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BIOCHEMICAL STUDIES OF THE EXCITABLE MEMBRANE OF *PARAMECIUM TETRAURELIA*

V. EFFECTS OF PROTEASES ON THE CILIARY MEMBRANE

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Summary

The swimming behavior of *Paramecium* is regulated by an excitable membrane that covers the body and cilia of the protozoan. In order to obtain information on the topology and function of ciliary membrane proteins, *Paramecia* were treated with trypsin, chymotrypsin or pronase and the effects of these proteases were analyzed using electron microscopy, gel electrophoresis of ciliary fractions and behavioral tests. At the concentrations used, trypsin and chymotrypsin had little or no effect on the cells while pronase removed the cell surface coat, visible as fuzzy material covering the cell membrane. The same pronase treatment caused the specific removal of a high molecular weight protein (250 000), as judged by sodium dodecyl sulfate polyacrylamide gel electrophoresis. This protein, the 'immobilization antigen', constitutes the major protein of the ciliary membrane. Although the immobilization antigen was removed (or markedly decreased), no marked and reproducible difference was observed in the swimming behavior of the treated cells. We also determined the effects of proteases on isolated ciliary fractions to explore the sidedness of ciliary membrane proteins. A set of proteins relatively resistant to protease digestion was identified; they may be intrinsic membrane proteins.

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Introduction

The ciliary membrane of *Paramecium* carries out three important and general biological functions. Firstly, the ciliary membrane plays a key role in the regulation of ciliary beat; it has been shown to contain the voltage-sensitive Ca^{2+} channels which control the orientation of the ciliary beat and it is responsible for the cell's excitability [1,2]. Many mutants displaying defects in excitability have been isolated (see Ref. 3) and the pattern of their ciliary membrane proteins and lipids is currently being analyzed. Secondly, it has been established that the agglutination of cells of opposite mating types at the first step of the mating reaction in *Paramecium* results from the interaction of 'substances' located on the ciliary membrane; purified ciliary membrane vesicles of one mating type can induce mating agglutination when added to cells of the opposite mating type [4]. Finally, the ciliary membrane contains the immobilization antigens, a set of high molecular weight (200 000—300 000) surface proteins [5—10] that have been studied for a long time because of the interesting genetic regulatory mechanisms governing their expression [5,8,11,12]. These surface antigens are called immobilization antigens (I-antigens) because antisera to these proteins immobilize the homologous *Paramecium*.

The pattern of proteins of the ciliary membrane of *Paramecium* has recently been established in detail by one- and two-dimensional electrophoresis [13]. However, no information is yet available as to the location and function(s) of the proteins thus identified. All of the three phenomena described above involve signals from the external environment that the cell utilizes as information. Proteins involved in transmembrane signalling are probably exposed on the outer surface of the membrane. Information on the topology of membrane proteins might aid in the identification of such proteins.

Wyroba [14] has previously studied the effects of proteases on the immobilization of cells with homologous antisera. She correlated the removal of the surface coat with accelerated immobilization.

We have studied the topology of the ciliary membrane proteins using protease treatment of whole cells as a vectorial probe of the cell surface and we sought to determine whether the removal of the surface coat of *Paramecium* had any effect on the behavior (excitability) of the cell. The effects of the protease treatment were analyzed by (i) protein patterns obtained using gel electrophoresis, (ii) electron microscopy of the treated cells or fractions, (iii) behavioral tests, and (iv) immobilization tests.

Controlled proteolysis of isolated ciliary fractions was also carried out. A set of membrane proteins resistant to protease digestion was identified.

Materials and Methods

Stocks. Paramecium tetraurelia, wild-type stock 51s (non-kappa bearing), was grown at 27 or 35°C in phosphate-buffered Cerophyl medium, bacterized with *Enterobacter aerogenes* as previously described [13]. Cells in the early stationary phase of growth were used for most of the experiments described.

Preparation of cilia and ciliary fractions. The procedures used to prepare cilia, membranes and other ciliary fractions have been described and discussed

in detail previously [13]. Cells were harvested by centrifugation at 200 × g_{max} for 1—2 min in an oil-testing HNS centrifuge and washed in 100 vol. of Dryl's solution (1 mM NaH_2PO_4, 1 mM Na_2HPO_4, 2 mM trisodium citrate, 1.5 mM $CaCl_2$, pH 6.8) at 4°C [15], two or three times. This procedure removed most of the bacteria and extruded trichocysts. The washed cells were resuspended in 50 vol. of a 1 : 1 mixture of Dryl's solution and buffer I (buffer I: 0.5 M sucrose, 20 mM Tris-HCl, 2 mM EDTA, 6 mM NaCl, pH 7.5) and kept at 4°C for 10 min. Cells were deciliated by the addition of $CaCl_2$ and KCl to a final concentration of 10 mM Ca^{2+} and 30 mM K^+. The cell bodies were pelleted by centrifugation at 850 × g_{max} for 2 min in the HNS centrifuge and cilia, which remained in the supernatant, were recovered by centrifuging at 28 000 × g_{max} for 20 min in a Beckman J-21C centrifuge.

The ciliary pellet was vortex mixed in a freshly prepared solution of 1 mM Tris, 0.1 mM EDTA, pH 8.3 (Tris-EDTA) for about 2 min to remove the ciliary membrane. This suspension was centrifuged at 48 000 × g_{max} for 30 min and the pellet resuspended in 10 mM Tris, pH 8.0, to a protein concentration of 5—10 mg/ml. 0.2—0.3 ml of this suspension was loaded on a sucrose step gradient consisting of 66% (w/w) (0.7 ml)/55% (1.7 ml)/45% (1.7 ml)/20% (0.7 ml) sucrose in 10 mM Tris, pH 8.0. Centrifugation at 45 000 rev./min in an SW 50.1 rotor for 1.5 h separated the mixture into three bands: ciliary membranes within the 45% sucrose, incompletely membrane-depleted cilia within the 55% sucrose and axonemes, trichocysts and bacteria at the 55/66% sucrose interface. The bands were collected and washed in 20—30 vol. of 10 mM Tris, pH 8.0, by centrifugation at 48 000 × g_{max} for 30 min.

