescence of the gamete plasma membranes and culminating in the formation of the zygote nucleus were investigated by light and electron microscopy in the sea urchin, *Arbacia punctulata*. Shortly after the spermatozoon passes through the fertilization cone, it rotates approximately 180° and comes to rest lateral to its point of entrance. Concomitantly, the nonperforated nuclear envelope of the sperm nucleus undergoes degeneration followed by dispersal of the sperm chromatin and development of the pronuclear envelope. During this reorganization of the sperm nucleus, the sperm aster is formed. The latter is composed of ooplasmic lamellar structures and fasciles of microtubules. The male pronucleus, sperm mitochondrion, and flagellum accompany the sperm aster during its migration. As the pronuclei encounter one another, the surface of the female pronucleus proximal to the advancing male pronucleus becomes highly convoluted. Subsequently, the formation of the zygote nucleus commences with the fusion of the outer and the inner membranes of the pronuclear envelopes, thereby producing a small internuclear bridge and one continuous, perforated zygote nuclear envelope.

INTRODUCTION

Much of what we know concerning the events of fertilization leading up to and associated with the formation of the zygote nucleus comes from studies made by a host of distinguished investigators utilizing the light microscope (6, 17–19, 30, 56, 84, 85). However, relatively few electron microscope studies dealing with various aspects of pronuclear development and fusion have been published. A list of these would include the following: formation of the male pronuclear envelope in the lamellibranch, *Barnea* (60, 61); sperm penetration in the echinoderms *Arbacia* and *Lytechinus* (34), in the polychaete *Hydroides*, in the enteropneust *Saccoglossus* (25), in the trematode *Haematoloechus* (16), and changes that take place in the penetrated mammalian ovum (74–77, 86, 87). In addition to the fact that the stage of maturation of the eggs at the time of sperm penetration differs among these organisms, only isolated phases of pronuclear development and fusion were studied. Thus, our knowledge of the fine structural transformations involving the development of the zygote nucleus is based on a limited number of investigations of various egg types. Moreover, aspects such as the formation of the sperm aster, its subsequent migration, and pronuclear fusion per se have not been documented in detail. Other than the information provided by the aforementioned investigations and additional observations of spermatozoa within eggs (64, 68, 78), the literature is scant concerning the ultrastructural details of those processes which occur subsequent to the fusion of the gamete

plasma membranes and prior to the first cleavage division of the zygote.

In an effort to elucidate the many facets which remain unsolved concerning the fertilization phenomenon, it now becomes necessary to undertake a fine structural study of those aspects leading up to and associated with the formation of the zygote nucleus. Considering that much of our current knowledge of fertilization is derived from experiments with the gametes of the sea urchin, it is desirable to take advantage of this wealth of information and make a detailed investigation of the events of pronuclear development and fusion in this group. Therefore, this paper deals with a fine structural analysis of the fertilization phenomenon in the sea urchin, *Arbacia punctulata*. It calls specific attention to the following morphogenetic events: (*a*) sperm incorporation, (*b*) development of the male pronucleus, (*c*) formation of the sperm aster, (*d*) migration of the pronuclei, and (*e*) encounter and fusion of the pronuclei.

MATERIALS AND METHODS

Arbacia punctulata obtained from the Marine Biological Laboratory, Woods Hole, Massachusetts during the months of July and August were induced to spawn by applying a 10 v alternating current across the test (see reference 40). The eggs were shed into seawater, and the sperm were collected "dry" according to the methods recommended by Costello et al. (26). The dry sperm were diluted with seawater according to the method prescribed by Just (45). After they had been washed in seawater, the eggs were fertilized and permitted to develop at 20°–23°C with constant stirring. To obtain a sequence of pronuclear development and fusion, eggs were collected at 2 min intervals for a 20 min period following insemination. The fertilized eggs were prefixed for light and electron microscopy for 1.5 hr in the paraformaldehyde-glutaraldehyde mixture of Karnovsky (46), were washed in seawater for 2 hr, and postfixed in a 1% solution of osmium tetroxide in seawater for 1.5 hr. Following fixation the fertilized eggs were rapidly dehydrated in a graded series of ethanol or acetone, infiltrated, and embedded in Epon (54). Thin sections were cut with a Porter-Blum MT-2 ultramicrotome, stained with uranyl acetate followed by the lead citrate stain of Venable and Coggeshall (83), and examined with the RCA EMU-3H electron microscope. Thick sections (1 μ) made for general light microscopy were stained according to the method of Ito and Winchester (43) and according to the method of Kurnick (49) for the demonstration of nucleic acids.

OBSERVATIONS

Sperm Incorporation

Concomitant with the cortical reaction in the eggs of *Arbacia* is the attachment of the spermatozoon to the egg's surface (see reference 4). At this site of conjugation, the plasma membranes of the gametes fuse, thereby producing a confluence of their contents (Fig. 1).

The ooplasm at the site of gamete fusion initially produces a slender protrusion; this region is the fertilization or entrance cone (Fig. 1, *FC*). Within 30–60 sec all of the sperm contents, i.e. its nucleus, mitochondrion, centrioles, and flagellum, are enveloped by the ooplasm of the fertilization cone, an area containing ribosomes and filaments. As the spermatozoon continues its movements into the ooplasm, the fertilization cone increases in size and becomes a large bleb with a flattened surface (Fig. 2). For convenience of description we have divided the fertilization cone into apical and basal regions. The apical region of the fertilization cone contains filaments that are oriented parallel to its long axis, whereas in the basal region there appear a number of vesicles, many of which contain rodlike elements (see reference 4) (Fig. 2, *F* and *VR*).

As the spermatozoon moves through the apical region of the fertilization cone, its apex points towards the center of the egg. Upon reaching the basal region, however, the spermatozoon begins to rotate 180°, an operation completed within 2–3 min from the time of insemination. When rotation is completed the spermatozoon is situated to one side of the fertilization cone, and its apex is directed towards the egg's surface (Fig. 3).

Rotation does not affect the relative positions that the sperm nucleus, centrioles, mitochondrion, and flagellum share with each other (Fig. 6). Associated with the base of the sperm nucleus are two centrioles. The sperm flagellum originates from the longitudinally oriented proximal centriole located in a deep conical indentation at the base of the nucleus. This recess is referred to here as the centriolar fossa (Figs. 1 and 6, *CF*). The distal centriole is perpendicular to the proximal one and does not appear to be anatomically associated with the flagellum. Adjacent to the base of the nucleus and surrounding the anterior region of the sperm flagellum is the doughnut-shaped mitochondrion. It contains a relatively electron-opaque matrix and long anastomosing cristae (Figs. 1 and 5, *SM*).

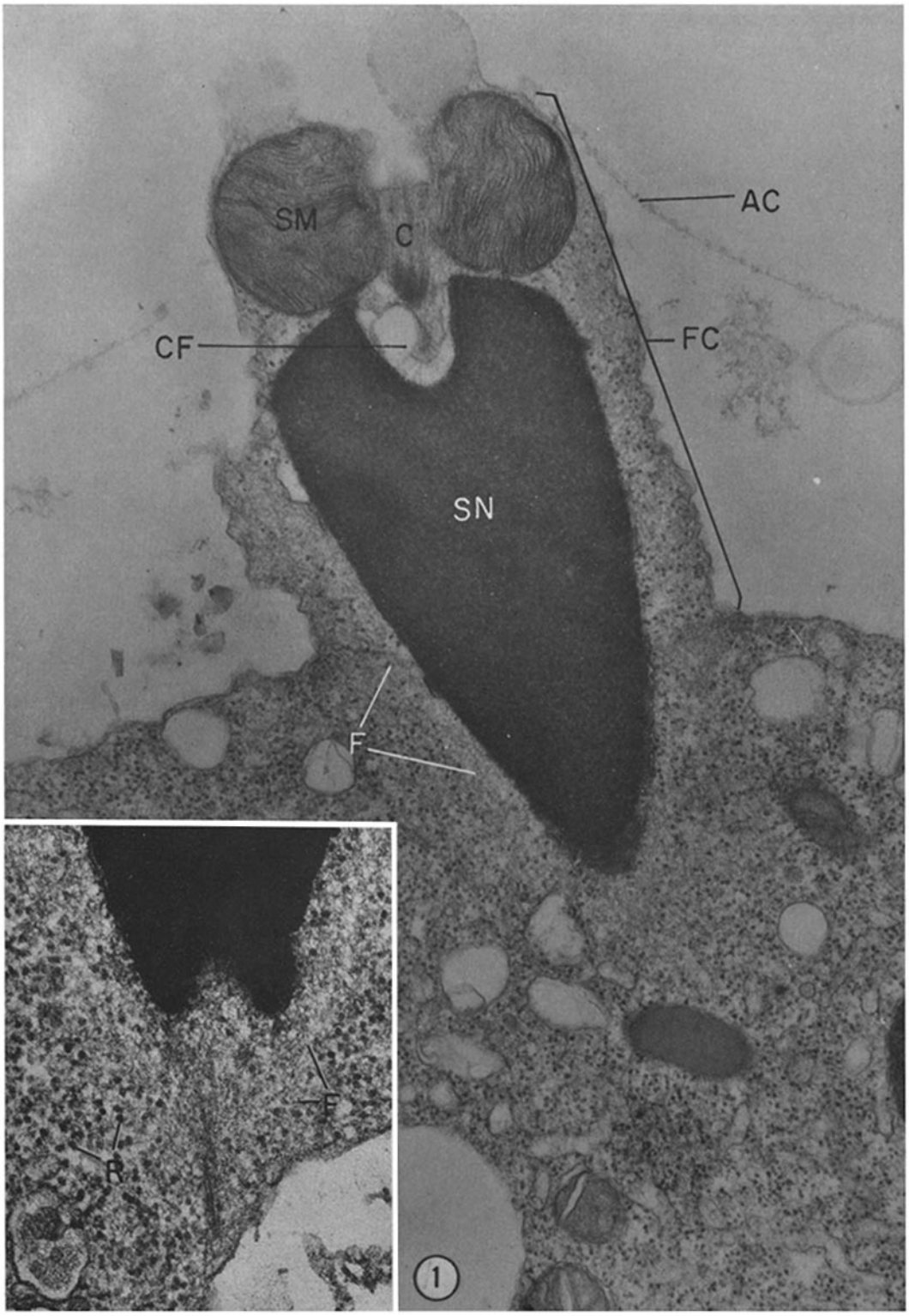


FIGURE 1 An early stage of the incorporation of the spermatozoon. *AC*, activation calyx; *FC*, fertilization cone; *SN*, sperm nucleus; *SM*, sperm mitochondrion; *C*, centriole; *CF*, centriolar fossa; *F*, filaments. The inset illustrates the ooplasmic matrix of the fertilization cone containing ribosomes (*R*) and filaments (*F*). × 24,000; Inset, × 53,000.

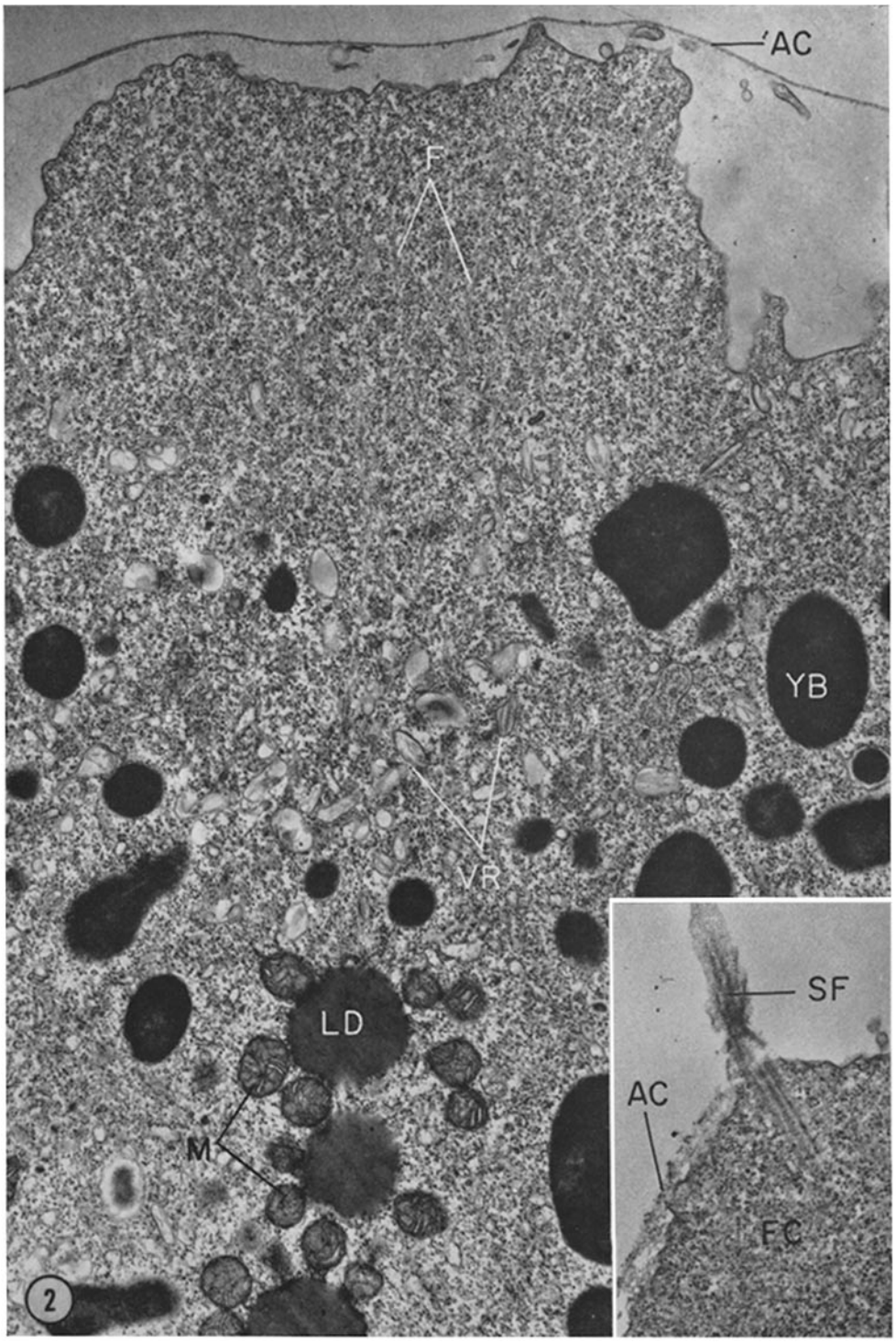


FIGURE 2 Fertilization cone about 4 min following insemination. The apical ooplasmic region of the fertilization cone is characterized by clusters of longitudinally oriented filaments (*F*) and the basal region by vesicles containing rodlike structures (*VR*). Inset, segment of the sperm flagellum (*SF*) projecting from the surface of the fertilization cone (*FC*). *AC*, activation calyx; *M*, mitochondria; *YB*, yolk bodies; *LD*, lipid droplet. × 15,500; Inset, × 18,000.

