

THE POLYMERIZATION OF ACTIN

II. How Nonfilamentous Actin Becomes Nonrandomly Distributed in Sperm: Evidence for the Association of this Actin with Membranes

LEWIS G. TILNEY

From the Department of Biology, University of Pennsylvania, Philadelphia, Pennsylvania 19174, and the Marine Biological Laboratory, Woods Hole, Massachusetts 02543

ABSTRACT

At an early stage in spermiogenesis the acrosomal vacuole and other organelles including ribosomes are located at the basal end of the cell. From here actin must be transported to its future location at the anterior end of the cell. At no stage in the accumulation of actin in the periacrosomal region is the actin sequestered in a membrane-bounded compartment such as a vacuole or vesicle. Since filaments are not present in the periacrosomal region during the accumulation of the actin even though the fixation of these cells is sufficiently good to distinguish actin filaments in thin section, the actin must accumulate in the nonfilamentous state. The membranes in the periacrosomal region, specifically a portion of the nuclear envelope and the basal half of the acrosomal vacuole membrane, become specialized morphologically in advance of the accumulation of actin in this region. My working hypothesis is that the actin in combination with other substances binds to these specialized membranes and to itself and thus can accumulate in the periacrosomal region by being trapped on these specialized membranes. Diffusion would then be sufficient to move these substances to this region. In support of this hypothesis are experiments in which I treated mature sperm with detergents, glycols, and hypotonic media, which solubilize or lift away the plasma membrane. The actin and its associated proteins remain attached to these specialized membranes. Thus actin can be nonrandomly distributed in cells in a nonfilamentous state presumably by its association with specialized membranes.

It is now clear that actin is one of the most common proteins in nonmuscle cells comprising from 10% to 15% of the total protein in cell types such as the amoeba, blood platelets, and cells of the nervous system (see 30 and 38 for references). Unlike those in skeletal muscle, the actin filaments

in most nonmuscle cells are transitory, appearing when needed, only to disappear at a later developmental stage. Frequently, these stages are separated by only a minute or two. For example, during cytokinesis filaments now known to be actin (14, 27, 33) appear in the cleavage furrow, yet

before and after cytokinesis these filaments disappear and cannot be found. Thus, the actin has to be capable of rapid assembly and disassembly. Since filaments are scarce in most cell types, e.g., platelets, marine eggs, echinoderm sperm, etc., even though the actin concentration is fantastically high, clearly a large percentage of the actin must be present in a nonfilamentous state. For example, when cells are broken, a large proportion of the actin is not sedimentable by conditions which should sediment actin filaments. This same actin can be polymerized into filaments under certain nonphysiological conditions (12, 16), and, if purified further, will polymerize readily if physiological concentrations of salt are added (see references 10, 12, 30, 34, and 38), indicating that the actin has not been altered by the fractionation procedures. Up to now, most investigators have concentrated on those parts of cells in which the actin is largely in the filamentous form, generally avoiding the nonfilamentous state. Since such rapid progress has been made in understanding how the more precise systems operate, namely, skeletal muscle and the flagellum, this is not surprising. Both muscle and flagella, however, are very specialized systems, and their movements are unrepresentative of the more general movements in cells exemplified by the erratic behavior of the footloose amoeba or the bewildering complexity of chromosomal movement. In both cases, there have to be precise controls over the assembly and disassembly of the fibrous proteins, microtubules and actin filaments. Since in many nonmuscle cells the bulk of the actin appears not to be filamentous in vivo, as defined by biochemical and morphological criteria, presumably being monomeric, yet purified actin, from either muscle or nonmuscle sources, will polymerize completely in vitro if physiological concentrations of salt are added, there must be some mechanism to maintain the actin in the nonfilamentous state.

In order to obtain more information on how the actin remains nonfilamentous, we chose as our model system the acrosomal reaction of certain echinoderm sperm (41). Echinoderm sperm, like the sperm of many aquatic species, must penetrate the protective coats which surround the egg. Penetration is achieved partly by a lytic process and partly mechanically. When, for example, *Thyone* sperm come into contact with the jelly of a *Thyone* egg, a remarkable reaction occurs. A process of up to 90 μm in length is generated from the anterior end of the sperm within about 30 s (see

reference 5). With this process the sperm pierces the jelly of the egg, the membrane at its tip then being able to fuse with the plasma membrane limiting the egg. Tilney et al. (41) demonstrated that the newly formed acrosomal process contains actin filaments. Before induction, no actin filaments could be found in the sperm. Since the plasma membrane has little mechanical rigidity, we concluded that the change in the state of the actin, transforming it from the monomeric state to the filamentous state, is responsible for this rapid cell extension. There is also biochemical evidence to support the fine structural observation of the absence of filamentous actin before induction. Most of the actin in unreacted sperm is released when sperm are lysed with the detergent, Triton X-100. Although this same detergent treatment has no effect on F-actin from *Thyone* muscle, nor does it inhibit its polymerization, over 85% of the sperm actin is unsedimentable under conditions which pellet F-actin, indicating that the actin in unreacted sperm does not exist as a filamentous polymer. If the actin is indeed nonfilamentous in the cell, we are faced with two unresolved problems: (a) how does the actin remain unpolymerized since the intracellular salt concentration in vivo is certainly sufficient to match the in vitro requirements for actin polymerization; and (b) how does the actin become sequestered in the anterior end of the sperm during spermiogenesis? The latter is particularly puzzling because in mature sperm the actin, instead of being enclosed in a membrane-limited compartment such as a vacuole or vesicle, is truly cytoplasmic. Thus, if the plasma membrane and the nuclear envelope were to separate, the actin, if not bound in some way, could and would diffuse around the nucleus and into the flagellum. In the accompanying and in subsequent papers, I will attempt to shed some light on the first question, i.e., how the actin remains unpolymerized in vivo. In this paper, I will wrestle with the second question which, stated in general terms, is: can nonfilamentous actin be specifically localized in a cell and, if so, how?

The first step in answering this difficult question is to examine the localization of actin at successive stages in spermiogenesis in *Thyone* sperm. This should give us sufficient information to eliminate several possible mechanisms. For example, in a cell such as a developing spermatid the actin "could" be sequestered in one particular region by polymerization from a specific focus; then, when spermiogenesis is nearly complete and the nuclear

envelope and the plasma membrane are juxtaposed, depolymerization could occur, "trapping" the actin in this specific region. Alternatively, the actin could be localized by its inclusion in a membrane-limited compartment, and so forth. As will be shown below, these mechanisms can be ruled out along with several others. Instead, the actin appears to be sequestered in the cell by its association with other proteins (see the accompanying paper) and with specific regions of membranes. The latter concept, if substantiated, may give us clues as to the organization of the cell cortex.

MATERIALS AND METHODS

Obtaining Material

Male *Thyone briareus* are ripe in Woods Hole from January until mid-June. The testis was removed from *Thyone* and cut into small pieces to allow the sperm to seep out. These pieces were placed in seawater; after a few minutes the seawater was strained through cheesecloth, and the sperm were concentrated by centrifugation (4,000 *g* for 5 min). For studies on spermiogenesis the testis was removed from *Thyone* and fixed immediately. Material was fixed several times during the course of the winter.

Preparation of Pellets of G- and F-Actin

An acetone powder of rabbit skeletal muscle was prepared by extracting the muscle residue after myosin removal with cold acetone at 4°C. The residue was extracted three times, with at least 10 volumes of cold acetone each time. The acetone powder was extracted with water for 30 min at 0°C (1 g was extracted with 25 ml of CO₂-free water at 0°C). The extract was centrifuged at 10,000 *g* for 10 min to remove the undissolved material and then filtered. One aliquot of the extract was then polymerized by the addition of 0.1 M KCl with 10 mM Tris HCl at pH 7.5. The F-actin was collected by centrifugation at 80,000 *g* for 2 h. The other aliquot, containing G-actin, was spun for 24 h at 165,000 *g*. By this time, a pellet is present in the bottom of the centrifuge tube. SDS-gel electrophoresis of this actin shows only a single band (see Fig. 1 of reference 40).

Electron microscopy

The testis was fixed by immersion in 2% glutaraldehyde (Electron Microscope Services, Fort Washington, Pa.) in seawater at room temperature for 1 h. The pH of the seawater (pH 8.0) remained unchanged. The testis was washed in seawater and postfixed in 1% OsO₄ in 0.1 M phosphate buffer for 1 h at 4°C at pH 6.8, washed repeatedly in distilled water, *en bloc* stained with uranyl acetate (0.5% for 2 h at 4°C), dehydrated in acetone, and embedded in Araldite.

The pellets of G- and F-actin were fixed in 2% glutaraldehyde in 0.05 M phosphate buffer at pH 6.8 for 1 h, washed in buffer, and postfixed in 1% OsO₄ in 0.1 M phosphate buffer at pH 6.8 for 45 min at 4°C, dehydrated rapidly in acetone, and embedded as described above.

Sperm which had been treated with distilled water or hexylene glycol were fixed in 1% glutaraldehyde in 0.05 M phosphate buffer at pH 6.8 for 30 min. They were then washed in buffer and postfixed in 1% OsO₄ in 0.1 M phosphate buffer at pH 6.0 for 1 h at 4°C. They were dehydrated and embedded as described above. Sections were cut on a Sorvall Porter-Blum II ultramicrotome (Dupont Instruments, Sorvall Operations, Newtown, Conn.) and stained with uranyl acetate and lead citrate. The sections were examined with a Philips 200 electron microscope.

RESULTS

The Morphology of G- and F-Actin in Thin Section

Although there have been many articles illustrating G- and F-actin by negative staining, to my knowledge no one has yet compared the morphology of G- and F-actin in thin section. Since we would like to know how actin accumulates in the anterior end of the sperm during spermiogenesis and since these stages can only be studied in thin section, it is important to be able to distinguish the morphology of G- and F-actin in thin section.

