The Development and Motility of Caenorhabditis elegans Spermatozoa[1]

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Nematode sperm have puzzled zoologists for nearly a century because they are not flagellated. Their morphology is amoeboid, but there have been only a few descriptions of their actual movement (8,9,17). We have undertaken an intensive examination of the spermatozoa of *Caenorhabditis elegans* in order to learn how they propel themselves and how they develop their specialized morphology. This nematode was chosen because it is easily cultured in the laboratory and it has become the subject of detailed studies of its genetics, development, and behavior (10). In this paper we summarize our recent results which demonstrate that these spermatozoa have a novel mechanism of amoeboid motility.

MORPHOLOGY AND MOVEMENT

C. elegans spermatozoa are bipolar cells about 4 μm wide and 6 μm long (14,15,16). They have a motile pseudopod on one end and a rigid hemispherical cell body on the other (Fig. 1). The pseudopod is studded with small projections and the cell body appears smooth. When sections of spermatozoa are viewed in the transmission electron microscope, the pseudopod is found to be filled with amorphous material; all cell organelles, mitochondria, nuclei, membranous organelles, and laminar membranes are confined to the cell body (Fig. 2). The membranous organelles (MO) are Golgi-derived vesicles characteristic of nematode sperm (3). During spermiogenesis these vesicles fuse with the plasma membrane forming stable pores around the cell body of spermatozoa (7,16).

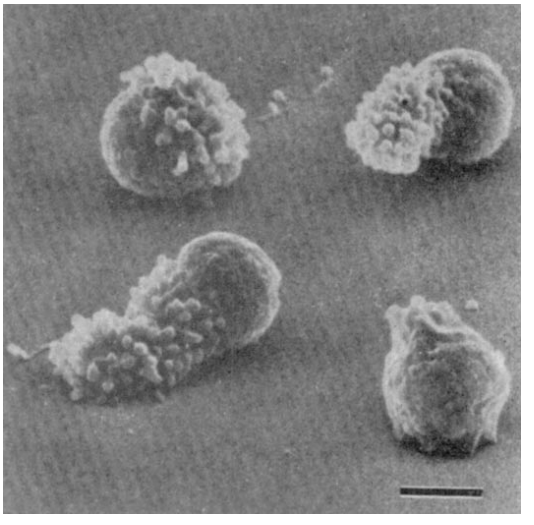

Fig. 1. Scanning electron micrograph of a field of spermatozoa on a cover slip coated with *Ascaris* uterine exudate. The movements of these cells were recorded on time lapse video tape before fixation. The only translocating cell was the one in the lower left with the flattened pseudopod. The other cells were wiggling their pseudopods but not moving over the substrate except for the cell on the lower right which is aberrant. Bar = 1 μm. Tilt 45°. Reprinted from (9) with permission of *J. Cell Biol.*

When spermatozoa are maintained in an appropriate low ionic strength medium their pseudopods will move for many hours (9,15). When viewed with Nomarski optics and examined with time lapse recording, clusters of projections form at the tip of the pseudopod and move back to its base at velocities of 20–45 μm/min (Fig. 3). This movement occurs whether or not the spermatozoa is attached to a substrate. Proper substrate attachment, however, is necessary for the spermatozoan to propel itself. Most conventional tissue culture substrates do not promote migration, but some spermatozoa will move over acid washed glass. A better substrate can be prepared

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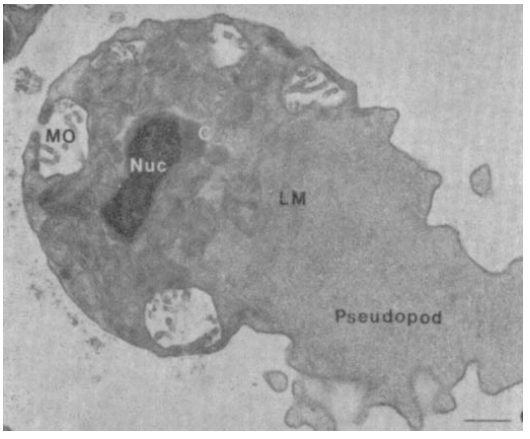