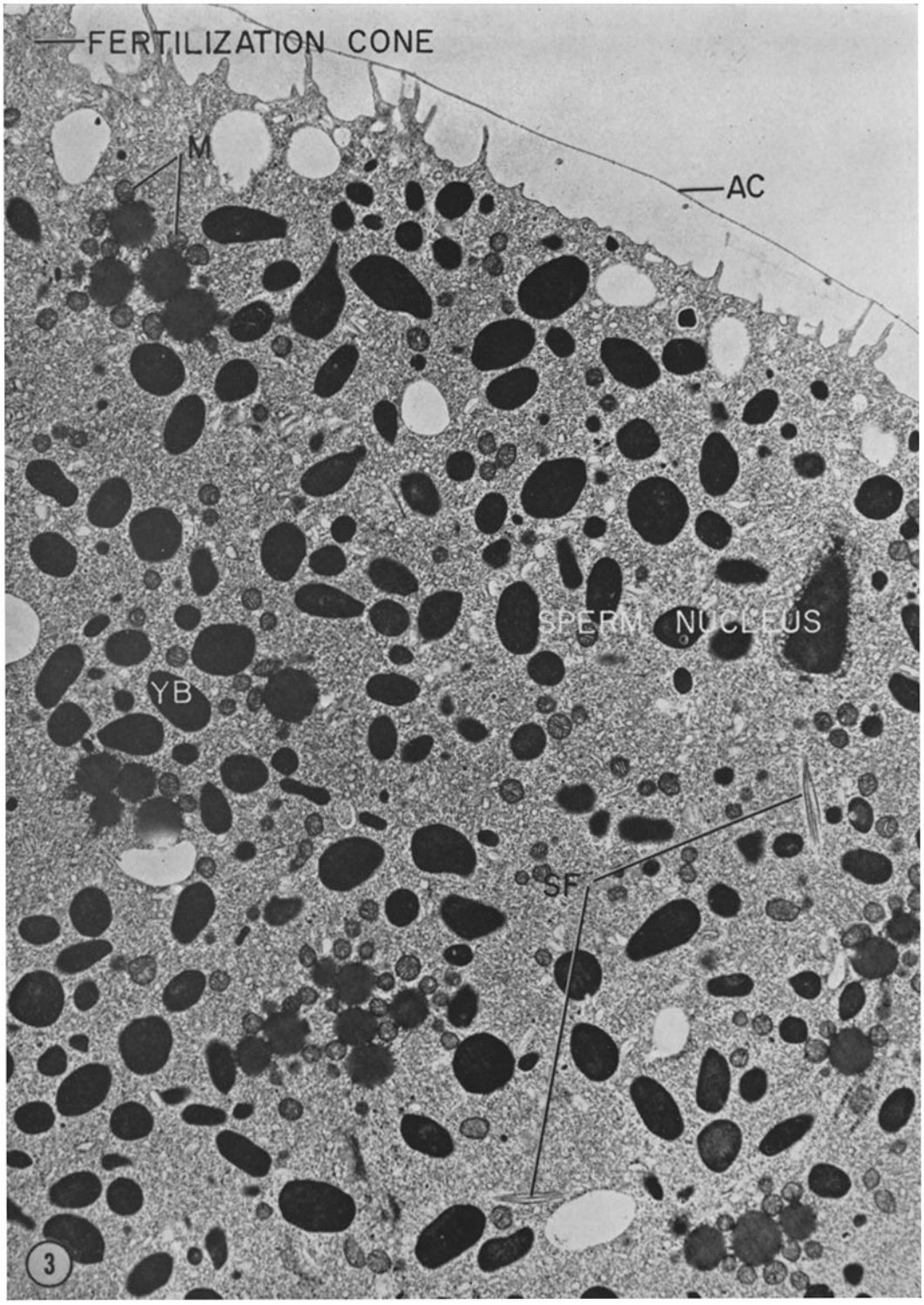


FIGURE 3 Section through an activated egg depicting the fertilization cone and the position of the sperm nucleus. *AC*, activation calyx; *SF*, sperm flagellum; *YB*, yolk body; *M*, mitochondria. × 9,000.

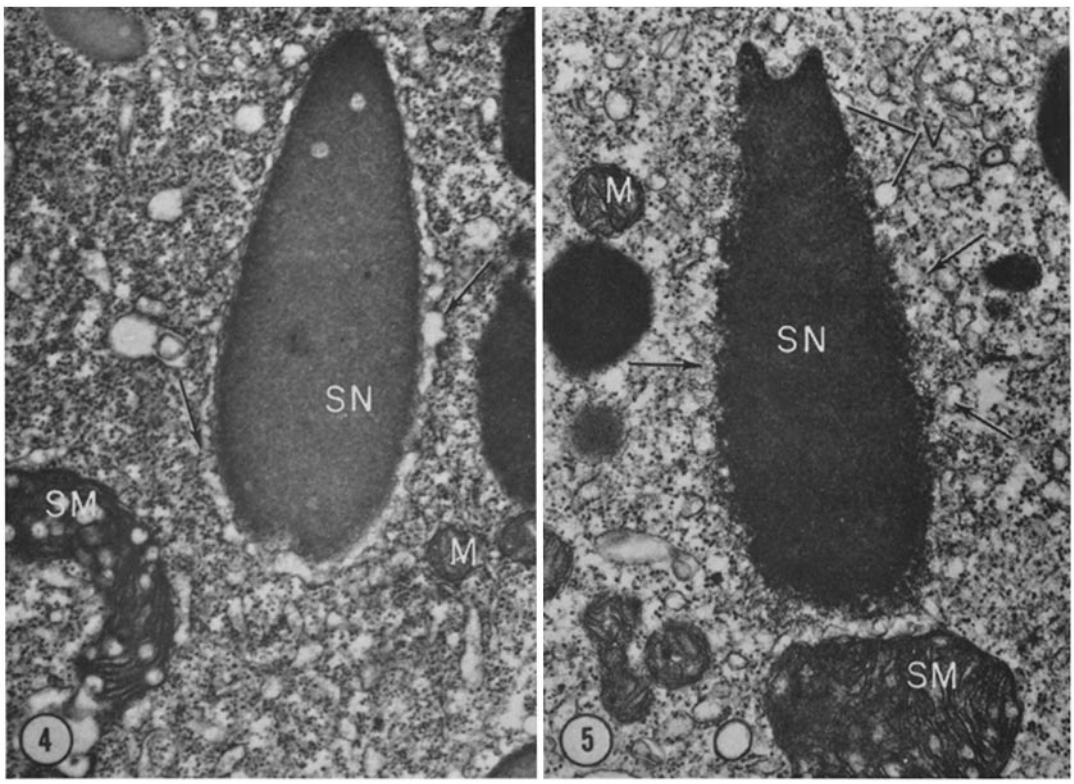


FIGURE 4 Rotated spermatozoon in an early stage of nuclear envelope degeneration. Note the vesicular appearance of the nuclear envelope (arrows). *SN*, sperm nucleus; *SM*, sperm mitochondrion; *M*, mitochondria of egg. × 18,000.

FIGURE 5 Rotated spermatozoon nearing the completion of nuclear envelope degeneration and the beginning chromatin dispersion (arrows). Vesicles (*V*) are present along the surface of the sperm nucleus (*SN*). *SM*, sperm mitochondrion; *M*, mitochondria of egg. × 18,000.

Profiles of the flagellum are observed extending from the rotated spermatozoon to the fertilization cone (Fig. 3, *SF*). Apart from the loss of its plasma membrane, the integrity of the sperm flagellum, i.e. its 9 + 2 tubular pattern, is maintained. In favorable sections, portions of the sperm tail with an intact plasma membrane were observed projecting from the surface of the fertilization cone directly above segments of the engulfed flagellum (Fig. 2, *SF*, inset).

Interestingly, we have never observed pores within the nuclear envelope of a spermatozoon before it enters the egg or immediately thereafter. However, as the spermatozoon passes through the basal region of the fertilization cone and subsequently undergoes rotation, the nuclear envelope appears vesicular (Fig. 4, arrows). The vesicles are interpreted as the product of the partial breakdown of the sperm's nuclear envelope and are the result of multiple fusions between the outer and inner membranes of the nuclear envelope (see reference 9). The anterior and posterior aspects of the sperm's nuclear envelope remain intact (Figs. 7, asterisk; and 6, *CF*).

Development of the Male Pronucleus

Formation of the male pronucleus begins subsequent to the rotation of the spermatozoon and entails the following two events: (*a*) the reorganization of the sperm chromatin and (*b*) the formation of the pronuclear envelope.

Chromatin reorganization is here defined as the dispersion of the tightly packed chromatin of the sperm nucleus. We have already called attention

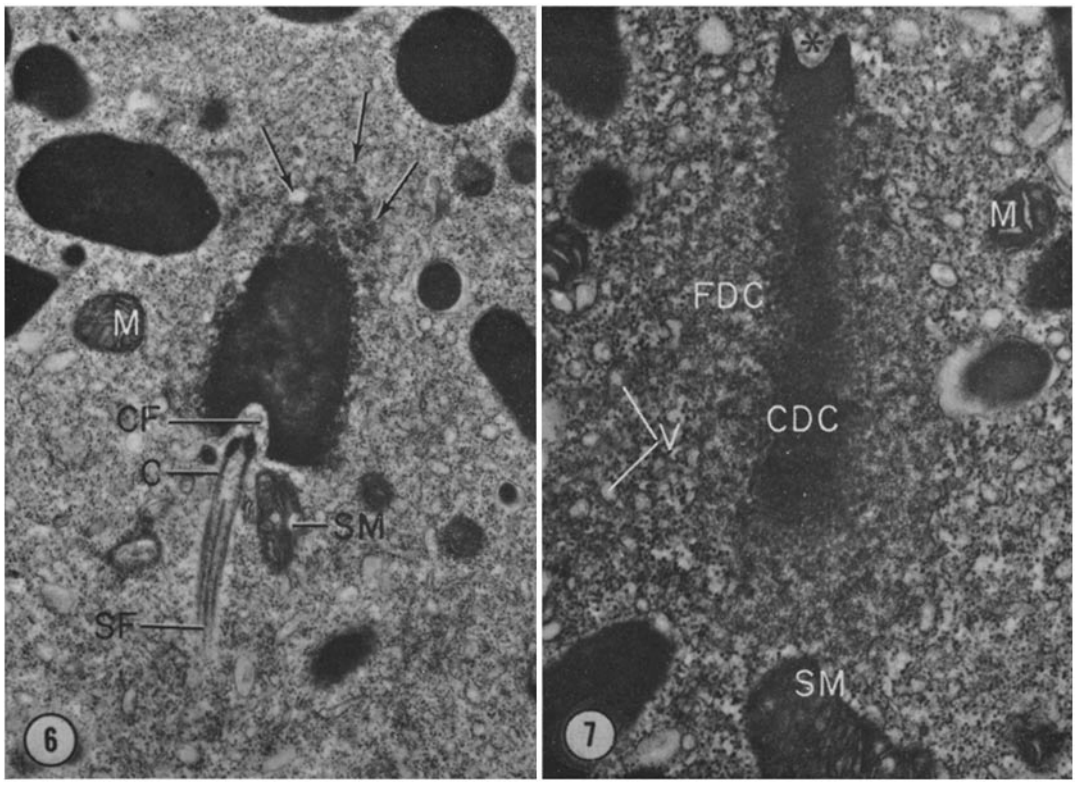


FIGURE 6 A further stage in the dispersal of the sperm chromatin (arrows). The nuclear envelope at the centriolar fossa (*CF*) is intact. *SM*, sperm mitochondrion; *SF*, sperm flagellum; *C*, centriole; *M*, mitochondria of egg. × 15,500.

FIGURE 7 Advanced stage in the dispersion of the sperm chromatin. Vesicles (*V*) are evident along the junction of finely dispersed chromatin (*FDC*) and the ooplasm. The nuclear envelope surrounding the anterior region of the sperm nucleus (asterisk) is intact. The more medial portion of the sperm nucleus is composed of coarse aggregates of chromatin (*CDC*). *SM*, sperm mitochondrion; *M*, mitochondria of egg. × 18,000.

to the fact that the nuclear envelope of the sperm nucleus vesiculates along its lateral margin. It is along this aspect of the sperm nucleus that the chromatin begins to disperse (Figs. 5 and 6, arrows). As a result of the presence of the nuclear envelope at the apex and base of the nucleus, there is a differential in the dispersion of the chromatin, which yields a heart-shaped male pronucleus (Figs. 7, 9, and 10).

