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Volume-regulatory Amino Acid Release from the Protozoan Parasite *Crithidia luciliae*

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Abstract. The unicellular protozoan parasite, *Crithidia luciliae*, responded to osmotic swelling by undergoing a regulatory volume decrease. This process was accompanied by the efflux of amino acids (predominantly alanine, proline and glycine). The relative loss of the electroneutral amino acids proline, valine, alanine and glycine was greater than that for the anionic amino acid, glutamate; there was negligible loss of the cationic amino acids, lysine, arginine and ornithine. The characteristics of amino acid release were investigated using a radiolabeled form of the nonmetabolized alanine analogue α-aminoisobutyrate. α-Aminoisobutyrate efflux was activated within a few seconds of a reduction of the osmolality, and inactivated rapidly (again within a few seconds) on restoration of isotonicity. The initial rate of efflux of α-aminoisobutyrate from cells in hypotonic medium was unaffected by the extracellular amino acid concentration. Hypotonically activated α-aminoisobutyrate efflux (as well as the associated regulatory volume decrease) was inhibited by the sulfhydryl reagent *N*-ethylmaleimide but was not inhibited by a range of anion transport blockers. As in the efflux experiments, unidirectional influx rates for α-aminoisobutyrate increased markedly following reduction of the osmolality, consistent with the swelling-activated amino acid release mechanism allowing the flux of solutes in both directions. Hypotonically activated α-aminoisobutyrate influx showed no tendency to saturate up to an extracellular concentration of 50 mM. The functional characteristics of the amino acid release mechanism are those of a channel, with a preference for electroneutral and anionic amino acids over cationic amino acids. However, the pharmacology of the system differs from that of the anion-selective channels that are thought to mediate the volume-regulatory efflux of organic osmolytes from vertebrate cells.

Key words: Volume regulation — Osmolyte — Amino acid — Anion channel

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Introduction

The trypanosomatids are unicellular protozoan parasites, most of which spend at least part of their life cycle in the gut of insects. Some genera (e.g., *Crithidia*) are exclusively insect parasites. Others (e.g., *Trypanosoma* and *Leishmania*) are also parasites of vertebrates. Such organisms are often pathogenic to their vertebrate hosts and are the causative agents of a number of major human and animal diseases.

During the course of their life cycles the trypanosomatids adapt to a wide range of physical and chemical environments. Their ability to do so indicates that they have powerful homeostatic mechanisms that enable them to control their basic physico-chemical properties. However, despite the severity and prevalence of the diseases caused by such organisms, and despite major advances over the last decade in understanding the regulatory mechanisms of vertebrate cells, the corresponding systems in protozoan parasites remain poorly understood.

In the context of cell volume control, it is clear from the few studies that have been carried out that the trypanosomatids are able to survive exposure to a much wider range of osmolalities than most animal cells (e.g., Cosgrove & Kessel, 1958; Darling, Burrows & Blum, 1990). This adaptive ability may involve several different mechanisms. At least some trypanosomatids have, like other protozoa, contractile vacuoles—organelles which actively pump water from the cell. The vacuolar

output increases with decreasing external osmolality (Cosgrove & Kessel, 1958), thereby countering, at least in part, the osmotic flow of water into the cell. An alternative strategy involves controlling the osmotic contents of the cell cytoplasm. A number of trypanosomatids that are thought to lack contractile vacuoles have been shown to respond to osmotic swelling by the loss of intracellular solutes (amino acids and K^+) and to undergo a regulatory volume decrease (RVD) (Andrade & Andrade, 1988; Darling et al., 1990; Blum, 1992; Vieira & Cabantchik, 1995). This process has been described in some detail in *Leishmania donovani* by Blum and coworkers who have shown that in the initial minute following cell swelling amino acid loss is much greater than K^+ loss (Blum, 1992) and that approximately half of the total amino acid loss is in the form of alanine (Darling et al., 1990). It was postulated that swelling-activated alanine loss may be mediated via the active transporter that is responsible for the concentrative uptake of this amino acid (Blum, 1992). However, this remains to be tested.

In this study, we have investigated the response to osmotic stress of the trypanosomatid parasite, *Crithidia luciliae. C. luciliae* is a nonpathogenic trypanosomatid that inhabits the gut of insects. It grows vigorously in standard culture media and has proven to be a valuable model system for studies of trypanosomatid biochemistry and physiology (McGhee & Cosgrove, 1980). Here we show that this organism responds to hypo-osmotic swelling by undergoing RVD. As in other protozoa, as well as many vertebrate cell types, this process involves the loss of cytoplasmic amino acids. The data are consistent with the amino acid release pathway being a channel that is selective for neutral and anionic solutes over cationic solutes.

Materials and Methods

Materials

[^{3}H]Water and [^{14}C]inulin were from Du Pont-New England Nuclear. [^{14}C]α-Aminoisobutyrate ([^{14}C]AIB) was from Amersham International. Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), carbonyl cyanide *p*-(trifluromethoxy)phenylhydrazone (FCCP), 4,4′-isothiocyanato-stilbene-2,2′-disulfonic acid disodium salt (DIDS), 2,4-dinitrophenol (DNP), *N*-ethylmaleimide (NEM), niflumate, ninhydrin (2,2-dihydroxy-1,3-indanedione; 1,2,3-indantrione hydrate) reagent solution, quinine and tamoxifen were from Sigma Chemicals. 5-Nitro-2-(3-phenylpropylamino)benzoic acid (NPPB) was a gift from Prof. R. Greger (Physiologisches Institut der Albert-Ludwigs-Universitat, Freiburg, Germany).

Growth and Harvesting of Parasites

C. luciliae were grown at 26°C in RPMI 1640 growth medium supplemented with 10% *v/v* fetal calf serum, 30 mM HEPES and 20 mM glutamine. Cells in late-log phase (at approximately 1.5×10^7 cells/ml) were harvested by centrifugation (700 × *g*, 5 min) and resuspended at a cell density of 0.5–2 × 10^9 cells/ml either in iso-osmotic saline (if the cells were to be used immediately for experimentation) or in RPMI adjusted to 300 mOsm/kg H_2O by the dropwise addition of a 1 M HEPES solution, pH 7.4 (if the cells were to be preloaded with radiolabeled solute prior to carrying out an efflux experiment).

