Caenorhabditis elegans Spermatozoan Locomotion: Amoeboid Movement with Almost No Actin

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ABSTRACT The pseudopods of Caenorhabditis elegans spermatozoa move actively causing some cells to translocate when the sperm are dissected into a low osmotic strength buffered salts solution. On time-lapse video tapes, pseudopodial projections can be seen moving at 20–45 μm/min from the tip to the base of the pseudopod. This movement occurs whether or not the cell is attached to a substrate. Translocation of the cell is dependent on the substrate. Some spermatozoa translocate on acid-washed glass, but a better substrate is prepared by drying an extract of Ascaris uteri (the normal site of nematode sperm motility) onto glass slides. On this substrate more than half the spermatozoa translocate at a velocity (21 μm/min) similar to that observed in vivo. Translocating cells attach to the substrate by their pseudopodial projections. They always move toward the pseudopod; changes in direction are caused by changes in pseudopod shape that determine points of detachment and reattachment of the cell to the substrate.

Actin comprises <0.02% of the proteins in sperm, and myosin is undetectable. No microfilaments are found in the sperm. Immunohistochemistry shows that some actin is localized in patches in the pseudopod. The movement of spermatozoa is unaffected by cytochalasins, however, so there is no evidence that actin participates in locomotion.

Fertilization-defective mutants in genes fer-2, fer-4, and fer-6 produce spermatozoa with defective pseudopodial projections, and these spermatozoa are largely immotile. Mutants in gene fer-1 have short pseudopods with normal projections, and these pseudopods move but the spermatozoa do not translocate. Thus pseudopod movement is correlated with the presence of normal projections. Twelve mutants with defective muscles have spermatozoa with normal movement, so these genes do not specify products needed for both muscle and nonmuscle cell motility.

Nematode sperm have intrigued biologists for over a century because they are nonflagellated and lack a conventional acrosome (4, 15). Their movement has been inferred to be amoeboid because they extend a pseudopod, but actual observations of translocating spermatozoa have been reported only in Nematospiroides dubius sperm in vitro (64) and in Caenorhabditis elegans sperm in vivo (60). We recently described the translocation of Ascaris lumbricoides spermatozoa in vitro and reported that these sperm have less actin than other amoeboid cells, ~0.5% of cell protein (32). We are especially interested in the motility of C. elegans spermatozoa because many fertilization-defective mutants that have infertile sperm can be isolated

(5, 17, 61). These mutants should help to identify gene products that are essential for cell motility.

In this paper we report in vitro conditions that have allowed us to study C. elegans sperm movements in detail. We compare the movements of spermatozoa from wild-type with those from fertilization-defective mutants and muscle-defective mutants. By biochemical analysis of isolated sperm we show that C. elegans sperm have even less actin than Ascaris sperm and have no detectable myosin. In the accompanying papers it is shown that the translocation of C. elegans spermatozoa can be explained by the flow of newly inserted membrane from the tip of the pseudopod backwards to the cell body (43, 44).

MATERIALS AND METHODS

Nematode Strains and Culture

All strains were maintained on petri dishes seeded with *E. coli* as described by Brenner (8). Wild-type is strain N2 from S. Brenner (Medical Research Council, Laboratory of Molecular Biology, Cambridge, England). The muscle-defective mutants listed in Table I were obtained from R. Waterston (Washington University, St. Louis, Mo.) or S. Brenner, except for *unc-90* which was isolated from strain ST-D29 obtained from R. Herman (University of Minnesota, Minneapolis). They are described in Waterston et al. (63). Strains CB1467: *him-5 (e1467)*V and CB1490: *him-5 (e1490)*V produce males at high frequency (18) and were used routinely as a source of normal sperm.

Juvenile males were picked from growth plates and maintained as virgins for 3 d at 25°C. They were then allowed to mate with an excess of hermaphrodites for 3 h just before dissection. This procedure ensures that there are >1,000 sperm per male and that up to 60% of them will be spermatozoa. Alternatively, spermatids were activated to spermatozoa with 0.5 μM monensin (31).

Light Microscopy

For observation and pharmacological experiments, sperm were obtained by cutting males with a razor blade fragment or fine tungsten needle in a drop of sperm medium (SM): 50 mM *N*-2-hydroxyethyl-piperazine-*N*'-2-ethanesulfonic acid (HEPES) titrated to pH 7.0 with NaOH, 50 mM NaCl, 25 mM KCl, 1 mM MgSO$_4$, 5 mM CaCl$_2$, 1 mg/ml bovine serum albumin (or 10 mg/ml polyvinyl pyrollidone, PVP 40) sometimes supplemented with 10 μg/ml gentamycin. Variations in this medium are described in Results.

For observation, sperm were placed on microscope slides that had been washed for 1-3 h in 1 N HCl, rinsed in distilled water and stored in 95% ethanol. The sperm were overlaid with a cover slip supported on two sides with Vaseline. All observations were made at room temperature, 20°-24°C. The effects of drugs were studied by dissecting sperm directly into medium supplemented with the test compound or by exchanging solutions through the open ends of the chambers. Control experiments with tracer dyes showed that all of the solution was exchanged within a few seconds.

Light microscope observations were made using Nomarski optics on a Zeiss Universal microscope equipped with a Panasonic WV-1350 television camera and model NV-8030 time-lapse videotape recorder. Sperm movements were analyzed either frame by frame, in real time, or speeded up 9- or 18-fold. Cell outlines were traced directly from the TV monitor onto transparent acetate film. All quantitative measurements were made from these tracings. Phase-contrast observations were made using a Zeiss standard microscope. Photography was done as previously described (60).

Preparation of Ascaris Uterine Contents as a Substrate for Sperm Motility

Intact uteri were dissected from mature *Ascaris* females (obtained from Esskay Quality Meats, Baltimore, Md.). The contents of the proximal 3 cm of each uterus were removed into SM salts (2 uteri/ml) by gentle massage with a rubber policeman. Eggs and large debris were removed by centrifugation and the supernatant was used immediately or stored frozen. For sperm motility tests, 5 μl of this solution were spread in an 8-mm Diam circle on an acid-washed glass slide and air-dried. Sperm were obtained by dissecting a *C. elegans* male in a drop of SM containing BSA and 0.5 μM monensin to activate them to spermatozoa (31), and these were placed on the dried extract. Preparations were overlaid with cover slips on Vaseline mounts as described above.

Electron Microscopy

For scanning electron microscopy, sperm were dissected into SM containing BSA on cover slips coated with *Ascaris* uterine extract and treated with monensin to activate spermatids to spermatozoa. These preparations were observed in a light microscope and recorded on video tape to identify translocating cells. These cells were rapidly fixed by perfusion of SM containing 1% formaldehyde plus 1.25% glutaraldehyde through the chamber and their positions on the cover slips were recorded. Samples were fixed overnight at 4°C, rinsed in SM, dehydrated in graded ethanols and critical point dried using CO$_2$ as the transition fluid. Preparations were coated with Au/PD and observed in a JEOL JSM-35 scanning electron microscope equipped with an LaB$_6$ filament operated at 8 kV. Cell positions recorded by light microscopy were used to identify those spermatozoa that were crawling when they were fixed.

Cells were routinely prepared for transmission electron microscopy (TEM) by glutaraldehyde-tannic acid followed by OsO$_4$ fixation and handled as described previously (59). Alternative fixation conditions are decribed in Results.

