Acta Protozool. (2003) 42: 19 - 29

ACTA
PROTOZOOLOGICA

How Cytotoxic is Zinc? A Study on Effects of Zinc on Cell Proliferation, Endocytosis, and Fine Structure of the Ciliate *Tetrahymena*

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Summary. Addition of 0.5-2.0 mM zinc (Zn^{2+}) to proliferating *Tetrahymena* cultures in 2% proteose peptone (PP) medium induced varying lag periods (~½-4 h) before proliferation resumed at almost normal (0.5, 0.75 mM) or decreased rates (1, 1.5, 2 mM). Initially cells in 1 mM Zn^{2+} moved slowly whereas in higher concentrations a dose-dependent number of cells ceased to move exhibiting rocking movements; the cells recovered, however, and regained motility during the lag period. All Zn-treated cells had small refractive granules which increased in number during the lag period, decreased when cell proliferation resumed, and increased again in non-proliferating cells in high density cultures. The endocytic capacity of the cells was elevated in the low concentrations but suppressed dose-dependently in 1-2 mM Zn^{2+}, concommitantly with an increase in cells not forming vacuoles (up to 80%), corresponding to the immobile cells mentioned above. *Tetrahymena* exposed to Zn^{2+} in dilute, 1% and 0.5% PP, medium showed the same trend of growth pattern and endocytosis as in 2% PP but at lower Zn concentrations. The maximum sublethal concentrations in 0.5%, 1%, and 2% PP growth medium permitting cell proliferation, were 0.2, 1.0, and 2.0 mM Zn^{2+}, respectively, a linear relationship between the amount of organic matter and toxicity. Fine structurally, Zn^{2+}-treated cells had dilated endoplasmic reticulum (induced protein synthesis), electron-dense granules (fused with food vacuoles), and a variety of vesicles, some containing electron-dense dots (Zn?). Apart from the early strong reaction, *Tetrahymena* adapted quickly to excess amounts of Zn^{2+}, perhaps because an ion regulation pathway for Zn^{2+} is already operating in the cells.

Key words: cell motility, cytoplasmic granules, homeostasis, *Tetrahymena pyriformis*, zinc.

INTRODUCTION

Zinc (Zn^{2+}) is an interesting metal nutritionally essential to living organisms in trace amount (μM). The element has multiple intracellular functions as a catalytic or structural constituent of more than 300 enzymes (Vallee and Falchuk 1993, Vallee and Maret 1993) and a cofactor of gene regulatory proteins and transcription factors (Zeng and Kägi 1994). The wide involvement of Zn in biological functions has resulted in an expanding interest in understanding the Zn metabolism.

A deficiency in Zn affects proper functioning of various organs, e.g., the nervous system, skin, intestine (Goyer 1991), and disturbs cell development, differentiation, and cell division (Vallee and Falchuk 1993). The latter disorders affect the immune system, mainly the T-cell population, causing disease advances of, e.g., AIDS, cancer G1 disorder, renal disorder, and aging heightening apoptosis (Fraker and King 2001). All these

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disorders, apart from inherited ones, are reversed by Zn supplementation (Vallee and Falchuk 1993). Excess amounts of Zn does not seem to have any adverse long time effects in humans (Vallee and Falchuk 1993) but whereas some reports indicate that Zn may inhibit apoptosis (Zalewski *et al*. 1991), others suggest that Zn actually induces apoptotic cell deaths (Haase *et al*. 2001, Iitaka *et al*. 2001) and propose Zn as a potential cytotoxic agent in treatment of thyroid cancer (Iitaka *et al*. 2001).

As an essential trace element in living organisms, regulation of the intracellular concentration of Zn^{2+} is of utmost importance. Homeostasis of the metal is mediated by metal-binding proteins and metallothioneins (MTs), small proteins binding heavy metals (Kojima and Kägi 1978, Brady 1982, Cherian and Chan 1993, Vallee and Maret 1993) and found in all organisms (Kojima and Kägi 1978, Piccinni 1989, Cherian and Chan 1993, Vallee and Falchuk 1993). Administration of Zn to rats caused a 3-fold increase in mRNA encoding for MT hence supporting a regulatory involvement of the protein in Zn homeostasis (Shapiro and Cousins 1980). MTs have been studied widely, not least in organisms exposured to cadmium (Cd) which induces synthesis of Cd-Zn metallothioneins (Kägi and Vallee 1960, Vallee and Maret 1993) which also happens in *Tetrahymena* (e.g. Yamagushi *et al*. 1978; Nakamura *et al*. 1981a, b; Piccinni *et al*. 1990), a ciliate used much as a model cell system in cytotoxicology (e.g. Nilsson 1989, Sauvant *et al*. 1999). On exposure to Cd, *Tetrahymena* homogenates contain a 3-fold increased amount of Zn in the supernatant but an unchanged amount in the sediment (Piccinni *et al*. 1990).

The role of MTs is not solely that of detoxication of heavy metals, as they are general regulators of the normal Zn-Cu metabolism in living organisms (Brady 1982, Bremner 1993, Cherian and Chan 1993). MTs are also present in untreated *Tetrahymena* and increase in amount when cell proliferation ceases at high cell density (Santovito *et al*. 2000).

In view of the general importance of understanding how cells regulate the intracellular concentration of Zn^{2+}, the present study was undertaken by exposing *Tetrahymena* to excess amounts of the metal. Early studies on effects of Zn on *Tetrahymena* were reviewed some years ago (Nilsson 1989). It was revealed that a high concentration of Zn^{2+} was tolerated by cells in media with a high content of organic matter, in fact, 100-fold higher in 2% proteose peptone (PP) than in distilled water (cf. Nilsson 1989). Moreover, Zn accumulated in small cytoplasmic granules (Dunlop and Chapman 1981, Jones *et al*. 1984). Such small refractive granules appear commonly in *Tetrahymena* under stress conditions and when growth ceases due to high cell density; they have been proposed to play a role as intracellular ion regulators in detoxication of heavy metals (cf. Nilsson 1976, 1989). Yet, how cytotoxic is Zn^{2+} really?

