Cell Surface Glycoconjugates of *Euglena gracilis* (Euglenozoa): Modifications under Potassium and Magnesium Deficiency

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Biochemical and ultrastructural examinations on the pellicle of autotrophically grown *Euglena gracilis* were carried out after three days under potassium and magnesium deficiency. Cell-surface changes were detected by lectin assay. Compared to cells grown in complete medium, deficient cells become larger in shape, accompanied by rising carbohydrate, chlorophyll and protein content, bind more and other lectin molecules: an increase of mainly galactose and N-acetylgalactosamine receptors was observed. Investigations with the mucilage stains alcian blue and ruthenium red indicated that mucilaginous material is released under deficient conditions, whereas the control cells show a strong precipitate of these stains well inside the cells beneath the pellicle.

Introduction

A model organism suitable for measuring the influence of deficient nutrition on the cell, and here especially on the cell membrane, is the green flagellated protist *Euglena gracilis* (Euglenozoa) with its complex and unusual surface structure. This unicellular organism possesses a cell membrane complex, the pellicle. It is composed of repetitive membrane domains – the ridges and grooves – in an undulating manner, underlaid with an epiplasmatic layer of fibrils and a regular pattern of microtubules which are interpreted as the peripheral membrane skeleton (Dubreuil and Bouck, 1988). The intussusceptive surface growth takes place by inserting a new ridge between two old ones (Hofmann and Bouck, 1976; Nakano *et al.,* 1987).

The complexity of this structure is also reflected in the chemical composition of the pellicle: the pellicle dry weight consists of about 70–80% protein, between 6 and 17% carbohydrates and 12–17% lipids (Barras and Stone, 1965; Hofmann and Bouck, 1976; Nakano *et al.,* 1987).

Some surfaces of euglenoid cells have already been tested in regard to their lectin binding capacity (Vannini *et al.,* 1981; Bré *et al.,* 1986; Strycek *et al.,* 1992). When the nutrition of *Euglena gracilis* is varied drastically, a change in the cell envelope with its glycoresidue-rich membrane can be detected. It was shown by lectin binding studies that a lack of cobalamine (vitamine B_{12}) leads to a loss of N-acetyl-galactosamine (GalNAc) residues on the one hand (Lefort-Tran *et al.,* 1980; Bré and Lefort-Tran, 1984; Bré *et al.,* 1986) and to a mucilaginous layer outside of the cell on the other hand (Bré and Lefort-Tran, 1978).

Previous studies using a complement bio-assay (Ruppel and Benninghoff, 1983; Benninghoff *et al.,* 1986) showed that cultivation of *Euglena gracilis* in magnesium (Mg^{2+}) and potassium (K^+) deficient media leads to a change in the complement binding capacity. A possible explanation is that the sugar residues vary according to the starving conditions so that the proteins of the human complement system cannot be activated via the alternative pathway, leading to complete inhibition of cytolysis by complement (Johnson, 1994).

Deficiency of potassium as main intracellular cation shows a direct influence on cells. Beside inhibition of cell proliferation, potassium starvation leads to a decrease of the deficient cation in the cell. Potassium effects through its important role

Abbreviations: BS-I, *Bandeiraea simplicifolia;* ConA, *Canavalia ensiformis;* HPA, *Helix pomatia;* LCH, *Lens culinaris;* LPH, *Limulus polyphemus;* RCA, *Ricinus communis;* TGA, *Tetragonolobus purpureas;* WGA, *Triticum vulgaris;* FITC, fluorescein-isothiocyanate

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as cofactor for a series of enzymatic reactions in photophosphorylation on photosynthesis, in glycolysis on carbohydrate metabolism and in binding of mRNA to ribosomes on protein synthesis (Lüttge and Clarkson, 1989; for review see Bhandel and Malik, 1988). Magnesium is cofactor for a lot of enzymatic reactions, too, structural component of ribosomes and nucleic acids and part of chlorophyll in photoautotrophic organisms (Hilt *et al.,* 1987). Deficiency leads to inhibition of cell division and shows direct effects on the cellular level of potassium ions because of its influence on Na-K-Cl-cotransport (Smith and Maguire, 1993). Both cations are considered to influence the permeability of membranes (Ryan, 1993).

