

Effects of Pluronic F-68 on *Tetrahymena* cells: protection against chemical and physical stress and prolongation of survival under toxic conditions

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Abstract

The effects of the non-ionic surfactant Pluronic F-68 (0.01% w/v) ¹ on *Tetrahymena* cells have been studied. A marked protection against chemical and physical stress was observed. The chemical stress effects were studied in cells suspended in buffer (starvation) or in buffers with added ingredients from a chemically defined medium (Ca²⁺, Mg²⁺, Na⁺, K⁺, trace metal ions). The physical stress was due to mechanical stress or hyperthermia. The data show that Pluronic: (a) prolongs the survival of low concentration cell suspensions during starvation; (b) prevents the cell death caused by low concentrations of Ca²⁺ (70 μM); (c) prolongs the survival of cells exposed to higher ion concentrations (10 mM Ca²⁺, or Na⁺ or K⁺); (d) postpones the death caused by trace metal ions like Zn²⁺, Fe³⁺ and, Cu²⁺; (e) protects cells from the death caused by shearing forces; and (f) prolongs the survival of cells exposed to hyperthermia (43°C). The cellular survival is increased at reduced temperatures (e.g. 4°C instead of 36°C) and at increased cellular concentrations (e.g. 100 cells ml⁻¹ instead of 25 or 10 cells ml⁻¹). There is no effect of pre-incubation with Pluronic. The protective effect of Pluronic towards *Tetrahymena* is observed for concentrations in the range from 0.001 to 0.1% w/v. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Cell death; Physical stress; Chemical stress; Surfactants

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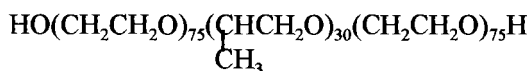
¹ Pluronic (0.01%) prolongs the survival of cells transferred from one medium to another (chemically defined medium (CDM) to HEPES, CDM to CDM diluted 100 times in water, CDM to broth medium (PY)). This probably means that it is also a protectant against cell death caused by osmotic pressure.

1. Introduction

Previously we have described a novel form of cell death in the unicellular ciliate *Tetrahymena* called interface-related death (Kristiansen et al., 1996). It occurs at the medium–air interface when cells are cultured at low inocula in a chemically defined medium (CDM). When the interface is removed by using completely filled tubes or when tubes are placed horizontally, this form of death is no longer observed. The addition of growth factors/hormones such as NGF (10^{-11} M) and insulin (10^{-7} M) or the addition of detergents/surfactants like SDS (0.002% w/v), NP-40 (0.001% w/v) and Pluronic F-68 (0.0001% w/v) prevent the interface-related death.

Recently, we found that Pluronic (0.01% w/v) has a strong influence on the survival period of low inocula (25 cells ml⁻¹) in HEPES buffer with and without additions (Hagemeister et al., 1999). When Ca²⁺ and Mg²⁺ were added in concentrations as in CDM, the survival period at 36°C is shortened from 4 to 2 days. However, the addition of Pluronic (0.01%) completely abolished the effect of these ions. When the trace metal ions of the medium (Fe³⁺, Mn²⁺, Co²⁺, Zn²⁺, Cu²⁺ and molybdate) were tested, they reduced the survival period when added individually. A mixture of these ions reduced the survival period at 36°C from 4 to 0 days. However, the simultaneous addition of Pluronic (0.01%) allowed the cells to survive 1 day (Hagemeister et al., 1999). Thus, Pluronic seems to be a substance with numerous positive effects with respect to cell survival.

Pluronic F-68, also called Pluronic PE 6800 (BASF Technical Information, 1989), is a low-foaming, non-ionic surfactant consisting of block polymers in which a central polypropylene oxide group is flanked by two polyethylene oxide groups:



Pluronic F-68 has a molecular mass of 8350 Daltons. It is a white powder, easily soluble in water resulting in a neutral pH. The percentage of

