# Biochemical Studies of the Excitable Membrane of *Paramecium tetraurelia*. IX. Antibodies against Ciliary Membrane Proteins

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ABSTRACT The excitable ciliary membrane of *Paramecium* regulates the direction of the ciliary beat, and thereby the swimming behavior of this organism. One approach to the problem of identifying the molecular components of the excitable membrane is to use antibodies as probes of function. We produced rabbit antisera against isolated ciliary membranes and against partially purified immobilization antigens derived from three serotypes (A, B, and H), and used these antisera as reagents to explore the role of specific membrane proteins in the immobilization reaction and in behavior. The immobilization characteristics and serotype cross-reactivities of the antisera were examined. We identified the antigens recognized by these sera using immunodiffusion and immunoprecipitation with $^{35}S$-labeled ciliary membranes. The major antigen recognized in homologous combinations of antigen-antiserum is the immobilization antigen (i-antigen), ~250,000 mol wt. Several secondary antigens, including a family of polypeptides of 42,000–45,000 mol wt, are common to the membranes of serotypes A, B, and H, and antibodies against these secondary antigens can apparently immobilize cells. This characterization of antiserum specificity has provided the basis for our studies on the effects of the antibodies on electrophysiological properties of cells and electron microscopic localization studies, which are reported in the accompanying paper. We have also used these antibodies to study the mechanism of cell immobilization by antibodies against the i-antigen. Monovalent fragments ($F_{ab}$) against purified i-antigens bound to, but did not immobilize, living cells. Subsequent addition of goat anti-$F_{ab}$ antibodies caused immediate immobilization, presumably by cross-linking $F_{ab}$ fragments already bound to the surface. We conclude that antigen-antibody interaction per se is not sufficient for immobilization, and that antibody bivalency, which allows antigen cross-linking, is essential.

*Paramecium tetraurelia* is a free-swimming ciliated protozoan in which the intraciliary concentration of $Ca^{++}$ regulates the direction of ciliary beating, and thus the swimming direction. The excitable surface membrane of *Paramecium* couples receptors for various stimuli to the cilia that cover its surface. Normally, *Paramecium* swims in a loose left-handed helix, propelled by the coordinated beating of its several thousand cilia. Stimuli produce the avoiding response in *Paramecium*; a transient reorientation of the power stroke of each cilium causes a temporary reversal of swimming direction and takes the cell away from the stimulus.

Each avoidance response is associated with an action potential that couples the stimulus to ciliary reversal. The action potential results from the opening of voltage-sensitive $Ca^{++}$ channels on the ciliary surface and the consequent inward flux of $Ca^{++}$; the resulting elevated levels of intracellular $Ca^{++}$ somehow trigger ciliary reversal and backward swimming (1, 2). Subsequent closing of these channels and extrusion of $Ca^{++}$ allow forward swimming to resume. A comparison of the electrical properties of ciliated, deciliated, and reciliated cells shows that the voltage-sensitive $Ca^{++}$ channels are located exclusively in the ciliary membrane (3, 4).

SDS PAGE of isolated ciliary membranes resolves ~70 polypeptides ranging from 15,000 to >250,000 mol wt. Two



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classes of polypeptides dominate the pattern: a group of acidic peptides of 42,000–45,000 mol wt, and a family of polypeptides of ~250,000 mol wt, called immobilization antigens (i-antigens) (5). The i-antigen constitutes the 200–300-Å-thick, electron-dense, "fuzzy" layer covering the surface of *Paramecium* (6, 7), whereas the 42,000–45,000-mol wt proteins appear to be intrinsic membrane proteins (7).

The immobilization antigens are polymorphic, surface-associated glycoproteins that have been studied extensively because of the interesting mechanisms by which their expression is regulated (8–11). Although they have the genetic potential to produce at least 12 different i-antigens, individual cells express only one i-antigen at a time, and this antigen defines their unique serotype. Antibodies against the purified i-antigen rapidly immobilize cells of the corresponding (homologous) serotype, but not cells of other (heterologous) serotypes (12–14).

The biological role of the immobilization antigens is unknown, and no peptide of the ciliary membrane has been shown directly to function in excitability. The ciliary membrane is specialized for the regulation of ciliary activity, and the i-antigen is the principal ciliary membrane protein. As part of our continuing efforts to identify the components in the ciliary membrane that are involved in excitability, we have raised antisera against ciliary membranes and against partially purified i-antigens. We report here our efforts to determine (*a*) the immobilization characteristics and corresponding antigenic specificities of these antisera, (*b*) whether immobilization is the result of general physical cross-linking of cilia or of specific functional alteration of the excitable membrane, and (*c*) the specificities and cross-reactivities of the serotypes used: A, B, and H. This information has been used for the application of these antisera in electrophysiological and immunocytochemical studies reported in the accompanying paper (15). A preliminary report of this work was published earlier (16).

## MATERIALS AND METHODS

*Stocks and Cultures:* *Paramecium tetraurelia* stock 51s was used. Lines expressing the serotypes A, B, and H were selected by using the antisera anti-A, anti-B, and anti-H after shifting cultures to 35, 15, and 15°C, respectively, as described (17, 18).

Cultures were checked on the day of harvest, and cultures showing <90% serotype uniformity were discarded. It was our experience that, of the three serotypes we worked with, A and B were stably maintained in culture, whereas H was sometimes transformed to a mixed population of H and B cells.

*[$^{35}$S]Sulfate Labeling:* *Aerobacter aerogenes* was grown for 12 h at 37°C in 100 ml of an inorganic medium of 4.6 mM Na phosphate, 100 mM Tris, 37 mM $NH_4Cl$, 27 mM KCl, 0.5% glucose, 50 μg/ml $MgCl_2$, 75 μM $Na_2SO_4$, pH 7.0, which contained 10 μCi/ml [$^{35}$S]$Na_2SO_4$ and was added to 900 ml of exhausted Cerophyl (Cerophyl Laboratories, Inc., Kansas City, MO), (spent culture fluid that was filtered, supplemented with stigmasterol [5 μg/ml], and adjusted to pH 7.0). To this was added a cell pellet of paramecia in logarithmic phase of growth in Cerophyl medium (100 ml). The cells were allowed to grow for 2–3 d and were then harvested as usual.

