MATING IN *CHLAMYDOMONAS*: A SYSTEM FOR THE STUDY OF SPECIFIC CELL ADHESION

II. A Radioactive Flagella-Binding Assay for Quantitation of Adhesion

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ABSTRACT

To measure the flagellar adhesiveness of *Chlamydomonas* gametes in a more quantitative manner than agglutination assays permit, a binding assay was developed which measured the binding of radioactive flagella of one mating type to unlabeled gametes of the opposite mating type. With the appropriate assay conditions, the number of [^{3}H]flagella specifically bound was shown to be proportional to the number of cells in the incubation mixture and, therefore, to the number of binding sites that were present. The assay was used to study the effects of trypsin treatment on the loss and development of flagellar binding sites. It was shown that after trypsin treatment at least 9 h were required for the return of a full complement of binding sites to the flagellar surface; moreover, the results indicated that these sites reappeared on existing, extended flagella.

The specificity of cell-cell interactions has been studied in a variety of systems: mating in yeast cells (4, 22) and protistans including *Chlamydomonas* (27, 29), *Tetrahymena*, and *Paramecium* (12); sorting out of numerous embryonic cells and tissue explants (24); and reaggregation of sponge cells (7, 14, 30). These interactions have been monitored with qualitative and semi-quantitative methods including direct examination of tissue aggregates (24, 25), determination of the number and size of cell aggregates (13, 15), and assessment of the percentage of cells involved in agglutination (4, 10, 28). More recently, assays have been developed that measure the binding of radioactive cells to tissues (2), cell aggregates (17), and tissue culture cell monolayers (26). Such radioactive binding assays permit a quantitative assessment of the specificity of cell interactions and can be useful in the isolation of surface moieties involved in the binding together of cells. For example, by use of a radioactive binding assay Barbera et al. (2) have shown that neuronal cells from a limited area of the embryonic chick retina will bind preferentially to that part of the optic tectum near their normal synapse. Similarly, Walther et al. (26) have measured the adhesiveness of several tissue culture cell lines by determining the rate of adhesion of radioactive single cells to cell monolayers. These radioactive binding assays can also be used to measure the adhesiveness of cell surface fractions and to isolate the adhesive components from these fractions. Taylor and Orton (23), Crandall and Brock (4), and Yen and Ballou (32) have used this approach to isolate and characterize yeast cell mating factors (isoagglutinins). Similarly, Merrell and Glaser (11) have isolated membranes from embryonic chick neural retina cells and have shown that radioactive membranes will specifically bind to the neural retina cells. Balsamo and Lilien (1), working with chick

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cells, have measured the binding of radioactive aggregation-enhancing factors to whole cells and have shown that the binding of the factors is specific and may be a cooperative process. Ultimately, the binding assays developed by these investigators can be used for the isolation and characterization of the molecules on the cell surface that are responsible for specific cell adhesion.

In previous studies on *Chlamydomonas* flagellar adhesion, the effects of proteases (9, 29), metabolic inhibitors (6, 8), and cell division (8, 18) on the development and loss of flagellar binding sites have been examined. However, precise quantitation of the number of binding sites has not been possible because of the indirect and qualitative nature of the assays used in these investigations. In this report a quantitative, reproducible, and sensitive assay is described which can be used to measure the binding of radioactive flagella of one mating type to unlabeled gametes of the opposite mating type. This flagella binding assay has been used to monitor the effects of trypsin on flagellar binding sites and to study the return of these sites to the flagellar surface. It is shown that recovery of a full complement of binding sites after trypsin treatment takes much longer than the agglutination assay (9) would indicate, and that the return of binding sites to the flagellar surface occurs through *in situ* modification or insertion of these sites into existing, extended flagella.

This flagella binding assay will now permit additional studies on the development and loss of flagellar adhesiveness and can be used for the isolation and characterization of the molecules on the flagellar surface that are responsible for adhesion.

MATERIALS AND METHODS

Culturing of Chlamydomonas

Strains 21gr [(+) mating type] and 6145c [(−) mating type] of *Chlamydomonas reinhardtii* were cultured as described in the previous report (20).

Incorporation of ^{3}H into Flagella

To obtain ^{3}H-labeled gametic flagella, 8 liters of cells (approximately 2×10^6 cells per ml) were collected from R medium as described in the previous report (20) and resuspended in 1 liter of nitrogen-free medium (M-N) (20). The pH of the suspension of cells was rapidly lowered to 4.5 with 0.5 M acetic acid for 30–90 s to deflagellate the cells and then raised back to 7.6 with 0.5 M potassium hydroxide. The cells and detached flagella were centrifuged at 1,900 *g* (3,000 rpm, International Equipment Co., Needham Heights, Mass. Pr-6 centrifuge, rotor no. 253) for 3 min at 25°C in 50 ml conical polycarbonate centrifuge tubes, sedimenting the cells and leaving the flagella in the supernate. The supernate was discarded and the deflagellated cells were resuspended in 500 ml of M-N, centrifuged again to separate any remaining flagella from the cell bodies, and then the cells were resuspended in 200 ml of M-N. 10 mCi of [^{3}H]sodium acetate (New England Nuclear, Boston, Mass. spec act 1,291 mCi/mmol) were added and the suspension was incubated at 25°C for 45 min with aeration. During this time the cells regenerated new flagella (16). After bringing the volume of cells up to the original 8 liters with M-N, the cultures were put in continuous light with aeration at 25°C. 15–18 h later, flagella were isolated using the sucrose-pH shock method described in the previous report (20).