G-Actin

G-actin in thin section appears as a finely granular material, the bulk of which seems homogeneous (Fig. 1). At high magnifications fine granules can be distinguished, each of which measures about 25 Å in diameter. However, scattered throughout this material are small islands of actin filaments. These filaments stand out from the G-actin whether or not they are oriented longitudinally, obliquely, or transversely. In transverse section they appear as dense dots about 55 Å in diameter (Fig. 1). The appearance of small numbers of filaments in this preparation may be due to minute traces of salt or contaminating tropomyosin, but more likely it is due to the extraordinary concentration of actin that is present in the pellet, a concentration that must exceed 100 mg/ml.

F-actin

When a pellet of F-actin is examined in thin section, instead of seeing a randomly oriented

population of filaments, a situation which does occur at the periphery of the pellet, we usually see strata of actin filaments like those illustrated in Fig. 2. Each stratum is composed of filaments of one orientation; the orientation of the filaments in the next stratum is at 90° to the first, and so on. The image is reminiscent of the packing of collagen fibers in a fish scale (26) and in the cornea (15). Yet, as is true for the small islands of filaments in the pellet of G-actin, the filaments, which measure 55 Å in diameter, are readily identifiable regardless of whether they are in transverse, longitudinal, or oblique section. Thin sections (grey in color) are preferable, as superposition of the filaments is minimal.

Recently, Pollard (personal communication) has demonstrated that he cannot preserve purified actin filaments in thin sections. Preservation of actin filaments is admittedly difficult. We have

found the following: (a) the source of glutaraldehyde is critical; the quality varies tremendously from supplier to supplier and, in fact, from batch to batch in the material from an individual supplier. When we buy a new batch of glutaraldehyde, we "bioassay" it on a biological system which we know has actin filaments, i.e., intestinal microvilli. (b) The *pH* of fixation is important. It is best to fix the specimens in glutaraldehyde at *pH* 7.0 and in osmium tetroxide at *pH* 6.0. (c) The temperature of the osmium tetroxide step also seems important. Undoubtedly, there is tissue variation as well. It is also true that actin filaments are stable to fixation if tropomyosin is present, as is evidenced by the extreme stability of thin filaments in skeletal muscle to any method of fixation. The F-actin described here is unlikely to be stable to fixation because of contamination with tropomyosin, for three reasons: (a) SDS-gel electrophoresis of this

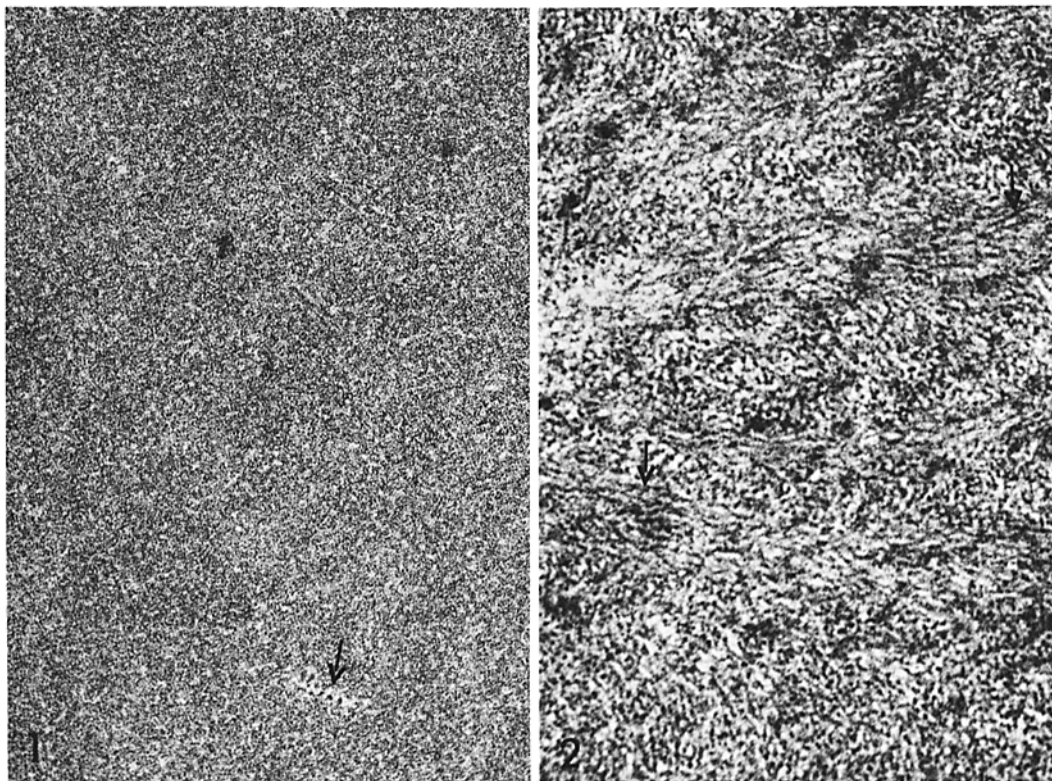


FIGURE 1 Thin section through a pellet of G-actin. The arrow indicates a small population of actin filaments cut in transverse section. $\times 96,000$.

FIGURE 2 Thin section through a pellet of F-actin. The pellet consists of strata composed of filaments of one orientation; the orientation of the filaments in the next stratum is at 90° to the first, and so on. Note that the filaments can be identified irrespective of the plane of the section. Filaments cut in transverse section appear as dense dots. The arrows indicate those cut in oblique section. $\times 96,000$.

material does not show contaminating tropomyosin (see Fig. 1, reference 40). (b) The filaments in this pellet measure 55 Å in diameter while thin filaments of *Limulus* muscle measure 70 Å and actin filaments in *Limulus* sperm with its associated proteins measure 85 Å in diameter. Thus, there is a difference in diameter. This point has been made already (39). (c) The filaments in the acrosomal process (Figs. 4 and 5) are readily observable. There is no tropomyosin associated with these filaments (see reference 41 and the following paper), yet these filaments are well preserved under our fixation conditions. In summary, then, actin filaments can be clearly distinguished from monomeric actin in thin sections (cf. Figs. 1 and 2; both are printed to the same magnification).

A Comparison of the Periacrosomal Material of Thyone Sperm with Pellets of G- and F-actin

The morphology of echinoderm sperm has been previously described (41); it is unnecessary to give more than a brief description here. Lying in an indentation in the nucleus is the spherical acrosomal vacuole; beneath and also lateral to it is the periacrosomal material (Fig. 3) which we know is composed largely of actin (41, 37). The periacrosomal material appears finely granular. Its morphology does not resemble that of the F-actin pellet (cf. Fig. 2 with Fig. 3), but instead is similar to that of the pellet of G-actin (cf. Fig. 1 with Fig. 3). A section through the acrosomal process (where the actin is polymerized), on the other hand, whether it is cut longitudinally (Fig. 4), transversely (Fig. 5), or obliquely, appears identical to portions of sections of the pellets of F-actin (cf. Fig. 2 with Figs. 4 and 5). These observations are consistent with our earlier studies (41) in which we concluded that before the acrosomal reaction the actin was stored in the nonfilamentous state. Since the actin is nonfilamentous in unreacted sperm but filamentous in reacted sperm, we will refer to the periacrosomal material subsequently as profilamentous actin or "profil. actin." This name refers not only to the actin, but also to its associated proteins as well. These proteins will be discussed in the following paper.

There is one exception to the nonfilamentous nature of profil. actin. If the plane of section passes precisely through the middle of the acrosomal vacuole and its associated periacrosomal region,

i.e. it runs through the center of the sperm from the anterior end to the posterior end, I frequently (because it is impossible to judge the exact plane of section, this phenomenon may be true in all sperm) see a small bundle of filaments extending posteriorly from the basal end of the acrosomal vacuole into the periacrosomal region. These filaments are only about 30–40 nm in length. They do not appear to contact the nuclear envelope. Similar filaments have been described in starfish sperm (36). More will be said about these filaments in the following paper in this series.

Membrane Specialization in Thyone Sperm

There are two membrane specializations to which I would like to draw attention as they pertain to the ensuing discussion of spermiogenesis. The first occurs on the membrane limiting the acrosomal vacuole. This membrane is differentiated into apical and basal halves. The basal half is thicker and more dense (Fig. 3) (9). The increased thickness of the basal half is due to a layer of dense material which is plastered against the inner or vacuolar surface. The second membrane specialization of interest is the nuclear envelope. This is particularly prominent in the periacrosomal region. Unlike the situation in most cells, in echinoderm sperm the outer and inner nuclear membranes approach each other and form a coupled complex which, at low magnification, appears as a single membrane. At higher resolution it is clear that the outer dense line of the inner nuclear "unit" membrane actually fuses with the inner dense line of the outer nuclear unit membrane (Fig. 6). I am using the word "fuse" here in a loose sense. Clearly, I do not know any details of the fusion process, i.e., do the lipids that make up the membrane actually mix or not? This fusion produces a central osmiophilic line corresponding to both fused membrane surfaces. Often, this fusion occurs at small foci adjacent to which the outer and inner nuclear envelopes approach each other but do not fuse (Figs. 3 and 6).

