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METABOLIC HEAT DISSIPATION AND INTERNAL SOLUTE LEVELS OF *ARTEMIA* EMBRYOS DURING CHANGES IN CELL-ASSOCIATED WATER

BY J. S. GLASHEEN AND STEVEN C. HAND

Department of Environmental, Population and Organismic Biology, University of Colorado, Boulder, CO 80309-334, USA

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Summary

Embryos (cysts) of the brine shrimp *Artemia* enter a profound, yet reversible, state of metabolic arrest in response to cellular dehydration. We have monitored metabolic activity during this transition in embryos from the Great Salt Lake population by using microcalorimetric measurements of heat dissipation. Embryo hydration states can be precisely controlled by immersing cysts in solutions of varying ionic strength. When developing embryos were incubated in a 2·0 mol l^{-1} NaCl solution, heat dissipation fell after 20 h to 1·13 mW g^{-1} dry mass, or 21 % of the value obtained when embryos were in control solutions of 0·25 mol l^{-1}. At higher ionic concentrations, heat dissipation declined to as low as 3 % of control values. Recovery from dehydration was rapid. Energy flow increased to 135 % of control values within 2 h after returning cysts to the control medium.

These metabolic transitions were correlated with embryo hydration levels measured across the same dehydration series. Total cyst water ranged from 112 ± 2·6 g H_2O 100 g^{-1} dry mass in 0·25 mol l^{-1} NaCl to 46 ± 0·6 g H_2O 100 g^{-1} dry mass in 5·0 mol l^{-1} NaCl. At the first point where heat dissipation was markedly suppressed (the 2·0 mol l^{-1} incubation), cyst water content was 72·8 ± 0·9 g H_2O 100 g^{-1} dry mass. This water content is similar to the 'critical' hydration level required to suppress carbohydrate catabolism and respiration in San Francisco Bay *Artemia* embryos (Clegg, 1976*a*,*b*). However, hydration characteristics of the two populations differed in solutions of lower ionic concentration.

Total osmotic pressure in fully hydrated cysts was 1300 mosmol kg^{-1} H_2O. A comprehensive inventory of the internal osmolytes indicated that inorganic ions (Na^+, K^+, Cl^-, Mg^{2+}, Ca^{2+}, Pi) accounted for 21 % of the osmotic activity and 1·48 % of embryo dry mass. Organic solutes (trehalose, glycerol, ninhydrin-positive substances, and trimethylamine-*N*-oxide+betaine) contributed 60 % of the osmotic pressure and 22 % of the dry mass. Macromolecular components (protein, lipids, glycogen and DNA) were also quantified and formed the bulk of embryo mass. Taken together, 97·4 % of the cyst dry mass was identified. At the cellular dehydration state promoting metabolic arrest, the concentrations of inorganic and organic osmolytes were 480–590 mmol kg^{-1} H_2O and

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1200–1480 mmol kg^{-1} H_2O, respectively. The influence of these osmolyte concentrations is considered in the context of macromolecular assembly and metabolic control.

Introduction

Post-diapause embryos (cysts) of the brine shrimp *Artemia* continue to be one of the most intensely studied anhydrobiotic systems (for reviews, see Crowe & Clegg, 1973; Clegg, 1981, 1984). In a previous communication, we examined mechanisms involved in the cessation of carbohydrate catabolism in dehydrated *Artemia* cysts from the Great Salt Lake (Glasheen & Hand, 1988). When hydrated, developing embryos were dehydrated by immersion in 5·0 mol l^{-1} NaCl, trehalose metabolism was inhibited at the series of reactions catalyzed by trehalase, hexokinase and phosphofructokinase. Intracellular pH (pHi) appeared to play no part in these metabolic transitions, in contrast to its pronounced regulatory role in *Artemia* embryos undergoing transitions into anaerobic dormancy (Busa *et al.* 1982; Busa & Crowe, 1983; Carpenter & Hand, 1986; Hand & Carpenter, 1986; Utterback & Hand, 1987; Hand & Gnaiger, 1988). Thus, entry into the quiescent state of anhydrobiosis in *Artemia* seems to be a strict function of cellular water content, as initially proposed by Clegg (1964, 1974, 1976*a*,*b*).

One deficiency currently preventing investigators from testing various mechanisms involved in dehydration-induced arrest of metabolism is the lack of a comprehensive analysis of the internal osmolyte system for *Artemia* embryos. Without such information, it is impossible to estimate the ionic and organic solute concentrations present in the embryo at the onset of metabolic arrest. Yet, in over two decades of research into the *Artemia* system, many of these important constituents of the intracellular milieu have never been quantified. The few values available (amino acid, trehalose, glycerol, sodium content; Emerson, 1967; Clegg, 1967; Conte *et al.* 1977) are taken from different populations of embryos and are often expressed in ways that make it difficult to estimate internal concentrations (e.g. per mg protein).

With the use of open-flow microcalorimetry, we characterize in this paper the dehydration-induced metabolic arrest in *Artemia* embryos from the Great Salt Lake population. Metabolic heat dissipation is a sensitive measure of total energy flow in the embryo (see Hand & Gnaiger, 1988) that can be applied in experiments where dehydration is accomplished with concentrated salt solutions. Use of calorimetry avoids the technical difficulties that arise when oxygen consumption is chosen as a means to estimate aerobic metabolism in solutions approaching saturation with NaCl; electrolyte solutions within the polarographic oxygen electrodes dehydrate under such conditions (Hale, 1983).

We relate the measured transitions in energy flow to the values for internal water content of embryos determined under identical experimental conditions. When the hydration values are coupled to the quantitative analyses of all major intracellular solutes, it is possible to estimate the composition of the internal

milieu of these embryos at any point along the graded series of dehydration. Potential mechanisms for metabolic arrest are considered in the light of these new findings. Metabolic arrest may possibly be the result of disruption of macromolecular assemblies (e.g. enzyme–cytostructural interaction) owing to the elevated ionic strengths of intracellular contents in dehydrated embryos.

