CRYOPROTECTANTS: A REVIEW OF THE ACTIONS AND APPLICATIONS OF CRYOPROTECTIVE SOLUTES THAT MODULATE CELL RECOVERY FROM ULTRA-LOW TEMPERATURES

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Abstract

Cryopreservation has become a central technology in many areas of clinical medicine, biotechnology, and species conservation within both plant and animal biology. Cryoprotective agents (CPAs) invariably play key roles in allowing cells to be processed for storage at deep cryogenic temperatures and to be recovered with high levels of appropriate functionality. As such, these CPA solutes possess a wide range of metabolic and biophysical effects that are both necessary for their modes of action, and potentially complicating for cell biological function. Early successes with cryopreservation were achieved by empirical methodology for choosing and applying CPAs. In recent decades, it has been possible to assemble objective information about CPA modes of action and to optimize their application to living systems, but there still remain significant gaps in our understanding. This review sets out the current status on the biological and chemical knowledge surrounding CPAs, and the conflicting effects of protection versus toxicity resulting from the use of these solutes, which are often required in molar concentrations, far exceeding levels found in normal metabolism. The biophysical properties of CPAs that allow them to facilitate different approaches to cryogenic storage, including vitrification, are highlighted. The topics are discussed with reference to the historical background of applying CPAs, and the relevance of cryoprotective solutes in natural freeze tolerant organisms. Improved cryopreservation success will be an essential step in many future areas such as regenerative medicine, seed banking, or stem cell technology. To achieve this, we will need to further improve our understanding of cryobiology, where better and safer CPAs will be key requirements.

1. Introduction

Within the community of cryobiologists, discussions surrounding the history, biochemical and biophysical modes of action of, and optimal applications for, the diverse range of solutes that constitute cryoprotective agents (CPAs) are central to the growing importance of cryobiology. Some of the early CPA discoveries carry almost legendary status, such as the serendipitous identification of glycerol as the essential solute allowing survival of spermatozoa from exposure to ultra low cryogenic temperatures by Polge and colleagues [233, 265]. However, as attributed to Albert Szent Giorgyi, *'a discovery is an accident meeting a prepared mind'*. Polge was already looking for freeze-protective substances (previously suggested to be sugars) in work on freezing fowl sperm, when the inadvertent use of a bottle containing a glycerol mixture led to instant success and ultimately the establishment of modern cryobiology [232]. For many scientists across a number of different fields (for example reproductive therapy, haemopoietic stem cell transplantation, banking of seeds and plant genetic resources, and now the rapidly expanding horizons for regenerative medicine), utilization of CPAs to support successful cryopreservation has become routine. Despite this prevalence, a clear understanding of the multifaceted actions of these solutes does not always accompany their pragmatic application.

Cryogenic banking of a variety of cell types to effectively 'halt the march of time' has become a very useful tool for many applications including managing resources, avoiding wastage, allowing testing and validation before release, matching different donor—patient pathways, as well as permitting shipping to distant end users. The selection of suitable CPAs and protocols for various applications may appear seductively straightforward, but more incisive testing of the effects of cryopreservation can often detect hidden cryopreservation failure that may take several hours to develop post-thawing [18, 307]. Cryopreserved cells may also fail to perform the full range of normal functions when assessments of recoveries are made on a more comprehensive basis [107]. The historical conceptualization of cryoprotection (as we now term it) from studies on freeze tolerance in natural

systems, linked with accumulation of protective solutes, goes back more than 100 years, and has been reviewed previously [105]. This was followed by a period of focused study from the 1950's onwards to identify solutes that could be applied in the laboratory to permit cell and tissue cryopreservation [143, 179]. The current review sets out to document what has been learned since the most recent previous overview a decade ago [105], on the range of available solutes, the benefits and challenges associated with CPAs, the chemical and physical bases for their abilities to modulate how water behaves during cryopreservation, and to shine a light on recent research in our understanding of cryoprotection.

2. The Challenges for Living Systems at Ultra-low temperatures

2.1 Water, Cooling, and Ice Nucleation.

In terms of the defined focus on cryopreservation, the discussion of solutes that permit cells to survive cryogenic exposure naturally lean towards the empirical. In fact, a classical definition of 'cryogenic' is a temperature below −150 °C, whereas cryopreservation requires cells to survive effects of exposure to a wide range of progressively lower temperatures, both above and below −150 °C, where different biophysical factors can play a role, as will be discussed later. In comparison, some biological systems survive exposure to the deep subzero range down to about −40 °C in the extreme. We have used the term 'ultra-low' to encompass all these temperature ranges where protective solutes are important. The definition of a *cryoprotectant* is any solute which, when added to cells in their medium, allows higher post-thaw recoveries than if it were not present [143]. However, before discussing the nature of CPAs and these applied definitions, it is worth considering the challenges that are imposed by cooling to ultra-low temperatures – i.e. the process of freezing. As has been well described, vital life processes depend on liquid water. The changing, subtle, multifaceted configurations that water molecules can assume under physiological conditions are necessary to solvate and stabilize the full gamut of essential biomolecules [98, 103, 272]. Discussions

on the position of water in life on this planet have often reached philosophical levels, as for example the quotation '*If there is magic on this planet, it is contained in water*' from anthropologist and science writer Loren Eisley. It is therefore not surprising that the phase transition that occurs during ice nucleation can be expected to have dramatic consequences for living organisms far beyond simple effects from temperature reduction. Above the freezing point, accepted evidence suggests that water exists as transient but inter-related networks of 'flickering clusters' momentarily constrained by hydrogen bonding and continually reorganizing on a picosecond timescale [292]. At the point of ice solidification, water molecules assume tetrahedral orientations where the hydrogen bonding leads to an open lattice network, with six interacting water molecules existing in a structured format (involving both nucleation events and growth of crystallites [204, 205]) sometimes referred to as 'the stacked chair structure' [101]. This process provides hexagonal ice as is routinely encountered in nature and the laboratory, and termed ice 1h by ice physicists [17]. These orientations have been confirmed by crystallographic and diffraction techniques, but it should also be pointed out that several other water molecule orientations can exist in ice {up to ice XVI that is a seventeenth crystalline ice phase with the least dense (0.81 g/cm^3) experimentally established by removing all guests using vacuum pumping on small partials of neon hydrate (of structure sII) first obtained in 2014 [94]} if physical conditions such as temperature and pressure change. Many of these forms are of a transitory nature and exist under relatively extreme conditions outside those of normal focus for biologists aiming to preserve life forms by cryopreservation. That said, the concepts do overlap because, as discussed below, the activities of cryoprotective solutes can influence the way that water molecules interact with each other and with other dissolved solutes as ice nucleation progresses.

One of the paradoxical facts concerning the phase transition of liquid water to ice is that, whilst we can discuss concepts of ice crystal structures and the multiplicity of variations in those, in practice, solutions rarely freeze (within any practical time frame) at the equilibrium phase change

temperature. This is important to the current discussion because one effect of many CPAs is to modify the phase change process. The driving mechanisms for mobile water molecules to become organized into ice nuclei, as temperature is lowered, are changes in thermal energy in the solution and, in biological systems, surfaces that can catalyze the event by a process called *heterogenous nucleation*. Many such catalysts exist in the environment such as microscopic dust particles, especially containing silicates, bacterial surfaces, chemicals in the crystalline form such as cholesterol, or indeed particular protein structures [102, 154, 310, 315]. Such heterogenous nucleation dominates ice crystal formation in biological systems over the temperature range between −5 °C to about −30 °C [310, 311]. Water molecules can undergo self-association to form structures that can nucleate ice with a process termed *homogenous nucleation* [311], which is surprisingly energetically unfavourable at high subzero temperatures (i.e. just below 0 °C where we normally assume environmental freezing to take place). The homogenous nucleation temperature for water occurs only at significantly lower temperatures close to −40 °C or below [102, 242, 310]. As discussed, in nearly all circumstances encountered when cooling dilute aqueous solutions, ice formation can be detected at intermediate temperatures (often between about −5 °C to −20 °C) depending on the solution, the cooling rate, the volume and container, to name only a few variables that influence the process called heterogenous ice nucleation [206]. This is equally true for ice nucleation in the environment, including the atmosphere [210]. The temperature difference between the true equilibrium melting point and that of heterogenous ice formation is termed 'undercooling' or 'supercooling' [67, 311]. This undercooling point or nucleation temperature can be measured by the release of the latent heat of ice formation and can be highly variable even between samples cooled within the same batch [206].

2.2 Osmotic Response to Ice Formation.

Chemists and natural scientists have known for centuries that one of the fundamental consequences of the water-ice transition is alteration of the ability of water to solvate salts and other solutes; this

was commented on by Boyle in his treatise on freezing [28], and confirmed by many observations since then [259, 271]. Solutes are excluded from ice crystal structures, leading to increases in concentration in the residual fraction of liquid water and colligative freezing point depression. For dilute salt solutions, as temperatures are progressively lowered, the fraction of ice increases and consequently the solute concentration increases, causing further freezing point depression [47]. In biological freezing, cells are invariably also excluded from the growing ice matrix. This inevitable consequence of increased solute concentration was conceptualized by Lovelock [179] as a major damaging factor during freezing of cells. He hypothesised that the increased salt concentration led at some point to irreversible membrane damage (by chemical mechanisms not fully defined) and subsequent cell lysis on thawing. He designed experiments which showed that in his studies of human red cells, sodium chloride, the major constituent in plasma, was the dominate solute. The osmotic effects that Lovelock described were subsequently accounted for in a quantitative way for cells in the presence of ice by Mazur [195] based on fundamental thermodynamics.

