FLAGELLAR TIP ACTIVATION STIMULATED BY MEMBRANE ADHESIONS IN *CHLAMYDOMONAS* GAMETES

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ABSTRACT

Membrane adhesions between the flagella of mating-type "plus" and "minus" gametes of *Chlamydomonas reinhardi* are shown to stimulate a rapid change in the ultrastructure of the flagellar tips, designated as flagellar tip activation (FTA). A dense substance, termed fibrous tip material (FTM), accumulates between the flagellar membrane and the nine single A microtubules of the tip. The A microtubules then elongate, growing into the distal region of the tip, increasing tip length by 30%. This study describes FTA kinetics during normal and mutant matings, presents experiments designed to probe its role in the mating reaction, and offers the following conclusions: (*a*) FTA is elicited by agents that cross-link flagellar membrane components (including natural sexual agglutinins, antiflagellar antisera, and concanavalin A) but not by flagellar adherence to polylysine-coated films. (*b*) FTA is reversed by flagellar disadhesion. (*c*) Gametes can undergo repeated cycles of FTA during successive rounds of adhesion/disadhesion. (*d*) FTA, flagellar tipping, and sexual signaling are simultaneously blocked by colchicine and by vinblastine, suggesting that tubulinlike molecules, perhaps exposed at the membrane surface, are involved in all three responses. (*e*) FTA is not blocked by short exposure to chymotrypsin, by cytochalasins B and D, nor by concanavalin A, even though all block cell fusion; the response is therefore autonomous and experimentally dissociable from later stages in the mating reaction. (*f*) Under no experimental conditions is mating-structure activation observed to occur unless FTA also occurs. This study concludes that FTA is a necessary event in the sexual signaling sequence, and presents a testable working model for its mechanism.

KEY WORDS microtubules · membranes · cell-cell recognition · sensory cilia · agglutination

The mating reaction of the biflagellate *Chlamydomonas reinhardi* (16) initiates when mating-type "plus" (mt^+) and "minus" (mt^-) gametes are mixed together. The resulting interactions can be subdivided into seven stages, all of which can be completed within 30 s. (*a*) The cells adhere to one another via *mt*-specific flagellar surface agglutinins, thought to be glycosylated membrane polypeptides (6, 57). (*b*) Pairs of adhering cells move their agglutinins out to their respective flagellar tips, a "tipping" response that brings the apical

cell surfaces into close proximity (14, 20, 38). (*c*) One or more "signals" are transmitted to the cell bodies of paired cells (14, 22, 39, 59). (*d*) As a first response to signaling, cells release an autolysin (9, 56) that mediates the shedding of cell walls (22, 59). (*e*) As a second response to signaling, cells activate their mating structures, the mt^+ gamete sending out a microvillarlike fertilization tubule (8, 15, 22, 63) and the mt^- gamete reorganizing the conformation of its mating-structure membrane (67). (*f*) The mating-structure membranes fuse together, creating a narrow cytoplasmic bridge that opens up to allow full cytoplasmic confluence (15). (*g*) The adhering flagella of the resultant quadriflagellated cell lose their agglutinative properties (33), presumably in response to a "signal to disadhere" transmitted at the time of cell fusion (16).

Because the tipping response appears to precede signal generation, and because bound ligands such as concanavalin A (Con A) and flagellar-directed antibodies also move to flagellar tips (20) and send mating signals to the cell bodies (9, 20), we examined flagellar tip ultrastructure during the course of the mating reaction. In this paper we report that a dense material accumulates beneath the flagellar tip membrane at the time of flagellar agglutination and is lost from the tip at the time flagella disagglutinate. An alteration in axoneme structure follows the accumulation of the material: the A microtubules increase in length, and the entire tip region elongates reversibly. We also present experimental evidence that such flagellar tip activation (FTA) is a critical feature of sexual signaling in *C. reinhardi*. A preliminary account of some of these studies has been presented (17).

MATERIALS AND METHODS

Strains and Culture Conditions

Clones of the wild-type (*wt*) strain 137c, mt^+ and mt^-, of *C. reinhardi* that exhibit high (near 100%) mating efficiencies were used in most experiments. The mutant strains *imp-1* mt^+ (18, 22) and *imp-5* mt^+ (6, 19) were used as indicated. The *cw-15* strain (lacking cell walls) (11) was used in several experiments in which mating-structure activation (MSA) was monitored so failure to shed cell walls would not bias scoring. Plate gametes (34) were harvested after 7–14 d on TAP-agar plates and suspended in nitrogen-free high-salt minimal medium (NFHSM) (34) for 1–2 h, or until strongly agglutinative.

Mating Test and Mating Efficiency

For mating experiments, equal numbers of mt^+ and mt^- cells were mixed in NFHSM at concentrations between 0.7 and 3 × 10^7 cells/ml, cell number being determined with a hemacytometer. At indicated times, aliquots were withdrawn and fixed with glutaraldehyde, and cells were scored by phase microscopy as being either biflagellated (BFC) or quadriflagellated (QFC) cells. Mating efficiency, as a percentage, was then calculated by the expression $2QFC \times 100/(2QFC + BFC)$.

Electron Microscopy

INTACT CRITICAL-POINT-DRIED FLAGELLA: Critical-point-dried cells were prepared by a modification of a method (38) that minimizes cell disruption. A 500-μl container was divided into two unequal but interconnected compartments by a removable stainless steel L-shaped holder. In the larger compartment, a 0.5% Formvar-coated EM grid was sandwiched between the holder and a stainless steel ring, creating a microcompartment just above the surface of the grid. Exchange of solutions occurred in the smaller compartment. A 100-μl sample of cells (10^7 cells/ml) was pipetted onto the grid and fixed after the appropriate experimental interval with 400 μl of cold fixative. Fixation scheme 1 consisted of 0.1% glutaraldehyde in NFHSM, for 60 min, followed by 2% OsO_4 in 10 mM HEPES buffer, pH 7.0, for 60 min; and fixation scheme 2 consisted of 2% OsO_4 in 10 mM HEPES buffer, pH 7.0, for 60 min, followed by 3% glutaraldehyde in the same buffer for 60 min. The 0.1% glutaraldehyde concentration was chosen after scanning EM studies revealed that higher concentrations caused extensive blebbing of both the flagellar and plasma membranes. Fixation scheme 2 was used specifically to study the microtubules in the flagellar tips.

After fixation, cells were washed in distilled water for 10 min, stained in 1% uranyl acetate in distilled water for 30–60 s, and washed again in distilled water for 10 min. Dehydration was carried out at room temperature with one change each of 30, 60, 90%, and two of 100% ethanol, 10 min each, and directly followed by critical-point drying in CO_2. Whole mount preparations were examined with a Philips EM300 at 60 kV or with a Hitachi HU-11C at 75 kV.

DETERGENT-EXTRACTED CRITICAL-POINT-DRIED FLAGELLA: These flagella were obtained using cells prepared by fixation scheme 1, except that after 30 min in glutaraldehyde the cells were extracted for 30 min with 30 mM octylglucoside (OG) (Calbiochem-Behring Corp., American Hoechst Corp., San Diego, Calif.) in NFHSM containing 0.1 mM dithiothreitol (DTT).

METHANOL-TREATED FLAGELLA: Cells fixed in 0.1% glutaraldehyde for 15 min were washed in distilled water for 10 min, treated twice with 100% methanol for 10 min each, then stained in 1% uranyl acetate in 100% methanol, 30–60 s, and immediately dried on No. 1 filter paper (Whatman, Inc., Clifton, N.J.), all at 4°C. The poor fixation, shock of dehydration, and air-drying adversely influence the shape of the cells, but flagellar morphology is similar to that visualized by the preceding method, and the procedure takes <1 h. Samples scored for FTA yielded the same results as those prepared by detergent treatment in a parallel experiment. The small fraction of the flagella that break during the procedure are easily distinguished and do not modify scoring data. The technique is not suitable for scoring MSA.

GRID-DEMEMBRANATED FLAGELLA: The procedure of Dentler and Rosenbaum (12) was followed, with minor modification. Intact cells were allowed to attach to polylysine-treated carbon-over-Formvar-coated grids for several seconds and were then treated with 30 mM OG in deflagellation buffer (5 mM $MgSO_4$, 0.35 mM DTT, 0.5 mM EDTA, 10 mM HEPES, pH 7.5). The procedure results in detachment of flagella from cells and a very mild demembranation of the flagella. Typical forms

of flagellar interaction (e.g., "tipped" flagella) could still be observed after preparation of mating cells.

THIN-SECTION ELECTRON MICROSCOPY: Cells were prepared by fixation scheme 1, dehydrated in ethanol, and embedded in an Epon-Araldite resin mixture (30).

Flagellar Isolation

Flagella were detached from ~2 × 10^{10} cells by the pH-shock method of Witman et al. (73). Cell bodies were pelleted by two brief centrifugations at 3,000 *g*; the supernate was layered over a 25% sucrose solution in 10 mM Tris, pH 7.4, and spun at 3,000 rpm for 15 min in an HB-4 rotor of a Sorvall RC-5B centrifuge (Du Pont Co., Sorvall Biomedical Div., Wilmington, Del.); the band of white flagella at the interface was collected, pelleted at 31,000 *g* for 20 min, and stored at −70°C. Upon thawing, the flagella exhibited strong isoagglutinability with gametes of opposite *mt*. Flagellar counts were made with a hemacytometer.

