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Cysteine as a fusion inhibitor in the sexual mating reaction of the green alga *Chlamydomonas eugametos*

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SUMMARY

Sexual mating of the biflagellated green alga *Chlamydomonas eugametos* is initiated by flagellar adhesion. This is followed by the formation of *vis-à-vis* pairs in which two cells of opposite mating type are connected by a slender cytoplasmic bridge. This neck slowly widens and, finally, zygotes are formed. *Vis-à-vis* pair formation was reversibly inhibited by cysteine. This effect was only observed when cysteine was present during the first few minutes of mating. Cysteine specifically prevented the protrusion of a plasma papilla between the flagellar bases; other early mating reactions tested were not affected. When cysteine was added to *vis-à-vis* pairs, the neck did not widen.

It is argued that cysteine inhibits an autolysin with a protease character involved in dissolving the cell wall between the flagellar bases during early mating and in widening the neck of *vis-à-vis* pairs.

Key-words: cell fusion, *Chlamydomonas*, sexual mating.

INTRODUCTION

In the unicellular biflagellated alga *Chlamydomonas*, sexual reproduction starts with a highly specific recognition reaction. The flagella of sexually competent cells (gametes) of opposite mating type (referred to as mt^+ and mt^-) adhere to each other; this reaction is mediated by mating type-specific macromolecules (agglutinins) at the flagellar surface. Several reports about the characterization of the agglutinins of *C. reinhardtii* (Adair *et al.* 1982; Saito & Matsuda 1984; Collin-Osdoby & Adair 1985), *C. eugametos* (Musgrave *et al.* 1981; Samson *et al.* 1987a) and *C. moewusii* (Samson *et al.* 1987b) have been published. They have been visualized by electronmicroscopy (Crabbendam *et al.* 1984; Goodenough *et al.* 1985; Samson *et al.* 1987b). Interaction of agglutinins with molecules at the flagellar surface of the partner cells is thought to generate the signals that induce further reactions. The early reactions prepare the gametes for the fusion of two cells of opposite mating type (for review see Van den Ende 1985 and Van den Ende *et al.* 1988). In *C. eugametos* the initial reactions include the adhesion of the gametes via their flagella (agglutination), formation of cell aggregates, twitching of the flagella of the agglutinating cells, contact-induced stimulation of the agglutinability, transformation of the flagellar tip, breakdown of cell wall material between the flagellar bases coupled with the protrusion of plasma papillae at the same location. Plasma papillae of two partner cells then fuse and the cells form a so-called *vis-à-vis* pair, which eventually becomes a zygote. To study the early, quickly proceeding steps of the mating process, it is useful to be able to inhibit the fusion

of the gametes. In *C. reinhardtii* several fusion-defective mutants have been used (Goodenough *et al.* 1976; Forest 1983). For *C. eugametos* and *C. moewusii* such mutants are unavailable, but several enzymes and chemical agents affect the fusion process (Wiese & Jones 1963; Wiese *et al.* 1984). However, some of these reagents (i.e. thiol inhibitors such as β-mercaptoethanol) also influence the initial agglutination when applied for a longer period of time, making them less suited for further study. Mesland & Van den Ende (1978) described a fusion inhibitor, released in the medium of acid-treated mt^- gametes of *C. eugametos*. However, no further details have been reported about the characterization or physiological significance of this inhibitor.

In this article we show that the amino acid cysteine inhibits the fusion of mating gametes of *C. eugametos* without affecting the early steps of the mating reaction that precedes the formation of plasma papillae. Addition of cysteine kept the cells arrested in the agglutination phase of the mating reaction. Furthermore, we discuss evidence that the effects of cysteine are related to the inhibitory effect on the gamete wall-autolysin, the enzyme which locally degrades the cell wall to allow the penetration of the plasma papillae and the subsequent widening of the plasma bridge.

MATERIALS AND METHODS

Materials

Cysteine was purchased as a free base preparation from Sigma, St. Louis (USA). Hippuryl-arginine was from Boehringer, Mannheim (FRG).