Scattering of the sperm nucleus produces the following three zones of chromatin conformation with varied densities: (*a*) a perimeter consisting of randomly oriented filaments, (*b*) a middle zone composed of aggregates of filaments that measure approximately 55 mμ in width, and (*c*) an inner zone that consists of the central region of the sperm nucleus composed of aggregations of electron-opaque chromatin (Figs. 7–9, *FDC*, *CDC*, and *CC*). At successively later stages of pronuclear development there is a continued diffusion of the chromatin of the male pronucleus until it finally appears as a delicate mesh of entangled filaments (Figs. 19, 21, and 22).

In some preparations there is an incomplete dispersion of chromatin of the male pronucleus prior to its fusion with the female pronucleus. Apparently, this condition depends upon the distance the male pronucleus must migrate in order to encounter the female pronucleus, for in those

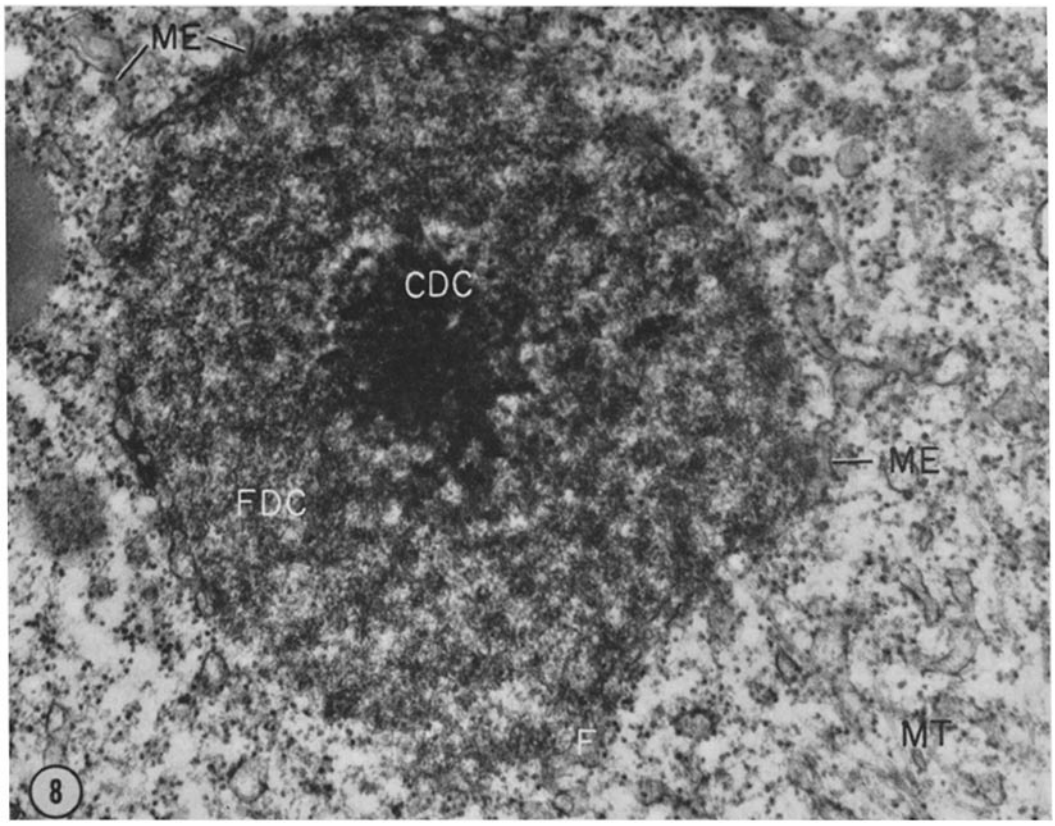


FIGURE 8 The male pronucleus. Membranous elements (*ME*) are located at the junction of the finely dispersed sperm chromatin (*FDC*) and ooplasm. *CDC*, coarse aggregates of sperm chromatin; *MT*, microtubules. × 24,000.

cases where the distance is short, much of the chromatin was found to be still somewhat compact.

The male pronuclear envelope is formed during the period of chromatin dispersion. At the junction of the fine filamentous sperm chromatin and the ooplasm, one notices a collection of smooth-surface vesicles (Fig. 7, *V*). These vesicles become confluent and form a double membrane structure characteristic of the nuclear envelope (Figs. 8–10, *PNE*). When the male pronucleus is cut tangentially, pores can be distinguished by their dense annuli (Fig. 9, inset). The pronuclear pores appear to be spaced more or less in a hexagonal array with a center to center spacing of about 200 mμ.

A small portion of the sperm nuclear envelope is structurally associated with the newly formed male pronuclear envelope. As previously stated, breakdown of the sperm nuclear envelope is not complete, for the nuclear membranes at the anterior and the posterior regions of the sperm nucleus remain essentially intact (Figs. 6 and 7). These portions of the original nuclear envelope are distinct throughout the formation of the male pronucleus and do not appear to be altered during subsequent morphogenesis (Figs. 9, 10, 12, and 21).

During the formation of the male pronucleus, the sperm mitochondrion and flagellum remain morphologically unchanged. Although these structures become spaced farther apart, they retain their positions relative to one another (Figs. 9 and 10, *SM* and *SF*). The centrioles are associated with a conoid indentation at one end of the pronucleus (Figs. 9, 10, and 12); this concavity presumably represents a remnant of the centriolar fossa.

Formation of the Sperm Aster

One of the first indications of sperm aster development is the dissociation of the proximal

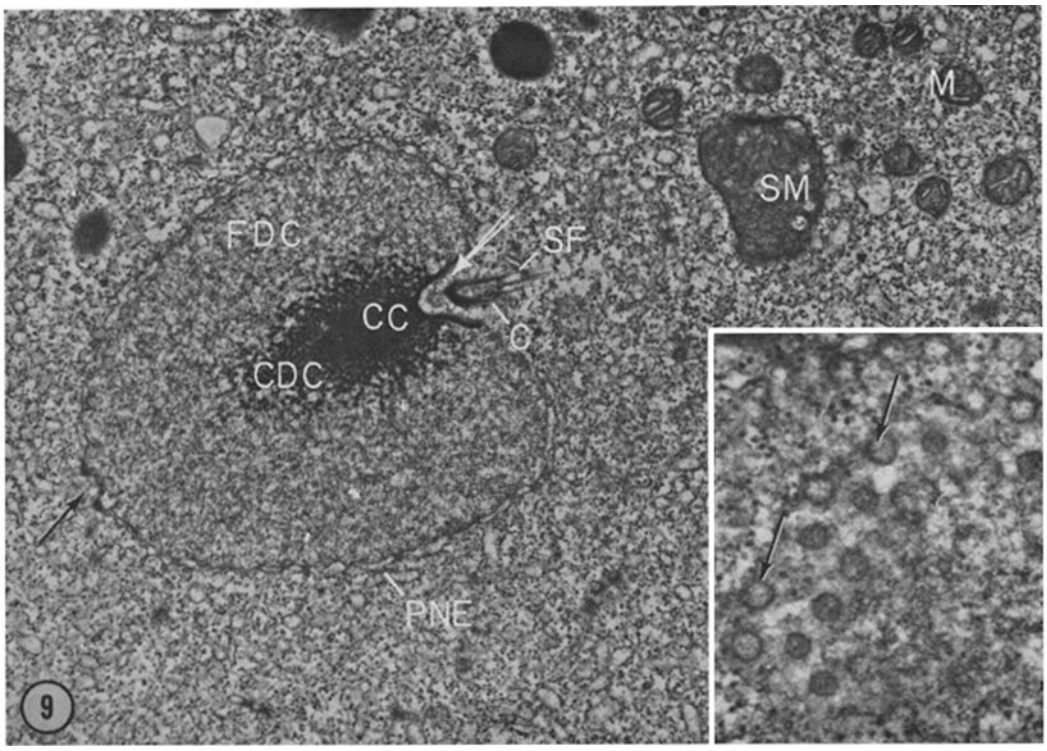


FIGURE 9 Heart-shaped male pronucleus delimited by a pronuclear envelope (*PNE*) perforated by pores (arrows, inset). The double- and single-stemmed arrows denote the portions of the male pronuclear envelope at the centriolar fossa and the apex of the sperm nucleus, respectively. A layer of electron-opaque material is associated with these regions. *FDC*, finely dispersed chromatin; *CDC*, coarsely dispersed chromatin; *CC*, condensed chromatin; *SM*, sperm mitochondrion; *C*, centriole; *SF*, sperm flagellum; *M*, mitochondria of egg. × 15,500; Inset, × 24,000.

centriole from the sperm flagellum. In cross-section the centrioles are seen to consist of nine pairs of tubules. Extending from one tubule of each pair is a curved arm. From the nine doublet tubules that comprise the centrioles emanate thin conical satellites (Fig. 11, *S*). In the immediate vicinity of the centrioles are short, randomly oriented segments of microtubules and elements of the endoplasmic reticulum (Fig. 11, *MT*). Distal to this area one finds longer microtubules separated from each other by ooplasmic components (Figs. 11 and 12, *MT*, *AL*, and *SER*).

Initially, parallel clusters of microtubules form a conical structure, referred to here as the sperm aster, the apex of which arises from the centriolar fossa of the pronucleus (Fig. 12, arrows; *SA*, inset). Microtubules are also distributed randomly within the ooplasmic matrix immediately surrounding the male pronucleus (Fig. 12, *MT*). This region contains ribosomes, endoplasmic reticulum of the smooth variety, and occasionally mitochondria and yolk bodies.

As the sperm aster continues to develop, the distal centriole becomes dissociated from the male pronucleus (Fig. 13, *C*). In the meantime the pericentriolar region of both centrioles becomes packed with segments of microtubules and endoplasmic reticulum of the smooth variety (Figs. 13 and 21, *MT*). This microtubular-endoplasmic reticulum system forms a dense matrix around the centrioles and constitutes what could be thought of as a centrosphere region (see reference 36). Formation of the centrosphere region and its subsequent growth tend to exclude ooplasmic constituents, such as yolk bodies, Golgi complexes, and mitochondria which are confined to its perimeter (Fig. 13, *YB* and *M*).

Radiating from the centrosphere region of the

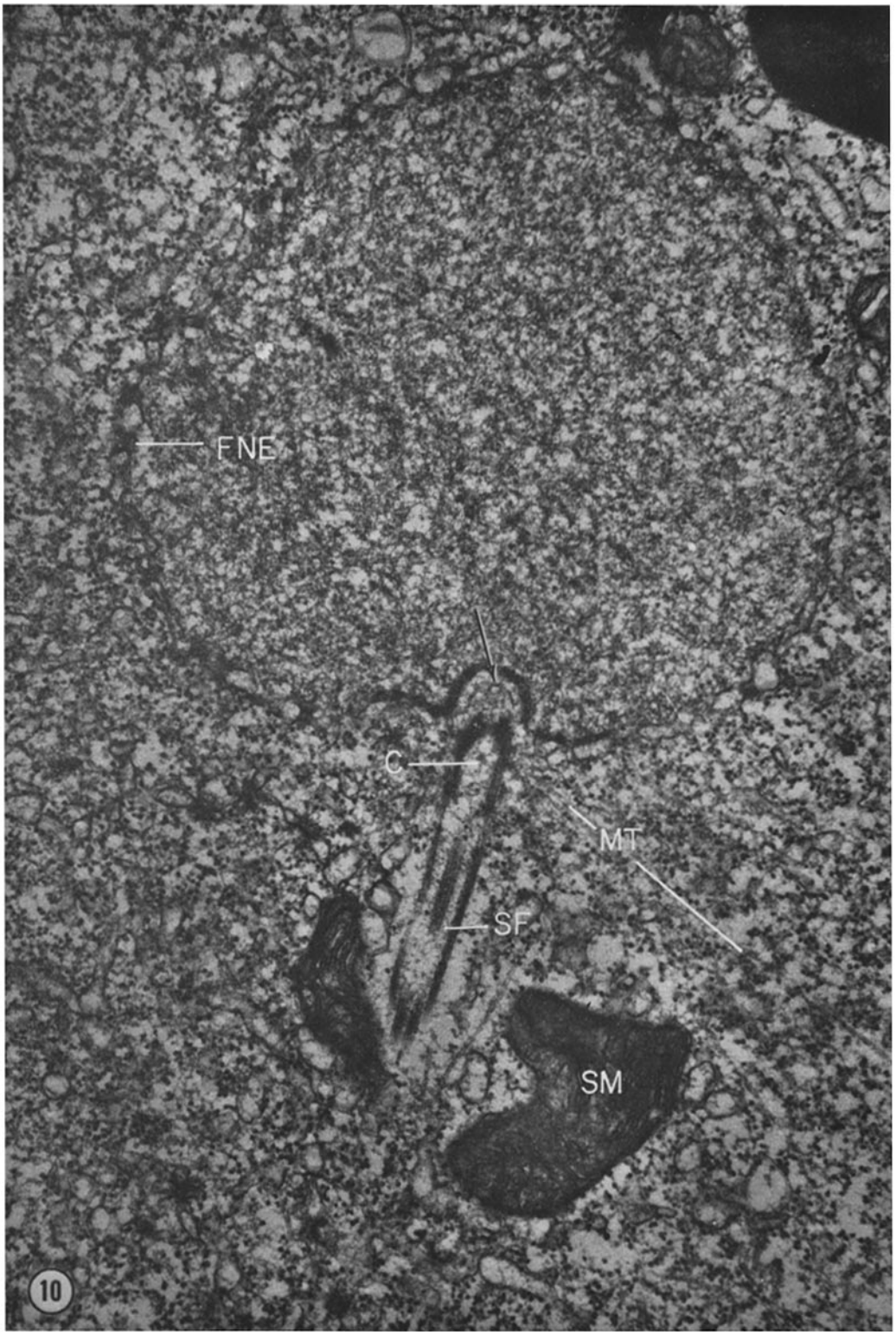


FIGURE 10 The male pronucleus associated with the sperm mitochondrion (*SM*), sperm flagellum (*SF*), and centriole (*C*). Microtubules (*MT*) emanate from the pericentrolar region. Arrow denotes the area of the male pronucleus that coincides with the centriolar fossa of the sperm nucleus. *PNE*, pronuclear envelope. × 24,000.

