

ANHYDROBIOSIS IN EMBRYOS OF THE BRINE SHRIMP *ARTEMIA*: CHARACTERIZATION OF METABOLIC ARREST DURING REDUCTIONS IN CELL-ASSOCIATED WATER

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SUMMARY

Upon entry into the state of anhydrobiosis, trehalose-based energy metabolism is arrested in *Artemia* embryos (cysts). We have compared changes in the levels of trehalose, glycogen, some glycolytic intermediates and adenylate nucleotides in hydrated embryos observed under conditions of aerobic development with those occurring after transfer to 5.0 mol l^{-1} NaCl. This treatment is known to reduce cell-associated water into a range previously referred to as the ametabolic domain. The trehalose utilization and glycogen synthesis that occur during development of fully hydrated cysts are both blocked during desiccation. Upon return to 0.25 mol l^{-1} NaCl both processes are resumed. Analysis of glycolytic intermediates suggests that the inhibition is localized at the trehalase, hexokinase and phosphofructokinase reactions. ATP level remains constant during the 6-h period of dehydration, as does the adenylate energy charge. An additional dehydration experiment was performed in 5.0 mol l^{-1} NaCl containing 50 mmol l^{-1} ammonium chloride (pH 9.0). The resulting level of gaseous ammonia in the medium has been shown to maintain an alkaline intracellular pH (pHi) in the embryos. The metabolic response to dehydration under these conditions was very similar to the previous dehydration series. Thus, these results are taken as strong evidence that the metabolic suppression observed during dehydration does not require cellular acidification, in contrast to the pronounced inhibitory role of low pHi during entry of hydrated embryos into the quiescent state of anaerobic dormancy. The arrest of carbohydrate metabolism seen during anhydrobiosis indeed appears to be a strict function of embryo water content.

INTRODUCTION

Organisms that are able to tolerate the removal of most or all of their cell-associated water without irreversible damage have been the focus of long-standing speculation (e.g. van Leeuwenhoek, 1702). In addition to the inherent fascination with such latent states, researchers have found anhydrobiotic organisms to be excellent

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biological systems for investigating the roles of cellular dehydration in metabolic arrest (Crowe & Clegg, 1973, 1978).

One of the best-characterized systems for such studies is the post-diapause embryo (cyst) of the brine shrimp *Artemia*, because its hydration state can be precisely controlled and because the embryo is commercially available in large quantities. In a superb series of papers (Clegg, 1974, 1976*a,b,c*, 1977; Clegg & Cavagnaro, 1976; Clegg & Lovallo, 1977) Clegg and associates elucidated many metabolic responses of brine shrimp embryos to graded levels of cellular dehydration. Of particular relevance to the present study are the observations that: (1) trehalose is the exclusive metabolic fuel used during pre-emergence development in *Artemia* embryos (Clegg, 1964); (2) carbohydrate metabolism is acutely suppressed when cellular water is reduced below $0.6 \text{ g H}_2\text{O g}^{-1}$ dry cyst (Clegg, 1976*a*); and (3) this influence on metabolism is fully reversible (Clegg, 1976*a*). While these metabolic transitions appear to be a function of water content, the controlling mechanisms involved in *Artemia* embryos, as well as in other anhydrobiotic organisms, are not understood (Clegg, 1976*a*).

In this communication we characterize more fully the biochemical events occurring during dehydration-induced arrest of carbohydrate metabolism in brine shrimp embryos. Specifically, we compared the changes in concentrations of trehalose, glycogen, glycolytic intermediates and adenylate nucleotides occurring during normal aerobic development of hydrated embryos with those occurring after transfer of embryos to concentrated solutions of NaCl. The hydration state of embryos can be controlled in this manner because the outer cuticular membrane is completely impermeable to inorganic ions, yet fully permeable to water (Conte, Droukas & Ewing, 1977; Clegg & Conte, 1980). Apart from water, only low molecular weight dissolved gases are capable of penetrating the embryo.

We also report results of similar dehydration experiments in which metabolite measurements were made on embryos after exposure to 5.0 mol l^{-1} NaCl containing ammonia. The presence of ammonia in the external medium has been shown by ^{31}P -NMR (nuclear magnetic resonance) to promote an alkaline intracellular pH (pHi) in *Artemia* cysts (Busa & Crowe, 1983). It is clear that embryo pHi declines dramatically during transitions into anaerobic dormancy (Busa, Crowe & Matson, 1982), a quiescent state brought about by anoxia. This reduction in pHi promotes suppression of various metabolic and developmental processes in these post-diapause embryos (Busa & Crowe, 1983; Carpenter & Hand, 1986; Hand & Carpenter, 1986; Utterback & Hand, 1987; Hofmann & Hand, 1987). Thus, to determine whether pHi also influences the arrest of cyst metabolism during dehydration, it was important to perform experiments in which any possible acidification of pHi was prevented.

MATERIALS AND METHODS

Dehydrated *Artemia* embryos (gastrula stage) from the Great Salt Lake in Utah were purchased from the Saunders Brine Shrimp Co. in 1984 (Ogden, UT, USA),

and stored at -20°C . Cysts were taken from the same 1.7 kg container for all experiments to avoid potential variation in quality among different commercial batches (see Clegg & Conte, 1980). Dry Wipe filters were a product of Chicopee Mills, Inc. (New Brunswick, NJ, USA). Glucose-6-phosphate dehydrogenase (Type VII), hexokinase (Type C-301), phosphoglucosomerase (Type III), lactic dehydrogenase (Type II), pyruvate kinase (Type II), myokinase (Grade III) and aldolase (Type IV) were purchased from Sigma Chemical Co. Glycerol-3-phosphate dehydrogenase and triosephosphate isomerase, both purified from rabbit muscle, were obtained from Boehringer Mannheim. All enzymes were dialysed prior to use to remove ammonium sulphate. Remaining chemicals and biochemicals were of reagent grade.

