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The Role of Glycerol and Inorganic Ions in Osmoregulatory Responses of the Euryhaline Flagellate *Chlamydomonas pulsatilla* Wollenweber[1]

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ABSTRACT

The green euryhaline flagellate *Chlamydomonas pulsatilla* Wollenweber, isolated from a coastal marine environment, was grown exponentially over the salinity range of 10 to 200% artificial seawater (ASW). The cellular volume and aqueous space of the alga, measured by [^{14}C] mannitol and ^3H_2O tracer analyses of centrifuged cell pellets, ranged between 2.3 and 3.1 picoliters and between 1.5 and 2.1 picoliters, respectively. The nonaqueous space determined in those analyses (28–35%) was consistent with the cell composition of the alga. The glycerol content of the alga increased almost linearly with increasing salinity; its contribution to intracellular osmolality at 200% ASW was about 57%. The contribution of amino acids and soluble carbohydrates to the cell osmotic balance was small. Intracellular ion concentrations determined by analyzing centrifuged cell pellets of known [^{14}C]mannitol space by atomic absorption spectrophotometry, and by neutron activation analyses of washed cells were similar. At 10% ASW, potassium and magnesium were the major cations, and chloride and phosphate were the major anions. The sodium and chloride content of the alga increased with increasing salinity; at 200% ASW the intracellular concentration of both sodium and chloride was about 400 millimolar. The intracellular osmolality (π_{int}) matched closely the external osmolality (π_{ext}) over the entire salinity range except at 10% ASW where π_{int} exceeded π_{ext} by 120 to 270 milliosmoles per kilogram H_2O.

Unicellular green biflagellates of the order Volvocales are widely distributed in saline and coastal marine habitats (18). Some of these microalgae, *e.g. Dunaliella* spp. (6–8), *Brachiomonas submarina* (3), and *Chlamydomonas pulsatilla* (21), can grow at salinities several times higher than that of seawater and survive even the saturation of seawater. Another feature of these flagellates, which are functionally unable to withstand significant turgor pressures as their flagellar membranes are directly exposed to the external environment, is that they maintain their protoplasm isoosmotic with the external solution except at extremely low salinities (26). While there is general agreement that glycerol plays an important osmoregulatory role in these flagellates, the extent to which this solute, which is highly soluble and compatible with metabolic reactions, contributes to the intracellular osmolarity is still uncertain. Conflicting reports regarding the relative importance of glycerol and inorganic ions in osmoregulation appear to be due to differences in methodology as well as to how the results obtained have been interpreted (3, 6, 12, 13, 22).

The present paper deals with osmoregulatory responses of the marine rockpool flagellate *C. pulsatilla* over a wide range of external salinity and discusses our findings in relation to those obtained by other investigators on similar salinity tolerant biflagellates.

MATERIALS AND METHODS

Algal Cultures. *Chlamydomonas pulsatilla* Wollenweber isolated by one of us (J. A. H.) from a marine supralittoral rockpool near St. Andrews, New Brunswick, Canada was grown axenically at 18°C on a 12 h light:12 h dark cycle with 24 W m^{-2} coolwhite light in ASW[2] with nutrient enrichments as described previously (19). The nitrogen source was 2 mM ammonium. For all experiments cells from exponential growth phase were harvested by centrifugation at 2000*g* and 15°C for 5 min, 2 to 3 h after the start of the light period. Cell densities were determined as described previously (1).

Measurement of Cell Carbon, Protein, Nitrogen, and Phosphate Content. For cell carbon analyses, 50 × 10^4 cells washed twice in organic carbon-free isoosmotic ASW were sonicated and assayed for total and inorganic carbon content using a carbon analyzer (Beckman model 915). The organic carbon content of the cells was calculated from the difference between total and inorganic carbon content of the sample. Cell protein and nitrogen contents were determined as described previously (2). Total and inorganic phosphates were determined by the methods described by Strickland and Parson (27).

