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Isolation of the membranes from secretory organelles (trichocysts) of *Paramecium tetraurelia*

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We present for the first time a method for isolation of the membranes of extrusive organelles (trichocysts) from sterile culture of different strains of *Paramecium tetraurelia*. First, trichocysts are isolated according to a new method (Glas-Albrecht, R. and Plattner, H. (1990) Eur. J. Cell Biol. 53, 164–172) with high purity and yield. Then the organelles are subjected to osmotic swelling. Since trichocysts then easily 'decondense' and entangle membranes, these cannot be isolated directly by centrifugation, but only by passage through a filter and subsequent centrifugation. Purity of membrane fractions is analysed by electron microscopy and SDS-PAGE, combined with silver staining or, after biotinylation, by avidin-peroxidase labelling. Molecular masses resolved in our gels are in a range from ≤ 15 to ≥ 105 kDa. Main bands obtained with *nd9-28°C* trichocyst membranes (most bands also being common to wild type trichocysts) are of about 16.5, 19–21, 27–29, 33–34, 44–45 (strong), 47–48 (strong), 57, 61, 65 (strong), 68–71, 75, 81, 94–95 (strong), 104 and ≥ 110 kDa, from a total of approx. 23 bands resolved. There is no remarkable occurrence of dominant protein bands from trichocyst contents ('trichynins'), though these might represent up to 10^3-times more of the total trichocyst proteins. The ratio of phospholipid/protein is approx. 0.2 mg/mg. The methodology developed might also be valuable for the isolation of extrusome membranes from some other protozoan species.

Introduction

Although the *Paramecium* cell, a ciliated protozoon, is a well established system for the study of exocytosis [1], its secretory organelles (trichocysts) surprisingly could be isolated in intact form only quite recently [2,3]. This enabled us to also develop a method for the isolation of trichocyst membranes. The problems we faced were the firm attachment of most trichocysts at the cell membrane [1,4–6] and, when the membrane was injured, the expansion ('decondensation') of trichocyst contents [7]; these then formed a network from which membranes cannot be isolated by the usual procedures.

These methodical problems are in striking contrast to other secretory systems for which it has already long been possible to isolate secretory vesicles, e.g. from exocrine [8,9] and endocrine glands [10,11], mast cells [12,13] and egg cells [14–16] etc. In most cases, organelle membranes could also be isolated [8,17–23]; (for reviews, see Refs. 24 and 25). For a more detailed account, see Discussion.

The isolation of secretory organelles or of their membranes is a prerequisite for an analysis of their chemical constituents and their possible relevance for stimulus-secretion coupling. Our work might also have some bearing on work with some other protozoa, including some parasitic sporozoa, which have similar extrusive organelles attached at the cell membrane (see Ref. 1).

Abbreviations: BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; kDa, kilodalton (apparent molecular mass); ME, 2-mercaptoethanol; *nd9-28°C*, non-discharge mutant 9 (cultivated at 28°C); PAGE, polyacrylamide gel electrophoresis; Pipes, piperazine-*N*,*N*'-bis(2-ethanesulfonic acid); PL, phospholipid; POX, (horseradish) peroxidase; SDS, sodium dodecylsulfate; Tris, tris(hydroxymethyl)aminomethane; wt, wild type.

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Materials and Methods

Cell cultures

The *nd9–28°C* mutant strain [26] (or the wildtype (*7S*) cells, for pilot experiments) of *Paramecium tetraurelia* were cultivated in a sterile medium according to Kaneshiro et al. [27] at 25°C or 28°C, respectively, and harvested at early stationary phase.

Isolation of trichocyst membranes

A survey of the method applied is presented in Fig. 1. Trichocysts were isolated essentially according to Glas-Albrecht and Plattner [2]. Briefly cells were washed twice in Pipes (piperazine-*N*,*N'*-bis[2-ethanesulfonic acid])-NaOH, 5 mM (pH 7.0), with 1 mM each of $CaCl_2$ and KCl added (= buffer I). Trichocysts were detached from the cell surface by exposure to 15 mM EDTA (ethylenediaminetetraacetic acid) in Tris (tris[hydroxymethyl]aminomethane)-HCl buffer, 50 mM (pH 7.0). Disintegration of cells was completed by homogenisation (approx. 20 strokes) at 0°C in a glass potter with a loosely fitting teflon pestle.

