

## GAMETIC DIFFERENTIATION

### IN *CHLAMYDOMONAS REINHARDTII*

#### II. Flagellar Membranes and the Agglutination Reaction

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#### ABSTRACT

A structural and biochemical study is presented concerning the agglutination of gametic flagella, the initial step in the mating reaction of *Chlamydomonas reinhardtii*. An alteration in the distribution of the intramembranous particles revealed by freeze-fracturing of flagellar membranes is shown to accompany gametic differentiation in both mating types. The isolation and electrophoretic analysis of flagellar membranes and mastigonemes are reported; no electrophoretic differences can be detected when the membrane or mastigoneme glycoproteins from vegetative and gametic cells are compared, nor when glycoproteins from the two mating types are compared, and no novel polypeptides are present in gametic preparations. The membrane vesicles, after they are freed of mastigonemes by sedimentation through a discontinuous sucrose gradient, are extremely active as an isoagglutinin, indicating a direct involvement of the membrane in the mating reaction.

As cells differentiate, they are often conferred with unique surface properties that allow extremely selective cellular interactions: the tissue-specific adhesion of dissociated embryonic cells (1), the species-specific reaggregation of dissociated sponge cells (16), and the developmental cycle of agglutinability in *Dictyostelium* (3) are three prominent examples. Such selective interactions have also been observed in or inferred from studies of simple eukaryotic organisms that undergo mating reactions. Thus, the composition or architecture of the cell surface appears to confer sexual discrimination in *Hansenula wingii* (7), in *Paramecium* (29), and in many of the volvocid phytoflagellates which agglutinate through the contact of their flagellar surfaces (31).

In this paper we present the results of our

observations on the flagellar surface of the phytoflagellate *Chlamydomonas reinhardtii*, which develops the capacity for such specific agglutination reactions after a simple alteration of its growth medium. Specifically, when vegetative *C. reinhardtii* cells of opposite mating types ( $mt^+$  and  $mt^-$ ) are induced to differentiate into gametes by nitrogen starvation (19, 25) and are mixed together, they agglutinate by their flagellar tips as a prelude to the zygotic cell fusion that occurs several minutes later (described in reference 13). The flagella of vegetative (nongametic) cells exhibit no such agglutinability, nor do certain nonmating mutant strains that have been isolated in this laboratory. Therefore, it should be possible to identify the agglutinins active in this reaction and to study both their mode of action and the

regulation of their appearance on the flagellar surface during gametic differentiation.

Previous morphological studies have established that the surface of the vegetative *C. reinhardtii* flagellum consists of a membrane bearing a fuzzy coat (24) and hairlike mastigonemes (5, 24, 33). We demonstrate similar components on the surface of gametic flagella but report a difference in the distribution of intramembrane particles, as revealed by freeze-cleave electron microscopy. We also present evidence that the agglutination reaction involves an interaction between flagellar membranes; there is no indication that the mastigonemes participate directly.

Previous studies have demonstrated that the gametic flagella of *C. reinhardtii* lose their agglutinability when treated with trypsin (32). We therefore analyzed active preparations of the flagellar surface components by polyacrylamide gel electrophoresis to determine whether the acquisition of agglutinability involves the addition of novel polypeptides to the flagellar surface. We find no new polypeptides associated with the gametic flagellar surface, a result that is discussed with regard to recent descriptions of the surfaces of gametic flagella from the related species *Chlamydomonas moewusii* (4, 20, 21).

## MATERIALS AND METHODS

### *Strains and Culture Conditions*

Wild-type *C. reinhardtii*, strain 137c, *mt*<sup>+</sup> and *mt*<sup>-</sup>, was used in most experiments. Where specified, the mutant strains, *imp-1*, *imp-2*, *imp-5*, *imp-6*, *imp-7*, *imp-8*, and *bald-2* were utilized. These were derived from the *mt*<sup>+</sup> wild-type strain by UV-mutagenesis and were initially isolated on the basis of their inability to mate (12, 14, 15). Methods for culturing vegetative and gametic cells are described in the preceding paper (19).

### *Scanning Electron Microscopy*

Gametes of opposite mating types 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<sub>4</sub> in 4 mM potassium-phosphate buffer, pH 7, was immediately added to a final concentration of 0.5% OsO<sub>4</sub>; 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 Pt-Pd, stabilized with carbon, and observed in an AMR-1000A scanning electron microscope operated at 20 kV with a specimen angle of 2°.

### *Negative-Staining Electron Microscopy*

To visualize whole cells, cells were centrifuged from their growth medium at 500 g for 2 min and washed twice in double-distilled water (gametes retain their agglutinability after such washes). A drop of cells was applied to a carbon-coated 400-mesh copper grid; excess water was removed by touching a piece of filter paper to the side of the grid; a drop of 2% uranyl acetate in water was applied; within 1 min, the excess stain was drawn off, a drop of water was applied, and excess liquid was again removed. The grid was then either air dried or vacuum evaporated in the specimen chamber of a Hitachi HU 11-C electron microscope.

Suspensions of isolated flagellar membranes and mastigonemes were negatively stained as described above for whole cells except that sucrose was removed by washing the grids before uranyl acetate was applied.

### *Freeze-Dry Electron Microscopy*

A concentrated suspension of unfixed cells, washed in distilled water, was applied to mica disks, frozen in Freon 22 cooled with liquid nitrogen, and dried under vacuum and shadowed in a Balzer's BAF 301 (Balzer's High Vacuum Corp., Santa Ana, Calif.).

### *Freeze-Fracture Electron Microscopy*

Flagella attached to whole cells were prepared for freeze-fracture electron microscopy by two procedures. (a) Cells were washed in cold phosphate buffer, fixed in cold 2% glutaraldehyde as described for negative staining, again washed in buffer, resuspended into cold 20% glycerol in buffer, and stored in the glycerol solution for 12–24 h. They were then centrifuged at 27,000 g for 10 min, and small drops of the resulting pellet, of pasty consistency, were pipetted onto 3-mm paper disks and frozen in Freon 22 cooled with liquid nitrogen. (b) Cells were washed in distilled water or in phosphate buffer, centrifuged at 3,000 g, and frozen as small drops at room temperature.

Isolated flagella were also examined by freeze-fracture electron microscopy. They were isolated essentially as described below and prepared for microscopy as described above for fixed cells except that the solutions were made 5% in sucrose.

Freeze fracturing was performed at –120°C in Balzer's BAF 301 device equipped with a platinum gun. Unfixed whole cells were, in certain cases, etched at –100°C for 1 min. Replicas were cleaned sequentially with methanol and Clorox, and mounted on uncoated 300-mesh grids.

### *Isolation of Flagella*

Flagella were isolated by a pH shock method essentially as described by Witman et al. (33).

## *Isolation of Flagellar Membranes and Mastigonemes*

Synchronously grown vegetative or gametic cells were harvested from 12–18 liters of culture and resuspended to a concentration of  $4 \times 10^8$  cells/ml in a solution containing 1 mM  $MgCl_2$ , 1 mM  $CaCl_2$ , and 0.250 M Tris-HCl, pH 7.4 (abbreviated MCT). The cell suspension was gently shaken for 10 min and then was chilled and centrifuged at 5,900 *g* for 8 min to remove the cells. A solution of 40% sucrose in MCT was added to this supernate to create a final sucrose concentration of 10%, and the supernate was centrifuged at 31,000 *g* for 30 min (Sorvall RC-2B centrifuge, SS-34 rotor, DuPont Instruments, Sorvall Operations, Newtown, Conn.) to sediment detached flagella and cell debris. The supernate was collected by aspiration and subjected to a second centrifugation at 31,000 *g* for 30 min. It was again collected by aspiration and passed through a sintered Pyrex glass filter (4–5.5- $\mu$ m pores). The filtrate was centrifuged at 105,000 *g* (Beckman Model L ultracentrifuge, type 40 rotor, Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) for 1 h and the supernate discarded. The pelleted material was suspended in MCT containing 10% sucrose (abbreviated SMCT), consolidated, and recentrifuged at 105,000 *g*. The pooled pellet, which we refer to as the *crude pellet* and which contained membrane vesicles and mastigonemes, was assayed for isoagglutinating activity (see below); it was then either subjected to further fractionation or frozen in liquid nitrogen and stored at  $-70^\circ C$ . Frozen samples were normally used for additional studies within 3 wk.

