Control of the Mating Competence Rhythm in *Chlamydomonas eugametos*

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Gametes of *Chlamydomonas eugametos* lose their mating competence during the light periods of a 24 h light/dark (LD) cycle, and reacquire it in the dark periods. A diurnal rhythm in sexual agglutinability of the flagella underlies these fluctuations. Under constant environmental conditions the oscillation persists as an endogenous circadian rhythm. Under natural environmental conditions the rhythm is cooperatively driven by endogenous and exogenous signals. Exogenous control includes a decline in agglutinability upon illumination, which can be countered by Diuron, and a rise in agglutinability upon lowering of the temperature. In LD, pronounced fluctuations of the external pH are found that can be blocked by Diuron. The pH rhythm does not interfere with the agglutinability rhythm. The control of other rhythms in *Chlamydomonas* is discussed. Assexual agglutinability is due to well-characterized agglutinins located on the flagellar membrane, this biological rhythm lends itself to further exploration at the molecular level.

INTRODUCTION

*Chlamydomonas eugametos* is a unicellular oval-shaped green alga, propelled by two flagella. It reproduces vegetatively by sporulation, whereby the cell divides into two, four or eight daughter cells (Demets et al., 1985). Upon nutrient deficiency, a final division takes place to produce gametes (Tomson et al., 1985). The gametes are morphologically identical to the vegetative parents, but exhibit a specific mating reaction when confronted with gametes of the opposite sex. The mating reaction comprises the following steps. As soon as partners recognize each other, using their flagella as sensors, the contact is consolidated by agglutination. By readjustments of the points of flagellar adhesion, the anterior ends of the cell bodies are brought to close proximity. Then, the two gametes partially fuse by the formation of a common plasma bridge, after which they resume swimming as a tandem organism, called a vis-à-vis pair. Several hours later, the paired cells fuse completely into a zygote (Lewin, 1952; Mesland, 1976).

*C. eugametos* is isogamous, which means that the gametes of the two sexes are morphologically identical. The sexes are referred to as mating type plus (*mt*⁺) and mating type minus (*mt*⁻). Each mating type possesses a specific set of agglutinins, located extrinsically on the flagellar membrane of the gametes, which have been purified and characterized (Musgrave et al., 1981; Homan et al., 1982; Klis et al., 1985). The agglutinins are large linear glycoproteins as shown by electron micrographs (Klis et al., 1985; K. J. Crabbendam, personal communication). They play a key role in the gamete–gamete contact.

Recently we reported regular daily fluctuations in mating competence of *C. eugametos* (Tomson et al., 1985). Evidence presented in this paper shows that these variations are caused by an endogenous rhythm in sexual agglutinability of the flagella.

Abbreviations: Diuron, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; LD, 12 h light/12 h dark; LL, continuous light; DD, continuous darkness.
METHODS

Gametes. Chlamydomonas eugametos strains UTEX 9 (mt+) and UTEX 10 (mt-) from the Algal Collection at the University of Texas at Austin, Texas, USA, were grown in Petri dishes on agar as described by Mesland (1976). Gamete suspensions were obtained by flooding 2-week-old cultures, grown under a 12 h light/12 h dark regimen, with distilled water.

Test conditions. All experiments were done at 19 °C unless stated otherwise. Illumination was provided by white fluorescent tubes at 5500 lx (65 μE s⁻¹ m⁻²). The gamete suspensions were placed, according to the experimental requirements, in a 12 h light/12 h dark regimen (LD), in continuous light (LL) or in constant darkness (DD). All experiments were repeated at least once, using different batches of gametes.

Quantification of flagellar agglutinability. Gametes were fixed in glutaraldehyde (final concn 1.25%, v/v) for 10 min and washed twice in double-distilled water. The killed cells were mixed on a slide with live gametes of the opposite mating type, giving an agglutination reaction that was observed under a light microscope. The reaction was quantified by testing a binary dilution series of the fixed material. When the agglutinability of a sample of fixed cells is referred to for instance as 2^2, this means that up to the fifth twofold dilution step of this sample, a positive agglutination reaction was scored when a constant quantity of living partner gametes was added. This semi-quantitative bioassay has proved to produce reproducible results.