Spermiogenesis

The single gonad of *Thyone* consists of numerous tubules which are united into a tuft attached at the left side of the dorsal mesentery (see 13 for details). When mature, the yellow testis occupies most of the body cavity. The tubules are covered by a serosal layer of epithelial cells beneath which are some muscle cells. The seminiferous tubules

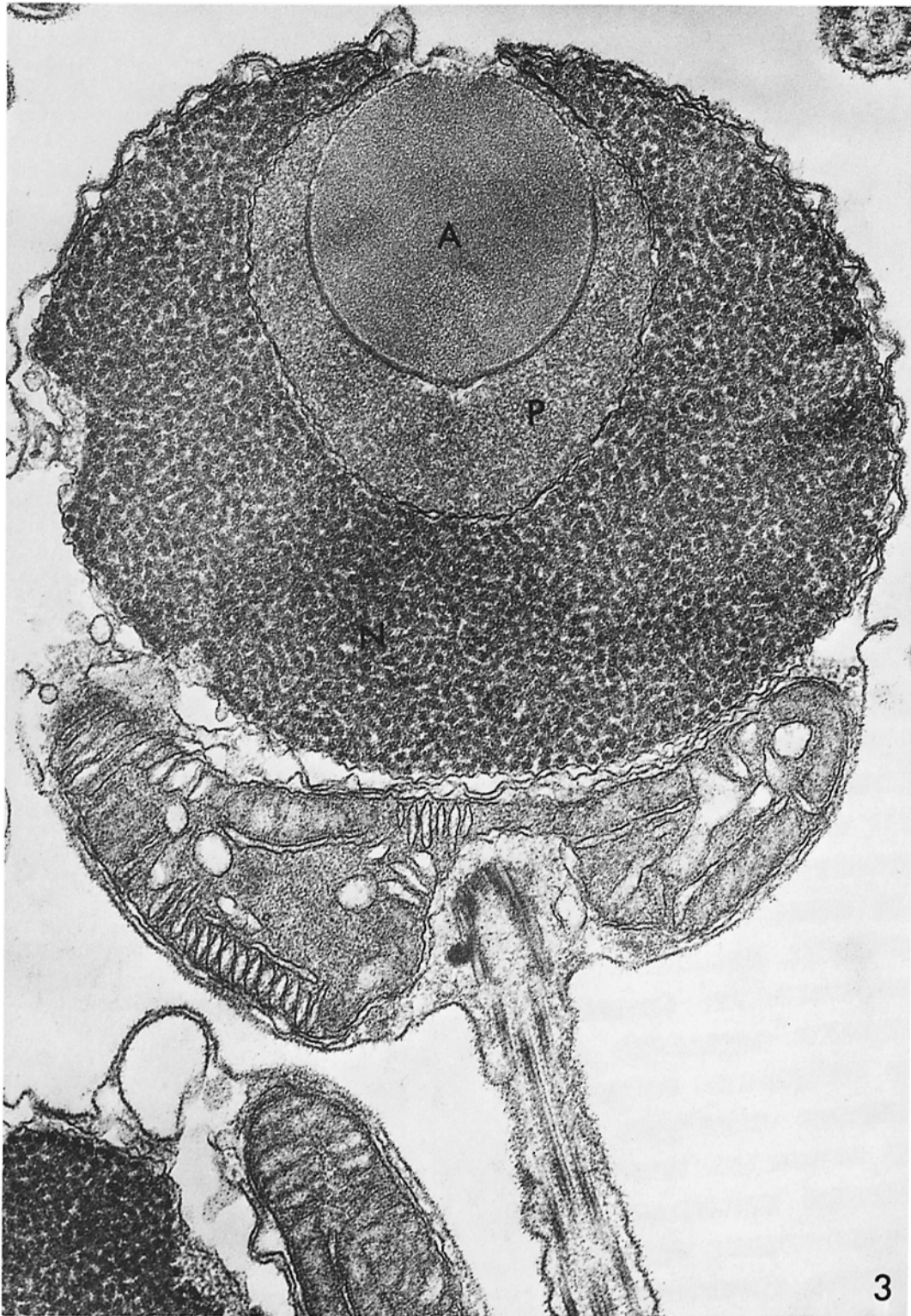


FIGURE 3 Thin section through a mature *Thyone* sperm. Lying within an indentation in the nucleus (*N*) is the spherical acrosomal vacuole (*A*), and beneath and lateral to it is the periacrosomal material, profil. actin (*P*). Note that the periacrosomal material closely resembles the granular material in the pellet of G-actin in Fig. 1. $\times 92,000$.

are separated from this covering layer by a thick connective tissue space. Directly beneath the seminiferous tubule is a basement membrane. As one progresses inward from the basement membrane

into the lumen of the seminiferous tubule, one can see cells in various stages of differentiation. Nearest the basement membrane are the large spermatogonia, and in the center of the lumen are mature sperm. We will confine our description to the maturation of the spermatids and in particular to the events related to the accumulation of the periacrosomal material because stages in the accumulation of the periacrosomal material occur only during spermatid maturation.

It is relatively easy to determine the stage of maturation of a spermatid in thin section, because a number of separate events occur simultaneously, e.g., a gradual loss of organelles, condensation of the nucleus, migration of the acrosomal vacuole, and indentation of the nucleus. These markers facilitate a reconstruction of the sequence of stages leading to the accumulation of actin in the periac-

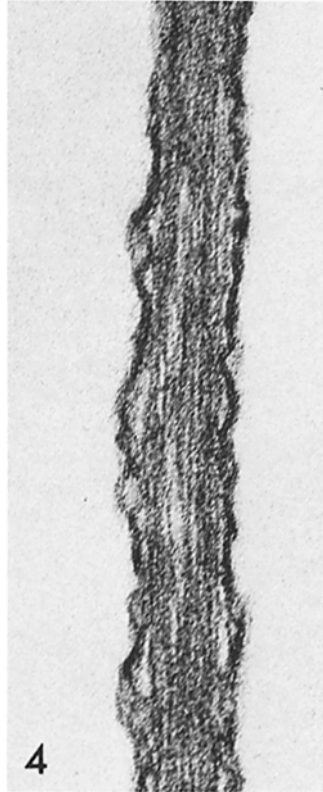


FIGURE 4 Longitudinal section through the acrosomal process. Beneath the limiting membrane is a large population of filaments. $\times 120,000$.

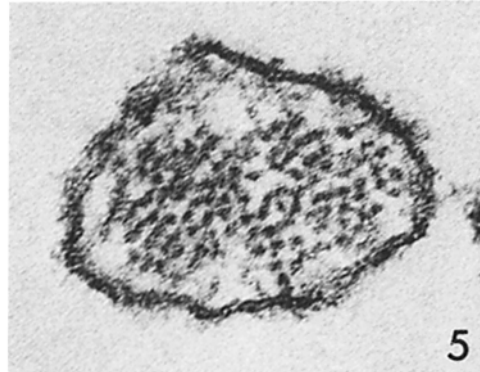


FIGURE 5 Cross section through the acrosomal process. The dense dots beneath the limiting membrane are cross sections through the actin filaments. $\times 210,000$.

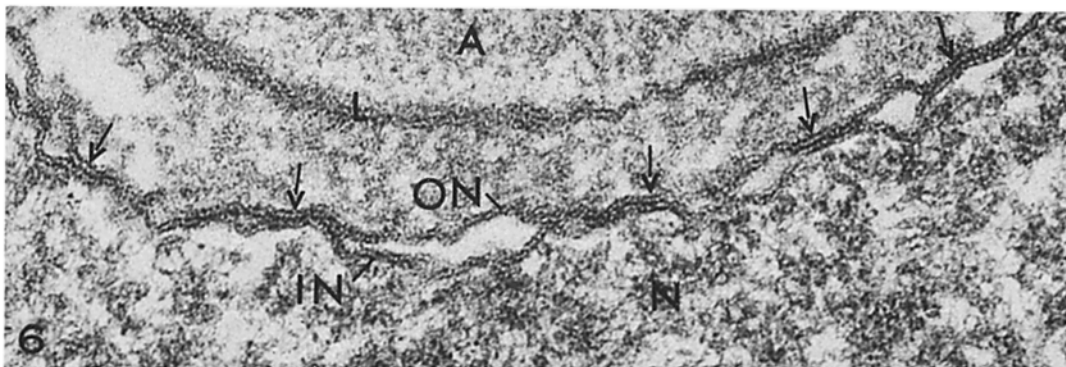


FIGURE 6 Thin section through a portion of the nuclear envelope and the acrosomal vacuole membrane in the periacrosomal region. The nucleus is identified by the letter, *N*; the contents of the acrosomal vacuole by the letter, *A*. Of particular importance is the relationship between the outer nuclear membrane (*ON*) and the inner nuclear membrane (*IN*). These membranes fuse at regions indicated by the arrows. $\times 220,000$.

rosomal region. In essence, each cell is labeled as to its position in spermatid differentiation.

EARLY SPERMATID DEVELOPMENT: Each developing spermatid is connected to at least one other by a slender cytoplasmic bridge. Usually, four spermatids are connected together; all four cells illustrate the same degree of maturity. At the very end of spermatid differentiation these bridges are broken.

One of the first events in spermatid differentiation is the formation of the acrosomal vacuole. As has been documented by many investigators beginning in the 1890's (3, 6, 23), this vacuole is formed by the coalescence of numerous small vesicles produced from the Golgi apparatus (see Fig. 7 *a*). An examination of many stages in the formation of the acrosomal vacuole demonstrates that even at the earliest stage the membrane limiting the vacuole is differentiated such that half of it is thicker than the other half. As already discussed, this is due to a deposition of dense material against the vacuolar surface of its limiting membrane. In the mature sperm it is this thickened surface that will face the periacrosomal region and the nucleus, while the thinner surface will face the anterior end of the sperm (9). During the acrosomal reaction, then, the thinner surface will fuse with the plasmalemma; the thicker surface will ultimately form the tip of the acrosomal process.

During the formation of the acrosomal vacuole the thickened surface characteristically faces away from the Golgi apparatus (Fig. 7 *a*) (6). Furthermore, the small Golgi-derived vesicles which fuse to form the acrosomal vacuole are generally found in association with, and in suitable micrographs, are seen fusing with only that surface of the vacuole which possesses the *thinner* or less dense membrane. As the vacuole increases in size, it also increases in electron density as if the material within it were being concentrated at the same time.

