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Ultrastructural Localization of Chitin in the Cystic Wall of *Euplotes muscicola* Kahl (Ciliata, Hypotrichia)

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Summary

The ultrastructural localization of chitin in the cyst walls of *Euplotes muscicola* was carried out after deproteinization and by specific labelling with WGA-gold complex.
The overall ectocyst contains chitin that alone can sustain the exact profile of this cystic layer.
In the endocyst, electron clear sublayers include chitin and alternate with other sublayers probably of proteic nature.

Key words*:* Chitin - Ciliata - Hypotrichia - Cyst – Ultrastructure

Introduction

Chitin is the structural proteoglycan of many protozoan cystic walls [4]. Nevertheless, the ultrastructural localization of chitin has been investigated only in *Oxytricha bifaria* [27] and *Histriculus muscorum* [22]. These studies attempted to reveal chitin with the help of enzymatic extractions. When sections of cysts were floated on chitinase solutions, the enzymatic treatment was ineffective or not specific. When whole cysts were successively treated by enzymatic solutions of protease and chitinases, the results are more suggestive. But, in our opinion, the modification of the cystic morphology, especially of the organization of cystic layers, can complicate the interpretation.

The cystic morphology of *Euplotes muscicola* is well-known [3, 4, 7, 23]. Using light microscopy, it has been suggested after examination of cysts submitted to alkaline and chitin extraction [4] that continuous layers of chitin constitute a great part of ectocyst and endocyst [3].

This paper deals with the ultrastructural distribution of chitin in the cystic walls of *Euplotes muscicola.*

At first, cysts were observed with scanning and transmission electron microscopes after deproteinization; this treatment should show the remaining chitinous continuous layers.

On the other hand, colloidal gold coupled to wheat germ agglutinin (WGA) was employed as a specific marker of chitin. Adjusted by Horisberger and Rosset [15, 16], this direct and acute method was used for the localization of chitin in many tissue sections and in unicellular organisms, such as the yeasts *Saccharomyces cerevisiae* and *Candida utilis* [17], and in cysts produced by the Rhizopod *Entamoeba invadens* [1]. We applied this treatment for the first time in a ciliate cyst, as announced in a preliminary note [30].

Material and Methods

1. 14 days old *Euplotes muscicola* cysts were obtained from a stock bred for several years in our laboratories. Encystment of trophonts was induced according to Yonezawa's technique [33] that consists in concentrating about thirty trophonts into a drop of culture medium. In such conditions, all individuals encyst within a few hours.

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2. Deproteinization

Cysts were immersed for 3 h in 1 N sodium hydroxyde solution, at 100 °C. Then, they were carefully washed and fixed in buffered glutaraldehyde (1.5%) and osmium tetroxide (1%) in Sorensen phosphate solution (0.1 M, pH 7.4). Sodium hydroxide treatment was omitted in controls.

Two series of samples were prepared. The first one was examined using a scanning electron microscope (Jeol JSM-840 A) after critical point drying and metallization in a gold-palladium sputtering apparatus (Balzers SCD-030). The second one was embedded in Epon. Thin sections, carried out with a Porter Blum MT_2B ultramicrotome, were contrasted with uranyl acetate and lead citrate [26] and viewed in a transmission electron microscope Jeol JEM-100 SX under a 80 kV accelerating voltage.

In addition, sections were collected on gold grids before contrasting and submitted to WGA-gold labelling of chitin following the method described below.

3. WGA-gold Labelling of Chitin

Colloidal gold particles of 15 mm diameter were obtained by reducing tetrachloroauric acid (from SIGMA) with sodium citrate according to Frens [9]. Because of its low molecular weight, WGA (from SIGMA) was cross-linked to Bovine Serum Albumin (BSA) with glutaraldehyde, as suggested by Horisberger and Rosset [16]. The optimal amount of WGA-BSA solution necessary to obtain the labelling solution was determined, with NaCl to flocculate, according to Faulk and Taylor [6]. Then 2 ml of WGA-BSA were mixed immediately with 100 ml of filtered gold solution.