Protease treatment. Whole cells: Cells harvested and washed in 28°C Dryl's solution were resuspended in Dryl's solution containing protease(s) and incubated at 28°C. Aliquots of the cell suspension were taken (i) for behavioral tests (see below) and (ii) for observation under the phase microscope to monitor the progress of proteolytic action and to ensure that cells did not blister or lyse. Experiments in which cell lysis exceeded 5% were not pursued further, since the results invariably reflected extensive non-specific proteolysis by endogenous proteases.

The proteolytic digestion was carried out for varying intervals of time (see Results) but in every case, the digestion was terminated by centrifuging the cells out of protease-containing solution (200 × g_{max} for 2 min in the HNS centrifuge) followed by washing in 100 vol. of 28°C Dryl's solution. In some experiments the digestion was terminated by the addition of 1/100 vol. of 0.2 M phenylmethylsulfonyl fluoride (PMSF) in absolute ethanol to the protease-containing suspension of cells. In all cases, the washed, protease-free cells were resuspended in a cold (4°C) 1 : 1 mixture of Dryl's solution and buffer I containing 2 mM PMSF and deciliated. The rest of the work-up procedure was as described above.

Control samples were treated in an identical fashion except for the omission of the protease.

Isolated ciliary fractions. Ciliary membranes, incompletely membrane-depleted cilia and axonemes were prepared using a sucrose step gradient as described above and washed once with 10 mM Tris, pH 8.0. The pellets were resuspended in 5 mM Tris, pH 7.5, at a protein concentration of 2—4 mg/ml.

The digestion was initiated by the addition of protease solution to a final concentration of 100 μg/ml. When proteolysis was carried out in the presence of detergent, the sample was resuspended in buffer containing 0.1% (w/v) Triton X-100. The total volume of the reaction was 50 μl. Samples were incubated at room temperature (24—26°C) for 0 to 60 min and the digestion was terminated by the addition of 4 mM PMSF/2% C_2H_5OH, and an equal volume (50 μl) of a two-fold concentrate of SDS-sample buffer (4% sodium dodecyl sulfate (SDS), 20% glycerol, 10% 2-mercaptoethanol, 125 mM Tris, 0.002% bromophenol blue, pH 6.8) kept at 100°C. The samples were immediately treated at 100°C for 5 min. Trypsin-treated samples were also terminated with 100 μg/ml of soybean trypsin inhibitor. Controls consisted of samples treated identically with the exception of protease addition and were incubated at room temperature for 0 and 60 min.

Gel electrophoresis. Samples were dissolved in 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 62.5 mM Tris-HCl, 0.001% bromophenol blue, pH 6.8 [16], and heated at 100°C for 5 min. Electrophoresis was carried out in SDS-polyacrylamide slab gels containing a linear 7.5—15% acrylamide gradient and a 3% stacking gel. The dimensions of the gels were 16 × 10 × 0.15 cm. Gels were electrophoresed at 20 mA constant current and dansylated cytochrome *c* was used to monitor the progress of the electrophoresis [17]. The gels were stained in 0.1% Coomassie brilliant blue R-250 in $C_2H_5OH/CH_3CO_2H/H_2O$ (9 : 2 : 9) and destained in 7.5% CH_3CO_2H.

Protein was determined by using the method of Lowry et al. [18] and phosphate according to the method of Chen et al. [19]. The proteolytic activity of the enzymes used was determined using azocasein as substrate by measuring the change in absorbance of acid-soluble material at 440 nm [20].

Electron microscopy. The procedures used were similar to those described previously [13]. The washed samples (whole cells, cilia or ciliary membranes) were fixed for 1 h in 2% glutaraldehyde in 0.08 M sym.-collidine buffer (pH 7.2) at room temperature. After several buffer rinses, the material was post-fixed in 1% OsO_4 for 45 min. It was subsequently washed and stained en bloc with 0.5% aqueous uranyl acetate for 2 h and was dehydrated in an ethanol series followed by propylene oxide and embedment in Spurr's resin [21]. Silver interference sections were taken on a Reichert Om U3 ultramicrotome, stained with lead citrate, and photographed with a Philips 300 electron microscope.

Behavioral tests. Aliquots of the cell suspension in protease-containing solution were transferred to a depression slide and observed under the dissecting microscope. After enzyme treatment for 30 min, the cells were washed two to three times with Dryl's solution and tested in Na^+ or Ba^{2+} solutions. Ba^{2+} solution: 8 mM $BaCl_2$ 1 mM $CaCl_2$, 1 mM Tris, pH 7.2; Na^+ solution: 20 mM NaCl, 0.3 mM $CaCl_2$, 1 mM Tris, pH 7.2 (see Ref. 3 for details).

Immobilization tests. Immobilization tests were carried out as described in Ref. 9. Aliquots containing about 50 protease-treated or control cells were washed in Dryl's solution and transferred into a series of dilutions of an immobilizing serum kindly provided by Yves Brygoo. This rabbit serum was obtained after immunization with isolated cilia from wild-type cells expressing I-antigen A. Cells were thereafter observed every 5 min.

Chemicals. Pronase used was protease from *Streptomyces griseus*, Sigma type

VI, lot No. 58C-0039, a preparation containing several distinct proteolytic activities [22]; chymotrypsin from bovine pancreas, Sigma type 1-S, lot No. 45C-8280-1; trypsin from bovine pancreas, Sigma type XII, lot No. 122C-6860; trypsin inhibitor from soybean, type 1-S from Sigma, lot No. 111C-8080; azocasein and PMSF were from Sigma and Triton X-100 from Research Products International.

Results

Protease treatment of whole cells

Concentrated cells were treated with either 10 μg/ml trypsin, 10 μg/ml chymotrypsin or 40 μg/ml pronase for 30 min. After washing, the cells were either fixed immediately for electron microscopy or deciliated. A portion of the iso-

TABLE I

SUMMARY OF PROTEASE EXPERIMENTS ON WHOLE CELLS

In all the experiments the cells were treated for 30 ± 5 min. The symbols —, +, ++, +++ are rough estimates of the amount of fuzzy material present over the ciliary membranes as observed at the electron microscope (E.M.) or of the staining intensity of the immobilization antigen band on the corresponding SDS-polyacrylamide gel patterns. —, indicates that the fuzzy material or the band is totally absent; +++ that it is of maximum intensity (control). The absence of an entry corresponds to experiments that have not been performed or to cases in which proteolytic degradation of the samples during preparation for electrophoresis precluded their analyses.