Visual Assay for Adhesion

The ability of gametes to agglutinate or isoagglutinate was determined according to the methods described in the previous report (20). Cell suspensions were subjectively scored for agglutinating activity according to the following qualitative scheme: (0), when 0% of the cells were clumped; (+), when 1–25% of the cells were clumped; (++), when 25–50% were clumped; (+++), when 50–75% of the cells were clumped; and (++++), when 75–100% of the cells were clumped.

Quantitative Flagella-Binding Assay for Adhesion

[^{3}H]Flagella isolated from 8 liters (approximately 6×10^6 cells per ml) of (+) gametes as described in the previous report (20) were resuspended in 6 ml of 7% sucrose in 10 mM Tris buffer, pH 7.8 at 25°C (TB) to give a protein concentration of approximately 1 mg/ml and either used immediately or stored at −16°C. Preparations of frozen flagella could be stored for many months without losing their adhesiveness.[1] To quantitate the amount of gametic flagella that would bind to flagellated cells of the same and opposite mating types, (+) gametic [^{3}H]flagella were incubated with 1-ml aliquots of washed cells in M-N in 50-ml conical

[1] Claes (3) has reported that flagella isolated from *C. reinhardtii* gametes using the pH shock method without sucrose were not specifically adhesive and would not cause cells of the opposite mating type to isoagglutinate. In the present investigations both (+) and (−) gametic flagella isolated with the sucrose-pH shock method of Witman et al. (31) were always specifically adhesive. The differences in results may reflect a difference between the strains used [Claes used strains 89 (−) and 90 (+)]. Alternatively, the difference could be due to the absence of sucrose in his isolation solutions or to some other differences in technique.

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polycarbonate centrifuge tubes at 25°C. The number of flagella added, incubation time, and cell density were varied in different experiments. The suspensions were incubated in the 50-ml tubes tilted at an angle of 75° to the vertical and shaken 90–100 times per minute with 3.7-cm strokes in a Dubnoff metabolic shaking incubator (Precision Scientific Co., Chicago, Ill.). 50 ml of ice-cold TB was added to dilute the suspension and stop the reaction. The tubes were centrifuged at 1,450 *g* (2,700 rpm, IEC PR-6 centrifuge, rotor no. 253) for 4 min at 4°C to sediment the cells and any bound flagella, and the supernate was removed by aspiration. This procedure left approximately 0.5 ml of the TB adhering to the walls of the centrifuge tubes. To force this residual TB to the bottom of the tubes so that it could be removed, the tubes were centrifuged again. After again removing the TB by aspiration, 0.1 ml of H_2O was added to the cells at the bottom of each tube to make a slurry. The entire slurry of cells from the bottom of each tube was then transferred to a glass scintillation vial to which was added 2.0 ml of Nuclear Chicago Solubilizer (NCS), Nuclear-Chicago Corp., Des Plaines, Ill., and the material was incubated at 50°C for 2 h or at room temperature for 10–15 h to completely digest the cells and flagella. Glacial acetic acid was added (0.075 ml) to each vial to inhibit chemiluminescence; 10 ml of fluor (6 g 2,5-diphenyloxazole, 0.078 g *p*-bis [2-(5-phenyloxazolyl)]-benzene per liter of toluene) was added, and the vials were counted by liquid scintillation. Counts per minute were converted to dpm using the channel's ratio method for determining counting efficiency.

Preparation of Inactivated Trypsin

Trypsin (218 U per mg, Worthington Biochemical Corp., Freehold, N.J.), was inactivated with 1-chloro-3-tosylamido-7-amino-2-heptanone (TLCK) (Sigma Chemical Co., St. Louis, Mo.) according to the method of Shaw et al. (19). Trypsin (50 mg) was added in small aliquots during 1 h to a stirred solution of TLCK (2.08 mg), freshly dissolved in 10 ml of 0.05 M Tris-maleate-calcium chloride buffer, pH 7.0 at 25°C. The solution was stirred continuously for an additional 3 h and then was adjusted to pH 4.0 with HCl. After dialysis at 4°C against several changes of 400 vol of distilled water at 4°C, the pH was readjusted to 4.0 and the solution lyophilized. Before use, the lyophilized TLCK-trypsin was dissolved (5 mg/ml) in 1 mM HCl.

