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Review

Cryptobiosis — a peculiar state of biological organization ☆

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Abstract

David Keilin (Proc. Roy. Soc. Lond. B, 150, 1959, 149–191) coined the term 'cryptobiosis' (hidden life) and defined it as 'the state of an organism when it shows no visible signs of life and when its metabolic activity becomes hardly measurable, or comes reversibly to a standstill.' I consider selected aspects of the 300 year history of research on this unusual state of biological organization. Cryptobiosis is peculiar in the sense that organisms capable of achieving it exhibit characteristics that differ dramatically from those of living ones, yet they are not dead either, so one may propose that cryptobiosis is a unique state of biological organization. I focus chiefly on animal anhydrobiosis, achieved by the reversible loss of almost all the organism's water. The adaptive biochemical and biophysical mechanisms allowing this to take place involve the participation of large concentrations of polyhydroxy compounds, chiefly the disaccharides trehalose or sucrose. Stress (heat shock) proteins might also be involved, although the details are poorly understood and seem to be organism-specific. Whether the removal of molecular oxygen (anoxybiosis) results in the reversible cessation of metabolism in adapted organisms is considered, with the result being 'yes and no', depending on how one defines metabolism. Basic research on cryptobiosis has resulted in unpredicted applications that are of substantial benefit to the human condition and a few of these are described briefly.

Keywords: Anhydrobiosis; Anoxybiosis; Biochemical adaptation; Trehalose; Vitrification; Water replacement hypothesis; Metabolic rate depression; p26; Molecular chaperone; Small heat shock/α-crystallin protein

1. Introduction and brief history

In 1959 Keilin (1959), published a benchmark review on 'cryptobiosis', a term he coined and defined as '..the state of an organism when it shows no visible signs of life and when its metabolic activity becomes hardly measurable, or comes reversibly to a standstill.' As we shall see, the difference between a 'hardly measurable' metabolism and one that is at a 'reversible standstill' is of considerable significance. Keilin noted that cryptobiosis resulted from such things as desiccation (anhydrobiosis), low temperature (cryobiosis), lack of oxygen (anoxybiosis) or combinations of these. I will focus chiefly on animal anhydrobiosis. This capability has been achieved by representatives of many invertebrate taxa, notably in embryonic cysts of primitive crustaceans, adult rotifers, nematodes and tardigrades

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and the eggs or embryos of some species in the last three taxa (Crowe and Clegg, 1973, 1978; Orstan, 1998; Ricci, 1998). Of course, many prokaryotes (Potts, 1994 and Potts, 1999; Seckbach, 1999), plant seeds and other propagules exhibit anhydrobiosis (Priestley, 1986; Vertucci and Farrant, 1995; Ingram and Bartels, 1996; Chandler and Bartels, 1999; Alpert, 2000) and even the vegetative tissues of certain higher plants have achieved this ability, the remarkable 'resurrection plants' (Tomos, 1992; Scott, 2000). No example of anhydrobiosis in the early developmental stages of vertebrates has been published. This is curious since so many invertebrate embryos have achieved this ability.

Although Keilin is best known for his work on cytochromes and cellular respiration, we are fortunate that he also was interested in the topic of this essay and described its history in such scholarly fashion (Keilin, 1959). I consider some highlights of this history, which goes back approximately 300 years to the time of Antony van Leeuwenhoek who apparently discovered the phenomenon. Using the microscope he developed, van Leeuwenhoek noticed that dried sediment from the gutters of houses gave rise to 'animalcules', as he called them (most likely rotifers or nematodes) shortly after the addition of water. Although he did a number of simple but ingenious experiments he neither claimed that the animalcules were completely desiccated nor did he raise the issue of their biological (metabolic) status.

