3

Principles of Cryopreservation

David E. Pegg

Summary

Cryopreservation is the use of very low temperatures to preserve structurally intact living cells and tissues. Unprotected freezing is normally lethal and this chapter seeks to analyze some of the mechanisms involved and to show how cooling can be used to produce stable conditions that preserve life. The biological effects of cooling are dominated by the freezing of water, which results in the concentration of the solutes that are dissolved in the remaining liquid phase. Rival theories of freezing injury have envisaged either that ice crystals pierce or tease apart the cells, destroying them by direct mechanical action, or that damage is from secondary effects via changes in the composition of the liquid phase. Cryoprotectants, simply by increasing the total concentration of all solutes in the system, reduce the amount of ice formed at any given temperature; but to be biologically acceptable they must be able to penetrate into the cells and have low toxicity. Many compounds have such properties, including glycerol, dimethyl sulfoxide, ethanediol, and propanediol.

In fact, both damaging mechanisms are important, their relative contributions depending on cell type, cooling rate, and warming rate. A consensus has developed that intracellular freezing is dangerous, whereas extracellular ice is harmless. If the water permeability of the cell membrane is known it is possible to predict the effect of cooling rate on cell survival and the optimum rate will be a tradeoff between the risk of intracellular freezing and effects of the concentrated solutes. However, extracellular ice is not always innocuous: densely packed cells are more likely to be damaged by mechanical stresses within the channels where they are sequestered and with complex multicellular systems it is imperative not only to secure cell survival but also to avoid damage to the extracellular structure. Ice can be avoided by vitrification—the production of a glassy state that is defined by the viscosity reaching a sufficiently high value (~10^{13} poises) to behave like a solid, but without any crystallization. Toxicity is the major problem in the use of vitrification methods.

Whether freezing is permitted (*conventional cryopreservation*) or prevented (*vitrification*), the cryoprotectant has to gain access to all parts of the system. However, there are numerous barriers to the free diffusion of solutes (membranes), and these can result in transient, and sometimes equilibrium, changes in compartment volumes and these can be damaging. Hence, the processes

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of diffusion and osmosis have important effects during the introduction of cryoprotectants, the removal of cryoprotectants, the freezing process, and during thawing. These phenomena are amenable to experiment and analysis, and this has made it possible to develop effective methods for the preservation of a very wide range of cells and some tissues; these methods have found widespread applications in biology and medicine.

Key Words: Cryopreservation; cryoprotectants; intracellular freezing; solution effects; supercooling; vitrification.

1. Freezing Injury and Cryoprotection

1.1. The Discovery of the Cryoprotective Effect of Glycerol

Writers of science fiction have been greatly attracted by the concept of "suspended animation," whereby the biochemistry of life could be reversibly suspended for long periods of time and then restored. Although such phenomena do occur in nature, though rarely, it is unfortunately a fact that freezing is normally lethal. In order to understand the effects of very low temperatures, we have to recognize that many structures and processes are temperature dependent and, consequently, cooling has extraordinarily complex effects that produce conditions that are far removed from normal physiology. When we cool below 0°C the biological effects are dominated by the freezing of water, which typically constitutes at least 80% of the tissue mass. Freezing is the conversion of liquid water to crystalline ice, which results in the concentration of dissolved solutes in the remaining liquid phase and the precipitation of any solutes that exceed their solubility limit. It was not until 1948 that a general method was discovered that permitted the freezing of many types of animal cells with subsequent restoration of structure and function. In 1949, Polge, Smith, and Parkes published their landmark paper ***(1)*** in which they showed that the inclusion of 10–20% of glycerol enabled the spermatozoa of the cock to survive prolonged freezing at –80°C. The theories of freezing injury then extant envisaged ice crystals piercing or teasing apart the cells and intracellular structures, destroying them by direct mechanical action. Glycerol, simply by increasing the total solute concentration, would reduce the amount of ice formed in the same way that antifreeze (ethanediol) reduces the amount of ice forming in the cooling system of an automobile engine. But it was also recognized very early on that one effect of freezing an aqueous solution was to increase the concentration of solutes in the dwindling volume of the remaining solution, and that this could be a fundamental cause of injury. In a series of classical papers published in the 1950s, James Lovelock ***(2,3)*** provided strong evidence that salt concentration, rather than ice, is the cause of freezing injury to cells, and that glycerol protects against this damage only to the extent that it modulates the rise in salt concentration during

freezing. It follows that the effectiveness of glycerol, or of any similar cryoprotectant, depends on a number of properties: (1) the compound must be highly soluble in water and remain so at low temperatures in order to produce a profound depression of the freezing temperature; (2) it must be able to penetrate into the cells; and (3) it must have a low toxicity so that it can be used in the high concentrations that are required to produce these effects. Many compounds have these properties. Those in common use include glycerol, dimethyl sulfoxide, ethanediol, and propanediol.