For the great majority of cell types studied, this osmotic dehydration during freezing has been consistently observed, by direct microscopy or more sophisticated electron microscopy [244]. At the relatively slow rates of cooling encountered during freezing in the environment, or for typical laboratory specimens (i.e. several ml of a cell suspension), this cell dehydration can reach extreme proportions. There remains sufficient time for the intracellular water to efflux across the cell membrane in the face of the increasing ice fraction, and as a consequence ice is not observed intracellularly. Studies by Meryman [199] provide more evidence for osmotic effects as major events in slow freezing cell injury. The same group proved additional support for the reduction of the osmotically driven cell injury seen during ice solidification by the colligative effects resulting from adding neutral solutes as CPA [202].

However, if cooling progresses at rates kinetically incompatible with full cell osmotic responsiveness, ice crystals can be detected within the body of the cells [288], at least in many cell systems. The sum

of these observations was combined by Mazur [193] to reflect the findings that for cells to withstand freezing processes, an optimal cooling rate can be defined, with increased cell injury both at very low cooling rates (equated to osmotic stress and other associated mechanisms during freezing) or very high cooling rates (where intracellular ice is produced). Mazur distilled these concepts into his seminal paper on the two factor hypothesis of freezing injury [194], which has largely withstood the test of time over many years of subsequent study. One important fact that has emerged has been the realization that the descriptors 'slow' or 'fast' in this context must be used with caution because there are many factors which combine to shift the optimum cooling rate for cell survival, including the biology of the particular cell in question, and the impact of CPAs, which will be discussed below.

2.3 Vitrification and the Avoidance of Ice.

At this point it should be recognized that over the same period, there was a growing understanding that, under very specific conditions, water can be cooled to cryogenic temperatures in a manner that avoids the process of ice nucleation all-together [183], a process called 'vitrification'. Vitrification in aqueous systems is a complex process in physical terms, but can be described as solidification of a solution at low temperatures by achieving an extremely high viscosity without ice nucleation and crystallization [301]. This has sometimes been referred to in the public parlance as the creation of 'amorphous ice', or 'glass water', but in the strict definition sense it cannot be ice as it displays no crystalline structure. The physics of vitrification of water are complex and beyond the scope of the current review [5]. Solids created by vitrification are called glasses. Water and dilute aqueous solutions must be cooled at extremely high cooling rates to vitrify, but the high levels of intracellular solutes attained when cells dehydrate in the presence of extracellular ice can make conditions more favourable for intracellular vitrification during typical cooling conditions. As will be discussed later, for larger cellular constructs (e.g. embryos and tissues), vitrification of both the intracellular and extracellular solutions is the preferred approach.

3. Cryoprotectants: Solutes that Modulate the Liquid Water to Ice Transition.

Given that intracellular ice formation is universally accepted as a severe cellular stress that tends to lead to irreversible injury, it is not surprising that CPAs have long been suspected of altering the manner in which liquid water transitions to ice. Indeed, freezing may be applied deliberately to deliver cell disruption in many biotechnological processes [236]. However, it is worth noting as an aside, that ice formation under normal conditions (e.g. for cells in normal growth media) does not result in immediate cell death. There is a kinetic aspect to the injury (itself generally attributed to dehydration stress), which for many mammalian cells can take several minutes to become irreversible [198]. For example, in work on suspensions of tumour cells, it was shown [130] that some 30% of cells would survive freezing to –15 °C for 10 minutes, but progressive injury manifested (e.g. a reduction to 14% survival) as the time was extended to 20 minutes.

Early in the history of cryoprotection, Nash [213] postulated that CPA action was linked to an ability to alter water properties as a result of hydrogen bonding between water and the CPA. This was a far-sighted contribution which has subsequently been taken to higher levels of sophistication with detailed molecular simulations [186], and remains a cornerstone to explain CPA activity today [293]. Water that is bound to solutes is considered 'osmotically inactive' and is not readily available to participate in the formation of ice crystals.

The nature of the dehydration injury following progressive slow freezing has been debated for many years [200] and has also been linked to the extent of bound versus free water. The discussions developed alongside the seemingly paradoxical observations that partial dehydration *before* freezing could permit survival of some cell types from deep cryogenic freezing [185]. In reality, the term bound water is an oversimplification of the complex nature of various water compartments within any cell [109], which respond in different ways to osmotic stresses such as freezing, but it serves as a useful way to develop discussions about cryoprotection. Indeed, the similarity between

anhydrobiosis and freezing tolerance was noted from environmental studies, which led to the concept that there are different water compartments within cells, containing freely diffusible (and freezable) water alongside restricted or 'bound' water [215]. Mazur's Two Factor hypothesis [194] was developed over several years from observations that different cooling rates could yield significantly different survival from cryopreservation across a varied range of cell types [171]. It was discussed that survival from cryopreservation required an 'optimal cellular dehydration' (in the presence of CPAs), which could be achieved by allowing regulated extracellular ice crystal growth (discussed in Section 5) during controlled cooling. The bound water compartment is associated with structural organization of membranes and organelles, and polymeric components such as proteins. As dehydrative freezing progresses to impinge on restricted water, cell structures can become unstable leading to irreversible injury under some slow cooling conditions [36, 45]. A second factor observed at higher cooling rates was related to intracellular ice formation (IIF), with the incidence of injury due to IIF increasing with increasing cooling rate [209]. The balance between these two factors results in a maximal survival for a particular cell type across a limited cooling rate profile, producing what came to be known as Mazur's bell shaped survival curves.

The status of water in the non-frozen solution could also play a role in cell injury. In a recent study of molecular structure in dehydrating trehalose glasses, as the free water diminished during dehydration, a highly networked trehalose glass was observed to develop. The appearance of this macrostructure coincided with the onset of significant cell death, suggesting a possible direct physical/compressive injury of cells below a critical moisture content [295]. Studies from nature suggesting that organisms that can survive dehydration (anhydrobiosis) can also withstand freezing, led to the 'water replacement' theory, which focused on the potential for certain protective solutes to moderate this kind of irreversible cellular destabilization by replacing water on the surface of critical biomolecules [46].

These discussions surrounding the importance of cellular water compartments progressed alongside the longer-term understanding that avoidance of biological freezing injury was often related to the presence of additives (in reasonably high concentrations) such as sugars, glycerol, or milk proteins in the mixture [152]. Ethylene glycol was seen to protect 'vinegar eels' (*Anguillula aceti*) against rapid cooling in liquid air [184], whilst sugars were observed to support the recovery of frozen erythrocytes [100]. The study from Polge et al. [233] combined aspects of this collective understanding and unified concepts of successful cell cryopreservation by application of glycerol as a CPA with low inherent toxicity. These ideas were quickly transferred to other cell types. For example, working within the same group in the subsequent decade, Lovelock further refined theories on the nature of freezing injury resulting from cell hypertonic stress that developed as ice progressively solidified as pure water from the extracellular solution, leaving cells bathed in residual fluid channels of increasing salt concentration [177]. He made important observations on the protective effects of neutral solutes such as glycerol during cryopreservation, and especially that dimethyl sulfoxide (Me$_2$SO – an agent not previously considered in freezing studies) was a very efficient solute in protecting red blood cells from freezing injury [179, 180]. He demonstrated that effective 'salt buffering' could be achieved during freezing by neutral solutes such as glycerol [178]. These protectants could be added in sufficiently high aqueous concentrations to depress the freezing point of the solution, thus avoiding cell exposure to injurious salt concentrations, and also penetrate the cell membrane to protect intracellular compartments, while remaining below threshold concentrations for toxicity [179].

In the mid-1960's Doebbler [65, 66] and Rowe [252] reviewed the contemporaneous literature to identify many of the CPAs that remain important in cryobiology even today. Doebbler further considered relationships between molecular structure and CPA activity. The ability of CPAs to hydrogen bond with water was proposed as a strong correlate with protective ability, whilst multiple hydrogen bonding sites per molecule magnified CPA efficiency, with the caveat that many of those

early studies were carried out using red blood cells as the model system. Comparisons of good aqueous hydrogen bonding molecules (e.g. Me_2SO) with structurally similar compounds that do not hydrogen bond with water (dimethyl sulfone) provided complementary evidence of the theory. Doebbler acknowledged the importance of biological differences across different cell types when comparing different CPAs. Doebbler also recognized that inherent physical properties of aqueous solutions of different CPAs, such as increasing viscosity with lowering temperature, impact the kinetics of ice nucleation and ice crystal growth. These properties could all play roles in the success of mitigating freezing injury, such that no one set of molecular properties would adequately predict CPA efficiency. During the same period, it was also observed that certain polymeric compounds, including dextran or polyvinylpyrrolidone could afford CPA activity during rapid cooling, and weakly so at slow cooling rates [66, 286]. It came to be understood that this protection could be attributed to the tendency of some polymers to enhance the formation of a glassy matrix during cryopreservation [273].