Fig. 2. Transmission electron micrograph of a mature spermatozoon fixed with GTA and tannic acid then OsO_4. Fused MOs can be seen expelling their fibrous contents which remains associated with the cell body surface. Laminar membranes (LM) are most pronounced in the region between the cell body. No microtubules or microfilaments are seen in this or thousands of other sections of spermatozoa examined. NUC = nucleus, C = centriole. Bar = 0.5 μm. Reprinted from (15) with permission of *J. Cell Biol.*

by drying an extract of *Ascaris* uteri on a slide. Spermatozoa attach to this surface by the projections on their pseudopods (Fig. 1) and then translocate at velocities up to 30 μm/min, comparable to that observed in vivo (9).

MEMBRANE MOVEMENTS

In order to find out how the visible movement of pseudopodial projections might propel the cell forward, we examined movement of the pseudopodial membrane using four different markers: latex beads, fluorescent lectins, the histochemical marker enzyme horseradish peroxidase, and fluorescent lipids.

When spermatozoa are exposed to positively charged latex beads, these beads stick avidly to both the pseudopod and the cell body. All beads that attach to the cell body remain in place, never moving over the cell surface at all. In contrast, beads that attach to the pseudopod are invariably transported back to the pseudopod-cell body junction where they become immobilized (Fig. 4). This centripetal bead transport oc-

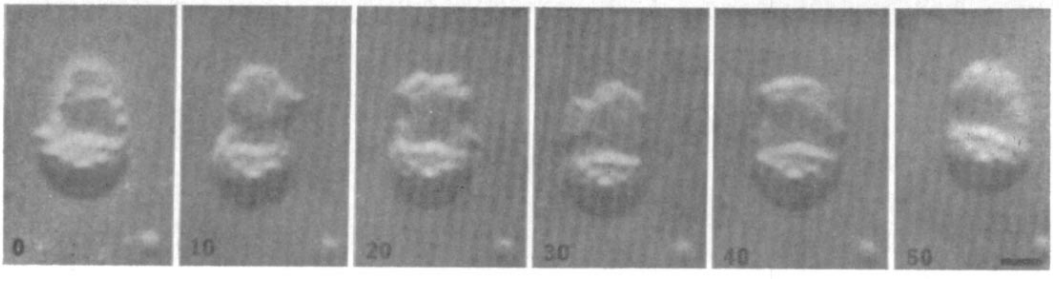

Fig. 3. Nomarski-microflash photos of a spermatozoon at 10-sec intervals illustrating changes in pseudopod shape during wiggling. Protrusion and retraction of different regions on the pseudopod are independent. The retrograde movement of pseudopodial projections is especially obvious between 10 and 20 seconds along the right hand side of the cell. Bar = 1 μm. Reprinted from (9) with permission of *J. Cell Biol.*

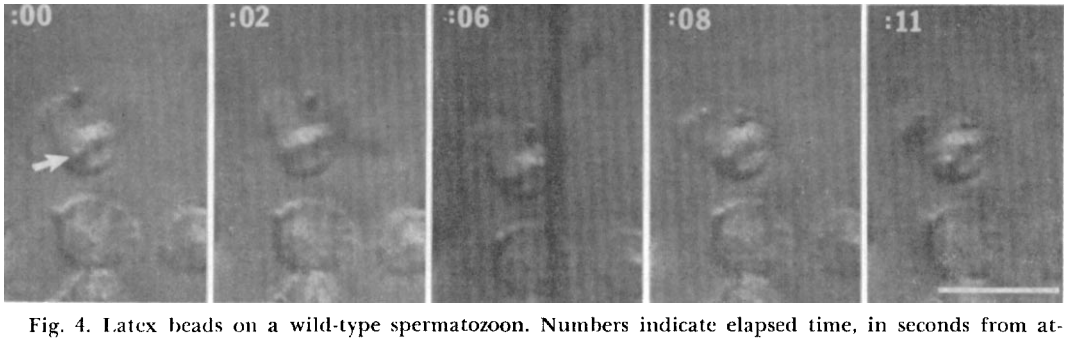

Fig. 4. Latex beads on a wild-type spermatozoon. Numbers indicate elapsed time, in seconds from attachment of bead at the tip of the pseudopod (:00) to its cessation of movement at the base of the pseudopod (:11). Arrow indicates a bead bound to the cell body which remained stationary throughout the 11-sec interval. Bar = 5 μm. Reprinted from (12) with permission of *J. Cell Biol.*