Sperm Isolation

Sperm were isolated by a procedure modified from that developed by M. Klass (21). Males were obtained by growing liquid cultures of *him-5* mutant strains that produce 35% males. Synchronous cultures were started with eggs obtained from adults isolated by flotation on 25% Ficoll and then treated with 20% Chlorox in 0.5 N NaOH for 8-12 min. 1-2 ml of eggs were placed in 2-liter baffled shake flasks (Bellco, Inc., Vineland, N. J., 2542 series) in S medium (52) with 0.1 M potassium phosphate. 20 ml of *E. coli* at 3 × 10^{11} cells/ml were added per ml of eggs, and the culture was shaken on a rotary table at 150 rev/min at 20°C. After 4 d, worms were collected by centrifugation and flotation on 25% Ficoll and resuspended on 35-μm pore size Nitex nylon filters (type HD3-35, Tetko, Inc., Elmsford, N. Y.) stretched on embroidery hoops. Hermaphrodites are retained by the filter and the males crawling through are separated from juveniles by collection on a 20-μm Nitex filter (HC3-20). The males are returned to culture for 1-3 additional days and refractionated if necessary so that cultures of 97-99% males are obtained. These males contain 600-2,000 sperm, depending on age. These sperm are 95-98% spermatids.

Males are washed by centrifugation and resuspended in a modified sperm medium in which Na$^+$ and K$^+$ have been replaced by choline to prevent activation to spermatozoa during centrifugation (31). This medium is made to 1 mg/ml BSA, 1 mM phenyl methyl sulfonyl fluoride, PMSF and 1 mM sodium bisulfite to minimize proteolysis during sperm isolation which is carried out at room temperature because the spermatids become fragile and partially activate when exposed to cold. Sperm are released from males by squashing between 16 × 22 cm lucite plates in a Carver laboratory Press at 13,000 lbs. Carcasses are removed by filtration through three layers of 10-μm pore size Nitex filter (HC-3-10). The sperm are separated from soluble material by layering them over 10 ml of 10% Percoll or 10% Ficoll (Pharmacia Fine Chemicals, Piscataway, N. J.) in choline substituted SM plus PVP and centrifuging the sperm into a pellet. The pellet is washed two times in the same medium and either quick frozen and stored at −70°C or lysed immediately with SDS containing gel sample buffer and boiled for gel electrophoresis or refrozen and stored at −70°C.

Recovery of sperm by this procedure is 60-90%, and >90% of the sperm remain as spermatids. 3 × 10^8 cells can be obtained from a 200-ml culture. 50-80% of these spermatids can be activated to spermatozoa if they are resuspended in SM and treated with monensin. By direct observation in a hemocytometer, spermatocytes and residual bodies are the only cellular contaminants, and these are only 0.05-0.1% the number of spermatids. There is also a visible granular contaminant that looks in TEM as if it came from the gut during squashing.

Cell Surface Labeling

Intact spermatids or spermatozoa were labeled in SM with ^{125}I-iododiazosulfanilic acid as described in (6). Lysed cells were treated with 0.1% SDS and frozen and thawed three times before labeling. The detailed results of this labeling will be presented elsewhere, but evidence that only surface components are labeled on intact cells is that the major sperm protein, which comprises 15% of the total sperm internal protein (21), is not labeled unless the cells are lysed.

Gel Electrophoresis

One-dimensional gel electrophoresis was performed according to Laemmli and Favre (24) using 10-20% acrylamide gradient gels. Two-dimensional gels were used according to O'Farrel (35), except that pH 5-7 ampholytes were used. Samples were prepared for gel electrophoresis either by sonicating and treating with DNAse (35) or by lysing in SDS, boiling, then precipitating with acetone and resuspending in sample buffer. Gels were Coomassie-Blue-stained as described by Fairbanks et al. (14) and silver-stained by the method of Switzer et al. (54) as modified by Oakley et al. (33). Molecular weight standards were nematode myosin (210,000), β-galactosidase (130,000), phosphorylase (94,000), BSA (68,000), pyruvate kinase (57,000), ovalbumin (43,000), pancreatic DNAse (31,000), soybean trypsin inhibitor (21,000), cytochrome C (12,400), and a cytochrome C doublet (25,000).

Quantitation of actin in sperm was done by scanning Coomassie-Blue-stained gels with a Joyce-Loebl densitometer (Durham, England). The area under the actin peak was determined and converted to micrograms of protein using a standard curve of purified rabbit muscle actin electrophoresed on gels that were stained and scanned in parallel. On 2-D gels the intensity of the actin spot was compared by eye to standard 2-D gels with various amounts of worm actomyosin (15% actin) electrophoresed and stained in parallel. Gels of ^{125}I-labeled proteins were autoradiographed as described in Swanstrom and Shank (53).

Partial peptide mapping of bands cut from Coomassie-Blue-stained gels was done as described by Cleveland et al. (11), except that the electrophoresis was not interrupted, the gels were 15-20% acrylamide, and the final peptide pattern was silver-stained as described above. This sensitive stain makes it possible to obtain a peptide map from a band on a gel with as little as 0.1 μg of protein.

Preparation of Actin Standards

Crude actomyosin for protein standards was prepared from whole worms (strain CB1490) according to Epstein et al. (13) and stored in 50% glycerol at −20°. By densitometry of Coomassie-Blue-stained gels, this preparation was 15% actin. Purified actin was prepared from an acetone powder of rabbit back and hind leg muscles (55) according to the procedure of Spudich and Watt (51) and stored at 4°C as G actin after filter sterilization. Protein determinations were made according to Lowry et al. (29) using BSA as standard.

Immunohistochemistry

Rabbit antifish muscle actin antiserum was the kind gift of Keigi Fujiwara. Rabbit antichicken gizzard actin antiserum was generously provided by Ira Herman. Its characterization is described in reference 16. Rabbit anti-*C. elegans* muscle myosin antiserum was kindly provided by Henry Epstein. It is described in reference 46. Sperm were fixed on cover slips or glass slides for 30 min in 4% formalin freshly prepared from paraformaldehyde. They were permeabilized by 1-min immersion in 100% acetone at −20°C, then rinsed and stained with antiactin at 100 μg/ml in PBS for 1 h, rinsed thoroughly, then stained with Rhodamine-conjugated goat anti-rabbit IgG (Cappel Laboratories, Cochranville, Pa.) diluted 1:80. To reduce background some preparations were treated with 5 mg/ml lysine after formaldehyde fixation, and the second antibody was diluted in 1:10 normal goat serum. Epi-fluorescence photographs were recorded on Kodak Tri-X film developed in HC-110 developer dilution B or in Diafine (Acufine Inc., Chicago, Ill.).

Heavy meromyosin fragments of rabbit myosin were generously provided by M. Rogers and W. Harrington. Sperm were lightly fixed in formaldehyde and permeabilized with glycerin (48) or saponin (34) and stained with HMM as described in reference 34.

Reagents

Cytochalasins B, D, and E were generously provided by Shin Lin and were stored as stock solutions in dimethyl sulfoxide (DMSO). They were biologically active when tested on nerve growth cones. Phalloidin (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) was also used as a DMSO stock solution. These drugs were diluted fresh in sperm medium, and the DMSO concentration never exceeded 1% although cell morphology and behavior was normal in up to 5% DMSO.

Colchicine (Sigma Chemical Co., St. Louis, Mo.) and Oncobendazole (Janssen R. & D., Inc. New Brunswick, N. J.) were made up fresh in SM and diluted just before use.

All other chemicals were of reagent grade and were obtained from Sigma Chemical Co., Worthington Biochemical Corp., Freehold, N. J., or Schwartz/Mann, Orangeburg, N. Y.

Terminology

The term "amoeboid" is used to describe the movement of *C. elegans* spermatozoa because it has been widely used to describe nematode spermatozoa (e.g. 4, 15).

RESULTS

Media for Studying Sperm Motility

Sperm from mated *C. elegans* males were used to develop a minimal maintenance medium that would sustain sperm motility for several hours and prevent cell lysis and degeneration. Many variations on standard tissue culture media were tested. The best results were obtained with the low osmotic strength SM described in Materials and Methods. Three essential requirements were identified. (*a*) The medium must be of low osmotic strength: 217 mOsm is the measured value for SM. Lysis and morphological defects occur in media above 300 mOsm. (*b*) SM must contain protein or other macromolecules such as polyvinylpyrollidone or Dextran 500 to prevent lysis although the salts alone are tolerated for short times and are used with some fixatives. (*c*) Pseudopods are maintained best at neutral pH although the range pH 6 to 8 is acceptable. A minor dependence on divalent cations is observed. In one experiment 52% of sperm in sperm medium (5 mM Ca^{++}, 1

mM Mg^{++}) had pseudopods *vs* 28–29% when calcium was absent or replaced with 1 mM EGTA. The removal of magnesium had even less effect. The ratio of sodium to potassium may be varied considerably. HEPES gave the best results as a buffer although Tris may be substituted. Phosphate buffers were less satisfactory.

