Elongation of the Fertilization Tubule in *Chlamydomonas*: New Observations on the Core Microfilaments and the Effect of Transient Intracellular Signals on Their Structural Integrity

PATRICIA A. DETMERS, URSULA W. GOODENOUGH, and JOHN CONDEELIS
Department of Anatomy, Albert Einstein College of Medicine, Bronx, New York 10461; and Department of Biology, Washington University, St. Louis, Missouri 63130

ABSTRACT Experimental manipulations of gametes of *Chlamydomonas reinhardi* and ultrastructural observation were used to examine the composition of the microfilaments in the fertilization tubule, their probable mode of formation, and their interaction with intracellular signals. Decoration with myosin subfragment-1 was used to demonstrate that the microfilaments in the fertilization tubule were actin filaments having uniform polarity: Myosin subfragment-1 arrowheads pointed away from the membrane at the tip of the process. Filaments were attached to the cone-shaped "doublet zone" at the base of the process by their pointed ends. Discrete attachment sites for filaments on the surface of the doublet zone were seen in stereo view. To test whether actin polymerization might accompany elongation of the fertilization tubule, mating gametes were exposed to cytochalasin D in an attempt to block actin polymerization. Treatment of mating type "plus" gametes with cytochalasin D prior to and during mating inhibited the appearance of actin filaments in fertilization tubules, suppressed fertilization tubule outgrowth, and lowered mating efficiency from 90 to 15%. The role of signals generated by flagellar adhesion in maintaining the structural integrity of the microfilament-doublet zone complex was examined by correlating flagellar disadhesion with the kinetics of breakdown of the complex. In zygotes, where flagellar disadhesion occurred after cell fusion, the complex disassembled within 3 h after mating. In gametes that had been agglutinated by isolated mating type "minus" flagella, microfilaments and fertilization tubules progressively disassembled over a 3-h time course following flagellar disadhesion. Disassembly of microfilaments was inhibited by maintaining flagellar agglutination, suggesting that signals generated by flagellar adhesion were necessary to maintain microfilaments intact.

Changes in the polymerization state of nonfilamentous actin in response to intracellular signals may result in altered cellular surface morphology. A dramatic example of this is the outgrowth of the acrosomal process in *Thyone* sperm that is mediated by very rapid polymerization of actin in response to a discrete signal (51). During the mating reaction of *Chlamydomonas reinhardi* a cellular extension of more modest proportions is generated by mating type plus (mt^+)[1] gametes. Activation of the mt^+ mating structure is accompanied by the elongation of the fertilization tubule, a process 1.5 μm long containing a microfilamentous cytoskeleton (8, 15, 18, 53). The microfilaments run the length of the fertilization tubule and insert into a cone-shaped electron-dense structure at its base. The site of microfilament attachment has been designated either the doublet zone (18) or the choanoid body (15), based on morphology.

Activation of the mating structure is but one step in the ordered sequence of events constituting the mating reaction of *Chlamydomonas* (16). Mating is initiated when gametes of opposite mating types are mixed. Agglutination via flagellar tips occurs (4, 36, 41, 57), and adherent flagellar tips become

[1] *Abbreviations used in this paper:* mt^+, mating type plus; mt^-, mating type minus; NFHSM, nitrogen-free high-salt minimal medium; S-1, myosin subfragment-1; TEM, transmission electron microscopy.

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"activated", a condition easily identified ultrastructurally by the accumulation of fibrous material along the cytoplasmic surfaces of the membrane at the tips of the flagella (31). Signals sent from the adhering flagella to the cell body (16, 44) result in the release of cell walls (9) and activation of the mating structures in both mating types (18, 57). Mating type minus (mt^-) gametes do not form a fertilization tubule, but their mating structure undergoes distinct morphological changes that can be recognized ultrastructurally (17). The outer surface of the tip of the mt^+ fertilization tubule is specialized to recognize and bind to the plasma membrane overlying the activated mating structure of the mt^- gametes (17). After binding, membrane fusion occurs at this specifically defined site (8, 15, 53, 56), and the flagella disadhere (16). The narrow cytoplasmic bridge formed between the mt^+ and mt^- gametes by the fertilization tubule becomes shorter and broader until quadriflagellate cells form by the complete fusion of the biflagellate gametes (8, 18). Thin section views of quadriflagellate cells reveal that the cytoskeletal component of the fertilization tubule remains visible in the apical end of zygotes 1–6 h (18, 15) after cell fusion occurs.

The de novo appearance of microfilaments within the fertilization tubule leads one to wonder how they are formed and what controls their stability, questions that have been asked repeatedly with regard to microfilaments in many other types of cells. The results we present here are a first approach at answering these questions for *Chlamydomonas*. Using an improved fixation technique and decoration with myosin subfragment-1 (S-1), we have made new observations on the structure, composition, and polarity of the microfilaments in the fertilization tubule. Further, we have examined the effects of cytochalasin D, a drug that perturbs actin polymerization, on the formation of the fertilization tubule. Finally, to address the factor(s) controlling the stability of the actin filaments, we examined the necessity for the presence of adhesions between flagellar membranes and signals generated by these adhesions in maintaining the structural integrity of the microfilament-doublet zone complex.

MATERIALS AND METHODS

Strains and Culture Conditions: Clones of the wild type strain 137c, mt^+ and mt^- of *C. reinhardi* were used for all experiments. The strain is available from the Chlamydomonas Genetics Center, Department of Botany, Duke University (Durham, NC).

Gametes were harvested from agar plates after 7–14 d and suspended in nitrogen-free high-salt minimal medium (NFHSM) for 1–2 h as previously described (30). NFHSM contains 13.6 mM $KHPO_4$ (pH 7.0), 68 μM $CaCl_2$, 81 μM $MgSO_4$, and trace amounts of $ZnSO_4$, $MnCl_2$, $FeSO_4$, $CoCl_2$, $CuSO_4$, and $(NH_4)_6Mo_7O_{24}$ (45). Cells were washed once in NFHSM and tested for their ability to agglutinate with gametes of the opposite mating type. Gametic agglutination was measured by eye under a light microscope and considered to be adequate if >90% of cells formed aggregated clumps. Prior to use, gametes were further tested for a mating efficiency of >90%. Mating efficiency (percent cell fusion) was determined by counting biflagellate cells (BFC) and quadriflagellate cells (QFC) in fixed samples after a 15-min mating of equal numbers ($1-2 \times 10^7$ cells/ml) of mt^+ and mt^- gametes and using the formula: Mating efficiency = (2QFC × 100)/(2QFC + BFC) (31).

Preparation of S-1 and Protein Determination: Myosin was a kind gift from Dr. David Kirk, who prepared it from rabbit skeletal muscle according to the method of Szent-Györgyi (46), as modified by Schloss et al. (38). The myosin was digested with papain (Sigma Chemical Co., St. Louis, MO) in the presence of ethylene diamine tetraacetic acid essentially as described by Lowey et al. (28), with modifications by Murray (34). S-1 preparations were assayed for actin-binding activity by making negative stained preparations of F actin (prepared according to Tilney and Detmers; [50]) mixed with S-1 and examining them by transmission electron microscopy (TEM) for the presence of decorated actin filaments. Protein concentrations were determined according to Lowry et al. (29), using BSA as a protein standard.