In our subsequent investigations on surface glycoconjugates of *Euglena gracilis* we examined by lectin binding studies whether lack of magnesium and potassium initiated a change of the carbohydrate residues. Electron microscopic investigations after treatment of cells with ruthenium red gave an insight into the mucilage deposition in the control and the "starved" pellicle.

Materials and Methods

Cultivation of Euglena gracilis

Euglena gracilis strain Z (Klebs 1224–5/25) obtained from Algensammlung Göttingen was grown under axenic conditions with a light/dark change of 14:10 h at 30 °C. Cell suspension was bubbled with air containing 3% CO_2. Every three days at the end of a dark period, the culture was diluted: 10 ml (0.5 x 10^6 cells/ml) of the suspension were transferred to 100 ml fresh medium (Cramer and Myers, 1952). The deficient media differed from the normal "control" media only in regard to the "starved" minerals: $MgSO_4$ x 7 H_2O was substituted with K_2SO_4 for the magnesium deficient medium; whereas KH_2PO_4 was replaced by NaH_2PO_4 for the potassium deficient medium. Higher concentrations of ions needed for substitution (sodium and potassium) did not influence the cells in previous investigations (Scholten-Beck, 1996).

Control cells were rinsed and resuspended with fresh deficient media. For further investigations cells were harvested after three days at the end of a dark period and rinsed three times with phosphate buffer (0.02 M KH_2PO_4-Na_2HPO_4, pH 7.2). In experiments with potassium deficient cells KH_2PO_4 was replaced in each buffer system by NaH_2PO_4. Cells were then resuspended in PBS (phosphate buffer containing 140 mM NaCl).

Growth analysis

Number of cells, protein content (Lowry *et al.,* 1951), carbohydrate (Roe, 1955) and chlorophyll content (Schmid, 1971) were determined every 24h at the end of dark period.

Lectin assay

For detection of carbohydrate residues on the pellicle of *Euglena gracilis* the following lectins (Table I) conjugated with fluoresceine isothiocyanate (FITC, Sigma, Deisenhofen, Germany) have been used.

200 µl cellsuspension (10^6 cells/ml PBS) were incubated with diluted FITC-lectin (final concentration of lectin 100 µl/ml in PBS, except WGA and LPH: 200 µl/ml) for 30 min at 25 °C. After rinsing three times with PBS cells were picked up in 200 µl PBS and measured in a FacScan flowcytometer counting the fluorescence of 5.000 cells each run (Becton Dickinson; Software Simulset, Hewlett-Packard). An aliquot was examined with an Orthoplan / Leitz Fluorescence Microscope, BP 450–490 nm; LP 515 nm. Images were taken with a Kodak Ektachrom slide film, 400 ASA. For determining the specifity of lectins a control was performed using the appropriate sugar(s) as inhibitors prior to lectin assay (lactose with RCA and BS-I; N-acetylgalactosaminewith HPA and BS-I; N-Acetylneuraminic acid with WGA and LPH; α-methyl-D-mannoside with ConA and LCH; glucose with ConA and fucose with TGA).

Staining of Euglena gracilis with alcian blue for light microscopy

Anionic groups of mucus can be demonstrated in the pellicle of *Euglena gracilis* (Cogburn and Schiff, 1984) by histochemical staining with alcian blue (Sigma). Cells were harvested after three days of cultivation in each media by centrifugation of 10 ml cellsuspension (10^6 cells/ml) immediately after the dark period. For the histochemical procedure cells were stained in a solution containing 0.1% alcian blue in 0.5 M acetic acid (pH 2.5) at room temperature for 15 min, rinsed three times

with water and examined with an Olympus BH microscope. Images were taken with a 200 ASA Kodak Ektachrom film with brightfield and phase contrast optics.

Staining of Euglena gracilis with ruthenium red for electron microscopy

Cells were harvested as described for alcian blue staining and prepared for electron microscopy as follows:

Ruthenium red (RR) powder (Sigma) was dissolved and a 0.5% (w/v) stock solution was prepared. A pellet of 10 ml cellsuspension (10^6 cells/ml) rinsed three times with EM buffer (0.05 M NaH_2PO_4-Na_2HPO_4, pH 7.0) was fixed with 2.5% glutaraldehyde (w/v; Serva, Heidelberg, Germany) and 0.1% ruthenium red (v/v) in 0.05 M EM buffer for 10 sec in the microwave oven at 700 W (Miele electronics M 720). Samples were rinsed three times with EM buffer and once with distilled water. Cells were placed into 2% osmium tetroxide (w/v; TAAB) containing 0.1% ruthenium red in EM buffer for two hours, rinsed three times with buffer and once with distilled water.