*Preparation of Antigens:* Immobilization antigens (A, B, and H) were prepared essentially as described by Preer (19). The 35–46% and 46–61% saturated ammonium sulfate precipitates were dissolved in 50 mM Tris, and 5 mM EDTA, pH 7.4, and were dialyzed, with two changes, against the same buffer at 4°C.

The procedure used to prepare detergent extracts of ciliary membranes has been described previously (18). Briefly, ciliary membranes (MV) were resuspended in a small volume (100–300 μl) of 50 mM Tris, 5 mM EDTA, and 4% (wt/vol) Triton X-100, pH 8.6, and agitated at 4°C for 2 h. The final protein concentrations were 6–15 mg/ml. After the incubation, samples were centrifuged in an Airfuge (Beckman Instruments, Inc., Fullerton, CA) at 120,000 *g* for 30 min at 25°C. The supernatant ($MV_1$) was removed and the pellet was extracted again, as described above, in the same volume of detergent solution to yield a second extract ($MV_2$). The extracts were divided into aliquots and stored at −20°C. Protein concentrations were determined by the method of Lowry et al. (20) with 1% SDS added to negate the interfering effects of Triton X-100 (21).

As shown before (18), 80–90% of the recovered protein was in the $MV_1$ fraction, whereas a second extraction ($MV_2$) contained 10–15%. Comparison on SDS gels (18) shows that $MV_1$ and $MV_2$ represented most of the membrane proteins in about the same relative proportions as they appeared in total membranes.

## Immunizations

ANTIBODIES AGAINST CILIARY MEMBRANE VESICLES (ANTI-$M_A$, ANTI-$M_B$, AND ANTI-$M_H$): Ciliary membranes of serotype A, B, or H, prepared as described previously (5), were emulsified into a mixture of 1 vol of incomplete Freund's adjuvant and 0.1 vol of Freund's complete adjuvant. For each membrane type, two rabbits were injected subcutaneously with 1.5–2.0 mg of membrane protein (1:1 in incomplete Freund's) as the primary dose, and 1.5 mg as a booster 3–6 wk later. The animals were bled 1 wk after the booster, then at 2–3-d intervals for 1 wk. In the case of anti-$M_A$ and anti-$M_H$, a second boost of 1.5 mg of membrane protein was given 10 wk after the priming dose.

ANTIBODIES AGAINST IMMOBILIZATION ANTIGENS (ANTI-A AND ANTI-H): i-antigen (0.5 mg) purified by the Preer (19) method from serotypes A (35°C) or H (15°C) was injected subcutaneously into each rabbit (two rabbits per antigen) as a 1:1 emulsion in complete Freund's adjuvant. After 4 wk the rabbits were boosted with 0.25 mg of i-antigen emulsified in incomplete Freund's adjuvant. 1 wk after the boost, the rabbits were bled every 3–4 d for 1 wk. Serum was decomplemented at 56°C for 30 min and stored frozen at −20°C. A portion of the serum was later used for IgG purification by 50% $(NH_4)_2SO_4$ precipitation and DEAE-cellulose chromatography in 30 mM sodium phosphate, pH 7.0 (22). Purified IgG fractions were stored in 0.1 M NaCl and 30 mM sodium phosphate, pH 7.0, containing 15 mM sodium azide and Aprotinin (500 trypsin inhibitor U/ml) (Sigma Chemical Co., St. Louis, MO) at −20° or −80°C.

*Immobilization Tests:* Cells, usually in early stationary phase, were washed and diluted in 1 mM HEPES, 1 mM $CaCl_2$, 0.5 mM KOH, 3.5 mM KCl, and 0.01 mM EDTA, pH 7.2, (resting solution) to 250–500 cells/ml. A cell suspension (80 μl) was transferred to each well of a depression slide and 20 μl of (diluted) antiserum was added. Cells were observed 0, 30, 60, and 120 min later, and the number of immobilized cells was determined at 60 and 120 min. At the end of the assay, a drop of Lugol's solution (2% KI, 1% $I_2$) was added and the cell number was determined. Immobilization was expressed as percent immobilized cells; an immobilizing titer of antibody is defined as the highest antibody dilution that immobilizes 50% of the cells in 2 h.

*Monovalent Fragments ($F_{ab}$) of Anti-A:* $F_{ab}$ fragments were prepared by papain digestion of a DEAE-cellulose-purified IgG fraction of anti-A using a modification of Porter's method (23). IgG (10 mg) was digested in 0.1 M NaCl and 10 mM sodium phosphate, pH 7.0, containing 2 mM EDTA, 10 mM β-mercaptoethanol, and 100 μg of papain (Calbiochem-Behring Corp., San Diego, CA). The digestion was carried out for 16.5 h at 37°C, and then 30 mM iodoacetamide was added to end the reaction. The products were dialyzed extensively against 0.1 M NaCl and 10 mM sodium phosphate, pH 7.0, and subjected to chromatography on a Sephadex G-100 column equlibrated with the same buffer.

*Immunoprecipitations:* 20 μg of membrane Triton extracts or 15 μg of i-antigen protein was reacted with various amounts of antisera (0–175 μl) in a total reaction volume of 200 μl in a buffer containing 1% Triton X-100, 50 mM Tris, and 5 mM EDTA, pH 8.6.

[$^{35}$S]$SO_4^=$-labeled antigen (Triton X-100 extracts [$MV_1$] or i-antigen) were diluted with cold antigen to obtain a specific activity of 2,000 cpm/μg protein (40,000 cpm/reaction for membrane samples and 30,000 cpm/reaction for i-antigen samples). Precipitation was allowed to occur at 37°C for 1 h, followed by incubation at 4°C for 12–16 h. The samples were then centrifuged in a microfuge (Beckman Instruments, Inc.) (10,000 *g*) for 3 min, and the supernatants were carefully removed. The precipitates were washed twice in 1 ml of Triton X-100 buffer containing 7.5 mg/ml of methionine and then in buffer without detergent. The samples were dissociated in 50 μl of 4% SDS and heated at 100°C for 5 min. An equal volume (50 μl) of 10% β-mercaptoethanol, 20% glycerol, and 125 mM Tris, pH 6.8 (24), was then added, and the samples were analyzed on 7.5–15% acrylamide gradient SDS gels as described (5). The gels were impregnated with EnHance solution (New England Nuclear, Boston, MA), dried under vacuum, and fluorographed at −70°C.