polypropylene oxide (% hydrophobicity) is about 20. It disperses calcium and magnesium salts (BASF Performance Chemicals, 1988; BASF Technical Information, 1989). The critical micelle concentration (CMC) is around 1.1 mM (Batrakova et al., 1998). Pluronic block polymers with higher hydrophobicity than F-68 such as L-81 and P-85 have CMC values of 24 and 67 μM, respectively. These polymers increase the accumulation of P-glycoprotein-dependent drugs (P-gp) in mammalian cells through inhibition of the P-gp-efflux system (Batrakova et al., 1998). Pluronic F-68 does not act by creating micelles, instead two to three layers of the block polymer attaches to the particle surface hereby effectively preventing aggregation (BASF Performance Chemicals, 1988; BASF Technical Information, 1989). Hitherto, the main use of Pluronic F-68 in cell biology is to control shear forces in dynamic culture situations (shaker and spinner cultures). Thus, Ramirez and Mutharasan (1990) suggested that the protective mechanism of Pluronic F-68 (0.5%) relies on its ability to decrease the plasma membrane fluidity through direct interaction with the plasma membrane. A similar interaction was also proposed by Murhammer and Goochee (1988). The percentage of the hydrophobic portion part of different Pluronic polyols correlated well with the effect on cell growth. Pluronics with low percentages (like F-68) had no effect on growth of tissue culture cells in spinner flasks (Murhammer and Goochee, 1990a), and 0.2% Pluronic F-68 protected cells from detrimental effects associated with both vortexing and cavitation (Murhammer and Goochee, 1990b). Goldblum et al. (1990) also used high concentrations (0.2 and 0.3%) of Pluronic F-68 to obtain increased resistance towards shear stress. They too believe that the protection is a result of polymer adsorption to the cell membrane. In a review Wu (1995) discussed the hydrodynamics of bubble rupture and cell–bubble interaction and the protective effects of pluronic polyols. Chattopadhyay et al. (1995) compared PEG, Pluronic, Methocel, and Dextrane with regard to protection efficiency in bubble capture experiments. They concluded that cells tend to adhere to medium–air interfaces and that the cell damage is mainly due to cell–

bubble interactions. These interactions are counteracted efficiently by Pluronic (0.1%) and Methocel E-50 (0.3%). Recently, Laouar et al. (1996) described different effects of Pluronic F-68 (0.1–1%) on yeast; A cell-permeabilizing effect was demonstrated in an *in situ* alcohol dehydrogenase assay and by enhancement of growth-inhibiting effect of sublethal doses of cycloheximide (0.1 $\mu\text{g ml}^{-1}$). However, Pluronic F-68 (1%) had no effect on growth or flocculation.

Here we report the effects of Pluronic F-68 (0.0001–0.2%) on many different properties of growing and non-growing *Tetrahymena* cells exposed to chemical stress and to a combination of chemical and physical stress. These data might elucidate some properties of *Tetrahymena* and at the same time illustrate the multiple effects of Pluronic F-68.

2. Materials and methods

2.1. Materials

Proteose peptone and yeast extract were purchased from Difco (Detroit, MI). All other chemicals were obtained from Gibco (Grand Island, NY), Sigma (St Louis, MO) or Merck (Darmstadt, Germany). Plastic tubes (capacity 5 ml, diameter 0.7 mm) were obtained from Greiner (Frickhausen, Germany).

2.2. Cells

Tetrahymena thermophila, CU 399 cells were used. For a presentation of different well-defined cellular states, acceptable temperature ranges, shift-up conditions and starvation kinetics see Hellung-Larsen and Andersen (1989) and Hellung-Larsen et al. (1993).

2.3. Cell culture in CDM

The CDM medium contains the four ribonucleosides, 19 L-amino acids, various salts, B-vitamins and 0.5% (w/v) D-glucose. It was prepared from stocks as described (Szablewski et al., 1991). The medium was adjusted to pH 6.5 and stored at

4°C in the dark. Cells were cultured at 36°C in 5 ml medium in 100-ml Erlenmeyer flasks. Cultures were diluted daily from densities around $2\text{--}7 \times 10^5 \text{ cells ml}^{-1}$ to $1\text{--}3 \times 10^3 \text{ cells ml}^{-1}$. The doubling time of the cells in CDM at 36°C is about 3 h.

2.4. Cell culture in broth medium (PY)

The PY medium contains proteose peptone 0.75% (w/v), yeast extract 0.75% (w/v), D-glucose 1.5% (w/v), various salts, pH 6.8 (Hellung-Larsen, 1988). The medium was primarily used to score surviving cells, since it is known that cells are able to proliferate from single cell level even in 100-fold aqueous dilution of PY (Hellung-Larsen and Lyhne, 1992). The doubling time of cells in PY at 36°C is about 2.6 h.

2.5. Cell concentration and cell size

Cell number/size was determined by the use of an electronic particle counter (Coulter, Hialeah, FL). A solution of 0.9% w/v NaCl + 0.1% w/v NaN_3 was used for paralyzing the cells. For further details see Hellung-Larsen and Andersen (1989). Cell proliferation, starvation kinetics and some of the toxicity assays were based on measurements of cell concentration and cell size.

2.6. Starvation and upshift

When *Tetrahymena* cells are transferred from PY or CDM medium to HEPES (10 mM, adjusted to pH 7.0 by KOH), starvation results in a dramatic decrease in cell size. The kinetics of this process depends on the temperature. Addition of PY medium to a final concentration of 1/20–1/6 \times PY (an upshift) leads to increased cell sizes and subsequently possibly to proliferation (Andersen and Hellung-Larsen, 1989).

