INTEGR. COMP. BIOL., 45:715–724 (2005)

Desiccation Tolerance in Encysted Embryos of the Animal Extremophile, *Artemia*[1]

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SYNOPSIS. Encysted embryos (cysts) of the primitive crustacean, *Artemia franciscana,* are among the most resistant of all animal life history stages to extremes of environmental stress. These embryos, extremophiles of the animal kingdom, are the main focus of this paper. Previous work has revealed the importance of biochemical and biophysical adaptations that provide a significant part of the basis of their resistance, and I consider some of these here. In the present paper the critical role played by the outer layer of the shell in desiccation tolerance will be one focus. Another involves studies on the response of dried cysts to high temperatures that, among other things, implicate one or more volatile factors released from the cysts that determines the extent of thermotolerance under a given heating regime. A hypothetical scheme is given to account for these peculiar results. Based on western immunoblotting analysis, and data from the literature, the scheme also implicates the heat-induced translocation of the stress protein p26 to nuclei as a potential cause of the reduction in hatching level.

INTRODUCTION

The word extremophile is usually applied to certain microbes; however, I see no reason why it cannot also apply to plant and animal taxa (Clegg and Trotman, 2002). The brine shrimp *Artemia* certainly qualifies in the animal category: the motile stages are arguably the best of all animal osmoregulators, enabling success in severely hypersaline environments, while the encysted embryos, or cysts, appear to be without equal in the animal kingdom when it comes to tolerating high doses of UV and ionizing radiation, surviving years of continuous anoxia while hydrated at physiological temperature, thermal extremes and desiccation-hydration cycles (reviewed by Clegg and Conte, 1980; MacRae *et al.*, 1989; Warner *et al.*, 1989; Liang and MacRae, 1999; Clegg, 2001; Clegg and Trotman, 2002). What emerges from the considerable amount of study on these cysts is their uniqueness as a model system for the study of resistance to severe stress by animals cells, including desiccation so severe that even measuring the amount of water retained becomes a difficult problem. A goal of the present paper is to emphasize (even promote) the usefulness of this model organism.

Among the biochemical adaptations involved in various ways with cyst stress resistance, the best studied are trehalose, the non-reducing disaccharide of glucose (see Crowe *et al.*, 1992, 1998), a large and unusual guanine nucleotide pool (Warner and Clegg, 2001) and two small heat shock (stress) proteins called p26 and artemin (for recent coverage see Willsie and Clegg, 2002; Chen *et al.*, 2003; Collins and Clegg, 2004; Qiu *et al.*, 2004; Warner *et al.*, 2004). Trehalose is critical to desiccation tolerance in these cysts (Clegg, 1986) and many studies from the laboratory of John and Lois Crowe have described the general mechanisms of its protection (see Crowe *et al.*, 1992, 1998, 2002, 2005; Wolkers *et al.*, 2002). Interestingly, based on studies *in vitro,* trehalose appears to work synergistically with the small heat shock protein and molecular chaperone, p26 (Viner and Clegg, 2001), a protein of some importance to results presented in this paper. Although animals able to undergo anhydrobiosis usually contain substantial concentrations of trehalose, and that is certainly true of *Artemia* cysts, there is apparently an interesting exception to the requirement for this sugar in animal anhydrobionts (Lapinski and Tunnacliffe, 2003) and more on this topic has emerged during the symposium (see the paper by Goyal *et al.*, 2005).

The tough shell of the cyst plays an important role in protecting the inner embryo from mechanical damage (Clegg and Conte, 1980) and more recent evidence demonstrates that the outer shell layer, known as the tertiary envelope, protects the embryo from intense UV radiation (Tanguay *et al.*, 2004), a stress that cysts normally encounter in nature. Some new results on the importance of this shell layer in determining the rate of water loss, as well as providing protection against mechanical damage, will be presented. A major part of the paper will consider some curious results on the thermotolerance of dried cysts that implicate at least one volatile substance that profoundly influences the heat resistance of dried cysts, based on their ability to hatch following heating exposure. Finally, the possible involvements of p26, artemin and hsp70 in the thermotolerance of dried cysts have been studied, but with little insight into the details of their participation. Strangely, there is reason to believe that one of these stress proteins, p26, might even play a negative role during the heating of dried cysts under certain conditions.

MATERIALS AND METHODS

Source of Artemia *encysted embryos (cysts) and their decapsulation*

Artemia franciscana cysts from salterns in the San Francisco Bay (SFB) were purchased from San Fran-

[1] From the Symposium *Drying Without Dying: The Comparative Mechanisms and Evolution of Desiccation Tolerance in Animals, Microbes, and Plants* presented at the Annual Meeting of the Society for Integrative and Comparative Biology, 4–8 January 2005, at San Diego, California.

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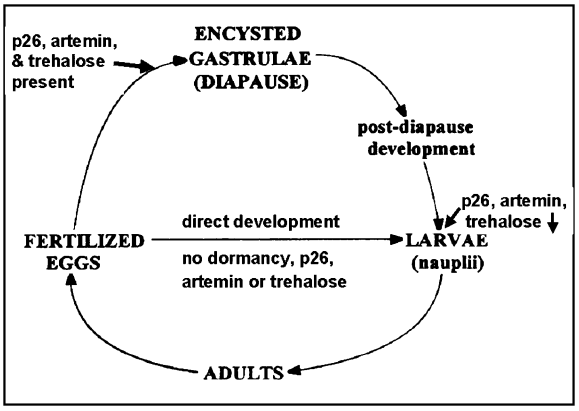

FIG. 1. The life cycle of *Artemia franciscana*. For a given clutch of embryos one or the other developmental path is taken. Modified from Clegg *et al.*, 1999.