Science moved slowly in those days and it took approximately half a century for a resurgence of interest, this time with the involvement of such luminaries of their day as John Turberville Needham, Henry Baker and Lazzaro Spallanzani (who seems to have been interested in just about everything). Much of this work focused on nematodes and rotifers and their ability to reversibly desiccate, often referred to as 'resuscitation' or 'resurrection'. During the last quarter of the 18th century, debates arose concerning the status of these dried organisms. Were they dead, but resurrected by restoration of their lost water? Did they never really stop living, but simply slowed, very greatly, their vital functions? Following a two decade decline of interest in these issues a heated discussion developed between two French biologists, F. Pouchet and P. Doyère, whose results on rotifers and tardigrades did not agree. Their positions were clear — Pouchet: no organism can survive complete desiccation, or return to life, once all life processes have been arrested; Doyère: such organisms can be revived after complete desiccation and the cessation of their life processes. The debate attracted interest, in part because, as Keilin (1959) documented, it was related to the major discussion over spontaneous generation in the mid-1800s. In 1859 the two protagonists approached the Biological Society of France to evaluate their claims. The Society agreed and appointed a special seven-man commission composed of such prominent scientists as Balbiani, Berthelot, Broca and Brown-Séquard. The commission submitted a 60 000 word report in March 1860, written by Paul Broca and signed by all seven members (Broca, 1860). Keilin refers to Broca's prose as 'written with great eloquence and a tendency to dramatization, which is already reflected in the motto 'To be or not to be, that is the question'.' Interestingly, the Commission did not take a firm position either way, although leaning toward the views of Doyère. Curiously, little attention was given in the report to the relationship between resuscitation and the debate over spontaneous generation, which was at its peak in 1860. Keilin makes the remarkable point that while these debates were at the forefront of French science, the appearance of Charles Darwin's 'Origin of Species' in 1859 'Produced scarely a ripple in the Académie des Sciences and in other learned societies of France' (Keilin, 1959).

Keilin's review seems to have restored interest in the phenomenon, a major meeting was held on cryptobiosis in 1961 (Grossowicz et al., 1961), followed approximately 10 years later by a review (Crowe, 1971) and book (Crowe and Clegg, 1973) and the published proceedings of two subsequent scientific meetings (Crowe and Clegg, 1978; Leopold, 1986). Since that time a large body of literature has accumulated, scattered amongst a variety of disciplines. In many cases, the authors do not use Keilin's terminology and some seem unaware of the phenomenon, per se, despite the relevance of their research to this topic.

2. Terminology-more than semantics

Keilin pointed out the ambiguities contained in most of the earlier terms and their definitions. He also made a distinction between dormancy (hypo-

metabolism) and latent life (ametabolism) for which the term cryptobiosis was proposed. At this point I consider an issue that is central to the significance of cryptobiosis and alluded to in the Introduction, namely, whether metabolism ever comes to a reversible *standstill*. I suspect Keilin qualified his definition by inserting 'and when its metabolic activity becomes *hardly measurable*' (my italics) in his definition. Perhaps he recognized the difficulty (impossibility?) of proving the absence of metabolism by experiment, although he never mentioned this problem. Before considering that point further, the conditions leading to cryptobiosis require attention. Keilin used the following terms and conditions: anhydrobiosis (dehydration); cryobiosis (cooling); anoxybiosis (oxygen lack); and osmobiosis (high salt concentration). Not choosing to haggle over the vagueness of the latter term, I adopt Keilin's terminology here.

