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METABOLIC HEAT PRODUCTION BY *ARTEMIA* EMBRYOS UNDER ANOXIC CONDITIONS

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Summary

The metabolic status of encysted *Artemia* embryos under anoxic conditions has been studied by calorimetry. Previous work demonstrated a very low, but still measurable, source of metabolic heat (31nWmg^{-1} drymass) after 6 days of anoxia, attributed to the catabolism of small amounts of carbohydrate storage compounds and the unusual nucleotide diguanosine tetraphosphate. Our results indicate that the heat measured drops below the detection limits of our calorimeter (11nWmg^{-1} drymass) after 1.5h of anoxia, in sharp contrast with previously published results. The possible sources of error that could explain this difference, and a re-evaluation of the metabolic status of cysts during anoxia, are discussed. We conclude that if the cysts are carefully protected from oxygen they become essentially ametabolic.

Introduction

In addition to their well-known ability to undergo nearly complete dehydration without being killed (Clegg, 1978), cysts of the brine shrimp, *Artemia*, can also survive extended periods (many months and perhaps several years) of complete anoxia (Clegg, 1992; Dutrieu and Chrestia-Blanchine, 1966). Under anoxia, metabolism in *Artemia* cysts is severely suppressed (Hand and Gnaiger, 1988; Hand, 1990; Clegg and Jackson, 1989*a*,*b*), but the extent of this diminution remains unclear. This question is important to our understanding of the mechanisms that the cells of *Artemia* cysts utilize to maintain the integrity of their biological structures and to recover from severe anoxic conditions.

This question was initially studied through analysis of different metabolites and how their levels vary during anoxia. For instance, Stocco *et al.* (1972) showed that during prolonged anoxia the unusual storage nucleotide diguanosine tetraphosphate (Gp_4G) was utilized slowly. More recently, Hand (1990) carried out calorimetric measurements of cysts under anoxia and reported the existence of metabolically related heat release, although at a very low rate, during 6 days in anoxia. In addition, the biochemical calculations presented by Hand and Gnaiger (1988) showed that the catabolism of Gp_4G at the rates measured by Stocco *et al.* (1972) could account for only 3% of the heat

Key words: *Artemia* cysts, metabolic heat, calorimetry, anoxia.

detected. They proposed that the source of this metabolic heat is the catabolism of small amounts of trehalose and glycogen. Hand (1990) reported a small decrease in carbohydrates after 3 days of anoxia under his experimental conditions, and he calculated that this catabolism accounted for 84% of the heat measured. Subsequently, using different experimental procedures from those of Hand and Gnaiger (1988), Clegg and Jackson (1989*a*,*b*) reported that catabolism of all major storage metabolites, including trehalose and glycogen, ceased during anoxia lasting more than 3 months.

Excellent reviews concerning both points of view can be found in Drinkwater and Clegg (1991) and Hand (1991). It has been pointed out that if carbohydrate or nucleotide metabolism continues during anoxia, even at very slow rates, the cysts would deplete all metabolic stores within several weeks (Drinkwater and Clegg, 1991). But it appears from the data of Clegg and Jackson (1989*a*,*b*) that this is not the case.

A potential explanation for this apparent discrepancy lies in the experimental methods used to induce anoxia by the two groups of investigators. Hand and Gnaiger (1988) used a flow calorimeter in their studies, providing a constant perfusion of the cysts with gases of known composition. Clegg and Jackson (1989*a*,*b*), in contrast, sealed the cysts in closed containers. Under these conditions the cysts apparently rapidly depleted any residual oxygen in the container and entered complete anoxia.

The aim of the present paper is to resolve this issue through a re-examination of calorimetric heat production by anoxic *Artemia* cysts under conditions similar to those used by Clegg and Jackson (1989*a*,*b*), providing new evidence about the metabolic status of anoxic *Artemia* cysts. In addition, we have explored the effect of the use of different cyst samples and preparation procedures on the calorimetric analysis in order to assess other sources of error in these studies.