Solutions

The standard iso-osmotic solution used throughout this study contained (in mM): 150 NaCl, 5 KCl, 10 HEPES, 1.3 $CaCl_2$ and 0.5 $MgCl_2$. In experiments in which cells were suspended in solutions containing high concentrations of alanine or α-aminoisobutyrate (AIB) the NaCl concentration was reduced to maintain the required osmolality. For all iso-osmotic solutions the pH was adjusted to 7.4 using 1 M NaOH and the osmolality was within the range 295–305 mOsm/kg H_2O. In all experiments in which cells were subjected to osmotic swelling, isotonic saline was diluted with a volume of hypo-osmotic solution (pH 7.4) containing (in mM): 5 KCl, 10 HEPES, 1.3 $CaCl_2$ and 0.5 $MgCl_2$, to give the required osmolality while maintaining a constant concentration of all components other than NaCl.

Analytical Measurements

Cell counts were carried out using a Coulter Multisizer and/or a hemocytometer with improved Neubauer ruling. For the latter method cells were diluted in a fixative solution (1% *w/v* paraformaldehyde and 0.5% *w/v* sucrose in phosphate-buffered saline). Total (primary and secondary) amino acid concentrations were measured using ninhydrin reagent (Lee & Takahashi, 1966) together with alanine standards. Full amino acid analyses were carried out using an ABI 420A derivatizer/analyzer which derivatized the amino acids with phenylisothiocyanate to form phenylthiocarbamyl amino acids prior to their passage through a narrow-bore HPLC column. The data were analyzed using an ABI 920A system (Perkin-Elmer, Lancashire, UK).

Protein concentrations were measured using the Coomassie-based Bio-Rad Protein Assay with bovine albumin standards. The osmolality of all solutions was measured using a Roebling freezing point osmometer (Berlin, Germany).

Cell Volume Measurements

Intracellular water volume was determined at room temperature (approximately 22°C) using a combination of ^3H_2O and [^{14}C]inulin as described by Knodler, Schofield & Edwards, (1992). Briefly, cells in iso-osmotic saline were added to tubes containing either an iso-osmotic or hypo-osmotic solution to which had been added either ^3H_2O or [^{14}C]inulin. The final cell density was typically 5×10^7 cells/ml and the final concentrations of radiolabel 0.5 μCi/ml or 0.05 μCi/ml, for ^3H_2O and [^{14}C]inulin, respectively. At the required times a 1-ml aliquot of suspension was removed and layered over 0.2 ml of oil (a blend of dibutylphthalate (Sigma Chemicals; 72% *v/v*) + di-′isononyl′phthalate (Fluka Chemicals; 28% *v/v*), with a density of 1.02 g/ml) in a microcentrifuge tube. The tube was centrifuged immediately, sedimenting the cells beneath the oil. A 0.05-ml aliquot of the supernatant solution was transferred to a scintillation vial for estimation of the concentration of radiolabel in the extracellular solution, then the remaining aqueous supernatant solution was aspirated. The radioactivity remaining on the walls of the tube was removed by rinsing the tubes four times with water and then the oil was aspirated. The cell pellet was lysed with 0.1% *v/v* Triton X-100 (0.5 ml) and deproteinized by the addition of 5% *w/v* trichloroacetic acid (TCA; 0.5 ml), followed by

centrifugation (10,000 × *g*, 10 min). Radioactivity was measured using a β-scintillation counter.

The amount of ^3H_2O in the pellet provided an estimate of the *total* water volume while the amount of [^{14}C]inulin provided an estimate of the *extracellular* water volume. The difference between them was taken to be the *intracellular* water volume. This was divided by the number of cells in the pellet (calculated from the cell counts in the original suspension) to give the individual mean cell water volume.

In one series of experiments *total* cell volumes were measured using A Coulter Multisizer (Coulter Electronics, Bedfordshire, UK) fitted with an aperture tube with a 100 μm diameter orifice. The relatively small size of the parasites (approximately 17 fl) meant that in some (though not all) experiments there was some overlap of the lower portion of the *Crithidia* cell volume distribution and the uppermost portion of the (somewhat variable) debris peak, not allowing a clear distinction between the two. In all experiments we therefore measured the mean volume of particles within a fixed range (7–200 fl). With this approach, there was some variation between the absolute values of the cell volume estimates on a day-to-day basis; however when the estimated mean volumes were expressed relative to that of cells in isotonic media the data were highly reproducible between experiments. The Coulter Multisizer lacked the facility for temperature control and the (online) measurements reported in the present work were carried out at a temperature of approximately 30°C, substantially higher than that at which the intracellular water and amino acid transport measurements were made (22°C).

Endogenous Amino Acid and Protein Release

In initial experiments, a ninhydrin assay was used to monitor the release of total (primary and secondary) amino acids from parasites suspended in media of different osmolalities. The cells were harvested then washed twice by centrifugation (700 × *g*, 5 min) in ice-cold iso-osmotic saline to remove amino acids from the extracellular solution while minimizing the loss of intracellular amino acids. Aliquots of the resulting suspension were then added to solutions of different osmolality at room temperature to give a final cell concentration of approximately 4×10^7 cells/ml. At predetermined intervals 1-ml aliquots of suspension were removed and layered over 0.2 ml of oil (as above) in a microcentrifuge tube which was centrifuged (10,000 × *g*, 20 sec) immediately. In these experiments only the supernatant (extracellular) solution was assayed for its amino acid content. A 0.4 ml aliquot of the solution was transferred from above the oil to a glass tube, to which was then added 0.2 ml ninhydrin reagent solution. The tubes were placed in a boiling water bath for exactly 10 min then cooled to room temperature. A 1-ml aliquot of ethanol (95% *v/v*) was added, then the concentration of ninhydrin positive substances in each sample was determined from the A_{570} values using alanine standards.

In later experiments, the amino acid composition of the cells and extracellular solution was analyzed before and after a 10-min exposure of the cells to isotonic or hypotonic medium. The protocol was similar to that used in the ninhydrin assay experiments, although in these experiments the free amino acid composition of both the extracellular solution and the cell pellet was analyzed. Cell extracts were prepared from the cell pellets by the addition of 0.8 ml H_2O followed by three cycles of rapid freezing and thawing. Both the cell extracts and extracellular solutions were filtered by centrifugation through a 10,000 MW cutoff filter (Millipore Ultrafree-MC 10,000 NMWL) prior to the analysis of their amino acid composition by HPLC.

In one set of experiments, estimates were made of the loss of protein from the cells following their exposure to media of differing osmolalities. The protocol for these experiments was again very similar to that used in the ninhydrin experiments. The protein contents of the cell pellets and the extracellular solution, sampled both before and after exposure of the cells to hypotonic medium, were determined. A 0.8 ml aliquot of extracellular solution was combined directly with 0.2 ml of Bio-Rad protein assay dye. Cell pellets were solubilized with 1 ml 0.5 M NaOH then neutralized with 0.5 ml 1 M HCl. A 0.01 ml aliquot of this solution was diluted with 0.79 ml H_2O then combined with 0.2 ml Bio-Rad protein assay dye. Protein concentrations in the solutions containing the dye were determined from A_{595} values, using bovine serum albumin standards.