After centrifugation (1500 g, 60 min), the pellet containing the WGA-BSA-gold complex was finally suspended in 10 ml of 0.02 M Sorensen phosphate buffer at pH 7.4. Just before use, the buffer containing $CaCl_2$, $MgCl_2$ (0.001 M) and 0.5 mg/ml polyethylene glycol (Carbowax 20 M, from SERVA) was filtered through a Millipore filter (0.45 µm pore size).

Thin sections of cysts fixed in glutaraldehyde (1.5%) were mounted on gold grids and floated for 45 min on a drop of five to twenty times diluted WGA-BSA-gold solution. In order to verify whether this treatment does not increase the accessibility of WGA to substrates embedded in epoxyresin, some sections were priorily etched with 10% H_2O_2 for 30 min.

The validity of the method applied for chitin labelling in *Euplotes* cysts was achieved in parallel with the following controls:

a. WGA-gold labelling of the cuticle of the shore crab *Carcinus maenas:* a strong gold labelling occurs in the procuticle of this species [30] where chitin is known to represent up to 60 to 70% of the total organic cuticular dry weight.

b. Inhibition of labelling by adding 10 mM N-N'-N"-triacetylchitotriose (from SIGMA) to the WGA-gold complex [10].

c. Inhibition of labelling by adding 0.4 M N-acetyl-D-glucosamine monomer (from UCB), an inhibitory about 4000 times less potent than the trimer [11], to the WGA-gold complex.

d. Incubation with PNA-gold complex. PNA is a lectin from *Arachis hypogaea* (Peanut) binding to galactosyl B-(1->3)-N-acetylgalactosamine and nonreducing terminal galactose. It shows no specificity for chitin or its oligomers. The gold labelled peanut lectin (10 nm gold) distributed by SIGMA was used. For comparison, the WGA gold complex (10 nm gold) from SIGMA was tested, too. The gold complexes were diluted five times in a 0.01 M phosphate buffer at pH 7.2, containing 0.15 M NaCl.

Note that, in chitinous and proteic structures as arthropodial cuticle and cysts of *Euplotes muscicola,* total chitin extraction is not possible without previous deproteinization; after both treatments, the cysts dislocate [3], preventing any control labelling on non-chitinous material.

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Results

1. Cyst Morphology after Deproteinization with Sodium Hydroxide

The ectocyst of control cysts is characterized by "dorsal" crests and parallel rows of ciliary pores, as well as by a marked cortical network corresponding to the argyrome. This argyrophilic network is seen only between the two median crests, in the excystment zone (Fig. 1). In thin sections, the ectocyst appears as a single microfibrillar layer, whereas the endocyst is composed of fine electron dense layers alternating with thicker granular electron clear ones (Fig. 2).

After hot sodium hydroxide treatment (Fig. 3), the outer organization of the cystic walls (crests, pores, argyrome) appears unchanged, with the exception of the excystment zone where the argyrome is cleared and appears in the shape of thick ribs. The intervals between these ribs contain fine fibres, more conspicuous than those of the non-treated cysts. The inner cystic wall can be observed in the spaces between fibres.

Outside the excystment zone, the whole ectocyst remains intact (Fig. 4), but its affinity for lead citrate and uranyl acetate is reduced. The electron dense layers of the endocyst appear completely digested whereas the electron clear ones do resist, but exhibit a comparatively reduced contrast.

The encysted cell is completely destroyed.

2. WGA-gold Labelling

Examination of contrasted ultrathin sections (Fig. 5) reveals that only the cystic walls react to WGA-gold. There isn't any labelling, neither at the cell nor at the embedding medium levels.

On stained sections (Fig. 6), the whole ectocyst and the electron clear layers of the endocyst bear gold particles, whereas the dense endocystic layers do not. Incubation in colloidal suspension diluted 5 or 20 times gives a similar labelling intensity.

Preliminary etching of sections by hydrogen peroxide does not increase the density of gold particles, nor indicates additional positive structures. As a result of this oxidizing agent it appears that some cellular structures are cleared.

Control sections of procuticle of *Carcinus maenas* show a similar characteristic labelling.

The control sections of cysts, incubated with WGA-BSA-gold inhibited by chitotriose, bear only a few scattered gold particles (Fig. 7).

The presence of N-acetyl-D-glucosamine does not seem to decrease the labelling.