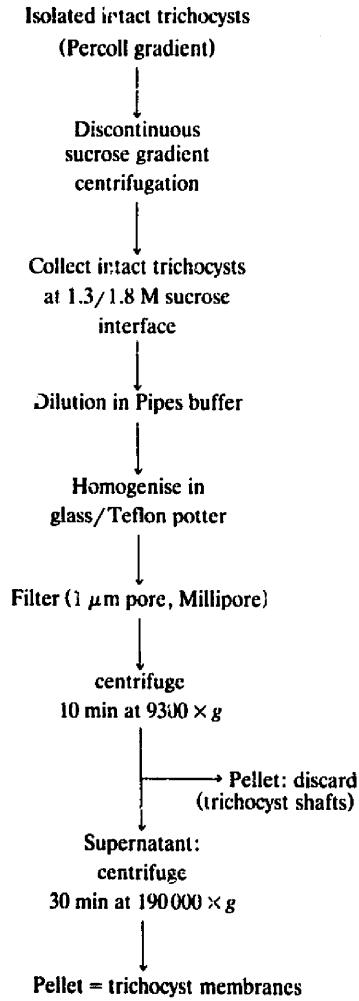

Fig. 1. Flow chart for the isolation of trichocyst membranes.

Trichocysts were collected from a self-generating 80% Percoll (Sigma, Steinheim, Germany) gradient (45 min at 50 000 × *g*, 4°C, Sorvall ultracentrifuge type OTB Combi equipped with a Ti 865 fixed angle rotor), suspended in Pipes-KOH buffer, 10 mM (pH 7.0), with 3 mM NaCl and 10 mM $MgCl_2$ added (= buffer II), layered on a discontinuous sucrose gradient (2.1 M, 1 ml; 1.8 M, 1.5 ml; 1.3 M, 1.5 ml) and centrifuged (45 min at 21 000 × *g*), again in the same ultracentrifuge, but with a swing-out rotor type AH 650. Trichocysts were collected from the 1.8/1.3 M sucrose interface.

Membranes were then disrupted by dilution (1:5) with buffer II, followed by homogenisation (under light microscope control) with the same type of potter (approx. 20 strokes) as indicated above. Membranes were separated from trichocyst contents ('shafts') by filtration through a Millipore filter (1 μm pore size) attached to a syringe. The filtrate was then centrifuged (10 min at 9300 × *g*, Sorvall centrifuge, rotor AH 650). The pellet (containing organelles, mainly trichocyst shafts) was discarded and the supernatant centrifuged again (30 min at 190 000 × *g*; same rotor). The resulting pellet contained trichocyst membranes which were analysed as follows.

Protein determination

Proteins were determined according to the Method of Bradford [28].

Protein biotinylation

Whole trichocysts were used to biotinylate membrane proteins according to Busch et al. [29]. Trichocysts were washed, incubated with 0.1 mg/ml (in 0.6% v/v dimethylsulfoxide) *N*-hydroxysulfo-succinimide ester of biotin (NHS-biotin, from Boehringer-Mannheim, Germany) using a w/w ratio of biotin to protein of 1:20, washed four times (the second time with 0.3% bovine serum albumin (BSA, from Paesel, Frankfurt/M, Germany) added to bind an excess of biotin). All these steps were carried out at 4°C in buffer II. Aliquots processed without BSA showed similar protein bands. Another control (with negative results) was the omission of biotinylation, but processing of blots as usual. (For SDS-PAGE see below, for visualisation of biotinylated proteins after SDS-PAGE, see 'Nitrocellulose blots'). Intactness of trichocyst membranes was tested, before and after biotinylation, by adding 5 mM $CaCl_2$ (causing visible decondensation of injured trichocysts) [2].

Polyacrylamide-gel electrophoresis (PAGE)

Samples were dissolved in boiling sample buffer consisting of 10% w/v sodium dodecylsulfate (SDS), 20% v/v glycerol (in 125 mM Tris-HCl (pH 6.8), 3 min), with 2-mercaptoethanol (ME) added. Some controls were also run without ME. We applied 2–6 μg of trichocyst membrane protein or 15 μg of trichocyst or of homogenate protein to 12.5% polyacrylamide gels [30]. Markers were the same as previously used [2]; for details, see Fig. 3. Gels were developed in a Mini Protean II (Bio-Rad, München, Germany) electrophoresis unit for 75 min, 150 V.