The present study describes effects of Zn^{2+} on cell proliferation, rate of endocytosis, and fine structure of *Tetrahymena* in proliferating cultures. The findings are discussed in relation to published literature on effects of Zn on the organism. The data show good agreement with previous findings but additional new information is reported, especially on the early reaction of cells to excess Zn.

MATERIALS AND METHODS

Tetrahymena pyriformis GL was grown axenically at 28°C in 2% proteose peptone (PP) enriched with 01% yeast extract and inorganic salts, including 0.4 µM Zn (Plesner *et al*. 1964). The experimental 100-ml cultures in 500-ml Fernbach flasks were agitated and aerated. Two cultures in exponentiel growth (20-40,000 cells/ml) were mixed and divided into 50-ml cultures of which 3 cultures received zinc chloride (stock: 100 mM) in an equal volume of distilled water to establish different Zn^{2+} concentrations, and the last culture, the control, received the same amount of water without Zn^{2+}. When addition of the metal decreased the pH of the growth medium (pH 7.0), pH was readjusted with NaOH. To test the effect of Zn^{2+} in reduced amounts of organic matter, cultures (50-80,000 cells/ml) were diluted with 10 mM Hepes buffer (pH 7.0) to obtain media of 1% and 0.5% PP, respectively; the dilution was made 1 h before testing. All cultures were followed for a 24-h period.

In vivo observations were made at low power microscopy of freely swimming cells (for cell shape and motility) and at high power of compressed cells (for cellular details) using a Reichert microscope with anoptral optics.

The cell density of the cultures was determined electronically (Coulter Multisizer II, Counter Electronics Ltd., England). Triplicate 0.5-ml cell samples were withdrawn from the cultures at hourly intervals and fixed with an equal volume of 1% glutaraldehyde in phosphate buffer (pH 7.2). The cell samples were diluted with 0.45% NaCl prior to counting.

The endocytic capacity was determined by a 10-min exposure of 2-ml cell samples to 2 ml carmine particles (0.4 mg/ml) suspended in the same medium as that of the cells. At the end of exposure, the cell suspension was fixed in 4 ml 1% buffered glutaraldehyde. After washing the cells, the number of labelled food vacuoles was counted in 100 cells (Nilsson 1976). The endocytic capacity of treated populations was expressed as the percent of that of control cells (100%).

For electron microscopy, 4-ml cell samples were fixed for 10 min in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2). The cells were washed in buffer and postfixed for 1 h in buffered

1% osmium tetroxide, washed, dehydrated in a graded series of ethanol, and embedded in epon. The sectioned material was contrasted with uranyl acetate and lead citrate before examination in a Zeiss EM 109 electron microscope.

RESULTS

Normal 2% proteose peptone medium

Addition of 0.5-2.0 mM Zn^{2+} to the medium affected growth of *Tetrahymena* by inducing a dose-dependent lag period before cell proliferation resumed (Fig. 1). After the lag period, cells in 0.5 and 0.75 mM Zn^{2+} resumed proliferation at almost normal rate, whereas cells in 1.0, 1.5, and 2.0 mM Zn^{2+} exhibited decreased rates of proliferation. After 24 h the cells in 0.5-1.0 mM Zn^{2+} had reached the maximum high cell density of control cultures (~ 1,000,000 cells/ml) and cells in 1.5 mM Zn^{2+} a little less (~ 700,000 cells/ml), whereas cells in 2 mM Zn^{2+} doubled 3 times only (~ 300,000 cells/ml).

In vivo observations revealed an initial dose-dependent effect of Zn^{2+} on cell motility. Visually swimming of cells in 0.5 and 0.75 mM was unaffected but in 1 mM their rate became more leisurely (~½ h) and some cells became immobile (~1 h); however, in 1.5 and 2 mM a high fraction of the cells (up to 80% in 2 mM Zn^{2+}) became motionless (with rocking movements) and a few cells had enlarged contractile vacuoles and were spherical of shape, yet a few cells behaved normally. Within the lag period, however, the cells had restored shape, regained motility, and were swimming normally before cell proliferation resumed. A fluffy precipitate formed within 1 h after addition of 2 mM (Fig. 3) and it was ingested by the cells; moreover, sometimes a few dead cells (< 0.1 %) were observed which indicates that 2 mM Zn^{2+} is a critical concentration, i.e. the highest Zn^{2+} concentration permitting cell proliferation in this medium.

The endocytic capacity of *Tetrahymena*, i.e. to form food vacuoles, was affected dose-dependently by Zn^{2+} (Fig. 2). The rate of endocytosis (closed symbols) was elevated in 0.5 and 0.75 mM Zn^{2+} but decreased in 1-2 mM Zn^{2+}, independently of a 1- or 3-h exposure. Whereas about 10% of the control cells (division stages) in proliferating populations do not form food vacuoles (e.g. Nilsson 1989), the number of cells not forming food

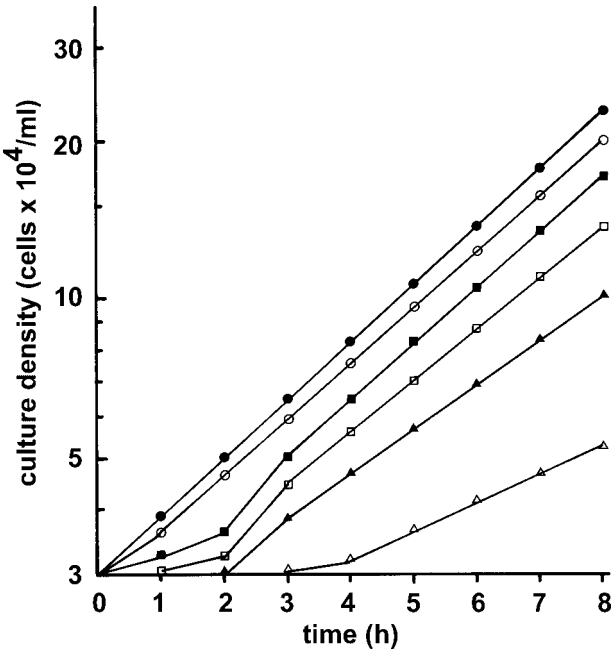


Fig. 1. Effect of Zn^{2+} on the growth rate of *Tetrahymena* in the normal 2% PP medium: control (closed circle), 0.5 mM (open circle), 0.7 mM (closed square), 1.0 mM (open square), 1.5 mM (closed triangle), 2.0 mM (open square). Means of 5-10 experiments per concentration.

