Chapter 2

Principles of Cryopreservation by Vitrification

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Abstract

Vitrification is an alternative approach to cryopreservation that enables hydrated living cells to be cooled to cryogenic temperatures in the absence of ice. Vitrification simplifies and frequently improves cryopreservation because it eliminates mechanical injury from ice, eliminates the need to find optimal cooling and warming rates, eliminates the importance of differing optimal cooling and warming rates for cells in mixed cell type populations, eliminates the need to find a frequently imperfect compromise between solution effects injury and intracellular ice formation, and enables cooling to be rapid enough to "outrun" chilling injury, but it complicates the osmotic effects of adding and removing cryoprotective agents and introduces a greater risk of cryoprotectant toxicity during the addition and removal of cryoprotectants. Fortunately, a large number of remedies for the latter problem have been discovered over the past 30+ years, and the former problem can in most cases be eliminated or adequately controlled by careful attention to technique. Vitrification is therefore beginning to realize its potential for enabling the superior and convenient cryopreservation of most types of biological systems (including molecules, cells, tissues, organs, and even some whole organisms), and vitrification is even beginning to be recognized as a successful strategy of nature for surviving harsh environmental conditions. However, many investigators who employ vitrification or what they incorrectly imagine to be vitrification have only a rudimentary understanding of the basic principles of this relatively new and emerging approach to cryopreservation, and this often limits the practical results that can be achieved. A better understanding may therefore help to improve present results while pointing the way to new strategies that may be yet more successful in the future. To assist this understanding, this chapter describes the basic principles of vitrification and indicates the broad potential biological relevance of vitrification.

Key words Vitrification, Freezing, Intracellular ice formation, Devitrification, Recrystallization, Chilling injury, Cryoprotective agents, Cryoprotectant toxicity, Osmotic limits, Protein denaturation, Biobanking, Glass transition, Glassy state, Optimal cooling rate, Organ preservation

1 Introduction and General Orientation

1.1 Overview

Vitrification is the solidification of a liquid into a noncrystalline or amorphous (meaning, literally, "without structure") solid known as a glass [1, 2]. The industrial significance of vitrification is well understood and long-standing. Beyond the manufacturing of familiar glassy items such as porcelain and windows, for example, obsidian, which is a vitrified form of lava [3], was used to make artifacts

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such as arrowheads as long ago as the Stone Age [3], and it is anticipated that, in the future, vitrification may be used to trap radioactive waste to prevent it from escaping into the biosphere [4, 5]. On the other hand, the potential biological significance of vitrification has been reasonably well appreciated for less than 80 years.

The possibility of vitrifying water was postulated as long ago as 1860 [6]. In 1980, the successful vitrification of a 0.1 M $CuCl_2$ solution as well as of pure water by ultrarapid cooling was reported [7], and in 1981, the vitrification of quenched 1 μm droplets of pure water was claimed on the basis of an absence of visible ice crystals in electron microscopic images [8]. But the successful use of vitrification to preserve biological viability or molecular stability in the vitreous state, which is the focus of this chapter, was most unambiguously achieved even earlier, in 1968, when human erythrocytes were vitrified in a rapidly cooled aqueous solution of 8.6 M glycerol and remained intact when rewarmed [9].

Vitrification is usually induced by cooling, which in nonfreezing aqueous solutions eventually elevates viscosity to ~10^{13} P, at which point the liquid is considered to have reverted to the glassy or vitreous state [2, 10]. But vitrification or something very close to it can also be achieved in nature or in the laboratory by drying, and some organisms [11, 12] and many proteins [13] can be preserved successfully in this way.

The ability of vitrification to preserve molecules, cells, tissues, whole organs, and even some whole organisms has many current and future agricultural, medical, scientific, and ecological ramifications. The application of vitrification to cryopreservation has been growing exponentially since the early 1980s ([14–16] and Fig. 1) and may eventually enable the preservation even of systems as complex and massive as whole human organs for transplantation [14, 17, 18]. Given the broad potential biological relevance of vitrification, which is illustrated in detail in the remaining contributions to the present volume, an understanding of the basic principles of vitrification is becoming increasingly important.

1.2 Basic Terminology

The "glass transition temperature," or T_G, is the temperature at which vitrification, the transition from a liquid-like state into the glassy state, takes place on cooling. T_G is usually defined on the basis of a change in heat capacity detected by, for example, differential scanning calorimetry (DSC). Additional discussion of the nature of the glass transition is given below. T_G can be measured during either cooling or warming, but there is no generally accepted word that describes the reverse of vitrification, i.e., the onset of liquid-like behavior as the temperature is raised from below T_G to above T_G. The terms "vitromelting" or "vitrofusion" were once suggested to describe this transition [19, 20], but they have not been adopted.

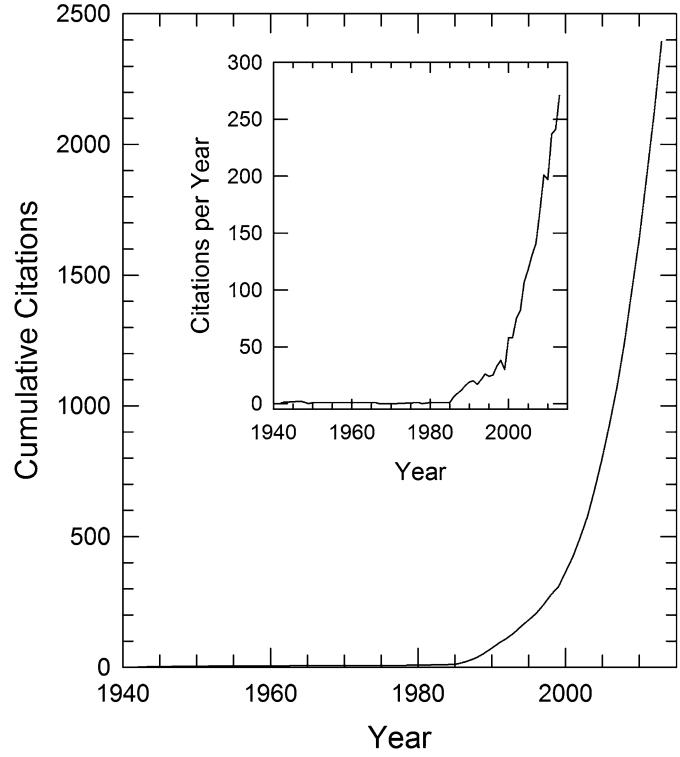

Fig. 1 Mentions of the word "vitrification" in PubMed over time. The data are censored prior to 1986 to avoid extraneous references, but are not censored thereafter

T_G' is a theoretical temperature reached when freezing is able to concentrate the unfrozen liquid portion of a solution until its melting point becomes equal to its glass transition temperature [21, 22]. Such extreme freeze concentration is rarely, if ever, achieved in real life in the case of aqueous solutions of the low-molecular-weight glass-forming solutes typically used for cryopreservation by vitrification [23] (Fig. 2), given that ice cannot continue to grow at a measurable rate as viscosities are approached that characterize the glass transition (>10^{10} P), and most literature depictions of T_G' as determined during warming probably actually represent the T_{IM}, the temperature of "incipient melting," which is the temperature of onset of melting well above T_G (at the extreme limit of the T_M curve in Fig. 2) [24–29]. No further comment is made about the somewhat controversial concept of T_G' in this review.

"Freezing" is the reorganization of water molecules into ice crystals [30]. Although "freezing" is often used to mean arresting motion or change, the use of this sense of the word in the context of vitrification, in which the object is to avoid ice crystallization, is misleading and inappropriate.

"Thawing" is the melting of ice. Reference to the "thawing" of vitreous systems is common but is inaccurate and is to be

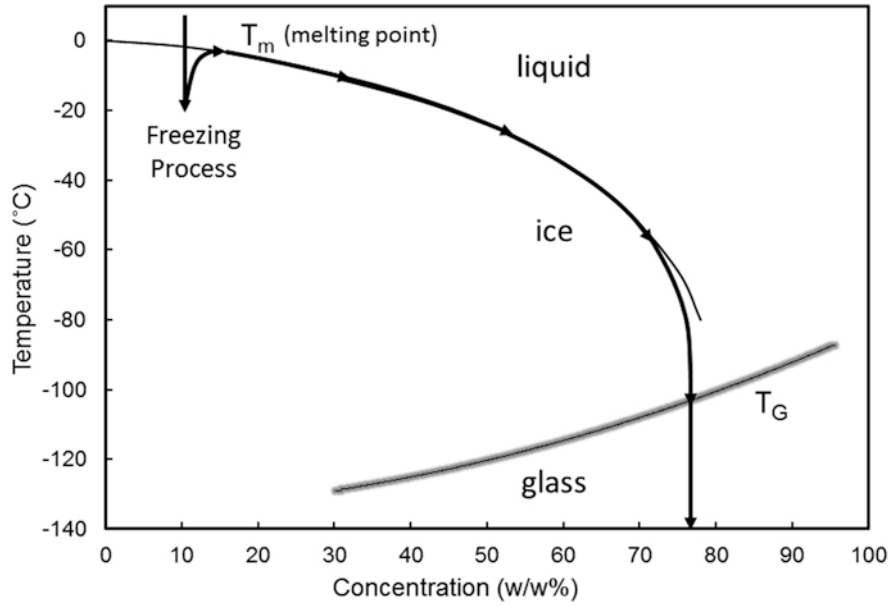

Fig. 2 The behavior of a frozen glycerol solution, leading to freeze concentration of the solution until the residual unfrozen portion of the solution becomes incapable of further freezing. Continuing cooling then leads to vitrification of the concentrated unfrozen solution. The *thin line*, denoted by T_m, is the solution melting temperature. The *thick shaded line* denoted by T_G is the glass transition temperature. The *thick arrowed line* shows the concentration of glycerol in the remaining unfrozen solution during slow (~1 °C/min) cooling. It tracks the melting temperature until increasing viscosity prevents sufficient ice growth to attain equilibrium. Below the T_G line, the sample consists of a mixture ice and glass

avoided. "Rewarming," or the warming of a previously cryopreserved system, whether frozen or vitrified, is a more accurate term than "thawing" in the context of vitrified systems.

"Devitrification" is not the reverse of vitrification. Instead, it is the formation of ice during warming after previous vitrification [31–33] and is explained in detail below.

"Recrystallization" is the transfer of water molecules from small ice crystals to larger crystals (for an early discussion, *see* [34]; for particularly illustrative photos, *see* [35]). This can happen under isothermal conditions, during which no net change in the total quantity of ice may occur, or during warming, in which case the quantity of ice may change even as recrystallization proceeds. Recrystallization tends to be damaging because it results in the conversion of a large number of relatively innocuous small ice crystals into a smaller number of larger and more damaging ice crystals. As noted below, recrystallization appears to be more important than devitrification per se in governing the fate of previously vitrified systems.

The ability of ice to form during either cooling or warming depends on how much time is available for ice nucleation and growth. Therefore, at sufficiently high cooling and warming rates,

it is possible to "outrun" the kinetics of ice formation. The "critical cooling rate" is the cooling rate above which appreciable ice formation is not observed [36], and the "critical warming rate" is the warming rate that completely or sufficiently suppresses ice formation during warming [36].

The critical cooling [37] and warming [38] rates for a given system depend very strongly on the total solute content of the system as well as on the chemical nature of the solute. The solutes used for vitrification are generally the same as or similar to those used to protect against freezing injury and are generally referred to as "cryoprotective agents" (CPAs) or "cryoprotectants" [39–41]. CPAs that are of sufficiently low molecular mass to pass reasonably quickly across cell membranes are referred to as "penetrating" or "permeating" CPAs (pCPAs), while those of higher molecular mass are referred to as "non-penetrating" CPAs (npCPAs). Glycerol, which has a molecular mass of 92.1 Da, is near the limit for defining the difference between pCPAs and npCPAs, although less hydrophilic CPAs may penetrate despite somewhat higher total masses. pCPAs include dimethyl sulfoxide, ethylene glycol, glycerol, and propylene glycol, but many others have been identified [40]. To cross cell membranes, pCPAs must not possess a net charge. npCPAs include polyvinylpyrrolidone, polyethylene glycol, sucrose, trehalose, and many others.

Although it has been argued that when cryoprotectants are used to enable vitrification they should be called "vitrificants" rather than "cryoprotectants" [42], cryoprotectants are defined to be agents that reduce or prevent freezing injury, so the term "cryoprotectants" remains proper in the context of vitrification because in fact these agents continue to prevent freezing injury, even if they do so by preventing ice formation altogether. However, the term "vitrificants," while not widely used, is also correctly descriptive of agents that facilitate vitrification.

A relatively new type of cryoprotectant is the "ice blocker," which is a molecule that is capable of undergoing specific interactions with ice or ice nucleating agents so as to reduce or prevent ice nucleation, ice growth, or both [16, 43–47]. "Antifreeze proteins" are proteins that can adsorb to the surface of ice crystals and prevent them from growing even when the temperature is lowered below the thermodynamic melting point of the ice [41, 48]. Although antifreeze proteins, or AFPs, were the first natural examples of ice blockers, ice blockers are usually thought of as being lower in mass and either synthetic or non-proteinaceous natural products.

A "vitrification solution" [49] is a solution of cryoprotectants sufficiently concentrated to enable extracellular and intracellular vitrification of the system at hand under the intended cooling conditions.

A "carrier solution" is the physiological support medium in which CPAs are dissolved to enable cells to be exposed to CPAs without injury beyond the injury associated with the CPAs themselves.

"Chilling injury" is injury caused by cooling per se. Although chilling injury is most conspicuous in the absence of ice, there has been some speculation and some strong evidence that it can occur also during freezing in specific cases [50–54]. "Thermal shock" or "cold shock" is injury caused by rapid cooling but not by slow cooling, whereas chilling injury is observed during slow cooling and may even be "outrun" by very rapid cooling if the system is not subject to injury from thermal shock.

"Anhydrobiosis" [55, 56] is the survival of life in a desiccated state. It is relevant to vitrification in the sense that sufficiently concentrated cytoplasm undergoes a glass transition that contributes to the survival of organisms, cells, or seeds that are adapted to or prepared for preservation by drying [57, 58]. Although it is of considerable potential applied and ecological significance [57–60], this chapter focuses primarily on low-temperature vitrification rather than on high-temperature anhydrobiosis or aestivation.

1.3 Vitrification and Molecular Stability at Low Temperatures

Vitrification is important for protecting cells and tissues against freezing damage, but it is not as important for preserving the basic molecular inventory of cells and tissues. Most molecular constituents of cells are reasonably stable under low-temperature conditions in situ even without special precautions, although there are exceptions. Generally speaking, neither freezing and thawing nor cooling per se causes the formation or breakage of covalent chemical bonds. The reversible formation of S-S cross-links in frozen thiogels [61], one particular protein (but not others) extracted from freeze-killed cabbage [62], and one of five SH groups in F-actin [63] has been reported, but no change in S-S or S-H content was found in lethally frozen sea urchin eggs [64], and an increase of S-H content in frozen-thawed bull spermatozoon membranes was observed [65].

Temperature reduction inhibits most chemical reactions (e.g., alkaline phosphatase catalysis is about 95 % slower at –25 °C than at 0 °C [66]), and although reactants can be concentrated greatly by the freezing process, chemical reactions are generally not quickly driven forward as a consequence, although enzymatic reaction rates may briefly increase at high subzero temperatures in frozen model systems [66]. Exothermic phase changes such as the crystallization of water or the formation of the liquid crystalline gel state [67, 68] or HEX_{II} state [69] of membrane lipids are favored over limited temperature ranges, but these phase changes do not destroy but only rearrange the participating molecules and in most cases are reversible. Protein cold denaturation, discussed in more detail below, may or may not be spontaneously reversible, but usually does not involve covalent modification of the protein.