Experiment No.	Treatment	Sample analyzed	Observation on:	
			Cell coat (E.M.)	I-antigen band (Gels)
1	control	whole cells	+++	
	chymotrypsin (10 μg/ml)		++/+++	
	pronase (40 μg/ml)		—	
2	control	whole cells	+++	
	pronase (40 μg/ml)		—	
3	control	cilia	++/+++	
	pronase (40 μg/ml)		—/+	
	pronase (80 μg/ml)		—	
4	control	cilia	++/+++	+++
	trypsin (10 μg/ml)		++	+/++
	trypsin (10 μg/ml) + soybean trypsin inhibitor (10 μg/ml)		++	++
	pronase (80 μg/ml)		—	—
5	control	cilia	++/+++	+++
	pronase (40 μg/ml) } pronase (80 μg/ml) }		+/— or —	—
6	control	cilia	+++	+++
	pronase (20 μg/ml)		++	++
	pronase (40 μg/ml)		+	+
7	control	cilia	++/+++	
		vesicles	++	
	pronase (40 μg/ml)	cilia	+/—	
		vesicles	+/—	
8	control	vesicles	++	+++
	pronase (80 μg/ml)		—/+	++/+

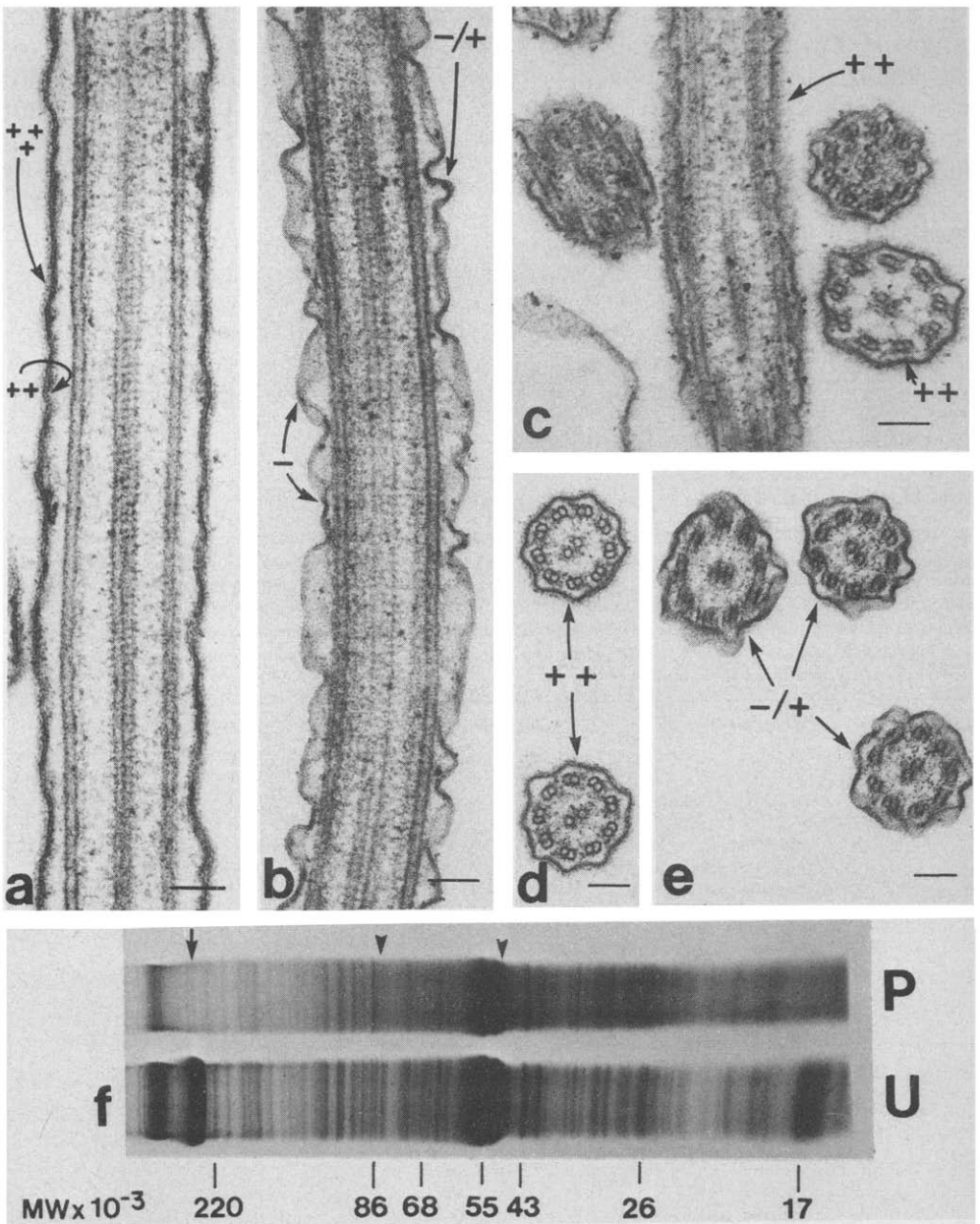

Fig. 1. Effects of protease treatment on whole cells. *Paramecia* were protease-treated, washed and the cilia isolated as described in the text and prepared for electron microscopy or SDS-polyacrylamide gel electrophoresis. Thin-section electron micrographs of cilia from: (a) untreated cells; (b) pronase-treated cells (40 μg/ml, 30 min at 27°C; (c) trypsin-treated cells (10 μg/ml, 30 min at 27°C); (d) untreated cells, (e) pronase-treated cells (40 μg/ml, 30 min at 27°C); bar, 0.1 μm. (f) SDS-polyacrylamide gel profile of cilia from: (U) untreated, and, (P) pronase-treated cells (40 μg/ml, 30 min at 27°C). Note the decrease (+/—) or absence (—) of the fuzzy material over the membranes in the samples from pronase-treated cells and the corresponding effect on a major protein band in the gels (arrow). Other non-reproducible differences in the protein patterns are indicated by arrowheads. The bars at the bottom of the gels denote the positions of the molecular weight standards used.

