Amino Acids (1997) 13: 281–297

Amino
Acids

Regulation of taurine accumulation in the ciliate protozoan *Tetrahymena pyriformis*

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Accepted May 13, 1997

Summary. *Tetrahymena pyriformis* suspended in an inorganic medium accumulates ^{3}H-labelled taurine against a concentration gradient resulting in a cellular to extracellular taurine gradient of about 3 within 2 hours. This is observed in spite of the fact that taurine influx in *Tetrahymena* via the saturable, Na^+-dependent system and the leak pathway becomes gradually reduced with time after transfer to an inorganic, taurine free medium. Taurine is not accumulated by *Tetrahymena* at pH below 7.0 or when protein synthesis is prevented by addition of cycloheximide. Addition of actinomycin D has no effect on the taurine accumulation. It is suggested that the transporter responsible for taurine accumulation in *Tetrahymena* has a pH threshold of 7.0 and that translation but not transcription is conditional for the emergence of the transporter following transfer to inorganic medium. Taurine accumulation is reduced when the phosphatase inhibitor calyculin A is added at the initiation of starvation but unaffected when the inhibitor is added once the cells are in the inorganic medium. Addition of forskolin significantly enhances taurine accumulation. It is proposed that phosphorylation as well as dephosphorylation are involved in the regulation of taurine accumulation in *Tetrahymena pyriformis*.

Keywords: Amino acids – Cycloheximide – Actinomycin D – Adenosine nucleotides – Calyculin A – Forskolin – pH-dependence – Taurine transport

Abbreviations: AIB: α-aminoisobutyric acid; DMSO: dimethyl sulfoxide; GABA: γ-aminobutyric acid; HEPES: N(2-hydroxyethyl)piperazine-N′-(2-ethanesulphonicacid); MOPS: 3-(N-morpholino)propanesulphonicacid; meAIB: N-methylaminoisobutyric acid; NMDG: n-methyl-d-glucamonium; TBAHS: tetrabutylammonium hydrogensulfate; TES: N-Tris (hydroxymethyl)-methyl-2-amino ethane sulphonic acid; TRIS: Tris (hydroxymethyl)amino methane.

Introduction

Taurine, 2-amino ethane sulphonic acid, is an important organic osmolyte in various vertebrate and invertebrate cells and the cellular taurine concentration is accordingly high in these cells (Huxtable, 1992). Taurine accumulation in mammalian cells, *e.g.*, Ehrlich ascites tumour cells is mediated by a Na^+- and Cl^--dependent transport system, designated the β-system, which has a high affinity for taurine and β-alanine (Lambert, 1984; 1985). Taurine is, on the other hand, released from mammalian cells via a swelling activated channel (Lambert and Hoffmann, 1994; Strange and Jackson, 1995).

Tetrahymena pyriformis, a ciliate found in freshwater environments, has been introduced as a model for the study of taurine transport because its taurine transporting systems resemble the systems seen in mammalian cells (Aomine, 1981; Kramhøft and Lambert, 1997). *Tetrahymena* has a taurine leak pathway, which resembles the swelling-activated taurine channel seen in the Ehrlich cells, *i.e.*, it is activated by unsaturated fatty acids and inhibited by inhibitors of anion transport systems (Kramhøft and Lambert, 1997). In addition, *Tetrahymena* has a saturable, Na^+-dependent system, which, however, is Cl^--independent and seems only to accept taurine as a substrate. It has been estimated that at low extracellular taurine concentrations 20% of the unidirectional taurine influx in *Tetrahymena* is via the saturable system and 80% via the leak pathway (Kramhøft and Lambert, 1997).

Taurine transport via the β-system and the taurine channels in mammalian cells, *e.g.*, the Ehrlich cells is sensitive to extracellular pH (Kromphardt, 1965; Lambert and Hoffmann, 1993) and active taurine transport is apparently maintained by a calyculin A sensitive phosphatase (Mollerup and Lambert, 1996). In the present paper we report a study on aspects of the regulation of taurine accumulation in *Tetrahymena* assuming that taurine accumulation in *Tetrahymena* reflects active taurine transport via the saturable, Na^+-dependent taurine transporter (Kramhøft and Lambert, 1997). Our results show that taurine accumulation in *Tetrahymena* depends on *de novo* protein synthesis, is strongly pH sensitive, and is regulated by processes involving phosphorylation as well as dephosphorylation.

Materials and methods

Culture conditions and media

Tetrahymena pyriformis, strain GL, was maintained and prepared as described previously (Kramhøft and Lambert, 1997). Briefly, stock cultures were maintained in PPY medium (0.75% proteose peptone, 0.75% yeast extract, 100μM ferric citrate, 50μM $CaCl_2$, and 1 mM $MgCl_2$ (Hellung-Larsen, 1988)). For experimental use cells were transferred to and grown at 28°C in PPY medium with 1% glucose for 24–26 hrs at which time the cultures had reached early stationary growth phase (cell density ≈ 0.5–$0.8 \cdot 10^6$ cells per ml). The cells were then transferred to an inorganic medium (IMP) by 3 times centrifugation (700 $\times$ g, 1 min) and resuspension in the medium. The final cell density was 30–60 mg cell wet weight per ml. IMP medium was modified from Hoffmann and Kramhøft (1969), and had the following composition (in mM): NaCl: 30; KH_2PO_4: 1; K_2HPO_4: 4; $MgCl_2$: 5; $CaCl_2$: 1; MOPS: 3.3; TES: 3.3, and HEPES: 3.3, pH 7.2. In most experiments the IMP medium also contained 5μM non-radioactive taurine (see legends to the figures). In Na^+-free medium

Na^+ was replaced by the equivalent concentration of Tris or NMDG-Cl. After the last centrifugation the cells were allowed to recover by standing in a thin layer (10–15 ml cell suspension in a 100 ml Ehrlenmeyer flask) at room temperature for $^1/_2$ hour, whereafter the pH of the cultures was readjusted to pH 7.2 and the experiment initiated.