### *Fractionation of the Flagellar Membranes and Mastigonemes in the Crude Pellet*

The crude pellet obtained as above could be fractionated into its components by two methods. The first involves isopycnic centrifugation and is an adaptation of the technique previously described by Witman et al. (33) for the purification of mastigonemes from vegetative cells. The crude pellet was suspended into a solution of 2.8 M CsCl in MCT and was centrifuged at 124,000 *g* (Beckman model L, SW50L rotor) for 24 h, causing the membrane vesicles and mastigonemes to band separately in the CsCl gradient (see Results). Fractions were collected, diluted with SMCT, and pelleted by centrifugation at 105,000 *g* for 1 h.

The second procedure does not yield mastigonemes but produces a relatively pure sample of membrane vesicles. The crude pellet was suspended in 1 ml of SMCT and layered over 4.25 ml of MCT containing 30% sucrose. The tube containing this discontinuous gradient was then centrifuged at 124,000 *g* in the SW50L rotor for 2 h; mastigonemes and some membrane vesicles remained near the interface of the two sucrose solutions while most of the membrane material was pelleted. The

pellet was resuspended in SMCT and recentrifuged at 105,000 *g* in a type 40 rotor for 1 h.

### *Isoagglutination Assay*

To measure the isoagglutinating activity of flagellar membranes and/or mastigonemes, a pellet containing these elements is suspended in its condensed moisture (typically 50–100  $\mu$ l) and 2–5  $\mu$ l of the suspension is added to a drop of test cells ( $\sim 5 \times 10^4$  cells in the drop) on a microscope slide. When the preparation is active and when the pellet derives from gametes of one mating type and the test cells are gametes of the opposite mating type, a widespread isoagglutination reaction develops, sometimes immediately and sometimes after a short delay, in which pairs and small clusters of cells agglutinate by distal flagellar adhesion. The SMCT alone is devoid of activity, and vegetative cells or gametes of the same mating type do not give such a response when used as test cells.

### *SDS Polyacrylamide Gel Electrophoresis*

The protein constituents of the various fractions isolated by the techniques described above were analyzed by electrophoresis in two different systems. One system involved cylindrical gels, 14 cm long, composed of 5% acrylamide, 0.175% methyl bisacrylamide, 25 mM Tris, 8 M urea, and 0.1% SDS. The samples were dissolved by heating in 8 M urea, 2% SDS, 10 mM Tris, and 5%  $\beta$ -mercaptoethanol, and 10–20  $\mu$ g up to 100  $\mu$ g of protein were loaded on each gel. The gels were subjected to electrophoresis at room temperature using an Ortec pulsed constant power supply for 10–12 h. They were then removed and stained with either Coomassie brilliant blue or periodic acid-Schiff (PAS) reagent, by the procedure of Glossmann and Neville (10). Appropriate protein and glycoprotein controls were also electrophoresed and stained for carbohydrate in order to confirm the absence of nonspecific SDS-Schiff stain reactions in the gels. Polypeptide mobilities and molecular weights were correlated on 5% and 8% gels using the following mixture of reduced and alkylated protein standards (11): myosin,  $\beta$ -galactosidase, phosphorylase A, bovine serum albumin, alpha globulin, actin, carbonic anhydrase, trypsin, RNase, and cytochrome *c*.

The second electrophoretic procedure was based on the discontinuous system described by Laemmli (17), adapted here for use in slab gels containing gradients of average pore size. The separating gel was 9 cm long by 15 cm wide, with a range of acrylamide concentrations from 5% to 15%, increasing from top to bottom, with a constant ratio of acrylamide to methyl bisacrylamide concentrations (30:0.8). The acrylamide gradient was created by mixing 5% and 15% acrylamide solutions in a two-chambered standard gradient mixer, including 10% glycerol as a stabilizing agent in the more concentrated acrylamide solution. A 3% stacking gel was poured above the separating gel and covered with a plastic spacer which

created 13 sample slots in the polymerized gel. The samples were dissolved by heating in 1% SDS, 0.25 M Tris, pH 6.8, 8 M urea, and 1%  $\beta$ -mercaptoethanol, and were run at 30 mA for 90 min, using a Canalco constant rate source. The gels were fixed and stained with Coomassie blue according to the procedure of Fairbanks (9) or with PAS. Protein concentrations were determined by the procedure of Lowry et al. (18).

## RESULTS

### *Visualization of the Mating Reaction*

Immediately after competent  $mt^+$  and  $mt^-$  gametes are mixed, groups of 2-10 cells cluster together, associating at the distal (tip) ends of their flagella. When 2-10% glutaraldehyde solutions are presented to these cells, most of the clumps break up; 0.03% glutaraldehyde, however, preserves the mating configuration (see Materials and Methods). Fig. 1 shows such a fixed mating pair as visualized by scanning electron microscopy. The opposed flagella are seen to entwine around one another and to make direct contact at their tips (Fig. 1, arrows), the tip of one flagellum usually extending out beyond the tip of the other. Similar images have been obtained in unfixed preparations of mating cells subjected to freeze-etch or to negative-stain electron microscopy. This spatial orientation is therefore used in the present study to distinguish agglutinating flagella from flagella that have made adventitious contact during specimen preparation.

### *Morphology of Vegetative and Gametic Flagellar Membranes*

The flagellar membrane of both gametes and vegetative cells appears in thin section (Fig. 2) to be surrounded by a fuzzy coat which has previously been termed the flagellar sheath (24). This material is similar in appearance to the carbohydrate coat associated with other surface membranes (23), and it is regarded here as an intrinsic carbohydrate component of the membrane rather than an extrinsic sheath.

When the flagellar membrane is freeze-fractured, the surface fuzz is again apparent (Figs. 3-5). Also revealed are the A (cytoplasmic) and B (extracellular) fracture faces of the flagellar membrane; these are studded with 90-140-Å diameter particles of the sort described for other biological membranes (6). The particles are occasionally seen to align in rows on the A fracture face (Figs. 3 and 5, arrows), and apparently complementary grooves

are present on the B face (Fig. 3, arrow). Such rows and grooves are encountered in replicas of both proximal (base) and distal (tip) regions of the membrane. Similar rows of particles have been

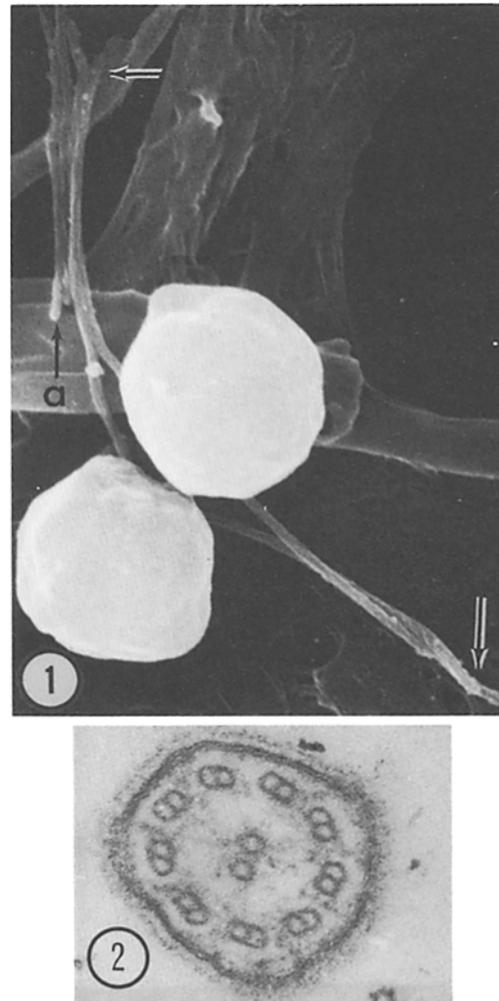


FIGURE 1 Mating pair of *C. reinhardtii* gametes, as seen by scanning electron microscopy, occupies the center of the field. The interacting flagella twist around one another and adhere closely at the tips (arrows), with one tip extending slightly beyond the other. Two agglutinating flagella from another pair are indicated at *a*. The background material is filter paper (see Materials and Methods). Photograph by Dr. Richard L. Weiss.  $\times 7,000$ .