Cell cultures

Chlamydomonas eugametos strains UTEX 9 (mt^+) and UTEX 10 (mt^-) from the Algal Collection at the University of Texas at Austin, USA, were cultivated in Petri dishes on agar-containing medium in a 12 h light/12 h dark regimen, as described by Mesland (1976). Gamete suspensions were obtained by flooding 2- to 3-week-old cultures with distilled water just before the dark period and harvesting the cells at the beginning of the next light period.

Determination of pair formation

To determine pair formation, a mixture of mating gametes was fixed, 1 h after mixing, with glutaraldehyde (final concentration 1·25% (v/v)), which disrupted agglutinating cell clumps but not *vis-à-vis* pairs. Free cells and pairs (about 300 cells) were counted in a haemocytometer and the percentage of paired cells was calculated. At room temperature formation of pairs was complete within 1 h.

Preparation of mating medium

Mt^+ and mt^- gamete suspensions were separately centrifuged at 200 *g* for 2 min. The concentrated suspensions (final density about 10^8 cells ml^{-1}) were mixed in a Petri dish and placed under a white fluorescent tube at 22°C. After 30 min the incubation was stopped and Tris-HCl (final concentration 10 mM, pH 7·5) was added. The cell-free supernatant, referred to as mating medium, was prepared by centrifugation at 10 000 *g* for 10 min at 4°C. Cell-free supernatants of gametes of both mating types were prepared in the same way.

Hydrolysis of gamete cell walls

Gamete cell walls were prepared as described by Musgrave *et al.* (1979) using 50 mM Tris-HCl, containing 1 mM EDTA and 5 mM phenylmethylsulphonyl fluoride at pH 7·8, as the buffer. The purified cell walls were labelled with ^{125}I by the chloramine-T method of Bailey (1984). Radioactively labelled cell walls were centrifuged at 10 000 *g* for 15 min through a 2·93 M CsCl cushion and the pellet was washed with ethanol, chloroform/methanol (2:1, v/v) and acetone, respectively, to remove membranous material. The washed cell walls were suspended in 0·1% (w/v) sodium azide solution and stored at 4°C. Freezing and thawing of the isolated cell walls was avoided, since it caused considerable breakdown into small fragments and solubilization of labelled material. Cell wall hydrolysis was assayed as follows: 0·5 ml mating medium was incubated with 40 µl radioactively labelled cell wall suspension (about 350 Bq per incubation) in a shaking water bath at 25°C. At the end of the reaction time the mixture was centrifuged at 10 000 *g* for 15 min and 50 µl was taken from the meniscus. Radioactivity was measured against appropriate controls.

Determination of gamete agglutinability

Agglutinability was assayed as described by Tomson *et al.* (1986). A suspension of gametes was fixed with glutaraldehyde (final concentration 1% (v/v)), washed and then diluted in a binary dilution series. Each dilution was tested with a constant amount of partner cells. Agglutinability was expressed as the titre of the last dilution that still showed agglutination.

Determination of papilla formation

Formation of papillae was examined as described by Musgrave *et al.* (1986) using indirect immunofluorescence as described by Lens *et al.* (1980). Monoclonal antibodies (mAb 44·2) specific for the used strain of mt^- gametes (Homan *et al.* 1987b) were used.

Microscopical observations

Specimens were observed and photographed using a Zeiss (Oberkochen, FRG) photomicroscope fitted with phase-contrast optics.

RESULTS

Inhibition of vis-à-vis *pair formation by cysteine*

Fusion of mating gametes of *C. eugametos* was inhibited in a concentration-dependent way by the amino acid cysteine. Figure 1 shows that the formation of *vis-à-vis* pairs is completely abolished at a concentration of 1 mM cysteine. *Vis-à-vis* pair formation could also be inhibited by hippuryl-arginine, which will be discussed in the next section. To investigate the reversibility of the inhibition by cysteine, gametes of opposite mating type were mixed in the presence of 1 mM cysteine. Fusion did not occur but the cells agglutinated normally. When cells were washed out of cysteine and resuspended in conditioned medium they partly recovered their ability to fuse. Figure 2 shows the formation of *vis-à-vis* pairs when cysteine was washed away at 0, 5, 20, 40 and 90 min after mixing the gametes. These results indicate that the inhibition by cysteine was reversible, but the percentage of *vis-à-vis* pairs clearly depended upon the length of the period that cysteine was present. As illustrated in the inset of Fig. 2, the percentage of *vis-à-vis* pairs decreased to about 45% of the control when cells had agglutinated in the presence of cysteine for 1 h