Not only is there a differentiation in the membrane limiting the acrosomal vacuole in these early spermatids, but also there is a local specialization of the nuclear envelope at this stage. That portion of the nuclear envelope farthest from the Golgi region and its associated acrosomal vacuole appears very dense at low magnification (Fig. 7 *a*). At higher magnification (Fig. 7 *b*) it is obvious that the increased density is due to the fusion of the inner and outer nuclear membranes which produces a membrane composed of three dense lines. Of interest, however, is that over the rest of the

nucleus the inner and outer membranes are separated by 200–300 Å. The local specialization persists throughout spermatid differentiation. Only at the last stages in the maturation of the sperm do the adjacent surfaces of the two nuclear membranes fuse along their entire lengths. What is particularly significant is that at this early stage in spermatid differentiation, these cells are programmed such that the future location of the profil. actin has already been determined. The acrosomal vacuole migrates to this specialized region as does the actin and its associated proteins.

Some differentiation of the cytoplasm is also obvious at this early stage. The nucleus is not centrally located, but in fact occupies one side of the cell, with the bulk of the cytoplasm lying near the Golgi region. What is remarkable is that the cytoplasm adjacent to the specialized portion of the nuclear envelope lacks organelles; there are no mitochondria or vesicles in this region, nor are there ribosomes (Fig. 7 *a*). Instead, the thin rim of cytoplasm associated with the specialized portion of the nucleus appears to consist of an amorphous material. Some filaments can be seen in this region as well, although they most frequently appear in extensions from the cell surface.

MID-SPERMATID DEVELOPMENT: The main event at this stage is the migration of the acrosomal vacuole from the Golgi region to the apical end of the cell (an event originally documented in hemipterans [3]) where it will be positioned with respect to the nuclear membrane specialization. How the movement of the acrosomal vacuole occurs remains unsolved. All that can be said is that in some cells at this stage I see large numbers of microtubules in locations where they might act in assisting the migration of the acrosomal vacuole (Fig. 8).

The acrosomal vacuole is positioned such that the dense membrane portion of the vacuole points towards the specialized portion of the nuclear envelope (Figs. 8 and 9). As before, the specialization of the nuclear envelope consists of the fusion of the outer and inner nuclear membranes to form a single membrane consisting of three dense lines. The remainder of the nucleus is unspecialized, with the inner and outer nuclear membranes being separated by 200–300 Å. In some spermatids the outer and inner membranes do not fuse along the whole length of the periacrosomal region. Instead, small points of fusion occur in this region. I suspect that the lack of complete fusion here may be

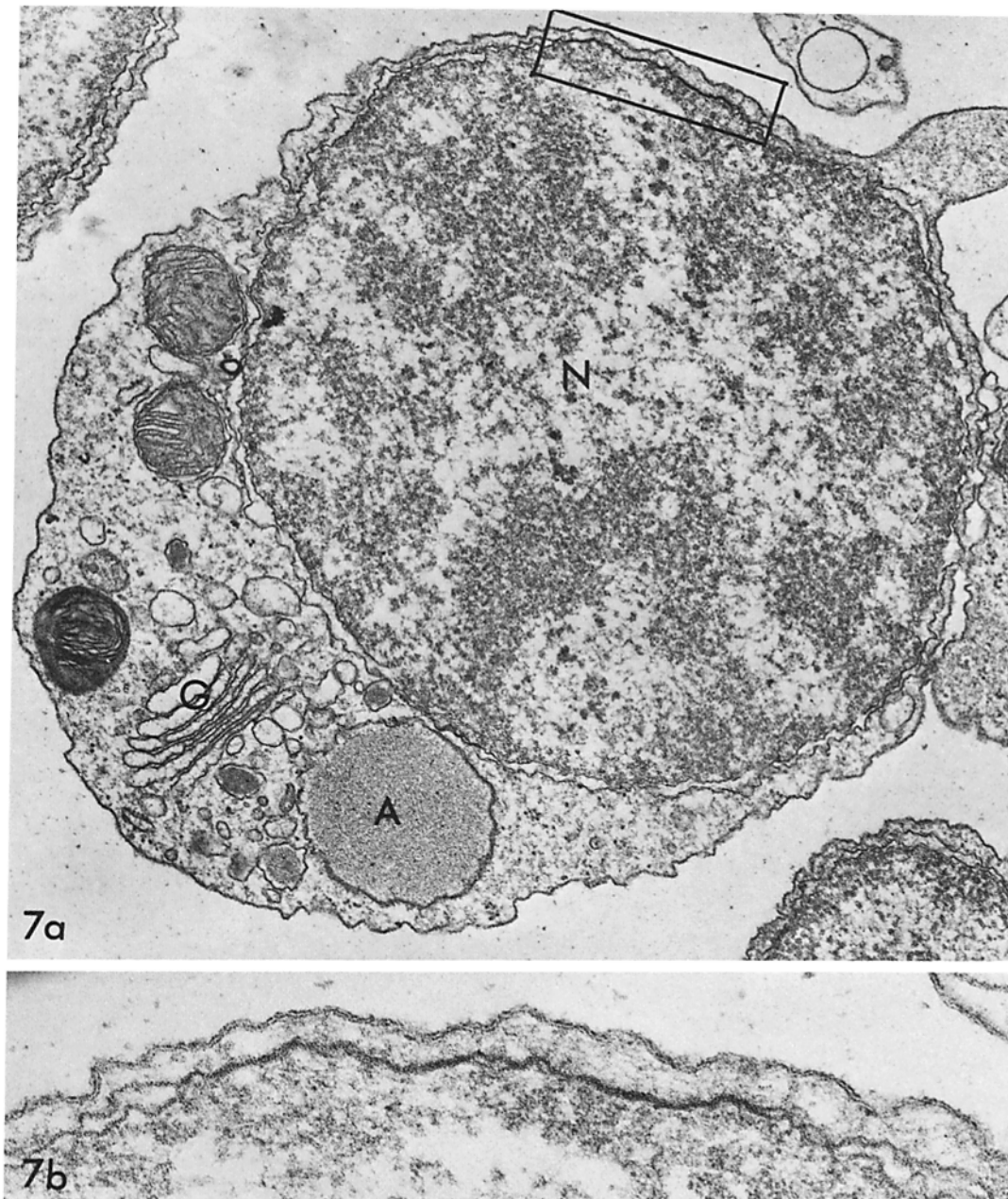


FIGURE 7 (a) Thin section through an early spermatid of *Thyone*. Beneath the nucleus (*N*) is the Golgi apparatus (*G*). Adjacent to the Golgi apparatus is the forming acrosomal vacuole (*A*). Note that the membrane which limits the acrosomal vacuole has a thickened region farthest from the Golgi apparatus. This thickened portion of the vacuole membrane will ultimately be in contact with the profil. actin. The nuclear envelope on the opposite side of the cell, away from the Golgi apparatus, is specialized. In this region (indicated by the box) the outer and inner nuclear membranes fuse. $\times 31,000$. (b) The region indicated by the box in figure 7 a at higher magnification. $\times 112,000$.



FIGURE 8 Thin section through a spermatid slightly later in development. By this stage in spermiogenesis the acrosomal vacuole (*A*) has migrated from the Golgi apparatus to the opposite pole of the cell. Note that the thickened portion of the acrosomal vacuole membrane points toward the nucleus (*N*). The nuclear envelope in the region of the acrosomal vacuole is also thicker due to the fusion of the outer and inner nuclear membranes. Along the lateral margin of the cell is a large population of microtubules (*mt*). $\times 44,000$.

related to fixation difficulties, since in some mature sperm a similar lack of fusion exists (Fig. 6).

Once the acrosomal vacuole has migrated and has been positioned, the nucleus begins to indent (Fig. 9). Between the acrosomal vacuole and the nucleus is an amorphous material. This material is much less densely packed than the periacrosomal

material in mature sperm (cf. Figs. 3 and 9), and no cytoplasmic organelles, even small ones such as ribosomes, are present. This material is continuous with the basal cytoplasm of the spermatid by a thin neck of cytoplasm which courses around the apicolateral margin of the nucleus (Fig. 9). Similar material is present throughout the rest of the

