Characterization of the Purified *Chlamydomonas minus* Agglutinin

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ABSTRACT *Chlamydomonas* flagellar sexual agglutinins are responsible for the adhesion of opposite mating-type (*plus* and *minus*) gametes during the first stages of mating. Purification and partial characterization of the *plus* agglutinin was previously reported (Adair, W. S., C. J. Hwang, and U. W. Goodenough, 1983, *Cell*, 33:183–193). Here we characterize the purified *minus* molecule. We show it to be a high molecular weight, hydroxyproline-rich glycoprotein that migrates in the 3% stacking region of an SDS-polyacrylamide gel and is absent from two nonagglutinating *minus* mutants. *Plus* and *minus* agglutinins are remarkably similar, although nonidentical, in amino acid composition, molecular morphology, and reactivity in vivo and in vitro with monoclonal antibodies raised against the *plus* agglutinin. Moreover, the adhesiveness of both *plus* and *minus* agglutinins, when coupled to agarose beads, is abolished by thermolysin, trypsin, periodate, alkaline borohydride, reducing agents, or heat, but unaffected by exo- or endoglycosidases. The *minus* agglutinin, however, migrates just ahead of the *plus* molecule on SDS PAGE, is excluded from an anion-exchange (Mono Q) column, elutes earlier during hydrophobic interaction (Bio-gel TSK Phenyl 5PW) chromatography, and is sensitive to chymotrypsin digestion (unlike the *plus* agglutinin); therefore, it differs from the *plus* agglutinin in apparent molecular weight, net charge, relative hydrophobicity and proteolytic susceptibility. Nevertheless, our results generally demonstrate a high degree of homology between these complementary cell-cell recognition/adhesion molecules, which suggests that they are specified by genes that have a common evolutionary origin.

Chlamydomonas reinhardi mating-type *plus* (mt^+) and mating-type *minus* (mt^-) gametes recognize and adhere to one another to initiate mating via sexual agglutinins located on their flagellar surfaces (reviewed in references 1 and 2). The processes of gametic recognition and flagellar adhesive interactions are extremely specific for both the mating-type and species of *Chlamydomonas* (3), features attributable to the agglutinin molecules themselves (4, 5). The *plus* and *minus* agglutinins can both be extracted in a biologically active form from mt^+ and mt^- gametes by EDTA (4, 6) and quantitated using an in vitro bioassay (4). The *plus* species has been purified by gel filtration chromatography and identified as a high molecular weight, hydroxyproline-containing, fibrous glycoprotein that is present as an extrinsic component of the mt^+ flagellar surface (4, 5, 7). Recently, Saito and Matsuda (8) have followed similar protocols to extract *minus* agglutinin and have fractionated the activity by hydroxyapatite chromatography. They report that the *minus* agglutinin is also a high molecular weight glycopolypeptide.

To investigate the molecular mechanism that governs agglutinin-mediated intracellular recognition and adhesion in *Chlamydomonas*, it is important that both adhesins be well characterized. This paper describes the characterization of the *minus* agglutinin, purified by Fractogel-75 gel filtration chromatography, and its comparison with the *plus* agglutinin. *Minus* agglutinin is shown to be highly homologous to its *plus* counterpart in amino acid composition, structural morphology, and immunological antigenicity, with both adhesins representing high molecular weight, extrinsic, flagellar glycoproteins, rich in serine and hydroxyproline, that are absent from nonagglutinating mutants. Furthermore, many agents or treatments that perturb the adhesive nature of the *plus* agglutinin are shown to have a similar effect on the *minus* species. Despite these homologies, the two agglutinins differ with respect to net charge, hydrophobicity, electrophoretic mobility, and susceptibility to chymotrypsin digestion.

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MATERIALS AND METHODS

Preparation of Agglutinin EDTA Extracts: *Minus* agglutinin was extracted from 3×10^{11} *C. reinhardi* mating-type *minus* plate gametes (strain CC-621) using buffered EDTA (Sigma Chemical Co., St. Louis, MO) according to the protocol routinely used for *plus* agglutinin extraction. This procedure has been modified from that previously described (4, 5) in the following ways. Washed gametes are pelleted by brief centrifugation at 8,000 *g* and resuspended in 15 mM EDTA, 20 mM PIPES (Research Organics, Inc., Cleveland, OH), pH 7.4, at a final cell density of 10^9 cells/ml. Extraction is performed at 25°C until the cells are nonagglutinative with untreated tester gametes of the opposite mating type; unlike Saito and Matsuda (6, 8), we find that our *minus* gametes require a somewhat longer incubation period (30–45 min) with EDTA than do *plus* gametes (20–30 min) for full agglutinin extraction. The suspension is then briefly centrifuged at 27,000 *g* to pellet cells, and the supernatant is centrifuged at 40,000 *g* for 20 min. Ammonium sulfate is added to this supernatant to 70% saturation for 30 min on ice. After a 15-min centrifugation at 40,000 *g*, the pellets are resuspended in 1.5 ml deionized water, dialyzed overnight at 4°C against the same, centrifuged at 100,000 *g* for 40 min to pellet large cell wall fragments, and the supernatant (crude preparation) is lyophilized and stored at −70°C. The nonagglutinating *minus* mutants *imp-10* (CC-1147) and *imp-12* (CC-1149) were treated in an identical fashion to the normal *minus* strain. All CC numbers denote stock cultures available from the *Chlamydomonas* Genetics Center, Department of Botany, Duke University (Durham, NC).

In Vitro Iodination of Agglutinin: A lyophilized crude EDTA extract was resuspended in 1.0 ml of column buffer A, which consisted of 20 mM PIPES (pH 7.4), 100 mM KCl (Sigma Chemical Co.), 5 mM EDTA, and 30 mM octyl-β-D-glucopyranoside (Calbiochem-Behring Corp., La Jolla, CA), centrifuged for 10 min at 40,000 *g*, and the supernatant was divided into 750 and 250-μl aliquots. The smaller sample was split into two portions, each of which was incubated with 200 μCi carrier-free $Na^{125}I$ (Amersham Corp., Arlington Heights, IL) and one IODO-bead (Pierce Chemical Co., Rockford, IL) for 15 min at 25°C (5). Unreacted label was removed by a rapid desalting (9) through 1 ml Bio-Gel P6-DG (Bio-Rad Laboratories, Richmond, CA) into column buffer A. Both labeled samples were added to the remaining (750 μl) agglutinin aliquot and centrifuged for 10 min at 40,000 *g*. The supernatant (900 μl), containing 1.5×10^9 dpm, was directly loaded onto the gel filtration column for agglutinin purification.