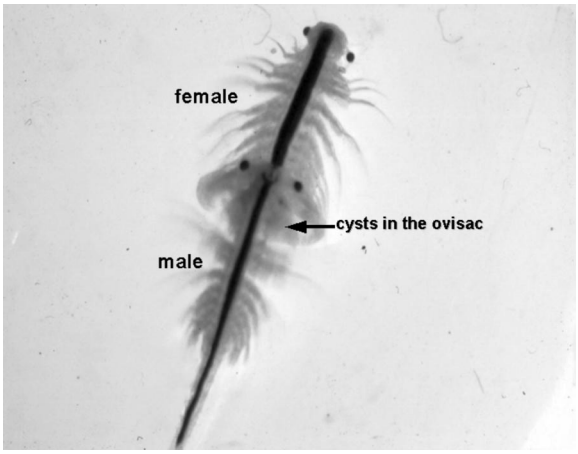

FIG. 2. An adult pair of *Artemia franciscana*. The male clasps the female by modified appendages. Cysts contained within the female's ventral ovisac are just visible, indicated by the arrow. Modified from Tanguay *et al.*, 2004.

cisco Bay Brand, Hayward, California, in 1984 and were stored at about −10°C under 100% N_2. Embryos, still under nitrogen gas, were placed at room temperature for 5 days before use, and had a final hatching percentage close to 90% when incubated in seawater (SW) at room temperature, in the light, for 48 hours. For stress protein analysis the dried embryos were first hydrated overnight in ice cold SW to suppress metabolism before further study. In some cases the outermost layer of the shell, the tertiary envelope (t.e. in Fig. 3 of this paper) was removed by decapsulation (Nakanishi *et al.*, 1962) prior to dehydration studies. That procedure is described in detail in a previous paper (Tanguay *et al.*, 2004). Decapsulated embryos were used immediately or stored at 2°C in 0.5 *M* NaCl overnight and then used.

Hatching assays

Groups of cysts were placed into 20-well plastic depression plates, each well usually containing 10–20 cysts in 300 μl of 0.2 mm-filtered seawater. The plates were covered, sealed with tape on the sides to prevent evaporation, and incubated in constant light at 21–24°C until hatching was complete. Groups of at least 100 cysts were routinely examined for hatching, each group being taken as an independent observation. Thus, an n of 3 represents the averages of three groups of at least 100 cysts each.

Dehydration of intact and decapsulated cysts

These methods have been published in detail (Clegg, 1978). Briefly, hydrated but blotted cysts were added essentially as a monolayer to tared aluminum foil cups and quickly weighed. Dehydration was carried out at 23°C and 66% relative humidity, conditions that prevailed in the open air of the laboratory. After drying under these conditions to steady state, the cups were heated at 103°C for 16–18 hr, then weighed quickly to estimate ''residual water'' in the cysts by weight loss. Intact cysts contained residual water contents, as defined above, of 0.08 g H_2O/g dried weight ± 0.01 SE (n = 5); decapsulated cysts contained slightly lower levels. The term ''residual water'' is ambiguous because some of the weight loss under these conditions could be due to the release of non-aqueous volatile cyst components. Thus, the value given above represents a maximum in terms of water content. Residual water is of relevance to current ideas about the mechanistic basis for cellular desiccation tolerance, notably the *water replacement hypothesis* to be considered briefly at the end of this paper.

Heating of dried cysts

Known weights of air-dried cysts were placed in microfuge tubes, usually of the ''secure lock'' type, of

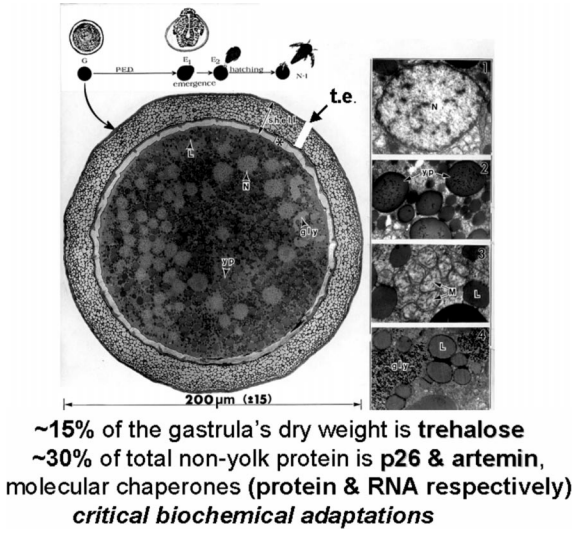

FIG. 3. Ultrastructure of an encysted embryo of *A. franciscana*. The panels to the right illustrate major organelles at higher magnification. L (lipid droplets), N (nuclei), yp (yolk platelets), gly (glycogen), M (mitochondria), asterisk (inner embryonic cuticle). The sequence at the top depicts the emergence of the embryo from the shell (E_1) and its subsequent development to the swimming nauplius (N-1). Modified from Clegg and Trotman, 2002.

capacity 1.75 ml when capped (measured). In some cases the tubes were first heated at 95°C for 22 and 40 hr and then cooled before adding cysts to evaluate the potential presence of volatile compounds in the plastic that might influence thermotolerance. Heating was carried out in an Eppendorf Thermomixer capable of swirling the tubes, which fit snugly in the heating block, at rates up to 1,400 rpm. The heater was either pre-set to a given temperature and the tubes then added directly, or the tubes were placed in the heating block at 25°C and the temperature increased at a rate of 5.5°C/min to the desired temperature which, in the present study, is 80°C. The basic method was the former, with the caps closed and without swirling. Variations on that procedure will be given with the results. In all cases hatching assays were carried out the same as for controls, within 3 hr after heating. However, as will be shown in this paper, storing post-heated cysts at room temperature for long periods did not change measured hatching percentages.

Cell fractionation

Dried cysts were hydrated at 0°C overnight in SW, quickly rinsed with ice cold distilled water and blotted (Clegg, 1978) then homogenized in ice cold buffer K (5 m*M* $MgSO_4$, 5 m*M* NaH_2PO_4, 40 m*M* Hepes, 70 m*M* potassium gluconate, 150 m*M* sorbitol, pH 7.4) at 100 mg wet weight of cysts per ml of buffer K. Two fractions were obtained by centrifuging homogenates at 1,600 g and 2°C for 5 min: a low speed supernatant (S) and a washed pellet (P) containing nuclei, yolk platelets and shell fragments. Further details about these methods have been published (Clegg *et al.*, 1994 and 1995; Willsie and Clegg, 2001 and 2002) where it has been demonstrated rigorously that p26 and hsp70 present in the pellet fraction are located exclusively in nuclei.

