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EMBRYOS OF *ARTEMIA FRANCISCANA* SURVIVE FOUR YEARS OF CONTINUOUS ANOXIA: THE CASE FOR COMPLETE METABOLIC RATE DEPRESSION

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Summary

Encysted gastrula embryos of the crustacean *Artemia franciscana* have acquired an array of adaptations that enable them to survive a wide variety of environmental extremes.The present paper shows that at least 60 % survive 4 years of continuous anoxia at physiological temperatures (20–23 °C) when fully hydrated. Although these embryos appear to carry on a metabolism during the first day of anoxia, no evidence for a continuing metabolism throughout the subsequent 4 years was obtained. During this period, there were no measurable changes in the levels of their stored, mobilizable carbohydrates (trehalose, glycogen, glycerol). Calculations indicate that, if these carbohydrates are being utilized at all during anoxia, the rate is at the least 50 000 times lower than the aerobic rate (lower limit of detection). Indications of proteolysis during prolonged anoxia were sought but not found. Under starvation conditions, the life span of larvae produced from embryos that had undergone 4 years of anoxia was not significantly different from that of larvae produced by embryos that had not experienced anoxia. Thus, all substrates and other metabolites required to support embryonic development to the nauplius, as well as endogenous (unfed) larval growth and molting, are retained during 4 years of anoxia. It is not possible to prove experimentally the absence of a metabolic rate in anoxic embryos under physiological conditions of hydration and temperature. Nevertheless, on the basis of the results presented here, I will make the case that the anoxic embryo brings its metabolism to a reversible standstill. Such a conclusion requires that these embryos maintain their structural integrity in the absence of measurable biosynthesis and free energy flow and are thus an exception to a major biological generality. Potential mechanisms involved in their stability are discussed.

Key words: *Artemia franciscana*, brine shrimp, embryo, anoxia, metabolic rate depression, trehalose, protein stability *in vivo*, stress proteins, bioenergetics.

Introduction

The great majority of free-living eukaryotic organisms cannot survive the absence of molecular oxygen (anoxia) for more than a day, and even those species well-adapted to resist anoxia die within a month or so (for books and reviews, see Hochachka and Guppy, 1987; Bryant, 1991; Hochachka *et al.* 1993; Grieshaber *et al.* 1994). In the latter cases, anoxic survival depends on an overall reduction in metabolic rate to conserve substrates and reduce the accumulation of toxic end-products, a phenomenon commonly referred to as ‘metabolic rate depression’ or MRD (reviewed in the references cited above and by Storey and Storey, 1990; Guppy *et al.* 1994; Hand and Hardewig, 1996). With one possible exception, MRD is not complete since the anoxic rate is easily measured, amounting to approximately 1 % or more of the aerobic rate. The potential exception is the encysted embryo of the brine shrimp *Artemia franciscana*, which survives years of anoxia and reduces its metabolism under anoxia to the extent that its very detection becomes a major experimental problem, documented in the present paper. Hand and colleagues (for example, Carpenter and Hand, 1986; Hand and Gnaiger, 1988; Hand, 1990, 1995) have produced substantial data indicating the existence of a continuing anoxic metabolism in these embryos during 6 days of anoxia, although the possibility that metabolism ceases under prolonged anoxia was recognized. Our results on this system provide no direct evidence for the presence of a continuing metabolism after a day or so of anoxia (see Clegg and Jackson, 1989; Clegg, 1992, 1993, 1994), a position also taken by Hontario *et al.* (1993). This matter seemed worthy of further study since there are important *qualitative* differences between the complete absence of metabolism and a greatly reduced one.

Materials and methods

Sources of Artemia franciscana

Unless stated otherwise, the encysted embryos of *Artemia franciscana* (Kellogg) were from the South San Francisco Bay salterns, purchased from San Francisco Bay Brand, Hayward,

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California. In some cases, embryos from the Great Salt Lake, Utah, were used (generously supplied by Professor Patrick Sorgeloos, *Artemia* Reference Center, Ghent, Belgium). Encysted embryos were stored dry, under nitrogen gas, at approximately –10 °C before use. In both cases, approximately 90 % of the embryos developed into swimming larvae when incubated in aerobic sea water at 23–25 °C for 72 h.

Anoxic incubations

In some cases, the dried embryos were hydrated under anoxic conditions by placing approximately 75 mg into an 8 ml screw-capped glass vial to which was added 6 ml of 0.4 mol l^{-1} NaCl buffered to pH 7.2 with 0.1 mol l^{-1} sodium/potassium phosphate and degassed for 5 h with nitrogen gas. The 2 ml air space was replaced with 100 % N_2, and the cap was screwed tight and wrapped with several layers of Parafilm to prevent loosening. The vial was oscillated (50 revs min^{-1}) for the first 24 h to allow the embryos to become uniformly hydrated, and thereafter stored on its side under ambient laboratory conditions (see Clegg, 1994, for additional details, including evidence that these conditions lead to anoxia). For some studies, the embryos were first hydrated in the same solution (aerobic) at 2 °C, and then given a 4 h incubation under aerobic conditions at 23 °C. These embryos were collected on cloth filters, blotted to remove interstitial solution and transferred to 8 ml vials (approximately 180 mg wet mass, equivalent to approximately 75 mg dry mass). Anoxic incubation medium was then added and the vials treated as described above.