OG Extraction of Flagellar Agglutinins

A gametic mt^- flagellar pellet was suspended in 1 ml of 30 mM OG in 10 mM Tris, pH 7.4, containing 10^{-4} M EGTA and 0.1 mM DTT. The detergent extract was dialyzed overnight against several changes of NFHSM. The dialyzed material was then applied to the surface of a Formvar-coated copper EM grid, allowed to dry, and presented with a drop of mt^+ gametes.

Antiflagellar Antisera

Antisera raised in rabbits against intact, unfixed, or glutaraldehyde-fixed mt^+ gametic flagella were presented to *wt* mt^+ or *imp-5* mt^+ gametic cells at the dilutions indicated. Additional information on the properties of these and related antisera is given in references 3 and 20.

Con A

A 5 mg/ml stock solution of Con A (Sigma Chemical Co., St. Louis, Mo.) was prepared in NFHSM and diluted with NFHSM as indicated for individual experiments. When presented to gametes at 1.25 × 10^7 cells/ml for 5 min, 100–200 μg/ml of the lectin gave maximal (near 100%) agglutination; 50 μg/ml gave 50% agglutination, and 12.5 μg/ml gave 10% agglutination, as scored by hemacytometer counts of fixed samples.

Drugs and Enzymes

Solutions of colchicine, vinblastine sulfate, cytochalasins B and D (Sigma Chemical Co.), and chymotrypsin (Worthington Biochemical Corp., Freehold, N.J.) were added to cells that had been spun out of their medium. Cytochalasins B and D were dissolved at 10 mg/ml in dimethyl sulfoxide (DMSO) (Sigma Chemical Co.), and this stock was diluted further with NFHSM; all other reagents were dissolved directly in NFHSM to the concentrations specified.

Milligram quantities of lumicolchicine (an inactive derivative of colchicine) were prepared by irradiating solutions of colchicine at 20 mg/liter (50 μM) in absolute ethanol (72). Irradiation took place in a hand-blown quartz 100-ml round-bottom flask with a Blak-Ray UV lamp (model B100A, Ultra-Violet Products, Inc., San Gabriel, Calif.). Aliquots of the solution were irradiated for 1 h, with completion of the reaction monitored by loss of absorbance at 350 nm and a concomitant rise in absorbance at 267 nm. Ethanol was removed by flash evaporation at 60°C; the flask was rinsed three times with 5 ml absolute ethanol, and the combined rinses were evaporated under a stream of nitrogen gas at 60°C. The yield of lumicolchicine was determined spectrophotometrically by absorbance at 267 nm.

RESULTS

Unactivated Gametic Flagella

The fine structure in the *Chlamydomonas* flagellum is described in numerous reports (12, 26, 30, 46, 49–51, 73, 74); noted here are features pertinent to this study. The organelle is differentiated longitudinally into two zones: an ~10–12-μm "shaft" region ending in an ~0.5–0.6-μm "tip" region. The shaft contains nine doublet microtubules bearing dynein arms, nexin links, and radial spokes, plus a central microtubule pair surrounded by its helical axial filament (the "sheath"). At the shaft-tip junction, the B microtubules of the doublets terminate, as do most of the other flagellar components. Continuing into the tip are the central pair and nine single A microtubules (Figs. 1 and 2). The central pair loses its axial filament and acquires a central wedge of connecting material (50 and Fig. 2); it terminates in a "cap" of two dense plates associated with at least three and possibly four spheres (12 and 50; Mesland, unpublished micrographs). The A microtubules radiate out toward and appear to make direct contact with the tip membrane (Figs. 1 and 2, small arrows), giving the tip axoneme a somewhat larger diameter (~270 nm) than the shaft axoneme (~200 nm). Each A tubule exhibits a short dense terminus (Fig. 1, *t*). The microtubule occupying position 3 of the axoneme (53) extends to the end of the flagellum and associates with the cap (Fig. 1). The remaining tubules terminate at more proximal levels of the tip region and differ considerably in length: in a representative detergent-treated axoneme (cf. references 12, 52), one A tubule extends 420 nm beyond the terminus of its B partner, whereas another extends only 90 nm.

In critical-point-dried preparations of intact flagella (Fig. 3), the electron-dense flagellar shaft (*S*) is readily distinguished from the electron-translucent tip (*T*), in which the "high" microtubule 3 and the terminating A microtubules (arrows) can be readily visualized.

Activated Gametic Flagella

Fig. 4 shows a critical-point-dried intact flagellum from a gamete that has been mated for 2 min and has undergone FTA. Two differences are apparent between this activated flagellum and the

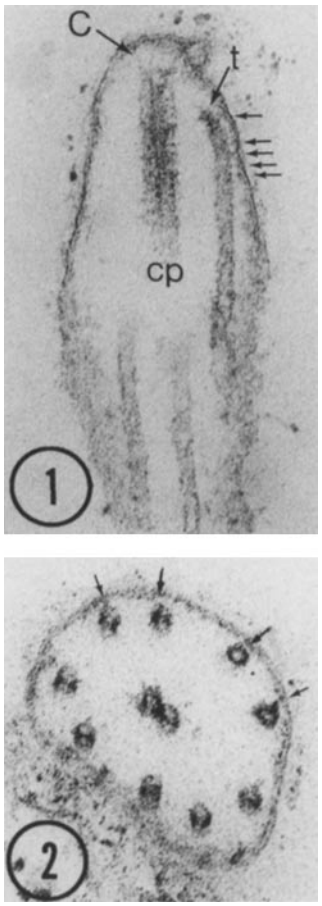


FIGURE 1 Unactivated flagellar tip from an mt^+ gamete seen in medial longitudinal section. Visible components of the cap (*C*) include two lightly stained spheres resting on a horizontal plate, which in turn rests on the central-pair microtubules (*cp*). The high A microtubule arches toward the cap and bears a blunt, dense terminus (*t*). Small arrows point to filamentous contacts between the microtubule and the flagellar membrane. × 110,000.

FIGURE 2 Unactivated flagellum from an mt^+ gamete, sectioned through a proximal portion of the tip. The nine A microtubules are positioned close to the flagellar membrane and appear, at arrows, to make contact via filamentous structures. × 110,000.

unactivated flagellum shown in Fig. 3: first, a zone of dense material (*D*) is present beneath the membrane of the tip region; second, the tip region is increased in length. These two changes are considered in turn below.

The dense material in activated flagellar tips is most readily examined in cross section. Fig. 5 shows an activated flagellum sectioned just below the cap region; Figs. 6–8 show successively more proximal images. A fibrous substance, absent from unactivated flagella (Figs. 1 and 2), has accumulated between the tip membrane and the A microtubules; until it is identified biochemically, we shall refer to it as fibrous tip material (FTM). In places the FTM appears to associate into ropelike strands which extend around the circumference of the tip (e.g., Figs. 6 and 7, arrows), but no other structural organization is apparent. FTM localizes in a highly invariant region of the flagellum: it begins at the level of the cap plates, forms a

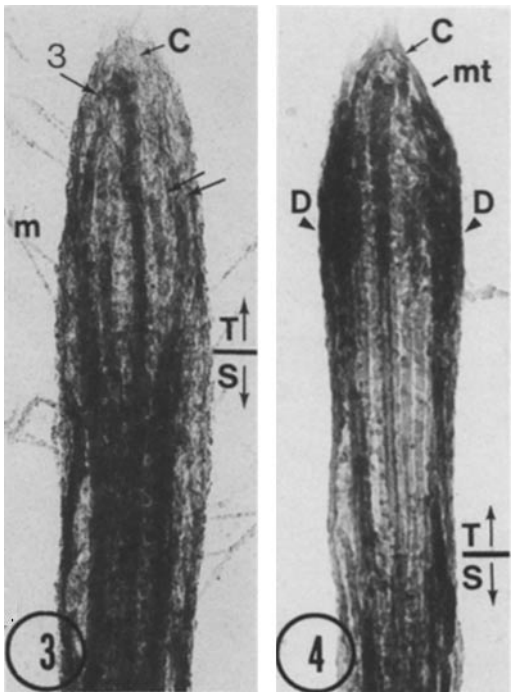
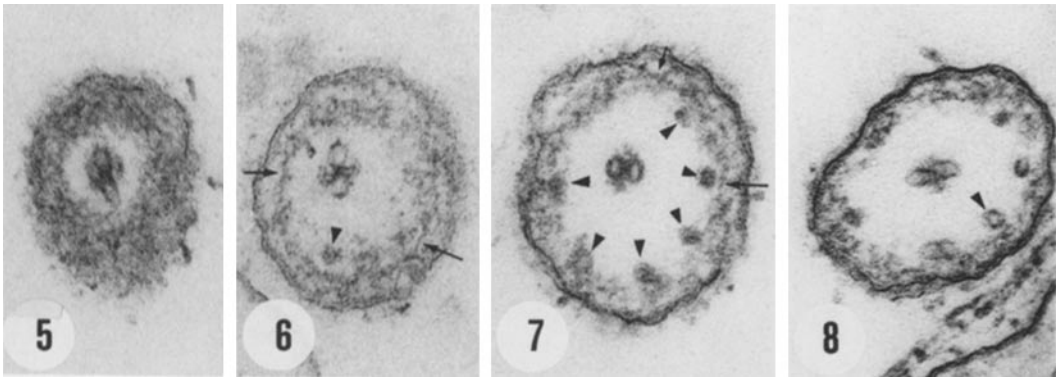


FIGURE 3 Unactivated mt^+ flagellum, prepared by fixation scheme 1 (see Materials and Methods) and critical-point dried. Transition from the dense shaft region (*S*) into the translucent tip region (*T*) is indicated. The high A microtubule (*3*) connects with the cap structure (*C*); other A microtubules terminate at lower levels (arrows). Mastigonemes are indicated by *m*. × 80,000.

