A QUANTITATIVE STUDY OF AGGLUTINATION IN *CHLAMYDOMONAS MOEWUSII*

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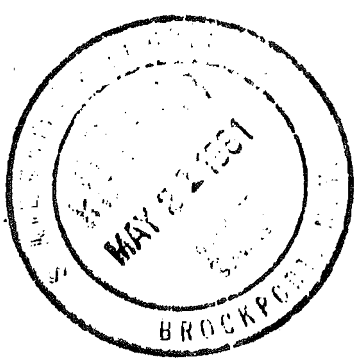

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ABSTRACT

A quantitative method for the measurement of agglutination among *Chlamydomonas moewusii* gametes has been presented. Using this technique, it was demonstrated that gametes possess a higher Con A isoagglutinability than vegetative cells and the + mating type is more isoagglutinable by Con A in both the vegetative and gametic states than the - mating type. Pairing is inhibited by both colchicine and trypsin treatments of gametes but agglutination is not significantly inhibited. Data from preliminary experiments in which ferritin Con A was used as a marker was suggestive of the fact that colchicine may have prevented the distal clustering of Con A receptor sites on flagellar tips during mating interactions. The involvement of Con A receptor sites in mating interactions is implicated and the mechanism of tipping is discussed in terms of the colchicine and trypsin data presented.

INTRODUCTION

The complexity, importance and beauty of cellular communication can be grasped when it is realized that it is the essence of our very being. Egg and sperm of the same species are able to recognize one another and the result is a living zygote. In the developing embryo, cells destined to form the same tissue remain together and those forming other tissues separate out by communicating with one another. In addition, the day to day well being of higher organisms is monitored and maintained by lymphocytes that are able to recognize foreign substances and aid in their removal.

One of the initial requirements in cell communication is the recognition of a message via receptor sites on the surface of the cell. Whether or not a cell can recognize a message therefore depends on the presence or absence of these receptors. Receptors seem to be a normal component of the cell surface. It does appear however, that the type of receptor on the surface may vary depending upon the level of development of the cell. Geiduschek and Singer (1979) have shown using fluorescein isothiocyanate conjugated Concanavalin A (FITC Con A) that Con A receptors were abundant in the nucleated

stages of mouse erythroid cells whereas spectrin binding sites were not prevalent. The mature, enucleated erythrocyte showed little binding of Con A but intense binding of spectrin. Oppenheimer (1978) has also demonstrated that the number of lectin receptor sites on the cell surfaces of sea urchin embryos changed during development. Agglutinability of these cells by Con A decreased with age. Changes in surface receptors are thus indicated as a normal course of development.

Cell surface changes have also been noted to occur during the transition from the normal to the transformed or malignant states. An obvious change was the disappearance of a high molecular weight protein, fibronectin, from fibroblasts following viral transformation (Hynes, 1973). When fibronectin was added back to Balb/c 3T3 cells transformed by Simian virus 40, rat kidney cells transformed by Kirsten sarcoma virus (Yamada et al, 1976), methylcholanthrene T Balb/c 3T3 cells (Willingham et al, 1977) and transformed hamster fibroblasts (Ali et al, 1977), the cells elongated and flattened and possessed an increased adhesion to the substrate. In other words, the morphology of the cells approached that of normal cells. Also, populations of surface glycoproteins from transformed hamster (Warren et al, 1973) and mouse cells (Sakiyama and Burge, 1972) possessed different gel migratory properties when compared to

those of their normal glycoproteins. Increased levels of glycosidase activity have been demonstrated in malignant human breast and colon tissue (Bosmann and Hall, 1974) and virally transformed mouse cells (Bosmann, 1972). Transformed cells have also been shown to lack the dense networks of plasma membrane-associated microfilaments and microtubules characteristic of normal cells (McNutt et al, 1971; McNutt et al, 1973). Transformed cells have been shown to lack contact inhibition of movement (Abercrombie and Heaysman, 1954) and to agglutinate in the presence of much lower concentrations of lectins than normal cells (wheat germ agglutinin - Burger and Goldberg, 1967; Burger, 1969; Con A - Inbar and Sachs, 1969a, 1969b; soybean agglutinin - Sela et al, 1970; Lis et al, 1970).

At first it was thought that the greater agglutinability of transformed cells by lectins was due to the clustered pattern of receptor sites on these cells as seen using ferritin Con A (Nicolson, 1971). It was later determined that the clustered pattern was induced by the lectin itself (Rosenblith et al, 1973).

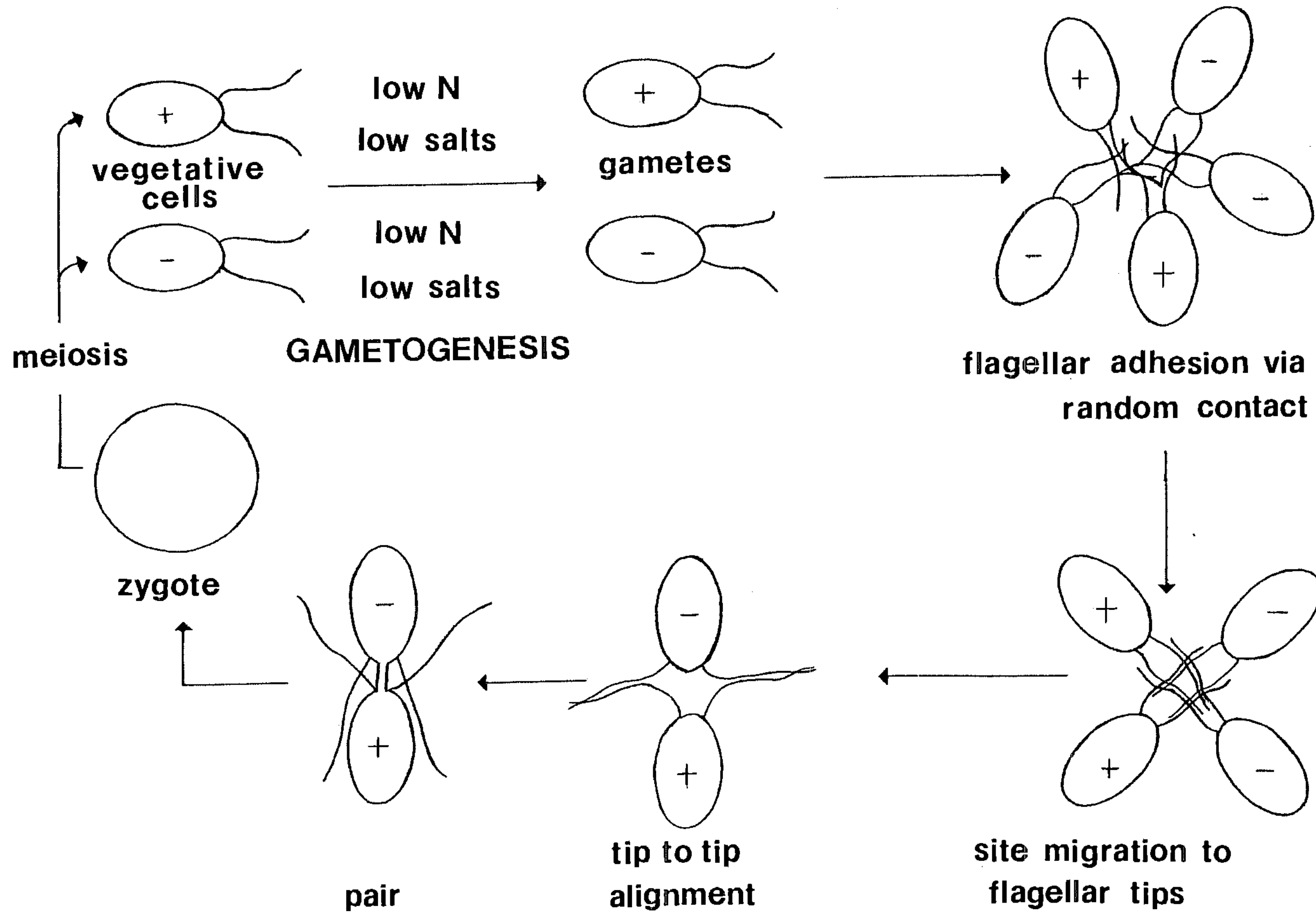
This short review of the data available concerning the surface changes that occur during transformation illustrates the complexity of the problem facing cancer researchers. In many cases, different cell types have been used making interpretation as to primary and

secondary characteristics very difficult. Before the implications of the data collected thus far can be fully realized, studies of surface changes in one system are warranted. It is difficult to elucidate the molecular basis for agglutination in part because of the lack of a model cell type that "transforms" from the nonagglutinable to the agglutinable state or vice versa. Van Veen et al (1976) have found that Chinese hamster ovary cells make the transition from the nonagglutinable to the agglutinable state by the addition of dibutyryl cyclic AMP.