Treatment of Cells with Trypsin

Gametes were washed and resuspended in 10 ml (5×10^6 cells per ml) of M-N in 50-ml conical polycarbonate centrifuge tubes. 100 μl of a 5 mg/ml solution of Trypsin (Worthington, 218 U per mg; contaminant chymotryptic activity inhibited with L-[1-tosylamido-2-phenyl] ethyl chloromethyl ketone [TPCK]) in 1 mM HCl was added to the cell suspension to give a final trypsin concentration of 0.005% and the mixture incubated at 25°C with shaking for 5 min. 40 ml of M-N was added to each tube to dilute the reaction and the cells were harvested by centrifugation at 1,300 *g* (2,500 rpm, IEC PR-6, rotor no. 253) at 25°C for 3 min. The supernatants were removed by aspiration and the cells were washed once by resuspension in 50 ml of M-N, harvested again, and resuspended in 10 ml of M-N.

Protein and Cell Density Determinations

Protein concentration and cell density determinations were performed according to the procedures described in the previous report (20).

Gel Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) was carried out according to the procedure of Fairbanks et al. (5) on 3% gels as described in the previous report (20).

Determination of Extent of Flagellation of Cells

Gametes (0.25 ml) in M-N were added to 0.25 ml of 4% glutaraldehyde in 0.01 potassium phosphate buffer, pH 7.0. The percent of biflagellated cells was determined by counting at least 400 cells per sample with a Zeiss phase-contrast microscope.

RESULTS

The Flagella Binding Assay

Fig. 1 presents a schematic diagram of the assay developed to quantitate the adhesiveness of gametic flagella. The assay measures the amount of (+) gametic [^{3}H]flagella that binds to the flagella of (−) cells firmly enough to sediment with the (−) cells upon centrifugation. [^{3}H]Flagella that do not bind remain in the supernate. Phase-contrast microscopy of sedimented (−) gametes that had been incubated with (+) gametic flagella showed the added flagella binding to the flagella of the (−) gametes; this confirmed that the radioactivity that was binding to the cells was, in fact, in the form of intact, labeled flagella. In the experiments described in this report, (+) gametic flagella were labeled with ^{3}H and specific binding is defined as the amount of (+) [^{3}H]flagella bound by (−) [6145c] gametes less the amount of (+) [^{3}H]flagella bound by (+) gametes. The range in maximum percent of flagella bound varied between experiments from 5% bound to 20% bound and depended on the adhesiveness of the flagella of the (−) gametes used in the incubation mixture as well as the adhesiveness of the isolated (+)

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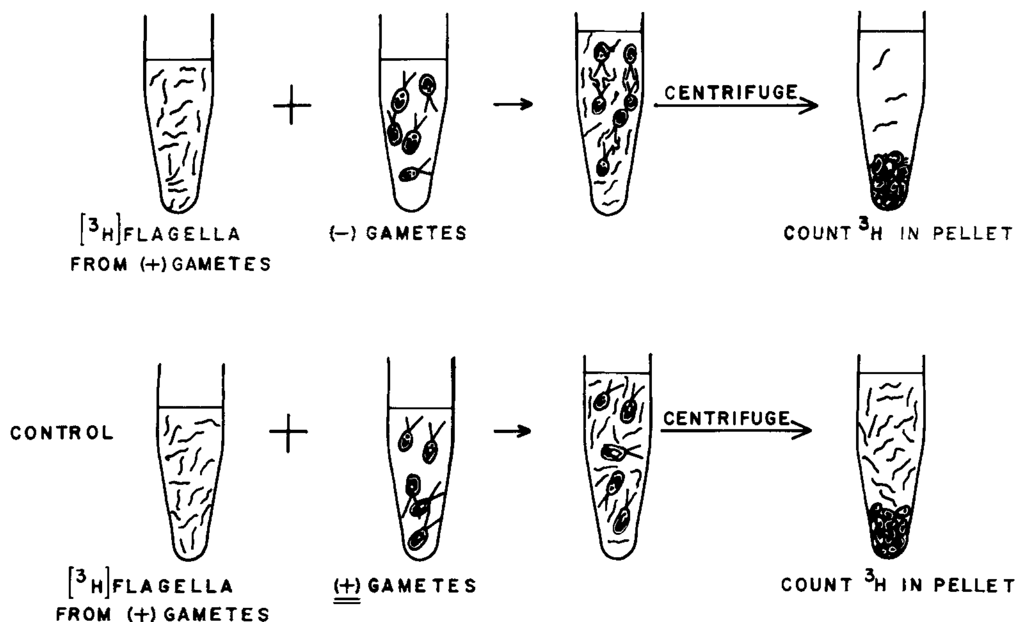

FIGURE 1 Schematic diagram of the flagella binding assay. Specific binding is defined as the amount of (+) [^{3}H]flagella bound by (–) gametes less the amount bound by (+) gametes.

