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The role of flagella in sexual reproduction of *Chlamydomonas eugametos*

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INTRODUCTION

Chlamydomonas, a unicellular green alga, is a very convenient organism to study plant cell biology in all its aspects. The organism is easy to handle in the laboratory and classroom alike. It grows autotrophically in large numbers on a simple salts medium in light, and it exhibits a multitude of cellular processes, harbouring numerous challenges to anyone interested in modern cell biology. It is a popular experimental system for research on photosynthesis, flagellar motility, phototaxis, cell wall metabolism and cell–cell interactions.

In this article we shall describe how *Chlamydomonas* cells interact with each other as a prelude to sexual reproduction. We shall concentrate on the major recent advances in our understanding of this process, but shall also indicate some of the outstanding problems that remain. The species *C. eugametos* (together with its subspecies *moewusii*) is the main object of this study, but reference will be made to *C. reinhardtii* when necessary. Both species differ in some significant details. The work on *C. reinhardtii* has recently been reviewed by Snell (1985).

Cell–cell interaction is a natural part of the sexual activity which precedes cell fusion. Cells do not fuse by chance; they have to be prepared for this act. This preparatory process usually follows several consecutive steps. Normally, the first step is the differentiation of vegetative cells into gametes, followed by some sort of mutual attraction between them.

The most common mode of attraction between gametes is by pheromones, specific low-molecular mass, diffusible substances. Some spectacular examples are found among the brown algae (Boland *et al.* 1984), where motile male cells are guided to their partners by a concentration gradient of a specific olefinic hydrocarbon, produced by the relatively immotile female gametes. When both sexual partners are motile, as in *Chlamydomonas eugametos*, a mutual attraction by pheromones is hard to visualize, and in fact there is no convincing evidence for its occurrence.

The next step in the sexual interaction involves the physical contact of partners. It is clear that components in the cell surface (either the cell wall or the plasma membrane) must play a decisive role in this process. Such surface components serve to stablize cellular contact, but they should also carry specific determinants, because cell fusion is a highly specific process: it is specific for gametes of the opposite sex but within one species. These compounds may also act as typical receptor molecules, which on contact generate a 'signal' by which the cells are triggered to prepare themselves for fusion. The acrosome reaction in echinoderm and vertebrate sperm cells is a typical example (e.g. Shapiro 1987). A similar process occurs during mating in *Chlamydomonas* (see the next section).

Cell–cell interactions are also part of the developmental processes in multicellular organisms. Again, diffusible substances play a key role, carrying morphogenetic signals that instruct changes in gene expression. Also, high-molecular weight compounds, immobilized in the cell surface, provide information about where cells are, whether they should stay together, or instead remain apart, and above all how they should coordinate their functions. The migration of cells from the neural crest to a multitude of specific regions in the developing vertebrate embryo is an impressive example of cellular activity, in which specific surface molecules are involved (Edelman 1986). Also in plants, there are many examples of cell–cell interactions which initiate developmental programmes. Examples are the pollen–stigma and *Rhizobium*–legume root hair interactions (e.g. Redmund *et al.* 1986; for reviews see Chadwick & Garrod 1986).

In the sexual interaction between gametes of *Chlamydomonas*, the flagella play a dominant role. Specific molecules, located at the surface of these organelles, called 'agglutinins', mediate sexual agglutination, in which partner cells adhere together prior to fusion. For a normal mating reaction to proceed, sexual agglutination is an indispensable process.

It is becoming clear that agglutinin-mediated adhesion is not a passive process, which is dictated only by the mutual affinities of the molecules involved, but is subject to regulatory influences from the cell. By some subtle mechanisms it can be modulated by altering the amount, distribution and/or chemical properties of the agglutinins. For this reason, special attention will be devoted to these processes.

THE SEXUAL SYSTEM IN *CHLAMYDOMONAS*

Chlamydomonas is a single-celled organism, with two flagella arising from the anterior end of the cell body, which protrude through the surrounding cell wall. Flagella are not present when the cells are grown on a solid medium. They provide the cells with locomotive force but during sexual interaction they function as sex organelles, mediating cell adhesion and orchestrating the preparations for cell fusion.

C. eugametos and *C. reinhardtii* are essentially haploid species. They are isogamous, which means that there are no morphological differences between the opposite sexes. Therefore, sex is designated as mating type *plus* (mt^+) and mating type *minus* (mt^-). They

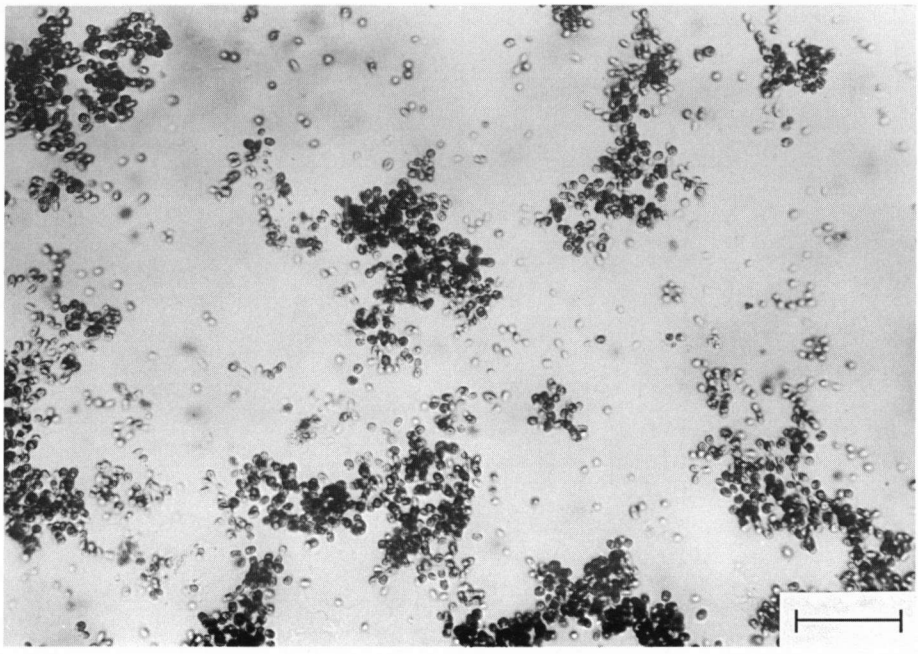

Fig. 1. Sexual agglutination of a mixture of mt^+ and mt^- gametes of *Chlamydomonas eugametos*; bar = 0.3 mm.

are heterothallic, which means that there is a stable inheritance of mating type. All the vegetative progeny of one parent have the mating type of that parent. In homothallic species, such as *C. monoica* (VanWinkle-Swift & Aubert 1983) and *C. geitleri* (Necas *et al.* 1980), the vegetative progeny of one cell contains cells of both mating types, which indicates that the mating type is frequently switched. Sexual phenotype is determined by a single nuclear gene or group of genes, the mt locus (Galloway & Goodenough 1985; Matagne 1987). This locus presumably becomes expressed when cells enter the gamete stage.

Sexual reproduction starts when two gamete suspensions of opposite mating type are mixed. The first and most rapid action is shown within seconds, when the gametes agglutinate due to a species- and sex-specific adhesiveness of the flagella (Fig. 1). At high cell densities agglutination results in large clumps. At low densities, cells agglutinate in small clusters, or simply in pairs. The flagella of agglutinating cells change their movement from the regular swimming beat to a vehement twitching movement which gives agglutinating clumps a vibrating appearance (Homan *et al.* 1980). Apparently the initial contacts are established by random collisions. There is no evidence for mutual attraction between the mating cells (Tomson *et al.* 1986). However, this raises the question of how the gametes produce the specific patterns that one observes in agglutinating cell suspensions. Soon after mixing gametes, a few clusters of interacting cells are formed which grow out into large cell aggregates. In the course of the following minutes, the number of such aggregates remains relatively constant, because free swimming cells will join existing clumps rather than instigate new nuclei. They seem to be attracted by, or become entrapped in, existing cell aggregates (Fig. 2). In the section on sexual agglutination some evidence for the latter alternative is given.

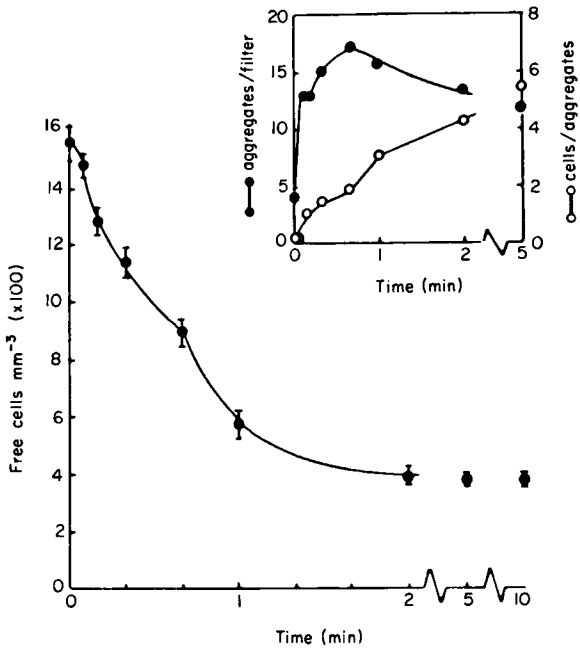

Fig. 2. Kinetics of the disappearance of free cells from a suspension of mating gametes of *Chlamydomonas eugametos*. Gametes of either mating type (1500 cells mm^{-3}) were mixed, immobilized at the times indicated by transferring aliquots of the cell mixtures to a water-moistened plastic membrane filter and counted. Each point represents the mean of nine individual measurements. Standard errors of means are shown. Inset: kinetics of the occurrence of aggregates and the calculated mean number of cells per aggregate of the same experiment. From Mesland (1978), with permission.

