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SHORT COMMUNICATION

LONG-TERM ANOXIA IN *ARTEMIA* CYSTS

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Among the many adaptations associated with the rigorous life history of the brine shrimp, *Artemia*, is the striking resistance of the encysted embryo (cyst) to oxygen lack (anoxia). Dutrieu and Chrestia-Blanchine (1966) reported that these cysts could be incubated in sea water under anoxic conditions for over 5 months without a decrease in viability. Subsequent work showed that anoxic cysts did not carry out a conventional lactate-producing metabolism and that the breakdown of trehalose, a disaccharide required for the energy metabolism of aerobic cysts, was not detected over an 8-h period of anoxia (Ewing and Clegg, 1969). Comprehensive studies of the nucleotide pool by Stocco *et al.* (1972) suggested that utilization of the unusual guanine nucleotide diguanosine tetraphosphate (Gp_4G) might provide the free energy presumably required to support the maintenance of anoxic cysts. Most recently, Hand and Gnaiger (1988) used calorimetric methods to show that anoxic energy metabolism, as reflected by heat production, was reduced to less than 2 % of aerobic values during short-term anoxia (10 h). Those authors also calculated that the utilization of Gp_4G could account for only about 2 % of anoxic heat production and suggested that the very slow catabolism of trehalose might be a more likely explanation for their results. We examined that suggestion by measuring the concentrations of trehalose and other carbohydrates in cysts incubated under anoxic conditions for 3 months (Clegg and Jackson, 1989). We found no measurable change in trehalose and glycerol content under these conditions, but did observe a statistically significant decrease in the glycogen content of anoxic cysts. In the present paper we report comparable data from additional studies on anoxic cysts incubated for 3 and 6 months, and we measure the free amino acid pool, cyst dry mass and hydration.

Cysts of *Artemia fransiscanus* were collected from the solar salt ponds near Hayward, California, in the summer of 1987 and were processed and dried as previously described (Clegg, 1986). Sea water containing penicillin (2000 units ml^{-1}) and streptomycin (0.1 mg ml^{-1}) was passed through 0.1 μm filters and gassed with 100 % N_2 for 3 h. Air-dried cysts (about 80 mg) were placed in 5 ml glass tubes and incubated dry at room temperature (about 23 °C) under four changes of 100 % N_2 for 3 days to remove air trapped in their shells. Anoxic sea

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water (3.5 ml) was added to tubes containing cysts which were then sealed with rubber stoppers covered by several layers of Parafilm and incubated on their sides at room temperature (20–23 °C) under laboratory conditions of lighting. Control cysts (zero time) were hydrated for 24 h in sea water at 0 °C. Cysts were harvested, extracted with 72 % ethanol, and centrifuged as described previously (Clegg and Jackson, 1989). Trehalose and glycerol in the ethanol-soluble fraction were measured with a high-performance liquid chromatography assembly equipped with a differential refractometer; glycogen was extracted from the ethanol-insoluble pellet and assayed colorimetrically. All these procedures, and determinations of cyst water content and viability, have been described in detail (Clegg, 1986; Clegg and Jackson, 1989). Free amino acids in the ethanol-soluble fraction were estimated with a fluorescamine colorimetric assay (Udenfriend *et al.* 1972).

Fig. 1 shows that glycogen was the only carbohydrate whose level changed during 3 months of anoxic incubation. Thus, it is possible that glycogen breakdown might contribute to the heat production observed by Hand and Gnaiger (1988) during short-term anoxia. However, no change was detected in the level of any carbohydrate between 3 and 6 months, indicating that no net catabolic utilization

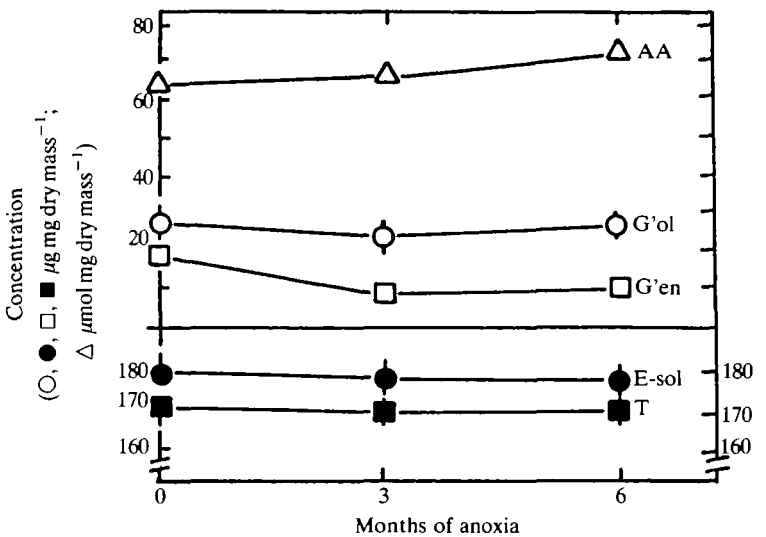

Fig. 1. Concentrations of various compounds in *Artemia* cysts incubated in sea water under anoxic conditions. AA, free amino acids; G'ol, glycerol; G'en, glycogen; E-sol, ethanol-soluble carbohydrate; T, trehalose. Each point is the mean (±standard deviation) for cysts in three independent incubation tubes. Lack of a vertical bar indicates the standard deviation is within the data point. One-way analyses of variance for each of the compounds measured, followed by appropriate *a posteriori* testing (SNK), revealed that glycogen contents in 3- and 6-month cysts, while not different from each other, were significantly less than values in unincubated cysts ($P<0.01$). Free amino acid content was greater in 6-month cysts than in 3-month and unincubated cysts ($P<0.01$), which were not significantly different from each other. No other significant differences were revealed.