Materials and methods

Samples utilized and preparation of cysts

Five different batches of dehydrated cysts were used in this study: commercial San Francisco Bay (California, USA) cysts (SFB); commercial Great Salt Lake (Utah, USA) cysts (GSL); La Mata (Alicante, Spain) cysts, collected in 1988 (L88); Ebro river delta (Tarragona, Spain) cysts, collected in 1984 (D84); and La Malá (Granada, Spain) cysts, collected in 1988 (M88). SFB and GSL are bisexual, belonging to the species *Artemia franciscana*; L88 and D84 are parthenogenetic populations, diploid and tetraploid, respectively; and M88 is a bisexual form of the species *A. tunisiana*.

Cysts were analyzed both untreated and treated with dechorionating procedures. The oxidation of the cyst's outer shell (dechorionation) with commercial bleach was carried out following the procedure of Clegg (1986), always maintaining the samples at 0°C. Cysts, untreated and dechorionated, were hydrated in distilled water for 6h at room temperature (22±1°C) prior to the calorimetric assays; they were then allowed to settle and all empty shells and accompanying debris (in the case of untreated material) was discarded. In order to determine whether the initial aerobic metabolism influences the heat release under anoxia, an experiment with 10h of aerobic incubation was performed.

Calorimetric analysis

After hydration, cysts were collected by filtration, and the required quantities were sealed in the 1ml measuring cells of the calorimeter. These cells are sealed with a rubber gasket that does not permit passage of gases. A Hart 7707 series differential scanning calorimeter (Hart Scientific, Provo, Utah) was used in isothermal mode at 25°C. The heat released is measured as the difference in voltage applied to a Peltier device necessary to maintain the preset temperature in the sample cell and in the empty reference cell. Voltages are converted to power (W) through calibration constants, calculated from the voltage applied to maintain the temperature when a known amount of energy is released to the cell. The heat dissipated was recorded every 180s. Baselines, blank experiments without cysts in the sample cells, were run and the results were subtracted from data collected with the embryos present.

Where anoxia was tested, experiments were performed by loading the cells completely (cyst samples ranged between 100 and 200mg dry mass), leaving a minimal volume for O_2 once the cells had been hermetically sealed. This ensured quick exhaustion of O_2 and rapid establishment of anoxia. Under these conditions, the samples were monitored for periods of up to 21 days. Control experiments under aerobic conditions were carried out, loading only 10–20% of the cell volume (20–40mg dry mass). In these cases, aerobic metabolism could be maintained for 12–14h after the cell had been sealed, which agrees with the oxygen consumption rates reported by Hand (1990).

Some experiments were performed under an argon atmosphere in order to avoid the presence of large amounts of CO_2 that could potentially interfere with the internal pH of cells and mask the metabolic results (Busa and Crowe, 1983).

Results

Calorimeter sensitivity

The calorimeter utilized showed a typical noise background in the baseline of 0.5 μW total heat released per measuring cell, and its variability in different baseline runs was found to be ±1 μW. Consequently, we can assume that the data collected will have the same magnitude of error. Since we measure minimal amounts of heat, it is necessary to calculate the lowest quantity of energy that can be discriminated from the baseline. Using a two-tailed *t*-test (Sokal and Rohlf, 1981) and the variation of repeated baseline runs, we determined that a minimum of 3 μW can be distinguished from the baseline at $P \leqslant 0.05$, using three replicates or more. Since the calorimeter has four different cells available, we performed all experiments with three replicates of the same sample, leaving the fourth cell as a reference.

If a minimum of 3 μW can be detected from every sample cell, the greater the amount of sample packed inside, the lower is the heat released per unit of mass that can be measured. With untreated whole cysts, about 15nWmg^{-1} drymass could be discriminated from the baseline. When dechorionated cysts are used, the minimal level decreases to 11nWmg^{-1} drymass, since more of the sample is metabolically active as a result of shell removal. Lower values than these cannot be measured with this apparatus.