Gels were either used for nitrocellulose blots (see below) or for staining by the following methods. (A) Standard Coomassie blue staining. Method (B) was precisely as indicated by Heukeshoven and Dernick [31]. Figures shown were obtained by method (A) or (B). Method (C) was a variation of the diamine silver staining method: Gels were fixed (concentrated acetic acid/methanol/water (1:4:5, v/v/v), 10 min), washed in water (10 min), fixed in 12.5% v/v glutardialdehyde (as proposed by Krystal [32] to increase Ag staining sensitivity) in water (15 min), washed in water (2 × 10 min) and then in 20% v/v ethanol (15 min). Gels were stained for 20 min in the following freshly prepared solution: 1 ml of 20% w/v $AgNO_3$, 1 ml of 25% v/v $NH_4(OH)$, 5 ml of 4% w/v NaOH and 93 ml of 20% v/v of ethanol (all dissolved in water). Then the preceding wash in ethanol was repeated twice for 10 min each time. Development in 100 ml 20% v/v ethanol plus 100 μl of 37% w/v formaldehyde and 25 μl of 2.3 M citric acid (all in water) was for 2–5 min, depending on band intensity vs. background achieved. The preserving solution (glycerol/acetic acid/water (5:10:85, v/v/v)) was applied for at least 5 min. All these steps were carried out at room temperature, before gels were dried.

Nitrocellulose blots

Using a Miniblot system (Bio-Rad; 1 h, 100 V) we followed Towbin and Gordon's method [33]. Biotinylated samples were transferred to nitrocellulose sheaths, washed in 'buffer III' (= 20 mM Tris-HCl (pH 7.6), with 500 mM NaCl and 0.5% w/v Tween 20 added), blocked with 5% fat-free milk powder in the same buffer (30 min), washed (four × 5 min) in buffer II, incubated for 1 h with streptavidin-POX (Pierce, Oud-Beijerland, Netherlands) diluted 1:1000, washed (four × 5 min) in buffer III and developed in 40 ml buffer III (without Tween 20) with 24 μl H_2O_2 (30% v/v) and 8 ml of 4-chloro-1-naphthol (0.3% w/v in methanol) added. Blots with marker proteins were processed in the same way, but stained with India ink.

Determination of phospholipid contents

Phospholipids (PL) were extracted according to standard methods [34]. The fractions to be analysed were dissolved in a five-fold excess of chloroform/methanol (2:3, v/v). Then 4–6 ml of 2 M KCl in 0.2 M phosphoric acid were added for phase separation. The chloroform phase containing the PL fraction was separated and the proteins in the aqueous phase were precipitated by trichloroacetic acid.

For PL determination, concentration series of 3-*sn*-phosphatidylcholine (98% pure, Fluka, Buchs, CH), dissolved in chloroform (with approx. 1% methanol added) were used as standards. PL staining according to Stewart [35] resulted in a concentration dependent, linear calibration curve. The PL contents of cell homogenates, isolated whole trichocysts and of trichocyst membranes was thus determined and referred to the protein content (see above) in the same fractions.

Electron microscopy

Fractions of trichocyst membranes were fixed with 1% v/v glutaraldehyde and then with 2% w/v osmiumtetroxide, both in Pipes buffer type II. After two washes in the same buffer, samples were dehydrated in graded acetone series, embedded in Spurr's low viscosity medium [36] and polymerised at 70°C for 20 h. Ultrathin sections were stained with 2% w/v aqueous uranyl acetate and with lead citrate (pH 12.0) [37], 3 min each.

Results

Trichocyst fractions, as starting material for membrane isolation, were obtained on a self-generating Percoll with high yield, as described before [2], i.e., by detaching trichocysts from the cell surface by EDTA. The percentage of membranes from other cell organelles was not determined separately, as we have previously found that these fractions contain only a small amount of contamination [2].

The method developed for the isolation of trichocyst membranes is summarised in 'Materials and Methods' and in Fig. 1. Essentially, isolated trichocysts are transferred to a discontinuous sucrose gradient, collected at the 1.3/1.8 M sucrose interface, diluted in buffer (1:5, for 10 min) and further disrupted in a glass/teflon potter. Upon subsequent filtration, most decondensed trichocyst shafts (contents) remain on the filter, whereas membrane fragments pass into the filtrate. Membranes are purified by two centrifugation steps (Fig. 1).

Electron micrographs reveal the presence of mostly unilamellar membrane profiles which in part form closed vesicles and in part open sheaths (Fig. 2). The amount of trichocyst contents or of other organelles is very low throughout the entire thickness of a membrane pellet.