An interesting problem is how many mutually adhering mt$^+$ and mt$^-$ cells in a random aggregate are still able to sort themselves out and generate cell pairs. When two cells of different mating type come into contact they have the tendency to become associated with both flagella, which gradually align over their whole length, tip-to-tip (Mesland 1976). The formation of pairs is further promoted by a mating-type specific reorientation of the flagella: as agglutination progresses, the mt$^-$ flagella become reflexed around their own cell body, while in contrast the mt$^+$ flagella are held forward in a complementary manner, as shown in Fig. 12. In this situation the two interacting cells are able to bring their anterior ends in apposition where they produce a specific protoplasmic protrusion, called mating papilla, between the bases of the flagella, which protrudes through the cell wall (Musgrave *et al.* 1985). Their eventual fusion results in a plasma connection that binds the two bodies together in cell tandems, called *vis-à-vis* pairs (Figs 3 and 4), which have a life time of several hours before they settle to the substrate, retract their flagella, and fuse completely by widening the pore in the cell walls through which the plasma connection extends. The flagella lose their sexual agglutinability within a few minutes after forming the plasma bridge (Musgrave *et al.* 1985). Complete cell fusion is followed by the formation of a common primary zygote wall, under which a thick and ornamented secondary zygote wall is eventually formed. Later, the primary wall is discarded as are the original gamete walls (Musgrave *et al.* 1983).

Flagellar adhesion between gametes thus serves different functions: (1) it allows the cells to sort themselves out into pairs; (2) it maintains the cells in close proximity; (3) it activates the cells for cell fusion (formation of the plasma papilla).

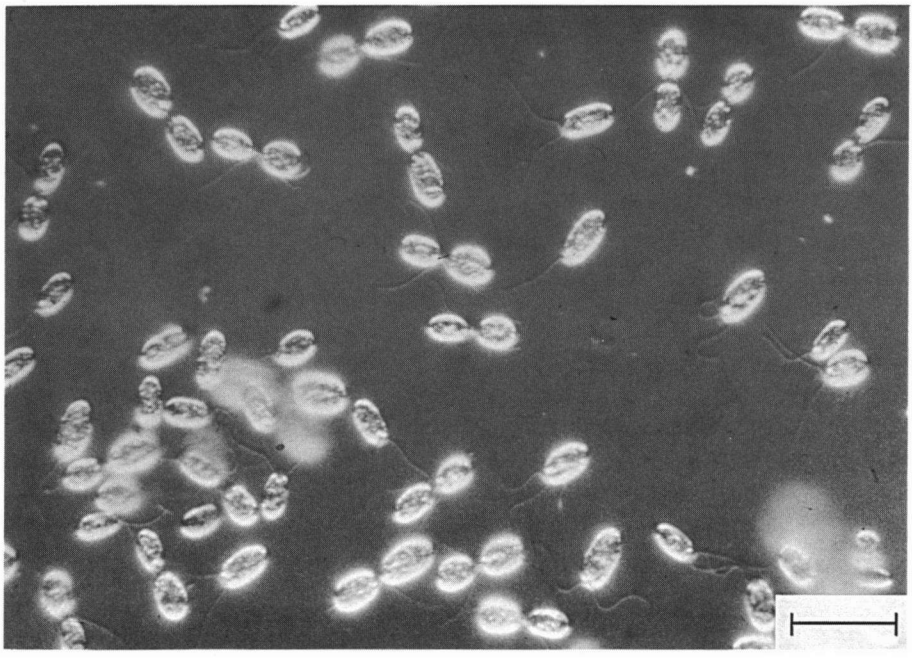

Fig. 3. *Vis-à-vis* pairs in a mixture of gametes of *Chlamydomonas eugametos*; bar = 0.03 mm.

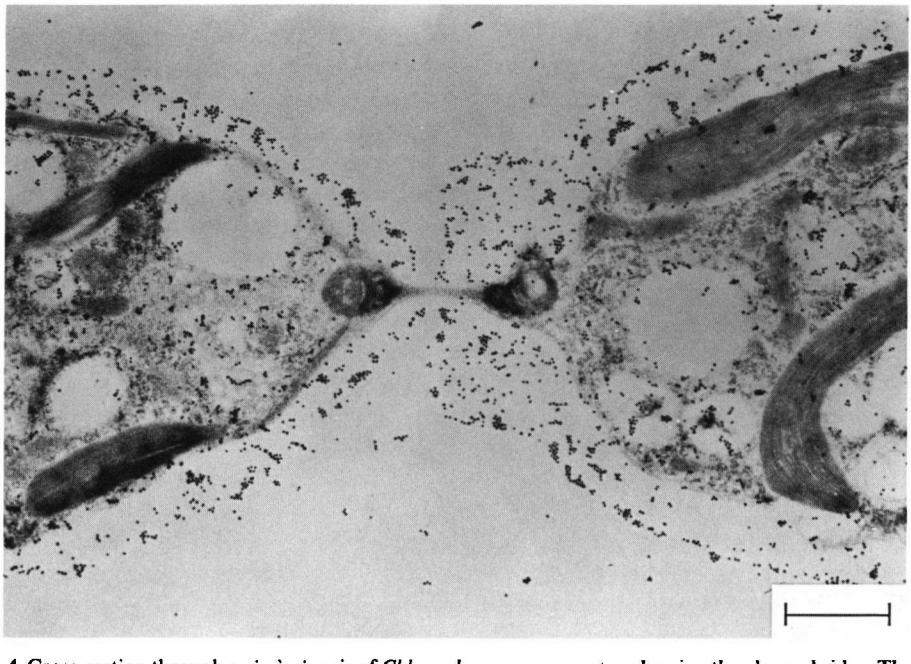

Fig. 4. Cross-section through a *vis-à-vis* pair of *Chlamydomonas eugametos*, showing the plasma bridge. The cell wall is visualized by gold-labelled anti-cell wall antibody; bar = 1 μm.

THE FLAGELLAR SURFACE

Chlamydomonas cells are propelled through the surrounding medium by their flagella. These have the classic structure, found essentially in all eukaryotic organisms, namely the cytoskeleton. This consists of nine-microtubule doublets and a central doublet, connected by nexin links and radial spokes, that is referred to as the axoneme. It is surrounded by a membrane which is an extension of the plasma membrane covering the cell body. The beating motion is based on the sliding movement of the microtubule doublets relative to one another, due to the action of a specific ATPase, dynein (Satir 1982). The membrane has a number of specialized functions (Bloodgood 1987). One of these is the regulation of the internal ionic environment of the flagella. For example, the internal free calcium level is regulated by voltage-dependent calcium channels. An increase in the free calcium level results in a modification of the bending pattern of the flagellum (Eckert & Brehm 1979). This shows that the flagellum can function as a sensory organelle with an excitable membrane.

A spectacular property of flagella is their ability to regenerate (reviewed by Lefebvre & Rosenbaum 1986). They can be easily amputated (by mechanical shear, osmotic or pH shock) under conditions in which the cell survives and is able to form new flagella in a time period, which considering the complexity of these organelles, is amazingly short (about 90 min). In the presence of cycloheximide, an inhibitor of protein synthesis, only one-half-length of the flagellum in *Chlamydomonas* is regenerated. This indicates that a pool of flagellar proteins, present in unassembled form in the cell body, has to be mobilized (Rosenbaum *et al.* 1969; cf. section on Gametogenesis). For full-length regeneration, there is a requirement for *de novo* protein and RNA synthesis. When flagella are amputated there is a rapid increase in the synthesis of flagellar proteins within the first 30 min after deflagellation. This is accompanied by an increase in the amounts of translatable mRNAs for these proteins (Lefebvre *et al.* 1980).

The site of incorporation of most newly synthesized axonemal proteins in regenerating flagella is at the distal tips (Dentler & Rosenbaum 1977) but very little is known about the mechanism of transport. The site of growth of the flagellar membrane is probably at the proximal end. In *Scherffelia dubia*, for example, flagellar membrane constituents are synthesized in the endoplasmic reticulum and Golgi complex and leave this system in membrane vesicles which are inserted in the membrane at a specific location at the base of the flagella during their regeneration (McFadden & Melkonian, 1986).