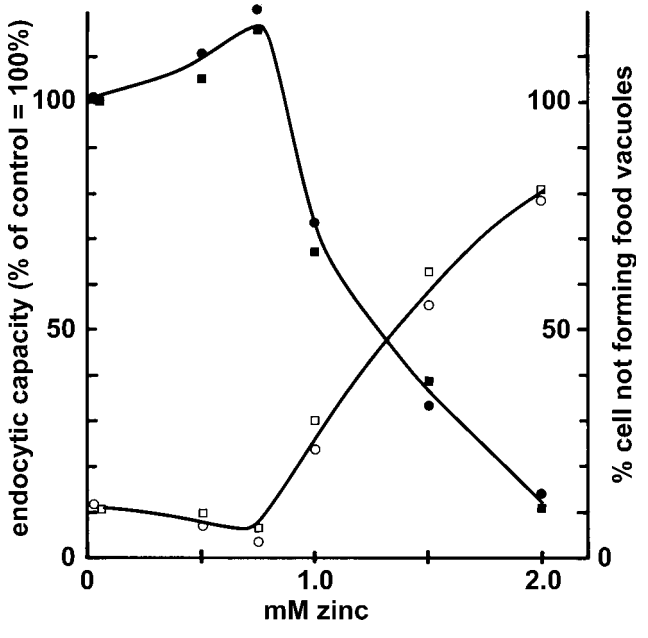
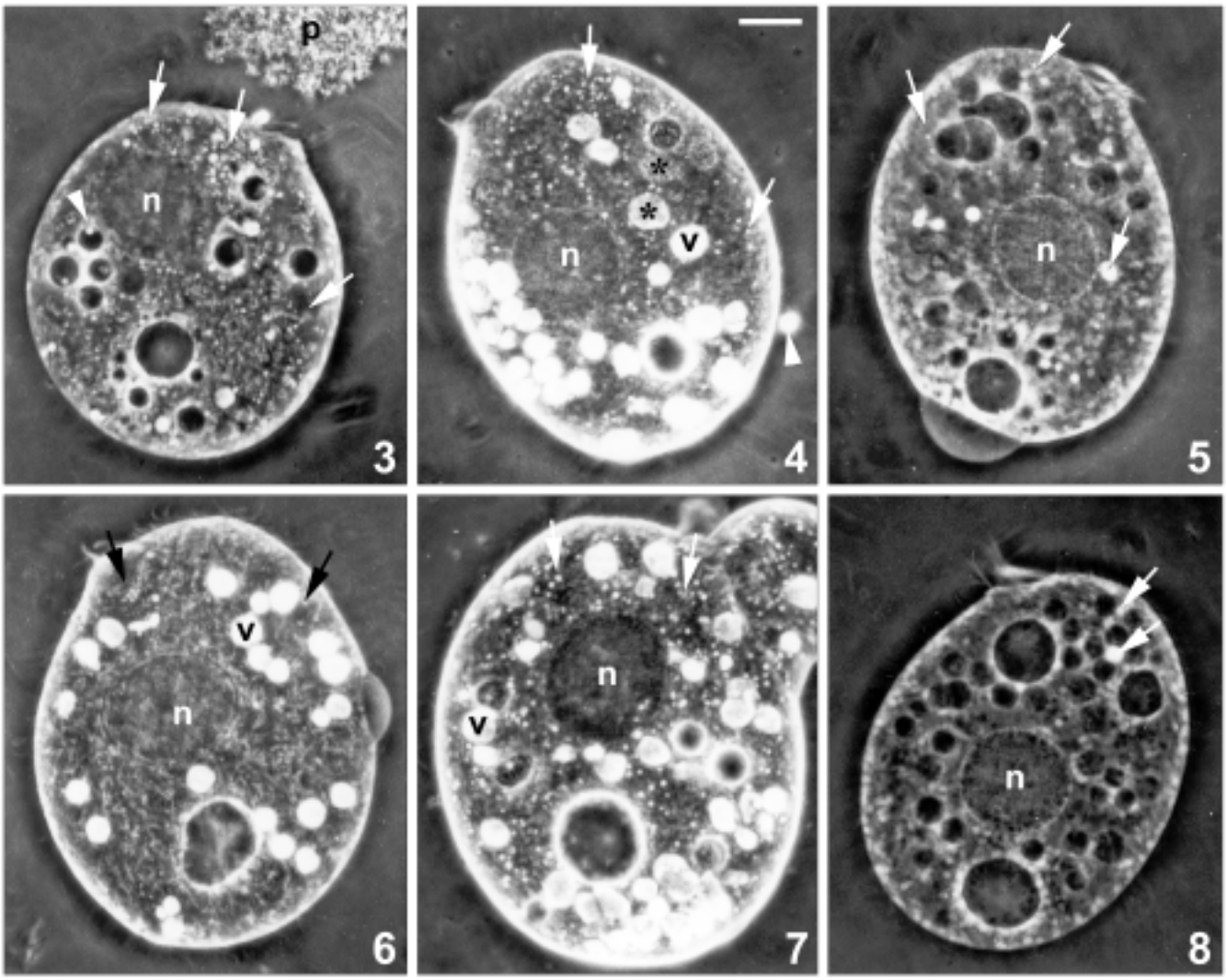