FIGURE 4 Activated flagellum fixed 2 min after initiation of mating, prepared by fixation scheme 1 and critical-point dried. The increased tip length from the tip/shaft junction (*T/S*) compared with Fig. 3 is apparent. The A microtubules terminate at a focus marked *mt*; several appear to connect to the cap (*C*). Electron-dense material is indicated at *D*. Absence of mastigonemes is not significant; their retention on the flagellar surface is highly variable in both mated and nonmated samples. × 80,000.



FIGURES 5–8 Activated flagellar tips viewed in progressively more proximal cross sections. Fig. 5 (fixed 15 s after initiation of mating) shows an extreme distal section, with a ring of FTM beneath the tip membrane (grazed during sectioning); the central-pair tubules are seen ending at the level of the cap plates. Fig. 6 (fixed 1 min after initiation of mating) shows the high filled microtubule at position three (arrow head) and strands of material within the ring of FTM (arrows). Fig. 7 (fixed 1.5 min after initiation of mating) shows six filled microtubules (arrowheads) and strands within the FTM (arrows). Fig. 8 (fixed 1.5 min after initiation of mating) is sectioned at a level where at least one microtubule appears hollow (arrowhead); the others are filled. × 110,000.

discrete 45-nm layer between the membrane and the ring of nine A microtubules, and terminates above the level of the tip/shaft junction (Fig. 4). Its presence creates a physical separation between the A microtubules and the flagellar membrane (Figs. 6–8).

The second structural change during activation—the increased length of the activated tip—has been quantitated in whole-mount preparations of the sort shown in Fig. 4. Fig. 9 plots the resulting measurements. Whereas a range of tip lengths is found for both unactivated and activated flagella, a statistically significant ($P \leq 0.001\%$) 30% increase in mean length is found to accompany FTA.

Two observations support the concept that the increase in length is effected by an elongation of the A microtubules.

(*a*) When flagella are fixed in OsO_4 (Figs. 10 and 11), FTM is largely extracted and the tips are highly transparent. As activation proceeds, the microtubules can be seen to elongate until all nine extend to the very end of the flagellum, converting the tapered unactivated tip (Fig. 10) into a rounded activated one (Fig. 11).

(*b*) In negatively stained axonemal preparations from activated flagella, the ends of the A tubules usually end in sheets of protofilaments topped by plugs (Fig. 12). Although such protofilament sheets are occasionally encountered on one or two of the nine microtubules in unactivated axonemes, their frequency is markedly enhanced during

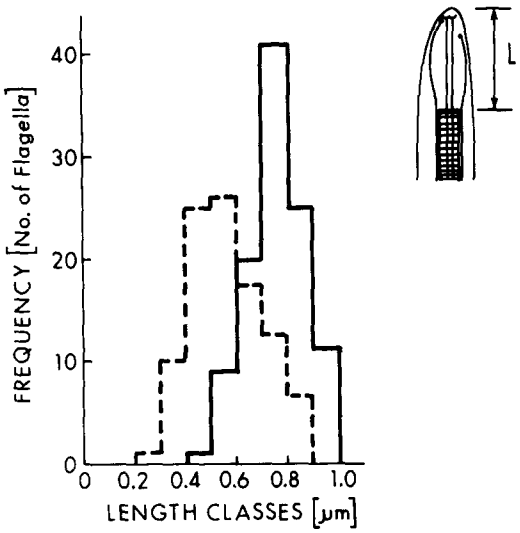


FIGURE 9 Distribution of tip-region lengths (see schematic drawing in *inset*) for unactivated (- - -) and activated (——) flagella. Samples were prepared by fixation scheme 1. Tips were measured directly from the microscope image using an ocular micrometer. Average length found: for unactivated tips, 570 nm (n = 100, SD = 14.1 nm); for activated tips (2 min of mating), 750 nm (n = 107, SD = 10.0 nm). $P \leq 0.001\%$ for the lengths to be equal.

FTA. A comparable increase in A tubule protofilament sheets is found in vegetative axonemes undergoing flagellar regeneration (12), suggesting that they represent the elongating ends of micro-

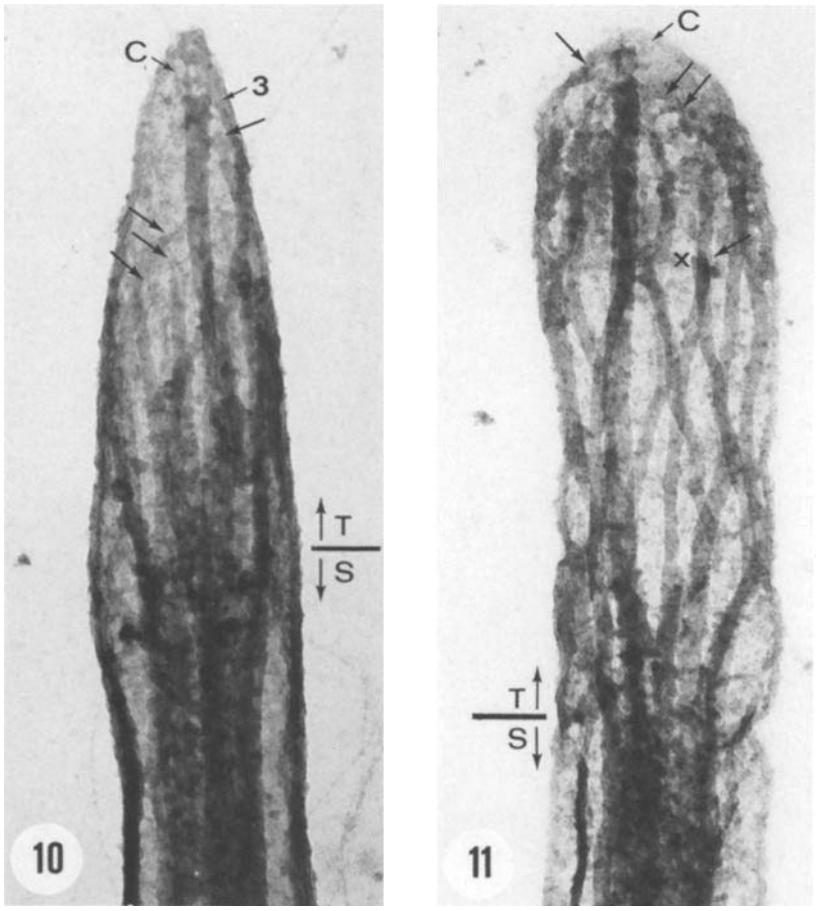


FIGURE 10 Unactivated mt^+ flagellum prepared by fixation scheme 2 (see Materials and Methods). The transition between shaft (*S*) and tip (*T*) region is indicated. Arrows point to the ends of A microtubules. The high A microtubule (*3*) connects to the cap structure (*C*). × 90,000.

FIGURE 11 Activated flagellum prepared by fixation scheme 2. The length of the tip region (*T*) has increased considerably. Most A microtubules terminate close to the cap structure (arrows), an exception being the microtubule marked *x*; their termini appear to associate with unstained spherical structures connected to the cap (*C*). × 90,000.

tubules. It is possible that the abundant "filled" microtubules in activated flagella (Figs. 5–8) also represent regions of active growth.

Kinetics of Activation

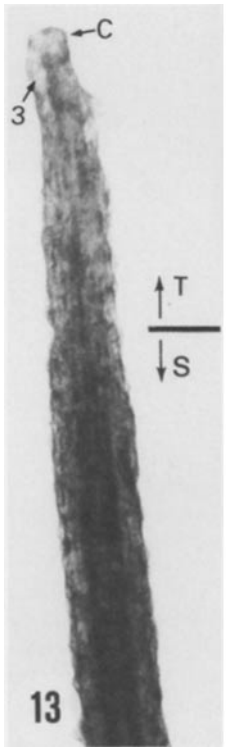
To study FTA kinetics during the mating reaction, we developed two preparative procedures which permit unactivated and activated flagella to be readily and accurately distinguished by low-magnification EM. In both procedures, cells are first fixed with dilute glutaraldehyde solutions; they are then treated either with methanol or with the detergent OG (see Materials and Methods). Figs. 13 and 14 compare unactivated and activated flagella prepared by the methanol procedure. The association of the A tubules with FTM in the activated state is preserved by glutaraldehyde fixation and retained during the subsequent extraction; the result is a distinctive distended tip and a narrow "neck" at the tip/shaft junction. Fig. 15 shows an OG-treated preparation and illustrates that unactivated (arrowheads) and activated (small arrows) tips can be easily identified at low magnification.

