GAMETIC DIFFERENTIATION IN *CHLAMYDOMONAS REINHARDTII*

III. Cell Wall Lysis and Microfilament-Associated Mating Structure Activation in Wild-Type and Mutant Strains

URSULA W. GOODENOUGH and RICHARD L. WEISS

From The Biological Laboratories, Harvard University, Cambridge, Massachusetts 02138

ABSTRACT

Cell fusion between mating type *plus* (mt^+) and *minus* (mt^-) gametes of *Chlamydomonas reinhardtii* is analyzed structurally and subjected to experimental manipulation. Cell wall lysis, a necessary prelude to fusion, is shown to require flagellar agglutination between competent gametes; glutaraldehyde-fixed gametes ("corpses") of one mating type will elicit both agglutination and cell wall lysis in the opposite mating type, whereas nonagglutinating *impotent* (*imp*) mutant strains are without effect. The fusion process is mediated by a narrow fertilization tubule which extends from the mt^+ gamete and establishes contact with the mt^- gamete. Formation of the tubule requires the "activation" of a specialized mating structure associated with the mt^+ cell membrane; activation causes microfilaments to polymerize from the mating structure into the growing fertilization tubule. Mating structure activation is shown to depend on gametic flagellar agglutination; isoagglutination mediated by the lectin concanavalin A has no effect. Gametes carrying the *imp-1* mt^+ mutation are able to agglutinate but not fuse with mt^- cells; the *imp-1* gametes are shown to have structurally defective mating structures that do not generate microfilaments in response to gametic agglutination.

The mating reaction of the biflagellate alga *Chlamydomonas reinhardtii* involves an elaborate sequence of events that can be presumed to depend on precise levels of cellular communication. The reaction is initiated by an agglutination between the flagellar tips of mating type *plus* (mt^+) and *minus* (mt^-) gametes (18), as illustrated in Fig. 1, an interaction that appears to involve surface components of the flagellar membranes (1, 24). Four events follow in quick succession: (*a*) a lytic activity digests the cell walls so that gametes are converted to naked protoplasts (2); (*b*) a cytoplasmic protuberance [the *fertilization tubule* (4)] extends from the mt^+ cell surface (Fig. 1, long arrow), an event mediated by the specialized *mating structure* first described by Friedmann et al. (4); (*c*) the fertilization tubule makes contact with an mt^- mating structure to form a narrow *cytoplasmic bridge* (4); and (*d*) the bridge expands in diameter until the mating pair is transformed into a single quadriflagellate zygote, an event that can be termed *zygotic cell fusion* (see Fig. 1). Quadriflagellate zygotes typically appear within 5 min after mt^+ and mt^- gametes are mixed; all of these events must therefore occur in a rapid and highly coordinated fashion.

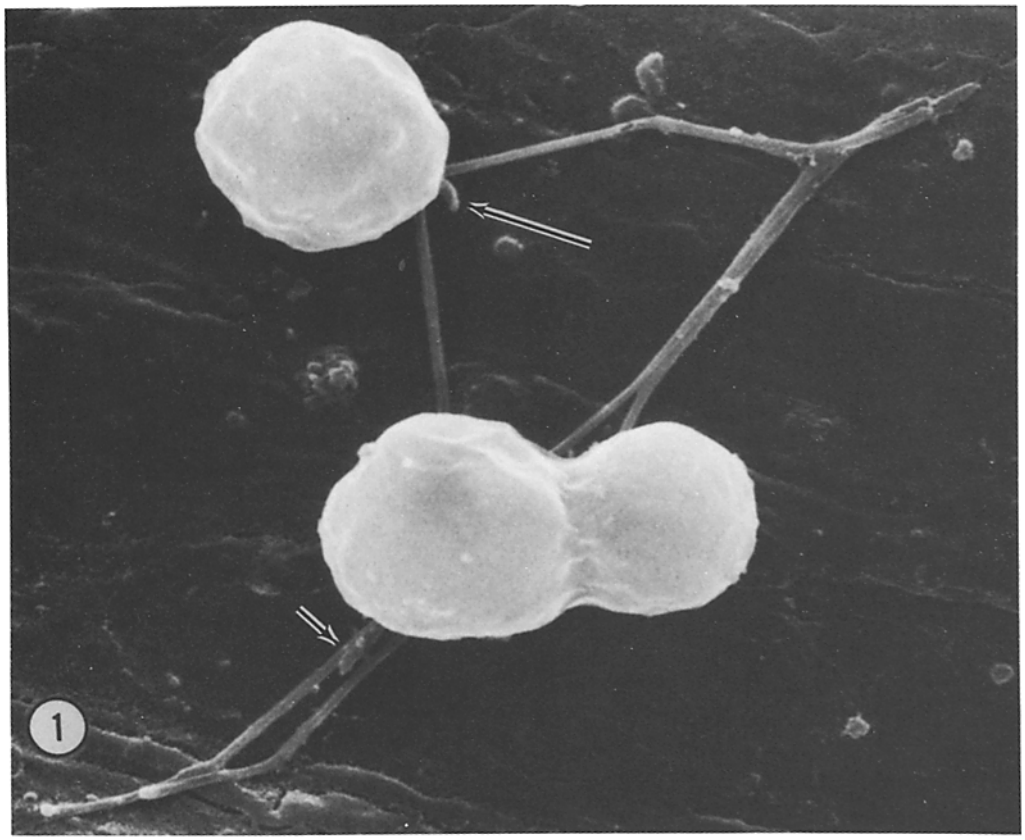


FIGURE 1 Mating in *C. reinhardtii* as visualized by scanning electron microscopy. A group of three cells is depicted: the single cell at the upper left is identified as an mt^+ gamete by the curved fertilization tubule (long arrow) that projects from its anterior; the two remaining cells are engaged in zygotic cell fusion. The flagellar tips that overlap at the upper right of the micrograph are in the true gametic-agglutinated configuration (see reference 1), with one of the flagella extending slightly beyond the other two. The flagellar tips at the lower left are no longer agglutinated; one of the zygotic flagella has slipped far down relative to the other, while the tip of the flagellum extending from the unmated mt^+ gamete (short arrow) has left the agglutinated region altogether. The images at the lower left presumably illustrate the loss of flagellar agglutinability that attends zygotic cell fusion (4, 24), a loss that allows unmated gametes to detach from fusing gametes and find free partners elsewhere. × 8700.

In this paper we report our observations on cell wall lysis and on the fine structural basis for the mating structure "activation" which results in cytoplasmic bridge formation and zygotic cell fusion. We also report experiments that focus on the relationship between the agglutination reaction and the subsequent events in the mating process. Using both mutant cells and experimentally treated cells that are either unable to agglutinate or unable to fuse, we have been able to establish that the release of the cell wall lytic activity and the activation of the mt^+ mating structure to form a fertilization tubule are independent events, both of which are elicited only by the mating type-specific adherence between flagellar membranes.

METHODS

Strains and Culture Conditions

Strain 137c, mt^+ and mt^-, of *C. reinhardtii* was used as wild type. The *impotent* (*imp*) mutant strains were derived from 137c mt^+ by UV-irradiation and were recovered by a screening procedure designed to detect nonmating cells (5, 6). The *imp-1* strain agglutinates normally but fuses only very rarely; the strains *imp-2*, *imp-5*, *imp-6*, *imp-7*, and *imp-8* are nonagglutinating and

can only be induced to fuse by centrifugation (5, 6). All *imp* strains have normal growth properties and motility.

Growth and gametogenesis conditions were as previously described (10). Both plate gametes and synchronous-light gametes (10) were investigated.

Electron Microscopy

Gametes to be examined by transmission electron microscopy were fixed either by the glutaraldehyde-osmium procedure described in reference 10 or by a more recently devised method which gives a superior fixation of the cytoplasm: cells are centrifuged at 3,700 *g*, and the pellet is suspended in 3% glutaraldehyde in 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid (HEPES), pH 7, at 23°C; the suspension is chilled to 0–4°C and allowed to fix overnight or up to 3 days; the cells are then pelleted at 3,700 *g*, resuspended in 1% OsO_4 in 4 mM potassium phosphate buffer, pH 7, and allowed to fix for at least 2 h or up to 48 h at 4°C; subsequent dehydration and embedding are as previously described (10). Negative staining was performed as described in reference 1. Microscopy was performed with a Hitachi HU-11C or with a Philips 300.