SDS-PAGE and immunoblotting

S and P fractions were added to equal volumes of 2× sample buffer (Laemmli, 1970) and boiled for 5 min. After low speed centrifugation (1,600 g, 5 min) to remove shell fragments, resulting supernatants were electrophoresed in 12% polyacrylamide gels, and proteins detected by Coomassie blue-G. Proteins from SDS-PAGE were also transferred to nitrocellulose sheets and prepared for immunodetection using a polyclonal anti-p26 and anti-artemin at 1:5,000 for 1 hr (Clegg *et al.*, 1994) as the primary antibodies, and horseradish peroxidase conjugated anti-rabbit IgG (1:5,000, 1 hr) as secondary. For the detection of hsp70, primary antibody was purchased from Affinity Bio-Reagents, Inc. (MA3-001; 1:1,250, 2 hr) and the secondary was horseradish peroxidase conjugated anti-rat IgG (1:5,000, 1 hr). Chemiluminescence was detected on blots in the Epi Chemi II Darkroom (UVP Laboratory Products) after incubation with Super Signal® West Pico (Pierce, Rockford, Illinois).

Results

Artemia franciscana

The research to be presented here relies on an understanding of this organism, so a brief description will be given. Of the seven described species of the genus *Artemia* (Triantapyllidis *et al.*, 1998; Van Stappen, 2002) most experimental research has used *A. franciscana* whose life cycle is outlined in Figure 1. This species is bisexual, with adults producing zygotes that proceed along one of two developmental paths, depending on conditions (Drinkwater and Crowe, 1987; Browne *et al.*, 1991; Drinkwater and Clegg, 1991; Van Stappen *et al.*, 1998). The path of direct development (Fig. 1) results in the release of fully-formed, swimming larvae produced from fertilized eggs in the female ovisac (Fig. 2, arrow). The alternative is the production of encysted gastrula embryos (cysts) that enter diapause, an obligate state of developmental arrest, and are then released into the usually hypersaline environment. These embryos remain in diapause until experiencing conditions that terminate it, and the resumption of development then ensues, given permissive conditions of temperature, water content and oxygen (Clegg and Conte, 1980; Drinkwater and Crowe, 1987; Drinkwater and Clegg, 1991; Van Stappen *et al.*, 1998). Of much importance is the synthesis of trehalose, artemin and p26 during the production of diapause embryos, events that do not occur during the direct pathway of development or, for that matter, in any other stage of the life cycle (Fig. 1). It appears that the genes that control these metabolic processes are effectively turned off under conditions of direct development, but virtually nothing is known about the details, an issue ripe for study.

A photograph of an adult couple is given in Figure 2. Encysted embryos (cysts) are barely visible in the female ovisac, located beneath the grasping male, as indicated by the arrow. Typically, 50–100 diapause cysts are produced at a time, although the number is highly variable, and then released into the environment. More information on the adults of *Artemia* is given in a recently published book (Abatzopoulos *et al.*, 2002).

The encysted embryo (cyst)

Figure 3 shows the general ultrastructure of an encysted gastrula in diapause, shortly after release from the maternal female. As mentioned, these embryos are arguably the most resistant of all animal life history stages to a wide variety of environmental stresses (see Clegg and Conte, 1980; Clegg and Jackson, 1998; Hand and Podrabsky, 2000; Clegg and Trotman, 2002). Encysted embryos normally encounter these conditions in nature when floating on the surface of their hypersaline environment and/or after being blown on shore where they frequently form windrows and are buried under masses of decaying biological material. In the latter case, the embryos experience severely hypoxic or anoxic conditions, as well as unpredictable

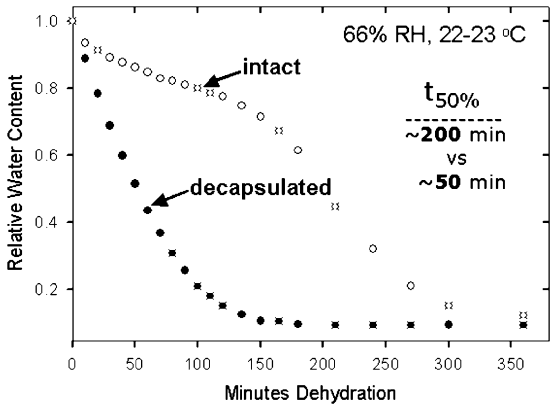

FIG. 4. The time course of dehydration of intact cysts and cysts previously decapsulated to remove the tertiary envelope of the shell (t.e. in Fig. 3). Dehydration conditions are given at the top of the figure and in the text. The time in minutes required for the cysts to lose one-half their initial water content ($t_{50\%}$) are also given.

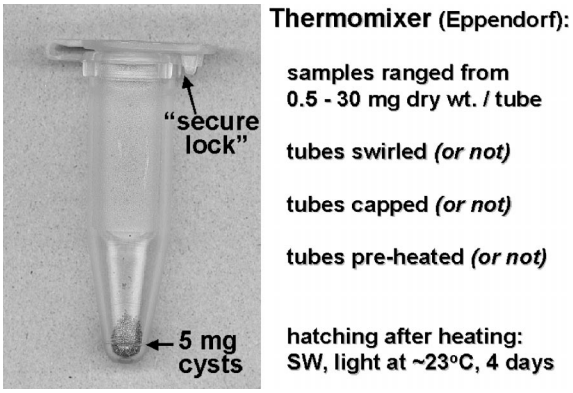

FIG. 5. Cysts were usually heated in ''secure lock'' microfuge tubes, one of which is shown here containing 5 mg of dry cysts. Some of the conditions examined are also listed. All studies were carried out in an Eppendorf Thermomixer.

and repeated bouts of desiccation/hydration due to fluctuations in humidity or rain. Thus, diapause embryos are well equipped to face a barrage of stresses that take place immediately after they are released from females, or much later.

