Actin Filaments Elongate from their Membrane-associated Ends

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ABSTRACT In *Limulus* sperm an actin filament bundle 55 μm in length extends from the acrosomal vacuole membrane through a canal in the nucleus and then coils in a regular fashion around the base of the nucleus. The bundle expands systematically from 15 filaments near the acrosomal vacuole to 85 filaments at the basal end. Thin sections of sperm fixed during stages in spermatid maturation reveal that the filament bundle begins to assemble on dense material attached to the acrosomal vacuole membrane. In micrographs of these early stages in maturation, short bundles are seen extending posteriorly from the dense material. The significance is that these short, developing bundles have about 85 filaments, suggesting that the 85-filament end of the bundle is assembled first. By using filament bundles isolated and incubated in vitro with G actin from muscle, we can determine the end "preferred" for addition of actin monomers during polymerization. The end that would be associated with the acrosomal vacuole membrane, a membrane destined to be continuous with the plasma membrane, is preferred about 10 times over the other, thicker end. Decoration of the newly polymerized portions of the filament bundle with subfragment 1 of myosin reveals that the arrowheads point away from the acrosomal vacuole membrane, as is true of other actin filament bundles attached to membranes. From these observations we conclude that the bundle is nucleated from the dense material associated with the acrosomal vacuole and that monomers are added to the membrane-associated end. As monomers are added at the dense material, the thick, first-made end of the filament bundle is pushed down through the nucleus where, upon reaching the base of the nucleus, it coils up. Tapering is brought about by the capping of the peripheral filaments in the bundle.

A common feature of actin filaments in nonmuscle cells is their association with membranes, in general the plasma membrane. This association is essential for such basic phenomena as cytokinesis, filopodial and microvillar movements, elongation of cell processes (e.g., the acrosomal process), and retraction of cell extensions such as occurs during clotting. To understand how the motility is generated and controlled we need to determine the polarity of the actin filaments, because this defines their possible direction of movement. We also want to know how the cell establishes this polarity with respect to the plasma membrane and how the filaments elongate. Are they, for example, nucleated from some membrane-associated protein, or are the filaments assembled from a cytoplasmic organelle and secondarily connected to a site on the membrane? Do these recently nucleated actin filaments, once connected to the membrane, elongate by the addition of monomers to the membrane-associated end of the filament, or do they elongate by the addition of monomers to the cytoplasmic end of the filament? There are reasonable arguments against both alternatives in the last question. For example, it is difficult to imagine how monomers can add onto the membrane-associated end and how at the same time the filament can maintain its association with the membrane. On the other hand, we know from the initial studies of Woodrum et al. (22), Hayashi and Ip (8), and Kondo and Ishiwata (10) and from the more recent studies of Pollard and Mooseker (personal communication), Wegner (21), and Tilney and Kallenbach (20) that monomers add much more readily to one end of an actin filament than the other in vitro. The "preferred" end for the addition of monomer corresponds to the barbed end of the arrowhead when the filament is decorated with subfragment 1. In filaments whose ends are membrane-associated, S1 decoration shows the

arrowheads pointing away from the membrane (1, 2, 5, 7, 12–14, 19, 20). This implies that the perferred end is at the membrane. If elongation were to occur from the cytoplasmic free end, a large pool of polymerizable actin would be required (9), because monomer addition must take place on the nonpreferred end.

Examination of the genesis of the filament bundle in *Limulus* sperm in vivo provides insight into the mechanisms of the initiation and elongation of actin filament bundles, whereas in the test tube situation many possibilities exist. Our in vivo studies provide evidence that initiation takes place on a specialized structure on a membrane. The filament bundle appears to elongate by addition of monomers onto filaments at the membrane-associated end. In vitro this is the preferred end for monomer addition.

MATERIALS AND METHODS

Material

Limulus polyphemus were collected by the Supply Department of the Marine Biological Laboratory, Woods Hole, Mass. and maintained in 100-gal Instant Ocean Tanks (Instant Ocean Synthetic Sea Salt, Aquarium Systems, Inc., Eastlake, Ohio). Sperm were collected as outlined by Tilney (16). The testis was exposed by sawing through the carapace with a hacksaw. The tissue near the carapace proved to be the best source of differentiating spermatids.

Preparation of G Actin and Subfragment 1 of Myosin

G actin was prepared according to the procedure of Spudich and Watt (15). The actin was gel filtered by the method of MacLean Fletcher and Pollard (11) to remove oligomers, yielding "pure actin."

Subfragment 1 (S1) of rabbit myosin was kindly donated by Dr. Annemarie Weber. It was prepared as indicated by Tilney and Kallenbach (20).

Isolation of the False Discharge and Incubation in G Actin and S1

Sperm were extracted in 1% Triton X-100, 30 mM Tris HCl, and 3 mM $MgCl_2$ at pH 8.0, which induces the formation of the false discharge (see Results). The suspension was then centrifuged at 5,000 *g* for 5 min to remove the chromatin, and then the supernate was centrifuged at 10,000 *g* for 10 min to pellet the false discharges. Contaminating the pellet of false discharges were flagellar axonemes. A portion of the pellet was suspended in a solution containing 1 mM $MgCl_2$, 75 mM KCl, and 10 mM imidazole at pH 7.3, and G actin was added so that the resulting solution contained 0.05 mg/ml G actin or 0.1 mg/ml G actin. The preparation was incubated for ~1 min at room temperature. Small drops of this preparation were deposited on colloidin-coated grids for negative staining. S1 decoration was similarly carried out by suspending the false discharges in the polymerizing buffer described above and adding G actin to a concentration of 0.1 mg/ml. After incubation for 5 min at room temperature, sufficient S1 was added to give an S1 concentration of 0.3 mg/ml. Incubation was continued at room temperature for 10 min. This suspension, which now contained false discharges with decorated actin filaments protruding from their ends, was used for negative staining.

Electron Microscope Procedures

NEGATIVE STAINING: Preparations were negative-stained with 0.5% uranyl acetate on collodion-coated grids stabilized with a thin carbon film.

PREPARATION OF THE TESTES FOR THIN SECTIONING: Small bits of the testes were fixed by immersion in 1% glutaraldehyde (8% stock obtained from Electron Microscopy Sciences, Fort Washington, Pa.) in seawater for 30 min at room temperature (pH 7.5), washed in seawater, and postfixed in 1% OsO_4 in 0.1 M phosphate buffer at pH 6.2 for 30 min at 0°C. The specimen was washed three times in distilled water and stained en bloc in 0.5% uranyl acetate. The specimen was then dehydrated in acetone and embedded in Epon. Thin sections were cut with a diamond knife on a Sorvall Porter-Blum microtome (DuPont Instruments, Sorvall, DuPont Co., Newtown, Conn.), stained with uranyl acetate and lead citrate, and examined with a Philips 200 electron microscope (Philips Electronic Instruments, Inc., Mahwah, N.J.).

RESULTS

Mature Sperm

In this section we would like to mention briefly certain features of mature *Limulus* sperm that are essential for understanding the experiments that follow and the changes that occur during sperminogenesis. Details can be found in the reports of Fahrenbach (6) and Tilney (16).