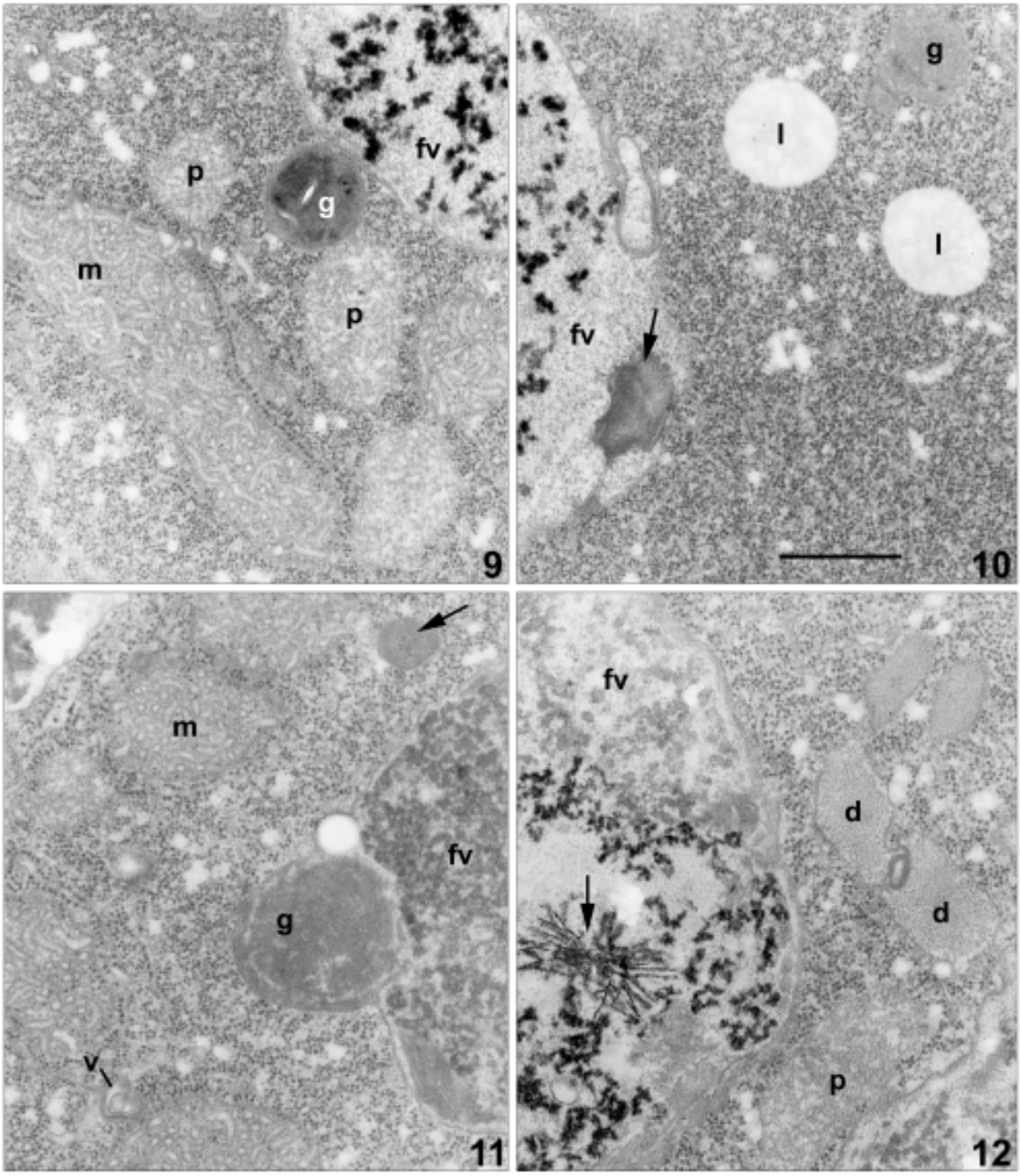
Fig. 2. Effect of a 1- (closed circle) and 3 h - (closed square) exposure to Zn^{2+} (abscissa) on the endocytic capacity of the *Tetrahymena* population (left ordinate, closed symbols) and on the fraction of cells not forming food vacuoles (right ordinate, open symbols). Normal 2% PP medium. Means of 5-10 experiments per concentration.