curs whether or not the cell is translocating over the substrate and occurs for beads attaching to the top, sides, or bottom of the pseudopod (12).

The movement of beads suggests that the surface membrane of the pseudopod, to which the beads are attached, is moving backwards from pseudopodial tip to base. This could be so only if there were a mechanism to recirculate or replace the membrane as it moves away from the tip. In order to identify such a mechanism, fluorescent lectins were used to ask whether new membrane components were being added at the tip of the pseudopod (12). Lectins are proteins that bind avidly to sugar residues in glycoproteins or glycolipids; wheat germ agglutinin (WGA) which binds to N-acetyl glucosamine residues was used for these experiments. Spermatozoa were first soaked in a high concentration of unlabeled WGA to block all lectin binding sites on the surface. Next, the excess unlabeled lectin was washed away. Then the cells were exposed to a brief pulse of fluorescently labeled WGA. Examination of these cells with the fluorescence microscope revealed bright fluorescence at the tip of the pseudopod (Fig. 5). This fluorescence must reflect new lectin binding components inserted in the membrane because controls showed that all preexisting lectin binding sites had been blocked (12). The fluorescence subsequently spread over the pseudopod and eventually disappeared, presumably because it was internalized and became too diffuse to detect.

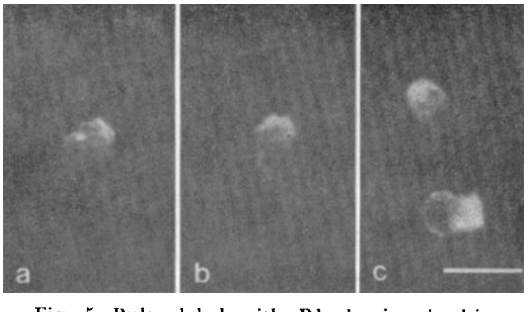

Fig. 5. Pulse label with Rhodamine isothiocyanate coupled WGA. Cells were first treated with unlabeled WGA (100 μg/ml) then rinsed and pulsed with labeled WGA for 8 sec (a) 15 sec (b) or 25 sec (c). Note progression of label from tip of pseudopod to base as length of pulse is increased. Bar = 5 μm. Reprinted from (12) with permission of *J. Cell Biol.*

Direct evidence for internalization of lectin binding components has not yet been obtained. Instead, we have found that the enzyme horseradish peroxidase, for which there is a sensitive electron microscope histochemical stain, is internalized in spermatozoa. It first binds to the surface membrane and then collects in the internal laminar membranes that are concentrated at the joint between the cell body and the pseudopod (Fig. 6a). Spermatids in the same field provide a control for the specificity of the enzyme uptake because no peroxidase activity is found in their laminar membranes, although it is evident on the cell surface (Fig. 6b).

The results described so far show that visible surface projections, attached latex beads, and lectins all move from the tip of the pseudopod to its base. The lectin pulse label further reveals that this movement is accompanied by the insertion of new membrane components at the tips of pseudopods, and the peroxidase uptake suggests that the internalization probably occurs at the base of the pseudopod.

The lectin labeling is eliminated if cells are treated with proteases so the lectin binding components inserted into the pseudopod are glycoproteins. These could be moved over the surface either by attachment to some cytoskeletal machinery which pulled them through the membrane or by bulk flow of the entire membrane. These two possibilities can be distinguished by observing the behavior of membrane lipids. If lipids move from the tip of the pseudopod together with glycoproteins, then bulk membrane flow must be occurring. In order to follow the behavior of membrane lipids, we inserted fluorescent phospholipid analogues into the membrane of spermatids and then observed the behavior of the pseudopod membrane after it formed during in vitro spermiogenesis (11). To explain this experiment it is first necessary to describe the process of spermiogenesis in vitro.