In some cases, the cryoprotectants used for vitrification may inhibit and in some cases may promote covalent or non-covalent changes in biomolecules, but their main purpose is to prevent

physical changes, particularly involving cell distortion, that precede and thus are generally far more important than chemical reactions, phase transitions, or protein denaturation for the survival of living cells during cryopreservation.

1.4 Cryopreservation by Vitrification: A Conceptual History

A historical introduction to the field of cryopreservation by vitrification will help to convey some of the key overall concepts in approximately the order in which they were originally developed. Additional reviews of the history of biological vitrification are available elsewhere [15, 16, 42, 70–72].

Cryopreservation by vitrification was apparently first introduced conceptually although without much clarity or influence by Stiles [73] in 1930. Apparently independently, the idea was reintroduced actively, clearly, and influentially by Luyet [31] in 1937. Luyet was inspired in part by Tammann's finding that 38 % of tested organic compounds could be vitrified by rapid cooling [74] and in part by indications that aqueous gelatin gels could be vitrified [75, 76]. The original concept was that if the water in living systems could be cooled rapidly enough, there would be insufficient time for crystals to form before reaching the glass transition temperature of water, and the living system could therefore be trapped in the vitreous state [31].

This approach in principle limited vitrification to samples that could be cooled and warmed very rapidly, but it was nevertheless pursued energetically by Luyet and his associates for 21 years [70]. In 1958, evidence emerged indicating that Luyet's primary indication of vitrification, optical transparency of thin films or thin living systems after cooling followed by opacification or continued transparency on warming, had been misleading and had deceived Luyet into believing he had attained complete vitrification when in fact what he had attained was partial vitrification involving predominantly the formation of ice crystals known as spherulites that were too small or too thin to scatter visible light enough to be visible to the naked eye [77, 78].

This setback apparently chastened Luyet, who never again advocated or claimed cryopreservation by vitrification. Instead, he turned his focus onto ice and studied its formation and morphology under an extensive array of conditions [70]. Eventually, he and his colleagues learned that the presence of very high concentrations of cryoprotectants, including both small molecules [27, 28, 79, 80] and larger polymers [26], could in fact enable vitrification even at low cooling rates [70, 81]. This is the key observation that has enabled most modern methods of vitrification, but ironically, Luyet himself never proposed using high concentrations of cryoprotectants to cryopreserve living systems by vitrification.

The ability of cryoprotectants to enable vitrification only began to be elucidated and disseminated by Luyet and his colleagues as of the end of 1966 [80] and continued until 1970 [26–28, 79].

At least in part for that reason, in 1965, a historical opportunity was missed. In that year, Farrant [82] reported that he could maintain guinea pig uteri in the liquid state at –79 °C (the temperature of subliming dry ice) using 55 % v/v dimethyl sulfoxide (Me_2SO) as the cryoprotectant and recover these whole organs with excellent contractile function after rewarming and removal of the Me_2SO. As later pointed out by Fahy et al. [83], 55 % v/v Me_2SO is sufficient for vitrification, so Farrant could have actually achieved successful vitrification of whole organs in 1965 merely by cooling them another 55 °C or so. However, in 1965, it was still assumed that cooling below –79 °C would lead to ice formation [82].

As noted above, the first definitive report of the successful vitrification of a living cell was published in 1968 by Rapatz and Luyet, who showed that erythrocytes cooled at high rates in the presence of 8.6 M glycerol remained intact (did not hemolyze) under conditions in which freeze-fracture electron microscopy demonstrated the absence of discernable intracellular and extracellular ice crystals [9]. Before this, it is possible that some of Luyet's successes in recovering life after very rapid cooling and warming in the presence of cryoprotectants (which were used to achieve dehydration prior to cooling so as to reduce the volume of water that required vitrification) [84–86] might have included some level of useful vitrification, but this is difficult to infer from available knowledge. Ironically, the vitrification of red cells by Rapatz and Luyet, which might have been regarded as the culmination of Luyet's life work, was noted little, if at all, outside of Luyet's laboratory for many years and is still almost never cited.

In 1970 [87] and 1972 [88], Rapatz reported successfully cooling frog hearts to –79 °C and rewarming them with good recovery using 11 M ethylene glycol (EG) and variations on Farrant's [82] pioneering method. He presumably could have vitrified and successfully recovered these hearts in a viable condition and, unlike Farrant, must have understood that this was possible, but he reported no attempts to do so. This is likely to be because, as he reported from the podium during his 1970 presentation [87] (but did not mention in his published abstract [87]), when hearts loaded with EG were transferred into liquid nitrogen, they "shattered," as he put it (a general problem that is discussed in detail below). He later reported that 10 M EG was the minimum concentration allowing recovery of frog hearts from –79 °C, but that rat hearts could not tolerate more than 5 M EG and therefore could not be successfully preserved [89]. Nevertheless, establishing that frog hearts, at least, can theoretically be vitrified and recovered remains one of the most outstanding achievements in biological vitrification.

From 1970 [90] to 1972 [91], Elford similarly worked out a method for preserving strips of intestinal smooth muscle in a supercooled state at –79 °C using variations of Farrant's method. In the 1972 work, although it was not reported formally, it has

been noted anecdotally (D.E. Pegg, personal communication) that some muscle strips cooled in liquid nitrogen (the ones that had not experienced the same kind of "shattering" or fracturing observed by Rapatz) recovered after warming and would therefore have been the first successfully and definitively vitrified organized tissues. However, once again, there was no suggestion that vitrification as opposed to deep supercooling might be used as a method of cryopreservation.

The first intimation that high concentrations of cryoprotectants might in theory be used to enable cryopreservation by vitrification came in 1978, when Pierre Boutron, a physicist who turned to cryobiology partly on the basis of the phase diagram work of Luyet and colleagues after previously having studied the structure of amorphous solid water [92], published a landmark paper that contemplated vitrification in a new way. This paper was the first to thoroughly describe the kinetics of ice formation in vitrifiable aqueous cryoprotectant solutions (containing glycerol, Me_2SO, and mixtures of the two) in view of the theoretical possibility of completely vitrifying cell suspensions using mixtures of cryoprotectants to facilitate vitrification and reduce toxicity [36]. It also pioneered the combined use of X-ray diffraction and differential scanning calorimetry (DSC) to investigate ice formation and glass transitions in aqueous solutions, introduced the concepts of the critical cooling rate and the critical warming rate, and introduced mathematical models of the kinetics of ice formation relevant to vitrifiable solutions [36]. However, Boutron's aim was "to find a very stable amorphous state of the whole solution *even for diluted solutions*" (emphasis added), whereas the accumulated work of Luyet and his colleagues had clearly established that amorphous solutions can only be stable against ice formation when they are concentrated, not when they are dilute. Indeed, Boutron's own paper showed that, for example, the critical warming rate of a 45 % w/w solution of glycerol is about 3×10^{11} °C/min, although the critical warming rate for 45 % w/w Me_2SO was found to be only 17,000 °C/min [36].

Undeterred, Boutron and Kaufmann went on to study, in 1978–1979, the stability of the amorphous state in aqueous solutions of ethanol [93], glycerol plus ethylene glycol [94], glycerol plus ethanol [95], and, most significantly, propylene glycol (PG, or 1,2-propanediol) [96]. Remarkably, 35 % w/w PG could be vitrified when cooled at only 320 °C/min, and 40 % PG vitrified when cooled at ~40 °C/min. The critical warming rate for 45 % w/w PG was a remarkable 260 °C/min, but the critical warming rate for 40 % w/w PG was extrapolated to be 76,000 °C/min.

In later years, Boutron and colleagues continued to seek solutions that could be vitrified at relatively low concentrations on the theory that such solutions would be less toxic than solutions that required higher concentrations to vitrify, regardless of the practical difficulties of escaping from ice formation during the warming of

these solutions from the vitreous state. Perhaps because such methods would not be applicable to systems much larger than single cells and because single cells can generally be preserved well by freezing using much lower and hence less toxic concentrations of cryoprotectants, Boutron's method did not seem to inspire much attention at the time.

In 1981–1984, Fahy [83, 97–102] proposed a different approach to vitrification that, in principle, is both definitive and universally applicable to almost all biological systems. Inspired by his desire to overcome mechanical injury from ice in whole organs [15, 49, 72, 83, 97], his method relies on the fact that at sufficiently high concentrations, both the critical cooling rates and the critical warming rates needed to suppress ice formation become low enough to enable, in principle, even the vitrification of objects as large as human organs. Unlike Boutron's approach of attempting to sidestep toxicity by using lower concentrations, Fahy elected to attack the problem of high-concentration toxicity head on [83, 103–106] so as to avoid the need for high cooling and warming rates. Also, unlike the approaches of Farrant, Elford, and Rapatz, which required introducing cryoprotectant at temperatures as low as −55 °C [87], Fahy's approach sought to enable the use of cryoprotectants at much higher temperatures that were more compatible with organ perfusion and the very temperature-dependent rate of passage of pCPAs across cell membranes.

This approach was shown to work when applied to mouse embryos by Rall and Fahy in 1985 [49]. This demonstration, which showed that vitrification was at long last a feasible general method for cryopreservation, inspired much subsequent research on a variety of living systems using a variety of cryoprotectant solutions and methods (Fig. 1). The emphasis since 1985 has been largely on refining the basic but complex parameters of cryoprotectant selection; the concentration, temperature, timing, and osmotic effects of each step of cryoprotectant introduction and removal; methods and equipment for cooling and warming; and methods for avoiding fracturing. These methods have been applied to a wide variety of living systems (*see*, e.g., the listings in [15]), including plant systems [107, 108]. The number of papers devoted to these topics since 1985 is beyond the scope of this introductory review, but their contents are reflected by the scope of the other contributions to this volume.

1.5 Advantages and Disadvantages of Vitrification

The overall purpose of vitrification is to avoid freezing. To understand the advantages and disadvantages of vitrification, it is therefore necessary to understand something about freezing injury.

Conventional cryopreservation by freezing involves, by definition, the formation and dissolution of ice during cooling and warming, respectively. Ice is an almost completely pure sub-

stance, so its formation subtracts solvent water from a freezing solution, leaving the dissolved solutes in a reduced volume of solvent. The effects of ice formation are in part due to this concentrating action, which increases both the osmotic concentration of the cellular environment and the individual concentrations of dissolved solutes such as electrolytes, buffers, etc. [109, 110]. If cooling proceeds sufficiently slowly, ice formation begins extracellularly [111, 112], there is time for cells to lose water down the transmembrane osmotic gradient established by the extracellular ice, and the cells will consequently shrink. If shrinkage proceeds too far, osmotic injury may result [113–116]. If cooling proceeds more rapidly, the rate of water subtraction from the cell fails to keep up with the rate of water subtraction from the extracellular environment, leaving the cell interior significantly more dilute than the extracellular solution [117]. This means that the thermodynamic freezing point of the cell fails to fall as rapidly as the prevailing temperature, i.e., that the temperature of the cytosol begins to fall farther and farther below its nominal freezing point. This defines a state of supercooling (cooling below the freezing point without ice formation), and as supercooling increases, the risk of ice formation within the cytosol increases.

In summary, cells cooled too slowly are liable to injury related to shrinkage and changes in solution composition ("solution effects" injury), whereas cells cooled too quickly are liable to injury related to intracellular ice formation (IIF) [117–121]. Between these two ends of the spectrum, there is an optimum cooling rate that minimizes both sources of injury [117–121].

These considerations are relevant to vitrification for three reasons. First, the existence of an optimum cooling rate is problematic. The optimum cooling rate can only be determined experimentally for every cell type of interest, which is inconvenient, particularly for multicellular tissues, which may contain not only multiple cell types but also cells in different relationships to each other and to the extracellular environment, all of which affect the optimal cooling rate [118]. Moreover, given the existence of a different optimal cooling rate for different cells, finding a compromise rate that gives high recoveries of all cells may be difficult and has been proposed as a limiting factor for cryopreserving complex systems [118]. Furthermore, even for a given type of cell, the optimum cooling rate very often fails to yield 100 % cell survival [118, 122]. And finally, the use of cryoprotective agents to increase survival at the optimal cooling rate also changes the optimal cooling rate itself [122], again in a way that will be cell type dependent. For these reasons, vitrification is advantageous in part because it transcends the need to find an optimal cooling rate, to compromise the survival of one cell type to ensure survival of another cell type, and to accept cell survival rates that are unlikely to approach 100 %.

The second point of relevance to vitrification is that, as noted, pCPAs must generally be used to obtain high survivals after freezing and thawing, since they mitigate solutions effects injury. Although relatively low concentrations of pCPAs are needed to prevent solution effects injury in many cells, the concentrating effect of freezing on dissolved solutes pertains just as much to pCPAs as it does to other solutes, the result being that pCPA concentrations may be driven high enough in the frozen state to induce toxic effects of their own [123–126]. Interestingly, the concentrations generated by freezing actually exceed the concentrations required for the vitrification of even large living systems [83, 101, 127], so the advantage of using lower concentrations for freezing is not necessarily as large as it at first appears.

The third reason the constraint of an optimum cooling rate is relevant to vitrification is that some important living systems such as oocytes are subject to chilling injury (see below), and attempts to cool more rapidly than the kinetics of chilling injury proceed are precluded if the result is death secondary to IIF (intracellular ice formation). Vitrification eliminates that obstacle by eliminating IIF at high cooling rates and has often been pursued for that reason [52, 54, 128, 129].

Beyond changes in solution composition and IIF, freezing can result in injury in at least two additional ways, both of them being mechanical in nature. First, the physical displacement of structures in organized tissues by the simple growth of extracellular ice can cause considerable damage to both the vascular bed and to parenchymal structures [98, 130–139]. In fact, it was the observation that dog kidneys frozen to −30 °C and stored for a week using 3 M glycerol could perfuse normally and respond well to pressors in vitro but urinated whole blood and stopped perfusing within 10–20 min of being transplanted (Fahy, Goldman, and Meryman, unpublished results) that inspired the proposal to investigate vitrification as a more promising approach to organ cryopreservation. Fortunately, Taylor and his colleagues have provided extensive microscopic evidence using freeze substitution methods that vitrifiable solutions successfully prevent tissue distortion by ice [16, 140, 141]. Second, even single cells can be injured by intracellular [118] and extracellular [134, 135] recrystallization. Both forms of mechanical injury secondary to ice formation argue for vitrification as a potentially less damaging preservation method, particularly for complex organized tissues and organs.

Vitrification does have significant disadvantages as well, however [142, 143]. First, the need to tolerate very high concentrations of CPA requires relatively sophisticated methods of adding and removing these agents and careful selection of the right CPA blend for the living system at hand. Second, it is not always clear

what CPA concentration and exposure time is needed to ensure vitrification and maintenance of an ice-free state upon warming in specific cases, and investigation of these points may be needed. Third, rapid cooling to below the glass transition temperature and/or rapid warming below T_G may induce fracturing of the glass in which the biological system is embedded [144, 145] (*see* also Subheading 2.7), which may cleave cells or tissues, causing irreversible injury [146] and additional ice nucleation [147]. Fourth, to avoid the third problem, in some cases, storage at "intermediate temperatures" [~−130 to −160 °C, i.e., below T_G but above the temperature of boiling liquid nitrogen (−196 °C)] may be needed to ensure long-term preservation without crack formation. Finally, although rapid warming is generally beneficial for frozen systems, it is even more important for vitrified ones due to the need to avoid injury from devitrification and subsequent recrystallization.