Using such extracted material, we can readily score the kinetics of activation. Fig. 16*A* plots

FIGURE 12 Axoneme from an activated flagellum, prepared by demembranating whole cells with detergent 1 min after the initiation of mating. Six A microtubules are visible, all terminating in sheets of protofilaments. Small arrows point to the sites of insertion of "plugs" (12) into the protofilament sheets. Large arrows point to the termini of two B microtubules. × 108,000.

results based on OG-extracted samples, and Fig. 17*A* shows a methanol-treated experiment. The first fused QFC vary somewhat in time of appearance in the two matings, but the overall kinetics are seen to be very similar. Three features of the activation process are to be noted in these experiments. First, FTA clearly precedes gametic cell fusion. Second, the maxima in cell pairing coincide with the maxima in FTA. Third, FTA is seen to be reversible, declining coincidentally with the rate of cell fusion.

FTA is found in such experiments to proceed through an "intermediate" stage: as illustrated in Fig. 18, many tips in early samples appear fuller than in unmated samples but do not yet display the bulbous ends and narrow necks of fully activated organelles. The development of this intermediate morphology precedes full activation in kinetic experiments (Fig. 16*B*), and intermediate flagella are at a minimum when full activation is maximal (Fig. 16*B*). In samples fixed from the same experiment plotted in Fig. 16 and analyzed by thin-section EM, 70% (19 of 27) of the tips photographed from the 15-s sample were found to contain some FTM. In whole-mount preparations, only 8% of the flagella in this 15-s sample were judged to be fully activated, whereas 26% were scored as intermediate (Fig. 16*B*). These observations suggest that the first activation event is the



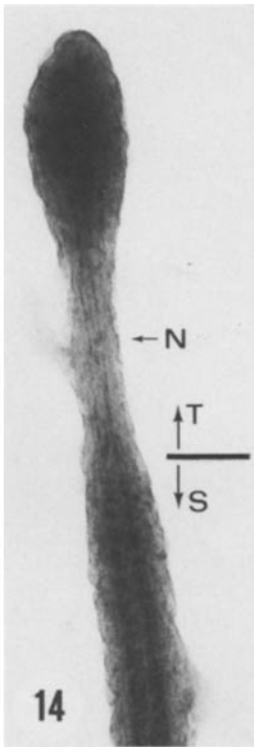


FIGURE 13 Unactivated mt^+ flagellum, fixed, methanol treated and air-dried. The central pair cap (*C*), its associated microtubule (*3*), the tapered tip region (*T*), and the shaft region (*S*) can be distinguished. Remnants of the matrix and/or membrane remain associated with the axoneme and form a double or triple semihelix. × 80,000.

FIGURE 14 Activated methanol-treated flagellum. The stretch caused by air-drying creates a very narrow neck zone (*N*) and accentuates the FTM-filled bulbous tip. The semihelical structure is visible around the whole length of the axoneme. *T*, tip; *S*, shaft. × 80,000.

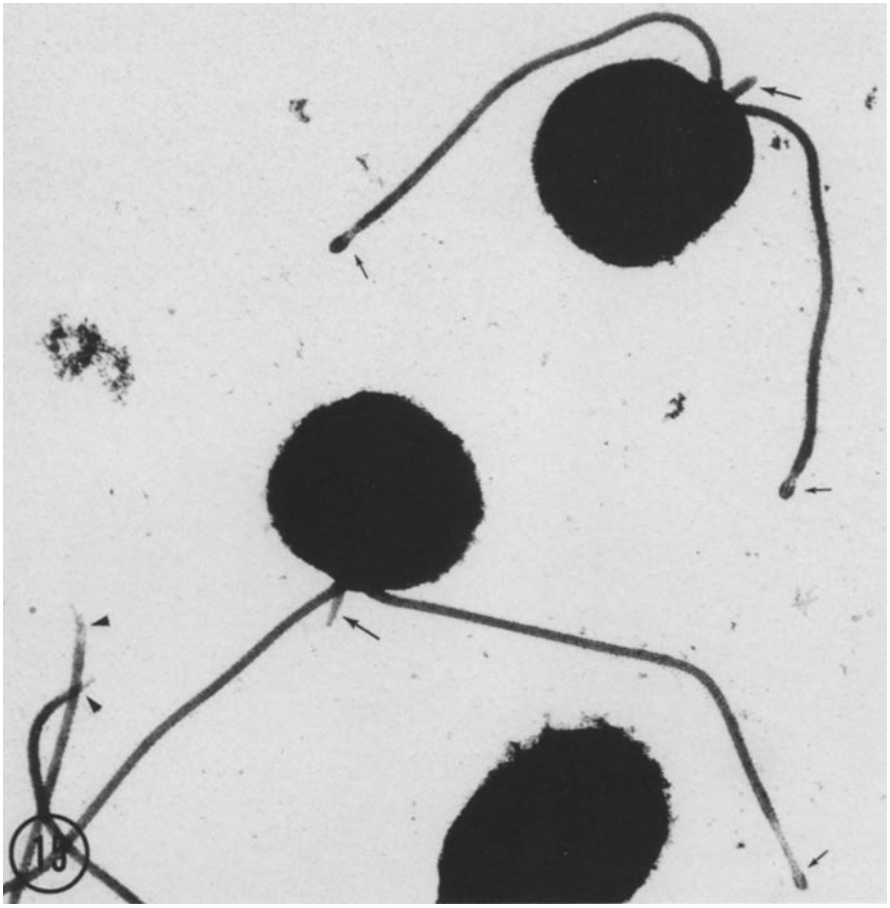


FIGURE 15 Activated flagella and mating structures in OG-treated, critical-point-dried mt^+ gametes. Gametes (2×10^7 cells/ml) were mixed with isolated mt^- flagella (2.7×10^7 flagella/ml) and fixed after 3 min. Activated flagellar tips (small arrows) and nonactivated flagellar tips (arrowheads) are indicated. Large arrows point to activated mating structures. × 8,100.

accumulation of FTM, which causes tips to change from a pointed (unactivated) to a rounded (intermediate) shape; this is followed by A tubule elongation, which generates the extreme morphological alteration scored as full tip activation.

That the reversal of FTA seen in Figs. 16*A* and 17*A* is a consequence of cell fusion and not a preprogrammed decline in activation ability has been demonstrated using *imp-1* mt^+ × *wt* mt^- matings. The *imp-1* mutation prevents gametic cell fusion and flagellar disadhesion but does not prevent the transmission of mating signals to the cell bodies; as a consequence, cells continue to agglutinate sexually for many hours (22). As seen in Figure 17*B*, flagellar tips remain fully activated in *imp-1* × *wt* matings for at least 30 min. The structure of such a tip, moreover, is indistinguishable from a tip that has been activated for only 1 min; the continued time of agglutination does not generate a tip that is either any longer or more filled with FTM.

FTA Induction by Agglutinins and Surface Cross-linking Agents

To determine whether it is essential for FTA that cells of both *mt* be present, we performed two kinds of experiments. First, isolated mt^- flagella were presented to mt^+ gametes. Second, mt^- flagella were extracted with OG (68), the detergent was removed by dialysis, a film of the dialyzed extract was allowed to form on a Formvar-coated EM grid, and a drop of mt^+ cells was then applied to the grid. Adair et al. (2) have found that gametes adhere by their flagellar tips to such films in an

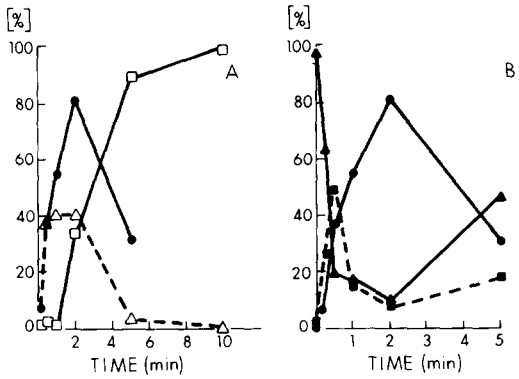


FIGURE 16 (*A*) Time-course of cell-pair formation (△), cell fusion (□), and FTA (●) in mating gametes. Duplicate samples were fixed at each time indicated. One was scored microscopically for pair formation and cell fusion; the other was OG treated and critical-point dried for scoring of FTA. (*B*) Time-course of the occurrence of unactivated (▲), intermediate (■), and activated flagella (●) in the same experiment as described for *A*.