Preparation of cysts for incubations

Throughout preparatory procedures, cysts were maintained at 0°C . Dried cysts were soaked for approximately 20 min in a modified antiformin solution (Nakanishi, Iwasaki, Okigaki & Kato, 1962), containing 0.75% (w/v) active hypochlorite, 0.4 mol l^{-1} NaOH and 60 mmol l^{-1} Na_2CO_3 . Following this treatment, floating cysts were decanted and discarded. Remaining embryos were washed repeatedly in 0.25 mol l^{-1} NaCl and collected by vacuum filtration onto Dry Wipe filters. Cysts were then soaked for 10 min in 0.25 mol l^{-1} NaCl containing 10 mmol l^{-1} HCl, followed by several washings in 0.25 mol l^{-1} NaCl. Embryos were stored overnight in 0.25 mol l^{-1} NaCl at 0°C .

Incubation procedures

All incubations were performed at 23°C in 125 ml Erlenmeyer flasks containing 60 ml of the desired aqueous medium. Flasks were continuously gassed using the procedure described by Gregg (1962), as modified by Carpenter & Hand (1986). 60 min prior to the addition of cysts, flasks containing incubation medium were equilibrated with a 60% N_2 :40% O_2 gas mixture and shaken continuously on a Labline rotary shaker at $110\text{ cycles min}^{-1}$. At the start of each experiment, hydrated cysts were quickly filtered onto Dry Wipe filters, and 1.5 g samples either placed into flasks or processed immediately as time 0 samples. The gas mixture was unchanged throughout all experiments, and flasks were shaken continuously to ensure even distribution of embryos in the medium. For control incubations, cysts were immersed in 0.25 mol l^{-1} NaCl throughout the 14-h period.

For dehydration experiments, it was first necessary to select a NaCl concentration that would dehydrate embryos sufficiently to cause complete cessation of carbohydrate metabolism. Thus, embryos were pre-equilibrated overnight at 0°C to one of four NaCl concentrations (0.25, 1.0, 2.0, 5.0 mol l^{-1}). At time 0 cysts were transferred to the same media at 23°C and incubated as above for 12 h. Cysts were then processed for the determination of trehalose levels (see below), and the resulting values were compared with the trehalose levels for time 0 cysts (Fig. 1). Based on

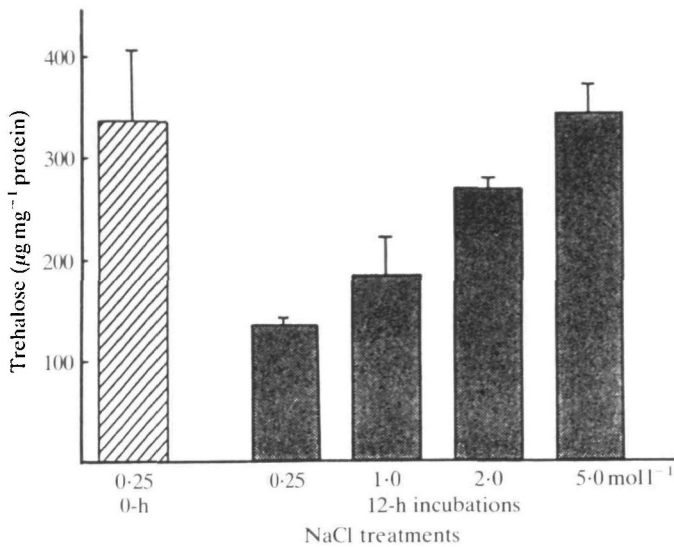


Fig. 1. Trehalose levels after 12 h of incubation in 60% N₂:40% O₂ at 23°C. All embryos were hydrated overnight at 0°C in their respective NaCl solutions prior to incubation. Histogram on the left represents time 0 controls (0.25 mol l⁻¹ NaCl, see Materials and Methods). Each vertical bar is the mean ± s.e. for three independent samples.

these data, 5.0 mol l⁻¹ NaCl was chosen for all further dehydration studies. Exposure of cysts to this concentration of NaCl has been shown to dehydrate San Francisco Bay embryos to a level of 0.23 g H₂O g⁻¹ dry cyst (compared with 1.28 g H₂O g⁻¹ dry cyst for embryos incubated in 0.25 mol l⁻¹ NaCl) (Clegg, 1974; Clegg & Cavagnaro, 1976).

To initiate dehydration experiments, embryos were incubated in 0.25 mol l⁻¹ NaCl until hour 4, when they were filtered and transferred to pre-equilibrated flasks containing 5.0 mol l⁻¹ NaCl. At hour 10, cysts were returned to 0.25 mol l⁻¹ NaCl, and the incubation continued until hour 14. This last transfer was made to determine whether the effects of dehydration on metabolism were reversible. Dehydration experiments that required simultaneous exposure to ammonia (see below) were performed identically.