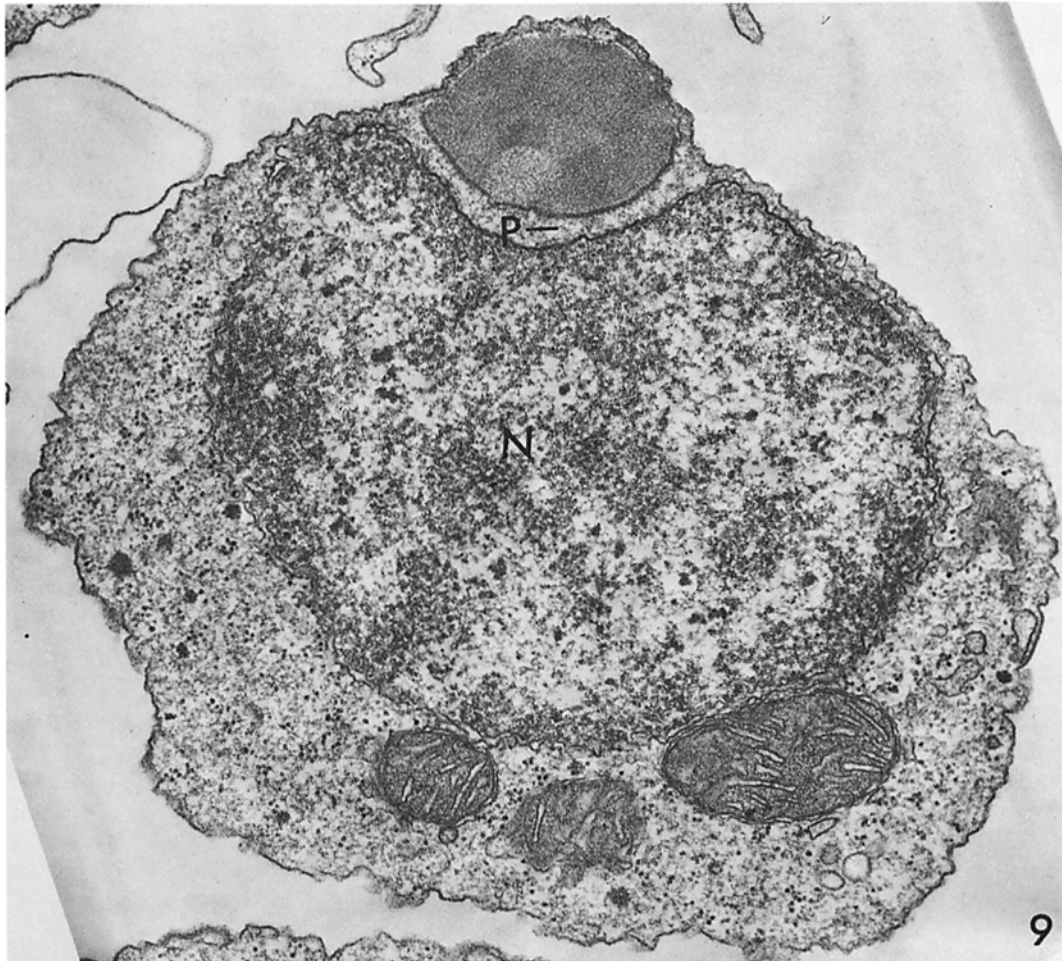


FIGURE 9 Thin section through a spermatid in a stage of development just preceding the accumulation of the periacrosomal material. By this stage the nucleus (*N*) has begun to indent beneath the acrosomal vacuole. Of particular interest is that the bulk of the cytoplasm, which includes the ribosomes, is found at the basal end of the cell. The periacrosomal region (*P*) is free of organelles. $\times 50,000$.

cytoplasm. There is no evidence of any filaments in the periacrosomal region.

LATE SPERMATID DEVELOPMENT: We are now in a position to inquire how the actin is transported to the periacrosomal region. There are three possibilities. Transportation could occur through the nucleus. This is extremely unlikely since there are no nuclear pores in the periacrosomal region and few around the remainder of the nuclear envelope. Secondly, transportation to the periacrosomal region might occur by the actin being carried within the acrosomal vacuole itself, only to be released into the periacrosomal region at a late stage. This possibility can also be

discounted because there is no change in the density or in the size of the acrosomal vacuole from the time of its formation in association with the Golgi apparatus until its appearance in mature sperm. Furthermore, the volume occupied by the periacrosomal material is greater than the volume occupied by the acrosomal vacuole, thus the formation of the periacrosomal material from the acrosomal vacuole is just not possible. Therefore, the periacrosomal material (largely actin) must be transported via the thin necks of cytoplasm which skirt the lateral surfaces of the nucleus. It is probably worthwhile to repeat at this point that since there are no ribosomes (and thus no protein

synthesis) in the periacrosomal region, even at an earlier stage in spermatid differentiation, the accumulation of the actin in this region must involve the movement of the actin itself, not just unsynthesized material.

An examination of many micrographs of sections through spermatids at various stages in the accumulation of the profil. actin reveals that at each stage the material that accumulates closely resembles the profil. actin seen in mature sperm (cf. Fig. 3 with Fig. 10). In fact, the material resembles the pellet of G-actin (cf. Fig. 1 with Fig. 10 b). *At no stage was I able to identify filaments in the periacrosomal region.* Thus the actin does not seem to accumulate in this region by its transformation into actin filaments.

Concomitant with the accumulation of the profil. actin is a further indentation of the nucleus and condensation of the chromatin. At this stage the basal cytoplasm is gradually being eliminated. Many of the organelles are sequestered in what appear to be myelin figures (Fig. 10 a). The latter are pinched off. Flagella begin to form from one of the centrioles which lie near the Golgi apparatus. The apical end of the nuclear envelope remains specialized in the periacrosomal region. This specialization now extends laterally somewhat so that the outer and inner nuclear membranes fuse over much of the anterior end of the nucleus. The final events of spermatid differentiation involve progressive condensation of the nucleus, sloughing off of the remaining basal cytoplasm, extension of the flagellum, and separation of the spermatids.

The Association of Nonfilamentous Actin with Membranes

From the above results, it is clear that the actin cannot accumulate in the periacrosomal region either by its conversion to F-actin or by its being sequestered in a membrane-bounded compartment. There is only one mechanism which could account for the transport and accumulation of the actin in this region of the differentiating spermatid. This mechanism takes into account the specializations on the acrosomal vacuole membrane and the nuclear envelope. If the actin were to associate and bind to these specialized membranes either by itself or with other substances, it would be effectively trapped in this region of the cell. With time, therefore, more and more actin would accumulate here by random diffusion. We now know that the

actin is, in fact, associated with two other proteins which maintain it as an isolatable aggregate (a description of these proteins is included in the following paper; see also 37 and 38). Thus, to strengthen this hypothesis, what is needed is some data demonstrating that actin and these two other proteins bind to the specialized membranes. A first step would be to see if the profil. actin remains associated with the specialized portions of the nuclear envelope and the acrosomal vacuole membrane after the removal of the plasma membrane.

TREATMENT OF THE SPERM WITH HYPOTONIC MEDIA: When a suspension of sperm in seawater is diluted with distilled water (one part seawater to five parts distilled water), the axoneme is rapidly retracted into the cell body where it wraps around the nucleus. At the same time the cell swells due to the increase in cell surface caused by the retraction of the axoneme (in deflagellated sperm, swelling is minimal). By phase-contrast microscopy three bodies can be distinguished within the swollen membrane. These are the mitochondrion, the nucleus, and a small, dense, spherical body which, in untreated sperm, is located adjacent to the mitochondrion. Generally, but not always, the acrosomal vacuole ruptures, releasing its contents. When it does not rupture, it remains attached to the nucleus via a phase-dense material which, of course, is the periacrosomal region. The important point is, however, that the phase-dense material remains associated with the nucleus for many hours even at room temperature; it neither disperses nor floats free as a unit (Fig. 11). In fact, it is this material which enables us to distinguish the nucleus from the mitochondrion. By electron microscopy it is easy to confirm the fact that the profil. actin remains intact. Its morphology is indistinguishable from that of the periacrosomal material seen in untreated cells (cf. Figs. 3 and 12). It remains associated on its basal surface with the nuclear membrane and on its apical surface with at least a portion of the acrosomal vacuole membrane. The latter tends to fragment. If profil. actin did not associate with itself as well as with the nuclear envelope and the acrosomal vacuole membrane, it should disperse into the large space that surrounds the nucleus and mitochondrion. In the situations where the acrosomal vacuole does not rupture, it is difficult to see why the vacuole and the nucleus do not separate unless they both interact with profil. actin since the vacuole no

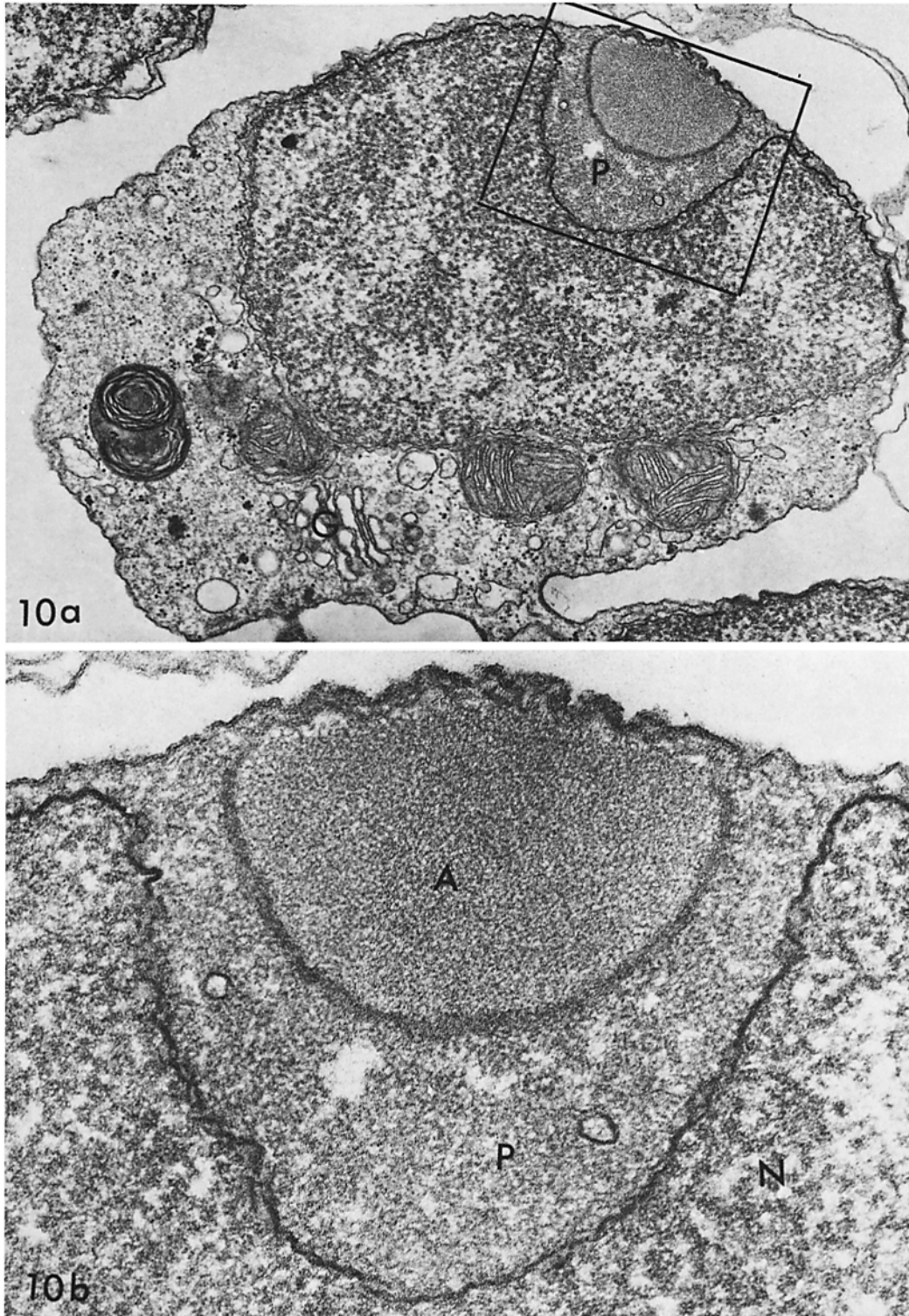


FIGURE 10 (a) Thin section through a spermatid midway in the accumulation of profil. actin (*P*). Note that the Golgi apparatus (*G*), the mitochondria and all the ribosomes are found at the basal end of the cell. The box indicates the region of the cell which is illustrated at higher magnification in Fig. 10 *b*. $\times 30,000$. (b) The periacrosomal region at higher magnification. Lying within an indentation within the nucleus (*N*) is the profil. actin (*P*) and the acrosomal vacuole (*A*). Of particular interest is the absence of filaments in the periacrosomal region. Instead of showing filaments, this region contains an amorphous material which closely resembles the pellet of G-actin illustrated in Fig. 1. The outer and inner nuclear membranes have fused in the periacrosomal region. $\times 96,000$.

longer adheres to the plasma membrane. On the other hand, after the plasma membrane has lifted away, the mitochondrion, which in treated sperm is located posterior to the nucleus, can be found anywhere in this membrane compartment, even anterior to the nucleus. Thus, unlike the acrosomal vacuole, this organelle *is not attached to the nucleus in situ*.