Intriguing questions are how flagellar amputation triggers specific gene expression and protein synthesis and how this synthesis is controlled. It is clear that there is some sort of interaction between cell body and flagellum.

Most important for the following discussion about flagellar agglutination is the dynamic character of the flagellar membrane. While continuous with the rest of the plasma membrane of the cell, it has (at least in *C. eugametos*) a different protein composition and different properties. At the base of each flagellum specialized arrays of intramembrane particles have been observed, which may play a role in maintaining the flagellar membrane as a separate plasma membrane domain (Weiss *et al.* 1977; Bray *et al.* 1983). One of the most spectacular properties of the flagellar membrane is that attached particles, such as bacteria or polystyrene beads, are translocated in a longitudinal direction (Bloodgood 1977). Their movement always occurs parallel to the long axis of the flagellum, in both directions, as if the particles are constrained to defined tracks within the flagellar membrane. This movement does not occur in dead cells or in isolated flagella.

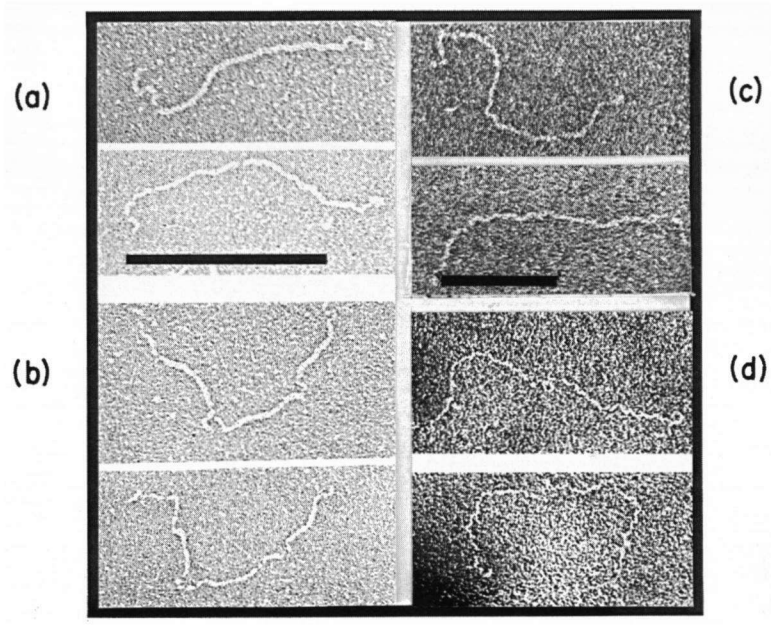

Fig. 5. Sexual agglutinins of *Chlamydomonas eugametos*, visualized by negative staining. (a) Agglutinins of *C. eugametos* mt^+; (b) agglutinins of *C. eugametos* mt^-; (c) agglutinins of *C. eugametos* syngen II, mt^+; (d) agglutinins of *C. eugametos* syngen II, mt^-. Bars = 100 and 200 nm, respectively. From Crabbendam *et al.* (1987) and Samson *et al.* (1987a) with permission.

Another motility phenomenon is whole-cell gliding. Cells are able to 'creep' along a solid substrate using their flagella (Lewin 1952). The cells have their flagella closely appressed to the substrate, oriented 180° with respect to one another. It seems to be the leading flagellum that provides the motive force (Bloodgood 1987). It is hypothesized that an energy-transducing system is anchored on the axoneme and is associated with transmembrane glycoproteins onto which particles or any other objects are adsorbed. The nature of this energy-transducing system is unknown.

With these properties of the flagellar membrane in mind, one is ready to understand some of the dynamics of sexual flagellar adhesion of flagella in *Chlamydomonas*. However, there must first be some understanding of the molecules involved in adhesion.

THE AGGLUTININS

The mt^+ and mt^- agglutinins are the 'receptors' at the flagellar surface of mt^+ and mt^- gametes, respectively, which are responsible for specific gamete–gamete adhesiveness. They have been identified and characterized as large (1·2–1·3 MDa) linear glycoproteins (Musgrave *et al.* 1981; Klis *et al.* 1985; Samson *et al.* 1987a,b). Their ability to bind to the flagellar surface of partner gametes is retained after isolation. This is demonstrated by adsorbing a preparation of isolated mt^+ or mt^- agglutinin to charcoal particles (Musgrave *et al.* 1981). Subsequently, partner cells become attached to these particles. The agglutinins have been visualized by electron microscopy (Crabbendam *et al.* 1987; Samson *et al.* 1987a). Figure 5 shows those of *C. eugametos*. In comparison, those of *C. moewusii* syngen II (also called *C. eugametos yapensis*, Samson *et al.* 1987a), which is

Table 1. Inactivation of isolated agglutinins of *C. eugametos* and *C. moewusii* syngen II by modification of their carbohydrate moieties

	Inactivation			
	C. eugametos		*C. moewusii* syngen II	
Treatment	mt^+	mt^-	mt^+	mt^-
Periodate (10 mM)	yes	yes	yes	yes*
Galactose oxidase	no	yes	—	—
α-Galactosidase	no	yes	no	no
α-Mannosidase	yes	no	no	yes
α-Glucosidase	—	—	yes	no

*After preincubation with either phosphatase or sulphatase this preincubation did not affect the agglutinative activity.

sexually incompatible with *C. eugametos*, are also shown. The mt^+ agglutinins differ considerably in length: 286 and 245 nm, respectively. In the mt^+ agglutinin of *C. eugametos*, two flexible regions are observed, one of which occurs 20 nm from the globular end and the other one 40 nm from the tapered end. In the mt^+ agglutinin of *C. moewusii* syngen II such flexible regions also occur but at various places. The mt^- agglutinins of both species are similar in length (340 nm) but they differ in form: while the mt^- agglutinin of *C. eugametos* has flexible regions at four well-defined sites, that of *C. moewusii* syngen II has a spiral form without well-defined joints.

There are gross similarities and subtle differences in their molecular composition. The agglutinins of both species are heavily glycosylated (50–60% protein, rich in hydroxyproline, serine and glycine) and contain arabinose and galactose as the predominant sugars (Samson *et al.* 1987a,b). Table 1 shows data on their susceptibility to glycosidic enzymes. These indicate that the carbohydrate part of the molecule is essential for the agglutinative activity of the agglutinins. Relatively small changes in the carbohydrate side chains cause a complete loss of activity. It is also clear from these data that the mt^- agglutinin of *C. moewusii* syngen II is distinguished by a much higher degree of phosphorylation and sulphation. Thus in these observations the sexual barrier between the two species is reflected by differences in form and composition of the molecules responsible for cell–cell adhesion.

The agglutinins of *C. reinhardtii* are similar to those of the mt^+ species described above (Adair *et al.* 1983; Adair 1985; Collin-Osdoby & Adair 1985; Goodenough *et al.* 1985). Both have a similar form with a total length of about 260 nm, possessing a bulbous end, a shaft and a terminal hook region. The molecules associate with the flagellar surface via the hook domain, so that the globular head is situated distal to the membrane. This reinforces the idea that the head domain is involved in sexual adhesion, which was originally based on the fact that treatment with thermolysin or reduction/alkylation inactivates the molecule and modifies the bulbous domain.

The agglutinins are extrinsic membrane molecules; presumably they are anchored to the membrane by intrinsic proteins. This is suggested by the ease with which these molecules become dissociated from the flagellar membrane (cf. Homan *et al.* 1982). The putative 'anchor' protein has not yet been identified.

There is a fundamental gap in our knowledge regarding the mode of action of the agglutinins. Clearly, they determine the specificity of the binding between the flagellar surfaces, but whether they bind to each other, or whether each agglutinin has its own receptor at the opposite membrane, has not been unequivocally established. There are two circumstantial arguments for the first option: (1) no other molecules than agglutinins have been found to bind to the flagellar surface of the opposite mating type and trigger a sexual response; (2) a monoclonal antibody which inhibits the activity of isolated mt^- agglutinin of *C. eugametos* specifically, also inhibits mt^- agglutination *in vivo* (see next section). This suggests that only one pair of *trans*-interacting surface molecules is involved in binding. Isolated mt^+ and mt^- flagella are able to clump together *in vitro* (Köhle *et al.* 1980; Goodenough 1986). However, an interaction between isolated mt^+ and mt^- agglutinins in solution has so far been undetected.