Substrate Requirements for Sperm Movement

A number of substrates were tested for their ability to promote cell attachment and to allow translocation. These include polystyrene, tissue culture plastic, acid-washed glass, magnesium acetate-impregnated glass, or the following materials dried onto glass slides: poly-L-lysine, poly-L-ornithine, BSA, ovalbumin, fetal calf serum, type IV collagen, fibronectin, and agar. Cells attach to many of these substrates but fail to translocate except on acid-washed glass where 5–15% of the spermatozoa will crawl forward.

Many of the observations on sperm motility described below were made on acid-washed glass, but a better substrate can be prepared by squeezing soluble material from the uteri of dissected *C. elegans* hermaphrodites and drying this onto glass slides. This was tried because the uterus is the normal site of sperm motility. It was found that material from the parasitic nematode *Ascaris* worked as well, so this was used for all quantitative studies because it was more easily obtained. At the appropriate concentration, this *Ascaris* uterine material increases the fraction of spermatozoa that translocate to 30–60%, and increases their velocity. The identity of the translocation-promoting material in the *Ascaris* extract is unknown, except that it is heat stable, trypsin insensitive, TCA soluble, not extractable by chloroform-methanol, and it chromatographs on a Bio-Gel P2 column (Bio-Rad Laboratories, Richmond, Calif.) as a single peak with apparent molecular weight of 600. It is not found in *Ascaris* coelomic fluid or extracted from tissue other than the uterus.

Observations of Sperm Motility

The mature spermatozoon of *C. elegans* is a bipolar amoeboid cell whose ultrastructure has been reported in detail (31, 59, 62). On one end of the cell is a hemispherical cell body containing the nucleus and major cell organelles; this is a stable structure unless deformed by substrate attachment. At the other end of the sperm is a rapidly moving pseudopod 0 to 5 μm long that is filled with an amorphous cytoplasm without organelles or microtubules or microfilaments (59, 62, and below). The pseudopod joins the cell body at a slight constriction. Dotting the surface of the pseudopod are 60 to 70 stubby projections 250 nm in Diam and 200 to 450 nm long that, like the pseudopod, are filled with amorphous cytoplasm (59).

When a spermatozoon is migrating across a treated substrate its pseudopod flattens down against the substrate which its projections contact (Fig. 1). TEM examination of spermatozoa cut perpendicular to the substrate suggests that only the projections are in contact with the substrate (not shown). In contrast, nontranslocating spermatozoa have more cylindrical, shorter pseudopods. They usually attach by their cell bodies with their pseudopods extending into the medium (Fig. 1). On strongly adherent substrates such as poly-L-lysine the whole cell flattens out against the substrate (data not shown) and no translocation is observed.

The movements of *C. elegans* spermatozoa on acid-washed glass or *Ascaris* extract resemble those of leukocytes (42),

embryonic cells (56) or *Ascaris* sperm (32). Two movements can be readily distinguished: alterations in pseudopod shape and movements of pseudopodial projections over the pseudopod surface. Both movements of the pseudopod are seen whether or not the cell is attached to the substrate.

Shape changes of pseudopods are produced by protrusion or retraction of the cell's leading edge or changes in contour along the sides of the pseudopod (Figs. 2 and 3). Clusters of pseudopodial projections form at the tip of the pseudopod and sweep backwards across the tops, bottoms, and sides of pseudopods, disappearing at the joint between the cell body and pseudopod. Their movements are independent of bulk shape changes. Groups of projections may form a transverse row across the pseudopod, not unlike a ruffle on a cultured fibroblast (20). Projections sweep backward on cells at 20–45 μm/min, slightly faster than the forward progress of the sperm.

During translocation, alterations in pseudopod size and shape continue both in the direction of motion and laterally or obliquely. Occasionally, translocation occurs without changes in pseudopod shape and size, suggesting that projection movements, not bulk pseudopod movements, propel the cell forward.

Turns are preceded by oblique or lateral changes in pseudopod shape that presumably lead to new substrate attachments (Fig. 4). If the trailing cell margin detaches abruptly, the cell body jolts forward or suddenly flips to the outside of the turn. If the cell margin is firmly attached, it distends into a flattened or conical form, sometimes trailing small retraction fibers. Detachment of these fibers is accompanied by a quick return to the usual hemispherical shape. Cells also turn when pseudopod detachment is followed by rotation. The new direction is established when new attachments are formed.

Fig. 5 illustrates a number of tracks of sperm moving on acid-washed glass slides as recorded on video tape. A typical sperm moves in a straight line or in a gentle arc for ~20 μm before turning. The velocity during this straight run averages 9.4 μm/min with a maximum of 30 μm/min on acid-washed glass. In vivo, sperm may move at up to 36 μm/min over eggs or along gonad walls. On *Ascaris* uterine extract, the mean velocity is 21 ± 4 μm/min, the range is 5–43 μm/min. Fluctuations in velocity occur both in the population of cells and in individuals. Fig. 6 shows the tracks and velocity profiles of three cells, illustrating variations in their behavior. Notice how moving sperm occasionally stop for up to two min before resuming their migration. Occasionally, pseudopods detach from the slide; the cells then remain in place and wiggle for many minutes before reattaching and moving off.

Numerous collisions between translocating sperm have been observed. Moving sperm sometimes dislodge sessile spermatids which, if firmly attached to the slide, may snap back into place as the moving cell passes. The same may happen to ruffling spermatozoa. Head-on collisions between spermatozoa provide
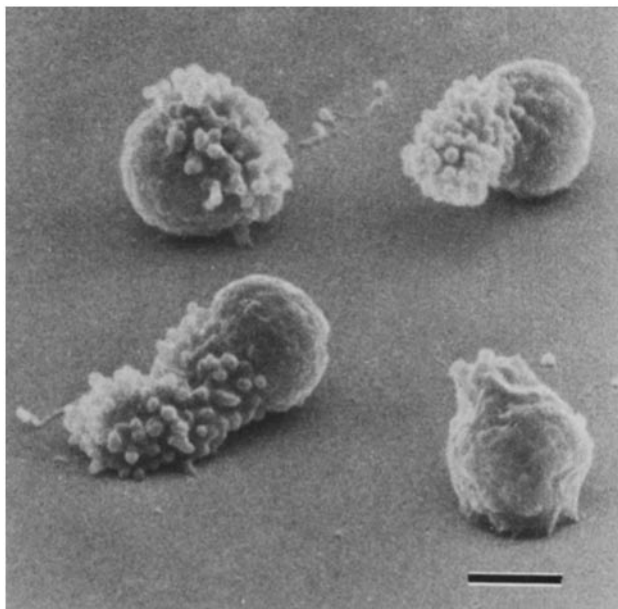

Figure 1 SEM image of a field of spermatozoa on a cover slip coated with *Ascaris* uterine exudate. The movements of these cells were recorded on time-lapse video tape before fixation. The only translocating cell was the one in the lower left with the flattened pseudopod. The other cells were moving their pseudopods but not migrating over the substrate. The cell on the lower right is aberrant. Bar, 2 μm. Tilt 45°. ×7,000.