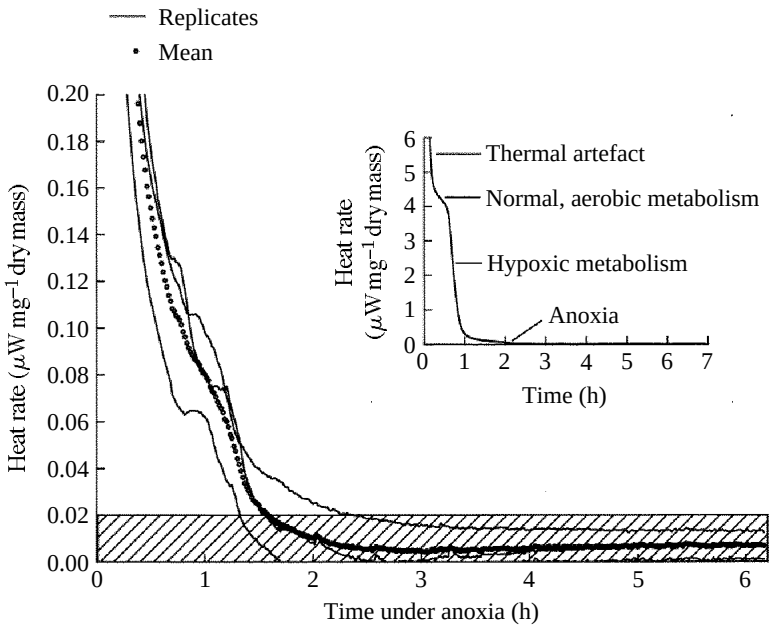

Fig. 1. Rate of metabolic heat dissipation by a sample of *Artemia* cysts from La Mata (Alicante, Spain) during the change from aerobic incubation to anoxia. The shaded area shows the heat values of the mean line that cannot be discriminated from zero ($P \leqslant 0.05$). Times under anoxia in this plot and all the remaining graphs have been computed starting with hypoxic metabolism. The inset shows an interpretation of the results.

Metabolic heat output: a typical experiment

Fig. 1 shows the results of a typical experiment. The inset plot represents the heat dissipated when cysts enter anoxia. The portion of the curve before the first inflection is a thermal artefact that occurs prior to stabilization of the calorimeter. It is followed by a brief period of aerobic metabolism (see Table 1 for details of the differences between aerobic and anoxic heat output). Thus, the heat production seen in Fig. 1 is in good agreement with that reported by Hand and Gnaiger (1988) for cysts under aerobic conditions. Following this brief period of aerobiosis, heat output decreases sharply, falling in about 1.5h below the minimal heat value (approximately $20\,nW\,mg^{-1}$ drymass) that can be statistically distinguished from zero ($P \leqslant 0.05$). After 9 h under anoxia, Hand (1990) reported a rate of heat release of $190\,nW\,mg^{-1}$ drymass in their flow calorimeter. When the cysts are sealed in closed pans, however, heat output falls rapidly, at a rate indistinguishable from that shown in Fig. 1. The effects of this prior aerobic incubation are summarized in Table 1. The pattern shown in Fig. 1 was observed, with small differences in timing, in the remainder of our experiments.

Population differences in metabolic heat output

Fig. 2 shows the rate of heat dissipation of untreated cysts from different samples during the shut down of aerobic metabolism. Samples L88, M88 and SFB show a clear decline below the sensitivity of the calorimeter, although in M88 the decline of metabolic

Table 1. *Heat dissipated from different samples of* Artemia *cysts after 6 or 10h of aerobic incubation followed by anoxic incubation*