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[^{3}H]flagella; however, within each experiment these variables could be controlled because the [^{3}H]flagella were from a single population of (+) gametes and (–) gametes were from a single population of cells.

Parameters of the Assay

INCUBATION TIME: When (+) gametic flagella were mixed with (–) gametes the isolated (+) flagella could be observed by phase-contrast microscopy to adhere almost immediately to the flagella of the (–) gametes. To determine the incubation time required for optimal binding, (–) gametes (0.63 and 2.1 × 10^6 cells per ml) were incubated with (+) [^{3}H]flagella (25 or 200 μl) for 2, 4, and 10 min at 25°C. As shown in Fig. 2, after 4 min the binding reaction was complete for the four combinations of cells and flagella used. As a result, an incubation time of 10 min was used in subsequent experiments when other parameters of the binding reaction were investigated.

CELL DENSITY AND FLAGELLAR NUMBER: The effect of cell density on the number of flagella bound is shown in Fig. 3. In this experiment the amount of flagella was held constant at either 25, 75 or 150 μl of (+) [^{3}H]flagella, while the density of cells was varied from 0.87 to 8.7 × 10^6 cells per ml. It can be seen that with 75 μl of (+) [^{3}H]flagella in the assay mixture, the number of flagella bound was proportional to the number of cells added. This indicated that the (+) [^{3}H]flagella were in excess at all four cell densities examined and that the binding sites on the flagella of the (–) gametes were saturated. Under these conditions, therefore, assuming that each (–) gamete had a relatively constant number of binding sites per flagellum, the number of (+) [^{3}H]flagella bound was proportional to the number of (–) gametic flagellar binding sites in the assay incubation mixture.

In this same experiment, when smaller amounts of (+) [^{3}H]flagella (25 μl) were used with cell concentrations below 4.3 × 10^6, the number of flagella bound was also proportional to the number of cells added. However, at higher cell densities, the curve began to plateau and adding more cells did not result in a proportionately greater number of (+) [^{3}H]flagella being bound. This indicated that at a cell density of 4.3 × 10^6 all of the flagella in the 25-μl aliquot that could be bound under these assay conditions were bound. Similarly, the curve shown for 75 μl of (+) [^{3}H]flagella would also have leveled off if enough cells had been added to react with all of the "bindable" flagella.

When 150 μl of (+) [^{3}H]flagella was used, it was expected that the amount of flagella bound would

have been the same as with 75 μl of flagella, since it was demonstrated that 75 μl of flagella was sufficient to saturate the binding sites on the (−) gamete flagella at all cell densities examined. However, with 150 μl of (+) [^{3}H]flagella there was an enhancement of binding at the highest cell density (8.7×10^6 cells per ml). This was probably due to a trapping effect in which large numbers of bound flagella formed a meshwork which caused unbound flagella to be sedimented in the assay. For this reason, in subsequent experiments with the flagella binding assay, low cell densities ($4–5 \times 10^6$ cells per ml) and saturating or near-saturating amounts of (+) [^{3}H]flagella were used; under these conditions the number of (+) [^{3}H]flagella bound was proportional to the number of flagellar binding sites present.

Utilization of the Radioactive Flagella Binding Assay to Quantitate the Loss and Return of Flagellar Adhesiveness after Trypsin Treatment

Trypsinization procedures can be useful for determining (*a*) the protein nature of flagella binding sites, (*b*) the timing of insertion of sites destroyed by the enzyme and, when coupled with metabolic inhibitors such as cycloheximide, (*c*) the requirement of new protein synthesis for the development of adhesiveness. By use of qualitative agglutination assays it has been shown that trypsin destroys the adhesiveness of gametic flagella (29) and that the gametes recover their agglutinability within 45 min after treatment (9). However, in order to quantitate the loss and development of flagellar adhesiveness and, eventually, to determine exactly what moieties are being removed by trypsin, a flagella-binding assay like that described above is essential. In the following trypsin experiments the conditions (cell density, number of (+) [^{3}H]flagella, and incubation time) used in the assay are given in the figure legends.

Effect of Trypsin and Inactivated Trypsin on the Adhesiveness of Flagella

It was important to demonstrate that the effects of trypsin were due to its enzymatic activity rather than to nonspecific masking of binding sites. Trypsin was inactivated as described in Materials and Methods with TLCK according to the method of Shaw et al. (19). SDS PAGE of the TLCK-treated trypsin demonstrated that the inactivation procedure did not result in proteolysis of the trypsin (Fig. 4).