Dehydration in the presence of NH₄Cl and the pK' of NH₃ in 5.0 mol l⁻¹ NaCl

In previous studies using ³¹P-NMR techniques, Busa & Crowe (1983) showed that incubating cysts in 0.16 mol l⁻¹ NaCl solution containing 40 mmol l⁻¹ NH₄Cl (buffered at pH 8.5 with 50 mmol l⁻¹ tricine) promoted an alkaline pHi (≥7.7), owing to the diffusion of ammonia gas across the cyst wall. Since, as discussed earlier, acidification of pHi in fully hydrated embryos is known to arrest carbohydrate metabolism, it would be informative to dehydrate cysts under conditions where acidification of pHi (if it were to occur) is precluded. Then, by comparing

Table 1. Determination of ammonia pK' value in 5.0 mol l^{-1} NaCl (pH 9.0, 23°C)

| $[\text{NH}_4^+]$ (mmol l^{-1}) | $[\text{NH}_3]$ (mmol l^{-1}) | Computed pK' | Mean \pm S.E. |
|---|---|-------------------|-------------------|
| 50 | 3.0 | 10.19 | 10.23 \pm 0.034 |
| 100 | 6.5 | 10.16 | |
| 150 | 7.5 | 10.28 | |
| 210 | 10.0 | 10.30 | |

with the effects of dehydration in the absence of ammonia, one could detect what role, if any, pH change plays in the metabolic arrest observed at low water activity. However, because the pK' of ammonia changes markedly with ionic strength (see Cameron & Heisler, 1983), it was first necessary to estimate the ammonia pK' in 5.0 mol l^{-1} NaCl in order to maintain NH_3 levels in our incubation medium comparable to those used by Busa & Crowe (1983).

Free ammonia concentration was measured at 23°C using a Corning 476130 ammonia combination electrode coupled to a Radiometer model 84 pH/volt meter. Two standard curves were prepared using known concentrations of NH_4Cl (one series prepared in 0.25 mol l^{-1} NaCl, $r = 0.999$; one in 5.0 mol l^{-1} NaCl, $r = 0.964$). These solutions were titrated to pH 12 immediately before measurement. Next, ammonia concentrations were determined for several NH_4Cl solutions prepared in 4.95 mol l^{-1} NaCl and buffered at pH 9.0 with 50 mmol l^{-1} tricine. These values and the resulting ammonia pK' , calculated from the Henderson-Hasselbach equation, are given in Table 1. Measurements indicated that a solution of 50 mmol l^{-1} NH_4Cl , 4.95 mol l^{-1} NaCl and 50 mmol l^{-1} tricine at pH 9.0 is equivalent in NH_3 concentration (3.0 mmol l^{-1}) to the solution of 40 mmol l^{-1} NH_4Cl , 0.16 mol l^{-1} NaCl and 50 mmol l^{-1} tricine at pH 8.5 used by Busa & Crowe (1983). Thus, the former solution was adopted for our studies. The small and reversed influence (relative to most other gases) of ionic strength on ammonia activity (Cameron, 1986) was not taken into account.

Finally, since our protocol called for a 6-h exposure to ammonia during the dehydration period, it was necessary to replenish the ammonia lost to the atmosphere during this time. Under our experimental conditions, the total ammonia pool ($\text{NH}_3 + \text{NH}_4^+$) dropped by 10% in 2 h (measured as per Chaney & Marbach, 1962). Thus, NH_4Cl was added at 2-h intervals throughout the dehydration period to restore the starting concentration.

Processing of cysts for biochemical analyses

At 2, 4, 6, 8, 10, 12 and 14 h during all incubations, triplicate flasks were removed from the gassing apparatus and plunged immediately into 0°C ice/water baths. Unfortunately, at hour 8 of the dehydration study in the presence of NH_4Cl , one of the flasks was broken. Thus, only two values are available for this sample time. Cysts were immediately taken to a 4°C cold-room where they were collected on Dry Wipe filters, transferred to precooled mortars, and ground to a fine powder under liquid

nitrogen. The powder was homogenized in 5 ml of 6% perchloric acid, and the extract was further processed and stored as described by Carpenter & Hand (1986).

Biochemical analyses

Ethanol-fractionated samples were analysed for trehalose and glycogen (see Carpenter & Hand, 1986). The remaining samples (those not fractionated with ethanol) were thawed and Millipore filtered (0.45 μm pore size). Fluorometric analyses were employed for determination of adenylate nucleotides and glycolytic intermediates using the NADH- and NADPH-linked enzymatic assays described by Lowry & Passoneau (1972), with certain modifications. ATP and glucose were determined by the addition of 0.28 units of hexokinase to a reaction mixture containing 0.06 units of glucose-6-phosphate dehydrogenase, 1 mmol l^{-1} MgCl_2 , 0.2 mmol l^{-1} dithiothreitol, 0.05 mmol l^{-1} NADP^+ in a 50 mmol l^{-1} Tris-HCl buffer (pH 8.1). For ATP determinations, the reaction mixture also contained 0.1 mmol l^{-1} glucose, and for glucose assays 0.3 mmol l^{-1} ATP was added. Glucose-6-phosphate and fructose-6-phosphate were measured by the successive additions of 0.06 units of glucose-6-phosphate dehydrogenase and 0.38 units of phosphoglucosomerase to a reaction mixture containing 0.5 mmol l^{-1} dithiothreitol, 0.05 mmol l^{-1} NADP^+ in a 50 mmol l^{-1} Tris-HCl buffer (pH 8.1). Dihydroxyacetone phosphate (DHAP), glyceraldehyde-3-phosphate and fructose-1,6-diphosphate were determined by the sequential additions of 4.0 units of glycerol-3-phosphate dehydrogenase, 0.31 units of triosephosphate isomerase and 0.02 units of aldolase to a reaction mixture containing 3 mmol l^{-1} dithiothreitol, 7.5 $\mu\text{mol l}^{-1}$ NADH, in a 50 mmol l^{-1} imidazole-HCl buffer (pH 7.0). Finally, ADP and AMP were assayed by the consecutive additions of 4.5 units of pyruvate kinase and 1.4 units of myokinase to a reaction mixture containing 2 mmol l^{-1} MgCl_2 , 75 mmol l^{-1} KCl, 5 $\mu\text{mol l}^{-1}$ ATP, 13 $\mu\text{mol l}^{-1}$ NADH, 40 $\mu\text{mol l}^{-1}$ phosphoenolpyruvate and 1.6 units of lactate dehydrogenase in a 50 mmol l^{-1} imidazole buffer (pH 7.1).