Gametes examined by scanning electron microscopy were fixed in a dilute glutaraldehyde solution which preserves cells in their agglutinated configurations. mt^+ and mt^- gametes in nitrogen-free high salt minimal medium (N-free HSM) (20) were mixed for 30 s at room temperature and then chilled on ice until the measured temperature reached 4°C; cold 3% glutaraldehyde in 10 mM HEPES, pH 7, was then added to a final concentration of 0.03% glutaraldehyde; cold 1% OsO_4 in 4 mM potassium phosphate buffer, pH 7, was immediately added to a final concentration of 0.5% OsO_4; fixed groups of mating cells were allowed to settle on Whatman no. 50 filter paper; cells on the filter paper were then dehydrated in ethanol and acetone, critical point dried, coated with platinum-palladium, stabilized with carbon, and observed in an AMR-1000A scanning electron microscope operated at 20 kV with a specimen angle of 2°.

Sorbitol Test for Wall-Less Cells

A known number of cells (determined with a hemacytometer) was placed in a test tube in 0.4 ml of culture medium. When information on the proportion of wall-less cells was desired, 0.4 ml of 1.5 M sorbitol was added, and the mixture was allowed to stand at room temperature for 5 min. 5 drops of a 4% glutaraldehyde solution were then added, and the proportion of swollen or broken cells (wall-less) to intact or slightly crenated (walled) cells was assessed with a hemacytometer. A total cell count was also made, and any reduction from the initial cell number was calculated. In the case of nonmating cells, such a reduction can be attributed to the fact that some wall-less cells swell and lyse into small fragments that are not scorable with the hemacytometer, and the proportion of wall-less cells is corrected accordingly.

Trypsinization of Gametes

A 1% stock solution of trypsin (Worthington Biochemical Corp., Freehold, N. J.) in 0.001 N HCl was added to a suspension of mt^+ or mt^- gametes in N-free HSM to a final concentration of 0.1% trypsin. An equal volume of 0.001 N HCl was added to controls. After 10 min, a 1% stock solution of soybean trypsin inhibitor (Sigma Chemical Co., St. Louis, Mo.) in 10 mM Tris, pH 7, was added to both experimental and control cells to a final concentration of 0.1%. Recovery from trypsinization, i.e. the re-acquisition of agglutinability, begins to occur within 20 min (see also reference 11); the trypsinized gametes were therefore used immediately.

Isoagglutination with Concanavalin A

A 0.5% stock solution of concanavalin A (Con A) (Sigma Chemical Co.) in N-free HSM was added to a suspension of mt^+ or mt^- gametes in N-free HSM to a final concn of 0.05% Con A, and isoagglutination was monitored by light microscopy. Prolonged exposure (>30 min) to the lectin was avoided since cells tend to become moribund and flagella detach from cells. All Con A effects were specifically inhibitable by the addition of α-methyl-mannoside.

Preparation of "Corpses"

Glutaraldehyde-fixed mt^+ or mt^- gametes (corpses) that retain their flagellar agglutinability were prepared by washing gametes once in fresh N-free HSM, adding an equal vol of cold 4% glutaraldehyde in 4 mM potassium phosphate buffer, pH 7, chilling the suspension, fixing on ice for 15–20 min, and washing carefully in buffer or medium, using gentle centrifugations (500 *g*), until all free glutaraldehyde is removed. Identical results are obtained if fixation is performed at room temperature or for at least 72 h. The corpse nature of such cells was demonstrated in two ways: (*a*) the fixed cells could not be broken when subjected to high pressures in a French pressure cell, indicating that they were extensively cross-linked, and (*b*) they exhibited no growth when plated on agar-containing growth medium.

RESULTS

In the sections that follow we first describe features of normal mating structure activation and cell wall lysis that have not previously been reported. We then describe how each event is expressed in mutant and in experimentally treated cells.

Morphology of Unactivated Mating Structures

Friedmann et al. (4) give an excellent description of many aspects of cell fusion in *C. reinhardtii*, but

certain fine structural features of the mating organelles are not reported.

Fig. 2 shows a medial section of the mating structure of an unmated mt^+ gamete. Two parallel zones of dense material are seen, a thin *membrane zone* (*mz*) immediately adjacent to the cell membrane, and a thicker *doublet zone* (*dz*) in the underlying cytoplasm, so-called because in favorable sections it appears as two narrow strips of material. The membrane-associated zone always appears in section as a continuous line; this zone is therefore visualized as an uninterrupted sheet of material, slightly curved, which adheres to the inner surface of the cell membrane. The doublet zone appears in section either as an uninterrupted region (Fig. 2) or as two sectors; serial sections reveal the zone to be a curved double layer of material with a hole to one side of its center, somewhat resembling a lopsided flattened collar. It is this zone that Friedmann et al. diagram in their paper (Fig. 4, reference 4) and refer to as a "choanoid body."

Fig. 3 shows the mating structure of an unmated mt^- gamete. It also occupies an anterior, and often protuberant, region of the cell to one side of the basal apparatus, and it possesses a curved, uninterrupted membrane zone. Several differences between this and the mt^+ structure are, however, present. First, the mt^- structure is considerably smaller, as determined by serial sections. Second, the membrane zone tends to be somewhat broader and more diffuse than the mt^+ membrane zone, particularly at its periphery. Finally, the mt^- mating structure has no doublet zone. The doublet zone, therefore, is restricted to mt^+ gametes, as indicated also by Friedmann et al. (4).

Morphology of mt^+ Mating Structure Activation

The activation of the mt^+ mating structure following sexual agglutination involves a transformation of the membrane zone and an extension of microfilaments from the doublet zone. The earliest stage in the activation process is shown in Fig. 4 (see also Fig. 7 of reference 4), where the cell membrane overlying the doublet zone is seen to have everted to form a small bud. The membrane zone can be identified at the base of the bud (Fig. 4, *mz*); it then becomes continuous with a broad band of dense, granular material adherent to the inner surface of the bud membrane [Fig. 4, "bud zone" (*bz*)]. Whether membrane zone components contribute to this bud zone or whether it consists solely of new material has not been determined, but it is clear that new, membrane-associated material appears during bud formation. The interior of the bud is seen to contain an electron-translucent material that is faintly reticulate (Fig. 4).

The next stage in the activation process is the elongation of the bud. Several features of this process are illustrated in Fig. 5. First, the membrane zone is seen to have opened up medially and to have lost its continuity with the bud zone (Fig. 5, arrow). Second, the membrane and doublet zones are no longer clearly separated by the electron-translucent region found in the unactivated mating structure (Fig. 2); instead, they appear to make contact, the zone of contact occasionally exhibiting a periodic structure (Figure 5, *p*). Finally, reticulate material continues to be present in the bud interior.

The elongated bud is next converted into a fertilization tubule which extends some 0.7 μm from the cell surface (Fig. 1). The fertilization tubule is curved (Fig. 6; see also Fig. 1) and is therefore usually encountered in section either at its tip (Fig. 7) or at its base (Fig. 8). The tip of the tubule retains the granular bud-zone material (Fig. 7), beneath which considerable additional membrane has been added, separating the tip from the base of the tubule. The open membrane zone and the doublet zone remain at the base of the tubule (Fig. 8), but they are now intermeshed to the point where it is difficult to resolve them as distinct zones (cf. Figs. 2 and 8). This "combined" zone appears to make direct contact with the cell membrane at the base of the tubule. Most dramatically, the combined zone is seen to give rise to parallel arrays of filaments (Figures 6–8), identical in structure to microfilaments (23), which extend into the fertilization tubule. In subsequent sections of this paper, the combined zone of dense material that attaches to the membrane and associates with microfilaments will be referred to as the *activated doublet zone*; this zone presumably contains membrane-zone material as well.

