

23 The Future of Cryobiology

Nick Lane

Department of Surgery, Royal Free and University College Medical School,
University of London, London, UK

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23.1 PROGRESS AND STASIS: 50 YEARS OF CRYOBIOLOGY

Few scientific problems have proved as intractable as cryopreservation. In his foreword to this book, Harry Meryman recalls the astonishing shifts in scientific paradigms since the 1950s. If we go back further, to the early 1940s, the differences become even more radical. When the "father" of cryobiology, Basil Luyet, published his seminal work *Life and Death at Low Temperatures* in 1940, Avery had yet to prove that genes are composed of nucleic acids. Even scientists of the calibre of J.B.S. Haldane wrote scornfully that DNA did not have the variability of structure necessary to encode genes and argued that histones were more likely candidates. Yet at this time, Luyet had a strikingly contemporary view of vitrification—still the holy grail of cryobiology. Compare Luyet and Gehenio writing in 1940 with Taylor in Chapter 22 in this volume:

Good vitrification is not injurious, there being no molecular disturbance, while an incomplete vitrification or devitrification and, a fortiori, crystallization, are injurious to the extent that they disrupt the living structure. (Luyet and Gehenio, 1940)

A vitrified liquid is essentially a liquid in molecular stasis. Vitrification does not have any of the biologically damaging effects associated with freezing because no degradation occurs over time in living matter trapped within a vitreous matrix. Vitrification is potentially applicable to all biological systems. (Taylor et al., 2004)

Conceptually, then, little has changed in our understanding of vitrification for more than 60 years. Yet these passages also serve to highlight the tremendous distance that cryobiology has actually covered since the 1940s. Luyet and Gehenio conceded failure to vitrify anything more complex than moss, listing what must have been a frustrating succession of failures to vitrify cell

suspensions, as well as plant and animal tissues, including leaves and muscle fibres. In contrast, in their chapter in this volume, Taylor et al. report success in vitrifying complex tissues, if not yet organs, including corneas, blood vessels, and articular cartilage.

It is not just vitrification that has leapt ahead. Chris Polge's felicitous discovery of the cryoprotective effects of glycerol in 1949 (which, incidentally, he referred to as vitrification) sparked a revolution in the science of cryobiology—indeed, some claim it was the beginning of scientific cryobiology. Since then, there have been tremendous developments in both the fundamental understanding and empirical practice of cryobiology. To give a single practical example, cited in the Chapter 18, fertility results following artificial insemination with donor cryopreserved semen have almost doubled in the United Kingdom in the last 8 years, whereas the number of treatment cycles has declined by over 50%. Similar far-reaching advances have transformed the prospects in almost all fields of cryobiology, from the apparently successful freeze-drying of platelets with trehalose (see Chapter 21), to the vitrification of meristems from recalcitrant tropical plants, such as bananas (see Chapter 10).

Perhaps the most remarkable fact about these achievements is that they are based, at least philosophically, on protocols and techniques pioneered in the 1950s by Polge, Audrey Smith, and James Lovelock. Not only are their early methodologies still in use today, but they are still regularly discussed. Several chapters in this book (1, 2, and 17) contain passages in which the validity of Lovelock's conclusions is debated with vigor, if not firm consensus. It is hard to think of many other fields of science in which the significance of experiments carried out 50 years ago is still in dispute today (though quantum physics is one good example, lest readers confound protracted problems with intellectual stagnation). If cryobiology seems to be running to stand still (or better, standing still while giving the appearance of running), the reason is that most practical advances have been built on incremental refinements in methodology. Thus, even though the triumphs might make the pioneers green with envy, remarkably few modern cryobiological methods would take them by surprise. The difficulty is that cryobiology has been strait-jacketed by its need to conform to the intractable laws of biophysics. For all its successes, cryobiology has been stuck in a rut.

23.2 LIMITATIONS OF THE BIOPHYSICAL APPROACH TO CRYOBIOLOGY

The incremental refinements in practical methodology over the last 50 years have been based on fundamental advances in our understanding of the biophysics of cryobiology, pioneered by Peter Mazur, Akira Sakai, and others. These biophysical principles are detailed in the chapters by Mazur, Muldrew et al., Taylor et al., and Sakai, in this book.

In essence, the survival of cells and simple tissues when subjected to cryogenic temperatures describes an "inverted U" according to the cooling rate. Very fast cooling rates supercool the intracellular environment below the homogeneous nucleation point (about -40°C for cytoplasm), typically causing lethal intracellular freezing. Conversely, very slow cooling (or "equilibrium" cooling) results in the osmotic dehydration of cells as ice crystallizes in the extracellular spaces. The osmotic stresses alone may be severe enough to cause lethal injury, for example, by "salting" proteins into solution and permuting biochemical reactions, whereas changes in membrane permeability to Na^+ may lead to swelling and rupture, as originally argued by Lovelock. Extracellular ice is likely to "seed" nucleation within cells and to directly injure cell membranes. Cells compressed together in the dehydrated state by an advancing ice front are also liable to interact in ways that are not easily reversible; for example, by the fusion of membranes. Even if ice formation is avoided by vitrification, the rewarming of metastable glasses can be critically rate-dependent, which poses the problems of devitrification and recrystallization. Although the precise mechanisms of injury in particular circumstances are often unproved, the outcome is clear: poor survival, especially of larger tissues, which are also stressed by purely mechanical forces.