Measurement of Organic Solutes, Cell Volume, Cell Water Content and Intracellular Ion Contents. Three to four L cultures (5–10 × 10^4 cells·ml^{-1}) were harvested, resuspended in 3 ml of isoosmotic ASW, and divided into 3 × 1 ml fractions. One fraction was extracted in methanol:chloroform:water (12:5:3) and analyzed for glycerol, amino acids, and soluble carbohydrates as described previously (3). The other two fractions were transferred to cytocrit tubes to which 0.1 μCi of either ^3H_2O or 10 mM [^{14}C]mannitol was added, and then centrifuged at 2000*g* for 5 min at 15°C; the pellet volume was determined. The pellet water content was calculated from the dilution of ^3H_2O in the supernatant solution. The supernatant solution in the cytocrit tube containing [^{14}C]mannitol was discarded, the sides of the tube wiped dry, and [^{14}C]mannitol trapped in the extracellular space was recovered by resuspending the cell pellet in 1 ml of isoosmotic ASW containing 10 mM mannitol and collecting the supernatant solution after centrifugation at 2000*g* for 5 min at 15°C. The pellet volume after the recovery of [^{14}C]mannitol was identical to that of the [^{14}C]mannitol pellet obtained after the

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[2] Abbreviation: ASW, artificial sea water.

first centrifugation in the cytocrit tube. The sides of the tube were wiped dry and the pellet was extracted in 5 ml of 10% HNO_3 overnight at 20°C. The extract was clarified by centrifugation at 2000*g* for 5 min at 15°C. The extracts were analyzed for Na^+, K^+, Ca^{2+}, Mg^{2+}, and Cl^- contents as described previously (3). The average cell size, cell water content, cell nonaqueous space, and intracellular ion contents were calculated as described previously (3).

Neutron Activation Analysis. One to two L culture (5–10 × 10^4 cells·ml^{-1}) was harvested and suspended to 1 ml in isoosmotic ASW in a cytocrit tube. The cell pellet volume was determined by centrifugation at 2000*g* for 5 min at 15°C, and the average cell size and cell water content were estimated from the ratios of pellet volume:[^{14}C]mannitol space:^3H_2O space determined in the above tracer analysis. The cell pellet was then washed twice in 1 ml of isoosmotic sorbitol solution containing 2.5 mM NaCl, 1 mM KCl, 1 mM $CaSO_4$, and 1 mM $MgSO_4$ by centrifugation at 2000*g* for 2 min at 15°C, and finally suspended to 1 ml in ion-free isoosmotic solution of sorbitol. Fifty μl fractions were dried at 80°C in ion-free polyethylene tubing and determined for inorganic ions by neutron activation analysis described previously (1).

Cell-Bound Cations. Permeabilized cells were dialyzed and determined for cell-bound cations as described previously (1).

RESULTS

Chlamydomonas pulsatilla grew best at 10% ASW, but showed good growth over the entire salinity range tested (Table I). At 200% ASW the growth rate was about 69% that at 10% ASW. Cell carbon, protein, nitrogen, and phosphorus increased with increasing salinity, with most of this increase taking place between 10 and 50% ASW. The C/N and N/P atomic ratios remained relatively constant at about 5 and 16, respectively. There was a gradual increase in Pi with increasing salinity.

It is evident from Table II that glycerol is the dominant osmoregulatory solute in *C. pulsatilla.* The cellular glycerol content increased almost linearly with increases in salinity. Although there were increases in free amino acids and soluble carbohydrates, these increases were less regular and the total contribution of these solutes to intracellular osmotic pressure was less than 10% that of glycerol at 200% ASW. The results for soluble carbohydrates were calculated based on the mol wt of glucose; the average mol wt of soluble carbohydrate is probably higher, and, therefore, the osmotic contribution of these solutes is likely to be somewhat less. Only trace amounts of quaternary ammonium compounds were detected. The values of π_{org} presented in Table II were calculated from the concentration of organic solutes based on the cell water content shown in Table III, using published values of osmolalities of glycerol solutions (9) and assuming an osmotic coefficient of 1 for amino acids and soluble carbohydrates. It is evident from Table II that organic solute accumulation plays a major osmoregulatory role in *C. pulsatilla.* At 200% ASW the calculated π_{org} was more than 50% of π_{ext} (Table II).