Denaturing PAGE: SDS PAGE, sample preparation, autoradiography, and gel stains (periodic acid-Schiff, [PAS],[1] Coomassie Blue, silver) were performed as described previously (4, 5). Gels containing 3–4 and 4–6% gradients of acrylamide and urea, respectively, were used without stacking regions in some experiments but run at lower voltages than the above gels (40 V for ~1 h, then 80 V until the bromophenol blue incorporated in the gradient gel migrated from the end of the gel).

Chromatographic Fractionation of Agglutinin Extracts: Fractionation of *minus* agglutinin extracts on Fractogel TSK HW-75 (F) (EM Science, Gibbstown, NJ) was performed in column buffer A (30 mM octylglucoside, 5 mM EDTA, 100 mM KCl, 20 mM PIPES, pH 7.4) as previously described for *plus* agglutinin (5), without prior chromatography on Sepharose 6B. For hydrophobic interaction chromatography, extracts (a crude preparation resuspended in 1.0 ml) were adsorbed to a nondenaturing hydrophobic phenyl column (Bio-Gel TSK Phenyl 5PW, Bio-Rad Laboratories), in 100 mM potassium phosphate, pH 7.0, containing 1.7 M ammonium sulfate. Bound species were eluted with a decreasing (1.7–0 M) ammonium sulfate gradient at a flow rate of 1.0 ml/min (10).

Anion-exchange chromatography was performed on a MonoQ HR5/5 column (Pharmacia Fine Chemicals, Piscataway, NJ) in 20 mM HEPES (Sigma Chemical Co.), 5 mM EDTA, pH 7.2. Elution of bound proteins was accomplished with a gradient of 100–500 mM KCl in the same buffer at a flow rate of 1.0 ml/min.

The high-pressure liquid chromatographic system employed for hydrophobic interaction and anion-exchange chromatography used a Micromeritics (Micromeritics Instrument Corp., Norcross, GA) solvent delivery system (model 750), gradient programmer (model 752), and variable wavelength ultraviolet detector (model E 54 U) on line to an IBM CS 9000 instruments computer. Aliquots (~200 μl) of column fractions were rapid-desalted through 1 ml of Bio-Gel P6-DG (9) and analyzed by SDS PAGE/autoradiography and/or PAS staining, microscopic bioassay (4), and radioactivity determinations in a Beckman Gamma 4000 Counting System (Beckman Instruments Inc., Palo Alto, CA). When required, pooled column fractions were concentrated over Aquacide II (Calbiochem-Behring Corp.).

Biochemical and Immunological Analyses: Amino acid analysis for *minus* agglutinin was conducted as described for *plus* agglutinin (7). *Minus* agglutinin was coupled to agarose beads for inactivation studies and treated as previously described (11).

Details of antibody production and properties of agglutinin-reactive monoclonal antibodies are presented in separate reports from our laboratory (1, 12). Immunoautoradiography was performed using the spaghetti overlay procedure (13).

Light, Fluorescence, and Electron Microscopy: Agglutinin bioassay by light microscopy was performed according to the method of Adair et al. (4). Quick-frozen, deep-etched samples of purified agglutinins were prepared for electron microscopy according to Heuser (14) and visualized as described previously (5, 7, 15).

Immunofluorescence microscopy of agglutinin-reactive monoclonal antibodies bound to cells was performed as follows. Cells were washed once with cold buffer of 10 mM HEPES, pH 7.5, 5 mM $MgSO_4$, 0.5 mM EGTA, 25 mM KCl, fixed for at least 2 h in the same buffer containing 2% paraformaldehyde (Eastman Laboratory and Specialty Chemicals, Rochester, NY), and washed three times with phosphate-buffered saline (PBS) before incubation for 30 min with monoclonal antibodies (25 μg/ml of a 50% ammonium sulfate cut of ascites fluids). Control samples were incubated with nonimmune mouse serum (Sigma Chemical Co.) or an irrelevant monoclonal antibody (D3, raised against *Chlamydomonas* dyneins, Mehard, B. B., and W. S. Adair, unpublished observation). After primary antibody incubations, cells were washed three times with PBS and incubated for 15 min with 5 μg/ml biotinylated horse anti-mouse Ig (Vector Laboratories, Burlingame, CA). After they were washed twice with PBS and once with 50 mM Na_2CO_3, 0.15 M NaCl, the cells were incubated for 5 min in the latter buffer containing 20 μg/ml fluorescein isothiocyanate-avidin D (Vector Laboratories). The labeled cells were then washed three times with 50 mM Na_2CO_3, 0.15 M NaCl and suspended in mounting buffer (25% glycerol in PBS). A Zeiss Photomicroscope I equipped with a III RS epifluorescence condenser was employed to visualize and photograph the labeled cells through a K550 barrier filter using Kodak Tri-X film, shot at ASA 400 and developed at ASA 800.