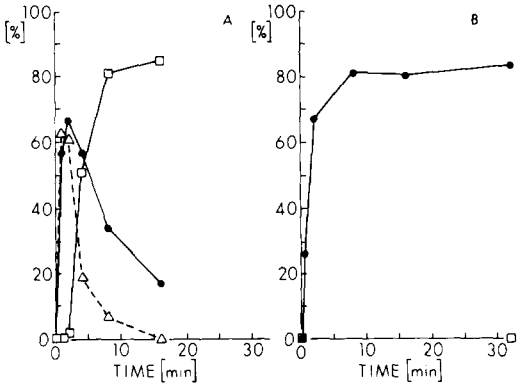


FIGURE 17 Kinetics of FTA (●), cell pairing (△), and cell fusion (□) scored in samples prepared by methanol treatment, examining *A*, a *wt* mating, and *B*, an *imp-1* $mt^+ \times$ *wt* mt^- mating. Cell pairing cannot be scored in *imp-1* matings, because the clump size becomes enormous.

mt-specific fashion; that is, mt^+ cells stick to mt^- but not mt^+ films, and vice versa.

As seen in Table I, the FTA response in mt^+ gametes is readily elicited by both the isolated flagella and the detergent-extracted mt^- agglutinin film. The same cell samples were also scored for MSA, seen in mt^+ whole mounts as the extension of a long, slender fertilization tubule from the cell anterior (Fig. 15, large arrows). As shown in Table I, the flagella and the extracted agglutinins are as effective in inducing MSA as they are in inducing FTA.

In control experiments designed to determine whether mt^+ gametes undergo activation if their flagella are simply tethered, cells were allowed to adhere to polylysine-coated EM grids and then examined. None of the flagella was activated by such adhesions, nor were mating structures activated (Table I). To rule out the possibility that the presence of polylysine might be inhibitory to the activation response, mt^+ cells tethered to polylysine films were subsequently presented with isolated mt^- flagella. As seen in Table I, the attached cells remained capable of normal FTA in response to these flagella.

Two experimental approaches were taken to determine whether sexual agglutinins are required to elicit FTA or whether the response can also be evoked by other ligands that cross-link flagellar surface components. First, an antiserum raised against isolated mt^+ flagella was presented to *wt* mt^+ gametes, causing them to isoagglutinate by their flagellar tips and to activate their mating structures (20). It is seen in Table I that 40% of the

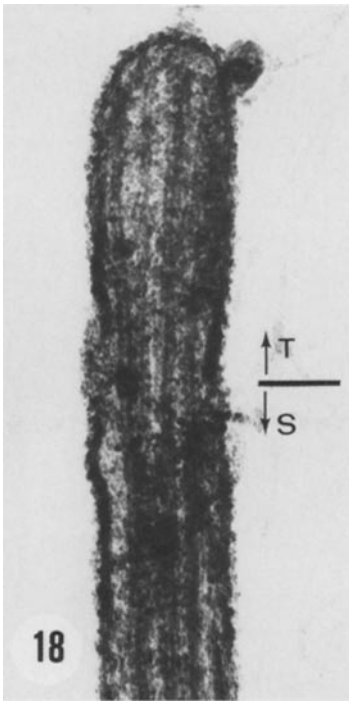


FIGURE 18 Intermediate stage of FTA in a flagellum fixed by scheme 1, 30 s after initiation of mating. Tip region (*T*) is not yet elongated but the tip is already rounded in shape. The tip is also moderately electron dense (cf. Fig. 3), presumably because it contains FTM. *S*, shaft. × 80,000.

TABLE I

Effect of Flagellar Membrane Agglutinins on Parameters of the Sexual Response in mt^+ Gametes

mt^+ Gametes presented with	Agglutination	Tipping	Cell wall release	FTA				MSA	
				Non	Inter*	Act	Total scored		Total scored
				%				%	
No additives	No	No	No	91	9	0	(98)	0	—
Polylysine-coated grid‡	Adhesion	No	No	76	24	0	(106)	2	(51)
Preimmune serum (1:6)	No	No	No	90	10	0	(102)	0	—
Isolated mt^- flagella (1.3 flagella/cell)	Yes	Yes	Yes	23	16	61	(140)	40	(77)
Isolated mt^- flagella after polylysine adhesion‡	Yes	Yes	—	18	23	59	(115)	44	(48)
OG^- extract§ (1.6 mg/ml)	Yes‖	Yes	Yes	28	25	47	(111)	47	(64)
αGG^+¶ (1:160)	Yes	Yes	Yes	15	44	41	(74)	45	(84)
Con A (1 mg/ml)	Yes	Yes	No	47	29	24	(72)	3	(59)
(200 μg/ml)‡	Yes	Yes	—	61	18	21	(106)	23	(56)

* Flagellar tips are scored intermediate (*inter*) when they cannot be scored as either unactivated (*non*) or activated (*act*). These include tips that have a clear intermediate morphology (see Figs. 16 *B*, 18, and 19); also included are tips that are difficult to score as, for example, when αGG^+ presentation has induced antibody/vesicle complexes (Fig. 19) that are resistant to OG removal.

‡ Experiment with the *cw-15* mt^+ mutant; occasionally a gamete of this mutant bears a spontaneously activated small mating structure.

‖ Gametes adhere to the film formed by the extract.

§ OG^- extract, dialyzed octylglucoside extract of mt^- gametic flagella.

¶ αGG^+, antiserum raised against glutaraldehyde-fixed isolated gametic mt^+ flagella.

flagella undergo unmistakable activation after 3 min, with a concomitant induction of MSA. In a parallel experiment, the mutant *imp-5*, which lacks a functional agglutinin (6), was isoagglutinated by a second antiserum raised against mt^+ gametic flagella, and the cells were examined by thin-section EM. Although meaningful quantitation of thin-sectioned material is impossible, many of the tips were activated (Fig. 19).

The second protocol for nonsexual agglutination used Con A, which isoagglutinates gametes of either mating type by their flagella (5, 35, 71). As seen in Table I, both FTA and MSA are elicited by the lectin in a parallel fashion. It should be noted that the fertilization tubules fail to elongate to their normal dimensions (Fig. 15) in the presence of Con A; instead, the activated structures are uniformly short, stubby protrusions (Fig. 20), as if the lectin generates a defective signal or interferes directly with the MSA response, or both.

FTA Reversal and Reinitiation

The kinetic experiments presented in Fig. 17 demonstrate that in an *imp-1* × mt^- mating in which cell fusion and zygotic flagellar disadhesion are blocked, the deactivation of flagellar tips is blocked as well, suggesting that tip deactivation is triggered either by cell fusion, by flagellar disadhesion, or by both. To determine which stimulus is operative, we took advantage of the phenomenon of reversible in vitro flagellar adhesion reported by Snell and Roseman (58). They discovered that when isolated flagella of one *mt* are mixed in a 1:1 ratio with gametes of opposite *mt*, an initial burst of isoagglutination is followed by a progressive loss of adhesion until only single cells and free flagella remain. The cells can be readily reagglutinated using a fresh sample of isolated flagella, whereas the initial set of flagella are rendered nonadhesive as a consequence of the agglutination reaction. Using this system, we were able to subject a sample of cells to successive rounds of adhesion/disadhesion under conditions in which no cell fusion occurs, and to ask whether activated tips undergo deactivation in response to disadhesion alone.

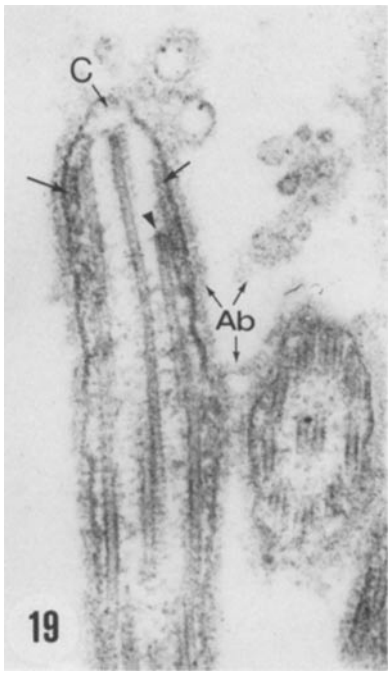


FIGURE 19 Antibody-mediated tip activation of an *imp-5 mt*$^+$ gamete. The flagellum is judged to be in an intermediate stage of activation, with FTM (arrows) localized between the axoneme and membrane but with tip elongation not yet initiated. Antibody (*Ab*) aggregates are associated with the membrane surfaces and with vesicles that bleb from the flagellar tips (20). Central-pair cap is at *C*; the high microtubule 3 arches toward the cap/central-pair junction. Arrowhead points to a dense microtubule terminus. × 66,000.

Fig. 21 shows the results of several experiments in which isolated *mt*$^-$ flagella were presented to *mt*$^+$ gametes. Fig. 21 *a* shows the kinetics of the agglutination/disagglutination response as judged by visual inspection; comparable kinetics are found by Coulter-counter assay (58). Fig. 21 *b* shows the extent of FTA in such samples; the deactivation process is seen to parallel disadhesion, ruling out a causative role for cell fusion in eliciting tip deactivation. The microfilament-filled fertilization tubules, it should be noted, remain extended during the disadhesion/deactivation phase of the experiment (Fig. 21 *c*), indicating that the polymerized state of the microfilaments is not dependent on sustained flagellar interactions.