One of the conditions leading to the termination of embryonic diapause in some populations of *A. franciscana* is desiccation (Drinkwater and Clegg, 1991). In this case the embryos are activated in the sense that diapause has ended, but they are quiescent since their development is prevented by lack of adequate amounts of water. When sufficient water is present, the embryos resume development (top sequence in Fig. 3), the only other requirements being suitable temperatures and adequate levels of molecular oxygen. The levels of artemin and p26 remain high during post-diapause development (Fig. 1) and are not substantially reduced in amount until about the time that emergence occurs (E1 in Fig. 3) (Clegg *et al.*, 1994 and 1995). After the first stage nauplius molts (Fig. 3, top), only small amounts of trehalose, artemin and p26 remain, and most of the latter seems to be restricted to a few cells that do not divide and eventually die (Liang and MacRae, 1999). Those three components clearly play vital roles in stress resistance of the encysted embryo, and do not appear elsewhere in the life cycle. Against this brief summary of the developmental biology of *A. franciscana* I present some results that add to what we know about the impressive adaptive repertoire of these cysts.

The shell and desiccation tolerance

Dehydration kinetics of intact and decapsulated cysts are shown in Figure 4, along with the estimated times for these cysts to lose half their initial water content ($t_{50\%}$). Decapsulated cysts lost water about 4× faster, overall, than intact ones under these conditions (Fig. 4). Note that the part of the shell removed by decapsulation, the tertiary envelope, is a significant part of the cyst volume (open bar in Fig. 3). These results were obtained from two groups of cysts at the same time, and are typical of many studies performed. This paired approach is more informative than lumping data to obtain means and errors from different runs in which modest differences in initial cyst hydration (difficult to avoid) have large influences on water contents over the course of dehydration, notably in the latter stages. Suffice to say that removing the tertiary envelope dramatically increased the rate of water loss from the embryo.

Decapsulation has substantial effects on survival, but only after dehydration because the decapsulation process, per se, had no effect on the hatching percentage (unpublished). However, after dehydration, carried out as shown in Figure 4, the hatching of decapsulated cysts was reduced to 48.3 ± 3.9 SE (n = 3) substantially lower than those not dehydrated (86.8 ± 2.5 SE, n = 3). In addition, some of the decapsulated cysts were obviously damaged mechanically, probably during their processing for hatching analysis. For the study described in Figure 4, a little over 8% of the cysts were damaged in this way. Clearly, cysts lacking the tertiary envelope are delicate and much more fragile than those containing complete shells.

Thermotolerance of dried cysts

The general method used in these studies is illustrated in Figure 5. ''Secure lock'' tubes that prevent loosening of caps during heating and swirling were usually used. A wide variety of conditions have been studied, some of which are listed in Figure 5 and will be considered later. Pre-heating the tubes at 95°C for 24 and 48 hr before heating cysts had no effect on hatching, either positive or negative, and microfuge tubes from 3 different suppliers were tested with the same outcome. Thus, the tubes can be considered as inert containers that do not contribute to the results presented here.

An unsuspected initial observation was the profound effect on hatching of the mass of dry cysts per tube after heating under precisely the same conditions (Fig.

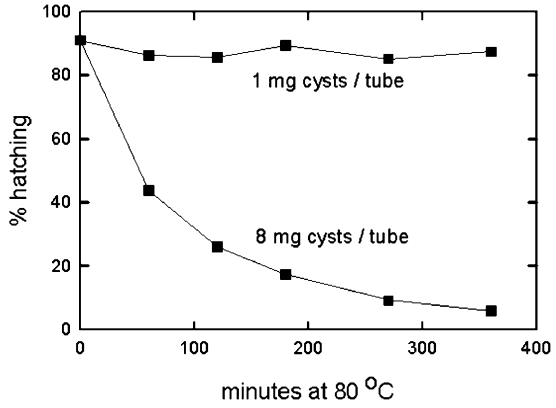

FIG. 6. Results of heating 1 and 8 mg of dried cysts in capped 1.75 ml tubes at 80°C for 1 hr on subsequent hatching. The tubes were placed directly into the Thermomixer, pre-set at 80°C.

6). In this typical example the use of 1 mg/tube resulted in no reduction of hatching, even after 15 hr at 80°C, whereas increasing cyst mass to 8 mg reduced hatching to only 3% over the same time (Fig. 6). All other conditions were the same: the tubes were capped from the beginning, placed directly into the Thermomixer pre-set to 80°C and the tubes were swirled at 500 rpm for the first two minutes. Thus, the only variable was the dried mass of cysts per tube, and this parameter strictly determined the level of hatching (Fig. 6). Subsequent study showed that the same results were obtained when the tubes were heated from 25 to 80°C at 5.5°C/minute, and by omitting the 2 minutes of swirling, so those variables are unimportant. Different temperatures have also been studied, and these results will be reported in detail in a future paper. For the present I note that the cysts are indeed thermotolerant when the mass of cysts/tube is optimal for heating studies. For example, the time required to kill 50% of cysts incubated at 90°C is close to 10 hours.

The effects of cyst mass have been examined in much more detail (Fig. 7): above 10 mg of dry cysts/tube, hatching was reduced close to zero. All other conditions in these experiments were the same: tubes

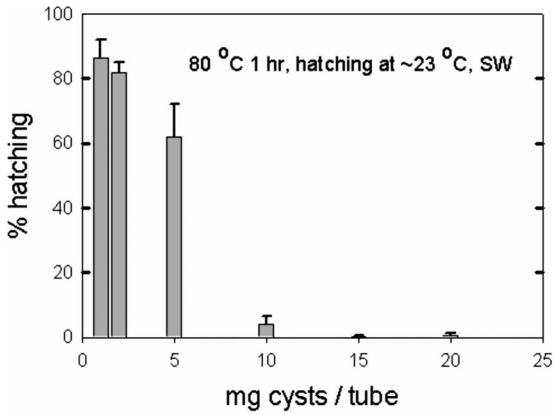

FIG. 7. The effect of cyst mass per tube (1.75 ml volume) on hatching after incubation at 80°C for 1 hr in capped tubes.