Mating competence test. To determine the percentage of sexually competent gametes in a given suspension, a sample was mixed with gametes of the opposite mating type in a ratio of 1:9, in triplicate, at 19 °C for 1 h, in the light. Total cell density was always 1-2 x 10⁶ cells ml⁻¹. The mixture was then fixed with glutaraldehyde (final concn 1.25%, v/v) and counted for pairs (p) and single cells (s). The quotient 10p/2p+s gives the fraction of competent gametes of the minor partner in the 1:9 mixtures. Full details of the test are given by Tomson et al. (1986).

Inhibition of photosynthesis. A 0.1 ml volume of 1 mM-Diuron in ethanol was added to 10 ml gamete suspension. This was sufficient to inhibit photosynthesis by at least 90%, as determined by the inhibition of O₂ production, using an oxygraph. The controls in these experiments also contained 1% (v/v) ethanol.

pH measurements. The external pH in gamete suspensions was continuously measured by a pH meter (Philips PW 9409), coupled to a recorder. A multiple set-up of eight pH electrodes registered pH oscillations in test suspensions and controls at the same time. The pH in each individual suspension was recorded every 16 min by an automated system.

RESULTS

Circadian rhythm in flagellar agglutinability

Under LD conditions the mating competence of the gametes of both mating types of C. eugametos shows a diurnal periodicity (Fig. 1a). At the beginning of each light period, most cells are able to form a pair with a gamete of the opposite mating type. During the course of the light period, the percentage of competent cells steadily declines, often to zero. In the dark periods, the...
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Gametes regain their ability to pair. To explain these fluctuations, the assumption was made that the competence was slowly suppressed by light. This idea originated from experiments on gametogenesis that showed that only minor amounts of competent gametes were produced as long as cultures were grown in LL (Tomson et al., 1985). If this hypothesis were valid, the mating competence should have stayed permanently high when the cells were placed in continuous darkness. In practice, the rhythm persisted in DD (Fig. 1). However, after a few cycles in DD, the oscillation faded out, coinciding with the loss of vitality of the phototrophic cells, being deprived of light. As all external conditions had been kept constant, this meant that the competence rhythm originated from an endogenous oscillation. Since the period of the rhythm was close to 24 h, the oscillation must be considered as an endogenous, circadian rhythm (Bünning, 1960).

We previously established that in LD, the fluctuations in mating competence ran parallel to fluctuations in sexual agglutinability (Tomson et al., 1985). Fig. 2 shows that in DD also, at a constant temperature of 19 °C, flagellar agglutinability and mating competence remained closely associated. As flagellar agglutinability is a prerequisite for successful mating, we concluded that we were in fact looking at just one endogenous rhythm, that of flagellar agglutinability.

Gametes were obtained from agar cultures that had been grown in LD. When the light and dark periods of this LD regimen were reversed, a disturbed, non-rhythmic competence pattern was found after one LD cycle, probably representing a transient stage of adjustment. After five reversed LD cycles, gametes showed a phase-reversal in the competence rhythm, corresponding to the newly imposed LD regime and running 12 h out of phase to the subjective LD regime (data not shown). This entrainability by external synchronizers is a generally accepted property of circadian rhythms (Edmunds, 1982).