Estimation of taurine uptake, taurine gradient and cell volume

The taurine influx was initiated by addition of ^{3}H-labelled taurine (3.7 kBq/ml). The final taurine concentration is indicated in the figure legends. Samples (1 ml) of the cell suspension were separated from the medium at the desired time points by differential centrifugation (20,000 × g, 1 min) through a mixture of dioctylsebacinate (14.73 w/w%) in dibutylphtalate (Ballentine and Burford, 1960) in preweighed Eppendorf vials. The upper water phase was used for estimation of ^{3}H-activity in the medium, the oil phase was discharged, whereafter the cell pellet was lysed, deproteinated and used for estimation of cellular ^{3}H-activity as described previously (Kramhøft and Lambert, 1997). Radioactivity was assessed by liquid scintillation counting. Cellular activity was corrected for trapped extracellular medium (11% of the cellular wet weight), determined in separate experiments, using ^{3}H-inulin as extracellular marker (Kramhøft and Jessen, 1992). Taurine uptake (nmoles per g cell dry weight) was calculated by division of cellular ^{3}H-taurine activity (cpm per g cell dry weight) with the specific activity of taurine (cpm per nmoles) in the extracellular compartment. The ^{3}H-taurine concentration gradient was calculated as the cellular ^{3}H-activity (cpm per ml cell water) divided by the extracellular ^{3}H-activity (cpm per ml medium). Cell volume is given as mg cell water per mg cell dry weight.

Estimation of cellular ATP, ADP and AMP by high pressure liquid chromatography

To estimate the cellular ATP, ADP and AMP contents, 1 ml of the cell suspension was transferred to preweighed Eppendorf vials and centrifuged (20,000 × g, 60 sec). The supernatant was removed by suction, the vials weighed and the nucleotides extracted with cold perchloric acid (300 μl, 0.6 M). The vials were weighed again in order to estimate the exact perchloric acid volume. After 20 min incubation, and another centrifugation (20,000 × g, 10 min), the acidic supernatant, containing the nucleotides, was transferred to new vials. Excess supernatant was removed and the perchloric acid cell pellet was dried (90°C, 48 hour) and weighed in order to estimate the cell dry weight. The acidic supernatant was neutralized by addition of 2.5 volumes of freshly prepared tri-N-octylamine (0.5 M) in freon, mixed 3 times 20 sec, left for 10 min, centrifuged (15,000 × g, 5 sec), and the neutralized supernatant finally filtered (0.22 μm Millex-GV, Millipore) before storage (−20°C). 10 μl of the samples were injected into a HPLC system (Pharmacia Biotech) and the adenosine nucleotides were separated on a C18-Superpac Sephasil column (4 × 250 mm, 5 μm particles). The mobile phase consisted of 0.3 mM TBAHS, 0.1 M KH_2PO_4/K_2HPO_4, pH 6.0 and 4% methanol (v/v). TBAHS was included as an ion pairing agent (see Perret, 1986). The flow velocity was 1 ml per min and the compounds were detected at 260 nm. The absolute nucleotide content was estimated from chromatograms of ATP, ADP, AMP standards in the range 10 μM–1 mM. The retention times were 10.8 min, 14.9 min and 23.2 min for AMP, ADP and ATP, respectively. Values for the adenosine nucleotides are given as µmoles per g cell dry weight and as µmoles per liter cell water.

Chemicals

Yeast extract and proteose peptone were from Difco (Detroit, Mi, USA). Calyculin A was from Alomone Labs (Jerusalem, Israel) and kept at −20°C as a 20 μM stock solution

in ethanol under a nitrogen atmosphere. All other chemicals were from Sigma (St. Louis, Mo, USA). ^{3}H-inulin was from Amersham (Little Chalfont, England). ^{3}H-taurine was from Amersham or NEN (Du Pont de Nemours, Dreiech, Germany).

Statistical evaluation

Error bars on the figures represent SEM values from at least 3 independent experiments. Unless otherwise indicated an observation is taken as significant when $P < 0.05$ by analysis of variance (ANOVA) or by a Student's t-test.

Results

It has previously been demonstrated that taurine uptake in *Tetrahymena* is dominated by taurine channels. However, a small active component amounting to about 20% of the total influx also contributed to the uptake (Kramhøft and Lambert, 1997). In those experiments taurine uptake was followed for 30 min and the active component could only be demonstrated if the cells had been grown in the presence of 1 mM taurine prior to the transfer to the inorganic medium. In the following we present results of experiments where taurine uptake was monitored for 2 hrs after addition of ^{3}H-labelled taurine to *Tetrahymena*, which were not preexposed to taurine. To avoid significant changes in the specific activity of taurine in the extracellular compartment during the experimental period, 5 μM non-radioactive taurine was added to the experimental medium. Figure 1A shows the cellular to extracellular ^{3}H-taurine gradient in *Tetrahymena* as a function of time following addition of ^{3}H-taurine. It is seen that within the initial 30 min the taurine gradient becomes equal to 1 (0.94 ± 0.07, $n = 15$ at time 20 min; 1.13 ± 0.1, $n = 13$ at time 30 min) and that the gradient in the subsequent 100 min increases linearly, resulting in a gradient of about 3 (2.6 ± 0.3, $n = 18$) after 2 hours exposure to the isotope. The time when isotope equilibrium was attained was not determined. An increased cellular to extracellular gradient could be secondary to a reduction in the cell volume. However, even though *Tetrahymena* actually shrink continuously during the experimental period, the cell shrinkage only amounts to about 25% after 2 hrs (Fig. 1B). The appearance of the taurine gradient seen in Fig. 1A is, therefore, taken to indicate that active taurine transport already becomes apparent within the first 2 hours following transfer to inorganic medium containing 5 μM taurine. It should be noted that a ^{3}H-taurine gradient of similar magnitude (2.83 ± 0.77, $n = 9$) was obtained with cells suspended for 2 hours in IMP without non-radioactive taurine added.