Materials and methods

Dehydrated *Artemia* embryos (gastrula stage) from the Great Salt Lake were purchased from the Sander's Brine Shrimp Co. in 1984 (Ogden, UT, USA), and stored at −20°C. For all experiments, cysts were taken from the same commercial batch. The hatching percentage for this batch of cysts has been previously reported (Glasheen & Hand, 1988) and is virtually identical to the percentages determined in other studies using embryos from the Great Salt Lake population (Conte *et al.* 1977; Peterson *et al.* 1978; Warner *et al.* 1979). Dura Wipe filters were a product of Chicopee Mills, Inc. (New Brunswick, NJ, USA). Viton tubing was obtained from Cole Palmer (Chicago, IL, USA). Glycerokinase (from *Candida mycoderma*), glycerophosphate dehydrogenase (Type I, rabbit muscle) and highly polymerized DNA (calf thymus) were purchased from Sigma Chemical Co. (St Louis, MO, USA). All other chemicals and biochemicals were reagent grade.

Washing and dechorionation of embryos

In general, preparatory procedures followed those of Clegg (1974) and were always performed at 0°C. Dried cysts were initially washed five times in 0·25 mol l^{-1} NaCl. After each wash, floating cysts were decanted and discarded. Embryos were then suspended in 5·0 mol l^{-1} NaCl and collected from the surface onto Dura Wipe filters; sand and other debris that had sunk to the bottom of the salt solution were discarded. At this point, washing procedures varied according to the type of experiments for which embryos were intended. For studies requiring the removal of the cyst outer shell (dechorionation), embryos were soaked for 30–40 min in a modified antiformin solution (Nakanishi *et al.* 1962) containing 2·5% (w/v) active hypochlorite, 0·4 mol l^{-1} NaOH and 60 mmol l^{-1} Na_2CO_3. Following dechorionation, embryos were washed five more times in 0·25 mol l^{-1} NaCl and either used immediately in experiments or stored overnight at 0°C in the same medium.

If cysts were to be used for Na^+ and Cl^- determinations, final washes were performed in deionized water (Milli-Q Reagent Water System; Millipore Corp.; resistance = 18 MΩ). Although it has been reported that soaking Great Salt Lake embryos in distilled water results in substantial lysis (Clegg, 1974), we found that if cysts were used immediately after brief washes in deionized water, the total percentage of ruptured cysts did not exceed that observed for cysts soaked in 0·25 mol l^{-1} NaCl (% ruptured cysts after deionized water wash = 9·5 ± 0·26; % ruptured after 0·25 mol l^{-1} NaCl wash = 13·1 ± 0·70; mean ± S.E.M., $N = 3$ determinations).

Microcalorimetry

Measurements of heat dissipation from *Artemia* embryos were made with an LKB 2277 thermal activity monitor equipped with a 3·5 ml perfusion chamber adapted to ensure uniform flow of medium through the sample of embryos (Hand & Gnaiger, 1988). Perfusion media were equilibrated with a 60 % N_2: 40 % O_2 gas mixture, calibrated with Tylan FC-260 mass flow meters. Flow lines for gases and for aqueous media were made of Viton and stainless steel, respectively. Flow rate of the perfusion media was set at 16·0 ml h^{-1}, and temperature was maintained at 23 °C for all experiments. Heat dissipation in microwatts was time-corrected by the calibration unit of the calorimeter; typical time constants were $t_1 = 600$ s and $t_2 = 17\,100$ s. Data were sampled every 60 s and stored on an IBM XT personal computer, as well as on a strip chart recorder for immediate visual inspection. For all experiments, blanks (no embryos in perfusion chamber) were run and subtracted from the measurements obtained with embryos present. To ensure that oxygen was not limiting for embryos, the O_2 in the gas mixture was increased above 40 %, and no significant change in heat dissipation was recorded.

Embryos, prepared as described above (with intact chorions), were allowed to hydrate overnight at 0 °C in 0·25 mol l^{-1} NaCl prior to the beginning of the calorimetry experiments. After calibration of the instrument, embryos were loaded into the perfusion chamber. Heat dissipation in 0·25 mol l^{-1} NaCl (control medium) was monitored for 4 h. Perfusion medium was then switched to one of five different NaCl solutions (1·0, 2·0, 3·0, 4·0, and 5·0 mol l^{-1}) and heat dissipation recorded for another 16 h on average. Finally, incubation medium was switched back to 0·25 mol l^{-1} NaCl and the recovery period monitored.

Measurement of embryo hydration

Hydration state in *Artemia* embryos can be precisely controlled by immersing cysts in solutions of varying ionic strength (Clegg, 1974), because the cyst wall (outer cuticular membrane) is impermeable to molecules other than low molecular weight dissolved gases and water (see Clegg & Conte, 1980, for a review). However, hydration values exist only for embryos from the San Francisco Bay population of *Artemia* (Clegg, 1974). Because significant interpopulational differences in hydration state may exist (Clegg, 1974), it was necessary for us to obtain these values for Great Salt Lake embryos to interpret more precisely the metabolic and intracellular solute data of this and previous studies (Glasheen & Hand, 1988).

First, to obtain a valid comparison with hydration values of San Francisco Bay embryos, experiments with the Great Salt Lake gastrulae were conducted in a virtually identical way to those in Clegg (1974), and the resulting data are presented as series I (see below). Second, hydration levels also had to be measured under conditions dictated by the various metabolic studies we have performed (series II, below).

Series I: hydration of dry embryos

Briefly, embryos were washed as described above and placed in desiccators over

$CaSO_4$ (Drierite, WA Hammond Co., Xenia, OH, USA) until gravimetric equilibrium was reached. Cysts dehydrated in this manner were used directly for incubations in the experimental salt solutions. Approximately 1 g of dry cysts was added to triplicate 125 ml flasks containing 60 ml of one of the following solutions: 0·25, 1·0, 2·0, 3·0, 4·0 or 5·0 mol l^{-1} NaCl. Flasks were then shaken at 110 cycles min^{-1} at 0°C for 24 h. Following incubation, 100–200 mg of cysts was filtered onto Dura Wipes and blotted between two sheets of Whatman no. 4 chromatography paper to remove interstitial water. Embryos were *not* washed in either deionized or dilute NaCl solutions prior to blotting. The amount of salt possibly adhering to the cysts after blotting was calculated to be negligible compared to the error introduced by even a 30 s washing procedure. Results of a preliminary study show a significant increase in water content of fully desiccated cysts following a 30 s rinse in 0·25 mol l^{-1} NaCl (embryo $H_2O = 11{\cdot}2 \pm 1{\cdot}09$ g 100 g^{-1} dry cyst; S.E.M., $N = 3$). Blotting was performed with a gentle circular motion until embryos attained the consistency of fine sand and flowed freely from the paper (Clegg, 1974). Cysts were then weighed on an analytical balance in tared aluminum weighing pans and placed over $CaSO_4$ in 1-l glass desiccators. To maintain consistency between treatments, blotting time for each sample was 3·5 min, with a total of 5 min for filtration, blotting and weighing. Groups of three replicates obtained for each NaCl incubation were placed in the individual desiccators for 72 h, after which time dry masses were obtained.