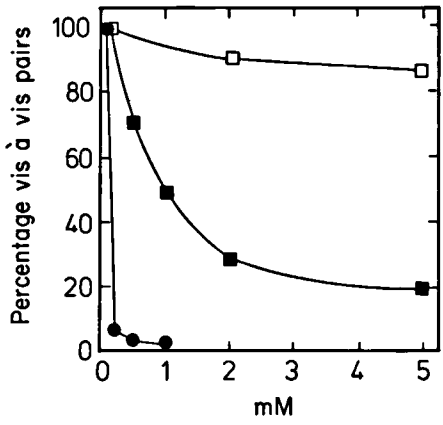

Fig. 1. Formation of *vis-à-vis* pairs after mixing gametes of opposite mating type of *C. eugametos* in the presence of various concentrations of cysteine (●) and hippuryl-arginine (□,■). Pair formation was scored after 1 h (●,■) or after 5 h (□).

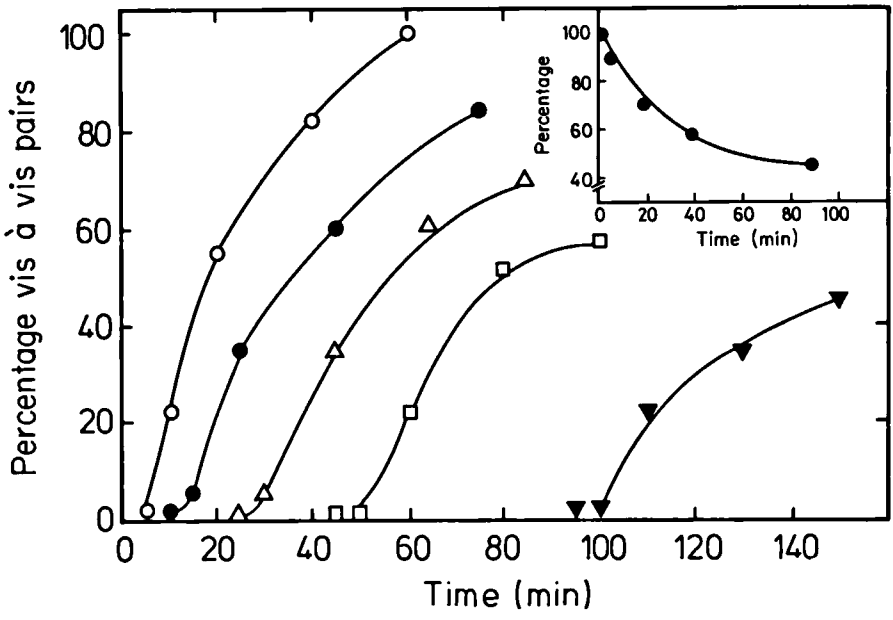

Fig. 2. *Vis-à-vis* pair formation in *C. eugametos* when cysteine was washed away at times 0 (○), 5 (●), 20 (△), 40 (□) and 90 min (▼) after mixing the gametes in the presence of 1 mM cysteine. The inset gives the maximal percentage of pairs formed 60 min after washing related to the point of time when cysteine was washed away.

or more. Removing cysteine at 120 min resulted in the formation of about 40% of the control (results not shown). To study the effect of cysteine in time, we added the amino acid to a final concentration of 2 mM at different periods after mixing the gametes of opposite mating type. Figure 3 indicates that the addition of cysteine at the very moment of mixing the gametes resulted in the total abolishment of fusion, but that addition 5–10 min later had a negligible effect.