Effect of cell density and pH on taurine accumulation

The average taurine gradient attained 2 hrs after addition of ^{3}H-labelled taurine is 2.6 (Fig. 1A) as calculated from 18 individual values, which varied from 1 to 8. Since the cell density is subject to some variation from experiment to experiment the variation in the taurine gradient could be related to the

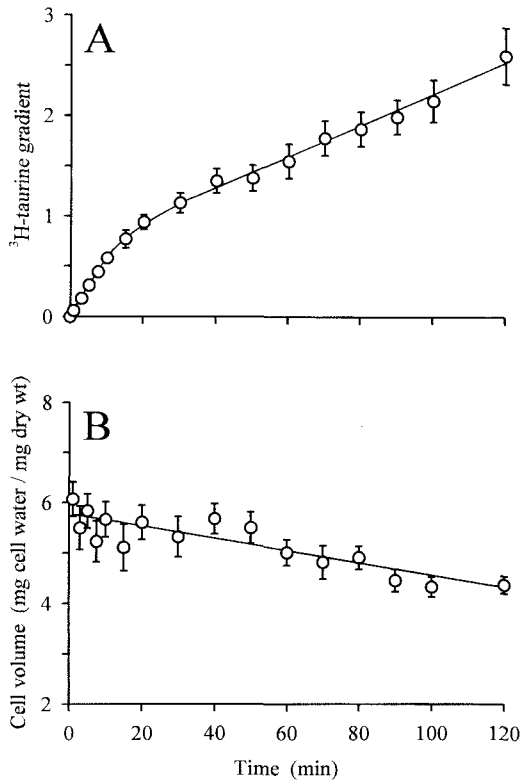

Fig. 1. Taurine accumulation and cell shrinkage in *Tetrahymena pyriformis* following suspension in inorganic medium containing taurine. Cells, grown in PPY medium, were transferred to inorganic medium (IMP) containing 5μM taurine as described in *Materials and methods*. ^{3}H-labelled taurine (3.7 kBq/ml) was added at time zero and samples were subsequently removed for estimation of the ^{3}H-taurine concentration gradient and the cell volume. **A** The gradient was estimated as the ^{3}H-labelled activity in the cellular acid soluble pool (cpm/ml) divided by the ^{3}H-labelled activity in the medium (cpm/ml). **B** The cell volume is given as the cellular water content relative to the dry weight. Each point represents the mean ± SEM of 10–18 values, estimated in separate experiments

actual cell density obtained after transfer of the cells to the experimental medium. A plot of the taurine concentration gradient obtained at time 2 hrs after addition of ^{3}H-labelled taurine versus the cell density in the experiments is given in Fig. 2. The plot clearly indicates that the less cells per ml culture, the larger is the taurine gradient obtained after 2 hrs, and that in cultures with a cell density above 70 mg wet weight per ml no taurine accumulation can be demonstrated. It should be noted though, that by far the most experiments presented in this paper are carried out at cell densities at 30–40 mg wet weight per ml, where concentration gradients above 2 were observed except in one case (see Fig. 2).

As a routine we readjusted the pH of the experimental cultures to 7.2 just prior to addition of ^{3}H-taurine. This was done because *Tetrahymena* acidifies the suspension medium and because active taurine transport in other cells, *e.g.*, Ehrlich cells has been shown to be pH-dependent, being inhibited by

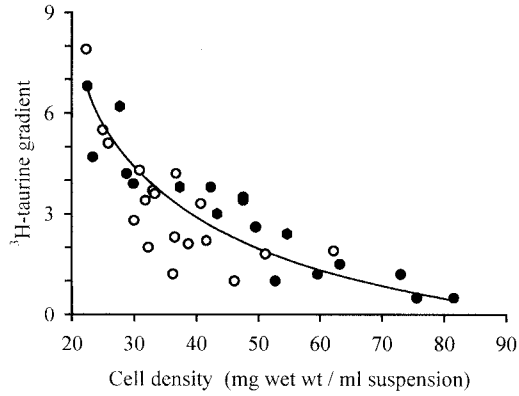

Fig. 2. Effect of cell density on taurine accumulation in *Tetrahymena pyriformis*. Cells, grown in PPY medium were transferred to inorganic medium (IMP) containing 5μM taurine. The final cell density was in the range 20–85 mg wet weight per ml cell suspension. ^{3}H-labelled taurine was added and the taurine gradient estimated after 120 min incubation as described in the legend to Fig. 1. The open symbols (○) represent the gradient values obtained at time 2 hrs and was used for calculation of the mean value in Fig. 1. The closed symbols (●) represent four sets of experiments where the correlation between cell density and the obtained taurine concentration gradient was systematically investigated. The line is drawn by hand

