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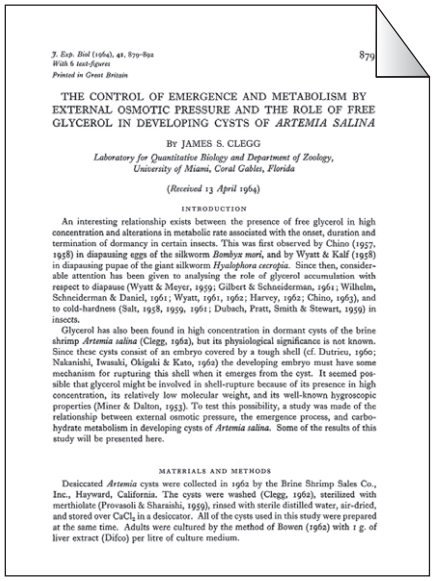
TREHALOSE AND ANHYDROBIOSIS: THE EARLY WORK OF J. S. CLEGG



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THE CONTROL OF EMERGENCE AND METABOLISM BY EXTERNAL OSMOTIC PRESSURE AND THE ROLE OF FREE GLYCEROL IN DEVELOPING CYSTS OF *ARTEMIA SALINA*

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INTRODUCTION

An interesting relationship exists between the presence of free glycerol in high concentration and alterations in metabolic rate associated with the onset, duration and termination of dormancy in certain insects. This was first observed by Chino (1957, 1958) in diapausing eggs of the silkworm *Bombyx mori*, and by Wyatt & Kalf (1958) in diapausing pupae of the giant silkworm *Hyalophora cecropia*. Since then, considerable attention has been given to analysing the role of glycerol accumulation with respect to diapause (Wyatt & Meyer, 1959; Gilbert & Schneiderman, 1961; Wilhelm, Schneiderman & Daniel, 1961; Wyatt, 1961, 1962; Harvey, 1962; Chino, 1963), and to cold-hardness (Salt, 1958, 1959, 1961; Dubach, Pratt, Smith & Stewart, 1959) in insects.

Glycerol has also been found in high concentration in dormant cysts of the brine shrimp *Artemia salina* (Clegg, 1962), but its physiological significance is not known. Since these cysts consist of an embryo covered by a tough shell (cf. Dutrieu, 1960; Nakanishi, Iwasaki, Okigaki & Kato, 1962) the developing embryo must have some mechanism for rupturing this shell when it emerges from the cyst. It seemed possible that glycerol might be involved in shell-rupture because of its presence in high concentration, its relatively low molecular weight, and its well-known hygroscopic properties (Miner & Dalton, 1953). To test this possibility, a study was made of the relationship between external osmotic pressure, the emergence process, and carbohydrate metabolism in developing cysts of *Artemia salina*. Some of the results of this study will be presented here.

MATERIALS AND METHODS

Desiccated *Artemia* cysts were collected in 1962 by the Brine Shrimp Sales Co., Inc., Hayward, California. The cysts were washed (Clegg, 1962), sterilized with merthiolate (Provasoli & Sharaishi, 1959), rinsed with sterile distilled water, air-dried, and stored over $CaCl_2$ in a desiccator. All of the cysts used in this study were prepared at the same time. Adults were cultured by the method of Bowen (1962) with 1 g. of liver extract (Difco) per litre of culture medium.

John Crowe discusses Jim Clegg's 1964 paper: The control of emergence and metabolism by external osmotic pressure and the role of free glycerol in developing cysts of Artemia salina. *A copy of the paper can be obtained from http://jeb.biologists.org/cgi/reprint/41/4/879.*

The brine shrimp, *Artemia salina*, produces encysted embryos that are capable of surviving complete dehydration – or at least as complete as is technically feasible; according to the best measurements available, they can be reduced to <0.007 g water g^{-1} dry mass (Clegg, 1978), at which water contents metabolism as we understand it ceases (Clegg, 1973). The dry cysts persist in this state, known as 'anhydrobiosis' for decades (reviewed in Clegg and Conte, 1980), but when they are rehydrated they rapidly imbibe water and resume active metabolism and development. Following further development, they then emerge from the cysts as free-swimming nauplii. Many of the important papers on these interesting animals have appeared in *The Journal of Experimental Biology*, including this JEB Classic from my friend and colleague Jim Clegg, in which he studied the metabolic events occurring during the resumption of development, implicating trehalose as a key molecule involved in the survival of desiccation, which eventually sparked a revolution in the preservation of biological systems.

It was already known that *Artemia* cysts are capable of undergoing development at a variety of salinities in nature. Clegg found under carefully controlled laboratory conditions that development proceeded normally under a wide range of external osmotic pressures; at pressures up to 30 atm (1 atm is ~101 kPa) the only effect on development was to decrease the rate, but not the final success at emergence. Some cysts were seen to complete development at pressures as high as 65 atm. This surprising discovery clearly meant that when the cysts were exposed to elevated external osmotic pressures they most likely balanced the osmotic gradient by synthesis of solutes, so he next studied the metabolic fates of the predominant osmotically active solutes – glycerol and trehalose – during development. He found that trehalose was utilized as a metabolite and at low external osmotic pressures was converted to glycogen. However, at high external osmotic pressures the trehalose was converted to glycerol, and under all hyperosmotic conditions a large net synthesis of glycerol was observed prior to emergence. So why was all this glycerol synthesized? Clegg suggested that not only was the glycerol used as an osmotic effector, but also it was accumulated near the end of development, thus increasing internal osmolarity to the point where the cysts swelled and ruptured, permitting emergence of the nauplii (Clegg, 1964).