*Behavioral Tests:* Paramecia, washed in resting solution (see above), were treated with antibody (IgG or $F_{ab}$) for 15–30 min at room temperature.

Treated cells were then washed twice with resting solution and tested in $K^+$ or $Ba^{++}$ solutions. $K^+$ solution: 20 mM KCl, 1 mM $CaCl_2$, 0.01 mM EDTA, and 1 mM HEPES, pH 7.2; $Ba^{++}$ solution: 8 mM $BaCl_2$, 1 mM $CaCl_2$, 0.01 mM EDTA, and 1 mM HEPES, pH 7.2 (see reference 25 for details). Cells normally show continuous ciliary reversal for 40–60 s when transferred to $K^+$ solution and undergo frequent avoiding reactions in $Ba^{++}$ solution.

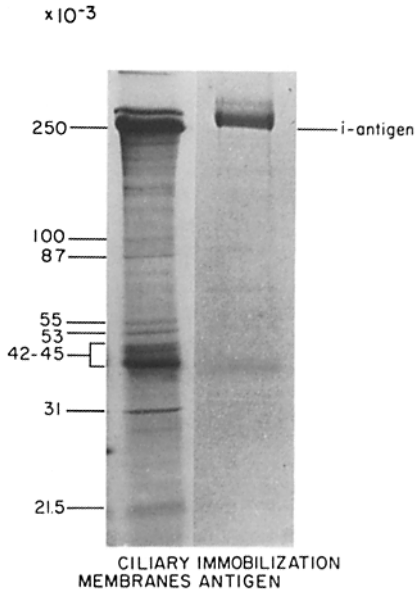


FIGURE 1 SDS gel of immunogens. Ciliary membranes (175 μg) and the immobilization antigen (50 μg) prepared as described, were electrophoresed on 7.5–15% gradient SDS gels. Note that the i-antigen preparation contains some protein with ~45,000 mol wt.

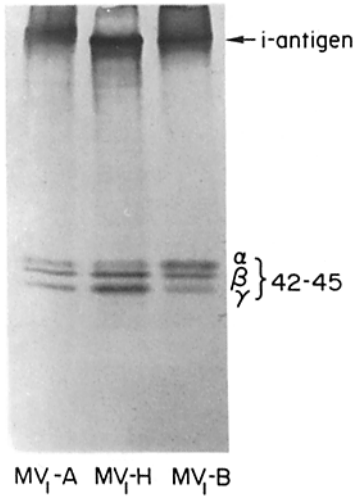


FIGURE 2 Comparison of A-, B-, and H-type ciliary membrane proteins. $^{35}S$-labeled ciliary membranes of A-, B-, and H-type cells were Triton-extracted, electrophoresed on 7.5–15% gradient SDS gels, and fluorographed. ($MV_1$-A) membrane extract of A-type cells; ($MV_1$-H) membrane extract of H-type cells; ($MV_1$-B) membrane extract of B-type cells. Molecular weight measurements, × $10^{-3}$.

## RESULTS

### *Immunogens*

Antibodies were produced in rabbits against (*a*) isolated ciliary membranes and (*b*) partially purified i-antigens. The protein profiles of these antigens, analyzed using SDS PAGE, are shown in Fig. 1. The pattern of ciliary membrane proteins is complex. Of particular relevance to the results here are the major membrane proteins: (*a*) the high-molecular-weight (~250,000–300,000) i-antigen, and (*b*) a group of three to four proteins with molecular weights of ~42,000–45,000. In agreement with previous results on isolated i-antigens (8), the i-antigen on ciliary membranes of serotype A appeared to be larger (~300,000 mol wt) than that on membranes of B and H cells (~250,000 mol wt) (Fig. 2). The relative amounts of the different proteins of 42,000–45,000 mol wt varied as a function of the serotype of the cell (Fig. 2 and references 26 and 27). As reported before (19), the purification scheme for the i-antigen yielded a final product that consisted mostly (~90%) of i-antigen (~250,000 mol wt); some minor proteins (including at least one of 42,000–45,000) appeared to co-purify with the i-antigen (Fig. 1).

### *Immobilization by Intact Antibodies*

The antisera raised against isolated ciliary membranes (anti-$M_A$, anti-$M_B$, and anti-$M_H$) as well as the antisera against isolated i-antigens (anti-A, anti-B, and anti-H) immobilized Paramecia. Low concentrations of these antisera showed immobilizing specificity towards cells of the homologous serotype, but at higher concentrations heterologous cells were also immobilized (Table I).

### *Monovalent Fragments ($F_{ab}$) of Anti-A: Binding and Immobilization*

We prepared monovalent $F_{ab}$ fragments of anti-A (anti-A [$F_{ab}$]) by papain digestion of intact IgG. Two included peaks of protein (I and II) from the Sephadex G-100 column were composed of mixtures of $F_{ab}$ and $F_c$. SDS PAGE of the pooled samples with or without β-mercaptoethanol showed that the papain digestion was essentially complete; no detectable intact IgG remained (data not shown). Immunodiffusion of peaks I and II against goat anti-rabbit $F_{ab}$ and goat anti-rabbit $F_c$ showed that the two column fractions of protein were active as antigens and were mixtures of $F_{ab}$ and $F_c$. In tests for antibody activity of the $F_{ab}$ fragments, all the samples showed precipitin lines (Fig. 3*a*) with goat anti-rabbit $F_{ab}$; but only samples containing peaks I and II (the pooled $F_{ab}/F_c$ fragments) showed radioactivity (from labeled antigen) bound to the precipitin lines, which corresponds to tertiary (goat anti-