acidification of the extracellular medium (see Lambert and Hoffmann, 1993). A systematic investigation of the relationship between the cell density, the actual extracellular pH, and the resulting ^{3}H-labelled taurine gradient, measured 2 hrs after readjustment of pH, was, therefore, carried out. Figure 3A demonstrates that, in spite of the pH adjustment just prior to initiation of the taurine uptake experiments, the cells continue to acidify the medium and, not surprisingly, the degree of acidification increases with the cell density. Figure 3B shows that a concentration gradient above 1 is only obtained provided the extracellular pH during the experimental period does not decrease below a pH of about 6.4. Thus, active taurine transport in *Tetrahymena* is like in other cells inhibited by acidification of the extracellular medium. Studying pH-sensitive processes in *Tetrahymena,* therefore, requires that extracellular pH is carefully controlled *e.g.* by adjusting the cell densities used to values below 30 mg wet weight per ml cell suspension.

Role of protein synthesis and RNA synthesis in taurine accumulation in Tetrahymena

Figure 4A shows the taurine uptake (nmoles per g dry weight) in *Tetrahymena* as a function of time after addition of ^{3}H-labelled taurine. The point on the control curve marked with an asterix denotes the taurine content in the cells at the time where the ^{3}H-taurine concentration gradient across the plasma membrane becomes 1. Besides confirming the data from Fig. 1, *i.e.*, that net accumulation of taurine occurs in *Tetrahymena* within the first two hours following transfer of the cells to the inorganic medium containing 5μM

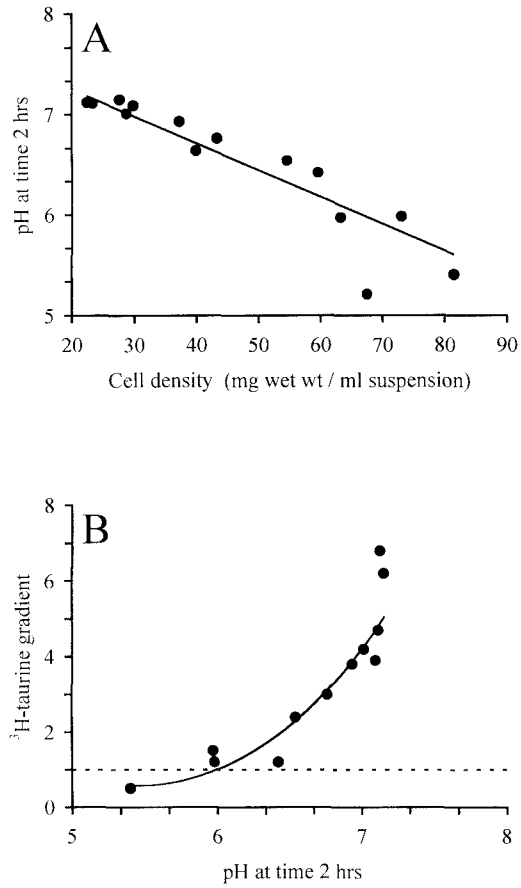

Fig. 3. Taurine accumulation in *Tetrahymena pyriformis* and its correlation with cell density and extracellular pH. Cells were transferred to inorganic medium containing 5 μM taurine, and the ^{3}H-labelled taurine added as described in the legend to Fig. 1. Each point represents a single experiment. **A** The pH of the cell suspension was measured after the two hours experimental period and plotted versus the cell density. **B** The taurine gradient obtained two hours after addition of ^{3}H-labelled taurine was calculated as described in the legend to Fig. 1 and plotted versus the pH. The lines are drawn by hand

taurine, Fig. 4A also shows that no accumulation of ^{3}H-labelled taurine occurs in the presence of the protein synthesis inhibitor cycloheximide (5 μM). As shown in Fig. 4B cycloheximide has no effect on the initial taurine influx, calculated by linear regression of measurements obtained within the initial 10 min after addition of the isotope. Thus, although cycloheximide does not affect the activity of the taurine transporting systems *per se*, accumulation of taurine against a concentration gradient apparently requires *de novo* protein synthesis either of the taurine transporter itself or of a regulator of the transporter. Inhibition of protein synthesis has previously been shown to reduce glycine and phenylalanine uptake in *Tetrahymena* (Wheatley and Walker, 1980).