S-1 Decoration of Cellular Microfilaments: Mating gametes were fixed for 5–7 min in 0.1% glutaraldehyde, washed twice, extracted for 10 min in 0.1% saponin (Sigma Chemical Co.) (35) and washed two more times; NFHSM (pH 7.0) was used as the buffer for all steps. Following the addition of 1–2 mg/ml S-1 in NFHSM containing 0.1 mM dithiothreitol, the cells were incubated for 20–30 min, washed twice with NFHSM, and fixed for TEM. All manipulations were performed at room temperature, and each step was followed by centrifugation at 3,000 *g* for 30 s to collect material.

Mating in the Presence of Cytochalasin D: The concentration of cytochalasin D used was selected on the basis of dose response curves, which indicated that 200 μg/ml (0.4 mM) cytochalasin D optimally lowers cell fusion during mating of *Chlamydomonas* but does not interfere with flagellar tip activation or cell wall loss (31). Cytochalasin D was added from a stock solution (10 mg/ml in DMSO) to mt^+ gametes at $2-4 \times 10^7$ cells/ml in NFHSM to achieve a final concentration of 200 μg/ml (2% DMSO). The gametes were incubated at room temperature for 1 h and then mixed with an equal number of mt^- gametes. Immediately before the mt^- gametes were introduced sufficient cytochalasin D stock solution was added to the mt^+ gametes to maintain the concentration of the drug at 200 μg/ml during mating. Mixtures of mating cells were either fixed for TEM after 2 min or scored for cell fusion after 70 min. The fixed cells were scored for cell wall loss, the presence of fibrous tip material (indicating flagellar tip activation), and the presence of elongated fertilization tubules containing microfilaments. Gametes mated in NFHSM or NFHSM plus 2% DMSO served as controls.

In an additional control experiment, mt^- gametes were incubated with 200 μg/ml cytochalasin D for 1 h at room temperature before adding mt^+ gametes that had not been preincubated with the drug. Again, the concentration of cytochalasin D was maintained at 200 μg/ml during mating.

Addition of mt^- Flagella to Elicit Elongation of Fertilization Tubule: Flagella were detached from mt^- gametes by a pH shock method and isolated essentially as described by Witman et al. (58). Prior to use they were stored as a pellet at −80° C.

Flagella were resuspended in 10 mM PIPES, 5% sucrose (grade II, Sigma Chemical Co.) pH 7.4 at a concentration of $2-5 \times 10^{10}$ flagella/ml, and an aliquot was added to mt^+ gametes (1.5×10^7 cells/ml in NFHSM) to give a final concentration of $1-2 \times 10^8$ flagella/ml. Agglutination of the gametes to the flagella was monitored using a light microscope, and fresh aliquots of mt^- flagella were added at 5-10-min intervals to maintain the agglutination response at >90%. Samples were fixed for TEM either after periods of continuous agglutination or at intervals after flagellar addition was discontinued. Samples were scored for the percentage of mating structures that had elongated into fertilization tubules containing microfilaments (referred to as percent mating structure activation).

Electron Microscopy: Samples were fixed for 30–60 min in 1% glutaraldehyde, 0.2% tannic acid (3) in NFHSM (pH 7.0) at room temperature, washed once in NFHSM, and postfixed for 20 min in 0.5% OsO_4 in 0.1 M $NaHPO_4$ (pH 6.0) on ice. A 15-min wash in distilled water at room temperature was followed by en bloc staining in 1% uranyl acetate (aqueous) for 30 min at room temperature, two washes in distilled water, dehydration through ethanol, and embedding in EPON-Araldite (25). Sections were cut either 50–60 nm thick (thin sections) or 120 nm thick (stereo views) on a Porter-Blum MT-2 ultramicrotome (Porter Instrument Co., Inc., Hatfield, PA) using a diamond knife, stained in 1% uranyl acetate (ethanolic) and lead citrate, and viewed on either a Hitachi HU-11C at 75 kV, a Zeiss EM 10 at 60 kV, or a JEOL 100CX at 60 kV.

RESULTS

Structure of the Cytoskeletal Component of the Fertilization Tubule

Improvements in the fixation of microfilaments together with the use of stereo micrographs have made it possible to make new structural observations on the cytoskeleton of the fertilization tubule. To examine the fertilization tubule before cell fusion had occurred, gametes of opposite mating type were mixed in equal numbers (2×10^7 cells/ml) and fixed for TEM after 2 min of mating. Cross-sectional views of the fertilization tubule revealed that it contained 80–100 microfilaments with an average center-to-center spacing of 12 nm (Fig. 1, *inset*). Cross-links between the filaments were not

apparent in cross-section, and no regular cross-periodicity was visible in longitudinal section (Fig. 1).

For observations of the microfilamentous remnant of the fertilization tubule in zygotes, mating was allowed to continue for 5 min prior to fixation. Stereo views of longitudinal sections through the zygote revealed that the microfilaments were not attached randomly over the surface of the cone-shaped doublet zone. Rather, the points of microfilament attachment were concentrated in circumferential thickenings or ridges, at least three of which are visible in Fig. 2. From their point of attachment on the cone the microfilaments fanned away from each other and appeared much less bundled than in the fertilization tubule. Although the microfilaments did not appear to be entirely bare, they were clearly not cross-linked at this stage, except at their point of attachment to the cone.

S-1 Decoration of Microfilaments

We found that the use of saponin as described in the Materials and Methods sufficiently permeabilized the plasma membranes of cells that had been lightly fixed in glutaraldehyde to allow both S-1 entry and some extraction of cytoplasm, which enhanced the visibility of decorated filaments. Most of the cellular organelles remained remarkably intact following this treatment.

Incubation with S-1 resulted in the appearance of a specific arrowhead pattern (22, 24) on the microfilaments of the fertilization tubule (Fig. 3), indicating that these microfilaments were composed of actin. The polarity of the S-1 arrowheads on the actin filaments was uniform throughout the fertilization tubule: All point away from the tip of the process and toward the doublet zone at its base.

Observation of early zygotes revealed that the same uniform polarity of the microfilaments was maintained during cell fusion. All the actin filaments remained anchored in the doublet zone by their pointed ends, even following dissociation of the doublet zone from the plasma membrane (Fig. 4).

Effect of Cytochalasin D on Mating of Chlamydomonas

Gametes mated in cytochalasin D were examined by TEM to determine what effect the drug had on both elongation of the fertilization tubule and the formation of microfilaments within fertilization tubules. The specific procedure is described above, and the results of these experiments are summarized in Table I. Under normal mating conditions in NFHSM, with or without DMSO, gametes exhibited at least an 80% positive response in all mating parameters measured. Whereas cytochalasin D did not affect cell wall loss or flagellar tip activation, it completely prevented the formation of well-organized actin filaments in fertilization tubules and significantly lowered mating efficiency.

Very short, filament-free fertilization tubules were, however, often observed in mating samples in which the mt^+ gametes had been preincubated with cytochalasin D (Fig. 5). Although the diameter of these fertilization tubules was similar to that of normally formed fertilization tubules (0.15 μm), the average length of these processes was only 0.3 μm (length distribution = 0.15–0.6 μm), ~20% of the average normal length. Although no orderly array of microfilaments was observed, the fertilization tubules were filled with electron-dense material, perhaps representing very short actin filaments that had polymerized in a nondirected fashion.