FIGURE 2 Flagellum from an  $mt^+$  vegetative cell fixed in glutaraldehyde and  $OsO_4$ , thin sectioned, and stained with uranyl acetate and lead citrate. A fuzzy surface coat projects from the flagellar membrane.  $\times 114,000$ .

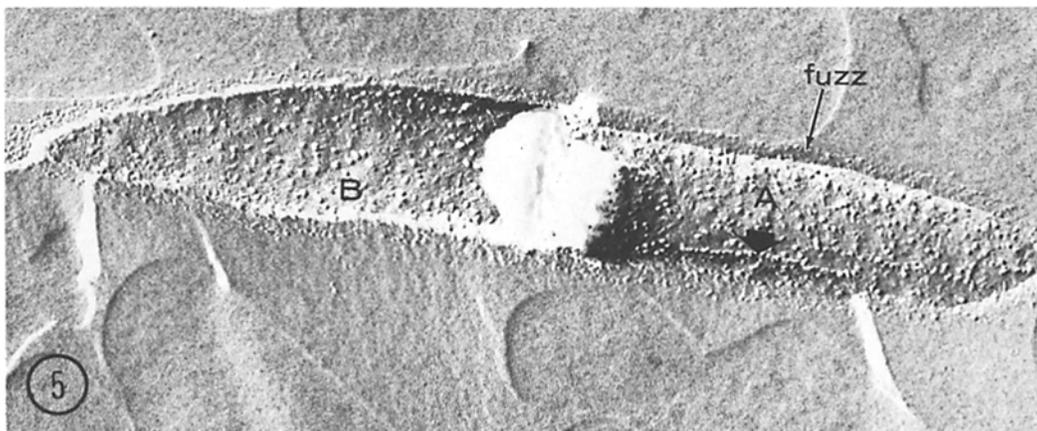
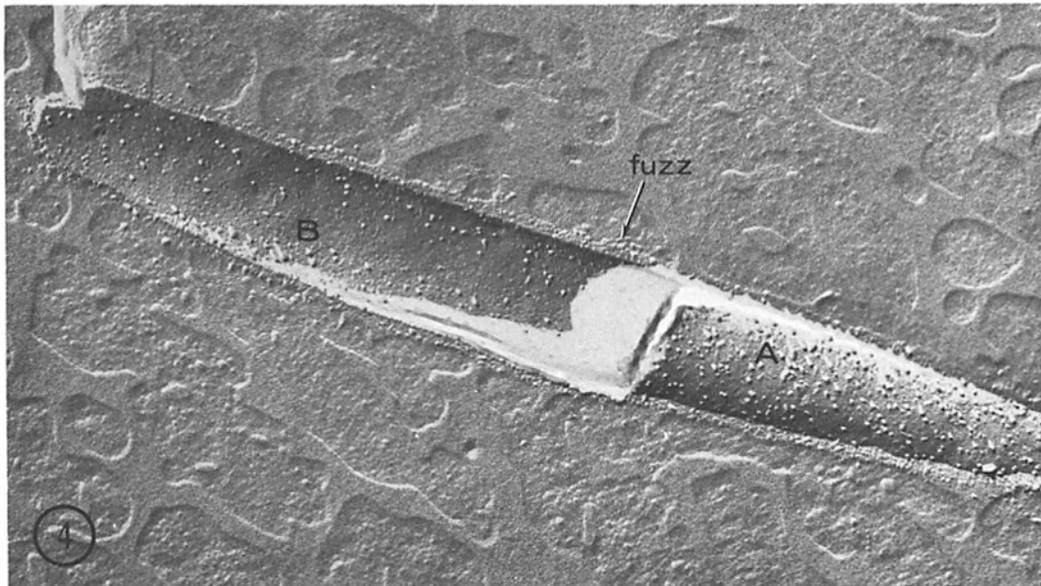
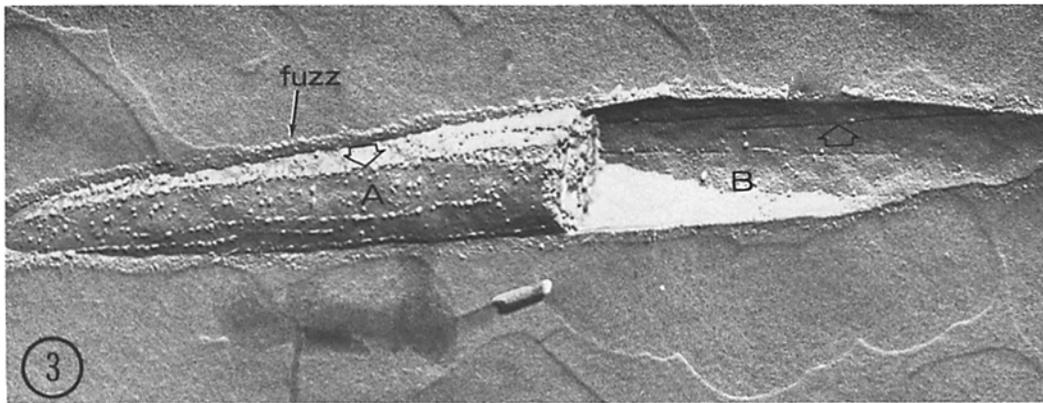


FIGURE 3 Flagellum from an  $mt^-$  vegetative cell as seen in freeze-fractured preparations showing aligned rows of intramembranous particles on the A face (arrow) and apparently complementary grooves on the B face (arrow) of the membrane. The B face carries very few particles.  $\times 53,000$ .

FIGURE 4 Flagellum from an unmated  $mt^-$  gamete as seen by freeze-fracture. The B face carries many particles.  $\times 53,000$ .

FIGURE 5 Flagellum from an unmated  $mt^+$  gamete as seen by freeze-fracture. Arrow indicates aligned row of intramembranous particles on the A face. The B face carries a high density of particles.  $\times 70,000$ .

described for sperm flagella (2, 8) and *Tetrahymena* cilia (26).

A striking difference has been observed between vegetative and gametic flagella in the particle density of membrane B faces. Whereas the B face of vegetative membranes is virtually devoid of particles (Fig. 3), the gamete membranes invariably carry an intermediate (Fig. 4) or high (Fig. 5) density of particles. The high particle density is encountered in both gametic mating types, occurs in both proximal and distal regions of the flagellar membrane, and is present in two nonagglutinating mutant strains (*imp-2* and *imp-8*).

No alteration in particle density or distribution was detectable in gametic flagella during the course of the agglutination reaction, either in fixed or in unfixed preparations.

#### *Morphology of Vegetative and Gametic Mastigonemes*

Mastigonemes are poorly visualized in freeze-fractured or freeze-etched replicas, but can be

studied by negative staining or in freeze-dried replicas. Negative staining reveals (Fig. 6) that arrays of mastigonemes project from the flagellar surface and cover the distal half to two-thirds of each flagellum. Considerable variation is found in the total number of mastigonemes associated with each flagellum. This variability is not significantly reduced by fixing cells in glutaraldehyde before negative staining, suggesting that a natural sloughing of mastigonemes may occur. The most dense population of mastigonemes encountered along a flagellar surface is found to be of the order of 13 mastigonemes per micrometer, a value found for vegetative flagella and for gametes of both mating types.