3. Anhydrobiosis

3.1. Biological significance

Anhydrobiosis tells us something fundamental about the basic nature of living systems. Consider that an organism in anhydrobiosis lacks all the dynamic features characteristic of living organisms, notably due to the lack of an ongoing metabolism to transduce energy and carry out biosynthesis. In that sense it is not 'alive,' yet neither is it 'dead' since suitable rehydration produces an obviously living organism. Therefore, we may deduce that it is the structural organization of cells and organisms, rather than their dynamics, that represents the most fundamental feature of living systems. However, to draw that conclusion one must prove that severe dehydration does indeed reversibly 'stop metabolism.' I have previously (see Crowe and Clegg, 1973; Clegg, 1986) given reasons why one is compelled to conclude that the removal of all but, say, 0.1 g H_2O/g dry weight (easily achieved by anhydrobionts), will inevitably result in the cessation of metabolism. For example, one can calculate that this amount of water is insufficient to hydrate intracellular proteins, without which a metabolism is obviously not possible. If such a desiccated organism has been adapted for this journey, it is anhydrobiotic, if not, it is dead. Central to these matters also is the definition of 'metabolism'. It should be appreciated (Clegg, 1986) that metabolism is not merely the presence of chemical reactions in anhydrobionts, indeed, those are inevitable at ordinary biological temperatures. It seems reasonable to require that a metabolism must consist of systematically controlled pathways of enzymatic reactions, governed in rate and direction, integrated and under the control of the cells in which they are found. An additional requirement concerns the transduction of free energy from the environment and its coupling to endergonic processes such as biosynthesis and ionic homeostasis. Thus, the severely desiccated anhydrobiont is indeed reversibly ametabolic and we may conclude that there are three states of biological organization: alive; dead; and cryptobiotic.

Less philosophical and more appropriate to current research, is the mechanistic basis of anhydrobiosis, how do these organisms survive the removal of virtually all cellular water, a condition that rapidly destroys non-adapted forms? We know something about the answer.

3.2. Mechanisms: the water replacement hypothesis (WRH) and vitrification

No attempt will be made to cover adaptations underlying anhydrobiosis at all levels of organismic organization. These are important, of course, involving such things as specialized integuments that slow water loss and provide mechanical protection. My coverage focuses on biochemical and biophysical adaptations that seem to be of general importance rather than organism-specific. In addition, I note that this will not be a detailed review of the large body of literature on the subject, but reviews and selected papers will be cited that allow ready access.

A generality emerging over the last three decades is the central involvement of high concentrations of various polyhydroxy compounds, sometimes referred to as 'compatible solutes' (Yancey et al., 1982; Somero and Yancey, 1997). Disaccharides play prominent roles, trehalose in microbes, animals and lower plants (Yancey et al., 1982; Vertucci and Farrant, 1995; Ingram and Bartels, 1996; Chandler and Bartels, 1999; Alpert, 2000) and sucrose in higher plants (Crowe and Clegg, 1973; Elbein, 1974; Majara et al., 1996;

Potts, 1994, 1999; Behm, 1997; Crowe et al., 1997; Goddijin and van Dun, 1999; Alpert, 2000), although other compounds are involved to a lesser extent. In general, these sugars protect macromolecules and membranes against the destructive effects of water removal by replacing the primary water of hydration and through the formation of amorphous glasses (vitrification). These mechanisms are not mutually exclusive and the evidence indicates that both are of adaptive importance in anhydrobiosis (Crowe et al., 1998a; Sun and Leopold, 1997).

Earlier studies revealed that trehalose was present in high concentrations in the dormant stages of various organisms, but not in their active life history stages (Clegg and Filosa, 1962; Birch, 1963), however, the full significance of that correlation was not realized at the time (Clegg, 1965). During the 1960s two seminal groups of observations were made, one by Sydney Webb who showed that the addition of certain polyhydroxy compounds could protect non-adapted bacteria against death due to desiccation (Webb, 1965) and the other by Donald Warner whose molecular modeling suggested that some of these same (or similar) molecules might 'fit' the hydration lattice of proteins and possibly other large molecules (Warner, 1962). The modeling was primitive compared with current methodology, yet, when combined with Webb's results and the biological correlation between dormant stages and trehalose accumulation, the stage was set for the next phase of study which would lead to the 'water replacement hypothesis' (WRH).