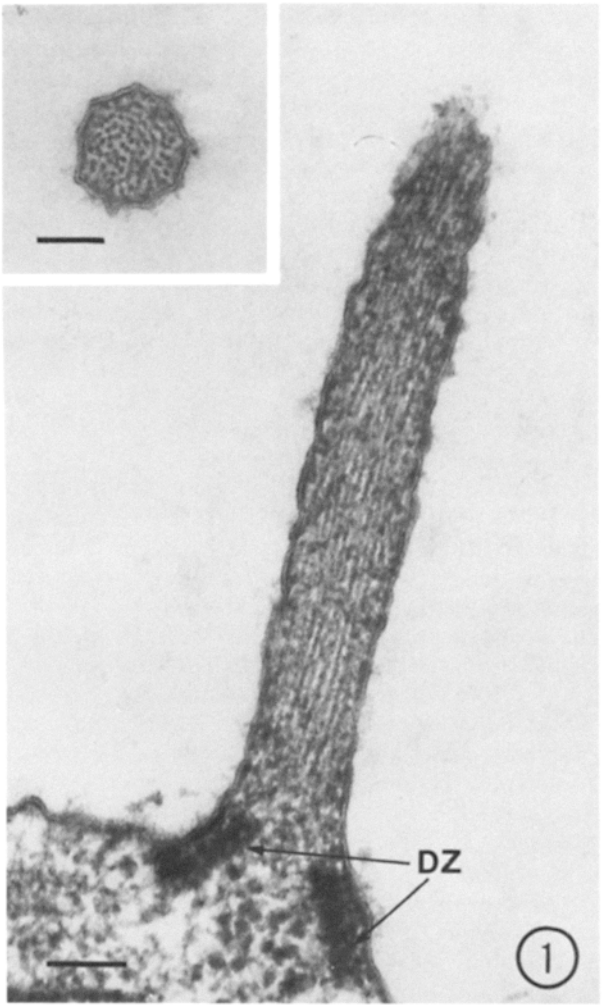


FIGURE 1 Longitudinal thin section through a fertilization tubule showing the arrangement of the microfilaments forming the core of the process. Although the filaments were tightly packed, no regular cross striations were observed. The section catches the middle of the doublet zone (*DZ*), revealing the medial discontinuity at the apex of this cone-shaped structure. Bar, 0.1 μm. × 106,350. *Inset*, cross section through a fertilization tubule showing that the microfilaments are packed in a random arrangement. Bar, 0.1 μm. × 88,200.

Fertilization tubules that fell at the longer end of the length distribution were able to make contact with activated mating structures of mt^- gametes (Fig. 6), offering an explanation for the observation that cytochalasin D did not completely eliminate cell fusion (Table I). Such images indicated that the drug did not interfere with the transmission of intracellular signals that elicited elongation of the fertilization tubule but rather directly blocked the formation of actin filaments of normal length.

Preincubation of mt^- gametes with cytochalasin D prior to mating only slightly inhibited cell fusion (Table I). Though mt^- gametes do not form a fertilization tubule, they do respond to the signals generated by sexual agglutination by activation of their mating structure (17, 56). Therefore, this experiment constituted independent evidence that cytochalasin D does not interfere with signals for activation of mating structures.

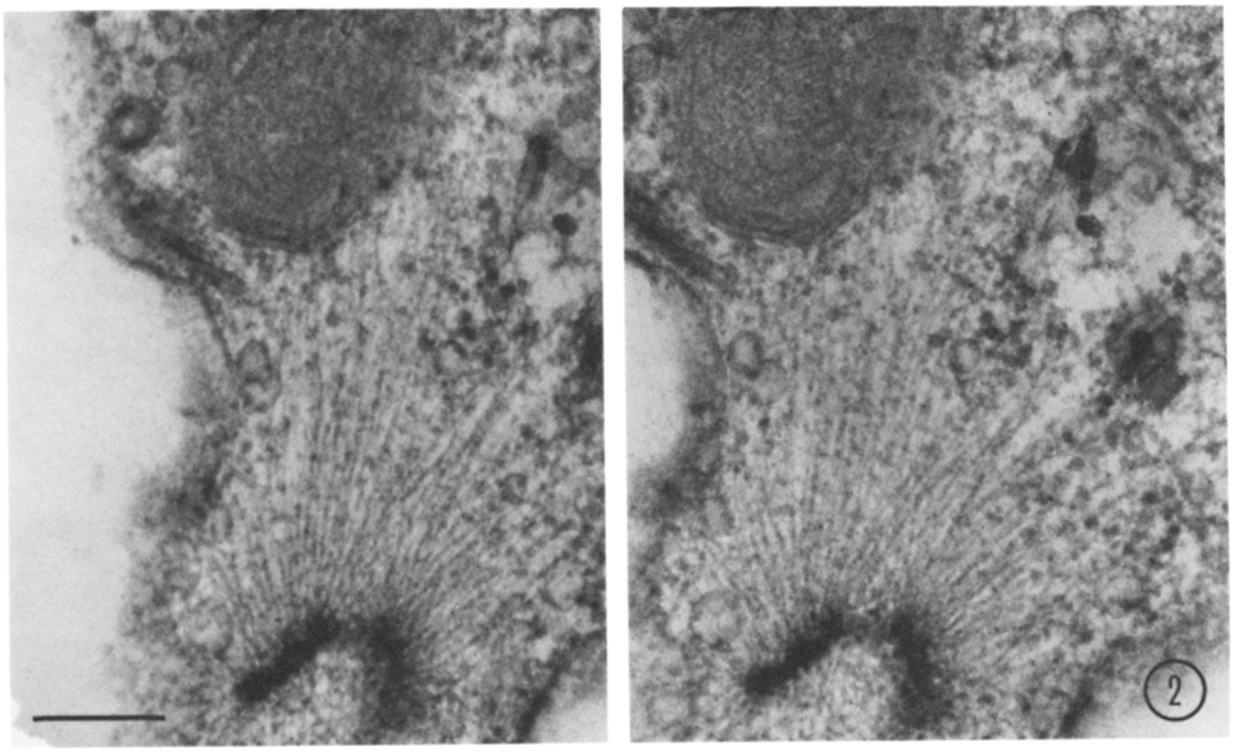


FIGURE 2 Longitudinal stereo view of the cytoskeletal remnant of the fertilization tubule in a zygote 5 min after mating. Three thickened areas are visible on the doublet zone, and microfilaments are attached to discrete sites on these thicker portions of the structure. Bar, 0.2 μm. × 85,500.