The distribution of mastigonemes of the flagellar surface is revealed in freeze-dried specimens. As seen in Fig. 7, a single row of mastigonemes projects from the flagellar surface; other replicas reveal a second row of mastigonemes on the opposite surface. The arrows in Fig. 7 point to sites along the row where mastigonemes may have broken off either naturally or perhaps during

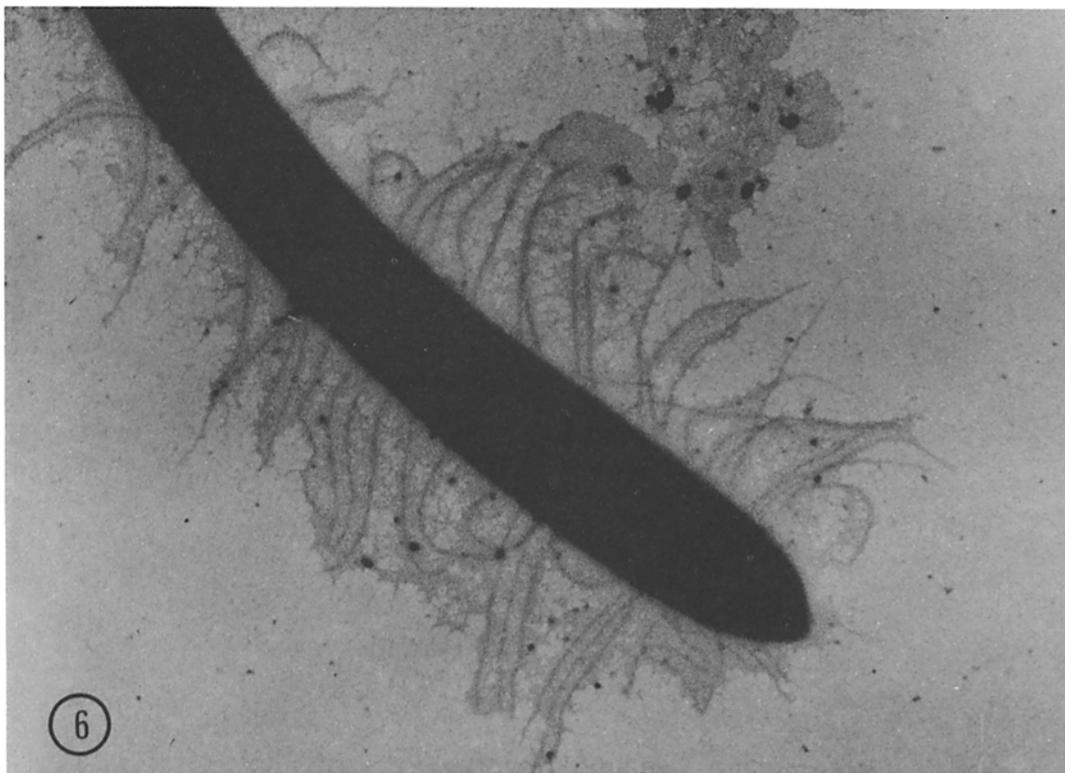


FIGURE 6 Flagellum from an unmated *mt<sup>-</sup>* gamete showing a particularly abundant array of mastigonemes. Negative stain.  $\times 36,000$ .

specimen preparation. Between the two rows of mastigonemes lie large expanses of free membrane surface, a fact that is not readily deduced from negatively stained preparations (Fig. 6).

Mastigonemes projecting from a negatively stained flagellar surface vary in length from 0.4 to 0.9  $\mu\text{m}$ , whereas isolated mastigonemes are virtually constant in length (reference 33 and our own observations), a discrepancy caused by variable angles of settling of mastigoneme rows onto the carbon film. No differences can be detected between the mastigoneme distributions of vegetative cells,  $mt^+$  and  $mt^-$  gametes, and the nonagglutinating mutant strains.

### Surface Components Involved in the Agglutination Reaction

Gametic flagella associated in the characteristic mating configuration shown in Fig. 1 appear by both negative staining and freeze-etch electron microscopy to make direct contact with one another; they are certainly not separated by the 1- $\mu\text{m}$  length of mastigonemes. The best morphological evidence for a membrane to membrane interaction during flagellar agglutination arises as an occasional artifact of negative staining in which, during rapid drying under vacuum, certain flagella lose their membranes and appear as splayed out axonemes. The lost membrane can then be located on a nearby intact flagellum and direct contact between the fuzzy membrane coats can be visualized (Fig. 8). That such associations represent vestiges of true agglutination and not adventitious associations is strongly indicated by the fact that quadri-flagellated zygotes (which are nonagglutinable) and nonmating gametes on the same grid do not show such associated membrane fragments.

In order to identify more specifically the agglutinins involved in the flagellar interaction, a preparation of membrane and mastigonemes derived from the flagellar tip is desirable. Fig. 9 illustrates the formation of just such a preparation from the flagellum of an unmated gamete. While the shedding process depicted presumably occurred during staining and drying, such images of shedding are seen to occur only from the flagellar tips, never from the sides. We believe that Fig. 9 depicts a shedding process that also occurs, for unknown reasons, *in vivo*; as described below, *C. reinhardtii* cells elaborate into their medium a small quantity of flagellar membranes and mastigonemes that can be isolated, purified, and analyzed biochemically.

### Isolated Flagellar Surface Components

Wiese and his colleagues (30) previously showed that the gametes of several species of *Chlamydomonas*, including *C. reinhardtii*, put into their medium a substance ("gamone") that causes an isoagglutination, but not cell fusion of gametes of the opposite mating type. Thus, the "gamone" derived from the growth medium of  $mt^+$  gametes was found to cause  $mt^-$  gametes to clump together in characteristic mating configurations (but had no effect on  $mt^-$  vegetative cells), and the gamone from  $mt^-$  gametes caused an isoagglutination only between  $mt^+$  gametes. We followed the gamone isolation procedure of Wiese (30), modified it to yield more reproducible results (as outlined in Materials and Methods), and examined the translucent pellet obtained when the culture medium is

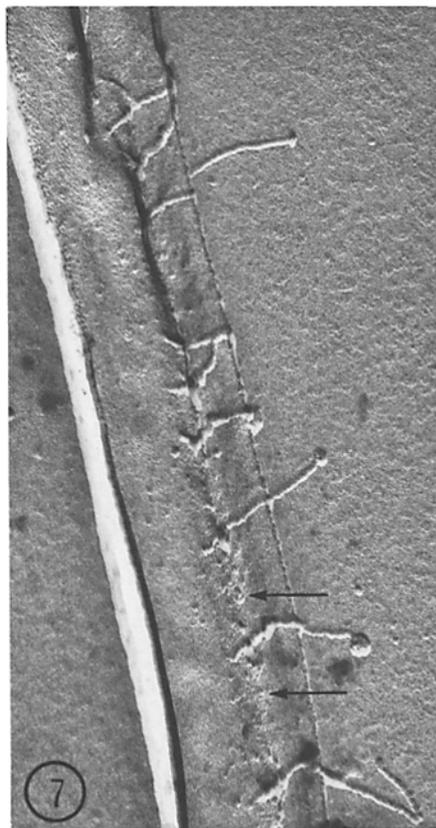


FIGURE 7 Flagellum from a vegetative  $mt^+$  cell, freeze-dried and shadowed, showing a medial row of mastigonemes. Several of the mastigonemes are balling up and others have become appressed on the surface, both effects probably being artifacts of specimen preparation. Arrows point to positions where mastigonemes have apparently broken off.  $\times 53,000$ .

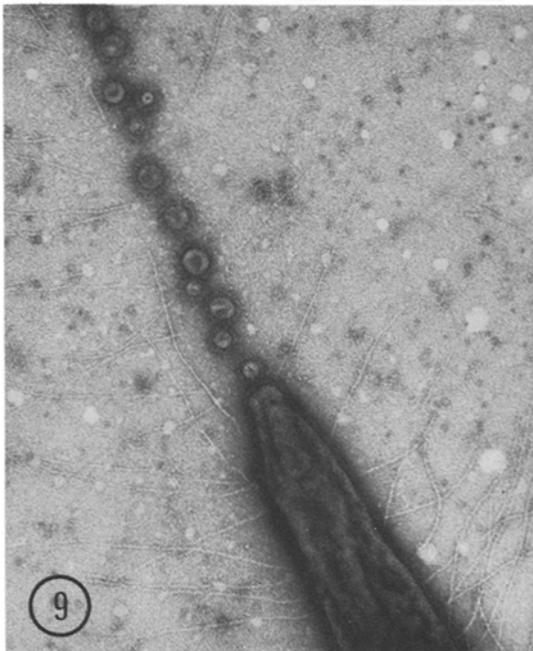
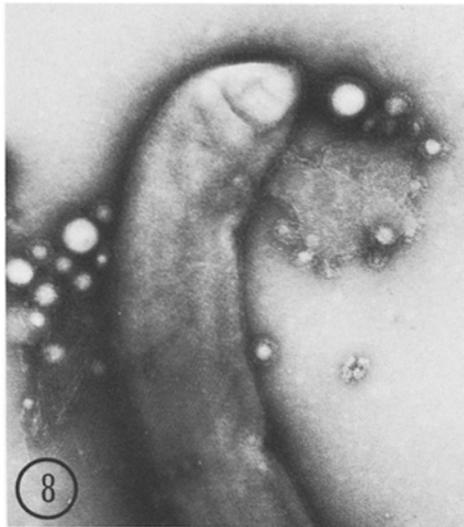


FIGURE 8 Flagellum from a mixture of mating gametes. Vesicles and a large, irregular piece of flagellar membrane adhere to the intact flagellum. The fragments appear to derive from a flagellum of opposite mating type that has lysed during specimen preparation (see text). The material in the lower left with an apparent moiré pattern is a flagellar collar (see reference 22 and Roy Gould, Ph.D. thesis, Harvard University, 1974) from a cell wall.  $\times 40,000$ .

