

Role of K⁺ and amino acids in osmoregulation by the free-living microaerophilic protozoon *Hexamita inflata*

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The primitive free-living protozoon *Hexamita inflata* was found to maintain a cell volume of approximately 260 fl under standard culture conditions. On increasing the extracellular osmolality the volume decreased and the cells remained shrunken for >30 min. By contrast, a decrease in the external osmolality resulted in a transient increase in cell volume which was followed by an efficient 'regulatory volume decrease' (RVD). *H. inflata* contains high concentrations of amino acids, with alanine constituting over 70% of the total amino acid pool. Exposure to hypo-osmotic medium resulted in the loss from the cell of both amino acids and K⁺, via one or more swelling-activated pathways. The efflux of amino acids and K⁺, together with a charge-balancing counter-anion, accounted almost fully for the observed RVD. The pharmacological properties of the swelling-activated pathways differ from those of volume-sensitive transporters and channels described previously in other cell types.

Keywords: anaerobic protozoa, osmoregulation, volume regulation, membrane transport, alanine

INTRODUCTION

Hexamita inflata, a free-living protozoon, is one of the most primitive extant protists as inferred by analysis of small-subunit rRNA coding region sequences (Leipe *et al.*, 1993; van Keulen *et al.*, 1993). It is a microaerobe with a high affinity for O₂ (apparent K_m for O₂ 13 µM) but it lacks mitochondria (Brugerolle, 1974) or detectable cytochromes and hence a respiratory electron chain (Biagini *et al.*, 1997). Energy requirements in *H. inflata* are believed to be met through substrate-level phosphorylation, with ethanol, lactate, acetate and alanine being the principal products of glucose fermentation (Biagini *et al.*, 1998). The arginine dihydrolase pathway is also believed to operate in *H. inflata*, providing ATP from the catabolism of arginine (Biagini *et al.*, 1998).

Free-living *Hexamita* species have been observed in low-

O₂ marine and freshwater environments (Fenchel *et al.*, 1995; Fenchel & Finlay, 1995). In addition, parasitic species of *Hexamita* (which may have free-living life stages) have been observed in a variety of organisms, both invertebrate (e.g. shellfish; Kulda & Nohýnková, 1978) and vertebrate (e.g. fish; Buchmann *et al.*, 1995; Ferguson, 1979); in this context, *Hexamita* spp. are of substantial economic significance.

In adapting to the variety of habitats in which it is found, *Hexamita* must cope with extreme variations in the osmolality of its environment. Such variations pose a challenge to cell volume. In many protozoa, osmoregulation and cell volume control is achieved via the operation of a contractile vacuole. However, *Hexamita* reportedly lacks this organelle (Brugerolle, 1974). Other protozoa rely on the modulation of intracellular levels of osmotically active solutes (osmolytes) via the operation of volume-sensitive membrane transport pathways. For example, the closely related diplomonad *Giardia intestinalis* possesses a large pool of free amino acids (Knodler *et al.*, 1994). Upon exposure to hypo-osmotic stress, *G. intestinalis* has been shown to release amino acids, principally alanine (present at approx. 50 mM;

Abbreviations: [1-¹⁴C]AIB, 2-amino[1-¹⁴C]isobutyrate; DID5, 4,4'-diisothiocyano-stilbene-2,2'-disulfonic acid disodium salt; NEM, *N*-ethylmaleimide; NPPB, 5-nitro-2-(3-phenylpropylamino)benzoic acid; RVD, regulatory volume decrease.