The single celled, eukaryotic, biflagellated, photosynthetic green alga, Chlamydomonas moewusii however is a far superior model system because during its life cycle, the transition from the vegetative to the gametic state is accompanied by surface receptor changes. Changes in the degree of lectin binding occur (Katz, 1979) as well as an appearance of recognition structures that function to draw opposite gametes together. The Chlamydomonas moewusii cell system is thus an excellent model for basic cancer research since surface changes occur normally during the life cycle and one of these changes is the acquisition of the ability to recognize gametes of the opposite mating type. This in particular correlates well with the changes in transformed cells in which members of tissues no longer recognize each

other and subsequently grow without limit. This system is also ideal because it is easily and inexpensively cultured and the membrane surface changes are localized on the flagellar membranes. Chlamydomonas moewusii exists as two mating types, + and -, which participate in a mating reaction when in the gametic state. Differentiation into gametes occurs when vegetative cells are placed in a minimal salt medium usually devoid of a nitrogen source. The cells are kept in darkness and then exposed to a short period of light (Lewin, 1956). The stages of sexual interaction of these gametes have been elucidated through the efforts of several workers. These stages include: an adhesion of opposite mating type flagella via random contacts, site migration to the distal portions or tips of the flagella, tip to tip alignment for body orientation (Mesland, 1976), flagellar tip activation (Mesland et al, 1980), wall lysis (degree varies with species), plasma papilla outgrowth, formation of papillar bridge, deadhesion of flagella from one another and other gametes and zygote formation (Mesland, 1976). A summary diagram of the Chlamydomonas moewusii sexual cycle is presented in Figure 1. Since the subsequent mating events are triggered by the first attachment of gametes via surface recognition sites and their subsequent tipping, these phenomena will be the main objectives of study.

Figure 1



Chlamydomonas moewusii Sexual Cycle

Following gametic differentiation, substances can be isolated from the cell free medium which are glycoprotein in nature and cause gametes of the opposite mating type to agglutinate when introduced into the vessel (Förster et al, 1956). These components (gamone, isoagglutinins) are similar to those receptors on living gametes in composition and activity (Wiese, 1965). The isoagglutinins are actually membrane vesicles containing the receptor sites (McLean et al, 1974; Bergman et al, 1975; Snell, 1976). The agglutinins or receptor sites for adhesion are thus assumed to be associated with the flagellar membrane and consist of a large molecular weight protein as revealed by SDS-polyacrylamide gel electrophoresis on C. reinhardtii membranes (Witman et al, 1972; Snell, 1976).

Evidence for the multiplicity of receptor sites on Chlamydomonas flagellar membranes has accumulated over the years. McLean and Brown (1974) reported that monovalent Con A bound to gametes could block subsequent isoagglutination by multivalent Con A but could not block the mating reaction. This indicated that there were at least two different sites that were characteristic of gametic flagella, the mating site and the Con A binding site. Claes (1975) observed that Con A did not interfere with flagellar agglutination between opposite gametes of C. reinhardtii but that the lectin

stimulated the release of the cell wall lytic factor from - gametes suggesting that at least two types of sites are on flagellar membranes. Two types of sites have also been indicated by two other pieces of evidence. Following trypsinization, the recovery of agglutinability and wall lysis occurred after different time periods (Solter and Gibor, 1978) and following deflagellation, the two sites appeared separately on the flagellar surface during regeneration (Ray et al, 1978). Forest and Goodenough (1977) have demonstrated that temperature sensitive mutants can sexually agglutinate without the subsequent formation of zygotes. At the non-permissive temperature (35°C), extension of the fertilization tube does not occur. The above data indicate that there are a number of sites that are associated with the gametic flagellar membrane, namely a site involving the adhesion of opposite gametes, a site that stimulates wall lysis, one that causes the formation of the fertilization tube and Con A binding sites.

The question of how receptor sites arise on the flagellar membrane during gametogenesis has received the attention of many researchers. McLean and Brown (1974) addressed themselves to the question of whether Con A receptor sites are present on the vegetative membranes of _C. moewusii_ and _C. reinhardtii_ but in the concealed state. Application of trypsin to vegetative

cells did not increase Con A binding. Bergman et al (1975) in their electrophoretic study of flagellar surface components did not detect any differences in the protein composition between vegetative cells and gametes or between mating types in C. reinhardtii. Snell (1976) detected a single major difference in his comparative electrophoretic analysis of isoagglutinins. A 70,000 dalton band was present in the isoagglutinin of the + gamete and sometimes in - membrane preparations. It is generally accepted now that this is a degradation product of a large glycoprotein since other investigators encounter it only occasionally (Goodenough, 1977).

Elucidation of a more specific description of the agglutinins has been sought using a variety of approaches including the treatment of flagellar membranes with lectins, enzymes, microtubule disruptors and chelators followed by the determination of the resulting effect upon mating ability.

Wiese and Shoemaker (1970) have reported that gametes are isoagglutinable by Con A at a concentration of 0.01% (0.1 mg/ml). Musgrave et al (1979) also reported that gametes are isoagglutinated by 0.01 - 0.1 mg Con A and vegetative cells by 0.1 mg or greater.

Trypsin has been used extensively in examining characteristics of the agglutinins. Wiese and Metz (1969) found that 0.025% trypsin whether in pretreatment

or added at the mixing of gametes significantly reduced pairing but had no effect on agglutination. A concentration of 0.1% trypsin was reported to destroy the agglutinability of only the - gamete while the + gamete was insensitive to the treatment (Wiese and Metz, 1969). McLean and Brown (1974) on the other hand reported that only the + gamete was sensitive to 0.1% trypsin. The mating ability of the - gamete was unaffected. Both types of C. reinhardtii seem to be affected in their mating ability by 0.05% and 0.1% trypsin (0.05%-Solter and Gibor, 1978; 0.1%-McLean and Brown, 1974). Study of sexual receptors on Chlamydomonas has also been aided by the use of chelators. Wiese and Jones (1963) treated C. moewusii with EDTA in concentrations ranging from 2×10^{-5} M to 2×10^{-4} M. The agglutination reaction was completely inhibited with no effect upon motility of the cells. It has also been reported that the sexual agglutinin of C. reinhardtii is released by treatment with EGTA (Adair et al, 1980).