As illustrated in Fig. 1, the acrosomal filament bundle extends from the basal surface of the acrosomal vacuole through a canal in the center of the nucleus to form, just anterior to the flagellum, a coil of five to seven turns. Each successive turn of the coil tends to lie more apical than the last; the posterior end of the bundle sometimes can extend from the top of the coil as an appendage that makes an acute angle with the flagellum. This appendage is the so-called false discharge. Thin transverse sections through the filament bundle at various positions have been assembled from random sections through a pellet of sperm. Where the filament bundle contacts the acrosomal vacuole, we find ~15 filaments in a ring at the periphery of a central homogeneous core (Fig. 1). At the posterior end of the nucleus we count 30 filaments, and transverse sections of each progressive turn of the coil show increasing numbers of filaments, i.e., 36, 39, 45, 54, 61, 77, until, in the false discharge, we count 85 (Fig. 1). Thus, the filament bundle expands gradually, with only 15 filaments at one end, and up to 85 at the other. The number of turns of the coil (5–7) and the greatest number of filaments in the coil or false discharge vary from sperm to sperm, but we encountered 70–100 filaments as a maximum number in the false discharge or in the last turn of the coil. The center-to-center separation of adjacent filaments in thin sections is 85 Å (3, 16) and in longitudinal sections of the acrosomal filament bundle, or in negative-stained preparations, adjacent filaments have their crossover points in register, thereby assuming a paracrystalline order (see references 3, 4, and 16).

Spermatid Differentiation

Sperminogenesis occurs within cysts lying within the seminiferous tubules. According to Fahrenbach (6), when the spermatids are mature, the cyst wall ruptures, the four spermatids, connected throughout meiosis and spermatid differentiation by cytoplasmic bridges, separate, and the sperm are liberated into the lumen of the seminiferous tubule. There are a number of clues that enable us to accurately determine the age of differentiating spermatids. These include the degree of condensation of the nucleus; the size and shape of the acrosomal vacuole; the amount, organelle constitution, and density of the cytoplasm; the obliteration of the space between the outer and inner nuclear envelopes in the region just beneath the acrosomal vacuole (the subacrosomal plate; see references 6 and 18); the formation of the flagellar axoneme and its extension outside the cell; and the length of the actin filament bundle or the number of turns this bundle makes beneath the nucleus. (For a more detailed discussion of these changes, see reference 6.)

Before the first appearance of the filament bundle, but in the region where the bundle will make contact with the acrosomal vacuole, some dense material, the acrosomal button (6), appears attached to the basal surface of the acrosomal vacuole (Fig. 2). Some dense material is also found in the acrosomal vacuole in this region. Both the acrosomal button and the dense material attached to the membrane facing the acrosomal vacuole tend to obscure the plasma membrane, which lies

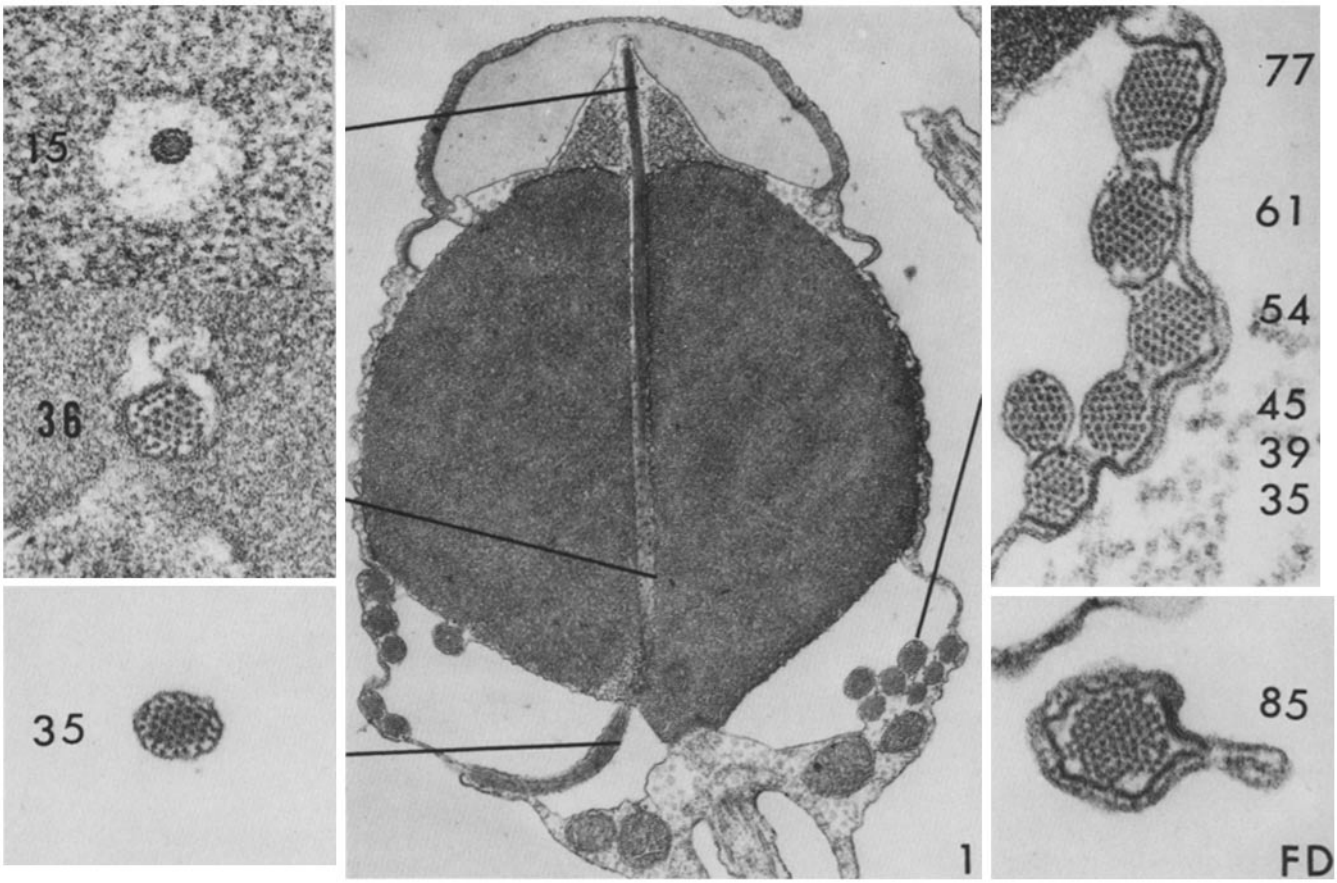


FIGURE 1 In the center of this plate is a longitudinal section through a *Limulus* sperm. To each side are transverse sections through the filament bundle at the position indicated on the longitudinal section. Note that the filament bundle gradually tapers, having 15 filaments near the acrosomal vacuole end and 85 at the other end, which exists in the false discharge (*FD*). The coil makes six to seven turns; each turn tends to lie apical to the last. Longitudinal sections, × 41,860; transverse sections, × 110,000.

between, making it difficult to resolve the membrane unless the photograph is printed very lightly or is "dodged" photographically.