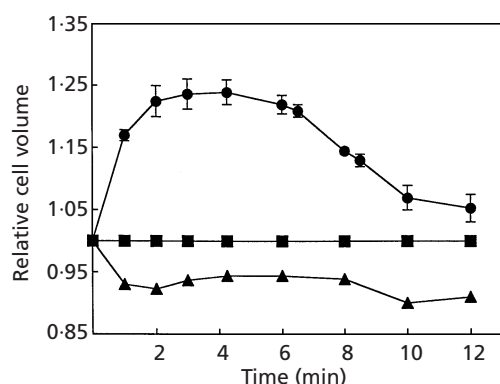


Fig. 1. Relative cell volume of *H. inflata* cells suspended in iso-osmotic (300 mosmol kg⁻¹; ■), hyper-osmotic (400 mosmol kg⁻¹; ▲) or hypo-osmotic (150 mosmol kg⁻¹; ●) media at *t* = 0. Error bars indicate SEM from three independent experiments (hyper-osmotic data represent the mean results from two experiments).

Knodler *et al.*, 1994), via swelling-activated transport pathways (Park *et al.*, 1995, 1997). The efflux of amino acids, perhaps together with other intracellular solutes, results in a net loss of water from the swollen cells and thereby allows *G. intestinalis* to undergo a regulatory volume decrease (RVD), back to its initial cell volume (Park *et al.*, 1995, 1997, 1998). The use of alanine as the principal organic osmolyte has also been observed in a range of other protozoa including *Crithidia luciliae* (Bursell *et al.*, 1996), *Acanthamoeba castellanii* (Geoffrion & Larochelle, 1984) and *Leishmania major* (Darling *et al.*, 1990; Vieira *et al.*, 1996).

In this study we have investigated the ability of *H. inflata* to regulate its volume in aniso-osmotic media and have characterized the mechanisms involved.

METHODS

Organism and solutions. *H. inflata* was grown as described previously (Biagini *et al.*, 1997). Cells were grown to late-exponential phase, harvested by centrifugation at 650 *g* for 5 min and resuspended in phosphate-buffered saline (PBS: 150 mM NaCl, 5 mM K₂HPO₄, 1.8 mM KH₂PO₄, pH 7.2). PBS was used as the standard iso-osmotic solution (300 mosmol kg⁻¹) and any change in the osmotic pressure was achieved either by the addition of appropriate solutes or by dilution with H₂O. The osmolality of all solutions was measured using an osmometer (Fiske). Cell counts were performed using a haemocytometer.

Cell volume measurements. Total cell volumes were measured using a Coulter Multisizer fitted with a 100 µm aperture tube. Signals from the Multisizer were collected on Multisizer AccuComp software. Results are expressed as the mean of the cell volume distribution from approximately 4000 cells for each time point. Intracellular water space was measured as described by Knodler *et al.* (1994), with the exception that [1-¹⁴C]mannitol was used rather than [1-¹⁴C]inulin to estimate the extracellular water volume in the cell pellets.

Amino acid analysis. Intracellular amino acid analysis was performed as described by Knodler *et al.* (1994), with β-alanine as the internal standard, on a Beckman 6300 amino acid analyser.

2-Amino[1-¹⁴C]isobutyrate influx. Volume-sensitive amino acid transport was investigated using 2-amino[1-¹⁴C]isobutyrate ([1-¹⁴C]AIB), a non-metabolizable analogue of alanine. [1-¹⁴C]AIB influx measurements were carried out essentially as described by Park *et al.* (1995, 1998). *H. inflata* cell suspensions (typically 2–4 × 10⁷ cells ml⁻¹) in PBS of varying osmolality were incubated with [1-¹⁴C]AIB [specific activity 0.5 µCi mol⁻¹ (37 kBq mol⁻¹)]. At specific time intervals aliquots of the suspension were overlaid onto oil (a mixture of dibutyl phthalate and diiso-octyl phthalate, 4:1, v/v; 1.03 g ml⁻¹) in a microcentrifuge tube and centrifuged at 10000 *g* for 20 s, sedimenting the cells below the oil. Cell pellets were lysed with 0.1% (v/v) Triton X-100, precipitated with 6% (w/v) trichloroacetic acid and centrifuged at 10000 *g* for 30 s. For scintillation counting, samples (1 ml) were mixed with scintillant (9 ml) containing 0.5% (w/v) 2,5-diphenyloxazole in Triton X-100/toluene (1:2, v/v).