Cytoplasmic Bridge Formation

A mating type-specific membrane fusion next occurs between the tip of the mt^+ fertilization tubule and the mt^- mating structure region (4). Little is known about how this occurs, but we should point out two components that may partici-

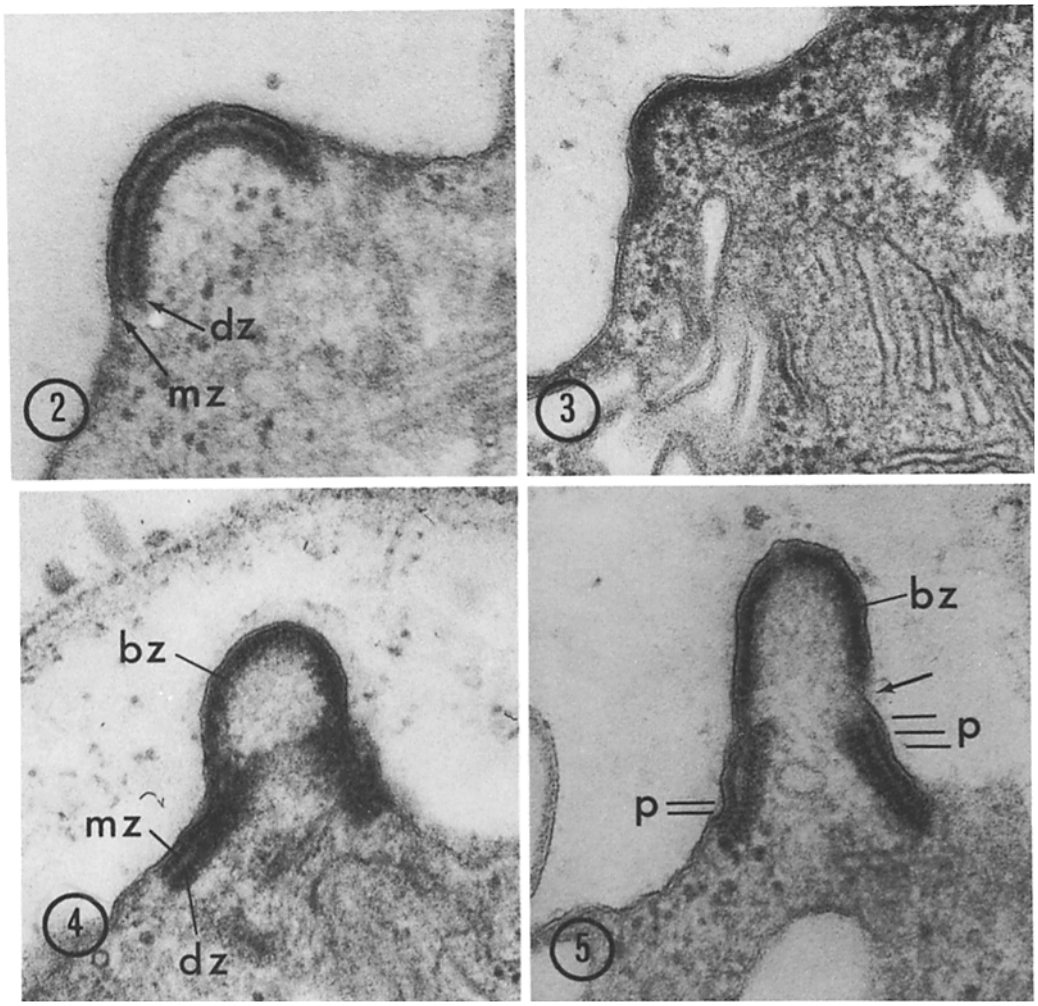


FIGURE 2 Mating structure from an unmated mt^+ gamete. *mz*, membrane zone; *dz*, doublet zone. × 100,000.

FIGURE 3 Mating structure from an unmated mt^- gamete showing a broad membrane zone. × 100,000.

FIGURE 4 Early bud stage in mt^+ mating structure activation. The bud interior is clear and contains some reticulate material. A zone of dense, aggregated material adheres to the inner surface of the bud membrane (*bz*); an amorphous material extends from the membrane's outer surface. An intact cell wall overlies the bud. *mz*, membrane zone; *dz*, doublet zone. × 100,000.

FIGURE 5 Late bud stage in mt^+ mating structure activation. The bud has lengthened, and the bud interior is somewhat denser and more fibrous than before (compare with Fig. 4). Amorphous material continues to associate with the external surface of the bud membrane and bud-zone material (*bz*) with its internal surface. Arrow points to a discontinuity between the membrane zone and the bud zone; the membrane zone has also opened up medially (compare with Fig. 2). Some periodically distributed material (*p*) extends from the doublet to the membrane zones. × 100,000.

pate in recognition and/or fusion at this stage: (1) the bud-zone material that associates with the inner surface of the fertilization-tubule tip membrane (Fig. 7); and (2) a dense material, similar in morphology to surface carbohydrates, which projects from the outer surface of the tip membrane (Fig. 7) and is seen also to overlie the unactivated mt^+ mating structure (Fig. 2) and the bud (Figs. 4

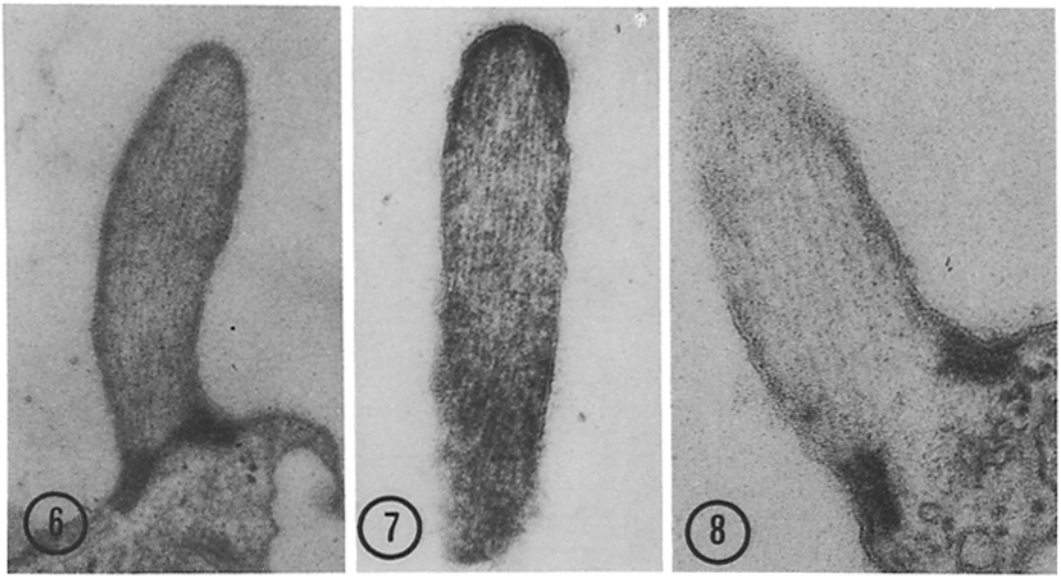


FIGURE 6 Fertilization tubule extending from a mated mt^+ gamete and caught in grazing section. Parallel arrays of microfilaments extend from the activated mating structure. The tip of the tubule curves out of section. The cell wall (not shown) appears fragmentary at this stage. × 70,000.

FIGURE 7 Tip of a fertilization tubule from an mt^+ gamete that has lost its wall. Bud-zone material adheres to the inner surface of the tip membrane (compare with Figs. 4 and 5, bz) and amorphous material is present on the external surface (compare with Figs. 4 and 5). Microfilaments fill the tubule interior. × 100,000.