Samples were then dehydrated in a graded ethanol series (15, 30, 45, 60, 75, 90 and 2 x 100% ethanol in distilled water). After infiltration, samples were embedded in water-mixable Transmit resin (TAAB), filled into BEEM capsules and left to polymerize for 24 hours at 70 °C.

Ultrathin sections of about 60–70 nm were made with a diamond knife (DuPont) on an Ultracut (Reichert Ultracut E) and put onto 200 mesh Cu-grids. Cells were counter-stained with 2% (w/v) aquaeous uranylacetate (Merck) and 2% (w/v) lead citrate (Merck).

Results

Growth analysis

Euglena gracilis grown in potassium and magnesium free media show rapid changes in physiological behaviour. Cells become a larger cell shape and the number of cells begins to decrease significantly after 24 hrs compared to control cells grown in complete media (Fig. 1a). The contents of carbohydrate (Fig. 1b), protein (Fig. 1c), and chlorophyll (Fig. 1d) show an increase beginning after 24 hrs of starvation and coming to maxima after 96 hrs compared to control cells. It was evident that starved cells produce significantly more paramylon.

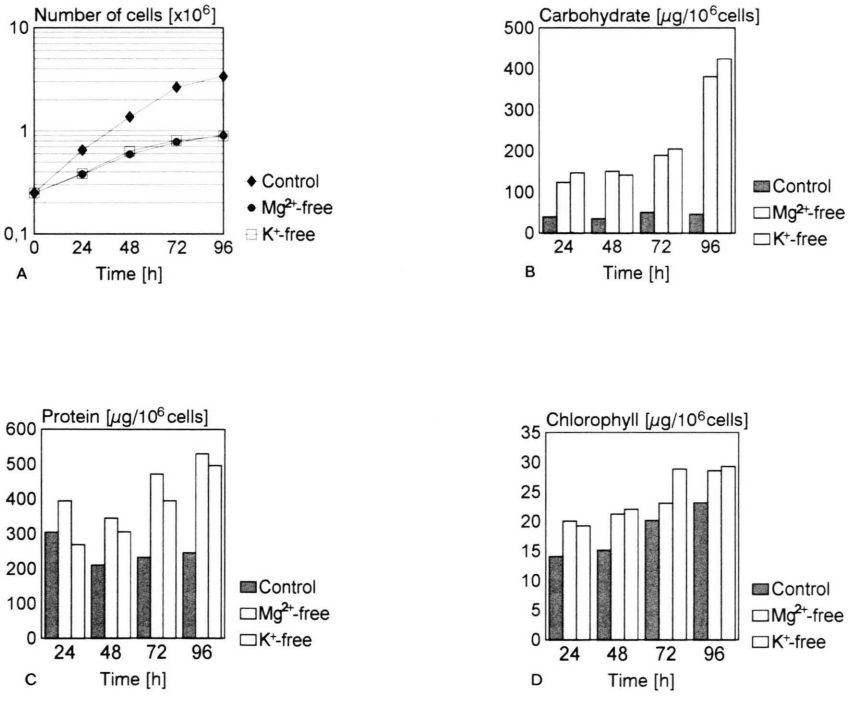

Fig 1. Growth analysis of *Euglena gracilis* cultures in control (complete medium), Mg^{2+}-free, and K^+-free media. Number of cells (A), content of carbohydrate (B), protein (C), and chlorophyll (D); $n = 10-12$.

Table I. FITC conjugated lectins and the specificities used in lectin assay.