ANTI-AGGLUTININ ANTIBODIES

The production of antibodies directed against cell surface components in *Chlamydomonas* contributes considerably to our understanding of sexual agglutination. One particular monoclonal antibody, specific for the mt^- agglutinin of *C. eugametos*, has been used extensively in the investigations discussed in this article, so its characteristics will be summarized here (Kooijman *et al.* 1988). This antibody, designated mAb 66·3, was selected because it blocks the biological activity of isolated mt^- agglutinin bound to Sepharose beads. It binds to the flagellar surface of both mt^+ and mt^- flagella, but it inhibits the agglutinability of only mt^- gametes. It does not bind to vegetative cells. The specificity of mAb 66·3 for flagellar components was tested by immunoblotting after separation of membrane glycoproteins in electrophoresis gels, by affinity chromatography and by immunoprecipitation. It was found that in a mixture of mt^- flagellar glycoproteins, the antibody binds only to mt^- agglutinin. Because it blocks agglutinin activity it presumably binds to, or nearby, the sexual agglutination site. However, mAb 66·3 cross-reacts with two surface proteins on the mt^+ flagellar surface, including and mt^+ agglutinin, but since it does not inhibit its agglutinability its epitope must be distinct from the mt^+ agglutination site.

Besides this agglutination-inhibiting antibody, several monoclonal antibodies (mAbs) were isolated, directed against flagellar glycoproteins which are not involved in sexual adhesion. These mAbs are very useful because their epitopes are strain-specific and can be used to distinguish mt^+ and mt^- gametes (Schuring *et al.* 1987; Homan *et al.* 1987a). Thus it is possible to design double labelling experiments in which two different mAbs (differentially labelled, e.g. with FITC and TRITC) are used: first, the mt^- gametes can be identified using a strain-specific antibody, and then using mAb 66·3, the distribution of the agglutinin within that mating type can be monitored (see section on Tipping).

GAMETOGENESIS

Chlamydomonas multiplies by vegetative cells division, until the nutrients are depleted. Upon starvation, cell division is arrested and the cells differentiate into gametes. This is illustrated in Fig. 6. The ability of *C. eugametos* to produce *vis-à-vis* pairs is attained at the end of the logarithmic growth phase in a liquid batch culture (Tomson *et al.* 1985). At this point there is also a rapid increase in agglutinin content. This can be observed by extracting cell samples at various times with SDS, and assaying the extracts (after removal of the SDS) by adsorbing them to charcoal and next examining the ability of the charcoal

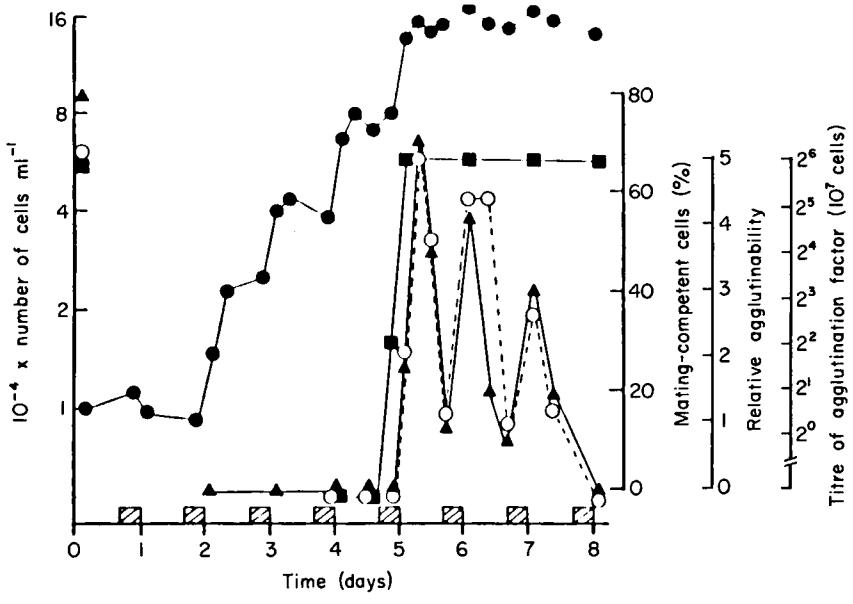

Fig. 6. Growth and gametogenesis of *Chlamydomonas eugametos* mt⁻ grown under a circadian LD-rhythm. Dark periods are represented as hatched rectangles. (●——●) Growth; (○——○) percentage of mating-competent cells; (▲——▲) relative agglutinability; (■——■) mt⁻ agglutinin content of the cells, determined after extraction with SDS. From Tomson *et al.* (1985), with permission.

particles to adsorb gametes of the opposite mating type. Thus gametogenesis is primarily demonstrated by the ability of the cells to synthesize agglutinins, which implies that specific genes are activated. Figure 6 also shows that there is a strong correlation between sexual agglutinability, which is a property of the flagellar surface, and the competence to produce *vis-à-vis* pairs, suggesting that sexual agglutination is the rate-limiting step that precedes sexual fusion. Thus mating competence is determined by the ability to confer sexual adhesivesness to the flagella, primarily by incorporating agglutinin molecules into the flagellar membrane. It should be noted that this incorporation is not only dependent on the amount of agglutinins synthesized. It is evident from Fig. 6 that there is a diurnal fluctuation in sexual adhesiveness of the flagella (together with a fluctuation in the ability to produce *vis-à-vis* pairs) while the overall agglutinin content in the cell is constant. Apparently, there is a considerable amount of agglutinin in the cell bodies. This has been demonstrated by extracting the cells after removal of the flagella by a pH shock (Pijst *et al.* 1983; Musgrave *et al.* 1986). The major part of this cellular agglutinin is present at the cell surface underlying the cell wall. The functional significance of this agglutinin compartment is unclear. Because it is always covered by the cell wall it is not expected to play a role in sexual interaction. It is also improbable that it plays a role in the transport of agglutinins to the flagella. Agglutinins are membrane glycoproteins, synthesized in the endoplasmic reticulum and Golgi complex, and are presumably transported via secretory vesicles and inserted in the membrane at the flagellar basis, as discussed in the section on The Flagellar Surface.

The dynamics of gametogenesis in *C. eugametos* are completely different when cells are cultivated on agar (Musgrave *et al.* 1986). The cells do not possess flagella when grown on

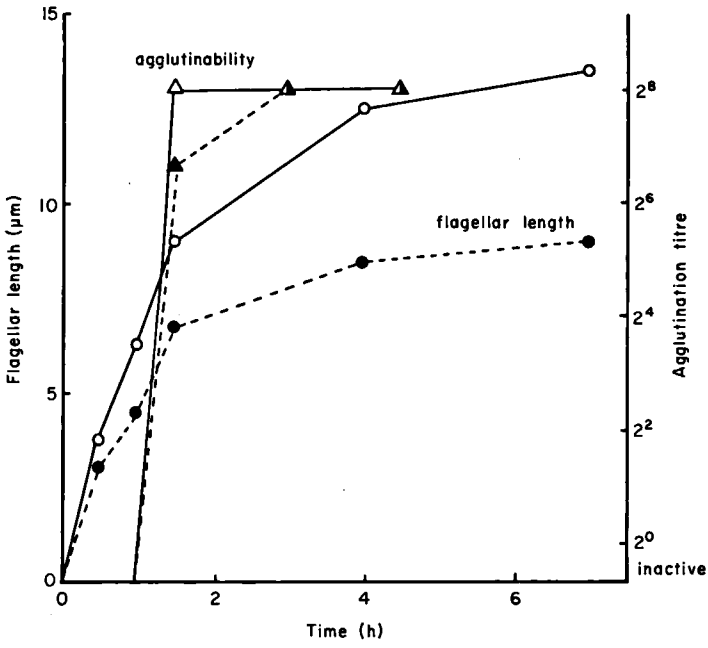

Fig. 7. Growth of flagella and development of agglutinability after suspending *Chlamydomonas eugametos* mt⁻ cells from agar culture in water or cycloheximide solution. (○——○) Length of the flagella; (△——△) agglutinability of the flagella. Cells treated with cycloheximide are represented by closed symbols and a broken line. From Musgrave *et al.* (1986), with permission.

a solid surface and when such cells are suspended in water or dilute buffer, flagellar growth and the development of flagellar adhesiveness can be followed with time (Fig. 7). If the cells are suspended in a cycloheximide-containing solution to prevent protein synthesis, it appears that flagellar biogenesis is partially inhibited and when the flagella of these cycloheximide-treated cells are amputated and regenerated, again in the presence of cycloheximide, the flagella only grow out as stumps of 3·5 μm. This indicates that while protein synthesis is inhibited the cells maintain a pool of flagellar components, by which they are able to generate flagella of approximately 60% of the length of the controls (cf. section on The Flagellar Surface). Treating agar cultures with cycloheximide does not affect the development of flagellar agglutinability. This implies that the cells also contain a supply of agglutinin, part of which is transported to and incorporated into the flagellar membrane during flagellar growth. This agglutinin can be extracted from dry cells by SDS, which dissolves the cell contents more or less completely. The agglutinin pool in the plasma membrane, which, as mentioned above, is not for convenience of the flagella, is developed only after the cells have been transferred to a liquid environment (Musgrave *et al.* 1986). It is unknown why *Chlamydomonas* cells grown on a solid surface, even in young cultures, are able to synthesize agglutinins. Maybe in such cells gametogenesis is induced by nutritional stress due to the slow diffusion of nutrients to the cellular colonies.