Series II: dehydration of hydrated embryos

Procedures employed for this series were identical to those for series I, except that the washed cysts were not dry, but rather pre-hydrated for 16 h in 0·25 mol l^{-1} NaCl, prior to starting the incubations in the experimental salt solutions. Following experimental incubations, the blotting, weighing and drying of cysts were performed exactly as they were in series I.

It is appropriate to note that the final hydration values reached after incubation in the experimental salt solutions are substantially different depending on whether one starts with dry cysts (series I) or cysts equilibrated with 0·25 mol l^{-1} NaCl (series II).

Ionic composition of embryos

Metallic ion content was determined by atomic absorption spectroscopy. Cysts were washed and then dechorionated to eliminate artifacts due to ion entrapment in the porous, acellular chorion. Embryos were filtered onto Whatman no. 41 paper, samples placed in tared crucibles, and dried to constant mass (24 h in a 60°C oven). Cysts dried in a 60°C oven exhibited little additional mass loss over embryos dehydrated 72 h over $CaSO_4$. Additional mass loss $= 0{\cdot}85 \pm 0{\cdot}17$ % (S.E.M., $N = 18$). Dry mass was measured, and embryos were placed in a Lindberg muffle furnace overnight at 550°C. The resultant ash was suspended in 5 % HNO_3. After addition of lanthanum oxide and cesium chloride, solutions were brought to a final concentration of 7·5 mmol l^{-1} CsCl, 1 % La_2O_3 and 0·5 % HNO_3. The

suspension was then centrifuged at 4000 *g* for 10 min. Supernatants were decanted and analyzed for ions. An additional wash of the pellet was not necessary for complete ion extraction.

Sodium was measured in a Varian Techtron atomic absorption spectrometer at 589·0 nm using an air–acetylene flame. Potassium was determined at 766·5 nm using the air–acetylene mixture. Magnesium was quantified at 285·2 nm with acetylene–N_2O as fuel-oxidant, and calcium was measured at 422·7 nm, again with nitrous oxide as oxidant. Calibration curves were generated from standard solutions (Environmental Protection Agency, US).

For inorganic phosphate and chloride analyses, the dechorionated cysts were filtered onto Dura Wipes and excess moisture wicked away. Two samples of approximately equal mass were removed and wet masses obtained. One sample was used for ion analysis, while a similar portion was dried to constant mass. For phosphate analyses, samples were homogenized in 5 ml of 6 % perchloric acid and centrifuged at 10 000 *g* for 15 min. Supernatants were neutralized with 5 mol l^{-1} K_2CO_3 and centrifuged again to remove perchlorate salts. Inorganic phosphate was determined with a modified version of the Piper & Lovell (1981) method. The colorimetric reagent was made by adding 37·5 g of guanidine–HCl to 25 ml of a 7·5 % ammonium molybdate/2·5 mol l^{-1} H_2SO_4 solution. This mixture was brought to a final volume of 100 ml with a 20 mg ml^{-1} bovine serum albumin solution. The reagent was added 3:1 (v/v) to both samples and standards, and after 30 min the absorbance was measured at 370 nm. For chloride measurement, samples were homogenized in deionized water and centrifuged at 10 000 *g* for 15 min. After addition of $Ba(OH)_2$ and $ZnSO_4$ to final concentrations of 0·025 mol l^{-1} and 1 %, respectively, supernatants were then left at room temperature for 48 h to precipitate interfering materials. Following centrifugation, supernatants were analyzed with a Haake–Buchler digital chloridometer.

Measurement of osmotic pressure

Dechorionated embryos were homogenized in two volumes of deionized water, and 175 μl of each homogenate was centrifuged at 140 000 *g* in a Beckman Airfuge for 30 min. The osmotic pressure of the supernatants was measured with a vapor pressure osmometer. In order to equate the osmotic pressure measured *in vitro* to the *in vivo* situation, this approach assumes, of course, that all solutes in the embryo are in solution. It is likely that this assumption could result in an overestimate of the actual *in vivo* osmotic pressure.

Analyses of organic solutes and macromolecular components

Trehalose, glycogen and glycerol were measured in dechorionated cysts as described by Hand & Carpenter (1986). Ninhydrin-positive substances (NPS) were measured in neutralized perchoric acid extracts by the colorimetric method of Lee & Takahashi (1966), with aspartic acid as the standard. Total methylamines were analyzed with a modified version of the reineckate precipitation method of Barnes & Blackstock (1974). Samples were homogenized in 4 vols of 70 % ethanol,

and then placed in a boiling water bath for 15 min. Homogenates were then centrifuged at 10000 *g* for 15 min, and the supernatants were collected and rotoevaporated. The residue was dissolved in 0·1 mol l^{-1} HCl and clarified by centrifugation. A volume of this supernatant containing approximately 20 μmol of methylamine (betaine standard) was diluted to 1·0 ml. Then 3·0 ml of ammonium reineckate reagent was added, the solution vigorously mixed, and precipitation allowed to occur overnight at 4°C. The reineckate solution consisted of 5 g of NH_4–$[Cr(SCN)_4(NH_3)_2]\cdot H_2O$ brought to 100 ml with 0·1 mol l^{-1} HCl. The solution was stirred for 45 min, titrated to pH 1·0, and filtered through Whatman no. 1 paper. The sample precipitate was collected by centrifugation at 3500 *g* and pellets were washed in diethyl ether three times. Final pellets were brought to 6 ml with 70 % acetone and absorbance read at 520 nm.