After incubation on a drop of the PNA-gold complex, cysts never present any labelling (Fig. 8), whereas labelling with WGA-gold from SIGMA is conspicuous (Fig. 9), even if gold particles are some less numerous when compared with samples treated with WGA-gold complexes produced in our laboratory.

3. WGA-gold Labelling of Deproteinizated Cysts

After deproteinization with hot hydroxide treatment, the residual material (ectocyst and electron clear layers of the endocyst) is obviously labelled by WGA-BSA-gold particles. Nevertheless the extraction procedure as well as non-osmication prevent any good contrast.

Discussion

Both of the ways used to localize chitin in cysts of *Euplotes muscicola* give rise to concordant conclusions. Indeed, the fact that the ectocyst and the electron clear layers of the endocyst are the structures resisting against treatment with hot sodium hydroxide and are digested by strong chitinases [3] as well as that they are obviously labelled with WGA greatly support the evidence that these cystic layers are composed of continuous laminae of chitin.

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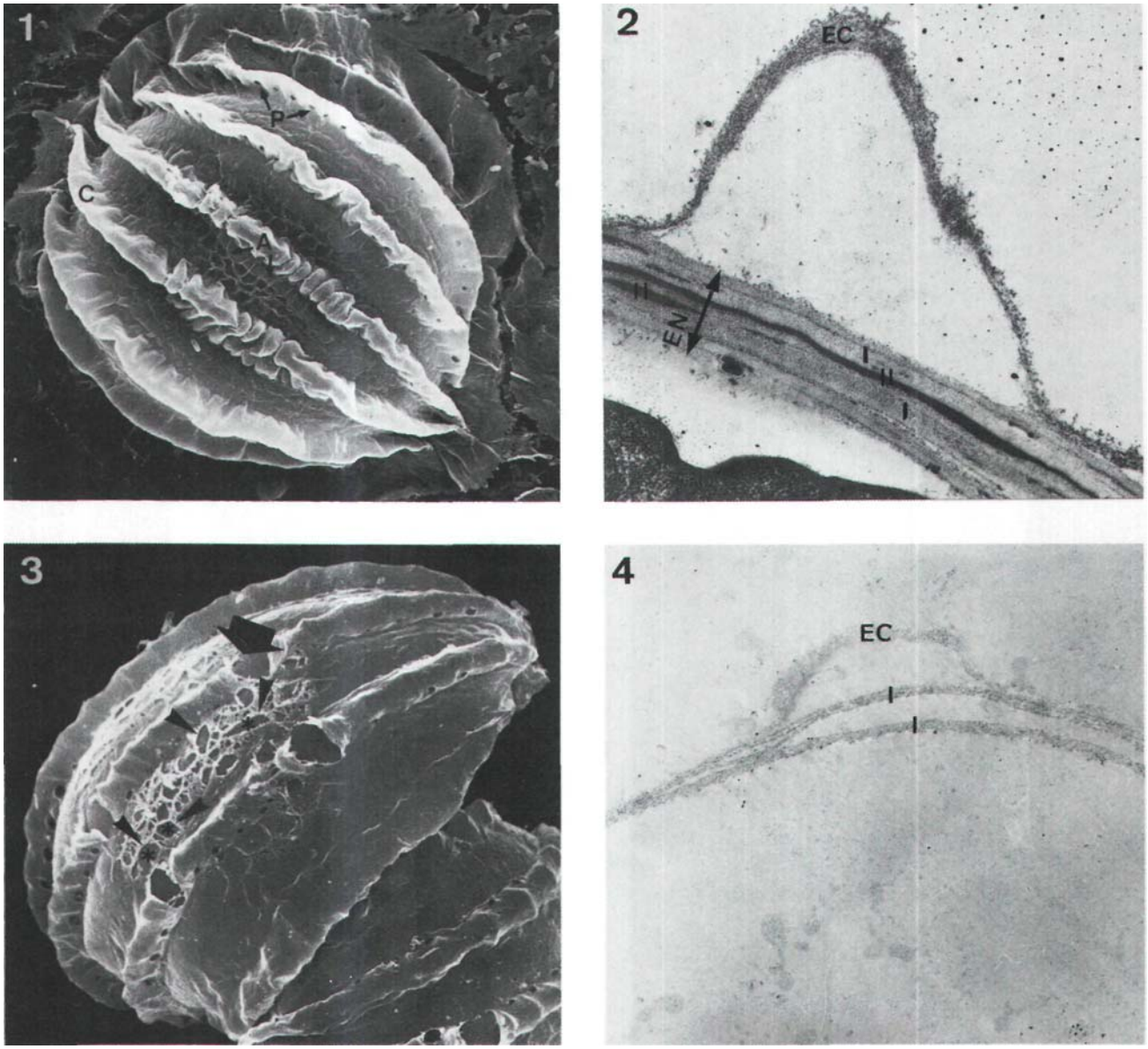
Morphology - **Fig. 1.** Ectocyst with caracteristic crests (C), pores (P) and argyrome (A). SEM, × 2500. **Fig. 2.** Ectocyst (EC) and endocyst (EN) which is composed of electron clear (I) layers alternating with electron dense ones (II). TEM, × 17000. -*Deproteinization* - **Fig. 3.** After treatment with hot sodium hydroxide solution, the profile of the ectocyst remains unchanged. In the excystment zone (large arrow), the argyrome appears in the form of thick ribs (arrow heads); on this network, slender fibers are overprinted. Between the ribs and the fibers, the subjacent layer is seen (asterisk). SEM, × 3500. - **Fig. 4**. The ectocyst (EC) and the electron clear layers (I) of the endocyst only subsist after deproteinization. TEM, × 7000.