FIGURE 8 Base of a fertilization tubule from an mt^+ gamete. The membrane zone is clearly discontinuous medially (i.e., it does not cross the lumen of the fertilization tubule); it also appears intermeshed with the doublet zone so that the two cannot be resolved as distinct regions. Microfilaments extend from this activated doublet zone. × 100,000.

and 5). Further information on the structural basis for the fusion reaction will be reported elsewhere.[1]

Once membrane fusion has occurred and a cytoplasmic bridge is established (Fig. 9), the activated doublet zone surface is no longer adherent and parallel to the membrane as it was at the base of the fertilization tubule (Figs. 6 and 8). Instead, the doublet zone remains attached to the bridge membrane only at its periphery (Figs. 10 and 11, *a* and *p*); the rest of the structure lies free in the bridge interior. The dense array of microfilaments attached to the convex surface of the activated doublet zone extends through the bridge and into the mt^- cell cytoplasm (Figs. 9 and 11); the orderly disposition of the filaments and their termination into the activated doublet zone are particularly apparent at this stage. A sparser population of filaments also extends backwards through a neck of cytoplasm that appears to pull out from the mt^+ gamete (Fig. 11, *n*; see also Fig. 9). Such images are consistent with the concept that tensile forces are generated in the bridge (see Discussion).

Morphology of mt^- Mating Structure Activation

The cytoplasmic bridge shown in Fig. 11 exhibits some dense material (arrows) at its junction with the mt^- cell, but it is difficult to determine whether this represents bud-zone material (Fig. 7) or an mt^- mating structure. Activated mt^- mating structures are therefore most readily identified when mt^- cells are mixed with mutant *imp-1* mt^+ cells that can agglutinate but not fuse (see below). In such matings, the membrane zone of the mt^- mating structure is found to have separated, and a

[1] Weiss, R. L., Goodenough, D. A., and U. W. Goodenough. Manuscript in preparation.

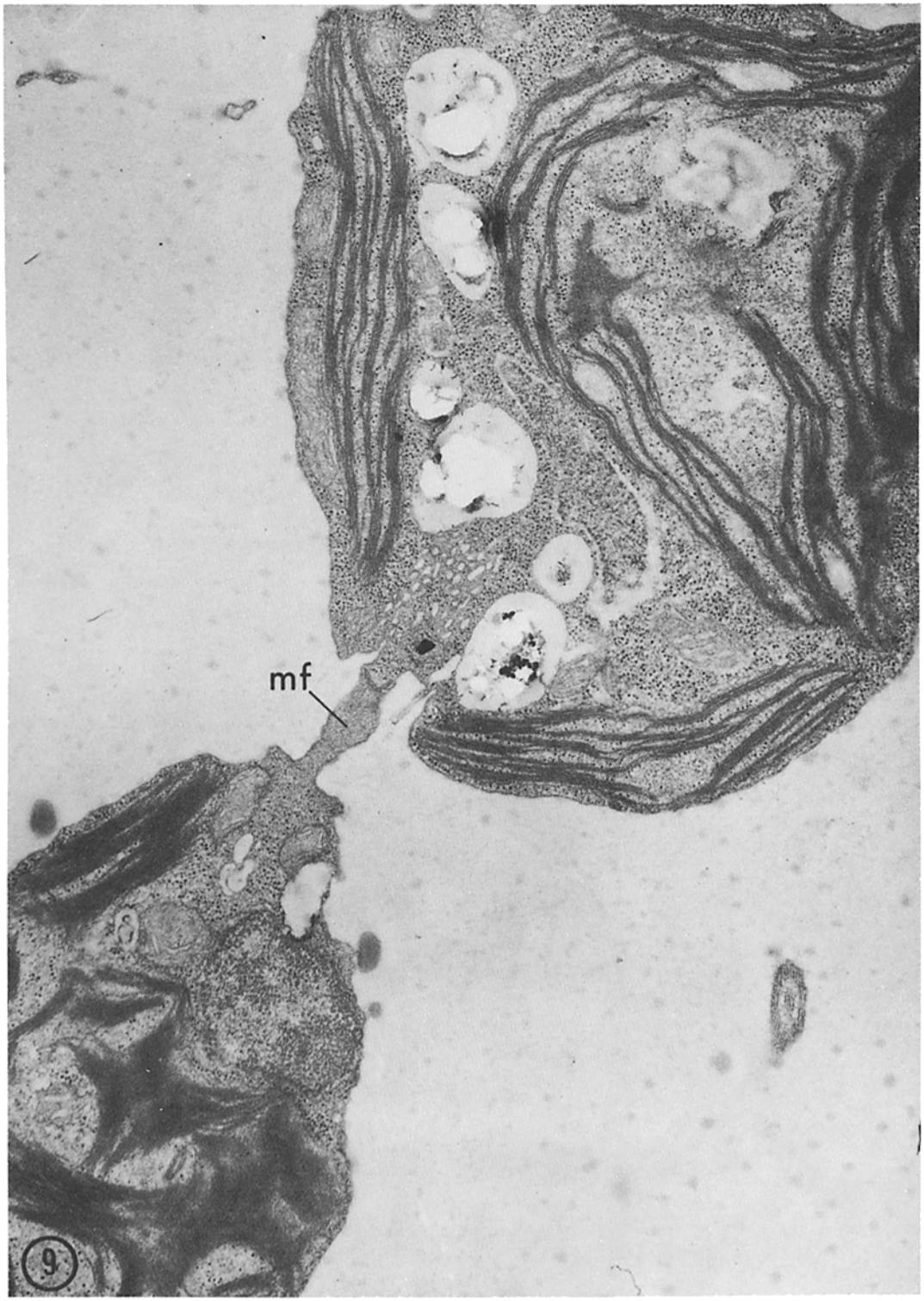


FIGURE 9 Mating gametes fixed 3 min after mixing, showing the disposition of the cytoplasmic bridge. Microfilaments (*mf*) are only faintly discernible at this magnification, but they can be seen to extend from the convex surface of the activated doublet zone which, in turn, spans a neck of cytoplasm extending from the mt^+ gamete (upper cell). × 20,000.

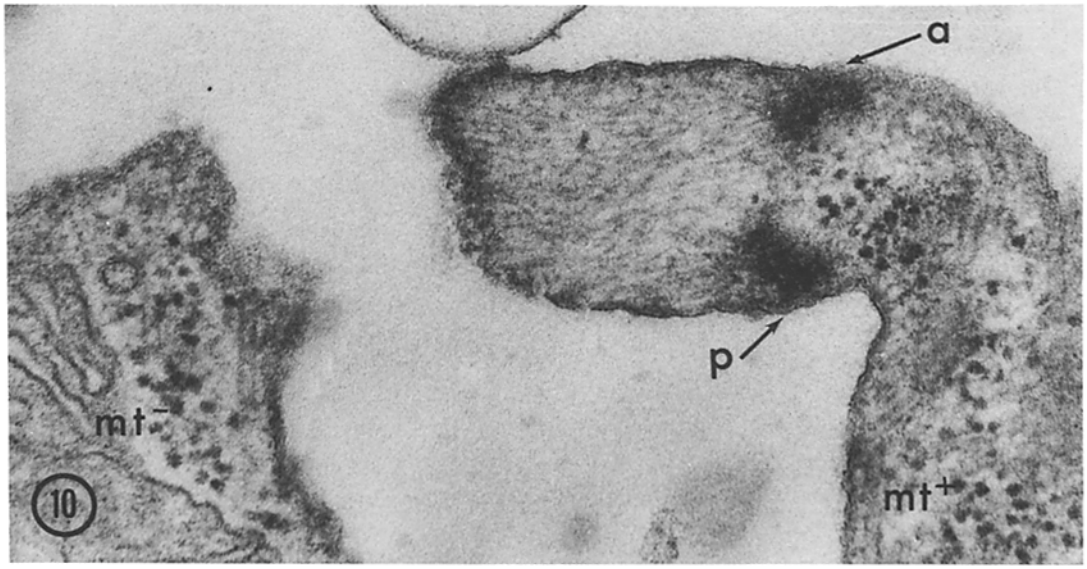


FIGURE 10 Cytoplasmic bridge between mt^+ and mt^- gametes; the bridge curves out of the plane of section. The two sectors of the activated mt^+ doublet zone attach to anterior (*a*) and posterior (*p*) aspects of the bridge membrane. Microfilaments extend into the bridge. × 100,000.

protuberant region of cytoplasm extends between the membrane-zone elements (Fig. 12). This protuberance bears little resemblance to the bud (Fig. 4) or the fertilization tubule (Figs. 6–8) of activated mt^+ gametes; it is irregular in outline and contains neither electron-translucent material nor microfilaments. Therefore, the mt^- contribution to the cytoplasmic bridge, if any, is believed to be minor compared to the mt^+ contribution.