Efflux of α-Aminoisobutyrate

The characteristics of amino acid release from *C. luciliae* were investigated in more detail using the nonmetabolized alanine analogue α-aminoisobutyrate (Midgely, 1978; Lepley & Mukkada, 1983). For efflux experiments, cells were harvested then resuspended (at approximately 1.5×10^9 cells/ml) in growth medium adjusted to an osmolality of 300 mOsm/kg H_2O and containing [^{14}C]AIB at an activity of 0.5 μCi/ml. After a 2-hr incubation at room temperature, with periodic mixing, extracellular radiolabel was removed by repeated centrifugation and resuspension of the cells in isotonic growth medium. Efflux time courses commenced with the addition of the cells to solutions of different osmolalities, giving a final cell concentration of approximately 2×10^7 cells/ml. At predetermined intervals 1-ml aliquots were removed from the suspension and layered over 0.2 ml of oil in a microcentrifuge tube. The tube was centrifuged (10,000 × *g*, 20 sec) then 0.8 ml of the supernatant solution was transferred to a scintillation vial for β-scintillation counting. The total amount of radiolabel loaded into the cells was estimated by transferring triplicate 0.2-ml aliquots of the efflux suspension (i.e., cells and medium) to microcentrifuge tubes to which were then added 0.2 ml 0.1% *v/v* Triton X-100 and 0.4 ml 5% *w/v* TCA. The tubes were kept on ice for 10 min then centrifuged (10,000 × *g*, 10 min), after which the supernatant solution was transferred to vials for β-scintillation counting.

Unidirectional efflux rate constants were estimated, where relevant, from the negative slope (at $t = 0$) of the graph of $\ln(X_i(t)/X_i(t = 0))$ *vs.* time, *t*, where $X_i(t = 0)$ denotes the total amount of [^{14}C]AIB present inside the cells at the beginning of the efflux time-course and $X_i(t)$ denotes the amount present inside the cells at time *t*.

Influx of α-Aminoisobutyrate

Cells were harvested then washed (×3) and resuspended in isotonic saline. Influx began with the addition of a 0.03-ml aliquot of cell suspension to a 0.47-ml solution containing both labeled and unlabeled AIB and layered over 0.4 ml of oil. The final cell count was 2×10^8 cells/ml, the concentration of radiolabel 0.2 μCi/ml, the extracellular AIB or alanine concentration in the range 10–50 mM, and the final osmolality either 300 or 100 mOsm/kg H_2O. The flux was terminated at appropriate times by centrifugation of the cells below the oil (10,000 × *g*, 30 sec) then the cell pellets were processed for β-scintillation counting as described above.

In all influx experiments the amount of radiolabel trapped in the extracellular space within the cell pellets was estimated from the amount of [^{14}C]AIB sampled within 2 sec of combining the cells and the radiolabel.

Inhibitors

N-ethylmaleimide was dissolved in iso-osmotic saline. Stock solutions of all other inhibitors were in ethanol following the discovery that an alternative organic solvent, dimethylsulfoxide, inhibited the phenom-

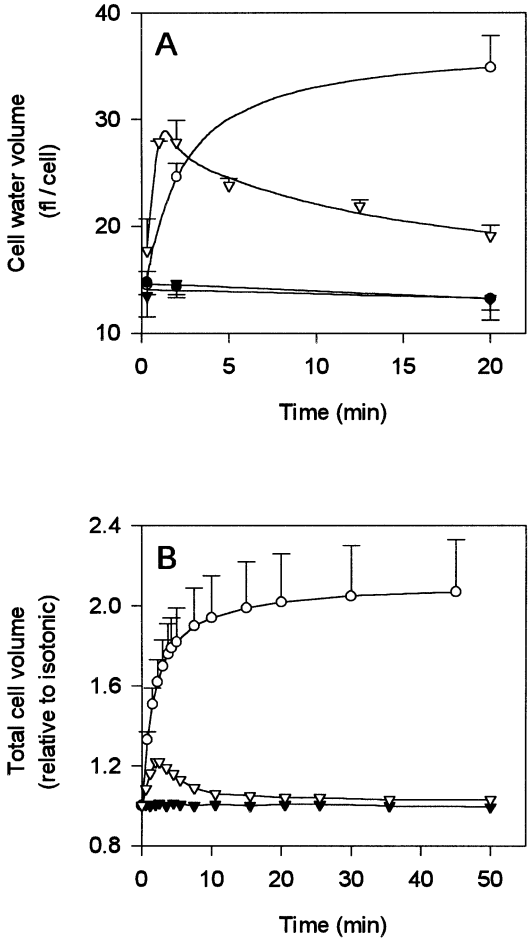

Fig. 1. The volume-response of *C. luciliae* suspended in hypotonic media. Time courses showing (*A*) the intracellular water volume and (*B*) the relative total cell volume of parasites following their suspension (at $t = 0$) in isotonic (300 mOsm/kg H_2O; ▼, ●) or hypotonic (100 mOsm/kg H_2O; ▽, ○) media with (●, ○), or without (▼, ▽) the sulfhydryl reagent NEM (2 mM). The cells in media containing NEM were pretreated with this sulfhydryl reagent (under isotonic conditions) for 15 min prior to beginning the time courses. The intracellular water data are averaged from three different experiments (carried out a temperature of approximately 22°C) and the total cell volume data from three different experiments (carried out a temperature of approximately 30°C). Error bars indicate SEM.

enon of interest (*see* Table 2). The final ethanol concentration in the cell suspension did not exceed 0.5% *v/v* at which concentration it had no effect on the system of interest. In all inhibitor experiments, cells (in RPMI adjusted to an osmolality of 300 mOsm/kg H_2O) were pre-incubated with inhibitors for 15 min prior to their suspension (at $t = 0$) in hypotonic or isotonic flux media (containing the inhibitors at the same concentrations as in the preincubation media).

Results

Volume Regulation in Hypotonic Medium

Figure 1*A* shows time courses for the mean cell water volume of *C. luciliae* following suspension of cells at $t = 0$ in either isotonic (300 mOsm/kg H_2O) or hypotonic (100 mOsm/kg H_2O) saline, in the presence and absence of the sulfhydryl reagent NEM. In isotonic media the cell water volume remained stable at approximately 14 fl throughout the 20-min incubation period and was unaffected by NEM. For cells exposed to hypotonic medium in the absence of NEM there was an initial increase in water volume to approximately 28 fl at 1–2 min, followed by a regulatory volume decrease (RVD) to a value of 20 ± 2 fl at 20 min. For cells pretreated with 2 mM NEM then suspended in hypotonic medium containing the sulfhydryl reagent the cell water volume increased progressively to a value of 34 ± 3 fl at 20 min. This value is some 2.5 times the isotonic cell water volume, slightly less than the threefold increase that might be expected of a cell showing perfect osmometric behavior on reduction of the osmolality to one third its original value (i.e., from 300 to 100 mOsm/kg H_2O). The initial rate of increase of the cell volume was slightly slower in the NEM-treated cells than in the untreated cells; however the difference was not statistically significant ($P = 0.27$; $n = 3$, paired *t*-test).