Biochemical assays

After anoxic incubation, the embryos were collected on cloth filters, washed rapidly with ice-cold distilled water, blotted 'dry' and measured wet weights were transferred to homogenizers. Some samples were placed in tared aluminum cups for determination of dry mass. For trehalose and glycerol determinations, embryos were homogenized in 75 % ethanol (approximately 100 mg wet mass ml^{-1}) and the supernatant obtained by centrifugation (2000 ***g***, 5 min). Samples were analyzed using a Waters high-performance liquid chromatography (HPLC) system (BioRad Aminex HPX-87H column) with a differential refractometer as detector and authentic compounds to determine retention times. Glycogen was extracted from the 75 % ethanol-insoluble pellet using 50 % KOH (95 °C, 1.5 h) and reprecipitated with ethanol (66 % final concentration at 2 °C for 24 h). The glycogen was dissolved with water and determined by a colorimetric method (Dubois *et al.* 1956) after chitin fragments had been removed by centrifugation at 2000 ***g*** for 5 min. Results are expressed as μg compound mg^{-1} dry mass.

For analysis of organic acids, embryos were homogenized in 6 % perchloric acid (PCA) at 0 °C. Samples of the supernatant (2000 ***g***, 5 min) were applied to the Aminex column described above and the effluent was monitored at 210 nm using a Kratos ultraviolet detector. Flow rate was 0.6 ml min^{-1} at 41 °C, and the mobile phase was 4 mequiv l^{-1}. Retention times were determined using authentic compounds which were also used, separately, to 'spike' PCA extracts. Results are expressed as μg organic acid mg^{-1} dry mass.

Incorporation of NaH[^{14}C]O_3 and electrophoresis

Encysted embryos are impermeable to nonvolatile solutes (De Chaffoy *et al.* 1978) but $^{14}CO_2$ (from H[^{14}C]O_3^-) is incorporated into a variety of amino acids (and other metabolites), enabling examination of protein synthesis (Clegg, 1976, 1977). In the present study, embryos hydrated at 2 °C were incubated at 23 °C under aerobic conditions for 4 h in 0.4 mol l^{-1} NaCl buffered to pH 7.2 with 0.1 mol l^{-1} sodium/potassium phosphate and containing 1.11 GBq ml^{-1} NaH[^{14}C]O_3 (1.94 GBq $mmol^{-1}$ from Amersham). After incubation, the embryos were collected on cloth filters, washed rapidly with ice-cold distilled water and either assayed immediately (see below) or transferred to 8 ml vials for anoxic incubation as described above.

For assay, the embryos were collected as usual and homogenized in a solution containing 150 mmol l^{-1} sorbitol, 70 mmol l^{-1} potassium gluconate, 5 mmol l^{-1} $MgCl_2$, 5 mmol l^{-1} NaH_2PO_4, 40 mmol l^{-1} Hepes at pH 7.9. Samples of these homogenates were added to equal volumes of 2× 'sample buffer' (Laemmli, 1970) heated at 100 °C for 5 min and electrophoresed on 12 % SDS–polyacrylamide gels. The gels were stained for proteins with Coomassie Blue, dried and exposed to X-ray film for autoradiography (see Clegg *et al.* 1994).

To quantify the amount of ^{14}C present in the proteins of embryos after the 4 h aerobic incubation with NaH[^{14}C]O_3 and after prolonged anoxia, samples of these same homogenates were processed as described in detail previously (Clegg *et al.* 1996). Briefly, homogenates were prepared in 5 % trichloroacetic acid (TCA) and the insoluble fraction was washed twice with 5 % TCA, then heated in 5 % TCA at 95 °C for 1 h. The hot-TCA-insoluble fraction was washed twice with 5 % TCA, resuspended in 88 % formic acid and samples were taken for scintillation counting. Previous work (Clegg, 1977) has shown that this treatment produces radioactive proteins and that little else is labeled by aerobic incubation with NaH[^{14}C]O_3.

Results

Anoxic longevity and post-anoxic developmental rate

Fig. 1 shows hatching levels for embryos that had not experienced anoxia (zero time controls) compared with those that had. Data for embryos undergoing 3 and 4 years of anoxia were obtained in the present study, the continuation of an earlier work (Clegg, 1994). Results from that study (0, 1 and 2 years of previous anoxia) are given here for comparison with the present findings.