Total protein was determined in dechorionated embryos as described by Hand & Carpenter (1986) using the method of Peterson (1977). Embryo DNA content was quantified with the diphenylamine method of Richards (1974). Pellets from cysts homogenized in 6 % perchloric acid (PCA) were resuspended in 10 ml of 1·5 mol l^{-1} PCA and heated at 70°C for 15 min with occasional mixing. Samples were then centrifuged at 10000 *g* for 15 min, and DNA measured in the supernatant. Total lipid content of dechorionated embryos was determined gravimetrically following the procedure of Folch *et al.* (1957), as modified by Ways & Hanahan (1964).

Results

Microcalorimetric measurements

This set of experiments was designed to quantify the relationship between reductions in cellular hydration and the suppression of total energy flow in *Artemia* embryos. Heat dissipation from developing embryos rose steadily during the first 4 h in control 0·25 mol l^{-1} NaCl. When the perfusion medium was switched to 1·0 mol l^{-1} NaCl, energy flow continued to rise, reaching 137 % of control (hour 4) values (Fig. 1; Table 1). However, when the control medium was switched to 2·0 mol l^{-1} NaCl, energy flow fell exponentially to 21 % of control values after approximately 18 h. In experiments with higher salt concentrations (3–5 mol l^{-1}), heat dissipation fell sharply to 6 % or less of the rates at hour 4 (Fig. 2; Table 1). Thus, it appears that metabolism in developing embryos is disrupted at a critical hydration level promoted by an external NaCl concentration between 2·0 and 3·0 mol l^{-1}. This range of external NaCl concentration needed for reduction of heat dissipation is comparable to that required for blockage of trehalose utilization (Clegg, 1976*a*; Glasheen & Hand, 1988) and the onset of respiration in *Artemia* (Clegg, 1976*b*). It is interesting to note that dehydration of embryos in 3·0 mol l^{-1} NaCl or higher yields an energy flow (0·20 mW g^{-1} dry mass) comparable to that observed in *Artemia* after several hours of anaerobic dormancy (Hand & Gnaiger, 1988). This low heat dissipation may reflect maintenance level metabolism in these

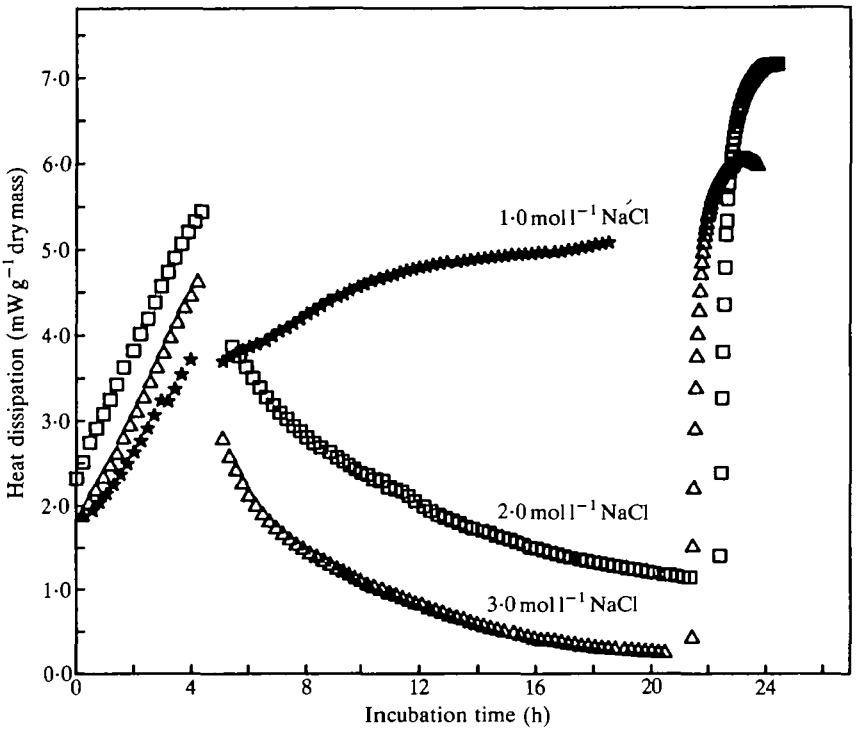

Fig. 1. Heat dissipation of *Artemia* embryos during incubations in various concentrations of NaCl. For all experiments, embryos were initially incubated for 4 h in 0·25 mol l^{-1} NaCl, after which the medium was changed to either 1·0 (★), 2·0 (□) or 3·0 (△) mol l^{-1} NaCl. After the 2·0 and 3·0 mol l^{-1} treatments, the incubation medium was switched to 0·25 mol l^{-1} NaCl, and the recovery period monitored.

Table 1. *Heat dissipation of* Artemia *embryos from the Great Salt Lake as a function of NaCl concentration*

Salt treatment (mol l^{-1} NaCl)	Heat dissipation			
	Control (4 h, 0·25 mol l^{-1}) (mW g^{-1} dry mass)	Dehydrated		Recovery (% control)
		(mW g^{-1} dry mass)	(% control)	
1·0	3·71*	5·08	137	–
2·0	5·36	1·13	21	133
3·0	4·54	0·26	6	133
4·0	4·69	0·14	3	134
5·0	4·41	0·21	5	139

* Average value for 20 min interval.

embryos. In any case, intracellular hydration levels induced by these high-salt media appear incapable of supporting conventional metabolism.

When embryos were returned to 0·25 mol l^{-1} NaCl perfusion after dehydration bouts, recovery of energy flow was rapid (Figs 1, 2). In each case (2, 3, 4 and 5 mol l^{-1} NaCl), heat dissipation reached values that corresponded to approximately 135 % of the pre-dehydrated value (Table 1). Note that every time the salt concentration was changed, there was a 30 min gap in the heat dissipation profile. This disruption was due to heats of mixing, which artifactually forced the calorimetric readings off scale for those periods.