Gametes were treated with trypsin and TLCK-trypsin as described in Materials and Methods. After incubation with the trypsin solutions, the

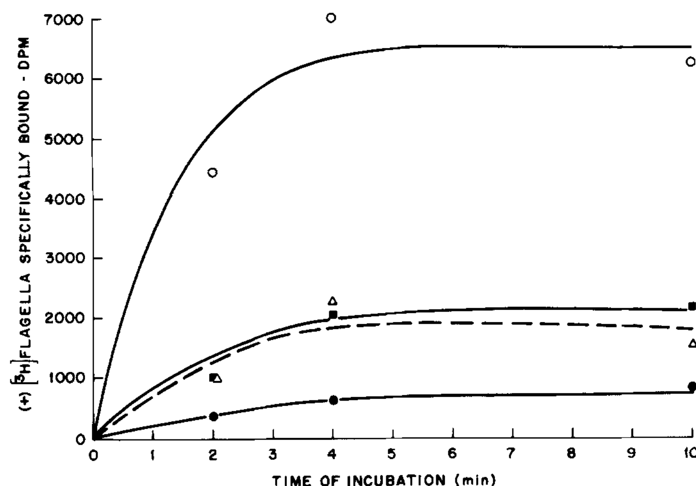

FIGURE 2 Dependence of dpm (+) [^{3}H]flagella specifically bound to (−) gametes on time of incubation at two different cell and (+) [^{3}H]flagella concentrations. ●, 25 μl of (+) [^{3}H]flagella, 0.63×10^6 cells/ml; ■, 25 μl of (+) [^{3}H]flagella, 2.1×10^6 cells/ml; Δ, 200 μl of (+) [^{3}H]flagella, 0.63×10^6 cells/ml; O, 200 μl of (+) [^{3}H]flagella, 2.1×10^6 cells/ml. Each point represents the average of triplicate determinations of dpm (+) [^{3}H]flagella bound by (−) gametes less the average of duplicate determinations of dpm (+) [^{3}H]flagella bound by (+) gametes. Incubation vol was 1.20 ml. Spec act of the (+) [^{3}H]flagella was 1,960 dpm/μl.

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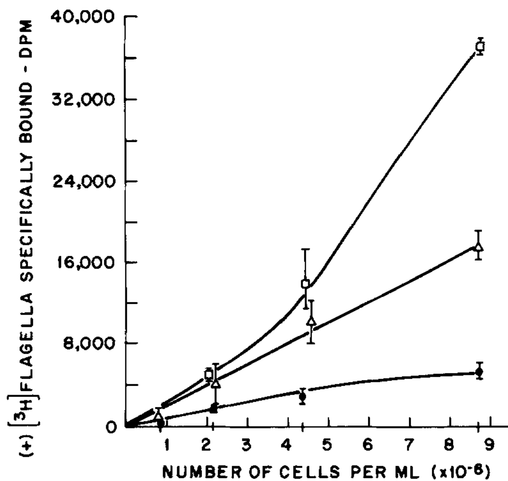

FIGURE 3 Dependence of dpm (+) [^{3}H]flagella specifically bound by (−) gametes on cell number. ●, 25 μl of (+) [^{3}H]flagella; Δ, 75 μl of (+) [^{3}H]flagella; □, 150 μl of (+) [^{3}H]flagella. Incubation time was 10 min; vol was 1.15 ml. Each bar represents the range of triplicate determinations of dpm (+) [^{3}H]flagella bound by (−) gametes less the average of duplicate determinations of dpm (+) [^{3}H]flagella bound by (+) gametes. Spec act of the (+) [^{3}H]flagella was 1,190 dpm/μl.

cells were washed twice with M-N, and their adhesiveness was determined by use of the flagella-binding assay. The results shown in Table I indicate that the active trypsin reduced the ability of the (−) gametes to bind (+) [^{3}H]flagella to background levels. On the other hand, the TLCK-trypsin-treated cells bound (+) [^{3}H]flagella to the same extent as the untreated control cells. These results showed that trypsin causes the loss of adhesiveness and that the effects of trypsin are due to its enzymatic activity, rather than to nonspecific masking of binding sites.

Monitoring the Return of Adhesiveness after Trypsin Treatment

Cells were treated with trypsin as described above, washed two times with M-N and resuspended in M-N. Adhesiveness was monitored at 0, 1, 4, and 9 h by use of a visual assay and the flagellar binding assay. In addition, the percentage of bi-flagellated cells was measured at each of these times to determine if there was any flagellar loss and regeneration during the experiment.

VISUAL ASSAY: Untreated (+) gametes were mixed with (−) gametes that had been trypsin-treated, and the amount of agglutination was scored qualitatively as described in Materials and Methods. As shown in Table II, immediately after treatment (t = 0) the trypsin-treated (−) cells had no agglutinating ability, whereas the untreated (−) cells showed full (++++) agglutination. At t = 1 h a few of the trypsin-treated (−) gametes were capable of agglutinating, and at t = 4 h the

FIGURE 4 SDS polyacrylamide gels of normal trypsin (A) and TLCK-treated, inactive trypsin (B).