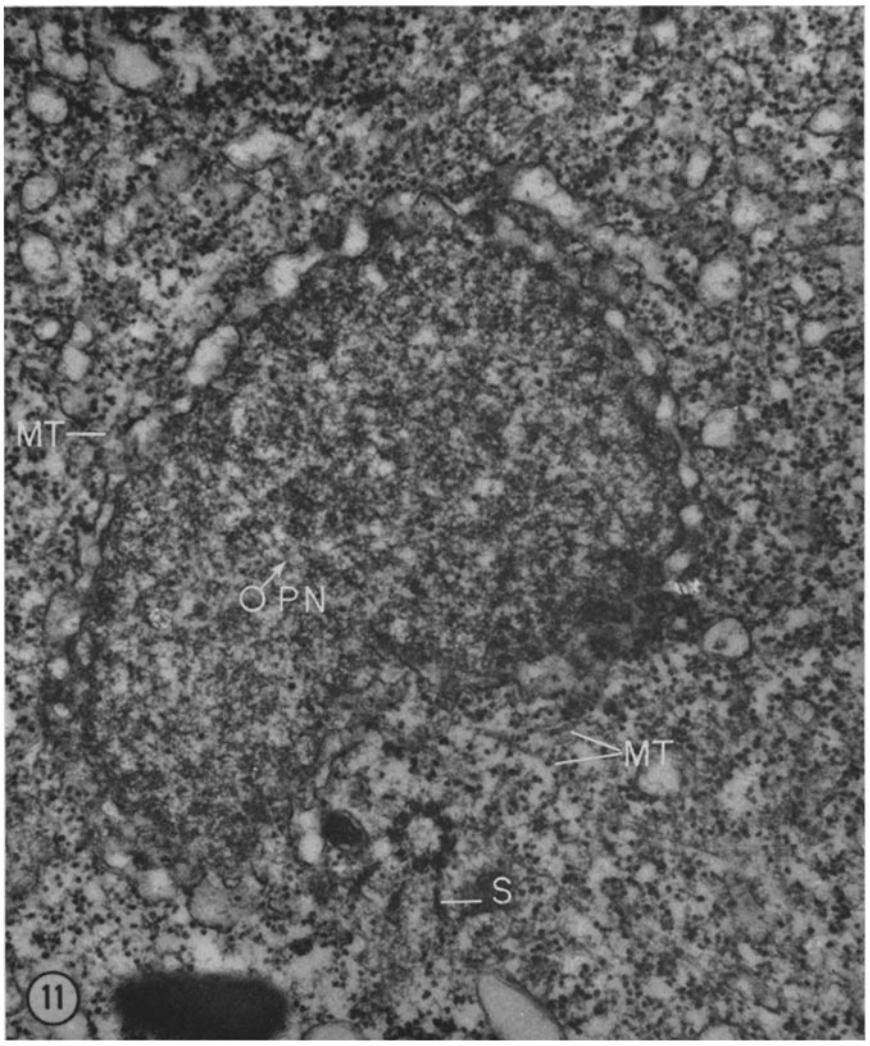


FIGURE 11 Electron micrograph illustrating pericentriolar satellites (*S*). Microtubules (*MT*) are adjacent to the male pronucleus (♂*PN*). × 24,000.

sperm aster are segments of microtubules which appear as small bundles separated from each other by ooplasmic components (Figs. 13 and 14, *MT*). Distal to the centrosphere the aster is composed of numerous profiles of endoplasmic reticulum of the smooth variety and annulate lamellae which extend into the peripheral aspect of the egg (Fig. 15, *SER*). Oriented parallel to these lamellar structures are clusters of yolk bodies, motochondria, and lipid droplets (Fig. 15, *YB* and *M*; inset).

During the later stages of sperm aster differentiation there is an increase in size of the aster (Fig. 15, inset). This transformation appears to be accompanied by an accumulation of large numbers of microtubules, endoplasmic reticulum, and annulate lamellae. Enlargement of the sperm aster continues throughout the migration of the male pronucleus. While these changes in the structure of the sperm aster are taking place, the heart-shaped male pronucleus transforms into a spheroid (Figs. 19–21).

Migration of the Male and Female Pronuclei

As a result of the rotation of the spermatozoon, the sperm aster, which is formed in the vicinity of

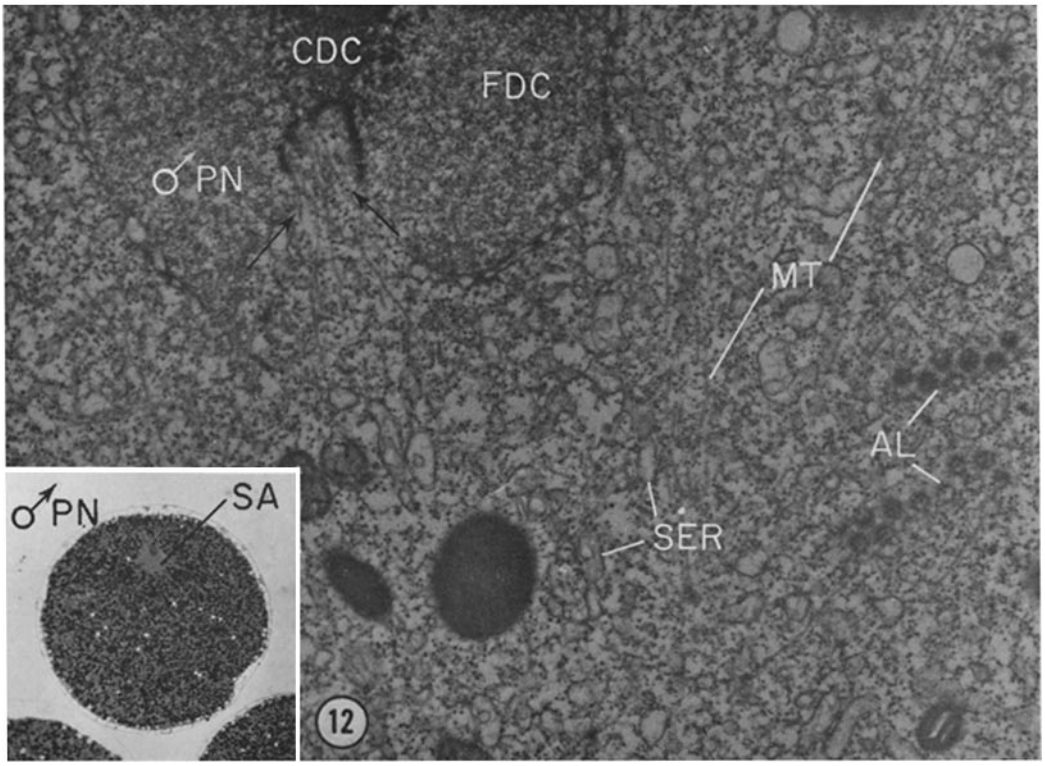


FIGURE 12 The electron micrograph is an early stage in the development of the sperm aster (see also inset, *SA*). A cluster of microtubules emanates from the centriolar fossa (arrows) of the male pronucleus (*♂PN*; also see inset). Microtubules are also present in the ooplasm surrounding the pronucleus. *FDC*, finely dispersed chromatin; *CDC*, coarsely dispersed chromatin; *AL*, annulate lamellae; *SA*, sperm aster; *SER*, endoplasmic reticulum of the smooth variety; *MT*, microtubule. × 18,000; Inset, × 400.

the sperm mitochondrion, lies in front of the male pronucleus and leads the way in migration to the female pronucleus (Fig. 12, inset).

The sperm aster and the male pronucleus remain associated with the sperm mitochondrion and flagellum during their movements. Throughout this period the male pronucleus continues to increase in size. This development is accompanied by the addition of smooth surface vesicular elements to the pronuclear envelope and the continued dispersion of chromatin.

Encounter and Fusion of Pronuclei

During the later stages of migration, the male pronucleus is a spheroid and contains fine, moderately compact, fibrillar chromatin (Figs. 20–22). In some cases, however, the male pronucleus may appear ellipsoidal or heart-shaped and invariably contains condensed chromatin in various stages of reorganization. Male pronuclei that are nonspheroidal at this stage of development are found in fertilized eggs in which the site of sperm entrance was very near the female pronucleus. This observation suggests that the distance of the female pronucleus from the site of sperm penetration may determine the time the male pronucleus spends in various stages of its development. Further demonstration of this temporal relation is given by Harvey (39).

Except for its migration in the direction of the advancing male pronucleus, the appearance of the female pronucleus remains essentially unchanged. From the time of insemination the female pronucleus is irregular in shape and is always limited by a perforated nuclear envelope (Fig. 16, *PNE*). Immediately surrounding the female pronucleus is an area containing ribosomes, endoplasmic reticulum of the smooth variety, and annulate lamellae

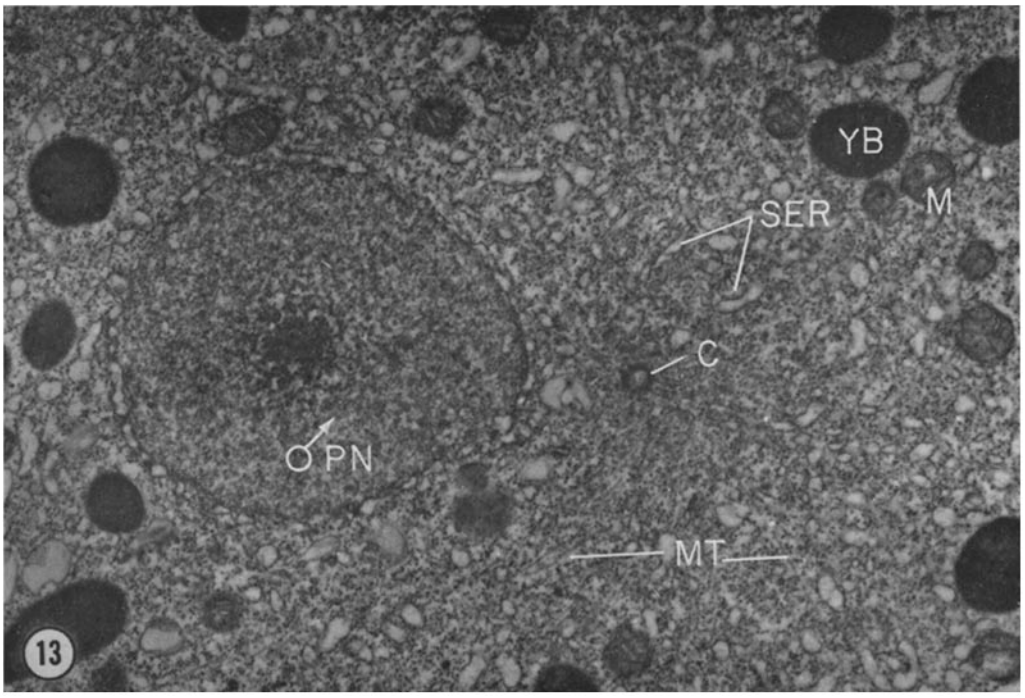


FIGURE 13 An electron micrograph of a centriole (*C*) and the sperm aster during the migration of the male pronucleus (♂*PN*; see also inset Figs. 14–15). Microtubules (*MT*) and endoplasmic reticulum of the smooth variety (*SER*) surround the centriole to form a centrosphere region which excludes larger ooplasmic constituents such as yolk bodies (*YB*) and mitochondria (*M*) from this area. × 15,500.

(Figs. 16–18). The female pronucleoplasm contains fibrillar chromatin and a variable number of electron-opaque spheroidal bodies. Each spheroidal body is composed of a compact, fine-textured material (Figs. 16 and 17, *NL*) and granules (Fig. 18, arrows). These spheroidal structures, hereinafter referred to as nucleoli-like bodies, are located randomly along the periphery of the pronucleus and protrude slightly into the surrounding ooplasm. The pores and the perinuclear space of the pronuclear envelope overlying the nucleoli-like bodies are exaggerated in size in comparison to those from other regions of the pronuclear envelope (Fig. 16). The perinuclear space is wider, about 100 mμ; and the diameter of the pronuclear pores is larger, approximately 100 mμ. In other regions of the pronuclear envelope the diameter of the pronuclear pores and the width of the perinuclear space are about 75 and 80 mμ, respectively.

When treated with methyl green–pyronin, the nucleoli-like bodies stain red, indicating that these structures contain RNA.

Nucleoli-like bodies similar to those found in the female pronucleus during pronuclear development and fusion have also been found in the nuclei of the young oocyte, the unfertilized egg, the two-cell stage, and in early prophase of the first cleavage division (Longo, F. J. and E. Anderson. Unpublished observations). These aggregations have not been observed at any time in the male pronucleus. Nucleoli-like structures have also been found in the fertilized and unfertilized eggs of various species of sea urchins by Millonig (57) and Harris (37, 38).

Occasionally the female pronucleus contains a number of intranuclear annulate lamellae which are similar to those found in ascidian oocytes (32, 41, 48). A section of such a lamellar structure with a circular configuration is shown in Fig. 16 (*IAL*).