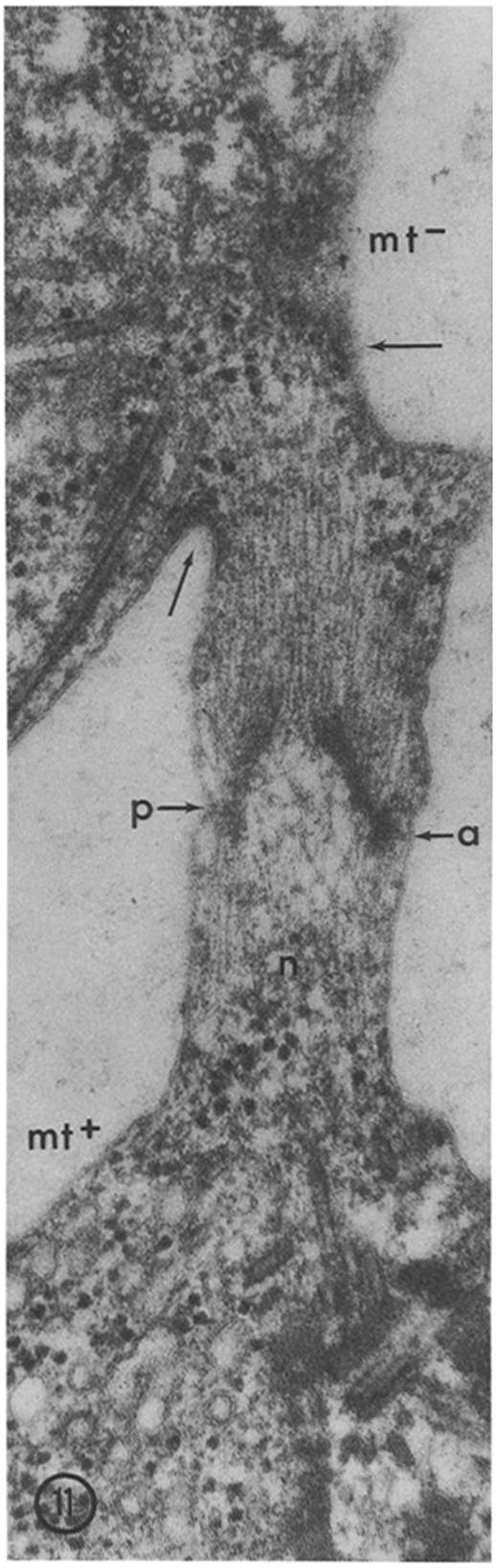
Zygotic Cell Fusion

A complete description of zygotic cell fusion, the conversion of two bridge-connected gametes to a single quadriflagellated zygote (see Fig. 1), is beyond the scope of this paper, but several observations on the fate of the mt^+ mating structure are relevant. As noted earlier, the activated doublet zone attaches to the bridge membrane at both anterior and posterior positions (Fig. 10, *a* and *p*). Zygotic cell fusion appears to initiate when the posterior attachment is broken. Once this occurs, a marked difference in the distribution of rigid structural components distinguishes the anterior from the posterior aspects of the conjoined cells: the anterior end contains two pairs of basal bodies that attach to the membrane (16), plus their associated bands of cortical microtubules (16), plus the membrane-attached activated doublet zone and its microfilaments, whereas the posterior is devoid of such rigid organelles and filamentous structures. As a result, cytoplasm flows preferentially through the posterior aspect of the opened bridge, causing the bridge to widen basally until it becomes as wide as the cells (Fig. 1). At this point, a single zygote can be said to have formed.

In the young zygote, the activated doublet zone commonly appears to lie free in the anterior cytoplasm (Fig. 13) between the two sets of basal bodies; serial sections reveal, however, that one end of the zone remains attached to the zygote's anterior surface membrane. Microfilaments continue to associate with the convex surface of the zygotic zone (Fig. 13) but do not exhibit the taut, ordered appearance that they assumed in the bridge (Fig. 11). Vestiges of such doublet zones are found in zygotes 1 h after mating but are not encountered in older zygotes.

Features of Cell Wall Lysis

In these mating mixtures, bud formation and elongation (Figs. 4 and 5) can occur while the cell wall still surrounds an mt^+ gamete, but cell wall lysis precedes or accompanies the extension of a fertilization tubule. Walls that have been shed by mating gametes are found to have ruptured at their anterior end (Fig. 14); since the entire wall is susceptible to lysis (see below), the lytic activity would appear to be released locally at the anterior



end of the gamete. The two obvious sites for such release are the contractile vacuoles or the bases of the flagella, but no experimental information on this point has yet been obtained. We should note that the specialized flagellar collars associated with the cell wall (16) are not digested by the lytic activity and instead slip down and then off the flagella (19).

Shortly after gametic agglutination begins, shed walls are abundant in the medium when examined by phase microscopy. Intact walls become increasingly scarce, however, as the released lytic activity continues to digest the walls, and eventually no vestiges remain. This phenomenon, coupled with the occasional rupture of cell walls by coverslip pressure alone, makes a visual assay of cell wall lysis such as that described by Claes (2) difficult to quantitate. We therefore monitored cell wall lysis by the sorbitol test described in Methods, which takes advantage of the osmotic fragility of wall-less cells relative to walled cells. In some cases, cell wall loss was also assessed by electron microscopy.

Induction of Cell Wall Lysis

Table I summarizes experiments investigating the induction of cell wall lysis. As indicated in the first section of the table, the unmated gametes used in this study yield a low percentage of swollen cells following sorbitol treatment. We should note that occasional suspensions of unmated gametes, more commonly mt^+ than mt^-, exhibit rather high, e.g. 25%, levels of sorbitol-induced swelling; a similar proportion of cells appears wall-less by electron microscopy. This observation presumably relates to reports of "naked" unmated gametes from other laboratories (4). The conditions that determine whether unmated gametes retain or lose their walls remain obscure; meanwhile, only walled gametes were selected to investigate the induction of wall lysis during mating.

The second section of Table I reveals that wall lysis is very rapid in a wild type mating. It is also extensive in a cross involving the mutant strain *imp-1* which agglutinates normally with mt^- gametes (see below). It does not occur, however, in

FIGURE 11 Cytoplasmic bridge between mt^+ and mt^- gametes. The activated doublet zone with its array of microfilaments lies in a neck (*n*) of cytoplasm that extends from the mt^+ gamete. The zone is attached to the bridge membrane at anterior (*a*) and posterior (*p*) positions. Arrows point to dense material at the juncture of the bridge and the mt^- gamete; this may represent bud-zone material (see Fig. 7), an opened mt^- mating structure (see Fig. 3), or some combination of the two. × 100,000.