2.7. Aero- and geotaxis

Tetrahymena cells tend to swim upwards due to a combination of positive aerotaxis and negative geotaxis. Cells ($2500 \text{ cells ml}^{-1}$) in CDM with and without Pluronic (0.01–0.0001% w/v) were

sucked up in capillary tubes allowing an air bubble to be present at the end(s). After about 1 h at room temperature the distribution of cells was registered by stereomicroscopy.

2.8. Assays for cell death in *Tetrahymena*

Five different forms of cell death were assayed (see Table 4).

1. Cell death mediated by a medium–air interface. Low inocula in CDM (typically lower than 100 cells ml⁻¹) were examined in vertically placed tubes or wells as described earlier (Kristiansen et al., 1996).
2. Cell death caused by certain ions. Cells in CDM (2–5 × 10⁵ cells ml⁻¹) were diluted in 1 mM HEPES to 25 cells ml⁻¹. To aliquots of this suspension Na⁺, K⁺ or Ca²⁺ (10 mM) ions were added with and without Pluronic. The resulting cell suspensions were then transferred to 5-ml Plexiglass tubes, which were completely filled in order to avoid the interface mediated cell death. The tubes were placed horizontally and incubated at 36°C. Two or more tubes of each suspension were upshifted daily by the addition of PY and reincubated. After a further 2–7 days of incubation, survivors were scored by light microscopy (Table 2).
3. Cell death caused by long term starvation.

This was performed as described in assay (b) but without added ions and with and without Pluronic. The cell death in this case is most likely due to lack of energy.

4. Cell death caused by mechanical stress. Cells in CDM (2–5 × 10⁵ cells ml⁻¹) were diluted in different media (Table 1) to a low cell concentration. Aliquots (2.5 ml) were transferred to 5-ml Plexiglass tubes, which were placed horizontally on a rocking apparatus and tilted eight times per minute to an angle of ± 40°C. This results in a bubble moving from side to side. After 3 h of tilting at 36 and 4°C with inocula of 10, 25 or 100 cells ml⁻¹ (Table 2) or after 2 and 3 days of tilting at 36°C with inocula of 25 cells ml⁻¹ (Table 3) the cells were upshifted by the addition of 0.5 ml PY. Thereafter cells were incubated 1–3 days at 36°C without tilting to allow any surviving cells to proliferate and tubes were scored for life. The results are presented as % survival; denoting the percentage of tubes showing surviving cells at the time of the upshift.
5. Cell death caused by hyperthermia (Fig. 1). Cells in CDM (2–5 × 10⁵ cells ml⁻¹) were diluted to 25 cells ml⁻¹ in 10 mM HEPES. Three milliliter suspensions with and without Pluronic (0.01%) were transferred to 5 ml plexiglass-tubes and immediately placed in a water

Table 1

Effect of combined chemical and mechanical stress at two different temperatures on survival of *Tetrahymena* cells^a

Cells in	Tilting at 36°C			Tilting at 4°C	
	100 cells ml ⁻¹	25 cells ml ⁻¹	10 cells ml ⁻¹	25 cells ml ⁻¹	10 cells ml ⁻¹
CDM	52 (40)	6 (50)	0 (20)	100 (10)	100 (10)
1/2 CDM in HEPES	–	31 (100)	–	100 (30)	100 (10)
1/10 CDM in H ₂ O	100 (20)	65 (20)	–	–	–
1/100 CDM in H ₂ O	100 (10)	100 (20)	100 (10)	–	–
CDM+Pluronic (0.01%) or HEPES or H ₂ O	100 (20)	100 (50)	100 (20)	100 (25)	–

^a *Tetrahymena* cells in CDM (2–500 000 cells ml⁻¹) were diluted directly in different solutions: CDM, diluted CDM, HEPES or water to three different final cell concentrations 100, 25 and 10 cells ml⁻¹. Pluronic (0.01%) was added in some cases. Cell suspensions (2.5 ml) in 5-ml Plexiglas tubes were tilted for 3 h at the temperatures indicated. A rocking apparatus tilted the tubes eight times per minute to an angle of ± 40°C. Then the cell suspensions were added 500 µl PY (an upshift) to allow proliferation of surviving cells. After 2–3 days of incubation at 36°C in a vertical position (no tilting) the tubes were scored microscopically for living cells. The numbers given represent % tubes with surviving cells (% survival). The numbers in parenthesis are the number of tubes. The data shown are based on three to eight different experiments.