Hatching experiments

To determine the viability of cysts after exposure to the experimental conditions of dehydration (with and without ammonia), embryos were subjected to the same 14-h protocols and then allowed to develop for an additional 40 h. The number of free-swimming nauplius larvae that had hatched was then counted under a dissecting microscope (after fixation with ethanol). It was especially important to determine if cysts exposed to ammonia accrued debilitating or lethal effects. Although little information is available concerning ammonia toxicity in invertebrates, blood concentrations as low as 30 $\mu\text{mol l}^{-1}$ can be lethal in rabbits (Eckert & Randall, 1983). Our results showed that the ammonia treatment did not compromise cyst viability; the hatching percentage for cysts dehydrated in the presence of NH_4Cl was $57.3 \pm 6.7\%$ (S.D., $N=3$), and for cysts dehydrated without NH_4Cl the value was $61.1 \pm 1.8\%$ (S.D., $N=3$). These values are virtually identical to percentages previously reported for normally hydrated embryos from the Great Salt Lake

population (Conte *et al.* 1977; Peterson, Ewing & Conte, 1978; Warner, MacRae & Wahba, 1979) as well as the San Francisco Bay population (Hand & Conte, 1982).

RESULTS

Fully hydrated development

Under aerobic conditions (60% N₂:40% O₂) in 0.25 mol l⁻¹ NaCl, there was a time-dependent mobilization by control cysts of trehalose stores beginning at time 0 and continuing throughout the 14-h experiment. Levels of the carbohydrate fell approximately 25% in the first 6 h, and then declined at an increased rate, reaching 75% utilization by hour 14 (Fig. 2). Concomitant with this decline in trehalose reserves was a four-fold increase in glycogen level, which showed a rapid surge beginning at hour 6 (Fig. 3). These shifts in storage carbohydrate pools are consistent with results of earlier studies of pre-emergence development in *Artemia* (Clegg, 1964; Ewing & Clegg, 1969; Carpenter & Hand, 1986). Concentrations of glucose and glucose-6-phosphate (Figs 4, 5) also showed patterns similar to those previously reported under these conditions (Carpenter & Hand, 1986). Levels of fructose-6-phosphate and fructose-1,6-diphosphate did not change significantly throughout the 14-h period. Glyceraldehyde-3-phosphate and DHAP concentrations declined up to hour 8, when they stabilized at about 15% and 25% of time zero levels, respectively, for the duration of the experiment (data not shown).

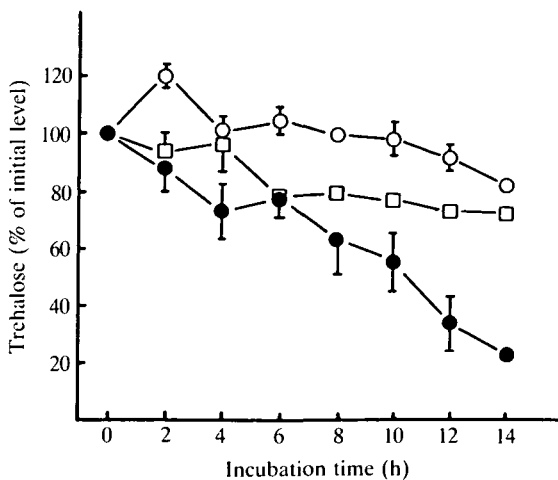


Fig. 2. Changes in the concentration of trehalose during 14-h incubations of *Artemia* embryos. Control cysts (●) were maintained in 0.25 mol l⁻¹ NaCl for the entire period. Dehydration in the absence or presence of ammonia was initiated at hour 4 by transferring cysts to either 5.0 mol l⁻¹ NaCl (○) or 5.0 mol l⁻¹ NaCl containing 50 mmol l⁻¹ NH₄Cl (□). In both of these incubations, embryos were returned to control (0.25 mol l⁻¹ NaCl) medium at hour 10, where they remained for the duration of the experiment. Each point is the mean \pm S.E. for three independent samples. Where vertical bars are absent, the S.E. is less than the size of the symbol.

ATP concentrations increased rather steadily up to hour 8, after which they remained relatively stable at an elevated level until hour 14 (Fig. 6A). AMP levels, apart from a slight upturn at hour 2, gradually fell throughout the incubation to 45% of hour 0 concentration (Fig. 6B). ADP concentrations fell 35% until hour 8, then remained stable for the duration of the experiment. These shifts in the adenylate nucleotide pool are reflected in an increase in adenylate energy charge (AEC)

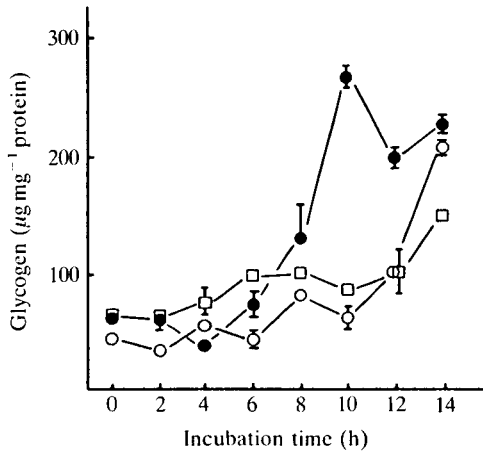


Fig. 3. Changes in the concentration of glycogen during 14-h incubations of *Artemia* embryos. Details for hydrated control (●), dehydrated (○) and dehydrated/ NH_4Cl (□) incubations are given in the legend to Fig. 2. Each point is the mean \pm s.e. for three independent samples. Where vertical bars are absent, the s.e. is less than the size of the symbol.