In the next stage, in longitudinal sections through developing spermatids, we see the filament bundle extending from the acrosomal button into the nucleus (Figs. 3 and 4). As the filament bundle penetrates into the nucleus, it carries both nuclear envelopes ahead of it. The outer nuclear envelope becomes the sheath of the filament bundle, and the inner, the conduit for the sheathed filament. Once the filament bundle has elongated so that it passes through the center of the nucleus, it begins to coil around the base of the nucleus.

In longitudinal sections through a differentiating spermatid, the number of turns of the coil and the number of filaments in each turn can be counted (see Fig. 3, *inset*). The number of filaments in the nuclear canal, however, can only be estimated by the diameter of the filament bundle because the bundle at that point appears in longitudinal section. By taking a transverse section of a filament bundle comparable in diameter to that of the longitudinal section, we can fairly accurately estimate the number of filaments in the longitudinal section. Thus, in a longitudinal section through a spermatid in which not one turn of the coil has been completed, we estimate that the bundle in the nuclear canal should contain 65–90 filaments (Fig. 4). These values coincide with a micrograph of a spermatid showing a transverse section of the filament bundle in the nuclear canal, before the bundle has elongated sufficiently to produce a single turn of the coil (Fig. 5). This filament bundle contains 70 filaments. We have several micrographs of spermatids that have completed two turns of the coil. We estimate that the filament number in the most apical turn of the coil is 95, in the next turn 80, and in the nuclear canal 65. As the number of turns of the coil increases, the number of filaments in the bundle in the nuclear canal decreases, so that when five turns have formed, there are 40–50 filaments in the nuclear canal, and when six turns have formed, ~30 filaments are found in the nuclear canal. These numbers can also be estimated by the diameters of the filament bundles in longitudinal section. They can also be determined by comparing adjacent spermatids in a cluster because the differentiation of the four spermatids that are linked together by intracellular bridges is synchronous and a section through one may be longitudinal, and through another, transverse. We have also examined many other sections in which, judging by the amount of nuclear condensation, the filament bundle has not made more than two turns, we count ~65 filaments in the nuclear canal. From all these cases, it is consistently clear that the bundle, when it first appears, contains ~70–90 filaments, the number present at the basal end of differentiated sperm. As this bundle elongates, the number gradually decreases in the nuclear canal so that, when six turns of the coil have been completed, there are ~80 filaments at the basal end that extends into the false discharge and only 30 filaments within the nuclear canal. Thus, the thick or basal end of the bundle forms

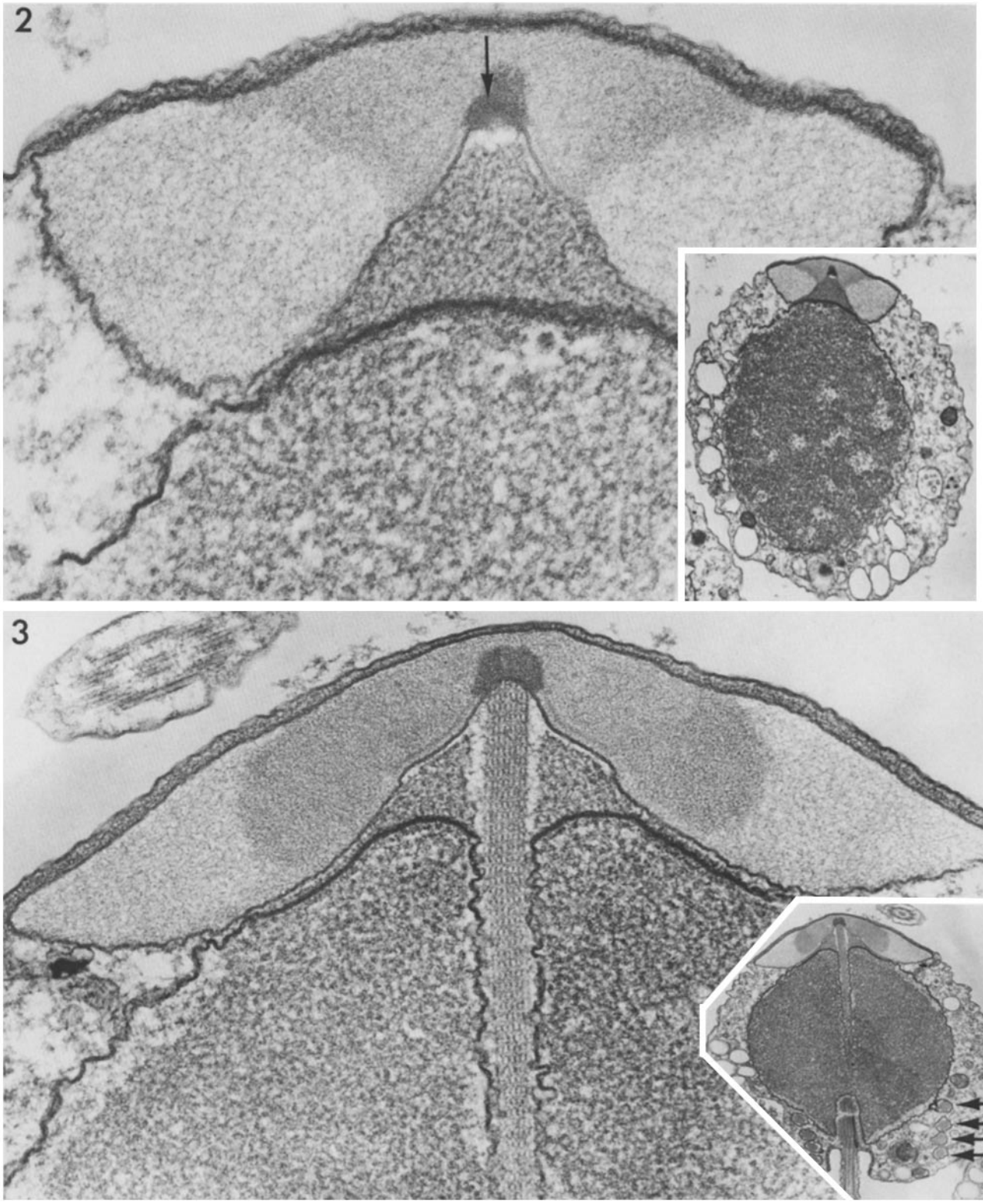


FIGURE 2 Thin section through the apical end of a *Limulus* sperm at a stage in spermatid differentiation in which the filament bundle has not yet assembled. At the position where the filament bundle will eventually contact the membrane, limiting the acrosomal vacuole, we see a dense material attached to both the cytoplasmic and vacuole surfaces of this membrane (arrow). This dense material has been called the acrosomal button. × 105,000; *inset*, × 14,000.