1.2. The Effect of Rate of Change of Temperature

That degree of understanding provided a starting point for the development of practical freeze-preservation techniques for a range of cells, but it soon became clear that reality was considerably more complex. First cooling rate, and then warming rate, was found to be important determinants of survival and Lovelock's theories did not account for such kinetic effects. In 1963, Mazur discovered that the rate of change of temperature was important because it controlled the transport of water across the cell membrane, and hence, indirectly, the probability of intracellular freezing ***(4)***. In general, intracellular freezing is lethal. Mazur argued that the rate of cooling controls the rate at which water is converted to ice; hence it controls the rate at which the concentration of the solution surrounding the cells changes; therefore, by controlling the osmolality of the surrounding fluid, the rate of change of temperature also influences the rate at which water is transported out of the cells during cooling and into the cells during warming. Providing water can leave the cells rapidly to maintain thermodynamic equilibrium across the cell membrane, the cytoplasm will not cool below its freezing point (supercool), and all the ice will be external to the cells. On the other hand, if the cooling rate is too rapid for the membrane of the cell in question to transport sufficient water out of the cell, then the protoplasm will become supercooled, and the greater the extent of supercooling, the more likely is the cell to freeze internally (**Fig. 1**). The combination of these two factors, solution effects and intracellular freezing, causes each cell to show maximal survival at a characteristic cooling rate; as the cooling rate increases from very low rates so does survival because the deleterious effects of exposure to high salt concentrations are reduced, but eventually survival drops off because intracellular freezing supervenes. Each cell has an optimum cooling rate (**Fig. 2**), although absolute survival is usually extremely low unless a cryoprotectant is present to reduce the damage at low cooling rates. Cryoprotectants, like glycerol, have the effect of reducing the solution effects, resulting in a lower optimal cooling rate and an increase in the maximum survival obtained (**Fig. 3**). We

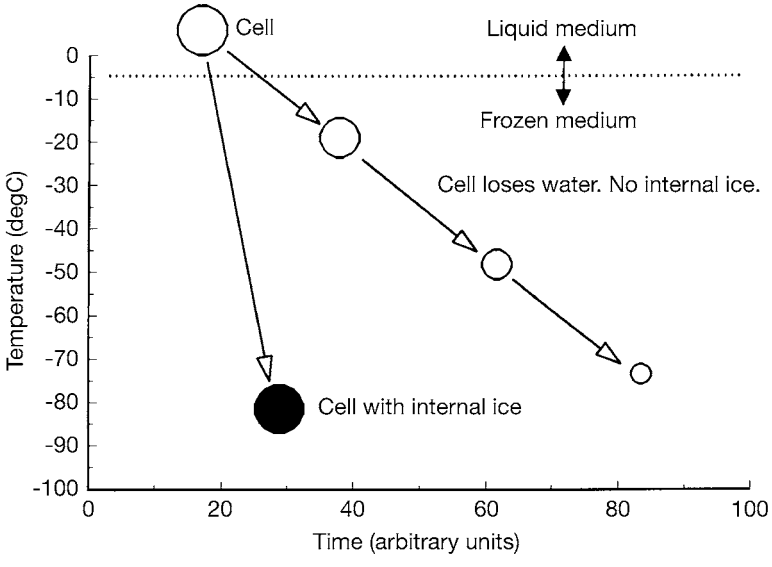

Fig. 1. Schematic representation of cells being cooled rapidly, and freezing internally or sufficiently slowly to lose water and avoid intracellular ice.