When the pronuclei are approximately 2 μ from each other, the surface of the female pronucleus undergoes a characteristic series of morphological alterations. The side of the female pronucleus proximal to the advancing male pronucleus

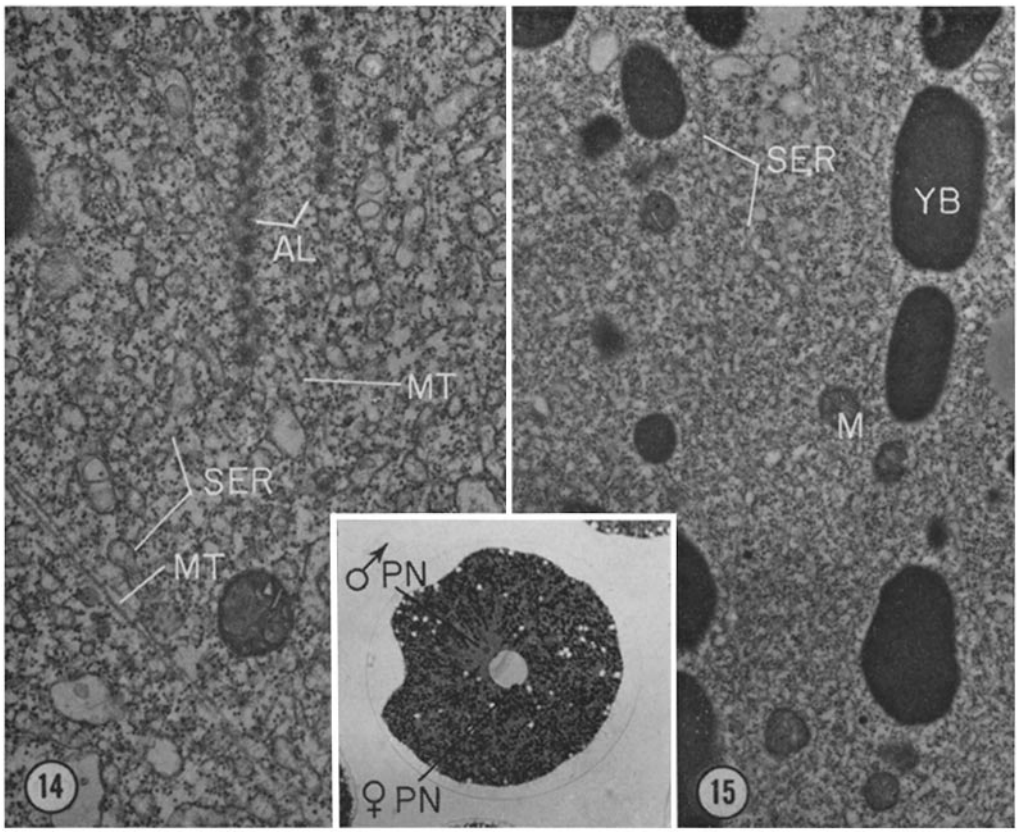


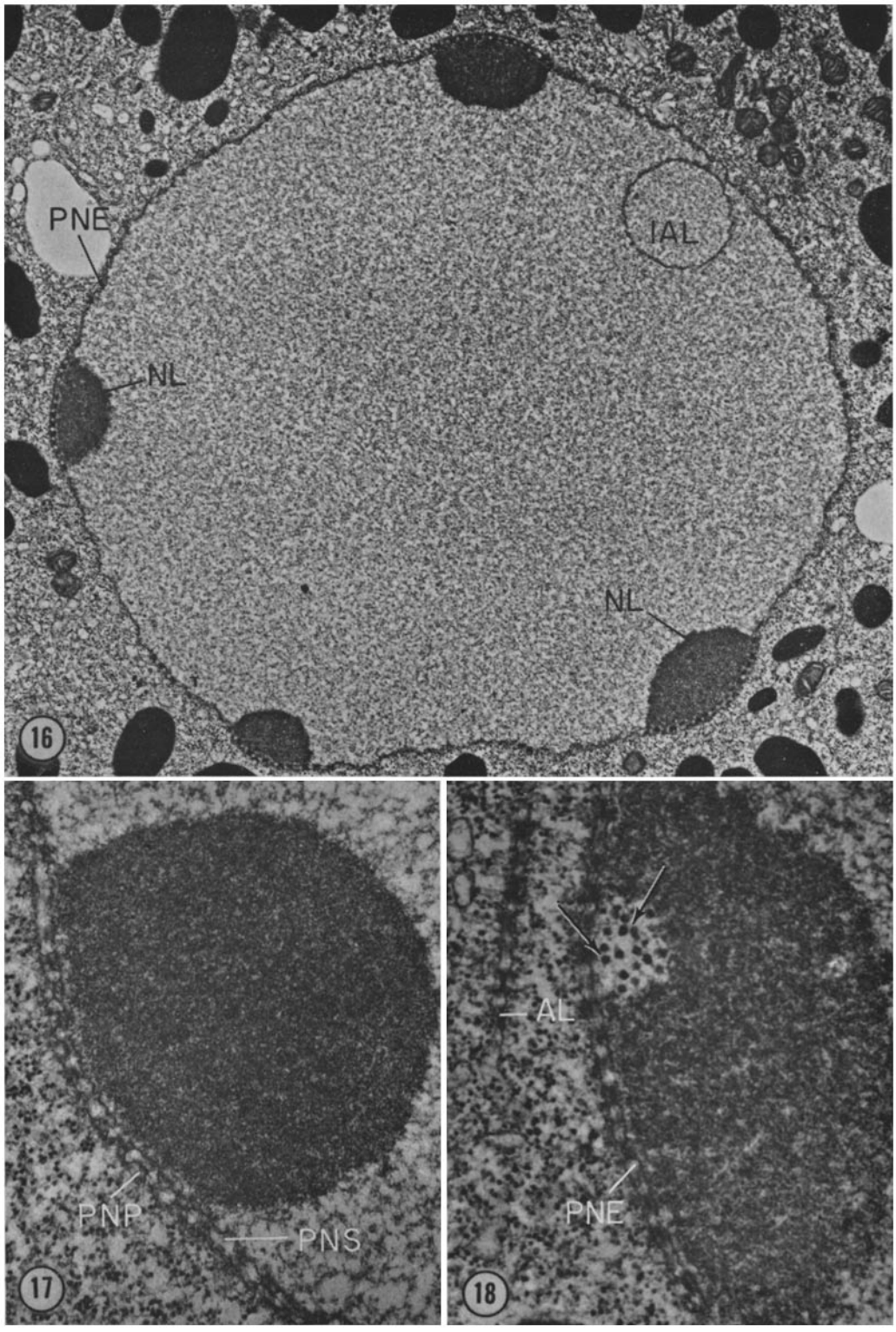
FIGURE 14 Peripheral region of the centrosphere showing the alignment of fascicles of microtubules (*MT*) and elements of the ooplasmic lamellar system. *SER*, smooth endoplasmic reticulum; *AL*, annulate lamellae. × 18,000.

FIGURE 15 Section of the peripheral region of the sperm aster demonstrating the orderly alignment of ooplasmic constituents (also see inset). *SER*, smooth endoplasmic reticulum; *YB*, yolk bodies; *M*, mitochondria. Inset, photomicrograph of the sperm aster and the male and female pronuclei (♂*PN* and ♀*PN*) during a stage in their migration. × 18,000; Inset, × 400.

becomes flattened and subsequently extends finger-like projections in the direction of the advancing male pronucleus (Fig. 19, *NP*).

Just prior to the fusion of the pronuclei, the elements of the sperm aster intermix with the constituents of the ooplasmic matrix surrounding the female pronucleus (Figs. 19–21 and inset). The sperm mitochondrion and flagellum move into this region and come to rest in an area adjacent to the pronuclei (Fig. 21, *SM* and *SF*). When the male pronucleus is about 500 mμ from the female pronucleus the centriolar fossa of the male pronucleus, along with its centriole, moves to one side of the female pronucleus (Fig. 22). The region of the male pronucleus proximal to the female pronucleus flattens so that the apposing pronuclear envelopes of both bodies are parallel (Fig. 22). The flattened surface of the male pronucleus does not produce projections like those of the female. Also located adjacent to the female pronucleus is a second centriole (Fig. 23, *C*). We believe that this centriole may represent the distal one which earlier had separated from the male pronucleus. Another interpretation, however, is that this centriole is of egg origin (see references 31, 84).

Formation of the zygote nucleus is initiated when the outer membranes of the pronuclear envelopes meet (Fig. 24, arrows). The area of contact appears to be restricted to a small locus. At this locus, the outer membranes of the male and the



FIGURES 16–18 Female pronucleus of an inseminated egg containing four nucleoli-like structures (*NL*) and an intranuclear annulate lamella (*IAL*). Note the difference in the structure of the perforated (*PNP*) pronuclear envelope (*PNE*) on the surface of these aggregations and that of other regions of the pronucleus. Some of the nucleolar-like structures contain dense granules (arrows, Fig. 18). *PNS*, perinuclear space, *AL*, annulate lamellae. Fig. 16, × 11,500; Figs. 17 and 18, × 24,000.

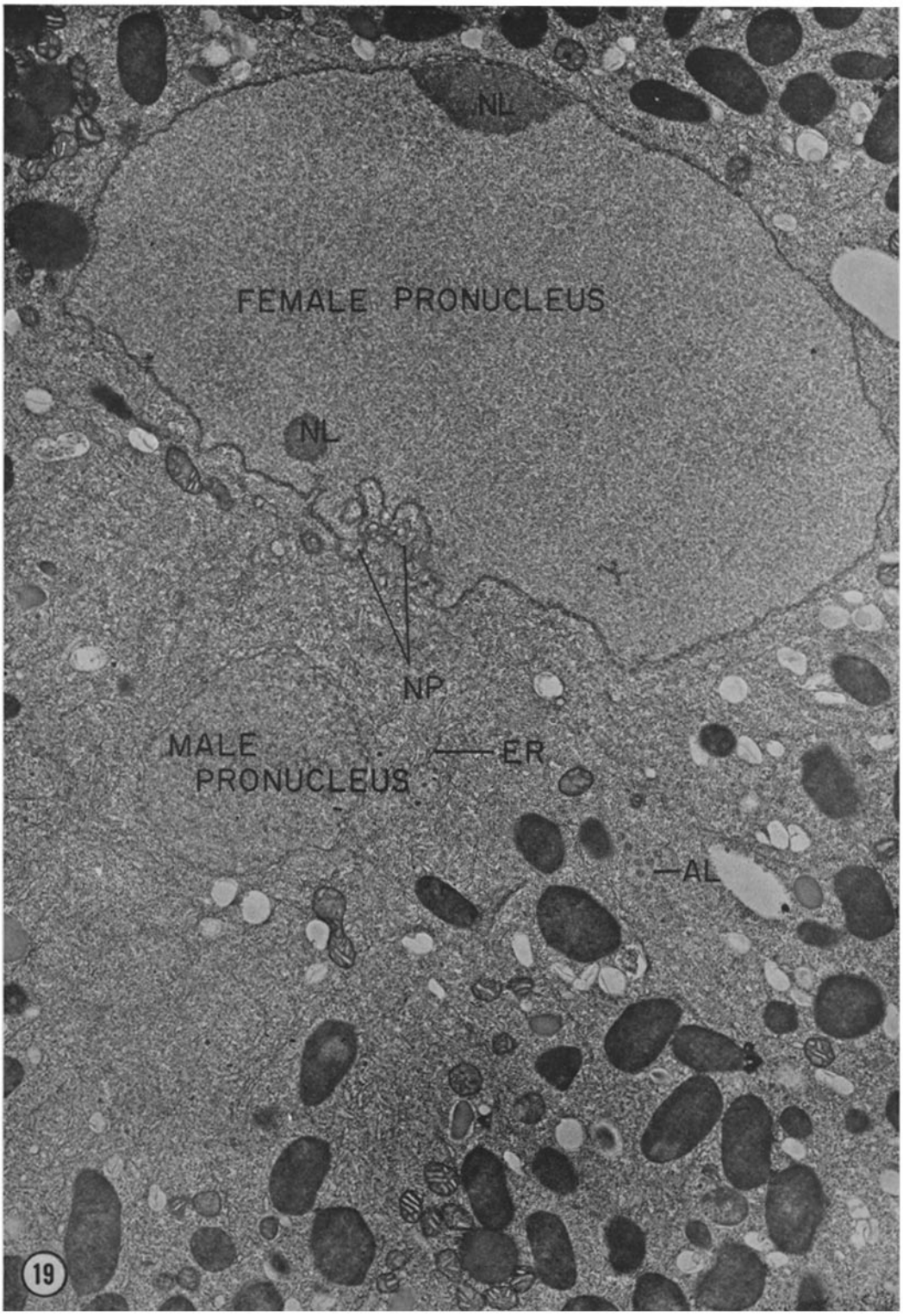


FIGURE 19 Encounter of the male and female pronuclei. The region of the female pronucleus proximal to the advancing male pronucleus has flattened and developed nucleoplasmic projections (*NP*). The endoplasmic reticulum (*ER*) surrounding the male pronucleus is continuous with the ooplasmic region encircling the female pronucleus. *AL*, annulate lamellae; *NL*, nucleolus-like body. × 11,500.