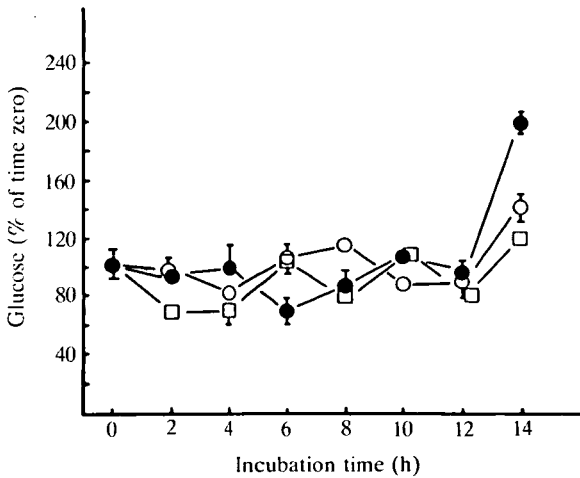


Fig. 4. Changes in the concentration of glucose during 14-h incubations of *Artemia* embryos. Details for hydrated control (●), dehydrated (○) and dehydrated/ NH_4Cl (□) incubations are given in the legend to Fig. 2. Each point is the mean \pm s.e. for three independent samples. Where vertical bars are absent, the s.e. is less than the size of the symbol.

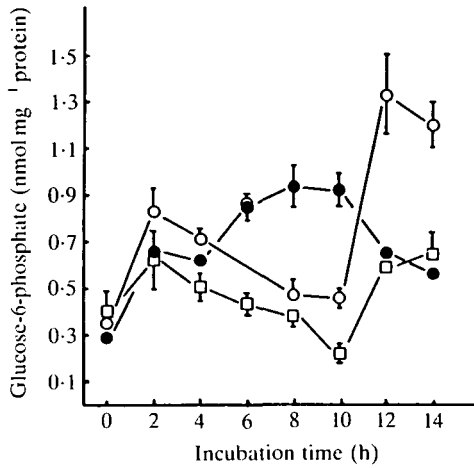


Fig. 5. Alterations in the levels of glucose-6-phosphate during 14-h incubations. Details for hydrated control (●), dehydrated (○) and dehydrated/ NH_4Cl (□) incubations are given in the legend to Fig. 2. Each point is the mean \pm s.e. for three independent samples. Where vertical bars are absent, the s.e. is less than the size of the symbol.

(Atkinson, 1977) from 0.49 to 0.72 during the 14-h period (Fig. 7). These results closely parallel those obtained by Carpenter & Hand (1986) for cysts incubated aerobically in 0.25 mol l^{-1} NaCl.

Dehydration in 5.0 mol l^{-1} NaCl

In contrast to the trends observed under control conditions, trehalose and glycogen levels did not change significantly during the 6-h period (hours 4–10) of dehydration (Figs 2, 3). These findings indicate that the mobilization of trehalose as well as the synthesis of glycogen are inhibited at this level of dehydration (see Clegg, 1976a). Upon return to control incubation (0.25 mol l^{-1} NaCl), trehalose levels began to decline again, and glycogen concentrations increased sharply. Thus, the blockage of both processes was reversed by rehydration.

The concentration of glucose remained virtually unchanged between hours 4 and 10 (Fig. 4). However, glucose-6-phosphate levels fell 34% within this same time interval, but rebounded rapidly when cysts were returned to control medium, actually overshooting hour 4 levels (Fig. 5). Fructose-6-phosphate concentrations likewise fell during the dehydration period, and returned to hour 4 levels after return to 0.25 mol l^{-1} NaCl medium. Fructose-1,6-diphosphate levels decreased by 60% during dehydration, and showed a 35% increase upon return to control conditions. Concentrations of glyceraldehyde-3-phosphate and DHAP did not change appreciably during dehydration (data not shown).

The concentration of ATP was constant over the 6 h of desiccation and then increased to near control levels upon rehydration (Fig. 6A). AMP level did not change between hours 4 and 14 (Fig. 6B), and our measurements of ADP showed no displacement from control levels at hours 4, 10 and 14 (data not shown). As a result

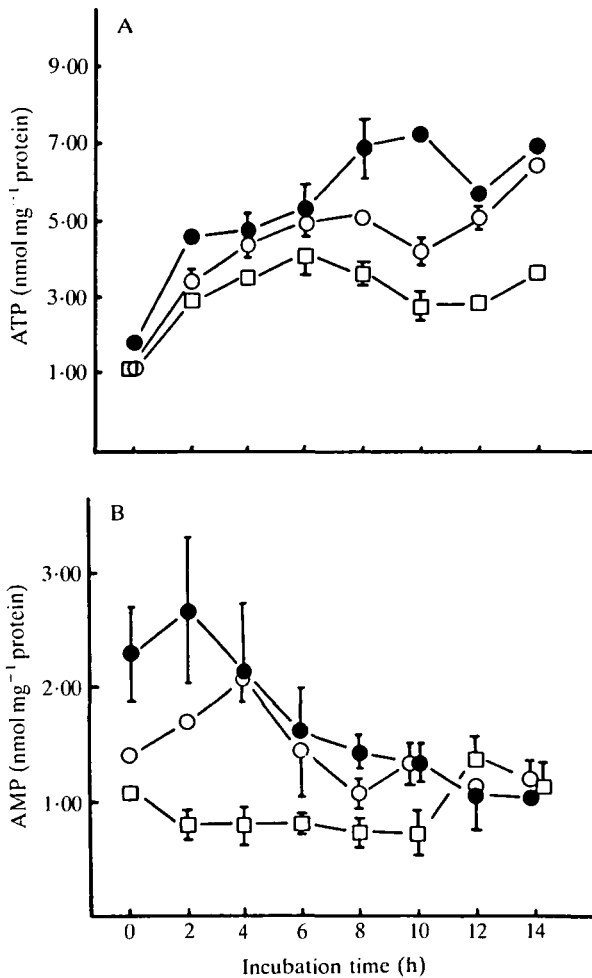


Fig. 6. Changes in the concentrations of ATP (A) and AMP (B) during 14-h incubations of *Artemia* embryos. Details for hydrated control (●), dehydrated (○) and dehydrated/ NH_4Cl (□) incubations are given in the legend to Fig. 2. Each point is the mean \pm S.E. for three independent samples. Where vertical bars are absent, the S.E. is less than the size of the symbol.