Cell membranes were identified by Rowe [252] as one of the key targets for freezing injury. He suggested that an 'association' between CPAs and cell membranes was important. Although this concept was developed further and expressed differently later by Clegg, the Crowes, and colleagues [36, 46] in their water replacement theory, this was a prescient observation on this additional property of CPA activity. Recent molecular modelling papers are enabling the study of CPA-membrane interactions in exquisite detail. For example, Hughes and colleagues have recently demonstrated membrane thinning in the presence of Me_2SO, pore formation at critical concentrations, and beyond these concentrations, loss of membrane stability [127]. As discussed later, these interactions can play a role in the relative toxicity of CPAs for any given cell type.

The study of the pharmacology of CPAs within cryobiology was championed by Karow [141, 143, 147]. He clearly defined the two major classes of CPAs (small molecular weight, *penetrating agents*, and high molecular weight *non-penetrating agents*). He listed some 56 chemicals (comprising both

CPA classes) which demonstrated efficacy defined as post-thaw survival of 40% or greater [147] in different cell types, although this depends to a large degree on the viability assays employed in the different studies. The majority of CPAs employed today appeared on that list, including dimethyl sulfoxide, propylene and ethylene glycols, methanol, some amino acids, amides, a number of oligosaccharides and some polymers such as albumin and PVP. Karow also highlighted the potential for CPA toxicity, especially for penetrating CPAs that can influence a variety of normal biochemical processes, but indicated that these toxic effects could be mitigated by developing CPA protocols where the final concentration, temperature, and time of exposure were optimized before cryopreservation. He introduced the concept of 'CPA pseudo-toxicity', whereby activities such as muscle excitation were shown to be significantly inhibited in the presence of agents such as dimethyl sulfoxide, but the inhibition was entirely reversible when the penetrating agents were washed out of the cells [141]. It has also become clear that there are several classes of effective, but chemically unrelated, CPAs that can produce desired effects (protection against freezing injury) by multiple mechanisms, not including unique drug / receptor or target interactions associated with specific drug classes.

Given the multiplicity of actions of CPAs (many of which have not yet been fully identified) and the need to mitigate toxicity, it was hypothesised early on that combinations of CPAs might be more effective. For example, the cryopreservation of corneas was enhanced by using combinations of dimethyl sulfoxide and sucrose compared to that achieved using dimethyl sulfoxide alone [32]. Importantly, in many of these early studies [170, 197], CPA combinations were developed by mixing both permeating and non-permeating CPAs, a philosophy that has been successfully exploited in many subsequent CPA protocols (see later Sections below). When studying CPA mixtures, within certain CPA classes (such as alcohols) it was noted that some alcohols (e.g. ethanol) could act as *cryosensitizers,* reducing post-thaw survival rather than acting as effective CPAs [157]. Kruuv's group attempted to produce an objective statistic (which they called tau - τ) to numerically define CPA

interactions [38], whereby synergism of individual CPA on outcome could be defined as τ > 0, whilst antagonism of CPA actions yielded τ < 0. However, given the continuing uncertainties about the multiple modes of CPA actions, it has so far been difficult to develop this concept further.

By the 1990s, activity in cryopreservation had expanded some 10-fold (as judged by cited publications) over that reported in the 1960s, which led to a pragmatic consolidation of the CPAs applied in the majority of the reported studies. Karow's 'list of 56' was reduced down to some 20 agents by Ashwood-Smith [11]. He produced a table listing CPAs on the basis of their expected efficacy, based on publications and his experiences. Of his 20 agents, only half were identified as potentially generally effective. We have updated Ashwood-Smith's list in Table 1, based on ongoing evidence since his publication. However, the majority of CPAs listed as effective by Ashwood-Smith [11] remain those of choice in contemporary scientific reports. In a similar vein to Karow, Hubalek [126] reviewed the CPAs used for cryopreservation of micro-organisms, and identified some 55 agents that had been studied to varying degrees. However, when a pair-wise analysis was applied to the CPAs based on survival of over 40% after freezing, the number again reduced down to 8 CPAs, and these same CPAs were represented by Ashwood-Smith [11]. It is fair to say that no new CPA classes have been discovered to replace the moderately or highly effective agents reported by Ashwood-Smith, Hubalek, and now shown in Table 1. What has changed over recent years has been a growing understanding of the biophysical factors that must be controlled to achieve survival from deep cryogenic temperatures, such as control of ice nucleation and/or crystal growth, and the potential for synergy between CPA mixtures, which are discussed in the following sections.

4. Cellular and Molecular Changes in the Presence of CPAs: Stability versus Toxicity

4.1 Protective Effect of CPAs on Membranes and Intracellular Organelles

Much of the discussion in this review has necessarily focused so far on the dominant effects of CPAs on ice formation, and cellular dehydration during freezing, both of which improve post-thaw survival. However, there have been a number of studies that have investigated interactions of CPAs directly with different cellular molecular structures, i.e. the specific effects of CPAs that contribute to survival.

Cell membrane stability in the presence of sugars was an early focus for Crowe's group [55], and they were one of the first to apply Fourier Transform Infrared (FTIR) spectroscopy to these kinds of questions [54]. In general, they identified hydrophobic interactions between sugars, amino acids, and membrane bilayers as important factors in cryoprotection [53, 255]. They also used membrane fusion studies in model systems as a target for freeze-induced dehydration injury, and provided information to support the concept that permeating CPAs such as glycerol and Me_2SO interact with polar head groups of phospholipids within the lipid bilayer [53, 54] to confer a degree of stability at concentrations similar to those used in cryopreservation. FTIR was subsequently applied as a way of defining freezing injury to the cell membrane and cell protein components by Bischof, Wolkers and colleagues [23]. Building from this, Wolkers' group applied FTIR to assess molecular alterations during cryopreservation of endothelial cells [267] in the presence of Me_2SO. They found that Me_2SO decreased the membrane conformational disorder encountered during freezing to −40 °C. There was a less clear effect of the CPA on cellular proteins during freezing, but Me_2SO did delay the onset of protein denaturation detected during warming. Wolkers, Oldenhof and colleagues recently combined FTIR with cryomicroscopy studies to link the ability of CPAs such as glycerol to facilitate continued membrane osmotic water flux out from the cells during transit to lower temperatures

through the high subzero range during progressive freezing of sperm. These observations were also linked to the interaction of the CPA with membrane phospholipids to mitigate the membrane lipid phase transitions occurring during transit through the subzero range [217, 218]. In their recent work, they used FTIR to study membrane phase transitions of fibroblasts during cooling at superzero temperatures as well as during freezing. It was found that membrane-impermeable trehalose could be taken up by mammalian cells during freezing-induced osmotic forces and fluid-to-gel membrane phase transitions. However, the range of cooling rates that can be used to cryopreserve fibroblasts with trehalose was observed to be much narrower compared to that when Me_2SO is used [313].

Because of the acknowledged importance of intracellular organelle stability for cryopreservation of mammalian oocytes (especially the tubulin-based network of the meiotic spindle which controls chromosomal distribution, and the actin-based network of the microfilaments which control normal fertilization processes), several investigators have focused on the potential for CPAs to stabilize (or indeed disrupt) these networks. The studies have been undertaken alongside the knowledge that cooling of oocytes *per se* can result in destabilization (depolymerization) of the meiotic spindle [231]. Addition of Me_2SO to mouse oocytes for a limited time was seen to correlate with appearance of cold-stable microtubule asters, whilst prolongation of exposure resulted in spindle disassembly [136]. Both propanediol and Me_2SO were found to stabilize the spindles of mouse oocytes in concentrations of 1.5M when the cells were cooled to room temperature [281], compared to control oocytes similarly cooled. Washing out of Me_2SO and return to physiological temperatures resulted in a return towards normal spindle morphology, although the same was not seen when using propanediol. Effects of propanediol on mouse oocytes were studied without cooling [137]; a differential was noted where meiotic spindles were stabilized when exposed to CPA concentrations of 1.5M and above, whilst application of < 1M CPA was associated with microtubule disruption. It was suggested that water-modifying CPA may be acting by reducing the critical concentration for microtubule polymerization, favoring stability. Gook and colleagues studied mouse and human

oocytes cooled to room temperature in 1.5M propanediol [110], and recorded a protective effect in human oocytes that was not seen in mouse oocytes. Further studies on CPAs at concentrations of 1.5M on human oocytes suggested that propanediol, ethylene glycol, and Me$_2$SO all stabilized human oocyte meiotic spindles during cooling to room temperature [306]. However a cautionary note was sounded by Larmen and colleagues [168]. In the context of studying exposure of mouse and human oocytes to much higher CPA concentrations, such as those used in vitrification techniques, exposure at room temperature was associated with spindle disruption, but this was not seen if the exposures were performed at 38 °C. Thus, there are complex interactions between CPA concentrations, temperature, time of exposure (i.e. the balance between stabilising effects of CPA versus increasing toxicity as exposure time is increased), and meiotic spindle stability, which are not currently easy to predict.