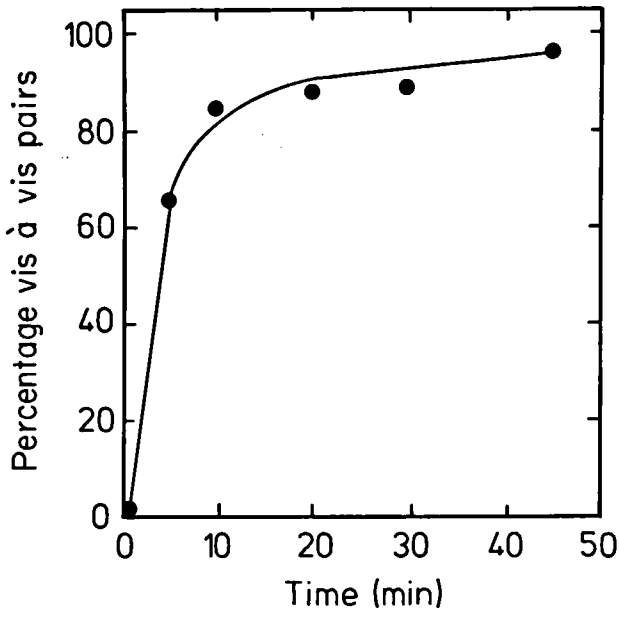

Fig. 3. Formation of *vis-à-vis* pairs of *C. eugametos* when cysteine (final concentration 2 mM) was added at the time indicated on the abscissa after mixing the gametes of opposite mating type. Pair formation was scored after 1 h and expressed as the percentage of the maximal pair formation in the absence of cysteine.

Table 1. Early mating reactions of gametes of *C. eugametos* in the presence of 1 mM cysteine

Adhesion	normal	⩾4 days
Stimulation of agglutinability	yes	⩾5 h
Twitching	normal	⩾4 days
Flagellar tip transformation	yes	⩾24 h
Orientation of cells in clump	normal	⩾4 h
Tipping	yes	⩾1 h
Formation of papillae	no	

Effect of adding cysteine upon early mating reactions

As indicated above, gametes agglutinated normally in the presence of cysteine. Table 1 shows that a concentration of 1 mM of cysteine did not impair most of the reactions that prepare the cells for fusion. Adhesion of the gametes of opposite mating type happened normally and continued for more than 4 days if the cells were placed in continuous light.

Contact-induced stimulation of gamete agglutinability took place as described by Tomson *et al.* (1986) and persisted for at least 5 h. Figure 4 represents the kinetics of the agglutinability when gametes of both mating types were mixed in the presence of 1 mM cysteine. In both mating types the agglutinability increased considerably within 5–10 min, as is invariably found in untreated mating cells. In the presence of cysteine the agglutinability persisted at an increased level for several hours.

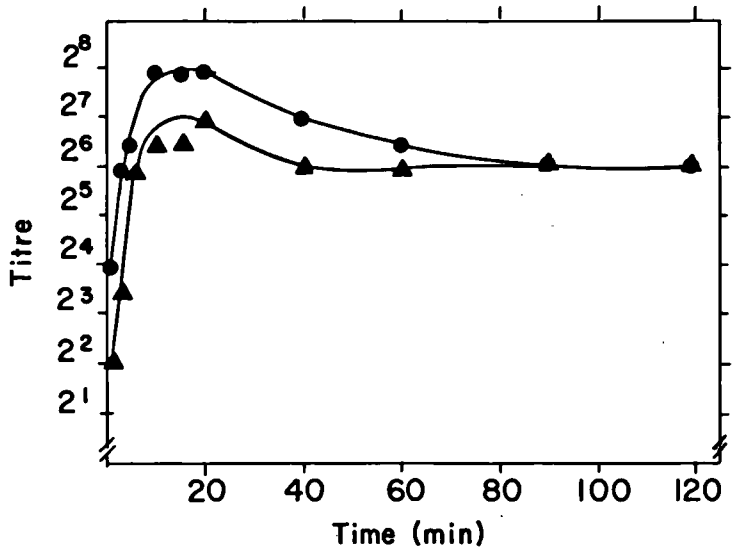

Fig. 4. Stimulation of gamete agglutinability of gametes of *C. eugametos*. Gametes of opposite mating type were mixed in the presence of 1 mM cysteine and the agglutinability of the mt$^-$ (●) and mt$^+$ (▲) gametes was tested at several time intervals after mixing.