of carbohydrate took place during this interval of anoxia. These results do not support the suggestion that trehalose may be a substrate for long-term anoxic energy metabolism, but indicate instead that such a metabolism, if indeed one exists in these cysts, is not based on carbohydrate breakdown after 3 months of incubation. Although we cannot rule out the presence of some unknown ethanol-insoluble carbohydrate that plays such a role, there is no evidence for the existence of such a compound.

Because several amino acids have been implicated as substrates for the anoxic metabolism of certain other invertebrates (for review see Hochachka and Guppy, 1987) we also examined the free amino acid content of anoxic *Artemia* cysts (Fig. 1). However, instead of a decrease, a small but statistically significant increase had occurred by the sixth month of anoxia. Little more can be said until further study is made.

Studies on the hydration and dry mass of cysts incubated under anoxic conditions revealed no measurable changes in these parameters (Table 1). The trivial decrease in dry mass (0.5–1 %) was similar for 3 and 6 months and can be attributed to a few cysts lost during sample processing [3.6 μg is the average dry mass per cyst (Clegg, 1974)]. Thus, if a long-term anoxic metabolism exists in these cells it does not result in the loss of detectable levels of a volatile end-product, or in the production of monomers from polymers that would be sufficient to increase cyst water content.

We also evaluated the viability of cysts after 6 months of anoxia by measuring nauplii production after 4 days of subsequent incubation in sea water under aerobic conditions. Previously anoxic cysts produced 55±2 % nauplii compared with 89±3 % for controls (standard deviations are given for three samples of at

Table 1. *Dry mass and hydration of* Artemia *cysts incubated in sea water under anoxic conditions*

Months of anoxia	Dry mass of cysts (mg)			Cyst hydration ($g H_2O$ g dry mass^{-1})
	At start	At finish	F/S	
0	–	–		1.315±0.041
3	51.39	51.13	0.995	1.307±0.020
	50.28	50.01	0.995	
	50.20	49.66	0.989	
6	50.68	50.38	0.994	1.310±0.010
	50.93	50.53	0.992	
	50.33	50.08	0.995	

The column 'At start' refers to the mass of cysts equilibrated over $CaSO_4$ which were then incubated in sea water for the period shown and then re-dried over $CaSO_4$ after washing the cysts free from sea water ('At finish').

F/S refers to the ratio of these dry masses.

Cyst hydrations are for means±standard deviations (N=3).

least 100 cysts). This mortality rate is slightly greater than that reported by Stocco *et al.* (1972) and much higher than observed by Dutrieu and Chrestia-Blanchine (1966).

In summary, we found no evidence in these studies for the utilization of trehalose during anoxia and no evidence that any carbohydrate is involved as a substrate for anoxic energy metabolism in these cysts between 3 and 6 months of anoxia. It is awkward, at best, to deal with experiments concerned with the potential absence of something, and we realize that no firm conclusion can be made in this regard. Nevertheless, the possibility clearly exists that these cells do indeed bring their metabolism to a complete standstill. That suggestion appears to be in conflict with widely accepted views which require a constant input of free energy (in the form of phosphoryl group transfer) for maintenance of cell integrity. In that regard, we note that the concentrations of the wide variety of nucleotides, and total phosphoryl-bond content, measured by Stocco *et al.* (1972) clearly are levelling off during the longest interval of anoxia they studied (2–3.7 months). A more direct test of this 'complete shut-down hypothesis' requires direct measurement of the turnover of phosphoryl-groups in anoxic cysts; unfortunately, the extraordinary impermeability of these cysts, even to inorganic phosphate, will make that approach very difficult.

Finally, we return to the excellent microcalorimetric measurements of Hand and Gnaiger (1988). The caveat must first be made that they used cysts from the Great Salt Lake, Utah, a source different from ours. Given that, they observed very low but measurable rates of heat production during relatively short periods (10 h) of anoxia; however, these rates were still falling when their study was terminated. Whether heat production actually reaches some low plateau or goes to zero during longer periods of anoxia can only be evaluated by further study. We wish to point out, however, that even the existence of a low rate of heat production in anoxic cysts is not by itself adequate proof for the existence of an 'energy metabolism', if by 'metabolism' we mean the operation of regulated and integrated enzyme-catalysed chemical reactions that are controlled by, and contribute to the maintenance of, the cells in which they occur. Thus, adventitious chemical reactions exhibiting negative enthalpies might account for very low rates of heat production, notably in cyst populations exhibiting large percentages of non-viable embryos. Such adventitious chemistry occurs in all materials, non-living and living, at ordinary temperatures, but hardly constitutes a metabolism; otherwise, the cosmos 'metabolizes' – an interesting idea, but one not usually entertained.

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