There is probably an ultrastructural difference between gametes and vegetative cells, however, this has been studied only in *C. reinhardtii.* It concerns the plasma papilla or 'mating structure', as it is called in this species. This is a specialized region of the plasma membrane between the flagellar bases. It is this part of the membrane which becomes

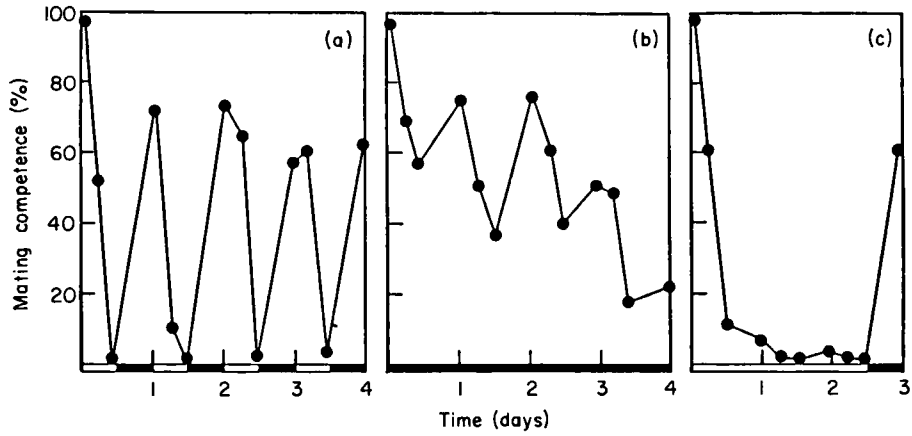

Fig. 8. Mating competence rhythm of *Chlamydomonas eugametos* mt^- in LD (a), DD (b) and LL (c), expressed as the percentage of cells able to produce *vis-à-vis* pairs. Similar rhythms were found for mt^+ gametes. (c) shows that the mating competence disappeared in LD but returned when the cells were placed in darkness again. From Demets *et al.* (1987), with permission.

extended by a reorganization of the underlying cytoskeleton in response to sexual interaction and by which the plasma bridge between the mating cells becomes established (cf. Detmers *et al.* 1983).

SEXUAL AGGLUTINABILITY HAS A CIRCADIAN RHYTHM

As shown above, gametes of *C. eugametos* show a diurnal periodicity in mating competence when subjected to a light–dark regime of, e.g. 12 h light/12 h dark. At the beginning of the light period this mating competence is high but it declines during the light period to increase again during the dark. As illustrated in Fig. 8, this rhythm persists in continuous dark while it rapidly damps out in continuous light (Demets *et al.* 1987). These fluctuations are paralleled by periodical changes in agglutinability, which again allows the interpretation that it is the sexual adhesiveness of the flagella which determines the mating competence. This fluctuation can be visualized by immuno cytochemistry. When mt^- gametes, fixed at different times while kept under constant environmental conditions in the dark, were treated with mAb 66·3 and then incubated with a secondary, gold-labelled antibody, a fluctuation in the density of the label was found on the flagella that coincided with the rhythm in agglutinability. This indicates that there is a correlation between sexual agglutinability and antigenicity towards mAb 66·3 (Tomson *et al.* 1988).

SDS-polyacrylamide gels of flagellar extracts exhibited a variation in the intensity of the agglutinin band, corresponding to the variation in flagellar agglutinability, while the intensity of all other flagellar proteins remained constant. These results suggest that the circadian fluctuations in agglutinability of *Chlamydomonas* gametes are the direct consequence of fluctuations in the concentration of agglutinins at the flagellar surface. Such a fluctuation could arise as the consequence of an oscillation in the incorporation or in the degradation of agglutinin. In the presence of cycloheximide, flagellar agglutinability declined at an exponential rate, which was independent of the moment of cycloheximide addition. This implies that there is a continuous turnover of flagellar agglutinins and also that the rate of agglutinin degradation, which is reflected by the decrease in

agglutin-ability, is constant throughout the day. Apparently it is the rate of incorporation of agglutinins into the flagellar surface which exhibits a daily fluctuation.

AGGLUTININS ARE INACTIVATED AS A RESULT OF SEXUAL ADHESION

We noted above that the aggultinins at the flagellar surface are subject to turnover. Several authors have shown that the rate of this turnover is considerably increased during the agglutination process in both *C. eugametos* and *C. reinhardtii* (Snell & Moore 1980; Pijst *et al.* 1984b). This is clearly illustrated by presenting living gametes with isolated flagella of the opposite mating type. The cells rapidly aggregate due to the cross-linking action of the added flagella but, after a short period, the aggregates dissolve because the isolated flagella lose their adhesiveness. By adding fresh flagella the process is repeated. In contrast, the addition of fresh gametes does not result in repeated aggregation. The interpretation is that during agglutination there is a rapid inactivation or loss of agglutinins in the flagellar membrane. This leads to a loss of adhesiveness of the isolated flagella because the inactivated molecules are not replaced by new ones, as occurs in living cells from a cellular pool. When a mixture of mt^+ and mt^- cells was treated with cycloheximide at various times after mixing in order to deplete this pool, the length of time before agglutinability was lost varied with the length of time the cells had been agglutinating. If we assume that the time until agglutinability is lost is related to the pool size of the agglutinins, one can see (Fig. 9) that immediately after adhesion started this agglutinin pool size diminished and apparently reached a minimum, after which it returned to its original level. This indicates that the gametes are able to adjust the size of their agglutinin pool by protein synthesis, even while it is rapidly utilized during sexual agglutination. In fact, gametes are able to detect the absence of agglutinins at the flagellar surface in a similar manner to which they are able to detect the absence of a flagellum in liquid medium (cf. Fig. 7).

We presently assume that the decrease in agglutinability observed on mixing gametes in the presence of cycloheximide is due to an inactivation or loss of agglutinin molecules from the flagellar surface. This is suggested by results obtained by Pijst *et al.* (1983), who used ^{35}S-labelled mt^- agglutinin and observed that it was sex-specifically bound to mt^+ gametes. This binding ability declined at a rate which was considerably less than when isolated mt^- flagella were incubated with living mt^+ cells. As argued by Snell (1985), it is also possible that cycloheximide affects not so much the synthesis of the agglutinins but rather the synthesis of a component involved in the expression of agglutinability and does not play an adhesive role itself.

The adhesiveness of the flagella declines minutes after the plasma bridge is formed between mating cells (Musgrave *et al.* 1985). This loss of agglutinability can be explained by the fact that agglutinating cells continuously inactivate each other's agglutinins, and by assuming that cell fusion blocks the synthesis and transport of new agglutinin molecules from the cell body to the flagellar surface. However, some flagellar adhesiveness remains. This is apparent from the fact that mt^- flagella isolated from gametes can aggregate motile *vis-à-vis* pairs, and that *vis-á-vis* pairs, particularly when they settle on the bottom of the incubation vessel, show a weak type of mutual adhesiveness (Musgrave *et al.* 1985).

SEXUAL AGGLUTINATION IS SELF-ENHANCING

A most remarkable property of sexual agglutination in *C. eugametos* was recently observed, namely that it is self-enhancing (Tomson *et al.* 1986; R. Demets &

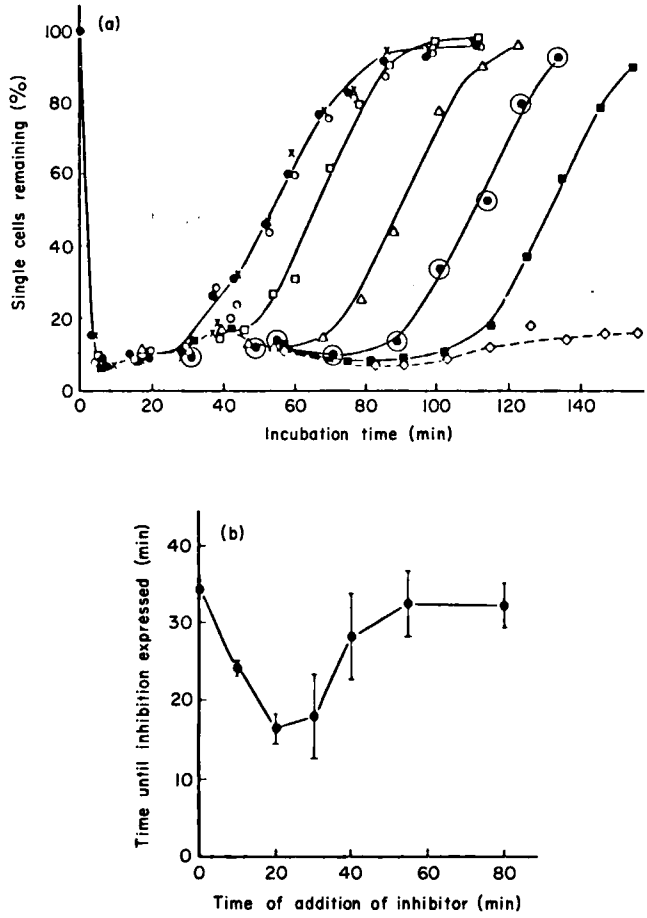

Fig. 9. (a) Effect of time of addition of cycloheximide on loss of agglutinability of *Chlamydomonas reinhardtii* gametes. Mt^+ and mt^- gametes were mixed and at 0 (●), 10(×), 20 (○), 30(□), 40(△), 55(⊙), and 80(■) min after mixing, cycloheximide was added. Control (◇). (b) Replot of the data in (a) averaged with data from two similar experiments performed on a different day, one performed with cycloheximide and one with anisomycin. Bars represent the average deviation from the mean. From Snell & Moore (1980), with permission.