Hydration experiments

Results of hydration studies are presented in Table 2 (series I) and Table 3 (series II). For series I data, our Great Salt Lake cysts were prepared identically to the San Francisco Bay embryos in the studies by Clegg (1974); i.e. embryos were hydrated in the experimental solutions directly from the dry state. Differences in

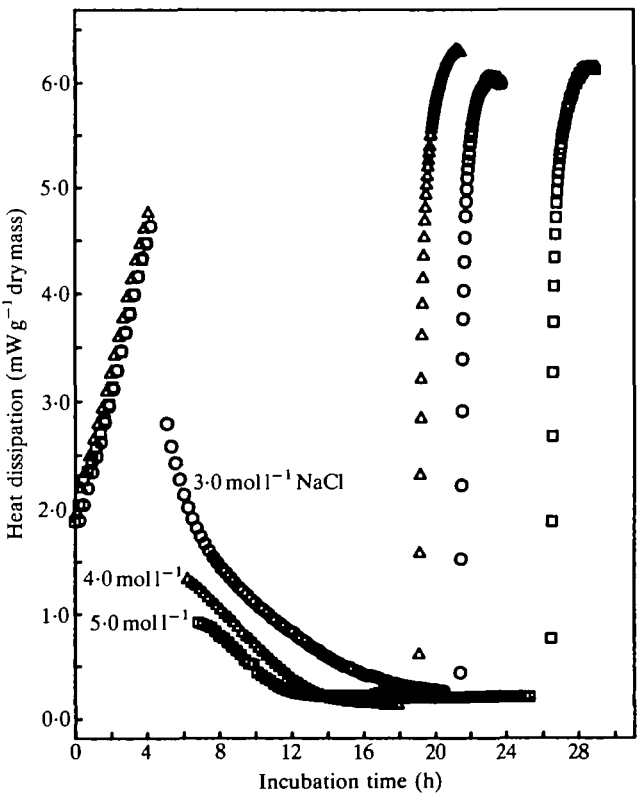

Fig. 2. Heat dissipation of *Artemia* embryos during incubations in various concentrations of NaCl. For all experiments, embryos were initially incubated for 4 h in 0·25 mol l^{-1} NaCl, after which the medium was changed to either 3·0 (○), 4·0 (△) or 5·0 (□) mol l^{-1} NaCl. After each treatment, the incubation medium was switched to 0·25 mol l^{-1} NaCl, and the recovery period monitored.

hydration behavior in response to external salinity exist between the two populations. The differences occurred primarily in the 1·0 and 2·0 mol l^{-1} NaCl incubations (Table 2); values were identical at the higher NaCl concentrations. These observations become particularly important when comparing the metabolic responses to dehydration between the two populations (e.g. relating microcalorimetric results with previous measurements of respiration).

Table 2. *Water content of* Artemia *embryos from the Great Salt Lake and San Francisco Bay*

	Great Salt Lake		San Francisco Bay*	
NaCl solution (mol l^{-1})	g H_2O 100 g^{-1} dry mass	% embryo H_2O	g H_2O 100 g^{-1} dry mass	% embryo H_2O
0·25	121·5 ± 1·1†	54·9 ± 0·5	–	–
0·5	–	–	119·3	54·4
1·0	99·0 ± 1·5	49·7 ± 0·7	88·1	46·8
2·0	60·5 ± 0·2	37·7 ± 0·1	51·8	34·1
3·0	42·1 ± 0·4	29·6 ± 0·3	41·0	29·1
4·0	31·7 ± 0·8	24·1 ± 0·6	32·2	24·4
5·0	24·4 ± 0·2	19·6 ± 0·2	23·0	18·7

Values were obtained by placing dry embryos directly into the indicated salt solutions at 0°C (series I, see text).

* Data for San Francisco Bay embryos are from Clegg (1974).

† Each value is the mean ± S.E.M. ($N = 3$).

Table 3. *Water content of* Artemia *embryos from the Great Salt Lake incubated in NaCl solutions at 0°C*

			Corrected data*	
NaCl solution (mol l^{-1})	g H_2O 100 g^{-1} dry mass	% embryo H_2O	g H_2O 100 g^{-1} dry mass	% embryo H_2O
0·25	112·0 ± 2·6†	52·8 ± 1·2	134·4 ± 3·1	57·3 ± 1·3
1·0	88·8 ± 0·1	47·0 ± 0·1	105·4 ± 0·1	51·3 ± 0·1
2·0	72·8 ± 0·9	42·1 ± 0·5	85·4 ± 1·0	46·1 ± 0·5
3·0	60·4 ± 1·6	37·6 ± 1·0	69·9 ± 1·9	41·1 ± 1·1
4·0	54·8 ± 0·5	35·4 ± 0·3	62·8 ± 0·6	38·6 ± 0·4
5·0	46·0 ± 0·6	31·5 ± 0·4	51·8 ± 0·6	34·1 ± 0·4

Embryos were prehydrated for 16 h in 0·25 mol l^{-1} NaCl (series II, see text).

* Corrected values represent the water content of metabolically active embryonic tissue. These values are arrived at in the following manner. Shells represent 20 % of the cyst dry mass, and their water content is minimal and essentially constant across NaCl treatments [≈4·54 g H_2O 20 g^{-1} shells (Clegg, 1974)]. Thus, subtracting shell H_2O from hydration values and dividing by 0·8 yields the hydration levels for metabolically active, dechorionated embryonic tissue.

† Each value is the mean ± S.E.M. ($N = 3$).

Series II data are from embryos pre-hydrated in 0·25 mol l^{-1} NaCl and then exposed to the increasing salt series (Table 3); this approach is the more practical for our metabolic studies. Note that in series II, embryos were effectively being dehydrated in order to reach the final hydration values, while in series I, the embryos were initially dry and gained water to reach the final hydration values. This directional difference in water movement apparently altered the final hydration levels obtained at 24 h; the water content was higher in embryos from series II. The reason for this difference has not been determined, but we suspect that numerous air spaces and channels in the chorion influence in some way the rate of embryo hydration *versus* dehydration. Preliminary studies indicated that hydration properties are markedly different with dechorionated embryos (data not shown).