Table 2
Survival under stress with and without Pluronic^a

Treatment period	Without Pluronic	With Pluronic
<i>Chemical stress (filled tubes)</i>		
Control		
2 days	100 (13)	100 (13)
3 days	67 (28)	100 (24)
Ca ²⁺ (10 mM)		
2 days	50 (8)	100 (8)
3 days	0 (10)	100 (10)
Na ⁺ (10 mM)		
2 days	100 (13)	100 (9)
3 days	63 (19)	100 (9)
K ⁺ (10 mM)		
2 days	44 (18)	50 (18)
3 days	13 (22)	53 (13)
<i>Chemical and physical stress (half-filled tubes tilted)</i>		
Control		
5 h	100 (24)	100 (15)
1 day	100 (16)	100 (16)
2 days	67 (21)	100 (20)
Ca ²⁺ (10 mM)		
5 h	33 (9)	100 (9)
1 day	0 (20)	100 (20)
2 days	0 (8)	100 (8)
Na ⁺ (10 mM)		
5 h	55 (11)	100 (11)
1 day	0 (19)	100 (19)
2 days	0 (18)	100 (13)
K ⁺ (10 mM)		
5 h	78 (18)	100 (13)
1 day	0 (23)	100 (23)
2 days	0 (15)	33 (15)

^a *Tetrahymena* cells in CDM (2–500 000 cells ml⁻¹) were diluted in 1 mM HEPES buffer pH 7.0 (neutralized with KOH) to 25 cells ml⁻¹. This cell suspension was divided into 4 flasks -to one was added no salts (control), to the others were added Na⁺, K⁺ or Ca²⁺ ions (final conc. 10 mM) from 1 M stocks of the respective chlorides. To one half of the resulting cell suspensions were added Pluronic F-68 (0.01% w/v). Five-milliliter Plexiglas tubes were filled with cell suspension and incubated at 36°C for 1, 2 or 3 days. At the end of these time periods 500 µl PY medium was added (upshift for scoring surviving cells). In other experiments tubes were half-filled with cell suspensions and tilted on a rocking apparatus (cf. text for Table 1) for 5 h, 1 or 2 days before upshift by addition of PY. The numbers given represent % tubes with surviving cells (% survival). The number in parenthesis indicate the number of tubes.

bath at 43°C. Upshifting was performed at time points ranging within a 0–8-h period by addition of PY. After a further incubation for

2–3 days at 36°C the tubes were scored for living cells. Since the only information available for single tubes are binary variables, the statistical model involved a binomial distribution for the number of living tubes treated alike, i.e. same treatment (Pluronic vs. No Pluronic), same experiment and same time. The binomial parameter (the probability of a tube being alive) was specified as linear on a logit scale with additive effects of Pluronic, experiment and time, all treated as categorical variables, i.e. without specifying a specific time-dependence.

3. Results

When low inocula (10 cells ml⁻¹) of *Tetrahymena* cells in CDM are tilted in half-filled tubes for 3 h at 36°C they all die. This is shown as 0% survival based on 20 different tubes (Table 1, upper line). However, the survival is strongly affected by the initial cell concentration (upper line) and the temperature during tilting (right part of Table 1). The data in Table 1 also show that dilution of the CDM medium increases the survival considerably resulting in 100% survival at 1/100 × CDM. A survival of 100% was also obtained using water or HEPES-buffer (not shown). If Pluronic (0.01% w/v) was added to CDM the survival was 100% at all conditions tested (Table 1, last line).

Thereafter the survival of cell suspensions in completely filled tubes incubated horizontally were investigated. Under these conditions we assume that the physical stress caused by the interface is negligible (upper part of Table 2). In these experiments the cells were diluted in 1 mM HEPES instead of 10 mM since we wanted to study the effect of three of the important ions in the CDM medium (Ca²⁺, Na⁺ and K⁺) on cell survival. It is evident that the control cells survive up to 2 days followed by a decline in survival (67%) observed after 3 days. Ca²⁺ (10 mM) and K⁺ (10 mM) strongly reduced the survival period whereas the effect of Na⁺ (10 mM) was less pronounced. When Pluronic (0.01%) was added the survival was 100% for the control cells as well

as for cells in Ca^{2+} and Na^{+} enriched HEPES, but Pluronic had only a minor effect on cell survival in K^{+} enriched HEPES.