In many of the studies mentioned above, isoagglutination or sexual agglutination were not measured quantitatively. Measurements either reflected visual estimates of agglutination or hemocytometer counts of percent pairing or zygote formation. One of the first quantitative assays for measuring the rate of cell adhesion was developed by Orr and Roseman (1969). They

developed a method based upon the determination of the decrease in single cells in an aggregating population with a Coulter Counter. Specifically, an aggregating population was placed on a shaker for a given period of time and then the suspension was diluted and counted. The reproducibility of this method was shown by the fact that two workers performing the same experiment obtained similar results. The cell volume used by Orr and Roseman (1969) was 3 ml. Oppenheimer and Odencrantz (1972) adapted the above method for use with volumes as small as 0.2 ml. Recently, Snell and Roseman (1979) have developed a quantitative method for measuring the agglutination reaction between Chlamydomonas gametes. Aliquots from a cell suspension were transferred into 50 ml of ice cold medium and poured into a 100 ml beaker, left undisturbed for 5 - 10 min at room temperature and then counted. To determine the total number of singles in the suspension, it was transferred back and forth between 100 and 50 ml beakers until no further increase in the number of singles occurred. In order that the numbers obtained represent only agglutination and not pairing, trypsin was added to the suspension at varying times to destroy adhesiveness.

Two types of flagellar surface motility have been identified on Chlamydomonas gametes. One type has been described by Bloodgood (1977) as being a rapid, saltatory movement of exogenous marker particles or microspheres

on the extracellular surface. This movement is dependent upon the continued presence of Ca^{++}, and the binding of the markers is inhibited by pronase treatment and inhibitors of protein synthesis such as cycloheximide. Movement is also inhibited by low temperatures but is not affected by either colchicine or cytochalasin B (Bloodgood et al, 1979). A second type of movement has been called "tipping" and refers to the movement of receptor sites to the distal portion of the flagella during sexual agglutination and preceeding pairing (Goodenough and Jurivich, 1978). This process was first observed by these investigators as the gametic response to antisera against flagellar membranes. Tipping has not been observed in vegetative cells. Mesland et al (1980) have reported that colchicine and vinblastine have no effect upon gametic motility or sexual agglutination but both block tipping.

The objective of this work was the application of an original quantitative assay for the measurement of agglutination to clarify fundamental aspects concerning the nature of flagellar receptor sites of C. moewusii. Measurements were made of the extent of Con A isoagglutination of vegetative and gametic cells and the effects of trypsin and colchicine pretreatments upon sexual agglutination and pairing. In addition, using modifications of the electron microscopic fixation and embedding techniques of Rittenburg et al (1979), the effect

of varying times of gametic mixing and colchicine treatment upon the interaction of gametes was studied using ferritin Con A as a marker.

METHODS AND MATERIALS

Culture Methods

Both mating types of *Chlamydomonas moewusii*, + (Utex 96) and - (Utex 97), were axenically maintained in the vegetative state in modified Bristol's liquid medium (Bold, 1942) under continuous illumination of 400 ft-c intensity. Modified Bristol's medium consisted of the following components:

$NaNO_3$	1.000 g/l
$CaCl_2$	0.025 g/l
$MgSO_4 \cdot 7H_2O$	0.075 g/l
K_2HPO_4	0.075 g/l
KH_2PO_4	0.175 g/l
NaCl	0.025 g/l
1% $FeCl_3$	0.29 mg/l
trace elements	1.0 ml/l
distilled H_2O	940.0 ml/l

The pH was adjusted to 7.0 ± 0.1. The trace element stock solution consisted of the following components and final concentrations per liter of medium (Provisional Algal Assay Procedure, 1969):

element	Bristol's	IM
H_3BO_3	0.6184 mg/l	0.0618 mg/l
$MnCl_2$	0.88 mg/l	0.088 mg/l
$ZnCl_2$	0.109 mg/l	0.0109 mg/l
$CoCl_2$	0.026 mg/l	0.0026 mg/l
$Na_2MoO_4 \cdot H_2O$	0.0242 mg/l	0.0024 mg/l
$CuCl_2$	0.03 µg/l	0.003 µg/l
$NaEDTA \cdot 2H_2O$	7.44 mg/l	0.744 mg/l

After two weeks, the cell suspensions were transferred to modified Bristol's agar plates (Bristol's supplemented with 13 g/l agar) and maintained for approximately one week on a cycle of 16 hr light and 8 hr dark.

Gametic Induction

Vegetative cells were induced to undergo gametogenesis in a 1:1 solution of soil extract (Starr, 1964) and induction medium (McLean and Brown, 1974). The cells were incubated in darkness for 14 - 15 hr followed by a 30 min exposure to light. Soil extract was prepared by autoclaving approximately 1/2 inch of garden soil with 2 l distilled H_2O in a 3 l Erlenmeyer flask. The mixture was then allowed to settle for several days after which the liquid was siphoned off and filtered at least 3x using Whatman #1 filter paper. The filtrate was divided into smaller fractions and reautoclaved. The soil was obtained from a cornfield on East Avenue in Clarkson, NY and an unfertilized garden on West Academy Street in Albion, NY. Induction medium was composed of the following:

$CaCl_2 \cdot 2H_2O$	0.005 g/l
$MgSO_4 \cdot 7H_2O$	0.03 g/l
phosphate buffer	
K_2HPO_4	0.717 g/l
KH_2PO_4	0.363 g/l
trace elements	0.01 ml/l
sodium citrate	0.05 g/l
1% $FeCl_3$	0.29 mg/l
distilled H_2O	946.0 ml/l

The pH was adjusted to 7.4 ± 0.1. Flagellated vegetative cells were obtained by dark incubation in Bristol's medium supplemented with 0.1% NH_4Cl (Wiese, 1965).

<u>Sexual Agglutination and Lectin Isoagglutination Assays</u>

Cell suspensions were adjusted to 5 x 10^6 cells/ml using the Coulter Counter, Model ZF (settings: 1/amplification = 1; threshold = 5; 1/aperture current = 1/2). The cell suspensions were diluted with induction medium for controls and induction medium supplemented with either trypsin, soybean trypsin inhibitor or colchicine in appropriate concentrations. Following the treatment, 3 - 4 ml of cells were pipeted into 12 x 100 mm test tubes or a 1 ml aliquot was added to 9 x 75 mm test tubes. Either test tube size was adequate for the assay. For mating studies, equal volumes of both mating types were mixed; for lectin studies the cell volume consisted of single mating types and appropriate concentrations of lectin. The test tubes were inverted once and left undisturbed for 15 min during which clustered cells settled out. Following incubation, 0.1 ml samples were removed from the suspensions just under the meniscus and counted in 9.9 ml Isoton II (Coulter Electronics). This procedure selected for the single cells that remained in suspension. Controls of individual mating types were included in order to discount any natural settling of cells in agglutination calculations. Calculations of sexual agglutination

were also corrected for total pairing in the vessel as well as for pairs in the 0.1 ml sample from the top. These pairing percentages were based upon hemocytometer counts. The procedures used in calculating agglutination and isoagglutination percentages are included at the end of this section.

Treatments of Cells with Potential Agglutination and Pairing Disruptors

Gametes were pretreated with 0.005 M colchicine (Sigma) for 1 hr before agglutination and pairing percentages were determined. The pretreatment period for varying concentrations of trypsin (Calbiochem) was 40 min followed by a 5 min incubation with approximately double concentrations of soybean trypsin inhibitor (Miles-Seravac). The cell suspensions were then centrifuged at 710 rpm in an International Clinical Centrifuge, Model CL (head # 221, International Equipment Co.) for 5 min. The cells were resuspended in induction medium and assayed for agglutination and pairing.

Ferritin Con A Labeling

Gametes were fixed in 0.1% glutaraldehyde and 0.5% osmium-0.45% potassium ferrocyanide for 45 min at room temperature. The cells were either fixed directly or the opposite mating types were mixed for a given time period and then fixed. When colchicine was used, treatment preceeded gametic interaction and fixation. Fixation

was followed by rinsing in 0.1 M phosphate buffer (pH 7.2) and incubation with appropriate concentrations of ferritin Con A (Miles-Yeda) for 15 min. The cells were rinsed 3x in buffer and then collected on a 3 µm Unipore polycarbonate filter (Bio-Rad). Dehydration was accomplished by passing an acetone series through the filter. The filter was then placed in 1 part Epon-Araldite and 2 parts acetone for 1 hr. This was followed by exposures to two thirds plastic and 100% plastic (2x) for 15 min each. The filters were cut and placed in molds for curing for 1.5 hr at 99°C. This procedure is modified after Rittenburg et al (1979).