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tryspin-treated cells agglutinated as well as the untreated cells, with almost every cell being involved in the agglutination reaction. Similarly, at 9 h the trypsin-treated (−) gametes were indistinguishable from the untreated (−) gametes with respect to their ability to agglutinate with the untreated (+) gametes. Therefore, the visual assay indicated that between 1 and 4 h were required for the full recovery of agglutinability.

FLAGELLA-BINDING ASSAY: The binding assay confirmed the results of the visual assay at $t = 0$ and $t = 1$ h. As can be seen in Table III, at both of these times the tryspin-treated (−) gametes bound essentially no (+) [^{3}H]flagella. However, at $t = 4$ h, when the visual assay showed no differences between the treated and untreated (−) gametes in their ability to clump with the (+) cells, the binding assay showed that the treated (−) cells were binding only 52% of the flagella bound by the untreated (−) cells. At $t = 9$ h, when the visual assay again showed no differences between the treated and the untreated cells, the binding assay revealed that the treated cells had 89% of the binding ability of the untreated cells. Therefore, the flagella binding assay indicated that at least 9 h were required for recovery of a full complement of binding sites.

TABLE I

*Effect of Trypsin and Inactivated Trypsin (TLCK-Trypsin) on Adhesiveness of (−) Gametes**

Treatment	(+) [^{3}H]flagella bound specifically	Binding ability remaining after treatment
	dpm	%
TLCK-Trypsin	4,073	106
Trypsin	64	1.7
Untreated	3,850	100

* 50 μl of (+) [^{3}H]flagella (1,190 dpm/μl) was incubated for 10 min with 5×10^6 cells; incubation vol was 1.05 ml. The dpm (+) [^{3}H]flagella specifically bound represents the average of triplicate determinations of dpm (+) [^{3}H]flagella bound to (−) gametes less the average of duplicate determinations of dpm (+) [^{3}H] flagella bound by (+) gametes.

TABLE II

Recovery of Agglutinating Ability After Trypsin Treatment

Time after trypsin treatment	Agglutinating ability	
	Trypsin-treated	Untreated
h		
0	0	++++
1	+	++++
4	++++	++++
9	++++	++++

TABLE III

*Recovery of (+) [^{3}H]Flagella Binding Ability after Trypsin Treatment**

Time after trypsin treatment	(+) [^{3}H]flagella bound specifically		Binding ability recovered	Cells with two flagella	
	Trypsin-treated	Untreated		Trypsin-treated	Untreated
h	*dpm*	*dpm*	%	%	%
0	0	3,714	0	94	95
1	319	3,381	9.4	96	97
4	1,620	3,141	52	92	95
9	2,749	3,093	89	99	99

* 40 μl of (+) [^{3}H]flagella (1,593 dpm/μl) was incubated for 10 min with 5×10^6 cells; incubation vol was 1.04 ml. The dpm [^{3}H]flagella specifically bound represents the average of triplicate determinations of dpm (+) [^{3}H]flagella bound to (−) gametes less the dpm of (+) [^{3}H]flagella bound to (+) gametes.

Also included in Table III is the percentage of cells with two flagella at each of the time points examined with the adhesion assay. It is clear that the trypsin treatment did not cause extensive deflagellation of the cells, since 94–95% of the treated and untreated cells had two flagella immediately after the trypsin treatment. In addition, at least 92% of the cells were fully flagellated at each of the other time points examined, indicating that the loss of adhesiveness of the cells was not due to deflagellation. Moreover, since the cells were fully flagellated during the course of the experiment, the results also showed that the return of binding sites to the flagellar surface was occurring through *in situ* modification or insertion of adhesive molecules.

DISCUSSION

In this report, a radioactive-binding assay has been described which was used to quantitate flagellar adhesiveness in *C. reinhardtii*. By use of the appropriate assay conditions (cell density, concentration of [^{3}H]flagella, incubation time),

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the number of [^{3}H]flagella specifically bound was shown to be proportional to the number of cells in the assay incubation mixture and, therefore, to the number of binding sites which were present. This binding assay will permit the measurement of the loss and development of flagellar binding sites in a more quantitative manner than has been heretofore possible with an agglutination assay.

By use of the radioactive binding assay it was shown that after trypsin treatment gametes required at least 9 h to regain [^{3}H]flagella binding ability equal to that of untreated gametes (Table III). This contrasted with the qualitative observations that only 4 h or less were required for the gametes to regain full agglutinating ability (Table II). Apparently, gametes are capable of agglutinating without their full complement of binding sites. This observation is relevant to studies on flagellar adhesion by others in which inhibition of agglutination has been used as an assay to determine whether supernates from trypsin-treated gametes (29), monosaccharides, or plant lectins (9) interact with binding sites on gametic flagella. The results in this report on the comparison of the [^{3}H]-flagella binding ability of trypsin-treated gametes to their agglutinating ability suggest that 50–90% (see Table III) of the flagellar binding sites would have to be interacting with these exogenous materials to affect agglutination; and, therefore, an inhibition of agglutination assay would not be very sensitive.