Figure 3 Rapid changes in contour of the pseudopodia of two spermatozoa (A and B). Tracings from videotape records of two sperm show changes in shape and retrograde movement of pseudopodial projections. The sequence in each frame is solid line, dotted line, dashed line, with the times shown in seconds. Bar, 5 μm.
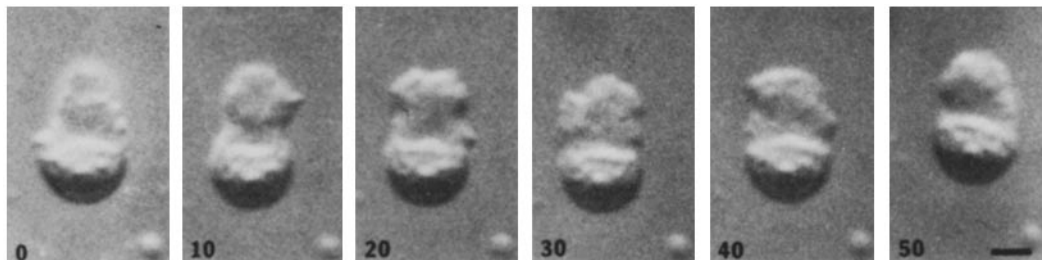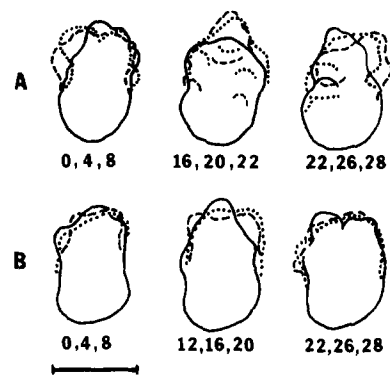

Figure 2 Nomarski-microflash photos of a spermatozoon at 10-s intervals, illustrating changes in pseudopod shape. Protrusion and retraction of different regions on the pseudopod are independent. The retrograde movement of pseudopodial projections is especially obvious between 10 and 20 s along the right hand side of the cell. Bar, 2 μm.

no evidence for contact inhibition of movement. Colliding cells continue to move forward and are minimally deflected as they pass. Moving sperm may drag one or two other cells (with or without their own pseudopods) behind. When this occurs, the attachments between cells are invariably between their cell bodies, not their pseudopods.

Sperm from males have been observed in vivo in the uteri and spermathecae of hermaphrodites as well as in the vas deferens of male gonads. The motility of these cells is indistinguishable from that of male sperm in vitro whether the cells use oocytes, shelled zygotes, or gonad walls as substrates. Fig. 4c shows a sperm moving through the lumen of the vas deferens of a male just after copulation. The in vivo and in vitro motility of endogenous hermaphrodite sperm is also indistinguishable from the in vitro motility of male sperm. Hermaphrodite and male sperm are known to be different in some respects because male sperm outcompete hermaphrodite sperm in fertilizing eggs (60). Visible differences in motility do not account for this property.

Actin and Myosin in Sperm

Because the amoeboid motility of spermatozoa appeared like that of other eucaryotic cells, we anticipated that actin would be a major polypeptide in the sperm. That is not the case. By SDS-polyacrylamide gel electrophoresis (SDS-PAGE), only a minor band is found at the mobility of worm muscle actin (Fig. 7). Two-dimensional gels show that this band is predominantly

a polypeptide that comigrates precisely with the major worm actin component (Fig. 8). Quantitation of Coomassie Brilliant Blue-stained 1-D gels or silver-stained 2-D gels reveals that this putative actin represents only 0.02% of the protein present in purified sperm.

Further evidence that the band visible on 1-D gels is actin comes from partial peptide mapping. Fig. 9 shows the silver-stained partial peptide map of the putative actin band cut from a Coomassie Blue-stained gel. The three peptides found in worm muscle actin are also found in the sperm actin, and the peptide at 25,000 is the most abundant. Because the sperm actin band is not homogeneous many other peptides are found as well. Attempts to peptide map the actin spot cut from 2-D gels have been unsuccessful.

Because of the small amount of actin detected biochemically we worried that actin originally present in the sperm could have been lost by proteolysis during preparation for gel electrophoresis. This is unlikely because sperm preparations lysed directly in hot SDS showed an intensity actin band on 1- and 2-D gels that was similar to that of cells lysed first by sonication (data not shown). In addition, the intensity of the actin band on 1-D and 2-D gels has been similar in more than 15 independent batches of isolated sperm in spite of slight variations in handling the cells. No proteolytic activity can be detected in sperm lysates when exogenous dye-coupled or isotope-labeled proteins are added. The major spot that migrates just below actin on 2-D gels (Fig. 8) does not have peptides corresponding to those in actin (not shown), and the major sperm protein is not an actin fragment (21, 32).

Again, because of the small amount of actin detected in purified sperm we were concerned whether this actin was in the sperm at all or whether it might be an impurity. We have used two different antiactin antisera to detect actin in fixed and permeabilized sperm by indirect immunofluorescence. Both sera gave the same result: dots of fluorescence are found in the pseudopods of spermatozoa (Figs. 10 and 11). Controls show that this punctate staining of spermatozoan pseudopods is not seen if the antiactin antiserum is omitted (Fig. 10 e, f) or if it is blocked by preincubation with purified rabbit actin (Fig. 11 c, d) or crude worm actomyosin (not shown). It is also not seen with another hyperimmune serum directed against nematode myosin (Fig. 11 e, f). Spermatids in the same field as the
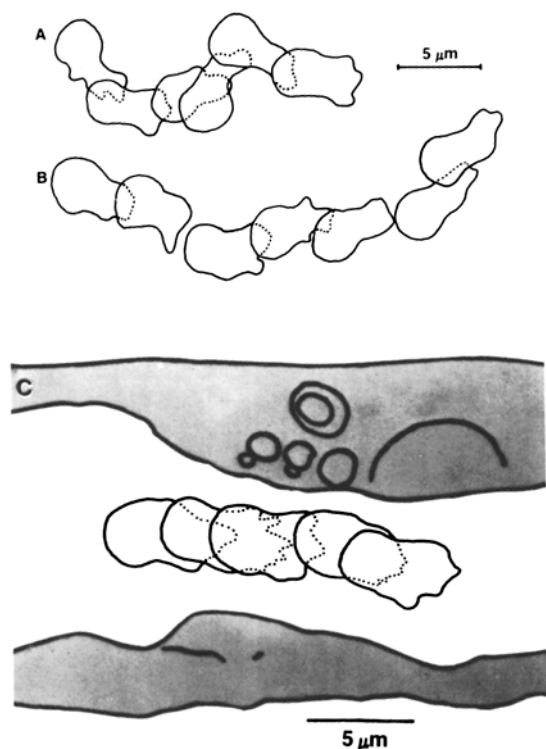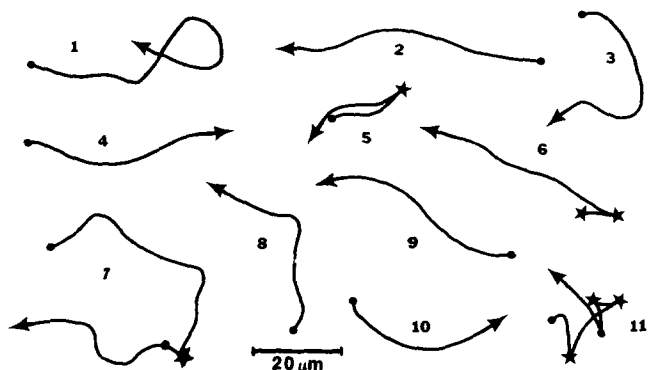

FIGURE 4 Outlines of spermatozoa moving on acid-washed glass and in the male vas deferens (A and B). Spermatozoa moving on glass for three min. Outlines are at approximately equal displacement intervals. Changes in contour of the leading pseudopodia produce changes in the direction of translocation. The cell body remains rounded and follows along behind. (C) A spermatozoon moving inside the lumen of the male vas deferens appears similar to sperm moving in vitro. Gonad walls are shaded. The average speed of the cell in (C) is 36 μm/min compared to 6.6 and 8.3 μm/min for cells A and B.