FIGURE 3 Thin section through the apical end of a *Limulus* sperm at a stage in differentiation in which the filament bundle has been partially assembled, forming four turns of the coil (arrows in the *inset*). It is of interest here that the filament bundle is attached to the acrosomal vacuole membrane at the same position as in Fig. 2. Where the filaments make contact with this membrane, there is a thin layer of dense material on the cytoplasmic side of the membrane that is greatly reduced in amount relative to that in Fig. 2. The dense material is still associated with the vacuole side of the membrane. × 81,000; *inset*, × 17,000.

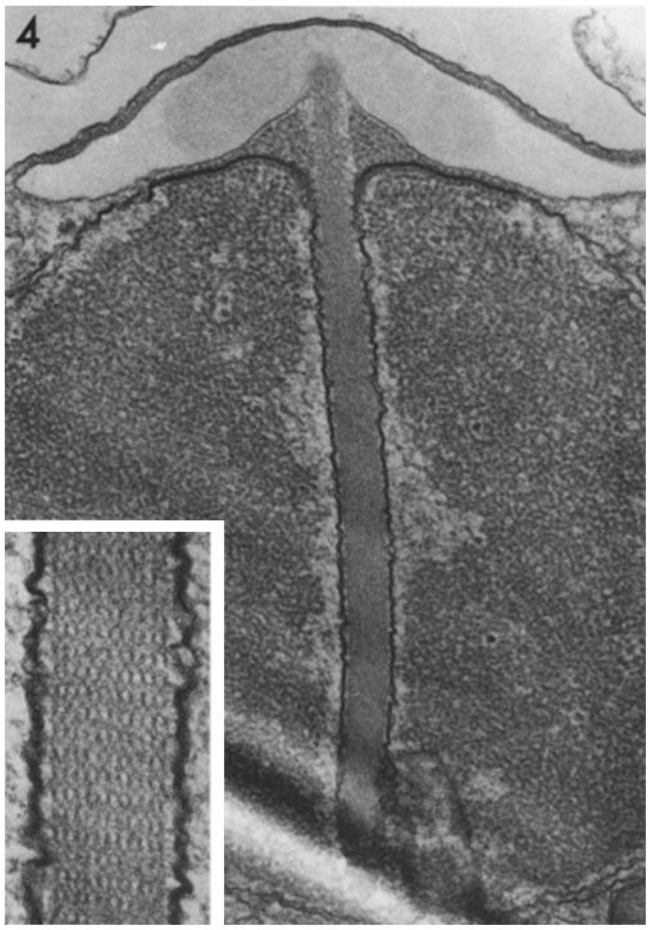


FIGURE 4 Thin section through an early spermatid in which only one-half turn of the coil has been made. Note that the filament bundle has a large diameter and contains many filaments, ~11 in this section. In transverse section this bundle would contain about 80 filaments. In the *inset* we can see that the filaments are organized in a paracrystalline array with their crossover points in register. × 39,000; *inset*, × 115,000.

first and, as it elongates, it gradually assumes the taper seen in mature sperm.

There are several additional points of interest. First, the packing of the filaments in the developing bundle is identical to that in the mature sperm. The filaments lie on a hexagonal lattice separated one from another by 85 Å (compare Fig. 1 to Figs. 5 and 6). In longitudinal sections adjacent filaments show paracrystalline order or, to be more specific, the crossover points of adjacent filaments are in register (see Fig. 3 and Fig. 4, *inset*); this is a characteristic feature of the filament bundle in mature sperm (3, 4, 16). Second, at all stages the apical end of the filament bundle appears to be attached to, or to make contact with, the acrosomal vacuole at the position of the acrosomal button (Fig. 3). Whereas before the assembly of the filament bundle the acrosomal button is very dense, as the number of turns of the coil increases the amount of dense material decreases so that, when four turns of the coil have been completed, the amount of dense material is very reduced (see Fig. 3). Third, where the filament bundle makes contact with the acrosomal vacuole, we see a single ring of filaments encircling a homogeneous core in mature sperm (Fig. 6). This core, encircled by the 15 actin filaments, extends basally ~1 μm, or at least halfway, through the nuclear canal (Fig. 7). The core does not appear in spermatids until the filament bundle is almost completely assembled. Thus, transverse sections through the filament bundle near the acrosomal vacuole or through the anterior half of the nucleus in spermatids whose filament bundles are only partially formed lack this core (see Fig. 6 *b*).

Actin Addition to the Filaments of the False Discharge

Close examination of segments of the false discharge that have been incubated in a solution of G actin and salt reveals that assembly of actin to this filament bundle is much more pronounced on one end than the other. The favored end is always the thin end, or the end that will be closest to the acrosomal vacuole in undischarged sperm (Fig. 8). Polarized addition of actin was true on all segments. We can easily distinguish the junction between the false discharge and the newly assembled actin, because the newly assembled actin filaments splay and do not assume the characteristic undulatory profile of the false discharge. We measured the relative lengths of polymerized actin at the two ends in >32 fragments of the false discharges. A simple estimate of the magnitude of the preference can be made by taking the ratio of the length of the longest actin filament on the preferred, or thin end to that of the longest filament extending from the unfavored, or thick end. We found the ratio to be ~10:1.

S1 Decoration of the Actin Assembled on the Filaments of the False Discharge

The actin filaments in the false discharge do not decorate with S1 because of competition with the 55,000-dalton protein (16). Therefore, to determine the polarity of the filaments in the false discharge, we must decorate the actin filaments assembled from the bundle of filaments in the false discharge. These newly formed filaments, polymerized from and continuous with the thin end filaments in the false discharge, are unidirectionally polarized with the arrowheads pointing toward the thick end (Fig. 9). Thus, in mature sperm, the arrowheads would point away from the acrosomal vacuole membrane and toward the flagellum.

DISCUSSION

Nucleation of the Filaments May Occur From a Specialization on the Acrosomal Vacuole Membrane

We have demonstrated that, before the appearance of the filament bundle in developing *Limulus* spermatids, a mass of dense material, the acrosomal button, appears on the acrosomal vacuole membrane. The newly formed filaments are embedded in this material and remain there as they elongate. If the filament bundle is isolated and monomeric actin is assembled on the ends of the filaments, and if then the newly assembled actin filaments are decorated with S1, all the filaments display unidirectional polarity with the arrowheads pointing away from the acrosomal button. (The filaments in the bundle proper do not decorate, because the S1 binding site is covered by a second protein [3, 16]). These two observations, namely, that the filaments appear in contact with some dense material attached to the membrane at an early stage in elongation and that they show unidirectional polarity, suggest to us that the