⁸⁶Rb⁺ and [1-¹⁴C]AIB efflux. The involvement of K⁺ in the volume regulatory response was investigated using ⁸⁶Rb⁺ as a congener for K⁺. Cells were harvested by gentle centrifugation (650 *g*) and preloaded with ⁸⁶Rb by incubation with ⁸⁶RbCl [1–2 µCi ml⁻¹ (37–74 kBq ml⁻¹)] for 2.5 h in fresh culture medium. After the pre-incubation the cells were washed (three times) to remove extracellular ⁸⁶Rb⁺ and resuspended in PBS. Efflux time-courses commenced with the addition of an aliquot of the ⁸⁶Rb⁺-loaded cells into either iso-osmotic PBS or hypo-osmotic (diluted with H₂O) PBS of varying osmolality. At predetermined time intervals aliquots were taken and centrifuged through the oil mix. Supernatant and pellet fractions were processed as described above.

For dual-labelling experiments, designed to monitor concomitantly the efflux of [1-¹⁴C]AIB and ⁸⁶Rb⁺, cells were loaded with [1-¹⁴C]AIB by subjecting them to a hypo-osmotic shock (150 mosmol kg⁻¹) in the presence of [1-¹⁴C]AIB (as described by Park *et al.*, 1998). The [1-¹⁴C]AIB-loaded cells were then washed in PBS, loaded with ⁸⁶Rb⁺ (1–2 µCi ml⁻¹; 2.5 h), and subjected to the efflux protocol described above. The concentrations of [1-¹⁴C]AIB and ⁸⁶Rb⁺ in the dual-label samples were estimated by measuring ¹⁴C and ⁸⁶Rb radioactivity over different ranges and subtracting the contribution of ⁸⁶Rb to the c.p.m. in the ¹⁴C range.

Suspension of cells in media having an osmolality of 150 mosmol kg⁻¹ caused a rapid lysis of a small proportion (14 ± 2%) of the cells (as estimated by total cell counts and confirmed in initial efflux experiments). The efflux time course data were corrected accordingly.

Estimation of intracellular K⁺ concentration. The intracellular K⁺ concentration of *H. inflata* under the growth conditions used in this study was estimated from the equilibrium distribution of ⁸⁶Rb⁺. The validity of this approach rests on the assumption that the equilibrium distribution of ⁸⁶Rb⁺ between the intra- and extracellular solutions is the same as that for K⁺. Cells were incubated with ⁸⁶RbCl (approx. 0.2 µCi ml⁻¹) overnight in culture medium. The amount of ⁸⁶Rb⁺ taken up by the cells was determined by centrifuging the cells through the oil mix and processing the cell pellet as described above. From the measured intracellular ⁸⁶Rb⁺ content, the intracellular water space (measured as above) and the extracellular ⁸⁶Rb⁺ concentration, it was possible to determine the transmembrane ⁸⁶Rb⁺ distribution ratio

$([^{86}\text{Rb}^+]_i/[^{86}\text{Rb}^+]_o)$, where $[^{86}\text{Rb}^+]_i$ and $[^{86}\text{Rb}^+]_o$ are the intra- and extracellular $^{86}\text{Rb}^+$ concentrations, respectively). The concentration of K^+ in the medium ($[\text{K}^+]_o$) was determined by flame photometry (Radiometer), and the intracellular K^+ concentration ($[\text{K}^+]_i$) calculated using the equation $[\text{K}^+]_i = [\text{K}^+]_o \times ([^{86}\text{Rb}^+]_i/[^{86}\text{Rb}^+]_o)$.