Research during the 1970s and 1980s added substantial but indirect evidence that trehalose might be serving as a water substitute in anhydrobiotic animals. Direct evidence that this can actually take place was obtained by Crowe and colleagues using model systems in vitro in an extensive series of studies (see Crowe et al., 1996, 1997, 1998a,b). Evidence for its occurrence in vivo was also obtained (Clegg, 1986) using encysted embryos (cysts) of the primitive crustacean, *Artemia franciscana*, which contain approximately 15% of their dried weight as trehalose (Clegg and Conte, 1980). These encysted embryos have been studied in detail (Persoone et al., 1980; Decleir et al., 1987; MacRae et al., 1989; Warner et al., 1989; Browne et al., 1991) and shown to exhibit phenomenal stress resistance (Clegg and Conte, 1980; Clegg and Jackson, 1992). They tolerate virtually complete desiccation (Clegg and Drost-Hansen, 1990), cycles of desiccation-rehydration (Morris, 1971) and when dry survive the conditions of outer space (Gaubin et al., 1983). Trehalose is clearly involved with the extraordinary stability of these embryos in the face of severe environmental stress. Later I consider their impressive abilities to undergo anoxybiosis.

The ability of trehalose and sucrose to form amorphous glasses at low water contents (vitrification) is a significant characteristic of these sugars (Crowe et al., 1996, 1998a; Sun and Leopold, 1997) although there is some discussion about the extent to which this occurs in vivo (see Reid, 1998). Of major importance is the enormous slowing of the diffusion coefficients of metabolites and macromolecules present in these glasses and it is possible that their translational motion could essentially cease (Potts, 1994, 1999). Vitrification taking place in anhydrobiotic forms would be of major importance in the avoidance of deleterious interactions between intracellular components that would otherwise take place as water is removed, something referred to previously as 'proximity effects' (Clegg, 1974).

An important feature of the WRH that has not been emphasized is that the same mechanisms that preserve cell structure, also prevent their function (Clegg, 1974, 1986). In the case of enzymes, that is of critical importance since uncontrolled catalytic activity would be disasterous over the often prolonged duration of anhydrobiosis. In addition, molecules embedded in a sugar/protein glass should gain substantial chemical stability compared to their counterparts diffusing freely in aqueous solution.

3.3. Are stress (heat shock) proteins involved in anhydrobiosis?

Extensive evidence shows that several families of heat shock/stress proteins (SPs) serve as molecular chaperones to assist the folding of newly synthesized proteins, protect them from stress-associated denaturation/aggregation, aid in their renaturation and influence the final intracellular location of mature proteins (Jakob et al., 1993; Parsell and Lindquist, 1993; Ellis and Hartl, 1999; Feder and Hofmann, 1999; Ellis, 2000; Feldman and Frydman, 2000; MacRae, 2000). In view of their critical involvement in protection of cellular proteins against damage from such things

as temperature extremes, strong oxidizing agents, anoxia and heavy metals it would seem reasonable to suppose that they would also be involved in the ability of organisms to reversibly desiccate. This potential relationship is particularly difficult to resolve, so the question asked in the heading to this section is by no means rhetorical. What follows refers exclusively to yeast and plants where it seems that virtually all of the work on this topic has been done. To my knowledge, no study has been published on the participation of SPs in animal anhydrobiosis.

The classic stress (heat shock) response is initiated by abnormal proteins, resulting eventually in increased SP levels and/or the synthesis of new isoforms, the usual result being the acquisition of enhanced stress tolerance (Parsell and Lindquist, 1993). The situation is less clear in cells adapted to undergo reversible desiccation, surely a major stress but complicated by several differences between water loss and the other stresses listed above. For a start, the metabolic shut-down in dried cells, seeds for example, does not permit them to launch a stress response. If protein denaturation occurs during desiccation or rehydration, we can expect that a stress response will be launched when the hydrated cells resume metabolism. However, it is by no means obvious how one sorts out pathways leading to desiccation tolerance and repair from those concerned with non-related aspects such as normal developmental processes.