FIGURE 9 Flagellum from an unmated *mt*<sup>+</sup> gamete bearing mastigonemes. Vesicles, many bearing mastigonemes, are seen to be blebbing off the flagellar tip: a total of 31 such vesicles is present in the unabridged micrograph. Negative stain.  $\times 37,000$ .

centrifuged at 105,000 *g*. This pellet contains small membrane vesicles and mastigonemes, as shown in Fig. 10; comparable preparations were obtained from both mating types. The vesicles carry the characteristic fuzzy coat of the flagellar membrane and are occasionally found to have mastigonemes inserted into them (Fig. 10, single arrow); moreover, no such vesicles (or mastigonemes) are obtained if cells of the mutant strain *bald-2*, which lack flagella, are subjected to this same isolation procedure. Finally, the preparation is identical in morphology to the material shed from flagellar tips during negative staining (Fig. 9). We therefore conclude that the gamone fraction contains flagellar mastigonemes and vesicles ( $\sim 100$  nm in diameter, including coat) derived from the flagellar membrane, a conclusion reached independently in two other laboratories (21, 28). Additional evidence for this conclusion is presented in a later section.

To determine whether the shedding of flagellar membrane and mastigonemes is a unique activity of gametic cells that might be relevant to the mating reaction, vegetative cells of both mating types were subjected to the same gamone isolation procedures. Both prove to shed mastigonemes and flagellar membrane vesicles into the buffer in the same fashion as gametes, as observed also by Snell et al. (28) and by McLean et al. (21) although the yield of material is generally lower. When these fractions from vegetative cells are isolated, however, they are completely inactive in promoting the isoagglutination of gametes. Moreover, pellets prepared from nitrogen-starved cultures of the nonagglutinating mutant *imp-2* are also inactive.

Because our experiments deal with flagellar fractions derived from both vegetative cells and gametes, and because the physiological significance of this shedding process is unknown, the term gamone seems inappropriate for our preparations; we shall therefore refer to the flagellar vesicles and mastigonemes that we harvest from the buffer and eventually pellet by 105,000 *g* centrifugation as the *crude pellet*.

We should note that the gamone procedure of Wiese (30) specifies that plates containing month-old cultures be flooded with a small volume of distilled water, that gametes be allowed to swim into the water until high cell densities are reached, and that these dense suspensions be left to stand, unagitated, for 24 h at room temperature. A similar procedure was used by McLean et al. (21). When we followed this procedure, we noted that considerable proteolysis took place in the cell

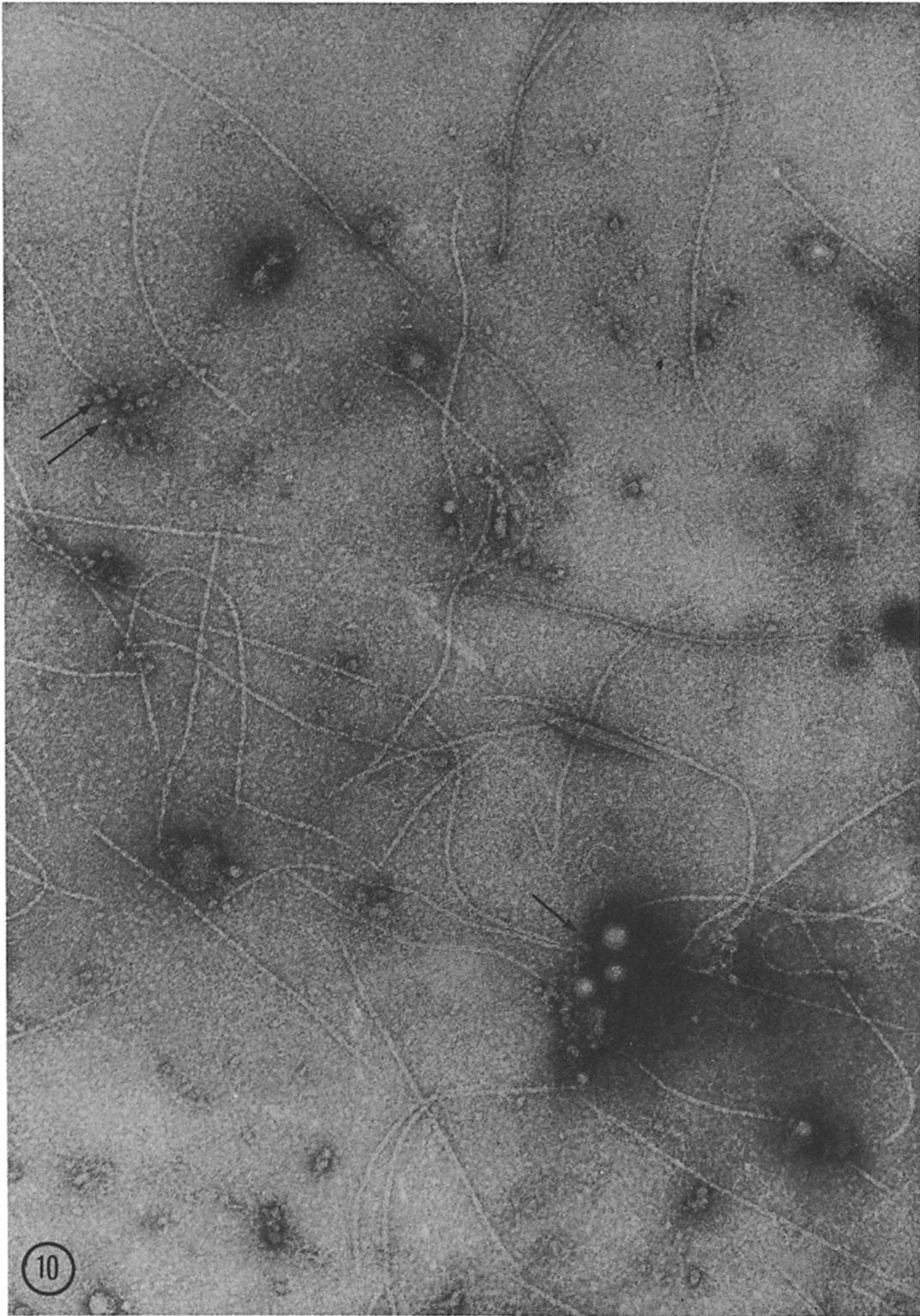


FIGURE 10 Crude pellet (see Materials and Methods) obtained from a culture of *mt*<sup>+</sup> gametes. Arrow indicates an aggregation of flagellar vesicles with attached mastigonemes. Free mastigonemes and single vesicles (bearing fuzzy coats) are also present. Double arrows indicate small rounded particles, often aggregated, of unknown origin.  $\times 70,000$ .

suspensions; specifically, when we added known amounts of bovine serum albumin to such suspensions and the liquid, freed of cells by high-speed centrifugation, was subjected to polyacrylamide gel electrophoresis 12 h later, most of the BSA had disappeared from its characteristic position on the gels, and proteolytic fragments were evident throughout the lower molecular weight region of the gels. To obtain gametic crude pellets that consistently exhibited high levels of isoagglutinating activity, gametes in the present study were prepared from synchronous liquid cultures (19), slowly agitated at high cell density with a rotary shaker, and maintained at high density for short periods. Inactive pellets were only infrequently obtained under such conditions; these were usually discarded, but were occasionally subjected to biochemical analysis as specified in a later section.