The effects of CPAs on the oocyte actin cytoskeleton have not been studied as much, but early work suggested that exposure to Me$_2$SO at room temperature caused little change in actin microfilaments, whereas exposure at 37 °C resulted in depolymerization [284]. In fact pharmacological *disassembly* was found to improve mouse oocyte survival after cryopreservation using Me$_2$SO [124]. Moving to higher CPA concentrations (as in vitrification), it was shown that exposure to ethylene glycol up to 6M for short times (3-5 min at room temperature) did not affect microfilament stability, whereas longer (7-10 min exposures) resulted in actin filament disruption [125]; however the suitability of short exposure times appears to be an avoidance of toxicity rather than any direct effect on microfilament stability.

4.2 Toxicity

It will be clear from the above that CPAs are multi-modal agents, which may be discussed loosely as 'non-specific' drugs. In fact CPAs were described as 'a new class of drugs' early in the history of cryobiology [143]. For example, CPA toxicities, similar to classical drug toxicities, can be described as

'manifestations of adverse effects'. However, CPAs differ quite significantly from most traditional drugs, which are often delivered in micromolar concentrations with a specific receptor target. In contrast, CPAs are applied in the molar concentration range, with multiple actions combining specific and non-specific targets. Within the pragmatic division of CPAs into *permeating* and *non-permeating* agents, the potential for adverse events or reactions is heavily skewed towards *permeating*, since these are obviously able to interact directly with a multitude of cellular activities such as enzymatic processes, transporter mechanisms, ion exchanges and the like. Very few toxicity events have been linked specifically with high molecular weight *non-permeating* agents.

4.2.1 Osmotic Injury during CPA Application and Removal

The differences in effective concentration ranges between normal drugs and CPAs however introduces an additional pathway for damage, resulting from osmotic effects. With most permeating CPAs being applied within the ranges of 5–10% w/v (equating often to about 1 mol l^{-1}) during slow cooling protocols (and at higher concentrations if vitrification is the chosen method), significant concentrations of the CPA will accumulate within intracellular compartments. Diluting cells post-thaw directly into isotonic tissue culture solutions (or injecting directly into a patient with normal isotonic body fluids) inevitably results in a significant uptake of water by the cells that in the extreme can destroy homeostatic mechanisms, including membrane barrier characteristics. This was observed early in applied cryopreservation, leading to the concept of gradual controlled dilution of CPAs in studies on red blood cells [201, 264] and embryos [296].

It was also understood from historical observations [113] that the thawing process itself might lead to injurious osmotic transients, whereby ice melting and the liberation of osmotically available water could induce localized dilution stress causing rupture and haemolysis during the warming of ice-embedded red blood cells as temperatures passed through the zone between −6 °C to −2 °C. Following on from these osmotic-dilution injury concepts, over the next two decades, osmotic buffers were successfully introduced into the initial CPA exposure solution [2, 64], returning focus,

and supplying explanation, to the concept of efficacious cryoprotectant mixtures (see later discussions). Agents such as sugars, or in some cases polymers, can assist in mitigating influx of water into CPA-loaded cells upon dilution, often at a similar but slightly lower concentration than the penetrating CPA (e.g. applying 0.5 mol l^{-1} sugar in the diluent) - based largely on empirical observations of the dilution steps. For example, glucose was used for CPA dilution after hepatocyte cryopreservation [107], whilst sucrose became popular for diluting CPA from embryos [216, 234, 238] and was also used in other cryo-protocols (e.g. for Plasmodium sp. by Mutetwa and James [211]). One example where optimization of sucrose as an osmotic buffer during CPA exposure has made a significant impact over the past decade has been in slow cooling of human oocytes. As large cells, oocytes may be uniquely sensitive to transmembrane water fluxes during CPA dilution [81] and addition of sucrose in the ranges of 0.2 or 0.3 mol l^{-1} improved post-thaw recoveries, leading to many reported live births following cryopreservation [22, 50, 81, 219]. It seemed to be understood that the CPA dilution step post-thaw was in fact preceded to some extent by the thawing process itself whereby ice (melting as pure water) could induce localized dilution stress within the cells trapped in the melting ice matrix.

Control of the osmotic consequences of CPA exposure using osmotic buffers has remained a consistent theme over the intervening period, not only for cellular systems [40, 164, 248, 308] but also when attempting CPA dilution during perfusion of vascularised organs [228]. As discussed by Pegg [222], cells can only tolerate moderate excursions in cell volume without significant damage, and this risk seems to be increased following sensitization by other steps (such as lowering temperatures and increasing exposure to high solute concentrations during ice formation) in cryopreservation, and therefore avoidance of CPA-derived osmotic injury has remained an important focus for study.

In reality, it can be difficult to decouple CPA-derived osmotic injury from direct pharmacological toxicity [84], so studies to optimize CPA addition and removal need to be undertaken with this

caveat in mind. Another factor to consider is that movement of neutral CPA solutes (and water) across cell membranes is temperature dependent in reproductive cells [108, 128, 220], as well as in other cell types [97, 173], so the kinetics of osmotically-driven cell volume readjustments can vary considerably. There is less information about these osmotic fluxes during rewarming, but Liu reported on these measurements for Me_2SO-exposed islet cells both during cooling and warming [173].

The ability to mathematically model osmotic consequences of CPA exposure or dilution, and then predict optimal CPA protocols to avoid osmotic injury, has been a focus for many years [78, 149, 172]. Historically osmotic cell responses (e.g. alterations in cell volumes during CPA exposure) were found to be relatively straightforward to measure in real time, and could thus form the basis for model development [220]. Different models have been suggested, which in some cases have been proposed to deal with the complexities of multi-solute systems [77, 79].

The application of modelling has advanced further in recent years with the efforts of the groups of Benson and Higgins. Redefining principles of cell volume histories against the kinetics and exposure concentrations of CPA, a mathematically optimized CPA equilibration procedure was developed for human oocytes [20]. The same group developed a CPA 'toxicity cost' function combining factors for both osmotic and chemical toxicities to endothelial cells [56]. Again, underpinning these approaches, the concepts of osmotic tolerance limits played a central role in developing the relevant assumptions. The temperature of CPA equilibration was equally important in proposing the formulation. Applying these combined parameters in their model, their numerically minimized cost function enabled high viable cell recoveries after exposure to and dilution from high concentrations of glycerol. The concentrations of glycerol achieved were capable of sustaining a vitrification approach [57].

4.2.2 Molecular and Pharmacological Toxicities

When considering the evidence pertaining to CPA toxicities in pharmacological terms, it is important to distinguish the types of cell exposures used to investigate the phenomena. Given the ability of many permeating CPAs to reach a range of different intracellular compartments, it is perhaps not surprising that effects of agents such as Me_2SO can be detected during long-term exposures. For example, early on Karow identified a number of toxic mechanisms in cardiac muscle [144, 145], and Conover [48] established the effects of neutral solutes (such as glycerol and ethylene glycol) on mitochondrial respiration at concentrations often used when they are applied as CPAs. More recently Yuan and colleagues [309] reported injurious changes to mitochondrial membrane potential following 24h exposure in astrocytes. However, these exposure conditions are far removed from the short exposures, often at reduced temperatures, followed by extensive post-thaw dilution, which form the basis of most cryopreservation protocols.

Expanding on Karow's studies, Fahy produced a number of important observations on CPA toxicities as a component of cryopreservation-related damage in its own right [84, 87]. He argued that Me_2SO had an impact on oxygen consumption and tissue sodium / potassium ratios [88], which he linked to glycolytic energy production, discussing the original concepts of Baxter and Lathe [19] who had shown specific actions on fructose 1,6 bisphosphatase, including destabilization of the enzyme. There was less clear evidence of non-specific protein denaturation [88]. Fahy also proposed that effects of 'toxicity neutralizers' could be explained by the ability of agents such as formamide to interact directly with Me_2SO, reducing relevant availability for toxic interactions with the enzyme. These studies linked back to earlier proposals from Baxter and Lathe [19]. CPA toxicity was also explained by disruptive protein interactions by Arakawa et al. [7]. They discussed the observations that CPA solvents such as Me_2SO and ethylene glycol exhibit hydrophobic interactions with proteins to destabilize them, at supra zero temperatures, in contrast to the stabilizing effect they produce on proteins by preferential exclusion at lower temperatures. Such concepts speak to the complexity of

defining CPA toxicity, and whether it is transitory, reversible, or long-lasting depending on the exposure conditions.

Fahy expanded ideas about CPA toxicity further by considering effects of the higher CPA concentrations now increasingly used in vitrification solutions. He proposed a formalism, qv^*, to quantify strength of potential hydrogen bonding between water and polar groups of CPA in solution, thence defining potential hydrogen bonding for disruptive protein interaction [92]. He further investigated links between amide toxicities, molecular self-interactions, and Me_2SO toxicity neutralization [82]. More recently gene expression profiling has been applied by Fahy and colleagues [49] to CPA toxicity studies in endothelial cells using exposure to 60% ethylene glycol for 2 hours followed by recovery for 24 and 72 hours. High CPA exposure produced a small decrease in cell growth, suggestive of a modest CPA toxicity in this model. A wide range of differences in expression profiles was documented indicative of a generalized cellular shock response at 24h post CPA exposure, and trending towards partial recovery by 72 hours. The importance of these transient changes in gene expression on long-term cell function post-thawing are yet to be determined.