TABLE I

*Antibody Titers for Immobilization in Homologous and Heterologous Combinations*

| | Antiserum | | | | |
|---|---|---|---|---|---|
| Cell type | Anti-A | Anti-$M_A$ | Anti-$M_B$ | Anti-H | Anti-$M_H$ |
| A | 1:500 (1) | 1:333 (1) | 1:125 (16) | 1:12 (4) | 1:17 (4) |
| B | 1:12 (42) | 1:17 (20) | 1:2,000 (1) | >1:10 (>5) | 1:20 (3) |
| H | >1:7 (>75) | 1:8 (40) | 1:67 (30) | 1:50 (1) | 1:67 (1) |

Antibody titers for immobilization in homologous and heterologous combinations. Washed A-, B-, and H-type cells were treated with a series of dilutions of each of the antisera shown. The highest dilution of antibody that caused 50% immobilization for each of the cell types (the immobilization titer), as well as the relative antibody titer (normalized to the homologous combination), is shown in the table.

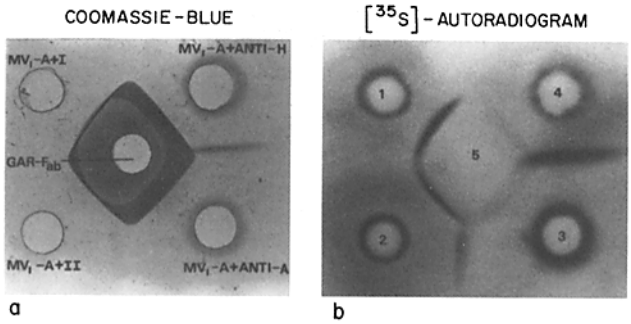


FIGURE 3 $F_{ab}$-fragments of anti-A. 4 μg of [35S]$SO_4^=$-labeled $MV_1$-A was preincubated with anti-A, anti-H, or anti-A ($F_{ab}$) (I and II) for 30 min at room temperature and the samples were tested for precipitation against goat anti-rabbit $F_{ab}$ (GAR-$F_{ab}$) on an Ouchterlony immunodiffusion plate. After diffusion for 36 h, the plate was washed, dried, stained, and destained to locate the proteins precipitated. Subsequently, the plate was autoradiographed for 2 d to locate the radioactive bands. (*a*) Coomassie Blue-staining pattern. (*b*) Autoradiograph.

$F_{ab}$) ($F_{ab}$) ($MV_1$-A) complexes (Fig. 3*b*). This confirmed that the anti-A ($F_{ab}$) retains its antigen ($MV_1$) binding capacity.

The monovalent fragments did not immobilize homologous A-type cells even at concentrations eight-fold higher (184 μg/ml) than the equivalent divalent antibody concentration needed to immobilize cells (23 μg/ml) (a protein concentration of 23 μg/ml corresponds to a 1:1,000 dilution of anti-A). A-type cells, washed in resting solution, were incubated for up to 6 h in $F_{ab}$ solution (diluted in resting solution). Although the swimming speed of the cells was somewhat reduced, no immobilization was obtained (Table II).

Cells treated with anti-A ($F_{ab}$) were immobilized immediately upon addition of a second antibody directed against the $F_{ab}$ fragments (goat anti-rabbit IgG/$F_{ab}$). When A-type cells washed in resting solution were preincubated in antibody I, only goat anti-rabbit IgG and goat anti-rabbit $F_{ab}$ were effective in immobilizing the $F_{ab}$-treated cells (Table II). Some slowing down of cells was also observed in control samples containing preimmune serum or azide, but no immobilization was observed.

## Behavioral Effects of Monovalent Antibodies

Monovalent antibodies did not detectably alter the swimming behavior of treated cells. A-type cells were treated with anti-A ($F_{ab}$) (46, 230, and 460 μg/ml) for 15–30 min and then subjected to behavioral tests. Cells treated with 46 μg/ml anti-A ($F_{ab}$) behaved like control cells, with no significant differences in the duration of backward swimming in $K^+$ solutions or the frequency of avoiding reactions in $Ba^{++}$ solutions. Cells treated with 230 or 460 μg/ml anti-A ($F_{ab}$) were slowed down considerably, and this reduced motility precluded meaningful behavioral tests.

## Antigenic Specificity by Double Immunodiffusion

Using immunodiffusion, two major precipitin lines, Z and A, were formed: (*a*) Z is an immunoprecipitate derived from an antigen present in membrane extracts of all serotypes but present in much lower amounts in the i-antigen samples. This precipitin line showed a reaction of complete identity (Fig. 4), which implies that these antigenic determinants are common and cross-reactive in A-, B-, and H-type cells, and hence

TABLE II

*Immobilization of Anti-A ($F_{ab}$)-treated Cells*

| | Percent immobilization after addition of antibody II | | | |
|---|---|---|---|---|
| | Antibody I | | | |
| Antibody II | Anti-A* | Anti-A ($F_{ab}$)* | Preimmune serum‡ | None |
| None | 100 | 0 | 0 | 0 |
| Goat anti-rabbit IgG§ | 100 | 95 | 0 | 0 |
| Goat anti-rabbit $F_{ab}$§ | 95 | 100 | 0 | 0 |
| Goat anti-rabbit $F_c$§ | 95 | 0 | 0 | 0 |
| 0.01% Sodium azide | 100 | 0 | 0 | 0 |

The azide controls were necessary because antibody II contained azide. A-type cells were washed in resting solution and preincubated in antibody I for 60 min, at which time antibody II was added. The percent of cells immobilized was determined and is shown in the table.

* 46 μg/ml anti-A, anti-A ($F_{ab}$).

‡ 750 μg/ml preimmune serum.

§ 276 μg IgG/ml.