FIGURE 5 The tracks of 11 spermatozoa moving on glass are shown. Cells may move in relatively straight lines or gentle arcs for up to 50 μm (tracks 2, 4, 9, 10) or may turn through small angles and even cross their own track (1, 3, 8, 11). Frequently cells cease translocating and wiggle in place for several minutes (stars, tracks 5, 6, 7, 11). They may then start moving again in any new direction. Occasionally, cells become quiescent (dots, tracks 7 and 11). This may last for up to 2 min before the cells begin the crawl again.
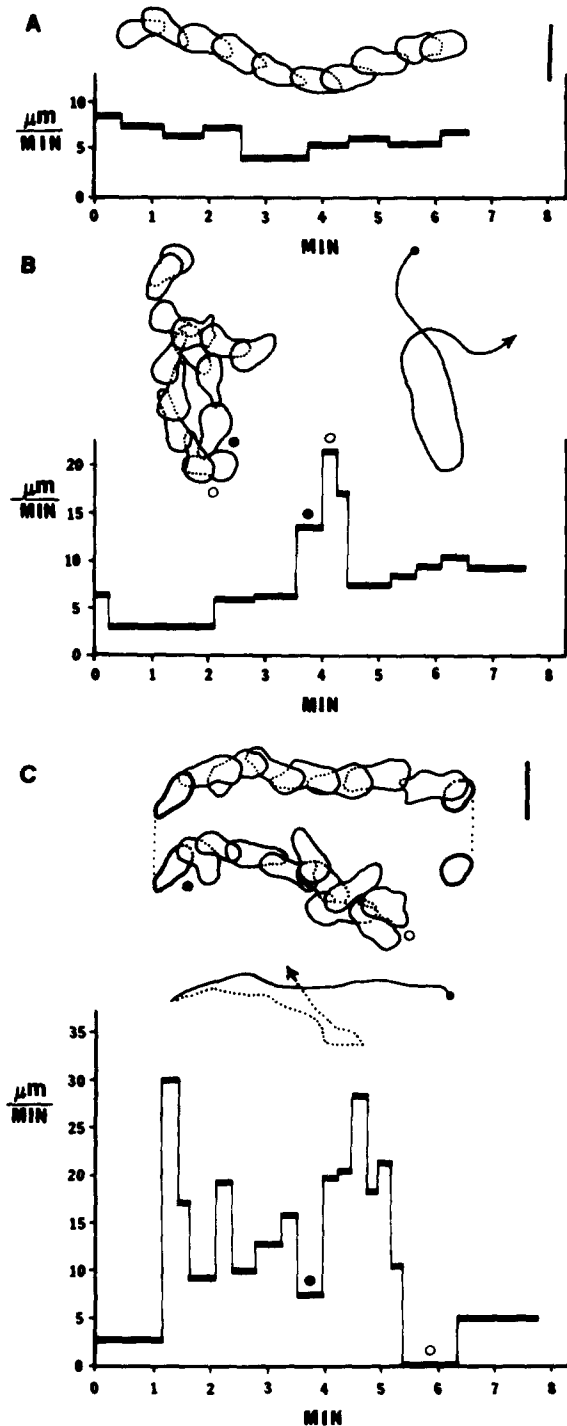
spermatozoa do not show punctate staining, and their uniform staining is difficult to distinguish from background. Consistent with the low amount of actin in the sperm, we were unable to detect fluorescence above background with a less sensitive, directly labeled antibody prepared from the same antichicken gizzard antiserum used in one of the indirect assays (not shown). This directly labeled serum readily detected actin in pseudopods of *Ascaris* spermatozoa, which have 0.5% of their protein as actin (32).

These immunofluorescent staining results show that at least some actin is present in spermatozoa but do not reveal whether this accounts for all of the 0.02% actin found in preparations of purified sperm. There are several potential sources of contaminating actin: fragments of worm carcasses; actin or actinomyosin released from carcasses during sperm isolation; other cell types containing actin. Carcasses or actomyosin are unlikely contaminants because no myosin (< 0.005%) is detected on gels (Figs. 7 and 12). When isolated sperm are labeled with the cell impermeant surface label ^{125}I-iododiazosulfanilic acid, a spot with the mobility of actin is not found by autoradiography of 2-D gels unless the sperm are lysed with detergent before labeling (Fig. 13). This shows that soluble actin or actomyosin or other actin outside of a cell is not contributing substantially to the actin found in purified sperm.

By direct examination of purified sperm preparations with the compound microscope, the only contaminating cells detected are spermatocytes and these are 0.05–0.1% the number of sperm. Spermatocytes do stain more brightly with antiactin antisera than do sperm in the same field (Fig. 14 a, b) and they do contain actin microfilaments (Fig. 14 c), so they must be contributing to the actin found in sperm preparations. Therefore 0.02% is a maximal estimate of the amount of actin in the sperm.

Microfilaments and Microtubules

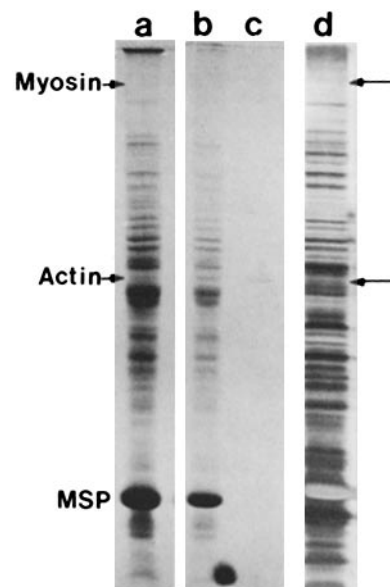With our conventional fixation procedure (1.3% glutaralde-

FIGURE 6 Tracks of three spermatozoa with accompanying velocity profiles. Each track is a tracing from videotape displaying the outline and position of a cell at approximately equal displacement intervals. In (b) and (c) overlap of cell outlines occurs, so the path of movement is shown by arrows also. In (c) the spermatozoon nearly retraces its tracks following a U-turn, so the second part of the track is displaced for clarity. Vertical bars, 10 μm.

Velocity profiles show the averages of speeds of leading and trailing cell margins between successive outlines. Circles indicate features of special interest, such as sharp turns or periods of quiescence.

Spermatozoon (A) moves in a gentle arc with approximately constant velocity. Cell (B) moves with relatively slow speed until turning (o). It then shows a burst of rapid movement (o) before slowly down again. Cell (c) moves with variable speed and wiggles without translocation for a minute (o), then it begins moving again.

FIGURE 7 SDS-PAGE of sperm protein and actin. Lanes a–c are Coomassie-Brilliant-Blue-stained. Lane d is silver stained. (a) 2 × 10^7 sperm, 320 μg total protein, (b) 5 × 10^6 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 (21).
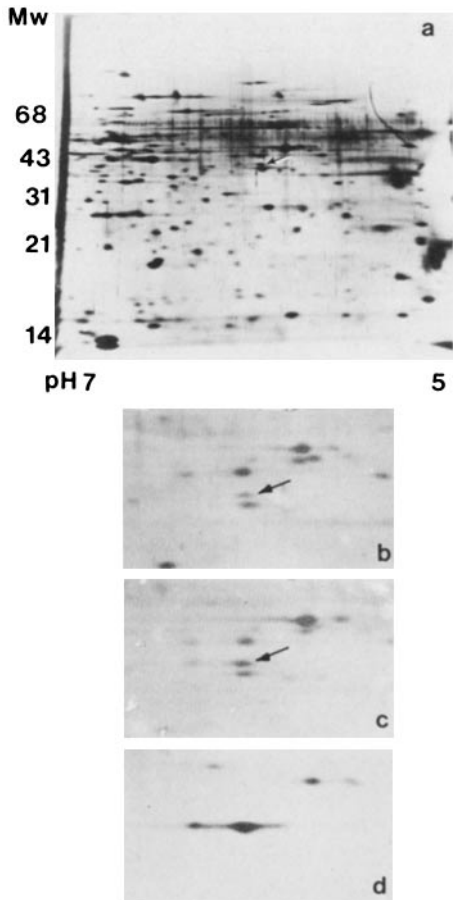
FIGURE 8 Two-dimensional gels of sperm proteins and standards. SDS-PAGE is vertical, the molecular weights ($\times$ 10^{-3}) of some standards are indicated. Isoelectric focusing (pH 5–7 ampholytes) is horizontal. All gels are silver-stained. (a) 10^7 sperm, the spot that comigrates with worm actin is indicated by arrow. (b–d) Actin region only. (b) 10^7 sperm. (c) 10^7 sperm plus worm actomyosin. Note enhancement of actin spot. (d) Worm actomyosin. Unlike Schachat et al. (47) we find that actin gives one major spot and a minor spot more basic. Sometimes the smear to the acidic side of the major actin spot forms another minor spot.