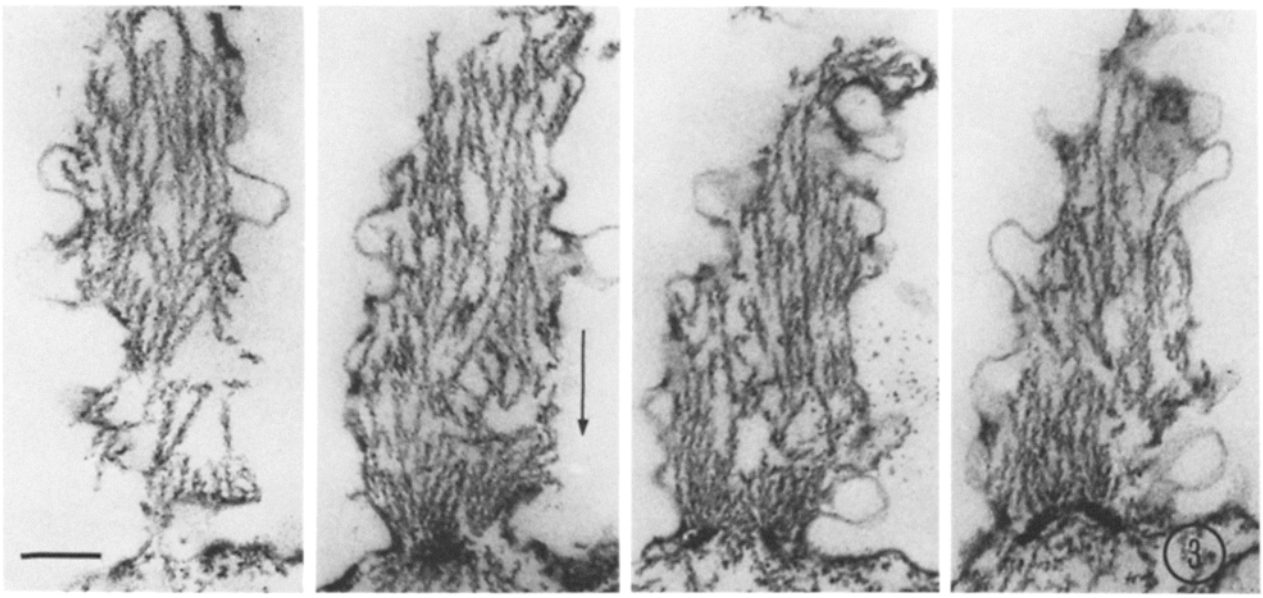


FIGURE 3 Serial sections through a fertilization tubule following S-1 decoration. Mating gametes were fixed briefly in glutaraldehyde, extracted in saponin, and exposed to S-1 prior to fixation for TEM, as described in Materials and Methods. S-1 arrowheads are clearly visible on all filaments within the fertilization tubule, and all arrowheads point away from the tip of the process. Arrow indicates the polarity of the filaments. Bar, 0.2 μm. × 52,500.

Breakdown of the Fertilization Tubule Cytoskeleton in the Zygote

Since formation of the actin filaments in the fertilization tubule is clearly dependent upon receipt of intracellular signals generated by adhesion of flagellar membranes (16, 44), examining the fate of filaments following the loss of flagellar adhesion could provide information on the interaction between such signals and the microfilament-doublet zone complex. A convenient method for making this observation was provided by mixes of mt^+ and mt^- gametes in the process of mating: Gametic flagella disagglutinate upon fusion of cells to form zygotes, and within a few hours the doublet zone with attached microfilaments disappears.

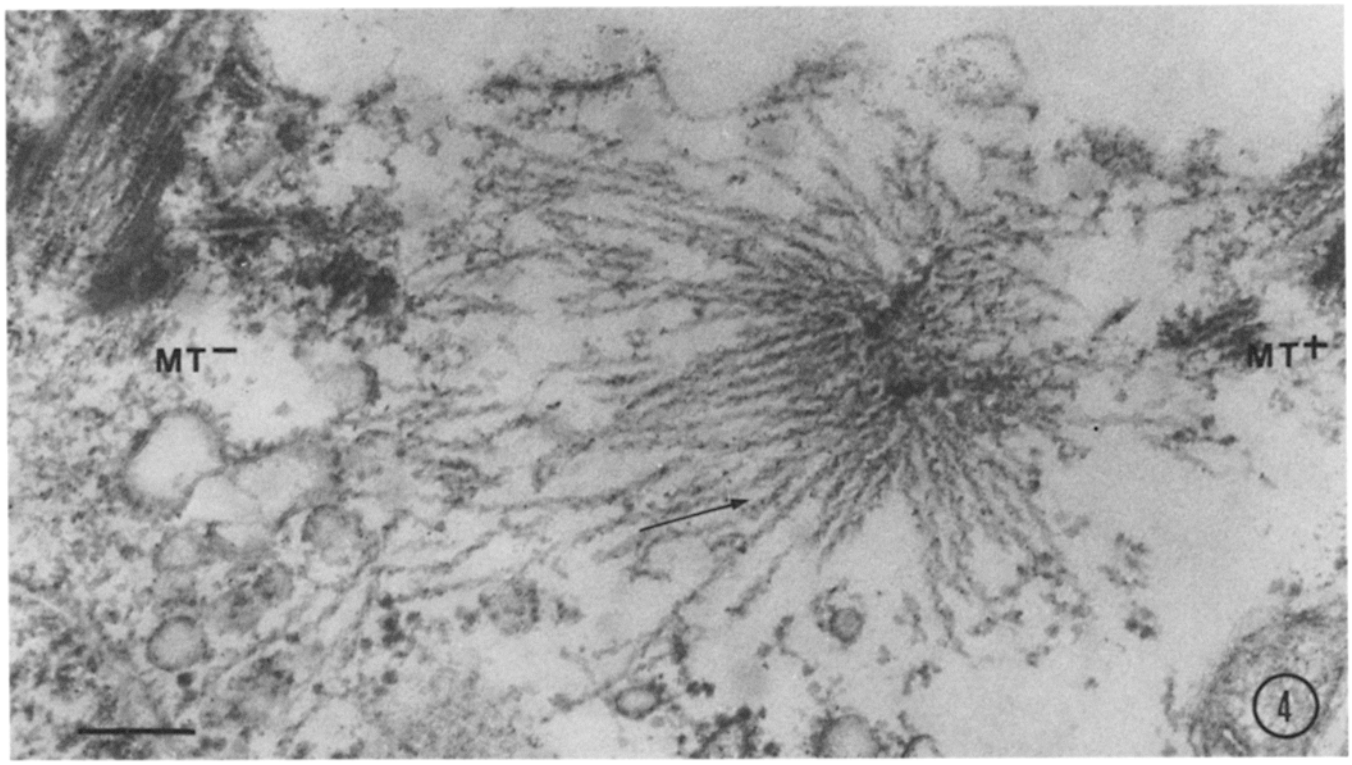


FIGURE 4 S-1 decoration of a fertilization tubule cytoskeleton in a zygote 5 min after mating. The actin filaments remain attached by their pointed ends to the doublet zone following cell fusion. Arrow indicates the polarity of the filaments. MT^+, basal body contributed by mt^+ gamete; MT^-, basal body contributed by the mt^- gamete. Bar, 0.2 μm. × 71,550.