Many proteins and nucleic acids are denatured by removal of water in vitro (Colaço et al., 1994; Aguilera and Karel, 1997; Crowe et al., 1997; Allison et al., 1999), but it is essential to determine whether or not they are denatured during the desiccation (and/or rehydration) of well-adapted anhydrobiotic organisms. That issue is of central importance, but a literature search turned up no information on the subject. My bias is that, in general, anhydrobiotic organisms prevent protein unfolding and other kinds of desiccation-damage, chiefly by participation of water substitutes and vitrification, rather than relying heavily on repair by molecular chaperones. No doubt there are exceptions to this sweeping generalization.

Nevertheless, evidence continues to accumulate on the potential involvement of certain proteins in desiccation tolerance, but not necessarily as molecular chaperones. Thus, early studies on plant life history stages correlated the accumulation of 'late-embryogenesis-abundant' (LEA) proteins with the acquisition of desiccation-tolerance during seed maturation (Vertucci and Farrant, 1995; Ingram and Bartels, 1996; Chandler and Bartels, 1999) and in the tissues of certain water-stressed angiosperms, the 'resurrection plants' (Tomos, 1992; Scott, 2000). A substantial amount of information is available on these proteins and I do not claim to have digested more than a sampling of this literature. My general impression is that LEA proteins, the related dehydrins (Close, 1997) and others (Garay-Arroyo et al., 2000) are involved more with the protection of membranes (including associated proteins) than with the classical molecular chaperoning of damaged globular proteins. Other roles have been proposed, including water binding and retention at specific intracellular sites and ion-trapping to mitigate high ionic strength during dehydration but, in my opinion, the evidence for these possibilities is modest.

The usual classes of SP families are present in these organisms, of course, but they need not be involved in desiccation tolerance as recent studies on a small heat shock protein in chestnut seeds indicate (Collada et al., 1997). Research on a LEA-like protein (hsp12) in yeast, *Saccharomyces cerevisiae,* provides support for the view that this particular protein is involved with membrane protection during dehydration (Sales et al., 2000) in general agreement with results on yeast (Eleutherio et al., 1993, 1998).

What is the initial signal involved in the production of these and other proteins during the acquisition of desiccation tolerance? In plants abcissic acid seems to be of major imortance in this regard (Vertucci and Farrant, 1995; Ingram and Bartels, 1996; Chandler and Bartels, 1999). Its appearance, resulting from decreases in tissue water levels, appears to initiate signal transduction pathways that result in the synthesis of a wide variety of proteins, including the ones mentioned in the previous paragraph. In the case of resurrection plants this process apparently begins in the root systems which, when sufficient water is lost, produce increased amounts of abcissic acid which are then transported to the rest of the plant (Scott, 2000).

Mostly overlooked has been the potential involvement of lipid-assisted protein folding (Bogdanov and Dowhan, 1999), but it appears that 'lipo-chaperones' could be important in pro-

tecting membranes during desiccation/rehydration, perhaps operating in concert with the protective effects of trehalose, sucrose or other sugars. Much effort has been devoted to understanding how the structure of membrane lipids is protected during water removal and addition, but less attention has been given to the participation of membrane lipids themselves, including the 'chaperoning' of membrane-associated proteins which has been demonstrated in hydrated systems (Bogdanov and Dowhan, 1999). It seems likely that these and other membrane properties are important as anhydrobiotic organisms undergo desiccation and rehydration. I am not aware of any studies that attempt to explore this possibility using, for example, membrane preparations from organisms able to undergo anhydrobiosis and those from non-adapted organisms. Of course, that is easier said than done.