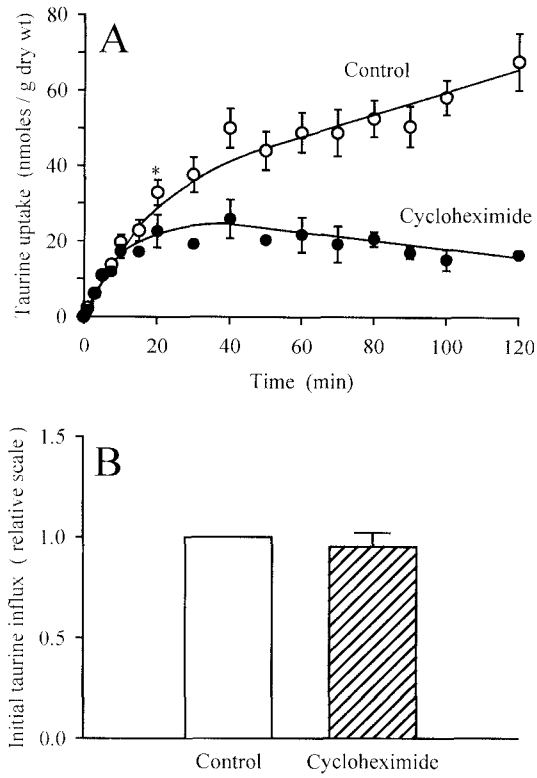

Fig. 4. Effect of cycloheximide on taurine uptake in *Tetrahymena pyriformis*. Cells, grown in PPY medium, were transferred to inorganic medium (IMP) containing 5μM taurine. Cycloheximide (5μM) was added just prior to the ^{3}H-labelled taurine (3.7 kBq/ml) which was added at time zero. Samples were subsequently removed for estimation of ^{3}H-taurine gradient. **A** Taurine uptake was calculated from the ^{3}H-labelled activity in the cellular acid soluble pool (cpm/mg cell dry wt) by division with the specific activity in the medium (cpm/mmole). Values in the control series (open symbols) and the cycloheximide series (closed symbols) represent 10–15 and 3 experiments, respectively and are given as mean values ± SEM. **B** The initial taurine influx was in three paired sets of experiments calculated by linear regression analysis from the taurine uptake measured within the first 8 to 10 min following addition of the isotope. Values are given relative to control values

A possible role of RNA synthesis for taurine accumulation in *Tetrahymena* was also investigated. Figure 5 demonstrates that the taurine concentration gradient obtained 2 hrs after addition of ^{3}H-labelled taurine in cells treated with actinomycin D (10μM) is not significantly different from the gradient obtained in the parallel controls. For comparison, Fig. 5 also shows the equivalent gradient obtained in the presence of cycloheximide (5μM). Thus, the mRNA encoding for the taurine transporter is not short-lived and apparently only translation and not transcription is conditional for the emergence of active taurine transport in *Tetrahymena* under the present conditions.

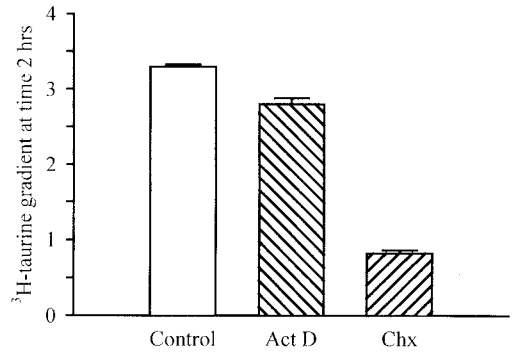

Fig. 5. Effect of actinomycin D and cycloheximide on taurine accumulation in *Tetrahymena pyriformis*. Cells were handled, ^{3}H-labelled taurine added, and the taurine gradient estimated two hours after addition of the isotope as described in the legend to Fig. 1. Actinomycin (10μM) and cycloheximide (5μM) were added just prior to the addition of ^{3}H-taurine. Values are given as mean values ± SEM and represents 4 control experiments, 3 experiments with cycloheximide and 4 experiments with actinomycin D

Effect of starvation on the initial taurine influx

It has previously been shown that *Tetrahymena pyriformis* is able to accumulate the non-metabolizable amino acid AIB against a concentration gradient and that this capacity is gradually reduced after transfer of the cells to a non-nutrient medium (Blum, 1982). The taurine influx in *Tetrahymena* was accordingly estimated at various time points after transfer of the cells from the PPY medium to taurine free IMP. The flux was in each case initiated by addition of ^{3}H-labelled taurine and the initial taurine influx calculated by linear regression of measurements obtained within the first 10 min after addition of the isotope. In all cases pH of the medium was adjusted to 7.2 just prior to initiation of the influx. It appears from Table 1 that the initial taurine influx in *Tetrahymena* is gradually reduced following transfer of the cells to the non-nutrient inorganic medium and after 3 hours starvation the initial taurine influx is reduced to 54% of the original value. Table 1 also gives the Na^+-dependent fraction of the taurine influx. It is seen that in accordance with previously published data (Kramhøft and Lambert, 1997) about 20% of the initial taurine influx is Na^+-dependent and that this Na^+-dependent fraction is slightly but not significantly increased after 3 hrs in IMP. Hence, assuming that taurine accumulation against a concentration gradient in *Tetrahymena* occurs via the Na^+-dependent transport system, this system as well as taurine transport via the taurine leak pathway, *i.e.*, taurine channel are down regulated in *Tetrahymena* under starvation.

Contents of adenosine nucleotides in Tetrahymena

Transfer of *Tetrahymena* cells from a nutrient medium to starvation medium could conceivably result in a decreased access to energy leading to ATP depletion and a concomitant increase in the AMP content. Table 2 shows the

Table 1. Initial taurine influx in *Tetrahymena pyriformis* measured at various time points after transfer from PPY medium to inorganic medium (IMP)

Time after transfer to IMP hours	Initial influx relative values	Na^+-dependent fraction of the initial influx % of total
0.5	1.0	20.4 ± 4 (13)
1	0.61 ± 0.08 (3)*	
2	0.48 ± 0.05 (3)*	
3	0.54 ± 0.08 (6)*	31.0 ± 11 (7)

Cells were harvested and suspended in inorganic medium as described in *Materials and methods* except that the non-radioactive taurine was omitted. At the indicated times pH was adjusted to 7.2 and ^{3}H-taurine (3.7 kBq/ml, final concentration 1.3 μM) was added. Samples were subsequently removed for estimation of the initial taurine uptake during the following 10 min. The initial influx, calculated by linear regression of at least 4 measurements obtained within this time period, is given relative to the initial taurine influx at 0.5 hrs, which was estimated at 0.38 ± 0.03 nmoles/g dry wt·min (n = 14). The Na^+-dependent fraction of the initial taurine influx, calculated as the difference between the initial taurine fluxes obtained from parallel cultures suspended in IMP and Na^+-free IMP, is given in % of the taurine influx in IMP medium. The numbers in parenthesis denotes the number of experiments. * indicates that the values are significantly different from 1.