So far, I have been unsuccessful in isolating the nucleus with its attached profil. actin free from the plasma membrane because of the rigidity of the plasma membrane. Sufficient shear to rupture this membrane (either a very tight-fitting glass homogenizer or an omnimixer must be used) ruptures the nucleus as well, thereby liberating the DNA which then forms a gel. Sonication is also unsuccessful.

TREATMENT OF THE SPERM WITH HEXYLENE GLYCOL: When sperm are immersed in 1 M hexylene glycol in 10 mM phosphate buffer at pH 6.4, the plasma membrane ruptures and in most sperm the nuclear envelope is lost as well, except in the periacrosomal region. In this region the nuclear envelope remains intact and is still associated with profil. actin. Fragments of the basal surface of the acrosomal vacuole membrane also remain associated with the profil. actin (Fig. 13). The apical portion of the acrosomal vacuole membrane is lost, however.

TREATMENT OF SPERM WITH THE DETERGENT, TRITON X-100: When sperm are treated with 1% Triton X-100 (Schwartz/ Mann Div., Becton, Dickinson & Co., Orangeburg, N.Y.) at alkaline pH (30 mM Tris HCl, 3 mM MgCl₂, and 0.1 mM EDTA at pH 8.0), the plasma membrane and acrosomal vacuole membrane are solubilized, but the nuclear envelope in the periacrosomal region remains intact. Outside of this region, the chromatin is sometimes covered by the inner nuclear membrane, the outer nuclear membrane being solubilized. In other regions both membranes are solubilized. The bulk of the profil. actin is solubilized as well (Fig. 14). Invariably, however, there is a thin layer of profil. actin associated with the nuclear envelope in the periacrosomal region. This material cannot be washed away even with repeated washings.

If sperm are treated with 1% Triton X-100 at acid pH (pH 6.4), all the membranes are solubilized. However, if 1 M hexylene glycol is added to a 1% solution of Triton X-100 in phosphate buffer at pH 6.4, all the membranes are solubilized, except the basal half of the acrosomal vacuole mem-

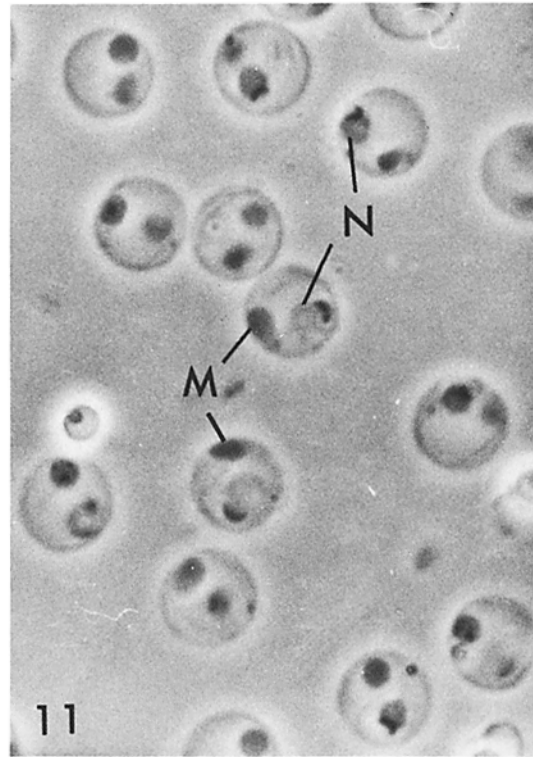


FIGURE 11 Phase-contrast micrograph of sperm which have been treated with distilled water. Within the membrane are two large bodies, the nucleus (*N*) and the mitochondrion (*M*). Attached to the nucleus is a phase-dense cup which is the periacrosomal material. $\times 3,300$.

brane and the nuclear envelope (both inner and outer membranes) in the region containing the profil. actin. In this region the membranes are usually unaffected as is the profil. actin (Fig. 15). In certain sperm the profil. actin cup pops out. On its surface remain remnants of the outer nuclear membrane and the acrosomal vacuole membrane (Fig. 16). Thus, the profil. actin remains associated with these specialized membranes.

From the preceding experiments with hypotonic media, detergents, and hexylene glycol, it is clear that the profil. actin remains associated with and presumably tightly bound to specialized portions of the nuclear envelope and the acrosomal vacuole membrane. These observations support the hypothesis that the actin is concentrated in the anterior end of the sperm during spermiogenesis by its association with these specialized membranes in the periacrosomal region.

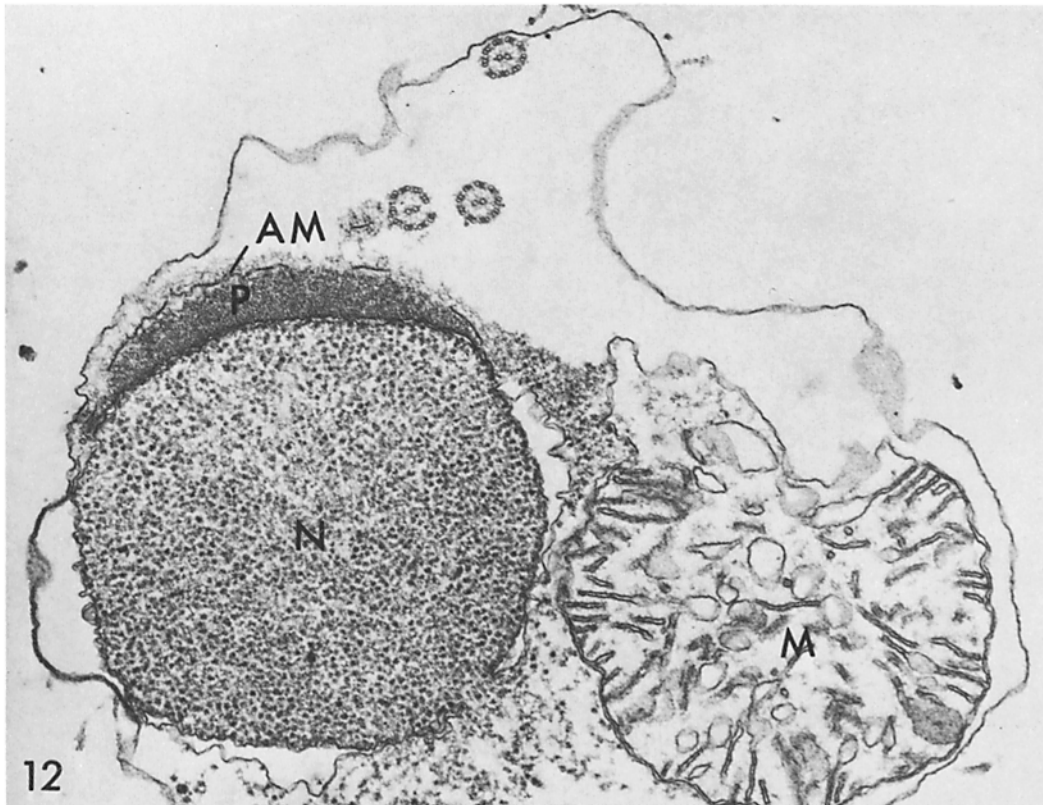


FIGURE 12 Thin section through a sperm which has been treated with distilled water. Remaining attached to the nucleus (*N*) is profil. actin (*P*). Fragments of the basal surface of the acrosomal vacuole membrane (*AM*) remain attached to this material. The swollen mitochondrion (*M*) is present here as well. $\times 33,000$.