Lectin	Specificity
Ricinus communis agglutinin (RCA_{120})	β-D-galactose (Gal)
Helix pomatia agglutinin (HPA)	N-acetylgalactosamine (GalNAc), α-D-galactose
Bandeiraea simplicifolia (BS-I)	β-D-galactose, N-acetylgalactosamine
Limulus polyphemus agglutinin (LPH)	N-acetylneuraminic acid (Neu5Ac), N-acetylgalactosamine, N-acetylglucosamine (GlcNAc)
Triticum vulgaris agglutinin (WGA)	$(N\text{-acetylglucosamine})_2$, N-acetylneuraminic acid
Canavalia ensiformis agglutinin (ConA)	mannose (Man), glucose (Glc),
Lens culinaris hemagglutinin (LCH)	mannose
Tetragonolobus purpureas agglutinin (TGA)	fucose (Fuc)

Carbohydrate residues

Compared to cultivation in complete medium, starved cells show a different degree of labeling after incubation in the presence of several lectins. Labeling pattern starts to change after 24 hrs of starvation, but significant data were achieved after 3 d under starving conditions. The labeling of starved cells with the FITC-conjugated lectins measured flowcytometrically is in every case much stronger than that of the cells grown in complete medium (Fig. 2a), the strongest labeling occurring with the lectins RCA_{120}, HPA, and BSI which all bind to Gal- and GalNAc-residues, similar to TGA which binds to fucose. These sugar residues increase during the starvation. LPH and WGA which both recognize Neu5Ac also show a stronger affinity to the starved cells. The Glc- and Man-residues of *Euglena* increase after starvation, too, indicated by a stronger labeling of the cells with ConA and partially with LCH for Man.

Specificity of binding was tested with the appropriate sugar(s) as inhibitors (Bonaly and Brochiero, 1994; v. Sengbusch and Müller, 1983; Bré *et al.*, 1986) and revealed expected data (Fig. 2b), represented by control cells, because no difference in inhibition between control and starved cells could be observed.

Microscopic observations led to the same results as cytometric measurement documented by FITC fluorescence of conjugated RCA_{120} (Fig. 3).

Mucilage

a) Alcian blue staining

After incubation of native cells with the mucilage stain alcian blue we found different results

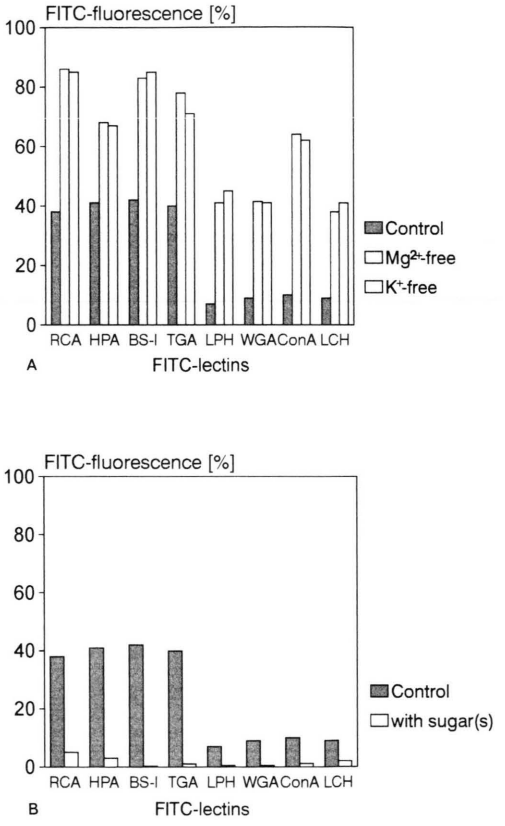

Fig 2. Lectin assay in percentage of fluorescence of FITC conjugated lectins measured flowcytometrically on 5.000 cells of *E. gracilis* each count. (RCA: *Ricinus communis*, HPA: *Helix pomatia*, BS-I: *Bandeiraea simplicifolia*, TGA: *Tetragonolobus purpureas*, LPH: *Limulus polyphemus*, WGA: *Triticum vulgaris*, ConA: *Canavalia ensiformis*, LCH: *Lens culinaris*)
A) control, Mg^{2+}-free, and K^{+}-free cultures. B) control culture with appropriate sugar(s) as inhibitors.