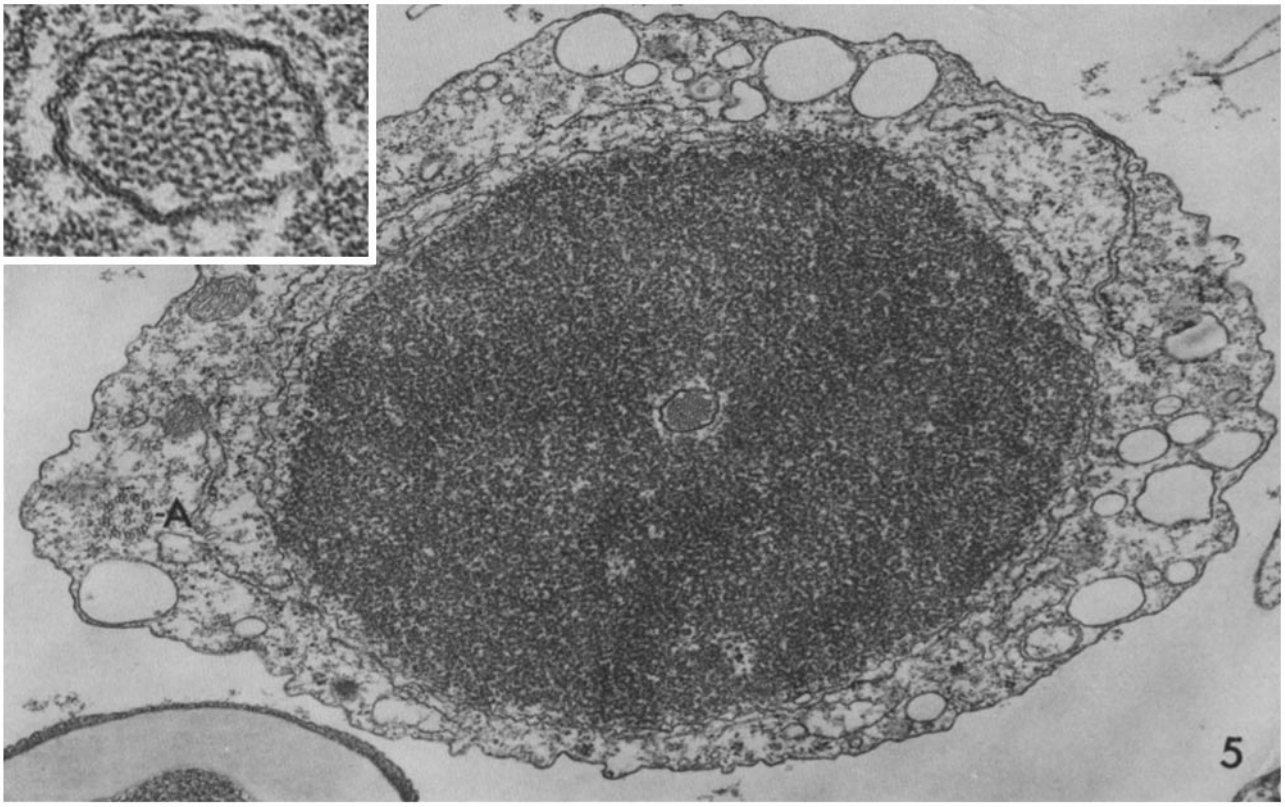


FIGURE 5 Transverse section through a developing spermatid of *Limulus* in which the filament bundle has begun to form. It is of interest that the filament bundle running down the nuclear canal contains ~70 filaments (see *inset*) organized on a hexagonal lattice. Also in this spermatid is a portion of the growing flagellar axoneme (*A*). × 37,000; *inset*, × 175,000.

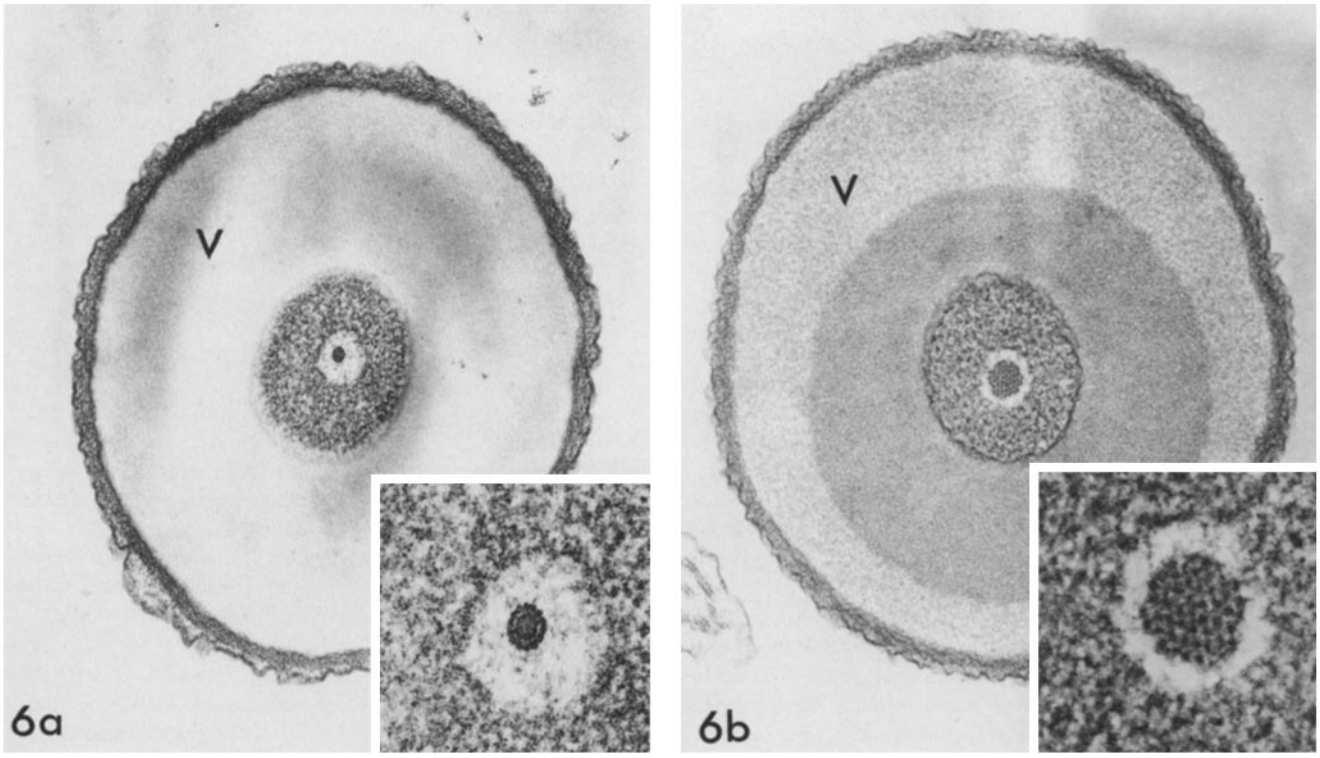


FIGURE 6 (*a*) Transverse section through the filament bundle in a mature *Limulus* sperm near where it terminates on the membrane, limiting the basal surface of the acrosomal vacuole (*V*). It is of interest that in the center of this bundle there is a homogeneous core surrounded by 15 filaments. × 46,000; *inset*, × 133,000. (*b*) Transverse section of a developing spermatid cut in the same region as *a*. Note that there are many filaments here (~52) and no central homogeneous core. × 46,000; *inset*, × 133,000.