The typical twitching reaction of agglutinating gametes was normal and persisted for many days as did the flagellar adhesion. We also observed normal flagellar tip transformation (also referred to as flagellar tip activation) (Mesland *et al.* 1980; Elzinga *et al.* 1982; Crabbendam *et al.* 1984). In the presence of cysteine all flagella remained transformed for at least 24 h. During agglutination, gametes acquired a special orientation in the clump and stayed exactly as described by Musgrave *et al.* (1985). The selective transport of agglutinins towards the flagellar tip (tipping), as described recently by Homan *et al.* (1987a), takes place in the presence of cysteine.

Using monoclonal antibodies (Homan *et al.* 1987b), it could be shown that in the presence of cysteine no wall-penetrating papillae were formed by agglutinating gametes (A. Musgrave, personal communication).

Effect of adding cysteine upon late mating reactions

Table 1 indicates that the early mating reactions before cell fusion were unaffected by cysteine with the exception of papilla formation. In the presence of cysteine it was observed that processes such as deadhesion, recovery of swimming behaviour, decrease of agglutinability to the basal level, recovery of the tapered tip of the mt$^+$ flagella and termination of tipping did not happen. These processes can be considered as the termination of the early mating reactions. In other words, the cells were kept indefinitely in the agglutination phase of the sexual mating.

Normally, many reactions concerning zygotogenesis start after fusion of the gametes. In *C. eugametos*, formation of zygotes implicates the further breakdown of the gamete cell wall. In *vis-à-vis* pairs the gamete wall remains intact except for the small pore through which the plasma bridge penetrates the wall. During zygote formation the narrow neck, which initially connects the two cells, is considerably widened to allow complete protoplast fusion. Figure 5 shows *vis-à-vis* pairs which were incubated in the absence or

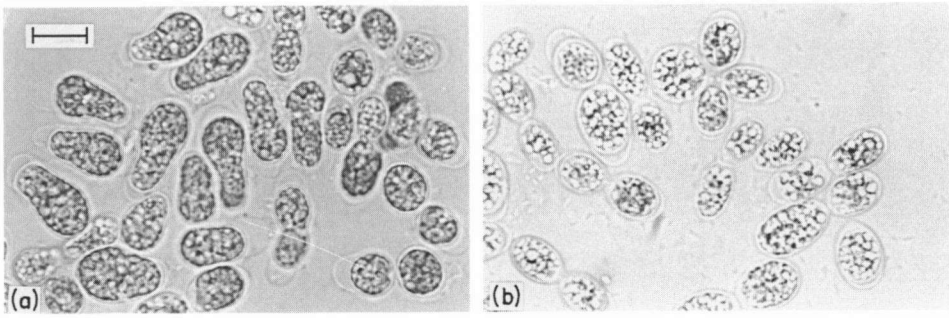

Fig. 5. Formation of zygotes of *C. eugametos* in the absence (a) and presence (b) of cysteine. Gametes of opposite mating type were mixed and allowed to form *vis-à-vis* pairs. One hour after mixing the cells, cysteine was added to a final concentration of 2 mM. The cells were placed in the light and photographs were taken 24 h after mixing the gametes. The bar indicates 10 μm.

presence of cysteine for 24 h. Without cysteine *vis-à-vis* pairs developed into somewhat rounded zygotes and a part of the cell wall was evidently degraded (Fig. 5a). In the presence of cysteine the original *vis-à-vis* pair morphology was maintained and although zygote wall material seemed to be deposited, no normal zygote formation took place (Fig. 5b).

Does cysteine act as an inhibitor of gamete-wall autolysin?