hyde plus 0.2% tannic acid followed by 1% OsO_4) no filaments with the dimension of actin-containing microfilaments or microtubules are found in sections of spermatozoa or spermatids (59). The small amount of actin present in spermatozoa would make microfilaments difficult to find in sections, but the immunofluorescence results suggest that there are local concentrations of actin, so filaments might be detected in the pseudopods adjacent to the membrane if they were present. We have examined sections cut both parallel and perpendicular to the substrate without finding microfilaments. We have tried numerous fixation conditions to preserve microfilaments or microtubules for detection in sections. These include first fixations with glutaraldehyde and formaldehyde, glutaraldehyde and OsO_4 combined, glutaraldehyde in various concentrations and from several suppliers and in several buffers, glutaraldehyde followed by thiocarbohydrazide, as well as glutaraldehyde plus tannic acid. Some of these first fixations were followed by osmication for 15 min to 1 h at pH values from 6 to 8 (30) in cold and at room temperature. In no case could microfilaments or microtubules be found in the pseudopods or elsewhere in the cells (data not shown).

Heavy meromyosin decoration of glycerinated or saponin-

permeabilized cells also failed to detect actin filaments in spermatids or spermatozoa whereas they were readily detected in spermatocytes in the same sections (Fig. 14 c).

A more sensitive way to detect actin filaments if they are attached to membranes or held in the cortex of a cell is to attach the cells to a microscope grid, blast off the top of the
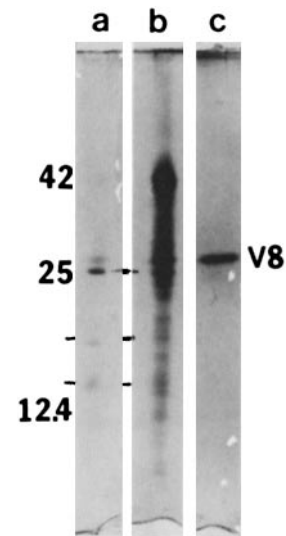

FIGURE 9 Partial peptide mapping of worm muscle actin and sperm actin. Lane (a) worm muscle actin cut out of a 1-D gel plus 12.5 ng staphylococcal V8 protease. (b) Sperm actin band cut out of a 1-D gel, such as shown in Fig. 7 a, plus 12.5 ng V8 protease. (c) 50 ng V8 protease (V8).
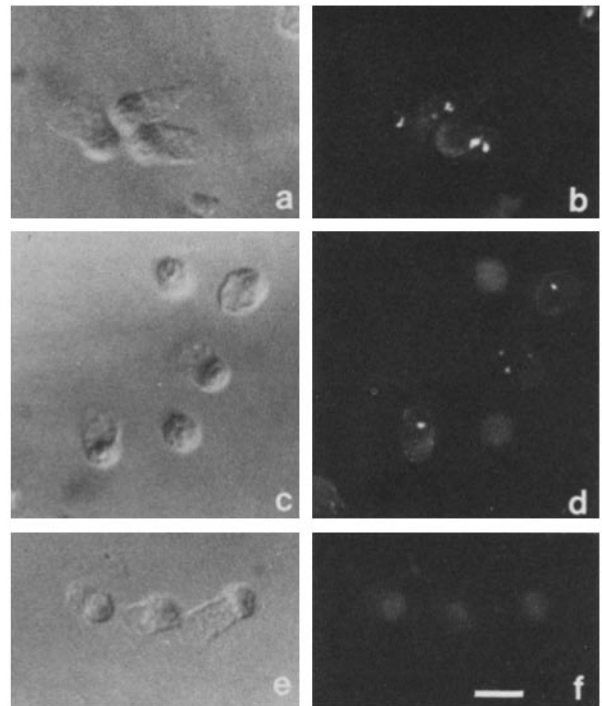

FIGURE 10 Antiactin immunofluorescence. (a–d) Spermatozoa fixed, permeabilized, treated with antichicken gizzard actin antiserum, then stained with rhodamine-conjugated goat antirabbit sera. (e–f) Control omitting antiactin serum. a, c, e Nomarski; b, d, f fluorescence. 90% of the spermatozoa with well preserved pseudopods have at least one bright spot of stain in experimentals (mean = 2.8/cell) whereas only 12% of control cells have any bright spots.
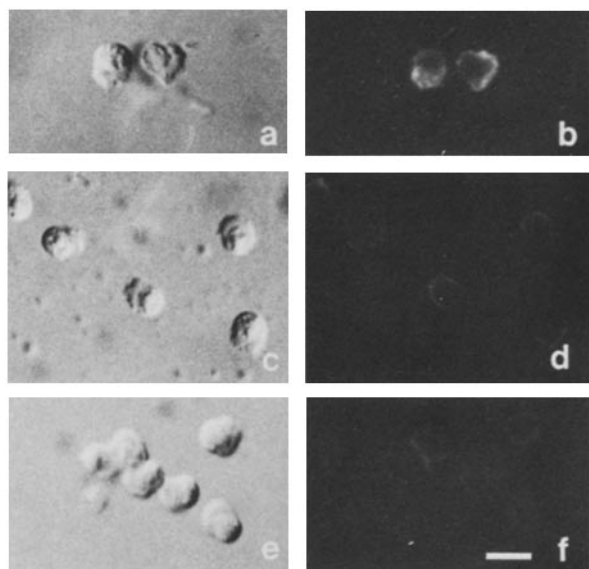
FIGURE 11 Antiactin immunofluorescence. (a, b) Spermatozoa fixed, permeabilized, treated with antifish muscle actin serum then stained with rhodamine-conjugated goat antirabbit serum. (c, d) Control spermatozoa prepared in parallel in which the first antiserum was mixed with 1 mg/ml rabbit actin. (e, f) Spermatozoa prepared in parallel treated with antinematode myosin serum. a, c, e Nomarski optics; b, d, f fluorescence. Bar, 5 μm.
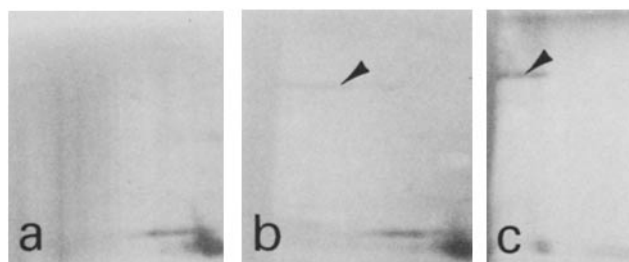

FIGURE 12 Two-dimensional gels of sperm proteins and standards, myosin region, silver stained. Myosin runs as a streak with variable lengths as indicated by arrows. (a) 10^7 sperm, (b) 10^7 sperm plus worm actomyosin, (c) worm actomyosin.

cell with a jet of buffer or detergent, and negatively stain the adhering membrane surface (10, 23). We have tried this with several buffers with and without calcium and have included heavy meromyosin or phalloidin in the washing solution to maximize the preservation of microfilaments. Only indistinct short filaments were found (Fig. 15). Repeated attempts to decorate these microfilaments with heavy meromyosin were unsuccessful, so they are unlikely to be actin microfilaments.

No structures corresponding to the dots of actin observed by immunofluorescence (Figs. 10 and 11) are found in TEM. We have asked whether or not the dotted staining pattern is induced by the attachment of cells to the substrate (poly-L-lysine-coated glass) before fixation. It is not. Cells fixed in suspension with formaldehyde before substrate attachment have about the same number of dots of stain (2–3/spermatozoa) and the same distribution (70% in pseudopod, 25% at base of pseudopod and 5% in cell body) as cells attached to the substrate before fixation.