Many of the triumphs of cryobiology over the last four decades stem from manipulating these forces, using controlled freezing and rewarming protocols, along with cryoprotective agents (CPAs). CPAs bring their own problems. At high concentrations, most are toxic in their own right. Exaggerating this toxicity, the equilibration of CPAs between the intracellular and extracellular compartments is slow, especially at subzero temperatures, in comparison with the osmotic movements of water (which may be orders of magnitude faster, even in the absence of aquaporins). The final distribution of CPAs, and the osmotic stresses generated, therefore depends on the permeability of the cell membranes and the temperature. Nonpermeating CPAs have the potential to exacerbate intracellular dehydration without stabilizing intracellular proteins or membranes, whereas permeating CPAs may not be cleared from cells quickly enough on rewarming, leading to swelling and possibly rupture (assuming that osmotic equilibration is rarely perfect, even when cells are subject to "equilibrium" cooling). This means that successful cryopreservation depends largely on the permeability of cells to water and CPAs, and on their sensitivity to the toxicity of high concentrations of CPAs. Sometimes these factors can be balanced to give an optimal protocol, sometimes not. Peter Mazur concludes his chapter with a beautifully concise summary of the practical limitations of the traditional biophysical approach to cryopreservation:

In some cases, difficulties in cryopreservation may stem from the complex concatenation of conflicting variables. Thus, cooling rates that are low enough to avoid intracellular ice formation may be so slow as to induce damage from solution effects or chilling. The use of higher CPA concentrations to minimize solution effects may introduce toxicity or exacerbate osmotic damage. Toxicity may be reduced at lower temperatures, but lower temperatures slow the permeation and further exacerbate osmotic damage. Damage from external ice can be prevented by vitrification, but the induction of the vitrified state requires high CPA concentrations that exacerbate both toxicity and osmotic problems. These incompatibilities may not be challenges to our understanding but in some cases they remain challenges to achieving successful cryopreservation. (Mazur 2004)

The problem today is that applying the basic principles of biophysics simply cannot solve many of the remaining challenges in cryobiology. The fact is that some cells or tissues deal with physical stresses better than others. Cells that are at once osmotically intolerant and sensitive to high concentrations of CPAs will fare badly under virtually any conventional cryopreservation protocol. Success is likely to be partial at best, and dependent on an empirical testing of different cooling and warming rates, as well as on the concentrations and toxicities of various CPAs. This is, in fact, exactly how some of the more cumbersome and difficult cryopreservation protocols have been developed over the last decades, but as the remaining challenges become steadily more refractory, we can predict that the successes will become correspondingly rare—especially in complex tissues in which different cell types have exacting and diverse requirements. The failure to vitrify large or complex mammalian tissues and organs using CPAs alone illustrates the intractable difficulties involved.

23.3 NATURE'S LABORATORY

There is another way out, and herein lies the probable future of cryobiology: a future that is closely tied in with the spectacular advances in molecular biology and genomics of the last decade. We are today on the verge of an explosion in our understanding of genetic adaptation. As advocated by Harry Meryman in the foreword, we can learn from nature's own laboratory: a marvelous arena with hundreds of specimens in each group—bacteria, algae, fungi, plants, invertebrates, fish, and amphibians—and millions of years run-time. The future of cryobiology is surely the dovetailing of formal biophysics with the study of life's adaptations to similar problems. In effect, if some cells and tissues are refractory to cryopreservation protocols, the trick is not necessarily to change the protocol but to change (precondition) the cells themselves. If the mountain will not come to Mohammed, then Mohammed must come to the mountain.

Even so, a number of failures show the difficulties involved in trying to apply the “tricks” of nature to the problems of cryobiology. Just as the incremental advances in conventional cryobiology have been achieved by the application of a mathematical formalism, so too we must in future apply, at the very least, a philosophical formalism to the remaining challenges. That is to say, we need to think carefully about the selection pressures involved in evolution and the extent to which they coincide with the biophysical pressures of cryogenic freezing. It is not necessarily true that “nature knows best,” when nature has never been called on to adapt to -196°C . In this final chapter, then, I will explore some of the most likely avenues of the future. The discussion is inspired by broad themes discussed in the foregoing chapters, and is not intended (or referenced) as a review; rather, it is a “preview” of future possible worlds.

A good example of enthusiasm running before formal analysis is the genetic engineering of tomatoes to express fish antifreeze proteins (AFPs), in the hope of protecting them against frost injury. This was first accomplished in the early 1990s, but proved a disappointment: The engineered tomatoes duly expressed the fish AFPs but were, if anything, more vulnerable to frost than normal tomatoes. Today we know the reason why: At high concentrations, fish AFPs alter the crystalline habit of ice, from dendritic to spicular, and sharp spicules of ice are more destructive of tissue structure than dendritic crystals. This outcome is worth dwelling on for a moment, as it highlights the uneasy relationship between the two Janus faces of science: the fundamental and the applied.

From a fundamental point of view, a detailed understanding of the mechanism of AFPs, apart from being fascinating in its own right, feeds back into applied science in unexpected ways. In this case, fish AFPs are now being used to potentiate damage in cryosurgery, as discussed in Chapter 3, by Elster and Benson, and Chapter 16, by Hoffmann and Bischoff. Moreover, close scrutiny of the mechanisms of spicule formation has underpinned the development of synthetic ice blockers (SIBs), such as 1,3-cyclohexanediol, discussed in the chapter by Taylor et al. By retarding ice formation in general, and by altering the crystalline habit of any ice that does form to hexagonal, rectangular, or trapezoid shapes, SIBs have the power to revolutionize vitrification. If they really can limit the devitrification and recrystallization of metastable glasses, SIBs may overcome the decades-old challenge of successfully rewarming vitrified organs (and, potentially, recalcitrant hydrated tropical seeds such as coconuts, as discussed in the chapter by Benson).