**Negative effect of light**

Comparison of Figs 1(a) and 1(b) shows that although the rhythm persisted in DD, the oscillations were more pronounced in LD. This supports the initial hypothesis, that light has a negative influence on mating competence. This idea was tested by placing gametes of high competence in LL. The competence declined to an almost steady 0%, indicating that there was indeed an adverse effect of light (Fig. 1c). Apparently in LD (Fig. 1a), the endogenously generated decline in mating competence was reinforced by the light. Upon placing the cells back in darkness, the competence returned to normal, showing that the prolonged illumination had done no irreversible damage (Fig. 1c). The association between agglutinability and competence...
Table 1. Effect of light and Diuron on flagellar agglutinability and mating competence in C. eugametos

Gamete suspensions were kept in LD. After a standard 12 h light period, series (c) and (d) were maintained in light and Diuron was added to series (b) and (d). Agglutinability and mating competence were determined 12 h later.

<table>
<thead>
<tr>
<th>Illumination</th>
<th>Addition of 10 μM-Diuron</th>
<th>Agglutinability titre</th>
<th>Mating competence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) LD</td>
<td>no</td>
<td>2&lt;sup&gt;o&lt;/sup&gt;</td>
<td>68</td>
</tr>
<tr>
<td>(b) LD</td>
<td>yes</td>
<td>2&lt;sup&gt;e&lt;/sup&gt;</td>
<td>71</td>
</tr>
<tr>
<td>(c) LL</td>
<td>no</td>
<td>2&lt;sup&gt;i&lt;/sup&gt;</td>
<td>40</td>
</tr>
<tr>
<td>(d) LL</td>
<td>yes</td>
<td>2&lt;sup&gt;e&lt;/sup&gt;</td>
<td>70</td>
</tr>
</tbody>
</table>

was also found in LL. Table 1 shows that the cells largely lost their agglutinability and competence in LL, though the negative effect in this experiment was less dramatic than that represented in Fig. 1(c). To investigate whether photosynthesis was involved in this light effect, gametes were placed in LL in the presence of Diuron to block photosynthetic activity. Agglutinability and competence were maintained at a significantly higher level than in the controls (Table 1). Therefore, it was concluded that the negative effect of light was linked to photosynthetic activity and that the Diuron treatment mimicked the effect of placing the cells in the dark.

**Rhythm in external pH**

Upon illumination of gamete suspensions, a strong rise in the external pH was found, with a decline to the original level in the dark periods. The rhythm was extremely pronounced: the fluctuations in the (non-buffered) gamete suspensions spanned more than four pH units (Fig. 3a). The rhythm was completely blocked by Diuron (Fig. 3b), indicating that it was caused by photosynthetic activity. No pH rhythm was found in LL or DD, where the pH was constant at 10.5 and 6.5 respectively (Fig. 3c, d). An attractive explanation for the photosynthesis-linked loss of agglutinability in LL was the possibility that flagellar agglutinability was destroyed by the excessively high external pH. However, when the mating process was tested over the pH range...
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Fig. 4. Effect of temperature on pair formation in C. eugametos. A batch of gametes was split into five equal portions that were mixed for 1 h at various temperatures with gametes of the opposite mating type. No pre-incubation had taken place, so the gametes were equally agglutinable at the beginning of the test.

Table 2. Combined effect of pre-incubation temperature and test temperature on pair formation in C. eugametos

Pre-incubation started at the beginning of a dark period of the standard LD regime and lasted for 18 h. The percentage of paired cells formed within 1 h upon mixing of the partners is given.

<table>
<thead>
<tr>
<th>Test temp.</th>
<th>Pre-incubation temp.:</th>
</tr>
</thead>
<tbody>
<tr>
<td>19 °C</td>
<td>9 °C</td>
</tr>
<tr>
<td>92%</td>
<td>43%</td>
</tr>
<tr>
<td>9 °C</td>
<td>42%</td>
</tr>
</tbody>
</table>

6–11 by resuspending gametes in various buffers (10 mM-Tris, HEPES, glycylglycine and histidine) a normal agglutination reaction was seen at all pH values, in all buffers. Even when the gametes had been pre-incubated for 12 h in a particular buffer, no inhibition of agglutination was detected. Moreover, gametes lost their competence in LL when the medium was buffered at pH 7.0 (data not shown). It was concluded that the loss of agglutinability induced by light is not due to changes in external pH.