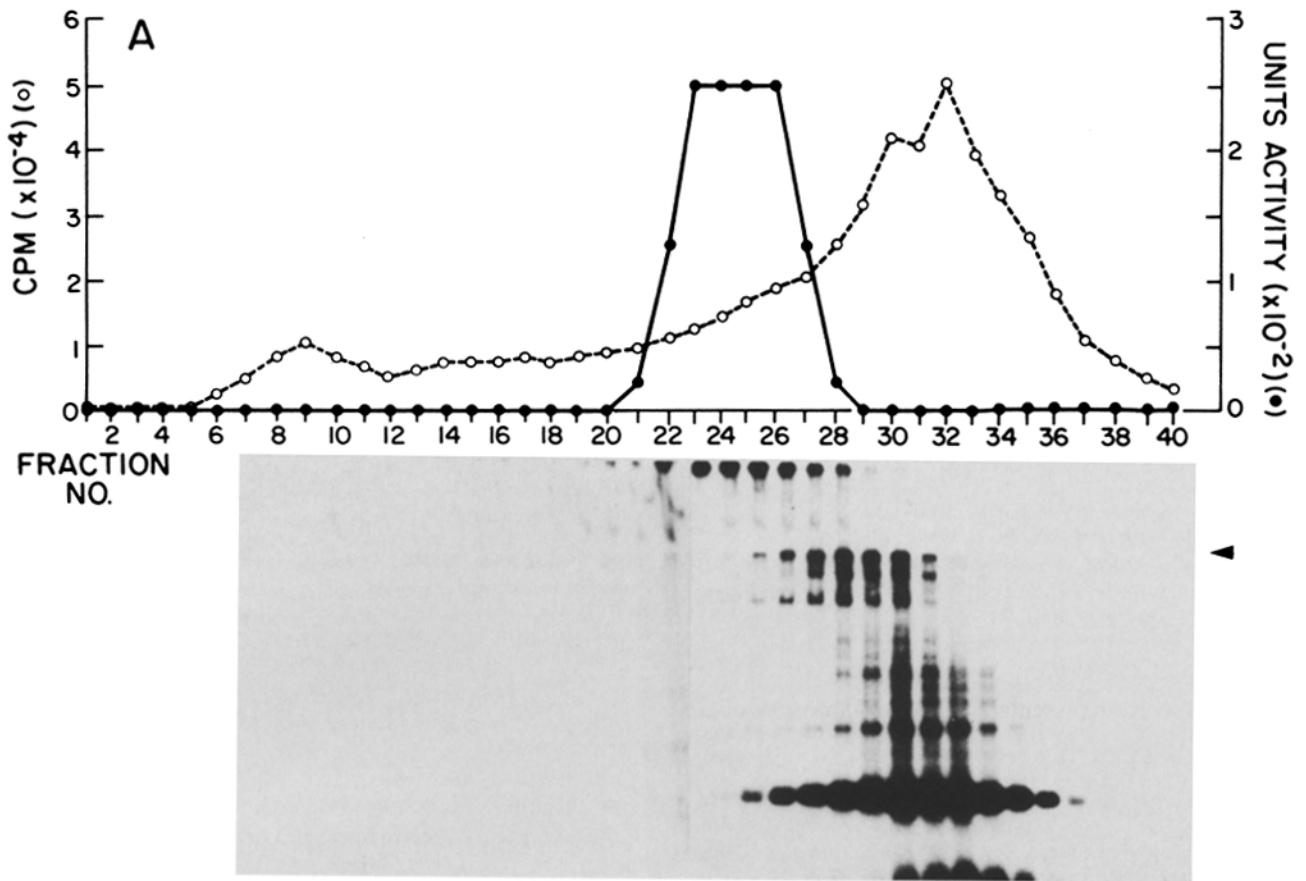
RESULTS AND DISCUSSION

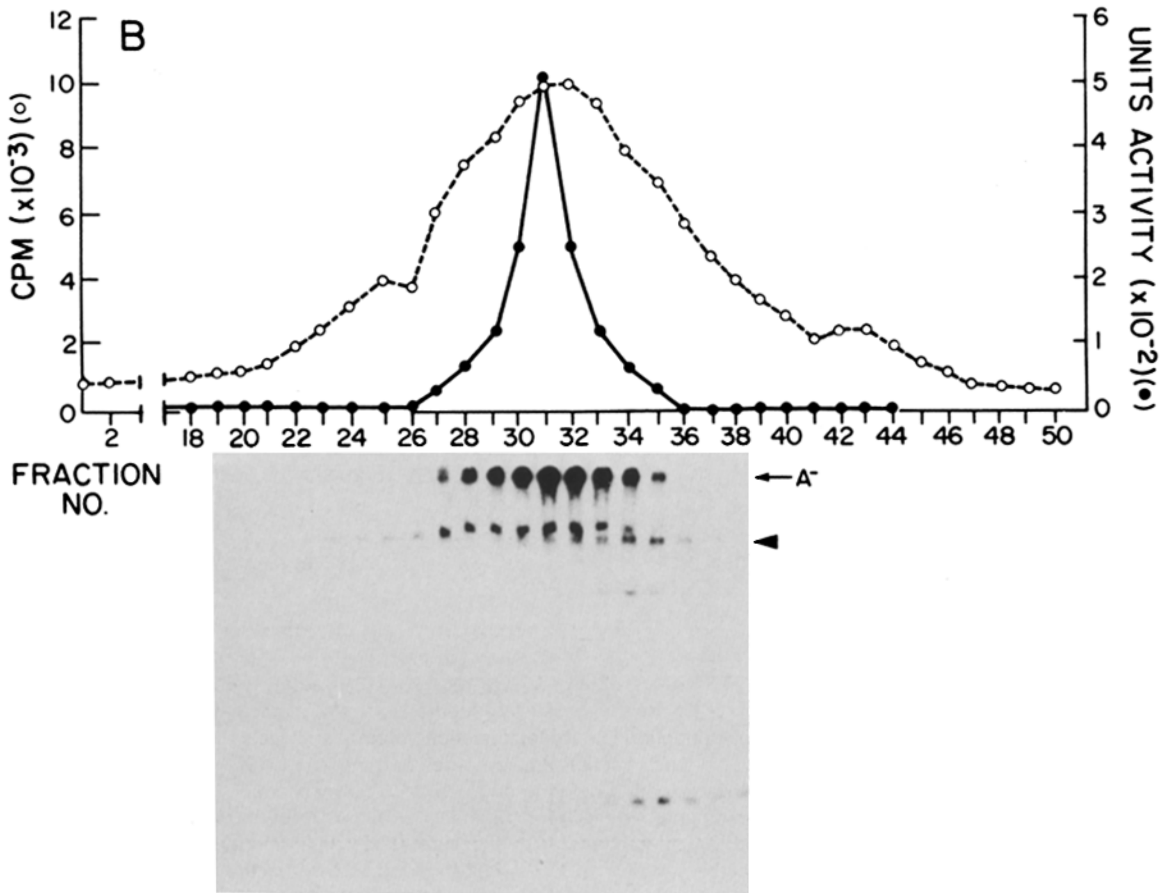
Purification of Mating-type Minus Agglutinin

Quantitative release of the *minus* agglutinin from gametic flagella is readily accomplished by incubation of living gametes with buffered 15 mM EDTA, analogous to the protocol employed for extraction of *plus* agglutinin (4, 5). Fig. 1 illustrates the purification of an in vitro ^{125}I-labeled *minus* agglutinin EDTA extract by Fractogel-75 sizing chromatography. Elution of labeled extract components was monitored by radioactive determination and a quantitative bioassay (4) was used to detect agglutinin biological activity. In the first cycle through the column matrix, a major portion of the labeled extract eluted in one broad peak (Fig. 1 *A*). Maximum *minus* agglutinin activity was predominantly associated with the shoulder of this peak. Autoradiography of column fractions after SDS PAGE revealed a number of labeled bands in the biologically active fractions.