From a strictly “applied” point of view, however, the engineering of tomatoes to express fish AFPs betrays a tendency toward an empirical “wishful thinking” that falls well short of the philosophical formalism discussed above. Any serious progress in resolving the future practical challenges in cryobiology will need to take a more structured approach. Empirical “shots in the dark” are no longer enough. Parallel fields can sometimes cloud the issue. The burgeoning interest in astrobiology, for example—the pursuit of life in space and on planets like Mars—has reinvigorated polar research, especially into microbial adaptations to extreme conditions, including extreme cold. As discussed in the chapters by Elster and Benson, and by Ponder et al., astrobiology shares common ground with cryobiology, but should not be conflated with it. The adaptations of life to cold take two broad forms: freeze tolerance and freeze avoidance. The latter is of profound importance to polar and permafrost ecology. Many permafrost bacteria, for example, avoid freezing and show signs of limited metabolism. In terms of the prospects of finding life actually living on Mars or Europa (rather than anabiotic in space), the study of metabolic adaptations and freeze avoidance is of far greater relevance than “mere” long-term preservation.

From the practical perspective, though, freeze avoidance at high subzero temperatures is a very different matter than freeze avoidance or freeze tolerance at cryogenic temperatures, where cellular metabolism certainly stops. (In both Antarctic sea-ice and permafrost bacteria, metabolic activity and protein synthesis are undetectable below about -20°C .) This is the trouble with fish AFPs: Their evolutionary “purpose” is to prevent fish from freezing without the need for high concentrations of osmolytes. This they do very effectively, within a restricted temperature range, by inducing a thermal hysteresis of 1 to 2°C (a lowering of the freezing point without affecting the melting point). Playing around with thermal hysteresis, however, is a dangerous game that can have

catastrophic consequences, such as the formation of spicular ice if the temperature supercools below the hysteresis point. In ecological terms, this is unlikely to happen at sea: Fish live in a stable, if icy, environment. Looking to the evolution of fishes for an answer to the very different problems of freeze tolerance or avoidance on land (where temperatures are far more variable and extreme), or worse, at cryogenic temperatures, is sloppy thinking.

23.4 THE CRUCIBLE OF EVOLUTION

Evolution is a grand experimental crucible, but it differs from human experimental research in two crucial respects: first, evolution is “applied” to a degree that would make purists blench, and second, it is inherently multivariate. By “applied” in this case I mean that life evolves to cope with very particular conditions, but only when this type of adaptation, rather than another, is advantageous. The lack of an adaptation may or may not be evidence that it cannot happen. Is the absence of frozen penguins in the Antarctic evidence that large, warm-blooded animals cannot be frozen, or merely that frozen penguins would make an easy meal for marauding seals? In North America, smaller animals, such as frogs and turtles, do indeed freeze. Does the fact that they are smaller and cold-blooded reflect ecological, physiological, or biophysical constraints on freezing tolerance (or all three)?

There are other problems with the use of adaptations as a window on future cryopreservation. Inherent in the very word “adaptation” is a stable environment; obviously no organism can adapt to an asteroid impact, but given a stable environment, life’s adaptations are often astonishingly precise. Conversely, the same adaptations are useless if the parameters are suddenly shifted. As discussed in the chapter by Kenneth and Janet Storey, wood frogs (*Rana sylvatica*) can survive freezing for days or even weeks at -4°C , but they cannot survive much longer at this temperature, or at all below about -6°C . Similarly, as discussed by Elster and Benson, the Antarctic alga *Zygnema* can endure repeated overnight exposures to temperatures of about -4°C and still maintain photosynthetic capacity during the day; but if exposed to more prolonged periods of freezing, or to lower temperatures, the cells leak solutes, lose their photosynthetic capacity, and die. The alga is in fact adapted to the Antarctic summer, when nocturnal temperatures rarely fall below -4°C . Wood frogs manage to restrict the temperature to within narrow limits by insulating themselves with snow, leaves, and moss, by which means they also contrive to limit evaporative water loss. In other words, both wood frogs and Antarctic algae have adapted with precision to expected average conditions, which they may help to regulate through their behavior, and natural selection has never been called on to protect them against lower temperatures. One of the most fascinating and difficult questions for cryobiology in future will be the extent to which adaptations to freezing at high subzero temperatures can help precondition against subsequent cryogenic storage. Certainly it is beyond our current competence to cryogenically preserve a frog already frozen at -4°C . We will need to learn from the spirit of the frog’s adaptations rather than the letter.

The wood frog is also a good example of the multivariate nature of evolution. As outlined by the Storeys, the expression of a large number of genes is either up-regulated or down-regulated in response to freezing. The function of several of these genes is still unknown, but those that have been identified do give some sense of the range of adaptations. Broadly speaking, the adaptive response falls into three categories: cryoprotection, suppression of metabolism, and stress response. Glucose is the main colligative CPA, and it probably also helps to stabilize cell proteins during the controlled dehydration of organs. Ice-nucleating agents (INAs) are found in the plasma and extracellular spaces, and presumably help direct extracellular ice formation and reduce osmotic stress. Central metabolism is selectively suppressed, especially ion-motive ATPase activity, muscle energy catabolism, and general biosynthetic pathways, with only a limited fermentative energy production to cover basal metabolism and specific biosynthetic pathways. Of these, the mitochondrial inner membrane ADP/ATP translocase is upregulated severalfold in response to freezing, but how this affects basal metabolism is as yet unknown.