Pharmacology

Many cells halt movement or change shape on exposure to

micromolar concentrations of cytochalasins (26, 27, 50). Cytochalasins B, D, and E had no effects on spermatozoan motility even at concentrations of 50–100 μM, even when applied in liposomes that fuse with the cell. In contrast, cell division during spermatogenesis was reversibly inhibited by 5 μM cytochalasin B (G. Nelson, unpublished observation) and embryonic cleavages are blocked by cytochalasin B (25).

The drug, phalloidin, induces polymerization of actin under conditions that normally favor the monomeric state of the protein. Alterations of cell shape and function accompany treatment of some live cells with the drug (40). Phalloidin had no noticeable effects on sperm motility, spermatogenesis, or cell morphology at 10–20 μg/ml.

Tubulin-depolymerizing drugs, colchicine and oncobendazole, were tested and found to have no visible effects on sperm motility at concentrations up to 100 μg/ml.

Spermatozoan Motility in Muscle-defective and Fertilization-defective Mutants

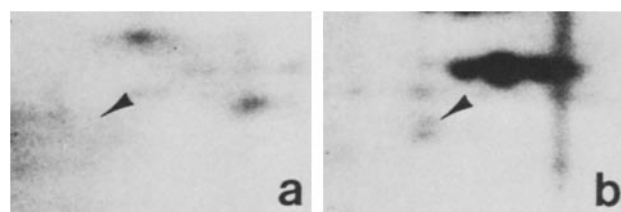To learn whether genes controlling muscle organization and

FIGURE 13 Autoradiographs of two-dimensional gels of labeled sperm protein, actin region only. (a) Intact spermatids labeled with [^{125}I]diazotized iodosulfanilic acid (10^6 cpm). (b) Lysed spermatids labeled in parallel (0.4 × 10^6 cpm). The position of the actin spot was located exactly from the stained gel patterns which were nearly identical for the two gels.
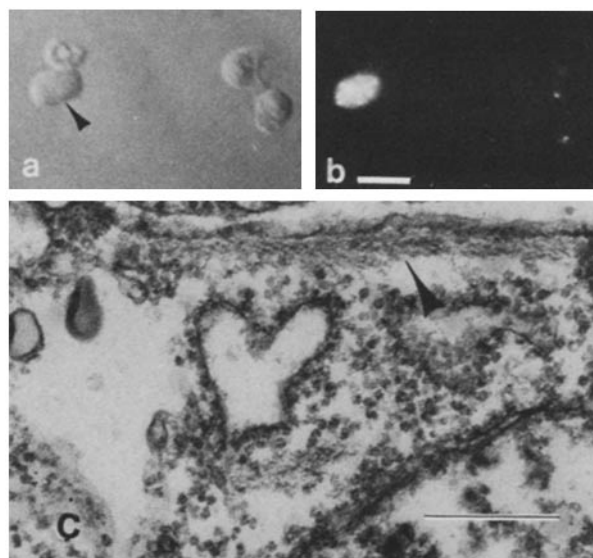

FIGURE 14 Spermatocyte. (a, b) Prepared as in Fig. 10 a–d, stained with antichicken gizzard actin serum. Arrow shows the spermatocyte. Note how much more intensely it is stained than are the two spermatozoa on the right. Bar, 5 μm. (c) Electron micrograph of spermatocyte cortex treated with heavy meromyosin showing arrowhead appearance of decorated actin filaments. No such decorated filaments were found in spermatids or spermatozoa on the same or other sections. Bar, 0.5 μg. ×36,000.
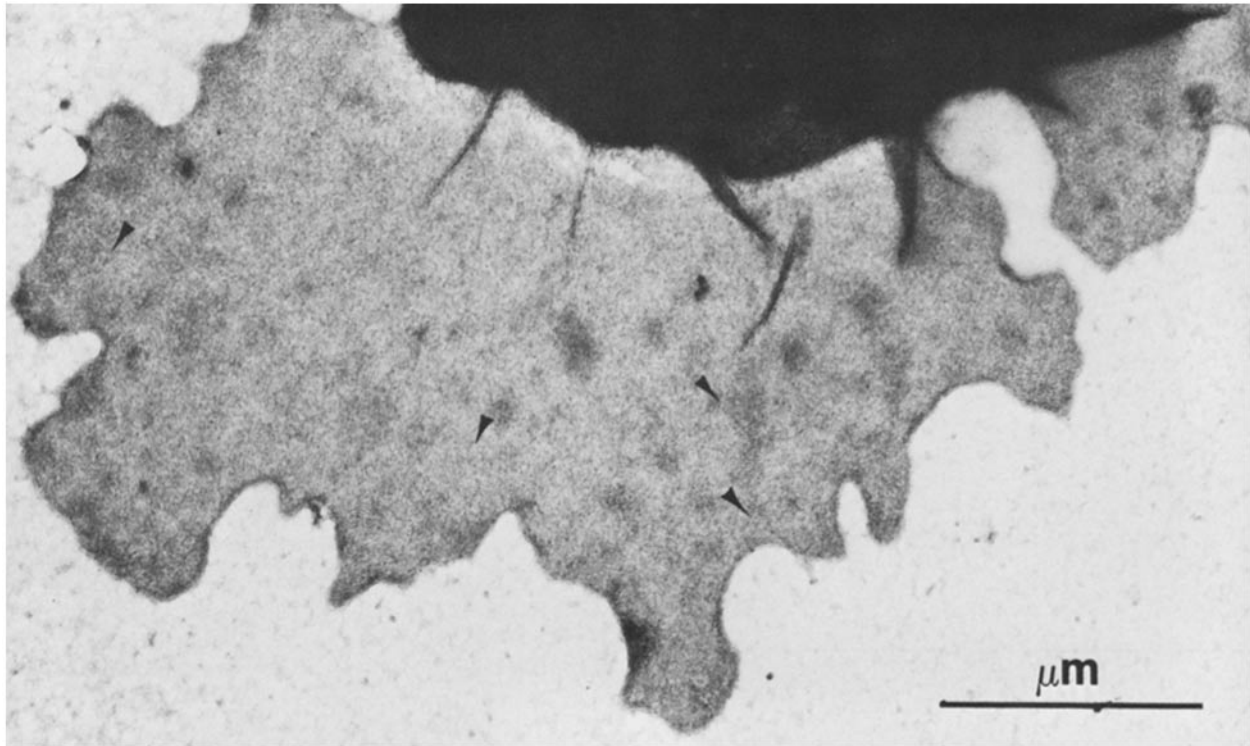
FIGURE 15 Spermatozoon sheared and negatively stained. Electron micrograph of a spermatozoon shared off a microscope grid with and negatively stained as described in Clarke et al. (10). Arrows mark short indistinct filaments that have a diameter expected for actin microfilaments. Repeated attempts to decorate these with heavy meromyosin have been unsuccessful. ×37,000.

function might also affect nonmuscle cell motility, we constructed males that were homozygous or hemizygous for each of 12 muscle-defective *unc* genes (63). These males were aged for 3–4 d and their sperm were dissected for the examination. Because these males were uncoordinated or paralyzed they could not mate. Consequently, the maturation of spermatids into spermatozoa, which is normally stimulated by copulation, was inefficient and only 0.3–10% of the cells bore pseudopods. However, in every case most of the mature spermatozoa were normal in morphology and ruffled and crawled normally (Table I). Those abnormal cells that were observed were similar to the abnormal spermatozoa found in old wild-type males (59).