A qualitatively similar pattern of behavior was seen for the *total* cell volume, measured using a Coulter Multisizer (Fig. 1*B*). Cells in hypotonic medium in the absence of NEM underwent an initial swelling, reaching a maximum volume at around 2 min, followed by an RVD. In cells pretreated with 2 mM NEM then suspended in hypotonic medium (also containing NEM) the RVD response was inhibited and the relative total cell volume increased progressively to approximately twice its normal value. The efficiency of the RVD response in the Coulter Multisizer experiment was substantially greater than that indicated in the intracellular water volume experiments, perhaps reflecting the increased activity of the cells' RVD mechanisms at the higher temperature at which the Coulter Multisizer experiments were carried out (*see* Materials and Methods).

Amino Acid Release

As is shown in Fig. 2, exposure of *C. luciliae* to hypo-osmotic medium (200 or 100 mOsm/kg H_2O) was followed by the release into the extracellular solution of amino acids. Under the same conditions there was a negligible loss of cell protein (*not shown*), ruling out the possibility that the amino acid loss was due to large-scale membrane damage.

The identities and quantities of amino acids released from *C. luciliae* in response to a hypo-osmotic shock was investigated in more detail using amino acid analysis. Qualitatively similar results were obtained from analyses of both the extracellular solution and the cell extracts, though for a number of the amino acids (particularly the cationic compounds ornithine, arginine and lysine) the

amount lost from the pellet exceeded the amount detected in the extracellular solution, perhaps indicating an increase in the intracellular oxidation of these amino acids under hypotonic conditions. Table 1 shows the amino acid composition of *C. luciliae* under normal (isotonic conditions) as well as the amount of each amino acid released into the extracellular solution during a 10-min incubation of the cells in isotonic (300 mOsm/kg H_2O) or hypotonic (100 mOsm/kg H_2O) conditions. As has been reported previously (Knodler, Edwards & Schofield, 1994), free amino acids constitute a significant fraction of the total osmotic contents of these cells, having a total intracellular concentration of 135 mM, with alanine accounting for over a third of this. For cells in isotonic medium there was little efflux of amino acids over 10 min. By contrast cells in hypotonic medium lost significant amounts of amino acids. The amino acids released in greatest abundance were alanine, proline and glycine which accounted for approximately 50, 15 and 7%, respectively, of the total amino acid efflux.

Figure 3 shows hypotonically activated losses of the ten most abundant intracellular amino acids, expressed as a percentage of the amount of each amino acid originally present inside the cells. It therefore provides some information about the selectivity of the amino acid release mechanism(s). The amino acids showing the greatest percentage loss were (in descending order) proline, valine, alanine, glycine and leucine, all small neutral solutes. The three cationic amino acids ornithine, arginine and lysine showed negligible hypotonically activated loss. For the anionic amino acid glutamate and for histidine (which has a pKa of 6.5 and for which there are presumably significant concentrations of both the neutral and cationic forms in the cell cytosol) the hypotonically activated loss was intermediate between that of the small neutral amino acids and those bearing a net positive charge.

Hypotonically Activated Efflux of α-Aminoisobutyrate

To investigate the characteristics of the amino acid release mechanism in more detail we made use of radiolabeled [^{14}C]AIB, a nonmetabolized alanine analogue which has been shown previously to be transported into several trypanasomatid species via a concentrative uptake mechanism (Midgely, 1978; Lepley & Mukkada, 1983; Darling et al., 1990). Figure 4 shows time courses for the efflux of [^{14}C]AIB from *C. luciliae,* following their suspension in saline having osmolalities ranging from 300 to 100 mOsm/kg H_2O. A decrease in the osmolality was followed by a rapid release of [^{14}C]AIB, the rate of which (as judged by the slope of the fitted curves in Fig. 4*A*) declined to that seen under isotonic conditions within a few minutes.

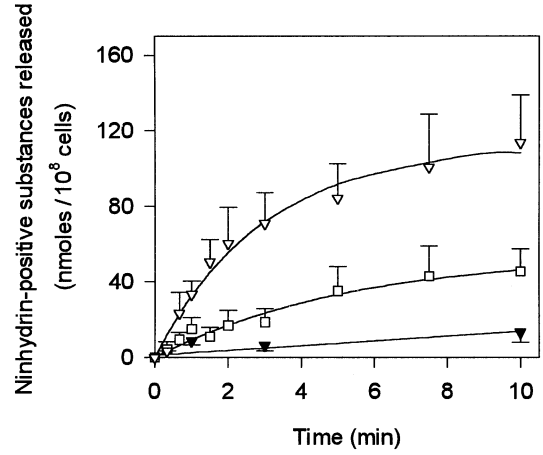

Fig. 2. Time courses for the release of ninhydrin-positive substances from *C. luciliae* into the extracellular medium following their suspension in solutions having an osmolality of 300 (▼), 200 (□) or 100 (▽) mOsm/kg H_2O. The data are averaged from three different experiments and are shown ± SEM.

Figure 4*B* shows both the rate constant for AIB efflux immediately following exposure of the cells to an-iso-osmotic media (calculated as described in Materials and Methods) and the fraction of the intracellular AIB lost after 10 min. Both varied as a sigmoidal function of the extracellular osmolality.

As with the activation of the amino acid release pathway following the reduction of the osmolality, the inactivation of the pathway on restoration of iso-osmotic conditions by the addition of a concentrated (1.6 M) NaCl solution was extremely rapid, occurring within a few seconds (Fig. 5). Similar results were obtained (i.e., the release of [^{14}C]AIB from cells in hypotonic medium was halted) when the external osmolality was restored to 300 mOsm/kg H_2O by the addition of an aliquot of a concentrated (1.4 M) sucrose solution (*data not shown*). However in this case, inactivation of the release pathway took several seconds longer, probably as a result of the longer time required to achieve full mixing of the concentrated (and much more viscous) sucrose solution with the cell suspension.