As described in Section 3.2, trehalose or sucrose (sometimes other sugars) are critical for the desiccation-tolerance of many anhydrobionts, raising the question of potential sugar-SP interactions in these organisms. The importance of interactions between trehalose and hsp104 in the heat shock response of yeast have been well-documented (Elliott et al., 1996; Iwahashi et al., 1998; Singer and Lindquist, 1998a,b). Trehalose plays the dominant protective role during exposure to high temperature, whereas, trehalose is rapidly degraded after heat shock because it interferes with chaperoning by hsp104 (Singer and Lindquist, 1998b; also Elliott et al. 1996). Whether this relationship holds for reversible desiccation of yeast is apparently not known, but seems worth studying. Relationships between water content, induction of desiccation tolerance, dehydrins and the oligosaccharide raffinose have been studied using wheat embryos (Black et al., 1999). In that system, small reductions in water content induce desiccation tolerance by starting processes in which dehydrins might participate, but not through interactions with raffinose. In resurrection plants initial dehydration leads to the production of large amounts of sucrose, arising from starch, photosynthesis or the 8-carbon sugar octulose, depending on the species (Scott, 2000). Interestingly, rehydration leads to the reversal of these pathways. One wonders whether there are antagonisms between high concentrations of sucrose and the stress proteins present in cells when they are rehydrated, comparable to the situation in the heat shock response of yeast as summarized above. The extent to which sugar-SP interactions are involved in the adaptive repertoire of organisms capable of anhydrobiosis remains an important topic for future study.

4. Anoxybiosis

It is easy to accept that anhydrobiosis and cryobiosis lead to a reversible metabolic standstill since the amounts of liquid water required to permit metabolism are removed in both cases. But what about other forms of cryptobiosis, notably anoxybiosis? Is it these that prompted Keilin to include the 'hardly measurable' in his definition? He apparently did not arrive at a conclusion as to whether oxygen lack can bring metabolism to a reversible standstill under physiological conditions of hydration and temperature. Here the definition of 'metabolism' becomes even more critical than in the other forms of cryptobiosis since there is an abundance of water and thermal energy that will favor chemistry, if not biochemistry.

A vast literature documents the ability of many multicellular organisms to undergo periods of oxygen lack of variable duration, however, to my knowledge no animal or higher plant can complete its life cycle under strictly anoxic conditions. It is indeed an aerobic world for all but certain microorganisms. Nevertheless, the ability of some animals to survive long bouts of continuous anoxia is impressive. That is particularly the case for a limited number of ectothermal vertebrates and a substantial number of invertebrates (Hochahchka and Guppy, 1987; Bryant, 1991; Hochahchka et al., 1993; Grieshaber et al., 1994; Hand and Hardewig, 1996; Storey, 1998; Hand and Podrabsky, 2000; Jackson, 2000). Well-adapted animals respond to anoxia by reducing their overall metabolic rates, commonly to between 1–10% of aerobic levels (Storey, 1998; Guppy and Withers, 1999; Hand and Podrabsky, 2000). This response, called metabolic rate depression (MRD), has been well-studied in sessile intertidal invertebrates and involves some interesting modifications in intermediary metabolism that are of obvious adaptive significance.

We may ask whether any of these organisms actually bring their metabolism to a reversible standstill under anoxia, while fully hydrated and

at physiological temperatures. The embryos of certain crustaceans are good candidates. Extensive studies on the encysted gastrula embryos of the branchiopod crustacean, *Artemia franciscana,* revealed no evidence of an ongoing metabolism during bouts of anoxia that lasted for periods of years (Clegg, 1997; Warner et al., 1997; Clegg et al., 1999). Recognizing that one cannot prove the absence of a rate by experiment, the case was made that, if metabolism was occurring at all, it would have to be at least 50 000 times slower than the aerobic rate (Clegg, 1997). Are encysted *Artemia* embryos unique in this regard? Other candidates include copepod embryos whose longevities in the anoxic benthos of marine (Marcus et al., 1994), brackish (Katajisto, 1996) and fresh water (Hairston et al., 1995) habitats, exceeded 10 years in all three studies and, in certain cases, possibly centuries (Hairston et al., 1995). Long-term anoxybiosis of a nematode species has been reported in which metabolic rate was reduced to undetectable levels for over 3 months of continuous anoxia (Crowe and Cooper, 1971). Fresh-water sponge gemmules subjected to anoxia at room temperatures in water survived almost 4 months of continuous anoxia and most of these were still alive when the study was terminated (Reiswig and Miller, 1998). It is difficult to explain these longevities without invoking complete anoxybiosis and I suspect that this phenomenon may be more common than currently believed.