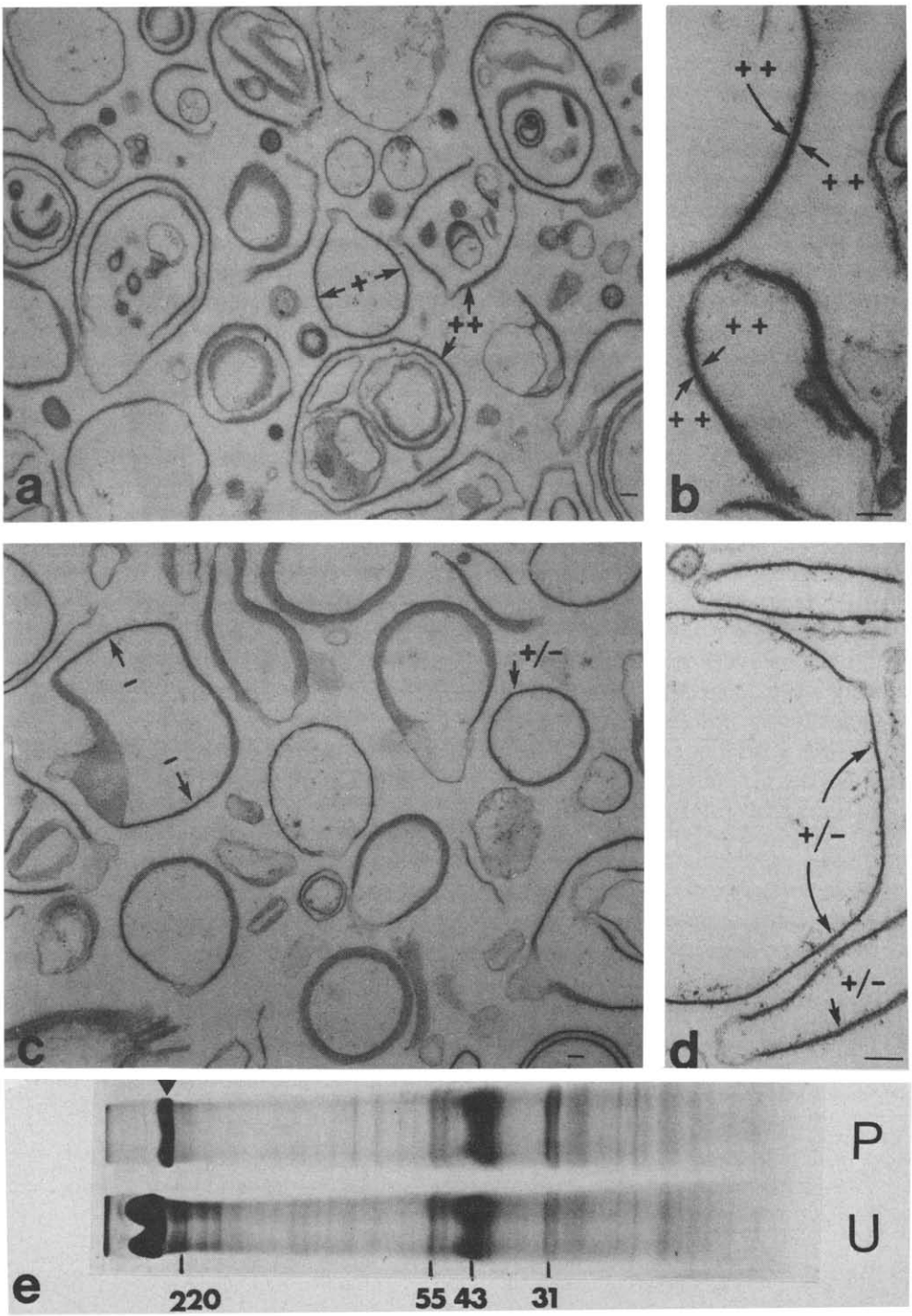

Fig. 2. Thin-section electron micrographs of ciliary membranes prepared from: (a, b) untreated cells; (c, d) pronase-treated cells (40 μg/ml, 30 min at 27°C). Bar, 0.1 μm. (e) SDS-polyacrylamide gel profile of ciliary membranes from: (U) untreated cells, and; (P) pronase-treated cells (40 μg/ml, 30 min at 27°C). Note the decrease (+/—) in the 'fuzzy cell coat' on both surfaces of the membrane in the pronase-treated samples.

lated ciliary fraction was fixed for electron microscopy, and a second portion processed for gel electrophoresis; in some experiments, a third portion was processed for the isolation of ciliary membranes. The ciliary membranes were fixed for electron microscopy and/or processed for gel electrophoresis. Representative results are summarized in Table I and illustrated in Figs. 1 and 2. In untreated cells, the outer membrane leaflet was covered by an electron-dense fuzzy material, the 'cell coat', which was about 200—300 Å thick and covered the entire cell, including the cilia.

At the concentrations used, trypsin and chymotrypsin did not have any significant effect on the appearance of the cell coat as judged by observation under the electron microscope (Fig. 1c). In contrast, pronase treatment caused striking effects: the fuzzy material was almost totally (or totally, depending on the pronase concentration) eliminated from the membrane of pronase-treated cells. This was true whether whole cells, isolated cilia or ciliary membrane vesicles were analyzed after treatment of whole cells. Furthermore, in isolated cilia, the ciliary membrane enclosing the axoneme appeared wrinkled (Fig. 1b) in contrast to the relatively smooth membrane of untreated controls. The amount of fuzz on the internal leaflet of the membrane was also reduced in the pronase-treated samples. This observation is best illustrated in the ciliary membrane vesicles; control vesicles have fuzzy material both on the external and internal faces (Fig. 2b) while vesicles from pronase-treated cells have lost the fuzzy material on both faces of the bilayer (Fig. 2d).