Finally, the 'corrected' hydration levels presented in Table 3 represent estimates of the actual water content of the metabolically active embryonic tissues. These data, in combination with the microcalorimetry results, showed that energy flow was severely disrupted when embryo water was reduced to 70–85 g H_2O 100 g^{-1} dry mass. This water content compares well with critical hydration levels required for the onset of carbohydrate catabolism and respiration, when corrected in a similar fashion (70 g H_2O 100 g^{-1} dry cyst; Clegg, 1976*a*,*b*).

Ionic composition of Artemia *embryos*

Ionic concentrations for dechorionated cysts are expressed as millimolal values in Table 4. Values calculated for each incubation condition were based on the corrected hydration values given in Table 3. The osmotic contribution of each ion reflect the embryo hydration levels in 0·25 mol l^{-1} NaCl and the indicated osmotic coefficient. Values for intracellular sodium, potassium and calcium were in good agreement with concentrations reported previously for barnacle muscle fibers (*Balanus nubilis*; Clark & Hinke, 1980). Inorganic phosphate levels were reasonably close to those reported for the decapod marine crustacean *Nephrops* (21 mmol l^{-1}; Robertson, 1961). Chloride was somewhat higher than the 30–35 mmol kg^{-1} H_2O reported by Hinke *et al.* (1973) and Hinke & Gayton (1971) for *Balanus* muscle. Intracellular magnesium levels were unexpectedly high in *Artemia* embryos, more than three times the concentration found in some crustacean muscle (e.g. *Balanus*; Page *et al.* 1971). These ions are calculated to contribute an osmotic pressure of 275 mosmol kg^{-1} H_2O when cysts are incubated in 0·25 mol l^{-1} NaCl. Since divalent ions were included in this value, their contribution to the osmotic pressure may be a slight overestimate, as the percentage of bound Mg^{2+}, Ca^{2+} and Pi is unknown. If cysts are dehydrated to the critical range of 70–85 g H_2O 100 g^{-1} dry mass (corrected), the intracellular osmotic pressure contributed by inorganic ions increases to over 500 mosmol kg^{-1} H_2O. Inorganic ions account for 1·5 % of the dry embryo mass (Table 5).

Organic solutes

Assuming that all the measured trehalose is soluble, this disaccharide accounted

Table 4. *Internal solute concentrations of* Artemia *embryos from the Great Salt Lake as a function of NaCl concentration*

Solute	mosmol kg^{-1} H_2O*	Osmotic coefficient	External medium ($mol\,l^{-1}$ NaCl) 0·25	1·0	2·0	3·0	4·0	5·0
			($mmol\,kg^{-1}$ H_2O)					
Ions								
Sodium	32·8	(0·9)	36·4 ± 1·0†	46·4 ± 0·7	57·2 ± 1·1	69·9 ± 2·1	77·9 ± 1·3	94·4 ± 1·8
Potassium	130·9	(0·9)	145·4 ± 3·9	185·4 ± 2·5	228·9 ± 4·1	279·6 ± 8·4	311·3 ± 5·0	377·4 ± 6·8
Magnesium	49·0	(0·9)	54·4 ± 1·6	69·4 ± 1·3	85·7 ± 1·9	104·7 ± 3·4	116·5 ± 2·4	141·2 ± 3·1
Calcium	5·3	(0·9)	5·9 ± 0·2	7·6 ± 0·2	9·3 ± 0·3	11·4 ± 0·5	12·7 ± 0·4	15·4 ± 0·5
Chloride	45·3	(0·9)	50·3 ± 3·2	64·1 ± 3·8	79·2 ± 4·8	96·7 ± 6·3	107·6 ± 6·5	130·5 ± 7·9
Phosphate	11·2	(0·9)	12·4 ± 1·1	15·8 ± 1·4	19·6 ± 1·7	23·9 ± 2·1	26·6 ± 2·3	32·2 ± 2·8
Subtotal (ions)	274·5							
Organics								
Trehalose	340·5	(1·0)	340·5 ± 16·9	434·3 ± 19·0	535·9 ± 24·3	654·7 ± 33·7	728·8 ± 32·6	883·7 ± 40·1
Glycerol	319·2	(1·0)	319·2 ± 30·7	407·0 ± 38·1	502·3 ± 47·4	613·7 ± 59·7	683·1 ± 64·1	828·2 ± 78·1
NPS	62·3	(1·0)	62·3 ± 3·4	79·4 ± 4·0	98·0 ± 5·0	119·7 ± 6·8	133·3 ± 6·8	161·6 ± 8·3
TMAO+betaine	56·4	(1·15)	49·0 ± 2·0	62·4 ± 2·0	77·0 ± 2·7	94·1 ± 4·0	104·8 ± 3·6	127·0 ± 4·4
Subtotal (organics)	778·4							
Total osmotically active solutes	1052·9							
Total osmotic pressure observed	1301·0							
Unidentified solutes (by subtraction)	248·1							

* Values for embryos hydrated in 0·25 $mol\,l^{-1}$ NaCl.
† Mean ± S.E.M. ($N = 3$).
NPS, ninhydrin-positive substances; TMAO, trimethylamine-*N*-oxide.

Table 5. *Internal composition of* Artemia *embryos from the Great Salt Lake*

Constituents	mg g^{-1} dry mass		% dry mass
Ions			
Sodium	1·12 ± 0·02*		0·11
Potassium	7·64 ± 0·10		0·76
Magnesium	1·78 ± 0·03		0·18
Calcium	0·32 ± 0·01		0·03
Chloride	2·40 ± 0·14		0·24
Phosphate	1·59 ± 0·14		0·16
		Subtotal (ions)	1·48
Organics			
Trehalose	164·9 ± 7·2		16·5
Glycerol	39·5 ± 3·7		4·0
NPS	11·1 ± 0·6		1·1
TMAO+betaine	7·7 ± 0·3		0·8
		Subtotal (organics)	22·4
Macromolecular components			
Protein	544 ± 13·0		54·5
Lipids	166·9 ± 3·4		16·7
Glycogen	16·2 ± 0·4		1·6
DNA	8·1 ± 0·2		0·8
		Subtotal (macromolecules)	73·5
		Total	97·4

*Means ± S.E.M. ($N = 3$).
TMAO, trimethylamine-*N*-oxide; NPS, ninhydrin-positive substances.

for 26 % of the osmotic pressure in *Artemia* embryos (Table 4), as well as 16·5 % of the dry mass (Table 5). This latter value is in close agreement with that reported by Clegg (1964). The polyalcohol glycerol also made a significant contribution to osmotic pressure (319 mosmol kg^{-1} H_2O), again in agreement with values reported by Clegg (1964).