In vertebrate cells, the swelling-activated transport of organic osmolytes is blocked by a range of anion transport inhibitors including DIDS, niflumate, NPPB, quinine and tamoxifen (Kirk, Ellory & Young 1992; Jackson & Strange, 1993; Goldstein & Davis, 1994; Kirk & Kirk, 1994). However, as shown in Table 2, these reagents were without significant inhibitory effect on the swelling-activated efflux of AIB from *C. luciliae.* DIDS at a concentration of 0.5 mM actually caused a significant increase in the initial rate of AIB efflux from cells in hypotonic medium though it had no effect on the efflux of AIB under isotonic conditions (*not shown*). A stimulation of AIB efflux from cells in hypotonic medium was

Table 1. Intracellular amino acid content and concentrations for *C. luciliae* in isotonic media and amounts of each amino acid lost from the cells during a 10-min incubation in isotonic (300 mOsm/kg H_2O) or hypotonic (100 mOsm/kg H_2O) media. The data are averaged from two separate experiments.

Amino acid	Intracellular concentration under isotonic conditions (mmoles/L cell water)	Intracellular content under isotonic conditions (nmol/10^8 cells)	Amount released during 10-min incubation in isotonic medium (nmol/10^8 cells)	Amount released during 10-min incubation in hypotonic medium (nmol/10^8 cells)
Asparate	2.40	3.39	0.15	0.54
Glutamate	6.03	8.53	0.43	3.46
Asparagine	0.32	0.45	0	0.02
Serine	2.25	3.18	1.38	1.76
Glutamine	1.63	2.30	0.11	2.11
Glycine	7.75	10.96	0.53	5.76
Histidine	4.61	6.52	0.67	2.56
Arginine	7.40	10.46	0.65	1.00
Threonine	0.70	0.98	0.30	1.47
Alanine	48.23	68.20	2.54	44.14
Proline	10.43	14.74	0.25	11.97
Tyrosine	0.78	1.10	0.01	0.55
Valine	3.67	5.21	0.23	3.44
Methionine	1.63	2.31	0	1.89
Isoleucine	1.81	2.55	0.14	1.04
Leucine	4.16	5.88	0	2.02
Phenylalanine	1.69	2.39	0.10	0.75
Ornithine	25.71	36.36	1.37	2.42
Lysine	4.06	5.72	0.12	0.30
Total	135.26	191.26	9.11	87.20

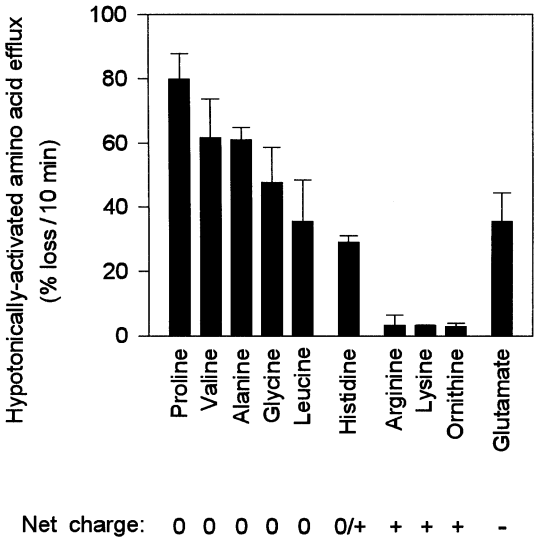

Fig. 3. Relative loss of each of the ten most abundant intracellular amino acids via a hypotonically activated mechanism. The hypotonically activated loss was calculated by subtracting the amount of each amino acid released into isotonic medium (300 mOsm/kg H_2O) during a 10-min incubation from that released into hypotonic medium (100 mOsm/kg H_2O), and is expressed as a percentage of the total amount of each amino acid present in the cells at $t = 0$. The data are averaged from two separate experiments.

also seen at lower DIDS concentrations (0.25 and 0.1 mM; *not shown*).

The sulfhydryl reagent, NEM (2 mM), inhibits swelling-activated amino acid transport both in mammalian (Martínez et al., 1994) and nonmammalian (Bursell & Kirk, 1995) vertebrate cells and was similarly effective in *C. luciliae.* NEM also abolished the enhanced efflux of AIB measured in DIDS-treated cells, consistent with the DIDS-induced increase in AIB efflux being due to increased flux via the osmotically activated pathway, rather than to increased leakage resulting from nonspecific membrane damage by this reagent. Surprisingly, the widely used organic solvent dimethylsulfoxide (at a concentration of 0.5% *v/v*) also proved an effective inhibitor of AIB release. However the protonophores DNP (1 mM), CCCP (0.005 mM) and FCCP (0.001 mM) did not.

Figure 6 shows time courses for [^{14}C]AIB release into media containing a high extracellular concentration (50 mM) of either AIB or alanine. Neither amino acid significantly affected the initial rate of [^{14}C]AIB efflux, demonstrating that the amino acid release mechanism is not prone to *'trans* stimulation'.

Hypotonically Activated Influx of AIB

The kinetics of the hypo-osmotically activated amino acid release mechanism were investigated by measuring the influx of AIB into *C. luciliae* suspended in isotonic

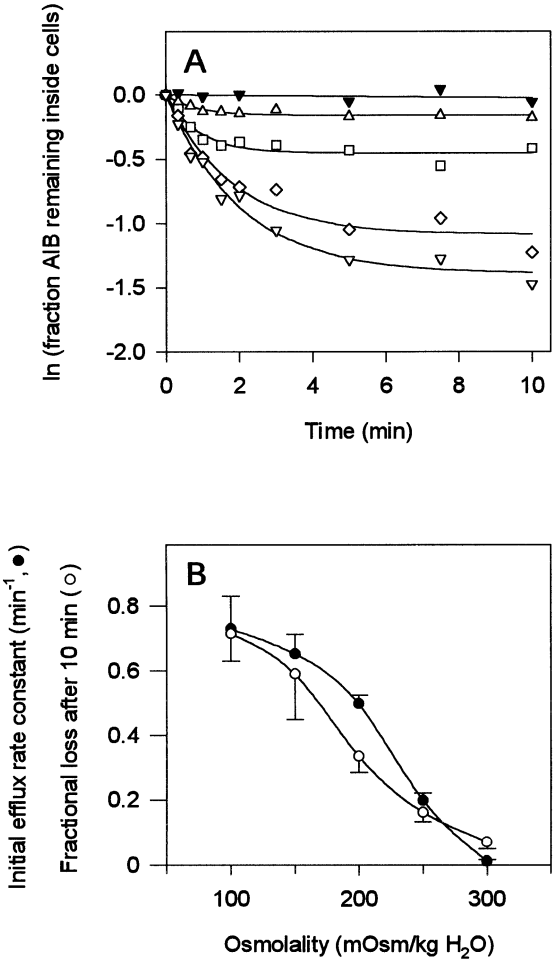

Fig. 4. Efflux of [^{14}C]AIB from *C. luciliae* in isotonic and hypotonic media. (*A*) Time courses for the loss of [^{14}C]AIB from *C. luciliae* following their suspension at $t = 0$ in media having osmolalities of 300 (▼), 250 (△), 200 (□), 150 (◇) or 100 (▽) mOsm/kg H_2O. The data are averaged from three different experiments. Error bars are omitted for clarity. (*B*) Fractional loss of [^{14}C]AIB after 10 min (○) and initial efflux rate constants for the loss of [^{14}C]AIB from *C. luciliae* immediately following their suspension in the different media (●). The data are averaged from three different experiments. Error bars indicate SEM.