Procedure for Calculating Agglutination and Pairing Percentages

Colchicine Data

$$\% \text{ aggl.} = \frac{[(\text{total cell \#} - \text{total \# pairs}) - \text{\# unmixed settlers}] - (\text{mix count} - \text{\# top pairs})}{\text{total cell \#}} \times 100$$

Trypsin Data

$$\% \text{ aggl.} = \frac{\text{average of settling controls} - (\text{mix count} - \text{\# top pairs})}{\text{total cell \#}} \times 100$$

Concanavalin A Data

$$\% \text{ isoaggl.} = \frac{\text{count from settling control} - \text{mix count}}{\text{total cell \#}} \times 100$$

$$\text{total \# pairs} = \text{total cell \#} \times \% \text{ total pairing}$$

$$\text{\# unmixed settlers} = (\text{total cell \#} - \text{total \# pairs}) \times \left(1 - \frac{\text{average settling control}}{\text{total cell \#}}\right)$$

$$\text{\# top pairs} = \text{mix count} \times \% \text{ pairing in 0.1 ml top sample}$$

In every case, background Isoton counts were subtracted from cell counts.

RESULTS

Quantification of Agglutination and Isoagglutination

The lack of a simple and reliable method for quantification of agglutination in *Chlamydomonas* led to investigations aimed at the development of such a method. The present method of agglutination quantification represents a culmination of the work of several students in this laboratory. Initial studies involved the measurement of optical density before and after agglutination (Lembo and McLean, unpublished data). The results obtained were erratic and not reliable. This led to the development of a filtration technique which separated clustered from single cells (Watson, McLean and Menoff, unpublished data). Filtration gave consistent results for sexual agglutination percentages. The work of the author began with an attempt at application of this filtration method to the agglutination reaction elicited by exposure of gametes to the lectin, Con A.

The filtration technique involved the incubation of opposite gametes for a given time period and then the cell mixture was poured through Whatman # 1 filter paper (7.0 cm) and the first five drops of the filtrate were collected. The cells in the filtrate were fixed in

iodine and counted using a hemocytometer. Percentages of agglutination were calculated from the initial and final cell concentrations and the fraction of gametes that passed through the filter in control experiments. The lectin-induced isoagglutination data obtained using this method was not consistent. Reasons for this inconsistency were examined. Since the filter paper consisted of cellulose, the possibility existed for the interference of cell passage due to the binding of flagella to the filter paper itself. Nitex filters were then substituted for the Whatman paper. Difficulties arose in the use of Nitex filters since clustered cells passed through into the filtrate in addition to single cells. The use of Nucleopore filters was hampered by the fact that a Millipore filter apparatus and syringe were required. In order to obtain a filtrate, the barrel had to be placed in the syringe and this seemed to force clustered cells through. Various pore sizes were tried in both the Nitex and Nucleopore brand filters all with the same results.

Another possible cause of the inconsistent lectin results was the fact that a directed movement of receptor sites toward the distal tip of gametic flagella, called tipping (Goodenough and Jurivich, 1978), could be responsible. Tipping had been suspected as the cause of lectin loss in previous electron microscopic studies (Katz, 1979).

Fixation of gametes prior to lectin treatment proved successful in the electron microscopic studies (Katz, 1979) and therefore it was applied to this situation. Results were inconclusive since the chemicals used in fixation seemed to incur a stickiness to the flagella of like gametes before lectin treatment. Like gametes were seen in clusters before the lectin was added. This stickiness was not observed in living gametes of the same mating type.

In view of the insolvable nature of the above difficulties, an entirely different approach to the quantification problem was applied. It was observed that agglutinated clusters settled out following the mixing of gametes. This natural process was employed to separate agglutinated clusters from single cells. Initial experiments were performed using a hemocytometer instead of the Coulter Counter, Model ZF, to obtain cell counts. The use of the Coulter Counter has proved to be a far more accurate method of counting since errors were no doubt introduced when counting a 0.1 ml sample or some dilution thereof using a hemocytometer.

Comparison of the data on sexual agglutination over a 2 hr period using the filtration method (Watson, McLean and Menoff, unpublished data) and the settling technique shows similar results. In both cases, percentages are high initially and then they drop off to below 20% after

60 min. The superiority of the settling technique is its applicability to lectin studies as well as mating reaction studies. This method however is not without its difficulties when being applied to lectin studies. One variable that seems uncontrollable at this time is the fact that cells of the - mating type at times show an attraction to the sides of the test tube at the top of the volume forming a ring. Accurate sampling is difficult because the ring of cells formed is not constant and varies from tube to tube and experiment to experiment. Samples taken from the center of the tube therefore contain low cell counts in some cases and high cell counts in others. This phenomenon does not seem to be due to an attraction to the light since the ring forms even if the cells are covered. In the few experiments in which this occurred, the data obtained was not included in the calculations of means.

The individual parameters of cell concentration, time, settling volume and Coulter Counter Model ZF settings as they pertain to the settling technique were established prior to investigation. Settings for the Coulter Counter were established based upon correlation of cell concentrations obtained using a hemocytometer. The settings which gave cell concentrations the most nearly comparable to hemocytometer results were 1/amplification = 1; threshold = 5; 1/aperture current = 1/2 (average size of a single Chlamydomonas moewusii cell is 15 µm in diameter).

The optimum time of incubation for sexual agglutination studies was determined to be 15 min. Data points obtained up to 60 min incubation showed a leveling at 15 min and following. Optimum pairing data was obtained after a 30 min incubation (Table 1). A 15 min incubation period was also suitable for Con A isoagglutination studies on both mating types (Table 2). Agglutination percentages versus cell concentration studies showed that maximum agglutination percentages were obtained between cell concentrations of 1×10^6 and 1×10^7 cells/ml. Similar results were obtained in lectin studies. A midpoint value of 5×10^6 cells/ml was chosen for this work (Tables 3 and 4). Comparable agglutination and pairing results were obtained with 4 ml and 3 ml settling volumes in 12 x 100 mm test tubes and a 1 ml settling volume in 9 x 75 mm test tubes (Table 5). Settling volumes then varied according to the nature of the experiment.

<u>Isoagglutination Data of Vegetative and Gametic Cells by Concanavalin A</u>

Con A concentration curves for both vegetative and gametic cells are shown in Figure 2. From these curves, it is evident that gametic cells were more responsive to Con A than their vegetative counterparts. It is also demonstrated that the + mating type in both the gametic and vegetative states was more agglutinable by Con A than the - mating type, with the + gamete being the most

Table 1

Determination of Optimum Incubation
Periods for Quantification of Sexual Agglutination
and Pairing Using the Settling Technique

Time, min	% Agglutination	% Pairing
5	43%	26%
15	62%	53%
30	61%	59%
45	60%	64%
60	60%	65%

Experimentation was carried out in 12 x 100 mm test tubes with 3 ml of a cell suspension at a concentration of 5×10^6 cells/ml.

Table 2

Determination of Optimum Incubation Period for Quantification of Con A Isoagglutination Using the Settling Technique

Time, min	96 % Isoagglutination	97 % Isoagglutination
1	0%	16%
3	33%	0%
5	30%	20%
8	47%	4%
10	44%	0%
15	54%	19%

Experimentation was carried out in 12 x 100 mm test tubes with 3 ml of a cell suspension at a concentration of 5 x 10^6 cells/ml. The Con A concentration was 100 µg/ml.