The experiments summarized in Fig. 21 reveal an additional feature of the FTA response, namely that flagella can undergo multiple activation/deactivation cycles. Thus in Fig. 21 *b*, 65% of the flagella were initially activated; this number can be "boosted" to nearly 90% if additional flagella are presented after 3 min (dashed lines). If additional flagella are added at 24 min (arrow), after roughly 50% deactivation has occurred, a full activation response is repeated, again in parallel with the development of a fresh set of adhesions.

Agents Blocking FTA but Not Adhesion

The foregoing experiments reveal that flagellar adhesion is ordinarily sufficient to trigger FTA; i.e., the two responses are ordinarily coupled. Presented here are experiments showing that colchicine and vinblastine, agents known to interact with tubulin (72), are effective in blocking the generation of FTA in response to adhesion.

Fig. 22 presents data on the sensitivity of the mating reaction to increasing concentrations of colchicine (*A*) and vinblastine (*B*). Both drugs, at concentrations in the range required for antitubulin effects with *Chlamydomonas* (13, 51), produce a sharp and complete inhibition of mating. Inspection of gametes pretreated with 10–15 mg/ml colchicine or 0.18 mg/ml vinblastine reveals that neither drug has any effect on motility or on the establishment of a vigorous agglutination reaction when the two *mt* are mixed. Both drugs, however, are found to block the tipping response: clumps of adhering gametes fail to move their adhesive sites out to the flagellar tips, the result

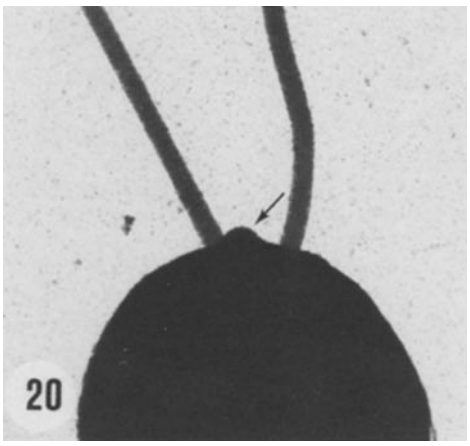


FIGURE 20 MSA by Con A in *mt*$^+$ gametes. Gametes (1.25×10^7 cells/ml) were incubated with 100 μg/ml Con A for 5 min, fixed, OG treated, and critical-point dried. A slight elevation of the mating structure (arrow) indicates activation, but no full outgrowth of the mating structure occurs (cf. Fig. 15). × 16,500.

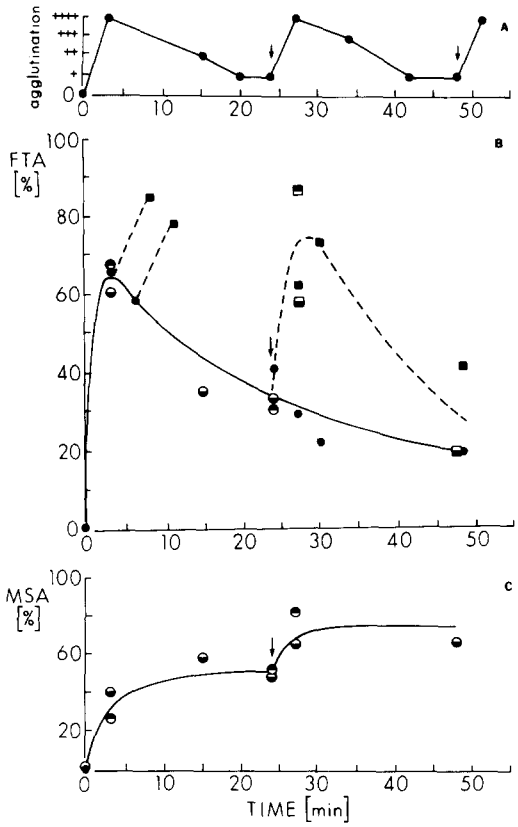


FIGURE 21 Influence of adhesion and disadhesion of isolated mt^- flagella to mt^+ gametes on FTA and MSA. Equal volumes of mt^+ gametes (2×10^7 cells/ml) and isolated mt^- flagella (2.7×10^7 flagella/ml) were mixed and aliquots were tested for agglutination (*A*), FTA (*B*), and MSA (*C*). At the times indicated (arrows), 2.7×10^7 flagella suspended in one-tenth the volume were added and the same parameters scored. (*A*) The adhesion/disadhesion cycle and its stimulation by adding fresh flagella. (*B*) FTA, scored in three different experiments (denoted by different symbol shadings). Circles and solid line: no additional mt^- flagella added; squares and broken lines: time-course after addition of fresh flagella. FTA can be boosted further by adding fresh flagella at 3 or at 6 min. (*C*) MSA, scored in the experiments indicated by equivalent symbols in *B*. The percentage of activated mating structures levels off at 45%, is stimulated further by addition of fresh flagella at 24 min, and plateaus at 70%.

being that the cell bodies pack in close together. When such agglutinating mixtures are fixed and examined by electron microscopy, an almost complete inhibition of FTA is observed both by whole-mount scoring (Table II) and by thin-section analysis (Fig. 23).

The experiment summarized in Table III was designed to determine whether a 30-min preincubation in colchicine is in fact required to inhibit mating, or whether shorter exposures are also effective. Cells of both *mt* were exposed to colchicine for only 30 s before being mixed, and were fixed and scored for QFC formation 5 min later. It is seen that the inhibition of fusion effected by this short exposure to the drug is comparable to the inhibition exerted by a prolonged exposure, whereas untreated controls undergo extensive fusion during the same interval.

In a reciprocal experiment summarized in Table IV, cells of opposite types were preincubated for 30 min in inhibitory concentrations of colchicine; they were then allowed to agglutinate in the presence of the drug for 5 min, after which the drug was diluted to a noninhibiting level for an additional 15 min. As seen in Table IV, significant recovery from the effect of the drug occurs in this short interval.

Lumicolchicine proved soluble in NFHSM only at 0.89 mg/ml. To perform a lumicolchicine control experiment, therefore, we developed the following protocol. We found that if gametes were incubated overnight in the presence of 0.89 mg/ml colchicine and 10 μl/ml DMSO, fusion was inhibited by about 40% relative to DMSO-incubated controls (44 vs. 69% for controls). When cells from these cultures were instead incubated overnight in the presence of 0.89 mg/ml lumicolchicine and 10 μl/ml DMSO, no inhibition of mating occurred (79 vs. 69% for controls).

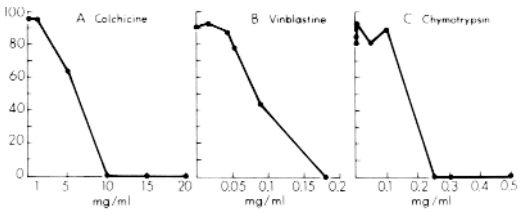


FIGURE 22 The effects of increasing concentrations of colchicine (*A*), vinblastine (*B*), and chymotrypsin (*C*) on the fusion of mt^+ and mt^- gametes. Cells were preincubated at 3×10^7 cells/ml in various concentrations of the reagents for 30 min, opposite *mt* were mixed, and fusion was allowed to proceed for 15 min (*A* and *B*) or 5 min (*C*), at which times fixative was added and the percentage of fusion determined. At 2–3 min after mixing, agglutination was assessed and judged to be as vigorous as in untreated controls except for 0.5 mg/ml chymotrypsin, in which agglutination was severely reduced.

TABLE II

Effect of Colchicine and Vinblastine on Parameters of the Sexual Response in Mating Gametes

	Agglutination	Tipping	Cell wall release	FTA				Cell fusion
				Non	Inter*	Act	Total scored	
					%			%
A. Colchicine treatment (10 mg/ml)								
Control	Yes	Yes	Yes	35	19	46	(139)	95
Experiment	Yes	No	No	85	11	4	(140)	0
B. Vinblastine treatment (0.12 mg/ml)								
Control	Yes	Yes	Yes	8	7	85	(102)	98
Experiment	Yes	No	Not scored	89	7	4	(109)	2

(*A*) Gametes of both *mt* (10^7 cells/ml) treated for 30 min, then mixed and fixed after 5 min for FTA and after 15 min for cell fusion determination. (*B*) Gametes of both *mt* (1.5×10^7 cells/ml) treated for 15 min, then mixed and fixed after 2 min for FTA and after 15 min for cell fusion determination.

* See Table I for details of scoring.

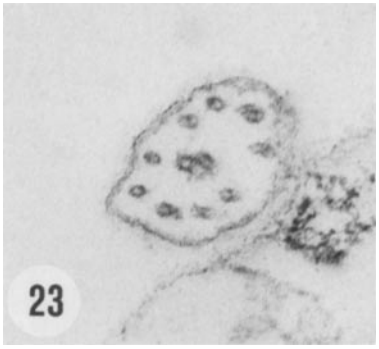


FIGURE 23 Tip cross section of a flagellum from a gamete incubated in 10 mg/ml colchicine for 1 min and allowed to agglutinate in the presence of colchicine for an additional minute. No FTM accumulation or other signs of activation were evident in seven tip sections photographed. × 81,000.