Figs. 3 and 4 represent SDS-PAGE profiles or blots from trichocyst membranes obtained from *nd9-28°C* trichocysts after silver staining (Fig. 3) of gels or after

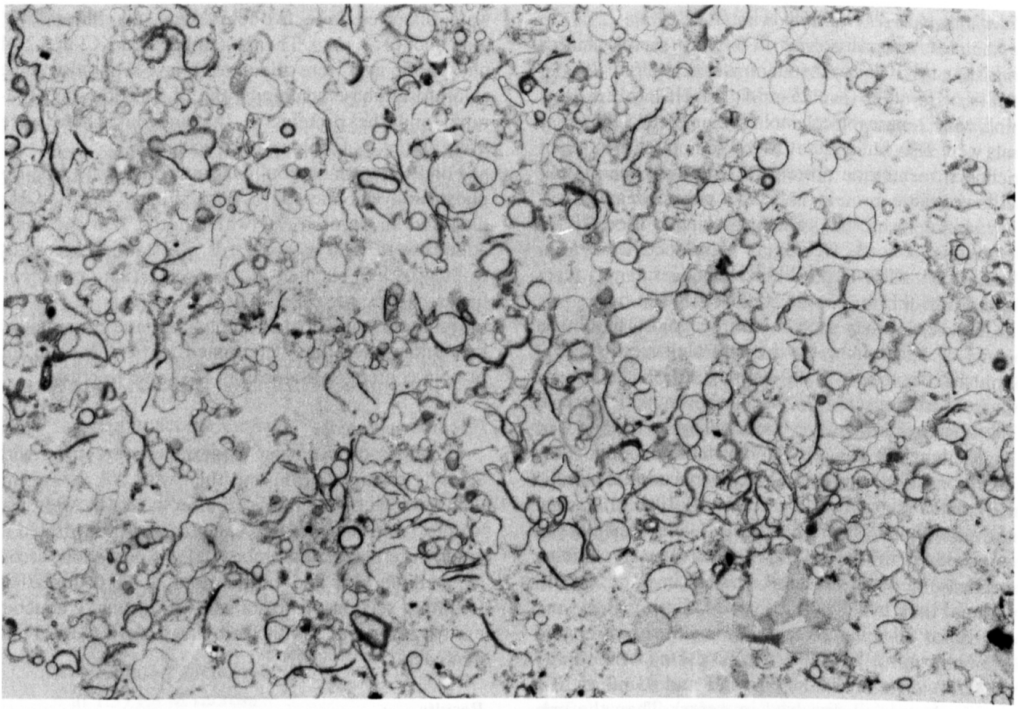

Fig. 2. Electron micrograph of an isolated trichocyst membrane fraction. Note the absence of any recognisable contaminants. Magnification 7500×.

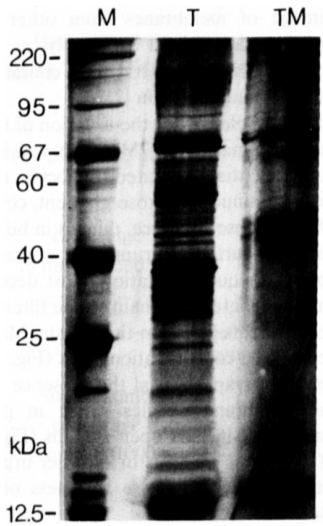

Fig. 3. SDS-PAGE (12.5% gel, +ME) from whole trichocyst fractions (T) or from trichocyst membranes (TM), both from strain *nd9-28°C*, after silver staining. M, markers.

biotinylation and subsequent streptavidin-POX labelling of nitrocellulose blots (Fig. 4), respectively. Both these methods were applied in order to visualise proteins as completely as possible and to account for the mostly very faint staining with silver reagent achievable with trichocyst membranes (see also Discussion). Results are summarised in Table I.