of the steady adenylate nucleotide pools during dehydration, energy charge remained relatively constant during dehydration (0.58 at hour 4, 0.61 at hour 10) (Fig. 7). These findings are in marked contrast to the changes in adenylate pools observed during anaerobiosis in *Artemia* cysts (Carpenter & Hand, 1986), where shifts in the adenylate nucleotides resulted in a drop in AEC from 0.72 to 0.42 (Fig. 7, triangles). Interestingly, in this same study, AEC remained constant when cysts were subjected to artificially induced aerobic acidosis (see Discussion).

Trehalase, hexokinase (HK) and phosphofructokinase (PFK) catalyse non-equilibrium reactions in *Artemia* embryos under conditions of aerobic development,

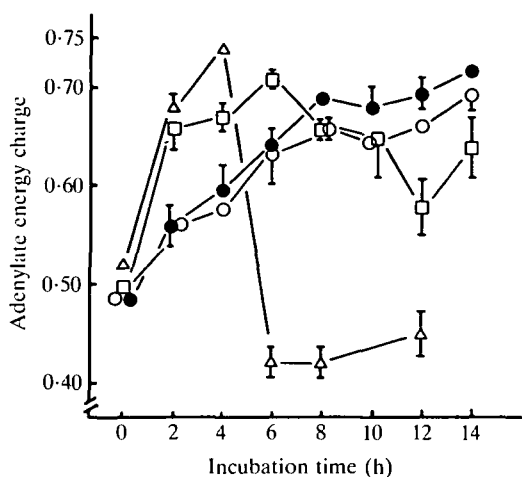


Fig. 7. Alterations in the adenylate energy charge (AEC) during 14-h incubations of *Artemia* embryos. Details for hydrated control (●), dehydrated (○) and dehydrated/ NH_4Cl (□) incubations are given in the legend to Fig. 2. Triangles represent AEC values for anaerobic incubation (after Carpenter & Hand, 1986). Each point is the mean \pm S.E. for three independent samples. Where vertical bars are absent, the S.E. is less than the size of the symbol.

Table 2. Mass action ratios (MAR) compared with equilibrium constants (K_{eq}) for the reactions catalysed by trehalase, hexokinase and phosphofructokinase

| Incubation | Trehalase ($K_{eq} = 1682$) | | Hexokinase ($K_{eq} = 3900$) | | Phosphofructokinase ($K_{eq} = 910$) | |
|---------------------------------------|----------------------------------|-----------------------|-----------------------------------|-----------------------|---|-----------------------|
| | MAR | MAR/ K_{eq} | MAR | MAR/ K_{eq} | MAR | MAR/ K_{eq} |
| Control | 2.85×10^{-3} | 1.70×10^{-6} | 2.83×10^{-1} | 7.25×10^{-5} | 2.96×10^{-1} | 3.25×10^{-4} |
| Dehydrated | 6.56×10^{-3} | 3.90×10^{-6} | 1.18×10^{-1} | 3.02×10^{-5} | 8.19×10^{-1} | 9.00×10^{-4} |
| Dehydrated/ NH_4Cl | 3.03×10^{-3} | 1.81×10^{-6} | 1.05×10^{-1} | 2.69×10^{-5} | 3.47 | 3.80×10^{-3} |

Mass action ratios are computed from metabolite levels observed experimentally.

K_{eq} values (using $\Delta G^\circ = -RT \ln K_{eq}$) were obtained from Cabib & Leloir (1958) for the trehalase reaction, and from Krebs & Kornberg (1957) for the hexokinase and phosphofructokinase reactions.

Values shown are for the hour 10 samples.

anaerobic dormancy and aerobic acidosis (Carpenter & Hand, 1986). Based on metabolite levels in the present study, we can extend this observation to the transition between hydrated and dehydrated states. Specifically, the quotient of the mass action ratio (MAR) divided by the equilibrium constant (K_{eq}) is far less than 0.05 in all cases (Table 2). It has been argued that, when an enzyme-catalysed reaction is displaced far from equilibrium ($\text{MAR}/K_{eq} < 0.05$), the step is more likely to have a regulatory role (e.g. Rolleston, 1972; Crabtree & Newsholme, 1985). In

this study, the trehalase reaction exhibited the most extreme displacement from equilibrium, with MAR/K_{eq} ratios between 1.7×10^{-6} and 3.9×10^{-6} .

Based on the blockage of trehalose mobilization and the constant glucose levels observed under anhydrobiotic conditions, our findings suggest that trehalase is inhibited during dehydration (see Discussion). Analysis of levels of other glycolytic intermediates also indicates inhibition at the HK and PFK reactions, as shown by the occurrence of negative crossover points. The crossover theorem (Chance, Holmes, Higgins & Connelly, 1958) predicts a decrease in the ratio of product to substrate for a reaction experiencing some form of inhibition. In dehydrated *Artemia* cysts, the glucose-6-phosphate/glucose ratio fell from 0.27 at hour 4 to 0.17 at hour 10, and upon restoration of hydrated conditions it returned to 0.27 at hour 14. At the PFK step, a decline in the fructose-1,6-diphosphate/fructose-6-phosphate ratio from 1.20 at hour 4 to 0.49 at hour 10 was noted. In the case of PFK, 4 h of rehydration raised this ratio to 0.55.