The stress response encompasses both biochemical and biomechanical adaptations, notably a rise in the activity of several antioxidant enzymes including glutathione peroxidase, and indeed a tenfold rise in glutathione levels in some organs, such as the brain. The most striking biomechanical adaptation is the production of fibrinogen peptides, which presumably aid in blood clotting in damaged capillaries following rewarming. The main point, however, is that the frog's cryoprotective strategy is exquisitely fine-tuned, both temporally and spatially. Within minutes, freezing triggers a carefully modulated chain of events, controlled by various signal-transduction pathways and transcription factors. Different genes are transcribed at different rates and in different organs. A loss of synchronization would in all likelihood undermine success. For example, fibrinogen is transcribed during the freezing period, rather than on rewarming—an energy-consuming task in difficult times. Presumably it is vital, literally, to block internal bleeding immediately on thawing, rather than potentially hours afterwards.

23.5 PARALLELS IN PLANTS

Plants orchestrate their responses to freezing in an equally complex manner, as discussed in the chapter by Roger Pearce. At least 60 plant genes are up-regulated or down-regulated in response to freezing, though not necessarily all at once in the same species (and indeed not necessarily all in response to freezing: Some may be adaptations to other aspects of the winter environment, such as wind and waterlogging). As in freeze-tolerant animals, the identity and function of many of these genes is still unknown, but the parallels in function of the known gene products are striking. Plant adaptations fall within the same three broad categories of cryoprotection, metabolic suppression, and stress response. In terms of cryoprotection, the classes of adaptation are again similar: raised concentrations of colligative cryoprotectants (such as raffinose, sucrose, or fructans) and nucleation of ice in extracellular compartments with INAs. In addition, some plants express AFPs, which (with the odd exception of the carrot) are evolutionarily distinct from fish and insect AFPs. Given my earlier remarks on fish AFPs, it is interesting to note that plant AFPs exert little thermal hysteresis, usually a few tenths of a degree, but are strong inhibitors of recrystallization on rewarming. They also tend to be present at lower concentrations than in animals, consistent with the lower thermodynamic driving force for recrystallization. Thus, in conjunction with INAs, the purpose of the AFPs seems to be to direct and restrict recrystallization, rather than to prevent ice formation at all, as is the case in fish and some insects.

Perhaps most important of all are the various plant adaptations to dehydration and desiccation. Intracellular desiccation, caused by the growth of extracellular ice, is the main cause of death in overwintering cereals and other crops. Plant adaptations to dehydration stress are orchestrated by plant hormones (such as abscisic acid) and transcription factors (such as drought-response element binding-factors, or DREBs). Several signal transduction pathways run in parallel or even converge. Overall, these raise the levels of proline, amino acids, and a number of late-embryogenesis-abundant proteins such as the dehydrins, which were first described in desiccating seeds. The exact mechanism of dehydrins is still unknown, but they are usually supposed to interact with hydrophilic groups on lipids and proteins to stabilize their structure during dehydration. Another interesting possibility is that they stabilize the glassy state in dehydrated tissues at freezing temperatures by raising the glass transition temperature (T_g). In seeds, this would reduce the likelihood of repeated cycles of vitrification and devitrification at freezing temperatures (which are associated with free radical-mediated decomposition). However, there is a danger here, as they also appear to lower the T_g at high water content (presumably preventing immature seeds from entering the glassy state, and again protecting against damaging cycles of vitrification and devitrification). Thus, at high water content, dehydrins seem to inhibit entry to glassy state, and at low water content, they stabilize the glassy state. If this is true, we can predict that the dehydrins should actually be detrimental to the cryopreservation of samples with high water content, as they would tend to destabilize the glassy state, especially at higher subzero temperatures (from -60° to -20°C). Again, we need to think

carefully about what dehydrins evolved to do. In applying them to cryopreservation protocols, there is no substitute for a deeper fundamental understanding of their mode of action and evolutionary purpose.

The metabolic response of plants to freezing depends on the broader strategy adopted: innate dormancy (as in woody species, which show the greatest known tolerance of freezing) or potential activity (as in plants that continue to grow during milder spells). In the latter case, metabolic rate is initially greatly suppressed by cold, but then partially recovers, with some degree of oxidative phosphorylation, photosynthesis, and protein synthesis taking place. Such changes are accompanied by other adaptations that facilitate metabolic activity, such as shifts in the spectrum of membrane lipids toward greater unsaturation, to maintain the fluid state. For example, active thylakoid membranes of chloroplasts consistently show a high level of lipid (e.g., phosphatidylglycerol) unsaturation in response to chill (low positive temperatures). In extreme cold, and therefore cryogenic preservation, such changes are probably counterproductive, as unsaturated lipid membranes are likely to lose their normal lamellar structure when dehydrated, and these lipids are also more vulnerable to lipid peroxidation on rewarming. Thus, adaptations favoring winter activity may prove detrimental to successful cryopreservation.