In Figs. 3 and 4 trichocyst membrane fractions are compared to fractions of whole trichocysts (secretory contents plus membranes). Evidently the typical trichynin (12–21 kDa, +ME) and some other bands (e.g., in the 30–40 kDa region; see Fig. 4) characteristic of trichocyst contents (see Discussion) are practically absent from trichocyst membrane fractions. This also holds true of the 76 kDa band occurring with very high intensity in trichocyst contents, partly in the soluble and partly in the insoluble fraction [38], but with less intensity in trichocyst membranes. Nevertheless, bands of ≤ 21 kDa should be regarded with caution because of their abundance in trichocyst contents. From silver stained gels we derive the occurrence of protein bands in trichocyst membranes as indicated in Table I. Most bands were found to be of identical kDa values and

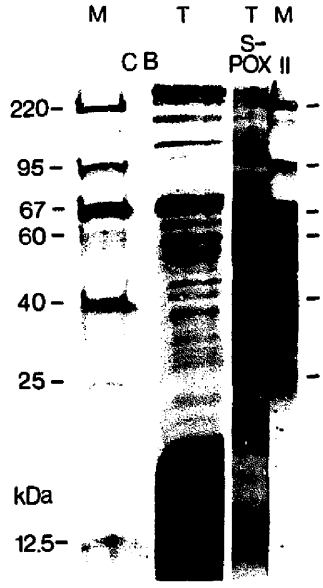

Fig. 4. SDS-PAGE gels (12.5%, +ME) obtained from biotinylated trichocysts (T) isolated from strain *nd9-28°C* and stained with Coomassie blue (CB) or, after blotting, stained with streptavidin (S-POX) or with Indian ink (II). The left and right halves of the figure were obtained from the same gel, when the gel was cut and used as such (left side) or for preparing blots (right side). Note the occurrence of intense 'trichynin' bands between 12 and approx. 20 kDa (from secretory contents) in the CB-stained gel and their absence from S-POX-stained blots after biotinylation of whole organelles. M, markers.

TABLE I

Protein bands in SDS-PAGE profiles (nd9-28°C cells) obtained by silver staining of trichocyst membranes or by biotinylation (streptavidin-POX labelling) of whole trichocysts

kDa	Ag-stain	Biotinylation
≥ 105	not analysed in detail	
104/103	+ to - +	+ + +
94–95	+ + to + + +	+ + + +
84	+	–
81	+ to - +	+
75/76	+ +	+
68–71	+ + +	(+) to +
65	+ +	+ + +
60–61	(+)	+ + +
57	+	+ + +
51/52	(+)	+
47–48	+ + + +	+ +
45/44	+ + +	+ + +
39	+ +	(+)
37	+ +	–
34/33–34	+	+ +
27/28–29	+	+
23–25	(+)	+
20/21	(+)	+
20/19	+	+ +
17	+ +	–
16/16.5	+	+
14.5	+	–
14	+ +	–
< 14	not analysed in detail	
n	13	6

n, number of experiments. + + + +, strongest; +, weakest; (+), inconsistent bands.

intensity, regardless of whether trichocysts or their membranes were isolated from *nd 9-28°C* (this paper) or from wildtype (*7S*) cells (data not shown).

Although biotinylation has been tried with originally intact trichocysts (tested according to Ref. 2, as analysed in Materials and Methods) at 4°C, membranes under these conditions may partly become leaky, at least for some small molecules. This can be concluded from some induction of decondensation by 5 mM $CaCl_2$, which can take place only when Ca^{2+} has access to the trichocyst matrix [7] through membrane leaks. This could explain the occurrence of largely identical bands as with silver stained gels, particularly when data from one and the same strain are compared (Table I). On the other hand, only a few additional or stronger bands are seen (while a few are weaker or missing) as compared to the stained gels (Fig. 4, Table I). None of the other prominent bands known to occur in secretory contents [38] are biotinylated. This holds particularly true of the prominent insoluble 'trichynin' bands (≤ 21 kDa, +ME), which are all shared by both strains analysed [38]. Although we have observed some membrane leakiness after (but not before) biotinylation, access of the biotinylation probe to the secretory contents must be very limited. Nevertheless, our data cannot stringently prove that all proteins biotinylated would face the cytoplasmic side.

The occurrence of the different bands essentially does not depend on the presence (Table I) or absence (not shown) of ME. (This is another criterion for the absence of any significant contamination by secretory contents; see Discussion).

Table II indicates the PL/protein ratios determined for cell homogenates, fractions of whole trichocysts and for trichocyst membranes, which reveals a considerable

TABLE II

Phospholipid / protein ratio in cell homogenates, isolated trichocyst fractions and isolated trichocyst membranes from P. tetraurelia

	PL/protein (mg/mg)
Homogenates	0.06
Trichocysts	0.01
Trichocyst membranes	0.20

increase of PL content after removing the secretory contents.