Sample	% hatching	Aerobic heat ($\mu W\,mg^{-1}$)	Anoxic heat ($\mu W\,mg^{-1}$)	Time under anoxia (h)	Anoxic heat as % of aerobic values	Lower limit of detection from zero* ($\mu W\,mg^{-1}$)
6 h of aerobic incubation						
L88 untreated	78±1 (3)	3.3±0.2 (5)	<0.016	519	<0.48	0.016
L88 dechorionated	−	−	<0.016	43	<0.48	0.016
L88 argon	−	−	<0.017	44	<0.51	0.017
GSL untreated	56±2 (3)	2.8±0.0 (2)	0.020±0.007 (3)	138	0.68	0.015
GSL dechorionated	−	−	<0.011	31	<0.39	0.011
M88 untreated	78±3 (3)	3.0±0.2 (4)	<0.017	9	<0.57	0.017
D84 untreated	71±2 (3)	3.2±0.2 (4)	0.050±0.001 (3)	45	1.56	0.014
SFB untreated	77±2 (3)	4.4±0.1 (3)	<0.027	9	<0.61	0.027
10h of aerobic incubation						
L88 untreated	−	5.6±0.1 (3)	<0.018	17	<0.32	0.018

Numbers of replicates are given in parentheses.

*Under the conditions of each analysis, this is the minimum value that can be statistically discriminated from zero ($P \leqslant 0.05$). See *Calorimeter sensitivity* in Results for more details.

Values are mean ± S.E.M.

Heat dissipated is given in $\mu W\,mg^{-1}$ drymass; anoxic heat is also given as a percentage of the aerobic value.

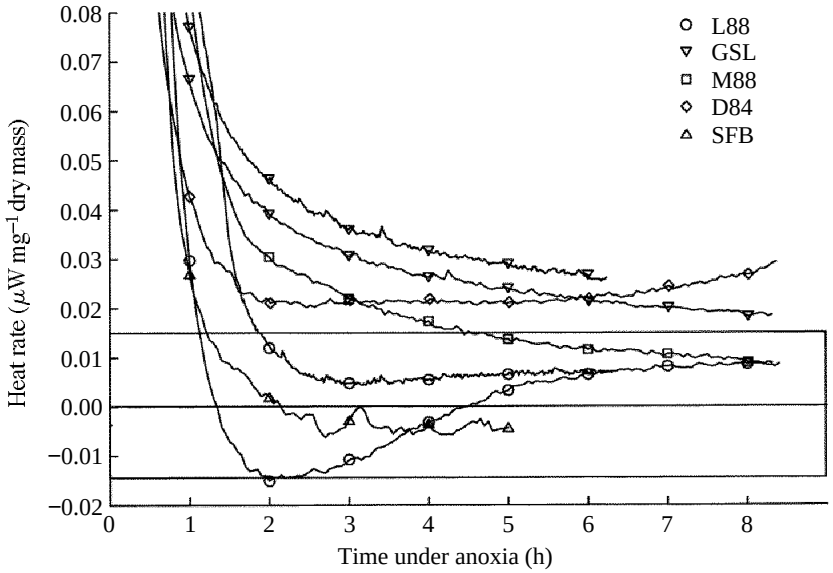

Fig. 2. Metabolic heat dissipated by different samples of untreated *Artemia* cysts during the transition from aerobic incubation to anoxia. Each line is the mean of three replicates. Different lines for the same sample indicate independent runs. The marked area around zero heat encloses the area within which heat values cannot be discriminated from zero ($P \leqslant 0.05$).