One factor that does seem to be common to both metabolically active and dormant (freeze-tolerant) species is the up-regulation of antioxidant enzymes and other stress proteins. A number of antioxidant enzymes are expressed at higher levels in cold-acclimated plants, such as freeze-tolerant wheat, including ascorbate peroxidase, glutathione reductase, and catalase. However, freezing inactivates enzymes such as superoxide dismutase, so high levels of simple antioxidants such as ascorbate and reduced glutathione may play a more important role at deep-freezing temperatures. Again, there is a striking parallel with the wood frog, in which reduced glutathione levels show the most marked rise. As noted in the chapter by Benson and Bremner, many colligative cryoprotectants, including glycerol, sucrose, and Me_2SO , are also excellent free radical scavengers, and may exert a part of their protective effect through this mechanism.

23.6 FIRST STEPS TO THE "NEW" CRYOBIOLOGY

Taken together, the main difficulty in basing the "new" cryobiology directly on nature's laboratory is that evolution selects for phenotypes adapted to specific conditions. Put another way, evolution selects blindly for multigene combinations, gambling on average conditions, whereas the scientific method prescribes the variation of single parameters, while maintaining other parameters at constant levels. It becomes very difficult to interpret the results of experiments in which multiple variables are changed simultaneously. This may be necessary, nonetheless, as individual changes can easily be counterproductive in isolation. For example, the seeding of small extracellular ice crystals with INAs to restrict supercooling prevents the sudden and inequitable osmotic stresses that accompany uncontrolled rapid freezing, whereas dehydration helps to promote vitrification of the intracellular environment. These changes, however, may be far from helpful if there are no compensating defenses against the mechanical injury caused by small extracellular ice crystals (e.g., raised fibrinogen levels in the frog), no defenses against recrystallization (for example AFPs), or no intracellular accumulation of compatible solutes to protect against osmotic stress, membrane fusion, and the precipitation of proteins. Most conventional cryopreservation protocols consider only dehydration and colligative cryoprotection, although INAs and AFPs are beginning to make an appearance in vitrification protocols. With a few exceptions, the other broad issues—the stress response and metabolic suppression—are yet to be incorporated in a systematic way into cryopreservation protocols.

This last consideration returns us to the question of which changes that are adaptive at high subzero temperatures will also protect cells and tissues at cryogenic temperatures. Clearly, colligative cryoprotection is a must. The similarity in the choice of CPAs in bacteria, protists, fungi, algae, plants, and animals, compared with cryopreservation protocols, is striking. Chris Polge's

accidental discovery of glycerol has been equally “accidentally” replicated in nature hundreds of times. Interestingly, most species use only one or two colligative CPAs, such as glucose in frogs, sucrose in many plants, and trehalose in many bacteria, yeasts, and fungi (see chapter by Tan and van Ingen). Why this should be the case has never been satisfactorily explained, but the precise choice does matter. A CPA that works well in one species may work badly in another. Thus glucose is an excellent CPA for frog erythrocytes but a poor CPA for human erythrocytes. The problem with glucose is presumably its reactivity with proteins in the Maillard (browning) reaction, as happens in people with diabetes. How wood frogs deal with such high levels of glucose is a fascinating question, with manifest medical applications, and the Storeys discuss a number of secondary adaptations to insulin structure and glucose transport that may have a bearing on the matter.

There is a broader point here—the evolutionary and energetic cost of multiple adaptations. The concentrations of CPA required to protect cells against dehydration, so as to enable vitrification, are likely to be toxic in their own right. Combining a number of CPAs at subtoxic levels is a possible solution to the problem (as in most vitrification protocols), but from a biological point of view, this requires potentially costly controls over the concentration and toxicity of individual components. Each must be homeostatically regulated within tightly defined limits. An alternative possibility is to guard against the toxicity of a single CPA, such as glucose, as in the wood frog. Here the evolutionary rationale is to precondition (in good time) against that CPA, to mitigate its toxic effects. Thus, the wood frog reacts within minutes to ice formation, but its tolerance of high glucose levels varies seasonally. Although such preconditioning involves quite a few concerted adaptations, it is easier to envisage these evolving from an existing stress response—to dehydration, say—than to imagine separate adaptations to subtoxic levels of numerous CPAs. How far we might be able to simulate preconditioning in organisms that (unlike frogs) have not had the benefit of millions of years of evolutionary honing to dehydration is an open question, but it certainly makes sense to amplify natural responses where possible, rather than to superimpose an “alien” protocol.

23.7 PRECONDITIONING DEHYDRATION TOLERANCE

Dehydration tolerance is critical in plants. As discussed in the chapter by Sakai, the twigs of extremely hardy plants such as *Salix sachalinensis*, *Populus maximowiczii*, and *Betula platyphylla* can even survive freezing to nearly absolute zero in liquid helium (at -269°C), if they are first equilibrium-cooled down to about -30°C . The procedure partially freeze-dries the twigs, with much of the freezable water in the cells extracted by equilibrium freezing. When plunged into liquid nitrogen, the remaining intracellular solution vitrifies, preventing further water loss to extracellular ice at lower temperatures, and so avoiding lethal dehydration damage. The stability of such glassy matrices depends on the T_g , which in turn varies with the concentration of the intracellular solution—in other words, the degree of dehydration. This depends on time, as well as the tolerance of cells to dehydration. If the cooling rate is too quick, or is held only a little below zero, then cells do not dehydrate sufficiently, and the intracellular solution may devitrify on rewarming, leading to intracellular ice formation and recrystallization. However, if the prefreezing phase is too protracted, or continues much below -30°C , then the intracellular environment desiccates too far and the cells are injured by dehydration. The “safe zone” varies according to the desiccation tolerance of the plant (and, as we have seen, can be modified by proteins such as dehydrins).