Table 2. Adenosine nucleotide contents and concentrations in *Tetrahymena pyriformis*

	ATP	ADP	AMP
Content		μmole/g dry wt	
PPY	5.1 ± 0.4#	4.8 ± 0.3	2.3 ± 0.03
IMP	6.2 ± 0.3#*	5.4 ± 0.4	2.5 ± 0.2
IMP + DOG	5.0 ± 0.4*	4.6 ± 0.3	2.0 ± 0.2
Concentration		μmoles/liter cell water	
PPY	695 ± 83#	659 ± 72	310 ± 17
IMP	992 ± 62#	860 ± 60	401 ± 33
IMP + DOG	827 ± 72	750 ± 46	327 ± 25

Cells were prepared for HPLC measurements as described in *Materials and methods*. DOG (deoxyglucose, 25 mM) was added just after transfer of the cells to IMP. Parallel samples for HPLC and the determination of cellular wet and dry weights were harvested about 50 min after resuspension of the cells in IMP. The pH of the cultures was adjusted to 7.2 at 30 min after resuspension in order to mimic the conditions prevailing in taurine uptake experiments. Data are from three sets of experiments and given as mean values ± SEM. # and * indicate that the values are significantly different as tested with analysis of variance (ANOVA).

ATP, ADP, and AMP contents as well as their equivalent cellular concentrations in *Tetrahymena* suspended in PPY medium, in IMP medium, and in IMP medium containing 25 mM deoxyglucose (DOG). It is seen that the cellular concentrations as well as the content of ATP are significantly larger in *Tetrahymena* suspended in IMP medium compared to cells suspended in the

Table 3. The effect of forskolin on ^{3}H-taurine accumulation in *Tetrahymena pyriformis*

	Taurine influx (relative figures)	^{3}H-taurine concentration gradient (relative figures)
Control	1.0	1.0
Forskolin (0.1 mM)	1.33 ± 0.05*	1.26 ± 0.04*

Cells were transferred to IMP without non-radioactive taurine added and pH was adjusted to 7.2 as described in *Materials and methods*. Forskolin (0.1 mM) was added to the one of two parallel cultures, whereafter the taurine influx was initiated by addition of ^{3}H-taurine (3.7 kBq/ml, final taurine concentration 1.3 μM). The taurine uptake (nmoles/g dry wt) and the taurine gradient (cpm per ml cell water/cpm per ml medium) were estimated from samples drawn 45–60 min after addition of ^{3}H-taurine and represents 8 individual sets of experiments. The taurine uptake and the taurine gradient in control cells were 4.8 ± 0.9 nmoles/g dry wt and 0.9 ± 0.1, respectively. Values from cells treated with forskolin are given relative to the control values. * indicates that values are significantly larger than 1.

PPY. The Table also shows that the increased concentrations of ADP and AMP at least partly reflect increased contents of the nucleotides. However, the cell shrinkage shown in Fig. 1 also contributes. It is also seen in Table 2 that the cellular content as well as the cellular concentrations are all decreased by about 20% as a result of preincubation for 50 min with DOG in IMP. Experiments where the ^{3}H-labelled taurine gradient was measured 2 hrs after addition of the isotope in the absence and in the presence of DOG showed, however, that addition of DOG had no effect on the taurine concentration gradient obtained (data not shown). Hence, the capacity for taurine accumulation in *Tetrahymena* does not seem to depend on the cellular content of adenosine nucleotides.

Role for phosphorylation processes in the accumulation of taurine

The observations presented in Table 1 support the notion by Blum (1982) that in the absence of any transportable amino acids, *Tetrahymena* not only stops or greatly reduces the rate of synthesis of its amino acid transporting systems but it also seems to rapidly inactivate or degrade these systems. The data in Fig. 1A and Fig. 4, on the other hand, indicate that in the presence of substrate this suppression of the synthesis of an appropriate transport systems seems to be lifted and the synthesis of the same systems might even be upregulated.

It has recently been demonstrated that addition of forskolin to Ehrlich ascites tumour cells leads to an increased cytosolic cAMP level and a concomitant stimulation of the taurine uptake via the taurine concentrating β-system (Mollerup and Lambert, 1996). *Tetrahymena* contains cAMP as well as the adenylate cyclase, which is the specific target of forskolin (Ramanathan and Chou, 1973; Laurenza et al., 1989). Addition of forskolin to *Tetrahymena* at a concentration, which increases the cytosolic cAMP concentration significantly in *e.g.* Ehrlich cells (Mollerup and Lambert, 1996), only increases the