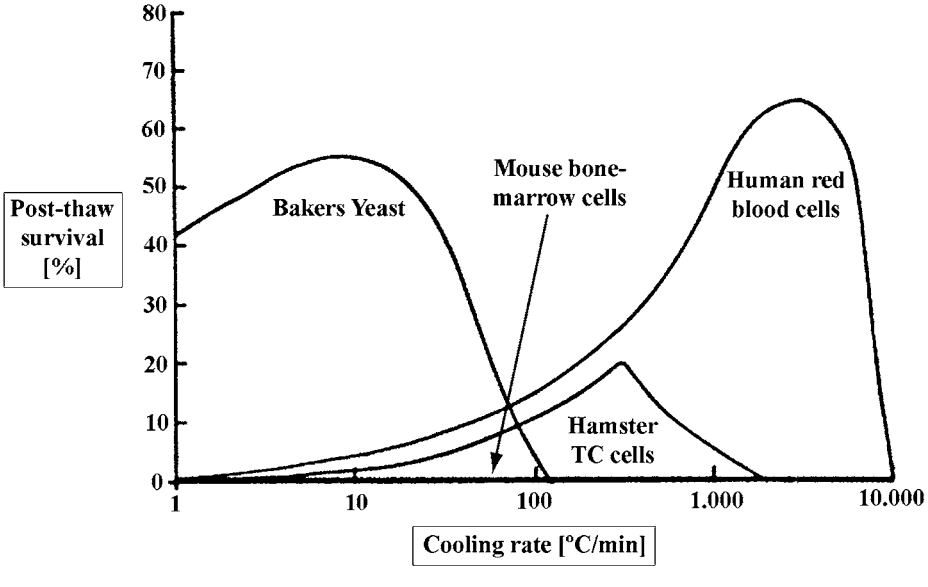

Fig. 2. The effect of cooling rate on the survival following freezing of four types of cell. (Based on **ref. 5**.)

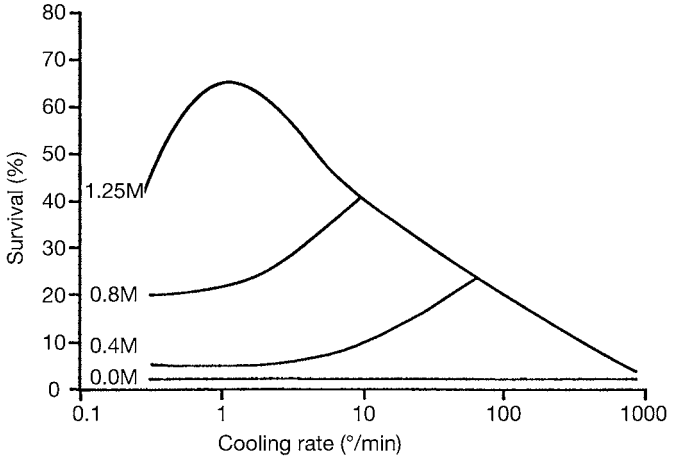

Fig. 3. The effect of cooling rate on the cryopreservation of mouse hemopoietic stem cells cooled in the presence of the indicated molar concentrations of glycerol. (Reprinted with permission from **ref. *6***.)

will now examine these mechanisms of cryoinjury and cryoprotection in a little more detail.

1.3. Solution Effects

Lovelock ***(2)*** had actually shown that the extent of hemolysis that occurred when a saline suspension of erythrocytes was cooled to and thawed from a given subzero temperature was similar to that suffered by a cell suspension that was exposed to the concentration of sodium chloride produced by freezing to that temperature and then returning to isotonic saline (**Fig. 4**). Lovelock also demonstrated ***(3)*** that when glycerol was present, hemolysis started at the (lower) temperature at which the same critical concentration of salt was produced (**Fig. 5**). Correlation does not prove causation, but in this case, if the solution changes were not causative of freezing injury, then the correspondence would be a remarkable coincidence indeed. It was these studies that led to the consensus that extracellular ice is harmless to cells and that freezing injury is caused by indirect effects of the formation of ice. However, the salt in the suspending medium is not the only solute to be concentrated—the cryoprotectant is concentrated to the same degree. Pegg and Diaper ***(7)*** showed that red blood cells actually suffer more damage when exposed to a given salt concentration in the presence of glycerol than in its absence, and this effect is dependent on the concentration of glycerol (**Fig. 6**). When red blood cells were frozen and thawed in the presence of a range of