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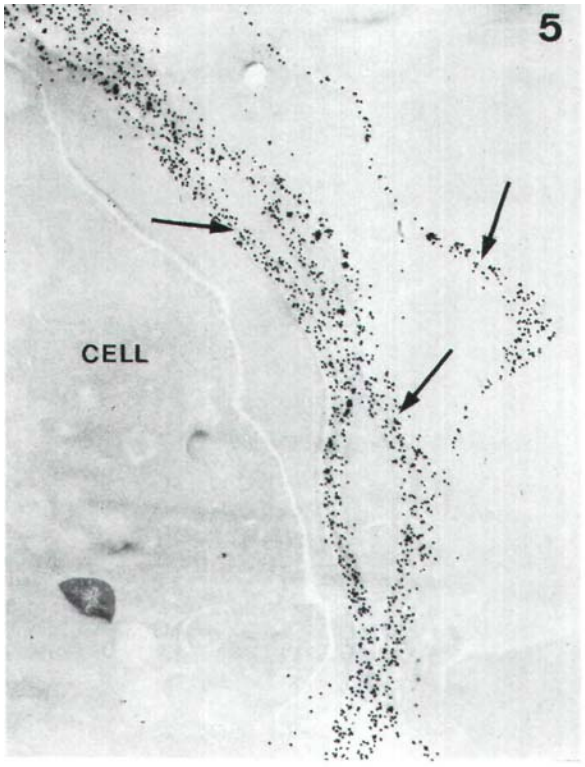
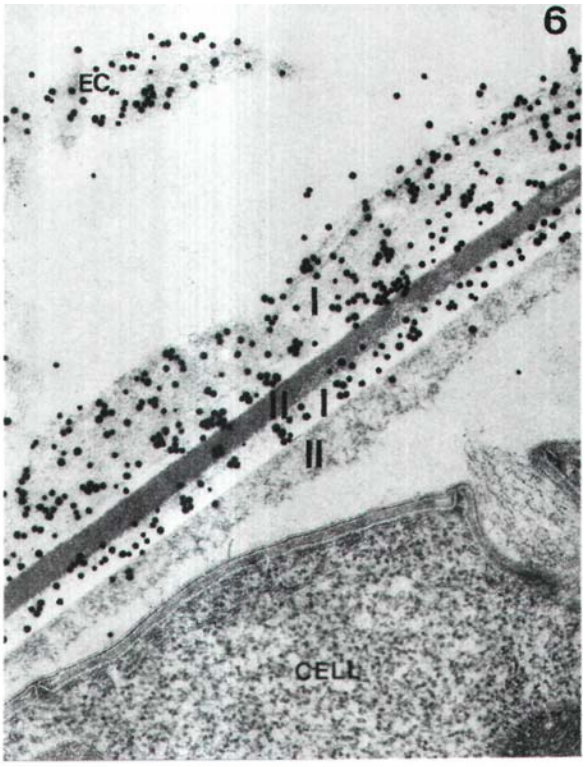
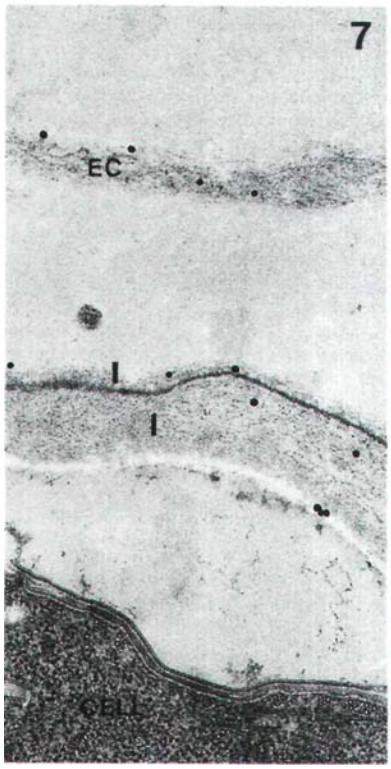
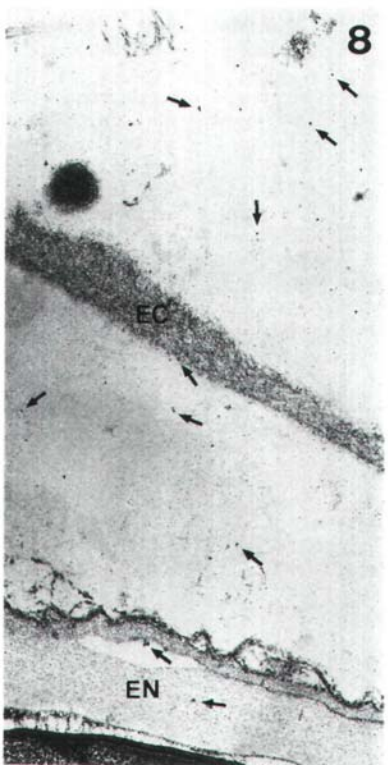
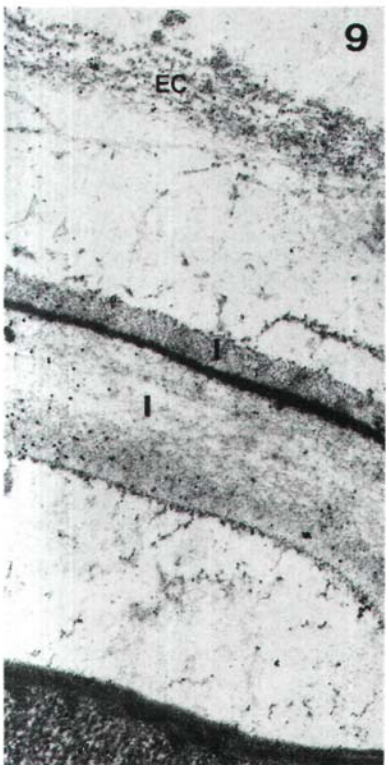
Colloidal gold labelling -**Fig. 5**. Uncontrasted section. The cystic wall (arrows) alone is labelled. TEM, × 9000.- **Fig. 6.** Theectocyst (EC) and the electron clear layers (I) of the endocyst are labelled, contrary to the electron dense layers (II). TEM, × 35 000. -**Fig. 7.** When trichitobiose is added to the WGA-gold solution, the ectocyst (EC) and the electron clear layers of the endocyst (I) are labelled with a few gold particles only. TEM, × 42 000. - **Fig. 8.** No specific labelling is obtained with the PNA-gold complex from SIGMA, arrows = gold particles, EC = ectocyst, EN = endocyst. TEM, × 18 500.-**Fig. 9**. Specific positive labelling with WGA-gold from SIGMA on the ectocyst (EC) and the electron clear layers (I) of the endocyst; the gold particles are slightly less numerous in comparison with WGA-gold solutions prepared in our laboratory. TEM, × 31 000.