#### *Fractionation of the Crude Pellet*

Two procedures have been developed to fractionate these crude pellets into pure membrane and pure mastigoneme fractions so that it could be determined whether one or both components were necessary for the isoagglutination reaction. The first procedure involves centrifugation of the crude pellet to equilibrium in CsCl (see Materials and Methods). This creates a gradient containing two well-separated bands of particles: a broad band at 1.305 g/ml (median density) and a narrow band at 1.35 g/ml. When the material from the broad band is collected and examined by negative staining, it proves to contain flagellar membrane vesicles, many of which still bear inserted mastigonemes; the attachment of these mastigonemes persists even when the crude pellet is passed through a Yeda press or subjected to sonication before suspension into CsCl. The narrow band is found to be a highly purified preparation of mastigonemes.

Membranes from both vegetative and gametic cells of *mt*<sup>+</sup> and *mt*<sup>-</sup> band at identical densities in the CsCl gradient and appear identical in ultrastructure. Similarly, no differences are found in the equilibrium densities or morphological characteristics of mastigonemes derived from gametic or vegetative cells of the two mating types.

When the fractions derived from the CsCl gradients were washed in SMCT and tested for isoagglutinating activity, neither the membranes nor the mastigonemes were found to be consistently active, nor was the native activity of the crude pellet restored by combining the isolated membrane and mastigoneme fractions. Moreover,

when an active crude pellet was suspended in 2.8 M CsCl (in MCT), kept at 4°C for 24 h without centrifugation, and then washed free of CsCl by dilution with SMCT and repeated centrifugation at 105,000 g, the exposure to high salt was found to have inactivated the preparation. Therefore, a method of isolating membrane vesicles from the crude pellet without a lengthy exposure to CsCl was devised.

This second procedure involves the sedimentation of the membranes through a layer of 30% sucrose, leaving the mastigonemes and some membrane at the interface of the denser and lighter sucrose solutions. The material at the interface is somewhat active as an isoagglutinin. The pelleted fraction, which contains virtually no mastigonemes as judged by electron microscopy (contrast Fig. 11 with Fig. 10), is highly active in isoagglutination assays, commonly exceeding the crude pellet from which it derives in the rapidity of its effects. This activity is retained for at least 24 h (as long as 5 days in one case) if the preparation is stored at 4°C and for at least 3 wk if the pellet is frozen and stored at -70°C.

#### *Electrophoretic Analysis*

A cylindrical gel system (see Materials and Methods) was used to identify the fractions containing membrane and mastigonemes and to implement their separation. Such gels of the different fractions just described reveal simple polypeptide patterns. The crude pellet, when electrophoresed in the presence of 8 M urea and 0.1% SDS and stained with Coomassie brilliant blue, displays two major polypeptides of very high apparent molecular weights (Fig. 12 A). A minor polypeptide band sometimes appears between these two (Fig. 12 B), but the presence of this band cannot be correlated with either mating type or with the cells' state of differentiation, and its significance is obscure. A broad zone, faintly stained by Coomassie blue, is often seen in the region corresponding to a molecular weight of approximately 70,000 daltons when the gels are heavily loaded, but the appearance of this region is identical in samples originating from vegetative and gametic cultures of both mating types. Briefer periods of electrophoresis failed to reveal any rapidly migrating species which might have run off the gel during the extended periods normally used to resolve the bands at the top of the gel, nor were any additional polypeptides observed when 8% acrylamide gels were employed. It should be noted that the gel origin frequently retained

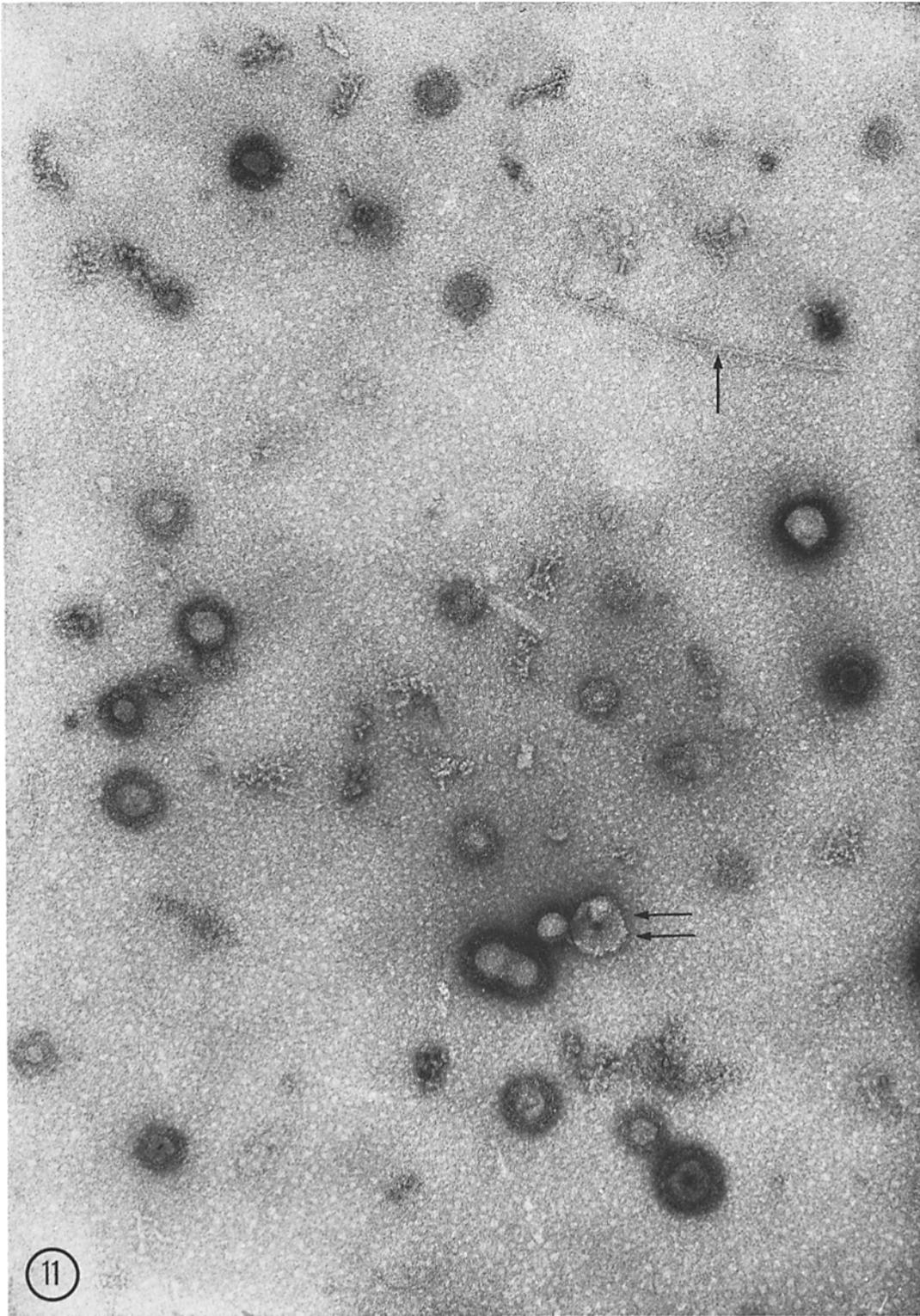


FIGURE 11 30% sucrose pellet (see Materials and Methods) obtained from the preparation depicted in Fig. 10. Arrow points to a single contaminating mastigoneme, intentionally included in the field to illustrate that defective staining does not explain the paucity of mastigonemes in the preparation. The small rounded particles found in the crude pellet are also not present. Double arrows indicate a membranous vesicle lacking a fuzzy coat; all other vesicles bear the coat, and are thereby of presumed flagellar origin.  $\times 70,000$ .

Coomassie blue-staining material, despite efforts to solubilize the samples completely and despite extended periods of electrophoresis.