IN VITRO SPERMIOGENESIS

Ascaris spermatids can be activated to form spermatozoa in vitro by a substance extracted from the glandular vas deferens (3,4) or by treatment with proteases (1). *C. elegans* spermiogenesis can be induced in

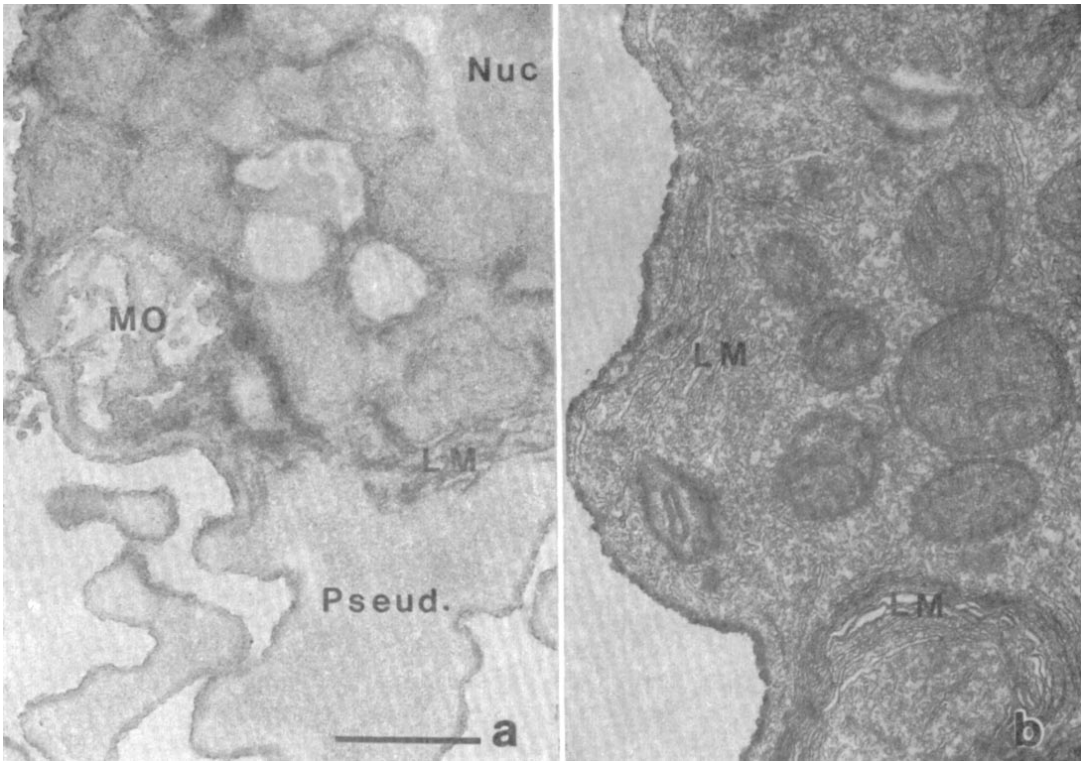

Fig. 6. Horseradish peroxidase uptake into spermatozoa. Sperm were incubated with 10 mg/ml HRP for 13 min, fixed with glutaraldehyde, and treated with diamino benzidine then peroxide. When a visible brown color developed, they were rinsed, post-fixed with OsO_4, and embedded and sectioned. Sections were stained with uranyl acetate only. a) Spermatozoon. b) Spermatid. The blackish deposit on the surface of cells is the HRP reaction product. In spermatozoa, but not spermatids, a similar deposit is found around the laminar membranes (LM).

vitro by treating spermatids with the Na^+ and K^+ transporting ionophore, monensin (7). In vitro spermiogenesis takes about 6 min and involves the rearrangement of cellular organelles and laminar membranes, the fusion of MOs with the plasma membrane on one side of the cell, the extension of the pseudopod from the opposite side of the cell, and the initiation of pseudopod motility (7). The changes in external morphology are shown by the scanning electron micrographs (Fig. 7) and the internal rearrangements are summarized diagramatically in Figure 8. By labeling the membrane of the spermatid and then initiating spermiogenesis in vitro, it is possible to follow the movements of membrane components during the formation of the pseudopod and during its initial movements.