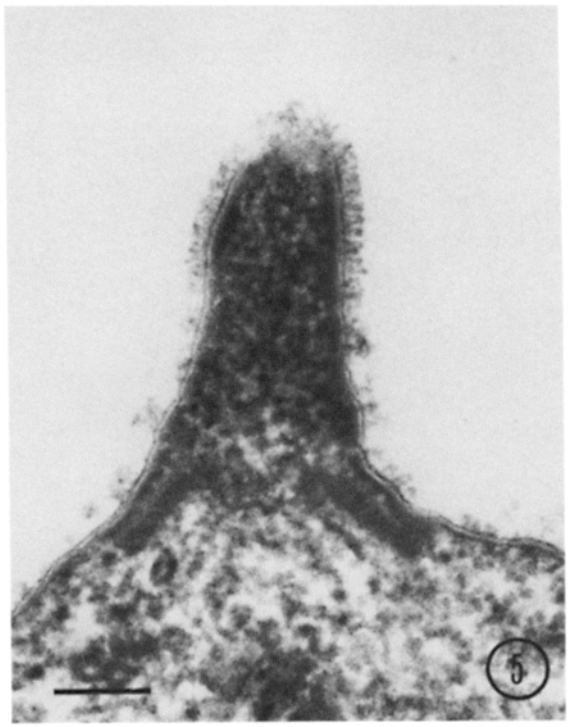


FIGURE 5 Fertilization tubule formed during mating in cytochalasin D. This fertilization tubule, which partially elongated in the presence of cytochalasin D, is filled with electron-dense material in place of the ordered array of microfilaments (compare with Fig. 1). Bar, 0.1 μm. × 126,700.

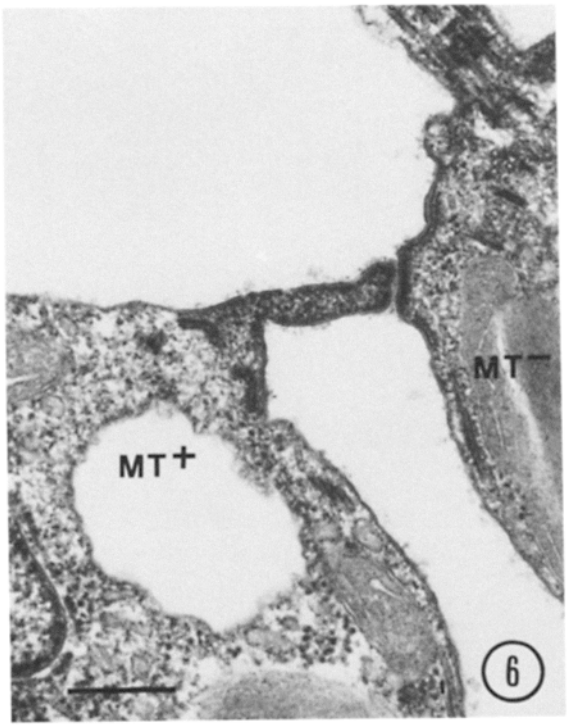
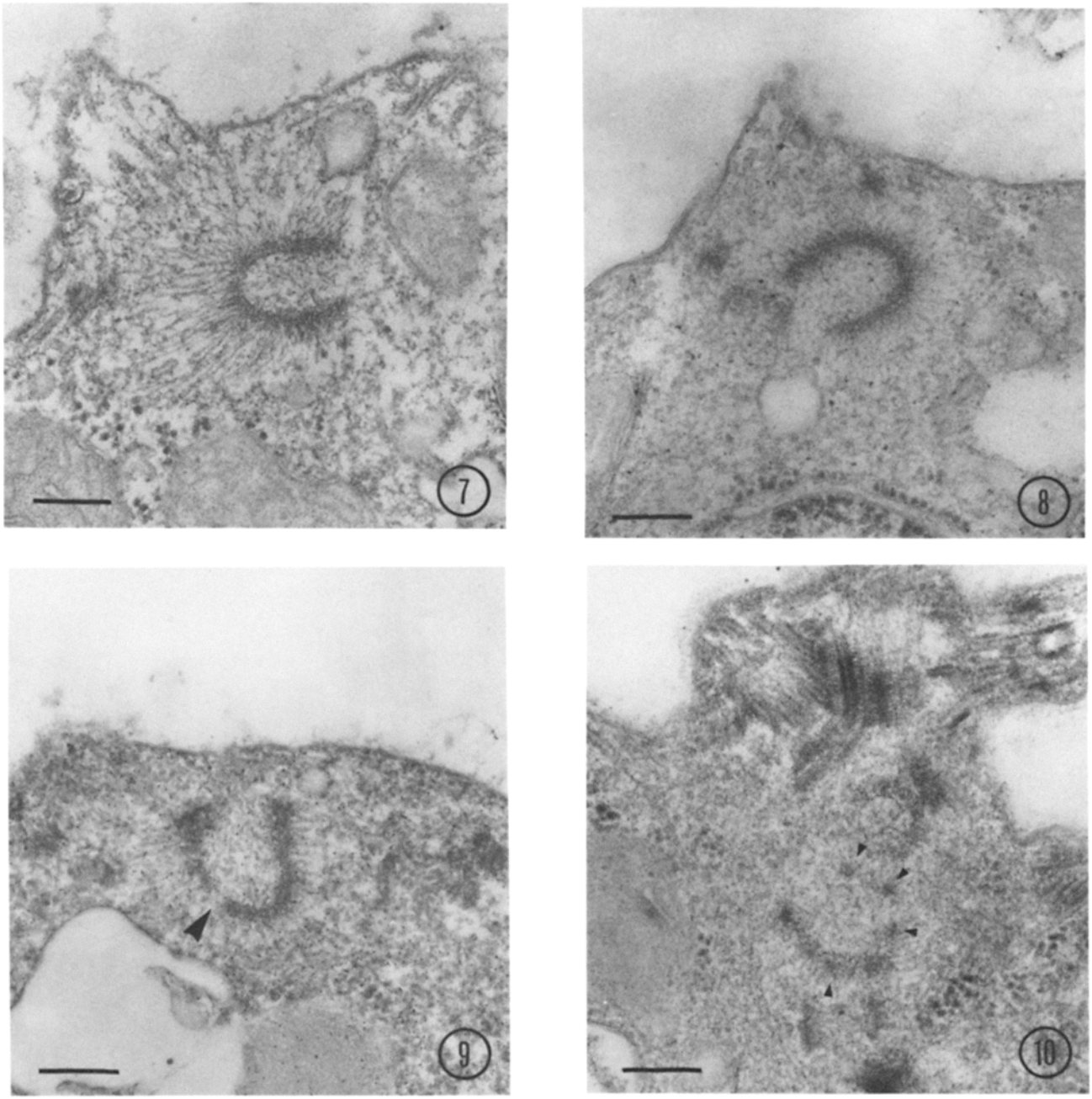


FIGURE 6 Fertilization tubule contacting an activated mt^- mating structure in the presence of cytochalasin D. The unusual length of this particular fertilization tubule apparently allowed it to make contact with the mt^- mating structure. The interaction between the mt^+ "fringe" material and that of the mt^- gamete is clearly visible. MT^+, mt^+ gamete; MT^-, mt^- gamete. Bar, 0.3 μm. × 46,500.