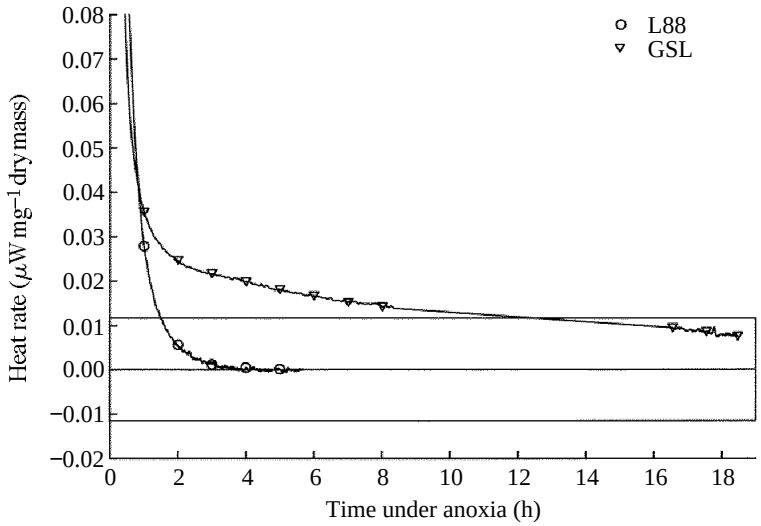

Fig. 3. Metabolic heat dissipated by dechorionated *Artemia* cysts from La Mata (Alicante, Spain) (L88) and Great Salt Lake (Utah, USA) (GSL) during the transition from aerobic incubation to anoxia. Each line is the mean of three replicates. The marked area around zero heat encloses the area within which heat values cannot be discriminated from zero ($P \leqslant 0.05$).

heat is delayed for 2.5h more than in L88 and SFB. This may be a consequence of the slower metabolic rate shown by populations of *A. tunisiana* at 25˚C, since previous studies have suggested that these populations are better adapted for life at lower temperatures (Browne *et al.* 1988; Hontoria, 1990; Vanhaecke *et al.* 1984). The rates of heat dissipation of D84 and GSL do not fall to the point where heat cannot be measured, and readings are always above the minimal statistically detectable value. We will offer an explanation for this seemingly anomalous behaviour below.

Effects of dechorionation on heat output

The chorion of *Artemia* is a set of investment coats that are highly porous. It seemed possible that air might be trapped in these structures and contribute to aerobic metabolism. We tested this possibility by measuring metabolic heat output in cysts from which the chorion had been removed. This treatment, if carefully done, does not affect the viability of the cysts (Bruggeman *et al.* 1980). Fig. 3 illustrates measurements in such cysts. In this case, the same cysts from GSL reach values below the limit of detection, but only after 12h of anoxia. Dechorionated L88 shows a similar pattern to that obtained with untreated L88 cysts (cf. Fig. 2).

Is the depression in metabolic heat due to CO_2 production?

Busa and Crowe (1983) previously reported that addition of CO_2 to *Artemia* cysts depresses intracellular pH, which, in turn, depresses oxygen uptake. Subsequently, Hand and Gnaiger (1988) found that addition of CO_2 depresses metabolic heat output as well. Thus, it seemed possible that the severe depression of metabolic heat output seen here is a result of the build-up of CO_2 in the closed vessels as the O_2 is utilized. In order to test this

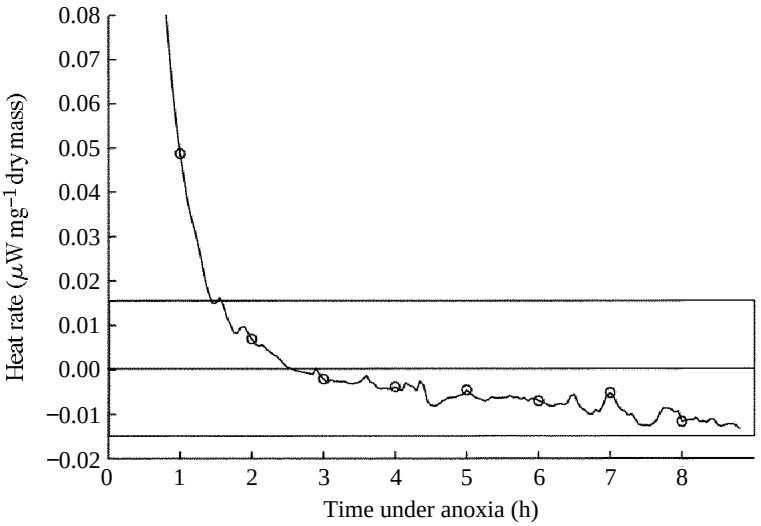

Fig. 4. Metabolic heat dissipated by untreated *Artemia* cysts from La Mata (Alicante, Spain) under anoxic conditions (argon atmosphere). The line is the mean of three replicates. The marked area around zero heat encloses the area within which heat values cannot be discriminated from zero ($P \leqslant 0.05$).

hypothesis, we packed the cysts in the pans after flushing them thoroughly with argon containing less than 0.005% O_2. Fig. 4 shows the results of measurements made under these conditions; the results are essentially identical to those shown in Fig. 1. On the basis of this result, we reject the hypothesis that a build-up of CO_2 is responsible for the depression in metabolic heat output under the conditions used here.