When latex beads are attached to spermatids and these spermatids are then activated to form spermatozoa, the beads move in fits and starts over the surface of the spermatid prior to pseudopod formation (11). There is no apparent pattern to the movements, and individual beads move independently of one another. When the pseudopod forms, beads are excluded from it and beads of the cell body cease movement entirely.

When spermatids are labeled with fluorescent WGA and then activated to spermatozoa, fluorescence is found on the initially formed pseudopod but is cleared from the pseduopod within a minute (11). This shows that the initial pseudopod is formed of membrane components that preexist on the spermatid and that these components are cleared from the pseudopodial surface when motility is initiated. This clearance appears to be the same as the clearance of the lectin binding sites labeled after the pseudopod formation previously described. This similarity allowed the use of fluorescent lipid analogues to study the be-

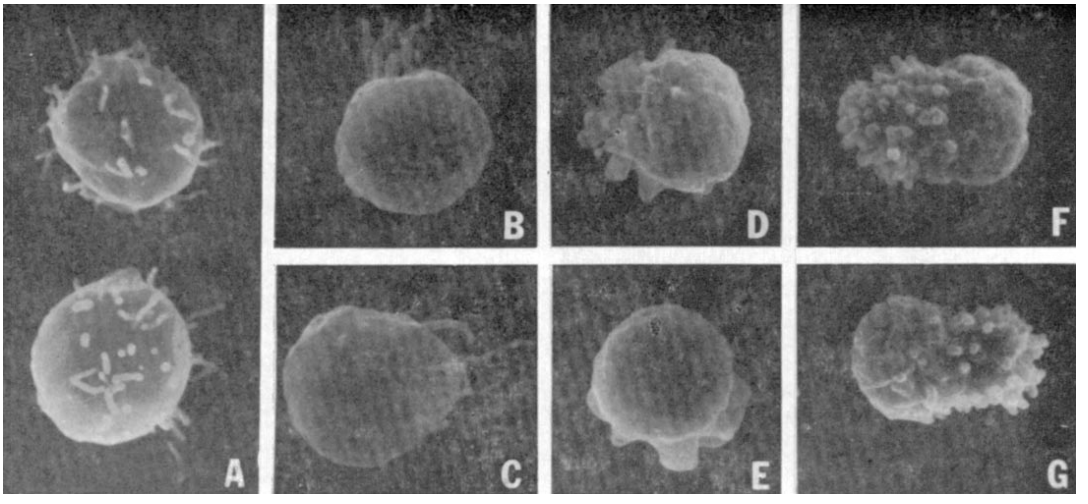

Fig. 7. Scanning electron micrographs of sperm fixed at various times after monensin treatment. A) Two spermatids fixed immediately after dissection are dotted by numerous microvilli. B–E) Sperm fixed 4 min after ionophore activation. B,C) Microspikes are clustered at one pole of the cell. D,E) Microspikes have been cleared from the cell surface and pseudopodia have begun to extend from the cell bodies. F–G) Sperm fixed 12 min after monensin treatment display large pseudopods covered by numerous pseudopodial projections. Magnification 6000 ×. Reprinted from (7) with permission of *Cell*.

havior of membrane lipids following spermiogenesis to find out if the whole membrane or only the glycoproteins were being cleared from the pseudopod (11).