Temperature effects

Temperature shifts can cause marked changes in endogenous rhythms, varying from phase-shifts to changes in the amplitude of the oscillation (Sweeney & Hastings, 1960). When gametes were transferred from 19 °C to 9 °C in DD, an unexpected strong increase in flagellar agglutinability was observed (Fig. 2). The fact that the work on Chlamydomonas gametes is done at 19 °C arises from the general experience that the cells tend to lose their mating competence at room temperature. Therefore, the effect of temperature on agglutinability may well span a wider range than had previously been assumed. The lower the temperature, the more agglutinable the cells seem to become. Fig. 2 shows that adaptation to the temperature drop took place rather slowly, and was interwoven with the diurnal rhythmicity. In view of the increase in agglutinability, it was expected that the sexual competence of the gametes would also be enhanced at 9 °C. This was confirmed by the simple test shown in Table 2. A batch of gametes was split into two; one half was kept overnight at 19 °C, the other at 9 °C, and mating competence was then tested at 19 °C in both cases. The gametes that had previously been kept in the cold formed about twice as many pairs (92%) as those that had been maintained at 19 °C (43%). The higher overall agglutinability in the pre-cooled population resulted, as expected, in a higher percentage of gametes being able to fuse with a partner. The experiment also revealed that the enhanced mating competence that had gradually built up in the cold was not immediately destroyed when the cells were re-exposed to 19 °C. This again suggests that the agglutinability adjusts only slowly to new temperatures. The standard test temperature of 19 °C was chosen in view of the fact that cell fusion is limited at lower temperatures (Weiss et al., 1977; Mesland & van den Ende, 1979; Fig. 4). This was also observed when gametes were pre-
incubated at 9 °C before fusion. Table 2 shows that in spite of the high agglutinability, pair formation within 1 h at 9 °C (42%) was almost equal to that in the control at 19 °C (43%). Obviously, at 9 °C the benefit of the increased agglutinability was nullified by the limited ability to fuse.

**DISCUSSION**

A diurnal periodicity in mating competence, characterized by a maximum at the start of the light period, has been found in numerous species within the Volvocales, including *Astrophomene gubernaculifera* (Brooks, 1966), *Chlamydomonas moewusii* (Wiese, 1984), *Chlamydomonas eugametos* (Tomson et al., 1985; Wiese, 1984), *Pandorina unicozza* (Rayburn, 1974), *Pandorina morum* (Coleman, 1979), *Volvulina steinii* (Carefoot, 1966) and *Volvulina pringsheimii* (Starr, 1962). As far as we know, there have been no reports on the control mechanism of these rhythms.

In *C. eugametos*, a circadian rhythm in sexual agglutinability of the flagella evidently underlies the mating competence rhythm. Like most biological rhythms, the agglutinability rhythm is driven endogenously in concert with exogenous influences (Aschoff, 1960). In DD, at a constant temperature, the rhythm is solely endogenously controlled. In LD, two exogenous effects interfere: (1) a suppressive effect of photosynthesis enhances the decline in agglutinability; (2) a temperature effect; since under non-laboratory conditions the light source modulates the temperature, diurnal temperature changes presumably play an additional role in the rhythm. Agglutinability rises upon lowering the temperature; this effect may cooperate with the endogenous component of the rhythm that restores agglutinability at night under natural day/night conditions.

Observations on various batches of gametes, of both mating types, suggested that the relative contributions of the three effects (the endogenous oscillation, the light effect, the temperature effect) may vary. Generally, all three effects were prominently present but sometimes one of them was almost lacking. In exceptional cases, the entire diurnal rhythm was hardly detectable.