To more fully resolve components eluting in this region, the most active fractions (fractions 20–26) were pooled, concentrated, and rechromatographed. The major agglutinin activity was now found within the peak of remaining ^{125}I-labeled EDTA-extracted material (Fig. 1 *B*). Peak *minus* agglutinin activity co-fractionated with two high molecular weight polypeptides, and these fractions otherwise contained only relatively minor amounts of other labeled components. When these two high molecular weight molecules were separated by high-performance liquid chromatography hydrophobic interaction chromatography, *minus* agglutinin activity was invariably associated with the slower-migrating, higher molecular weight gel band (as described below); this molecule is therefore considered to be the active *minus* agglutinin. The gel-band species has been named polypeptide A^-, by analogy with the similarly migrating gel band of active *plus* agglutinin (5), which we henceforth designate polypeptide A^+.

[1] *Abbreviation used in this paper:* PAS, periodic acid-Schiff.


A
CPM (x10^-4) (○)
UNITS ACTIVITY (x10^-2) (●)
FRACTION NO.


B
CPM (x10^-3) (○)
UNITS ACTIVITY (x10^-2) (●)
FRACTION NO.
A⁻

Minus Agglutination Mutants

Chlamydomonas mutants unable to agglutinate sexually with gametes of the opposite mating-type have been isolated in both *plus* and *minus* strains and genetically characterized (22, 23). Adair et al. (5) have shown that EDTA extracts from nonagglutinating *plus* mutants lack the agglutinin polypeptide A^+. To learn whether *minus* nonagglutinating mutants lack polypeptide A^-, EDTA extracts (inactive) were prepared from *imp-10* and *imp-12* strains, and subjected to Fractogel-75 purification in a manner analogous to that employed for active *minus* extracts, and the fractions were analyzed by SDS PAGE. Fig. 2 (lanes *2* and *3*) demonstrates that both mutants lack a polypeptide A^- component, which supports the identification of this high molecular weight glycoprotein as the active *minus* agglutinin molecule. In addition, molecules with the morphology of A^- are present on the surface of gametic, but not vegetative, *minus* cells when observed in situ (15). The mutants do, however, produce glycopolypeptides that migrate farther into the stacker region of the gel (Fig. 2). These species, which are also produced by wild-type gametes, derive from the flagellum but are not involved in adhesion; they are described in detail elsewhere (15).

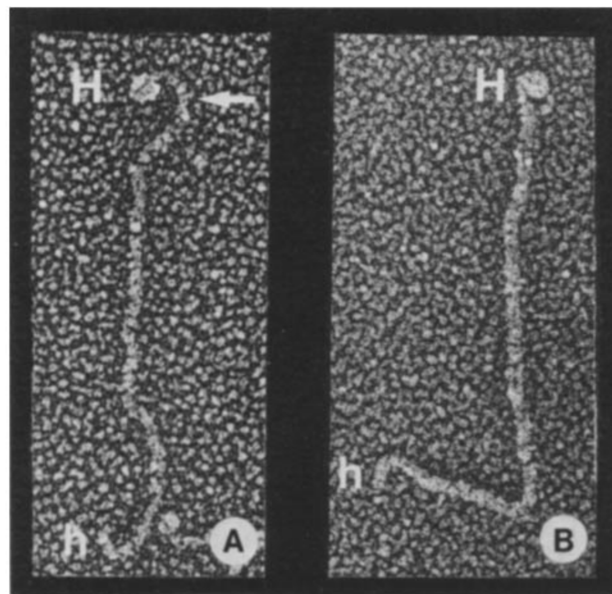


FIGURE 3 Comparative morphology of mt^- and mt^+ agglutinins. Purified agglutinins were adsorbed to pulverized mica and examined by the rapid-freeze deep-etch procedure of Heuser (14). *A*, mt^- agglutinin; *B*, mt^+ agglutinin. *H*, head domain; *h*, hook domain; arrow, distinctive crook conformation of the mt^- molecule. × 250,000. Micrographs courtesy of Dr. John Heuser.

Visualization of Purified Minus Agglutinin

Fig. 3 compares representative isolated molecules of *plus* and *minus* agglutinins as visualized by the quick-freeze, deep-etch technique. Clearly, the *plus* and *minus* agglutinins are homologous. Both have four morphologically distinct regions: a terminal head, a straight portion, a flexible domain, and a terminal hook. The *minus* agglutinin however, displays a "shepherd's crook" conformation near the head region, and its head is somewhat larger. A detailed structural comparison of these proteins is provided in a separate report (15).

FIGURE 2 SDS PAGE (3–6% acrylamide, 4–6% urea) of purified mt^- agglutinin and corresponding fractions from *imp-10* and *imp-12* extracts. EDTA extracts prepared from wild-type mt^- and agglutination-defective mutant (*imp-10* and *imp-12*) "gametes" (3×10^{11} cells per sample) were fractionated by two chromatographic runs over Fractogel-75 (as in Fig. 1, 2.6-ml fractions), and examined by urea/polyacrylamide SDS PAGE/PAS staining (see Materials and Methods). For gel analysis, 1.0 ml of a peak activity mt^- fraction and 1.0 ml of corresponding fractions from the mutants were dialyzed extensively against dH_2O, lyophilized to dryness, and resuspended in 20 μl SDS sample buffer (4). 6 μl of each sample was loaded per lane. Lane *1*, fraction of mt^- extract displaying peak activity; lanes *2* and *3*, corresponding fractions from *imp-10* and *imp-12*, respectively. All other *imp* fractions also lacked a detectable polypeptide A^- band (not shown).