A derivative of the phospholipid, phosphatidylcholine, with a fluorescent group attached to one lipid side chain was used as the lipid label for these experiments. This molecule was introduced into the spermatid membrane by exchange from lipid vesicles. This was done at 4 C in order to prevent uptake of the lipid into the cell interior. The excess label was then washed out and the cells warmed to room temperature and activated to form pseudopods. When this process was observed in the fluorescence microscope, it was found that the initial pseudopod was fluorescent, but the fluorescence cleared rapidly from the pseudopod as motility began (Fig. 9). This clearance occurred at a rate similar to that observed for the clearance of fluorescent lectins from the surface. Controls indicated that the lipid was properly inserted in the membrane

Fig. 8. Diagram of internal morphological changes occurring during spermiogenesis. Reprinted from (15) with permission of *J. Cell Biol.*

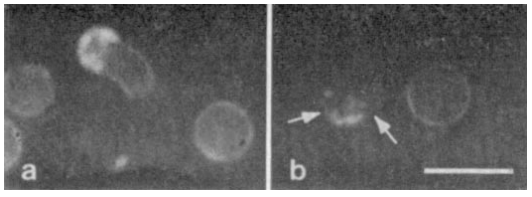

Fig. 9. Pattern of fluorescence on live sperm incubated in NBD-PC vesicles. a) Round cells are spermatids exhibiting a uniform ring of fluorescence and no staining of cytoplasmic organelles; spermatozoon photographed 15 sec after pseudopod extension. Fluorescence intensity of pseudopod membrane is about the same as that on nearby spermatids. Bright dots in the cell body are fused membranous organelles. b) Spermatid (right); spermatozoon (left) photographed 1 min after extending its pseudopod. Intensity of fluorescence in cell body membrane (arrows), visible among the membranous organelles, is about the same as on the spermatid membrane but fluorescence is barely detectable in the pseudopod. Bar = 5 μm. Reprinted from (11) with the permission of *J. Cell Biol.*

and was free to diffuse back over the pseudopod if motility was stopped by addition of metabolic inhibitors or fixative (11). These results show that lipids are cleared from the pseudopod at the same rate as glycoproteins. Thus they support the bulk membrane flow mechanism of surface movement.

A MOTILITY MODEL

The results of all the experiments described above can be explained if new membrane components are continually inserted at the tip of the spermatozoon's pseudopod. These components flow back over the pseudopod surface, and they become internalized at the pseudopod-cell body junction. Since both lipids and glycoproteins flow back over the pseudopod surface at the same rate, the flow must involve the entire membrane. Since the mature spermatozoon has no ribosomes, no protein synthesis can occur; therefore this flow must be accompanied by recirculation of membrane components.

This membrane flow would propel the spermatozoon forward whenever the pseudopod was attached to the substrate and the cell body was not. This suggests a function for the fusion of the membranous organelles with the cell body during spermiogenesis: release of a material to prevent cell-body adhesion to the substrate.

SPERM MUTANTS

One reason for pursuing this analysis of spermatozoan motility and development in *C. elegans* is that mutants altering these processes can be readily obtained because they have a distinctive sterile phenotype. We have isolated 34 mutant strains in which the sperm are defective. These strains define at least 16 different genes whose products are necessary to produce fertile sperm (2,5,13; D. Burke and T. Roberts, unpublished). The morphological and developmental defects caused by mutations in six of these genes have been described in detail (15). Two of them will be described here to illustrate some types of alterations induced by mutations in a single gene.

Mutations in the gene *fer-1* cause production of spermatozoa with short pseudopods and no MO fusions (Fig. 10). Apparently the pseudopods wiggle normally, but the spermatozoa cannot translocate. Consequently they get swept out of the spermatheca of hermaphrodites and cannot penetrate eggs (14). When these mutant spermatozoa are pulse labeled with fluorescent lectins to study their membrane flow, it is found that they insert new components at the tips of their short pseudopods