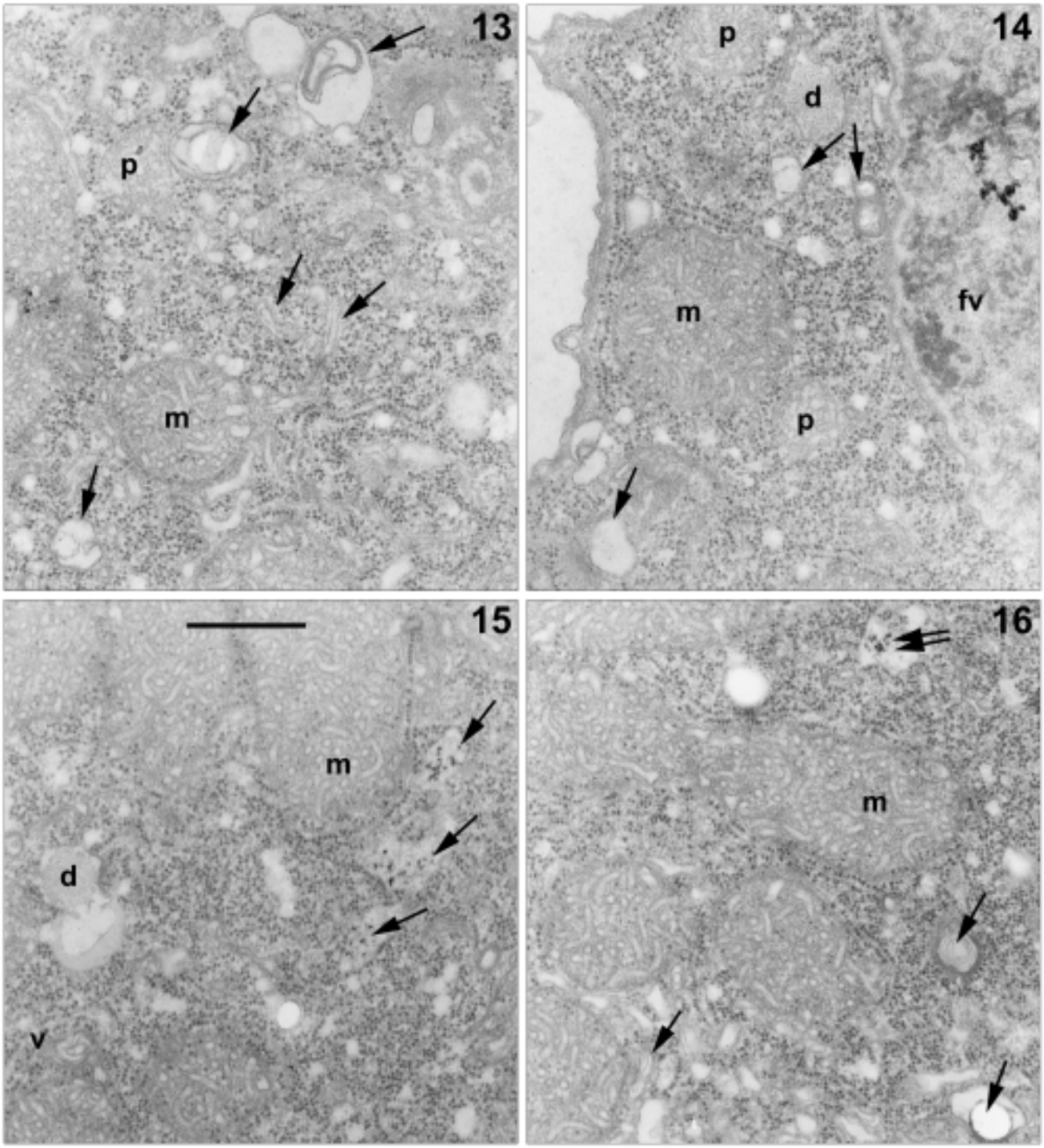
Figs 3-8. Appearance, and turnover, of small refractive granules in *Tetrahymena* (compressed *in vivo*) exposed to Zn in the normal 2% PP; (Figs 3-6) and dilute 1% PP (Fig. 7) growth medium as compared to untreated control (Fig. 8). **3 -** after 1 h in 2 mM Zn the cell contains many small granules (arrows), some have fused with food vacuoles (arrowheads); outside cell, a patch of Zn-PP precipitate (p); **4 -** after 5 h in 2 mM Zn cell contains many small granules (arrows) but also refractive food vacuoles (v) some of composite nature (star) as formed after fusion with granules, a defecation ball (arrowhead) outside cell; **5 -** after 5 h in 0.5 mM Zn, the population proliferates and the cell has few small refractive granules (arrows) but also some larger ones; **6 -** after 5 h in 1 mM Zn the proliferating cell has few small granules (arrows) but still refractive food vacuoles (v); **7 -** after 5 h in 1 mM Zn in 1% PP medium the cell contains numerous small granules (arrows), as well as refractive food vacuoles (v), resembling cell in 2 mM Zn in the normal 2% PP medium (Fig. 4); **8 -** the 5-h control cell has initiated granule formation due to high population density: small refractive granules and a single larger one (arrows). Nucleus (n). Scale bar 10 µm.

vacuoles during the 10-min test increased markedly in high Zn^{2+} concentrations (open symbols); these cells were not in division but in number they correspond to that of the immobile cells mentioned above. Interestingly, cells forming food vacuoles did so at the normal rate, i.e. up to 6 vacuoles/10 min (cell cycle stage dependent); the finding indicates a close relationship between cell motility and capacity for endocytosis. By 24 h all Zn-treated cells had recovered and formed food vacuoles at the same rate as that of control cells.

Conspicuously, all Zn-treated cells contained small refractive granules which accumulated during the lag period (Figs 3, 4). The granules remained present at a low value in proliferating cells but increased again in number at high cell density (Figs 5, 6). Such small refractive granules also appear in control cells when



Figs 9-12. Turnover of dense (refractive) granules in *Tetrahymena* exposed to 1.5 mM Zn^{2+} for 1 h (Figs 9, 10) and 5 h (Fig. 11) and to 1.0 mM Zn^{2+} for 3 h (Fig. 12) in 2% PP medium. **9** - dense granule (g) close to a food vacuole (fv) indicative of near fusion; **10** - opposite side af the same food vacuole (fv) as shown in Fig. 9 containing a laminated structure (arrow) indicative of completed fusion with a dense granule, in the cytoplasm a less dense granule (g) and lipid droplets (l), growth inhibited related structures; **11** - after 5 h in 1.5 mM Zn^{2+} cell proliferation has initiated (see Fig. 1) and a granule (g) is fusing with a food vacuole (fv), electron-dense material (arrow) within the grazing section of another granule and a vesicle (v) with laminated substructure; **12** - part of food vacuole (fv) in cell after 3 h in 1 mM Zn^{2+} containing electron-dense material after uptake of Zn-PP precipitate, note the crystal-like structure (arrow), in the cytoplasm delated endoplasmic structures (d). Mitochondria (m), peroxisomes (p). Scale bar 0.5 µm.



Figs 13-16. Cytoplasmic structures possibly involved in lowering and controlling the intracellular Zn^{2+} concentration in *Tetrahymena* exposed for 3 h to 1 mM (Figs 13, 14) and for 5 h to 1.5 mM Zn^{2+} (Figs 15, 16) in 2% PP medium. **13** - various vesicles (arrows) and tubular structures believed to be accumulating the metal; **14** - dilated endoplasmic structure with amorphous material (d), laminated structure, and vesicles (arrows) with fine dense dots (Zn?); **15** - dilated structures (d) with distinct dense (Zn?) material (arrows), vesicle with laminated structure (v); **16** - dilated structure with dense (Zn?) material (double arrow), vesicles with laminated contents or "empty" ones and tubular structures (arrows). Mitochondria (m), peroxisomes (p). Scale bar 0.5 µm.

proliferation ceased due to high cell density (Fig. 8). The early appearance of the granules on exposure to excess Zn indicates a rapid entry of the metal, increasing the intracellular concentration, followed by an efficient mechanism of removal or capture of the ions. The later decrease in number of granules before proliferation is resumed, seems to relate to an apparent fusion of granules with food vacuoles (e.g. Figs 3, 4), giving rise to a refractive content of these, unrelated to ingestion of the fluffy precipitate, and in turn to extrusion via refractive defecation balls (Fig. 4) which accummulated with time at the bottom of the cultures.