DISCUSSION

Once the acrosomal vacuole has migrated to the anterior end of the sperm and the nucleus has begun to indent, the profil. actin begins to accumulate. Filaments cannot be found in the periacrosomal region during the accumulation of this material. Since I can preserve filaments in the acrosomal process of reacted sperm, it cannot be argued that I am not seeing filaments because of imperfections in fixation techniques. In fact, I showed that the accumulating profil. actin, which is largely actin (37, 38), appears morphologically similar to a pellet of G-actin. Since all the protein synthetic machinery (ribosomes and mitochondria) as well as almost all the cytoplasm is located in the basal end of the cell, the actin, not precursors to actin, i.e., amino acids, must be transported from here to the anterior end of the sperm. How this transportation is effected remains unestablished. Yet, not

only can the actin be concentrated in one part of the cell, but it can be sequestered there; for, if the plasma membrane is removed by any of a variety of treatments, the actin does not disperse, but remains aggregated. In fact, the profil. actin remains associated with a specialized portion of the nuclear envelope and a thickened portion of the acrosomal vacuole membrane. Both of these specialized membranes are formed before the accumulation of profil. actin. There is one model which is consistent with all the data presented here and which explains how the actin is transported and sequestered in one localized part of the cell. The model is as follows: early in spermatid differentiation, that portion of the nuclear envelope which will face the periacrosomal region becomes specialized as does that portion of the acrosomal vacuole membrane which is associated with the periacrosomal region, such that they have attached to their surfaces substances that will bind actin

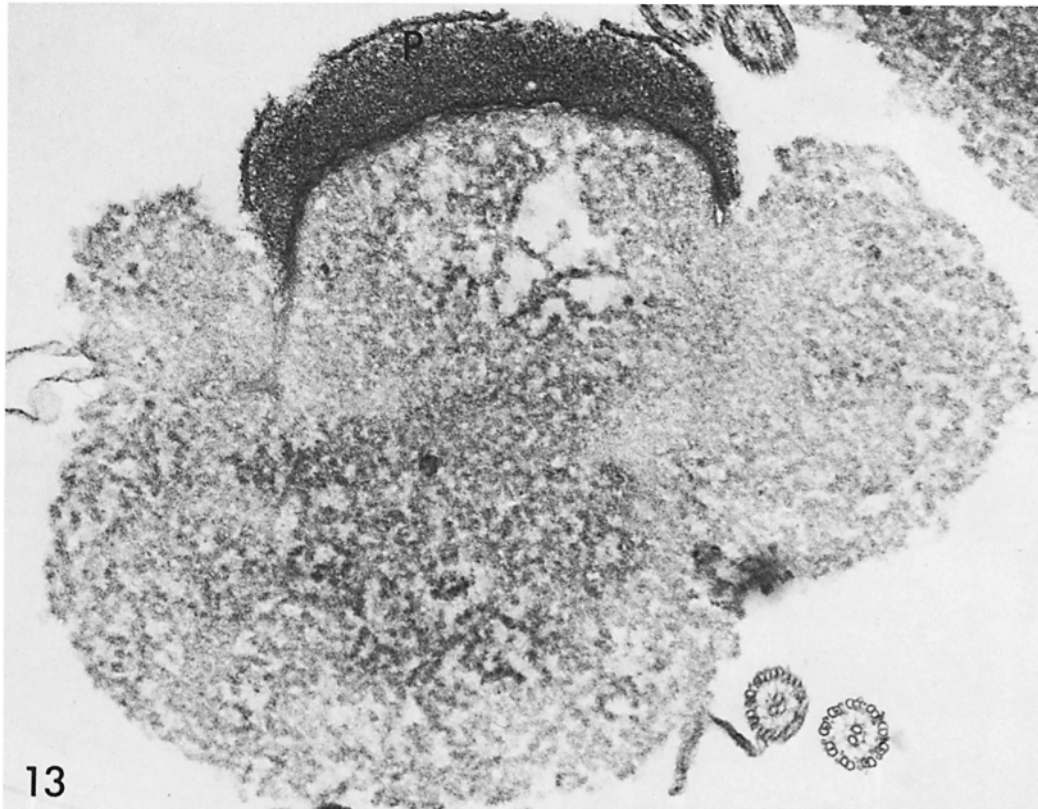


FIGURE 13 Thin section through a sperm which has been treated with 1 M hexylene glycol. The plasma membrane is solubilized by this treatment. The profil. actin (*P*) remains attached to the nuclear envelope. Fragments of the acrosomal vacuole membrane remain as well. Except in the periacrosomal region, the nuclear envelope is solubilized. $\times 52,000$.

and/or its associated proteins. We now know that the profil. actin is composed of actin and a pair of proteins whose mol wt are 250,000 and 230,000 (37, 38). Whenever actin and this pair of proteins approach these specialized membranes by random diffusion from the basal end of the cell, they will bind. Gradually, they accumulate here, being in essence trapped by their association with these specialized membranes or attached substances so that back diffusion cannot occur. This model is consistent, of course, with the observation that when the plasma membrane is solubilized or pulled away from the nucleus by a variety of agents such as hypotonic media, detergents, or glycols, the profil. actin remains associated with these specialized membranes. In fact, when the profil. actin floats free from the nucleus, it remains attached to fragments of these specialized membranes.

To test this model further, it is necessary to

demonstrate that the profil. actin will bind to these specialized membranes *in vitro*, yet to no other membranes such as the basal portion of the nuclear envelope or to mitochondrial membranes, etc. At present, all I can say is that profil. actin is closely associated with these specialized membranes. To carry out the *in vitro* binding of actin and its associated proteins requires that these specialized membranes be stripped absolutely clean of residual profil. actin, for, if a small residuum of this material remains, then in the reassociation experiments we can conclude only that the profil. actin is capable of sticking to itself, a fact which we already know (see the next paper in this series). So far, I have been unable to "wash off" all traces of the profil. actin (see Fig. 14). A thin layer of this material always remains tightly associated with the specialized portion of the nuclear envelope. Of course, the fact that this material cannot be



FIGURE 14 Thin section through a sperm which has been treated with 1% Triton X-100 in 30 mM Tris, 3 mM $MgCl_2$, and 0.1 mM EDTA at pH 8.0. This treatment solubilizes the plasma membrane and the nuclear envelope except in the periacrosomal region. In this region the nuclear envelope remains intact. Whereas the bulk of the periacrosomal material is solubilized under these conditions, a thin layer of this material always remains associated with the intact portion of the nuclear envelope. $\times 73,000$.

completely washed off indicates that it probably is, in fact, bound.

Even though this model is very attractive and, in fact, must be at least partially correct, there are many unresolved problems. The two major ones are: (a) how can a membrane store positional information, a subject that has intrigued many investigators, but for which there is no information available, and (b) if actin and its associated proteins interact with each other, why do they not just form an aggregate anywhere in the sperm instead of attaching only to these specialized membranes? An analogous situation exists in red cell ghosts. In that system the actin is associated with two proteins, spectrin (40), which will be shown in the following paper to have molecular

weights and characteristics very similar to those of the two proteins associated with the actin in the periacrosomal region in the sperm. Whereas spectrin and actin associate *in vitro* as determined by viscometry and light scattering (40) *in vivo* they associate with the plasma membrane as well. There are a number of possible mechanisms that could account for the association of these proteins with the plasma membrane in both the red cell and the sperm, but since there is no data available, I will not burden the reader with speculation.

The specialization of the nuclear envelope in early spermatids deserves additional comment. Besides asymmetries in the nuclear envelope caused by an unequal distribution of nuclear pores, evidence is gradually accumulating which demon-

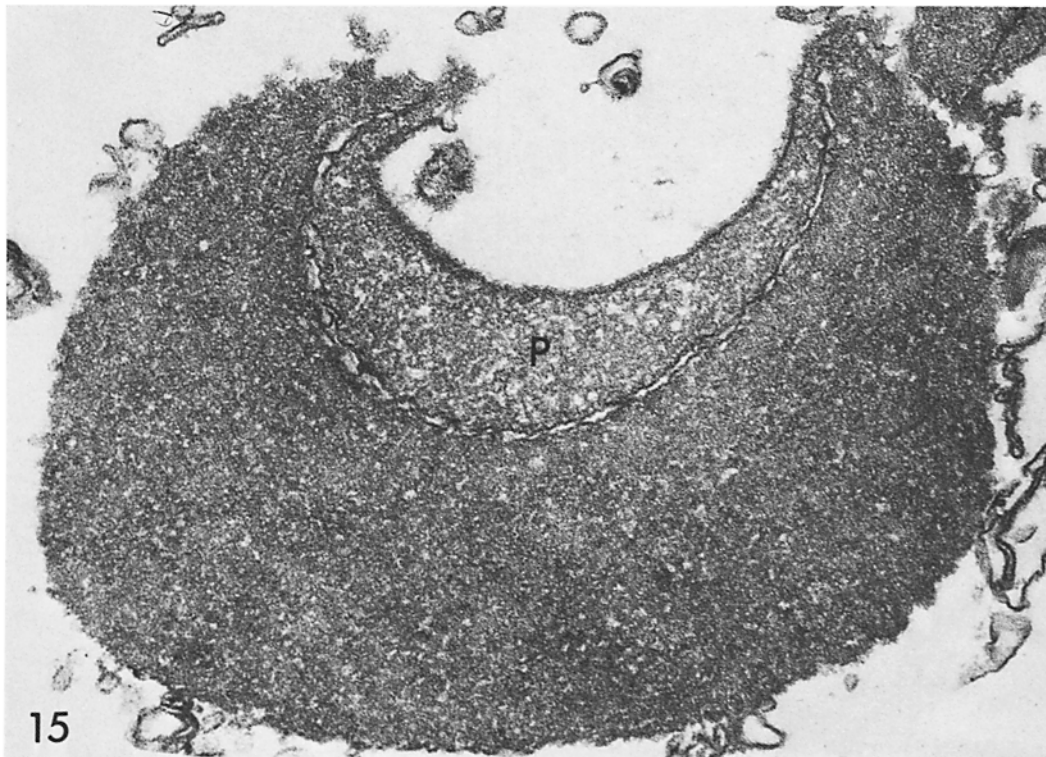


FIGURE 15 Thin section through a sperm which has been treated with 1% Triton X-100 in 10 mM phosphate buffer at pH 6.4. This treatment solubilizes all the membranes except the nuclear envelope in the periacrosomal region and the basal portion of the acrosomal vacuole membrane. The profil. actin (*P*) remains associated with these remaining membrane fragments. $\times 57,000$.

strates that the nuclear envelope should not be thought of as an inert bag surrounding the chromatin, but actually may contain positional information. Thus, portions of the nuclear envelope can attach to chromatin or structural proteins located within the nucleus; they can attach to certain structural proteins outside the nucleus and, what is perhaps even more surprising, there is now evidence that structures within the nucleus communicate through the nuclear envelope with structural proteins outside the nucleus (2). Since, in most cases, the associations with the nuclear envelope are not random, but instead are located at discrete points along the nucleus, we must conclude that the nucleus can contain positional information which in turn might lead to the nonrandom distribution of other materials, i.e. actin. Let me give examples to illustrate this point. In yeasts, certain algae, fungi, and certain protozoans, an intranuclear spindle forms whose microtubules attach to plaques that are attached to the nuclear

envelope. In certain Heliozoa such as *Actinophrys* (20) and *Echinospaerium* (21, 31, 32, 42) the ends of the microtubules in the axoneme make direct contact with the outer nuclear membrane. During spermiogenesis in many organisms the chromatin condenses while it is attached to discrete portions of the nucleus envelope (1, 19, 22, 35); microtubules are associated with the outer nuclear membrane in these regions. In certain arthropod sperm, the microtubules are attached by short bridges to specialized regions derived from the nuclear membrane (29, my unpublished observations). During spermiogenesis in dragon fly spermatids (18) and in the squid *Loligo* (2), microtubules are connected to the nuclear envelope by short arms or bridges.