These observations on the removal of the fuzzy material are reproducible, although the extent of material removed varied in independent experiments. A quantitative comparison of untreated and treated cells based on measurements of stretches of membranes covered by fuzzy material in randomly selected samples indicates that the differences are highly significant (Table II).

The electron microscopic analysis was paralleled by a comparison of the pro-

TABLE II

TABLE SHOWING QUANTITATION OF DATA FROM ELECTRON MICROSCOPIC ANALYSIS DEMONSTRATING A SIGNIFICANT DECREASE IN THE AMOUNT OF FUZZY MATERIAL ON THE SURFACE OF PRONASE-TREATED CELLS

Data presented are from four independent experiments. In each case, the pronase concentration was 40 μg/ml. For each sample, six random fields of view were photographed. Using a compensating planimeter, the total length of membrane represented and the length of membrane in section covered with fuzz were measured. Only regions of the photographs wherein the 'railroad track configuration' of the membrane leaflet could be clearly discerned were included in the measurements.

Experiment No.	Cilia sample analyzed	Length of membrane bearing fuzz	Total length of membrane	% membrane bearing fuzzy coat
1	untreated	51.2	113.3	45
	pronase-treated	15.3	215.9	7
2	untreated	84.6	113.4	75
	pronase-treated	0.0	199.5	0
3	untreated	128.6	139.5	92
	pronase-treated	33.7	101.0	33
4	untreated	191.6	266.7	78
	pronase-treated	73.0	279.8	26

tein profiles obtained using SDS-polyacrylamide gel electrophoresis of the untreated and pronase-treated samples. No difference from the controls was detected in the fractions derived from trypsin-treated cells. In the case of pronase, the electron microscopic observations were matched by a marked decrease (or total absence) of a major protein band from the electrophoretic pattern of cilia isolated from treated cells (Fig. 1f). We also found a good correlation between the amount of fuzzy material remaining after pronase treatment and the intensity of this high molecular weight band on the gels (Table I). This band, M_r 250 000, is known to correspond to the immobilization antigen [6,7,9,10,13] and represents the major protein of the ciliary membrane [7,13]. While there were other minor differences observed between the pattern (e.g., see Fig. 1f) of pronase-treated cilia and untreated controls, these were not reproducibly obtained. In particular, a set of proteins of M_r 42 000—44 000 (which are also major components of the membrane) were unaffected by the treatment as was also a protein of M_r 31 000. The latter protein is of interest, since it exhibits a growth stage variation which parallels the sexual reactivity of the cell [13].

It was often observed that ciliary membranes prepared from pronase-treated cells aggregated on the sucrose gradient used to separate them; the protein-to-lipid ratio of these stripped membranes was found to be lower than that of untreated ciliary membranes (1.2 ± 0.4 μg protein/nmol P_i and 2.5 ± 0.5 μg protein/nmol P_i, respectively).

We encountered serious difficulties in obtaining the electrophoretic patterns of cilia and ciliary membranes from protease-treated cells; the preparations often displayed generalized proteolytic degradation, complicating the identification of the specific effects of the protease. There are two likely sources of these difficulties which have also been encountered by others working on different systems [23,24]:

(1) Although the cells were washed after protease treatment, some residual enzyme probably remains bound to the cell surface and is carried over into the SDS-solubilized sample. It is known that proteolytic enzymes can retain some activity in SDS while most proteins are unfolded and denatured and hence very susceptible to degradation. The inclusion of PMSF in all steps subsequent to washing the cells and rapid dissolution of the samples in boiling SDS, as suggested by Bender et al. [23], reduces such proteolytic artifacts.

(2) When the pronase treatment is drastic (greater than 40 μg/ml), or extended (greater than 30—45 min), the cells become fragile, and during the deciliation procedure a fraction of the cells lyse, releasing potent intracellular proteases contained in the digestive vacuoles of the cell. These difficulties prevented us from obtaining reliable electrophoretic patterns of ciliary membranes from cells treated with high concentrations of pronase or for extended periods of time. Only membrane patterns from 'moderately' treated cells could therefore be obtained and those only showed a decrease in the immobilization antigen (Fig. 2e).

Behavioral observations. Cells in protease solution (40 μg/ml) showed normal swimming behavior with occasional continuous ciliary reversals. After 30 min in pronase solution the cells slowed down considerably. When tested in Na^+ or Ba^{2+} solutions, the pronase-treated cells behaved in a manner similar to that

of untreated control cells, both sets showing repeated avoiding reactions. The frequency of the avoiding reaction and the duration of backward swimming was not significantly different from those of controls.

Immobilization tests. Cells grown at 27°C were pronase-treated (40 μg/ml) at room temperature for intervals of time ranging from 15 min to 3 h and then tested in a range of dilutions (1/10, 1/50, 1/100, 1/200, 1/500 and 1/1000) of a high-titer antiserum (completely immobilizes untreated cells in 20 min at 1/200 dilution). No marked difference was found between control and treated cells in the speed and extent of immobilization and no clear case of 'escape' from immobilization was obtained after pronase treatment.

Cells grown at 35°C as well as a number of mutant strains grown at 27°C express a different antigenic type (Refs. 5 and 8; and Adoutte, A., Ling, K.-Y. and Kung, C., unpublished observations) and are totally insensitive to the serum used above; they continue to swim for hours at the 1/200 dilution of this serum. These cells when treated with pronase as described above were found to be immobilized by the serum as early as 15 min after pronase treatment. It therefore appears that pronase unmasks some antigenic determinants in the 35°C-grown cells and in the mutant strains which interact with serum antibodies leading to immobilization. The same phenomenon probably occurs in the 27°C-grown cells, rendering escape from immobilization a priori impossible. These results agree with the findings of Capdeville [25] in *Paramecium primaurelia*. Preliminary results obtained show that the antigenic determinants being unmasked might correspond to the set of proteins with M_r 42 000—44 000 (Ramanathan, R., Eisenbach, L. and Nelson, D.L., unpublished observation).