In the previous study (Clegg, 1994), it was shown that drying embryos after anoxia increased the hatching levels when they were rehydrated in aerobic sea water. This suggested that anoxia caused these embryos to re-enter diapause since this treatment is known to terminate diapause

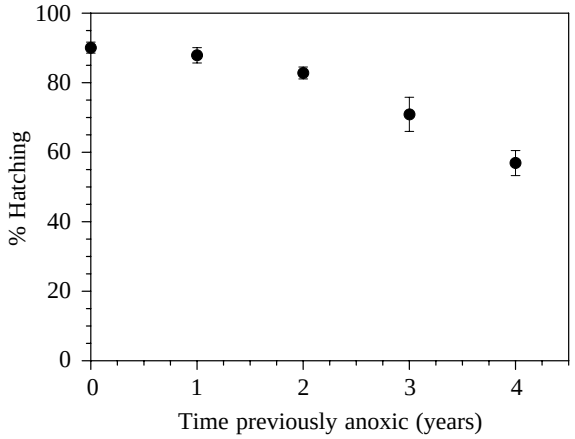

Fig. 1. Effect of previous anoxic incubation of encysted embryos on their subsequent production of nauplius larvae (hatching) when returned to aerobic conditions and incubated for 10 days. Bars are standard deviations for 3 groups of at least 200 embryos for each data point (mean).

in embryos from San Francisco Bay (i.e. those used in Fig. 1). However, drying embryos that had undergone 4 years of anoxia did not increase the proportion that hatched in aerobic sea water (data not shown).

Fig. 2 describes the onset and rate of hatching by embryos that had previously undergone anoxia, compared with those of controls. As the duration of anoxia increased, the onset of post-anoxic hatching was markedly delayed, amounting to a 10-fold difference between controls and the 4 year anoxic sample. The *rate* of hatching was also slowed in relation to the duration of the anoxic period. Thus, the time required for 50 % of the final hatching level to be achieved can be used as an overall measure of developmental rate: these times are approximately 22 h for control animals, 81 h after 2 years of anoxia, 106 h after 3 years of anoxia and 167 h after 4 years of anoxia.

Hatching in the latter two samples did not appear to be complete when the assay was terminated (Fig. 2) and,

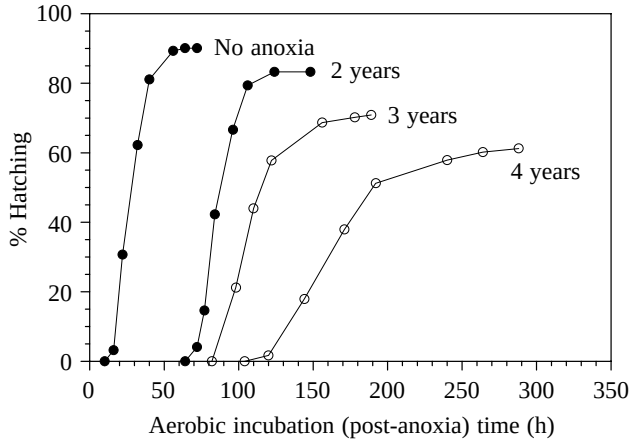

Fig. 2. Production of nauplius larvae (hatching) as a function of aerobic incubation time after different periods of embryonic anoxia, as indicated. The data points represent means of at least 200 embryos.

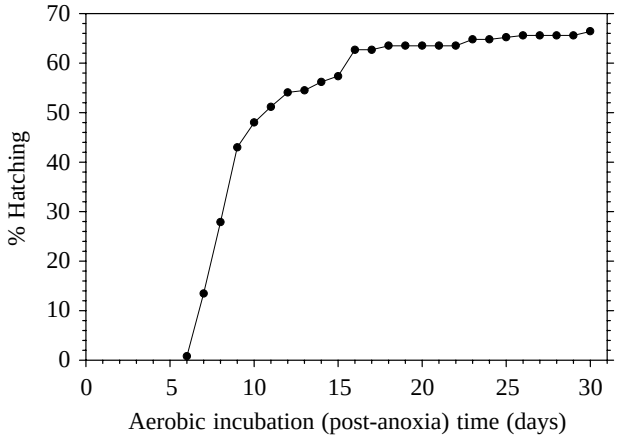

Fig. 3. Long-term aerobic incubation of encysted embryos previously made anoxic for 4 years. The data points are means for 367 embryos and represent cumulative hatching.

therefore, longer hatching assays were carried out for the 4 year anoxic sample. Typical results (Fig. 3) showed clearly that hatching was still continuing in this sample, albeit at a very slow rate, even when the study was ended after 30 days of post-anoxic, aerobic incubation. Therefore, the results shown in Figs 1 and 2 represent minimal hatching levels for post-anoxic embryos. Fig. 3 also raises the curious problem of identifying 'dead' embryos in anoxic populations.