Elliott, McGann and colleagues undertook studies on CPA toxicity in relation to cryopreservation of articular cartilage. In intact tissues, Me_2SO was investigated across a range of concentrations (between 1 and 6M) and exposure time and temperature [80]. As expected there was a relationship between temperature and toxicity, at concentrations above 1M (which itself was relatively innocuous). The studies were extended to chondrocytes, as the cellular component of cartilage, and were used to model coefficients of toxicity for a range of single CPAs, and for combinations of CPAs of 2, 3 or 4 mixed components [3]. The coefficients were modelled to derive positive or negative values for the mixtures, relating to whether toxicities were increased or decreased over and above those for single CPAs at the same concentrations. Within relatively high concentration (6M) ranges used for vitrification, a 4-component mix (Me_2SO-ethylene glycol-glycerol-formamide) was least toxic. However, a cautionary note was sounded about the choice of CPA to combine into multi-

component solution. Including propylene glycol with any of the other CPAs studied resulted in greater toxicities. When studying single component Me$_2$SO toxicities, Puschmann and colleagues [237] found that temperatures of exposure for a range of Me$_2$SO concentrations (up to 50% w/v) as required for a liquidus tracking approach to cryoconservation, were again important for CPA exposure to encapsulated liver cells. In this model, it was possible to assess CPA exposures down to $-20\,°C$ in non-frozen samples, where again the lowest temperature best mitigated toxicity.

Occasionally organelle-specific CPA toxicities have been suggested. For example, mitochondria were identified as sensitive targets of CPA toxicity in aquatic species [119, 312]. Whether such specific CPA sensitivities are seen across a broad range of species remains as yet unknown.

Other chemical mechanisms for CPA toxicity have been proposed. In a recent comprehensive review, Best [21] discussed evidence that oxidative damage may be another important injurious effect. For example, in plant vitrification protocols, lipid peroxidation was detected which could be abrogated by inclusion of antioxidants such as glutathione in the mixture [278]. Best also discussed potential membrane perturbations resulting from effects such as differences between water-water or Me$_2$SO-water hydrogen bonding, and CPA solution dielectric constant relative to membrane dielectric constant [13, 21].

There have also recently been studies on the potential for epigenetic changes induced by CPAs. It is known that Me$_2$SO at low concentrations [1% or less] can stimulate alterations in the epigenetic profile of mouse embryonic stem cells [129], but this was observed after several hours of exposure under cell culture conditions. Changes in histone acetylation were detected in bovine oocytes following cryopreservation or vitrification in Me$_2$SO / ethylene glycol mixtures [269] over 12 hours post thawing, but these were in response to the entire cryopreservation protocol, and may not be linked only to CPA exposure. Genomic effects of cryopreservation in gametes and embryos has recently been reviewed by Kopeika and colleagues [155], but again direct effects relating only to CPA

exposure have not often been reported. This area of CPA toxicity will likely be studied more in the near future as more accessible methods to study epigenetic changes become available.

4.2.3 Minimizing CPA Toxicity

We have discussed above the studies from Fahy & colleagues [82, 84, 88] and McGann & colleagues [3, 80] where chemical agents (which would not be recognized as main line CPAs – such as acetamide and formamide) have been combined with traditional CPAs to yield a reduction in toxicity. Best [21] recently reviewed these toxicity minimizing agents. Sugars, both monosaccharides, such as galactose, or disaccharides, such as sucrose or trehalose, figured prominently in these categories. For example, Petrenko and colleagues showed that it was possible to partially substitute sucrose for Me_2SO in the cryopreservation of a range of cells [229, 230]. Polymers such as polyethylene glycol have also been used. Exact modes of action are often unclear, but positive results following their application in cryopreservation mixtures have often been reported, perhaps mainly by reducing toxic concentrations of agents such as Me_2SO. The extracellular CPA of themselves are unlikely to have a direct impact on the intracellular targets of toxicity of the penetrating CPA, but by maintaining a suitable total CPA solute concentration (with less permeating CPA), successful modulation of ice formation, and cell protection during cryopreservation can be achieved.

There are also several agents that can be used to reverse the effects of CPA exposure and cryopreservation in post-thaw culture. Oxidative stress has been identified as a negative consequence of cryopreservation in both plant [39, 99, 187] and animal cells, especially sperm [95, 116, 274] across a range of species. Application of antioxidants during cryopreservation and/or, in post-thaw recovery periods, has in many cases, improved outcomes [4, 6, 278]. Other drugs that can interrupt cell death pathways, such as caspase inhibitors, have been used following cryopreservation of hematopoietic stem cells [24]. Inhibitors of Rho associated kinases (ROCK inhibitors) have been applied successfully to enhance recovery of stem cell populations after cryopreservation [135, 251].

It should be pointed out, that in many of these instances the agents have been used to reverse injury from the cumulative injuries across the whole cryopreservation protocol, not just direct CPA toxicity.

It will be clear from the above discussions that we have only a nascent understanding of the multimodal effects of CPAs on cell molecular stability *per se* (even without effects of ultralow temperatures). The new technologies now coming on-stream (such as FTIR [217, 218]) should help in developing better approaches to CPA toxicity and its avoidance.

5. Beyond the Single Cell: Application of CPAs to Large Tissues or Organs

When considering cryopreservation of multicellular systems or vascularized organs, all of the fundamental concerns of CPA toxicity and efficacy remain important, with the additional significant challenge of ensuring that CPAs can reach, equilibrate with, and be removed from, all parts of the biological construct. Whilst single cell suspensions can invariably achieve rapid CPA equilibration within minutes of exposure by surface diffusion, this cannot be assumed for even relatively small tissues, such as cornea, without proper evaluation. Whilst it might be anatomically correct to consider discussions of CPA permeation first in tissues, in this section we have elected to discuss organ exposure first, since both in historical terms and in understanding the associated problems, organ studies provided the best early appreciation of the challenges for CPA exposure in complex systems.

5.1 Organs

In general, CPA permeation into solid organs cannot be achieved by topical exposure and diffusion alone. Perfusion into the vascular bed of an organ can theoretically reduce the CPA diffusion pathway from several cm (for topical exposure) down to a few microns, as long as the perfusion process is both efficient and non-injurious, and reaches the microvascular network. From the earliest investigations, it was appreciated that vascular perfusion of intact organs was a potentially efficient method to deliver CPA into the whole biomass [117, 142, 262] before cryopreservation, using Me_2SO

as a CPA. It also became evident that the composition of the carrier solution used for CPA perfusion was also of importance, because the movements of CPA into (and out of) internal cell compartments within an organ generated similar osmotic cell volume changes as those seen in single cell suspensions. However, these changes could alter perfusion resistance and organ weight gain during CPA perfusion, which could damage the vascular compartments even in the absence of freezing. These changes could be partially mitigated by choosing specific perfusate formulations, adding osmotic buffers, and perfusing at hypothermic temperatures [140]. Pegg and colleagues used perfusion at 10 °C over 30 min to deliver 1.4M Me$_2$SO to canine kidneys before cryopreservation [225]. Karow [146] and Guttman [258] also considered the important problem of being able to confirm good CPA distribution throughout the entire organ mass following perfusion, and used radio-labelled Me$_2$SO to assess Me$_2$SO permeation kinetics. Karow [146] investigated Me$_2$SO permeation by perfusion at 37, 25, and 10 °C, and, as might be predicted, although hypothermic perfusion was chosen from previous studies to mitigate problems of CPA toxicity, CPA permeation was slowest at low temperatures. Their results also identified another important variable, that organ osmotic injury could be better controlled by gradually increasing Me$_2$SO concentration during perfusion, rather than immediately starting perfusion with 1.4M CPA.

Studies on renal perfusion with Me$_2$SO, ethylene glycol and glycerol were undertaken by Pegg [223] at a range of perfusion temperatures. Based on his various measurements, he suggested that glycerol and ethylene glycol were worthy of further evaluation as organ CPAs. Pegg and Jacobsen went on to undertake a series of studies on rabbit kidney CPA perfusion with glycerol [132, 134, 226]. Glycerol distribution in perfused kidneys was measured by freezing point depression of biopsied renal tissues [131]. They developed a non-injurious hypothermic CPA perfusion system, with inclusion of osmotic buffers, which allowed perfusion with glycerol up to 3M with subsequent CPA removal and successful auto-transplantation [133]. In the same studies they used propylene glycol as CPA, but found that this CPA was less-well tolerated during perfusions at 2, 3 or 4M. It

should be pointed out however, that none of the CPA-equilibrated kidneys survived cooling and ice formation during slow cooling to −80 °C and rewarming. This mirrored unsuccessful attempts to apply Me$_2$SO as CPA for kidneys by similar perfusion methods ahead of cooling to −80 °C [225].