Dehydration in the presence of NH_4Cl

When cysts were challenged with dehydration in 5.0 mol l^{-1} NaCl containing 50 mmol l^{-1} NH_4Cl at pH 9.0 (conditions fostering an alkaline pHi), the results closely paralleled those observed in 5.0 mol l^{-1} NaCl alone. Trehalose mobilization, after an initial 2 h lag, was effectively shut down (Fig. 2). Glycogen synthesis was similarly suppressed and then resumed after the return to control conditions (Fig. 3). Glucose levels remained essentially constant during dehydration, whereas glucose-6-phosphate concentration fell nearly 60% (Figs 4, 5). Thus, once again, inhibition was indicated at the trehalase and hexokinase reactions. The glucose-6-phosphate/glucose ratio decreased from 0.28 to 0.08 between hours 4 and 10. The ratio returned to 0.21 by hour 14. Between hours 4 and 8, both fructose-6-phosphate and fructose-1,6-diphosphate levels fell. However, owing to a rise in fructose-1,6-diphosphate concentration between hours 8 and 10, the negative crossover point previously exhibited in the 5.0 mol l^{-1} NaCl treatment (without ammonia) was not observed here for PFK. [If the hour 8 value (0.54) is compared with the hour 4 value (1.43) a negative crossover point is observed.] Upon rehydration at hour 10, concentrations of both fructose-6-phosphate and fructose-1,6-diphosphate increased in a fashion reminiscent of that seen in simple 5.0 mol l^{-1} NaCl incubations. Significant changes were not observed in levels of glyceraldehyde-3-phosphate and DHAP (data not shown).

Finally, the concentrations of AMP and ADP did not change significantly between hours 4 and 10, although ATP levels fell 23% (Fig. 6A,B). The resulting change in adenylate energy charge was slight (0.67 to 0.65, Fig. 7). Once again, we observed a relatively high AEC maintained during the 6 h of dehydration (compared with AEC for anaerobically incubated cysts; Carpenter & Hand, 1986; Fig. 7, triangles). Taken together, the dehydration experiments (with and without ammonia) indicated that acidification of pHi was not required to achieve metabolic arrest during anhydrobiosis.

DISCUSSION

Results of the present study extend our appreciation of the role of cellular dehydration in metabolic arrest in several ways. In addition to confirming that induction of anhydrobiosis in *Artemia* cysts is occasioned by a reversible blockage in the mobilization of trehalose, the analyses of glycolytic intermediates indicate that this suppression of carbohydrate catabolism is focused at specific reactions, or a sequence of reactions, in the pathway. Thus, restriction of cellular water may preferentially influence the catalytic performance of some enzymes more than others. The results of the dehydration experiment in the presence of ammonia strongly suggest that the metabolic arrest at low water activities is not a result of a depression of pH_i. In contrast to the situation during anaerobic dormancy (see below), measurements of adenylate nucleotides revealed that adenylate energy charge remained high at least for the short-term dehydration period tested here. Apparently, dehydration imposes an acute blockage of both synthetic (energy-requiring) and catabolic (energy-producing) processes. Consistent with these findings, our 5.0 mol l⁻¹ NaCl desiccation treatment is known to reduce cellular water into a range termed the ametabolic domain (Clegg, 1981).

When the metabolite measurements are interpreted in the context of crossover point theory (Chance *et al.* 1958; Rolleston, 1972; Crabtree & Newsholme, 1985), inhibition of trehalase and hexokinase is indicated during both dehydration experiments (with and without ammonia). A strong negative crossover point at the HK reaction is clear. Yet levels of glucose (the product of the trehalase reaction) did not rise appreciably during the entire period of dehydration, as might be expected considering the blockage of the HK step. Consequently, the observations of a constant glucose level and the acute arrest of trehalose hydrolysis strongly support simultaneous inhibition of trehalase. In addition, both dehydration treatments resulted in blockage of the PFK reaction until hour 8. (Owing to an unexplained rise in fructose-1,6-diphosphate level between hours 8 and 10 of the experiment with NH₄Cl, the negative crossover point was not observed for that time interval.) This approach to experimental analysis of metabolic control (Crabtree & Newsholme, 1985), which includes the identification of non-equilibrium reactions, the flux-generating step and the external regulators and their roles, has proved useful in our previous studies (see Carpenter & Hand, 1986; Hand & Carpenter, 1986). Certainly, other quantitative approaches exist, involving, for example, the use of control coefficients (Kacser & Burns, 1973; Rapoport, Heinrich, Jacobasch & Rapoport, 1974). The relative merits of the above methods have been thoroughly debated elsewhere (Crabtree & Newsholme, 1987; Kacser & Porteous, 1987). However, neither of these concepts of metabolic control incorporates the influence of compartmentation and enzyme-cytoskeletal interaction (e.g. Masters, 1985; see below).

The dehydration-induced shutdown of carbohydrate catabolism can be highly adaptive in *Artemia* cysts (which undergo severe bouts of desiccation in nature), as it certainly represents a means of conserving vital energy stores during dormancy.