Cell volume and aqueous space of the alga increased with increases in external salinity from 10 to 100% ASW, but decreased markedly when the salinity was increased further to 200% ASW (Table III). Over the salinity range of 10 to 200% ASW, between 28 and 35% of the cell volume was occupied by nonaqueous space (Table III). The glycerol space was calculated from the alga's glycerol content (Table II) and from published values for the displacement of water by glycerol in an aqueous system (9). At 200% of ASW about 7% of the cell volume was occupied by glycerol. The cell volumes of *C. pulsatilla* presented in Table III were in close agreement with estimates of cell volumes from microscopic measurements of cell size (results not shown).

Table IV shows the intracellular ion contents calculated from the analyses of centrifuged cell pellets. Potassium and magnesium were the major cations and chloride the major anion in cells growing at 10% ASW. A significant amount of sodium was also present in these cells. The sodium and chloride content of the alga increased markedly with increases in the external salt concentration. Some increases in the potassium and magnesium contents were also observed when the external salinity increased from 10 to 100% ASW. Calcium was present in small amounts and showed little change over the salinity range 10 to 200% ASW. Our study of cations bound to cell macromolecules revealed that almost all of the calcium was present in a bound (nondialyzable, see "Materials and Methods") state. About 55% of cellular magnesium was bound to macromolecules over the salinity range 10 to 50% ASW, and about 46% over the range 100 to 200% ASW. An average of 6% intracellular sodium and potassium were bound to cell macromolecules over the entire salinity range tested. The osmolalities of inorganic ions (π_{inorg}) were calculated from the concentration of both free and bound cations, chloride, and Pi (Table I) based on cell water contents presented in Table III. The values of π_{inorg} shown in Table IV reveal an important contribution of inorganic ions in osmoregulation of *C. pulsatilla.* At 10% ASW the alga accumulated inorganic ions in excess of that required to equal the external osmolality. At higher salinities the uptake of inorganic ions remained major solutes for the cellular osmotic balance. The calculated π_{inorg} at 200% ASW was close to 50% of π_{ext} (Table IV).

Table V shows the intracellular ion content of *C. pulsatilla* determined by neutron activation analysis of cells washed in isoosmotic sorbitol solutions which contained 1 to 3.5 mM concentrations of major inorganic ions to prevent ion leakage during the washing period (1). It can be seen from Tables IV and V that the intracellular ion contents and the values of π_{inorg} determined in the two experiments were similar.

DISCUSSION

Chlamydomonas pulsatilla is a true euryhaline flagellate well adapted to a wide salinity range. Its growth is optimal at low

Table I. *Effect of Salinity on Growth Rate and Cell Composition*

Growth rates are given as means of triplicate experiments. Cell protein, carbon, nitrogen, and phosphate contents are means of 2 to 3 experiments. The variation between experiments was less than 10%.

Salinity	Growth Rate[a]	Protein	C	N	P	Pi	C/N	N/P
% ASW	*h^{-1}*	*pg cell^{-1}*		*pmol cell^{-1}*		*fmol cell^{-1}*	*ratio*	
10	0.035	348	18.9	4.17	0.259	62	4.5	16
50	0.028	464	25.0	5.13	0.355	81	4.9	14
100	0.023	450	28.9	5.20	0.340	83	5.5	15
200	0.020	433	27.6	5.30	0.351	98	5.2	15

[a] Growth rates were calculated as ln $2/t_d$, where t_d is cell doubling time in hours of cultures growing from early- to mid-exponential growth phase.

Table II. *Effect of Salinity on Intracellular Organic Solutes*

Cells washed in nitrogen-free isoosmotic ASW were extracted in methanol:chloroform:water, and the water soluble fraction was collected for analysis. Amino acids were determined on a Beckman amino acid analyzer model 121 using lithium citrate buffers, glycerol was determined enzymically, and carbohydrates were determined by the anthron-sulfuric acid procedure. Other amino acids detected (each <1 fmol·cell^{-1}) were aspartic acid, glutamine, glycine, valine, leucine, tyrosine, phenylalanine, and lysine. π_{org} was calculated from the concentration of organic solutes based on cell water content shown in Table III, using osmolalities of glycerol solution published by Brown *et al.* (9) and assuming an osmotic coefficient of 1 for all other organic solutions. π_{ext} was determined with a Wescor's dew point osmometer.