The vitrification protocols pioneered by Akira Sakai replace the prefreezing stage with either solute dehydration or air desiccation, to prevent the formation of ice in both the intracellular and extracellular compartments. Again, tolerance of dehydration is critical. Freeze-drying, solute dehydration, and air-desiccation all require either an innate or an acquired dehydration tolerance. Extremely hardy species have an innate dehydration tolerance, but, excitingly, it seems that even the meristems of tropical monocots, such as bananas and orchids, can acquire dehydration tolerance. By taking advantage of the plant’s own stress response, the procedure is remarkably simple, if time-consuming. Thus, preconditioning of meristem donor plants can be achieved simply by

supplementing their growth medium with rising concentrations of sucrose over a period of a month before vitrification. Similar protocols, and others such as transient heat shock (at about +45°C), also precondition fungi and yeasts, enhancing freeze-tolerance and survival (see chapter by Tan and van Ingen).

Although cause for celebration, there are greater difficulties in applying such elegant and simple methods to mammalian tissues. For a start, animal cells do not have a cell wall. The extent to which plant cell walls protect against osmotic dehydration is uncertain, but vitrified plant cells are considerably plasmolyzed (their cytoplasm contracts away from the cell wall). This probably protects in two ways, neither of which applies to animal cells: first, the CPA solution fills the periplasmic space, protecting membranes and preventing collapse of cell volume; and second, the cell walls preserve tissue structure and integrity, and prevent fusion of dehydrated cells. However, even though there is no equivalent to cell walls in mammalian cells, the microscopic structure of mammalian tissues is bulwarked by collagen scaffolding, and the absence of cell walls has not prevented the successful vitrification, using SIBs, of quite complex tissues, as discussed earlier (or see the chapter by Taylor *et al.*). Indeed, the cryo-banking of many mammalian tissues does not even require cell survival, but rather the preservation of structural integrity, which acts as a framework for the regrowth of cells from the perimeter (see the chapter by Wusteman and Hunt). Of course, this approach only applies to small grafts with a periphery that is contiguous with the adjoining tissues, from which the appropriate cells can grow, and not to whole organs (although cryopreserved cells can be "seeded" into an organs, as in hepatocyte or islet cell transplants, which repopulate parts of damaged or dysfunctional organs).

A second problem may pose more of an obstacle to the successful vitrification of mammalian cells in organs: the lack of an appropriate stress response to cold. Even in plants, this can be a problem. The strongest protection against freezing injury in plants is produced by up-regulating the DREB transcription factors. However, DREBs regulate the downstream transcription of many genes, each of which has an incremental effect on frost tolerance. The difficulty is that DREBs are also found in tropical or temperate plants. In these cases, they seem to control the transcription of a different or overlapping suite of genes in response to other stresses. Rice, for example, expresses DREBs but has no tolerance to cold below +15°C. In mammals and other warm-blooded animals, the problem is even more acute: Whereas mammalian cells are perfectly capable of mounting stress responses, they are extremely sensitive to changes in temperature and do not produce any systematic stress response to chilling below about +32°C. Worse, mammalian homeostasis regulates salt balance strictly, and mammals are never subject to the kind of salt stress that is a major selective pressure for amphibians like frogs. For these reasons there has been little interest in preconditioning mammalian cells to freezing.

Even so, such preconditioning might still be possible. Not all mammalian cells are so molly-coddled. For example, the cells of the renal medulla can tolerate fluctuating concentrations of salts in the molar range as part of the urine-concentrating countercurrent multiplication system. They do so by accumulating compatible solutes, including sorbitol, myo-inositol, glycine betaine, and taurine, via transcription factors acting on osmotic response elements. The system appears to be an ancient throwback to similar mechanisms in bacteria and protists. Interestingly, the renal medulla cells are not the only mammalian cells that can respond in this way to osmotic stress. There is some evidence that glucose triggers an osmotic response in vulnerable tissues (such as the micro-circulation) in people with type II diabetes. Other cells show similar responses in cell culture, implying that the osmotic response elements are still functional in many mammalian cells. Whether preconditioning with glucose or salts, or genetic manipulation of the osmotic response elements, might improve CPA loading and tolerance before vitrification is a fascinating question for the future. Certainly, harnessing endogenous osmotic response elements to the task of CPA loading has the potential to overcome many of the difficulties encountered in the engineering of desiccation tolerance in mammalian cells (such as transfection of trehalose-synthase genes, or membrane poration), discussed in the chapter by Acker *et al.*

23.8 STABILITY OF GLASSES

Assuming that these methods do ultimately enable the stabilization and vitrification of dehydrated organs, one question looms: How stable are metastable glasses? Is there an upper limit to the temperature at which they can be stored, or might it be possible to desiccate to glass and store at room temperature? Predictions about the future have a knack of returning to haunt those reckless enough to make them, but it would be unfair to readers to address the future of cryobiology without venturing an opinion. As befits one of the editors of *Life in the Frozen State*, I hazard the view that desiccation followed by storage at room temperature will prove less fruitful than cryogenic vitrification. Quite apart from the hazards associated with the "collapse temperature" (the temperature at which freeze-dried preparations exhibit observable deformation and cannot easily be rehydrated) the most interesting reasons relate to the stability of glasses below T_g . As discussed by Acker et al., it is not true to say that "molecular motion stops" below T_g . On the contrary, although molecular motion and chemical reactions are much slower in the glassy state than in aqueous solution, membrane degradation can take place in a matter of weeks, even at 50°C below T_g .