(300 mOsm/kg H_2O) and hypotonic (100 mOsm/kg H_2O) media. For cells in isotonic medium (containing 50 mM alanine) there was negligible influx of AIB over a 1 min incubation period (Fig. 7). By contrast, suspension of the cells in hypotonic media was followed by an immediate and marked increase in the unidirectional influx rate of AIB. Hypotonically activated AIB influx was inhibited by NEM, consistent with it being via the same pathway that mediated volume-regulatory amino acid release. The unidirectional influx of AIB into osmotically swollen cells increased almost linearly with extracellular concentration, showing no tendency to saturate in the range of 10–50 mM (Fig. 8).

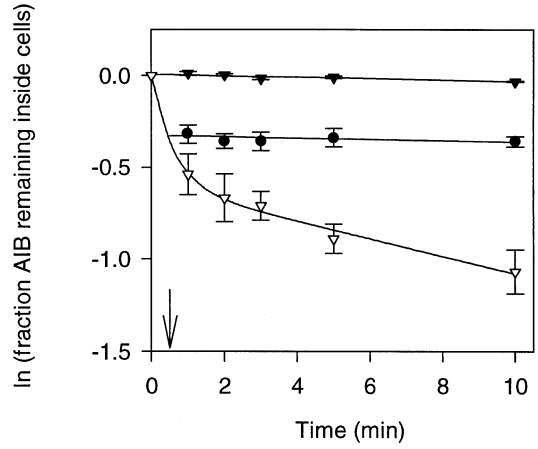

Fig. 5. Rapid inactivation of the hypo-osmotically activated amino acid release mechanism following restoration of isotonicity. Cells preloaded with [^{14}C]AIB were transferred at $t = 0$ from isotonic (300 mOsm/kg H_2O) to either hypotonic (100 mOsm/kg H_2O; ▽, ●) or isotonic (300 mOsm/kg H_2O; ▼) solution. At $t = 30$ sec (indicated by the arrow) an aliquot of either hypertonic solution (1.6 M NaCl in hypotonic saline) or hypotonic solution was added to the hypotonic flux suspensions, thereby either restoring the osmolality to 300 mOsm/kg H_2O (●) or maintaining it at 100 mOsm/kg H_2O (▽). The data are averaged from three different experiments and are shown ± SEM.

Discussion

Cell Swelling in Hypotonic Media

The initial swelling of *C. luciliae* exposed to a hypotonic medium occurred on a time scale of several minutes, somewhat slower than that typically seen on exposure of vertebrate cells to hypotonic conditions. This difference may be accounted for by the protozoan having a relatively low plasma membrane water permeability and/or a membrane/cytoskeleton able to support substantial transient osmotic pressure gradients. The observation in Fig. 1*A* that the rate of swelling of NEM-treated cells was somewhat less than that of untreated cells might be accounted for by the sulfhydryl reagent reducing the water permeability of the cell membrane or interacting with (and thereby increasing the rigidity of) the cytoskeleton.

The Contribution of Amino Acids to RVD

C. luciliae parasites, like other protozoa (Knodler et al., 1994; Vieira & Cabantchik, 1995), contain high concentrations of free amino acids. The value obtained here for the total intracellular amino acid concentration of 135 mM for cells in normal (isotonic) growth medium (Table 1) is similar to the value of 148 mM estimated previously by Knodler et al. (1994), with good agreement between the two studies for the estimated intracellular concentra-

Table 2. Effect of transport inhibitors on the release of AIB from *C. luciliae* during a 1-min incubation

Inhibitor	Osmolality	Concentration (mM)	*n*	AIB release (% of control)	*P*
—	100	—	—	100	—
—	300	—	13	2 ± 2	≪0.001
DIDS	100	0.5	7	143 ± 14	0.01
Niflumate	100	0.5	3	83 ± 6	NS
NPPB	100	0.1	3	99 ± 12	NS
Quinine	100	1	3	87 ± 5	NS
Tamoxifen	100	0.01	3	99 ± 11	NS
DNP	100	1	3	87 ± 10	NS
CCCP	100	0.005	3	81 ± 10	NS
FCCP	100	0.001	3	91 ± 14	NS
DMSO	100	70 (i.e., 0.5% *v/v*)	4	34 ± 16	0.03
NEM	100	2	3	2 ± 1	<0.01
DIDS + NEM	100	0.5 (DIDS) + 2 (NEM)	3	1 ± 2	0.03

In all cases cells in isotonic media were pretreated with the appropriate reagents for 15 min prior to their suspension in iso- or hypotonic media containing the reagents at the same concentration. The amount of AIB released is expressed as a percentage of that lost from cells suspended in hypotonic medium (100 mOsm/kg H_2O) in the absence of inhibitors. The *P* values indicate the significance of the difference between the AIB release measured in the presence of inhibitor (or under isotonic conditions) with that measured in cells in hypotonic medium in the absence of inhibitor (paired, two-tailed *t* test). NS indicates $P > 0.05$.

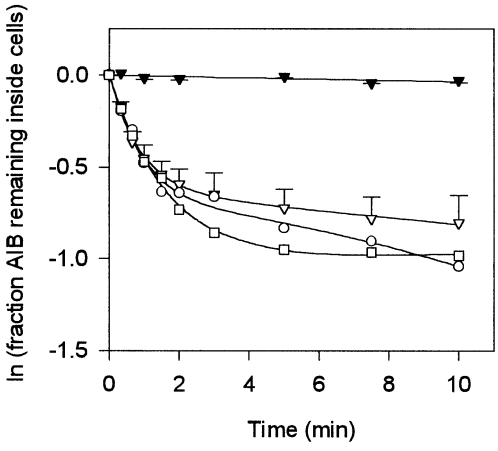

Fig. 6. Effect of a high (50 mM) extracellular concentration of either alanine (□) or AIB (○) on the efflux of [^{14}C]AIB from cells suspended in hypotonic media (100 mOsm/kg H_2O). The triangles show time courses for AIB efflux into isotonic (▼) or hypotonic (▽) media in the absence of extracellular amino acids. The data are averaged from three different experiments. Error bars (indicating SEM) are, for clarity, shown only for the time courses measured in the absence of extracellular amino acids.