Dilute (1.0, 0.5%) proteose peptone media

In order to establish a relationship between the tolerated concentration of Zn^{2+} and the amount of organic matter, the normal growth medium (2% PP) was diluted to 1.0 and 0.5% PP, respectively. In these media Zn^{2+} was tolerated only at lower concentrations by *Tetrahymena* than in the 2% PP medium but otherwise the effects on the cells followed the same trends (data not shown) as shown for cells in 2% PP (Figs 1, 2).

In 1% PP, 0.1 mM Zn^{2+} affected cell proliferation only slightly (like 0.75 mM Zn^{2+} in 2% PP). A concentration of 0.5 mM Zn^{2+} induced a 3-h lag period before proliferation resumed (almost like 1.5 mM in 2% PP). In 1.0 mM Zn, a 5-h lag period was induced but even though the cells were affected more (Fig. 7) than cells in 2 mM Zn^{2+} in 2% PP (Fig. 4), the cell population had doubled twice by 24 h. Hence in 1% PP medium, 1.0 mM Zn is considered the highest tolerated concentration.

In 0.5% PP, 0.1 mM Zn^{2+} induced a 2-h lag period before the cells started to proliferate (like in 1 mM Zn^{2+} in 2% PP). In 0.2 mM, the cells exhibited a 4-h lag period before proliferation resumed (almost like 2 mM Zn^{2+} in 2% PP) but the percentage of immobile cells was high during the lag period. Cells in 0.5 mM Zn^{2+} were affected severely and many had grossly enlarged contractile vacuoles and were spherical of shape and some cells died; the population had not increased in density after 24 h but the cells had regained motility and normal shape. The highest Zn^{2+} concentration permitting cell proliferation in the 0.5% PP medium was then 0.2 mM.

In both dilute media *Tetrahymena* was affected by Zn^{2+} in the same manner as that described above for cells in the normal 2% PP medium but at respective lower concentrations. With respect to the effect of Zn^{2+} on cell motility, appearance of the small refractive granules (see Figs 4, 7), and on the capacity of cells to form food vacuoles, all findings showed the same trends as those described above for cells in 2% PP medium but at the respective, low concentrations of Zn^{2+}.

Fine structure

Fine structurally, Zn-treated cells showed signs of suppressed growth initially in the high concentrations as reflected in some change in nucleolar structure (not shown), appearance of lipid droplets, glycogen islands, and not least of small, electron-dense granules (Figs 9-11). The dense granules were especially numerous early on exposure to Zn^{2+} during the lag period; however, already at this stage, and throughout exposure to Zn^{2+}, the granules appeared to fuse with food vacuoles and remnants of their contents appeared within the food vacuoles (Figs 9-11) which contained varying amounts of electron-dense (Zn?) matter (Figs 9-10, 12) after ingestion of soluble Zn or Zn-containing precipitate. The finding indicates an intracellular turnover of the granules and thereby elimination of accumulated Zn through defecation as suggested light microscopically (Fig. 4). Moreover, the cells had dilated endoplasmic reticulum, indicative of induced protein synthesis (Figs 12, 14) and an abundance of various small vesicles and tubular structures (Figs 13-16), some with electron-dense dots (Zn?), not seen in the control cells. With time in Zn, some of these structures (Figs 15, 16) contained distinct electron-dense material (Zn?), resembling the contents of food vacuoles (Figs 9-10, 12) in which electron-dense material, even crystal-like structures (Fig. 12), could derive from ingestion of the fluffy Zn-containing precipitate formed with the PP medium at high Zn^{2+} concentrations.

DISCUSSION

The present findings on how excess amount of Zn^{2+} affects growth of *Tetrahymena* are in good agreement with earlier reports (summerized in Table 1) using growth media of 2% proteose peptone (PP) (Jones *et al*. 1984, Larsen and Svensmark 1991) and 1% PP (Dunlop and Chapman 1981). The unusual short lag periods noted in the present study in up to 1 mM Zn^{2+}, indicate a moderate effect only on, or high adaptation of, the cells as they resume growth at almost normal rate. The phenomenon is likely to relate to Zn^{2+} being an essential metal for which the cells already have a regulatory mechanism. The present findings confirm also that toxicity of Zn^{2+} increases when the amount of organic

Table 1. Effects of zinc chloride on *Tetrahymena.*

Medium	Zn^{2+} amount	Lag/growth rate	Specific remarks	Reference
2% PP	0.5 mM	no inhibition	granules (Zn)	Jones *et al*.1984
	1.0 mM	reduced rate	granules (Zn)	
	5.0 mM	kills cells/5 days	granules (Zn)	
2% PP	0.5 mM	unaffected	granules	Larsen and Svensmark 1991
	1.0 mM	2-h/decreased	granules	
	2.0 mM	5-h/decreased	granules	
2% PP	0.5 mM	~½-h/unaffected	granules	Present study
	0.75 mM	~1-h/affected	granules	
	1.0 mM	2-h/decreased	granules	
	1.5 mM	~2-h/decreased	granules/immobility	
	2.0 mM	4-h/decreased	granules/immobility	
1% PP	94 µM	undisturbed	granules (Zn)	Dunlop and Chapman 1981
	0.9 mM	decreased	granules (Zn)	
	1.8 mM	kills all cells		
1% PP	0.1 mM	~1-h/decreased	granules	Present study
	0.5 mM	3-h/decreased	granules/immobility	
	1.0 mM	>5-h/recovery	granules/immobility	
0.5% PP	0.1 mM	2-h/decreased	granules/immobility	Present study
	0.2 mM	5-h/decreased	granules/immobility	
	0.5 mM	24-h/ceased	granules/immobility	
0.2% PP	130 µM	50% inhibition		Yamagushi *et al*. 1973
	1.0 mM	growth ceases		
Tap water	~4 µM	4-h survival		Ruthven and Cairns 1973
	19 µM	kills in10 min		
Distill. w.	20 µM	96-h survival		Carter and Cameron 1973
	40 µM	24-h/30% mortality		
Hard w.*	0.4 mM	8-h LD_{50}		Chapman and Dunlop 1981
IM**	8 µM	1-h LD_{50}		Svensmark and Larsen 1988