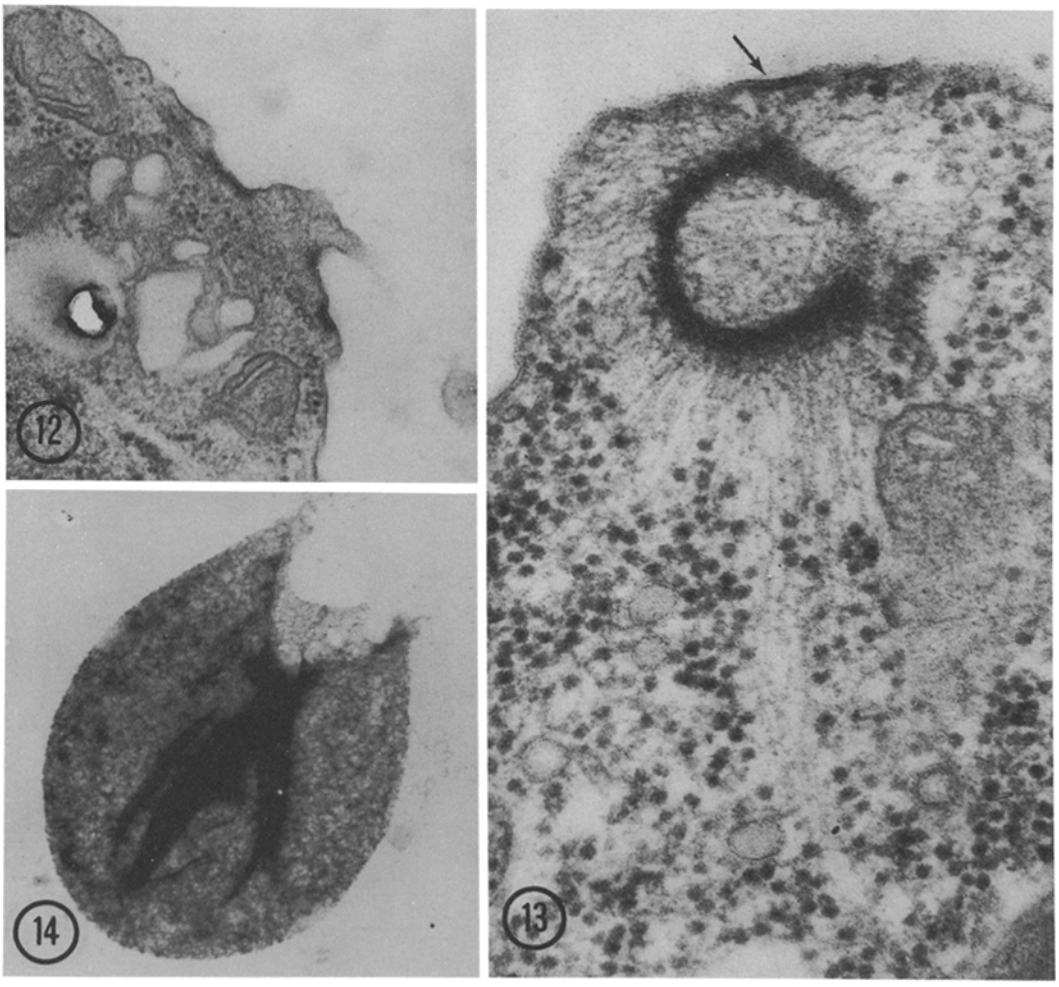


FIGURE 12 The mating structure of an mt^- gamete fixed after being mixed with *imp-1* gametes for 16 h. The membrane zone is open medially and a portion of the cytoplasm extrudes through the opening. The cytoplasm is differentiated from its surroundings in that it is free of ribosomes, but it lacks any evidence of microfilaments. × 52,000.

FIGURE 13 Activated mt^+ doublet zone in a young zygote. Serial sections reveal that the zone attaches to the anterior zygote membrane at the position indicated by an arrow. The microfilaments that radiate from the convex surface of the structure appear meandering rather than taut (compare with Fig. 11). × 100,000.

FIGURE 14 Negatively stained cell wall from which a mated gamete has emerged through the ruptured anterior end. × 10,000.

matings involving the nonagglutinating strains *imp-5*, *imp-7*, and *imp-8*, nor does it occur when wild-type cells are rendered nonagglutinable by trypsinization (26), as noted also by Claes (2).

When mt^+ gametes are fixed with glutaraldehyde, they remain normally agglutinable (see also reference 25) with mt^- cells; similarly, glutaraldehyde-fixed mt^- corpses retain normal agglutinability with mt^+ cells (no agglutination is detected, however, when mt^+ and mt^- corpses are mixed; see also reference 17). The data in Table I show that 50% of the cells in each "corpse mating" lose their walls; that these are the unfixed members of such matings has been ascertained by identifying the mating type of the wall-less cells by electron microscopy using the morphology of the mating structure as a marker. If it is assumed that the fixed cells are unable to release lytic activity, these

results also demonstrate that both mating types can be induced to liberate the cell wall lytic activity, thus ruling out the possibility that only one gamete type releases activity into the medium where it acts on the cell wall of the other type.

Finally, the effect of isoagglutination by Con A was investigated. It is known (11, 27) that Con A will cause unmated *mt*$^+$ *C. reinhardtii* gametes to adhere to one another by their flagellar tips in apparent mating configurations, although no cell fusion ensues; an identical effect occurs with Con A-treated *mt*$^-$ gametes, indicating that in *C. reinhardtii* the lectin does not interact with some flagellar surface component that is restricted to one mating type. Nonetheless, we investigated whether a flagellar-tip agglutination, albeit nonspecific, could elicit a cell wall lysis. As seen in Table I, no lytic activity is elicited during Con A isoagglutination of either mating type (an interpretation that assumes that con A has no inhibitory effect on wall lysis, an assumption that requires experimental demonstration).

Induction of Mating Structure Activation

The third column of Table I summarizes electron microscope investigations that examined whether mating structures were activated when various classes of gametes were mixed. It is apparent, first, that mixtures of gametes that produce no agglutination and no cell wall lysis (the non-agglutinating and the trypsinized gametes) also produce no activated mating structures. Similarly, Con A-isoagglutination of *mt*$^+$ cells has no effect on the mating structure. In contrast, the agglutination of *mt*$^+$ cells by *mt*$^-$ corpses results in normal bud formation although not, interestingly, in any apparent microfilament production. That this is not caused by wall removal per se is demonstrated by microscopy of the naked unmated *mt*$^+$ gametes that occasionally arise (see previous section); in none of these gametes is the mating structure found to be activated despite the lack of cell walls.

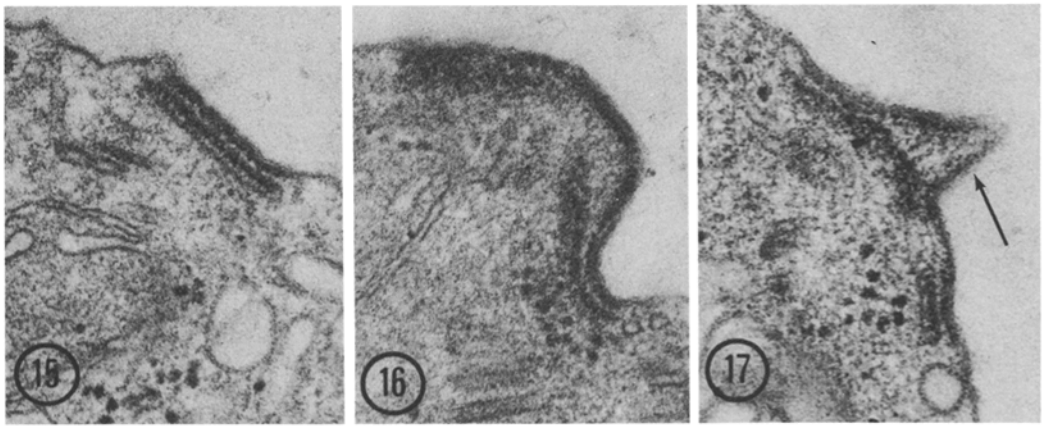
The imp-1 Mating Structure

A major discrepancy in Table I concerns the *imp-1* × *mt*$^-$ cross. Whereas wall removal is normal and the agglutination reaction is capable of transmitting the "activation signal" to the *mt*$^-$ gametes in the mixture (see Fig. 12), the *imp-1* mating structure remains unactivated even after 18 h of mating. Two possible explanations for such

TABLE I

Presence or Absence of Cell Wall Lysis and Mating Structure Activation in Various C. reinhardtii Cells and Cell Mixtures

	Time before sorbitol addition	Swollen (wall-less) cells	Mating structure activation
		%	
Single Strains			
mt$^+$ (wild type)	5 min	1	No
mt$^-$ (wild type)	5 min	2	No
imp-1	5 min	2	No
imp-5	5 min	4	No
imp-7	5 min	4	No
imp-8	5 min	4	No
Mixtures (1:1 proportions)			
mt$^+$ × *mt*$^-$	5 min	61	Yes
imp-1 × *mt*$^-$	30 min	71	No *imp-1*, Yes *mt*$^-$
imp-5 × *mt*$^-$	1.5 h	3	No
imp-7 × *mt*$^-$	1.5 h	6	No
imp-8 × *mt*$^-$	1.5 h	5	No
mt$^+$ (trypsin) × *mt*$^-$ (trypsin)	15 min	5	No
mt$^+$ × *mt*$^-$ corpse	30 min	49	Partial
mt$^+$ corpse × *mt*$^-$	30 min	51	Not determined
Con A			
mt$^+$ + Con A	15 min	1	No
mt$^-$ + Con A	15 min	1	Not determined



FIGURES 15–17 Three representative images of the *imp-1* mt^+ mating structure (see text for details). Arrow in Fig. 17 indicates a discontinuity in the membrane zone. × 100,000.

refractory behavior present themselves: either the signal is not transmitted normally to the *imp-1* cells, or the *imp-1* mating structure is defective in its ability to respond to the signal.