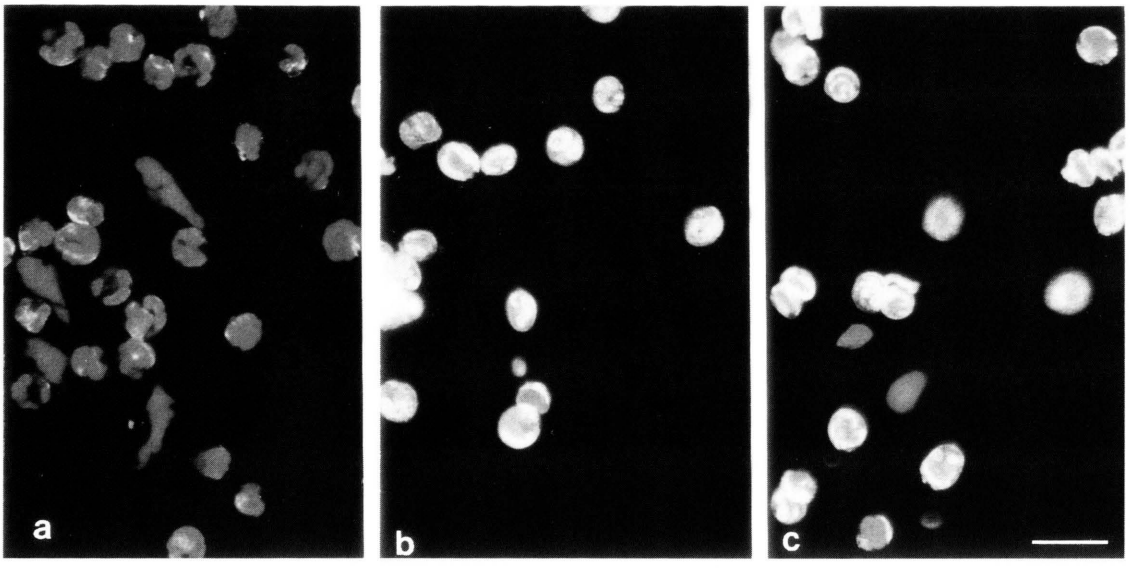

Fig 3. Fluorescence images of cells of *E. gracilis* with FITC conjugated RCA_{120}
a) control, b) Mg^{2+}-free, c) K^{+}-free cultures. Bar: 40 μm.

for the cells of investigated culture conditions: the control cells do not show significant staining of the envelope (Fig. 4a), whereas the magnesium-starved cells reveal a staining (Fig. 4b) in form of small knots. The potassium starved cells expose a distinct layer of staining material on the cell surface (Fig. 4c). It is clearly evident that the stained mucilaginous material is situated outside the cells.

b) Ruthenium red staining

For more detailed information about the mucilage we incubated the cells with ruthenium red (RR). Although treatment with RR causes a slight damage of the starved cells, precipitate of RR was impressive. On the electron microscope cross sections show a significant labeling only when grown in complete medium (Fig. 5b). The precipitate can be found beneath the cell membrane close to the ridges, but always only on one side of each groove, thus giving a strong impression of polarity. The areas with a strong RR precipitate are visible on the side where the microtubules are situated and the epiplasmatic layer is rather thin, most clearly in the control cells. Starved cells give a different picture: magnesium starved cells show a labeling of only every other ridge, and the precipitate of RR is much weaker (Fig. 5d). The potassium depleted cells only show a very weak labeling of even less ridges (Fig. 5f). The inserted new ridges, observed in the potassium starvation, do not show a precipitate of RR. Cells without treatment of RR do not show any visible accumulation of mucilaginous material (Fig. 5a,c,e).

Discussion

Cells of *Euglena gracilis* grown in depleted media rapidly change their growth behaviour and surface glycoresidues. While population of control cells are labeled strongly by only a few lectins, the magnesium and potassium starved populations show a strong and unequivocal labeling with the majority of used lectins.