When aliquots of the cell suspensions are present in half-filled tubes and tilted (Table 1) we assume that now the cells are subjected to a combination of a chemical stress exerted by the medium itself and a physical stress exerted by the interface. It is important to notice that in this experiment (Table 2) the up-shift for the scoring of surviving cells was performed at the time points 5 h, 1 or 2 days. Under the tilting conditions the control cells survive for 1 day whereas a decline in survival is observed after 2 days (67%) (cf. 100% after 2 days without physical stress). The addition of Ca^{2+} , Na^{+} or K^{+} has strong effects so that no surviving cells were observed after just 1 day, and a decline in survival is observed even after 5 h—pronounced for Ca^{2+} (33%) and less pronounced for Na^{+} (55%) and K^{+} (78%). The simultaneous addition of Pluronic (0.01%) resulted in 100% survival in all cases except for K^{+} (2 days).

Table 3 shows the maximal survival period for cells in 10 mM HEPES plus additions with and without Pluronic (0.01%) at three different tem-

peratures. The subtext of the table explains how the maximal survival periods were determined. As expected the survival period is increased considerably when the temperature is reduced from the optimal growth temperature (36°C) to 28 or 16°C. This holds true at all conditions. The salts decrease the survival period by 1 day at 36°C, by 3 days at 28°C, and by 4 days at 16°C. However, the simultaneous addition of Pluronic completely eliminates the effect of the salts. At 16°C, cells in HEPES with salts and Pluronic actually live longer (43 days) than cells in HEPES with Pluronic alone (35 days). Table 3 also shows that the mixture of six different trace metal ions is highly toxic to the cells and that Pluronic in this case roughly increases the survival period by 1 day. A high concentration of Pluronic (0.1%) was also tested but the effect was not improved (data not shown).

The *T. thermophila* cells used in this study have an optimal temperature 36–37°C for proliferation (Elliot, 1973). If cells are diluted to 25 cells ml^{-1} in HEPES (10 mM, pH 7.0) and exposed to 43°C (hyperthermia; Fig. 1) they die. As shown, the control cells died within 8 h of exposure whereas the addition of Pluronic F-68 (0.01% w/v) resulted

Table 3
Maximal survival periods in HEPES plus additions^a

	Survival period (days)					
	Without Pluronic			With Pluronic (0.01% w/v)		
	36°C	28°C	16°C	36°C	28°C	16°C
None	4 (31)	8 (7)	21 (8)	17 (5)	28 (4)	35 (4)
Salts ^b	3 (16)	5 (3)	17 (2)	16 (6)	27 (3)	43 (2)
Trace metals ^c	0 (4)	2 (3)	3 (3)	1 (4)	2 (3)	4 (3)

^a *Tetrahymena* cells were diluted to 25 cells ml^{-1} in 10 mM HEPES containing either 'salts' or 'trace metal ions' with or without Pluronic F-68 (0.01% w/v). Aliquots of 5 ml were incubated in filled tubes (i.e. no medium–air interface present) at 36, 28 and 16°C, and upshifted by the addition of 0.5 ml PY every 1 or 2 days for up to 45 days. The upshifted tubes were incubated at 36°C for 2–7 days before microscopic scoring of surviving cells. The numbers indicate the maximal survival period (days), cf. the sub-text. The numbers in parentheses indicate the number of experiments carried out at each condition. The maximum survival period of 4 days for control cells, 36°C, without Pluronic was based on 31 different experiments comprising a total number of 124 tubes: 39 of 41 tubes (91%) showed living cells after 2 days; 33 of 54 tubes (61%) showed living cells after 3 days; 3 of 17 tubes (18%) showed living cells after 4 days; 0 of 10 tubes (0%) showed living cells after 5 days. The maximum survival period of 3 days for cells in HEPES+salts, 36°C, without Pluronic was based on 16 different experiments comprising a total number of 58 tubes: six of six tubes (100%) showed living cells after 1 day; seven of 30 tubes (23%) showed living cells after 2 days; two of 16 tubes (13%) showed living cells after 3 days; none of six tubes showed living cells after 4 days.

^b CaCl_2 (0.07 mM), MgCl_2 (2.0 mM), KH_2PO_4 (1.8 mM), K_2HPO_4 (1.1 mM), pH 7.0 (Hagemeister et al., 1999).

^c Mixture of FeCl_3 , MnSO_4 , $\text{Co}(\text{NO}_3)_2$, ZnSO_4 , CuSO_4 , $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}$ (0.08–36 μM) pH 7.0 (Hagemeister et al., 1999).