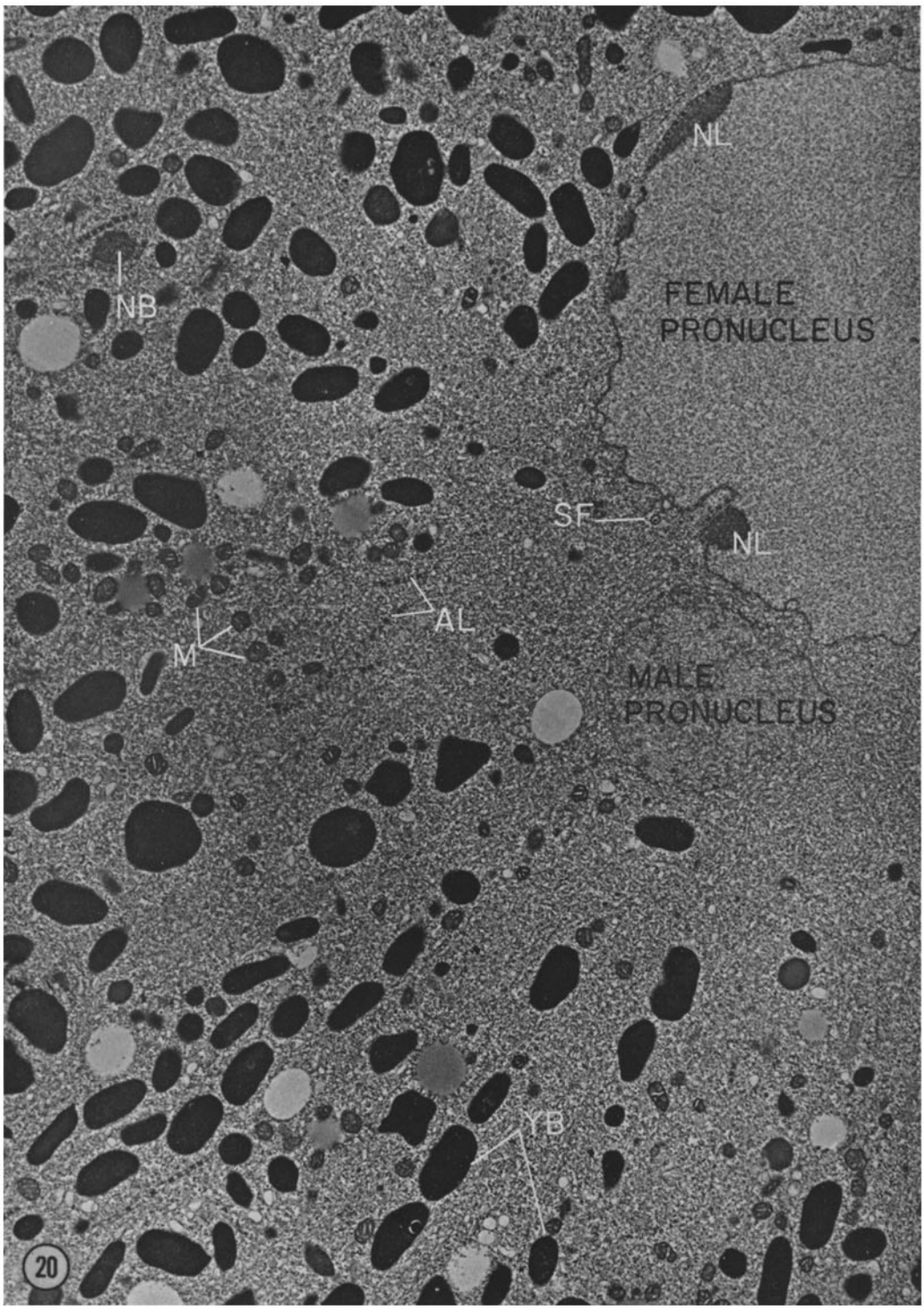


FIGURE 20 Male and female pronuclei prior to fusion. The sperm aster has merged with the ooplasmic region surrounding the female pronucleus. *AL*, annulate lamellae; *M*, mitochondria; *YB*, yolk bodies; *HB*, heavy body; *NL*, nucleoli-like structures; *SF*, sperm flagellum. × 9,500.

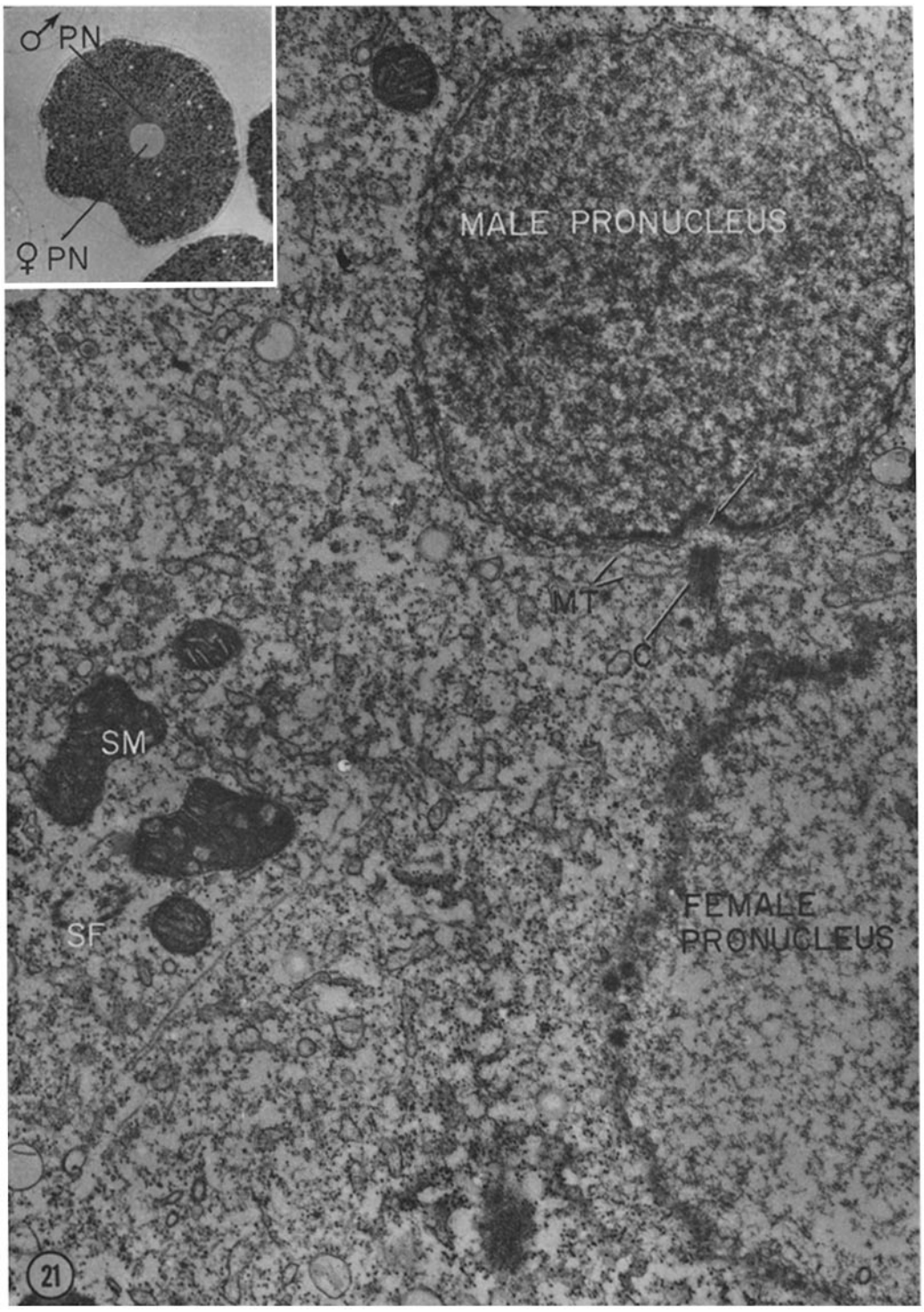


FIGURE 21 Male and female pronuclei prior to their fusion (♂*PN* and ♀*PN*, inset). The centriole (*C*) and associated microtubules (*MT*) are located between the pronuclei. The sperm mitochondrion (*SM*) and a portion of the sperm flagellum (*SF*) are to one side of the pronuclei. Arrow, centriolar fossa. × 18,000; Inset, × 400.

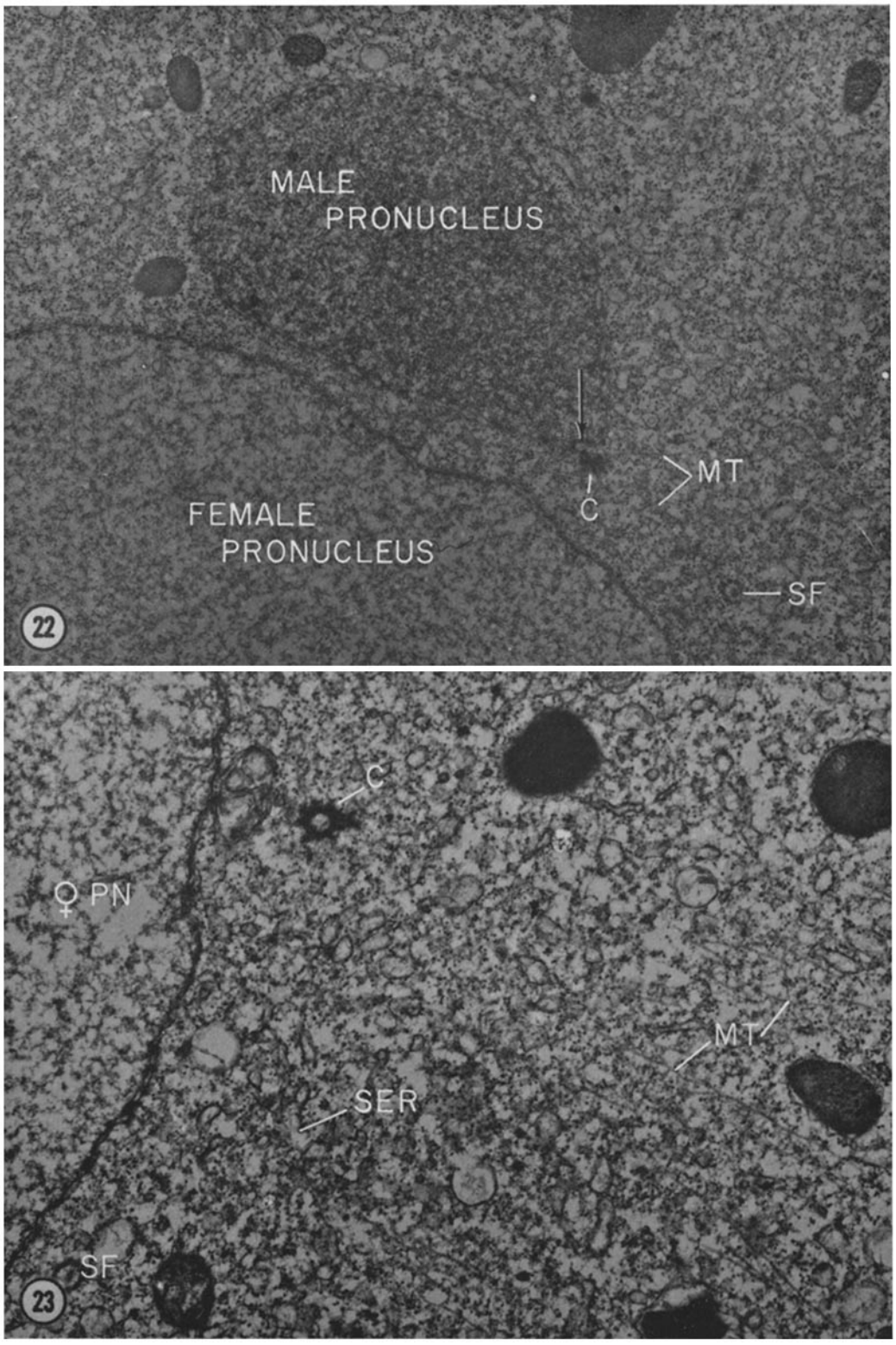


FIGURE 22 A section through the male and female pronuclei in apposition to one another. The centriolar fossa (arrow), its associated centriole (*C*), and microtubules (*MT*) are to one side of the pronuclei. *SF*, sperm flagellum. × 15,500.

FIGURE 23 Section of the centriole (*C*) adjacent to the female pronucleus (♀ *PN*). *MT*, microtubules; *SER*, endoplasmic reticulum of the smooth variety; *SF*, sperm flagellum. × 18,000.