Longevity of nauplii obtained from controls and previously anoxic embryos

It has long been established that embryonic trehalose, a disaccharide of glucose stored in very large amounts, provides the energy and carbon source needed to support embryonic development to the nauplius (see Clegg and Conte, 1980; Slegers, 1991). However, the nauplius still contains substantial amounts of glycogen, glycerol and yolk that enable it to molt, undergo further morphogenesis and survive for 3–4 days in the absence of food (see references cited above). If a metabolism occurs in encysted embryos during such prolonged anoxia, one might expect these and/or any other vital endogenous substrates to be utilized, thus reducing the endogenous longevity of nauplii derived from them. Such an effect should be most marked in nauplii after 4 years of anoxia. However, the longevity of nauplii hatched from control embryos (3.6±0.7 days, mean ± S.D., N=33) did not differ from that of nauplii obtained from embryos that had previously undergone 4 years of continuous anoxia (3.8±0.7 days, mean ± S.D., N=36).

An increase in the proportion of nauplii from anoxic embryos that exhibited obvious developmental defects was observed in this study. In a sample of 368 control nauplii, 4 were abnormal (1 %); of 401 nauplii from the 4 year anoxic embryos, 16 showed abnormalities (4 %). The most common defects involved stunted appendages and abnormal abdomens. Abnormal nauplii were not used in the longevity study.

Stored energy sources in control and anoxic embryos

Although these longevity studies suggested that endogenous

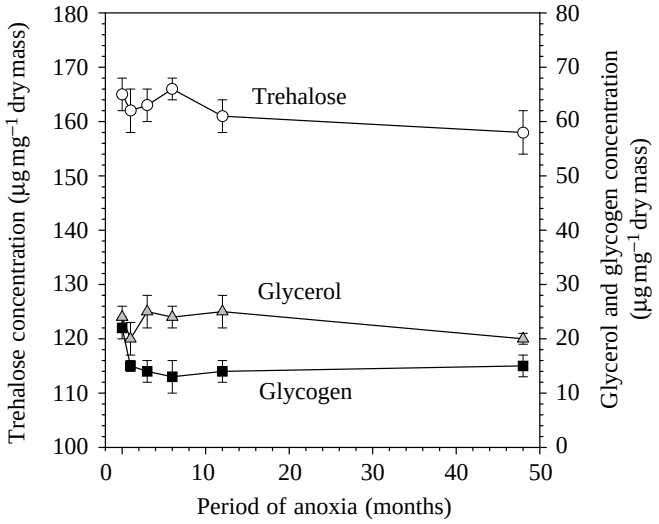

Fig. 4. Concentrations of mobilizable carbohydrates in dried encysted embryos that were hydrated and incubated under anoxic conditions for the times shown. Data points are means ± S.D. (N=3 independent samples). The only significant change is the decrease in glycogen concentration during the first month of anoxia (see text for statistical analysis).

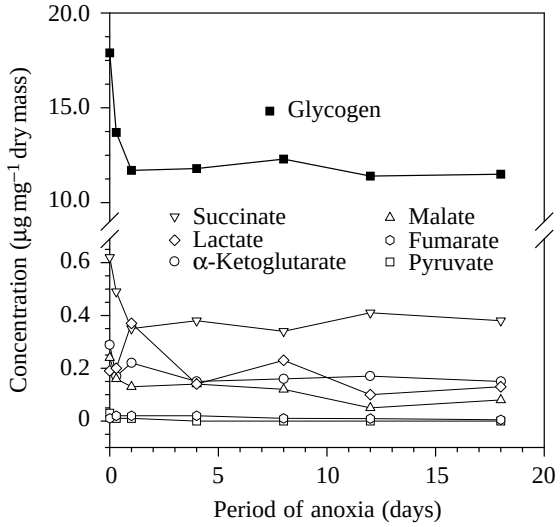

Fig. 5. Concentrations of glycogen and various potential end-products of anoxic metabolism in dried encysted embryos hydrated and incubated under anoxic conditions. Note the different scale for the glycogen data (N=1).

stores were not being used during embryonic anoxia, I compared the concentrations of trehalose, glycerol and glycogen in embryos after 4 years of anoxia with levels in those not made anoxic (Fig. 4). Results for each compound were subjected to a one-way analysis of variance (ANOVA) followed by pairwise multiple comparisons (Student–Newman–Keuls test) where appropriate. The results of the analysis indicate that levels of trehalose and glycerol did not change significantly with time under anoxia (P=0.58 for trehalose and P=0.52 for glycerol). Glycogen levels, however, dropped significantly (P=0.002) during the first month of anoxia, but did not change significantly from that level over the subsequent 4 years of anoxia.

Fig. 5 plots the initial decrease in glycogen concentration during shorter exposures to anoxia. Evidently, this decrease occurred only during the first day of anoxia. Also shown in Fig. 5 are concentrations of metabolites considered to be probable end-products of an anoxic energy metabolism. Levels of all these metabolites either decreased or did not change during anoxia. Since propionate has been reported to accumulate as an end-product during the first 5 days of anoxia (Hand, 1990), I looked for this compound. A peak was detected by HPLC that was close to that of authentic propionate, but since its retention time was not identical and the peaks separated when co-applied to the HPLC column, further study was not carried out.