Many sperm-defective mutants have been identified in our laboratory by isolation of fertilization-defective mutants (5, 61; T. Roberts, unpublished observation). Some of these have distinctive morphological abnormalities (59). The spermatozoa of four mutants have defective pseudopods; they are also defective in motility. *fer-1* mutant spermatozoa have short pseudopods with normal projections that appear to move normally, but the cells do not translocate. *fer-2* mutant spermatozoa are variable, but most are totally immotile. A few move their pseudopods slowly but do not translocate. *fer-4* and *fer-6* mutant spermatozoa have only a few short, thin pseudopodial projections and nearly all the spermatozoa are immotile. *fer-3* mutant spermatozoa are mostly normal but some are immotile. *fer-14* mutant spermatozoa move normally. Further analysis of movement in some of these mutant sperm is given in the following paper (44).

DISCUSSION

C. elegans spermatozoan motility appears similar to that of several "amoeboid" cell types. Pseudopods are the source of movement and by changing their contours they control the direction of movement. This is like the behavior of pseudopods on myxomycete amoebae, leukocytes, and other nematode sperm (3, 32, 42, 49, 64). Unlike the situation in fibroblasts (1, 20), there is no extension/retraction cycle of the leading margin of spermatozoan pseudopods and no cytoplasmic streaming is detected in sperm as it is in free-living amoebae and leukocytes (3, 41).

The motility of sperm is also quantitatively similar to that of other amoeboid cells. Forward motion averages 9.4 μm/min on glass or 21 μm/min on *Ascaris* extract with a maximum of 42 μm/min. This can be compared with 6–11 μm/min for other nematode sperm (32, 37, 64), 15–25 μg/min for leukocytes (42), 6–12 μm/min for *Fundulus* "deep" cells (56), and 6–14 μm/min for *Dictyostelium mucoroides* (45). Fibroblasts move significantly more slowly (1).

During turns, projections of the pseudopod lead the new direction; the cell body plays little or no role in establishing the direction of motion. It is likely that the progressive formation of new attachment sites on the pseudopod sets the new direction. Only when relatively old attachments beneath the cell dissolve or break does the cell body shift into alignment. The rapid rounding up and the position shifts of the cell body show that tension develops between leading and trailing portions of the cell.

The pseudopodial projections of *C. elegans* spermatozoa are stubby but may be analogous to filopodia, microspikes, or ruffles on other cells (2, 57). They appear to form the attachments of the pseudopod to the substrate. Translocation of sperm does not require alterations in bulk pseudopod morphology, suggesting that projection movement or membrane movement is responsible for forward motion (see following paper, 44). Observations on mutant sperm support the view that projections are required for movement. *fer-1* mutant sper-

Muscle Mutant Strains and Sperm Phenotypes *

Gene, Allele, Linkage group	Muscle and Body phenotype	Sperm with pseudopods	Spermatozoan morphology
		%	
unc-15 (e73) I	paralyzed, paramyosin structural gene	3.1	a few smooth pseudopods
unc-22 (e66) IV	twitches thin filament defect	~10	normal
unc-23 (e25) V	progressive head muscle dystrophy	9.7	a few long pseudopodia
unc-45 (e286) III	slow, disorganized body muscles	3.4	some cells have filaments
unc-52 (e444) II	progressive body muscle dystrophy adults paralyzed	0.8	normal
unc-54 (e675) I; him-5 (e1467) V	paralyzed, myosin heavy chain structural gene	0.4	normal
unc-60 (e723) V	paralyzed, disorganized body muscles	0.7	normal
unc-78 (e1217) X sperm from hemizygous males	sluggish, disorganized body muscles	1.4	normal
unc-82 (e1220) IV	sluggish, disorganized body muscles	2.3	some pseudopods irregular
unc-87 (e1216) I	slow, thin filament defect	<5	normal
unc-89 (e1460) I	uncoordinated, disorganized body muscles	0.3–3.0	some pseudopods irregular
unc-90 (e1463) X sperm from hemizygous males	paralyzed, disorganized body muscles	5.9	normal

* Muscle mutant phenotypes and genetic map assignments are described in references 8, 13, and 64. The abnormal spermatozoa observed were similar to abnormal spermatozoa seen in old wild-type males (62).

matozoa have pseudopods that are abnormally short but they bear normal-looking projections. These cells twitch and wiggle their pseudopods but cannot direct their movement. In contrast, fer-4, fer-6, and fer-2 mutant spermatozoa have normal-length pseudopods but lack normal projections. These pseudopods are nearly all immotile.

Although C. elegans sperm are capable of rapid motility and shape changes they contain astonishingly little actin when compared to other amoeboid cells whose protein is typically 5–20% actin (7, 22, 36, 58), although Ascaris sperm are only 0.5% actin (32). Gel electrophoresis shows that actin comprises about 0.02% of total protein in sperm preparations. The 0.1% contaminating spermatocytes could make a substantial contribution to this actin. Spermatocytes are ~ 5 times as large as sperm. If they contained 4% actin they could account for all the actin found in the isolated sperm. Thus the immunofluorescence results are the only evidence that sperm contain any actin, and the 0.02% is a maximal estimate of the amount.

We cannot prove that actin does not participate in sperm motility, but all attempts to demonstrate a necessary role for actin have been negative. A single spermatozoon has ~ 16 pg of total proteins, so 0.02% represents a maximum of 3.2 fg of actin per cell. This amount of actin represents ~ 50,000 molecules per cell which, if distributed uniformly in the pseudopod, would be at 200 μg/ml. If all this actin were polymerized into thin filaments there would sufficient actin to make 150 μm of filaments. Distributing these to each of the pseudopodial projections would provide six microfilaments in each projection. Such filaments should have been detected in some of our fixation conditions, especially the negative staining which reveals the pseudopodial projections clearly (Fig. 15). Therefore either the amount of actin is overestimated or the actin is not in filamentous form or the actin is not concentrated against the membrane of the pseudopodial projections that appear to be the motile elements of the pseudopod. Any of these possibilities argues against the involvement of actin in motility. We do not understand the significance of the dots of actin staining seen by means of immunofluorescence. No corresponding concentration of filaments or other material has been seen in TEM.

The absence of any cytochalasin effect on motility when these drugs do affect cell division in spermatocytes argues again against actin's participation in pseudopodial movement. It is unlikely that the sperm are insensitive to cytochalasins due to lack of penetration because cytochalasins penetrate spermatocytes, liposome delivery of cytochalasin was ineffective, and the spermatozoa are sensitive to many other drugs such as chloramphenicol, oligomycin, dinitrophenol, and are readily permeable to labeled amino acids and other metabolites (S. Ward, unpublished observations). Therefore if actin does participate in pseudopodial movements it must do so in a cytochalasin-insensitive manner. If current in vitro models are correct (9, 28), the actin must participate without forming new microfilaments.

The absence of myosin adds additional support to a novel mechanism of cell motility. But because myosin is normally less abundant than actin (e.g. reference 39) and myosins of low molecular weight are known (38), the possibility of myosin-like proteins in sperm needs further examination.

We screened most of the known collection of muscle-defective uncoordinated mutants in the hope of finding evidence that some component of muscle-cell contraction or assembly was involved in nonmuscle motility. None of the muscle-defective mutants showed any defect in amoeboid sperm motility. This is not surprising, for several reasons. First, the muscle mutants are maintained as homozygous hermaphrodites, so they must contain some fertile sperm. Second, there may be multiple genes coding for myofibrillar proteins as is known for actin in other organisms (12, 19) and for myosin in C. elegans (13). Third, not all muscle contractile proteins have been identified by mutants. It may be necessary to devise specific screening procedures to find genes needed for both muscle and nonmuscle motility, if they exist.

The fertilization-defective mutants that are defective in sperm motility should be altered in gene products that are involved in this amoeboid cell's unusual motility. Biochemical analysis of these mutants is in progress in the hope of identifying these gene products.

In the following paper (44) it is shown that membrane glycoproteins are inserted at the tip of the pseudopod and move back along the pseudopod to its base at a rate similar to the movement of pseudopodial projections. In addition, lipids move backward at a similar rate after spermiogenesis (43). This

flow provides a direct mechanism for amoeboid movement that might not require actin or myosin. A cell properly attached to the substrate by its pseudopodial projections would move forward as the membrane flowed backward.

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