Table 1 shows that cysteine inhibited the formation of a plasma papilla. This raises the question whether cysteine directly or indirectly inhibits the gamete wall-autolysin, which is the enzyme involved in the local degradation of the cell wall. The cell wall of *Chlamydomonas* consists of glycoproteins (Roberts 1974) and the gamete wall-autolysin of *C. reinhardtii* appears to have a protease character (Matsuda *et al.* 1984, 1985). Since the gamete wall-autolysin of *C. reinhardtii* can be inhibited by cysteine, histidine and glutamic acid (Matsuda *et al.* 1985), we examined the effect of these and several other protease inhibitors (including EDTA, sodium pyrophosphate, sodium oxalate, dithiothreitol and phenylmethylsulphonyl fluoride) upon the *vis-à-vis* pair formation in *C. eugametos*. Most of these inhibitors, however, affected the initial agglutination and were unsuited for this test. As shown above, cysteine had an extreme inhibitory effect and histidine also impaired the *vis-à-vis* pair formation strongly but about 20% cell fusion was still observed at 10 mM histidine (results not shown). On the other hand, phenylmethylsulphonyl fluoride, an inhibitor of serine proteases, did not show any effect upon the formation of *vis-à-vis* pairs. We also investigated the effect of hippuryl-arginine upon the cell fusion. Hippuryl-arginine is used as an artificial substrate to test the hydrolytic activity of carboxypeptidases (zinc containing metalloproteases; Folk 1970). As shown in Fig. 1, hippuryl-arginine affected the cell fusion, which suggests that this artificial substrate competes with the natural substrate, i.e. cell wall material. When cell fusion was scored after a longer incubation period, inhibition of *vis-à-vis* pair formation was much less apparent, suggesting that hippuryl-arginine was hydrolysed by enzymic activity and that the reaction products did not influence cell fusion. This also indicates that hippuryl-arginine did not impair the initial recognition reaction.

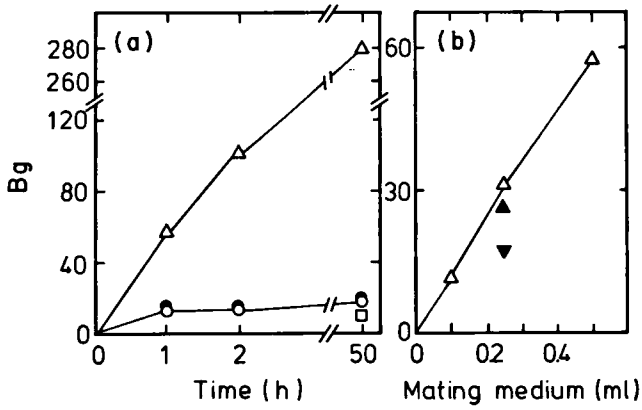

Fig. 6. Solubilization of radioactive material from isolated, ^{125}I-labelled cell walls of gametes of *C. eugametos*. Cell walls (350 Bq/assay) were added to the mating medium, cell free medium or buffer and incubated under different conditions. Cell wall degradation was determined by counting radioactivity that could not be precipitated in 15 min at 10 000 *g*. (a) Incubation of 0·5 ml mating medium (△) or cell free medium of mt$^+$ (●) and mt$^-$ (○) gametes at pH 7·4 and 25°C for different incubation times. (□) Incubation of 0·5 ml mating medium at pH 7·4 and 0°C. (b) Incubation of different concentrations of mating medium for 1 h, at 25°C and at pH 4·5 (▼), 7·4 (△) and 9·0 (▲), respectively.

As shown above, the fusion of mating gametes of *C. eugametos* can be impaired by adding protease inhibitors. This raises the question of whether the medium of normally mating gametes contains an enzymic activity capable of releasing components from gamete cell walls. Using isolated, radioactively labelled gamete walls, we examined whether compounds present in the medium of mating cells could solubilize material from these walls. Figure 6 shows that release of wall components, determined as the amount of radioactivity that could not be precipitated at 10 000 *g* for 15 min, was accomplished in a time- and concentration-dependent manner by mating medium. About 70% of the radioactivity could be solubilized. Incubation at 0°C resulted in a very low rate of solubilization, which suggests that an enzyme was involved in the release at higher temperatures. Incubation with a cell-free medium of non-agglutinating gametes also seemed to cause some hydrolysis of wall components but since non-precipitable radioactivity hardly increased with time, we suppose that these values are the result of spontaneous, non-enzymic wall disintegration. Incubation at different pH values showed that breakdown was comparable at pH 7·5 and pH 9·0, and much lower at pH 4·5. We found that 10 mM EDTA and 10 mM $HgCl_2$ completely inhibited the solubilization of cell wall material in our assay, while release of wall components decreased considerably in 2 mM cysteine. These results suggest the presence of cell wall lytic activity in the medium of mating gametes.