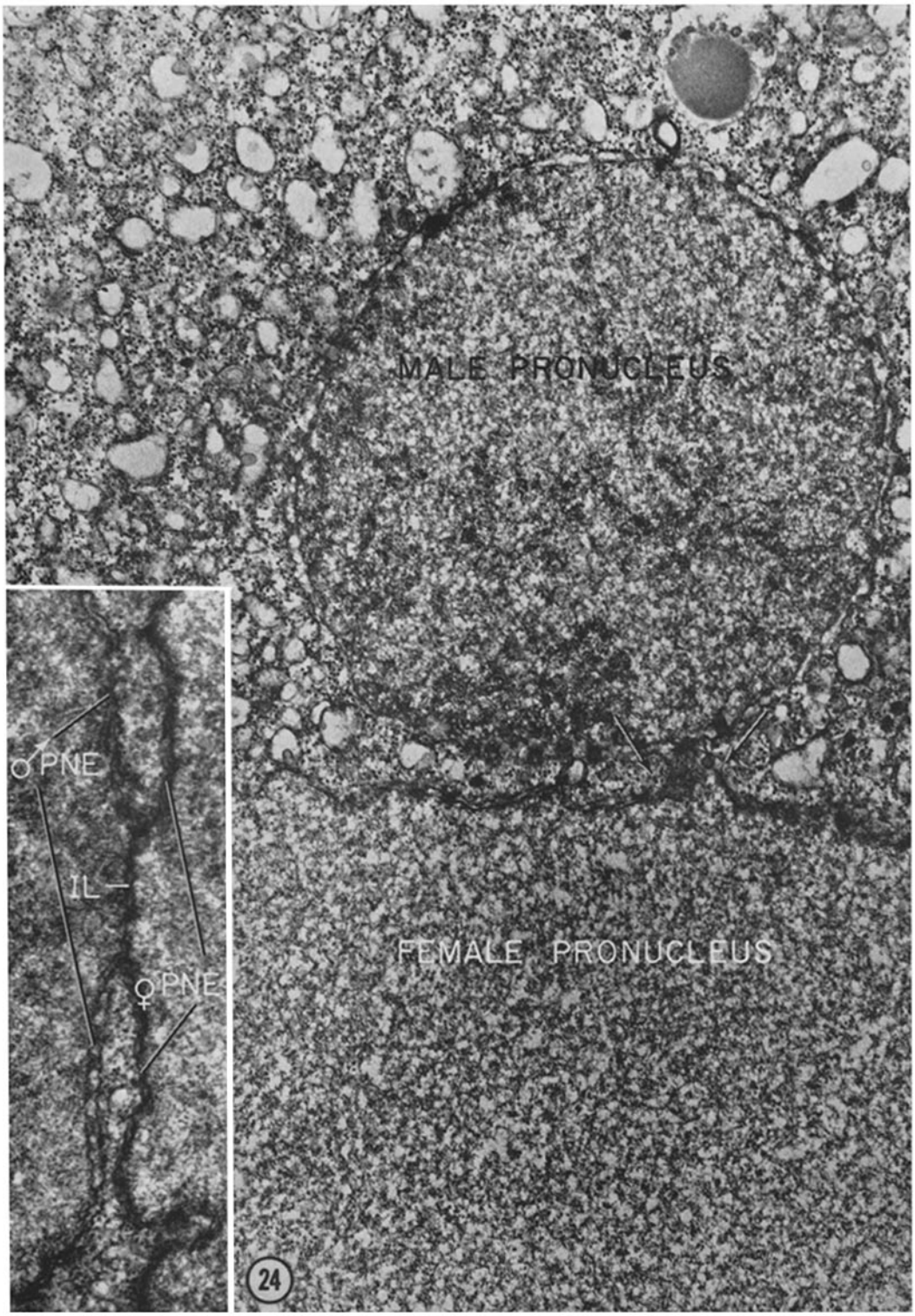


FIGURE 24 Initial stage in the fusion of the pronuclei (arrows denote site). Inset, initial stage of pronuclear fusion. The outer lamina have fused and the inner lamina (*IL*) are parallel to one another. ♂*PNE* and ♀ *PNE*, male and female pronuclear envelopes. × 18,000; Inset, × 20,000.

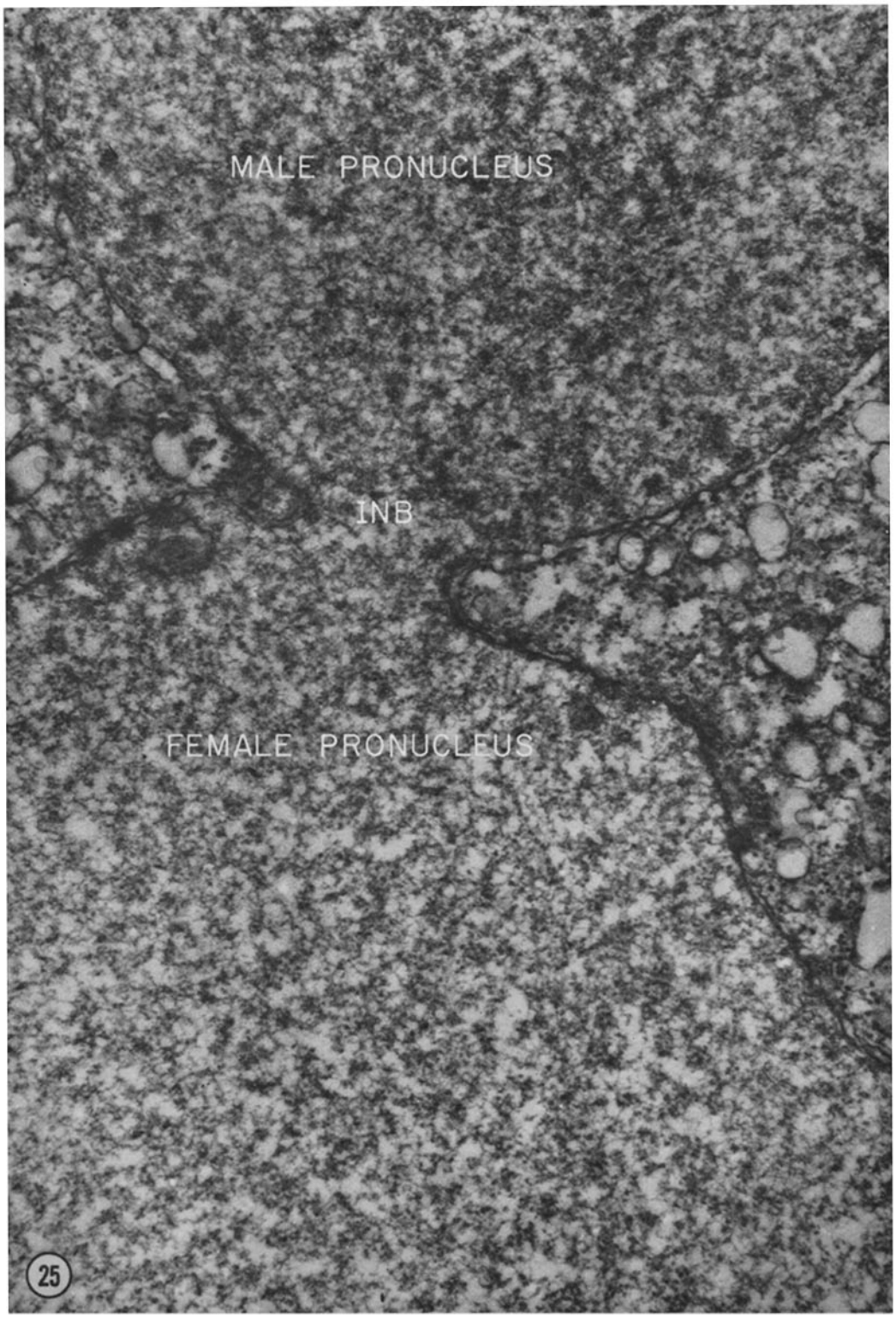


FIGURE 25 Completion of the coalescence of the inner and the outer lamina of the male and female pronuclear envelopes. An internuclear bridge (*INB*) connects the two pronuclei. × 24,000.

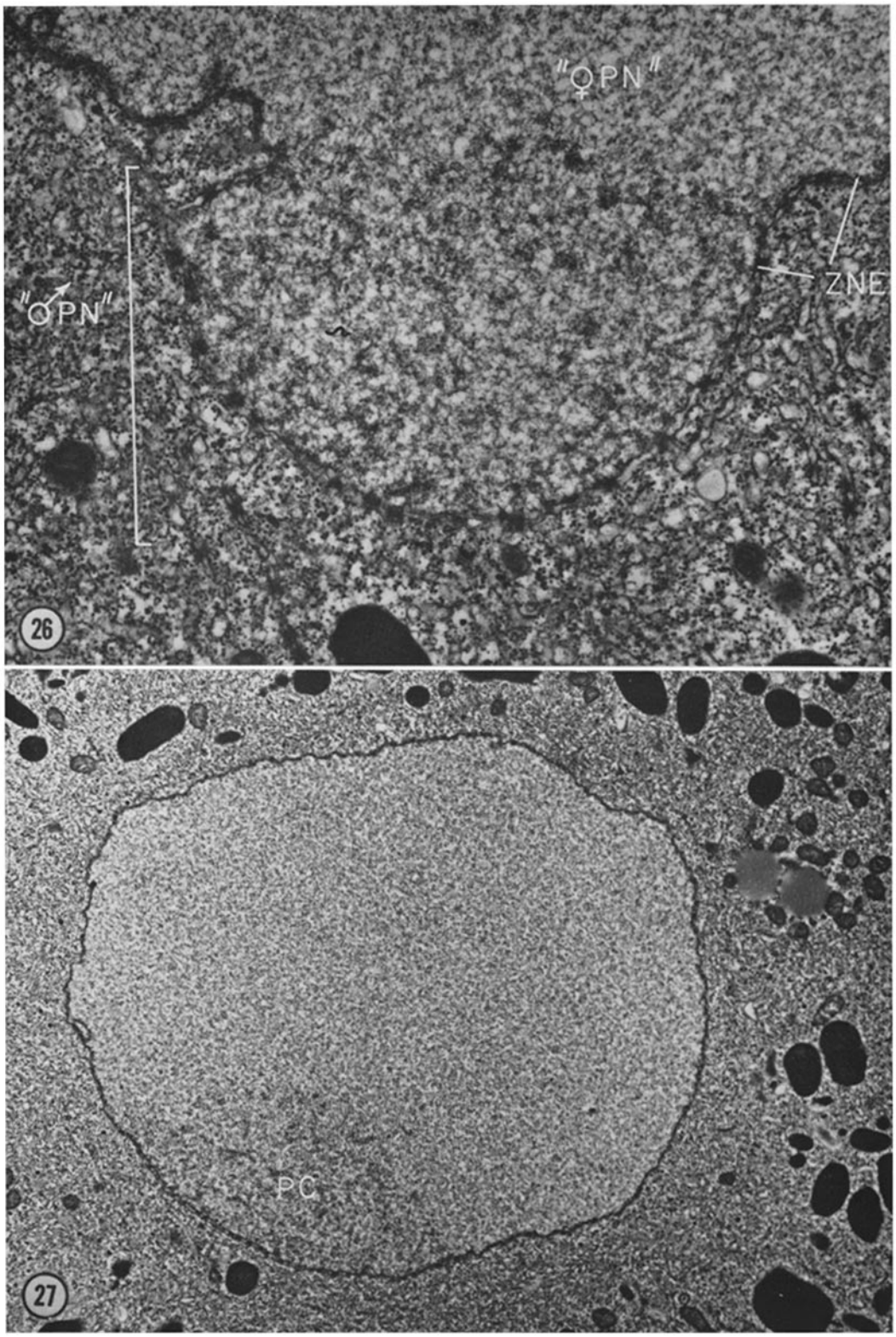


FIGURE 26 Portion of the zygote nucleus containing a protuberance that was formerly the male pronucleus ("♂*PN*"). The perforated zygote nuclear envelope (*ZNE*) is continuous. "♀*PN*," portion of the zygote nucleus that was formerly the female pronucleus. × 18,000.

FIGURE 27 Zygote nucleus containing a dense complement of paternal chromatin (*PC*). × 9,500.

female pronuclei fuse (Fig. 24, inset). Subsequently, the inner lamina contact each other and fuse (Figs. 24, inset, and 25), thereby forming an internuclear bridge between the two pronuclei (Fig. 25, *INB*). At first the bridge is small; however, it gradually increases in diameter (Figs. 25 and 26). Continued enlargement of the internuclear bridge yields an ellipsoid body containing a protuberance that was formerly the male pronucleus (Fig. 26, "♂*PN*"). This large ellipsoid structure is the zygote nucleus. Later the zygote nucleus becomes a spheroid (Fig. 27).

Following the formation of the zygote nucleus, the paternal chromatin is seen as a dense fibrillar band at the site of fusion (Fig. 27, *PC*). However, this material eventually diffuses throughout the zygote nucleus and becomes indistinguishable from that of the female.

Throughout the fusion of the pronuclei, the sperm mitochondrion and the flagellum remain adjacent to the female pronucleus (Fig. 21). Whether or not these structures take part in the process of pronuclear development and fusion is unknown.

DISCUSSION

Formation of the Fertilization Cone

The present fine structural investigation of fertilization in the eggs of *Arbacia punctulata* has shown that at the site of gamete conjugation a fertilization cone is produced. This observation confirms previous microscopic analysis of this phenomena (22, 23, 34, 78, 84, 85). Development of this region appears to be a rather specific surface response of the egg, which involves a segregation of certain ooplasmic components. This segregation is best seen in the production of the apical and basal regions of the fertilization cone. Similar regions have been observed in the entrance cones of inseminated sea urchin oocytes by Runnström (70), Lönning (52), and Franklin (34).

The density and the longitudinal orientation of the fibrils within the fertilization cone of inseminated *Arbacia* eggs suggest a supportive role for these structures. Filaments found within the surface specializations of many cells, for example the microvilli of the intestinal epithelium, suggest a similar function (33). Filaments oriented parallel to the long axis of the fertilization cone have also been observed in the eggs of *Urechis caupo* by Tyler (82), who suggests that these structures may be concerned in the further progression of the sperm into the egg.

Vesicles lining the base of the fertilization cone have been seen in sea urchin oocytes by Runnström (70), Lönning (52), and Franklin (34); however, these authors did not observe rodlike inclusions within these structures.

Entrance and Rotation of the Spermatozoon

It is apparent that the movement of the incorporated spermatozoon is rather complex and not simply one of turning about a fixed axis at the base of the fertilization cone. Upon penetrating the egg, the spermatozoon undergoes a series of movements which include its rotation and lateral displacement from the site of penetration. Furthermore, in favorable sections, the rotated spermatozoon is seen associated with segments of the sperm flagellum which appear to be portions of a broad loop. These observations support previous investigations of sperm incorporation by Dan (28), Allen (3), and the Chambers (17, 19).

With regard to the movements of echinoderm sperm within the egg, Dan (28) found that despite the length of the sperm flagellum in the various species studied, in no case did a sperm nucleus move more than 20 μ from its point of entrance before the development of the sperm aster. These observations suggest that if, upon penetration, the sperm flagellum were freer to move in the ooplasm than were the other sperm organelles, the former would be expected to bend into a broad loop which would bring about the turning of the spermatozoon by 180° (3). In this instance, the spermatozoon would appear to travel through the fertilization cone and peripheral ooplasm along an arched path. Investigations by the Chambers (17, 19) have also demonstrated that the site of sperm aster formation is frequently displaced from the position of the fertilization cone.