TABLE 1. *Effect of incubation after heating on hatching.*

mg cysts/ tube	% hatching Day 1		Day 22		Day 40	
5	51.7	65.2	55.9	58.7	60.3	59.0
10	7.5	5.2	7.1	6.3	6.1	6.9
20	1.2	1.0	1.1	1.4	1.3	0.8

After heating at 80°C for 1 hr in capped tubes using the weights of cysts shown, the cysts were stored at room temperature for the three periods. Two sets of results are shown for each storage time. At least 100 cysts were used for each of the 18 measurements of hatching.

were capped and placed directly into the Thermomixer, pre-set to 80°C. The same results were obtained using a gradual increase in temperature, and the lack of swirling. The study shown in Table 1 explored the possibility that the effect of dried cyst mass/tube was spontaneously reversible after heating. Results in Table 1 were obtained for cysts incubated at ~23°C for 1, 22 and 40 days, after heating as described in Figure 7. The outcome of two independent studies showed that the effects of heating were not spontaneously reversible, at least under these conditions. Furthermore, treatments that are known to terminate diapause in at least some cysts (Clegg and Jackson, 1998) were examined: 3% H_2O_2 for 15 min, incubation in 0.1 *M* NH_4Cl (pH 8.5) for 6 hr, and a dehydration-hydration cycle. However, none of these treatments reversed the effects of heating (Figs. 6 and 7).

Table 2 shows results on the effects of heating cysts in open *versus* capped tubes, and with or without swirling. Once again, there was a large effect of cyst mass/tube; however, the other variables also had big effects. Heating while the tubes were not capped led to a major increase in hatching (presumably from cysts in close contact with the air in the tube), and swirling completely removed the inhibitory effect of cyst mass/tube. However, swirling itself had no effect when the caps were closed (Table 2). These results indicated that the release of some volatile factor(s) from the cysts influenced in a major way the outcome of the heating experiments. The origin of the factor(s) is most likely the embryonic cell mass because decapsulated cysts lacking the tertiary envelope (Fig. 3) behaved like intact cysts when heated in the same way (results not

TABLE 2. *Effects of uncapping and swirling during heating at 80°C for 1 hr.*

mg cysts/ tube	% hatching after heating as described Open, static		Open, swirl		Capped, swirl	
1	88.7	91.0	87.3	88.6	85.8	90.2
10	86.4	85.5	91.4	87.7	2.1	4.3
20	33.7	29.2	88.4	85.9	0.5	1.2

The results of two independent experiments are shown. At least 150 cysts were used to determine hatching in all cases. ''Swirl'' refers to the tubes being oscillated at 500 rpm in the Thermomixer for the entire 1 hr incubation. Results for capped and static are shown in Figure 7, being very similar to capped and swirl as shown here.

TABLE 3. *Effect of gas space on hatching after heating at 80°C for 1 hr.*

ml gas space	mg cysts/tube	mg cysts/ml	% hatching
0.65	0.5	0.77	90.1
	1.0	1.54	85.4
	2.0	3.08	66.7
	5.0	7.69	8.2
1.75	2.0	1.14	80.3
	5.0	2.86	61.5
	10.0	5.72	6.4
	15.0	8.57	1.1

Gas space refers to the volume in empty tubes after the caps were closed. All cysts were heated under static conditions in capped tubes.

shown). Another study asked whether the critical parameter was the mass of dry cysts *per unit volume* of the gas phase in the tube, rather than the mass of cysts, per se. The answer is the former, determined by using tubes with different total volumes (Table 3). Evident decreases in hatching took place when the cysts mass/tube volume (mg/ml) exceeded 2, and became of major importance when the ratio was greater than 5, regardless of cyst mass and tube size per se.

Stress proteins

During the initial phases of the heating studies just described I examined cysts previously heated under various conditions, for the stress proteins artemin, p26 and hsp 70. One such study involved cysts that had been heated for 1 hr at 80°C using 30 mg cysts/tube (Fig. 8). At that time it was not known that, under these conditions, no hatching would occur in such preparations after heating. An important consideration is that the dried cysts, after heating, had to be hydrated before processing them for stress protein analysis. That was done for 16 hr at zero degrees to suppress metabolic activity. With that caveat in mind, Figure 8 shows a Coomassie-stained gel (part A), and a western immunoblot from a comparable gel (part B). Three conditions were examined: C, cysts not previously heated; 1. cysts heated for 1 hour at 80°C, then prehydrated at 0°C; 2. as in sample 1, followed by incubation for 4 hr at 23°C. All three samples showed similar protein profiles (Fig. 8A) except for p26 which was increased substantially in the nuclei (pellets) of samples 1 and 2, from heated cysts. Notice that p26 was not translocated out of nuclei after incubation at a temperature permitting metabolism (sample 2), an outcome of importance to the observed inverse correlation between the level of hatching and the amount of nuclear p26. Further results are given in Figure 9 where a similar study used different masses of cysts/tube, and the amounts and locations of p26 and artemin determined after heating at 80°C for 1 hour, then processing as above. In this case, heating resulted in the translocation of similar amounts of p26 to nuclei of cysts heated at 20 mg/tube (sample 1), and 15 mg/tube (sample 2), but slightly less when 10 mg cysts/tube were used (sample 3). These results also showed that the level of artemin remained about the same, and