Salinity	π_{ext}	Glycerol	Amino Acids									Soluble CHO[a]	π_{org}
			Pro	Ala	Glu	Asn	Ser	Met	Thr	Other	Total		
% ASW	*mOsm·kg* H_2O^{-1}						*fmol·cell*$^{-1}$						*mOsm·kg* H_2O^{-1}
10	120	40	0.1	9.8	8.7	1.8	0.7	1.0	0.9	3.1	26.1	8	53
50	490	210	6.2	12.6	13.8	2.9	1.1	1.0	1.1	4.5	43.2	19	127
100	975	600	14.3	20.1	19.8	3.8	2.4	2.1	1.6	7.7	71.4	33	354
200	1940	1450	13.2	16.5	17.8	3.6	2.2	1.5	1.2	6.3	62.3	48	1133

[a] Soluble carbohydrate (CHO) based on mol wt of glucose.

Table III. *Effect of Salinity on Cell Volume, Water Content, and Nonaqueous Space*

Average cell volume and aqueous and nonaqueous fractions of the cells were calculated from [^{14}C]mannitol and ^3H_2O tracer analyses of the centrifuged cell pellet. Glycerol contents shown in Table II were used to calculate nonaqueous space occupied by glycerol. Values in parentheses are the percentage of cell volume.

Salinity	Cell Volume	Aqueous Space	Total Nonaqueous Space	Glycerol Space
% ASW			*fl*	
10	2470	1670 (68)	800 (32)	8
50	2730	1960 (72)	770 (28)	18 (<1)
100	3070	2140 (70)	930 (30)	65 (2)
200	2260	1470 (65)	790 (35)	151 (8)

Table IV. *Estimation of Intracellular Ion Content from Atomic Absorption Spectrophotometry of Cell Pellet Extracts and Cell Osmotic Balance*

The cell content of inorganic ions was calculated from the analyses of centrifuged cell pellets. π_{inorg} was calculated from concentrations of Na^+, K^+, Ca^{2+}, Mg^{2+}, Cl^-, and Pi (Table I) based on cell water content shown in Table III, assuming an osmotic coefficient of 0.9 for free cations and chloride, 0.8 for bound monovalent cations, 0.2 for bound Ca^{2+} and Mg^{2+}, and 0.2 for Pi (3). Cations bound to cell macromolecules were determined after 12 h dialyses of permeabilized cells against water. π_{int} is the sum of $\pi_{inorg} + \pi_{org}$ shown in Table III. π_{ext} was determined as described in Table III.

Salinity	π_{ext}	Na^+	K^+	Ca^{2+}	Mg^{2+}	Cl^-	π_{inorg}	π_{int}
% ASW	*mOsm·kg* H_2O^{-1}			*fmol·cell*$^{-1}$[a]			*mOsm·kg* H_2O^{-1}	
10	120	70	190	10	176	230	326	379
50	490	148	224	13	232	439	445	572
100	975	377	286	20	274	697	652	1006
200	1940	591	191	35	239	597	942	2075

[a] Calculated from the difference between total ion content of centrifuged cell pellet and the amount of ion trapped in the extracellular space. The volume of extracellular space ranged between 23 and 26%.

Table V. *Neutron Activation Analysis of Washed Cells*

Cells washed twice in isoosmotic sorbitol solution containing 1 to 3.5 mM concentrations of major salts and resuspended in salt-free isoosmotic sorbitol solution were analyzed for inorganic ion content by neutron activation. The values of ion content are averages of six determinations with ±SE given in parentheses. Concentrations of inorganic ions were calculated from cell water content estimated from the volume of centrifuged cell pellets after subtracting 25% extracellular volume, and using percent cell aqueous space shown in Table III. π_{inorg} was calculated as described in Table IV.