Amino Acid Composition of the Minus Agglutinin

Table I presents the amino acid composition of the *minus* agglutinin. Included for comparison purposes are profiles previously published for the *plus* agglutinin (7) and for 2BI, one of the major *C. reinhardi* cell wall glycoproteins (24). The three *Chlamydomonas* high molecular weight glycoproteins are remarkably similar in overall composition. Furthermore, values for the *C. reinhardi* sexual agglutinins closely resemble those reported for the *minus* agglutinin of *C. eugamatos* (25). As expected, the *plus* and *minus* agglutinins are more similar to one another than to the cell wall glycoprotein, presumably reflecting their more similar evolutionary histories. Note that the *minus* agglutinin, like its *plus* homologue and 2BI, contains high levels of hydroxyproline and serine residues. As discussed previously, these two amino acids serve as points

FIGURE 1 Purification of mt^- agglutinin. A crude EDTA extract, obtained from 3×10^{11} mt^- gametes, was iodinated in vitro and fractionated by two cycles of Fractogel-75 chromatography (see Materials and Methods). Agglutinin activity was determined by quantitative bioassay (4). Radioactivity of 100 μl of each fraction (2.6 ml total fraction volume for cycle 1, 2.2 ml for cycle 2) was determined by gamma-counting. For SDS PAGE, 50 μl of each fraction was rapid-desalted into dH_2O (9), lyophilized to dryness, and resuspended in 10 μl SDS sample buffer (4). 5 μl was loaded per lane on a 3:5–15% polyacrylamide gel (see Materials and Methods). After fixation and drying, gels were autoradiographed (4, 5) for the indicated times. Arrowheads denote stacking gel interfaces. (*A*) Cycle 1: 1.46×10^9 dpm column load; 2.6 ml fractions; 19 h exposure. (*B*) Cycle 2: fractions 20–26 from cycle 1 pooled, concentrated to 1.5 ml and re-chromatographed; 2.25×10^6 dpm column load; 2.2 ml fractions; 24-h exposure; A^-, polypeptide A^-.

TABLE I. *Amino Acid Compositions of the mt^+ and mt^- Sexual Agglutinins*

Amino acid	Amino acid composition (No. of residues per 1,000)		
	(+) Agglutinin*	(−) Agglutinin	Wall protein‡
Lys	22 ± 8.5	41	39
His	31 ± 9.2	20	6
Arg	36 ± 3.8	22	35
Asx	95 ± 4.0	104	112
Thr	59 ± 5.7	67	75
Ser	103 ± 13.3	113	78
Glx	88 ± 17.5	83	64
Pro	43 ± 9.3	56	67
Gly	88 ± 11.2	88	71
Ala	77 ± 7.8	78	86
Val	46 ± 8.0	62	68
Cys	50.6	ND	ND
Met	10 ± 4.0	8	12
Ile	27 ± 3.8	35	38
Leu	51 ± 9.9	63	69
Trp	ND	ND	ND
Tyr	15 ± 1.2	11	31
Phe	28 ± 8.3	31	38
Hyp	123 ± 13.6	120	112

ND, not determined

* Values expressed are mean ± SD from three separate analyses (except ½ cysteine).

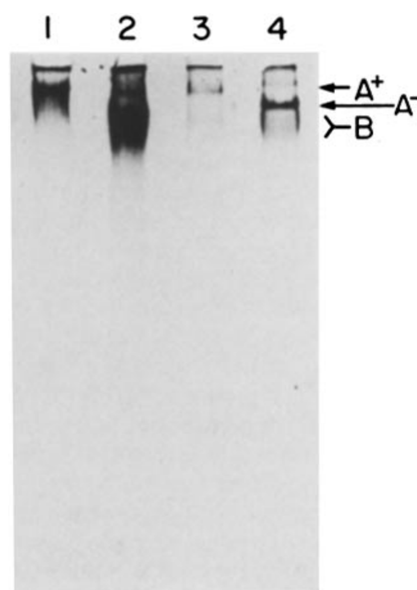


FIGURE 4 Electrophoretic mobilities of polypeptides A^+ and A^-. Purified mt^+ and mt^- agglutinins were analyzed by urea/polyacrylamide SDS PAGE (4–6 and 3–6%, respectively). 1.0-ml fractions, dialyzed extensively against dH_2O and lyophilized to dryness, were resuspended in 20 µl SDS sample buffer (4). 6 µl of each sample was loaded per lane. Quantitative agglutinin bioassays (4) of the purified fractions were performed before lyophilization. Lane *1*, peak Fractogel-75 purified mt^+ agglutinin (1,024 U activity); lane *2*, Fractogel-75 fraction enriched for "band" B; lane *3*, peak MonoQ/Fractogel-75 purified mt^+ agglutinin; lane *4*, peak Fractogel-75 purified mt^- agglutinin. Arrows indicate the mt^+ agglutinin (A^+), mt^- agglutinin (A^-), and heterogenous "band" B (*B*) region. Note that the slowest-migrating PAS-reactive band in lane *4* does not correspond to polypeptide A^+; it is frequently found in both mt^+ and mt^- extracts, and when isolated by hydrophobic interaction chromatography it lacks agglutinin activity.

for oligosaccharide attachment in a variety of plant glycoproteins, including *Chlamydomonas* cell wall glycoproteins, and the hydroxyproline residues are thought to confer on the protein its fibrous properties (7).

Differential Electrophoretic Mobility of Plus and Minus Agglutinins

Both *plus* and *minus* agglutinins migrate slowly in SDS PAGE gels (Fig. 4, lanes *1* and *4*). As described in detail elsewhere (15), one or more faster-migrating glycopolypeptides, collectively designated B (Fig. 4, lanes *2–4*), often contaminate peak agglutinin fractions; since the B material alone has no adhesive activity, however, it is not relevant to the present study. Agglutinin fractions enriched for A^+ (Fig. 4, lane *1*) consistently display maximal activity, whereas fractions containing abundant band B (lane *2*) are much less active. Of interest here is that the polypeptide A^- band reproducibly migrates farther into the 3% stacking region of SDS PAGE gels than does polypeptide A^+, regardless of the purification method. This difference is most easily observed with longer electrophoretic runs on urea/polyacrylamide gels containing gradients of 3–4% acrylamide and 4–6% urea, conditions that provide improved separation as well as increased migration (without loss of resolution) of both bands A and B (Fig. 4, lanes *3* and *4*). The increased migration of active *minus* agglutinin relative to active *plus* agglutinin presumably reflects a structural disparity under denaturing conditions between the *plus* and *minus* sexual adhesins.