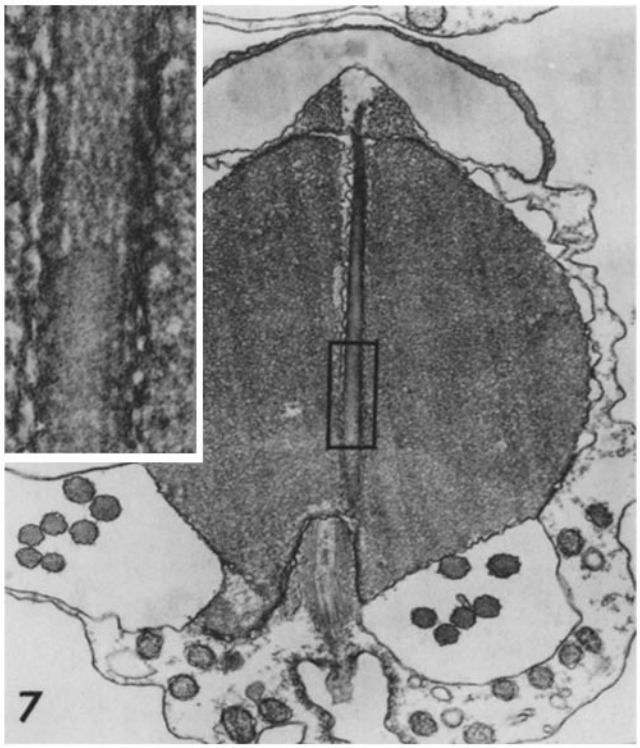


FIGURE 7 Longitudinal section through a mature sperm. A portion of the filament bundle shown in the inset is indicated by the box. It is of interest that the core extends from the basal surface of the acrosomal vacuole to the region indicated by the box. × 40,500; *inset*, × 192,000.

dense material may nucleate the filaments and thereby control their polarity. A similar argument has been applied to the reformation of microvilli after pressure-induced disassembly, in which the actin filaments appear to nucleate from a dense membrane-associated site (17). In neither case has it been proved that nucleation occurs at the membrane-associated site, since it is possible that short filaments of actin, nucleated either spontaneously or from some cytoplasmic material, have secondarily associated with these specialized membrane sites with the requisite polarity. However, these observations are consistent with such an interpretation. More important is the observation that the elongation of the filaments in vivo must certainly take place where these filaments contact the membrane. This is the subject of the next part of the Discussion.

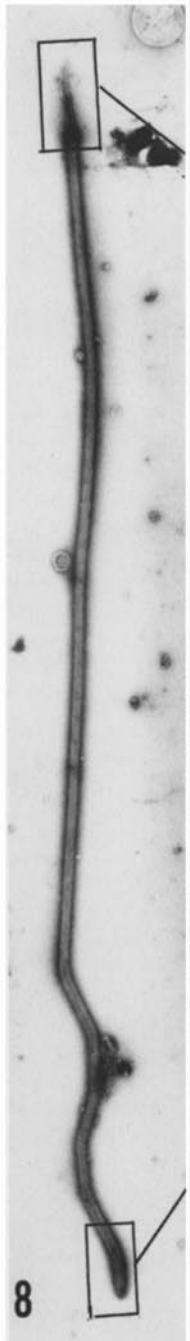
Actin Filaments Must Elongate From Their Membrane-associated Ends

In the mature sperm the actin filament bundle is tapered, having 15 filaments at the end associated with the acrosomal vacuole, and >85 filaments at the basal end, the end that extends into the false discharge. We determined that, at the earliest stages in the genesis of the bundle, there are approximately 85 filaments in the nuclear canal and, as the bundle elongates, this number steadily decreases to 30, the number present in the nuclear canal of mature sperm. We think that these pictures provide convincing evidence that the basal end of the bundle is made first (Fig. 10), elongation subsequently occurring by the addition of monomers to the end associated with the acrosomal vacuole membrane (see Fig. 11 *A*). In this model, monomer addition occurs at the preferred end, and tapering is achieved by the progressive capping of the peripheral filaments in the bundle, which possibly is related to the reduction in density of the subacrosomal button. When the filament bundle has elongated to within 1 μm of its final length (~55 μm), the homogeneous core, which extends from halfway down the nuclear canal up to the acrosomal vacuole, will be added, and the remaining 15 filaments that cover its surface will elongate.

To establish the correctness of this model, we must consider the possibility that the addition of monomers is at the basal, nonpreferred end. As well as requiring assembly on the nonpreferred end, this alternative has the obvious handicap of requiring an elaborate mechanism for generating the taper seen in mature sperm. One possibility (Fig. 11 *B*) is that the taper is produced by sliding of the outer filaments in the bundle relative to the inner ones. As the apical ends of the sliding filaments move away from the acrosomal button, they are capped to prevent any polymerization onto them. To generate the gradual taper, the outer filaments must either slide at different rates, depending on their radial position, or start sliding at different times. Because the outer filaments slide, the rate of polymerization to their growing, nonpreferred ends must be less than that to the central filaments; otherwise a hollow tube would be formed, something never seen. Thus, to achieve the final structure, a coordinated mechanism would be required to correlate the rate of polymerization with the rate of sliding and the amount of sliding as well as selective filament capping. This sliding model also would require that the filaments not be cross-linked to one another during the genesis of the bundle. In an earlier publication (3) we demonstrated that the paracrystalline order of filaments in mature sperm is the result of each filament in the bundle being linked to its neighbors by some 5,000 cross-links. Because the bundle in the developing spermatid has the same paracrystalline order found in the adult sperm, it too must be cross-linked.

Given this information, we think that model *B* does not represent a feasible mechanism, for the following reasons: (*a*) addition of actin takes place at the nonpreferred end; (*b*) the rate of addition at these ends, if it did take place, would have to be different for different filaments and would have to be such that different rates of growth of the filaments exactly compensate the effects of filament sliding; (*c*) to slide, all interfilament bonds would have to be broken and made for each increment of the motion, and we think that this is highly unlikely.