Is depression of metabolic heat output reversible?

Fig. 5 shows the results of experiments in which the cysts were kept under anoxia for extended periods. Except for D84, none of them showed a major change in the pattern of heat dissipation in anoxic conditions after the first sharp decrease. The rate of heat production thereafter appears to remain stable for the duration of the experiment. In some cases, after completing the anoxic experiments, the measuring cells were opened, allowing aeration of cysts, and the measurements were resumed. In every case, the initial metabolic heat output resumed at levels close to those seen in aerobic cysts (data not shown).

Summary of magnitude of depression of metabolic heat output

The differences between the aerobic rate of heat production after 6 or 10h in aerobically hydrated cysts and the rate of heat production after long periods of anoxia are summarized in Table 1. The data shown here represent either a detectable heat output or, if it is below the limit of detection, the lowest rate that could be detected. Heat released after different periods of anoxia ranges between 0.32 and 1.56% of the heat released under normoxia. No effect of the duration of the initial aerobic incubation is observed. Hand (1990) reported comparable values of 0.4% after 6 days of anoxia. In our

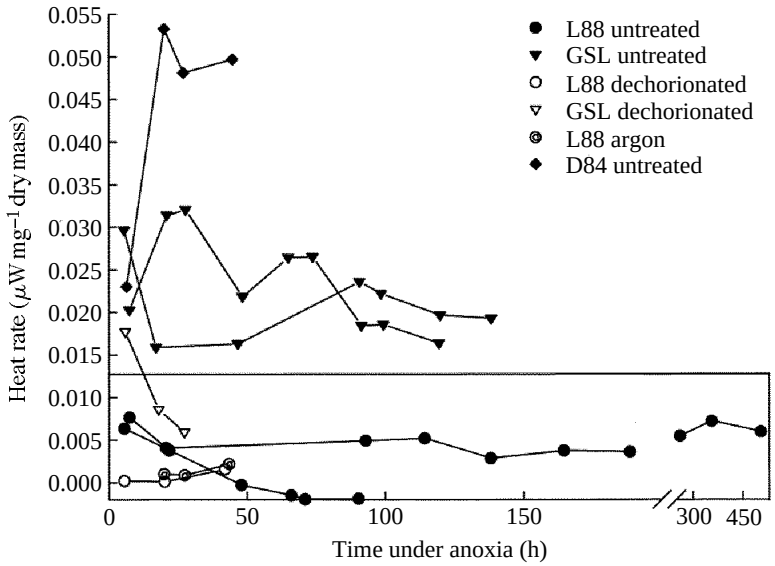

Fig. 5. Metabolic heat dissipated by different samples of *Artemia* cysts during long anoxic incubations. Each point is the mean of three replicates. Different lines for the same sample indicate independent runs. The marked area around zero heat encloses the area within which heat values cannot be discriminated from zero ($P \leqslant 0.05$).

experiments, we studied a wide range of samples and utilized a variety of preparation procedures.

Discussion

The findings presented here show that the results of calorimetric analysis can vary from sample to sample and under different cyst preparation procedures. Therefore, this must be kept in mind when evaluating the metabolic activity of *Artemia* cysts in anoxia.

Although some of the results presented here seem to disagree with the measurements of Hand and Gnaiger (1988) and Hand (1990) in fundamental aspects of the carbohydrate utilization and metabolism by *Artemia* cysts during anoxia, we think that these discrepancies are due to differences in the sources and preparation of the cysts and in differences in the experimental methods used. We summarize those sources of error in the following paragraphs.