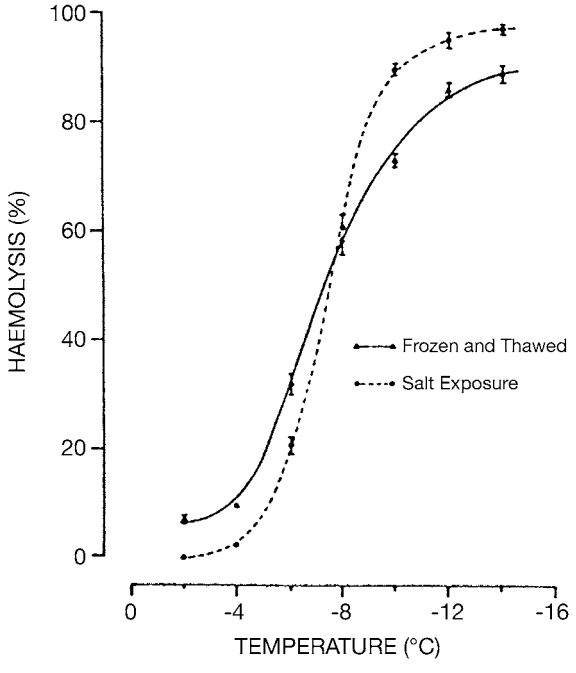

Fig. 4. Human erythrocytes were frozen to the indicated temperatures and then thawed (triangles) compared with exposure to equivalent salt concentrations and then being returned to isotonic conditions (circles). (Reprinted with permission from **ref. *8***.)

concentrations of glycerol, they demonstrated that the correspondence between the effects of salt exposure and of freezing was retained. This observation is important for two reasons: it shows that cryoprotectants are not innocuous—overall they are protective but at a price; second, the observation adds powerful support to the solution-effect theory.

1.4. Intracellular Freezing

If the water permeability of the cell membrane is known, and the temperature coefficient of water permeability can be estimated, then it is possible to predict the effect of cooling rate on cell survival (**Fig. 7**). The calculated degree of supercooling for different rates of cooling shows that intracellular freezing is unlikely at 1°C/min but is highly probable at a cooling rate of 10°C/min. For hepatocytes a cooling rate around 1°C/min will essentially eliminate the risk of intracellular freezing and faster cooling will be preferred only if solution effects are a problem. The optimum rate will be a tradeoff between those two factors. Of course, other cells have different water permeabilities and it has been shown by direct experiment that the cooling rate that

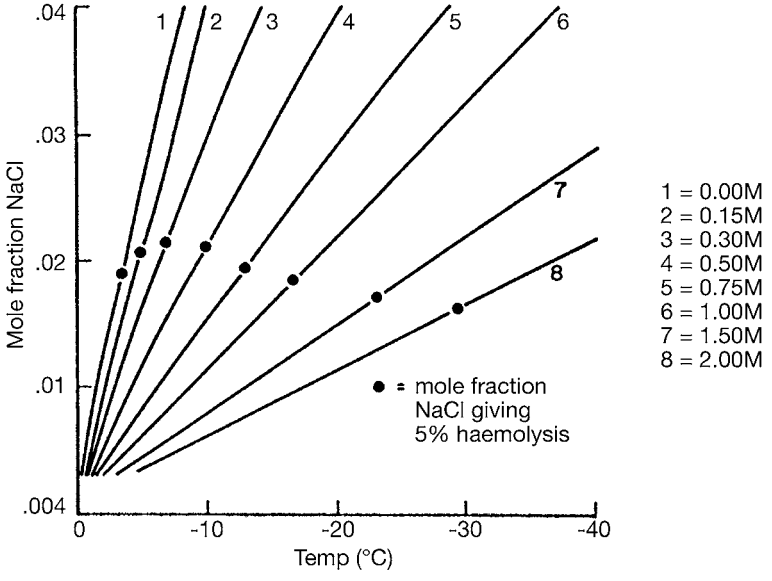

Fig. 5. The increase in mole fraction of NaCl in solutions that have the indicated molality of glycerol and are isotonic with respect to NaCl. Five percent hemolysis was observed at a mole fraction of NaCI = 0.016 – 0.021. (Reprinted with permission from **ref. *9***.)

produces intracellular freezing on a cryomicroscope corresponds with the cooling rate that produces significant intracellular supercooling (***[10]***; **Fig. 8**). In fact, very small amounts of intracellular ice are compatible with recovery, and this is one reason why the warming rate has a profound effect. The behavior of very small intracellular ice crystals differs between slow and rapid warming: slow warming allows the crystals to recrystallize, to coalesce, and to grow. This has been demonstrated to damage the cells in which it occurs; however, during rapid warming there is insufficient time for this to happen and the ice simply melts. Because the cooling rate influences the formation of intracellular ice, while warming rate controls what happens to that ice subsequently, and because cells differ in their water permeability and probably also in their susceptibility to intracellular ice, then it follows that cells will differ in their cooling and warming requirements and cooling rate will interact with warming rate.