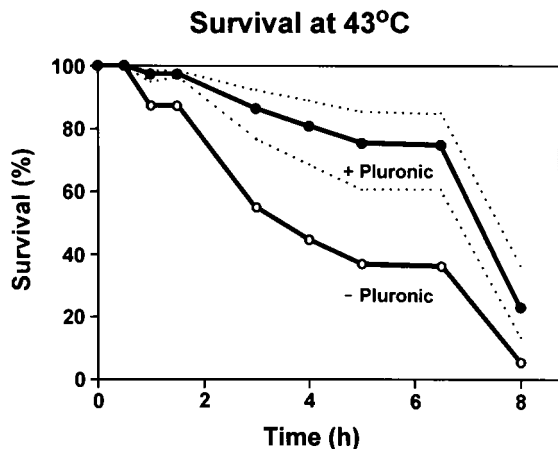


Fig. 1. *Tetrahymena* cells were grown in CDM medium from 2×10^3 to about 5×10^5 cells ml^{-1} during overnight incubation at 36°C . Aliquots were diluted to 25 cells ml^{-1} in HEPES (10 mM, pH 7.0). Pluronic (0.01% w/v) was added to 3 ml cell suspension in 5-ml tubes. At time zero the tubes were placed at 43°C . At the time points indicated the tubes were transferred back to 36°C and 500 μl PY was added. Surviving cells were scored after a further 2 days incubation at 36°C . A total of 388 tubes were used, 216 serving as controls. Eight independent experiments were performed each with a specific representation of the time points. Each time point was studied by at least three different experiments using at least 28 tubes. The figure shows the estimated probability of tube survival as a function of time, for experiments with and without Pluronic added. In addition, the dotted lines show the 95% confidence bands arising from the variability in the effect of Pluronic only, i.e. the uncertainty in the prediction of an experiment with Pluronic from a corresponding experiment without Pluronic.

in a prolongation of the survival period. Cultures with Pluronic showed no survivors after 20 h of incubation (not shown). When cells were diluted to 25 cells ml^{-1} in broth medium (PY) the effect of the hypothermia was also strongly reduced (not shown). The statistical analysis showed the effect of Pluronic to be highly significant, $P < 0.0001$, with an estimated log odds of 1.66 (SD = 0.33), or a corresponding estimated odds of 5.27 with 95% confidence interval (2.74, 10.14). This means, that the odds of a tube surviving up to a given time is somewhere between three and ten times as high if Pluronic is added compared to when Pluronic is not added.

As mentioned elsewhere the efficient concentration of Pluronic to inhibit cell death was in the

range 0.001–0.01%. We tested the toxicity of Pluronic F-68 itself by adding it to low inocula cultures in CDM (2 cells ml^{-1}) in the presence of a surface. Under these sensitive conditions—perhaps only under these conditions—it was observed that Pluronic (0.2% w/v) is toxic whereas 0.02% and 0.002% protected the cells from liquid–air interface mediated death.

When Pluronic (0.01%) was added to cells proliferating in PY or CDM or dilutions thereof we observed no effect on proliferation rate at 36°C . We also studied proliferation in CDM at a suboptimal temperature of 16°C and no effect of Pluronic was observed (data not shown).

Here arises the question whether Pluronic has to be present all the time to exert its effect and whether there is an effect of pre-incubation. For this purpose a number of experiments using the most sensitive assay (Table 4, assay a) were performed. Cells were pre-incubated with 10, 1 or 0.1% Pluronic for different time periods (1–4 h) and then transferred to Pluronic-free conditions. In all cases pre-incubation clearly showed no effect.

When *Tetrahymena* cells were transferred from PY medium to HEPES buffer (10 mM, pH 7.0) Pluronic (0.01%) had no effect on the decline in cell number or the decline in cell size during starvation. This was studied by using 100 000 cells ml^{-1} and a temperature of 36°C . The cells were followed daily over a period of 11 days.

After 11 days of starvation the cellular diameter is about 10 μm . These cells can be upshifted by addition of PY (final conc. $1/25$ – $1/10 \times \text{PY}$). This results in cytoplasmic growth until the cells reach their minimal volume of cell division (Andersen and Hellung-Larsen, 1989). It was tested if Pluronic affects PY-induced cytoplasmic growth. This was not the case (not shown). It was also found that the addition of Pluronic alone (i.e. in the absence of PY) did not cause any volume increase. Thus, Pluronic does not serve as an energy-source for *Tetrahymena*.

4. Discussion

This study primarily deals with the protective effects of Pluronic against cell death of *Tetrahymena*.

mena induced in many different ways (Table 4). It is important to notice that all assays (except for assay j) were performed using low cell concentrations, 10, 25 and 100 cells ml⁻¹ (Tables 1–3) in order to avoid conditioning of the medium. Kristiansen et al. (1996) showed in a so-called reinoculation experiment that the death of about 100 cells in 1 ml could modify the medium, thus allowing the survival of 25 new cells in 1 ml against cell death mediated by a liquid–air-interface (Table 4, assay a). To exclude the above mentioned liquid–air interface mediated cell death form, the tubes were completely filled with cell suspension (Table 2), and to expose the cells to a well-defined mechanical stress a moving bubble was created by tilting half-filled tubes for 3 h (Table 1) or longer (Table 2).