Chromatographic Behavior of Plus and Minus Agglutinins

Comparable elution profiles are obtained for *plus* or *minus* agglutinin preparations fractionated on a variety of gel-filtration sizing columns, including Fractogel-75. In contrast, the adhesins behave quite differently on anion-exchange (MonoQ) and hydrophobic interaction (Bio-Gel TSK Phenyl 5PW) columns. Whereas *plus* activity is retained on a MonoQ column (20 mM HEPES, 5 mM EDTA, pH 7.2) and subsequently eluted with a 100–500 mM KCl gradient (Fig. 5), *minus* activity is excluded from the anion-exchange resin. Therefore, it appears that the agglutinins differ in net charge, a feature that might facilitate their interaction in vivo. Note that this column is also useful for separating bands A and B of *plus* agglutinin.

Although both *plus* and *minus* agglutinins are efficiently adsorbed to the hydrophobic phenyl matrix (1.7 M ammonium sulfate, 100 mM potassium phosphate, pH 7.0), each elutes (with decreasing ammonium sulfate concentration) at an independent region of the gradient (Fig. 6), a characteristic retained when *plus* and *minus* extracts are combined and cochromatographed (not shown). Because *minus* activity elutes before the major extract contaminants (Fig. 6*A*), and *plus* activity is retained until after most of the wall material has been released (Fig. 6*B*), both agglutinins can be effectively purified to near homogeneity on the nondenaturing hydrophobic phenyl column. In addition, the high molecular weight gel band that co-elutes during F-75 purification with the *minus* agglutinin can be separated from the active adhesin by such hydrophobic interaction chromatography, as it elutes at a region intermediate to the elution positions of the *minus*

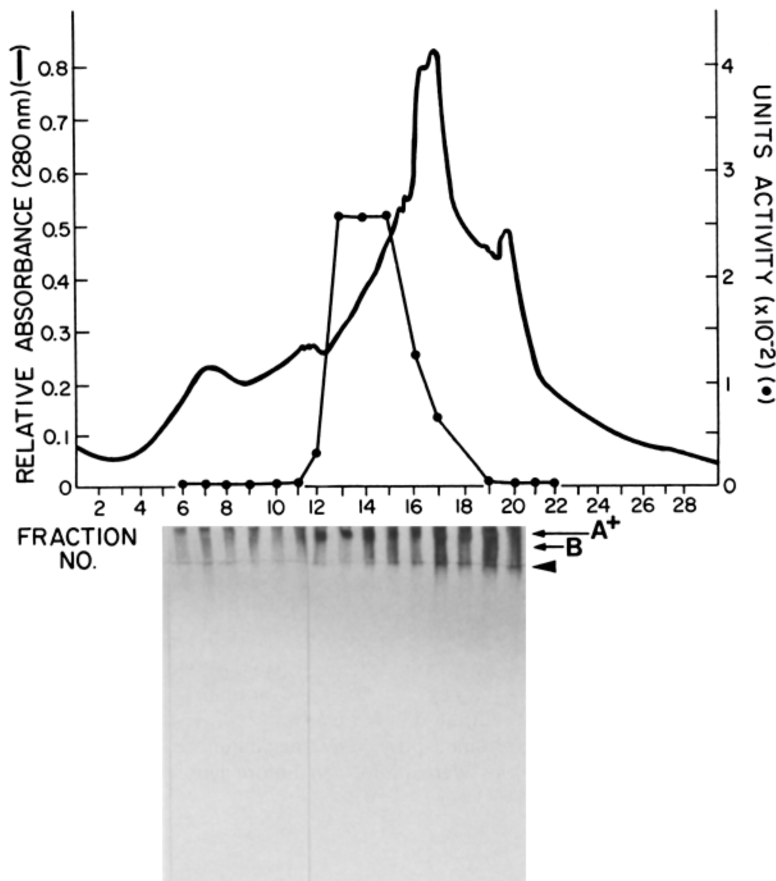


FIGURE 5 Purification of mt^+ agglutinin by anion-exchange chromatography. A crude mt^+ agglutinin extract (1.5×10^{11} cells), resuspended in 1.0 ml 20 mM HEPES, pH 7.2, buffer containing 5 mM EDTA, was adsorbed to a MonoQ column, and eluted with a 100–500 mM KCl gradient (see Materials and Methods). 150-μl aliquots of each fraction (1.0 ml total) were rapid-desalted (9) and analyzed by quantitative bioassay (4). For SDS PAGE (3:5–15%), the desalted aliquots were lyophilized and resuspended in 15 μl SDS sample buffer; 6 μl was loaded per lane. Fixed gels were PAS stained (see Materials and Methods). Note that polypeptides A^+ and B are resolved and that agglutinin activity is associated with A^+ alone. The arrowhead denotes the stacking gel interface.

and *plus* agglutinins (not shown). Since selective desorption depends on the strength of the hydrophobic interaction between the nonpolar amino acid groups of the protein and the phenyl substituents of the matrix (10), the lengthy retention time of *plus* agglutinin on this column is suggestive of its being relatively more hydrophobic than the *minus* agglutinin.

Inactivation of Minus Agglutinin Conjugated to Agarose Beads

Purified agglutinin, when covalently attached to an inert support such as agarose beads, retains potent biological activity in assays using living gametes of the complementary mating type (11). Such immobilized preparations provide a sensitive and rapid in vitro assay system for assessing the effects of various agents (chemical, enzymatic, etc.) on agglutinin activity (11).

Table II compares data obtained for immobilized *minus* agglutinin subjected to various treatments with results previously published (11) for the effects of these treatments on beads coupled with *plus* agglutinin. Most of the treatments have a similar effect on both agglutinins. Thus, both species are sensitive to digestion by thermolysin or trypsin, alkaline borohydride reductive modification of carbohydrate, heating above 65°C (although *minus* may be slightly more heat labile than *plus*), periodate oxidation (see also reference 6), and thiol reduction. Unlike *C. eugamatos* (16–18), *minus* agglutinin is not inactivated by α-galactosidase, nor is *plus* activity destroyed by α-mannosidase. An interesting difference is seen with α-chymotrypsin: *minus* agglutinin activity demonstrates a partial loss of activity at 0.1 mg/ml and a complete loss of activity at 1 mg/ml, whereas *plus* activity is unaffected by even 10 mg/ml of this protease. Since the overall amino acid composition of *minus* agglutinin, as compared with *plus* agglutinin, is not enriched for aromatic amino acids (see Table I), it is unlikely that the chymotrypsin-induced loss of *minus* biological activity results from a generalized degradation of *minus* agglutinin by this protease. If the selective inactivation of *minus* agglutinin results from chymotryptic cleavage at or near the *minus* adhesive site, this enzyme should be useful for probing the differences in the *plus* and *minus* mating-type-specific adhesion sites.