A potential disadvantage of certain techniques of vitrification comes from the use of container-free cooling methods to accelerate the cooling and warming rate and thereby enable the use of minimal concentrations of cryoprotectant. The lack of a container may result in contamination of the sample being preserved [142]. The need for such methods is questionable, however, and it seems likely that closed-system vitrification will eventually remove the risk of contamination.

1.6 Vitrification in Nature

It is reassuring that nature has often drawn the same conclusion as the cryobiologist in pursuing ice-free cryopreservation in preference to freezing.

A number of insects, for example, survive the winter by freeze avoidance [57, 58, 148, 149], achieved by suppressing the presence of ice nucleating substances, synthesizing high concentrations of cryoprotectants such as glycerol, and producing "antifreeze proteins" that bind to ice and prevent it from growing (*see* Subheading 2.6 below for more discussion of antifreeze proteins and ice blockers). In one case, that of the larval Alaskan red flat bark beetle (*Cucujus clavipes puniceus*) [149], more than half of the individuals tested supercooled to below −60 to −70 °C and none showed exotherms indicative of freezing when cooled to −150 °C in a DSC. All showed large whole body glass transitions between −58 and −76 °C (DSC curve inflection points; mean T_G, −71 °C). Two large larvae had a second small T_G at −96 or −98 °C. When unselected larvae were cooled to −71.5 ± 1.5 °C or to −100 °C, the survival rates were about 50 % and 7 %, respectively, although the latter rate was probably reduced by mechanical damage sustained by the methods used.

In any case, at least some larvae appear able to survive cooling to below even the lowest observed T_Gs. Further, the coldest temperatures recorded in nature (–79.8 °C in Alaska [150], –89.2 °C at the Russian Vostok Station in Antarctica [151], and most recently –94.7 °C in eastern Antarctica [150]) are all below this insect's main T_G and well below the highest measured T_G of –58 °C. This suggests that some organisms have actually survived low temperatures in a vitreous state under natural conditions using endogenous cryoprotectants similar in both molecular weight and concentration to those being used for artificial vitrification in cryobiological laboratories to those being used for artificial vitrification in cryobiological laboratories (in *C. c. puniceus*, up to 6.5 M glycerol was measured in the cited study, and up to 10 M glycerol has been reported elsewhere [152]).

C. c. puniceus, in addition to elaborating protective substances, concentrates them to vitrifiable levels in part by water loss to the environment. Species whose moisture content varies with the ambient humidity are said to be "poikilohydric," and this water loss can be sufficient to induce cytoplasmic vitrification [108, 151, 153, 154] and may even make vitrification under natural conditions more common than survival by freezing tolerance [151, 155]. As one example, soil nematodes dried to below 0.3 g of water per g dry weight survived cooling in liquid nitrogen and showed no evidence of freezing [156]. However, vitrification by dehydration enables survival at high temperatures as well as at low temperatures [11, 12].

The lowest common terrestrial temperatures are typically between about –30 and –60 °C [149, 151], but the cells of many species have been shown to have glass transition temperatures above –50 °C [57, 153, 157]. Twigs of *Populus balsamifera* were shown to undergo a glass transition at about –45 °C and are known to be able to survive immersion in liquid nitrogen [157]. Highly frost-hardy plants in general, according to Sakai, survive in conjunction with their ability to form intracellular glasses [108].

1.7 Vitrification During Freezing of Living Cells

When ice forms in the presence of cryoprotectants, their concentrations are elevated by loss of water from the solution into the ice phase, until eventually they preclude further ice formation during continued cooling, resulting ultimately in vitrification of the residual unfrozen solution [79, 83] (*see* Fig. 2; also Subheading 2.1). Similarly, freeze concentration of the extracellular solution and concomitant osmotic reduction of cell volume (*see* also Subheading 1.5 above) result in vitrification of cytoplasm when cooling is slow enough to preclude IIF [83, 158–161]. Therefore,

most cells survive cryopreservation as a result of vitrification, even if the medium surrounding them is not completely vitrified.

2 The Physical Principles of Vitrification

2.1 Vitrification Depends on the Solute Concentration of Aqueous Solutions

Figure 2 illustrates the process described in Subheading 1.7, showing the example of the slow freezing of a 10 % glycerol solution in water on a glycerol-water phase diagram. The solution may initially supercool before the first ice crystal forms, but thereafter the concentration of the remaining unfrozen solution follows the melting temperature line (T_m) with continued cooling. Increasing solution viscosity during cooling eventually inhibits ice growth, causing a small departure from thermodynamic equilibrium [23]. Final cooling then continues with little change in concentration until the glass transition temperature (T_G) is reached [83]. Below T_G, the viscosity of unfrozen solution between ice crystals becomes so high (>10^{13} P) that the solution behaves as a solid. It does so while retaining the random molecular arrangement of a liquid. A solid with the same unstructured molecular arrangement as a liquid is called a glass [1].

With a sufficiently high solute concentration and/or cooling rate, it is possible to cool all the way to the glass transition temperature without significant ice formation [83, 101]. This is the basis of cryopreservation by vitrification. During cryopreservation by vitrification, the entire sample volume remains substantially free of ice during cooling. As shown in Fig. 3, this can be achieved by using either low solute concentrations and fast cooling rates or higher concentrations and slower cooling rates. Heat transfer limitations necessitate the use of high solute concentration and slow cooling rates when vitrifying large volumes, such as tissues and organs.

As hinted at in Fig. 3, there is a way to predict the concentration of CPA that will enable vitrification at moderate cooling rates (around 10 °C/min). The curve labeled "T_h" designates the homogeneous nucleation temperature, which is further described in Subheading 2.3. T_h sets the limit beyond which the solution cannot be supercooled without ice nucleation. Careful analysis of the threshold concentration required for vitrification (C_V or CNV, the "concentration needed for vitrification") based on visual inspection of ~8 ml volumes of CPA solutions cooled at about 10 °C/min showed that, for glycerol-water, ethylene glycol-water, dimethyl sulfoxide-water, and propylene glycol-water systems (and the latter at 1, 1,000, and 1,500 atm of applied pressure), C_V coincided with the concentration required to make T_h equal to T_G in every case [83].

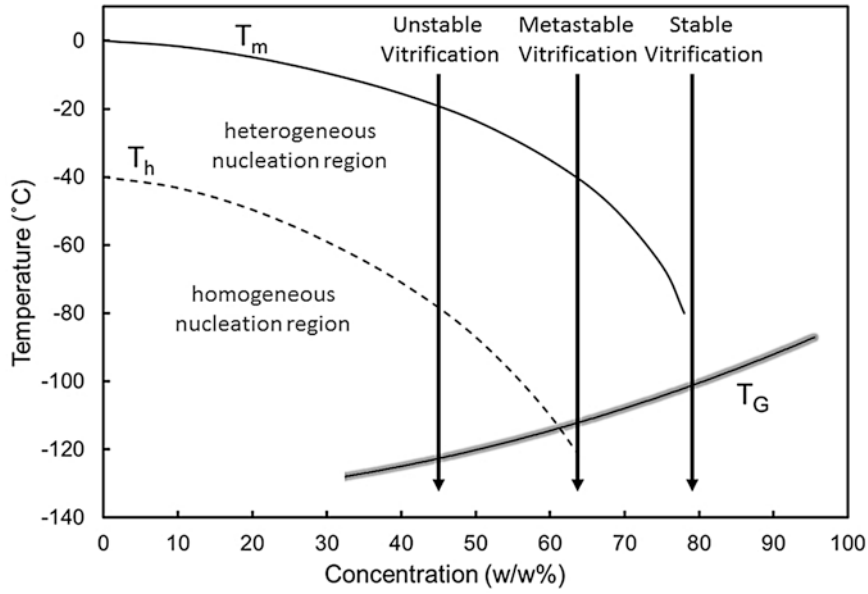

Fig. 3 Vitrification at three different concentrations of glycerol in water. Unlike freezing, with vitrification the solution concentration remains constant during cooling because cooling is too rapid for ice to form or grow appreciably. Unstable vitrification requires cooling at thousands of degrees per minute, or more, due to high ice nucleation and growth rates associated with homogeneous nucleation. Metastable vitrification is typically possible at cooling rates on the order of 10 °C/min. Stable vitrification ("equilibrium" vitrification) is possible at arbitrarily low cooling rates

2.2 The Physical Nature and Basis of Vitrification

Vitrification occurs when thermal energy becomes insufficient for molecules to overcome potential energy barriers that must be overcome for translational rearrangements within a liquid. Below the glass transition temperature, molecules lose the ability to wander among other molecules over the timescale of measurements being made. They instead vibrate in place. As a consequence, the measured values of thermodynamic response functions such as heat capacity, thermal expansion coefficient, and compressibility fall from those of a liquid to those of a solid. The glass transition is typically detected calorimetrically by an observed drop in heat capacity during cooling. In contrast, thermodynamic state variables such as volume, energy, and entropy do not change at the glass transition. Only their slope as a function of temperature undergoes change [2].

Although the glass transition is a material phase change (liquid to solid), it is not a thermodynamic phase change from one equilibrium state to another. The glass transition is a kinetic phenomenon in which viscosity delays intermolecular rearrangements that are thermodynamically favored. In essence, vitrification "locks in" a nonequilibrium thermodynamic state. As cooling rates are varied by orders of magnitude, measured glass transition temperatures can vary by several degrees Celsius [162], with slower cooling rates resulting in lower measured glass transition temperatures. This is

due to the kinetic nature of the glass transition. Slower cooling rates provide more time for intermolecular rearrangements that release heat, contract volume, and otherwise approach equilibrium before rising viscosity stops liquid-like behavior.

The measured decrease in heat capacity that occurs during passage below the glass transition temperature provides a more abstract interpretation of the glass transition. The heat capacity of a liquid above the glass transition temperature is greater than that of a crystal at the same temperature. Entropy varies as heat flow divided by temperature. Therefore, during cooling, the entropy of a liquid decreases faster than the entropy of a crystal of the same composition. This leads to a projected temperature called the Kauzmann temperature (T_K) below which the liquid is extrapolated to have a lower entropy than the crystal [163]. Since a disordered liquid state is supposed to have higher entropy than an ordered crystal, cooling a liquid to T_K would create a paradox. The decrease in heat capacity at the glass transition prevents this thermodynamic paradox. Although kinetic in nature, the eventual occurrence of a glass transition can be viewed as a thermodynamic necessity for crystallizable liquids with a Kauzmann temperature greater than absolute zero.

As mentioned above, thermodynamic nonequilibrium is intrinsic to glasses at the time of their formation. Lack of equilibrium pertains to the glassy phase in which cryopreserved cells are suspended irrespective of whether cryopreservation is by freezing or vitrification.

Four types of nonequilibrium are noteworthy in the context of cryopreservation. First, there is vapor pressure nonequilibrium between ice and the unfrozen sample volume. This is important for cryopreservation by vitrification because the aim is for the sample to remain substantially free of ice during cooling and storage despite strong thermodynamic driving forces (vapor pressure nonequilibrium) favoring ice growth. Unequal vapor pressures between ice crystals of different sizes also play a role in ice recrystallization [34, 164], which can damage cells during rewarming of frozen samples or vitrified samples that form ice during rewarming [134, 165, 166]. Second, there is chemical nonequilibrium, in the sense of chemical instability and change. At relatively high temperatures, chemical or protein conformational changes in cells are able to proceed beyond the controls of normal metabolism, but these changes can be slowed and ultimately arrested as temperatures decline and increasing viscosity combined with insufficient activation energies prevents chemical reactions. Chemical nonequilibrium may relate to the rate of viability loss during cooling. Third, there is chemical potential nonequilibrium. Nonequilibrium of cryoprotectant concentration between different regions of a vitrified sample [17] or across the cell membrane [83, 167] can make the difference between satisfactory and unsatisfactory preservation. Fourth, there is

mechanical nonequilibrium. Mechanical nonequilibrium is relevant to mechanical stress and strain during cryopreservation, which is an especially important consideration for large samples such as tissues and organs, and is discussed in more detail in Subheading 2.7.

2.3 Ice Nucleation

Ice formation begins with a process called nucleation [168]. There are two kinds of nucleation, homogeneous and heterogeneous.

During nucleation, water molecules begin organizing into the structure of ice on a nanometer scale. The resulting nascent ice crystals, or ice nuclei, tend to be unstable. In accordance with the Gibbs-Thomson equation [169], ice crystals of small size have low melting temperatures (high vapor pressure) caused by sharp curvature of the crystal surface. Consequently, newly formed ice nuclei tend to melt at any temperatures warmer than about −38 °C in pure water. This defines the homogeneous nucleation temperature (T_h), the lowest temperature to which small [30, 170] samples can be cooled under normal conditions without ice formation and the highest temperature at which small samples are likely to form ice when preexisting ice crystals or contaminants that mimic ice crystals (heterogeneous nucleators, discussed below) are absent [30, 70, 170–173]. As shown in Fig. 3, T_h decreases with increasing solute concentration [79].

Ice can form at temperatures above T_h in the presence of heterogeneous nucleators [30, 168, 174]. Heterogeneous nucleators are particles or surfaces that mimic the structure of ice on a molecular scale or otherwise induce water to assume a more icelike configuration with a larger radius of curvature. Ice crystals with a larger radius of curvature upon their initial formation have a lower surface energy, allowing them to avoid melting at warmer temperatures [174]. The most potent heterogeneous nucleators can cause ice to form at temperatures only 1 °C below T_m [175, 176]. Heterogeneous nucleators are ubiquitous environmental contaminants [168, 174]. They are usually responsible for initial ice nucleation events in volumes larger than ~10 μm diameter water droplets prepared as aerosols or emulsions to study homogeneous nucleation [177].

The phase diagram of a cryoprotectant solution can be divided into heterogeneous and homogeneous nucleation zones (Fig. 3). These zones give rise to at least three distinct types of vitrification.

Vitrification using solute concentrations insufficient to prevent passage through the homogeneous nucleation zone will nucleate at an innumerably large number of points in the solution [164] (e.g., ≥2,500 nuclei/μm³ in one case [178]). Since in the homogeneous nucleation zone water is self-nucleating, passage through this zone should be considered unstable vitrification. (In glass science, solutions that homogeneously nucleate are said to be "doubly unstable" due to instability with respect to both crystal nucleation and growth [179].) Unstable vitrification can be survived if warming is sufficiently rapid, as discussed below.

Vitrification using a solute concentration high enough to avoid homogeneous nucleation but low enough that ice formation is still thermodynamically favored ($T < T_m$) can be defined as metastable vitrification [83, 180, 181]. It is metastable because such a solution can supercool all the way to the glass transition without ice nucleation necessarily occurring, but ice is still thermodynamically favored. Ice nucleation events will essentially be confined to discrete locations where heterogeneous nucleators are present.

A third form of vitrification is stable vitrification, or "equilibrium vitrification" [16], which uses solute concentrations so high that ice cannot exist in the solution [2, 83]. A practical approximation to the edge of the stable vitrification zone can be defined as the concentration that is sufficient to make devitrification vanish during slow warming [18, 83], but complete stability requires the higher concentrations that are typical of those that preclude ice growth during previous slow freezing (the "unfreezable concentration," or C_U [15, 72]).

Ice nucleation occurs by local reorientation of water molecules. The nucleation rate is therefore driven primarily by the free energy difference (thermodynamic driving force) between ice and liquid water in solution. Rates of nucleation rise as temperature decreases, reaching a maximum near the glass transition temperature [2, 182]. Only below the glass transition temperature do astronomical viscosity and loss of rotational freedom begin to slow ice nucleation [2, 182, 183].