The cell death observed in all these assays is probably induced by a stress which can be: (1)

chemical due to certain ions (Table 4, assay b), starvation (assay c) or trace metal ions (assay f); and (2) physical due to liquid–air-interface (Table 4, assay a), mechanical stress (assay d), or hyperthermia (assay e).

The CDM, which is an excellent proliferation medium for *Tetrahymena* in concentrations above about 500 cells ml⁻¹ (the minimal inoculum depends on the medium height and the temperature; Kristiansen et al., 1996), is toxic for low concentrations of cells (Table 1). The toxicity can be observed and quantitated at 36°C but is absent at 4°C. It is probably related to cellular uptake of the medium substances, since tilting as well as diluted CDM (1/100 × CDM) are harmless. When cells are suspended in HEPES or water they survive the mechanical stress at both temperatures while cells suspended in CDM die. In the last case Pluronic protects the cells from the death caused by the moving bubble.

Table 4
Effect of Pluronic F-68 on *Tetrahymena* cells

<i>Strong effect</i>	
a. Cell death mediated by a medium–air-interface	Pluronic-F-68 (0.0001% w/v $\sim 10^{-7}$ M) prevents the surface mediated death of low inocula cultures of <i>Tetrahymena</i> in CDM (Kristiansen et al., 1996).
b. Cell death caused by certain ions	Pluronic (0.01%) prevents the death of low concentration suspensions in buffer when Ca ²⁺ (70 μ M) and Mg ²⁺ (2 mM) are added (Table 3). It also prolongs survival of cells exposed to 10 mM Ca ⁺ , Na ⁺ or K ⁺ (Table 2).
c. Cell death caused by long-term starvation	Pluronic (0.01%) prolongs survival of low concentration cell suspensions in HEPES buffer (Table 2).
d. Cell death caused by mechanical stress	Pluronic (0.01%) protects low concentration cell suspensions in chemically defined medium from cell death caused by a combination of chemical and mechanical stress (Tables 1 and 2).
e. Cell death caused by hyperthermia	Pluronic (0.01%) prolongs survival of low concentration cell suspensions in buffer at 43°C (Fig. 1).
<i>Slight effect</i>	
f. Cell death caused by trace metal ions	Pluronic F-68 (0.01%) postpones the cell death caused by a number of trace metal ions (Table 3 and Hagemester et al., 1999).
g. Toxicity	A slightly toxic effect of Pluronic (0.2%) was observed. Lower concentrations had no effect.
<i>No effect</i>	
h. Cell proliferation	No effect of Pluronic (0.01%) on rate of proliferation of cells on PY or CDM at 36 and 16°C.
i. Pre-incubation	No effect of pre-incubation with Pluronic (10, 1 or 0.1% w/v).
j. Starvation kinetics	No effect of Pluronic (0.01%) on the decline in cell number (range 10 ⁴ –10 ⁵ cells ml ⁻¹) and cell size during starvation.
k. Up-shift	Pluronic (0.01%) does not interfere with the up-shift caused by the addition of PY or CDM.
l. Aero- and Geotaxis	Pluronic (0.01%) has no effect.

Two of the most harmful ingredients of CDM are the salts (Mg^{2+} , Ca^{2+}) and the trace metal ions, which were studied in some detail (Table 3). As expected the control cells (cells in HEPES) survive the starvation for 4 days at 36°C and for a longer period of time at reduced temperatures. This is most likely due to a reduced cellular metabolism, thus allowing the cells to conserve energy.

The effect of Pluronic (0.01%) on the 'salts' is very strong. Pluronic actually abolishes the effect of the salts, whereas it can only extend the survival period for a day when the trace metal ions are added.

It was found that not only Ca^{2+} and Mg^{2+} are toxic to low concentrations of *Tetrahymena* but also Na^+ and K^+ (Table 2). The cells were suspended in 1 mM HEPES instead of 10 mM (Tables 1 and 3) and we found that Pluronic (0.01%) protects the cells while the toxicity of K^+ (10 mM) was not completely overcome (Table 2). Furthermore, it seems that the combination of a chemical and a physical stress compared to chemical stress alone accelerates the cell death. At 2 days without Pluronic the controls are reduced from 100% (chemical stress) to 67% (combined stress), the surviving cultures in HEPES + Ca^{2+} from 50 to 0%, the surviving cultures in HEPES + Na^+ from 100 to 0%, and the surviving cultures in HEPES + K^+ from 44 to 0%. The controls were cells suspended in 1 mM HEPES which does not create any chemical stress but 'only' results in starvation for 3–4 days before cell death (Table 3). Thus, it is likely that the decrease in % survival of control cells is due to the physical stress.