Although the utilization of glycogen occurs during the first day of anoxia, indicating an anoxic metabolism, its metabolic fate remains to be determined. The decrease in glycogen concentration is not due to a simple hydrolysis since no significant increase in free glucose concentration was observed (data not shown).

Effects of previous aerobic incubation on carbohydrate levels during subsequent anoxia

The preceding results were obtained from dried embryos that were hydrated from the start under anoxic conditions. To enable comparison with other work on anoxia in these embryos, the effects of an initial aerobic incubation on carbohydrate levels during anoxia (Fig. 6) were also examined. As mentioned, previous studies have shown that, during subsequent aerobic incubations, trehalose concentrations fall while glycogen and glycerol concentrations increase (reviewed by Clegg and Conte, 1980; Slegers, 1991), a result confirmed

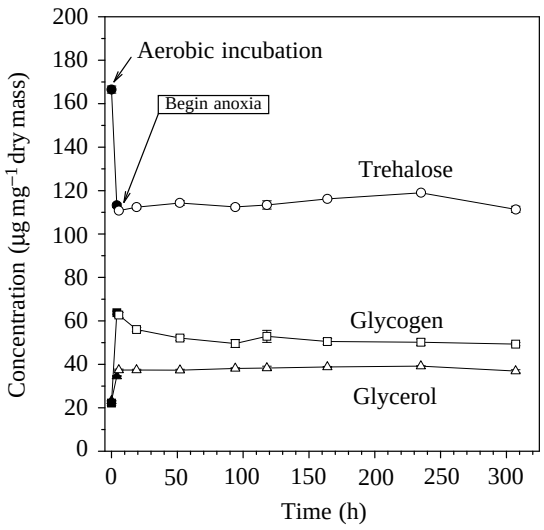

Fig. 6. Concentrations of mobilizable carbohydrates in encysted embryos first incubated under aerobic conditions for 4 h (filled symbols) followed by transfer to anoxic conditions (open symbols). Data points are means ± S.D., most of which are within the data point (N=3 independent measurements).

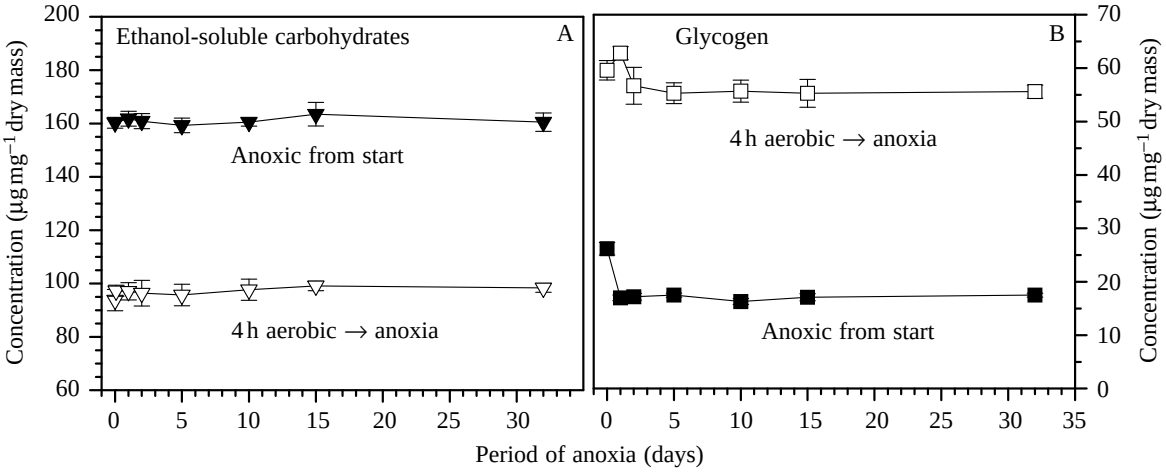

Fig. 7. Concentrations of 75 % ethanol-soluble carbohydrates (A) and glycogen (B) during anoxia of encysted embryos from the Great Salt Lake. The dried embryos were either hydrated and incubated under anoxic conditions or first hydrated at 2 °C, given a 4 h aerobic incubation and then transferred to anoxic conditions. All data points are means ± S.D. (*N*=3 independent measurements). In some cases, the standard deviations are contained within the data points.

in the present work (Fig. 6). When these aerobic embryos were then transferred to anoxic conditions, results qualitatively similar to those shown in Figs 4 and 5 were obtained: namely, glycogen levels decreased initially, while no change was observed in the levels of trehalose or glycerol (Fig. 6). Thus, an initial period of aerobic metabolism had no qualitative effect on the fate of these carbohydrates during subsequent embryonic anoxia.