Immunologic Relatedness of Plus and Minus Agglutinins

Monoclonal antibodies directed against determinants found on the *plus* agglutinin have been generated in this laboratory and are described in detail elsewhere (1, 12). Three classes of agglutinin-reactive monoclonal antibodies have been identified. Two are partially (class III) or extensively (class I) cross-reactive with other *Chlamydomonas* flagellar or wall components; the third (class II) reacts specifically with polypeptide A and the polypeptide B family (1, 12, 15).

All three monoclonal antibody classes, although initially selected for their ability to isoagglutinate *plus* gametes by their flagella (1), prove to be equally effective at isoagglutinating *minus* gametes. Fig. 7 illustrates this cross-reactivity for two of these antibodies using immunofluorescence microscopy. Like the patterns observed for *plus* (1, 12), the *minus* gametes display highly labeled flagella and cell walls with class I antibody (Fig. 7*A*), and punctate flagellar staining and unlabeled cell walls with class II antibody (Fig. 7*B*). Similar mating-type cross-reactivity is also observed with Class III

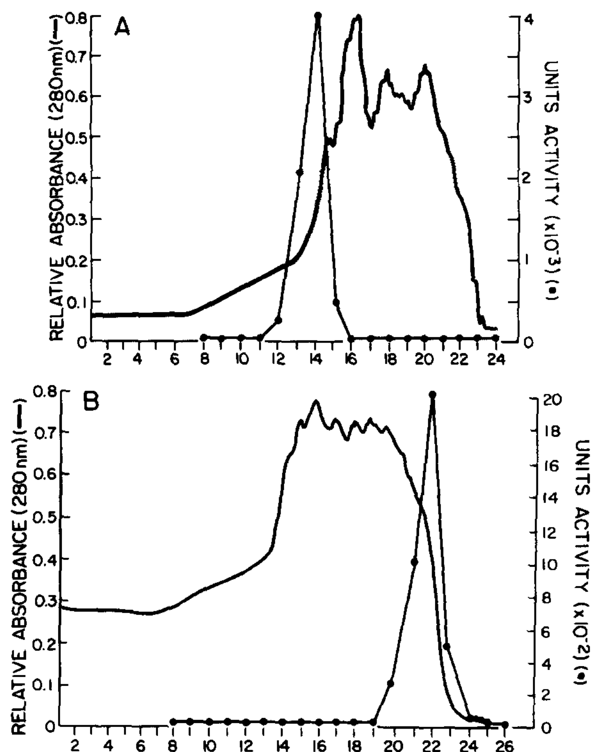


FIGURE 6 Purification of mt^+ and mt^- agglutinins by hydrophobic interaction chromatography. A crude mt^+ or mt^- extract (3.0×10^{11} cells) was resuspended in 2.0 ml 1.7 M ammonium sulfate, 0.1 M potassium phosphate, pH 7.0, and adsorbed to a Bio-Gel TSK Phenyl 5PW column. Elution was performed with a decreasing ammonium sulfate gradient (see Materials and Methods). 75-μl samples of each mt^- fraction (1.0 ml total) and 135 μl of each mt^+ fraction (1.0 ml total) were rapid-desalted into dH_2O (9) and agglutinin activity was determined by quantitative bioassay (4). SDS PAGE analysis of the column fractions confirmed the bioassay results, demonstrating the presence of polypeptide A^+ or A^- only in the active fractions (not shown).

monoclonal antibodies (not shown).

To learn whether the antibodies were recognizing the same surface molecules on both *plus* and *minus* gametes, class I and II monoclonal antibodies were used to probe a partially purified *minus* agglutinin preparation, after SDS PAGE, by spaghetti overlay immunoautoradiography (25). Antigen–antibody complexes were detected using ^{125}I-sheep anti-mouse Ig, $F(ab)_2$, followed by autoradiography. Fig. 8 shows that both class I (lane *1*) and class II (lane *2*) monoclonal antibodies recognize the *minus* agglutinin. Since recent immunotopographical mapping studies have demonstrated that the class II monoclonal antibodies bind to a repeating determinant along the length of the *plus* agglutinin rod (1, 12), the *plus* and *minus* agglutinins may have common features in their polypeptide backbones, consistent with their highly similar amino acid compositions and morphologies.

In conclusion the *minus* sexual agglutinin has been extracted from *C. reinhardi minus* gametes with 15 mM EDTA, and purified by Fractogel-75 sizing chromatography, and the purified adhesin was characterized. These studies have demonstrated that, overall, *minus* agglutinin is remarkably similar to the *plus* sexual adhesin in amino acid composition, immunological antigenicity, structural morphology, and sensitivity or resistance to various agents or treatments.

Such homology between the *C. reinhardi* sexual agglutinins

TABLE II. *Inactivation of Bead-conjugated Agglutinins*

Agent or treatment*	*Plus* inactivation	*Minus* inactivation
Thermolysin		
10 μg/ml	Partial	ND
100 μg/ml	Complete	ND
1 mg/ml	Complete	Complete
Trypsin		
100 μg/ml	Partial	ND
1 mg/ml	Complete	Complete
Chymotrypsin		
100 μg/ml	No effect	Partial
1 mg/ml	No effect	Complete
10 mg/ml	No effect	Complete
Heating		
45°C	No effect	No effect
55°C	No effect	ND
60°C	No effect	Complete
65°C	Complete	Complete
Periodate (10 mM)	Complete	Complete
Alkaline sodium borohydride (1.0 M)	Complete	Complete
Endoglucosaminidase H (0.005 U)	No effect	ND
α-Galactosidase (1.0 U)	No effect	No effect
β-Galactosidase (0.8 U)	No effect	ND
α-L-Fucosidase (0.02 U)	No effect	ND
β-*N*-Acetylglucosaminidase B (0.142 U)	No effect	ND
α-Mannosidase (0.20 U)	No effect	ND
Alkaline phosphatase (0.23 U)	No effect	ND
Neuraminidase (0.05 U)	No effect	ND
Mixed exoglycosidases‡	No effect	ND
Dithiothreitol (100 mM)	Complete	ND
β-mercaptoethanol		
10 mM	Partial	ND
120 mM	Complete	Complete
Spontaneous reoxidation	None	None
β-Mercaptoethanol (100 mM) + iodoacetamide (100 mM)	Complete	Complete
Iodoacetamide (100 mM)	No effect	No effect
β-Mercaptoethanol (100 mM) + *N*-ethyl maleimide (100 mM)	Complete	ND
N-Ethyl maleimide (100 mM)	No effect	ND
minus agglutinin	No effect	ND