*Hard water - 500 ppm of Ca^{2+} (12.5 mM), Mg^{2+} (20.6 mM), or Ca^{2+} + Mg^{2+} (16.5 mM); **IM - inorganic salts (Plesner *et al*. 1964).

matter in the medium (2-0.5% PP) decreases (Table 1). Tolerance is low in the absence of organic matter (~20 µM Zn^{2+}) (Carter and Cameron 1973, Ruthven and Cairns 1973, Svensmark and Larsen 1988) but increases (Table 1) in presence of high amounts of inorganic calcium (Ca) and magnesium (Mg) salts (Chapman and Dunlop 1981). The protective power of organic matter may relate to the high affinity of Zn for forming more or less labile complexes with ligands, such as, amino acids, peptides, and various proteins (Albert 1981, Vallee and Falchuk 1993). In 2% PP medium, the amounts of 0.5, 1, and 2 mM Zn^{2+} found as free ions or very labile complexes, were 17, 15, and 11%, respectively, (Larsen and Svensmark 1991); the decreasing percentage may relate to formation of the fluffy precipitate seen at high Zn^{2+} concentrations. The Zn-PP precipitate was preferentially ingested by the cells which then became exposed to a higher (unknown) amount of the metal than that added to the medium.

Metallothionein (MT) is an important protein which binds Zn as already mentioned. The amount of MT is low in proliferating *Tetrahymena* but increases when growth ceases in old cultures (Santovito *et al*. 2000). Although it remains to be established, MT is expected to increase markedly in *Tetrahymena* exposured to excess Zn, as happens in other organisms (Shapiro and Cousins 1980, Cherian and Chan 1993), and when the ciliate is exposed to Cd (Piccinni *et al*. 1990). With a high binding affinity for Zn, 7 Zn atoms per MT-molecule (e.g. Vallee and Falchuk 1993), MT may act as a modulator as it captures and releases Zn readily (Brady 1982, Cherian and Chan 1993, Vallee and Maret 1993). In the cytoplasm MT may function by releasing Zn when needed (Brady 1982, Bremner 1993, Cherian and Chan 1993), whereas storage of Zn would be expected to be within membrane-bound compartments.

Tetrahymena exposed to Zn^{2+} had small refractive granules as also reported by other investigators (see

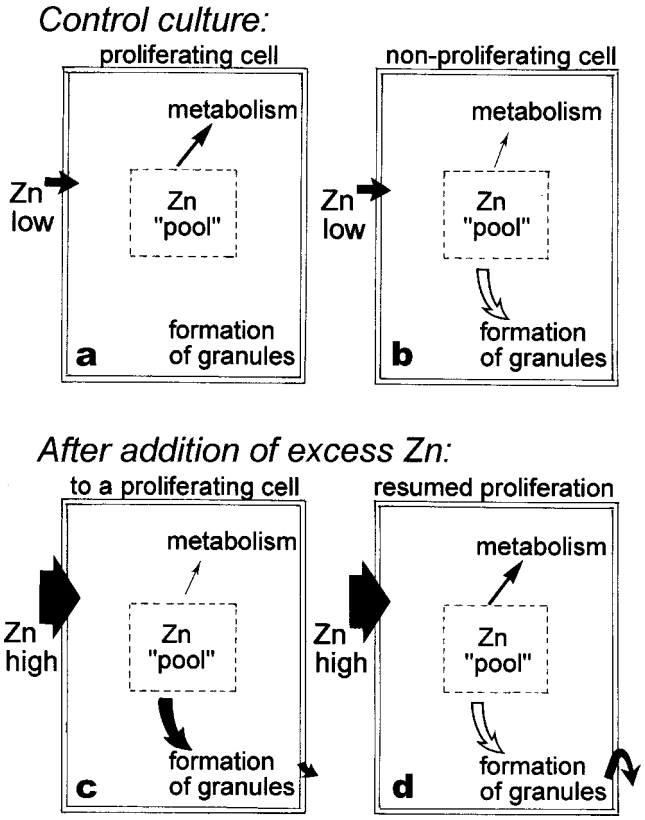


Fig. 17. Proposed intracellular state of Zn homeostasis in *Tetrahymena* assuming a constant intracellular "Zn pool". Control cells at low external Zn^{2+} concentration (a, b) and proliferating cells exposured to excess amounts of Zn^{2+} (c, d). **a** - proliferating control cell, Zn^{2+} enters plasma membrane in balance with an intracellular "pool" of available Zn^{2+} and drainage of remaining Zn^{2+} through metabolism; **b** - non-proliferating control cell: entry of Zn^{2+} through plasma membrane is unchanged but intracellular drainage through metabolism is decreased, excess Zn will be exported through pathway of granule formation for storage as a minor component with Ca, Mg, K, and P; **c** - on exposure of proliferating cells to excess Zn^{2+}, massive entry of Zn^{2+} through plasma membrane rising the intracellular concentration (causing immobility), less Zn is drawn through metabolism (proliferation ceased), the inbalance activates the pathway to granule formation where Zn may become a major cation in the refractive granules, some Zn is passed out of the cell *via* defecation balls, the chaotic state is stabilized during lag period; **d** - end of lag period with restored homeostasis, massive entry of Zn^{2+} through plasma membrane unchanged, intracellular "Zn pool" restored, drainage through metabolism increased to normal as cells proliferate, but not enough to eliminate the pathway of granule formation completely, turnover of accumulated Zn *via* defecation. - When high cell density has been reached the situation resembles that of (b) but with large increase in number of granules.