Effect of inhibitors on $^{86}\text{Rb}^+$ and $[1\text{-}^{14}\text{C}]\text{AIB}$ efflux. A number of inhibitors of ion and organic osmolyte channels were tested for their ability to inhibit $[1\text{-}^{14}\text{C}]\text{AIB}$ and $^{86}\text{Rb}^+$ efflux. These included BaCl_2 (2 mM), quinine (1 mM), tamoxifen (10 μM), 4,4'-diisothiocyanato-stilbene-2,2'-disulfonic acid disodium salt (DIDS, 500 μM), niflumate (200 μM), 5-nitro-2-(3-phenyl-propylamino)benzoic acid (NPPB, 100 μM) and *N*-ethyl-maleimide (NEM, 200 μM). Cells were preloaded with AIB and $^{86}\text{Rb}^+$ as described above and incubated for 15 min with the inhibitors before being subjected to a hypo-osmotic shock

(150 mosmol kg^{-1}) for 5 min. The inhibitor concentration was maintained pre- and post-osmotic shock. Cells were then processed as described above. Experiments were performed in triplicate and repeated at least twice.

RESULTS

Cell volume regulation in aniso-osmotic media

In initial experiments we investigated the ability of *H. inflata* to regulate its volume in response to an acute increase or decrease in the osmolality of the suspending medium. Cells in PBS had an estimated mean total volume of 262 ± 12 fl ($n = 15$). This compares to an estimated mean intracellular water volume of 214 ± 5 fl ($n = 5$), some 80 % of the total volume. Cells in PBS maintained a constant volume for 30 min.

Table 1. Intracellular amino acid concentrations of *H. inflata* under iso-osmotic and hypo-osmotic conditions, and hypo-osmotically induced amino acid release

The results are means \pm SD from three experiments.

Amino acid	Iso-osmotic conditions		Hypo-osmotic conditions [nmol (10^8 cells) $^{-1}$] \dagger	Hypo-osmotically induced amino acid release [nmol (10^8 cells) $^{-1}$] \ddagger
	[nmol (10^8 cells) $^{-1}$]	(mM)*		
Alanine	1482.4 ± 40.0	68.95 ± 1.82	664.1 ± 20.1	818.3
α -Aminobutyrate	15.7 ± 1.6	0.74 ± 0.07	8.5 ± 0.5	7.2
Arginine	3.0 ± 2.0	0.14 ± 0.09	2.5 ± 0.3	—
Asparagine	8.9 ± 3.3	0.41 ± 0.15	12.3 ± 3.0	—
Aspartate	10.0 ± 0.8	0.47 ± 0.04	10.3 ± 0.8	—
Citrulline	2.8 ± 1.2	0.13 ± 0.05	2.5 ± 0.5	—
Cystine	10.8 ± 2.1	0.50 ± 0.09	10.1 ± 4.3	—
Glutamate	42.9 ± 2.6	2.00 ± 0.12	32.7 ± 1.3	10.2
Glutamine	0.6 ± 0.6	0.03 ± 0.03	2.5 ± 1.7	—
Glycine	67.8 ± 2.6	3.15 ± 0.12	39.5 ± 5.8	28.3
Histidine	3.4 ± 0.1	0.15 ± 0.01	2.0 ± 0.5	1.4
Isoleucine	37.7 ± 1.1	1.76 ± 0.05	10.5 ± 0.5	27.2
Leucine	92.9 ± 2.1	4.32 ± 0.09	25.9 ± 1.0	67.0
Lysine	10.5 ± 0.5	0.49 ± 0.02	7.8 ± 0.8	2.7
Methionine	8.6 ± 0.4	0.40 ± 0.02	5.5 ± 0.5	3.1
Ornithine	2.4 ± 0.1	0.11 ± 0.00	2.5 ± 0.5	—
Phenylalanine	30.2 ± 1.0	1.40 ± 0.04	7.3 ± 0.8	22.9
O-Phosphoserine	6.7 ± 0.5	0.32 ± 0.02	6.0 ± 0.3	—
Proline	107.1 ± 54.6	4.87 ± 2.48	64.9 ± 35.2	—
Serine	8.5 ± 0.7	0.40 ± 0.03	6.5 ± 1.3	2.0
Threonine	12.4 ± 0.7	0.58 ± 0.03	7.8 ± 0.8	4.6
Tryptophan	8.1 ± 2.4	0.38 ± 0.11	1.8 ± 2.0	6.3
Tyrosine	20.6 ± 2.9	0.96 ± 0.13	5.8 ± 0.5	14.8
Valine	48.1 ± 3.4	2.24 ± 0.16	13.0 ± 0.5	35.1
Total	2042.2 ± 127.2	94.91 ± 5.77	951.6 ± 98.9	1046.3