Other studies on application of Me$_2$SO to renal tissue cryopreservation were undertaken, again focusing on the importance of the CPA carrier solution by Clark, Fahy and Karow [43, 44]. Fahy developed a computer-controlled perfusion system to deliver Me$_2$SO-based CPA mixture to rabbit kidneys, allowing repeatable incremental concentrations of CPA or osmotic agents to be infused or washed out [83]. Addition and removal of the Me$_2$SO-based solution using this perfusion equipment yielded good function for CPA concentrations up to 4M [86]. Refractrometry measurements were used to determine efflux concentrations of Me$_2$SO, and by inference, the accomplishment of full CPA equilibration. The high concentrations of Me$_2$SO were chosen to achieve cryo-banking by vitrification. Fahy and colleagues went on to evaluate a number of CPA mixtures, mainly based on Me$_2$SO, propylene or ethylene glycol as major components but also containing acetamide, formamide, polyvinyl pyrrolidone and polyethylene glycol to improve vitrification properties, using both tissue slice models and organ perfusion technology [92]. A mixture of CPA (called M22) with the main components of Me$_2$SO, ethylene glycol, and formamide was introduced by perfusion to rabbit kidneys [93], and it proved possible to cool the organs and recover them from −45 °C, with evidence of good consistent graft survival, but also of renal injury. More recent information on kidney responses to such CPA mixtures has been provided [91]. More specifically, it was shown that an animal could survive indefinitely after autologous transplantation of its kidney after it had been vitrified. A critical factor for success was how well the CPA had distributed within the kidney, and therefore had prevented localized ice formation.

5.2 Tissues
Standard CPAs such as glycerol or Me$_2$SO have been used for cryopreservation of non-vascularised tissues such as skin [25, 151, 192] for many years, on the basis that surface exposure to CPA will

provide sufficient permeation as long as exposure time is prolonged (in the region of 30 min). This often begs the question about whether complete permeation with CPA has been achieved. Interestingly, transplant outcomes from tissue cryopreservation may be equally good if low recoveries of the cellular components follow cryopreservation [121], suggesting that other issues such as sterility or tissue matrix preservation have been maintained by the process. In fact, similar types of outcome have been found across a range of composite tissues cryopreserved using CPAs, glycerol or other permeating CPAs and slow cooling [14, 221, 224] where partial cell loss was seen after thawing e.g. in cornea, blood vessels, cartilage etc. Currently cryopreservation of cornea is not a routine clinical therapy because of such partial cryo-injury using slow cooling with Me_2SO [8], whilst even cryopreservation of small corneal slices (lenticules) was found to result in some cell injury post thawing [203]. In situations where corneas have been cryopreserved using Me_2SO, it was shown that a reduction in passenger infectious agents could be detected [27], probably relating to the washing steps for CPA exposure and removal; never-the-less this could be a helpful outcome for cornea cryopreserved for emergency therapy, which is currently the most common indication for their use.

A number of methods have been developed to measure CPA permeation into small 3-dimensional tissues to optimize CPA penetration. Chemical NMR measurements were shown to detect Me_2SO permeated into liver tissues [106] and this was subsequently used to study CPA permeation into corneas [287], ovarian tissues [214, 276] and heart valve leaflets [165]. Sharma and colleagues measured Me_2SO permeation into cartilage by solute extraction of biopsies and measurement of solution osmolality [260]. Wolkers and his colleagues established a diffusion model based on ATR-FTIR spectroscopy to follow temperature-dependent diffusion kinetics of glycerol, glucose, sucrose, and HES molecules, and to estimate the time needed for complete infiltration of different types of decellularized heart valve tissues with glycerol as well as sucrose solutions [290, 291].

As with organ cryopreservation, there has been an increased focus on vitrification methods for tissues, applying the essential higher CPA concentrations and mixtures. Vitrification of blood vessels using a mixture of Me_2SO, propanediol, and formamide provided promising results [266]. Four CPAs (Me_2SO, ethylene glycol, propanediol and glycerol) were investigated in concentrations up to 6M for cartilage vitrification [138], where Me_2SO and EG were found to have the highest coefficients of permeation. The expected relationship between temperature and rate of permeation was observed. Wusteman and colleagues investigated a range of CPA mixtures in a model tissue system [304], investigating dielectric properties of the CPA mixtures that may have relevance if electromagnetic re-warming is employed for vitrified constructs. They found that propanediol performed well in this respect and in other measures of viable cell recovery. It has also become evident that there are important low temperature thermo-mechanical properties of CPAs that need to be considered when applying vitrification to large structures, and methods have been developed to measure these properties [250]. Rabin and his colleagues investigated a single CPA (Me_2SO) at vitrifying concentrations and VS55 (a mixture of Me_2SO, propanediol and formamide). The same group has pursued studies into the behaviour of a range of vitrifying CPA mixtures [74] to address issues such as physical cracking during vitrification and rewarming of large tissues. A similar vitrification approach has been applied to heart valve leaflets by Brockbank and colleagues [30], where the mixtures of the 3 CPAs in VS55 were increased (from a total concentration of 55%) to 83% to give VS83. Again successful recoveries have gone hand in hand with a reduced viable cell yield, as another example of where tissue matrix preservation seems more important than global viable cell function following cryo-banking. Another approach for vitrification of cartilage (liquidus tracking) using a single CPA (Me_2SO) added in sequential steps has been investigated by Pegg's group [150, 227] as an approach both to avoiding ice formation by colligative freezing point depression and, from early studies in smooth muscle preservation [75], to minimizing CPA toxicity encountered with the necessarily high concentrations required. Liquidus tracking has been discussed as a method to

introduce vitrifiable concentrations of Me$_2$SO into cartilage [1] and was based on original observations by Farrant [96] and technical demonstrations by Elford & Walter [76]. This has also been applied to large volumes of tissue engineered liver constructs, although not as successfully yet as for cryopreservation in small scales [237]. In general, whilst vitrification provides a very exciting perspective for tissue preservation in the coming years, as yet no large-scale clinical trials have been undertaken.

6. Nature, Evolution, and Avoiding Ice Formation Injury: Antifreeze Proteins, Synthetic Peptides, Small Molecule Recrystallization Inhibitors, and Ice Nucleating Agents

The importance of naturally-derived CPAs in applied cryopreservation, such as various sugars, has been discussed in Sections 2 and 3 above. Evolution has provided additional strategies to avoid ice-related injury for organisms living in cold environments, which have recently become of wider interest. These strategies include antifreeze proteins and ice nucleation agents, which have become almost a sub-speciality in cryobiology in their own right.

Antifreeze proteins and antifreeze glycopeptides, AF(G)Ps, are a category of natural cryoprotectants utilized by freeze-avoiding animals and amphibians that limit the formation of ice upon cooling below the equilibrium phase change temperature of the bulk liquid phase, a phenomenon called 'thermal hysteresis' [156] or 'freezing hysteresis' [37]. AFPs in antarctic fish have been shown to provide protection from freezing down to −2 °C [61, 62]. Insects also use AFPs to avoid ice formation at low temperatures [69, 70] and AFPs also exist in freeze-tolerant plants [111, 114]. Although the presence of AFPs in solution can lead to some colligative freezing point depression [69], the primary role they play in ice-avoidance is by adsorbing to the surface of the propagating ice plane and inhibiting further ice crystal growth. In AFPs from fish this adsorption has been attributed to hydrogen bonding between polar residues on the peptide and the oxygen atoms on the prism face

of the ice [61, 63, 153, 196, 245, 246, 263] but the specific nature of the interaction between AFPs and the ice planes remains an active area of investigation [156]. Direct observations by fluorescence microscopy of AFP – ice surface interactions in a temperature-controlled microfluidics device have indicated that there was a practically irreversible binding of AFP to ice surfaces, with a demonstrable kinetic to achieve maximum freezing hysteresis of several minutes within the observed samples [37]. There have been numerous investigations to determine the structural motifs that give rise to the ice-binding phenomena responsible for the protective effects of AFPs and AGFPs, but considerable structural diversity exists amongst the various classes [58]. The commonality is that all natural AFPs bind the ice surface in some way to prevent growth of the ice crystal. Recent molecular dynamics simulations of AFP from the spruce budworm *Chorisoneura fumiferana* suggest that this protein binds indirectly to the prism ice face through an arrangement of ordered water molecules surrounding the ice [158]. Future simulation work should reveal if this behaviour characterizes all AFPs or if this is a phenomenon that is unique to this budworm AFP structure and its associated hysteresis behaviour. The scientific understanding of the range of AFP across many different phyla, and of their modes of action, has increased significantly over the past 5 years [16, 37, 68, 176].

Although AFPs have been investigated as additives for cryopreservation solutions, when pushed below the thermal hysteresis freezing point, solutions containing AFPs will form ice crystals with shapes that are oftentimes more lethal than hexagonal ice [15, 58]. Whether or not AFPs are protective or damaging depends mainly on formulation characteristics involving the concentrations and types of AFPs and cryoprotectants [289]. For example, Halwani et al. [118] have shown that AFPs from the beetle *Dendroides Canadensis* can be beneficial in preservation compositions containing Me_2SO. AFPs have also been shown to interact with cell membranes in a way that is protective for chill-sensitive cell types [253, 254].