Table 3

Determination of Optimum Cell Concentration for Quantification of Sexual Agglutination and Pairing Using the Settling Technique

Cell Concentration	% Agglutination	% Pairing
1×10^5	0%	66%
5×10^5	57%	57%
1×10^6	67%	63%
5×10^6	77%	57%
1×10^7	61%	56%

Experimentation was carried out in 12 x 100 mm test tubes with 3 ml of a cell suspension of varying cell concentration. Aliquots were withdrawn after 15 min for agglutination data and after 30 min for pairing data.

Table 4

Determination of Optimum Cell Concentration for Quantification of Con A Isoagglutination Using the Settling Technique

Cell Concentration	96 % Isoagglutination	97 % Isoagglutination
1×10^5	0%	0%
5×10^5	4%	0%
1×10^6	9%	17%
5×10^6	36%	6%
1×10^7	36%	5%

Experimentation was carried out in 12 x 100 mm test tubes with 3 ml of a cell suspension of varying cell concentration. Aliquots were withdrawn after 15 min for isoagglutination data. The Con A concentration was 50 µg/ml.

Table 5

The Effect of Cell Volume and Test Tube Size on

Sexual Agglutination and Pairing Percentages

Test Tube Size	Volume, ml	% Agglutination	% Pairing
12 x 100 mm	4	60%	50%
12 x 100 mm	3	60%	53%
12 x 100 mm	2	49%	44%
9 x 75 mm	1	55%	59%

Experimentation was carried out using a cell concentration of 5×10^6 cells/ml and incubation periods of 15 min and 30 min respectively for agglutination and pairing data.

Figure 2

Con A isoagglutination percentages revealed the greater isoagglutinability of gametes as compared to vegetative cells and the greater isoagglutinability of the + mating type as compared to the - mating type in both the vegetative and gametic states. Calculation of the standard error of the mean revealed that the dip in the 96 gametic isoagglutination curve at 250 μg Con A/ml is not significant and the dip in the 97 gametic isoagglutination curve at 300 μg Con A/ml may be significant.

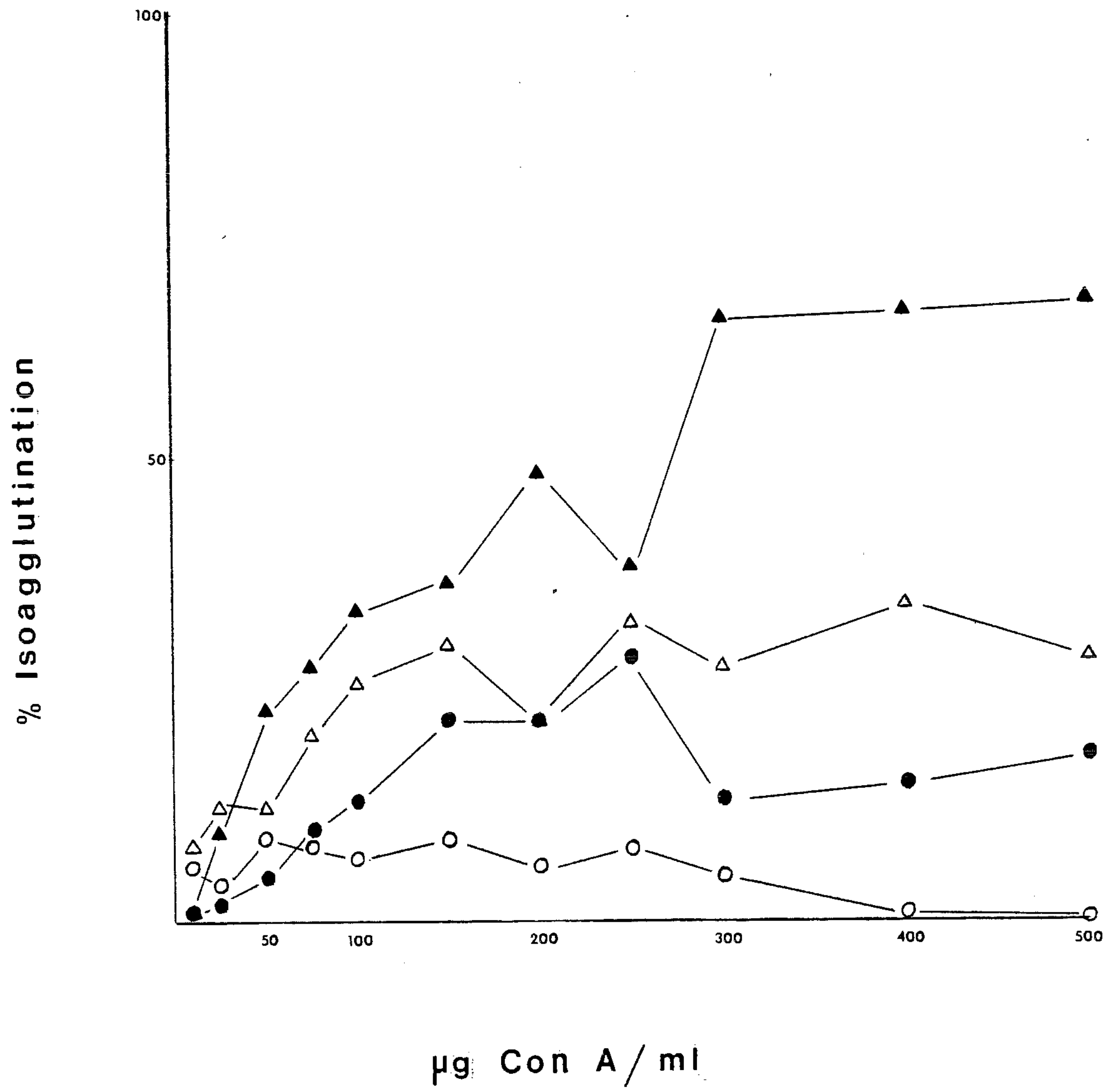
(△) = + vegetative cell

(▲) = + gamete

(○) = - vegetative cell

(●) = - gamete

Figure 2



agglutinable. A leveling of responses occurred in all cell types at 300 µg Con A/ml.

The Effect of 0.005 M Colchicine Upon Mating Ability and Con A Isoagglutinability

Treatment of gametes with 0.005 M colchicine for 1 hr had no effect upon agglutination but pairing was inhibited almost entirely (Figure 3). As time progressed agglutination in both the control and colchicine treated cells began to decline by 45 min after mixing. Agglutination after 2 hr was negligible in both cases. Initial control agglutination percentages appear lower than in the colchicine treated cells because some control cells have progressed to the pairing stage and the colchicine treated cells never make this progression. Percentages in both instances have been calculated to represent actual agglutination and pairing. Colchicine had no effect upon the isoagglutinability of vegetative and gametic cells by Con A (Figure 4). Results for the - gametic cells are suggestive of an isoagglutinability increase after colchicine treatment but in light of the standard errors of the data points, further experimentation is warranted.