FIGURES 7–10 Fig. 7: Cross-section through a microfilamentous cytoskeleton from a zygote 10 min after mating. A dense array of microfilaments is attached to the very base of the cone-shaped doublet zone. Bar, 0.2 μm. × 60,750. Fig. 8: Cross-section through a microfilamentous cytoskeleton from a zygote 1 h after mating. Microfilaments were attached to the doublet zone in approximately the same density. They were, however, much shorter, as is apparent from the decrease in the area around the doublet zone from which organelles are excluded. Bar, 0.2 μm. × 60,750. Fig. 9: Cross-section through a microfilamentous cytoskeleton from a zygote 2 h after mating. The microfilaments have shortened further, and a discontinuity in the doublet zone is visible (large arrowhead). Bar, 0.2 μm. × 60,750. Fig. 10: Cross-section through a microfilamentous cytoskeleton from a zygote 3 h after mating. Discontinuities in the doublet zone became more numerous and extensive, and the structure broke into pieces (small arrowheads) with a few very short microfilaments still attached. At times later than 3 h the complex was unrecognizable in the zygote. Bar, 0.2 μm. × 60,750.

Previous reports regarding the transience of the microfilament-doublet zone complex in the zygote (15, 18) have not mentioned details of the process by which the complex disassembles. By comparing the structure of the complex at different time intervals after the beginning of mating, a pattern for disassembly can be discerned. Serial sections were made of each time point, and the same area of the doublet zone was compared among time points. Figs. 7–10 illustrate the temporal sequence of the disassembly of the microfilament-doublet zone complex in the zygote. Disassembly of this cytoskeletal component occurred in two distinct steps. First, the microfilaments became shorter while maintaining their

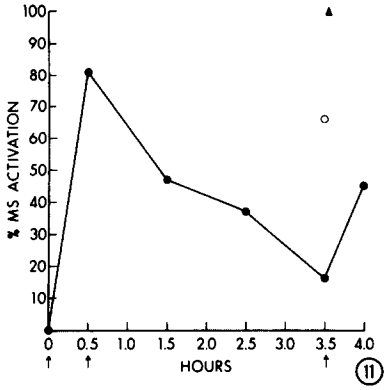
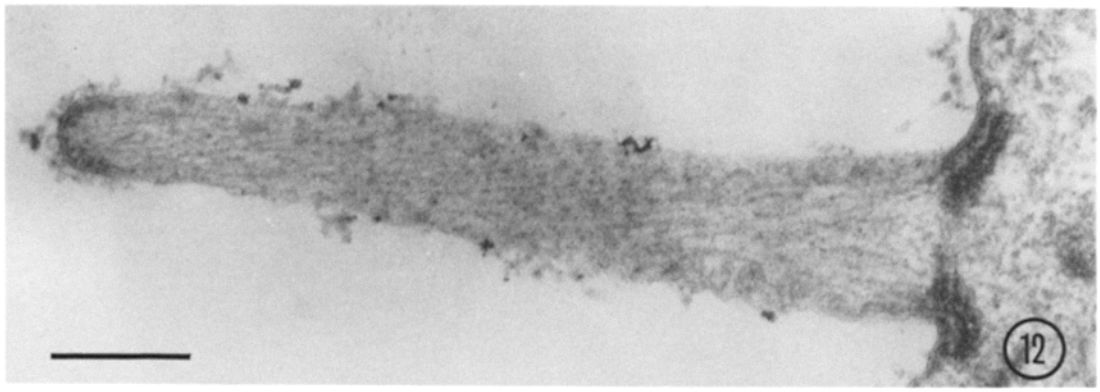


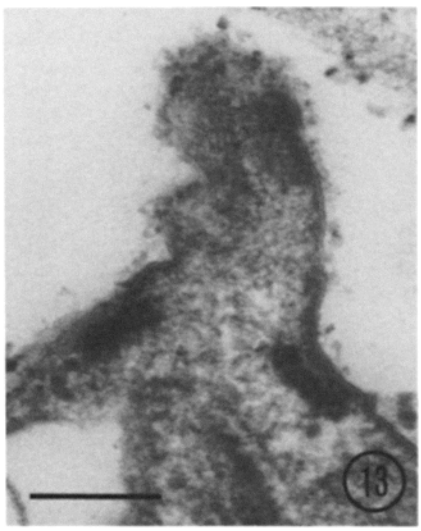
FIGURE 11 Activation of the mt^+ mating structure by mt^- flagella and subsequent reversal. Addition of mt^- flagella to mt^+ gametes (described in Materials and Methods) was begun at time = 0 (first arrow) and continued for 30 min (second arrow). At this time flagellar addition was discontinued for 3 h then resumed for 30 min (third arrow). % mating structure (*MS*) activation was followed by thin section (see Figs. 12–15) over time (closed circles). At 3.5 hours mt^- gametes were added to a sample of mt^+ gametes previously activated by exposure to mt^- flagella for 30 min, and mating was allowed to continue for 2 min before the sample was fixed and scored for % MS activation (closed triangle). A control sample of mt^+ gametes was exposed to agglutinatively active mt^- flagella for 3.5 h (open circle).

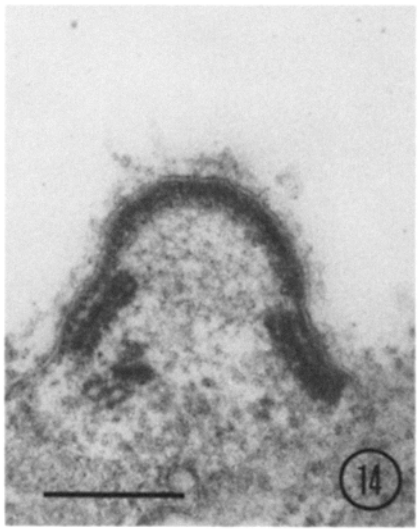
connection to the doublet zone. When the microfilaments became very short the doublet zone began to disassemble. Throughout this process the complex remained in the apical cytoplasm of the zygote, suggesting that the doublet zone may have retained its connection to the mt^+ basal body via a fibrous rootlet (19).

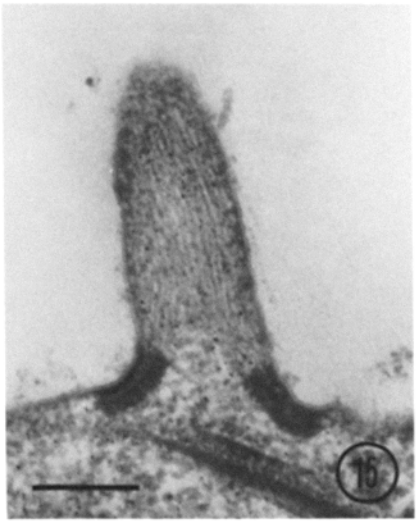
Elongation of the Fertilization Tubule Was Reversible

The disassembly of the microfilamentous complex in the zygote following loss of flagellar agglutination suggested that the continuous presence of a signal generated by flagellar adhesion was necessary for maintaining the structural integrity of the microfilaments. To test this hypothesis, outgrowth of the fertilization tubule was elicited by exposing mt^+ gametes to agglutinative flagella isolated from mt^- gametes (31). As the agglutinative activity of the added flagella was depleted, the mt^+ gametes disadhered (43), and the effect of flagellar disadhesion on the structure of the fertilization tubule was