Support for the latter hypothesis has come from a fine structural analysis of *imp-1* gametes: the morphology of their mating structures is found to be distinctly abnormal. First, the *imp-1* mating structure tends to be considerably smaller than the normal mt^+ mating structure. Second, it appears to be structurally unstable, in that it exhibits a variety of aberrant structural configurations, in contrast to the highly invariant phenotype of the wild-type organelle. Several representative images are shown in Figs. 15–17. In Fig. 15, the density of the lower doublet zone band is seen to be lighter staining than the upper band, and the structure is flat rather than protuberant (compare with Fig. 2). In Figs. 16 and 17, the two sectors of the doublet zone in the region of the "collar opening" are of unequal length and dissimilar orientation, as contrasted to the very symmetrical aspect of the normal mt^+ mating structure in this region. Finally, the membrane zone is seen in Fig. 17 to have everted and to exhibit a discontinuity (arrow); in other *imp-1* mating structures the membrane zone appears to collapse over the "collar opening." Similarly aberrant *imp-1* mating structures are encountered following two quite different fixation regimes (see Methods), and they are found in both plate and synchronous-light gametes (10). No such images are obtained either from wild-type cells or from the mt^+ nonagglutinating mutant strains.

DISCUSSION

Properties of Gametic Mating Structures

The act of cell fusion in *C. reinhardtii* is shown here to involve specific morphological changes in the two gametic mating structures. Following true gametic agglutination at the flagellar tips, the continuous membrane zones of both mating structures develop medial discontinuities. In mt^- cells, this discontinuity permits a small outpocketing of membrane-covered cytoplasm (Fig. 12). In mt^+ cells, the formation of the discontinuity is coupled with an apparent intermeshing between the membrane zone and the underlying doublet zone; microfilaments then polymerize from the combined membrane-doublet zone, and a long slender protrusion, the fertilization tubule, extends out from the cell anterior (Fig. 6). This tubule soon establishes contact with the mt^- outpocketing and a cytoplasmic bridge forms, by some unknown mechanism, to connect the mating cells (Fig. 9).

That the doublet zone is responsible for fertilization-tubule formation is indicated here in two ways. First, we show that mt^- gametes lack both a doublet zone and the ability to form a fertilization tubule during mating. Second, we show that the *imp-1* mutation produces both structural defects in the mt^+ mating organelle (Figs. 15–17) and a functional inability to form a fertilization tubule despite an apparently normal agglutination with mt^- gametes. As reported elsewhere (5, 6), the *imp-1* mutation is closely linked to, and may reside within, the mt^+ locus, suggesting that *imp-1* marks a gene involved with the mt^+-specific

production of a doublet zone. We should point out that there is no obvious structural feature of the aberrant *imp-1* mating organelle that suggests why its function is impaired. It seems most likely to us that the *imp-1* mutation affects some process in the assembly of a doublet zone such that both its structural integrity and its ability to generate microfilaments during the mating process are defective.

Certain resemblances exist between the desmosome, which establishes an intercellular attachment between animal cells (3, 12), and the activated mt^+ mating structure of *C. reinhardtii*: in addition to functional analogies, both appear in the electron microscope as small dense regions that associate with membranes and with filaments. The desmosome and its tonofilaments are stable rather than transient structures, in contrast to the mating organelle, and the functions each organelle serves share only superficial features. Nonetheless, it is interesting to speculate that primitive mating structures were ancestors to modern junctional complexes and, thereby, to the Z lines of striated muscle which derive embryologically from desmosomes (7).

Microfilament Involvement in the Mating Region

The rapid polymerization of microfilaments from the activated mt^+ doublet zone into the fertilization tubule presumably mediates the outgrowth of the tubule; moreover, the microfilaments presumably lend structural support to the narrow cytoplasmic bridge that connects the two relatively large and intensely gyrating cells (Fig. 9). Whether the microfilaments act other than as structural supports is not established, but several observations suggest that the filaments may exert a tensile force in the cytoplasmic bridge. The bridge filaments insert into the activated doublet zone which, in turn, is anchored into the bridge membrane. The filaments appear taut in the bridge (Fig. 11), whereas they seem to meander in the zygote (Fig. 13). Finally, bridge formation causes a neck of cytoplasm to pull out from the mt^+ gamete (Figs. 9 and 11), and there is some indication that the bridge may actually shorten and widen before zygotic cell fusion (4). An active, force-generating role for the microfilaments during mating, as during other cellular processes (reviewed in references 8 and 14), therefore seems plausible, although it has not yet been demonstrated experimentally.

Two possible relationships between the doublet zone and the microfilaments are suggested by our observations. (*a*) The doublet zone may act as a scaffolding onto which microfilaments polymerize. Specifically, the reticulate material that accumulates in the bud (Figs. 4 and 5) may represent microfilament precursor material, sent into the bud from the cytoplasm in response to flagellar agglutination, which then polymerizes in response to some template property of the activated doublet zone. (*b*) The unactivated doublet zone may itself contain microfilament precursors which do not polymerize until flagellar agglutination occurs. Supporting this concept is the fact that the unactivated doublet zone appears dense and compact (Fig. 2), whereas the activated zone appears diffuse (Figs. 10, 11, and 13). This structural transformation may, however, reflect other aspects of doublet-zone activation, e.g. its attachment to the membrane. The most direct way to distinguish between these two possibilities will be to isolate mt^+ mating structures and analyze their protein compositions electrophoretically before and after activation; such experiments are now in progress in our laboratory.

The polymerization of microfilaments during the mating reaction is reminiscent of acrosome elongation in certain echinoderm sperm as reported by Tilney et al. (22). It should be noted that the mitotic cleavage furrow of *C. reinhardtii* contains microtubules but apparently no microfilaments (9), in contrast to most furrowing cells where microfilaments abound (14, 21, 23). It would appear, therefore, that *C. reinhardtii* restricts its ability to generate microfilaments to a brief but critical stage in its life cycle, and this ability is apparently dependent on the presence of a normal doublet zone.

Communication between Flagellar Membranes and Cells

The observations reported here establish several additional features of the mating reaction. First, it is shown that gametic flagellar agglutination is both necessary and sufficient to trigger the subsequent events of cell fusion in wild type cells. Thus nonagglutinating *imp* mutant strains and trypsinized gametes are unable to elicit either cell wall lysis or mating structure activation when mixed with gametes of opposite mating type, indicating

that neither event is stimulated by some activity, e.g. the secretion of a hormone, that occurs independently of flagellar agglutination in gametic mixtures. Moreover, if it is assumed that the glutaraldehyde-fixed corpses are incapable of secretory or metabolic activity, then the fact that corpse agglutination can trigger both cell wall lysis and mating structure activation indicates that a gamete requires no soluble or metabolic products from its mating partners to prepare itself for cell fusion; the agglutination signal alone appears to be sufficient.