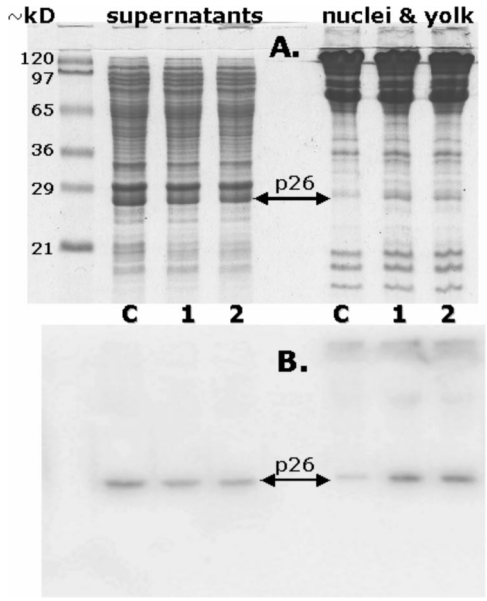

FIG. 8. SDS-PAGE showing a Coomassie stained gel (A.) and western immunoblot (B.) using fractions from dried cysts that had previously been heated at 30 mg/capped tube at 80°C for 1 hr (1 and 2) or not heated (C). In all cases the cysts were hydrated at 0°C for 16 hours prior to homogenizing to obtain the two fractions (see MATERIALS AND METHODS) indicated at the top of the figure. Sample 2 was also incubated for 4 hr at 23°C before homogenizing.

did not undergo heat-induced translocation to nuclei as did p26. Thus, reductions in hatching levels due to increasing cyst mass/tube were correlated with the translocation of p26 to nuclei.

Since heating dried cysts appeared to release a volatile factor that played a major role in reducing subsequent hatching (Table 2) the question arose concerning the location of p26 and artemin under those con-

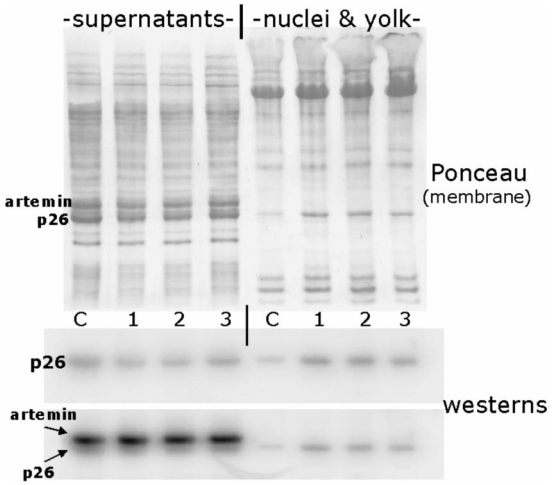

FIG. 9. Western blot of fractions from unheated dried cysts (C) and cysts that had been incubated at 80°C for 1 hr in capped tubes. Masses of dried cysts per tube were 20 mg (1), 15 mg (2) and 10 mg (3). All dried cysts were prehydrated before homogenizing (see Fig. 8 legend). Artemin and p26 were detected by Ponceau staining of the membrane after transfer (top part) and by immunoblotting in the bottom sections.

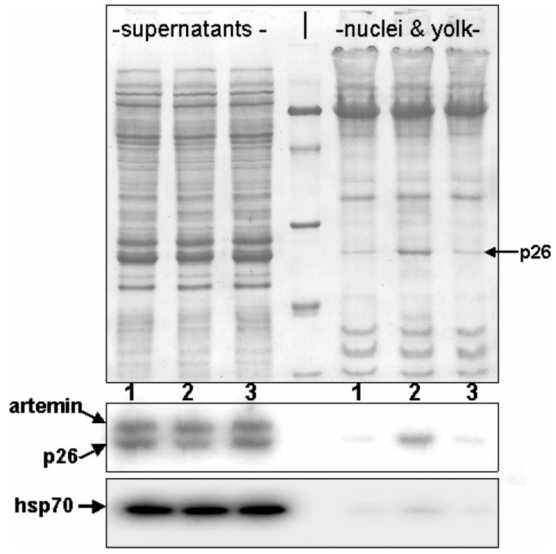

Fig. 10. Western blotting to detect artemin, p26 and hsp70 in cysts that had not been heated (1) and in cysts (20 mg dried weight/tube) that had been incubated at 80°C in capped tubes under static conditions (2) or in open tubes with swirling at 500 rpm (3).

ditions. As expected from Figures 8 and 9, substantial amounts of p26 were translocated to nuclei in cysts heated in capped, un-swirled tubes (Fig. 10). In contrast, analyses of the same mass of cysts per tube revealed no such p26 translocation when the cysts were heated in uncapped tubes and swirled during heating, conditions that also did not reduce hatching (Table 2). Also shown in Figure 10 are results for hsp70, being qualitatively similar to those for p26. These results added strong support to the suspicion that a volatile factor from the cysts was involved in the effects of heating on a decrease in hatching, as well as on the translocation of p26 and hsp70 to nuclei. Interestingly, it turned out that other considerations were also involved.

The air phase in the microfuge tube is necessary for heat-caused decreases in hatching

Given the evidence that the release of a volatile factor from the cysts is important, this did not, by itself, explain the effect of cyst mass/tube on post-heating hatching since, other things being equal, the results from the use of 1 mg should not differ from the use of 10 mg. As a result, it seemed that the air phase in the microfuge could be involved. To test that possibility, microfuge tubes containing 20 mg of dried cysts were placed in a flow-through chamber and perfused with 100% nitrogen gas for 1 hr at room temperature to remove the original volume of air from the tubes and to saturate the spaces in the tertiary envelope. Individual tubes were removed and the caps closed while continuing to gently flush their air spaces with nitrogen. After placing parafilm around the ''secure lock'' cap (Fig. 5) the tubes were heated at 80°C for 1 hr, and hatching determined as usual. The results were striking: cysts heated in nitrogen-perfused tubes produced a hatching level of 82.4% ± 4.2 SE (n = 3), essentially that of unheated controls, whereas hatching from the non-perfused controls was, as expected, very low (2.1% ± 1.1 SE, n = 3). Clearly, some component or components of the air phase are as important as the ratio of cyst mass to the volume of the air phase. Therefore, it seemed necessary to consider that an interaction between this component, or components, and the volatile factor(s) released from heated cysts was taking place, and that it was the product(s) that played a key role in reducing hatching and translocating p26 to nuclei.