Development of the Male Pronucleus

Data collected in the present study have permitted us to divide the events of pronuclear differentiation into the following three steps: (*a*) breakdown of the sperm nuclear envelope, (*b*) dispersion of chromatin, and (*c*) formation of male pronuclear envelope.

BREAKDOWN OF THE SPERM NUCLEAR ENVELOPE: Following the incorporation of the spermatozoon within the ooplasm of the mature egg, there is an incomplete breakdown of the non-

perforated nuclear envelope. This degeneration was observed as the production of vesicular structures along the lateral aspect of the sperm nucleus, while portions of the envelope at the base and apex remain intact. We view the vesicles to be the end result of a process akin to membrane vesiculation as described by Barros et al. (9). Barros and his associates have used the term "membrane vesiculation" to denote "the occurrence of multiple unions between two cellular membranes lying in close apposition, with the formation first of a double-walled fenestrated layer and ultimately an array of separated membrane-bounded vesicles" (see also reference 25). Similar observations have been made by Franklin (34) for the degeneration of the sperm nuclear envelope in the inseminated oocytes of sea urchins. However, the study made by Franklin dealing with sperm incorporation into oocytes is in conflict with the present study with regard to the time at which this breakdown occurs. For example, Franklin (34) observed that the breakdown of the nuclear envelope starts during early stages of penetration and continues as the sperm passes through the fertilization cone of the oocyte. Degeneration of the sperm nuclear envelope in inseminated mature eggs of *Arbacia*, as found in this study, commences at a later stage of incorporation, i.e. just prior to the rotation of the spermatozoon. This temporal difference may be related to the presence of an exaggerated fertilization cone in oocytes used by Franklin (34).

Investigations of sperm incorporation (polyspermic) into mature eggs of the annelid *Hydroides* (22) and the enteropneust *Saccoglossus* (23) demonstrate various nuclear changes which presumably lead to the formation of the male pronucleus. Unlike the situation in *Arbacia*, breakdown of the sperm nuclear envelope in both organisms occurs as the spermatozoon passes through the fertilization cone. This difference in morphogenesis may also be related to the presence of exaggerated entrance cones. Colwin and Colwin (24) have stated that, "in both species (*Hydroides* and *Saccoglossus*) extensive and detailed studies of living material (20, 21) showed that, with the exception of the size and duration of the fertilization cone, there were no discernible differences between monospermy and polyspermy with respect so the early events of syngamy."

As already mentioned, the sperm nuclear envelope remains intact at the apex and the base of the sperm nucleus. One wonders if these membranous structures are specialized portions of the nuclear envelope—specializations brought about by their previous association with the acrosome and centriole. Intact portions of the nuclear envelope located along the surface of incorporated sea urchin sperm nuclei have been previously reported by Rothschild (68) and Franklin (34). Colwin and Colwin (22) have also noted the persistence of the nuclear envelope at both the apex and the base of the incorporated sperm nucleus in *Hydroides*.

DISPERSION OF CHROMATIN: The breakdown of the sperm nuclear envelope exposes the compact sperm chromatin to the ooplasm. It also permits the dispersion of the sperm chromatin, a process that continues throughout the development of the pronucleus. At the end of the dispersal process, the male pronucleus is composed of a rather homogeneous nucleoplasm. Just prior to the conclusion of chromatin dispersion, three zones were recognized on the basis of their densities. The presence of such a pattern of chromatin dispersion suggests that the factor(s) governing this mode of dispersion is peripherally disposed. Pasteels (60, 61) has followed the reorganization of the spermatozoon subsequent to incorporation in the clam *Barnea* and has shown that dispersion of the chromatin appears to occur simultaneously throughout the whole of the sperm nucleus. The difference in scattering of the sperm chromatin in *Barnea* and in *Arbacia* indicates the need for more comparative studies. Indeed, it would be important for future experiments to determine if there were any correlation between the pattern of chromatin condensation during spermiogenesis and the pattern of chromatin dispersion during pronuclear development. Observations of sperm incorporation in the rat egg by Szollosi and Ris (77) also indicate the importance of future correlative experiments, because these investigators have reported various conformational changes of the incorporated sperm chromatin which are reminiscent of those found during spermiogenesis.

The functional significance of the dispersion of the sperm chromatin for the succeeding events is unknown. The relation which exists between DNA synthesis and the pronuclear fusion is uncertain; however, it is possible that dispersion of the sperm chromatin during pronuclear development is necessary for DNA synthesis. It has been demonstrated that the DNA concentration within the pronucleus decreases during its morphogenesis (1, 7, 13), possibly as a result of the dispersion phenom-

enon; however, the total DNA content does double at the end of this developmental period (53). Evidence that DNA replication precedes the first cleavage division has come from various investigators. For example, Alfert (1) reported doubling of the DNA content of pronuclei prior to the first cleavage division in the mouse embryo. Pasteels and associates (27, 62) have confirmed the observation of Alfert in rodents (27) and have demonstrated similar results in the annelid *Sabellaria* (62). Furthermore, DNA synthesis has been found to occur prior to pronuclear fusion in fertilized eggs of the sand dollar *Echinarachnius* (72). Bucher and Mazia (14) have shown that DNA synthesis in the sea urchin, *Strongylocentrotus*, is normally preceded by pronuclear fusion. However, if the pronuclei in the inseminated eggs of the sea urchin *Arbacia* are not allowed to fuse by treatment with either high pressure or Colcemid, the individual pronuclei are capable of DNA synthesis (88, 89).

FORMATION OF THE MALE PRONUCLEAR ENVELOPE: The electron micrographs accompanying this paper reveal that there is an accumulation of smooth-surfaced vesicles along the periphery of the dispersing chromatin prior to the appearance of the pronuclear envelope. We think that these smooth-surfaced vesicles are the forerunners of a portion of the pronuclear envelope. While these structures are smooth surfaced, we have no evidence that they were derived from endoplasmic reticulum of the smooth variety. In any event, it is possible that the vesicles fuse, thereby giving rise to a structure that is initially reminiscent of annulate lamellae (see reference 47). One can imagine that the annulate lamella-like structures become associated with the original portions of the sperm nuclear envelope that remain (the apex and base of the sperm nucleus) throughout the development of the pronuclear envelope. It is not known whether the degenerated portions of the sperm nucleus are destroyed or stored within the ooplasm or whether the observed vesicles are synthesized anew by the egg.

Other investigators in discussing the reformation of the nuclear envelope and the pronuclear envelope have indicated that the endoplasmic reticulum is responsible for the formation of this perforated lamellar structure (8, 15, 35, 42, 59, 61, 66, 86).

Development of the Sperm Aster

It will be recalled that the sperm aster is essentially composed of the following three parts: (*a*) centrioles, (*b*) a centrosphere region composed of disoriented microtubules and vesicular structures, and (*c*) bundles of microtubules and aggregations of lamellar structures radially oriented about the centrosphere. The foremention organization of the sperm aster is equivalent to the mitotic aster described by Harris (36) for the zygote of the sea urchin, *Strongylocentrotus*. The earliest indication of the initiation of sperm aster development is the deployment of pericentriolar structures, accompanied by the separation of the sperm flagellum from its attached centriole. The association of the microtubules and the centrioles is similar to that observed in various cell types (33).

The origin of the microtubules making up the sperm aster is uncertain. Ledbetter and Porter (50) have concluded that "centrioles are apparently not essential to their development, for they are absent in *Pelomyxa* and in plant cells." Nevertheless, Slautterback (73) has described microtubules arising from the periphery of a special centriolar formation in *Hydra*. In addition, investigations by de Thé (29) and André and Bernhard (5) have demonstrated the attachment of microtubules to centriolar satellites. Recently, Rebhun and Bernstein (65) have reported an augmentation in the size of the sperm aster of *Lytechinus pictis* when fertilized eggs are exposed to hexylene glycol. This increase in size is accompanied by an increase in the number of microtubules. Rebhun and Bernstein (65) indicate that augmentation of volume and microtubules in the sperm aster due to hexylene glycol is independent of protein synthesis. Roth and Daniels (67) studied the nature of the mitotic apparatus in ameba and concluded "that the spindle fibers (microtubules) are composed of polymerized, oriented protein molecules that are in equilibrium with and bathed in non-oriented molecules of the same protein."

The pattern of the lamellar and tubular systems found in the sperm aster of *Arbacia* is similar to the relationship that the endoplasmic reticulum assumes with the asters in dividing cells. In a study of lamellar systems in *Drosophila virilis* meiosis, Ito (42) found that the central region of the aster is composed of tubular elements, while the outer astral region is formed of radiating membranous lamellae. Finger-like projections of endoplasmic reticulum extending from the center of the aster have also been observed in dividing sea urchin blastomeres (35). Rebhun (63) has demonstrated the presence of elements of the endoplasmic reticulum in the aster of dividing *Spisula* eggs.

In the present study, elements of the microtubular-endoplasmic reticulum system of the sperm aster were seen to extend to the periphery of the inseminated egg. Whether these structures assist the migration of the sperm aster and male pronucleus and by what mechanism is unknown. However, observations by Chambers (17) and Allen (2) suggest that the growth of the sperm aster may propel the male pronucleus towards the female pronucleus, particularly since the rays of the aster extend to the cortex of the egg (see references 69, 84). Such an astral mechanism for migration has also been proposed by Brachet (12) and Allen (2), based on sperm aster movements in dispermic eggs. In such cases the male pronuclei are "pushed" apart by the development of their asters.

Zimmerman and Silberman (88) and Zimmerman and Zimmerman (89) have demonstrated the importance of microtubular construction for the migration and fusion of pronuclei in the sea urchin. Colcemid and high pressure, which have been employed extensively for the disruption of microtubular structure (55, 79, 80), also prevent the formation of the zygote nucleus (88, 89). Since microtubules may be involved in cytoplasmic motion and elongation (81), high pressure and Colcemid may influence the movement of the pronuclei by affecting the microtubule structure of the sperm aster. Zimmerman and Zimmerman (89) state that "this view is compatible with the concept that either Colcemid or colchicine exerts its effect through interference with polymerization of molecular subunits, which are essential for the formation of fibrous protein structures used in cellular activity."

Encounter of the Pronuclei and Their Fusion

It will be recalled that when the pronuclei meet at the close of their migration, the region of the female pronucleus proximal to the male pronucleus and accessory structures (sperm aster, mitochondrion, and flagellum) becomes flattened and highly convoluted. Whether the response of the female pronucleus is necessarily initiated by the male pronucleus or a more general reaction to the centrioles or the microtubules that make up the sperm aster is unknown. However, a similar phenomenon has been described in a wide number of mitotic cells where the nuclear surface adjacent to the centrioles and pericentriolar microtubules becomes convoluted (15, 35, 59, 66). In any event we view the morphological reaction of the female pronucleus as a type of induction, "nuclear induction."

The specificity of the induction of the male pronucleus and accompanying structures on the surface of the female pronucleus are demonstrated quite clearly in the case of dispermic eggs (Longo, F. J. Unpublished observations). Fine structural studies have shown that when the female pronucleus is encountered on opposite sides by male pronuclei, their sperm aster, mitochondria, and flagella, both regions of the female pronucleus exposed to the advancing male pronuclei and accessory structures undergo the same morphogenesis as found in monospermic eggs.

Deformations of the female pronucleus during migration have also been reported by previous investigators in other echinoderms (see reference 58). For example, observations by Allen (2) on *Psammechinus* eggs fertilized in glass capillaries demonstrate similar morphological changes in the female pronucleus during migration as found in the eggs of *Arbacia*. Allen (2) observed that, following fertilization in *Psammechinus*, the female pronucleus moves toward and elongates in the axis of the capillary tube before it migrates to the male pronucleus. Migration of the female pronucleus is accompanied by a bulging of its surface proximal to the male pronucleus. Monroy (58) has suggested that the surface alterations demonstrated by the female pronucleus during its movements within the ooplasm may indicate the presence of active and rapid exchanges across the pronuclear envelope.

The formation of numerous nucleoplasmic projections by the female pronucleus prior to fusion does not appear to play an important role in this process. The function of these morphological specializations remains obscure.

Fusion of the pronuclei in *Arbacia* is a well-ordered process involving a sequence of membrane fusion, similar to the pattern reported by Jensen (44) for the nuclei of the cotton plant, *Gossypium hirsutum*. In the present study there was no evidence that mass breakdown of the pronuclear envelopes occurs, nor was there any indication of the mechanism by which the inner and the outer membranes of the pronuclear envelopes fuse. Initially, the inner and the outer membranes of the two pronuclei are seen in close apposition, and later they completely join. It is probable that the mechanism of pronuclear fusion is similar to that observed in gamete membrane fusion (25).

According to Boveri (10, 11), the essential point in the fertilization phenomenon is the introduction of the centriole with the sperm, and its subsequent morphogenesis. More specifically, apparently the centriole has to interact with the cortical ooplasm in order to develop and display its activity in a normal manner (71). Lillie (51) expressed the dependency of pronuclear development and the function of the centrioles on an ooplasmic factor very succinctly in the following statement: "the spermatozoon needs itself to be fertilized." What this ooplasmic factor(s) or capacity is remains moot. However, many of the ultrastructural aspects that have been explored in this study demonstrate its capacity to initiate a rather intricate series of events necessary for the formation of the zygote nucleus—hence, the beginning of a new generation.

This investigation was supported by grants (1-F2-GM-36,162-01, and GM-08776) from the National Institutes of Health, the United States Public Health Service.

Received for publication 9 May 1968, and in revised form 21 June 1968.

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