Those lectins which may recognize galactose residues or their derivates, i. e. RCA, HPA, and BSI, labeled control cells clearly. The existence of galactose at the pellicle of *Euglena* is in accordance with investigations by v. Sengbusch and Müller (1983) who treated control cells with RCA. Bré and Lefort-Tran (1984) used HPA for determining galactose residues at the surface of *Euglena* and found a strong labeling which disappeared under vitamine B_{12} starvation. Obviously the receptor sites for galactose or N-acetylgalactosamine disappear during this type of starvation. The presence of galactose and N-acetylgalactosamine at the surface of *Euglena* control cells is generally

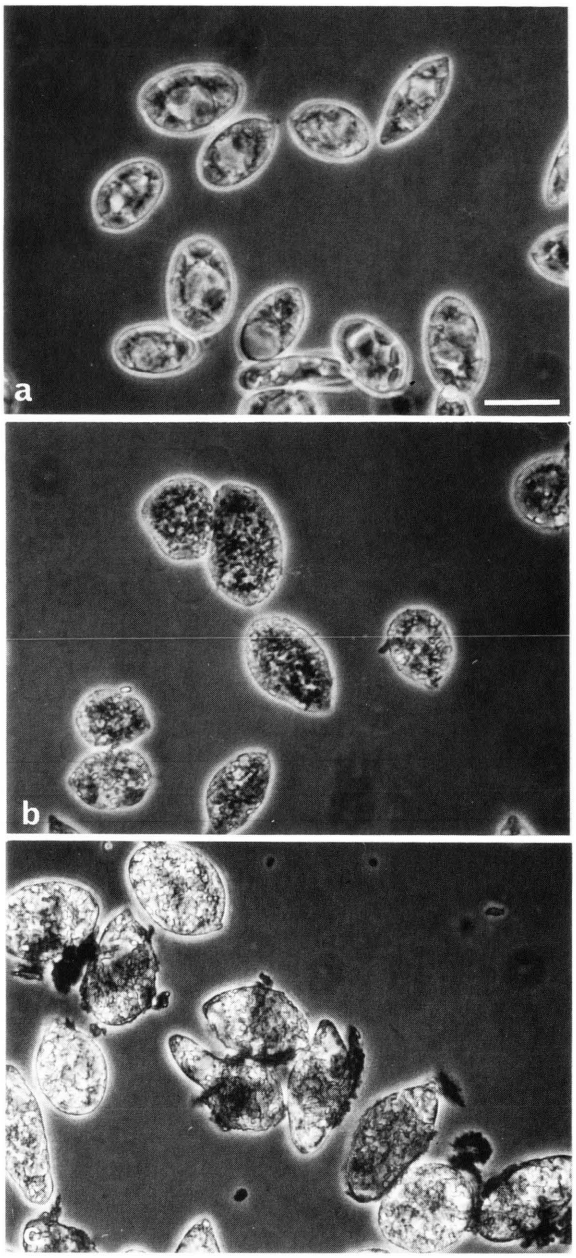

Fig 4. Cells of *E. gracilis* after treatment with Alcian blue dye.
a) control cells, b) Mg^{2+}-free cultures, c) K^{+}-free cultures; extracellular mucilage is stained as outer envelope. Bar: 15 μm.

accepted (Bré and Lefort-Tran, 1984; Bré *et al.*, 1986). During the potassium and magnesium starvation, however, we found an increase of these receptors.

The lectins LCH, specific for mannose, and ConA with its specificity for mannose and, although to a lesser degree, for glucose also labeled control cells definitely. Using ConA and LCH we found the majority of cells with a strong labeling at the flagellum and the reservoir region according to others (Bouck *et al.*, 1978; Vannini *et al.*, 1981; Rogalski and Bouck, 1982; v. Sengbusch and Müller, 1983). A weak labeling, however, was found over the whole cell as well.

The two lectins specific for N-acetylneuraminic acid (Neu5Ac) -a sialic acid- are LPH and WGA. WGA was also tested by v. Sengbusch and Müller (1983), but without gaining a label with any of the *Euglena* strains examined. We found a weak labeling with both lectins, but only when we applied a higher amount of these lectins (200 μg/ml). V. Sengbusch and Müller (1983) generally used lesser concentrations for their investigations (50 μg/ml). So it seems that Neu5Ac, GalNAc and GlcNAc residues are either exposed at the surface of *Euglena gracilis* in a very small amount or are difficult to be reached by the lectins. These findings are in accordance to previous investigations, in which Neu5Ac was found to be a part of a glycosphingolipid in the membrane of *E. gracilis* (Preisfeld and Ruppel, 1995).

Responsible for the differences in labeling might be variant culture and test conditions used by different authors, like cultivation temperature, autotrophical or heterotrophical growth, time of light/dark change and composition of media (Triemer, 1980).