Discussion

The study of adaptation to severe stress in the cysts of *Artemia* has focused primarily on biochemical processes, and these have been reviewed frequently over the years (Clegg and Conte, 1980; MacRae *et al.*, 1989; Warner *et al.*, 1989; Browne *et al.*, 1991; Clegg and Trotman, 2002). The complex shell of these cysts (Fig. 3) is of obvious value in protection against mechanical damage and that has been documented further in the present study that showed decapsulated cysts were prone to lethal mechanical damage even though only the tertiary envelope (Fig. 3) was removed. In addition, this same layer determines the rate at which water is lost from the embryo during dehydration (Fig. 4). Although diapause cysts contain what seems to be a complete set of components needed for desiccation tolerance, including trehalose, the present studies revealed that the *rate* of dehydration is also important since rapid water loss significantly reduces the hatching level of dehydrated cysts. The contribution of a complete shell to the optimal rate of water loss and desiccation tolerance is as important as it is to UV resistance (Tanguay *et al.*, 2004).

Previous studies on the thermotolerance of dried cysts indicated their impressive resistance to temperatures close to 100°C (Hinton, 1954; Iwasaki, 1973). The results of those investigators were highly variable, even though they used cysts from the same origin, the South San Francisco Bay. For example, after 1 hr close to 100°C, hatching levels (normalized to controls) were found to be 105% (Hinton, 1954) and 70% (Iwasaki, 1973); after 2 hr the outcome was 9% and 55%, and after 4 hr, 0.05% and 33%, respectively. One motivation to undertake the work on thermotolerance of dried cysts came from this variation in reported results (also see Clegg and Conte, 1980) and our own unpublished studies that also revealed large variation and difficulty in replication. Something seemed to be missing in the conduct of these studies. Work presented here (Figs. 6, 7, Tables 1–3) provides a possible explanation for that variability; namely, the effects of cyst mass per unit volume of the surrounding air space, as well as the extent of confinement, during heating. Importantly, it was the ratio of the dried mass of cysts to the total volume of the airspace that proved to be a critical determinant of the effect of heat on subsequent hatching at 23°C. Why should mass have any effect at

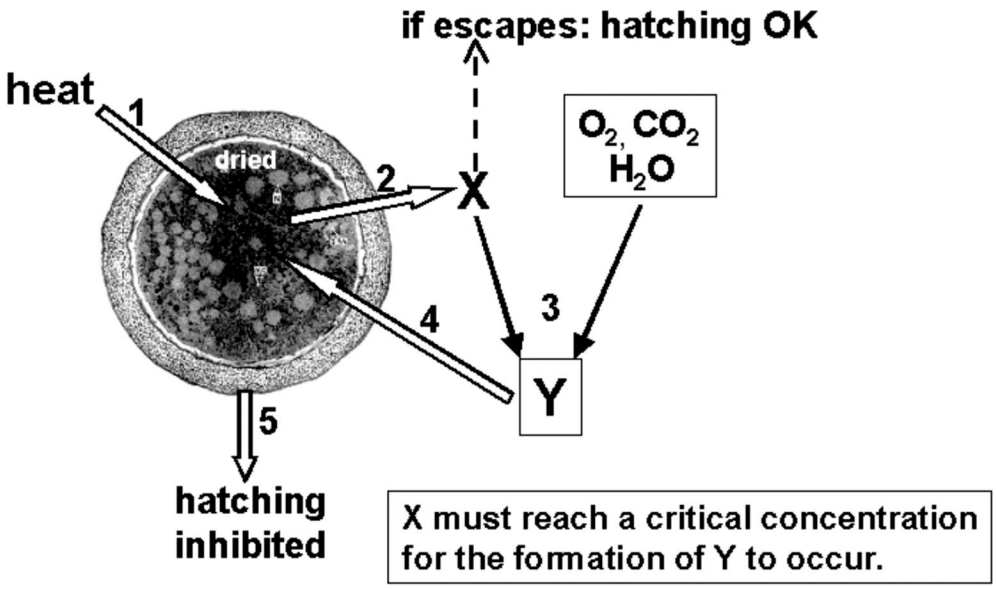

FIG. 11. Hypothetical scheme to account for the effects of heating dry cysts at 80°C for 1 hr under various conditions. Heat applied to the dried embryo (1) releases a factor X into the gas phase (2). If the tube containing the cysts is not capped, X escapes and there is no reduction in hatching after heating. If the tube is capped, X is postulated to react (3) with some or all of the components in the gas phase (CO_2, O_2, H_2O) of the tube to produce Y. The latter then enters the cyst (4), leading to the inhibition of development and hatching (5). X and Y need not be single substances. Because of the effect of cyst mass per tube (Figs. 6 and 7) it is necessary to suppose that a threshold concentration of X, proportional to dry cyst mass, must be reached in order to produce Y.

all, notably since the differences in cyst mass are so small compared to the total tube volume? Thus, the density of dried cysts has been measured to be 1.3 g/ml, with a specific volume of 0.77 ml/g (Clegg, 1984). Therefore, 1 mg of dried cysts (0.77 μl) makes up only about 0.04% of the total tube air space (1.75 ml) and 10 mg of dried cysts only 0.4%. Yet, when heated at 80°C for an hour in capped tubes of 1.75 ml total volume, cysts from the 1 mg sample yielded about 85 percent hatching, similar to unheated controls, whereas hatching from the 10 mg sample was reduced to a few percent (Fig. 7). Those results were obtained even though the percentage of the cyst volume to total tube volume is very small in both cases.