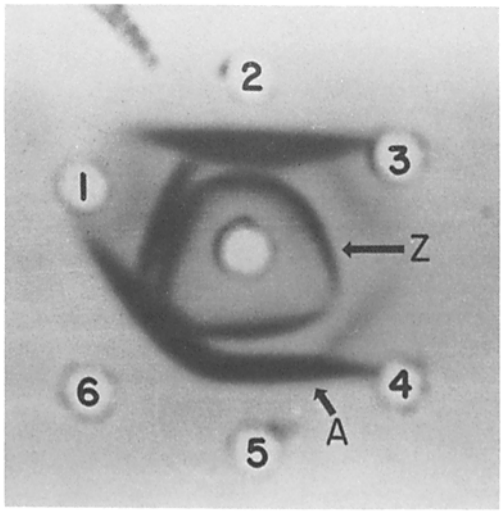


FIGURE 4 Double-immunodiffusion of antigens in 1% (wt/vol) agarose in 50 mM barbital, 1 mM EDTA, 1 mM ε-amino caproic acid, pH 8.5, containing 1% (wt/vol) Triton X-100. 10 microliters of the samples in 1% (wt/vol) Triton X-100 was added to the wells and diffusion was allowed to proceed for 36 h. The slide was washed, dried, stained, destained, and photographed. The Coomassie Blue-stained gel is shown. Center well: anti-A. *1*: $MV_1$-B, 20 μg; *2* and *5*: $MV_1$-A, 20 μg; *3*: $MV_1$-H, 20 μg; *4*: H i-antigen, 15 μg; *6*: A i-antigen, 15 μg. Note that two precipitin lines, *Z* and *A*, are formed. *Z* forms a line of complete identity while *A* exhibits a reaction of partial identity.

are immunologically indistinguishable in homologous/heterologous combinations. (*b*) A is present in large amounts in homologous cell antigens (wells 2, 5, and 6; Fig. 4). The A precipitin line almost exclusively contributes to the reaction of i-antigen samples. This precipitin line clearly showed a reaction of partial identity, i.e., a comparison of the patterns obtained with A and B cell extracts (well 1 with wells 2 and 6) or A and H cell extracts (wells 2 and 5 with wells 3 and 4) shows a spur formed; this spur is formed by the determinants exclusively present in wells 2, 5, and 6 (homologous cell extracts). The fusion of the lines is obtained because of

common determinants present in all three cell extracts. The cross-reaction is greater for the B cell extracts than for the H cell extracts (compare wells 1 and 3).

From the positions of the two precipitin lines, A and Z, we infer that the antigen contributing to A is larger than that of Z and/or the amount of antibody to A is greater than that to Z in the antisera. Both of these factors are probably operative; from the results of radioimmunoprecipitations (see below) analyzed by SDS PAGE, it seems very likely that band Z corresponds to the 42,000–45,000-mol-wt proteins, whereas band A corresponds to the 250,000–300,000-mol-wt i-antigen.

## *Antibody Specificity by Radioimmunoprecipitation*

Since the antisera formed precipitable antigen-antibody complexes without the use of a second antibody, the antigens must be multivalent. Fig. 5 shows the results obtained in an "equivalence" immunoprecipitation. The major proteins precipitated using anti-$M_A$ (homologous combination) were the i-antigens (>250,000 mol wt) and three proteins in the 42,000–45,000-mol-wt range (Fig. 5). Several minor proteins were also precipitated using anti-$M_A$, prominent among which are proteins of 150,000 and 19,000 mol wt. Anti-$M_H$ (heterologous combination) precipitated the 42,000–45,000-mol-wt proteins, but little, if any, i-antigen (Fig. 5). Some protein of ~150,000 mol wt was also precipitated.

A similar equivalence immunoprecipitation was done using the anti-(i-antigen) antisera (anti-A and anti-H). The A i-antigen was precipitated only by homologous anti-A and not by heterologous anti-H, whereas the 42,000–45,000-mol-wt proteins were immunoprecipitated by both anti-A and anti-H (Fig. 5).

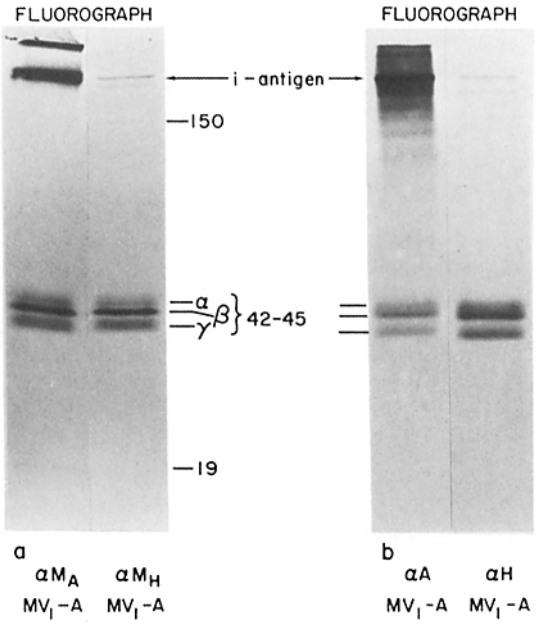


FIGURE 5 Radioimmunoprecipitation. [$^{35}$S]$SO_4^=$-labeled Triton extracts of ciliary membranes of A-type cells ($MV_1$-A) were immunoprecipitated with anti-$M_A$ ($\alpha M_A$), anti-$M_H$ ($\alpha M_H$), anti-A ($\alpha A$), and anti-H ($\alpha H$), and the precipitates were run on 7.5–15% gradient SDS gels and fluorographed to locate the radioactive proteins precipitated. The Ag/Ab ratio used was within the equivalence zone.

To determine which of the antigens are recognized at antibody-limiting concentrations, we carried out titrations (Figs. 6 and 7). Low levels of anti-A (5 μl) preferentially precipitated the A i-antigen, whereas increasing amounts of antibody precipitated the β, α, and γ (42,000–45,000) proteins, in that order (Fig. 6). Low levels (5 μl) of anti-H precipitated essentially nothing from $MV_1$-A, whereas increasing amounts of serum preferentially precipitated the β, α, and γ (42,000–45,00-mol-wt) proteins.