Nitrogenous solutes account for 12 % of the osmotic activity in *Artemia*. Ninhydrin-positive substances contribute 62·3 mosmol kg^{-1} H_2O (Table 4) and 1·11 % of the dry mass (Table 5). This value is significantly lower than for most marine invertebrates (Yancey *et al.* 1982), but it agrees well with data reported by Emerson (1967) and Clegg & Lovallo (1977) for *Artemia* embryos. Total methylamines (calculated using betaine as the standard) were present at 56·4 mosmol kg^{-1} H_2O. This is the first report documenting the presence of these compounds in *Artemia* embryos. The beneficial effects of these osmolytes on enzyme structure and function have been documented (Clark & Zounes, 1977; Bowlus & Somero, 1979). In *Artemia*, trehalose and glycerol should also be considered as compatible solutes (Crowe, 1971; Crowe *et al.* 1987).

For the soluble components, osmotic coefficients have been assigned in the

following manner: inorganic ions, 0·9 (Scatchard *et al.* 1938); ninhydrin-positive substances (NPS), 1·0 (Robertson, 1975); trimethylamine-*N*-oxide (TMAO)+ betaine, 1·15 (Robertson, 1975); glycerol, 1·0 (Scatchard *et al.* 1938); and trehalose has been assumed to behave identically to sucrose (1·0). In summary, soluble organic compounds account for 778 mosmol kg^{-1} H_2O, or 73 % of the osmotic pressure observed in *Artemia* embryos. The measured organic solutes comprise 22·4 % of cyst dry mass (Table 5).

Osmotic pressure

Total osmotic pressure, measured by vapor pressure depression in dechorionated embryos, was 1300 mosmol kg^{-1} H_2O at the level of hydration induced in *Artemia* by immersion in 0·25 mol l^{-1} NaCl (Table 4). This leaves an unidentified osmotic pressure component of 248 mosmol kg^{-1} H_2O or 19 % of the total. It is quite possible that much of this unknown component consists of osmotically active protein, oligopeptides, or polysaccharide moieties of glycoproteins, as was previously suggested for barnacle muscle (Clark & Hinke, 1980). When cyst hydration is decreased to 85·4 g H_2O 100 g^{-1} dry mass (immersion in 2·0 mol l^{-1} NaCl), total osmotic pressure increases to 2048 mosmol kg^{-1} H_2O. It is interesting to note that the internal osmotic pressure of *Artemia* embryos is substantially below that of the external medium for all incubations $\geq$1·0 mol l^{-1} NaCl (see Discussion).

Macromolecular components

PCA-insoluble protein made up the majority of embryo dry mass in *Artemia*. The value of 54·4 % agreed well with the protein component (58·5 %) found in *Balanus* muscle (Clark & Hinke, 1980). Chloroform/methanol-extracted lipids also formed a large component of cyst dry mass (16·7 %). These results were similar to those reported by Dutrieu (1960) for intact (chorionated) embryos. Glycogen values were low (1·6 %) in *Artemia*, as would be expected at this point in development based on previous studies of carbohydrate metabolism in dormant cysts (Glasheen & Hand, 1988; Carpenter & Hand, 1986; Clegg, 1976*a*). DNA content (0·81 %) was in line with values reported for most eukaryotes at resting stages in the cell cycle (e.g. blue crab, Wang & Stickle, 1986). Taken together, 97·4 % of the dry mass in *Artemia* embryos has been identified and assigned to ionic, organic solute or macromolecular fractions (Table 5).

Discussion

The results of the present study characterize more fully the metabolic arrest and the alterations in internal solute composition that occur as *Artemia* embryos enter and exit the state of anhydrobiosis. The use of microcalorimetry improves our understanding of these transitions by providing an estimate of total energy flow in the embryo. The comprehensive inventory of intracellular solutes, in conjunction

with embryo hydration values, offers an opportunity to estimate more precisely the status of the internal milieu at the critical hydration point where metabolic arrest begins. Considering the numerous metabolic studies performed with *Artemia* embryos, it is surprising that a complete characterization of the internal osmolyte system has not been available until now. We will discuss below selected possibilities as to how changes in intracellular solution conditions might bring about the metabolic arrest observed during restricted water availability.

Our measurements of heat dissipation show that total energy flow is acutely suppressed when water content of the metabolically active tissue falls to 85 g H_2O 100 g^{-1} dry mass. At 70 g H_2O 100 g^{-1} dry mass and below, heat dissipation has dropped to only a few percent of the control (0·25 mol l^{-1} incubation) values or, in absolute terms, 0·14–0·26 mW g^{-1} dry mass. During the quiescent state induced by anaerobiosis, *Artemia* embryos exhibit similar values during short-term (several hour) experiments (Hand & Gnaiger, 1988), although the mechanism of the arrest is apparently quite different. Substantial evidence supports a primary role for acidotic pHi in anaerobic dormancy; but during dehydration, metabolic arrest proceeds normally under conditions where pHi does not change (Glasheen & Hand, 1988). Both these forms of hypometabolism are fully reversible. Upon restoration of intracellular water, energy flow in embryos rises rapidly and dramatically to levels comparable to those in fully hydrated, developing embryos (Figs 1, 2). In previous studies, embryo respiration and trehalose usage displayed similar patterns (Clegg, 1976*a*,*b*; Glasheen & Hand, 1988).