The basis of this degeneration is little known, but it may relate to oxidative reactions, especially if the glass is stored not far below T_g and the temperature or moisture content fluctuates (which promotes cycles of vitrification and devitrification and accompanying free radical reactions). Certainly, oxidative damage is known to take place in viable desiccated seeds stored at -20°C. The degree of damage is influenced by the moisture content and the prestorage status of seeds, such as age and lipid composition. Cell survival improves when steps are taken to minimize oxidative damage; for example, by lowering the moisture content, or by storing under vacuum, under nitrogen, or in the absence of light. In this context, Crowe et al. suggest one reason why trehalose seems to be superior to other nonreducing sugars like sucrose, at least under non-ideal conditions: The glycosidic bond between the sugar monomers in trehalose is far less vulnerable to hydrolysis than that in sucrose. As a result of such hydrolysis, the rate of Maillard browning in samples stored with sucrose approaches that of glucose and is up to 2000 times faster than with trehalose. Even with trehalose, however, it is safer to assume that oxidative degradation will continue to some extent at ambient temperatures. For example, lyophilization of some yeasts with trehalose gives rise to genetic variants, such as the petite, respiratory-deficient variants that lack part of their mitochondrial DNA (see chapter by Tan and van Ingen). For long-term storage, it may be necessary to preserve at temperatures well below T_g .

The extent to which oxidative damage can occur at cryogenic temperatures, in either the frozen or the vitrified state, is an open question discussed in the chapter by Benson and Bremner. Gamma irradiation of cells stored in liquid nitrogen produces a detectable hydroxyl-radical signature on electron paramagnetic resonance spectroscopy, but the lack of decay implies that the radicals are "trapped" in the matrix and do not react (at least until rewarmed). Interestingly, the hydroxyl-radical signal does decay at -162°C, which presumably means that hydroxyl-radical reactions can take place in the vapor phase of liquid nitrogen, especially if the storage facility is regularly accessed (see Chapter 15). Similar reactions may take place in the permafrost, which is exposed to ultraviolet and gamma radiations. Even deeply buried permafrost bacteria express catalase, possibly to protect against gamma radiation from radioactive potassium-40 in the surrounding rocks; see the chapter by Ponder et al. Polar bacteria appear to repair DNA at temperatures as low as -17°C, as judged by the incorporation of radio-labeled thymidine, presumably implying that it is damaged at these temperatures, perhaps by oxidative reactions. Whether some degree of DNA damage or epigenetic instability (such as changes in DNA methylation) can be caused by oxidative reactions at cryogenic temperatures is unknown (and seems unlikely), but the stability of genes and genomes over the whole course of cryogenic storage is an issue of growing interest, recently christened "cryobionomics" by Keith Harding. The apparent genetic variability of freeze tolerance between individuals implies that selection for cryogenically stable genotypes might take place before, during, or after

storage. Such selection could reflect natural variations in resistance to oxidative stress rather than other, perhaps ultimately more desirable, phenotypic traits related to wild-type vigor.

Nonetheless, if suitable precautions are taken (such as shielding against radiation or light, and safeguarding against large temperature fluctuations in the vapor phase, to prevent degenerative cycles of vitrification and devitrification), it seems unlikely that hydroxyl radicals would be generated by oxidative reactions during cryogenic storage. There is little detectable activity of oxidative enzymes, such as lipoxygenase, below about -20°C . At lower temperatures, the concentration of reactants by ice can promote nonenzymatic oxidative reactions and lipid peroxidation. Data from the frozen food industry give an indication of how far such reactions can proceed: In frozen parsley, for example, nearly 20% of phospholipids are lost within a month of deep freezing at -32°C , and remarkably, 70% are lost within 3 d at -18°C . Thus, cryogenic temperatures may be required to inhibit oxidative reactions altogether.

23.9 STABILIZING ENERGY-TRANSDUCING MEMBRANES

Regardless of how many oxidative reactions take place during cryogenic storage, the most serious threat posed by oxidative injury is postponed until rewarming. This damage is a sequel of the spatial and organizational disruption of energy-transducing membranes during cryopreservation. The vulnerability of mitochondrial membranes was demonstrated by Tappell and others during the 1960s, when they showed that ATP synthesis is uncoupled by cycles of freezing and thawing. The chloroplast membranes are similarly (if not more) vulnerable in plants. Damage to the photosynthetic and respiratory redox proteins (which are very densely packed, accounting for 60% of membrane composition), and to the highly unsaturated lipid bilayer in which they are embedded, is likely to be manifest only on rewarming, when electron transport begins again. Now, oxygen is freely available as an electron acceptor, but the integrity of the electron transport chains is compromised. Such conditions are practically guaranteed to generate superoxide radicals, hydrogen peroxide, and hydroxyl radicals. Apart from the ensuing failure to restore primary energy metabolism when most critically needed, mitochondrial and chloroplast free radical production distorts cellular redox signaling, and if unrestrained, it may promote apoptosis. Release of cytochrome c from damaged mitochondria is redox dependent, and a critical component of the apoptosis execution machinery.