As indicated by growth analysis starvation leads to a reduction of cell division and subsequently to an increase of volume about 150%. So a possible explanation for a stronger labeling of starved cell populations might be that the pellicle is stretched in such a manner that glycoresidues hidden in the grooves are now available for the detection by the lectins. Hofmann and Bouck (1976) examined the intussusceptive surface growth of *Euglena* and found that new surface material is inserted just before mitosis starts. We found a lot of starved cells which were inserting new pellicular strips (see Fig. 5f), probably exposing other sugar residues as the control cells.

Obviously magnesium and potassium starvation becomes perceptible during mitosis before nucleus division. This interpretation is in accordance with

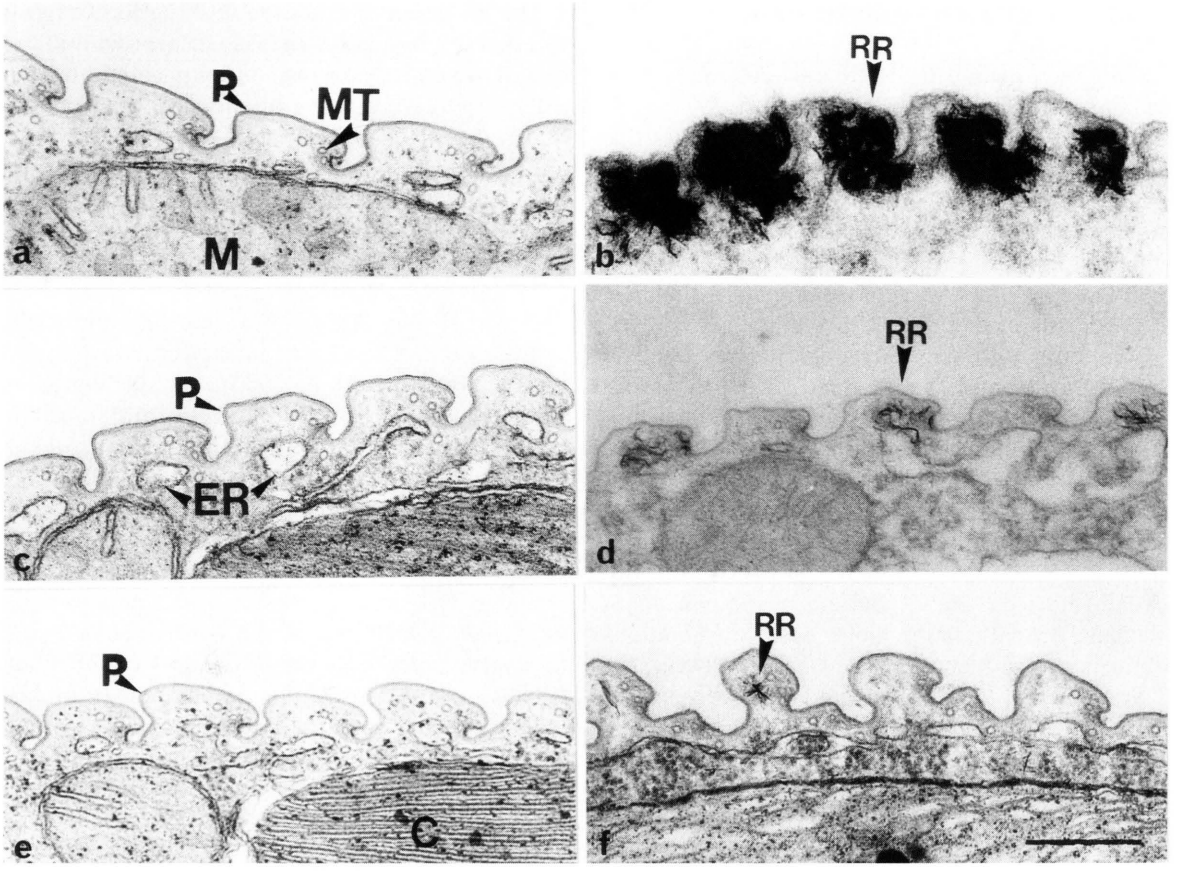

Fig 5: Ultrathin sections of *E. gracilis.*
Control cells a: without RR; b: with RR; Mg^{2+}*-starved cells* c: without RR; d: with RR; K^{+}*-starved cells* e: without RR; f: with RR. C: Chloroplast, ER: Endoplasmatic reticulum, M: Mitochondrion, MT: Microtubules, P: Pellicle, RR: Ruthenium red. Bar: 0.25 μm.