Traditional views regarding this metabolic arrest in *Artemia* entering anhydrobiosis have centered upon the role of water in maintaining the structural and functional integrity of metabolic pathways. Minimum water content has been established for San Francisco Bay embryos that permit the onset of various metabolic functions (for a review, see Clegg, 1981). Hydration values for Great Salt Lake embryos have not been available heretofore, but now some interpopulation comparisons can be made. Critical hydration levels necessary for the onset of major metabolic events are similar for each population. A water content of 70 g H_2O 100 g^{-1} dry mass is needed for respiration in the San Francisco Bay population (Clegg, 1974), while 70–85 g H_2O 100 g^{-1} dry mass is required for substantial heat dissipation in Great Salt Lake embryos (Table 2; Fig. 1). However, differences in hydration response are seen in solutions of low ionic strength. This metabolic shutdown observed during progressive dehydration/rehydration has been explained by perturbation of 'vicinal' or protein-associated water (Clegg, 1984). Cellular regions of vicinal *versus* bulk water were described by Porter (1986) as 'protein-rich' and 'water-rich' phases, and this distinction was suggested by experiments performed as early as 1938 (Kopac, 1938; Chambers, 1940). In the present study, there is at least indirect evidence that the properties of intracyst water may be quite different from those of the external medium. For example, the difference between external and internal osmotic pressure for embryos incubated in 5·0 mol l^{-1} NaCl approaches 10 000 mosmol kg^{-1} H_2O. It is

unlikely that a lack of osmotic equilibrium can explain this discrepancy, but rather that much of the intracellular water is restricted and not osmotically available.

While interpretations based on *solvent* status have proved highly useful, the important corollary of intracellular *solute* content has been relatively neglected *vis à vis* metabolic organization and control in *Artemia*. As a direct consequence of reductions in cell-associated water, intracellular solute concentrations rise dramatically. In *Artemia*, as water activity decreases to the critical level (as demonstrated by metabolic arrest), the concentration of inorganic ions approaches 500 mmol kg^{-1} H_2O. In most marine species studied, levels above 100–200 mmol kg^{-1} H_2O of univalent ions are severely deleterious to enzyme function, as judged by lowering of reaction velocities as well as elevation of K_m (Borowitzka & Brown, 1974; Bowlus & Somero, 1979; Yancey *et al.* 1982). In addition to these direct influences on enzyme function, evidence suggests that protein binding to subcellular components may be disrupted by elevated levels of inorganic ions. In mammalian muscle, the adsorption of the glycolytic enzymes aldolase, lactate dehydrogenase and pyruvate kinase to an F-actin–tropomyosin–troponin complex was significantly reduced in 150 mmol l^{-1} KCl (Clarke & Masters, 1975). Likewise, 150 mmol l^{-1} NaCl resulted in desorption of glyceraldehyde-3-phosphate dehydrogenase from Band 3 protein in erythrocytes (Yu & Steck, 1975). Mammalian brain hexokinase, in its more active 'bound' form, is almost completely solubilized in 350 mmol l^{-1} KCl or NaCl (Wilson, 1968). Partitioning of enzymes into soluble and particulate fractions (with differing kinetic properties) or altering the cohesiveness of metabolons (multienzyme associations) are attractive models for effective means of metabolic regulation (for reviews, see Welch, 1977; Kurganov, 1985; Welch & Keleti, 1981; Srere, 1987; Ovadi, 1988; G. N. Somero & S. C. Hand, in preparation).

Thus, in dehydrated *Artemia*, ionic perturbation of protein–protein or protein–cytoskeletal interactions may account for at least part of the observed metabolic arrest. Previous experiments have indicated simultaneous inhibition of three major enzymes during the glycolytic shutdown in dehydrated embryos, namely trehalase, hexokinase and phosphofructokinase (Glasheen & Hand, 1988). The catalytic properties of *Artemia* trehalase are significantly altered by modifications of macromolecular assembly state (Hand & Carpenter, 1986). While *Artemia* hexokinase does not reversibly associate with isolated mitochondria (Rees *et al.* 1989), as does the rat brain homologue, we have preliminary evidence that phosphofructokinase does bind to a particulate fraction in homogenates of these embryos (J. S. Glasheen & S. C. Hand, unpublished observations). The fact that the dehydration-induced metabolic shutdown in *Artemia* is fully reversible is consistent with the above view of metabolic organization. Such non-covalent assembly/disassembly transitions could alter enzymic function without compromising native protein structure. Indeed, it seems reasonable to suggest that conservation of macromolecular integrity is an important prerequisite for organisms capable of undergoing such drastic reductions in cell-associated water.

It is well established now that many organisms that frequently face water stress

possess systems of 'compatible' solutes (Brown & Simpson, 1972), i.e. compounds either non-perturbing or actually stabilizing to macromolecular structure and function (Yancey *et al.* 1982; Somero, 1986). Generally, these organic solutes are either uncharged or zwitterionic, and quite often structurally resemble the stabilizing ions of the Hofmeister series (Clark & Zounes, 1977). Our analyses of intracellular solutes in *Artemia* reveal significant levels of methylamines (50–127 mmol kg^{-1} H_2O, depending on hydration state), a family of compatible solutes known to exert stabilizing influences at concentrations as low as 50 mmol kg^{-1} H_2O (e.g. Hand & Somero, 1982). In addition, as dehydration reaches critical levels in the *Artemia* embryos, concentrations of the osmolytes trehalose and glycerol exceed 1000 mmol kg^{-1} H_2O. In the case of biological membranes, Crowe *et al.* (1987) have suggested that trehalose serves a 'water replacement' function, stabilizing membrane structure through physical intercalation with phospholipids. In combination with transition metal ions, trehalose also protects proteins against denaturation incurred during freezing (Carpenter *et al.* 1986) and drying (Carpenter & Crowe, 1988).

Based on the comprehensive inventory of embryo solutes presented herein, we suggest that the role of intracellular osmolytes should be more fully integrated into hypotheses explaining the dehydration-induced arrest of *Artemia* metabolism. At low water activities, concentrations of inorganic ions rise appreciably, which could result in the disruption of protein–protein associations and the attendant loss of metabolic function. The substantial quantities of the organic solutes trehalose, glycerol and the newly detected methylamines may be involved in fostering reversibility of these anhydrobiotic bouts by preventing macromolecular denaturation during drying. Our current *in vitro* studies are incorporating these solute data to quantify the relative influences of inorganic and organic osmolytes on protein assembly state. The eventual goal is to clarify the importance of the internal milieu in regulating metabolic events, not only in terms of solvent interactions but also in terms of the nature and quantity of critical solutes.

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