A similar immunotitration of anti-$M_H$ against $MV_1$-A is shown in Fig. 7. Low levels of anti-$M_H$ did not precipitate any proteins but increasing anti-$M_H$ complexed the β, α, and γ (42,000–45,000-mol-wt) proteins; note that even 175 μl of anti-$M_H$ did not bring down any significant amount of A i-antigen. In comparison, anti-$M_A$ added to a sample containing anti-$M_H$ and $MV_1$-A (Fig. 7, lane *i*) quantitatively precipitated the A i-antigen and the 42,000–45,000-mol-wt proteins.

These results show that, in (*a*) antibody-limiting cases (corresponding to "low" levels of antibody in the titration experiments), the results are biased towards the most abundant antibody population in the polyspecific antisera, and (*b*) in antibody-saturation cases (corresponding to "high" levels), the results reflect the total potential (in terms of precipitability) of the antisera.

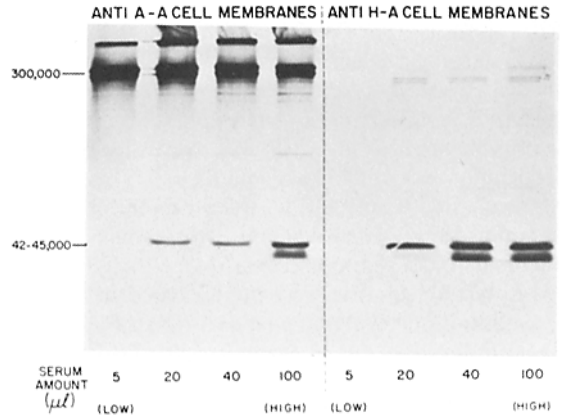


FIGURE 6 Immunotitration of anti-A and anti-H. 20 μg of a Triton-extract of A-ciliary membranes ($MV_1$-A) was titrated with 5, 20, 40, and 100 μl of anti-A and anti-H, and the immunoprecipitates formed were run on 7.5–15% gradient SDS gels. The fluorograph of the SDS gel is shown.

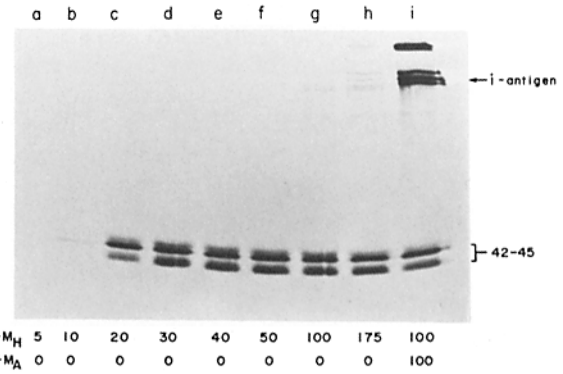


FIGURE 7 Immunotitration of anti-$M_H$. 20 μg of $MV_1$-A was reacted with different amounts (5, 10, 20, 30, 40, 50, 100, and 175 μl) of anti-$M_H$ and the immunoprecipitates were analyzed by SDS PAGE and fluorography. Lane *i*: 100 μl of anti-$M_A$ added to a sample containing 100 μl of anti-$M_H$ and 20 μg of $MV_1$-A.

With immunoprecipitations at low and high serum concentrations for the other antigen-antibody combinations, we determined the immunoprecipitability of the several membrane antigens by the different antibodies. Examples of this set of data are presented in Fig. 8. In all cases, low serum concentrations selectively precipitated the homologous i-antigen, e.g., anti-$M_A$ and anti-A recognized A i-antigen only. However, at high serum concentrations, all the antisera precipitated the 42,000–45,000-mol-wt proteins. Heterologous precipitation of the i-antigen was obtained only at high serum concentrations and only in the case of A and B cell types; there appears to be cross-reactivity between the A and B i-antigens. Heterologous precipitation of the H i-antigen or heterologous precipitation by the anti-H sera were not obtained. The same results were obtained with partially purified i-antigen and with membrane extracts containing i-antigen. These observations are summarized in Table III.

In controls with preimmune serum, <5% as much $^{35}S$ protein was precipitated as with immune serum. A second control sample containing anti-BSA, BSA, and 20 μg of $^{35}S$-labeled $MV_1$-A showed no nonspecific binding of ciliary membrane proteins to a nonimmune precipitate. Immunoprecipitates incubated in the presence of 4 mM phenylmethylsulfonyl fluoride to control for any possible proteolytic activity were found to be identical to samples without phenylmethylsulfonyl fluoride.

In short, immunoprecipitation revealed three major populations of precipitating antibodies in anti-$M_A$; in order of decreasing abundance, they are: (*a*) antibodies unique to A i-antigen, (*b*) antibodies to 42,000–45,000 mol wt (α, β, and γ) proteins, and (*c*) antibodies that react with A and B-type i-antigens, but not with H i-antigens. We have shown (18) that only homologous absorptions (using A-type cells or A i-antigens) absorbed antibodies specific to A cells, with a consequent loss of immobilizing activity; conversely, the cross-reactive anti-(42,000–45,000-mol-wt) antibodies were nonselectively absorbed by all cell types. These results show that both the i-antigens and the 42,000–45,000-mol wt proteins are accessible in vivo and confirm the inferred antigenic specificities. However, we were unable to demonstrate in these immunoabsorption experiments the existence of a class of antibodies cross-reactive to A- and B-type i-antigens. This discrepancy may be due, in part, to the different sets of conditions under which the cell absorptions and immunoprecipitations were done, e.g., the accessibilities of antigenic determinants may be dissimilar under the in vivo and in vitro situations.