Other environmental insults (e.g. anoxia) have been shown to foster a similar reversible interruption of carbohydrate utilization (Ewing & Clegg, 1969; Carpenter & Hand, 1986). As discussed previously, depression of pHi is a primary effector of the latter type of metabolic arrest. Thus, one of the primary goals in the present study was to determine if pHi played any role in the hydrated–dehydrated transitions in *Artemia* cysts, or if water content itself was the primary regulator of carbohydrate catabolism. We observed that the arrest of carbohydrate metabolism during dehydration in the absence of ammonia was essentially identical to that observed during dehydration with ammonia (conditions in which pHi was certainly alkaline). Consequently, it is highly unlikely that acidification of pHi has any role in the dehydration-induced metabolic shutdown. Likewise, Drinkwater & Crowe (1987) have shown that diapause (endogenously controlled dormancy) is not imposed by acidic pHi in *Artemia* embryos.

Another interesting result from the present study is that adenylate energy charge did not change significantly during either of the 5.0 mol l^{-1} NaCl dehydration treatments, and AEC was maintained at a relatively high level throughout the 6-h period of desiccation. Thus, we conclude that changes in AEC are not necessary to promote the initial shutdown of carbohydrate metabolism in desiccated *Artemia* embryos. This observation has a parallel in the plant kingdom. In studies of the desiccation-tolerant moss, *Tortula ruralis*, it was noted that ATP levels remained high in rapidly dried specimens (Bewley & Pacey, 1978; Bewley & Gwozdz, 1975). Those workers concluded that a rapid dehydration resulted in a simultaneous shutdown of both energy-producing and energy-consuming pathways. This may well be the case in *Artemia* and deserves further investigation.

The AEC pattern seen during short-term dehydration of brine shrimp cysts contrasts with the acute decline seen in embryos during entry into anaerobic dormancy (Carpenter & Hand, 1986). However, the artificially induced quiescent state of aerobic acidosis (achieved by rapidly acidifying cysts with exogenous CO_2) also results in depression of metabolism without a drop in AEC, similar to the effect of short-term dehydration (Carpenter & Hand, 1986). It should be noted that ATP levels do fall slowly in cysts maintained at a hydration level of $0.18 \text{ g H}_2\text{O g}^{-1}$ dry cyst (in the vapour phase) for long periods (48–168 h) (Clegg & Cavagnaro, 1976). However, there is no direct evidence that this decline is the result of metabolic (enzymatic) activity (Clegg & Cavagnaro, 1976).

Our results clearly support the suggestion that the arrest of carbohydrate metabolism during anhydrobiosis is a strict function of water content (see Clegg, 1981). However, because there are several viable mechanisms by which alterations in embryo water content could promote metabolic suppression, it is appropriate to consider the relationship of water to cellular metabolic control. Currently, the notion that enzymes function in an aqueous cytoplasm, at the mercy of simple diffusion of substrates, is being sharply contested. Evidence suggests that metabolite transfer, at least for segments of metabolic pathways, may take place *via* direct enzyme–enzyme transfer without involving the aqueous cytoplasm to an appreciable extent (Welch,

1977; Srivastava & Bernhard, 1986). Furthermore, these functional proteins (including glycolytic enzymes) formerly thought to be 'soluble' have been shown to exist in complexes, or metabolons (Srere, 1985), intimately associated with the cell cytoskeleton. Considerable evidence has been amassed for the segregation of cellular water into at least two phases – a bulk compartment and a restricted, macromolecularly-bound phase – each exhibiting distinctly different physical properties (for a review see Clegg, 1984). Certainly, water associated with metabolons belongs in the 'bound' category.

The above interpretation of cellular water and metabolic organization is consistent with the observation that metabolic processes in *Artemia* cysts are unaffected by reducing hydration levels from 1.4 to 0.65 g H₂O g⁻¹ dry cyst (Clegg, 1981). Nuclear magnetic resonance and microwave dielectric studies suggest that, across this range of embryo water contents, only bulk water is being removed (Seitz *et al.* 1981; Clegg *et al.* 1982). Below the hydration level of 0.65 g H₂O g⁻¹ dry cyst metabolic interference commences and bound water is perturbed. We suggest that these conditions may promote an attendant disruption/alteration of metabolon organization and other protein-protein associations, including polymerization reactions (see Clegg, 1984). These interactions are highly dependent on water structure, ionic strength, ligand concentration, etc. The simultaneous inhibition of trehalase and hexokinase suggested by our metabolite analyses in this study could easily be explained by a dehydration-induced disruption of an enzyme-enzyme association (metabolon). It is also appropriate to mention in this context that the catalytic performance of trehalase from *Artemia* cysts is markedly influenced by alteration of macromolecular assembly state (Hand & Carpenter, 1986).

In conclusion, the reversible arrest of carbohydrate catabolism during dehydration of *Artemia* embryos appears to be a strict function of cellular water content, and acidification of pH_i is not required to promote the metabolic depression. Interpretation of metabolite levels indicates that the inhibition can be explained by simultaneous inhibition of the trehalase and hexokinase reactions. Phosphofructokinase may also be inhibited, but the data are less conclusive. The maintenance of high adenylate energy charge in cysts dehydrated for 6 h suggests a water-mediated shutdown of both the energy-consuming biosynthetic machinery and the energy-producing catabolic pathways. The reversibility of this arrest implies that the macromolecular integrity is not compromised. The fact that trehalose is the major metabolic fuel in *Artemia* embryos (up to 17% of dry mass) is of great significance (Clegg, 1964). Overwhelming evidence now suggests that trehalose serves as a natural stabilizer of biological membranes and proteins against damage incurred during dehydration (Crowe, Crowe, Carpenter & Wistrom, 1987), thus supporting a dual role for this disaccharide. Finally, the molecular mechanism of the arrest may well be explained by perturbation of vicinal water, which results in alterations in metabolon organization and protein polymerization states. Experiments are under way to test this hypothesis. Unquestionably, a greater understanding of the regulatory events involved in tolerance to desiccation will require appreciation of the intricate dynamics of water-protein as well as water-cytoskeletal interactions.

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