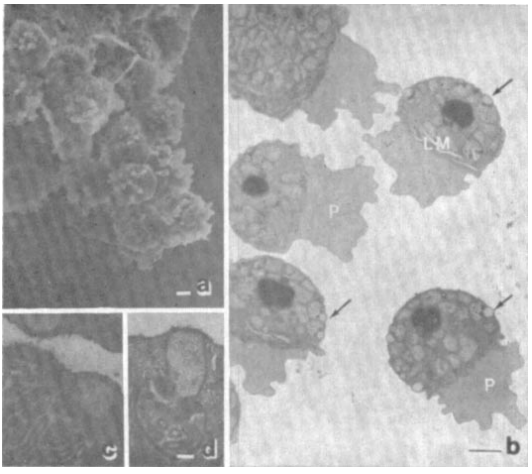

Fig. 10. *fer-1* spermatozoa. a) SEM of a fragment of male seminal vesicle showing many spermatozoa with rounded cell bodies and short pseudopods with normal projections. Bar = 1 μm. b) TEM of spermatozoa. Laminar membranes (LM) separate these pseudopods (P) from the cell body as in wild-type. Arrows show unfused MOs. Bar = 1 μm. c,d) Spermatozoa. MOs abutting plasma membrane but not fusing. Bar = 0.1 μm. Reprinted from (15) with permission of *J. Cell Biol.*

but do not take these up at the base (Fig. 11). Instead, fluorescence is found all over the surface. Similarly, latex beads move all over the surface of spermatozoa, from cell body to pseudopod and over the cell body, unlike the polarized movement of wild-type spermatozoa (11). Thus this mutation prevents the spermatozoon from establishing its topographical asymmetry and prevents uptake of membrane components.

A second mutant, in the gene *fer-2*, causes production of infertile sperm with grossly defective pseudopods (Fig. 12). Many cells have no pseudopods at all. When these cells are labeled with fluorescent lectins, it is found that most of them do not clear lectin binding components from the pseudopod at all, although a few can clear them slowly (12). This mutant appears to be defective in both pseudopod formation and motility.

The biochemical defects in these and the other mutants have not yet been identified. Sufficient sperm can be isolated for biochemical analysis, however, so it should be possible to find the defective gene products and thus interpret the morphological and motility defects in molecular terms.

WHAT PROPELS MOVEMENTS?

We anticipated that the actin, a ubiquitous protein in motile systems, would be a major component of the sperm. It is not. By gel electrophoresis, actin is less than 0.02% of the sperm's total protein (Fig. 13). This is a thousandfold less than other amoeboid cells except for *Ascaris* sperm which we have found to be only 0.5% actin (8). By two-dimensional gels and by immunofluorescence localization, some of this actin is indeed in the spermatozoa, but all attempts to demonstrate its participation in motility have been negative (9).

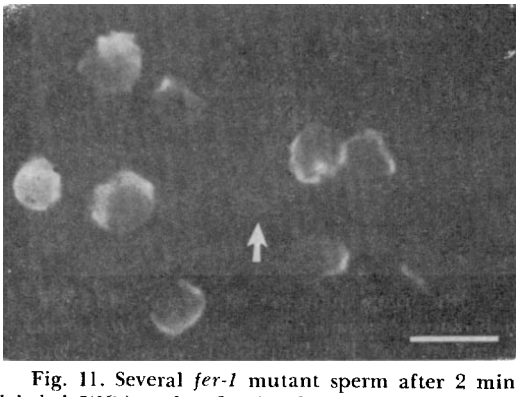

Fig. 11. Several *fer-1* mutant sperm after 2 min labeled WGA pulse, 3 min chase. Spermatid indicated by arrow, remaining cells are spermatozoa. Note the variety of labeling patterns. Bar = 5 μm. Reprinted from (12) with permission of *J. Cell Biol.*