Phase-contrast observations of trypsin-treated gametes showed that 92–99% of the cells were fully flagellated as they regained their adhesiveness (Table III). This indicated that the return of binding sites to the flagellar surface occurred through *in situ* modification or insertion of these sites on existing, extended flagella. In related studies by Schmeisser et al. (18), it was shown that cell division, during which time new flagella are regenerated, was not required for the development of adhesive gametes. Similarly, Kates (8) has shown that loss of mating competency could occur in the absence of cell division. However, in the studies by Kates and by Schmeisser et al. it was not possible to determine whether the development and loss of adhesiveness was occurring on intact flagella, since neither investigator reported whether the cells maintained their flagella as their adhesiveness changed. It might have been that existing flagella were resorbed and new flagella regenerated.

Other investigators have studied the effects of RNA and protein synthesis inhibitors on gametogenesis and the maintenance of mating competency. Hipkiss (6) has shown that streptomycin, which interferes with chloroplast protein synthesis, did not interfere with the development of flagellar adhesiveness when present during gametogenesis, but did prevent gametic fusion. Kates (8) has shown that gametes lose their ability to fuse and form zygotes 8 h after actinomycin-D treatment. In Kates' studies the ability of gametes to fuse and form zygotes was assessed, rather than adhesiveness, and it may have been possible that actinomycin-D interfered with some step in zygote formation other than flagellar adhesion.

The radioactive binding assay described in this report will now make it possible (*a*) to quantitate the development of flagellar adhesiveness as cells differentiate into gametes, (*b*) to measure the loss of flagellar adhesiveness when gametes are re-fed nitrogen, (*c*) to investigate the effects of RNA and protein synthesis inhibitors on loss and development of adhesiveness, (*d*) to determine if flagellar amputation and regeneration have any effect on flagellar binding sites, and (*e*) to determine if zygotic flagella, which are no longer adhesive to each other or to the flagella of other zygotes, have lost all flagella binding sites.

Moreover, the assay, or modifications of such an assay should permit the identification of flagellar surface fractions involved in adhesion. In the previous report on *Chlamydomonas* flagellar adhesion (20) it was shown by electron microscope and electrophoretic analyses that gamone, the specifically adhesive material released into the medium by gametes, is composed of flagellar surface components. This was substantiated by demonstrating that isolated flagellar membranes from gametes were capable of causing isoagglutination of gametes of the opposite mating type. When the gamone was separated into membranes and mastigonemes, only the membrane fraction (which contained a few mastigonemes) caused isoagglutination; the mastigoneme fraction was inactive.

Because the assay used in these studies was based on an assessment of cell isoagglutination, it would not permit the identification of structures which could bind to gametic flagella but not cause isoagglutination, i.e. univalent structures. By use of the radioactive binding assay described above, it should now be possible to directly measure the

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binding ability of both the membrane and mastigoneme fractions of gamone to determine if the mastigoneme fraction, which is not active in causing isoagglutination, is capable of binding to cells of the opposite mating type.