* Assuming an intracellular water space of $21.5 \mu\text{l}$ (10^8 cells) $^{-1}$ for cells in iso-osmotic conditions.

\dagger Intracellular amino acid concentration after 7 min exposure to a hypo-osmotic shock (150 mosmol kg^{-1}).

\ddagger Amounts only given where the difference is greater than the sum of the SD values before and after the hypo-osmotic challenge.

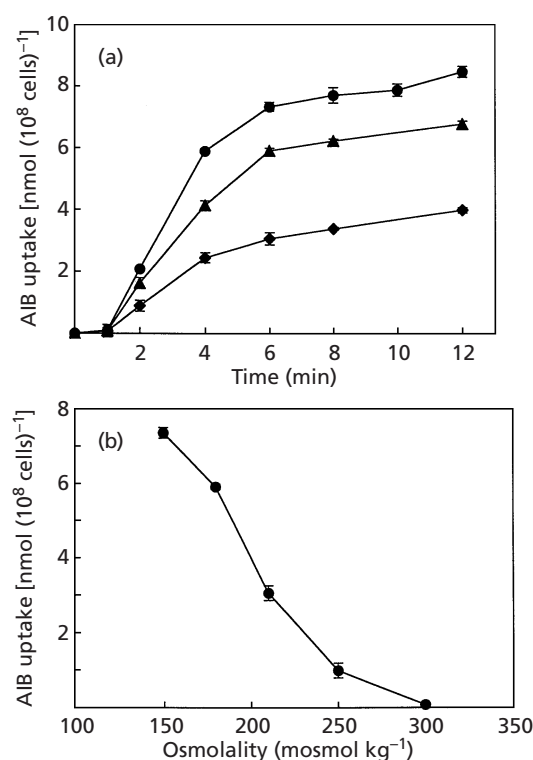


Fig. 2. (a) Time courses for the uptake of the alanine analogue AIB into *H. inflata* cells, after suspension at $t = 0$ in buffer with osmolalities 210 mosmol kg⁻¹ (◆), 180 mosmol kg⁻¹ (▲) or 150 mosmol kg⁻¹ (●). (b) AIB uptake by *H. inflata* during 6 min exposure to buffer of varying osmolalities. Error bars indicate SEM from three independent experiments.

An increase in the extracellular osmolality, from 300 mosmol kg⁻¹ to 350–400 mosmol kg⁻¹, achieved by the addition of appropriate amounts of mannitol, choline chloride, NaCl or KCl, resulted in a marked reduction of cell volume within 1–2 min. In all cases the cells failed to show a volume recovery and remained shrunken for up to 30 min (Fig. 1).

A reduction in the external osmolality from 300 to 150 mosmol kg⁻¹ caused the cells to swell, attaining a maximum volume approximately 1.25 times their original value 3–4 min after the osmotic challenge (Fig. 1). The initial swelling was followed by an RVD and by 12 min the cell volume had returned to approximately 1.05 times its original value.