In Fig. 11 *C*, addition of monomers takes place also at the basal, nonpreferred end. Tapering is produced by the selective depolymerization of the outer filaments in the bundle at the preferred end. To achieve depolymerization at the preferred end, a depolymerase would be needed because of the high concentration of monomers necessary for addition at the nonpreferred end. The depolymerase, in effect, ensures that depolymerization takes place under conditions that are highly favorable for polymerization. At each stage in development of the bundle the taper is similar, if not identical, to that of mature sperm. To achieve this result with model *C*, it is necessary to make the rates of polymerization and depolymerization the same. Model *C*, therefore, is unsatisfactory for the following reasons: (*a*) as with model *B*, addition of monomers takes place at the nonpreferred end of the filaments; (*b*) an energy-requiring depolymerase is needed to carry out depolymerization at the preferred end; (*c*) the rate of polymerization at the nonpreferred end must exactly equal the rate of depolymerization at the preferred end.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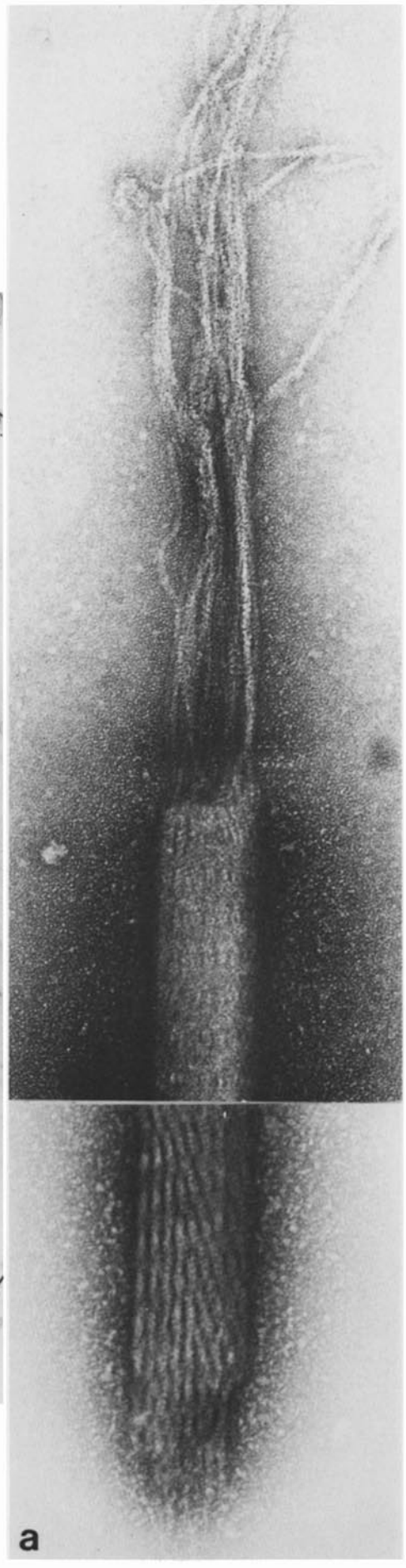
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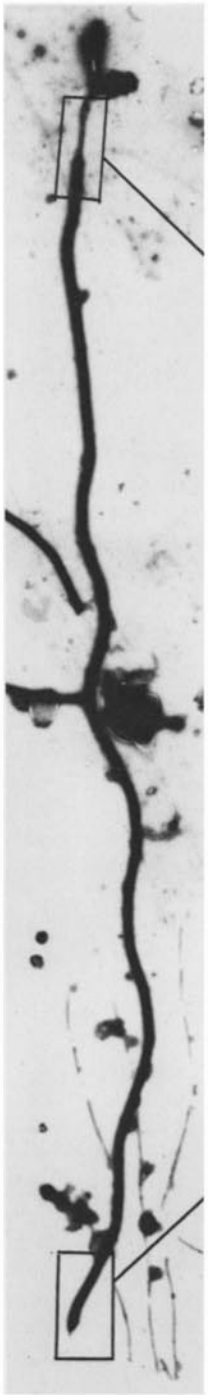

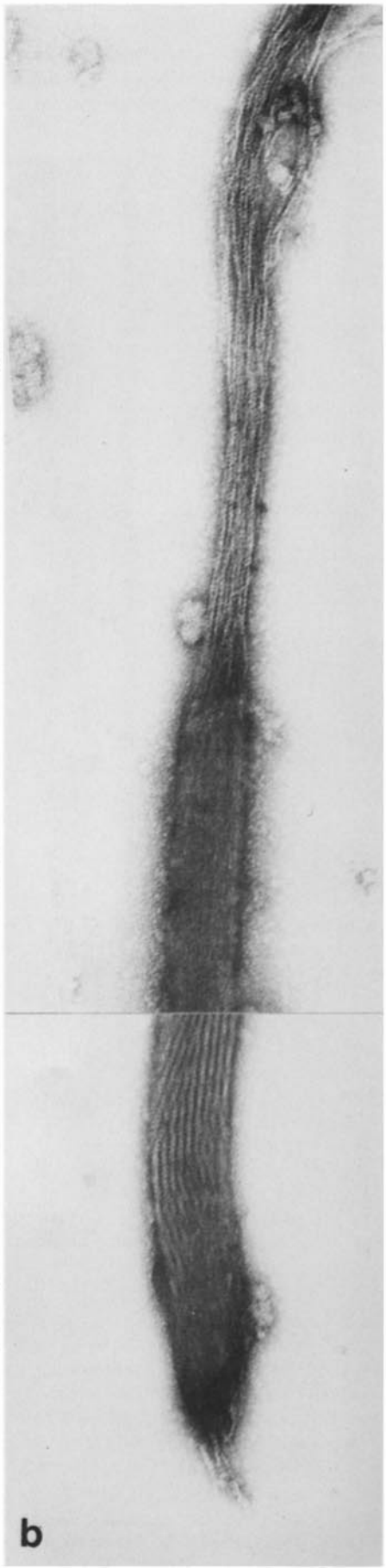
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An additional complexity arises in the case of models *B* and *C* but not model *A*. In brief, as the final step in the production of the bundle, a homogeneous rod >1 μm long, is generated at the apical end of the bundle. In the mature sperm this rod appears as a core surrounded by a ring of 15 filaments. In model *A* such a structure can arise as the natural consequence of termination of bundle elongation. At a late stage in bundle formation a new protein is made which polymerizes on the apical growing end of the bundle, thus forming the rod and terminating the addition of actin. As the core is polymerizing, it pushes the bundle through the canal into the coil in the same way that the polymerizing bundle subunits pushed the bundle from the nuclear canal into the coil.

In models *B* and *C*, however, the bundle is not pushed by polymerization. Thus, a more elaborate mechanism is required to generate the core. It will be necessary to depolymerize actin at the apical end to a depth of ~1 μm, then assemble the core, and finally repolymerize the 15 outer filaments.

The most compelling arguments for accepting model *A* are (*a*) the observed structural features of the bundle can arise naturally from the mechanism of its assembly and (*b*) the polymerization takes place at the preferred end. In models *B* and *C*, on the other hand, both the tapering and the addition of the core do not arise as natural consequences of the polymerization but would require elaborate, highly coordinated additional mechanisms.