TABLE III

Effect of Colchicine Preincubation Time on Cell Fusion

Duration of preincubation	No. of QFC	No. of BFC	Fusion in 5 min
			%
30 s	2	282	1
5 min	2	170	2
Control	114	147	61

We preincubated mt^+ and mt^- cells separately at 3×10^7 cells/ml in 15 mg/ml colchicine in NFHSM for the times indicated. Control cells were preincubated for 5 min in NFHSM. Opposite *mt* were mixed, and fusion was allowed to proceed for 5 min; the mating reaction was then stopped by the addition of 3% glutaraldehyde solution. Cells were scored in the light microscope as quadriflagellated cells (QFC) or biflagellated cells (BFC) and the percentage of fusion was calculated (29).

Agents Blocking Cell Fusion but Not FTA

We have to date identified four ways to block the *Chlamydomonas* mating reaction at a stage between FTA and cell fusion. The first is chymotrypsin digestion. The sensitivity of the mating reaction to this enzyme is displayed in Fig. 22 *C*. When gametes treated with 0.25 mg/ml chymotrypsin are mixed and examined, flagellar adhesiveness, flagellar tipping, and wall loss during the first 5 min of mating appear completely normal (Table V). The FTA response, moreover, develops to the same extent as in the controls (Table V). As assessed either by thin-section or whole-mount electron microscopy, however, neither activation of mt^+ mating structures nor cell fusion occurs (Table V). It should be noted that if the gametes are allowed to continue mating for an additional 10 min in the presence of the enzyme and are then examined, the agglutination response is found to have greatly abated, possibly because chymotrypsin has at this point destroyed a critical number of agglutinins (cf. reference 70). In this case, a concomitant reduction is found to have occurred in FTA (Table V), much as in experiments with isolated flagella (Fig. 21).

The second class of agents that blocks "downstream" from FTA is that including the cytocha-

lasins B and D. High concentrations of these drugs, although without effect on FTA, inhibit both MSA and cell fusion (Table VI). Whether they act on the microfilaments of the mt^+ mating structures (22) has not yet been determined.

The remaining two conditions that inhibit mating at a stage between FTA and cell fusion are the *imp-1* mutation, which affects MSA (22; Fig. 17 *B*), and the lectin Con A. When *cw-15* gametes are preincubated in low concentrations (12 μg/ml) of the lectin and are then mixed, fusion is inhibited by 40%; such inhibition is not observed if gametes are instead pre-isoagglutinated by flagella or if the lectin is first mixed with its hapten α-Me-D-glucose.

TABLE IV

Effect of Colchicine Dilution on Cell-Fusion Inhibition

Preincubation solution	Dilution solution	No. of QFC	No. of BFC	Fusion in 15 min after dilution
				%
NFHSM	NFHSM	222	47	90
Colchicine (15 mg/ml)	NFHSM	60	191	39
Colchicine (15 mg/ml)	Colchicine (15 mg/ml)	1	200	0

We preincubated mt^+ and mt^- cells separately at 3×10^7 cells/ml for 30 min in the solutions indicated. Opposite *mt* were mixed, and mating was allowed to proceed for 5 min, at which time the mating mixtures were diluted threefold as shown. Fusion was allowed to continue for an additional 15 min at which time the mating reaction was stopped and the percentage of fusion determined as described in Table III.

DISCUSSION

Morphology of FTA

The flagella of *Chlamydomonas* are reported in this paper to undergo a rapid and reversible change in morphology, diagrammed in Fig. 24, during the course of the mating reaction. EM observations of specimens prepared by a variety of protocols reveal FTA to be a highly invariant process: FTM localizes in a specific region of the flagellum, A microtubules elongate by a constant percentage, and the process commences and reverses in response to defined stimuli (adhesion and disadhesion). Such precision argues strongly that we are observing a carefully controlled biological process.

We have surveyed literature on ciliated sensory cells and mechanoreceptors (69) for evidence of tip specializations analogous to those reported here. An enormous variation in microtubule patterns is encountered in sensory cilia, but the presence of filamentous structures organized in parallel with the membrane is one invariant theme. For mechanoreceptors, a second theme is that the tip is anchored into an immobilized structure, be it a cuticular extracellular cap in the case of insects (e.g., 40) or the otolithic membrane in the case of the vestibular apparatus in vertebrates (25, 28). In only one case, however, has any change in ciliary structure been correlated with signal transmission; Moran et al. (40) report a pronounced bend at the base of insect mechanoreceptor cilia under conditions of maximal stimulation. It will be intriguing to learn whether morphological changes can also be detected at the tips of sensory cilia in response to sensory stimulation, or whether this elaborate

TABLE V

Effect of Chymotrypsin Digestion on Parameters of the Sexual Response in Mating Gametes

	Mating time	Agglutination	Tipping	Cell wall release	FTA				Cell fusion
					Non	Inter*	Act	Total scored	
	min					%			%
Control	2	Yes	Yes	Yes	13	13	74	(117)	86
Chymotrypsin	2	Yes	Yes	Yes	18	11	71	(115)	
(0.25 mg/ml)	5	Yes	Yes	Yes	18	10	72	(124)	
	15	Slight	Slight	—	64	25	11	(128)	1

Gametes of both *mt* (10^7 cells/ml) were treated separately for 15 min, then mixed and fixed after the time indicated for FTA and after 15 min for cell fusion determinations.

* See Table I for details of scoring.

TABLE VI

Effects of Cytochalasins B and D on Parameters of the Sexual Response in Mating Gametes

	Agglutination	Tipping	Cell wall release	FTA Non	FTA Inter*	FTA Act	FTA Total scored	Cell fusion
				%				%
Control	Yes	Yes	Yes	4	14	82	(121)	88
Control DMSO	Yes	Yes	Yes	15	15	70	(134)	85
Cytochalasin B (200 μg/ml)	Yes	Yes	Yes	21	19	60	(114)	37
Cytochalasin D (200 μg/ml)	Yes	Yes	Yes	21	15	64	(117)	15

Gametes of both *mt* (10^7 cells/ml) were treated for 30 min, then mixed and fixed after 2 min for FTA and after 15 min for cell fusion determinations. In the critical-point-dried preparations of the 15-min samples in both experiments, none of the single cells exhibited an activated mating structure.

* See Table I for details of scoring.

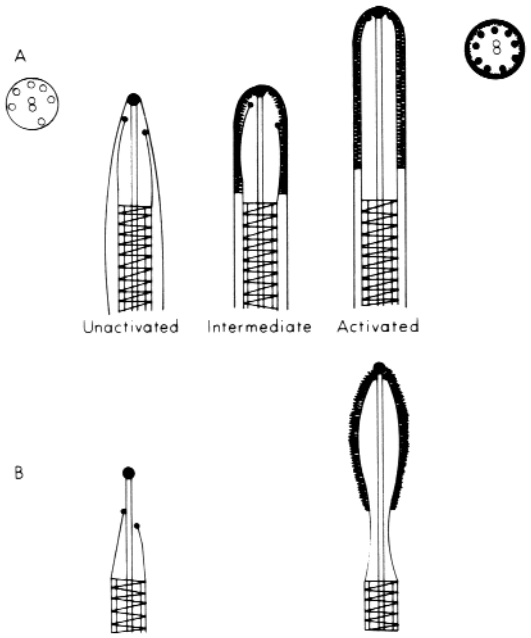


FIGURE 24 (*A*) Diagram of the FTA sequence showing unactivated, intermediate, and activated flagella as in critical-point-dried whole mounts, plus unactivated and activated tips in cross section. (*B*) Unactivated and activated flagella as revealed in extracted preparations. Microtubules are represented by single lines; the spiral represents a structure we believe may encircle the axoneme (Figs. 13 and 14). In the unactivated tip, A tubules spread out toward the membrane and perhaps anchor there by filamentous contacts (Figs. 1 and 2); when, however, the membrane is removed with detergent or methanol, the tubules collapse on one another. In the activated tip, on the other hand, mild glutaraldehyde fixation stabilizes associations between the FTM and the microtubules; therefore, membrane removal does not alter the bulging outlines of the tip, and the dense FTM

response to external stimuli is unique to *Chlamydomonas.*

The biochemical composition of FTM is unknown, but possible identifications can be considered based on morphological criteria. Plaques of dense material are known to localize between the shaft axoneme and the membrane in both vegetative (50) and gametic (17) flagella of *C. reinhardi,* but the plaque material is considerably more electron dense than is FTM. Moreover, the plaques always occupy only an arc of the flagellar circumference rather than the entire circumference, and they remain present in the shaft while FTM is accumulating in the tips. Therefore, there is no reason to believe that plaques and FTM are identical or even homologous.

A second possibility, that FTM represents a precursor form of tubulin destined to participate in A-tubule elongation, is also discouraged on morphological grounds. In studies of both regenerating and degenerating flagella (27, 30, 49, 51) and basal bodies (21) in *Chlamydomonas*, EM images of apparent tubulin precursor pools reveal a light-staining, homogeneous area that is very different from FTM.