The treatment with H_2O_2 before incubation does not increase the labelling. Furthermore it clears material because a proteic extraction. For these reasons, and in agreement with Bendayen [2], we do not recommend etching for sections of non-osmicated material embedded in Epon.

In regard to the controls, the intense labelling of *Carcinus maenas* procuticle is one of the arguments supporting the specificity of our WGA complex to chitin.

The almost complete inhibition of the labelling with trichitobiose and the absence of inhibition with N-acetyl-D-glucosamine agree that the labelling is chiefly due to the high affinity of WGA for the trimer [11].

The complete lack of gold particles on Epon and other cell structures than the cystic wall when the WGA-BSA-gold complex is used, or on the whole sections when the PNA gold complex is employed, also supports the idea of specificity. It should be observed that the labelling obtained with commercial WGA-BSA-gold complex is relatively weak, probably because of a poorer particle concentration.

The lack of contrast of chitinous layers after hot hydroxide treatment is probably explained by alkaline extraction of the proteins previously associated with chitin. The good WGA-labelling indicates that, in spite of this drastic extraction, at least a part of the lectin binding sites of the chitin (acetamido-groups, [11]) are maintained.

Besides, disappearance of the electron dense layers of the endocyst after deproteinization, and their lack of reaction with WGA-gold, points out the absence of chitin. These results and previous demonstrations of the presence of proteins in the endocyst [3, 7] suggest the proteic nature of these electron dense layers.

A quantitative determination of chitin in the cyst walls of *Euplotes muscicola* cannot be considered nowadays, because cultural methods do not produce a sufficient amount for this purpose. Nevertheless the weak morphological deterioration of the ectocyst and of the chitinous layers of the endocyst after deproteinization, and their strong WGA-gold-labelling (in spite of the embedding in non-hydrosoluble resin) argue for a high quantitative, and may be morphogenetic, importance of chitin in the cystic walls of *Euplotes muscicola.* The excystment zone is the only one that could be poorer in chitin: indeed, the appearance of the argyrome and the apparition of fine fibres would be explained by a loss of non-chitinous material.

It is an outstanding observation that chitin alone may sustain the exact profile of the cyst wall, as it does in the cuticle of Arthropods.

To our knowledge, the regular alternance of morphologically and chemically different layers within a cystic wall, like the endocyst of *Euplotes muscicola,* has never been described in other ciliates. This peculiar organization, and the typical structure of the ectocyst [3], lead to suspect that building of the cystic walls is very special.

Works in progress of our laboratory support this idea; none of the cystic precursors appearing in the cytoplasm (granules or vacuoles), as described in hypotrichous ciliates (*Oxytricha fallax* [12], *Pleurotricha* [21], *Gastro-styla steinii* [32], *Laurentiella acuminata* [13], *Oxytricha bifaria* [31], *Paraurostyla weissei* [5]) are found during the encystment of *Euplotes muscicola.* In contrast, it seems that the synthesis of chitino-proteic microfibrils should take place at the plasmic membrane level, according to the process observed in arthropodial cuticles [10]. None of the systems involved for chitin synthesis in other unicellular organisms, such chitosomes in yeast or microvesicles from the ciliate *Eufolliculina uhligi* [14], have been observed. Furthermore we never found any confirmation of a delamination of the hypolemma [3].

It may be of interest to note that the special way of cystic wall building in *Euplotes muscicola* is in good agreement with the recent assertion of a large phyletic distance between Euplotidae and other hypotrichs, greater than the distances between the families of Hypotrichida [8,19,20, 24, 25, 29].

Addendum

After registration of this paper, we have received the very good study of Mulisch and Hausmann, concerning WGA-labelling of chitin in cystic walls of *Blepharisma undulans* and *Pseudomicro-thorax dubius.*

Mulisch M. and Hausmann K. (1989): Localization of chitin on ultrathin sections of cysts of two ciliated protozoa, *Blepharisma undulans* and *Fseudomicrothorax dubius,* using colloidal gold conjugated wheat germ agglutinin. Protoplasma, *152,* 77-86.

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