2.4 Kinetic Aspects of Ice Formation in Vitrification Solutions During Cooling

Boutron was the first to characterize the quantitative relationships between the cooling rate of CPA-water solutions of different concentrations and their ability to escape from ice formation on cooling, as evidenced by the lack of exotherms recorded using differential scanning calorimetry [94]. His modeling eventually led [37] to an equation

$$-\ln\left(1-x^{1/3}\right)+0.5\ln\left(1+x^{1/3}+x^{2/3}\right)+\sqrt{3}\,arctg\left(\left(\sqrt{3}\,x^{1/3}\right)/\left(2+x^{1/3}\right)\right)=\mathrm{k4}/|v|$$

that accurately predicts the amount of ice formed during the cooling of cryoprotectant solutions at different rates given certain starting information, such as the amount of ice that forms at very low cooling rates, in which ice formation is maximum. In this equation, k4 is a constant and x is q/q_{max}, where q is the calorimetrically determined amount of ice observed to form at cooling rate v and q_{max} is the similarly determined maximum amount of ice that can form at very low cooling rates. When k4 equals v, $x = 0.036$, so k4 is equivalent to the cooling rate that reduces ice formation to 3.6 % of the maximum amount that can form [184].

The critical cooling rate can be defined as the cooling rate sufficient to reduce x to whatever any particular living system can

tolerate. In the limit of low x, which is the limit of interest for vitrification, the above equation becomes simplified. If $x = 10^{-6}$, then

$$v = 100\mathrm{k}4 \,/\, 3.$$

If x is set to a value reflecting 0.2 % w/w ice, then the equation becomes [184]

$$v = \mathrm{k}4 \,/\, \left[3\left(0.2 \,/\, q_{max}\right)^{(1/3)}\right],$$

where $q_{\max}$ is the maximum mass percent of the solution calorimetrically observed to freeze while cooling very slowly (arbitrarily using the 334 J/g heat of fusion of pure water at 0 °C to convert the heat of the exotherm into the mass of ice formed) and the 0.2 factor represents a mass percent of solution crystallized of ~0.2 % (which should be low enough even for the survival of a vitrified kidney [17]). The convention of choosing 0.2 % w/w ice as a standard for defining the critical cooling rate is based in part on the fact that this is the minimum amount of ice that has been considered to be quantifiable by DSC [184].

Figure 4 shows the effect of k4 when the critical cooling rate is defined as the rate that reduces x to 0.1, 0.2, 0.5, or 1 % of the maximum amount of ice that can form in the system. Using

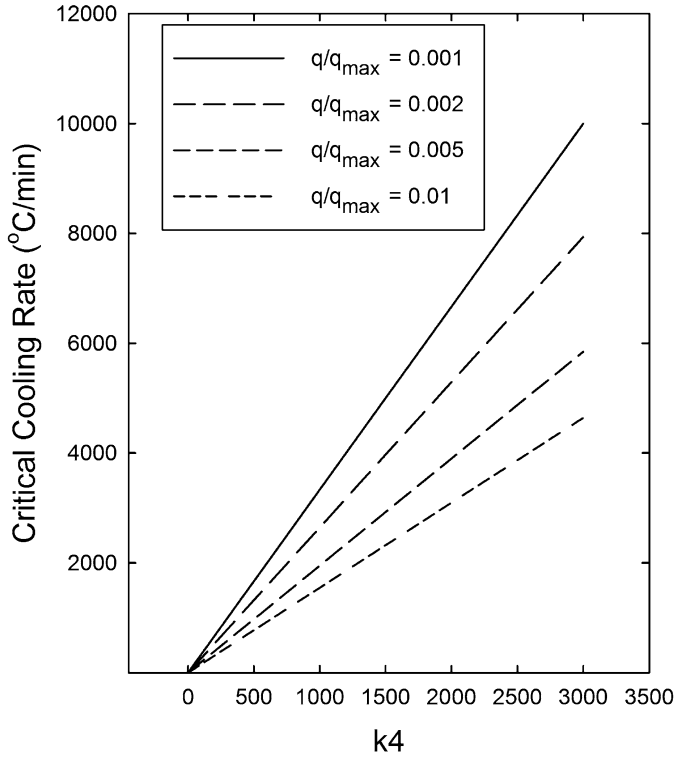

Fig. 4 Universal relationship between Boutron's k4 values and the critical cooling rate for vitrification based on the choice of x (0.1–1 % of the maximum possible amount of ice)

$x = 0.2/q_{max}$, Baudot et al. have calculated critical cooling rates for many cryoprotectant solutions and compared them to the critical warming rates for the same solutions [185].

2.5 Devitrification and Recrystallization

Once ice has nucleated into stable nanoscale [186] nascent ice crystals, it tends to grow. However, unlike nucleation, which depends on local molecular motion, ice growth requires diffusion to supply water molecules to a growing ice front and dissipate solutes not incorporated into the crystal. This dependence on diffusion makes the rate of ice growth strongly, and inversely, dependent upon solution viscosity. Consequently, ice in vitrification solutions grows most rapidly at temperatures not far below T_m [2]. This is the opposite of the behavior of ice nucleation, which has a maximum rate at temperatures near T_G. This separation between the temperature optima for ice nucleation and growth has important implications for determining the cooling and warming rates necessary for avoiding appreciable ice formation.

During cooling, ice growth will be limited by the small number of nuclei (typically those arising from heterogeneous nucleation) that form at higher temperatures where the growth rate is significant. Nucleation continues and accelerates during cooling until T_G is passed, but at those temperatures, the nuclei are too cold to grow. Upon warming, nucleation resumes when the temperature range near T_G is again traversed. This means not only that many more nuclei are present during warming than are

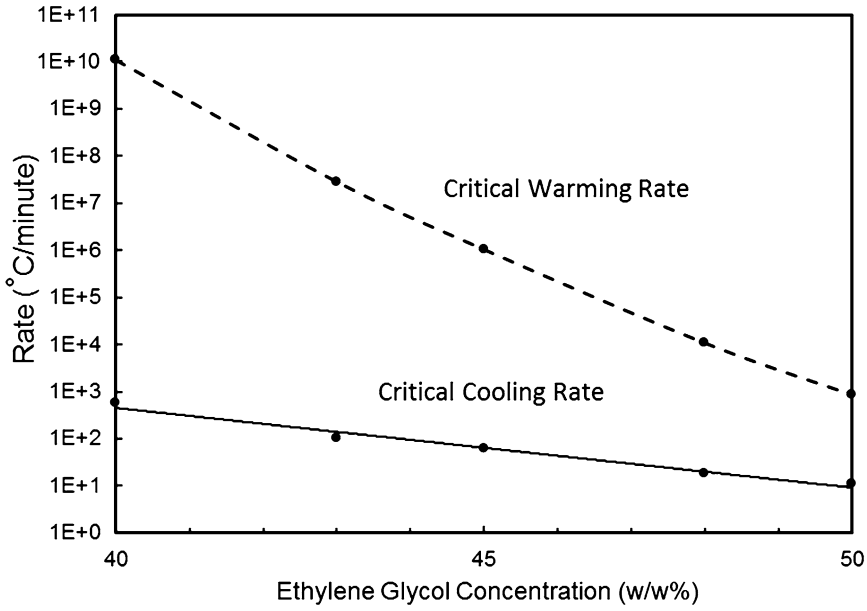

Fig. 5 Critical cooling and critical warming rates to avoid ice formation during cooling or warming various concentrations of ethylene glycol in water. Rates increase exponentially as concentration decreases, with the calculated critical warming rate becoming practically unattainable at low concentrations. Data from Baudot and Odagescu [188]

present during cooling [2, 182, 187] but also that during warming, vastly more nuclei will be present at temperatures that favor rapid ice growth. This means that total ice development is much more rapid during warming, and hence warming rates required to avoid significant devitrification are found to be far higher than cooling rates initially required to achieve vitrification (e.g., Fig. 5, [188], and [38]). For glycerol, propylene glycol, and ethylene glycol, as the critical cooling rate increases from 10 °C/min to 100 °C/min, the critical warming rate increases from ~10^2–10^3 °C/min to ~10^5–2×10^7 °C/min [15, 72] (a 5-log increase for ethylene glycol and glycerol and perhaps a 3-log increase for propylene glycol). Fahy found that for vitrification solutions of propylene glycol and Me_2SO, which vitrify at about 10 °C/min, the critical warming rate is 1,000 °C/min (ignoring the effect of the carrier on the latter) [189, 190].

The nucleation [191] and growth [192] rates of ice in vitrification solutions are also dependent on the carrier solution. Carrier solutions based on NaCl, which include many typical culture media, have minimal effects, but carrier solutions based on sugars, which displace more water and have a stronger effect on solution viscosity, are comparable in effect to CPAs, gram for gram, in increasing vitrification tendency and reducing the critical warming rate [72, 191].

Another feature of rewarming after forming large numbers of small nuclei on cooling has to do with the visual appearance of the solution. Converting a relatively dilute CPA solution into a heavily nucleated glass does not necessarily change the visual appearance of the solution because the crystals can be too small to scatter light, making it possible for the solution to remain transparent, albeit there may be a blue coloration as a telltale sign of the presence of ice nuclei that may be overlooked [164]. During rewarming, such dilute solutions tend to opacify at a temperature higher than the temperature shown by differential scanning calorimetry (DSC) to be the temperature of devitrification (Fig. 6) because opacification requires the small crystals formed during devitrification to recrystallize until the prevailing crystal size is larger than the wavelength of light [164]. At higher CPA concentrations, the onset of devitrification is synonymous with the onset of opacification because, at a lower nucleation density, each nucleus must grow to a larger size to evolve a detectable amount of heat. At rapid warming rates, opacification may not be observed if insufficient recrystallization takes place even if devitrification is extensive. Therefore, using visual appearance as evidence of vitrification and the lack of devitrification is valid only for solutions that can be vitrified at modest cooling rates (near 10 °C/min) and is not reliable for significantly more dilute solutions [164].

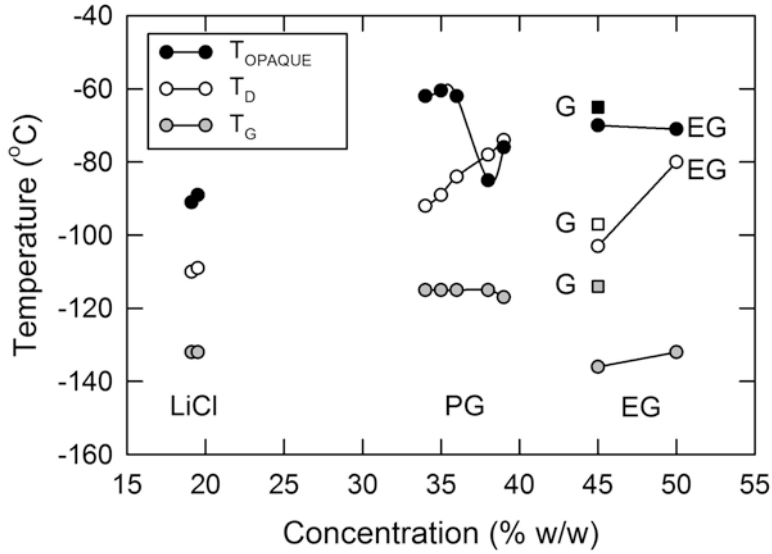

Fig. 6 Lack of equality of solution opacification and devitrification when solution concentrations are below the concentrations needed for metastable vitrification. T_{OPAQUE} is the temperature of maximum opacity during warming at 80 °C/min. T_D is the temperature of the maximum of the devitrification peak during warming at 80 °C/min, and T_G is the temperature of onset of the glass transition of the quenched solution as measured during rewarming at the same rate. All solutes are dissolved in water. For propylene glycol (PG), ethylene glycol (EG), and glycerol (G, *squares*), C_V at a cooling rate of ~10 °C/min is ~43–46 % w/w, 52–55 % w/w, and 57–60 % w/w, respectively. T_{OPAQUE} and T_D are therefore projected to converge ~5–8 % w/w below C_V for PG and ~0–3 % w/w below C_V for EG. Drawn from the data of [164]

On the opposite end of the spectrum is the use, for whole organs, of a vitrification solution (M22) that has a critical cooling rate of less than 0.1 °C/min and a critical warming rate of around 0.4 °C/min [14, 193]. For kidneys vitrified with M22, warming at ~10 °C/min has resulted in appreciable ice formation due to incomplete distribution of the cryoprotectant into all parts of the organ [17, 18]. In this case, the critical warming rates are low enough to be addressed by radiofrequency or microwave frequency electromagnetic warming [194]. Such warming has been achieved by either electric coupling to ions and polar molecules in cryoprotected tissues [195–198] or magnetic coupling to exogenous magnetic nanoparticles [199].

2.6 Antinucleation and Specific Ice Growth Inhibition

In recent years, there has been growing interest in agents able to inhibit ice nucleation or ice growth by specific molecular recognition of ice or ice nucleators, sometimes called "ice blockers." Antifreeze proteins and antifreeze glycoproteins are natural examples, but they are difficult to obtain in useful quantities and at affordable cost for tissue cryopreservation applications.

As alternatives, the synthetic polymers polyvinyl alcohol (PVA) and polyglycerol (PGL) have been recognized as ice growth and ice nucleation inhibitors, respectively, for cryopreservation applications [43, 44], with PGL specifically effective against heterogeneous nucleators of bacterial origin. The utility of ice blocking compounds is that they can have large effects on ice formation even while present in small quantities (Fig. 7), even quantities as small as one part per million [43]. They can also inhibit ice recrystallization [200], making them useful if devitrification can't be avoided during warming or if cryopreservation by conventional freezing is used [201]. They are being productively used in an increasing number of vitrification applications [202–208].

A flavonol glycoside antinucleator also significantly improved survival of vitrified shoot apices at a concentration of only 0.05 % [209], and a number of other small molecules may have future applications as practical ice blockers for vitrification [16, 45–47], but currently they have not been tested for this purpose. A new synthetic molecule, polylysine with about 65 mol% of its side chains replaced with carboxyl groups, has shown recrystallization inhibition and good cryopreservation by freezing [210, 211] as well as successful applications to vitrification [212–215].

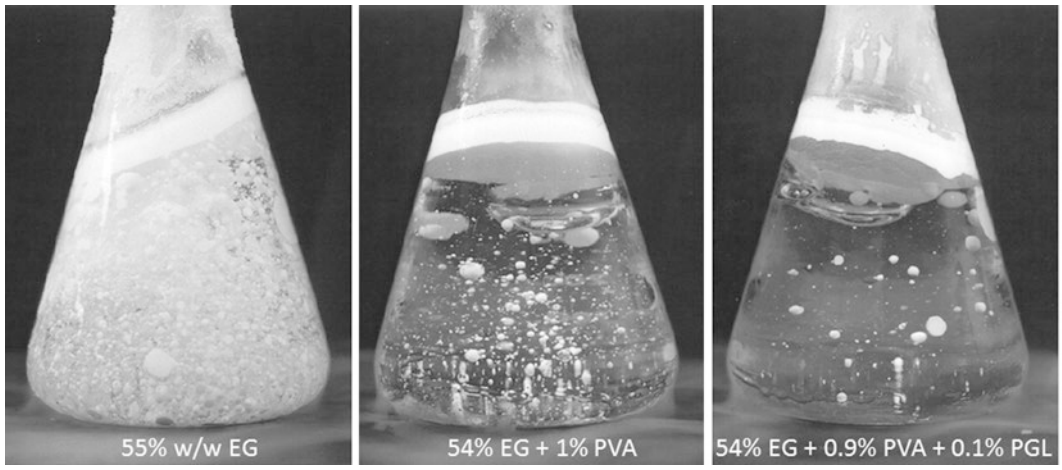

Fig. 7 Semivitrified 500 g samples of ethylene glycol (EG) solutions cooled to −128 °C. Small quantities of polyvinyl alcohol (PVA) and polyglycerol (PGL) ice blockers dramatically reduce the amount of ice formed during cooling. The PVA and PGL used were, respectively, X-1000 and Z-1000 ice blockers from 21st Century Medicine, Inc.