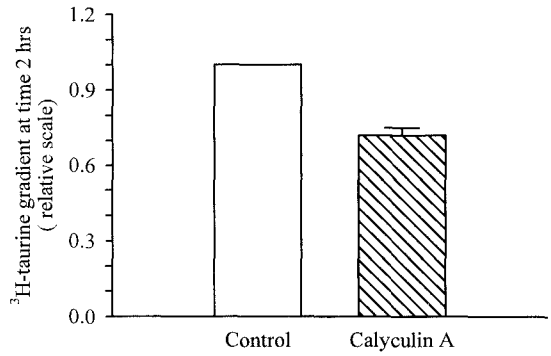

Fig. 6. Effect of calyculin A on taurine accumulation in *Tetrahymena pyriformis*. The cells were handled, ^{3}H-labelled taurine added and the taurine gradient estimated two hours after addition of the isotope as described in the legend to Fig. 1. The cell concentration was about 20 mg wet wt/ml. Calyculin A (100 nM) was added at the time of transfer of the cells to the inorganic medium. The taurine gradient in calyculin A treated cells is given relative to the gradient in untreated control cells. The cell volumes after 2 hours were estimated at 4.0 ± 0.2 and 4.1 ± 0.5 mg cell water/mg dry weight for control cells and cells treated with calyculin A, respectively. The values represents 3 paired sets of experiments

initial taurine influx of taurine in *Tetrahymena* insignificantly (≈15%, see Kramhøft and Lambert, 1997). However, addition of forskolin actually improves taurine accumulation in *Tetrahymena* measured after about 1 hour significantly by 30% (Table 3.). Preliminary data (not shown) indicate that the cAMP level slowly decreases with time in *Tetrahymena* after transfer to inorganic medium. Thus, the increased taurine accumulation seen in *Tetrahymena* after transfer to the inorganic medium containing taurine (see Fig. 1A and Fig. 4) seems not to involve cAMP or cAMP mediated phosphorylation.

Mollerup and Lambert (1996) have recently shown that the activity of the taurine transporting β-system in the Ehrlich cells is maintained by a calyculin A sensitive phosphatase. Figure 6 shows that the taurine concentration gradient in *Tetrahymena,* obtained 2 hrs after addition of ^{3}H-labelled taurine, is significantly reduced by about 30% following treatment with 100 nM calyculin A. From experiments with duck erythrocytes it appears that 200 nM is required for 100% inhibition of the KCl cotransporter (Palfrey, 1994). The effect of the 100 nM calyculin A on taurine influx in *Tetrahymena* might, therefore, not represent the maximal effect of the inhibitor. The calyculin A induced reduction in the taurine gradient is not secondary to inhibition of the cell shrinkage normally seen in inorganic medium, because the degree of cell shrinkage is unaffected by addition of calyculin A (see legend to Fig. 6). In the experiments shown in Fig. 6 we added calyculin A immediately, *i.e.*, within 3 min after removal of the PPY medium. However, if calyculin A was added later, *e.g.*, 15 min after transfer of the cells to IMP or just prior to addition of the ^{3}H-labelled taurine, no effect on taurine accumulation was observed (data

not shown). Thus, dephosphorylation of the taurine accumulating system by a calyculin A sensitive phosphatase could well be an early event involved in the accumulation of taurine in *Tetrahymena* following transfer to inorganic medium containing taurine.

Discussion

It has previously been demonstrated that *Tetrahymena pyriformis* cultivated in the presence of taurine express a capacity for concentrative taurine uptake (Kramhøft and Lambert, 1997). The system responsible for the active taurine transport is Na^+-dependent, but differs from the taurine transporting β-system seen in mammalian cells (Huxtable, 1992) in being Cl^--independent and not inhibitable with structural analogues to taurine, *e.g.*, hypotaurine, β-alanine or GABA (Kramhøft and Lambert, 1997). The results presented in this paper show that taurine is also accumulated against a concentration gradient in *Tetrahymena* without previous growth in the presence of taurine, provided the taurine uptake was followed for a prolonged period of time (Figs. 1 and 4).

The active taurine transport system in Tetrahymena is pH sensitive

Figures 2 and 3 demonstrate that the size of the taurine concentration gradient obtained 2 hrs after addition of ^{3}H-taurine depends on the cell density and/or on the actual pH of the experimental medium during the experiment. A possible decreased access to O_2 in cultures with high cell densities might be a factor determining the function of an active transport system. However, since taurine transport in other systems is strongly reduced at low pH (Huxtable, 1992), we suggest that an increased acidification of the extracellular compartment at high cell densities is the direct cause of the concomitant reduction of the taurine accumulation. From Fig. 3B it may be estimated that if extracellular pH has decreased from 7.2 to 6.4 during the 2 hrs experimental period, taurine accumulation in *Tetrahymena* is completely abolished. It should be noted, that such a decrease in extracellular pH is practically without effect on the intracellular pH (Kramhøft and Jessen, 1992). In additional experiments it was found that the decrease in pH during the 2 hours experimental period is practically linear (data not shown). Since taurine accumulation becomes measurable from 30 mins after addition of ^{3}H-labelled taurine, *i.e.*, the taurine gradient becomes larger than 1 (see Fig. 1A), it may thus be estimated that the pH "threshold" value for cessation of active taurine transport in *Tetrahymena* is close to pH 7.0. This threshold is more alkaline than the threshold for taurine uptake in mammalian cells, *e.g.*, Ehrlich cells, where active taurine transport via the β-system decreases gradually with decreased pH of the extracellular medium (Lambert and Hoffmann, 1993). The system in the Ehrlich cells, however, still operates at a significant rate at pH 7.0 (Lambert and Hoffmann, 1993) and is only completely blocked at extracellular below pH 5 (Kromphardt, 1963). Thus, the active taurine trans-

porter in *Tetrahymena* also differs from the mammalian β-system in being inactive at pH below 7.0.

The above observations might also explain why no cellular to extracellular taurine gradient was observed in *Tetrahymena* grown in PPY overnight in the presence of 1 mM taurine (Kramhøft and Lambert, 1996). Measurements of the pH of the PPY cultures just prior to transfer of the cells to the inorganic experimental medium revealed that extracellular pH was 6.58 ± 0.02 ($n = 10$), i.e. the extracellular pH during growth in PPY was below the apparent pH threshold value for the taurine accumulating system.