Recent advances in combinatorial chemistry and peptide science are enabling the development of libraries of synthetic 'AFP-like' molecules that can be screened for their effects on ice formation and

growth [282]. These library studies are enabling delineation between thermal hysteresis effects, generally correlated with ice-binding and ice-shaping, and ice recrystallization inhibition (IRI), which doesn't necessarily require ice-binding. The latter would be preferred for cryopreservation formulations, as it can avoid problems with lethal ice crystal structures below the hysteresis freezing point. Structure-property studies of synthetic analogies of AFPs have revealed that thermal hysteresis and IRI can indeed be uncoupled even though both commonly occur together in AFPs. Capicciotti and colleagues have identified classes of molecules, such as hydrogelators, that have high IRI activity but do not exhibit thermal hysteresis effects. They also observed that a very subtle difference in the positioning of hydroxyl groups on carbohydrate moieties can drastically affect the ability of the molecule to suppress ice recrystallization, even though the molecules are otherwise structurally identical [34, 35]. They posit that these subtle differences in structure influence how the molecule interacts with water in the vicinity of ice crystals, reducing water mobility at the interface in a way that affects diffusion of water from small to large ice crystals, thus hindering the recrystallization process [34]. High-throughput screening techniques with large chemical libraries are enabling exciting discoveries that can dramatically influence preservation science. For example, Capicciotti's group has shown that these newly identified small molecule recrystallization inhibitors can enable cryopreservation of red blood cells with reduced glycerol contents [33]. The potential value of IRI activity in vitrification approaches is now becoming more widely recognized.

On the other hand, in evolving survival in cold extremes, nature has developed the additional (and what may seem scientifically antagonistic) strategy to encourage ice formation by employing ice nucleating agents (INAs) in some circumstances. However, this becomes understandable by reflecting on Mazur's Two Factor hypothesis (see Section 1), where optimal cell dehydration can enhance survival. This has been discussed as the concept of 'regulated ice formation' [268] to avoid intracellular ice formation in cells and tissues essential for survival. For example, it has been appreciated for some time that overwintering insect species show a seasonally adaptive elevation of

their supercooling points, explained by allowing ice formation at higher subzero temperatures [310, 311]. Similar events can be seen in cold tolerant amphibians and marine intertidal molluscs [268]. In many cases, INA activities appear to be related to foreign sources such as resident bacteria; for example, INA in an intertidal mollusc was associated with presence of *Pseudomonas fulva* [175].

INA activities have also been linked to specific proteins and lipoproteins (also known as INPs). Knowledge about the molecular pathways for direct seasonal adaptation by synthesis of these agents remains incomplete. A protein in the plasma of cold tolerant *Rana sylvatica* has been shown to have INA properties [298]. A 74Kd protein has been purified and characterised from the overwintering insect species, *Vespula maculate* [71], whilst a much larger 800 Kd lipoprotein has been linked to INP activity in *Tipula trivittata* [72].

To our knowledge, there have not yet been recorded attempts to modify natural INPs, as has been done for AFPs. The importance of controlling ice nucleation across a broad range of cryobiological applications beyond natural cold tolerance has been recently reviewed [206]. Inert chemical structures such as activated charcoal or zeolites can demonstrate INA properties [207] but such processes are beyond the remit of this review. The importance of INA activities, in many cases linked with chemical or biological micro-particles, has also been recognized in the atmospheric cloud cycle [12, 297].

7. CPA Mixtures: Trading Synergy and Avoidance of Ice Damage Against Complexity

The concept of vitrification has been touched upon in several of the earlier sections. The concept of restricting the ice nucleation process in aqueous solutions at deep cryogenic temperatures has been of interest for many decades. The presence of high concentrations of solutes can stabilize the amorphous patterns of water molecules in solution on a kinetic basis, and increase viscosity, favoring vitrification during cooling. High pressures can also inhibit ice nucleation. Luyet and

colleagues worked on this phenomenon in aqueous biological systems for several decades from the 1930s [90, 182, 183]. Luyet [181] discussed such concepts as 'amorphization', whilst also accepting that the generally accepted terminology is 'vitrification'. To quote from Luyet [181] 'We are up against a wall (*viz.* vitrification in biology). Our best chance to break through is probably to use high concentrations of solutes'. They and others were aware that in dilute aqueous systems (such as 20% glycerol), extremely high cooling rates (>10^4 °C/second) were necessary for vitrification. Fahy and colleagues discussed these physical parameters in the context of cryobiology [92, 93, 301], in particular the notion that by increasing the solute concentrations greatly, it was theoretically and demonstrably possible to kinetically interfere with ice nucleation and crystal growth and achieve a 'glassy state' at extremely low temperatures, in systems that may be biologically tolerated. There are many excellent reviews on the physical bases for vitrification that are beyond the scope of the current discussion [5, 59, 90, 188, 294, 301]. The seminal work on vitrifying mouse embryos by Rall and Fahy 30 years ago [241] placed vitrification clearly in the field of practical cryopreservation.

Here we shall discuss the CPA mixtures that have been most commonly used in applied vitrification. Details on common vitrification solutions, including composition, glass transition temperature (T_g), critical cooling/warming rates, and applications for each solution have been summarized in Table 2.

It is worth revisiting at this point the role of *non-permeating* CPAs in the development of practical vitrification approaches. It was recognized early on that extremely high concentrations of CPAs (in the region of 60% w/v) would be needed to achieve vitrification, which would likely cause significant toxic effects if achieved using agents such as Me$_2$SO. By using non-penetrating CPAs (with inherently lower toxicities) to partially replace the amounts of permeating CPA, total solute concentrations could be maintained whilst reducing chemically-derived cell injury [89]. In addition, it was pointed out that cells naturally contain significant amounts of intracellular polymeric solutes such as proteins, and non-permeating CPAs could be used in the vitrification solution to balance the effective toxicities of the total extracellular and intracellular compartments, reducing to some

degree the intracellular concentrations of permeating CPA required to achieve vitrification for a given critical cooling rate [89, 240]. Polymers such as hydroxyethyl starch, polyethylene glycol and polyvinyl pyrrolidone were discussed in this context [89]. Shaw and colleagues [261] undertook studies investigating the chemical nature of vitrification in ethylene glycol mixtures supplemented with polymers including dextran, Ficoll and PVP, in which they showed that indeed polymers could effectively replace a significant amount of ethylene glycol whilst still achieving a glassy state in the solutions during cooling. In mouse embryo vitrification, some of these same polymers (dextran or Ficoll) were successfully used to replace up to half of the concentration of ethylene glycol used as permeating CPAs [160] with good success. Sugars such as trehalose, sucrose or raffinose can also be used in a similar way to achieve practical vitrification with lower concentrations of permeating agents such as ethylene glycol [159]. These classes of non-permeating CPA also tend to increase the viscosity of the carrier solutions, which tends to favour vitrification of the external solution and in some cases results in raised glass transition temperature (T_g), as discussed by Fahy and Wowk [90].

Further details on the CPA mixtures, methods of exposure and dilution used in the early embryo vitrification experiments were given by Rall [243]. VS1 is an equimolar mixture of Me_2SO and acetamide (2.62M), with propanediol (1.3M), and polyethelyne glycol (MW 8000; 6% w/vol), a mixture described by Fahy & colleagues [89]. PEG, which was added as the polymer, had been shown to improve tendency towards vitrification [89]. The cooling rates in the small samples were high (<2000 °C/min), and rapid rewarming (>200 °C/min) was beneficial. These approaches were rapidly repeated in other embryo and oocyte vitrification studies [104, 212]. From that point onwards, vitrification within reproductive medicine spawned an entire industry within cryobiology, with a number of technical innovations, such as small sample holders (e.g. cryoloop, cryotop, open pulled straws [161, 174, 247]) developed specifically for embryos or oocytes. A sampling of current vitrification systems used for small volume samples is shown in Table 3. Many combinations of CPAs have been evaluated, leading to the widely used vitrification solution based on (w/v) 15% Me_2SO,

15% ethylene glycol, 0.5M sucrose [161]. A pre-loading phase (7.5% Me$_2$SO, 7.5% EG) is usually performed for about 12 min, followed by a 1 min exposure to full vitrification mix before rapid cooling [249]. These approaches are by far the widest global clinical application of vitrification today.

The increased interest in vitrification has also led, in other species, to the development of modified CPA mixtures, chosen for specific reasons such as toxicity or ease of permeation, while based on the same overall philosophy. For example, in aquatic species (Coral), propylene glycol (3.5M), ethylene glycol (1.5M) and methanol (2M) has been used [277]. In plant tissue vitrification, a CPA mixture of 7M ethylene glycol, 0.9M sorbitol and 6% bovine serum albumin was applied to rye protoplasts [167]. Sakai's group [257] developed a number of plant vitrification solutions, and PVS2 was seen to be effective – with a CPA mixture of (w/v) 30% glycerol, 15% ethylene glycol and 15% Me$_2$SO in Murashige-Tucker medium (MT) containing 0.15 M sucrose. As in other protocols, pre-loading was performed with 60% PVS2 at 25°C for 5 min and before exposure to full chilled PVS2 at 0°C for 3 min. In plant systems, additional manipulations such as encapsulation dehydration of the tissues, which reduces free water content capable of nucleating ice, has been used to improve outcomes with PVS2 [115]. In some systems, modifications such as PVS3 (50% glycerol; 50% sucrose) have been proposed to improve the toxicity profile [285]. The PVS range of CPA mixtures remain the most widely used agents for plant tissue vitrification [256]. It is also worth highlighting that whilst these laboratory developments for plant vitrification have been made over the past 20 years, there is a parallel set of studies in environmental plant cold tolerance which indicate that natural vitrification processes may occur during overwintering [122].