2.7 Thermally Induced Volume Changes, Strain, and Fracture Formation

Like most matter, cryoprotectant solutions contract with cooling, possessing a linear thermal expansion coefficient of ~90 ppm/°C [216]. Below the glass transition temperature, the thermal expansion coefficient is observed to fall to ~40 ppm/°C [216] due to kinetic inhibition of molecular movements required to continue liquid behavior. This causes mechanical stress in samples during vitrification. Colder outer parts of a sample will solidify (take on a lower thermal expansion coefficient) before warmer inner parts of a sample do. The resulting tendency of the interior to contract more than the exterior after passage through the glass transition and subsequent approach to thermal equilibrium creates stress. Cryoprotectant glasses have a fracture strain of ~0.3 % and fracture stress of ~3 MPa [217], which is only one tenth that of silica glass (due to hydrogen bonding in the former versus covalent bonding in the latter). This makes samples prone to fracturing during vitrification [144, 145, 147]. Fracturing is undesirable for cell samples because fracture planes nucleate ice [147] and unacceptable for tissue or organs.

Stress during cooling to temperatures far below T_G has been found to be proportional to the cooling rate and the square of the linear sample size [218]. It is best managed by slowing the cooling rate as T_G is approached [145] and ensuring sample temperature uniformity as best as reasonably possible during passage below T_G.

Ensuring that samples don't adhere to container surfaces during cooling is especially important for fracture avoidance [71] because containers typically respond differently to cooling than cryoprotectant samples. Hydrophobic polymers that permit sample retraction away from container walls during cooling are to be preferred over hydrophilic materials such as borosilicate glass.

3 The Biological Principles of Vitrification

3.1 Are Cryoprotectants Necessary for Vitrification?

As reviewed above, high-temperature vitrification can be achieved in some cells and organisms simply by drying, and some organisms can also dry into a glassy state during the winter at low terrestrial temperatures. But low-temperature vitrification has also been attempted in the absence of added cryoprotectants for some cells that are not desiccation tolerant. It has been known for many years that very small biological systems and even pure water can be cooled rapidly enough to avoid ice crystals that are visible in the electron microscope [8], but that is a different problem than vitrifying cells and recovering them in a viable state upon rewarming. The problem of vitrifying cells without pCPAs is a topic of some current interest and discussion, and a detailed examination of this question is appropriate for illustrating additional principles of cryopreservation by vitrification.

The vitrification tendency of cryoprotectant solutions has been studied extensively as a function of cryoprotectant concentration, but living cells contain solutes in addition to any cryoprotectants they may take up, and these natural solutes definitely augment the vitrification tendency of cytoplasm in the presence of added pCPAs [9, 83, 143, 219]. The quantitative contribution of intracellular solutes to intracellular vitrification in different cells or in different organelles has, however, not been well studied. Cytoplasm typically contains 15 % w/w protein and just 80 % water, the endoplasmic reticulum contains only 65 % water, nuclei contain only 61 % water, and mitochondria contain only 59 % water [189]. Exactly how these low water contents influence vitrification tendency deserves to be considered more carefully. However, since most cells and their organelles are in osmotic equilibrium at ~300 milliosmolal (mOsm), they do not have an extraordinary water activity and readily experience IIF and behave very much as though their water is present as an ideal dilute solution [117–119, 220].

Ice formation is also affected by the presence of solid surfaces, whose organization of local water structure tends to inhibit ice formation. According to Meryman's discussion [221] of the work of Hori (in 1956), the spontaneous freezing temperature of water between glass plates separated by 10 μm is –30 °C, and a plate separation on the order of 0.14 μm results in no ice formation even at –100 °C and a negligible water vapor pressure. Water structuring by other types of surface has been investigated at some length [222, 223]. The water in intracellular compartments may be influenced by this same general type of effect near the plasma membrane, organelle membranes, and the cytoskeleton, further favoring vitrification, and the effect might be particularly significant for sperm considering their highly elongated, thin shape and packed DNA.

The water content of sperm, as estimated from their osmotically inactive volume (the b value in the Boyle-van't Hoff equation described below), is just 23–55 % according to Isachenko et al. [224] or 60 % according to Morris [225]. This has led to speculation that sperm might be vitrifiable internally in the absence of pCPAs even at relatively low cooling rates (~150–250 °C/min [224]), but direct evidence for this speculation is lacking. Others have cooled sperm at high rates without pCPAs and obtained survival, but again have not verified that the survival was due to intracellular vitrification and the prevention of devitrification [226–229], and another report notes ice formation in the sperm tail, midpiece, and neck after rapid freezing [230]. The possibility of intracellular vitrification given cooling rates of up to 7.2×10^5 °C/min [228] seems plausible but has to be compared to the fact that the estimated cooling rate required to vitrify 0.3 M glycerol in an isotonic salt solution, which would have a lower activity of water than isotonic cells including sperm cells, can be estimated from curves

originating from the work of Toner to be on the order of 2×10^6–2×10^7 °C/min or more [15]. In addition, viability was found to be more or less independent of cooling rate, which is consistent with the survival of IIF.

A general problem with attempting to prove that cells can survive vitrification without cryoprotectants is that if cooling is sufficiently rapid, IIF leads to ice crystals that are too small to kill the cell outright, and if warming is sufficiently rapid to preclude recrystallization, these crystals will continue to be innocuous during rewarming, so IIF can be mistaken for vitrification if the main criterion for vitrification is survival [15, 189] (see further discussion and examples in the next section). Although the water content of sperm is limited, the extent to which that water is immobilized by contact with intracellular solutes and structures may not be reflected by the water content per se.

Morris [231] checked human sperm for intracellular ice using freeze-fracture/freeze-etch electron microscopy and freeze substitution after freezing at various rates after previously equilibrating them with ice at –7 °C with and without glycerol pretreatment. He observed no clear evidence for intracellular ice, even after rewarming to –40 °C. The results, though, do not speak directly to the question of whether sperm can be vitrified without pCPAs, because cells pre-equilibrated at –7 °C were also equilibrated, prior to rapid cooling, with either about 20 % glycerol, both extracellularly and intracellularly, or with an osmoticum about 13 times more concentrated (~3.8 Osm) than native sperm contents, and both exposures would dramatically increase resistance to IIF compared to sperm quenched under isotonic conditions [225]. Moreover, sperm frozen to –7 °C and below without glycerol were not viable. In this regard, sperm are no different than muscle: slow freezing of muscle results in vitrification of the unfrozen residual liquid once the water content has been reduced to about 20 % by mass [29], but muscle cells rendered vitrifiable by such extreme dehydration would not be viable.

Morris has estimated intracellular T_h values for isotonic, pCPA-free sperm as a function of intracellular protein concentration, finding $T_h \sim -43$ °C for 30 % protein and $T_h \sim -58$ °C for 60 % protein [225]. However, a T_h of –58 °C would be the equivalent of ~30 % w/w glycerol, which would require a T_m of ~–7 °C, vs. the –0.5 °C characteristic of isotonic cells, and Morris estimates the intracellular protein concentration in real cells at around 20 %. He measured the T_G of a *previously freeze-concentrated solution* of 10 % glycerol + 40 % bovine serum albumin at about –29 °C, but did not (and could not) measure the T_G of the unfrozen solution. For comparison, the T_G of isotonic muscle tissue is well below –100 °C [29] and probably close to –130 °C.

Another footnote to this discussion is that reference to "vitrifying sperm" in the absence of CPAs would normally imply vitrifying

not just the cells but their environment. Proponents of this method, however, are not normally aiming to vitrify the extracellular milieu, so their use of the term "vitrify" is not synonymous with the use of this term for other forms of biological vitrification. If cooling were done sufficiently rapidly to vitrify the extracellular solution, it would presumably also be rapid enough to vitrify cells within the solution as well, but devitrification would still be expected.

The titles of many papers cited in this section assert the achievement of vitrification in the absence of any proof. It is one thing to postulate vitrification but something else to claim it. Luyet made the error of claiming vitrification only to be disappointed when his suppositions were disproven. As a matter of scientific rigor, unsubstantiated claims should in general not be made.

3.2 "Vitrification" into Doubly Unstable Glasses and "One-Way" Vitrification

As noted above, doubly unstable solutions are nucleated homogeneously and therefore require very high warming rates to prevent devitrification, if devitrification can be prevented at all. Warming vitrified samples as fast as possible, such as by immersion in a warm bath at temperatures as high as +50 °C [232] for dilute vitrification solutions of low toxicity, may be hazardous yet still insufficient to prevent devitrification.

However, it has been apparent for some time [189] that strict avoidance of devitrification is not necessary [15, 72], and this has been underscored in recent times by, for example, the successful "vitrification" of cells using solute concentrations as low as 2 M (15 % w/v) propylene glycol plus 0.5 M (17 % w/v) trehalose [233]. This solution has a critical cooling rate on the order of 300,000 °C/min, the limit of the equipment used (unstable vitrification). If we assume that the achievable warming rate is within an order of magnitude of this rate, then considering that, for example, 40 % w/w ethylene glycol, which can be expected to be much more resistant to devitrification, has a critical warming rate of 10^{10} °C/min (Fig. 5), it seems clear that the cells in these experiments survived despite extensive devitrification.

Figure 8 documents the effect of warming rate on the survival of cells that were rapidly cooled under conditions that led to IIF when vitrification solutions were not used and vitrification was not the objective. As can be seen, despite extensive IIF, cells were able to survive in high proportions as long as they were warmed at rates in the vicinity of 1,000 °C/min, which presumably "rescued" these cells from the recrystallization of intracellular and perhaps extracellular [134] ice.

Similarly, survival of devitrification is presumably explained by limitation of the sizes of ice crystals. Homogeneous nucleation will result in very large numbers of very small ice crystals, but below a critical size on the order of 100 [234] to 300 [235] nanometers, intracellular ice crystals have been found to be survivable. Recrystallization of these small ice crystals is rapid and can kill the cell [117, 118] when crystal sizes exceed ~400 nm [235], so pre-

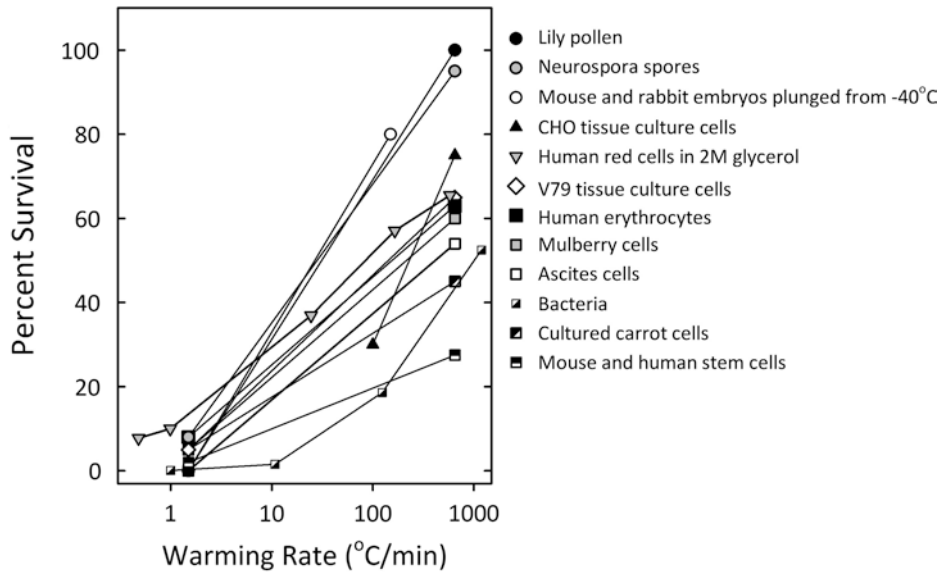

Fig. 8 "Rescue" of a wide variety of intracellularly frozen cells by rapid warming. For original references, *see* [189]. Drawn from tabular data in [189]

sumably the critical warming rate necessary to survive devitrification is equal to the warming rate necessary to avoid fatal recrystallization, which is why it can be orders of magnitude lower than the warming rate necessary to avoid devitrification per se [15, 72, 165, 181]. An interesting benchmark for future research to define is the critical warming rate necessary to survive devitrification, which to date has not been systematically evaluated or defined.

Methods that preserve cells by forming partially crystallized glasses [72, 236] that can further devitrify upon rewarming deserve to be distinguished from methods that achieve and maintain a predominantly amorphous state. "Quench-limited freezing" may be a suitable term for such methods if ice formation on cooling is particularly extensive; "doubly unstable vitrification" [72] or, as suggested here, "unstable vitrification" (Fig. 3) would be descriptive when ice formation on cooling is more subtle. If, in such cases, significant devitrification cannot be avoided, the term "one-way vitrification" has been suggested to describe the fact that the living system or its environment does not remain amorphous during warming [72].

Describing rapid freezing or unstable vitrification methods simply as "vitrification" obscures the true physical nature of the process being employed.

3.3 Carrier Solutions and Cryoprotectants

All cells normally survive in an environment to which they have been adapted. In the mammalian body, this environment has some common characteristics, including, typically, a total osmolality a little less than 300 mOsm (which depresses the freezing point to

about –0.55 °C), a high sodium concentration (~145 mM), a low potassium concentration (~4.5 mM), a high chloride content, a pH in the vicinity of 7.4, and a variety of other electrolytes, proteins, signaling molecules, etc. To survive exposure to low-temperature conditions, this environment must in some form be maintained within viable limits, and that is the function of the "carrier solution," which is the physiological support medium in which cryoprotectants are dissolved. Cells suspended in pCPAs alone would not be able to maintain their volume, pH, ionic content, and membrane integrity, so there must be a basic solution that is maintained around the cells in both the presence and absence of cryoprotectants. The carrier solution in a sense carries the cryoprotectants to and from living cells while allowing them to avoid injury that is unrelated to the cryoprotectants per se. Carrier solution compositions vary widely, but all are designed with this basic supportive role in mind.

Cryoprotectants must generally be added to carrier solutions to enable vitrification. To be an effective pCPA, the agent must be miscible or soluble in water to high concentrations, be of low toxicity, be able to remain in solution even at very low temperatures, and, as noted above, be able to cross the cell membrane [39, 125]. npCPAs have similar requirements but are normally used at much lower concentrations and by definition do not enter cells. More details on the correct use of pCPAs and npCPAs are provided below.

The toxic effects of cryoprotectants have been shown to depend on the choice of the carrier solution [141, 237–239]. Often, the reason for this dependence is unclear, but carriers that do not support cell viability under hypothermic conditions may add extraneous hypothermic injury to any injury that may be due to CPA exposure per se.

As noted above (Subheading 2.5), the carrier solution also plays an important role in limiting ice nucleation and ice growth rates during both cooling and warming. An influence of this effect on survival rates, though, remains to be demonstrated.

A major function of the carrier solution in addition to maintaining viability is to control cell volume. The principles of preparing carrier solutions to accomplish this in the presence and absence of cryoprotectants are described in the next section.