Taurine uptake is reduced in Tetrahymena following starvation in inorganic medium

Tetrahymena accumulates various amino acids against a concentration gradient and it appears that most if not all of the transport occurs across the plasma membrane via distinct amino acid transporting systems and not by endocytosis (Hoffmann and Rasmussen, 1972; Blum, 1982; Davis and Stephens, 1983, 1986; see Dunham and Kropp, 1973; Blum, 1982). The influx of certain amino acids in *Tetrahymena* is affected by the presence of other amino acids in the extracellular compartment (see Dunham and Kropp, 1973) and besides the taurine transporter, *Tetrahymena* also has two other amino acid transporting systems which can be distinguished by their ability to transport AIB and meAIB (Blum, 1982). However, even though influx of free amino acids into *Tetrahymena* might only play a minor role to the contribution of nutrients (Davis and Stephens, 1983) the cells lose their capacity to take up amino acids following transfer to a non-nutrient medium (Blum, 1982). Similarly, starvation of *Tetrahymena* causes a reduction in the initial taurine influx, *i.e.*, a reduction in transport via the Na^+-dependent taurine transporter as well as via the taurine leak pathway (Table 1). The reduced ability to transport taurine is not a result of a reduced availability of ATP, because the content ATP and ADP are larger in cells suspended in inorganic medium compared to cells in PPY (Table 2). The same is true for the concentration of both ATP, ADP and AMP. The increased AMP concentration might, on the other hand, lead to activation of a AMP-dependent kinase and subsequently inactivation of taurine transport. Alternatively, loss of transport capacity could well be due to removal of active carriers from the plasma membrane. Jonassen and Grinde (1986) have demonstrated that upon transfer of *Tetrahymena* to starvation conditions, the cells respond by an increased protein degradation, and it was estimated that the rate of degradation amounts to 10–14% per 3 hrs. Crockett and coworkers (1965), on the other hand, have demonstrated that a considerable *de novo* protein synthesis takes place under starvation conditions in *Tetrahymena*. Thus, turnover of cellular proteins occur during starvation and the loss of transport capacity could be a result of the break down of transport systems being faster than their synthesis and incorporation into the cell membrane. This is also supported by the observation that cycloheximide completely blocks taurine accumulation (Fig. 4B). Assuming that protease activity is a prerequisite for protein degradation

leading to the loss of amino acid transport capacity, this activity is insensitive to leupeptin and pepstatin according to Blum (1982). PPY itself is a protease inhibitor (Jonasssen and Grinde, 1986) and the gradual loss of taurine transport capacity seen after transfer from PPY to inorganic medium is, therefore, likely to result from an increased action of proteases on the transport systems.

Regulation of taurine accumulation in Tetrahymena

Tetrahymena cultivated for 24 hours in the presence of taurine prior to transfer to the inorganic medium were previously shown to express a capacity for concentrative taurine uptake as compared to cells grown without added taurine (Kramhøft and Lambert, 1997). From the data in Figs. 1A and 4 it is seen that *Tetrahymena*, grown in the absence of taurine, are in fact able to accumulate taurine against a concentration gradient. Figures 4A and 5 show that the taurine accumulation is completely abolished by addition of cycloheximide (inhibitor of protein synthesis), whereas actinomycin D (inhibitor of RNA synthesis) has no effect. Cycloheximide does not affect the initial taurine influx (Fig. 4B), *i.e.*, the transporter *per se*, indicating that *de novo* synthesis of the taurine transporter or a regulator of the system seems to be a prerequisite for the taurine accumulation in *Tetrahymena*. It is, therefore, suggested that the active taurine transporter in *Tetrahymena* has a short lifetime and in the presence of substrate resynthesis of the taurine transporter still takes place and might even exceed the breakdown. The rate of synthesis and the rate of loss of the AIB and meAIB transporters also seem in part to be controlled by the presence of exogenous amino acids (Blum, 1982).

It has recently been demonstrated that taurine influx via the β-system in Ehrlich cells is stimulated by addition of forskolin, which stimulates the adenylate cyclase but inhibited by addition of calyculin A (Mollerup and Lambert, 1996), which is reported to be a potent inhibitor of protein phosphatases 1, 2A and 3 (Honkanen et al., 1994). Forskolin also stimulates taurine accumulation in *Tetrahymena* (Table 3), indicating that cAMP either directly or indirectly via a cAMP-dependent kinase improves taurine accumulation in *Tetrahymena*. On the other hand, addition of calyculin A at the time of the transfer of cells from PPY to the inorganic medium reduces the ability of *Tetrahymena* to accumulate taurine (Fig. 6). However, calyculin A has no effect on taurine accumulation if added after the cells have been transferred to the IMP medium (data not shown). This is taken to indicate that it is not the taurine transporter in *Tetrahymena per se* but rather an activator of the system which is more active in a dephosphorylated state.

In conclusion, *Tetrahymena pyriformis* accumulates taurine via a Na^+-dependent, saturable transporter which is inactivated when extracellular pH decreases below 7.0. cAMP and/or a cAMP-dependent kinase as well as a calyculin A sensitive phosphatase play a role in the regulation of taurine accumulation in *Tetrahymena*. It is proposed that it is the activity of a regulator of the taurine transporter which is reduced by phosphorylation following transfer from PPY to inorganic medium.

Acknowledgements

The work was supported by the Carlsberg Foundation (B. Kramhøft) and by The Danish Natural Research Council (J. Mollerup).

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Received March 11, 1997