data of growth analysis which points out that inhibition of cell division starts after 24 hrs but increases strongly after a long time. Also protein, carbohydrate and chlorophyll content increase with time of starvation. We suppose that starved cations are available for the cells from an inside pool during the first 24 hrs but after inner cations are consumed starvation works on metabolism. Because cells with two nuclei were not found like described for manganese starved cells (Hilt *et al.*, 1987), we assume that all synthesis necessary for duplication are performed but divison is blocked.

The pattern of glycoprotein does not change during starvation (Scholten-Beck, 1996), but sugar residues increase obviously. It is known that cells under physiological stress tend to expose mucilage (Leedale, 1967, Arnott and Walne, 1967, Triemer, 1980). Our investigations with alcian blue, capable to detect mucilage outside at the surface of *Euglena*, point out a distinct difference between the populations of control and starved cells. None of the control cells show a reaction with alcian blue whereas all starved cells are clearly stained. Cogburn and Schiff (1984) demonstrated anionic groups in the mucilage of *Euglena gracilis* var. bacillaris by histochemical staining with alcian blue. This variety releases a lot of mucilage droplets when cultures reach the stationary phase of growth in contrast to the cells of our culture conditions. Rosowski (1977) found droplets of mucilage between the pellicular strips of *E. gracilis* in small amounts. We also found some staining at the posterior tip of control cells but an overall strong labeling of starved cells.

Ultrastructural investigation of *Euglena* treated with ruthenium red (RR) reveals a strong inside labeling of control cells. RR leads to cytochemical staining of mucilaginous material (Luft, 1971; Strycek et al. 1992). According to our results, this seems due to an accumulation of mucilage which is not secreted yet, located in the cytoplasm beneath the cell membrane. However, a structure which encloses the mucilage could not be found. A difficulty to find these structures was that RR leads to a slight damage of the cells.

Compared with some investigations by Leedale (1967), Arnott and Walne (1967), Buetow (1968) and Triemer (1980) with euglenoid flagellates, mucilage is accumulated beneath the cell membrane in "mucus bodies" thought to be involved in mucilage secretion (Cogburn and Schiff, 1984). Staining with RR has not been tested previously on *Euglena gracilis* strain Z. Our investigations did not reveal "mucus bodies", but an accumulation of mucilage beneath the pellicle. After observations of mucilage production in *Euglena gracilis* var. bacillaris, Triemer (1980) suspected a direct secretion of mucilage from the Golgi apparatus into the reservoir region. Leedale (1967) considered that muciferous bodies are continuous with the endoplasmic reticulum and function in the secretion of mucilage. It might be that the release of mucilage takes place at the side of each ridge where the notches are and in case of existing muciferous bodies they will presumable fuse with the cell membrane. At this point we find the strong precipetate of RR. A precipitate of RR in vesicles of the Golgi apparatus was not found, but we found a lot of cells with a strong precipitate in the reservoir region, too.

The secretion of mucilage and the inhibition of cell division beside an increase of protein and carbohydrate under starving conditions might be considered as the beginning of encystation or palmelloid status (Rosowski and Willey, 1977; Cogburn and Schiff, 1984). Our lectin studies, concerning potassium and magnesium starvation, support this interpretation, as starved cell populations expose more receptors, i.e. sugar residues, to bind lectins, especially at the anterior end of the cells near the reservoir region. Although magnesium and potassium starvation can lead to different physiological effects (Fry and Hall, 1990), the influence on growth parameters, glycoresidues, and mucilage only differs quantitatively. Both deficient cells express more sugar residues, but less mucilage is stained beneath the pellicle. Staining with alcian blue shows an increase of detectable mucilaginous material outside the cell during starvation, whereas RR reveals detectable substances beneath the pellicle inside the control cells. Inside the starved cells only small amounts of RR could be observed. These results are supported by preliminary results on a definite increase of carbohydrate content in the medium and lead to the interpretation that during starvation cells release their mucilage usually "stored" beneath the pellicle.

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