But there is a troublesome aspect about a metabolic standstill during anoxybiosis. If that is true, this would represent an exception to a major generality of biology, namely, that a constant flow of free-energy is required to maintain cellular integrity, involving biosynthesis and homeostasis. Are these organisms actually exceptions and, if so, then how do they accomplish that extraordinary feat? In attempting to answer this question in *Artemia* embryos, we examined the stability of proteins during long term anoxia and found no evidence for protein unfolding and aggregation over years of continuous oxygen lack, in fully hydrated embryos at room temperature (Clegg, 1997; Clegg et al., 1999). That was particularly surprising in view of the absence of detectable protein synthesis in anoxic embryos and the lability of hydrated globular proteins, in general (Somero, 1995). During the early phases of this work we found massive concentrations of a protein, named p26, restricted to the encysted embryo stage of the life history (Jackson and Clegg, 1996; Liang and MacRae, 1999). We also showed that p26 underwent extensive stress-induced translocation to nuclei and other sites (Clegg et al., 1994) and that these exhibited strong pH dependance (Clegg et al., 1995) in a fashion consistent with intracellular pH changes in vivo (see Hand and Hardewig, 1996; Hand, 1998; van Breukelen and Hand, 2000; van Breukelen et al., 2000). Subsequent study (Liang et al., 1997a,b) showed that p26 belonged to the small heat shock/α-crystallin family of proteins (de Jong et al., 1998) and demonstrated that this protein exhibited molecular chaperone activity in vitro (Liang et al., 1997a) and probably in vivo (Liang and MacRae, 1999). Importantly, chaperone activity in vitro did not require ATP or GTP, allowing us to suggest that p26 maintained protein stability during anoxia, which provided at least some explanation for the stability of proteins and survival of anoxic embryos in the 'absence' of metabolism.

Although the participation of p26 as a major biochemical adaptation is now established, we were troubled by the exception noted above and continued to seek the existence of some sort of ongoing metabolic activity in anoxic embryos. A hint came from earlier work on their guanine nucleotide pool (Stocco et al., 1972), which is unusually large and diverse (Warner, 1992), suggesting that one of these, Gp_4G (P^1,P^4-diguanosine 5′-teraphosphate), might be utilized during anoxia (Stocco et al., 1972). We recently re-examined this possibility and now have good evidence that this nucleotide is indeed metabolized during prolonged anoxia, albeit extremely slowly (Warner and Clegg, unpublished results). A metabolic pathway leading to production of GTP (and ATP) from Gp_4G has been proposed, based on these results and substantial evidence from previous work (Warner, 1992). Thus, it appears that the great majority of intermediary metabolism is indeed brought to a reversible standstill by anoxia, but that a specialized and limited guanine polynucleotide pathway continues to provide free energy for these embryos during anoxia. At this point it appears that these embryos might not violate the 'free energy rule' after all, although they seem to come very close.

Now another problem arises, what are the processes in anoxic embryos that require and/or use free energy? Curiously, the answer is not

obvious. Protein biosynthesis demands large percentages of the free energy budget of cells in general but, as described above, we are confident that this is not taking place during anoxia. Another major free energy requirement of cells involves ionic and cell volume homeostasis, but encysted embryos have avoided this by becoming impermeable to non-volatile molecules (see Clegg and Conte, 1980) and, of course, by acquiring the ability to undergo anhydrobiosis.