3.4 Osmosis, Osmotic Limits, and Osmotic Protocols

Osmosis is the movement of water from a region of high water concentration or vapor pressure to an area of low water concentration or vapor pressure. The importance of osmosis is particularly high for vitrification protocols because of the comparatively enormous concentrations of cryoprotectants required in comparison to freezing protocols, which necessarily reduce water concentration greatly, and even to close to the limits of compatibility

with life. Cavalier use of both pCPAs and npCPAs without adequate avoidance of osmotic shifts is a frequent preventable cause of injury associated with the use of vitrifiable concentrations of cryoprotectants. More detailed descriptions of osmotic effects during the introduction and removal of cryoprotectants are available in many publications (e.g., [116, 121, 240, 241]), but here we will simply describe the basic phenomena of relevance.

The rate of water movement across the cell membrane depends on the transmembrane difference in water vapor pressure, or osmolality, without regard to the nature of the solutes whose presence generates the transmembrane vapor pressure gradient. For pCPAs, the effect of the CPA on the transmembrane osmotic gradient is transient. Initially, since water moves more rapidly than the CPA, extracellular pCPA raises extracellular osmolality more than intracellular osmolality, and the cell loses water in response. Later, as the pCPA enters the cell down its own transmembrane concentration gradient, water diffuses back into the cell to maintain osmotic equilibrium. This sequence of events is often referred to as the "shrink-swell cycle."

The end result of this process then depends on the carrier solution [121]. When the pCPA concentration is (nominally) the same, per unit liquid volume, on both sides of the membrane, the cell will have returned to its original volume provided the osmotic effect of the carrier solution, which was equal to the osmotic effect of intracellular molecules before CPA addition, is also the same, per unit solution volume, as it was prior to pCPA addition. Although cells contain proteins and many other complex molecules whose osmotic coefficient might be expected to differ from the osmotic coefficient of the carrier solution, in practice, this difference is small, and the cell can reasonably be modeled, to a first approximation, as a dilute salt solution having the osmolality of plasma (~285 mOsm) [117, 121, 220]. If the osmotic coefficient of the carrier is about the same as the osmotic coefficient of cytosolic solutes, then for the cell to return to its original volume, the carrier must have the same concentration per unit volume of extracellular solution as it did prior to the addition of the cryoprotectant [121].

In other words, the correct way to compose a cryoprotectant solution, if the goal is no volume change after pCPA equilibration, is to add all the carrier solutes needed for a given volume, add all the CPAs needed for the same given volume, and then bring the solution to the final desired volume by the addition of water. This method essentially replaces water, volume for volume, with pCPA, such that the molar concentration of carrier solution solutes is unchanged.

This is important to note for three reasons. The first reason is that making vitrification solutions on a % w/w basis, which may be

meaningful for physical reasons, is not meaningful for biological purposes, because carrier solution and other impermeant solutes included on this basis are not readily maintained at a molar concentration that can be evaluated with respect to the effect of the solution on the volumes of cells that may be placed into it. A procedure for preparing solutions on a % w/w basis while still maintaining isotonicity has been described [196] and should be employed, with suitable modifications if a defined tonicity other than isotonic is desired. The procedure to maintain isotonicity in the presence of X% w/w pCPA is: (a) prepare a concentrate of the carrier solution, such as dissolving the nonaqueous components of 1 L of carrier in water so as to obtain a final total volume of 200 ml (this would be a 5× carrier concentrate); (b) weigh the carrier solution concentrate; (c) add a weight of pCPA equal to the weight of the carrier times $(X/(100-X))$ (thus creating a solution that has the correct % w/w concentration of pCPA); and (d) q.s. to 1 L with X% w/w pCPA in water (thus creating an isotonic solution with the correct final % w/w).

The second reason to understand the principles of isotonicity is that it is common for cell freezing labs to add a pure cryoprotectant to a cell culture medium prior to freezing, which dilutes the culture medium. Since cell freezing often employs, for example, 10 % v/v Me_2SO, the error is not very significant in that case. But when composing a much more concentrated vitrification solution, diluting the carrier by, for example, 50 % would require intracellular solutes to be diluted to the same extent to maintain osmotic equilibrium at the end of pCPA equilibration, which means a doubling of cell liquid space [121]. Not only might this be harmful for the cell, but it will also dilute the intracellular solutes that also contribute to vitrification tendency of the cytoplasm [83, 167], making the cell at greater risk of IIF during both cooling and subsequent warming. In fact, it seems desirable to vitrify cells when they are still in the shrinkage phase of the shrink-swell cycle rather than after complete pCPA equilibrium, in part to reduce exposure time to the pCPA and in part because it is at that time that the cytoplasm is the most concentrated [49, 83, 167], but this strategy will not be completely efficient if the carrier solution has been diluted during the shrinkage phase. See the next section for a special case in which deliberate hypotonicity during vitrification solution loading can be used to actually expedite CPA equilibration.

The third reason to understand how to adjust tonicity in the presence of CPAs is that chilling injury and its avoidance or minimization depend on the effective tonicity of the vitrification solution [18]. More details about this topic are given below.

When the pCPA concentration is diluted, extracellular water at first rushes into the cell down its concentration gradient, causing the cell to swell. Over time, as the pCPA in the cell diffuses to

the extracellular space, taking water with it to maintain equilibrium, the cell volume begins to return back toward normal. Eventually, if the extracellular carrier solution is isotonic (unchanged in molarity from its molarity in the absence of pCPA), the cell will in principle return to its original volume. This "swell-shrink" sequence is the reverse of the "shrink-swell" behavior observed during pCPA administration.

Although cells can be damaged by either excessive shrinkage or excessive swelling, cells can generally withstand about a fourfold increase in extracellular osmolality but only around a twofold decrease (the "osmotic limits" of the cell) [241]. This means that a given fold change in extracellular concentration is more hazardous during the removal of pCPA than during its introduction. To offset cell swelling during pCPA removal, a non-penetrating agent such as mannitol, sucrose, or trehalose can be included in the carrier solution to increase the effective osmolality of the extracellular medium and thus offset, to some extent, the decrease in effective osmolality caused by dilution of the pCPA, thus limiting the extent of swelling [241]. Used in this way, extracellular agents are sometimes referred to as "osmotic buffers."

At equilibrium, the relationship between the volume of intracellular water and the *net* extracellular osmolality, Π, is given by the Boyle-van't Hoff equation [242], which, under isothermal conditions, can be written as

$$\mathrm{Vc} = b + V_o \Pi_o / \Pi,$$

where Vc is the cell volume, V_o is the volume of intracellular water under normal (isotonic) conditions, b is the osmotically inactive volume of the cell, and Π_o is the isotonic extracellular osmolality. This equation says that the volume of a cell is a linear function of the reciprocal of the effective extracellular osmolality and that when the extracellular osmolality approaches infinity, the volume of the cell approaches its non-osmotic (usually its dry) volume, b.

Since the volume of intracellular water, V_w, is given by $V_w = \mathrm{Vc} - b$, we can rewrite the equation as

$$V_w / V_o = \Pi_o / \Pi,$$

which shows that the volume of intracellular water changes in inverse proportion to the extracellular osmolality. For example, if the extracellular osmolality decreases by a factor of 2, the cell water content will double, and if the extracellular osmolality increases by a factor of 2, the cell water content will be halved. This rough rule of thumb is handy for estimating acceptable changes in concentration, assuming b is relatively small.

When pCPAs are added in steps, each step contributes to the value of b, because as successive steps of concentration are added, previously added intracellular pCPA cannot leave the cell, since intracellular concentrations can never exceed extracellular concentrations.

As b increases to a maximum following each step of pCPA addition, the redefined volume of intracellular water under isotonic conditions (in the presence of the pCPA) correspondingly decreases ($V_o = Vc - b$). When the next step of pCPA addition takes place, there is less intracellular water to extract, so the osmotic consequence of a given step change in pCPA concentration is reduced even if the fold change in water content is the same. This enables the step size to be safely increased as pCPA administration proceeds. In addition, since, from the above expression, V_w responds to the fold change in effective extracellular osmolality and not to the absolute change, the concentration of pCPA can be incremented exponentially rather than linearly. This procedure, by speeding pCPA addition and thus reducing total exposure time to the cryoprotectant, can actually result in less toxicity [105].

During washout, intracellular water content is again responsive to the fold change in extracellular osmolality, so washout can proceed exponentially as well, but the b value decreases rather than increasing with time as intracellular pCPA is subtracted, which is one reason the hazard of excessive cell swelling increases as the concentration approaches zero. For this reason, including an osmotic buffer throughout the pCPA washout process, including the step when the pCPA concentration reaches zero, is generally recommended.

A non-penetrating solute, unlike a pCPA, cannot be added without lowering cell volume relative to what it would otherwise have been. If the pCPA that accompanies an npCPA happens to reach transmembrane concentration equilibrium (which it will generally not), the npCPA will have just as much of an osmotic effect on the cell in the presence of the pCPA as it would have had in the absence of the pCPA, even if the npCPA contributes a very small fraction of the total extracellular osmolality, since the effects of intracellular and extracellular pCPA cancel each other out. Also, under the influence of the npCPA, pCPAs behave like water: any decrease in cell volume caused by the npCPA will concentrate the intracellular pCPA, causing efflux to the more dilute extracellular pCPA pool and thus a reduction in that part of the b value that is contributed by the pCPA.

With the above concepts in mind, it is possible to explore unusual osmotic protocols that are intended to enable more rapid cryoprotectant addition and removal without exceeding but while taking advantage of the osmotic limits of the cell. For example, in a scheme by Meryman [241], a cell is exposed to a concentration of pCPA that brings the cell transiently to its hypertonic osmotic limit (about four times isotonic) but contains a carrier solution that is only half as concentrated as an isotonic solution. After equilibration, the cell swells to twice its isotonic volume (neglecting b), which then allows the osmotic concentration of the medium to be raised by a factor of 8 in the next step rather than 4: an

increase of twofold would merely bring the cell back to its isotonic volume, so an increase of fourfold in addition to that is needed to once again bring the cell to its hypertonic osmotic limit (again, neglecting the b value of the cell, which would allow the fold change to be even greater). Upon washout, the cell is placed into a solution containing a four times isotonic carrier plus whatever pCPA is needed to avoid the hypotonic transient osmotic limit of the cell during swelling, after which the cell comes to its hypertonic osmotic limit prior to the next dilution step. The next dilution step can then reduce total extracellular osmolality by a factor of 8. In principle, the steps involved in this scheme could be even greater, even beyond accounting for the b value, because in practice significant permeation of the pCPA will usually take place during the shrinkage or swelling phases of the process, thus limiting volume extremes.

Although the above guidelines are helpful for determining the final equilibrium state after concentration changes and boundary conditions on volume excursions during transient shrinking or swelling, the design of a cryoprotectant addition and removal protocol also generally requires some knowledge of the permeability of the system to the cryoprotectants employed. The timing, magnitude, and temperature of concentration steps can be considerably improved by using computer modeling of the shrink-swell and swell-shrink processes under a variety of virtual conditions (e.g., [243–246]), and this approach is recommended whenever possible. When this is not possible, our experience has been that a protocol in which pCPA concentration is doubled on every addition step and halved on every dilution step is effective in avoiding osmotic injury during the preparation of rabbit renal cortical slices [190].

Clever computer-modeled schemes have been proposed to exploit known permeation rates and the osmotic limits of the cell so as to maximally accelerate CPA addition and removal [247, 248], though most have not been tried. One particularly interesting and innovative current scheme that also factors in toxicity minimization is described in the next section.

The problem of introducing and removing cryoprotectants is more difficult when the pCPA and npCPA must be administered by perfusion. In a kidney, for example, exponential addition of pCPA is counterproductive because lags in distribution of the CPA to the medulla result in little benefit of the exponential addition rate, whereas accelerating exposure of the renal cortex to the CPA and then holding the cortex at the highest concentrations long enough for the medulla to “catch up” can be lethal [17, 194]. As a general rule, the osmotic protocol for each system should be tailored to the specific needs and characteristics of that system.

3.5 Procedures for Avoiding Cryoprotectant Toxicity

The central problem of vitrification has always been inducing living cells to tolerate enormous concentrations of CPAs and low concentrations of water. In 1984, Fahy et al. suggested seven approaches to controlling cryoprotectant toxicity in vitrification procedures, i.e., avoid osmotic injury; employ cryoprotectant mixtures so their mutual dilution minimizes specific sources of toxicity; use one or more npCPAs to allow reduction in the intracellular pCPA concentration (see next section for more details); maintain temperature as low as possible; select an appropriate carrier solution; keep exposure time to the CPA to a minimum; and, when possible, employ cryoprotectant toxicity neutralization (CTN; *see* Subheading 3.7 below).

Implicit in this list at the time was also the very first step in designing a vitrification solution, and that is to determine exactly how much CPA is needed (C_V). Extensive lists of solutions that are, within 1 % w/v total concentration, exactly sufficient in concentration to avoid visible ice crystals (i.e., that are at their C_V) on cooling at about 10 °C/min have been published (e.g., [70, 83, 127]), and means of interpolating between known solutions to estimate the C_Vs of arbitrary mixtures of CPAs have also been described (e.g., [143, 190]). However, some investigators determine C_V only to the nearest 5 %, potentially exposing their cells to as much as 4 % w/v or v/v (or w/w) more CPAs than needed, which may have strong [249] and unnecessary effects on the total toxicity observed. C_V is a function of solution composition, cooling rate, and applied pressure [70, 97, 99, 127, 191, 250], so it must frequently be redetermined for new circumstances.

These methods have been used successfully, but since 1984, five additional approaches have been added: use ice blockers to reduce the overall quantity of pCPA required [43, 44], use methoxylated CPAs in moderation [251], employ creative addition and washout methods to minimize the overall "cost function" of toxicity for the solution in question when the system is a cell suspension or simple tissue [252, 253], preferentially employ weak glass formers [203], and use a combination of constant pressure and constant flow methods when CPA must be introduced by perfusion so as to speed equilibration of poorly circulated tissues [194]. Organ perfusion techniques are still being demonstrated, so further elaboration will be provided here only on the use of ice blockers, methoxylated CPAs, cost function optimization, and preferential use of weak glass formers. The latter is further discussed from a theoretical point of view in Subheading 3.8 below.

As noted above, ice blockers interact with, and inhibit, extracellular ice, but they do not have access to the intracellular space. This inaccessibility is immaterial, however, because cells do not appear to contain heterogeneous nucleating agents of any significant effectiveness. Evidence for this conclusion comes from a

variety of observations (*see*, e.g., Table 1 and accompanying discussion in [34]), but the most direct have been experiments in which single cells were contained in the type of emulsions used to detect homogeneous nucleation and were found to have minimal nucleating activity in this system [171, 254]. The fact remains, however, that relying on the use of excessive extracellular ice growth inhibition, whether by adding ice blockers or by adding viscosity-enhancing agents, runs the theoretical risk of making the extracellular medium more resistant to ice nucleation than the cytoplasm and thus potentially deceptive in predicting the fate of cells cooled in it.

Replacing hydroxyl groups on OH-bearing pCPAs with methoxy groups has two favorable effects: glass-forming tendency is greatly improved and membrane permeability is greatly increased [251]. There are, however, two counterbalancing negative effects: increasing the glass-forming tendency of pCPAs generally increases toxicity in the context of vitrification (*see* Subheading 3.8), and increased membrane permeability is due to increased hydrophobicity, which also tends to make pCPAs more toxic [255]. However, with at least one methoxylated pCPA, "methoxy-glycerol" (3-methoxy-1,2-propanediol), a net positive effect can be obtained [18]. Methoxy groups are more effective for vitrification not entirely because of their superior ability to hydrogen bond with water but also because interactions between the methoxy groups are impossible, since these agents present only the lone pair electrons of their oxygen atoms to the solution for bonding, which do not hydrogen bond to one another, and therefore these oxygens are more free to interact with water and therefore are not "wasted." Hydroxyl groups, on the other hand, can hydrogen bond to each other, and these self-associations reduce interaction with water, thus requiring more pCPA to induce vitrification. In 3-methoxy-1,2-propanediol, two hydroxyls are present to maintain reasonable hydrophilicity, helping to offset the hydrophobic effect of the methoxy group. This modality for toxicity control appears effective [18] but has been little investigated to date.