That a partial signal is communicated to a living gamete when it agglutinates with a glutaraldehyde-fixed corpse indicates that at least some of the components involved in agglutination are not inactivated by glutaraldehyde cross-linking. This result is consistent with the possibility that polysaccharide chains associated with the two prominent glycoproteins of the gametic flagellar surface (1) are involved in the agglutination reaction, since most carbohydrates lack the free amino groups that participate in glutaraldehyde cross-linking (15).

Whatever the nature of the agglutination reaction, our observations indicate that the interaction must be very precise if the subsequent mating responses in the cell body are to be elicited. Thus the Con A-induced isoagglutination between gametes of the same mating type entails an interaction between flagellar tips which presumably involves surface carbohydrates (13) and which cannot be distinguished morphologically from true mating (27), but we demonstrate that this interaction fails to induce either cell wall lysis or mating structure activation. Therefore, assuming that the lectin is not itself inhibiting any stages in the lytic and/or activation processes, these experiments indicate that the gamete is somehow able to discriminate between specific and nonspecific perturbations of its flagellar surface.

Once mating type-specific agglutination has occurred, a signal must be sent along some 11 μm of flagellum to reach its target site(s) in the cell body. While analogies to nerve conduction should be considered, nothing is yet known about how the signal is propagated. It is known, however, that an additional kind of cell-to-surface communication occurs once zygotic cell fusion has taken place: zygote flagella lose their agglutinability (see Fig. 1), beat freely, and fail to interact with the flagella of unmated gametes in the mating mixture (4, 24), despite the fact that they remain agglutinable by Con A (our unpublished observations). That this loss of mating type-specific agglutinability is dependent on the occurrence of cell fusion rather than, for example, on some "aging" process is demonstrated by the fact that agglutinating *imp-1* and *mt*$^-$ gametes, being unable to fuse, continue to agglutinate for at least 48 h. Therefore, just as events involving the gametic flagellar membrane lead to changes in the gametic cells, so does zygote formation elicit a major modification of the flagellar surface.

Carol Hwang and Joyce Zern provided invaluable assistance during the preparation of this and the preceding manuscripts in this series, and Kenneth Bergman helped with some of the experiments and many of the concepts presented.

Supported by National Institutes of Health grants GM-18824, GM-06637, and 5-T01-HD00415, and by a grant from the Maria Moors Cabot Foundation for Botanical Research, Harvard University. Aid was also provided by Sigma Xi, The Scientific Society of North America.

Received for publication 24 June 1975, and in revised form 4 August 1975.

REFERENCES

1. BERGMAN, K. D., U. W. GOODENOUGH, D. A. GOODENOUGH, J. JAWITZ, and H. MARTIN. 1975. Gametic differentiation in *Chlamydomonas reinhardtii*. II. Flagellar membranes and the agglutination reaction. *J. Cell Biol.* **67**:606–622.
2. CLAES, H. 1971. Autolyse der Zellwand bei den gameten von *Chlamydomonas reinhardii*. *Arch. Microbiol.* **78**:180–188.
3. FARQUHAR, M. G., and G. E. PALADE. 1963. Junctional complexes in various epithelia. *J. Cell Biol.* **17**:375–412.
4. FRIEDMANN, I., A. L. COLWIN, and L. H. COLWIN. 1968. Fine-structural aspects of fertilization in *Chlamydomonas reinhardi*. *J. Cell Sci.* **3**:115–128.
5. GOODENOUGH, U. W. 1974. Mutations affecting gametogenesis in *Chlamydomonas reinhardi*. *J. Cell Biol.* **63**(2, Pt. 2):116 *a*. (Abstr.).
6. GOODENOUGH, U. W., C. HWANG, and H. MARTIN. 1976. Isolation and genetic analysis of mutant strains of *Chlamydomonas reinhardi* defective in gametic differentiation. *Genetics*. In press.
7. HAGOPIAN, M., and D. SPIRO. 1970. Derivation of the Z-line in the embryonic chick heart. *J. Cell Biol.* **44**:683–687.
8. HUXLEY, H. E. 1973. Muscular contraction and cell motility. *Nature* (*Lond.*). **243**:445–449.
9. JOHNSON, U. G., and K. R. PORTER. 1968. Fine structure of cell division in *Chlamydomonas*

reinhardi: basal bodies and microtubules. *J. Cell Biol.* **38**:403–425.

10. MARTIN, N. M., and U. W., GOODENOUGH. 1975. Gametic differentiation in *Chlamydomonas reinhardtii*. I. Production of gametes and their fine structure. *J. Cell Biol.* **67**:587–605.
11. MCLEAN, R. J., and R. M. BROWN. 1974. Cell surface differentiation of *Chlamydomonas* during gametogenesis. I. Mating and concanavalin A agglutinability. *Dev. Biol.* **36**:279–285.
12. MCNUTT, N. S., and R. S. WEINSTEIN. 1973. Membrane ultrastructure and mammalian intercellular junctions. *Prog. Biophys. Mol. Biol.* **26**:45–101.
13. NICOLSON, G. L. 1974. The interaction of lectins with animal cell surfaces. *Int. Rev. Cytol.* **39**:89–190.
14. POLLARD, T. D., and R. R. WEIHING. 1974. Actin and myosin and cell movement. *C. R. C. Critical Rev. Biochem.* **2**:1–65.
15. RICHARDS, F. M., and J. R. Knowles. 1968. Glutaraldehyde as a protein cross-linking reagent. *J. Mol. Biol.* **37**:231–233.
16. RINGO, D. L. 1967. Flagellar motion and fine structure of the flagellar apparatus in *Chlamydomonas*. *J. Cell Biol.* **33**:543–571.
17. RUTISHAUSER, U., and L. SACHS. 1974. Receptor mobility and the mechanism of cell-cell binding induced by concanavalin A. *Proc. Natl. Acad. Sci. U. S. A.* **71**:2456–2460.
18. SAGER, R., and S. GRANICK. 1954. Nutritional control of sexuality in *Chlamydomonas reinhardi*. *J. Gen. Physiol.* **37**:729–742.
19. SNELL, W. J. 1975. Mating in *Chlamydomonas*. A system in the study of specific cell adhesion. Ph.D. Thesis, Yale University, New Haven, Conn.
20. SUEOKA, N., K. S. CHIANG, and J. R. KATES. 1967. Deoxyribonucleic acid replication in meiosis of *Chlamydomonas reinhardi*. I. Isotope transfer experiments with a strain producing eight zoospores. *J. Mol. Biol.* **25**:47–66.
21. SZOLLOSI, D. 1970. Cortical cytoplasmic filaments of cleaving eggs: a structural element corresponding to the contractile ring. *J. Cell Biol.* **44**:192–209.
22. TILNEY, L. G., S. HATANO, H. ISHIKAWA, and M. S. MOOSEKER. 1973. The polymerization of actin: its role in the generation of the acrosomal process of certain echinoderm sperm. *J. Cell Biol.* **59**:109–126.
23. WESSELS, N. K., B. S. SPOONER, J. F. ASH, M. O. BRADLEY, M. A. LUDUENA, E. L. TAYLOR, J. T. WRENN, and K. M. KAMADA. 1971. Microfilaments in cellular and developmental processes. *Science (Wash. D. C.).* **171**:135–143.
24. WIESE, L. 1969. Algae. *In* Fertilization: Comparative Morphology, Biochemistry, and Immunology. II. C. B. Metz and A. Monroy, editors. Academic Press Inc., New York. 135.
25. WIESE, L., and R. F. JONES. 1963. Studies on gamete copulation in heterothalic Chlamydomonads. *J. Cell. Comp. Physiol.* **61**:265–274.
26. WIESE, L., and C. B. METZ. 1969. On the trypsin sensitivity of gamete contact at fertilization as studied with living gametes in *Chlamydomonas*. *Biol. Bull. (Woods Hole).* **136**:483–493.
27. WIESE, L., and D. W. SHOEMAKER. 1970. On sexual agglutination and mating-type substances (gamones) in isogamous heterothallic Chlamydomonads. II. The effect of concanavalin A upon the mating-type reaction. *Biol. Bull. (Woods Hole).* **138**:88–95.