A. M. Tomson, personal communication). An immediate agglutination is observed when gametes of opposite mating type are mixed. By fixing cells at different times during this agglutination process, and testing cells of either mating type by presenting them with living partners in a dilution series, it was found that the adhesiveness of the flagella increased considerably. A maximum value was reached 10–15 min after mixing, when the rate of *vis-à-vis* pair formation was maximal. Thereafter, the agglutinability of the cells decreased, caused by the formation of *vis-à-vis* pairs which were no longer adhesive. When cells were mixed in 10 mM Tris buffer, pH 9, by which cell fusion but not flagellar adhesion is inhibited, the agglutination reaction persisted for several hours and a constant high level of agglutinability was maintained. If the agglutination reaction was disturbed by vigorous shaking, the cells disadhered and the agglutintability in both mating types dropped within minutes to the original level. This procedure could be repeated several times, which demonstrates the reversibility of this phenomenon. An increase in adhesiveness of gamete

flagella could also be obtained by presenting gametes of a single mating type with purified agglutinin of the opposite mating type.

The rise in sexual adhesiveness of the flagella of mating cells was paralleled by an increased ability to bind the monoclonal antibody mAb 66.3. When mating cell mixtures were fixed with glutaraldehyde at various times and after washing presented with mAb 66.3, the antibody was shown to bind more abundantly to flagella of gametes that had been agglutinating for 15 min, compared to flagella of gametes that had just been mixed or had fused to form *vis-à-vis* pairs. These results show that the increase in adhesiveness is correlated with an increase in antibody binding during sexual agglutination in mt^+ as well as in mt^- flagella. However, we were unable to assess whether during sexual interaction more agglutinin molecules were incorporated into the membrane of the flagella or whether the affinity of the molecules, already present, was raised.

These results help to explain a phenomenon, referred to in the section on The Sexual System in *Chlamydomonas*, namely the formation of a specific agglutination pattern when complementary cell suspensions of high cell density are mixed. One then observes the rapid formation of a number of clumps, but although in the course of time more and more cells become engaged in agglutination, the number of clumps does not increase, they just become larger and larger. Our present interpretation is that cells engaged in sexual contacts are more adhesive than single, swimming cells. Therefore, free swimming gametes have a greater chance of adhering to potential partners in an agglutinating cell clump than to those that are still swimming free.

SEXUAL AGGLUTINABILITY IS MODULATED BY LIGHT

Förster & Wiese (1954) discovered that the mating ability of *C. eugametos* is dependent on light. This property, which is unrelated to the entraining influence of light on the circadian oscillation of agglutinability described in the section on Sexual Agglutinability, appears to be inherited in a Mendelian fashion and is unlinked to mating type. Thus, strains of either mating type may, or may not, require light for sexual activity (Kooijman *et al.* 1986). Light-requiring strains are completely incompetent to mate in the dark but attain this ability when illuminated with a weak dose of light. This mating ability is closely correlated with agglutinability. Thus, some strains are non-agglutinable in the dark but when illuminated they rapidly become agglutinable, as shown in Fig. 10. Their agglutininability is quickly lost when they are returned to the dark. The conclusion is that it is the agglutinability which is the light-sensitive property of some strains. How do we explain this phenomenon? Since no other properties of the sensitive cells are apparently affected by illumination, one must conclude that it is the agglutinins themselves or their concentration at the flaggellar surface that is influenced by light. Indeed, when flagella of dark-exposed cells were separated from the cell bodies and extracted with detergent, no active agglutinin was found, in contrast to those of illuminated cells.

The activation and inactivation of agglutinability are completely reversible in the presence of inhibitors like cycloheximide and tunicamycin, which suggests that they do not reflect protein synthesis or turnover. Apparently there is some light-controlled process by which agglutinins in the flagellar membrane are activated or de-activated. This process must be operative in the flagella only, since agglutinins present in the cell body (mainly at the plasma membrane, cf. section on Gametogenesis) are unaffected by illumination.

To show that there is an *in situ* modification of agglutinins that is controlled by light, equal batches of flagella obtained from light- and dark-exposed cells of a light-sensitive

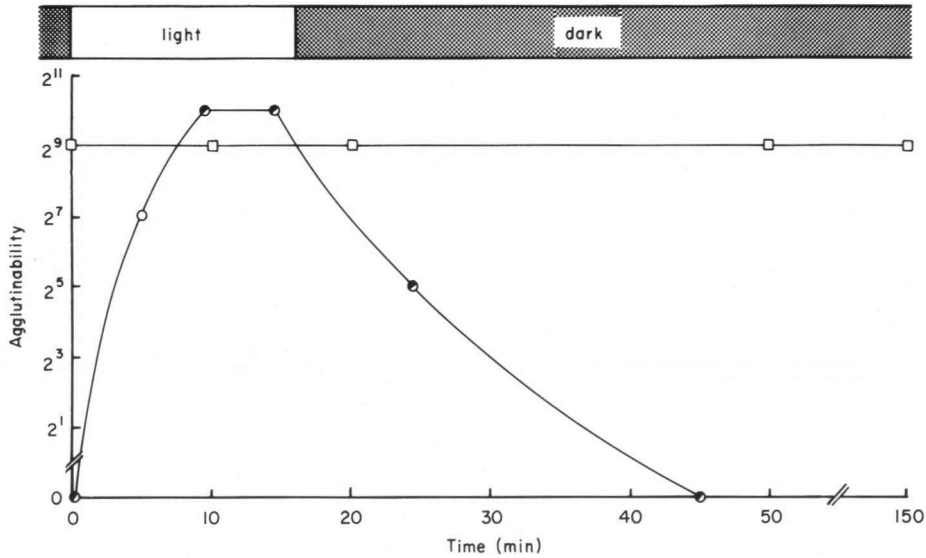

Fig. 10. Effect of light and darkness on the agglutinability of a light-sensitive mt$^+$ strain (○——○) and a light-insensitive mt$^-$ strain (□——□) of *Chlamydomonas eugametos*. (●——●) Mt$^+$ cells, preincubated with cycloheximide 50 min before illumination. Light bar = light period; shaded bar = dark period. From Kooijman *et al.* (1986) with permission.

mt$^-$ strain were extracted and subjected to fractionation by ion exchange chromatography, using a discontinuous salt gradient. Equivalent fractions enriched in agglutinin, taken from illuminated and non-illuminated cells, were subjected to SDS$^-$ polyacrylamide gel electrophoresis (Kooijman *et al.* 1988). Figure 11 shows that a typical agglutinin band was present in both fractions. The identity of the mt$^-$ agglutinin in extracts of flagella of non-illuminated cells was further confirmed by immunoblotting using mAb 66.3 that binds specifically to the agglutinin among the mt$^-$ flagellar components. It was shown that the mAb bound just as effectively to an agglutinin band from flagella of non-illuminated cells as to that from flagella of illuminated cells. We conclude that during activation/de-activation, agglutinins are neither incorporated nor lost, but are modified while remaining on the flagellar membrane. To test whether the mt$^-$ agglutinin from dark flagella was indeed inactive when completely free of all other flagellar proteins, it was purified by gel filtration in the presence of SDS. It remained inactive while the retention time was equivalent to that of purified active agglutinin. Therefore, the modification by light does not involve the formation of a complex with other high molecular mass components, but a structural modification that does not affect its molecular mass.