Comparison of embryos from Great Salt Lake (GSL) and San Francisco Bay (SFB)

All our previous work on *A. franciscana*, anoxia has involved embryos collected from SFB, while others have used embryos from GSL with different results. Although these animals are the same species, it was possible that they differed in their response to embryonic anoxia. Therefore, a limited study was carried out on GSL embryos (Fig. 7). In this case, we examined changes in concentrations of glycogen and total carbohydrates soluble in 75 % ethanol. It is known that trehalose makes up approximately 90 % of the latter fraction (Clegg and Jackson, 1989) so the use of this rapid colorimetric assay provided a convenient estimate for the amount of trehalose in these embryos. As for the studies on SFB embryos, two cases were examined: embryos were either made anoxic from the dried state or given an aerobic incubation prior to anoxia. The results of this study on GSL embryos (Fig. 7) were comparable to those obtained with SFB embryos, indicating that geographic origin was not of importance, at least in terms of the effects of anoxia on ‘trehalose’ and glycogen levels.

Proteins in control and anoxic embryos

Encysted embryos are impermeable to amino acids, and probably to all non-volatile compounds (De Chaffoy *et al.* 1978; Clegg and Conte, 1980), so NaH[^{14}C]O$_3$ ([^{14}C]O$_2$) was used because it is known to label a number of metabolites including several amino acids (Clegg, 1976, 1977). Embryos hydrated at 2 °C for 24 h were incubated under aerobic conditions for 4 h at 25 °C with NaH[^{14}C]O$_3$ as described in Materials and methods. After aerobic incubation, embryos were rapidly washed free of external radioactivity with ice-cold distilled water and samples of known wet mass (approximately 100 mg) were either frozen at once (–72±2 °C) or transferred to anoxic NaH[^{14}C]O$_3$-free incubation medium (phosphate-buffered 0.4 mmol l^{-1} NaCl) for further incubation at 20–23 °C for periods of up to 1 year. All samples were analyzed at the same time, as described in Materials and methods.

Fig. 8A is a Coomassie-stained gel (SDS–PAGE) of proteins extracted from embryos after the 4 h aerobic incubation (‘0 anoxia’) and after subsequent anoxic incubations. At this level of resolution, the profiles appear to be the same, with no indication of protein loss or degradation. Embryos containing ^{14}C-labeled proteins were studied in an attempt to increase the sensitivity of detection of protein hydrolysis. Fig. 8B is an autoradiogram of this gel after drying. Once again, the profiles are all remarkably similar, with no evidence of protein hydrolysis over an entire year of anoxia.

The total amount of radioactive protein in the same samples used in Fig. 8 was also determined (Table 1). Analysis of variance of these data revealed that no significant differences exist between the proteins of embryos after aerobic incubation and those undergoing anoxic incubation. These results confirm the visual impression given in Fig. 8 and document the extraordinary resistance of the primary structures of proteins in anoxic embryos.

Discussion

It is remarkable that at least 60 % of these embryos give rise to viable nauplii after experiencing continuous anoxia for 4 years (Fig. 1). To my knowledge, no laboratory study has demonstrated this level of anoxic survival for any free-living eukaryote, while fully hydrated at temperatures of 20–23 °C.

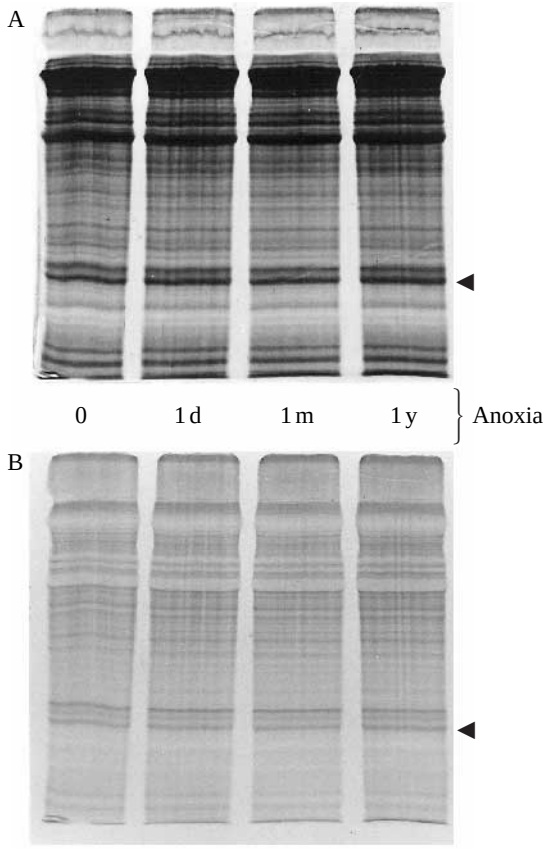

Fig. 8. Analysis of proteins in homogenates of encysted embryos by denaturing polyacrylamide gel electrophoresis (SDS–PAGE). Encysted embryos were hydrated at 2 °C in sea water, then incubated with NaH[^{14}C]O_3 under aerobic conditions, as described, for 4 h. After removal of external NaH[^{14}C]O_3, the embryos were incubated under anoxic conditions for the times shown. (A) Coomassie-stained proteins; (B) autoradiogram of this same gel. The arrowhead points to an abundant protein of molecular mass 26 kDa referred to in the text as p26. 1 d, 1 day; 1 m, 1 month; 1 y, 1 year.