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REFERENCES

1. BALSAMO, J., and J. LILIEN. 1974. Embryonic cell aggregation: kinetics and specificity of binding of enhancing factors. *Proc. Natl. Acad. Sci. U.S.A.* **3:**727-731.
2. BARBERA, A. J., R. B. MARCHASE, and S. ROTH. 1973. Adhesive recognition and retinotectal specificity. *Proc. Natl. Acad. Sci. U.S.A.* **70:**2482-2486.
3. CLAES, H. 1971. Autolyse der zellwand bei den gameten von *Chlamydomonas reinhardi. Arch. Mikrobiol.* **78:**180-188.
4. CRANDALL, M. A., and T. D. BROCK. 1968. Molecular aspects of specific cell contact. *Science (Wash. D.C.).* **161:**473-475.
5. FAIRBANKS, G., T. L. STECK, and D. F. H. WALLACH. 1971. Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. *Biochemistry* **10:**2606-2616.
6. HIPKISS, A. R. 1967. The effect of streptomycin on gametogenesis in *Chlamydomonas reinhardi. Life Sci.* **6:**669-672.
7. HUMPHREYS, T. 1963. Chemical dissolution and *in vitro* reconstruction of sponge cell adhesions. I. Isolation and functional demonstration of the components involved. *Dev. Biol.* **8:**27-47.
8. KATES, J. R. 1966. Biochemical Aspects of Synchronized Growth and Differentiation in *Chlamydomonas reinhardi.* Ph.D. Thesis. Princeton University, Princeton, N. J.
9. MCLEAN, R. J., and R. M. BROWN. 1974. Cell surface differentiation of *Chlamydomonas* during gametogenesis. I. Mating and concanavalin A agglutinability. *Dev. Biol.* **36:**279-285.
10. MCLEAN, R. J., C. J. LAURENDI, and R. M. BROWN, Jr. 1974. The relationship of gamone to the mating reaction in *Chlamydomonas moewusii. Proc. Natl. Acad. Sci. U.S.A.* **71:**2610-2613.
11. MERRELL, R., and L. GLASER. 1973. Specific recognition of plasma membranes by embryonic cells. *Proc. Natl. Acad. Sci. U.S.A.* **70:**2794-2798.
12. METZ, C. B. 1954. Mating substance and the physiology of fertilization in ciliates. *In* Sex in Microorganisms. C. B. Metz and A. Monroy, editors. Amer. Assn. for the Advancement of Science, Washington, D.C. 284-334.
13. MOSCONA, A. 1962. Analysis of cell recombination in experimental synthesis of tissues *in vitro. J. Cell Comp. Physiol.* **60:**65-80.
14. MOSCONA, A. A. 1963. Studies of cell aggregation: Demonstration of materials with selective cell binding activity. *Proc. Natl. Acad. Sci. U.S.A.* **49:**742-747.
15. MOSCONA, A. A. 1968. Cell aggregation: properties of specific cell-ligands and their role in the formation of multicellular systems. *Dev. Biol.* **18:**250-277.
16. ROSENBAUM, J. L., J. E. MOULDER, and D. L. RINGO. 1969. Flagellar elongation and shortening in *Chlamydomonas.* The use of cycloheximide and colchicine to study the synthesis and assembly of flagellar proteins. *J. Cell Biol.* **41:**600-619.
17. ROTH, S. A., and J. A. WESTON. 1967. The measurement of intercellular adhesion. *Zoology.* **58:**974-980.
18. SCHMEISSER, E. T., D. M. BAUMGARTEL, and S. H. HOWELL. 1973. Gametic differentiation in *Chlamydomonas reinhardi.* Cell cycle dependency and rates in attainment of mating competency. *Dev. Biol.* **31:**31-37.
19. SHAW, E., M. MARES-GUIA and W. COHEN. 1965. Evidence for an active-center histidine in trypsin through use of a specific reagent, 1-chloro-3-tosylamido-7-amino-2-heptanone, the chloromethyl ketone derived from N-tosyl-L-lysine. *Biochemistry.* **4:**2219-2224.
20. SNELL, W. J. 1976. Mating in *Chlamydomonas.* I. Ultrastructural and electrophoretic analysis of flagellar surface components involved in adhesion. *J. Cell Biol.* **68:**48-69.
21. SNELL, W. J., S. A. KROOP, and J. L. ROSENBAUM. 1973. Characterization of adhesive substances on the surface of *Chlamydomonas* gamete flagella. *J. Cell Biol.* **59:**327*a* (Abstr.).
22. TAYLOR, N. W. 1964. Specific, soluble factor involved in sexual agglutination of the yeast *Hansenula wingei. J. Bacteriol.* **87:**863-866.

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23. TAYLOR, N. W., and W. L. ORTON. 1968. Sexual agglutination in yeast. VII. Significance of the 1.7S component from reduced 5-agglutinin. *Arch. Biochem. Biophys.* **126**:912–921.
24. TRINKAUS, J. P. 1969. Cells into Organs: The Forces that Shape the Embryo. Prentice-Hall, Inc., Englewood Cliffs, N. J.
25. TRINKAUS, J. P., and J. P. LENTZ. 1964. Direct observation of type-specific segregation in mixed cell aggregates. *Dev. Biol.* **9**:115–136.
26. WALTHER, B. T., R. OHMAN, and S. ROSEMAN. 1973. A quantitative assay for intercellular adhesion. *Proc. Natl. Acad. Sci. U.S.A.* **70**: 1569–1573.
27. WIESE, L. 1969. Algae, *In* Fertilization. C. B. Metz and A. Monroy, editors. Vol. 2. Academic Press, Inc., New York. 135–188.
28. WIESE, L. 1974. Nature of sex specific glycoprotein agglutinins in *Chlamydomonas*. *Ann. N.Y. Acad. Sci.* **234**:383–394.
29. WIESE, L., and C. B. METZ. 1969. On the trypsin sensitivity of gamete contact at fertilization as studied with living gametes in *Chlamydomonas*. *Biol. Bull.* **136**:483–493.
30. WILSON, H. V. 1907. On some phenomena of coalescence and regeneration in sponge. *J. Exp. Zool.* **5**:245–258.
31. WITMAN, G. B., K. CARLSON, J. BERLINER, and J. L. ROSENBAUM. 1972. *Chlamydomonas* flagella. I. Isolation and electrophoretic analysis of microtubules, matrix, membranes, and mastigonemes. *J. Cell Biol.* **54**:507–539.
32. YEN, P. H., and C. E. BALLOU. 1973. Composition of a specific intercellular agglutination factor. *J. Biol. Chem.* **248**:8316–8318.

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