Swelling-activated amino acid release

Analysis of the free intracellular amino acid pool of *H. inflata* revealed alanine to be the major component, present at a concentration of 69 mM under iso-osmotic conditions (Table 1). Leucine, glycine, valine and glutamate were also found at high (>2 mM) concentrations. The concentration of proline fluctuated between samples to a greater extent than that of any of the other amino acids, as reflected in the relatively high standard deviation. The total free amino acid pool of *H.*

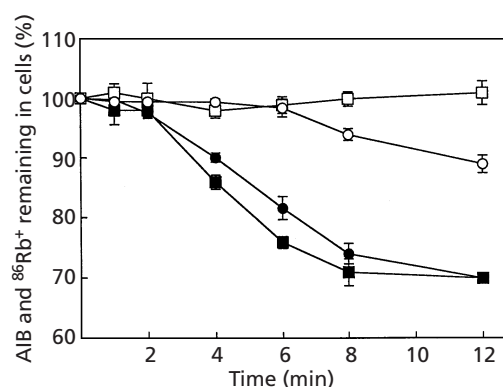


Fig. 3. Time courses for the efflux of ⁸⁶Rb⁺ (○, ●) and [1-¹⁴C]AIB (□, ■) from *H. inflata* following suspension in iso-osmotic (300 mosmol kg⁻¹; ○, □) and hypo-osmotic (150 mosmol kg⁻¹; ●, ■) media at $t = 0$. The data are corrected to account for the lysis of a small proportion of cells (14%) within the first few seconds of suspension in the hypo-osmotic medium. Error bars indicate SEM from three independent experiments.

inflata, under iso-osmotic conditions, was estimated to be approximately 95 mM.

The extent of involvement of amino acids in the RVD response noted in Fig. 1 was assessed by analysing the amino acid content of cells exposed to hypo-osmotic medium. Cells suspended in medium with an osmolality of 150 mosmol kg⁻¹ for 7 min lost over half of their total intracellular amino acid pool (Table 1). Alanine was the major contributor, constituting 78 % of the total amino acid loss. Other significant contributions to the total amino acid loss were made by (in descending order) the other neutral amino acids leucine, valine, glycine, isoleucine, phenylalanine and tyrosine, and the anionic amino acid glutamate.

Swelling-activated transport of [1-¹⁴C]AIB and ⁸⁶Rb⁺

The pathway(s) involved in swelling-activated amino acid release from *H. inflata* were investigated in more detail by measuring the transmembrane flux of [1-¹⁴C]AIB, a structural non-metabolizable analogue of alanine. [1-¹⁴C]AIB did not enter the cells under iso-osmotic conditions. However, on decreasing the osmolality, there was a slight (approx. 2 min) lag, after which [1-¹⁴C]AIB was taken up (Fig. 2a). The rate of uptake and final intracellular [1-¹⁴C]AIB concentration were dependent upon the magnitude of the osmotic stress to which the cells were subjected, with the highest rate of uptake observed at the lowest osmolality tested. The elevated rate of uptake was maintained for 4–6 min, after which it decreased. A plot of the amount of AIB taken up by cells after 6 min exposure to solutions of varying osmolalities shows a sigmoidal dependence on the extracellular osmolality (Fig. 2b).

The swelling-activated efflux of [1-¹⁴C]AIB and ⁸⁶Rb⁺ from *H. inflata* was investigated by preloading cells with

one or both of the radiolabelled solutes as described in Methods, then resuspending them in either iso-osmotic or hypo-osmotic medium and monitoring the loss of the two radiolabels from the cells. Fig. 3 shows time courses from a typical efflux experiment. Under iso-osmotic conditions, $^{86}\text{Rb}^+$ was lost from the cells at a slow, but significant rate, whereas $[1\text{-}^{14}\text{C}]\text{AIB}$ levels remained approximately constant. On exposure of cells to hypo-osmotic medium there was a brief (approx. 2 min) lag, followed by a progressive loss of both solutes from the cells. The time-courses for the efflux of $^{86}\text{Rb}^+$ and $[1\text{-}^{14}\text{C}]\text{AIB}$ from cells in a hypo-osmotic medium were very similar, with the intracellular concentrations of the two solutes dropping to a similar extent.