Ferritin Con A Labeling

A study of ferritin Con A binding following colchicine treatment of gametes and gametic interaction was undertaken to examine the relationship if any of Con A

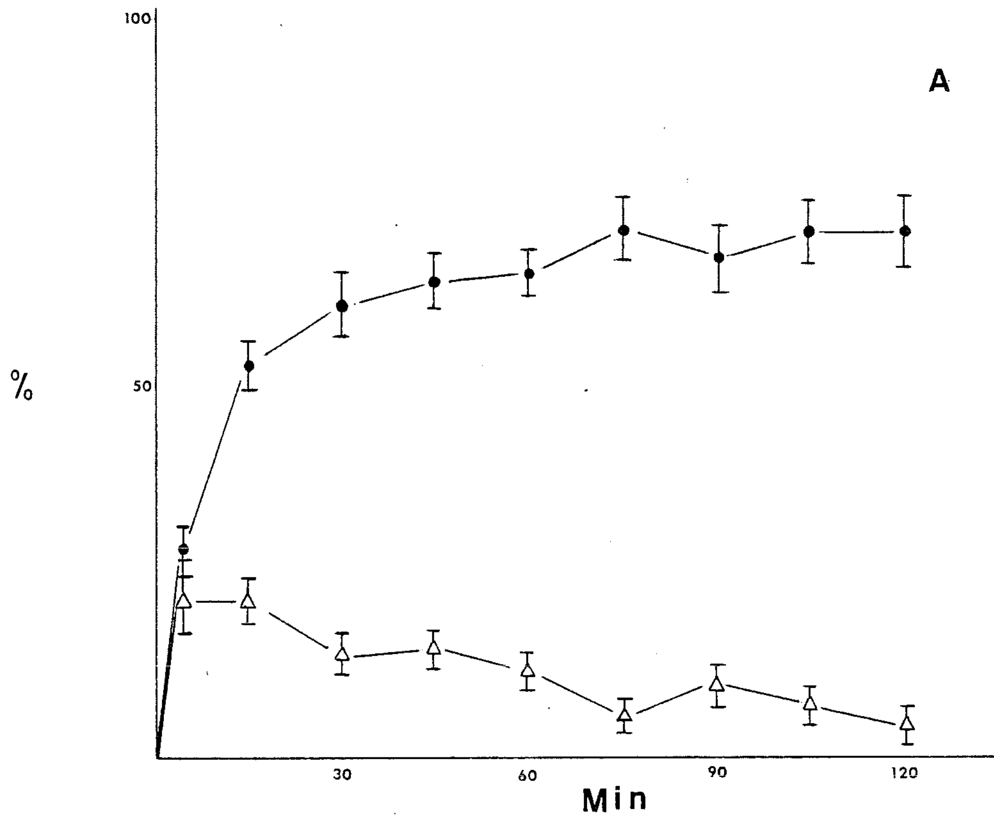
Figure 3

0.005 M colchicine had no effect upon sexual agglutination but inhibited pairing almost completely.

A. Control Gametes
   (△) = % Agglutination
   (●) = % Pairing

B. Colchicine treated Gametes
   (△) = % Agglutination
   (●) = % Pairing

Figure 3



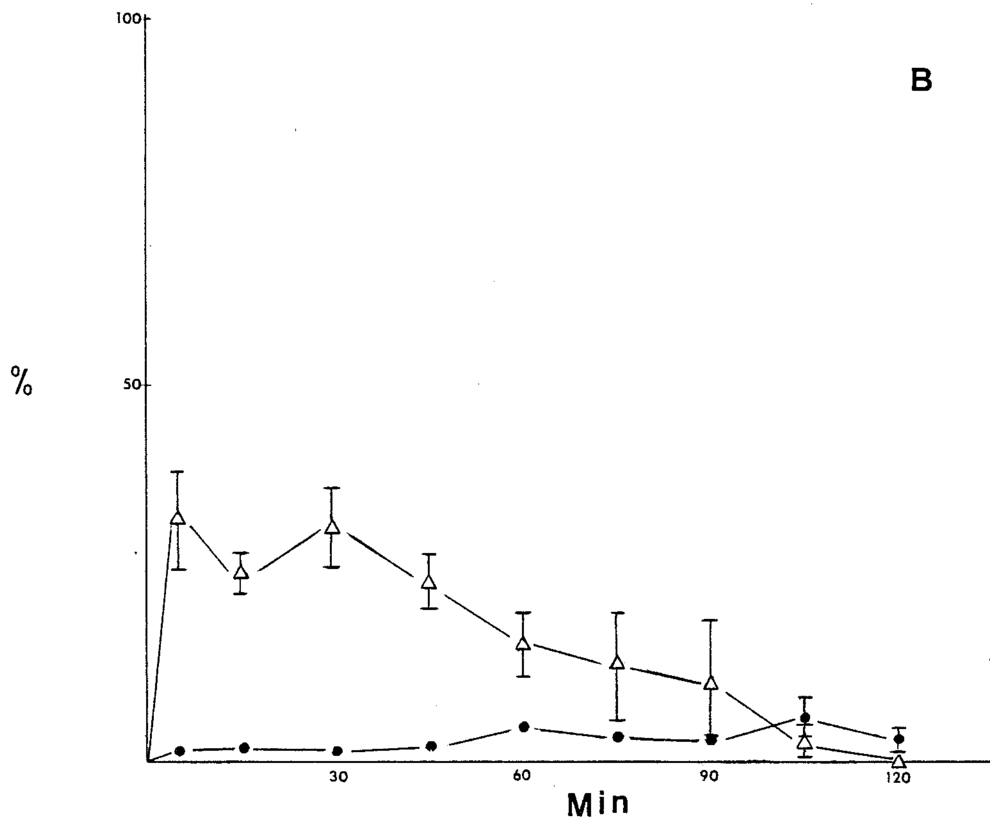


Figure 4

Colchicine treatment had no effect upon Con A isoagglutinability.

A. = % isoagglutination of control cells in the presence of 200 µg Con A/ml

B. = % isoagglutination of cells treated with 0.005 M colchicine followed by the addition of 200 µg Con A/ml

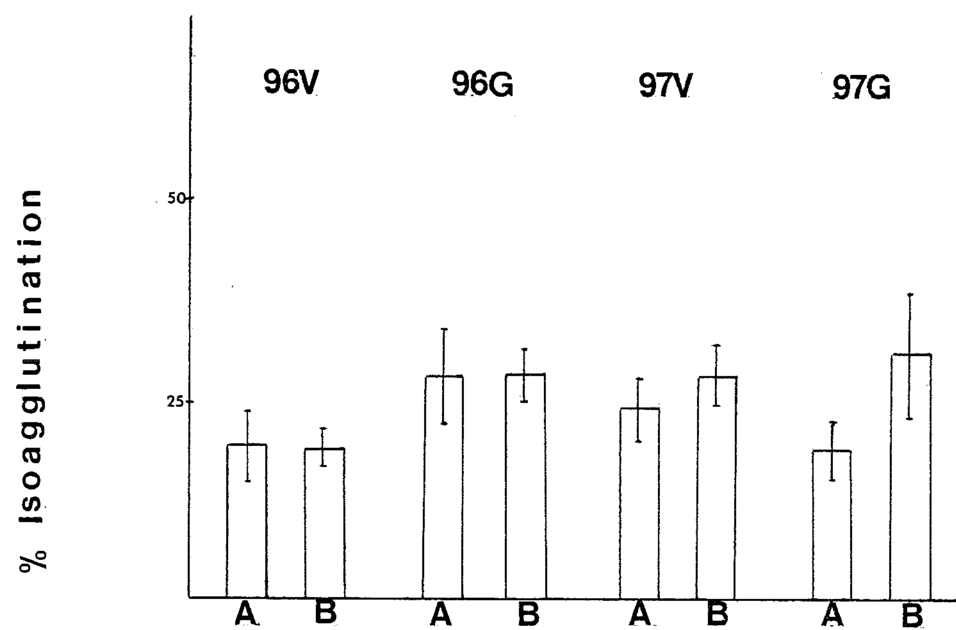
96V = + vegetative cells

96G = + gametes

97V = - vegetative cells

97G = - gametes

Figure 4



receptor sites to the tipping phenomenon. Gametes were prefixed prior to lectin treatment in order to discount any rearrangement of sites due to lectin perturbation. Unreacted gametes showed a random distribution of ferritin grains along the flagella (Table 6). Interaction of gametes revealed localizations of ferritin grains along the lengths of the flagella that changed position with time. A 30 sec interaction of gametes resulted in a localization of ferritin in the intermediate zone of the flagella (Table 6). A 30 min interaction revealed a localization at the distal tip of the flagella. Colchicine treatment and subsequent gametic interaction revealed no localization of ferritin grains.