Another interesting outcome of this study is that the affinity towards mAb 66.3 is modulated by light. The antibody binds to inactive as well as to active mt$^-$ agglutinin on immunoblots but only after denaturation in SDS. In their native state, however, active and inactive agglutinins are readily distinguished by this antibody. When intact dark- and light-exposed mt$^-$ gametes were treated with FITC-labelled mAb 66.3, only the flagella of illuminated cells gave a positive reaction. In strains that are not light-requiring, the flagella of gametes could be labelled using this antibody at all times, whether illuminated or not. In other words, there is a strong correlation between the binding of the antibody and the activity of the agglutinin. In the native state, the antigenic site for mAb 66.3 is exposed when the molecule is active. When the native molecule is non-adhesive, the

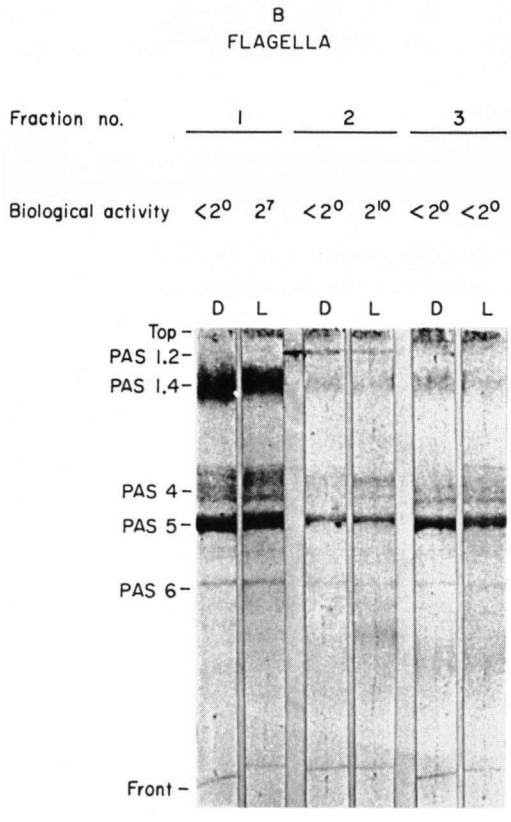

Fig. 11. Polyacrylamide gel electrophoresis in SDS of extracts of flagella of illuminated (L) and non-illuminated (D) gametes of *Chlamydomonas eugametos*. A cell suspension was divided into two equal parts. One suspension was placed in the dark and the other exposed to a regime of 12 h light and 12 h dark. After 40 h, the cells of both suspensions were isolated by centrifugation and extracted with 3 M guanidine thiocyanate. The extracts were partially purified by anion exchange chromatography. Two agglutinin-containing fractions (no. 1 and 2), and one fraction containing no agglutinin (no. 3), were desalted, freeze-dried and resuspended in water. After determination of the biological activity, the fractions were freeze-dried again and taken up in sample buffer and subjected to gel electrophoresis. The gel was stained with Periodic Acid Schiff Reagent. PAS 1.2 has been identified as the mt^- agglutinin. From Kooyman *et al.* (1988), with permission.

antigenic site is cryptic. When the molecule is denatured, the antigenic site becomes exposed.

The nature of the light-controlled modification of agglutinins is unknown. We hypothesize that it concerns a covalent modification, mediated by an ectoenzymic activity, such as a protein kinase and/or phosphatase, which becomes irreversible once the cell is killed or the agglutinin is extracted. The modification must be a subtle one, since it does not affect the mobility of agglutinins during SDS-polyacrylamide gel electrophoresis. It must result in a modification of the molecule by which the antigenic epitope becomes cryptic and at the same time the sexual adhesion site becomes inaccessible. The fact that the light-requirement can occur in mt^+ as well as mt^- strains suggests that the modified domain is common to both the mt^+ and mt^- agglutinin.

As yet, there is no answer to the question what is the nature and location of the photoreceptor. The action spectra, published by Lewin (1956) and Foerster (1957), and observations by R. Kooijman (personal communication), indicating peaks at 460 and

590 nm, gives no clue. It is logical to consider that the photosynthetic system is involved. However, the activation of gametes is achieved with fairly low fluence rates (0.2 W/ m^2), and is not inhibited by Diuron (3(3,4-dichlorophenyl)-1,1-dimethylurea), a well-known inhibitor of Photosystem II.

TIPPING

Goodenough & Jurivich (1978) observed that when gametes of *C. reinhardtii* were exposed to antisera raised against the flagellar surface, the antibodies did not bind uniformly over the flagella, but were concentrated at the tips. The authors interpreted this by assuming that bound antibodies are transported along the flagellar surface to the tip region. Recent work, using monoclonal antibodies against *C. eugametos* glycoporteins, has considerably extended this observation. Since it is possible to label specifically the agglutinins on flagella of mt^- gametes, it is possible to visualize how they are redistributed during sexual agglutination (Homan *et al.* 1987b). When gametes are mixed and at various times fixed with glutaraldehyde, one can then visualize the distribution of the mt^- agglutinin, using mAb 66.3 labelled with FITC. It then appears that before agglutination, the mt^- agglutinin is evenly distributed over the flagella but in the course of the agglutination process becomes concentrated at the flagellar tips. This change in antigen distribution is apparently conserved by fixation. After cell fusion, when gamete flagella de-agglutinate, this concentration of the label at the tips also disappears. The tip-oriented transport of antigens is not a mass-flow phenomenon. Abundant flagellar glycoproteins that are not involved in sexual adhesion can be labelled with other monoclonal antibodies (Homan *et al.* 1987a; see section on Anti-agglutin Antibodies). These do not appear to migrate laterally. So tipping is a specific process restricted to a few flagellar glycoproteins, including the agglutinins (Fig. 12).

Tipping can also be achieved artificially by treating living mt^- gametes with labelled mAb 66.3. One can then observe that the label is first reorganized into small patches over the entire length of the flagellar surface, which later (when the cells have been incubated for more than 5 min) become concentrated at the tips. The conclusion is that the agglutinins are transported to the flagellar tips as the result of the cross-linking action of the antibody (monovalent Fab fragments are ineffective). The extent to which cross-linking of agglutinins also occurs during sexual agglutination is not yet known.

Even though the mechanism of agglutination-dependent redistribution is obscure, it helps us to explain an outstanding feature of sexual agglutination. We have discussed the Sexual System in *Chlamydomonas* and that agglutination progresses from random spot contacts, involving any part of the flagellar surface, to a stage where partner flagella are adhered over their whole legnth, with their tips always associated. We can now explain this phenomenon by an adhesion-triggered redistribution of the agglutinins in the plane of the flagellar membrane to the tips. It is interesting to note that, notwithstanding the tip-oriented transport, a substantial portion of the agglutinin molecules remains distributed over the entire flagellum. This can be rationalized by assuming that this ensures that the complete flagellar surface remains agglutinable, because that is what we observe during sexual agglutination (see Fig. 12).

SEXUAL AGGLUTINATION TRIGGERS CELLULAR REACTIONS

The interaction between the flagellar surfaces of mating partners elicits a number of conspicuous cellular responses. The most predominant reactions in *C. eugametos* are: (1)

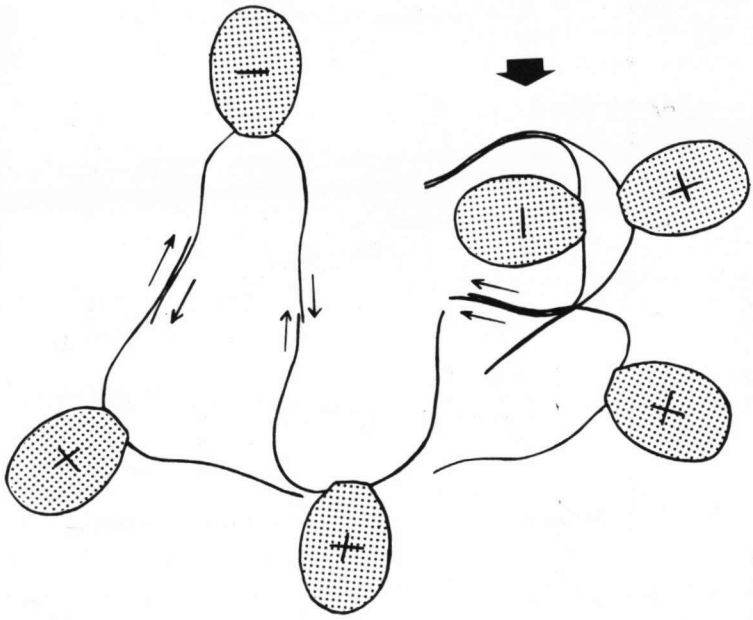

Fig. 12. Diagram to illustrate how tipping helps gametes of *Chlamydomonas eugametos* to sort themselves into pairs. The most stable contacts are realized by the alignment and the specific orientation of mt^+ and mt^- flagella (arrow). From Homan *et al.* (1987), with permission.

the arrest of the normal flagellar beat: the flagella start to 'twitch', the resulting loss of cell motility might assist in clump formation; (2) the accumulation of agglutinin molecules at the flagellar tips during sexual agglutination, called 'tipping' and; (3) the protrusion of a plasma papilla, which involves the local hydrolysis of the cell wall at the anterior end of the cells by which fusion eventually occurs.