A third possibility is that FTM represents a microtubule-organization center (MTOC) (44), imported to or organized within the tip to mediate A-tubule elongation. MTOC (e.g., centriolar satellites and spermatid nuclear rings) are typically dense, amorphous aggregates (43–45, 55, 60) sim-

is readily observed. The narrow neck contains that sector of the axoneme that is stabilized neither by the spoke/dynein/spiral system nor by the FTM system; it may therefore be differentially subject to stretch during specimen preparation.

ilar to FTM, but because *Chlamydomonas* flagella normally grow without the apparent participation of tip MTOC material (30, 49, 51), it is not obvious why an MTOC should be recruited to mediate A-tubule outgrowth during mating. Moreover, whereas microtubules are typically found to be completely embedded within MTOC material (cf. reference 43), their association with FTM involves only a discrete portion of their outer surfaces (Figs. 5–8).

Because an actinlike protein has recently been identified in the *Chlamydomonas* flagellum (47), it is intriguing to speculate that FTM may be a polymerized form of actin, α-actinin, or both. The demonstrated association of actinlike molecules with the cytoplasmic surfaces of various plasma membranes (48) and the poor preservation of FTM by OsO_4 fixation (cf. reference 48) lend credence to this speculation, but it is otherwise without experimental support.

Finally, of course, FTM may represent a substance that has yet to be identified and/or described in other organelles or organisms. Experiments to isolate and characterize the material from activated *imp-1* flagella are planned in this laboratory.

Stimulation of FTA

The experiments reported in this paper establish that the adhesive cross-linking of flagellar surface components is sufficient to cause the FTA response. Because detergent-extracted agglutinins, antiflagellar antiserum, Con A, and isolated flagella can all elicit FTA in gametes of a single *mt*, the response is clearly not mediated by possibly hormonelike factors produced by opposite-type cells during the mating reaction. Moreover, because a monolayer of agglutinin on an EM grid will induce activation of an adherent monolayer of cells, it is clearly not necessary that pairs of cells interact with one another. Finally, that cells tethered to polylysine films do not activate either their flagellar tips or their mating structures demonstrates that the response is not due to tip immobilization per se.

Con A and the antiflagellar antisera are capable of agglutinating flagella of either *mt* (5, 20, 35, 71). Such type nonspecificity cannot, however, be used to argue that the FTA response is similarly nonspecific. Thus the fluoresceinated lectin interacts with a number of flagellar membrane polypeptides in SDS gels (W. S. Adair, unpublished observations), any of which may prove with further analysis to be *mt*-specific. Similarly, the antisera have been shown (3) to carry antibodies directed against at least 20 flagellar polypeptides, and both specific and nonspecific adhesions may be generated. The *imp-5* strain, whose flagellar tips are activated by antiserum, cannot carry out sexual agglutination, but it is not yet known whether the *imp-5* mutation affects the sexual adhesins directly or the (membrane) component(s) required for them to function. Therefore, until the sexual adhesin or adhesins are identified biochemically and agents are found that cause isoagglutination without associating with these adhesins, it will not be possible to ascertain whether FTA requires that the sexual adhesins per se be agglutinated (cf. references 31, 54) or whether the agglutination of other surface components will also generate the response.

Reversal and Reinitiation of FTA

The experiments with isolated flagella (Fig. 21) demonstrate two additional properties of FTA. First, it is clear that reversal of FTA occurs in response to flagellar disadhesion and not in response to cell fusion or to possibly hormonelike factors produced by opposite-type gametes. Second, it is seen that the response can cycle: activated tips can develop, reverse, and redevelop in the same flagellum. This observation rules out models in which a gametic flagellum is "primed" for the reaction and undergoes a one-shot activation in response to adhesion. Instead, the agents responsible for tip elongation and FTM accumulation must be thought of as being repeatedly responsive to the agglutinated status of the organelle.

Agents That Fail to Block FTA

The FTA response occurs in cells that have been treated with agents affecting subsequent steps in the mating reaction. Specifically, chymotrypsin digestion, which markedly alters the electrophoretic mobility of several flagellar membrane glycopolypeptides (B. Monk, unpublished observations) and which inhibits transmission of mating signals and/or the MSA response, has no effect on the FTA response. The cytochalasins B and D, at concentrations in great excess of those used to perturb such parameters as locomotion and sugar transport (4, 62), also inhibit cell fusion without blocking FTA. And finally, the presence of Con A markedly suppresses zygote formation but, again, does not perturb FTA. The FTA response is thus

not generally susceptible to agents that interfere with the mating reaction. This point becomes important in considering the FTA block exerted by antitubulin agents: one cannot dismiss this block as a nonspecific perturbation of a biological process readily disrupted by external agents.

Blocking FTA with Antitubulin Agents

Experiments with the antitubulin agents reveal that it is possible to block the development of tipping and FTA without disrupting sexual adhesions. The concentrations of colchicine and vinblastine used are far higher than those used in studies of mammalian cells (72), raising the question of the tubulin specificity of these effects. Because most protozoa and plant cells are highly resistant to both drugs and require high drug concentrations for such responses as mitotic inhibition, cell-shape change, and flagellar-regeneration inhibition (reviewed in reference 24), the concentrations employed here are of less concern than they would be in a mammalian study.

Two additional considerations support the notion that the drugs are interacting with tubulins. First, vinblastine and colchicine inhibit the mating reaction in an identical fashion, causing blocks in both the tipping response and in FTA; yet each drug interacts with a different domain of the tubulin protein (72). Therefore, if a nontubulin molecule or molecules are proposed as being the nonspecific targets of drug action, they must be postulated to be susceptible to both colchicine and vinblastine (but not to very high concentrations of cytochalasins). Second, we find that lumicolchicine fails to inhibit the mating reaction even after prolonged exposures. We recognize that these observations are consistent with, but do not prove, a tubulin involvement in the FTA response, and we are presently pursuing more direct approaches to this question.

FTM Accumulation and A-Tubule Elongation

Two basic alternatives can be entertained to explain the accumulation of FTM and the elongation of A microtubules during the FTA response. The first possibility is that FTM and microtubule precursors preexist in gametic flagellar tips and that these are induced to aggregate or polymerize by some adhesion-related stimulus. Thus the occurrence of adhesions might stimulate an efflux of Ca^{++} from the tip matrix, and the lower concentration of Ca^{++} might favor both aggregation and polymerization, with disadhesion stimulating a reversal of these steps (see, e.g., reference 10).

The alternative possibility is that FTM and microtubule precursors are brought to the flagellar tips as a consequence of the adhesion/tipping reaction. If, as is argued in reference 20, sexual agglutinins are distributed throughout the length of the unmated flagellar surface, if these agglutinins span the membrane or are associated with a transmembrane complex, and if this complex in turn associates with FTM and microtubule precursors, then the tipping of agglutinins would be accompanied by the tipping of these precursors. Once concentrated in the tip region, the FTM precursors would proceed to aggregate or polymerize in a fibrous mass, while the microtubule precursors would participate in A-tubule elongation.

We have not yet found a means to dissociate tipping from FTA experimentally, and therefore we do not know whether FTA induces tipping or tipping induces FTA. If we argue that tipping must precede FTA, as supposed in the second model above, then colchicine and vinblastine must somehow act to prevent the migration, the accumulation of adhesive sites in flagellar tips, or both. Candidate targets for these drugs are tubulin molcules, which several laboratories, including our own, suggest may associate with the cell surface (1, 7, 32, 61). If the putative membrane-tubulin monomers associate with agglutinins (as in the second model), if surface adhesions bring these monomers together into aggregates ("patches"), and if this patching is essential for the tipping reaction (as in the lymphocyte patching/capping sequence), then the antitubulin drugs may prevent tipping by preventing patch formation. The rapid onset and reversal of the colchicine block to mating (Tables III and IV) is consistent with a cell-surface target for the drug action, but more direct experiments are clearly needed.

Nature of the Signal

Because under all experiments conditions reported here, MSA and gametic cell fusion occur only under conditions in which FTA is allowed to occur, we propose that FTA is necessary to signal MSA. The signal is presumably transmitted via the axoneme, the flagellar membrane, the flagellar matrix, or a combination of the three. Because paralyzed mutants of *Chlamydomonas* lacking

central-pair microtubules (66) or radial spokes (46, 74) can mate normally, we conclude that neither swimming motility nor an intact axoneme is necessary for signal transmission. On the other hand, because short flagella are sexually agglutinable but reportedly fail to signal (36, 37, 59), it is possible that a critical flagellar length is important to the signaling mechanism.

Two fundamental problems have been left unsolved. We need to determine first whether it is FTM accumulation, axoneme elongation, or both that contribute to "the signal," and second, how these tip events are perceived as signals-to-activate by the cell body. We can postulate, as a working model, that the pivotal event may be the simultaneous elongation of all nine A tubules in association with the FTM, making them unable to slide past one another (cf. reference 65). The flagellum would continue its ATP-driven dynein-bridge formation but to no avail, the result being possibly (ion-mediated) changes at the flagellar bases (29, 41, 42), possibly a deformation of cellular microtubules or fibers (23, 40, 64), and the activation of mating structures. The model predicts that pressure applied to the flagellar tips of one *mt* might lead to MSA in the absence of any adhesion, a prediction that can be tested. It is interesting to speculate that in certain ciliary-based mechanoreceptors where the ciliary tip is closely associated with a pressure-transducing structure (reviewed in 69), analogous primary events may be occurring.

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