Salinity	Cell Water Content	Na^+	K^+	Ca^{2+}	Mg^{2+}	Cl^-	π_{inorg}
% ASW	*pl*			*fmol·cell*$^{-1}$			*mOsm·kg* H_2O^{-1}
10	1.73	46 (±6)	142 (±11)	11 (±2)	128 (±14)	188 (±12)	237
50	2.13	171 (±12)	202 (±23)	16 (±2)	180 (±16)	391 (±27)	393
100	2.45	391 (±27)	267 (±19)	22 (±2)	242 (±19)	658 (±53)	548
200	1.54	548 (±33)	170 (±18)	18 (±3)	229 (±12)	606 (±47)	873

salinities (about 10% ASW), but it can maintain high division rates with normal cell carbon, protein, nitrogen, and phosphate contents at salinities as high as 200% ASW. The alga can osmoregulate without major water loss over the salinity range of 10 to 200% ASW. It maintained a cell water content of 65 to 72% over this salinity range. The nonaqueous space of *C. pulsatilla* determined by the tracer analysis was consistent with our study of the cell composition of this microalga. The cells contain high levels of protein which constitute between 15 and 19% of their fresh weight. Our data on cell protein are consistent with the measurements of cell carbon and nitrogen contents; the ratios of protein/C and protein/N calculated from these results are similar to those reported for other microalgae (25). Moreover, some of the intracellular space in *C. pulsatilla* is occupied by glycerol which at 200% corresponds to about 7% of the cell volume. These results agree with an intracellular nonaqueous space of about 30%. In *Dunaliella salina*, the nonaqueous (nonosmotic) fraction was reported to constitute less than 10% of the cell volume (22). This appears unlikely, however, as *D. salina* is shown (25) to have a cell composition similar to that shown for *C. pulsatilla* here. Furthermore, the glycerol contents reported for *D. salina* (6, 22) indicate substantial displacement of water by glycerol in these cells.

In *C. pulsatilla* the accumulation of glycerol plays a major osmoregulatory role at high salinities. At 200% ASW the alga contain about 990 mM glycerol on a cell water basis. This corresponds to an osmolality of only 1100 mOsm (9) and is, therefore, insufficient to make a complete osmotic adjustment in cells exposed to 1940 mOsm. Furthermore, the alga grows with little accumulation of glycerol at salinities lower than 100% ASW. We have found that other potential osmoregulatory solutes such as amino acids and soluble carbohydrates make only a minor contribution to cell osmolality of this flagellate.

Our data on intracellular ion concentrations reveal a marked capacity of the flagellate to increase sodium and chloride and to a lesser extent potassium, magnesium, and Pi contents, in response to increases in external salinity. The intracellular osmolality (π_{int}), calculated as the sum of osmolality of intracellular inorganic ions (π_{inorg}) and the osmolality of organic solutes (π_{org}), matched closely the external osmolality (π_{ext}) over the salinity range of 50 to 200% ASW. At the lower salinity of 10% ASW, however, the alga contained about 150 to 270 mOsm (Tables IV and V) solutes in excess of that required to equal the external osmolality. A significant turgor pressure, however, may not exist in these cells, as *C. pulsatilla* contains four functional contractile vacuoles at 10% ASW (20) Contractile vacuoles in *Chlamydomonas* spp. are shown to be associated with the avoidance of turgor pressure at low salinities (15).