TABLE III

SUMMARY OF PROTEASE EXPERIMENTS ON ISOLATED CILIARY MEMBRANES

Controlled proteolysis of membranes was carried out in 100 μg/ml protease for 0—60 min at room temperature. ++, +, —, denote the susceptibilities (to proteolytic digestion) of 10 proteins identified in the SDS-polyacrylamide gels; ++ indicates complete susceptibility while — indicates that it is resistant to proteolysis. Not all proteins found in the ciliary membrane fraction have been tabulated; only proteins which could be reproducibly identified have been included.

Protein (M_r) ($\times 10^{-3}$)	Effect of		
	Trypsin	Chymotrypsin	Pronase
200—250 I-antigen	+	+	++
100	++	+	
87	+/—	—	++
64	++	+/—	
55 α-tubulin	++	++	++
53 β-tubulin	+/—	+/—	++
42—44 *	—	—	—/+
40	++		
31	++	—	++
21.5	—	—	—

* This constitutes a set of at least four closely migrating proteins.

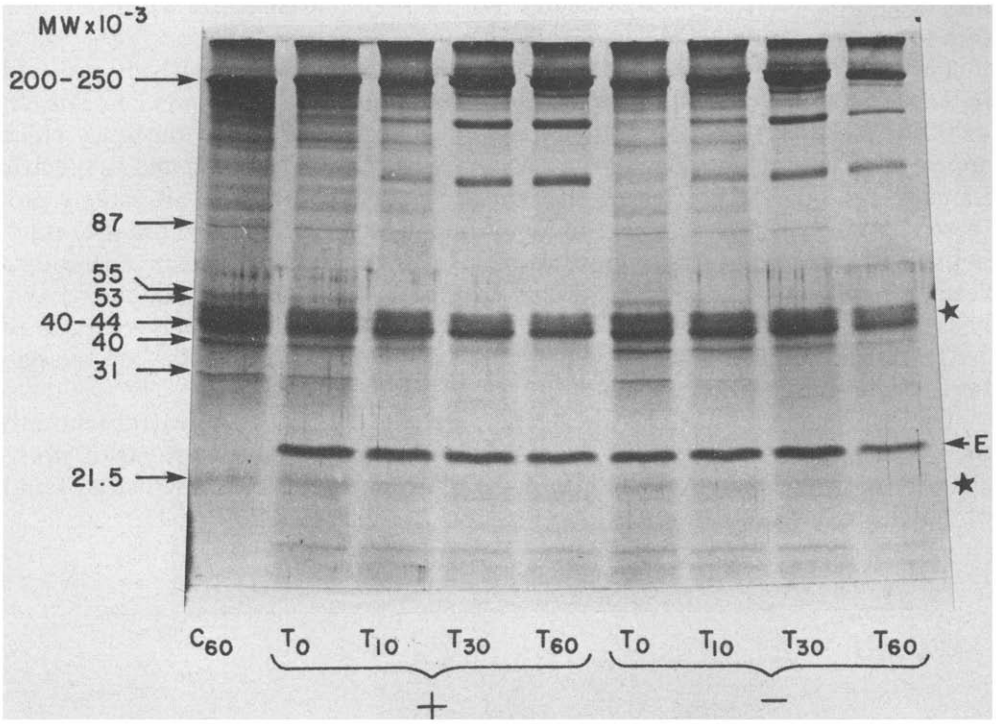

Fig. 3. SDS-polyacrylamide gel showing the results of trypsin treatment of isolated ciliary membranes in the presence (+) and absence (—) of 0.1% (w/v) Triton X-100. C_{60} refers to a control membrane sample (no enzyme added) incubated at room temperature for 60 min. T_0, T_{10}, T_{30} and T_{60} refer to membrane samples incubated with 100 μg/ml trypsin for 0, 10, 30 and 60 min, respectively. The molecular weights of several membrane proteins (Table III) are indicated on the left of the figure while the asterisks refer to membrane proteins that are relatively resistant to digestion. E denotes the position of added trypsin.

Protease treatment of ciliary membranes

Ciliary membranes contain up to 45 proteins as detected by Coomassie staining on one-dimensional SDS-polyacrylamide gels [13]. We chose 10 reproducibly identifiable proteins and determined their susceptibilities to the proteases used. Isolated ciliary membranes were treated with 100 μg/ml trypsin, chymotrypsin or pronase and the reaction terminated after incubating for 0, 10, 30 and 60 min at room temperature. The protein patterns were determined using SDS-polyacrylamide gel electrophoresis. These results are summarized in Table III and illustrated in Figs. 3—5. Control membrane samples (no protease) showed little internal proteolysis after incubation for 60 min at room temperature (Figs. 3 and 4). The effects of trypsin, chymotrypsin or pronase were marked and distinct for each enzyme, e.g., the I-antigen was totally removed by pronase but only partially removed by trypsin and chymotrypsin, while the M_r 31 000 protein was trypsin- and pronase-sensitive but chymotrypsin-resistant. The time course of proteolysis also showed that the membrane proteins exhibit different degrees of susceptibility to a given protease (Figs. 3 and 4); for exam-

ple, the M_r 31 000 protein was clearly more susceptible than the M_r 87 000 protein to trypsin degradation.

Two sets of proteins, M_r 42 000—44 000 and 21 500, were relatively resistant to proteolysis by trypsin, chymotrypsin and pronase (Figs. 3—5). The M_r 42 000—44 000 region constitutes a set of at least four acidic proteins which appear as a cluster both in one-dimensional SDS-polyacrylamide and isoelectric focusing gels [13]. The finding that these two sets of proteins are largely protease-resistant suggested to us that they might be intrinsic membrane components. To test whether sequestration of these proteins within membrane vesicles might make them inaccessible to proteases, we carried out the proteolysis in the presence of 0.1% (w/v) Triton X-100 (protein: detergent ratio of 2—3). As shown in Fig. 3, the inclusion of detergent had no effect on the pattern of trypsin digestion obtained.

The experiments illustrated here compare the effects of equivalent amounts of protease (as micrograms of protein); we also compared the effects of equivalent activities of enzymes (determined by using azocasein as a substrate) and obtained similar results.