On balance, the results implicate the involvement of a volatile factor or factors that are released from the encysted embryos when they are heated, and that this somehow leads to a reduction in hatching. The key observations seem to be (1) the huge effect of dried cyst mass, but *only* when heated in capped tubes, and (2) the importance of the gas phase (air) when dried cysts are heated in capped tubes. A working hypothesis to account for these results is summarized in Figure 11. Heating dried cysts (1) leads to the release of some volatile factor or factors from the cysts (2) indicated as X in Figure 11. If the caps are open and the tubes swirled (Table 2) X escapes and no reduction in hatching occurs. But if X is retained (capped tubes) it is postulated to react with something in the gas phase, presumably CO_2, O_2 and/or water vapor, as indicated by 3. Both carbon dioxide and molecular oxygen are sufficiently reactive to present a host of possibilities involving such things as, for example, NH_3 released from the cells of the embryo by heat. In any case, the result is the formation of another unknown, Y, which is assumed to enter the embryo (4) resulting in the inhibition of development and hatching (5). Because this sequence depends directly and strongly on cyst mass per tube it is also necessary to propose that a critical threshold of X must be reached before the inhibitory effect is achieved. That threshold is not achieved until cyst mass per tube exceeds about 2 mg (Fig. 7), presumably releasing enough of the postulated X to reach critical concentration.

Little can be said about the participation of p26, artemin and hsp70 in the tolerance of these cysts to desiccation. Based on the involvement of stress proteins in the desiccation of other systems (see Tsvetkova *et al.*, 2002) including the well known LEA proteins (see Wise and Tunnacliffe, 2004 for review, and the recent paper by Grelet *et al.*, 2005, and references therein) it would seem likely that all three of these proteins are somehow involved. However, the present study has not produced any insight into this issue in the case of *Artemia* cysts undergoing desiccation and rehydration. Previous work on *hydrated* cysts has shown that heat shock and anoxia result in the translocation of cytoplasmic hsp70 and p26 to nuclei, and that these proteins play very important roles in the protection of nuclei against thermal and anoxic damage (see Willsie and Clegg, 2001, 2002). But that work also demonstrated that in order for stressed embryos, or those in diapause, to resume development, nuclear p26 *must* be translocated from nucleus to cytoplasm (Willsie and Clegg, 2001). A corollary finding is that all the nuclei in embryos that will *not* produce nauplii

contain a large amount of p26. It seems possible that these embryos are dead, being ''locked'' in a state of diapause, at least in part because they can not remove p26 from their nuclei. Thus, there seem to be two faces to p26: on the one hand, it is an excellent and important molecular chaperone (Liang *et al.*, 1997; Collins and Clegg, 2004); on the other, it has the potential to lead cysts into permanent diapause (*i.e.,* death).

In contrast to the above, the same mass of cysts per tube when heated with swirling in uncapped tubes that produced high hatching levels (Table 2), also produced embryos that contained no more p26 in their nuclei than in unheated controls (Fig. 10, sample 3). That outcome is consistent with the hypothesis that any condition that translocates p26 to nuclei in an *irreversible* way will prevent development and hatching, even when otherwise favorable conditions prevail.

There is an interesting uncertainty concerning the *timing* of p26 translocation to nuclei in dried cysts that have been heated under conditions that reduce hatching (Figs. 8–10). Thus, it is not possible to directly examine the location of p26 (and hsp70) in heated, dried cysts since they must first be hydrated. That was done at 0°C, the assumption being that active metabolic processes would be suppressed while full hydration was achieved during 16 hr. The question is whether the translocation of p26 to nuclei induced by heating dried cysts takes place in cells *before* their prehydration, when they contain only about 0.08 g H_2O/g dry weight—essentially a ''solid state'' situation. That would be remarkable, if true, but there is an alternative possibility—that heating, perhaps by means of the unknown Y (Fig. 11) results directly or indirectly in the acidification of the *dried* embryonic cells. Then, only after or during their hydration at 0°C for 16 hours, does the translocation of p26 to nuclei take place. That is plausible because it has long been known that acidic pH_i is a major determinant of metabolism in these cysts (Busa and Crowe, 1983; Carpenter and Hand, 1986) and that includes p26 translocation to nuclei, a process that can occur rapidly under certain conditions, including *in vitro* at low temperatures (Clegg *et al.*, 1994 and 1995). I know of no way to decide between these two interesting alternatives, given present results.

CONCLUDING REMARKS

A few comments are in order concerning the extent to which these cysts, and other anhydrobiotic organisms, can lose intracellular water. How dry is *dry*? In the case of *Artemia* cysts it seems that there is no lower limit to desiccation using methods that do not involve very high (damaging) temperatures. For example, treatments designed to remove *all* cyst water had no effect on viability (Clegg *et al.*, 1978). In that study, Brunauer-Emmett-Teller gas bombardment techniques were applied to cysts in 12 consecutive cycles of vacuum and gas bombardment, a treatment that reduced kangaroo tendon to a powder (Clegg *et al.*, 1978). The conclusion was drawn that virtually all of the water was removed, yet all the embryos survived. That seemed strange at the time, and even much earlier (page 808 in Clegg, 1967), considering the importance of a minimal amount of water to macromolecular integrity. Based on seminal work by Sydney Webb and D.H. Warner (see Crowe and Clegg, 1973) the speculation seemed warranted that molecules other than water might substitute for, or replace this structural water in organisms capable of anhydrobiosis, a notion that has come to be known as the ''water replacement hypothesis'' subsequently well documented in extensive research by John and Lois Crowe and their many colleagues (cited previously in this paper and others in this symposium).

Finally, I note that Antony van Leeuwenhoek initiated the study of anhydrobiosis 303 years ago at the age of 70 with the publication in 1702 of his letter number 144 ''On certain animalcules found in the sediment in gutters of the roofs of houses.'' I dedicate my modest paper to his memory, the Father of anhydrobiosis whose initial studies on the subject revealed an objectivity and intellectual discipline that were rarely seen in scientists of his time (see Keilin, 1959) and perhaps ours.

ACKNOWLEDGMENTS

Some of the research considered here was supported by grants from NSF (MCB-98 07762) and the Naval Research Laboratory (N00173-01-1-G016) which are acknowledged with thanks, as is the award of a Senior Fulbright Research Fellowship while at Ghent University, Belgium. I thank Professor Herman Slegers for a gift of the polyclonal antibody against artemin, and Joshua Tanguay, Crista Collins and Russell Brunet for previous technical assistance.

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