Discussion

The starting material for the isolation of trichocyst membranes was purified trichocyst fractions prepared according to the method of Glas-Albrecht and Plattner [2]. Since this method proved applicable to different strains (particularly to those with trichocysts firmly anchored at the cell surface), the method described now for the isolation of trichocyst membranes appears appropriate for all the different *Paramecium* strains (although we analysed in more detail only *nd9-28°C* cells). Several standard preparation procedures otherwise used to isolate secretory organelle membranes proved unsuccessful.

Trichocyst membranes are detached by osmotic swelling and filtration to avoid interference of decondensed trichocyst contents (see Introduction). The membrane fractions thus obtained are pure according to the following criteria: (a) the trichocyst fractions contain only little contamination by other cell organelles [2]; (b) EM analysis reveals almost exclusively membranes; (c) SDS-PAGE profiles are very different from intact trichocysts; in particular the ME-sensitive main secretory components, i.e. 'trichynins' [38–40] of 12–21 kDa (+ME) are scarce; this also holds true of the abundant 76 kDa secretory protein [38]. (d) Electrophoresis bands are also different from ciliary membranes [41,42], from gullets [43] or from surface membrane fractions [44,45]. In detail we have calculated from our previous data [44] the following protein bands occurring in isolated 'pellicles' (surface membranes; +ME, Coomassie blue staining; data pooled from *7S* and *nd9-28°C* cells because of close similarity): In pellicles the strongest band has 56 kDa (probably representing tubulin [2]), strong bands are of 38 and 79.5 kDa, medium bands of 14.5, 20.5, 29, 31.5, 75, 101 and ≥ 110 kDa, weak bands of 12.5, 17, 23, 49, 67, 90.5 kDa, with traces of 42 kDa. Hence, there is no systematic overlap with major proteins in membranes from trichocysts, although these also form part of the cell cortex. Furthermore, tubulin has been shown to be absent from trichocyst fractions [2] even before processing to membrane fractions.

Silver staining of trichocyst membranes always resulted in only a faint protein pattern, with the exception of only a few bands, probably because of the relatively low protein content (see below).

The typical structures ('collar') attached to the upper region of trichocyst membranes in situ [4,5,26] are not seen on electron micrographs from isolated organelles [2] and, thus, cannot contribute to the membrane material isolated here.

Adsorption of soluble secretory components is a notorious problem in the isolation of secretory organelle membranes [46,47] and trichocysts do contain such components [38]. Yet a comparison of bands shows that only very few bands coincide with secretory (glyco-)proteins. This holds true for bands in the 12–21 kDa region (+ME). In this case cross-contamination appears particularly unlikely, since these secretory proteins are mostly insoluble and since proteins of this size are insensitive to ME in our membrane fractions, in contrast to 'trichynins' [38–40].

Trichocysts contain large crystalline packages of secretory proteins. Therefore, their PL/protein ratio is much lower than in cell homogenates (Table II). In contrast to the intact organelles, trichocyst membranes have a 20-times higher PL/protein ratio. They are presumably poor in proteins, since they display only a few freeze-fracture particles [5,48]. This is not unusual, since a high PL/protein ratio is characteristic also of the secretory organelle membranes of a variety of other systems (e.g., endocrine [24,49,50] or exocrine gland vesicles [9,17,18]; cortical granules of egg cells [23] and neuronal transmitter vesicles [51]; for review, see Ref. 25). Assuming 10% protein content in membranes, a rough estimation shows that proteins contained in trichocyst membranes might amount to only 0.1% of total trichocyst protein. Purity of the membrane fractions isolated, therefore, also follows from the high PL/protein ratio (Table II). It is not unexpected, however, that protein profiles (Table I) are widely different from secretory organelle membranes from other-cells.

The isolation method presented also fills a gap in the analysis of the *Paramecium* cell. Beyond nuclei and the cytoplasmic components mentioned above, it has been possible so far only to isolate cilia and their membranes [41,42], mitochondria [52], crystal vacuoles [53] and enriched phagolysosomal fractions [54] from this cell type. However, for reasons given in the Introduction, the membranes of extrusive organelles (trichocysts) could not be isolated previously. We also expect our methodology to be applicable to those protozoa (including some parasitic ones) which also have their extrusive organelles firmly attached at the cell surface [1], since this turned out to be the main obstacle in the isolation of such organelles [2] and, consequently, of their membranes.

Furthermore, the isolation of trichocyst membranes will allow a detailed analysis of biochemical properties, of possible constituents in common with other secretory organelles and of components of possible relevance for secretory activity.

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