1.5. The Cell Packing Effect

Most studies of freezing injury have been carried out with relatively dilute cell suspensions, whereas the cells are quite densely packed in some systems that have to be preserved, for example red blood cells for transfusion, and

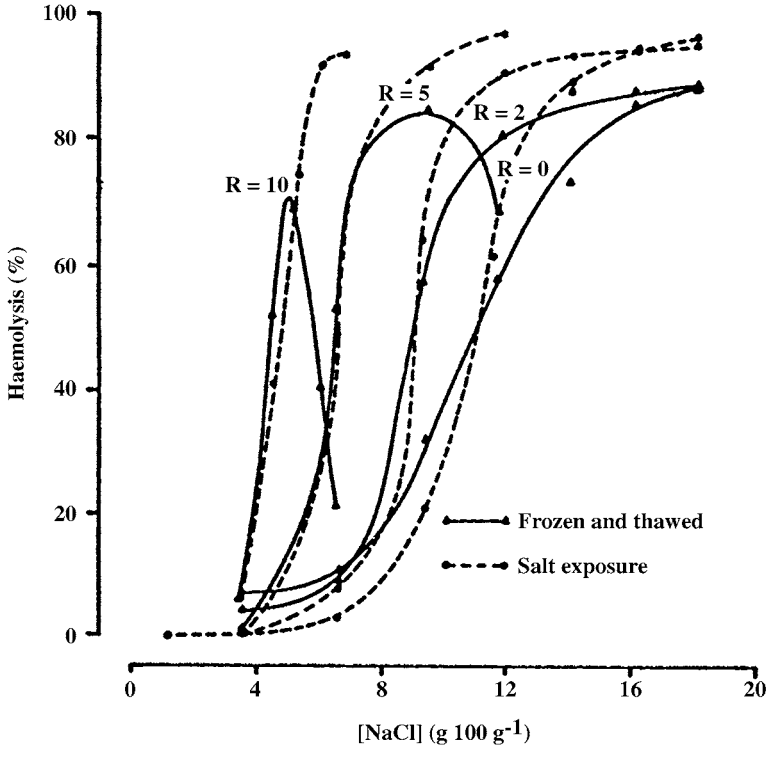

Fig. 6. Hemolysis observed when human erythrocytes were frozen to temperatures that produce the indicated concentrations of NaCl, compared with exposure to equivalent salt concentrations followed by return to isotonic conditions. The R values are the weight ratio of glycerol to NaCl in each solution. (Reprinted with permission from **ref. *8***.)

particularly in tissues and organs. Experiment has shown that the proportion of red blood cells suspended in 2.5 *M* glycerol solution that are hemolyzed during freezing and thawing is strongly dependent on hematocrit (the percentage of cells by volume) when the hematocrit exceeds 50%. The increase in hemolysis as the hematocrit is increased is ameliorated by increasing the glycerol concentration. At 2 *M* glycerol concentration, hemolysis is inversely dependent on warming rate when the cooling rate is less than 1000°C/min and is directly dependent on cooling rates at higher cooling rates ***(11)***.

These observations cannot be accounted for by the classical mechanisms of cryoinjury—solution effects and intracellular freezing. The most likely explanation is that densely packed cells are more likely to be damaged by mechanical stresses when the channels within which they are sequestered change shape as a result of recrystallization of the ice that form their boundaries.

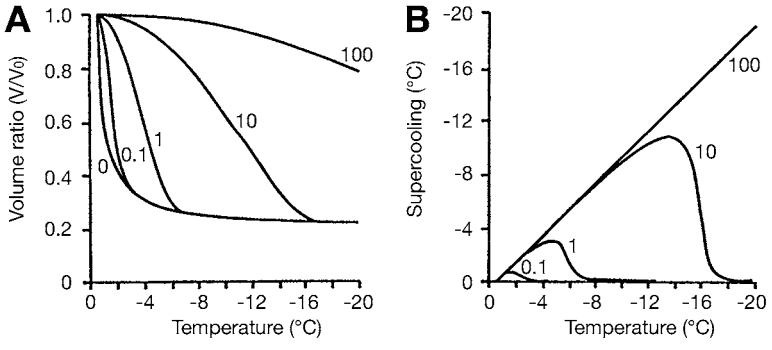

Fig. 7. The calculated effect of cooling rate on the volume of hepatocytes and the extent of supercooling of the cell contents. **(A)** Relative volume (v/v_0) of cells cooled at the indicated rates (°C/min). The line labeled 0 is the equilibrium line. **(B)** From the same calculations as in **A**, the calculated degree of supercooling of the cell contents at the indicated cooling rates. At 10°C/min the cells are supercooled by 10°C and therefore likely to freeze internally. (Reproduced with permission from **ref.** ***12***.)