The identity of the two high molecular weight polypeptides in the crude pellet was established from the fractionated preparations. The mastigonemes isolated from CsCl gradients yielded a single polypeptide band with the same mobility as the faster of the two major bands of the crude pellet (Fig. 12 E). Membrane protein, whether derived from a CsCl gradient or from the material sedimented through 30% sucrose, also appeared as a single band on gels, comigrating with the slower of the two major components of the crude pellet (Fig. 12 C, D). The membrane fractions prepared by either of the two methods were frequently contaminated by slight amounts of mastigoneme protein. This contamination is minimal if the crude pellet is derived from cells suspended in MCT for only 10 min and if the pellet is fractionated on sucrose gradients, in which case the mastigoneme band contributes insignificantly to the total Coomassie blue stain on the gel. Again, an ill-defined Coomassie blue-stained region is sometimes visible in gels of the membrane fraction, in the vicinity of 70,000 daltons molecular weight. Since such purified membrane preparations are extremely active isoagglutinins when obtained from competent gametes, it seems likely that the membrane vesicles found in these fractions account for the isoagglutination of gametes of the opposite mating type.

We should note that membranes collected by sedimentation through 30% sucrose can be suspended into 2.8 M CsCl and subjected to equilibrium density centrifugation, in which case they band at the same density as membrane vesicles isolated directly by isopycnic centrifugation of crude pellets; moreover, they show the same unique major polypeptide band on gels. We should also note that when intact gametic flagella are isolated, and solubilized and subjected to electrophoresis in the presence of urea and SDS, the resulting gels (Fig. 13) exhibit a high molecular weight protein with the same mobility as that of the membranes prepared by isopycnic centrifugation or sedimentation through 30% sucrose. Thus, it appears likely that all of our membrane fractions derive from the same source.

The membrane polypeptides of vegetative and gametic flagella of both *mt*<sup>+</sup> and *mt*<sup>-</sup> behave identically in this gel system and cannot be distinguished even if run on the same gel. Similarly, the mastigoneme proteins of vegetative and gametic

cells, and of both mating types, migrate with the same electrophoretic mobilities. Thus, no unique polypeptide profile can be assigned to the flagella of either mating type nor to vegetative vs. gametic flagella by this procedure.

Witman et al. (33) report that mastigonemes from *mt*<sup>+</sup> vegetative cells contain glycoprotein as determined by PAS staining of gels. When gels of the crude pellet or of membrane and mastigoneme fractions from gametes are stained by the PAS procedure, both the membrane and the mastigoneme proteins are found to contain carbohydrate, the membrane band staining relatively more intensely than the mastigoneme band. The subunit molecular weight of mastigonemes from *mt*<sup>+</sup> vegetative cells was determined by Witman et al. (33) to be approximately 170,000, whereas we find that both the membrane and the mastigoneme glycoproteins migrate more slowly in our gel system than does myosin (*M* = 200,000 daltons), whether subjected to electrophoresis separately or on the same gel as the standards (Fig. 14). They were still less mobile than myosin when run on gels containing 8% acrylamide and 0.28% methyl bisacrylamide. Since the molecular weights of glycoproteins cannot be reliably estimated by SDS-polyacrylamide gel electrophoresis (28), these observations serve only to establish that both the membrane and mastigoneme glycoproteins are probably rather large.

The slab gel system utilized (see Materials and Methods) yields more detailed patterns and allows more accurate comparisons between samples. In this gel system, all Coomassie blue or PAS-staining material entered the separating gel, eliminating ambiguities due to the incomplete penetration of the gel by the sample. The migrating protein was subject to continuous stacking effects by the conditions of electrophoresis, creating distinct bands where only blurred zones had been obtained in the tube gels. The use of the slab gel conferred greater sensitivity to minor components, an effect which could be enhanced by drying the gel and thus concentrating the protein-bound dye.

Fig. 15 shows the electrophoretic patterns exhibited by crude pellets obtained from vegetative and gametic cells. The gels are heavily overloaded for the major membrane glycoprotein, beneath which lies the mastigoneme glycoprotein. Both are seen to migrate more slowly than myosin (present as the largest of the molecular weight standards).

Several classes of minor polypeptides are also apparent in Fig. 15. Close to the origin lie two or

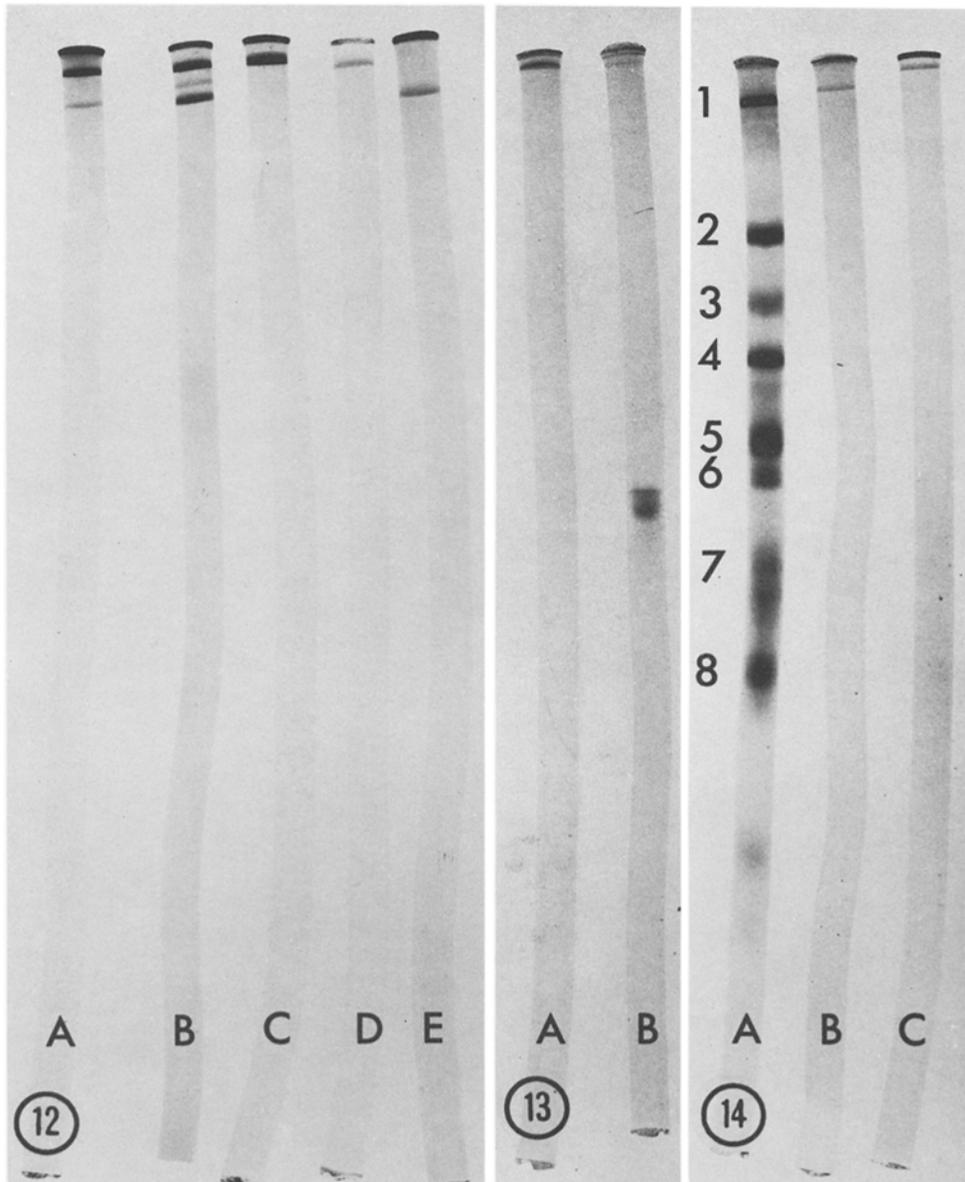


FIGURE 12 Protein composition of flagellar surface fractions. The fractions were prepared from wild-type gametes, *mt*<sup>+</sup>, although identical results were obtained with vegetative and gametic cells of both mating types. Electrophoresis was performed as described in Materials and Methods. (A) Crude pellet; (B) crude pellet, displaying minor third component; (C) membrane isolated by CsCl density gradient centrifugation; (D) membrane isolated by sedimentation through a 30% sucrose layer; (E) mastigonemes isolated from CsCl gradients.