Regarding cost function minimization [252], the toxicity of a cryoprotectant is a function of its concentration in the cell and its time of exposure. Both aspects of toxicity can be minimized by adding cryoprotectant while the cell is maintained at its hypotonic volume limit: this increases the gradient for pCPA uptake while keeping the intracellular concentration as low as possible. The intracellular concentration is then abruptly raised to the target value by osmotically shrinking the cell, which is a rapid process since water crosses the cell membrane very quickly. To remove the pCPA, the cell is again immediately brought to its hypotonic volume limit, which immediately dilutes the intracellular pCPA, and the cell is maintained at this hypotonic limit while pCPA concentrations are lowered, after which the cell is restored to its desired

volume, which transiently increases the lingering intracellular pCPA concentration, thus speeding its exit from the cell, while not raising its concentration to a toxic level. This approach has yielded positive results with oocytes, which were able to be loaded with Me_2SO in a remarkably short time yet recover very well [253]. Applicability to other systems remains to be explored.

Both empirically and theoretically, pCPAs that interact most weakly with water are generally the ones that are the least damaging when included in a vitrification solution (*see* also Subheading 3.8). The principle of favoring weak glass formers for the formulation of vitrification solutions (VSs) has enabled the development of less toxic VSs [18, 203], but there are limits on its application. For example, the weak glass former, ethylene glycol, may have specific toxic effects at higher concentrations [203] or may be required in such high concentrations as to begin to deplete water to the point where water becomes inadequately available despite weak interactions with the pCPA. Glycerol, which is another weak glass former, is frequently too impermeable, too viscous, or too toxic for other reasons to be used in concentrations that might be more ideal in theory. Despite such limitations, however, favoring weak glass formers has, in combination with the other modalities discussed above, including CTN, the use of 3-methoxy-1,2-propanediol, ice blockers, and the use of another npCPA, enabled the creation of M22, a 9.4 M solution whose critical cooling and warming rates are extremely low (~0.1 °C/min and 0.4 °C/min, respectively) 14, yet which can be perfused through a kidney with only transient dysfunction after transplantation [18].

Despite these advances, there is still both an academic and an applied need to better understand CPA toxicity so as to enable still more effective countermeasures to it. This topic is discussed in Subheading 3.8 below.

3.6 Extracellular Agents in Vitrification

As noted in Subheading 3.1, living cells contain significant amounts of protein, metabolic intermediates, nucleic acids, and other solutes. These solutes might, when water content is significantly reduced by the use of pCPAs, contribute more to glass-forming tendency of the cytosol than the carrier solution contributes to the stability of the extracellular solution. In this case, the use of sufficient pCPA to ensure vitrification of the extracellular solution would actually require more pCPA than needed to vitrify the intracellular solutions and would therefore be more toxic than necessary. To correct this problem, it was suggested in 1981 [99] and reported in 1982 [100] that extracellular pCPA concentrations could be reduced by using npCPAs as counterparts to intracellular solutes, thus maintaining the vitrification tendency of the vitrification solution with less pCPA and still allowing cells in contact with this

solution to vitrify. This was later documented photographically, and many examples of the use of npCPAs were presented [83].

npCPAs, in addition to facilitating vitrification, also tend to increase the viscosity and the effective tonicity of the solution. The effect on viscosity is beneficial for vitrification but may become counterproductive when pCPA diffusion rates need to be maximized or when pCPAs have to be introduced by perfusion [17]. The effect on tonicity may be limiting if the living system to be preserved is sensitive to chilling injury and chilling injury is a function of the tonicity of the medium [18] (*see* Subheading 3.9 below). When neither of these drawbacks intervenes, in principle, the amount of npCPA that can be used is limited only by the upper osmotic limit of the cell in question, the extent to which pCPA levels can be reduced without compromising the vitrification tendency of the VS, and the extent to which the npCPA may induce specific toxic effects [206, 256] (see also below). Although npCPAs do not directly perturb cytosolic proteins, interactions with the external leaflet of the plasma membrane and with integral membrane proteins that communicate with the cytoplasm are still possible. In principle, the amount of npCPA that can be used might be increased by reducing carrier solution solutes in favor of more npCPAs should cell shrinkage be the limiting factor.

By increasing npCPA concentration, cell volume will be reduced and intracellular solutes will become more concentrated, thus further stabilizing the cell against ice formation. In retrospect, in fact, cell shrinkage may be a primary mechanism of action of npCPAs in vitrification, since, as noted above, the effect of the carrier solution on ice nucleation extracellularly may not be greatly different than the effect of the intracellular solutes intracellularly. In addition, because the cell is shrunken, the absolute amount of pCPA inside the cell will also be reduced, and this means that, after vitrification, the cell can be diluted more abruptly and more extensively before the hypotonic osmotic limit of the cell will be reached (*see* Subheading 3.4).

A study by Shaw et al. [257] brought out many important facets to the use of npCPA that should be kept in mind. Excessive amounts of high-molecular-mass npCPAs tend to raise T_m and increase the total solute concentration needed to vitrify (both potentially adverse effects) while having minimal effects on T_G. However, T_D (the temperature of devitrification) tended to be raised, which is beneficial.

Kuleshova et al. studied sugars as npCPAs for vitrification of oocytes and embryos [258]. Glucose, fructose, and sorbitol were equal in effectiveness to ethylene glycol, mole for mole, in supporting vitrification and raised T_G. Trehalose, sucrose, and raffinose, being larger molecules, required an increase in total solute concentration, when measured on a weight percent basis, to replace EG

mole for mole, thus depleting the solution of extra water, but also raised T_G, raffinose being particularly active in the latter respect.

3.7 Toxicity Neutralization

There are cases in which the addition of a nontoxic concentration of one cryoprotectant to a toxic concentration of another can neutralize the toxicity of the latter [259, 260], while the then-acceptable presence of the latter can then allow the former to be maintained at a nontoxic concentration and still allow the solution to vitrify [18, 190, 203, 206, 249]. The same principle has also allowed improved recovery after freezing and thawing [104].

Cryoprotectant toxicity neutralization (CTN) is thus far restricted to the neutralization of amide toxicity by Me_2SO, and even for amides, the effect is not universal [260]. CTN is strong for formamide and urea, weak for acetamide and *N*-methylformamide, and nonexistent for dimethylformamide and *N*-methylacetamide [260].

The mechanisms that underlie these effects are presently unknown. The idea that a "compatible solute" effect might be involved analogous to the protection of proteins against urea by solutes in nature that have a protein-stabilizing effect sufficient to offset the protein-denaturing effect of urea [190, 261–264] was not experimentally supported [249]. Pursuant to the original suggestion [265] that CTN may involve physical interaction between amides and Me_2SO, it was determined that the magnitude of the exothermic heats of mixing when Me_2SO is mixed with formamide, ethylene glycol, and propylene glycol has the same rank order as the viability of kidney slices exposed to mixtures of Me_2SO with these same solutes (formamide > ethylene glycol > propylene glycol), and the heat of mixing between Me_2SO and *N*-methylformamide, whose toxicity is only marginally neutralized by Me_2SO [260], was minimal [190]. On the other hand, when similar experiments were done in the presence of water, the affinity between Me_2SO and water is so strong that the interaction between Me_2SO and formamide in aqueous solution is "thermochemically repulsive" (endothermic) [249]. Still, there remains a correlation between the effectiveness of CTN for a given amide and its strength of interaction with Me_2SO in aqueous solutions: as the interaction becomes more thermochemically repulsive, CTN becomes weaker and disappears (cf. [249] and [260]).

Despite the current lack of information about the molecular basis of CTN, a specific molecular example of CTN is available that may be instructive in future studies. The inactivation of membrane Na^+,K^+-ATPase by urea is blocked by Me_2SO [266].

Although the combination of amides and Me_2SO was originally proposed to neutralize the toxic effects of Me_2SO [249, 265], this effect has not been observed subsequently [249, 267]. Although there is no known toxicity neutralizer that can protect against Me_2SO toxicity at this time, acetylcholinesterase inhibition

by Me_2SO can be physiologically blocked by atropine [268], some oxidative effects of Me_2SO can be reversed with reducing agents [259, 260], and there is one still-unconfirmed report that glucose can prevent irreversible binding of Me_2SO to proteins and also reduce its toxicity [237].

So far, few systems have been evaluated for their ability to benefit from CTN. CTN is known to apply to rabbit renal cortical, liver, and brain slices and to murine osteoblasts [206] and seems very likely to pertain as well to rat liver [207], renal cortical and medullary [269], and brain [205] slices, and amide + Me_2SO mixtures have also been successfully applied to other systems ([16, 140], and unpublished results). The only tested systems that so far do not seem to benefit from CTN are human oocytes and early stage embryos (S.F. Mullen, unpublished results).

3.8 Specific and Nonspecific Biochemical Effects of Cryoprotectants

Ironically, the central problem of vitrification, cryoprotectant toxicity, remains, in 2014, practically unexplored mechanistically. This attests to the decades-long lack of interest in cryobiological problems that has prevailed on the part of biochemists, with very rare exceptions (most notably, [265]). Criteria proposed 24 years ago now [249] for showing that a given biochemical modification is an important cause of observed cryoprotectant toxicity under practical cryobiological conditions have rarely, if ever, been met. Fortunately, however, this picture is finally beginning to change.

Examples of cryoprotectant metabolism in vivo or in vitro at elevated temperatures, such as the phosphorylation of glycerol leading to ATP depletion [270, 271] or the transformation of ethylene glycol into toxic by-products [272, 273], have no demonstrated relevance to events at the lower temperatures at which pCPAs are usually administered or in tissues or cells whose metabolic activities are different than those studied. Me_2SO, which has a largely undeserved reputation as being particularly toxic and has numerous pharmacological effects at body temperature [274] and can chemically react with tissue sulfhydryl groups [275], understandably has no demonstrated pharmacological effects at 0 °C, at which temperature its reaction with sulfhydryl groups may be too slow to be meaningful for cellular viability [206, 259]. It has been shown to react with steroids and triterpenoids [276], but never to do so under biologically meaningful conditions. As summarized elsewhere [249] (references given therein), pCPAs have been shown to elevate membrane phase transition temperatures; rearrange the cytoskeleton, including most significantly the meiotic spindle; cause membrane blistering; fuse cell membranes; change gene expression; alter RNA polymerase; weaken DNA-nucleosome binding; destabilize nucleic acid duplexes; impair ribosome assembly; and induce many other adverse changes, but for the most part, the relevance of these observed changes, if any, to most cells being prepared for cryopreservation is currently unknown. There seems

to be little or no generalized effect of vitrification solutions on passive membrane permeability to sodium and potassium under practical conditions [190]. Some pCPAs can induce differentiation of leukemia cells [277] and can change their chromatin and DNA conformation [278], but these are not what would normally be considered toxic effects. In summary, it seems that the literature on the biochemical effects of cryoprotectants and the literature on the toxic effects of CPAs in cryobiological applications are mostly disconnected from one another.

A proposed protein-altering mechanism of Me_2SO toxicity involving specific interaction between Me_2SO and protein surface lysine residues [265] has not been supported by subsequent investigations [249, 259] and is not favored by the general observation that small molecules, including Me_2SO [279], tend to be preferentially excluded from the hydration layer surrounding protein surfaces, thus stabilizing them against denaturation [263, 280–282] and even enhancing renaturation after previous denaturation [283, 284]. Mixtures of pCPAs in vitrification solutions intended for use at high hydrostatic pressures did show increased protein destabilization tendencies, but these effects were correlated *inversely* with toxicity [249]. In addition, the toxic effects of individual commonly used pCPAs do not appear to be accounted for by their general protein denaturation tendency [249, 260, 279, 285, 286] or by their ability to increase the permeability of membranes when used below 15 °C [267], and Arakawa et al. have argued that disruption of the hydration layer surrounding proteins and membranes by pCPAs may account for pCPA toxicity at high temperatures but not at low temperatures [286].

Nevertheless, protein denaturation has not been ruled out in ambient pressure vitrification solutions composed of mixtures of pCPAs, which is the most common type of VS in use today. Even though each individual pCPA in the VS mixture may be at a nominally non-denaturing concentration, the sum total of all of the pCPAs might still have a cumulative denaturing effect, especially when cold destabilization of proteins is also factored in, and some evidence indicates that pCPAs do not protect effectively against cold denaturation [287]. There is now, in fact, some evidence for the possibility that high-concentration VSs might induce protein denaturation under low-temperature exposure conditions. This evidence has been derived in two independent ways.

The first line of evidence is indirect and arises from the finding that the toxicity of a large number of VSs can be correlated strongly with a compositional variable, qv*****, which defines the number of moles of water associated, on average, with each water-bonding polar group on the pCPAs of a VS when that solution is exactly at its C_V [203]. The evidence indicates that pCPAs that interact

strongly with water [288], such as 1,2-propanediol, are more toxic precisely because they interact strongly with water, presumably competing with biological molecules for access to water, whereas more weakly bonding solutes, such as ethylene glycol, are required in higher concentrations to vitrify water because of their weaker interaction with water, yet leave the water that remains more free to hydrate biomolecules despite a lower overall concentration of water in the solution [203]. It is unclear whether this mechanism of nonspecific toxicity arises as a result of protein denaturation or some other water-dependent effect(s), but it is compatible with a role for protein denaturation in the manifestation of toxicity.

The second line of evidence is more direct and is based on a recent microarray analysis of alterations in transcription following exposure of rat liver slices to two candidate (8.8 and 8.9 M) VSs [207]. It was published as part of an analysis of chilling injury (see next section), and so full results were not provided, but the results that were presented are illuminating.

Although neither VS reduced slice ATP, 1985 transcripts were changed, of which 92 increased at least 1.5-fold and 49 decreased at least 1.5-fold. The VSs increased transcripts for 11 heat shock genes, and one particular Hsp70 family member, Hspa1b, was elevated 12.6-fold, although transcription of Dnajc12 (an Hsp40 homolog) was slightly decreased. Also consistent with the loss of some proteins to denaturation, eight genes associated with ribosome biogenesis were induced as well, especially the 5S rRNA gene, whose transcripts increased 4.5-fold. VS exposure decreased transcription for genes in the p38 signaling pathway about 25 % and depressed TGFβ-1 and TGFβ-3 transcripts, which normally lead to stimulation of the p38 pathway. In contrast, the VSs greatly increased transcription related to the ERK and especially to the JNK pathway, a third finding consistent with the possibility of protein denaturation. Though not clearly related to denaturation, it was also of note that expression of Hmox-1 [heme oxygenase (decycling) 1], which functions primarily as a major defense against oxidative stress and injury [289], was decreased by 1.9-fold.