The efflux of $^{86}\text{Rb}^+$ both in iso-osmotic and in hypo-osmotic medium was unaffected by whether the cells had been subjected to the $[1\text{-}^{14}\text{C}]\text{AIB}$ preloading procedure (results not shown).

Intracellular K^+ concentration

To gain some insight into the extent to which the swelling-activated efflux of K^+ contributed to the RVD response of *H. inflata* following a hypo-osmotic challenge, the intracellular K^+ content was estimated from the equilibrium distribution of $^{86}\text{Rb}^+$. Cells preincubated overnight in iso-osmotic growth medium containing $^{86}\text{Rb}^+$ accumulated the radioisotope to a concentration some 2.5 times that in the extracellular solution, yielding an estimate for intracellular K^+ concentration of $84 \pm 13 \text{ mM}$ (SEM, $n = 5$).

Effect of inhibitors on the swelling-activated efflux of $[1\text{-}^{14}\text{C}]\text{AIB}$ and $^{86}\text{Rb}^+$

A range of reagents shown previously to inhibit the swelling-activated transport of solutes from various cell types were tested for their effects on the efflux of $[1\text{-}^{14}\text{C}]\text{AIB}$ and $^{86}\text{Rb}^+$ from *H. inflata* after hypo-osmotic challenge ($150 \text{ mosmol kg}^{-1}$). The thiol reagent NEM and the anion-transport inhibitor niflumic acid both lysed the cells at concentrations down to $200 \mu\text{M}$ and their effect on efflux could therefore not be evaluated. The K^+ -channel blockers BaCl_2 (2 mM) and quinine (1 mM) and the anion-channel blockers DIDS ($500 \mu\text{M}$), NPPB ($100 \mu\text{M}$) and tamoxifen ($10 \mu\text{M}$) were all without significant inhibitory effect.

DISCUSSION

The free-living microaerobic diplomonad *H. inflata* is capable of adapting to a wide range of osmotic environments. In this study we have investigated the osmoregulatory strategies that underlie its ability to do so.

Increasing the extracellular osmolality by the addition of various solutes caused *H. inflata* to shrink and it failed to show any volume recovery over a 30 min period. Failure to regulate cell volume in response to

osmotic shrinkage is not uncommon amongst the cells of higher eukaryotes (Hallow & Knauf, 1994), and has also been observed in other protozoa (e.g. *Giardia*; Park *et al.*, 1995). It could be argued that cell shrinkage is unlikely to be life-threatening, at least in the short-term, and *H. inflata* does not expend resources on mounting a rapid volume-regulatory response under these conditions.

Osmotic swelling poses a more serious challenge for a cell as, unless it is countered, the cell is at risk of bursting. We have demonstrated that *H. inflata* can regulate its volume in response to a hypo-osmotic challenge and that it does so through the loss of both amino acids and K^+ .

A simple calculation enables a semi-quantitative assessment of the relative contribution of amino acids and K^+ loss to the overall RVD. In the absence of any RVD mechanisms operating (i.e. if the cells behaved as 'perfect osmometers') a 50% reduction of the osmolality should have resulted in a doubling of the cell water volume, from 214 fl to 428 fl. As is evident from Fig. 1, 7 min after the reduction of the osmolality from 300 to $150 \text{ mosmol kg}^{-1}$ the total cell volume was approximately 312 fl and the cell water volume therefore approximately 264 fl (assuming that the contribution of cell solids to the total volume was unaffected by osmolality), some 164 fl less than the volume predicted had the cell lacked the capacity to volume regulate. From Table 1, during 7 min in hypo-osmotic medium each cell lost 10.5 fmol of amino acids. Over the same period each lost approximately 6 fmol of K^+ (approx. 30% of the total intracellular K^+ content; from Fig. 3). If it is assumed that the loss of amino acids and K^+ was accompanied by sufficient water to produce a solution of osmolality equivalent to that of the extracellular solution, and that the osmotic coefficients of the amino acids and K^+ are 1 and 0.95, respectively, then it can be calculated that the loss of amino acids from the cell resulted in the net loss of approximately 70 fl of water, and the loss of K^+ resulted in the net loss of approximately 40 fl of water. The loss of K^+ from the cell must necessarily have been accompanied by the loss of an equivalent amount of a counter-balancing ion; and assuming that this was either Cl^- or another monovalent anion, this accounts for a further 40 fl of water loss.