FIGURE 13 Comparison of the isolated membrane fraction and the major flagellar components. (A) Membrane vesicles derived from the crude pellet after sedimentation through a layer of 30% sucrose (see Materials and Methods); (B) flagellar components. The three major bands are the membrane protein (top) and the paired tubulins (lower) (29).

FIGURE 14 Nominal molecular weight estimation by SDS-urea-polyacrylamide gel electrophoresis (see Materials and Methods). (A) Molecular weight standards: 1. myosin (M 200,000) 2.  $\beta$ -galactosidase (M 130,000) 3. phosphorylase A (M 95,000) 4. bovine serum albumin (M 68,000) 5.  $\gamma$ -globulin (M 50,000) 6. actin (M 45,000) 7. carbonic anhydrase (M 29,000) 8. trypsin (M 23,000); (B) mastigoneme fraction from CsCl gradient; (C) membrane fraction pelleted through 30% sucrose layer.

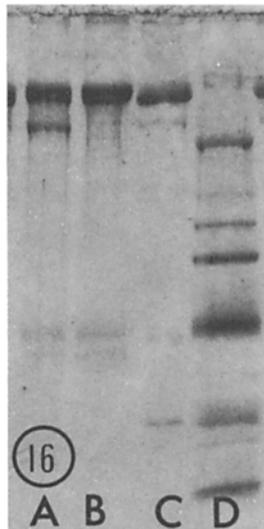
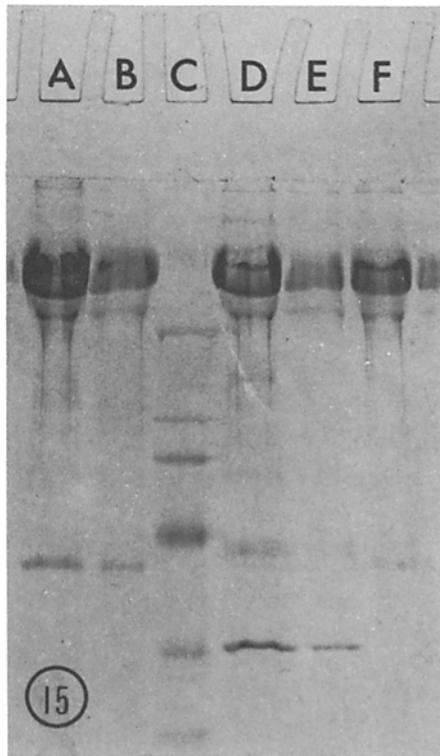


FIGURE 15 Comparison of crude pellets from vegetative and gametic cells. (A) and (B)  $mt^-$  gametic crude pellet; (C) molecular weight standards (from top to bottom: myosin,  $\beta$ -galactosidase, phosphorylase A, bovine serum albumin, ovalbumin, and chymotrypsin); (D) and (E)  $mt^+$  vegetative crude pellet; (F)  $mt^+$  gametic crude pellet.

FIGURE 16 Purification of flagellar membranes. (A)

three minor polypeptide species of extremely high molecular weight, all of which are PAS positive and all of which are present in both vegetative and gametic samples. At least two of these are also present in membranes purified by sucrose gradient centrifugation (gels not shown).

In the vicinity of the bovine serum albumin standard ( $M = 68,000$ ), one or two distinct minor bands are present in all four preparations. These bands are PAS positive, copurify with the membrane vesicles (Fig. 16), and presumably correspond to the ill-defined region of staining observed in tube gels. The bands are seen in Fig. 15 to differ slightly in electrophoretic mobility, but both the prominence and the position of these bands vary slightly from preparation to preparation (compare, for example, tracks B and C in Fig. 16) and we have not been able to correlate such apparent molecular weight differences with mating type or with state of differentiation. We find that bands in this region are particularly prominent when inactive preparations are subjected to electrophoresis, raising the possibility that they arise through proteolysis of a higher molecular weight polypeptide.

Finally, the vegetative sample in Fig. 15 exhibits a band comigrating with ovalbumin ( $M = 45,000$ ) which is absent from either gametic preparation. The band is not apparent in purified vegetative membrane preparations (Fig. 16 B) nor in a mastigoneme-containing vegetative preparation (Fig. 16 A), nor has it ever been observed in tube gels of crude pellets. Since the vegetative sample shown in Fig. 15 was stored for several weeks while the gametic samples for this gel were being prepared, the band is most likely to be a consequence of proteolysis.

## DISCUSSION

The acquisition of mating type-specific flagellar agglutinability during gametogenesis must be the result of subtle biochemical or structural modifications of the flagellar surface. The fuzzy surface coat and its associated mastigonemes appear identical in all types of cells, and the electrophoretic

$mt^-$  vegetative preparation containing both membrane vesicles and mastigonemes; (B)  $mt^+$  vegetative membrane vesicles purified by the sucrose gradient method; (C)  $mt^+$  vegetative crude pellet—the same samples as in Fig. 15 D; (D) molecular weight standards—the same sample as in Fig. 15 C.

analysis of flagellar surface components indicates that their protein composition is alike in all types of cells, despite the obvious functional differentiation of gametic flagella. The only apparent structural changes accompanying gametogenesis are an increase in flagellar length (22) and the alteration in the distribution of intramembranous particles reported here. It is not clear that either of these differences is causally related to membrane agglutinability. The change in particle distribution, for example, is not confined to that distal region of the gametic flagellum involved in agglutination, and occurs even in the flagellar membrane of nonagglutinating mutant cells when they are deprived of nitrogen. Therefore, while it is possible that this change is directly related to agglutinability, it may also simply reflect alterations in the chemical and physical properties of membrane lipids caused by nitrogen starvation (cf. reference 19), alterations perhaps unrelated to other changes in the flagellar surface required for agglutinability.

Given the surface architecture of gametic flagella, three possible modes of flagellar adhesion must be considered: membranes could interact directly; mastigonemes could interact directly; or the mastigonemes from one flagellum could associate with the membrane of the other flagellum. The evidence presented here does not rigorously exclude any of these models, but it does suggest that the ultimate form of agglutination required for subsequent events in mating probably involves membrane-membrane interactions. Flagellar membrane vesicles from gametes of one mating type can isoagglutinate gametes of the opposite mating type, and when the adhering flagella of mating cells are examined by electron microscopy, one finds that the intimacy of surface contact is uninterrupted by mastigoneme-filled gaps. The enormous proportion of flagellar surface unoccupied by mastigonemes and the great variation in mastigoneme abundance among equally agglutinable gametes also discourage hypotheses of mastigoneme involvement. The difficulty of obtaining mastigonemes totally free of flagellar membrane under conditions that do not destroy the isoagglutinating activity of the crude pellet has thus far precluded a definitive test of their capacity to isoagglutinate or to inhibit agglutination as functionally univalent entities.

McLean and co-workers (21) also conclude that membranes mediate flagellar agglutination in the distantly related species *C. moewusii*. Certain dissimilarities should be noted, however. The

membranous isoagglutinating fraction prepared from the gametic culture medium of this organism contains membrane vesicles much larger (up to 0.5  $\mu\text{m}$  in diameter) than those isolated from *C. reinhardtii*. These are reported to exhibit differences in buoyant density when derived from different mating types, to retain isoagglutinating activity when isolated in CsCl gradients, and, unlike the membranes described here, to be only loosely attached to their mastigonemes. They are also reported to exhibit six distinct glycosyl transferase activities, all of which are enhanced during mating (4, 20), an observation that may not be compatible with our electrophoretic analysis of the *C. reinhardtii* membrane. These various dissimilarities may relate in part to the fact that the mating process in *C. moewusii* differs in some detail from that in *C. reinhardtii* (31).

The present work demonstrates that gametic isoagglutination in *C. reinhardtii* is mediated by vesicles derived from the flagellar membrane. The extreme prominence of a single glycoprotein in this membrane and the highly restricted distribution of mastigonemes on its surface are also documented and perhaps reflect an unusual membrane structure. It seems reasonable to propose that mating type-specific agglutinability may be produced by slight changes in the amino acid composition of the proteins or glycoproteins associated with this membrane, and/or by changes in the carbohydrate compositions of its surface coat, possibilities that we are now in a position to test, particularly in view of the availability of five nonagglutinating mutant strains.

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