The maximum survival periods for control cells (starvation in 10 mM HEPES) and for cells cultured in HEPES + salts or trace metal ions are given in Table 3. The table sub-text explains in detail how the survival period is defined.

Interestingly, Pluronic F-68 in concentrations from 0.0001 to 0.01% has a number of positive effects on *Tetrahymena* (Table 4). In fact, the only negative effect of Pluronic is a slight toxicity at a final concentration of 0.2%.

In five assays (Table 4, h–l) we observed no effects of Pluronic. The absence of effect on pro-

liferation (assay h) was studied in the broth medium (PY) as well as in the CDM at the optimal growth temperature (36°C) and at a reduced temperature (16°C). These results suggest that Pluronic does not interfere with uptake of nutrients. This conclusion is in concordance with data obtained from upshift-experiments (assay k) in which Pluronic has no effect. These experiments also showed that Pluronic itself does not create any changes in cell number or cell size. In other words, Pluronic is not taken up by the cells or metabolized.

No effect of pre-incubation (Table 4, assay i) of cells with Pluronic was observed. This was tested with different concentrations of Pluronic and with different periods of time before the removal of Pluronic. The Pluronic was removed by spinning the cells and transferring them to medium without Pluronic. In some cases the pre-incubated cell suspensions were only diluted with medium or buffer to yield a concentration of Pluronic of 0.01 or 0.001%. In these cases the cells reacted exactly as did those to which Pluronic was added directly. Thus, there is no evidence for strong binding of Pluronic to the cells or uptake by the cells. On the contrary, Pluronic has to be present in the medium to produce the observed effects.

Pluronic clearly prolongs the survival period of starving cells when low cell concentrations in 10 mM HEPES are studied (Table 3, upper line). However, when much higher concentrations of cells are studied (Table 4, assay j). Pluronic has no effect on the kinetics of cell number decline or cell size reduction. Thus, Pluronic is probably not reducing the well-described exudation of enzymes, etc. during starvation (Nielsen and Villadsen, 1985) but is rather stabilizing non-conditioned cells (25 cells ml^{-1}) without affecting the conditioned or conditioning cultures (10^4 – $10^5 \text{ cells ml}^{-1}$).

As expected Pluronic has no effect on other intrinsic properties of *Tetrahymena* such as aero- and geotaxis (Table 4, assay l). As indicated in assay a, Pluronic prevents cell death mediated by a liquid–air interface. In this case it is efficient in a concentration of 0.0001%, which is much lower than the concentration resulting in a measurable effect on the surface tension—i.e. 0.01% Pluronic

reduces the weight of a drop whereas 0.001% has no effect. Therefore we concluded that the protective effect of Pluronic on this form of cell death is rather caused by an effect on the cell membranes (Kristiansen et al., 1996). For the other assays presented in this study a Pluronic concentration of 0.01% was used.

The mechanism of action of Pluronic on living cells is unknown. Based on this study we would suggest the following: (1) Pluronic F-68 surrounds the ions Ca^{2+} and Mg^{2+} (and to a less extent probably some of the trace metal ions?) thereby reducing the toxicity of these ions; (2) Pluronic F-68 binds loosely to the cell membranes without affecting uptake of low molecular weight substances and without affecting the exudation of molecules during starvation; (3) Pluronic does not enter the cells; (4) Pluronic stabilizes the cells resulting in prolonged survival during natural starvation and in the presence of stress (chemical or a combination of chemical and mechanical stress). The mechanism behind this effect remains an open question, although it has been suggested that Pluronic stabilizes the cell membrane (Ramirez and Mutharasan, 1990). Recently Massaro (personal communication) reported that the acetylcholine-induced cytosolic Ca^{2+} increase in HEK cells transfected with the mouse muscarinic acetylcholine receptor is affected by Pluronic F-68 apparently by reversibly blocking the uptake of extracellular Ca^{2+} . It is not known whether Pluronic affects the uptake of nutrients or drugs. We do not think so, since it does not affect proliferation (Table 4, assay h). It could affect protein secretion, pH tolerance, and tolerance to ethanol and detergents. This is not known as yet.

A protection against the combined chemical and mechanical stress (Table 1) and the cell death caused by a liquid–air-interface is also obtained by addition of SDS (0.002%), NP 40 (0.001%) and Tween 80 (0.001%) (data not shown). However, these compounds do not affect long term starvation (Table 4, assay c).

One of the practical aspects of the present study is that it is probably now possible to create stress-free conditions thereby reducing/abolishing ‘natural’ cell death. The potential for novel studies on

specific signal molecules has thus been improved considerably.

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