In summary, 7 min after the suspension of *H. inflata* in a medium of half the original osmolality, the parasite had an intracellular water volume some 164 fl less than that predicted had the cells been unable to undergo RVD. Of this 164 fl, approximately 70 fl may be attributed to the loss of amino acids and 80 fl to the loss of K^+ together with a charge-balancing counter-anion, accounting almost fully for the observed RVD.

The volume-regulatory efflux of amino acids has been described in a number of different protozoa, including *Giardia* (Park *et al.*, 1995), *Crithidia* (Bursell *et al.*, 1996), *Acanthamoeba* (Geoffrion & Larochelle, 1984) and *Leishmania* (Darling *et al.*, 1990; Vieira *et al.*, 1996).

In all cases, as with *H. inflata*, the predominant intracellular amino acid that was lost in the greatest quantity during RVD was alanine. In *Leishmania*, hypo-osmotic challenge was reported to result in increased $^{86}\text{Rb}^+$ efflux; however, it was concluded that this made only a minor (<4%) contribution to the RVD (Blum, 1992). This contrasts with the situation in *H. inflata*, in which the efflux of K^+ , together with its counter-anion, contributes approximately half of the total RVD. We have observed a similar substantial contribution by K^+ to the total RVD in *Giardia* pertaining to hypo-osmotic challenge (S. Maroulis & M. R. Edwards, unpublished). To our knowledge, these observations of the diplomonads *Hexamita* and *Giardia* represent the first reports of the loss of K^+ making a significant contribution to the RVD response in a protozoon.

The strategy of releasing a combination of organic and inorganic solutes following cell swelling is common in cells from higher eukaryotes. Cells from vertebrates typically contain high cytosolic concentrations of the sulfonic amino acid taurine and/or polyols such as *myo*-inositol and sorbitol and these are released in response to cell swelling via broad-specificity 'osmolyte channels' (Kirk, 1997). Such cells commonly also possess one or more swelling-activated K^+ and Cl^- transport pathways (channels and/or cotransporters) which mediate the volume-regulatory efflux of KCl (Hallow & Knauf, 1994).

The finding that the volume-regulatory efflux in *H. inflata* was not inhibited by reagents that inhibit swelling-activated K^+ and anion channels in vertebrate cells is consistent with the pathway(s) operating in *H. inflata* being quite different from their vertebrate counterparts. The observation that osmotic swelling induces an increase in both the unidirectional influx (Fig. 2) and efflux (Fig. 3) of the alanine analogue AIB is consistent with the pathway being bi-directional, with the net flux of amino acids from the cell being determined simply by the large outward concentration gradient for these solutes. If a single pathway mediates the efflux of all of the amino acids lost from the cell, it is clearly one with a broad substrate specificity, though the relative loss of the different amino acids from the cell does suggest a degree of selectivity, with the pathway having an apparent preference for neutral amino acids (Table 1). The volume-regulatory loss of amino acids from other protozoa has been attributed to a swelling-activated channel (Vieira *et al.*, 1996; Bursell *et al.*, 1996; Park *et al.*, 1998) and the data are consistent with a similar pathway operating in *H. inflata*. Whether it is this same pathway that mediates the volume-regulatory efflux of K^+ remains to be established.

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