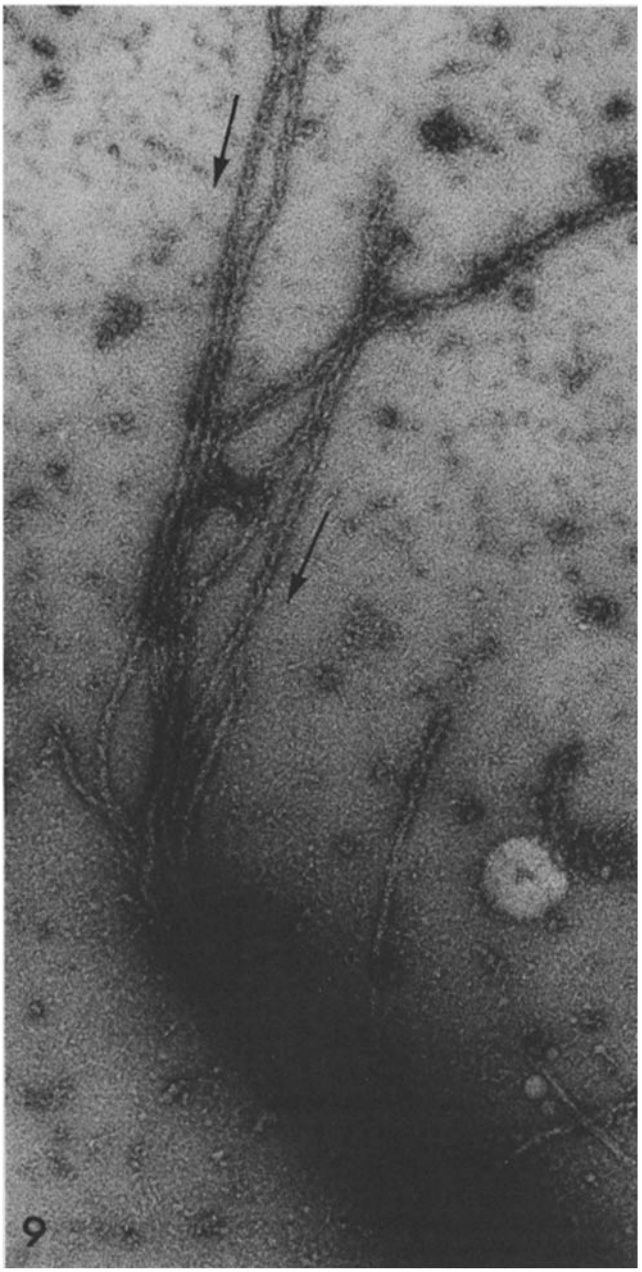

FIGURE 9 The false discharge was incubated in monomeric actin, which assembled primarily on the end nearest the acrosomal vacuole, and then these newly polymerized filaments were incubated in S1 of myosin. Note that the newly polymerized filaments attached to the false discharge have unidirectional polarity (see arrows); the arrows point away from the thin end, which is associated with the acrosomal vacuole membrane. × 77,000.

CONCLUSIONS

The development of sperm provides insight into the mechanism of the assembly of actin bundles in vivo. The polarity of the actin filaments attached to the acrosomal membrane in *Limulus* sperm (actually, the membrane becomes confluent with the plasma membrane during the acrosomal reaction and is thus a plasma membrane) is exactly the same as the polarity of the actin filaments attached to the plasma membrane in microvilli (1, 2, 12), stereocilia (7, 19), filopodia (5, 13, 14), and the acrosomal process (20). Because the acrosomal vacuole membrane becomes continuous with the plasma membrane, our results open up the possibility that, in those systems as well as in *Limulus* sperm, addition of actin monomers occurs at the end of the filament associated with (attached to) the plasma

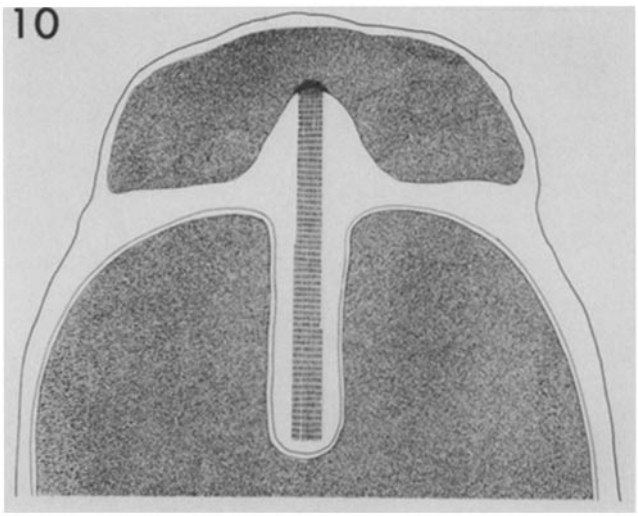

FIGURE 10 Drawing illustrating how the elongation of the filament bundle in developing spermatids proceeds. Monomers add to the end associated with the vacuole, thereby pushing the filament bundle down through the center of the nucleus.

FIGURE 8 The false discharge was isolated and incubated in monomeric actin. The monomers assemble primarily from one end, the thin end, or the end that will be nearest the acrosomal vacuole. (*a*) × 280,000 and × 14,000; (*b*) × 156,000 and × 26,000.

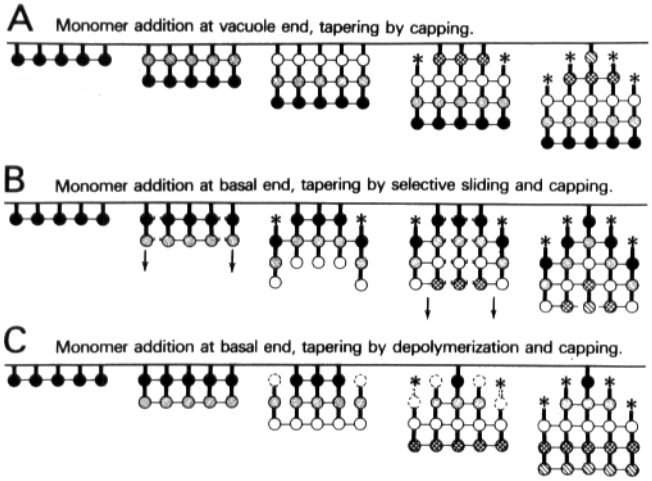

FIGURE 11 Models for filament bundle formation in *Limulus* sperm. The black dots represent the monomers that are first assembled. The grey dots are the next monomers that add to the already assembled monomers. These are followed by more monomers (white dots) and so forth (cross-hatched dots).

membrane. This is the preferred end for assembly in vitro as seen by monomer addition and S1 decoration. Consistent with our conclusion are some recent observations on the elongation of the acrosomal process of *Thyone* sperm where, by a kinetic argument, it appears that actin monomers add to the membrane-associated end of preexisting filaments (Tilney and Inoué, unpublished observations). Also, in vitro studies by Mooseker (personal communication) on isolated microvilli show that actin monomers tend to add at the membrane-associated end of the filament.

One reviewer of this manuscript was disturbed by the fact that, because we have not proved that nucleation occurs on the acrosomal vacuole membrane, we have no evidence that the actin filaments are ever attached to the acrosomal vacuole membrane. Let us assume, said he, that nucleation occurs elsewhere, i.e., on the nuclear envelope, and that the acrosomal vacuole membrane acts only as a passive cap to block filament elongation until the ends are pulled away from the dense material so that monomer can insert and elongation can continue. His point is justified—we do not have any evidence for *attachment* of actin filaments to membranes, and that is why we have used the word "associate." In fact, in no system is there any evidence for end-on attachment of actin filaments to membranes. In order to use the word "attach" we must know to what protein, lipid or polysaccharide the actin monomer is bound and how tightly (specifically, with what value of a binding constant) it is bound. It may, in fact, not be "bound" at all. To study such attachments, it would be necessary to develop an in vitro system in which such binding could be measured. An in vitro study, however, will always leave some doubt, because one would never know how much the membrane was perturbed during isolation. The importance of our study is that we have shown that actin filament elongation in vivo occurs from the end "associated" with the membrane, the preferred end for addition of monomer.

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