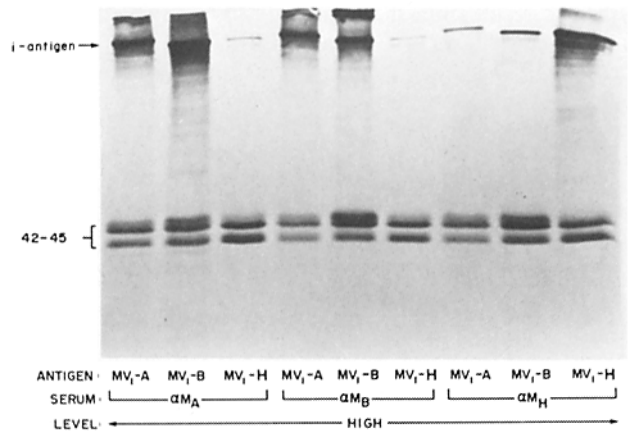


FIGURE 8 Radioimmunoprecipitations of membrane extracts with high levels of homologous and heterologous antisera. 20 μg of $^{35}S$-labeled Triton-extract of A, B, or H-type cells ($MV_1$-A, $MV_1$-B, or $MV_1$-H, respectively) was reacted with high levels of anti-$M_A$ ($\alpha M_a$), anti-$M_B$ ($\alpha M_B$), or anti-$M_H$ ($\alpha M_H$). The precipitates obtained were washed and run on 7.5–15% gradient SDS gels. The fluorograph of the gel is shown. Note that all antigen-antibody combinations precipitated the 42,000–45,000-mol-wt proteins while only the A and B i-antigens cross-reacted with each other (lanes *1*, *2*, *4*, and *5*). Cross-reaction *of* the H i-antigen by the A- and B-type antisera and precipitation of the A- and B-type i-antigen *by* anti-$M_H$ was not obtained. The minor amounts of protein that appear at the i-antigen region of the gel in such cases is thought to be derived from contaminating antigens and/or contaminating antibodies (see Discussion).

TABLE III

*Specificity of Antibodies in Immunoprecipitating Membrane Antigens*

| Antibody | Amount | Membrane antigens: A i-antigen (300,000 mol wt) | B i-antigen (250,000 mol wt) | H i-antigen (250,000 mol wt) | α, β, γ (42,000–45,000 mol wt) |
|---|---|---|---|---|---|
| Anti-A and Anti-$M_A$ | Low | + | − | − | − |
| | High | + | + | − | + |
| Anti-$M_B$ | Low | − | + | − | − |
| | High | + | + | − | + |
| Anti-H and Anti-$M_H$ | Low | − | − | + | − |
| | High | − | − | + | + |

Specificity of antibodies in immunoprecipitating membrane antigens. Immunotitrations at low and high serum concentrations (see text) were carried out for all the antigen-antibody combinations shown. The table shows the capacity of the different antisera to precipitate A, B, and H i-antigens and the 42,000–45,000-mol wt (α, β, and γ) proteins. +, denotes that in the given antigen-antibody combination recognition and precipitation occurred. The antigens used were $^{35}S$-labeled Triton extracts of ciliary membranes of A-, B-, and H-type cells ($MV_1$-A, $MV_1$-B, and $MV_1$-H) and i-antigens of A-, B-, and H-type cells. (Compare with Figs. 5–8.)

## DISCUSSION

### *Paramecium's Surface Contains Both Common and Serotypically Unique Antigens*

Both sets of antisera, one raised against the semipurified i-antigens and the other raised against ciliary membranes, immobilized Paramecia. The major antigenic specificities of the antisera were similar; in $^{35}S$ radioimmunoprecipitations, two prominent sets of proteins, the i-antigens and 42,000–45,000-mol wt proteins, were recognized. At low antiserum concentrations, serotype-specific immobilization was obtained, presumably because of the effect on unique determinants on the cell surface. Radioimmunotitration, used as an in vitro analogue of cell immobilization, suggested that selective interaction with the homologous i-antigen under low serum concentrations was responsible for the serotype-specific immobilization.

At higher serum concentrations, nonselective immobilization was obtained, presumably because of cross-reactive antibodies to common determinants present on cells of all serotypes. Radioimmunoprecipitations suggest that the immobilization at high serum levels of cells of serotype A or B by

anti-H, or of H cells by anti-A or anti-B, is due to antibodies that recognize the ubiquitous 42,000–45,000-mol-wt proteins. The immobilization of serotype A by anti-B, or of serotype B by anti-A, is the result of antibody interaction with the common determinant present in the i-antigens of both serotypes A and B, or of interaction with the 42,000–45,000-mol-wt proteins, or of a combination of these effects. These results confirm earlier experiments that placed serotypes A and B in a serotype subgroup from which serotype H is excluded (6, 28).

Two further observations are consistent with this interpretation. First, we found that immunoabsorptions in heterologous combinations led to a loss of "heterologous" immobilizing activity with no significant loss of homologous immobilizing activity (18). Second, we have shown (7) that mild protease treatment of living Paramecia removed the i-antigen, but had little or no effect on the 42,000–45,000-mol-wt proteins. Such protease-treated cells showed a loss in serotype-specific immobilization; before protease treatment, cell immobilization at low antiserum concentrations was serotype specific, but removal of the i-antigen led to immobilization by heterologous antibody. One possible explanation for this effect is that the i-antigen covers cryptic, cross-reactive determinants on the 42,000–45,000-mol-wt proteins in the ciliary surface.

### Relation of i-Antigens to 42,000–45,000-Mol-Wt Proteins

Several independent lines of evidence show that the i-antigens and 42,000–45,000-mol-wt proteins are externally exposed, glycosylated proteins (7, 18, 29, 30). The effects of externally applied antibodies to these proteins on cell physiology (immobilization and electrophysiology) and cell absorption experiments also suggest an external location for these antigens (15, 18).

Although there appears to be a concerted regulation of expression of the i-antigens and the 42,000–45,000-mol-wt proteins (Fig. 2 and references 26 and 27), these latter proteins were found to be totally cross-reactive across serotypes (Fig. 8 and Table III; A. Adoutte, personal communication).