DISCUSSION

In this report we show that at a concentration of 1 mM cysteine the fusion of mating gametes of *C. eugametos* is inhibited. It is a reversible effect and it is essential that the inhibitor is present during the first few minutes of the mating reaction. We suggest that the inhibitory effect of cysteine in cell fusion is due to an inhibition of the gamete wall-autolysin. This enzyme locally degrades the cell wall as an early mating reaction, permitting the papillae to penetrate the wall. We have the following arguments for this proposal:

1. Cysteine did not affect any of the tested early mating reactions but did affect the formation of a plasma papilla.

2. In the medium of mating gametes enzymic activity appears to be released, which solubilized compounds from isolated gamete walls. We presume that this enzymic activity is related to the gamete wall-autolysin, since the activity is not found in the cell-free medium of gametes of a single mating type.

3. The enzymic activity in the medium could be inhibited by cysteine and by EDTA and $HgCl_2$, which are metalloprotease inhibitors. These compounds also inhibit the activity of the autolysin of *C. reinhardtii* (Matsuda *et al.* 1985).

4. Cysteine has an inhibitory effect upon the activity of the gamete wall-autolysin of *C. reinhardtii*; this activity has been identified as that of a metalloprotease (Matsuda *et al.* 1984, 1985). In our experiments histidine also, but not phenylmethylsulphonyl fluoride, a serine protease inhibitor, shows an inhibitory effect.

5. Cell fusion could also be inhibited by hippuryl-arginine, an artificial substrate for certain metalloproteases.

These observations indicate the presence of a gamete wall-autolysin in *C. eugametos* which resembles the autolysin of *C. reinhardtii* in its metalloprotease character. The inhibition by low concentrations of hippuryl-arginine (80% inhibition at 5 mM artificial substrate) suggests that the specificity of the enzyme for its natural substrate is not extremely high, which has also been reported for the autolysin of *C. reinhardtii* (Schlösser 1981).

Figure 5 shows that cysteine had an effect on cell wall degradation in a later stage of the mating during zygotogenesis. Thus, cysteine seemed to interfere with both the local breakdown of gamete wall in the initial phase of the mating reaction and the more extensive degradation later during zygote formation. We assume that in *C. eugametos* the autolytic activity that permits the penetration of papillae through the cell wall between the flagellar bases is also responsible for the degradation of the wall in a later stage. This would mean that autolytic activity is available not only directly after mixing the gametes, but also during the rather extended time span of zygote formation.

As indicated in Fig. 3, the inhibitory effect of cysteine upon cell fusion was only observed when it was present during the first few minutes of mixing the gametes. Snell (1982) reported that in *C. reinhardtii* all lytic enzyme was released in the first 3 min after mixing. Our results suggest that in *C. eugametos*, which fuses more slowly than *C. reinhardtii*, autolysin release and/or activation takes place in the first few minutes after the gametes recognize cells of the opposite mating type. The results shown in Fig. 3 also exclude the possibility that cysteine interacts in one way or another with the membrane fusion itself, for example by interacting with the molecules on the membrane surface of the papillae. The presence of cysteine hardly influenced the cell fusion in case the amino acid was added later than 5–10 min after mixing, when no or very few pairs had so far been formed (see Fig. 2, ○).

The abolishment of fusion by cysteine caused a number of processes, which, in a normal mating, proceeded for a relatively short period, to continue for an extremely long time. As discussed by Goodenough (1977) and Mesland & Van den Ende (1979), the plasma membrane of the mating structures possibly carries recognition molecules. We suppose that interaction of these molecules might be responsible for a 'start' signal resulting in, for example, membrane fusion and the synthesis of zygote-specific mRNAs (Ferris & Goodenough 1987). It might also be the trigger for many 'stop' messages with regard to

the early mating reactions. Most terminating reactions will be symmetric for both mating types. A mating type specific reaction must be considered for the inversion of the flagellar tip transformation in *C. eugametos*, since the flagella of the mt^- cell remains transformed after cell fusion but not the mt^+ flagella.

In the sexual mating reaction of *Chlamydomonas*, a number of reactions were observed as a prelude to cell fusion. A low concentration of the amino acid cysteine probably interferes specifically with the cell wall degrading enzyme since it did not influence any of the other tested early reactions (Table 1) nor did it appear to interact with the fusion of the membrane itself (Fig. 3). This makes cysteine a very attractive fusion inhibitor in *C. eugametos*.

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