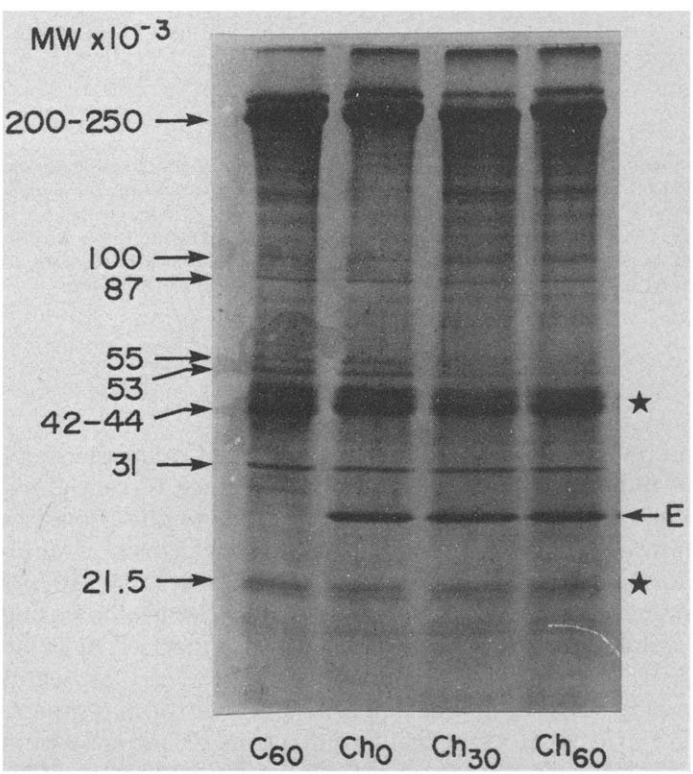

Fig. 4. SDS-polycrylamide gel showing the effects of chymotrypsin on isolated ciliary membranes. C_{60} is a control sample incubated in the absence of enzyme for 60 min and Ch_0, Ch_{30} and Ch_{60} refer to membrane samples incubated with 100 μg/ml chymotrypsin for 0, 30 and 60 min. As in Fig. 3, asterisks indicate membrane proteins relatively resistant to proteolysis and E indicates the position of added chymotrypsin.

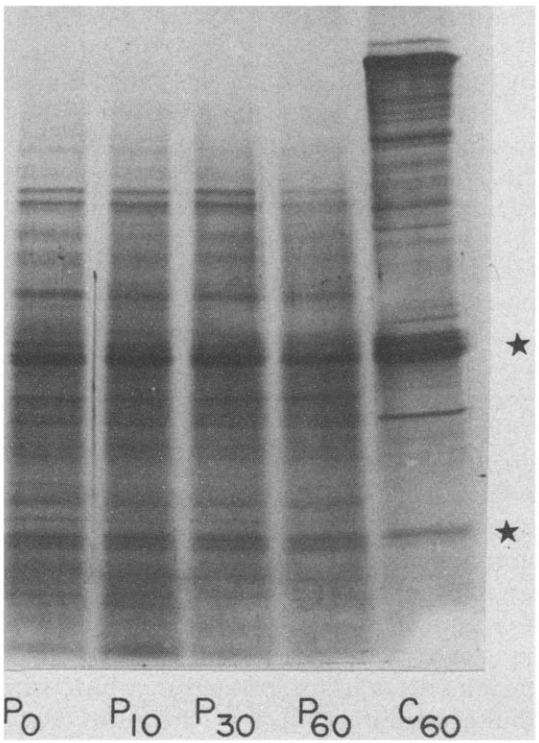

Fig. 5. SDS-polyacrylamide gel showing the results of pronase digestion of isolated ciliary membranes, P_0, P_{10}, P_{30} and P_{60} refer to membrane samples incubated with 100 μg/ml pronase for 0, 10, 30 and 60 min. C_{60} denotes the pattern of a control membrane sample incubated at room temperature for 60 min. Note as in Figs. 3 and 4, that the M_r 42 000—44 000 and 21 500 proteins (★) are relatively resistant to protease digestion.

Discussion

The fuzzy coat of Paramecium contains immobilization antigen. Previous studies by two sets of workers have indicated the external location of the immobilization antigens and suggested the possible identity of these proteins with the cell coat. Beale and coworkers [26—28] have shown that fluorescent and ferritin-labeled antibodies to the I-antigen elicit the immobilization reaction and react specifically with the entire outer surface of *Paramecium*. Wyroba and Przelecka [29] have also shown that the surface coat can be stained with ruthenium red, that the intensity of stain is decreased after treatment with trypsin, pronase or neuraminidase, and that these treatments accelerate the immobilization reaction when the cells are challenged with homologous antisera [14]. The present study definitely establishes the identity of the surface coat with the immobilization antigen by showing that a decrease or removal of the surface coat (seen as fuzzy material under the electron microscope) correlates with a decrease or absence on SDS-polyacrylamide gels of the major membrane protein, a high molecular weight protein band, known from previous work [7] to correspond to the immobilization antigen. Under the conditions

used, only pronase was effective in removing the cell coat; trypsin and chymotrypsin were ineffective.

Many other protozoans are similarly covered by a cell coat. In several cases it appears to be made up essentially (or exclusively) of a major glycoprotein species. Such is the situation in Trypanosomes [30—32], *Gregarina* [33] and possibly in *Chlamydomonas* [34,35]. The analogy with the Trypanosomes is striking in that both *Paramecium* and Trypanosomes undergo 'antigenic variations' which correspond to the presence, at the cell surface, of a different type of major coat protein [5,8,30,36]. There is also similarity between the I-antigen of *Paramecium* and the fibronectins (or LETS protein) found at the surface of adherent vertebrate cells [37—39]. Both are high molecular weight (subunit molecular weight 200 000—250 000) polymorphic, surface-associated glycoproteins. Fibronectins are extensively cross-bridged by disulfide bonds [37] and the same is probably true of the I-antigen which contains up to 10% cysteine [5,10] and is particularly prone to form aggregates. Fibronectin is a transformation-sensitive protein, possibly involved in cell-cell contact [37,38]; while no role in cell-cell interactions can be attributed to the I-antigens, their expression at the cell surface is regulated by environmental conditions.