FIGURES 12–15 Fig. 12: Fertilization tubule formed after exposure of mt^+ gametes to mt^- flagella for 30 min. This corresponds to the second arrow in Fig. 11 (closed circle). Microfilaments are clearly visible in fertilization tubules from this sample. Bar, 0.2 μm. × 91,000. Fig. 13: Fertilization tubule in the process of collapsing 1 h following discontinuation of addition of mt^- flagella. Bar, 0.2 μm. × 86,600. Fig. 14: Fertilization tubule collapsed after 3 h without agglutinative mt^- flagella. This corresponds to the third arrow in Fig. 11 (closed circle). Following the loss of agglutination between mt^+ gametes and mt^- flagella, the fertilization tubule returned to the morphology of the unactivated mating structure. Only very short filamentous material remained. Bar, 0.2 μm. × 93,600. Fig. 15: Fertilization tubule formed during activation by mt^- gametes following 3 h without agglutinative mt^- flagella. This corresponds to the closed triangle in Fig. 11. Microfilaments have reappeared, and the process once again was elongated. The distal portion of the fertilization tubule curved out of the plane of section. Bar, 0.2 μm. × 70,000.

TABLE I

Effect of Cytochalasin D on Chlamydomonas Mating

	Wall loss	Flagellar tip activation	Mating structure* activation (mt^+)	Mating† efficiency
	%	%	%	%
Control	80 (n = 158)	85 (n = 54)	92 (n = 51)	92 (n = 102)
DMSO	82 (n = 149)	90 (n = 44)	90 (n = 30)	90 (n = 114)
Cytochalasin D mt^+ preincubation	83 (n = 139)	82 (n = 51)	0§ (n = 31)	15 (n = 88)
Cytochalasin D mt^- preincubation				75‖ (n = 153)

* Percent of mt^+ gametes having normally elongated fertilization tubules.
† Calculated as described in Materials and Methods.
§ The short fertilization tubules observed in the presence of cytochalasin D were devoid of actin filaments.
‖ Of DMSO control, separate experiment.

observed by TEM. Since cell fusion did not occur, agglutination could be regenerated by adding more flagella, and the reversibility of the effects of flagellar disadhesion could also be examined.

Agglutination of mt^+ gametes by adding mt^- flagella for 30 min, as described in Materials and Methods, resulted in 80% mating structure activation (Figs. 11 and 12). Continuing agglutination for 3 h longer did not increase this figure (open circle, Fig. 11). If the addition of fresh mt^- flagella was discontinued after 30 min, the number of agglutinated mt^+ gametes decreased over time until all cells had disadhered by 30 min. Samples fixed at intervals after cessation of flagellar addition showed a progressive decrease in the number of fertilization tubules present (Fig. 11). Very rare sections showed fertilization tubules that could be interpreted as being in the process of collapse (Fig. 13), because the tip appeared deflated rather than round. By 3 h, 84% of gametes exhibited mating structures having an unactivated morphology (Fig. 14), indicating that microfilaments had disassembled and that fertilization tubules had collapsed.

A second round of fertilization tubule outgrowth could be elicited by adding either mt^- flagella or mt^- gametes (Figs. 11 and 15). Isolated flagella were somewhat less effective than the gametes in promoting the formation of fertilization tubules. Apparently, the flagella lost activity while stored at 4°C during the course of the experiment, because flagella stored for 3.5 h at this temperature were capable of eliciting only 65% mating structure activation in fresh mt^+ gametes.

DISCUSSION

Structure and Function of the Fertilization Tubule

During mating of *C. reinhardi* the mt^+ mating structure undergoes a dramatic change in morphology to become the fertilization tubule (15, 18). This transformation from a slight protrusion on the apical end of the gamete to a 2 μm long process occurs in essentially two stages. First the electron-dense membrane zone separates from the cone-shaped doublet zone beneath it to form a bud. The bud rapidly elongates into the fertilization tubule, which contains microfilaments in a longitudinal array. The separation between the membrane zone and the doublet zone increases during elongation of the fertilization tubule, with the doublet zone remaining at the base of the process while the membrane zone travels with the tip. The external surface of the tip is specialized for recognition of the membrane overlying the mt^- mating structure (17), and membrane fusion occurs when these two areas contact.

The elongation of the fertilization tubule is a crucial step in the mating sequence of *Chlamydomonas*. Gametes of opposite mating type may form mating pairs with their mating structures on either the same side or on opposite sides of their flagellar apparatuses (in either the "cis" or "trans" position; [15]). Formation of an elongated process is therefore necessary for contact to occur between the fusion-specific sites on the plasma membranes of mt^+ and mt^- gametes. The core of microfilaments within the fertilization tubule must certainly provide it with the structural strength necessary to make contact with the correct spot on a rapidly jerking partner.

Using an improved method for the fixation of microfilaments which combined fixation in glutaraldehyde-tannic acid with en bloc staining in aqueous uranyl acetate, we have been able to make new observations on the structure of the microfilamentous core of the fertilization tubule. The microfilaments within this cellular process were not arranged for maximum rigidity: They were not hexagonally packed and did not appear to be highly cross-linked. The random packing arrangement of the filaments seen in cross-section does not, however, preclude the possibility that some cross-links may exist between filaments (11).

Stereo micrographs of the microfilaments from the fertilization tubule remaining after cell fusion clearly demonstrated

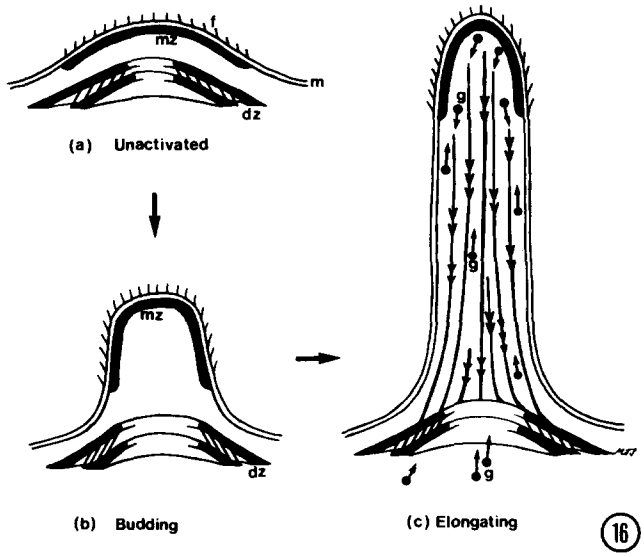


FIGURE 16 Model for elongation of the fertilization tubule. (a) In the unactivated mt^+ mating structure an electron dense membrane zone (*mz*) overlies a cone-shaped doublet zone (*dz*). The exterior surface of the mating structure is provided with "fringe" (*f*; [17]), which appears to mediate recognition of and binding to the mt^- mating structure prior to fusion. (*b*) Following receipt of mating signals the membrane zone separates from the central portion of the doublet zone, and a bud is formed. (c) Nucleation of actin polymerization then occurs at the doublet zone, assuring the uniform polarity of the filaments, which grow by monomer addition to the barbed end (*g*, monomers adding in this manner). When elongation is complete the barbed ends of the filaments are embedded in the membrane zone, which may help promote stability of the filaments.

that the filaments were inserted in the doublet zone. There were apparently specific attachment sites for microfilaments on the outer surface of this cone-shaped structure.