Particularly interesting to this report is Kessel's (18) elegant study on dragonfly spermatids. Kessel showed that in developing spermatids the nuclear envelope presents a scalloped appearance brought about by the nuclear membrane being thrown into

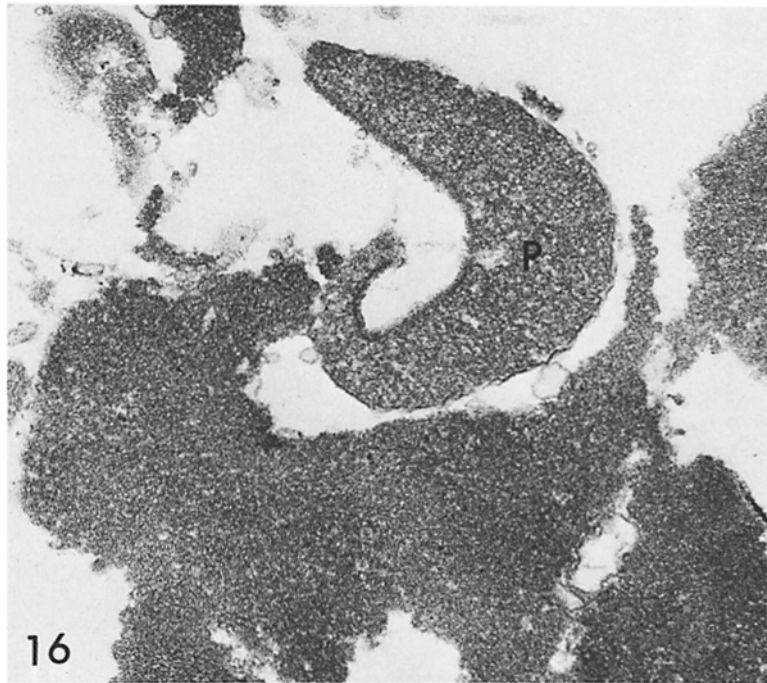


FIGURE 16 Thin section through a portion of a sperm treated as described for Fig. 15. In certain sperm such as this one, the profil. actin (*P*) becomes free from the chromatin. On its surface remain remnants of the nuclear envelope and the acrosomal vacuole membrane. The profil. actin maintains its characteristic cup shape. $\times 62,000$.

alternate furrows and ridges. Microtubules are restricted to the furrow regions where they are connected to the outer nuclear membrane by short arms. It is only in the furrow that the two nuclear membranes (outer and inner) approach each other and fuse and thus, at low magnification, appear more dense. At the ridges where there are no microtubules, the outer and inner nuclear membranes are separated by their normal distance of 200–300 Å. During spermatid differentiation in certain annelid worms such as *Tubifex* (7) or *Spirobis* (31), as in the dragon fly, the microtubules are associated only with that portion of the nuclear envelope in which fusion of the inner and outer membranes has taken place. The similarity between the observations on dragon fly and annelid spermiogenesis and those on spermiogenesis in *Thyone* where the profil. actin is associated with only that portion of the nuclear envelope where the outer and inner nuclear membranes fuse is striking. Thus, the nuclear envelope and the plasma membrane (the acrosomal vacuole membrane is a latent form of the plasma membrane, as

during the acrosomal reaction it forms the tip of the acrosomal process) are capable of differentiation along their surfaces. These membrane specializations suggest that in many diverse systems membranes may be coded in order to influence the nonrandom distribution of fibrous proteins in cells.

One wonders if the specialized portions of the nuclear envelope and the acrosomal vacuole membrane contain an unusual distribution of particles as determined with freeze-fracture techniques. Logically, there is no reason why these membranes should have an unusual distribution of particles. First, actin and/or its associated proteins need not necessarily attach to substances which are embedded within the lipid bilayer. It may be sufficient for the proteins to attach to material that is connected to the polar regions of the lipid molecules on the cytoplasmic surface of the plasma membrane. Secondly, the technique of freeze fracturing does not have resolution adequate enough to detect anything but dense aggregates of proteins. If small protein molecules or polypeptides were to span the

membrane, these would be "invisible" by this technique. Based upon the molecular weight of proteins known to cross or be embedded in the lipid bilayer, i.e., glycoporphin in red cell ghosts, rhodopsin in artificial vesicles, and the six connexin molecules which form each particle in a gap junction), the minimum size for a visible particle must be at least 100,000 daltons. Thus, profil. actin may be bound to these specialized membranes either by its association with molecules outside the lipid bilayer or by its association with molecules which pass through the lipid bilayer but whose mol wt are less than 100,000, or both of the above. When I examined *Thyone* sperm by freeze-fracture techniques, it was clear that there is not an unusual distribution of particles on either of the nuclear membranes (my unpublished observations). This disappointing result is not surprising, for the reasons given above. In fact, it is becoming increasingly apparent that particles are not related to the intra- or extracellular distribution of materials. For example, the "split diaphragm" connecting adjacent podocytes in the glomerulus are not related to a special distribution of particles (17), nor during capping is the concanavalin A receptor associated with particles in the membranes (27, 28). Recently, we (Mooseker and Tilney [25]) examined three systems in which actin filaments are attached to membranes. These included the attachment of actin filaments to the membrane at the tips of the microvillus and the attachment of actin filaments to the basal surface of the acrosomal membrane in *Mytilus* and *Limulus* sperm. In all three cases there are no particles in the region where these filaments make contact with the membrane.

Profil. actin also appears to be associated with the basal or thickened half of the acrosomal vacuole membrane. This Golgi-derived membrane will become confluent with the plasma membrane when the sperm is induced to undergo the acrosomal reaction. Thus the basal or thickened portions of this membrane will ultimately be located at the tip of the newly formed acrosomal process, with its thinner or apical portion contributing to the lateral surface of the acrosomal process. The acrosomal vacuole membrane, therefore, can be considered as a stored part of the plasma membrane. Thus, the profil. actin is associated with the future plasma membrane. This concept is intriguing when we realize that most cells have a gelated surface layer or cortex. This is particularly true in marine eggs

on which much work has been carried out in an attempt to define the gelated cortex. One mechanism to account for the gelation of the cortex involves a profil. actin-like material, i.e., actin and its associated high molecular weight proteins associating with the plasma membrane. In fact, the isolated cortex of sea urchin eggs contains actin and a high molecular weight protein (my unpublished observations). Thus, the interwoven network that has been so elegantly described by Buckley and Porter (4) in preparations of tissue culture cells that were fixed, critically point dried, and examined with high voltage microscopy may in fact be actin and its associated proteins. It may also be no coincidence that when the plasma membrane fraction of cultured cells, namely HeLa cells, is run by SDS-gel electrophoresis, not only is actin present (8), but also there is a pair of bands whose molecular weights resemble that of the proteins associated with the actin in sperm. Similar proteins are present in myoblasts, chondroblasts (my unpublished observations), and human erythrocytes (40). The human erythrocyte is a particularly interesting case, as the actin in the red cell appears to function in conjunction with spectrin (a pair of proteins similar in molecular weight to the proteins in *Thyone* sperm) to form an amorphous network beneath the cell surface which could help to stabilize the plasma membrane. In essence, the actin and the spectrin in erythrocytes form a thin cortical layer attached to the membrane. The attachment of nonfilamentous actin and its associated proteins may give support to the plasma membrane and as well may have other important biological functions. The most obvious is that almost all the important activities with which actin is associated in animal cells occur in relation to membranes. For example, cells undergo cytokinesis, they phagocytose, they secrete, they send out long processes such as the acrosomal process or exploratory pseudopodia, they transport substances by increasing the surface area for absorption by moving microvilli (24), they contract parts of their surfaces in clot retraction, etc. All these functions require that the actin filaments must be bound to the plasma membrane either directly or by some intermediate molecule such as α -actinin or myosin. Thus, by storing the non-polymerized actin in a position where it is available for most of the important cell functions, the cell is capable of rapidly changing from one state to another. I do not mean to imply that actin is not

used in the medullary regions of cells, for, clearly in plant cells and "primitive" cells such as the amoeba, movement can be provided by such medullary actin. Yet, the fact remains that most of the work done by the animal cell requires the contractile apparatus to be tightly coupled to the plasma membrane. Thus, the attachment of non-filamentous actin to the plasma membrane may serve two purposes, that of stabilizing the cell surface and that of storing the actin in a location where it is readily available to polymerize and perform work.

It is my great pleasure to thank Dr. John Fuseler for numerous enthusiastic discussions. He pointed out to me the membrane specialization of the nuclear envelope in the periacrosomal region in my micrographs and was instrumental in helping to develop the interpretation presented here. I also wish to thank Robert Kyrka for his preparation of the pellet of G-actin and Mrs. Doris Bush for her unfailing assistance in cutting suitable sections for electron microscopy. My appreciation also goes to Ray Stephens, Mark Mooseker, Robert Kyrka, and Patricia Detmers for their participation in active discussions.

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