Of interest, however, is the work of Marcus *et al.* (1994), who isolated copepod embryos from 40-year-old anoxic marine sediments that hatched into nauplii when placed under aerobic conditions. Even more remarkable are the results of Hairston *et al.* (1995), who found that viable freshwater copepod embryos could be isolated from 332-year-old anoxic, freshwater sediments. If the ages of these embryos are the same as those of the sediments (an assumption not currently subject to test), then these embryos must either certainly be ametabolic or are permeable to compounds in the interstitial water of these sediments that support an anoxic metabolism. The latter possibility seems unlikely, but appears to be testable. Nevertheless, these observations on copepod embryos suggest that a reversible metabolic standstill under anoxia is not limited to *Artemia* embryos.

An important question in the present study concerns those embryos that do *not* hatch after anoxia is terminated. It has generally been assumed that embryos are dead if they do not hatch after a period of incubation comparable to controls; however, that is a risky assumption in view of the results shown in Figs 2 and 3, which demonstrate that some anoxic embryos require at least a month to complete the hatching process when returned to aerobic conditions. These results raise the question of how to distinguish between anoxic embryos that are dead and those that simply require an extremely prolonged aerobic incubation to hatch. I am not aware of a way to do that at present by direct inspection.

Table 1. *Radioactive proteins in encysted embryos during anoxia*

Duration of anoxia	Radioactivity in protein (disints min^{-1} mg^{-1} wet mass embryo)
None	3408±135
1 day	3497±206
1 month	3658±196
1 year	3444±162

Embryos were incubated with NaH[^{14}C]O_3 under aerobic conditions for 4 h at 23 °C and then transferred to anoxic conditions, as described in Materials and methods. The amount of ^{14}C in the total protein fraction was determined after the periods of anoxia shown above.

Results are means ± S.E.M. (N=3).

Why should prolonged anoxia cause such lengthy delays in hatching for those embryos that do eventually hatch (Figs 2, 3)? One explanation is the need to repair damage that might have accumulated during anoxia. Another possibility could be the time needed to reverse the regulatory mechanisms that bring metabolism to a standstill during anoxia and/or that are involved in protecting the anoxic embryo from damage. It is known that overall metabolic rates of post-anoxic embryos are reduced compared with those of controls (Clegg, 1993). Consequently, the return to a complete metabolic and developmental program after prolonged anoxia is not a simple reversal of the events involved in the aerobic–anoxic transition which is, by comparison, very rapid. Further study of this matter is needed.

No evidence was found for a continuing metabolism during prolonged anoxia. Although a metabolic rate cannot be proved experimentally to be zero at temperatures of 20–23 °C, the results from the present study provide strong support for that possibility in anoxic embryos. If these embryos are metabolizing under anoxia, the rate is truly minuscule. For instance, under aerobic conditions, trehalose is metabolized at a rate of about 15 μg mg^{-1} dry mass h^{-1} (Fig. 6) but no change in the content of this sugar was detected during 4 *years* of anoxia (Fig. 4). Since a decrease in trehalose content of 10 μg mg^{-1} dry mass could easily be measured, this will be used as the lower limit of detection: 10 μg mg^{-1} dry mass 4 $years^{-1}$ is equivalent to the loss of 0.3 ng mg^{-1} dry mass h^{-1}, which is at least 50 000 times slower than the aerobic rate.

The existence of an anoxic metabolism not involving the use of carbohydrate as substrate cannot be excluded, but this would be a unique process since we know of no example of an anoxic metabolism in any free-living organism that uses lipids or amino acids without concomitant carbohydrate mobilization.

The conclusion that anoxia in excess of 1 day reduces metabolism in these embryos to a reversible standstill should be compared with the results of Hand and colleagues (cited in the Introduction). They reported significant anoxic decreases in levels of trehalose and glycogen, the accumulation of propionate, lactate and other end-products, and the production of small but easily measurable amounts of heat, that persisted over at least 6 days of anoxia and were interpreted as a continuing anoxic metabolism. However, these workers also recognized that metabolism might come to a standstill during prolonged anoxia. Hontario *et al.* (1993), also using microcalorimetry, showed that heat production (and therefore metabolism) reached undetectable levels within hours of anoxic incubation. They proposed that the very small amounts of heat measured by Hand and colleagues during the first 6 days of anoxia could be accounted for by a combination of microbial contamination and instrumental problems. Hand (1995) responded by controlling contamination and increasing the sensitivity of the microcalorimetric technique employed, and concluded that heat continues to be produced by anoxic embryos. Most recently, Hand (1995) accepted the possibility that at least some of this heat may not be coupled to metabolic activity, but rather could be the result of non-metabolic chemical (exothermic) reactions taking place in dead embryos (Clegg and Jackson, 1989). This is relevant since Hand and colleagues have utilized embryos from Great Salt Lake, Utah, whose hatching levels were typically in the range 65–75 % (hatching levels were not reported in Hand, 1995). Pursuing the possibility that at least some of the heat production represented a continuing metabolism, Hand (1995) proposed that if only 10 % of the minuscule amount of heat measured in anoxic embryos were coupled to the metabolism of trehalose (or any carbohydrate) it would take 18 years of anoxia to utilize this sugar completely. Assuming that these numbers are correct, and knowing that encysted embryos contain approximately 165 μg trehalose mg^{-1} dry mass (Fig. 4), the amount of trehalose that would be utilized during 4 years of this postulated anoxic metabolism can be calculated to be 35 μg trehalose mg^{-1} dry mass, a change that would easily be detected if it took place. Similar results are obtained for glycogen and glycerol, apparently the only other mobilizable carbohydrates (Glasheen and Hand, 1989). Thus, I believe the preponderance of evidence supports the view that metabolism in these embryos is brought to a standstill after the first day of anoxia.