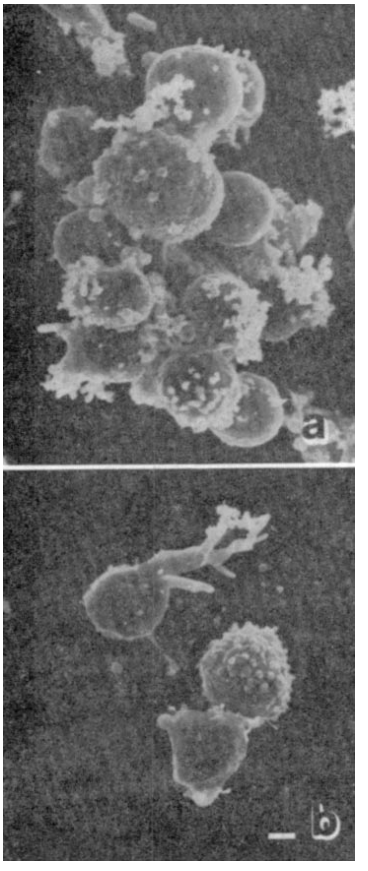

Fig. 12. SEM of *fer-2* spermatozoa. a) Cluster of sperm spermatozoa have short or aberrant pseudopods with projections, spermatids are smooth with few microspikes. b) Additional aberrant spermatozoa. Reprinted from (15) with the permission of *J. Cell. Biol.*

There is another protein that is the major component of both *C. elegans* and *Ascaris* sperm. This is a small (15,500 dalton) polypeptide which comprises 15% of the sperm's total protein (6,8,9). The function of this protein is unknown, but it makes up the fibrous bodies during spermatogenesis and becomes concentrated in the pseudopod during spermiogenesis (S. Ward and M. Klass, submitted for publication). Its abundance and location suggests that it participates in motility, but how it does so is unknown.

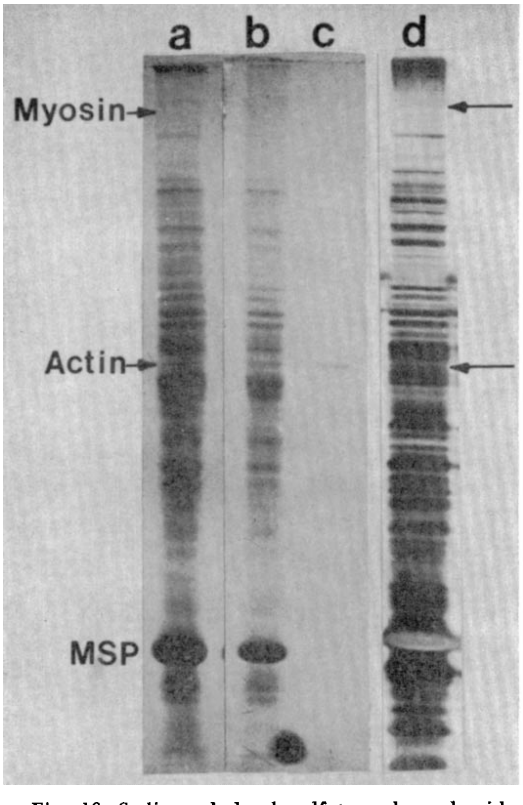

Fig. 13. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of sperm protein and actin. Lanes a–c are Coomassie-brilliant-blue stained. Lane d is silver stained. (a) 2×7^{10} sperm, 320 μg total protein, (b) 5×10^8 sperm, 80 μg protein, plus 0.2 μg rabbit actin, (c) 0.2 μg rabbit actin, (d) 10^7 sperm. The positions of worm actin and myosin from parallel gels of worm actomyosin are shown by arrows. MSP is the major sperm protein (6). Reprinted from (9) with permission of *J. Cell Biol.*

CONCLUSIONS

These results demonstrate that *C. elegans* spermatozoa have a novel mechanism of motility, propulsion by bulk membrane flow. The molecular mechanisms driving this flow are not yet understood, but they are unlikely to be dependent on an actin-myosin contractile system. The availability of a large collection of immotile mutants will allow further dissection of the molecular mechanisms for building the spermatozoon and for propelling its membrane flow.

There is an important practical implication of this apparently novel mechanism of spermatozoan motility, if it occurs in parasitic nematodes. It should be possible to find chemicals that interfere specifically with nematode sperm motility. These could then be used to sterilize populations of worms without harming their hosts or other organisms.

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