The validity of the two techniques used for intracellular ion analyses in our present study has been discussed elsewhere (1, 3). Our results of intracellular ion content of *C. pulsatilla* are in a marked contrast with the recent report by Katz and Avron (22) of a virtually complete exclusion of salt in the cells of *D. salina* growing at high salinities. The intracellular salt concentrations of *D. salina* were calculated from analyses of centrifuged cell pellets (23,000*g* for 15 min) which were reported to contain about 70% extracellular space (22). In contrast, micrographs of *D. parva* cell pellets (13,000*g* × 2 min) indicate less than 10% of extracellular space (28). In the present study, cell pellets of *C. pulsatilla* obtained by low speed centrifugation (2,000*g* × 5 min) contained 23 to 26% extracellular space. The cell size of *D. salina* estimated by Katz and Avron (22) from their measurements of cell pellet and extracellular volumes was between 90 and 92 fl. In other studies, the cell size estimates from light microscopic examination of *D. salina* ranged between 400 and 680 fl (13, 23, 25). However, if one agrees that the *D. salina* strain studied by Katz and Avron was only 92 fl in size, then this strain is too small to contain the reported 11, 24, and 36 pg glycerol cell^{-1} at 0.5, 1.0, and 1.5 M NaCl, respectively (22). These levels of glycerol, if contained in a cell volume of 92 fl, correspond to biophysically impossible turgor pressures of 1.3, 4.5, and 10.3 MPa at 0.5, 1.0, and 1.5 M NaCl, respectively. *Dunaliella* spp. from saline habitats are not known to possess contractile vacuoles (20) or any other mechanism for avoiding turgor pressure. It appears likely that an underestimation of the cellular volume of *D. salina* cell pellet, and thus an overestimation of its extracellular space, was made in the work of Katz and Avron (22). This raises serious questions about the accuracy of the data on the ion content of *D. salina* calculated by these workers from the analyses of cell pellet, as an overestimation of external salt solution trapped in the cell pellet would result in an underestimation of intracellular ions.

The results of the present study of *C. pulsatilla* are consistent with our previous work on *B. submarina* (3) and *Chlorella autotrophica* (1) in showing major contributions by both organic and inorganic solutes in osmotic adjustment. Several workers have stressed that inorganic ions cannot play a significant osmoregulatory role in slightly vacuolated euryhaline microalgae and the cell cytoplasm of higher plant halophytes, as the enzymes isolated from these organisms are inhibited when tested in the presence of high salt concentrations (7, 8, 10, 24). *C. pulsatilla*, *B. submarina* (3), and *C. autotrophica* (1) growing at 200% ASW contain between 1000 and 1200 mM ions, most of which must be localized in the cytoplasmic fraction of these slightly vacuolated microalgae. Similarly high concentrations of ions in cytosolic solution and chloroplasts have been estimated from x-ray microanalyses of leaf mesophyll cells from plants grown in saline conditions (16, 17). We accept that these ion concentrations may not be compatible with optimal metabolic functions. Our studies show that the accumulation of salt by *C. pulsatilla* and other microalgae at high salinities is accompanied by reduction in growth rates (1, 3). Our knowledge of salt compatibility of metabolic functions in eukaryotic organisms is limited to *in vitro* study of enzymes, and appears far from being conclusive on several accounts. First, as Munns *et al.* (24) have pointed out, the salt sensitivity of both plants and algal enzymes has so far been tested in experimental systems using enzyme concentrations hundreds of times lower than those present in the intact cells. Second, although there is evidence that an elevation of substrate levels can to a considerable extent relieve salt inhibition of enzyme activities (4, 14), little attention has been paid to determining changes in endogenous substrate pools in cells adapted to varying salinities (11). Finally, and perhaps most importantly, we must consider the salt compatibility of inorganic ions in euryhaline microalgae in relation to the levels of endogenous osmoregulatory organic solutes. It has been demonstrated that enzymes can be protected from inhibitory effects of inorganic ions in the presence of compatible organic solutes (5). This suggests that the synthesis of glycerol in *C. pulsatilla* not only provides an important source of noninhibitory organic solutes under saline conditions, but also increases the cell's ability to use inorganic ions as osmoregulatory solutes. It may be noted that it is only after considerable buildup of intracellular ions at salinities greater than 50% ASW that a large accumulation of glycerol takes place in this flagellate. Thus, the uptake of ions and the synthesis of glycerol appear to be the two closely associated osmoregulatory responses in *C. pulsatilla*. Evidently, some detailed biochemical work on the interrelationships among ion distribution, organic solute accumulation, and metabolic processes in microalgae is urgently needed. We believe it is only through the use of experimental systems truly representing *in vivo* conditions that we can ascertain the salt compatibility of metabolic functions in salt-tolerant plants and microorganisms unequivocally.

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