Such vicious circles are well known in the context of ischemia-reperfusion injury in transplanted organs, and although cryogenically preserved tissues are not ischemic (because metabolism has ceased altogether), membrane disruption is likely to provoke a similar outcome. That this really is the case is illustrated not only by studies of cryopreservation (e.g., many studies of oocyte cryopreservation show mitochondrial degeneration) but also by research into the mechanisms of tissue destruction following cryosurgery. For many years a "Cinderella" subject, modern cryosurgery is coming into its own as a sophisticated molecular science with direct relevance to cryopreservation as well as surgery. As discussed in the chapter by Hoffmann and Bischoff, free radical-mediated mechanisms underpin both microvascular and immune injury, which in turn produce indirect cryosurgical damage well beyond the confines of the ice-ball itself. Although the intention of cryosurgery is to maximize damage, it seems probable that the only reason such injury is not routinely detected following suboptimal cryopreservation is that it is not routinely looked for. In this context, Taylor et al. speculate that the "80% ceiling" of success in the vitrification of mammalian tissues may be a consequence of apoptotic cell death and membrane instability. The situation is even more extreme in conventional cryopreservation. For example, the number of live chondrocytes in cryopreserved articular cartilage (40% survival immediately after thawing) is "dramatically lower" 3 months after transplantation, a loss that Muldrew et al. ascribe to apoptosis. The question is, Can we learn from nature's laboratory how to limit the disruption of the energy-transducing mitochondrial and chloroplast membranes?

Plants provide a clue. In photosynthesis, several alternative pathways of cyclic, noncyclic, and pseudo-cyclic photo-phosphorylation coexist, and it seems likely that their joint operation ensures redox poise under extremely variable ambient conditions. Similar, albeit less sophisticated, controls are found in mitochondria. Indeed, the existence of independent platoons of mitochondrial and chloroplast genes may be a testament to the importance of rapid genetic responses to environmental changes, to maintain redox poise. Regarding freeze-tolerance, the most interesting adaptation of plant mitochondria is the rapid expression of an alternative oxidase in response to cold, which bifurcates the respiratory chain. The alternative oxidase generates little energy, as it bypasses complexes II, III, and IV, but it does have two important effects: first, it quickly reduces excess oxygen to water, and second, it leaves the respiratory complexes in a relatively oxidized state. Thus, in response to the sudden onset of cold, the mitochondria are effectively “parked” in an oxidized state, with few respiratory electrons kicking around to cause trouble and with low levels of oxygen available to react on rewarming. At the same time, the pool of reduced antioxidants, in particular glutathione and ascorbate, is built up. These antioxidants are concentrated in the mitochondria and chloroplasts. Whatever else happens there deserves closer scrutiny. For example, sugars such as trehalose have been shown to stabilize the respiratory complexes (such as ATPase activity) following freeze-thaw of isolated mitochondria, but whether sugars—or proline and dehydrins—do concentrate in the organelles of freeze-tolerant plants is unknown. Clearly we need to learn a lot more about the subcellular compartmentalization of freezing tolerance.

Given that animal cells do not benefit from the plant’s alternative oxidase pathway, is it possible to “park” animal mitochondria in a stable, relatively oxidized state before freezing? It is certainly feasible. Presumably this happens naturally in starvation. Many freeze-tolerant insects survive the polar winters by voiding their guts. Such behavior is usually interpreted in terms of the ice-nucleating properties of feces—removing the feces removes the most potent ice-nucleators—but the lack of sustenance may provide the additional benefit of “parking” respiratory chains in a relatively oxidized state. Certainly, as discussed in the chapter by Elster and Benson, the larvae of the Arctic diptera *Heleomyza borealis* enter a preconditioned hypometabolic state before the onset of winter, and this dormant state appears to be its main protective strategy. It is conceivable that we could stimulate similar changes in mammalian cells. For example, calorie restriction in mammals promotes major changes in gene expression, which regulate mitochondrial function and suppress free radical leakage. It would be interesting to see whether calorie restriction, perhaps for a period of a few weeks before freezing, could improve mitochondrial integrity during and after cryopreservation.

The other beneficial effect of expressing an alternative oxidase in plants is the removal of excess oxygen. This may be less easy to manipulate in mammals, as oxygen delivery to cells is very tightly regulated. However, there are some signs that elimination of oxygen is desirable. Mazur’s group have shown that the motility of mouse sperm is nearly doubled after cryopreservation with an *Escherichia coli* membrane preparation called Oxyrase, which reduces the oxygen tension to less than 3% of atmospheric pressure. Although such preparations are hardly applicable to whole tissues, it is already known that the outcome of vitrification is enhanced by storage under nitrogen, as discussed earlier. Thus, experimental manipulations to reduce the oxygen tension in stored tissues may well be a profitable avenue in future.

23.10 THE FUTURE OF CRYOBIOLOGY

So where does all this leave the future of cryobiology? We have much to learn from Mother Nature, but if we consider the spirit rather than the letter of evolutionary adaptations, and how to dovetail these adaptations with the formal biophysics of cryobiology, the years ahead look rich with possibilities. The fields that seem especially rewarding relate to the stability of glasses, the preconditioning of dehydration tolerance, and the preservation of metabolic integrity during and after

cryogenic storage. If each of these aspects can be incorporated into cryopreservation protocols, I see no reason why the future challenges of cryobiology should not be successfully overcome. Above all, it is time to cast off the old biomechanical view of cells as passive osmometers and to embrace the dynamism of the new biology.

ACKNOWLEDGMENTS

I would like to thank Professor Barry Fuller, Dr. Erica Benson, Dr. Ana Hidalgo, Dr. Keith Harding, and Dr. David Bremner for stimulating discussions on the content of this chapter.