In a closely related paper published the following year he studied metabolism in *Artemia* embryos of two types: one enters a dormant stage, encysts, and can survive dehydration; the other undergoes direct development without a dormant stage and does not survive drying (Clegg, 1965). He discovered in this technically difficult study that the pre-dormant cysts synthesized large quantities of trehalose (in fact, they converted as much as 15% of their dry mass to this sugar), while the non-dormant ones contained essentially no trehalose. The dormant and non-dormant cysts also contained glycerol, but the dormant ones had about twice as much as the non-dormant ones. He speculated at the time that the trehalose was an energy source and was utilized mainly because of its high stability, but he offered no explanation for the presence of the massive amounts of glycerol other than for osmotic rupturing of the cyst at the completion of development. However, the clear correlation between the presence of glycerol and trehalose in the dormant cysts and survival in the dry state suggested in retrospect that these molecules might be related to the cysts' ability to survive drying. At the time, the thinking of all of us interested in the phenomenon of anhydrobiosis was influenced by the well-known discovery in the field of cryobiology that glycerol is a potent cryoprotectant, so we assumed – incorrectly, as it turned out –

that glycerol would be the important molecule in stabilizing biological structures during anhydrobiosis.

Where did Clegg go from there? He continued to work with *Artemia*, but mostly as a model for investigations on the status of intracellular water, profoundly influencing our understanding of metabolic complexes and the channeling of metabolism (see Clegg, 1991a; Clegg, 1991b). His work from the 1960s (Clegg, 1964; Clegg, 1965), which gave us an interesting metabolic and developmental picture in its own right, might well have been forgotten, except that it turned out to have more general significance than he realized at the time.

A decade passed before much more progress was made in sorting out the roles of glycerol and trehalose. In the 1970s we discovered that biological membranes can be stabilized during drying in the presence of trehalose, but not glycerol; in fact, trehalose was clearly superior to every other sugar tested [but see some addenda to these findings in Crowe et al. (Crowe et al., 2001)]. We and others then promptly found that liposomes and proteins can be preserved by drying with trehalose, both of which findings led to many commercial products, particularly in the pharmaceutical industry. For instance, Ambisome is a liposomal based therapeutic agent for treating systemic fungal infections that is delivered as a dry powder, using technology that came out of our studies on the stabilization of liposomes by trehalose.

By the mid-1980s we had worked out a possible mechanism for these stabilizing effects, which is still being tested experimentally and by molecular dynamics (reviewed in Crowe, 2007). Based on experimental biophysical data, we proposed that trehalose serves as a water replacement in the dry state, conferring on membranes and proteins physical properties that resemble those seen in the fully hydrated state. Further, we proposed that this effect involves direct interaction between the trehalose and polar groups on membrane lipids and proteins by hydrogen bonding, a suggestion that came to be known as the water replacement hypothesis. Experimental data have shown that higher order structures in membranes such as rafts can be stabilized by drying with trehalose, a phenomenon that can be explained by the water replacement hypothesis (Leidy et al., 2004).

In fact, the level of complexity that can be preserved in the dry state is startling; we have applied these findings to human blood cells, particularly platelets. We found a way of introducing trehalose into platelets and discovered that the trehalose-loaded platelets survive freeze drying, with a greatly extended shelf life. The rehydrated platelets show complex responses such as Ca^{2+} and H^{+} transport in response to agonists and clot formation (reviewed in Crowe et al., 2001; Crowe, 2007). These higher order responses require that several receptor and signaling processes be maintained intact. Numerous laboratories around the world are involved in developing this technology for the preservation of a variety of cells of interest in biology, agriculture and medicine, and several trehalose-stabilized freeze-dried products are currently in various stages of development and clinical trials for human use.

In recent years, Clegg returned to the field of cellular preservation, and his discovery and purification of certain stress proteins in *Artemia* (e.g. Clegg, 2005) are beginning to play a role in the stabilization of eukaryotic cells in the presence of trehalose (Ma et al., 2005). One word of caution: trehalose is being applied in many areas where it is inappropriate; for example, the cosmetics industry is adding trehalose to their products, often with no clear rationale for doing so. Also, while trehalose does have some special properties that make it useful (reviewed in Crowe et al., 2001; Crowe, 2007), the same result can be achieved with other sugars under ideal conditions.

Clegg's papers from the early 1960s pointed the way to a new field of inquiry that is currently remarkably active, with implications as far ranging as ecology and human medicine. His 1964 paper is a classic in many realms, which will continue to inspire.

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