It was postulated earlier that the membrane-bound i-antigen(s) may be a precursor of the soluble surface i-antigens (30). We have preliminary evidence that the membrane-bound form of the i-antigen contains a hydrophobic region not found in the soluble protein (R. Ramanathan, S. B. Arthur, and D. L. Nelson, unpublished data). All of the above findings are suggestive of an association between the i-antigens and the 42,000–45,000-mol-wt polypeptides in the membrane, which may be of physiological significance. Perhaps the 42,000–45,000-mol-wt proteins serve as membrane anchors for the i-antigen molecules. Capdeville (31, 32) postulated, on the basis of serological analyses of a group of allelic i-antigens, that the surface antigen may consist of a constant (common) portion and a variable (specific) portion. Such a model suggests that, by analogy with immunoglobulin synthesis, regulation of antigen expression might be achieved by genetic recombination. Genomic recombination is also known to occur in the expression of the polymorphic surface antigens of a group of parasitic protozoa, the trypanosomes (33, 34). However, Forney et al. (35) have recently cloned fragments of two i-antigen genes (for serotypes A and C) and have used the clones as probes of the genomic sequences that flank the A gene. Their results give no evidence of expression-linked DNA rearrangements of the sort known to occur with surface antigen genes of trypanosomes (33, 34).

### Divalent Antibodies Are Required to Immobilize Cells

Beale and Kacser (36) observed that treatment of Paramecia with immobilizing antisera led to the adherence of the distal tips of cilia, presumably via antigen-antibody complexes, whereas proximal regions of the cilia retained motility. Using fluorescein-conjugated antibodies, they also showed that, in *Paramecia* fixed before antibody addition, i-antigen was distributed uniformly over the entire surface of the cell, whereas in cells fixed after immobilization, the antigen-antibody complex was present in globules at the tips of cilia (36). Beale and Kacser suggested that the accumulation of antigen-antibody complexes at the tips of cilia occurred via exudation of the antigen into the medium and its subsequent precipitation onto the tips of the cilia. This model implies that antigen cross-linking and adherence of ciliary tips cause immobilization; in such a model, divalent antibodies are required for immobilization. We find that $F_{ab}$ fragments alone do not immobilize cells, but that subsequent cross-linking of bound $F_{ab}$ by a second antibody does cause immobilization, which demonstrates the necessity for cross-linking in the immobilization reaction. Mere chemical liganding or occupation of antigenic sites on the membrane do not cause immobilization. Our results, however, do not rule out the possibility that bridging of intraciliary antigenic sites or the lateral movement of surface antigens causes immobilization.

Paramecia and other ciliates (*Tetrahymena* and *Colpoda*) have been observed to recover from immobilization when treated with sublethal levels of antibody. This has been assumed to result from the shedding of accumulated antigen-antibody products (12). Eisen and Tallan (37) have shown that *Tetrahymena* can recover from immobilization by proteolytically producing monovalent fragments in situ. Our data are consistent with this finding, and it appears that antigen-antibody cross-linking is necessary for cell immobilization.

Divalent antibodies against the isolated ciliary membranes and purified i-antigen immobilize cells and, when present above threshold concentrations, lyse the cells. Behavioral observations of antibody-treated cells were consequently limited to cells treated with sublethal levels of antibody for short periods of time. We expected that monovalent fragments would enable us to distinguish effects of antibody binding per se from effects of cell immobilization and would allow us to study the effects of higher concentrations of antibody on the behavior of cells and probe the role of the i-antigen in excitability. No significant behavioral differences were detected in cells treated with low concentrations of $F_{ab}$; at high $F_{ab}$ concentrations, reduced cell motility precluded behavioral studies. We also examined the electrophysiological properties of antibody-treated cells (both $F_{ab}$ fragments and bivalent antibodies) and found that the voltage-sensitive, inward Ca current is specifically reduced in such cells when compared with control cells. These results and their significance are discussed elsewhere (15); we were able to distinguish between the direct effects of antibody binding and cell immobilization by using monovalent fragments. It is possible that we were unable to detect behavioral differences in antibody-treated cells because of insufficient sensitivity of behavioral assays as compared

with electrophysiological experiments. Such apparent inconsistencies between behavioral and electrophysiological results have also been observed in experiments using temperature-sensitive pawn mutants at permissive temperatures (38) and protease-treated cells (7).

We used only single-antibody precipitation techniques to determine the antigenic specificities of the antisera. The success of such a technique requires that the antigens be multivalent and that the descriptions of the antisera reflect the minimum complexity of the antisera; there may be other antibodies not detected by the immunoprecipitation techniques that have other effects on cell physiology, e.g., mating reactions. For determining the roles of specific membrane proteins in immobilization, single-antibody precipitation is probably a sufficient description; immobilization requires divalent antibodies, and perhaps only multivalent antigens and the antigen-antibody complexes they form can be expected to participate in immobilization.

Any method using polyclonal antibodies to analyze the cross-reactivities of the polymorphic i-antigen(s) must rely heavily on the availability of homogeneous preparations of antibodies and antigens that can be used as analytical reagents. Although we ensured, as far as possible, that only serotypically homogeneous (>90%) populations of cells were used for the preparation of antigenic extracts and immunogens, it was technically impossible to achieve absolute purity in these preparations. This, added to the fact that the H serotype was inherently unstable, complicated our analysis of cross-reaction. For example, the homologous/heterologous immunoprecipitations (see Figs. 5–8) did not give all-or-none precipitation patterns of the i-antigen, as might have been expected for analytically pure reagents; the small amounts of precipitated proteins in non–cross-reactive heterologous cases (for example, see Fig. 8, lanes *7* and *8*) could be derived from contaminating antigens in the extracts used or contaminated immunogens used. We were unable to use preabsorption of antigenic extracts or antisera to rid them of contaminating serotypes because we wanted to analyze both the common and the serotype-specific determinants present; furthermore, i-antigen preparations contained small amounts of the 42,000–45,000-mol wt proteins.

We expect that in addition to their applications in electrophysiological studies and electron microscopic localization studies, the polyclonal antisera described here, will be useful in exploring the relationship between the i-antigen and the 42,000–45,000-mol-wt proteins, in exploring the topography of ciliary membrane vesicles, and possibly in the purification by immunoaffinity chromatography of specific ciliary membrane proteins.

Since the completion of the experiments using $F_{ab}$, we learned that A. Barnett and E. Steers (39) have also demonstrated that divalent antibodies are required for immobilization using *Paramecium multimicronucleatum* and mono- and divalent anti-C antibodies.

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