Effects of viability

The results obtained with untreated GSL and D84 (Figs 2 and 5) were completely different from those obtained from our other samples, but are comparable to the results published by Hand (1990), who also worked with GSL cysts. At first, we found no apparent reason for these strain differences, but further analyses showed that both samples have a lower hatching percentage than the rest of our samples. In our studies, GSL samples gave a hatching rate of only 56%, comparable to the 60% reported by Hand (1990). Thus, the low hatchability might explain the long delay of GSL cysts in achieving

heat dissipation below detection limits (Fig. 3). We suggest that these samples simply contained fewer metabolizing cysts and that the supply of O_2 in the sealed pans was, therefore, utilized more slowly. At the same time, the high rate of heat release in these samples could be due to hydrolytic exothermic reactions taking place in dead cysts.

Effects of cleaning the cysts

Dechorionated GSL cysts gave results comparable to those for untreated high-hatching-rate samples; unlike the anomalous intact GSL cysts, heat output in the dechorionated ones declined below the limit of detection (cf. Figs 2 and 3). Dechorionation probably eliminates some of the dead, broken cysts, so some of the delay in reaching a low rate of heat output may, again, be attributed to fewer metabolically active cysts in the sample. A related source of error that may be even more important is the presence of external microflora on the cysts, which may release low amounts of heat even under anoxia. Commercial cyst harvesting and processing results in a dirtier material than in the laboratory-scale, small-quantity processing. This seems to be the case for GSL cysts. D84 is an older sample that was not as clean as the newer ones, so it can also be considered to exhibit these characteristics. This accompanying material could also be responsible for the increase in the rate of heat dissipation in D84 with longer exposure to anoxia. If we dechorionate GSL, eliminating the external microflora, the 'anoxic' heat detected in untreated cysts decreases considerably.

Maintenance of anoxic conditions

Another likely source of error is the maintenance of truly anoxic conditions. In our system, inadvertent admission of O_2 is out of the question, since in the closed and isolated environment of the measuring cell of the calorimeter, once the oxygen is exhausted, anoxia cannot be avoided. We suggest that maintenance of such strictly anoxic conditions in the open-flow system used by Hand and Gnaiger (1988) and Hand (1990) would be extremely difficult and that this could be a major source of error.

In the light of the results reported here, we conclude that the measurement of heat released from anoxic *Artemia* cysts is affected by the sample viability and accompanying materials, as well as the preparation procedures and methodology utilized. Attention must be paid to all these points when evaluating the cyst metabolic activity. The heat dissipated by different samples of anoxic cysts, according to our results, is much lower than previously reported (Hand, 1990). Residual metabolic activity cannot be ruled out, but in any case it is lower than $11 nW mg^{-1}$ drymass at 25°C and probably very close to zero. This rate of energy production is not consistent with an ATP-producing anaerobic glycolytic pathway, diverted to the maintenance of membranes and synthesis of macromolecules. The required energy for all these processes would exceed, by several orders of magnitude, that supplied by a catabolism with the heat dissipation measured in this study. Transcription and protein synthesis under these conditions is suppressed, so the ATP would not be needed (Hochachka and Guppy, 1987; Clegg and Conte, 1980).

The suggestion that the anoxic cysts are ametabolic is clearly consistent with the observation that the embryos can survive extended periods of anoxia (Dutrieu and Chrestia-Blanchine, 1966; Clegg, 1992) and with direct observations of the lack of

substrate utilization (Clegg and Jackson, 1989*a*,*b*) and the lack of an oxygen debt (Drinkwater and Clegg, 1991) in cysts following extended periods of anoxia. However, the intriguing question of how higher-order cellular structure is maintained in such ametabolic cysts awaits further investigation that should be directed to non-energy-consuming mechanisms, since the currently accepted explanations based on maintenance of low metabolic activity under anoxia are not consistent with the evidence presented here.

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