Table 1). The granules increased in number during the lag period and remained present throughout the exposure to Zn^{2+}. However, when cell proliferation resumed, the number of granules decreased, indicating a turnover, but it increased again at high cell density when cell proliferation ceased. The small refractive granules are not seen in proliferating control cells but they appear normally in *Tetrahymena* in high density cultures when cell proliferation is decreasing (Rosenberg 1966; Munk and Rosenberg 1969; Nilsson 1976, 1989), a cyclic event. The granules contain Ca, Mg, potassium (K) and phosphorus (P) (Rosenberg 1966) with a constant cations/P ratio in an organic matrix (Coleman *et al.* 1972) and they have been proposed to be involved in the intracellular ion regulation as storage sites for excess metals. The cyclic appearance/disappearance of the granules relates to the activity of an associated pyrophosphatase with an optimum at pH 6.0 which is different from the optimum of pH 7.5 of a soluble pyrophosphatase in *Tetrahymena* (Munk and Rosenberg 1969). In the presence of excess Zn, the refractive granules also contained Zn in addition to Ca and P (Dunlop and Chapman 1981) and Mg and K (Jones *et al.* 1984) and after 24 h, Zn became the major cation with Ca and Mg as minor components (Jones *et al.* 1984). As would be expected, Zn was also detected in minor amount in granules in old control cells (Dunlop and Chapman 1981). In the present study, the dense granules fused with food vacuoles, hence any Zn accumulated within them, would together with ingested Zn, become defecated from the cells; this finding indicates a constant turnover of accumulated Zn in *Tetrahymena* exposed to excess amount of Zn. In humans several Zn-transporter proteins (ZnTs) have been identified to be involved in Zn efflux (ZnT1), in vesicular sequestration of the cation (ZnT2, ZnT3), and in export of Zn (ZnT4) out of cells (Milon *et al.* 2001). Moreover, in human HeLa cells excess Zn accumulates in intracellular vesicles (Chimienti *et al.* 2001) like the granules in *Tetrahymena*. Whether specific Zn transporter proteins are involved in Zn metabolism in ciliates remains to be shown.

The initial, dramatic effect of high Zn^{2+} concentrations on motility of *Tetrahymena* has not been reported previously. Immobility, associated with rocking movements of the cells, was a gradual effect developing within ~0.5 h and strongly indicates an interference of Zn with Ca metabolism. Availability and change in the intracellular ionic Ca concentration play an important role in proper functioning of ciliary movement (Machemer 1988). In a related ciliate *Paramecium* fixed while actively swimming, excess Zn deposited at high Ca sites (Fischer *et al.* 1976), a finding suggesting entry of Zn^{2+} through Ca^{2+} gates. In *Tetrahymena* a cation channel for K^+ and Ca^{2+} has been isolated from ciliary membranes and the channel was found to be permeated also by other cations (Oosawa *et al.* 1988); Zn was not

tested but it is likely to enter the cells *via* these Ca^{2+} channels. That Zn interferes with Ca metabolism is also indicated by suppressed uptake of ^{45}Ca in *Tetrahymena* exposured to 0.5 and 1 mM Zn^{2+} (Jones *et al*. 1984). Moreover, in rat liver cells 0.5 mM Zn^{2+} stimulates the activity of Ca, Mg ATPases in plasma and lysosomal membranes (Adachi *et al*. 1996), and Zn enters rat neurons through Ca channels (Büsselberg 1995). Inactivation of cilia, i. e. cell immobility, is Ca-dependent and induced by membrane depolarization which causes a slight rise in the intraciliary Ca concentration (Machemer 1988). It seems likely that the immobility induced in *Tetrahymena* by exposure to excess Zn, is caused by massive entry of the metal through Ca channels in the ciliary membrane whereby Ca homeostasis is disturbed, causing a slight rise in the intraciliary Ca^{2+} concentration whereby ciliary beating is inactivated. The cells recovered, however, and regained motility during the lag period, i.e. they adapted to the high external Zn concentration, indicating restoration of Zn homeostasis.

In proliferating cultures *Tetrahymena* is assumed to have an established Zn homeostasis. In homeostasis, a balance exists between the amount of Zn entering the cell and a constant cytoplasmic "pool" of available Zn, ionic and temporary in binding proteins, and drainage of the metal through metabolism as proposed in Fig. 17a. When proliferation ceases, the same amount of Zn will enter the cell but as less Zn is drawn through metabolism, excess Zn is directed through the pathway of granule formation (Fig. 17b) where it is stored together with Ca, Mg, K and P in small refractive granules. In excess amount of Zn, the element enters the cell massively which disturbs homeostasis causing an increase in the intracellular Zn^{2+} concentration, cell proliferation ceases so less Zn is drawn through metabolism, and the increased Zn^{2+} concentration is lowered by activating (induced protein synthesis?) the pathway to granule formation (Fig. 17c). Once a new balance is established between entry of Zn and the intracellular handling of the metal, the cells will proliferate and drainage of Zn through metabolism will return to previous value (Fig. 17a) but not enough to eliminate the pathway of granule formation (Fig. 17d), i.e. cells proliferating in excess Zn will still have granules, but in a lower number than during the lag period (Fig. 17c) because accumulated Zn is turned over and defecated (Fig. 17d). Likewise, when the culture reaches high cell density, proliferation ceases and less Zn is drawn through metabolism so the number of granules increases in the non-proliferating cells. In fact, if non-proliferating cells, i.e. active in granule formation (Fig. 17b), were exposed to excess Zn, they would probably adapt more quickly with less effect on cell motility than that observed in the present study.

In conclusion, proliferating *Tetrahymena* exposed to high sublethal amounts of Zn^{2+} experiences a major hazard initially. Due to a massive entry of Zn, cell motility is affected but once the cells have circumvented the hurdle and restored homeostasis (during the lag period), they can resume proliferation. Hence, apart from the initial hazard, excess amount of Zn has no adverse long time effects on *Tetrahymena*, only a somewhat prolonged generation time, i.e. the metabolic cost of the intracellular handling of excess Zn.

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Received on 17th July, 2002; accepted on 2nd October, 2002