Several diurnal rhythms have been discovered in *Chlamydomonas*. It is well known that the vegetative division cycle can be phased in LD, resulting in the synchronous release of daughters from the mother cells at every dark/light transition (Lloyd et al., 1982; Demets et al., 1985). While Straley & Bruce (1979) have demonstrated that the division rhythm is endogenously controlled, as it persists in DD (provided the cells are fed supplemental acetate), Spudich & Sager (1980) have stated that the rhythm is brought about exogenously by direct (phasing) effects of the alternating light and dark periods on the progress of the cells through the division cycle. It seems more likely that each report covers a complementary part of the control mechanism and that the cell division rhythm in LD is controlled by a combination of endogenous and exogenous signals. A combined endogenous/exogenous control is very common in biological rhythms, but the phenomenon is often overlooked (Hoffmann, 1976).

Other well-known diurnal rhythms in *Chlamydomonas* include photosynthesis and phototaxis. Of course, both rhythms are, in LD, primarily exogenously controlled, for both are strictly light-dependent processes. Still, in continuous darkness (interrupted by short exposures to light) persisting endogenous rhythms of photosynthetic capacity (Hoffmans-Hohn et al., 1984) and phototaxis (Bruce, 1970) have been found.

In the cases of the cell division cycle, photosynthesis and phototaxis, the phasing of the endogenous control corresponds to the phasing of the exogenous influences. The functional significance of this co-phasing is evident: *Chlamydomonas* adapts to the LD cycles in order to make optimal use of the offered light. To rationalize the mating competence rhythm is more difficult. Although the endogenous control perfectly matches the exogenous influences, the functional significance of the mating competence rhythm with respect to the sexuality is obscure, as the daily decline of competence undoubtedly diminishes the rate of sexual reproduction.

In LD, a marked rhythm in the external pH was detected. This pH rhythm was found in vegetative cultures as well. Similar pH rhythms have been reported for other species of the
Volvox Volvocales (Rayburn & Starr, 1974; Coleman, 1979; Hoffmans-Hohn et al., 1984). In C. eugametos, this rhythm proved to be linked to photosynthetic activity. We have found no indications of the involvement of nitrogen salts in this rhythm, as was suggested by Coleman (1979) to explain the pH rhythm in *P. morum*. We suppose, instead, that the cells convert HCO₃⁻ into CO₂ and OH⁻ ions during the light periods, just as *C. reinhardtii* does (Tsuzuki, 1983). The OH⁻ ions are exported, causing the rise in pH, while the CO₂ is retained for assimilation.

No clear-cut explanation for the rise in agglutinability upon a temperature drop has been found, but the turnover of the agglutinins may play a role in this process. A slowed-down degradation of the agglutinins in the cold perhaps leads to (temporary) accumulations of agglutinins at the flagellar surface. Preliminary results indicate that after a shift-down in temperature the agglutinability rhythm persists in DD without much alteration in the period. A temperature-compensated period length is a common feature in many endogenous rhythms (Sweeney & Hastings, 1960).

Besides the slow decline in agglutinability upon prolonged illumination reported here, some strains of *C. eugametos* interestingly show an additional, rapid reaction to light (Kooijman et al., 1987; Wiese, 1984). In these cases light is needed to activate the agglutinin molecules before sexual agglutinability can be expressed. These strains are, consequently, sexually inactive in DD. By interrupting the DD regime with short light intervals, enough to activate the cell (15 min), we established that the endogenous rhythm is present in these strains as well. The conclusion is that this light-dependent on/off switch must be envisaged as a final regulatory step, that results in the expression of agglutinability at a level that is pre-set by the circadian rhythm.

The circadian rhythm of flagellar agglutinability in *Chlamydomonas* reported here may be a useful, simple model system. In contrast to the cell-cycle-related phenomena (Straley & Bruce, 1979; Spudich & Sager, 1980; Donnan & John, 1983) the rhythm can be studied in one generation of non-dividing individuals. Moreover, monoclonal antibodies have recently been raised against the agglutinins and other components of the flagellar surface. This offers the attractive possibility of probing this biological rhythm at the molecular level.

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**REFERENCES**