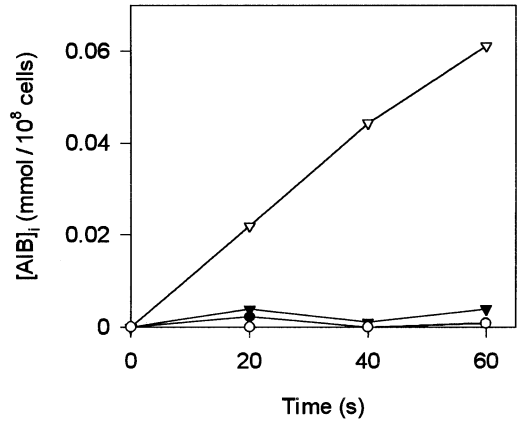

Fig. 7. Time course for the influx of AIB into *C. luciliae* suspended (at $t = 0$) in isotonic (300 mOsm/kg H_2O; ▼, ●) or hypotonic (100 mOsm/kg H_2O; ▽, ○) media with (●, ○) or without (▼, ▽) the sulfhydryl reagent NEM (2 mM). The cells were suspended in media containing 50 mM alanine in order to minimize the uptake of radiolabeled substrate via the saturable, constitutively active AIB uptake system. The NEM-treated cells were preincubated in normal isotonic saline containing 2 mM NEM for 15 min prior to beginning the time course. The data are from a single experiment and are representative of those obtained in five similar experiments.

tions of all of the individual amino acids. Amino acids probably account for nearly half of the total intracellular osmolality under isotonic conditions.

On exposure to hypotonic media *C. luciliae* responded with a rapid release of amino acids. The observation that the sulfhydryl reagent NEM inhibited both the efflux of AIB (Table 2) and RVD (Fig. 1) is consistent with amino acid efflux playing a significant role in the volume regulatory response of this organism to hypotonic swelling. This is borne out by the calculation of the fractional water loss that might be attributed to the net efflux of amino acids. On reduction of the osmolality from 300 to 100 mOsm/kg H_2O, a cell showing 'perfect osmometric behavior' would be expected to undergo a threefold increase in its intracellular water volume (Hallows & Knauf, 1994). In the case of *C. luciliae* this would mean an increase in the individual cell water vol-

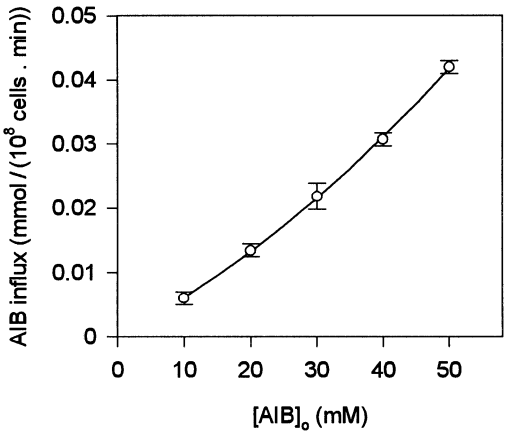

Fig. 8. Concentration dependence of the unidirectional influx of AIB into *C. luciliae* suspended in hypotonic medium (100 mOsm/kg H_2O). The data are averaged from three different experiments and are shown ± SEM.

ume from 14 to 42 fl (assuming that all of the intracellular water is 'osmotically active'). Yet 10 min after a shock of this magnitude the cell water volume was approximately 22 fl (Fig. 1*A*), some 20 fl less than that predicted for a perfect osmometer. The total amount of amino acids released from a single cell during 10-min exposure to a medium having an osmolality of 100 mOsm/kg H_2O was approximately 0.9 fmoles (i.e., 87.20 mmol/10^8 cells in Table 1). If the amino acids leaving the cell are assumed to have an osmotic coefficient of 1 then the volume of solvent water accompanying them from the cell will have been approximately 9 fl. The release of amino acids therefore accounts for approximately half of the deviation of these cells from perfect osmometric behavior (after 10-min exposure to 100 mOsm/kg H_2O) and is therefore a major, though probably not the only, volume-regulatory mechanism used by *C. luciliae* under hypotonic conditions.

This is similar to the situation in a number of other protozoa. In the free-living protozoan *Acanthamoeba castellani*, reduction of the extracellular osmolality from 240 to 40 mOsm/kg H_2O was followed by the loss of free amino acids (predominantly alanine and proline) and this was estimated to account for around 60% of the total loss of intracellular osmolytes (Geoffrion & Larochelle, 1984). In the trypanasomatid *Leishmania major*, osmotic swelling was followed by an immediate release of ninhydrin-positive substances, approximately 40% of which was alanine (Darling et al., 1990). A comparison of the relative loss of alanine with that of K^+ ($^{86}Rb^+$) suggested that the former contributed some tenfold more than the latter to the total loss of intracellular osmolytes, and hence to RVD (Blum, 1992). Similarly, in the protozoan parasite *Giardia intestinalis*, the loss of amino acids (predominantly alanine) was shown to make a major contribution to the RVD response to these cells to osmotic swelling (Park, Schofield & Edwards, 1995).

The Nature of the Amino Acid Release Mechanism

The release of amino acids from *C. luciliae* in hypotonic media does not reflect a general loss of cytoplasmic contents as there was no significant loss of protein from the cell on exposure to hypotonic media. It has been postulated that the release of alanine and perhaps other amino acids from *Leishmania donovani* may be via the carrier system which, under isotonic conditions, mediates the active accumulation of alanine and AIB (Blum, 1992). Like the hypotonically activated amino acid release mechanism in *C. luciliae* (Table 2) the system which mediates AIB uptake into the closely related organism *C. fasciculata* is inhibited by NEM (Midgely, 1978). However the available data suggest that the systems differ in a number of important respects. Firstly, the AIB uptake system is saturable (Midgely, 1978; Lepley & Mukkada, 1983), which is why in this study there was negligible uptake of radiolabeled AIB (over 2 min) into cells in isotonic media containing 50 mM alanine (Fig. 7). By contrast the AIB transport via the hypotonically activated system showed no tendency to saturate in the concentration range 10–50 mM (Fig. 8) and there was a substantial unidirectional influx of AIB via this system in cells exposed to 50 mM alanine (Fig. 7). Secondly, AIB uptake into *C. fasciculata* (Midgely, 1978) and into *L. tropica* (Lepley & Mukkada, 1983) is inhibited by proton ionophores (e.g., 1–2 mM DNP) whereas hypotonically activated AIB release from *C. luciliae* was not (Table 2). Finally, competition experiments with *L. tropica* suggest that the AIB uptake system in this trypanasomatid transports a range of different amino acids but does not transport valine. By contrast valine is a 'preferred substrate' for the hypotonically activated system of *C. luciliae* (Fig. 3). In view of these differences it seems unlikely that active amino acid uptake and hypotonically activated amino acid release is via the same route.