Removal of I-antigen does not alter swimming behavior. The function of the cell coat in *Paramecium* is at present unknown. Since the cell can be submitted to rapid changes in its environmental conditions, it is tempting to speculate that it might play a 'protective' role in *Paramecium;* for instance, by 'buffering' the immediate ionic environment of the membrane. It was, therefore, disappointing that no clear-cut, reproducible alteration in cell behavior was detected after the removal of the surface coat protein. It is possible that the lack of any behavioral abnormality is related to the adaptive capabilities of the membrane of *Paramecium*, a recently proposed idea to account for a number of peculiarities in the electrophysiological properties of the membrane [40]. We are presently investigating the possibility that the electrophysiological properties of the cell membrane might have been altered by removal of I-antigen.

Structures on the inside face of the membrane are affected by external proteolysis. Our observations that the internal leaflet of the ciliary membrane also contained fuzzy material which was removed by external application of pronase (most clearly seen in membrane vesicles, Fig. 2) is intriguing. The possibility that the enzyme was penetrating the lipid bilayer seemed unlikely, since (i) tubulins, the major axonemal proteins, were not degraded, and (ii) the I-antigen degradation was specific, reflecting vectorial proteolysis. It is therefore possible that the pronase-mediated removal of externally disposed I-antigen also releases some protein(s) that constitutes the fuzz on the internal leaflet of the membrane. This could mean that the I-antigen is a transmembrane protein and/or that this large external protein serves as an anchor for some internal protein(s). If pronase treatment caused the transmembrane links (anchoring points) to be destroyed, both external and internal fuzz would be removed. These kinds of complex interactions between cell-surface proteins and cytoplasmic components have been seen in other systems, e.g., fibronectin-actin interactions in cultured mammalian cells [38,39] and major sialoglycoprotein-spectrin interactions in erythrocyte membranes [24]. In *Paramecium* the nature and identity of the material constituting the internal fuzz are not known; the only protein

reproducibly absent in cilia prepared from pronase-treated cells was the I-antigen (Fig. 1). The altered morphology of the ciliary membrane (wrinkling seen in Fig. 1) which results from pronase treatment may also be related to the loss of such anchoring points.

Use of limited proteolysis to explore membrane topology. This study clearly shows that the I-antigen is located on the external face of the ciliary membrane. It was, however, disappointing that no other externally disposed protein could be identified using proteolysis of intact cells. We believe that this inability is due to two reasons. (i) The I-antigen constitutes approx. 75% of the ciliary membrane protein and covers the entire surface of the cell, possibly hindering the accessibility of added reagents to other external proteins. (ii) We were unable to use drastic proteolysis conditions (high protease concentrations or extended treatment) because the cells lyse under these conditions.

We are currently pursuing the study of the topology of the membrane components using other approaches and have found that the I-antigen, M_r 42 000—44 000 and 31 000 proteins are all glycosylated proteins and can be vectorially iodinated using lactoperoxidase-catalyzed radioactive iodination (Ramanathan, R. and Nelson, D.L., unpublished observation).

Limited proteolysis to study topology of cilia, incompletely membrane-depleted cilia and vesicles. As discussed above, the 'vectorial' information obtained in this study concerns only the I-antigen. To avoid the complications that result from the release of digestive proteases during cell lysis (see Results), we attempted to use isolated cilia and incompletely membrane-depleted cilia as systems for controlled proteolysis. It is not known whether these organelles are closed structures, a prerequisite for their usefulness in topological studies. Incompletely membrane-depleted cilia consist of axonemes enclosed within a membrane and appear as discrete, structurally stable units under the conditions used [13]. The membrane associated with the incompletely membrane-depleted cilia seems to be similar to the ciliary membrane [13]. Treatment of a suspension of isolated cilia (in Dryl's-buffer I solution) with 10—70 μg/ml trypsin or pronase caused degradation of many ciliary proteins including tubulin and other axonemal proteins. Similar results were obtained when a suspension of incompletely membrane-depleted cilia was treated with 10—100 μg/ml trypsin or pronase (data not shown). This suggests that at least a fraction of the isolated cilia or incompletely membrane-depleted cilia are not closed structures, obviating their potential usefulness in studying the disposition of ciliary membrane proteins. Independent work done in our laboratories (Schobert, C. and Kung, C., unpublished observations) also indicates that cilia isolated by the current method are osmotically inactive.

The sidedness of our membrane vesicle preparations is not known; electron micrographs show that the vesicles are of heterogeneous size and some multilamellar vesicles can be seen [13]. An amorphous fuzzy layer was seen on both surfaces of the membranes. Our observation that the pattern of protease digestion was not altered by the inclusion of detergent (Fig. 3) suggests that the membrane vesicles are heterogeneous with respect to sidedness.

Our ciliary membrane preparations invariably contained small amounts of tubulin (Fig. 4) and we have included the tubulins in our analysis of the susceptibilities of ciliary membrane proteins to proteases (Table III). It is not

known whether this small amount of tubulin represents an axonemal component. One other report [41] shows a small amount of tubulin in specific association with the flagellar membrane of *Chlamydomonas*. In this light, it is curious to note, as Table III shows, that α- and β-tubulin show different susceptibilities to proteases and that one of the minor modifications derived from pronase treatment of intact cells is the splitting of β-tubulin (Fig. 1f, arrowhead). Data are also presented to show that two sets of proteins, M_r 42 000—44 000 and 21 500, are largely protease-resistant; these proteins may represent intrinsic membrane proteins. We are presently continuing to investigate this possibility. Whether the M_r 42 000—44 000 set of proteins correspond to dimeric forms of the M_r 21 500 proteins is not known at this time; such SDS-resistant polypeptide associations have been reported for PAS 1 and PAS 2 (PAS, periodic acid-Schiff band) in the erythrocyte membrane [42].

In conclusion, in this paper we have clearly established the identity of the I-antigen with the surface coat. The absence of any detectable behavioral abnormality in treated cells leaves open the possible role, if any, played by the I-antigen in the excitability of the cell. We have also identified a set of protease-resistant proteins possibly representing intrinsic membrane proteins. Other studies of membrane protein topology and membrane subfractions are currently under way.

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