It is important to point out that, even if protein denaturation is involved in the mechanism of VS toxicity, the proteins undergoing denaturation may be a small subset of the total [190]. Cryoprotectants decrease the solubility of tyrosine, leucine, alanine, cystine, and glycine [190, 279], which should have a generally inhibitory effect on denaturation, although 20 % Me_2SO increases the solubility of tryptophan by about 40 % [279]. Different proteins respond differently to cryoprotectants [255, 290], suggesting that the most susceptible proteins may be selectively involved in toxic responses. More globally, and dramatically, closely related tissues as a whole also react differently to cryoprotectants despite the fact that the same basic

"housekeeping" enzymes are essentially common to all cells. For example, guinea pig uteri [291] and intestinal smooth muscle [91] can tolerate the levels of Me_2SO required for metastable cooling to dry ice temperature without freezing, whereas rabbit renal cortex cannot [292]. Similarly, frog hearts [87, 88] but not rat hearts [89] can tolerate the 10–11 M level of ethylene glycol required for the same purpose.

At least two additional biochemical studies on the nature of cryoprotectant toxicity are currently underway, and a third is being planned. In addition, efforts are being made to develop better methods for measuring protein denaturation on a global basis to enable direct examination of the role of protein denaturation in both cryoprotectant toxicity and chilling injury. The potential contributions of molecular biology to cryobiology could be revolutionary and have been long awaited.

3.9 Chilling Injury and Its Modification or Avoidance

Chilling injury is observed both in nature, at temperatures above 0 °C [293–295], and in the laboratory, at temperatures well below zero [18, 52, 53, 207]. It has been linked to phase changes in membranes [67, 68, 296–299] and associated defects in membrane permeability [298, 300, 301] and can be blocked in some cases by directly modifying cell membrane composition [68, 302, 303], by using antifreeze proteins [300, 304], or by using genetic engineering [305, 306] to block these phase transitions and inhibit membrane leakage. Chilling injury may also result from protein denaturation based on protection by prior heat shock [307], the production of heat shock proteins in response to chilling [307], and the production of "cold shock proteins" in response to chilling as well [308, 309]. Also suggestive is the observation that the unfolded protein response (ER stress response) is induced by 18 h of cold storage of human corneal endothelial cells and inhibited using the specific blocking agent, salubrinal [310]. However, chilling injury is generally observed to be an immediate response rather than a delayed response to temperature reduction [18, 52, 53].

In none of the above mechanistic and interventional studies was the system of interest saturated with a multi-molar concentration of cryoprotectant before the induction of chilling injury. Further, chilling injury associated with vitrification is typically observed primarily or exclusively at temperatures far below those of the lipid phase transition temperatures and denaturation phenomena noted above [18, 52, 53, 128, 207].

Nevertheless, one DNA microarray study of chilling injury in the context of vitrification has recently been done, and it verified changes suggestive of the ER stress/unfolded protein response and altered lipid metabolism [207]. Precision-cut rat liver slices loaded with either of two vitrification solutions showed no drop in ATP content compared to controls when held at 0 °C, but a 20–30 % drop after cooling without freezing to –15 °C for 10 min

(as detected after incubation at 37 °C following CPA washout). Principal component analysis indicated clear separation between the effects of CPA administration and the effects of chilling in the presence of CPA. Comparing CPA treatment to CPA treatment plus chilling, 1,108 transcripts changed in abundance with chilling, but of these, only 31 increased more than 1.5-fold and only 6 decreased more than 1.5-fold, so the changes observed were in general mild, in keeping with the mild change in ATP content, and likely were indicative of the first changes induced by chilling. The primary observations were an increase in heat shock protein and heat shock factor transcripts, an increase in ribosomal RNA transcripts (which would favor more protein synthesis to replace denatured proteins), a lack of activation of apoptotic pathways (suggesting ER stress did not reach levels sufficient to induce apoptosis), activation of DNA damage sensing genes, activation of two of the three MAP kinase stress pathways (involving increased JNK and ERK signaling without increased p38 signaling), changes that tend to reduce cholesterol synthesis and remove it from the cell membrane and transfer it to the endoplasmic reticulum, and, rather paradoxically, changes that tend to reduce synthesis of polyunsaturated fatty acids and increase synthesis of saturated fatty acids as well as reduce fatty acid oxidation and metabolism. A number of other changes in expression were seen whose significance is less easy to interpret. In summary, the effects of chilling and the effects of VS exposure were remarkably similar, as though chilling injury were an extension of VS toxicity.

Pig oocytes [311] and embryos [312] are particularly sensitive to chilling injury in part as a consequence of containing globules of cytoplasmic fat that cause damage to the cell membrane on cooling. This problem has been reduced by various lipid removal or segregation techniques [311, 313], although birth rates have tended to be low despite adding additional interventions [311, 314, 315].

Oocytes are in general quite susceptible to cooling injury, and much of this susceptibility is related to disassembly of the meiotic spindle and subsequent abnormal or incomplete reassembly [316, 317]. Cryoprotectants can stabilize [318] but can also damage [319] the spindle. Nevertheless, with proper methodology, oocytes can be preserved without significant spindle damage [319].

The vitrification of oocytes is motivated in no small part by the utility of vitrification for "outrunning" chilling injury [54]. The successful cryopreservation of *Drosophila* embryos was also enabled by the ability of vitrification to allow rapid chilling injury to be "outrun" in this species as well [52, 53, 128].

The demonstrated methods mentioned above for altering chilling injury (consisting of modifying plasma membrane composition or using antifreeze proteins to prevent membrane leakage) are generally inconvenient or impractical for many applications and may not be pertinent to chilling injury below 0 °C. McGrath [320], in

non-cryoprotected systems, and Fahy et al. [18, 321], in cryoprotected systems, showed that chilling injury can be reduced or prevented by an increase in medium tonicity. In the latter case, the optimum tonicity for avoiding chilling injury during vitrification was found to be between about 1.3 and 1.5 times isotonic, whereas for porcine embryos a tonicity of ~2.8 times isotonic was effective [320].

Chilling injury is not universally observed in systems prepared for vitrification [206]. It is seen in rabbit but not rat renal cortical slices, is seen in rabbit and rat liver slices but not in monkey liver slices, and may be absent in rat or rabbit hippocampal slices. Comparing susceptible and non-susceptible tissues of the same type might offer another way of understanding chilling injury and seeking new mitigation strategies for it.

3.10 Storage in the Vitreous or Near-Vitreous State

Little is known about the safety of various durations of storage in the vitreous state at temperatures in the vicinity of T_G, but this is an important topic for several reasons. First, the risk of fracture formation increases as vitreous samples are cooled to the temperature of liquid nitrogen (*see* Subheading 2.7 above) and fractures may damage organs, tissues, oocytes, embryos, and other systems as well as creating sites of ice nucleation [147] that may indirectly damage vitrified cells during warming. Second, liquid nitrogen immersion has a number of practical, safety, and potential contamination issues that could be avoided by storing in the vapor phase if this were known to be safe. Therefore, one would like to know how far below T_G a sample must be cooled to protect it for long-term storage and to verify that this temperature is still warm enough to minimize the risk of fracturing.

Song et al. [322] reported that vitrified rabbit jugular veins (T_G ~ −123 °C) stored at −130 °C for 4 weeks or for 4 months or stored below −160 °C in liquid nitrogen vapor for either 4 weeks or for 4 months all recovered as well as veins stored for only 24 h and approached the functionality of fresh controls. Heart valves and cartilage yielded similar results [322]. Our laboratory has stored rabbit hippocampal slices under isothermal conditions in the vicinity of −145 °C (T_G ~ −124 °C) for months as well and has seen no deterioration [206]. In Song et al.'s experience, there was no visual development of ice during storage, and freeze substitution showed no ice development after 5 months of storage in liquid nitrogen vapor [322].

Fahy and Rall [15] and Mullen and Fahy [72] reported calculated safe storage times based on the assumption that the rate of biological deterioration is governed by the mobility of molecules near T_G and therefore by the viscosity of the cryoprotectant solution. For 40 % w/v DAP_{10} + 6 % PEG 6000 in an RPS-2 carrier solution (40 % DAP_{10} = 10 % w/v 1,2-propanediol plus 30 % w/v of a mixture of Me_2SO and acetamide in a 1:1 mol ratio; T_G assumed to be about −122 °C), the amount of diffusion equivalent to 1 min at −20 °C was found to require 1 week at −88 °C, 1 month

at –93 °C, 1 year at –100 °C, 10 years at –106 °C, and 10,000 years at –116 °C [15]. For the intensively studied and used M22 vitrification solution (T_G ~ –123 °C [18]), diffusion requiring 10 s at 0 °C was found to require 1 week at ~–93 °C, 1 month at ~–97 °C, 1 year at ~–103 °C, 10 years at ~–109 °C, and 10,000 years at ~–119 °C [72].

If not for the likelihood of nucleation above T_G [72, 182], these times suggest that storage near but actually above T_G would provide sufficiently long storage times for all practical purposes. According to one scheme [99], intensive nucleation above T_G might not be a problem for the survival of a living system because homogeneous nucleation will generate ice crystals that are too small to be harmful. If the liquids in the sample are allowed to nucleate completely, the lack of heterogeneity in crystal size would preclude recrystallization, and warming would then simply melt the ice nuclei, perhaps without significant grain growth. The workability of this scheme has never been experimentally tested and might lead to interesting results when tested in the future.

If storage is to be below T_G to minimize nucleation, how far below T_G is cold enough for this purpose? In the M22 vitrification solution, detectable nucleation can be extrapolated to be extinguished at about –136 to –137 °C or about 13–14 °C below T_G [2]. Mehl [182], comparing the devitrification tendency of the VS41A vitrification solution after 6 months of storage at –135 ± 3 °C (a mean of 12 °C below T_G) to that of unstored samples, found that the warming rate required to observe zero ice on warming increased from 50 °C/min without storage to only 100–150 °C/min after storage, which supports the idea that extensive nucleation during holding near T_G may not lead to insurmountable problems on warming. Mehl also pointed out that the number of nuclei may not matter if the aim is to suppress their growth on warming and they are all the same size at the beginning of warming, which will tend to be the case since ice crystals nucleated near or below T_G are not likely to grow until warming begins.

Although it may be academic given the above long projected storage times near T_G, relaxation times below T_G take on the form of Arrhenius kinetics, rising exponentially rather than super-exponentially as temperature continues to fall [2]. Essentially, the extension of logarithmic viscosity plots above T_G to temperatures below T_G is described approximately by the tangent to the curve at T_G.

Empirically, Rowe found no difference in stability of rapidly frozen red cells between –165 °C and –196 °C over 16 years [323]. Valeri and Pivacek found no difference in the recovery of frozen peripheral blood mononuclear cells stored at –135, –150, and –196 °C for 2–2.4 years [324]. And red cells frozen in 40–45 % w/v glycerol were stored successfully for 21 years even at –80 °C [325]. Although indirect, these observations are consistent with the possibility of storing vitrified systems not far below T_G.

From time to time, investigators who correlate the glass transition with the stability of dried systems have noted that deterioration of those systems can take place even very far below the nominal glass transition temperature(s) of those systems [326–329]. The reason for this is unknown, but it must be remembered that the physical state of dried systems is far different from that of hydrated vitrified systems. The former may be more susceptible to mechanical or chemical sources of injury, the nature and meaning of the glassy state may be different, and deterioration may be measured at much higher absolute temperatures than for the case of hydrated vitrified systems.

According to Sun, however, deterioration of dried liposomes can sometimes be stopped by using more sugar prior to drying, perhaps because the extra sugar provides space between the liposomes so that they don't fuse [326]. This may be more analogous to a hydrated system, in which water, the smallest major biological molecule, fills in more gaps and bonds the system together more strongly.

3.11 Proteins at Low Temperatures

We conclude by noting a little-referenced area of molecular biology that has a direct bearing on the principles of biological vitrification and that may one day provide additional insights that will help to guide the cryobiologist to more successful vitrification methods. This field of research, called "cryoenzymology," has provided a significant body of literature documenting the ability of mixed cosolvent systems comprising water and molecules that depress its freezing point to maintain protein secondary, tertiary, and quaternary structure sufficiently well in some cases to enable normal enzymatic catalysis to proceed, albeit at greatly reduced reaction rates, at temperatures as low as −70 °C [330, 331]. Because vitrification preserves cells and molecules in the absence of ice, cryoenzymology offers many direct windows on phenomena that may affect the success of vitrification, including changing dielectric constants and p*K*a values with decreasing temperature and increasing cryoprotectant concentrations [331], which may bear on such phenomena as cold denaturation and chilling injury during vitrification. On the other hand, one of the observations made is that although protein cold denaturation is a real phenomenon [332–334], it can be prevented in some cases by rapid cooling to temperatures too low to favor it kinetically [331, 332], and cryoprotectants such as sucrose can prevent protein denaturation during freezing [332], while glycerol, for example, has been shown to prevent enzymes such as glucose-6-phosphate dehydrogenase, carbamyl phosphate synthetase, and pyruvate decarboxylase from dissociating into subunits due to cooling [331].

So far, such observations have not been applied to events taking place in living cells. Perhaps, in the future, an alliance between cryoenzymologists and cryobiologists could lead to interesting and potentially utilitarian results. By the same token, there may be lessons to be learned from those who freeze proteins either in purified form or in situ [66, 335].

4 Summary and Conclusions

Successful biological vitrification is the result of reconciling the physics of aqueous solutions with the biology of surviving exposure to very water-poor mixed solvent systems. The toxicity of a cryoprotectant solution in the context of vitrification has meaning only in the context of the physical properties of the solution, and the physical properties of the solution, in turn, have meaning only in the context of their compatibility with life and cellular health. From the mutual embrace of these two very different scientific realms has come an extremely broad, beneficial, and growing technology for preserving life at low temperatures. For much of the modern history of this now-united field, the major scientific emphasis has been on intensive study of the physics of vitrification and devitrification, with the biological side of the equation being confined more or less to the modeling of pCPA permeation rates in relatively simple living systems and to hypothesis-driven process optimization without the benefit of a mechanistic understanding of cryoprotectant toxicity, but that mechanistic understanding is now beginning to emerge.

The use of vitrification to preserve living systems and the constituents thereof is currently growing at an exponential rate, and the occurrence of vitrification in the natural world is becoming increasingly appreciated. Successful applications have been reported for a wide variety of mammalian cells and tissues and even for an intact mammalian kidney but extend as well to the plant kingdom and to the preservation of insects and other invertebrates of scientific, medical, and commercial interest.

The success of applied vitrification depends on the choice and final concentrations of cryoprotectants employed, the avoidance of osmotic damage during their introduction and removal, the selection of appropriate temperatures and durations of each phase of treatment with cryoprotectants, the avoidance of chilling injury, the avoidance of fracturing (especially but not exclusively in larger systems), and, increasingly, the use of new types of chemical agent that can interact specifically and directly with ice to inhibit nucleation and growth of ice crystals. Fortunately, much is currently known about all of these factors. Mathematical modeling is also allowing increasingly sophisticated and creative protocols for speeding the introduction and washout of pCPAs while minimizing toxicity throughout, but equipment for continuous rather than stepwise introduction and removal of cryoprotectants, which is needed for the most efficient protocols, is not yet widely available.

The most important barrier to vitrification since the inception of the concept of using high concentrations of cryoprotectants to achieve it remains the same: the intrinsic toxicity of the vitrification solution. Spectacular improvements in the control of vitrification

solution toxicity have already been achieved, but more progress is still needed. Fortunately, for the first time, the powerful and comprehensive "'omics" tools, as well as the more focused and selective tools, of modern molecular biology and enzymology are finally beginning to be applied to the problem of gaining a comprehensive understanding of the still-mysterious and pivotal problems of cryoprotectant toxicity and chilling injury.

There is no guarantee that deeper understanding will lead to fundamental new applied breakthroughs, but the insights gained to date are already providing useful clues to potentially more effective interventions. All in all, the future of cryopreservation by vitrification looks bright and full of exciting new possibilities.

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