We recently uncovered a potential free energy requiring candidate, namely, p26 and its nuclear translocation. Although not requiring ATP or GTP for molecular chaperone activity in vitro, that might not be the case in vivo, particularly for the extensive nuclear translocation that this protein undergoes during anoxia and other imposed stresses (Clegg et al., 1994, 1999). Pursuing that possibility, we recently found that p26 is a GTP-binding protein based on intrinsic fluorescence spectroscopy (Viner and Clegg, unpublished results) and have demonstrated that this protein is also a GTPase (Warner and Clegg, unpublished results). Our working hypothesis is that anoxic embryos undergo a massive and almost complete metabolic shutdown during anoxia, but have to support the energetic requirements of their molecular chaperone, p26, which they achieve by mobilizing Gp_4G and eventually producing GTP and/or ATP. Of course, there may be other endergonic processes in anoxic embryos of which we are unaware.

5. Some surprising applications

The history of science reveals that well-executed basic research sometimes results in completely unpredictable outcomes that prove to be of substantial importance to the human condition. The study of cryptobiosis provides such examples.

Nature has exploited the properties of trehalose to achieve anhydrobiosis by what appears to be a remarkably simple process (Section 3), select a good water substitute and produce it in amounts sufficent to protect structure but prevent function. Some of the early work leading to that realization was done in the mid-1960s by John Crowe and associates who studied the ability of an interesting but little-studied group of animals, the tardigrades, to achieve anhydrobiosis (Crowe and Higgins, 1967). As mentioned (Section 3) trehalose became an important focus in the early 1970s and the Crowe laboratory turned its attention to the involvement of that sugar in nematode anhydrobiosis (Crowe and Clegg, 1978). The next phase extended these findings by showing that this disaccharide stabilized liposomes and other membrane preparations, as well as proteins against dehydration damage in vitro (see Crowe et al., 1997, 1998b; Allison et al., 1999). Many laboratories have been actively studying these and other aspects of 'trehalose protection' and I do not wish to minimize these important contributions (examples are Aguilera and Karel, 1997; Collada et al., 1997; Sun and Leopold, 1997; Eleutherio et al., 1993, 1998; Reid, 1998; Koster et al., 2000). But I think it is fair to say that the Crowe laboratory has led the way on a relentless quest to answer the question 'how does trehalose work?' More recently, their research resulted in the use of trehalose to reduce chilling injury in bovine oocytes (Arav et al., 1996), enhance recovery and preserve function in human pancreatic islets (Beattie et al., 1997) and to greatly increase the shelf-life of human blood platelets (Tablin et al., 2000) including their lyophilization (personal communication from John Crowe). These contributions are obviously of enormous economical and clinical importance. This brief account represents a good example of the value of basic science, indeed this section might be subtitled 'from tardigrades to platelets and beyond'.

Others have used trehalose to improve substantially the survival of cryopreserved mammalian cells (Eroglu et al., 2000) and even to enable their reversible desiccation (Guo et al., 2000). The latter result is astonishing and supports the idea expressed here and elsewhere (Crowe et al., 1998b), that the achievement of anhydrobiosis during evolution seems to have been a remarkably simple process, simply select the best 'water substitute' and synthesize it in concentrations sufficient for the purpose.

6. Future prospects

The statement just made needs further critical study, is it really that simple? Although possible, particularly in view of the results on mammalian cell desiccation, one would like to know more about the details. Anoxybiosis presents the chal-

lenge of evaluating the significance of extremely low metabolic rates and the long-term survival of eukaryotic cells and organisms with such minimal metabolic support. I think that increased attention should be given to organisms living part of their life history in anoxic environments since these are good candidates to achieve complete anoxybiosis. The utility of trehalose, sucrose and other sugars in the preservation of macromolecules, cells and tissues from non-adapted organisms deserves and is receiving intense examination. Largely overlooked thus far, is the value of some cryptobiotic organisms as useful model systems for the investigation of research areas of a more general nature, such as the basic mechanisms involved in the regulation of metabolism, study of the physical properties of intracellular water and the stability of biological materials in situ. Finally, further thought about what cryptobiosis can tell us about the basic nature of cellular life seems worthwhile. One wonders what Antony van Leeuwenhoek would have to say about the progress achieved in understanding the peculiar phenomenon he discovered some 300 years ago.

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