It is important to attempt to determine whether metabolism comes to a reversible standstill in anoxic embryos since cells are viewed as highly unstable systems operating far from equilibrium. Thus, it is a general and important rule of biology that living cells require a continuous and substantial flow of free energy to maintain their integrity when fully hydrated at physiological temperatures. Eukaryotic cells do indeed seem to obey this rule, in general, but those in the anoxic *Artemia franciscana* embryo may not. In the final section of this paper, the significance of that result is examined further.

Consider the proteins of these embryos during prolonged anoxia. Protein turnover utilizes a very large fraction of the free energy budget of cells (Hand and Hardewig, 1996). Thus, it can be expected that proteolytic activity must be turned off in *Artemia franciscana* embryos during anoxia, and the results shown in Fig. 8 and Table 1 support that expectation. Further support comes from the study of Anchordoguy and Hand (1994), who showed that the ubiquitin-dependent pathway of proteolysis was profoundly reduced during anoxia in this system (also see Anchordoguy and Hand, 1995). It appears from the present results (Fig. 8; Table 1) that non-ubiquitin pathways of proteolysis in these embryos (see Warner, 1987, 1989) must also be turned off during anoxia. It should be noted that Anchordoguy *et al.* (1993) found that the rate of degradation of cytochrome oxidase in anoxic embryos was reduced, but still measurable, over 28 days of anoxia (half-life, 101 days). If this rate continued over 4 years of anoxia (Fig. 1) little, if any, cytochrome oxidase would remain (10 half-lives) to support the respiration that takes place immediately after anoxic embryos are returned to aerobic conditions (Clegg, 1993).

A more difficult problem for the anoxic embryo, in my opinion, concerns the inherent instability of proteins, notably their tertiary and quaternary structures. When hydrated, globular proteins will tend to denature/unfold and participate in deleterious aggregation reactions (Dill, 1990; Somero, 1995). It does not seem likely that the *Artemia franciscana* embryo has evolved proteins whose structures intrinsically resist unfolding and aggregation since it has been pointed out that globular proteins must be unstable at *physiological temperatures* in order to be sufficiently flexible to function (Somero, 1995; Shoichet *et al.* 1995). How is protein conformational integrity maintained in these embryos over years of anoxia, during which replacement by synthesis does not occur?

One possibility could involve the participation of molecular chaperones that reduce unfolding and/or renature unfolded proteins (for recent reviews, see Ellis, 1994; Buchner, 1996; Hartl, 1996). We have been studying an extremely abundant protein in these embryos (p26, Fig. 8) that exhibits a number of features characteristic of the small heat shock (stress) protein family (Clegg *et al.* 1994, 1995, 1996; Jackson and Clegg, 1996). This protein, whose native relative molecular mass is approximately 500 000 (subunits of 26 000), makes up 10–15 % of the total non-yolk protein, partially translocates into the nucleus and other cellular compartments during anoxia and other stresses, and is restricted to the encysted embryo stage of the life cycle. It is noteworthy that the small stress protein family does not require nucleoside triphosphate(s) to perform chaperone functions *in vitro* (Parsell and Lindquist, 1993; Jakob *et al.* 1993; Waters *et al.* 1996). Further study of the potential role of p26 during prolonged anoxia in these embryos is needed.

A second possibility concerns the presence of large amounts of trehalose and glycerol in these embryos (Fig. 4). These compatible solutes are well-known stabilizers of proteins and other cellular constituents (reviewed by Yancey *et al.* 1983; Crowe *et al.* 1992; Winzor *et al.* 1992; Somero, 1995; also see

Hottiger *et al.* 1994). Consequently, these compounds might generate an intracellular environment that stabilizes membranes and macromolecules during prolonged anoxia without the need for other participants. Of course, the involvements of molecular chaperones and compatible solutes are not mutually exclusive possibilities.

Although the mechanism(s) of stabilization remains to be described, the results of this study suggest that the anoxic embryos of *Artemia franciscana* provide an exception to one of the most pervasive generalities of biology: the need for a constant flow of metabolic free energy to maintain integrity under physiological conditions of hydration and temperature.

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