The Effect of Trypsin Treatment Upon Mating Ability

The quantitative assay was utilized to determine the effects of other treatments such as trypsin digestion upon agglutination and pairing. The effect of trypsin upon the mating types was investigated by interaction of treated gametes of one mating type with untreated opposite gametes. The sexual agglutinability of the - gamete was affected to a greater extent than that of the + gamete (Figure 5). Agglutinability of both mating types decreased when treated with trypsin in concentrations of greater than 0.025%. The decrease in agglutinability of the - gamete however was more dramatic than that of the + gamete. The pairing ability of both

Table 6

Longitudinal sections of complete flagella (12 - 15 µm in length) could not be obtained. Tips and bases whose lengths vary could only be identified. Intermediate sections were eliminated because it could not be determined whether they were closer to the base or the tip. Numbers of ferritin grains per 0.33 µm contiguous segment were counted and comparisons made between tips and bases and among the different treatments. Each number represents the mean of counts from 3 - 6 flagella.

Table 6

Ferritin Con A Concentrations at Proximal and Distal Ends of Gametic Flagella

	+ Gamete	- Gamete	Gametes Mixed 30 sec	Gametes Mixed 30 min	Colchicine treated Gametes Mixed 4 min
Distal End	9.9	5.0	7.7	43.7	11.0
	3.8	3.8	7.7	42.3	6.5
	4.8	0.7	24.6	15.0	3.8
	2.0	12.0	6.0	5.0	
	3.0		23.0	8.0	
	5.0				
		12.5			
		3.0		10.0	
	2.0	2.7	1.0	9.0	8.0
	1.0	1.4	3.3	9.5	9.3
	6.5	3.0	3.5	3.5	8.4
Proximal End	18.0	1.0	1.5	4.3	7.0

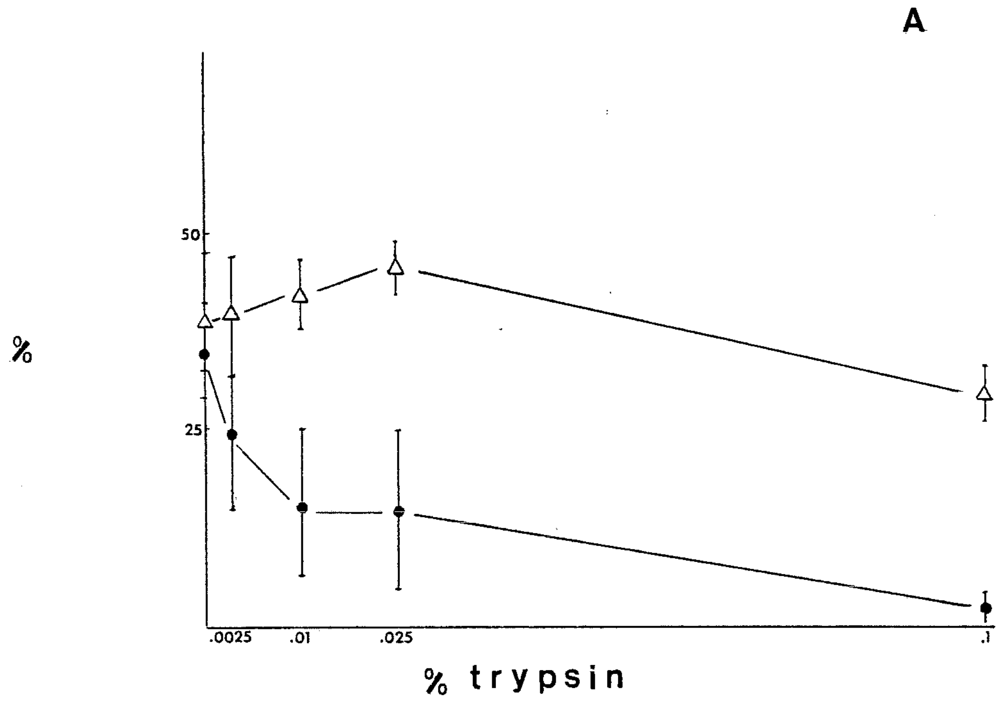
Figure 5

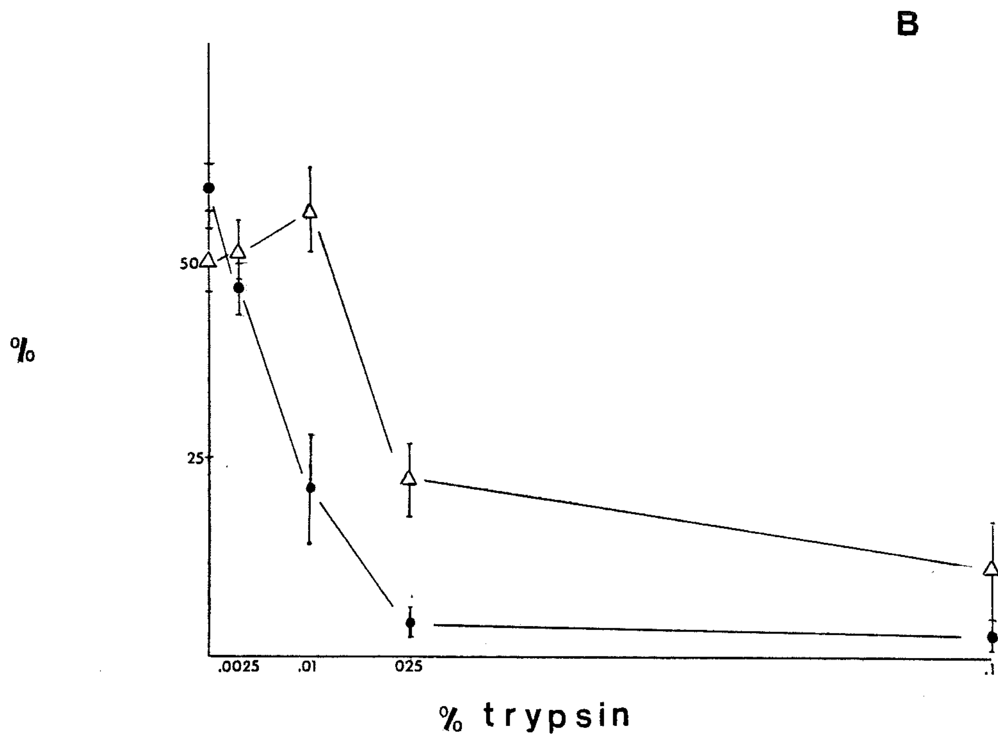
The agglutinability of the - gamete is more sensitive to trypsin digestion than that of the + gamete. High concentrations of trypsin affected the pairing ability of both gametes.

A. + gamete treated with varying concentrations of trypsin
(△) = % agglutination
(•) = % pairing

B. - gamete treated with varying concentrations of trypsin
(△) = % agglutination
(•) = % pairing

Figure 5





mating types was inhibited by trypsin treatments in concentrations of 0.025% and greater.

DISCUSSION

A simple and reliable method for quantification of the agglutination reaction of *Chlamydomonas moewusii* has been presented. Another Coulter Counter assay has been developed for the *Chlamydomonas reinhardtii* system by Snell and Roseman (1979). Examination of both methods reveals that each seems well suited to the cell system for which it was developed. The basic premises are the same for both assays. Percent agglutination is based upon the disappearance of single cells from a cell suspension. The total number of single cells in the sample was determined in our assay be taking representative samples from the single mating types and taking an average. In Snell and Roseman's assay (1979), the total number was determined by pouring the suspension back and forth between beakers until no further increase in cell number was obtained. Also in our assay, Coulter Counter counts from the mixed sample were corrected for the number of pairs that were included in the counts. This was accomplished by determining the percent pairing in a similar sample. Snell and Roseman (1979) performed calibration experiments initially and determined that the upper and lower threshold settings on their Coulter

Counter excluded most of the zygotes from the counts. They reported that the number of single cells obtained from the Counter was overestimated by 10% due to the zygotes. The pairing percentages were determined in our assay by hemocytometer counts of samples treated with iodine which serves to break up clustered cells and fix the cells. Pairing or percentages of zygote formation were determined by Snell and Roseman (1979) by trypsin treatment to destroy cell adhesiveness and microscopic counts of zygotes.