One area of focus for a supportive role for solute additives in CPA mixtures, especially in intended vitrification, is ice nucleation inhibition (see also Section 6). Early on, the concept of using AFP (as ice nucleation inhibitors) to improve the tendency for achieving and stabilising the vitrified state in bulk aqueous samples was investigated by Sutton and Pegg [270]. They particularly commented on the ability of AFP to reduce the critical warming rate to avoid devitrification and potential ice nucleation

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in their model CPA mixture during warming. In relation to control of damaging ice formation, particularly during rewarming of vitrified biological samples, Fahy [85] discussed concepts related to creating synthetic 'ice blocker' molecules. This was taken further by Wowk et al. [300, 302, 303] who studied synthetic variants of polyvinyl alcohol and polyglycerol which could reduce ice crystal nucleation and growth in experimental vitrification studies. A naturally-occurring flavonol glycoside has been identified in cold tolerant tree species and investigated in laboratory vitrification experiments [139]. The topic has recently been reviewed by Wowk [90, 301]. Recently, the application of iron-centred nanoparticles as a component of a vitrification mixture to enhance uniform warming in vitrified samples has shown some success [190]. This may open up the possibility for future classes of CPAs to be developed on biophysical rather than biochemical properties.

In terms of CPA mixtures for vitrification of larger tissues, the combinations have often largely been based around the original recipes from Rall and Fahy using Me_2SO, and acetamide [241], or by Song and colleagues using Me_2SO, formamide and propylene glycol already discussed [241, 266]. Butanediol has been suggested as a polyol with favourable glass forming tendencies [26], but this agent has not found widespread application due to concerns about toxicity at the required concentrations [21]. In chondrocyte studies, a CPA mixture (final solute concentration of 6M) based on equal concentrations of Me_2SO, formamide, ethylene glycol has been proposed [3]. The increase in CPA total concentration (to 12.6M) used recently for heart valve biopreservation [31] again was based on a similar 3 CPA mixture – in this case, Me_2SO – 4.65M, formamide – 4.65M, propylene glycol – 3 31M, but these extremely high concentrations were selected for specific purposes. Whilst these developments offer great promise for the future, we could find no published clinical trials of their use so far.

8. Summary

Whilst applications of CPAs in cryobiology and cryo-technologies have progressed into many widely used applications, a greater understanding of the molecular actions of these agents has been achieved over the past decade. New techniques are now being applied to study both the biological and biophysical properties of CPAs, which will take cryobiology further forward. Additionally, the ice modifying properties of certain CPAs are becoming of interest in many diverse areas, such as food freezing processes [29]. In addition, new ideas are coming forward to resolve or control central issues, such as ice nucleation and crystal growth, or achievement of vitrification in biological samples at cryogenic temperatures. Taken together, these show great promise to improve and widen the application of cryogenic preservation as a significant enhancing technology for the future.

Acknowledgements

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1 Tables

Alcohols and Derivatives	Sugars & Sugar alcohols	Polymers	Sulfoxides & Amides	Amines
Methanol*	Glucose*	Polyethylene glycol (PEG)**	Dimethyl sulfoxide****	Proline**
Ethanol~	Galactose*	Polyvinyl pyrrolidone (PVP)*	Acetamide*	Glutamine*
Glycerol***	Lactose~	Dextrans**	Formamide*	Betaine*
Propylene glycol**	Sucrose~**	Ficoll**	Dimethyl acetamide~	
Ethylene Glycol**	Trehalose**	Hydroxeythyl starch**		
	Raffinose**	Serum proteins (complex mix)**		
	Mannitol~*	Milk proteins (complex mix)~*		
	Sorbitol~	Peptones~		

~	Effective in prokaryotic cells
*	Effective to a limited degree in eukaryotic cells
**	Moderately effective in eukaryotes; often in combination
***	Very effective in a defined number of cell types
****	Highly effective and widely used across all classes of cells

2   Table 1. Cryoprotective agents

3

Preserved samples	Common Name	Composition	Comments	Source
Embryos up to blastocysts				
Mouse	**VS1**	Me$_2$SO: 2.62 M; Acetamide: 2.62 M; PG: 1.3 M; PEG: 6.0% (w/v) (8000 MW)	T_g of VS1, VS2 and VS3 range between −108 and −113 °C. CCR of VS1 is 5 °C/min.	[240, 241, 243]
	VS2	PG: 5.5; PEG: 6% (w/v)		[240]
	VS3	Glycerol: 6.5 M; PEG: 6% (w/v)		[240]
	EFS	EG: 40% (v/v); Ficoll 70: 30% (w/v); Sucrose: 0.5 M		[148, 314]

Preserved samples	Common Name	Composition	Comments	Source
Bovine	**EFS**	EG:40% (v/v); Ficoll 70: 18% (w/v); Sucrose: 10.26% (w/v)		[189]
Human	--	EG: 15%; Me$_2$SO: 15%; Sucrose: 0.5M		[162]
	--	EG: 5.5 M; Sucrose: 1.0 M		[123]
	--	Me$_2$SO: 15%; EG: 15%; Ficoll 70: 10 mg/mL; Sucrose: 0.65 M		[208]
Oocytes				
Mouse	--	Me$_2$SO: 6 M		[299]
	--	EG: 1.4 M (8%); Me$_2$SO: 1.1 M (8%); Trehalose: 1 M		[42]
	--	PG: 1.5 M; Trehalose: 0.5 M		[169]
Bovine	--	EG: 15%; PG: 15%; Sucrose: 0.5M		[41]
	--	EG: 6.8 M; Sucrose: 1.0 M; TCM199 medium		[163]
	--	EG: 15%; Me$_2$SO: 15%; Sucrose: 17.1%; Calf serum: 20%; TCM199 medium		[235]
Human	--	EG: 15%; Me$_2$SO: 15%; Sucrose: 0.5M		[249]
	--	EG: 5.0 M; Sucrose: 1.0 M		[163]
Gorgonian coral (*Junceela juncea*)	--	EG: 1.5M; PG: 3.5M; Methanol: 2M		[277]
Zerbrafish (*Danio rerio*)	--	Me$_2$SO: 3M; PG: 2M; Methanol: 2M		[112]
Tissues				
Ewe ovarian arteries	**VS4**	Me$_2$SO: 2.75 M; PG: 1.97 M; Formamide: 2.76 M	T_g: −125°C; CCR: 14.3°C/min	[51, 52]
Rabbit venous blood vessels/Rabbit kidneys	**VS55 (VS41A)**	Me$_2$SO: 3.1M; Formamide: 3.1M; PG: 2.2M	T_g: −123°C; CCR: 2.5°C/min. CWR: 50 °C/min.	[10, 266]
Rabbit carotid arteries/corneas	**LSV/40/15**	PG: 40%; Trehalose: 15%; LSV: 5% of the total salt concentration of PBS	T_g: −100 °C.	[305]
Bovine blood vessel and muscle	**DP6**	Me$_2$SO: 3M; PG: 3M	T_g: −119 °C; CCR: 40°C/min.	[73, 239]

Preserved samples	Common Name	Composition	Comments	Source
Rabbit/Human corneas		PG: 50% (v/v); Sucrose: 0.25M; PEG: 6%; Chondroitin sulfate: 2.5%[6]	T_g: −107 °C	[9]
Heart valves (sheep; rat)	**VS83**	Me$_2$SO: 363.2 g/l; Formamide: 209.3 g/l; PG: 252.6 g/l	T_g: −119 °C	[31]
Rabbit kidney	**M22**	Me$_2$SO: 2.855 M; Formamide: 2.855 M; EG: 2.713 M; N-Methylformamide: 0.508 M; 3-Methoxy,1,2-propanediol: 0.377 M; PVP K12: 2.8% (w/v); X-1000 ice blocker (PVA): 1% w/v; Z-1000 ice blocker (PGL): 2% W/V	T_g: −124 °C; CWR ≤ 1 °C/min	[93]
Plants				
Cultured cells and somatic embryos from mesophyll tissue of asparagus	**PVS**	Glycerol: 22%; EG: 15%; PG: 15%; Me$_2$SO: 7%	T_g of 85% PVS: −115.3 °C	[279]
Orange (*Citrus sinensis*) cells	**PVS2**	Glycerol: 30%; EG: 15%; Me$_2$SO: 15%; Sucrose: 0.15M	T_g: −115 °C	[257]
Mint plant shoot tips	**PVS3**	Glycerol: 50%; Sucrose: 50%	T_g: −90 °C	[275, 285]

1 Table 2. Vitrification solutions in common use, including Glass transition temperature (T_g), Critical

2 Cooling Rates (CCR) and Critical Warming Rates (CWR), where known.

3

Small volume vitrification systems	Sample volume (µL)	Achievable cooling/ warming rate (°C/min)	Source
French straw	250	2500 / 2500	[243]
Electron microscopic grids (EM)	< 1	180,000 / --	[191]
Open pulled straws (OPS)	1 to 2	20,000 / 20,000	[280]
Cryoloop	< 1	15,000 / 45,000	[60, 166]

Hemi-straw	~ 0.3	> 20,000 / --	[60, 283]
Cryotop (open system)	< 0.1	23,000 / 42,000	[161, 162]
CryoTip (closed system)	~ 1	12,000 / 24, 000	[162]
Quartz micro-capillary (QMC)	< 1	250,000 / --	[120, 169]

1   Table 3. Vitrification systems for small volume samples.