ND, not determined.
* Conditions for enzyme and chemical incubations are given in Materials and Methods.
‡ β-Galactosidase (0.4 U), α-L-fucosidase (0.025 U), and β-*N*-acetylglucosaminidase B (0.5 U).

contrasts with the sexual agglutination factors purified from three genera of ascomycetous yeast. The latter differ markedly in the two yeast mating types: one factor is a large, heavily-glycosylated, heat-stable component that is inactivated by reducing agents, whereas the other is a smaller, less-glycosylated, heat-labile component that is unaffected by reducing agents (19, 20).

A second difference between the agglutinins of *Chlamydomonas* and yeast lies in their in vitro reactivity. The purified yeast factors can interact in vitro to form stable complexes. Although the molecular mechanism for this recognition/adhesion process is not yet clear, the factors clearly have enough molecular information to effect these biological functions. By contrast, purified (or other nonliving) agglutinin preparations

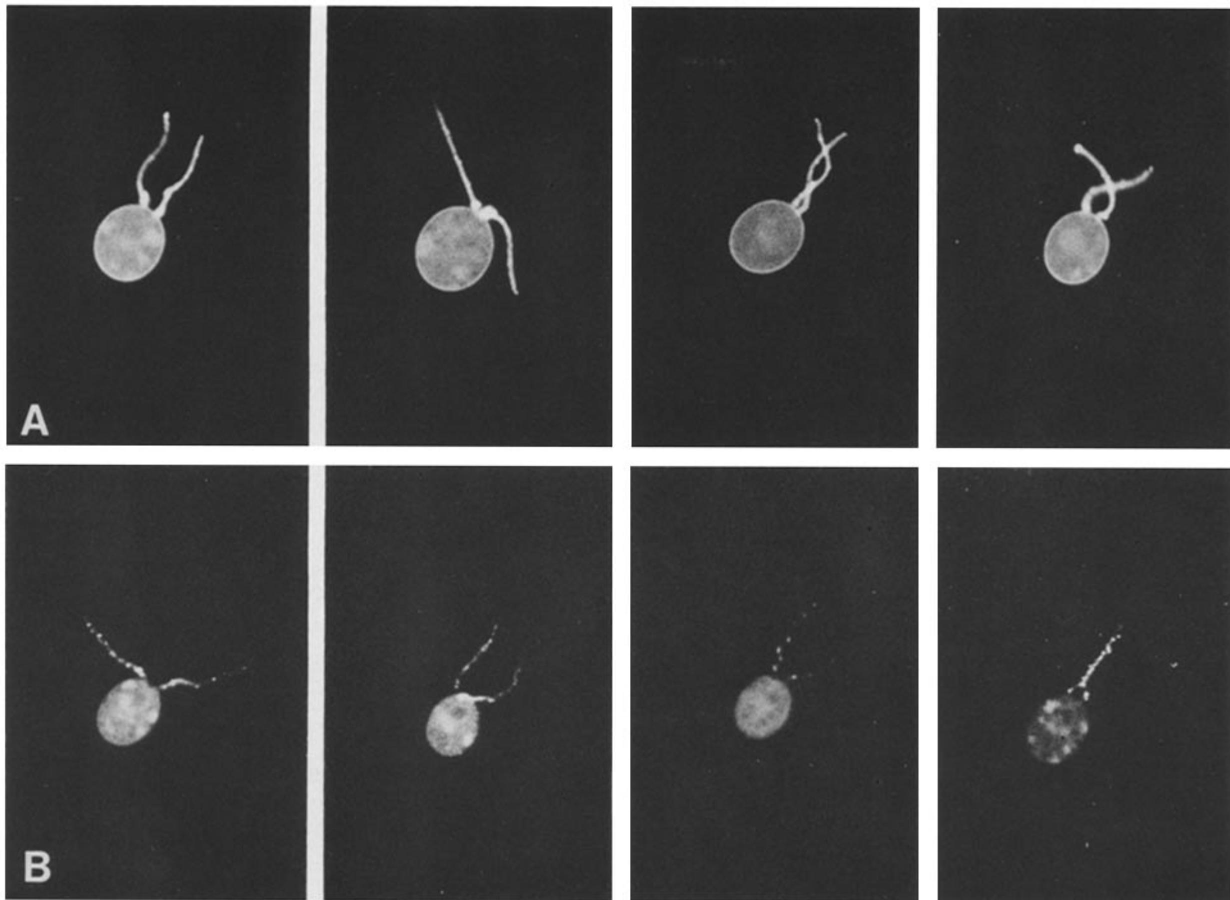


FIGURE 7 Immunofluorescence staining of mt^- gametes. Paraformaldehyde-fixed gametes were stained with Class I monoclonal A15 (row *A*) and Class II monoclonal A12 (row *B*) as described (references 1 and 12, and in Materials and Methods). Cell body fluorescence in row *B* derives from chlorophyll (most clearly seen in color photographs, in which chlorophyll-related fluorescence appears red), whereas cells in row A display additional antibody-associated fluorescence (green in color photographs) due to cross-reaction of Class I antibodies with cell wall glycoproteins (1, 19). Control cells, stained with preimmune or irrelevant antisera, reproducibly showed no antibody-associated fluorescence (1, 19). × 1,200.

FIGURE 8 Immunolabeling of mt^- agglutinin. *Minus* agglutinin, partially purified by Fractogel-75 chromatography, was subjected to SDS PAGE (4–8% gel) and probed with Class I monoclonal A15 (lane *1*) and Class II monoclonal A12 (lane *2*) by the spaghetti overlay procedure (13). Antigen-antibody complexes were detected with ^{125}I-sheep anti-mouse Ig, F(ab)$_2$. 24-h exposure.

from *Chlamydomonas* show no evidence of interacting with each other, but instead require the participation of one living partner (26). Thus, it appears that the *Chlamydomonas* cell–cell recognition/adhesion event involves more than the simple presentation of the appropriate mating-type-specific agglutinins on the flagellar surfaces.

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