This analysis of the two methods of agglutination quantification reveals that the Snell and Roseman method (1979) could not easily be used for our cell system because of the incomplete action of trypsin in the destruction of cell adhesiveness (Figure 5). In addition, the accuracy of Snell and Roseman's assay (1979) must be questioned because of the interference of zygotes in counts of single cells. It seems that the 10% overestimation obtained in calibration was used to adjust the counts obtained in all aggregation studies. A criticism of this work is the generalization that zygote interference is constant. In our experiments, the percent pairing interference in agglutination counts was determined each time. Our data shows that the percentage of pairs included in agglutination counts varies with each experiment. In terms of accuracy for

the Chlamydomonas moewusii system then, it is apparent that the settling technique is superior.

The greater Con A isoagglutinability of the gametes of C. moewusii as compared to their vegetative counterparts correlates with the greater binding of Con A by gametes as opposed to vegetative cells (Katz, 1979). This does not agree with the contention of Musgrave et al (1979) that there is no correlation between the extent of Con A binding and isoagglutination of C. eugametos. Initially, we reported (McLean et al, in press) an agreement with Musgrave et al (1979) because we saw no significant difference between Con A isoagglutinability of gametes and their vegetative counterparts at a Con A concentration of 200 µg/ml. The Con A concentration curves (Figure 2) of gametes and vegetative cells show a significant difference between isoagglutinability at Con A concentrations of 300 µg/ml and greater. The data upon which Musgrave et al (1979) have based their conclusions may be results of artifacts of their procedure. Our experience (Katz, 1979) has shown that extended incubations of gametes with Con A at room temperature has resulted in a loss of binding probably due to the tipping phenomenon. In addition, we have found that the pH shock method of flagellar detachment (Witman et al, 1972) used by Musgrave and coworkers causes fraying of flagellar membranes (McLean, Jamieson,

Sedita, unpublished data) resulting in the loss of membrane bound receptor sites. Therefore, Musgrave and coworkers could have lost some of the radioactivity from their gametic flagella through tipping and damage to the flagellar membranes during deflagellation.

Mesland et al (1980) have reported that 0.0375 mM colchicine prevented tipping, flagellar tip activation and gamete fusion of C. reinhardtii. Menoff (1980) reported that 0.005 M colchicine inhibited mating structure activation and gamete fusion in C. moewusii because treated cells were not able to properly align their flagellar tips and the anteriors of their cell bodies. Sexual agglutination was not affected by colchicine treatment in either of these cases (Mesland et al, 1980; Menoff, 1980). The results reported here using the quantitative agglutination assay agree with both of these groups. Agglutination percentages for treated cells do not differ from those of control cells. Colchicine treatment does however inhibit pairing almost completely.

Inhibition of tipping has been the implicated action of colchicine treatment of gametic cells (Mesland et al, 1980) with the subsequent inhibition of flagellar tip activation and gamete fusion. The glycosylated tubulin located in the flagellar membrane of Chlamydomonas reinhardtii by Adair and Goodenough (1978) has been

postulated as a possible site of action of colchicine. Con A, when bound to gametic flagella, is tipped (Goodenough and Jurivich, 1978) and can elicit flagellar tip activation and mating structure activation (Mesland et al, 1980). Con A sites as marked by ferritin Con A were seen to be localized at different points along the flagella as the time of gametic interactions progressed. After 30 min, the localization of ferritin grains was found on the distal tip of the flagella. No localization of sites occurred in colchicine treated cells. This data is suggestive of the fact that colchicine is responsible for the inhibition of site migration or tipping because of the apparent lack of a clustering or localization of Con A sites on colchicine treated cells. Substantiation of this claim could only be obtained by further examination of flagellar sections particularly on colchicine treated cells since the possibility of a localization exists beyond the segments counted in Table 6 in the intermediary zone between the proximal and distal ends. In addition, the localizations of ferritin grains may not be due to site migration. Gametic interaction could somehow cause the opening up or exposure of terminal mannose or glucose residues on sites which would then be bound by ferritin Con A. Another cause of the increase in Con A sites following gametic contact could be the stimulation of their synthesis by gametic interaction.

Exposure of additional sites may also occur during the fixation procedures.

Bloodgood (1977) and coworkers (1979) have described a surface motility characteristic of C. reinhardtii flagellar membranes. This motility involves the translocation of polystyrene beads along the length of the flagella. Hoffman and Goodenough (1980) have dissected this surface motility into at least two components, namely that of a bead binding or tubulin-like component and a bead translocation component on the flagellar surface. The bead binding component is suggested to be tubulin-like because it is affected by colchicine and vinblastine treatment (Hoffman and Goodenough, 1980). The bead translocator is affected by trypsin treatment (Hoffman and Goodenough, 1980). These workers have also noted that bead translocation is inhibited when the flagellar tips of gametes are "locked in." This observation seems to link together both the tipping and bead translocating mechanisms. A common driving force or motor possibly involving a membrane bound ATPase (Watanabe and Flavin, 1976) is implicated (Hoffman and Goodenough, 1980).

In light of the fact that a trypsin sensitive driving force may be involved in tipping, experiments were undertaken to quantitate the effects of trypsin digestion on both sexual agglutination and pairing in

C. moewusii. Pairing is greatly reduced in both mating types at a concentration of trypsin of 0.025% and is completely inhibited at concentrations of 0.1%. Agglutination however, occurred between trypsin treated gametes. One of the sites of action of trypsin therefore must involve the events between agglutination and pairing, one of which is tipping. These findings then support the contention of Hoffman and Goodenough (1980) that a trypsin sensitive component may be involved in tipping. In other words, trypsin may affect the translocating mechanism and thus prevent tipping and subsequent pairing.

Results also show that the sexual agglutinability of the - gamete of C. moewusii is sensitive to trypsin in concentrations of 0.025% and greater. Wiese and Metz (1969) reported that pairing in C. moewusii is sensitive to trypsin at 0.025% but agglutination is unaffected. They also reported that the agglutinability of the - gamete is reduced in the presence of 0.1% trypsin. There is agreement between the two sets of data in general trends but there is disparity concerning the concentration of trypsin at which agglutinability becomes sensitive. One possible explanation may be a difference in the purity of the enzymes used.

In conclusion, the Con A data presented here strengthens the case for further lectin studies with Chlamydomonas in order to more fully understand the

mating events. Gametogenesis is accompanied by an increase in Con A isoagglutinability and Con A receptor sites may be tipped during mating interactions. Collectively, the data also suggest a mechanism for the tipping phenomenon. Since ferritin binding on colchicine treated cells was not noticeably less than in control gametes and the Con A sites are localized at progressive points along the flagella toward the distal tips as the time of gametic interaction increased, the receptor sites could possibly be attached to the "binding component of the flagellar motor" (Hoffman and Goodenough, 1980) perhaps by a tubulin-like molecule and the entire complex may then be moved by the translocator (Hoffman and Goodenough, 1980). The binding component and the translocator seem to move up and down the length of the flagellum as traced by polystyrene beads (Hoffman and Goodenough, 1980; Bloodgood, 1977; Bloodgood et al, 1979). The binding of material such as Con A or the agglutinins of opposite mating types could trigger a signal that directs the motor complex to concentrate movement toward the tips. The result would be the accumulation of material at the flagellar tips which then in turn may play a role in triggering subsequent mating events. This model is reminiscent of that proposed by Menoff (1980) in which a tubulin component within the membrane is associated with receptor sites on the cell surface and an ATPase on the cytoplasmic side of the membrane.

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