Flagellar Signals and Turnover of Microfilaments

The fertilization tubule is a transient structure, existing solely to form a cytoplasmic bridge between gametes of opposite mating types. Following cell fusion the cytoskeletal component of the fertilization tubule is visible in the apical cytoplasm of the zygote until it gradually is broken down (15, 18). We have followed the morphology and kinetics of the microfilament-doublet zone complex in zygotes and found that it occurred in two stages: (*a*) disassembly of microfilaments, and (*b*) breakdown of the doublet zone.

Reversible agglutination of mt^+ gametes with mt^- flagella demonstrated that the loss of flagellar adhesions was causal in the collapse of elongated fertilization tubules. Disassembly of the microfilament-doublet zone complex then appeared to result from a reversal of the process by which it formed, and special factors that might be present only in the zygote were not necessary for disassembling at least the microfilamentous portion of the defunct fertilization tubule. Interestingly, the time required for most of the fertilization tubules to collapse to the unactivated morphology was the same as that required to complete the shortening of microfilaments in the zygote.

Whereas microfilaments disappeared during the collapse of fertilization tubules following flagellar disadhesion, the doublet zone remained completely intact. This contrasted to the situation in the zygote where the doublet zone was disassembled along with the microfilaments. So whereas the loss of the signal generated by flagellar adhesion was sufficient to initiate disassembly of microfilaments, an additional signal or factor present in zygotes but not in gametes was required for breakdown of the doublet zone.

Two mating mutants, *imp-1* and *imp-11*, have been described that are capable of erecting a fertilization tubule but are incapable of fusing with mt^- gametes (17). Despite continued flagellar agglutination of these mutants with mt^- gametes, their fertilization tubules collapse within 30 min of formation. This suggests that the signal for formation of the fertilization tubule is different, either qualitatively or quantitatively, from that necessary to maintain its structural integrity, although both appear to be mediated by specific membrane adhesions. Alternatively, the target for the signal that maintains the fertilization tubule may be lacking in the mutants. The most noticeable deficiency in these mutants is the lack or diminution of the membrane zone, which apparently functions in cell fusion. A second function of the material of the membrane zone may be to cap the ends of microfilaments to prevent their disassembly. The exact nature of the signal for activation of mating structures is at present unknown, although there is evidence that calcium and/or magnesium ion fluxes across flagellar membranes may be involved (42).

Actin in the Fertilization Tubule

The dimensions of the microfilaments (6–8 nm wide) in the fertilization tubule strongly suggested that they are actin filaments. To demonstrate this directly and to determine the polarity of the filaments we decorated them with myosin S-1. S-1 bound to the filaments with the same polarity with respect to the membrane as has been observed in several other cellular systems (3, 7, 13, 23, 33, 39, 40, 49, 52), suggesting that common organizing principles may exist.

Microfilaments became visible in the elongating fertilization tubule only after the mating structure had formed a bud. To test whether elongation of the fertilization tubule was dependent on de novo polymerization of actin filaments we treated mating gametes with cytochalasin D. Cytochalasin D would presumably bind to the barbed ends (5, 6, 14, 27) of nascent actin filaments within the fertilization tubule, thereby preventing growth of filaments from this end. Cytochalasin D did inhibit the formation of organized actin filaments in the fertilization tubule, although it did not inhibit the generation, transmission, or receipt of mating signals. In the presence of the drug, mating structures did undergo budding, in which the membrane zone and doublet zone separated, but the elongation of the fertilization tubule from the bud was blocked. Cytochalasin D thus uncoupled budding from elongation, suggesting that actin polymerization was necessary for the latter but not the former.

It is not completely clear why it is necessary to use a concentration of cytochalasin D (200 μg/ml) over two orders of magnitude higher than that necessary to obtain an effect in vertebrate cells (1, 32). Since *Chlamydomonas* inhabits an environment in the wild where it may come into contact with fungal metabolites such as the cytochalasins, it is possible that these cells have evolved mechanisms for neutralizing the effect of this drug, for example decreased permeability. There is no reason to expect that actin from *Chlamydomonas* is in itself more resistant than usual to the drug, for actins isolated from diverse sources behave biochemically in a very similar manner (20, 54).

Model for Elongation of the Fertilization Tubule

The observations reported here taken together with previously reported work are consistent with the model for elongation of the fertilization tubule illustrated in Fig. 16. The unactivated mating structure and bud stage, in which the membrane zone separates from the doublet zone, have been described elsewhere (15, 18). In the model, outgrowth of the fertilization tubule is accompanied by the directed polymerization of actin filaments from nucleation sites located within the doublet zone. The specific attachment of the pointed ends of the actin filaments to discrete sites on the doublet zone suggests that this structure might serve as the nucleation center for actin polymerization. Following nucleation, filaments could elongate by addition of monomers onto the barbed ends, the preferred end for monomer addition (21, 26, 55, 59). The source of the monomeric actin has not yet been identified, although it is clear that actin exists in the cytoplasm of mt^+ gametes (12).

Two alternative models suggested by observations made on the elongation of other cellular processes containing actin are also worth considering. The first alternative involves assembly of actin filaments being directed by proteins at their membrane-associated ends as appears to occur during the formation of the future acrosomal process in *Limulus* sperm (48). In such a model the membrane zone at the tip of the fertilization tubule would act as the assembly site and determine the polarity of the filaments. An objection to the membrane-zone-assembly model is raised, however, by the observation that actin polymerization can occur normally in a mating

mutant (*imp*-11) that is deficient in the membrane zone component (17). A second alternative would resemble the model suggested by Begg and et al. (2) for elongation of microvilli in sea urchin eggs. In this case actin polymerizes randomly in the elongating process and is then cross-linked into ordered arrays having uniform polarity. The image of the cytochalasin D-treated fertilization tubule in Fig. 6 may be reminiscent of a microvillus in the first "floppy" stage of elongation. The degree of elongation illustrated here in the presence of the drug is, however, relatively rare. Also under normal mating conditions actin filaments are aligned longitudinally within the fertilization tubule at all stages of elongation and appear to originate within the doublet zone (17). Although not conclusive, the observations accumulated to date, then, tend to support the first model presented above and illustrated in Fig. 16.

The observation of a discrete structure that is a potential nucleation site is striking because very few such structures have been described. Short actin filaments within the actomere of *Thyone* sperm function as it appears the doublet zone of *Chlamydomonas* does: They nucleate actin polymerization and determine the polarity of the growing filaments (47). In both *Chlamydomonas* and *Thyone* actin polymerizes rapidly at a single discrete location. One might then expect organizing centers such as the actomere and doublet zone to occur only in cells where the location of directed polymerization of actin must be predetermined. In cells where actin is diffusely localized in the cortex, nucleation of actin polymerization may be controlled by individual classes of proteins rather than organized structures. A number of such controlling proteins have been isolated and characterized (reviewed in 37), including a 120,000-dalton protein from *Dictyostelium* that nucleates growth of actin filaments and is diffusely localized in the cortex of the amebae (10). It will be of interest to investigate the nucleation activity of the doublet zone in vitro and compare it biochemically with actin-associated proteins isolated from other cells.

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