It appears that one or more signals that trigger these cellular reactions are generated during sexual agglutination. We do not know how this happens but the results of using the monoclonal antibody mAb 66.3, described above, lead to some speculations. Treating mt^+ or mt^- gametes with this antibody evokes the same responses as does sexual agglutination. The amount of antibody needed to induce papilla formation is about 40 times higher than that required to induce twitching or tipping. This amount is similar to that needed to block the agglutinability of living mt^- gametes, implying that only after occupation of many agglutinin molecules is the induction signal strong enough to trigger the outgrowth of the papilla. On the other hand, monovalent Fab fragments do not exhibit any of these reactions. This indicates that it is the cross-linking of the agglutinins that activates the gametes. Since the described reactions could also be obtained with mAbs that interact with the agglutinins outside the binding region, it seems that general cross-linking is sufficient to produce the responses. Similarly, Goodenough & Jurivich (1978) found in *C. reinhardtii* that a polyclonal antiserum, raised against isolated flagella, induced a sexual response in gametes, whereas the Fab fragments from the same serum had no effect (equivalent polyclonal antisera raised against *C. eugametos* were not effective in this way). These results suggest that during sexual interaction, the agglutinins reorganize laterally in the plane of the flagellar membrane by mutual association. Somehow this results in the induction of an intracellular signal that induces the subsequent responses needed for fusion.

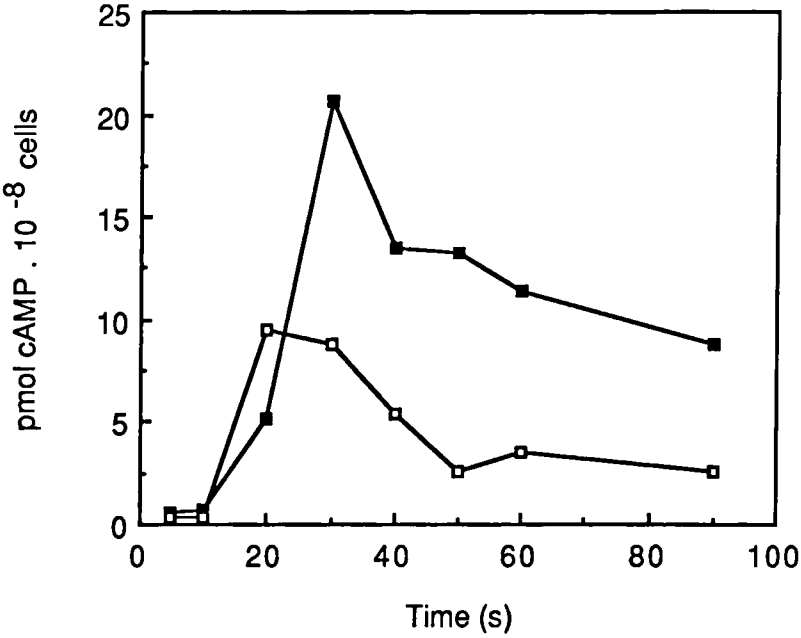

Fig. 13. Changes in intracellular cyclic AMP content in gametes of *Chlamydomonas eugametos*, incubated with isolated flagella of the other mating type. (■——■) mt^+ cells incubated with isolated mt^- flagella; (□——□) mt^- cells incubated with mt^+ flagella. From Pijst *et al.* (1984a), with permission.

What is the nature of the signal? Pijst *et al.* (1984a) observed a very rapid transient rise in the cellular content of cyclic AMP on mixing gametes of *C. eugametos*. This response could also be brought about by presenting isolated mt^+ flagella to intact mt^- gametes and vice versa, indicating that the rise is a direct consequence of flagellar interaction (Fig. 13). It preceded all morphological and physiological changes in the cells, prior to cell fusion. Significantly, a cyclic AMP-dependent protein kinase was found in cell homogenates, which led to the suggestion that cyclic AMP-mediated processes are part of a signal transduction system generated by sexual agglutination. Recently, these observations were extended by Pasquale & Goodenough (1987) in *C. reinhardtii*. They found a similar rise in cyclic AMP content after mixing mt^+ and mt^- gametes. The return of the cyclic AMP level to its original level coincided with the completion of the mating process by cell fusion. When a non-agglutinating mutant (*imp*-5 mt^+) was mixed with wild-type mt^- gametes, neither agglutination occurred nor was a cyclic-AMP increase observed. On the other hand, when a non-fusing mutant (*imp*-1 mt^+) was mixed with wild-type mt^- gametes, the cells agglutinated for a prolonged time because cell fusion was blocked. In this mixture the cyclic AMP level did not return to its original level. This again demonstrates that the rise in cyclic AMP is a direct result of sexual agglutination and that cyclic AMP is an intracellular messenger in *Chlamydomonas*. This is made even more probable by the finding that single mt^+ and mt^- gametes respond to exogenous cyclic AMP (accompanied by inhibitors of phosphodiesterase) by exhibiting various responses, including tipping and papilla formation, and, in the case of flagella-less mutants, are able to fuse without flagellar agglutination.

The free calcium level also rises rapidly upon agglutination, as has been observed in *C. reinhardtii* by Bloodgood & Levin (1983). Kaska *et al.* (1985) obtained evidence by

X-ray analysis that calcium is concentrated in a few cellular locations (presumably granules), and is dissipated soon after the start of sexual agglutination. In *C. eugametos* an increase in intracellular free calcium content might be functional in the arrest of the flagellar beat, at the start of sexual agglutination, because the calcium level in flagella is known to affect their motility (Hyams & Borisy, 1978). It is also possible that intracellular free calcium plays a role in relatively late processes before cell fusion, particularly in the secretion and/or activation of enzymes involved in cell wall hydrolysis.

CONCLUSIONS AND SPECULATIONS

Agglutinin molecules are redistributed to the flagellar tips directly after the start of sexual agglutination in *C. eugametos*. We argue that this redistribution is the consequence of an aggregation of agglutinin molecules, since it can be mimicked by treating flagella with antibodies, which by their multivalent character have a cross-linking action. Since it is hard to understand how this redistribution could arise as a consequence of cell–cell contacts *per se*, one must assume that submembrane cytoskeletal elements are involved that are associated with integral membrane constituents connected to the agglutinins. The reorganization of these elements, triggered by sexual contact, would result in the lateral movement, aggregation and ultimately tipping of agglutinins at the flagellar surface. Therefore, we envisage that a signal, elicited by sexual contact, has an inductive action on this cytoskeleton-mediated agglutinin redistribution. Moreover, it appears that artificially induced clustering of agglutinins, by agglutinin-directed antibodies, produces all the responses that are also invoked by normal sexual agglutination. This leads to the view that agglutinin aggregation is an essential part of signalling in *Chlamydomonas*. There are several other systems where receptor aggregation has been shown to be involved in signalling, such as in insulin action in vertebrate cells (for example Kahn *et al.* 1978; Schlessinger 1980).

One could envisage that gametic contact-induced and cytoskeleton-driven agglutinin aggregation leads to an amplification of signal generation. This would explain the fact, emphasized by Pasquale and Goodenough (1987), that although isolated flagella of different mating type adhere together, no detectable signal (in the form of a rise in cyclic AMP content) is observed. In such flagella, the cytoskeleton-linked agglutinin redistribution is probably inoperative, and therefore, no signal amplification would occur. Agglutinin aggregation in the plane of the membrane might also be functional in enhancing flagellar adhesiveness, in a similar way to that found in human neutrophil cells by Detmers *et al.* (1987). These authors found that in such cells a transient stimulation of the ability to bind complement, induced by treatment with phorbol ester, is tightly correlated with the state of aggregation of the complement receptor in the plane of the membrane. The authors hypothesize that clustered receptors and ligands have a higher apparent affinity towards each other because their dissociation rate is decreased. Dissociation of a cluster of ligand molecules from a cluster of receptors will only occur after simultaneous dissociation of all the individual receptor-ligand pairs. If a similar system is operative in *Chlamydomonas*, it would explain the rise in agglutinability after the start of the mating process (described in the section on Sexual Agglutination Triggers Cellular Reactions). Of course, such a view can only be maintained if it is established that this rise in agglutinability is not caused by an increase in agglutinin content of the flagella.

Since sexual agglutination can be by-passed by administering cyclic AMP derivatives, the conclusion seems warranted that cyclic AMP is one of the first constituents of the

signal transduction pathway. How the transient intracellular rise in cyclic AMP content is brought about by sexual agglutination must await further experimentation. As discussed above, agglutinin aggregation might be an important element in this process, which could lead to the activation of calcium channels (cf. section on The Flagellar Surface). The resultant increase in calcium influx in the flagella of agglutinating cells could have a regulatory effect on the adenylate cyclase and/or phosphodiesterase activity on the flagellar surface. Trifluoroperazine, an inhibitor of calcium–calmodulin dependent reactions, inhibits the cyclic AMP release as well as the mating response (Pasquale & Goodenough 1987). Another possibility is that agglutinin aggregation results in the activation of adenylate cyclase molecules via G proteins (e.g. Levitsky 1987). In addition, the target of cyclic AMP as a second messenger must be explored. Pijst *et al.* (1984a) found evidence for a cyclic AMP-dependent protein kinase in *C. eugametos*, while Pasquale & Goodenough (1987) observed an inhibitory action of the specific protein kinase inhibitor H-8 in *C. reinhardtii*. This supports the contention that in both species protein kinase-mediated phosphorylation reactions are operative in the signal transduction, which ultimately result in the reactions which prepare the cells for sexual fusion.

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