The apparent ability of the hypotonically activated pathway to accommodate a range of amino acids, its ability to mediate the flux of solutes in both directions across the cell membrane, and its failure to saturate or to show *trans* stimulation are all consistent with the hypothesis that the volume-regulatory amino acid release pathway in *C. luciliae* is a type of channel, rather than a conventional transporter. Although it remains to be established whether the hypotonically activated release of the different amino acids is via pathways of a single type it is revealing to compare the percentage losses of each of the major amino acids within the cell. Of the ten most abundant intracellular amino acids, those showing the greatest relative hypotonically activated loss during 10-min exposure to 100 mOsm/kg H_2O (Fig. 3) were the

neutral amino acids proline (80%), valine (62%) and alanine (61%). Glycine, a smaller compound, showed a somewhat lower fractional loss (48%), indicating that the release pathway is not a simple molecular sieve, discriminating between solutes on the basis of size alone. There was negligible hypotonically activated efflux of the cationic amino acids lysine (3%), arginine (3%) and ornithine (3%). The relative loss of the anionic amino acid, glutamate (35%), fell in between those for the neutral and cationic amino acids, as did that of histidine (29%) which is present in both the neutral and cationic forms at physiological pH. The data are therefore consistent with the pathway being selective for neutral and anionic amino acids over cationic amino acids.

There is now substantial evidence that the swelling-activated transport of organic solutes such as amino acids in vertebrate cells is via broad-specificity, anion-selective channels (reviewed by Strange, Emma & Jackson, 1996). The results of the present study are consistent with an anion-selective channel mediating the swelling-activated efflux of amino acids from *C. luciliae.* However, there are significant differences between swelling-activated organic osmolyte transport in *C. luciliae* and that in vertebrate cells. In *C. luciliae,* activation of the pathway occurred immediately following the reduction of the osmolality (Fig. 4*A*) whereas in vertebrate cells there is typically a lag of several min (e.g., Kirk et al., 1992; Kirk & Kirk, 1993). Similarly, the inactivation of the osmolyte channel of vertebrate cells on restoration of isotonicity takes several min to occur (e.g., Hall et al., 1996), whereas in *C. luciliae* inactivation of the pathway occurred within a few seconds (Fig. 5). There are also substantial differences in the pharmacological characteristics of the vertebrate and protozoan systems. Although both are effectively inhibited by NEM, the swelling-activated transport of organic osmolytes in vertebrate cells is blocked by a range of anion transport inhibitors which were found here not to inhibit the pathway in *C. luciliae.* However, it is questionable whether at least some of the anion transport inhibitors used here exert their effect on swelling-activated transport in vertebrate cells via a direct interaction with the channel (e.g., Ballatori et al., 1995) and the differences may reflect differences in the regulation of the systems rather than fundamental differences between the proteins mediating the flux.

The characteristics of hypotonically activated amino acid release from *C. luciliae* are very similar to those for several other protozoan parasites. In *L. major* (Darling et al., 1990) and in *G. intestinalis* (Park et al., 1995), as in *C. luciliae,* exposure to hypotonic media is followed immediately by a rapid release of amino acids (predominantly alanine), lasting for 1–2 min. In all three organisms, the efflux rate shows a sigmoidal dependence on the extracellular osmolality. In *G. intestinalis* osmotically activated alanine efflux is inhibited by the thiol reagent *p*-hydroxymercuribenzoate but is not inhibited by 0.1 mM niflumate (Park et al., 1995), similar to the findings in the present study that osmotically activated alanine efflux from *C. luciliae* was inhibited by NEM but not by 0.5 mM niflumate. It was noted that in *G. intestinalis* the amino acid release mechanism favored neutral amino acids over cationic and anionic amino acids and it was postulated that the efflux was via a carrier-type transport protein or uniport (Park et al., 1995). The available data are equally consistent with the involvement of a channel as is postulated here to mediate volume-regulatory amino acid release from *C. luciliae.*

The Nature of the Volume Sensor

A comparison of the cell volume time courses of Fig. 1 with the time courses for amino acid (Fig. 2) or AIB (Fig. 4) release provides some insight into the nature of the mechanism by which *C. luciliae* senses the need to activate its volume-regulatory amino acid release mechanism(s). It is clear both from the cell water volume estimates (Fig. 1*A*) and the Coulter Multisizer data (Fig. 1*B*) that following a decrease in the osmolality from 300 to 100 mOsm/kg H_2O the cells take several minutes to swell, reaching a maximum (in the absence of NEM) at 1–2 min. By contrast, the rate of release of amino acids from cells exposed to hypotonic conditions is maximal within a few seconds (i.e., long before the cell has reached its maximum volume; Figs. 2 and 4). By 2 min, at which point the cell volume *is* maximal, the rate of amino acid efflux (as judged from the slope of the relevant curves in Figs. 4–6) is actually decreasing (Fig. 4). The activity of the amino acid release pathway is therefore not determined by the actual volume of the cell. This would seem to rule out the possibility that the pathway is regulated by the degree of macromolecular crowding in the cell cytosol as has been proposed for volume-regulatory transport systems in other cells (Parker, 1993).

The observation that the pathway is inactivated following the addition to a hypotonic suspension of sufficient sucrose to restore the osmolality to 300 mOsm/kg H_2O indicates that activation of the pathway in dilute saline is not due to the decreased extracellular Na^+ concentration, nor to decreased extracellular ionic strength. It is possible that the activity of the amino acid release mechanism may be modulated in direct response to the extracellular osmolality or tonicity and that the cell therefore has some means of detecting these. Alternatively, the cell may sense the rate of change of its volume (rather than volume *per se*) and modulate the activity of the amino acid release mechanism accordingly.

Conclusion

Exposure of the unicellular protozoan parasite *C. luciliae* to hypotonic medium is followed by an immediate loss of amino acids. This process accounts for a large part of the cells' RVD response. The data are consistent with the hypothesis that the pathway which mediates volume-regulatory amino acid release from this organism is a channel that has substrate-selectivity characteristics similar to those of the anion-selective channels which mediate the swelling-activated transport of organic solutes in vertebrate cells. However, the pharmacological characteristics of the pathway, as well as its rate (and perhaps mechanism) of activation differ from those of its vertebrate counterparts.

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