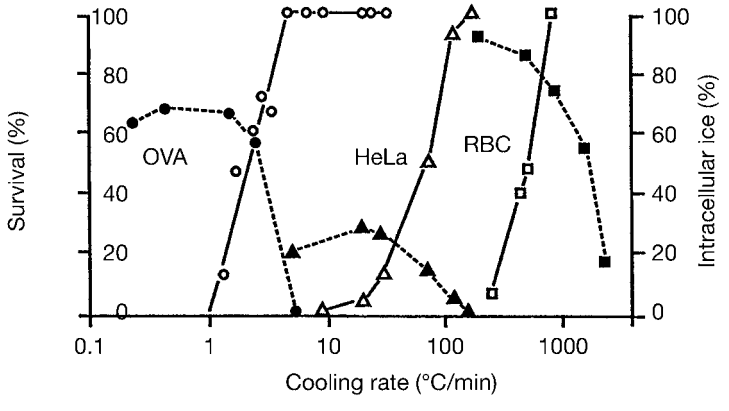

Fig. 8. The survival of three types of cells plotted against cooling rate and correlated with the observed occurrence of intracellular freezing. (Reproduced with permission from **ref.** ***10***.)

2. Cyroprotection

2.1. General Considerations

Cryoprotection usually involves treatment of the cells or tissues with cryoprotectant solutes, often in high concentration, and this produces a driving force for the movement of water by osmosis and of solutes by diffusion. Freezing involves changes in the concentration and composition of aqueous solutions and this also produces driving forces for the movement of water and

solutes. Biological systems contain numerous barriers to the free diffusion of solutes (membranes), and these can result in transient, and sometimes equilibrium, changes in compartment volumes; if excessive, these changes can be damaging. Hence, the processes of diffusion and osmosis are very important for cryopreservation. Fortunately, the quantitative description of mass transfer processes is well developed ***(13,14)***.

The driving force for flow is pressure. Thus, the flow of water, J_v, through a membrane is given by

$$J_v = k.P$$

where k is a constant that is characteristic of the membrane-and-water-combination and P is the pressure difference across the filter. J is given the subscript v to signify volumetric flux. When the driving force for the flow of water through a membrane is osmotic pressure rather than hydrostatic pressure, flow can be described by the same equation if osmotic pressure, π, is substituted for hydrostatic pressure, thus,

$$J_v = k.\pi$$

The constant k has the same value in the two equations, providing only that the membrane and the solvent, water in this case, remain the same. Thus, both hydrostatic and osmotic pressure differences can be incorporated into a single equation. The constant k is then known as the hydraulic conductivity L_p. (The units are cm/atm/s.)

$$J_v = L_p.(P + \pi)$$

Under the conditions prevailing in cryobiology the hydrostatic pressure term will normally be zero and π can be calculated from concentration by multiplication by the product of the universal gas constant R, and the absolute temperature T. If the area of the membrane is A, and internal and external osmolalities are denoted by C_i and C_e, then we obtain

$$J_v = L_p.A.R.T.(C_i - C_e)$$

The solute flux J_s is described by

$$J_s = \omega_s.A.R.T.(S_e - S_i)$$

This equation states that flux across unit area of membrane is proportional to the solute permeability ω_s, and the difference in concentration of the solute across the membrane. The more familiar solute permeability P_s (units are cm/s) is equal to $\omega_s RT$. The constant RT is 23,235 atm.cm^3/mole. The convention for the direction of flux is that outside $\rightarrow$ inside is positive. A somewhat more complex formalism was elaborated by Kedem and Katchalsky ***(13)*** in 1958 and their equations are often used in cryobiology where they are usually referred to as the

K–K equations. Kedem and Katchalsky assumed that the solvent and solute used a common channel through the membrane and they therefore added a solvent/solute interaction term, σ, known as the reflection coefficient. This led to modification of the equations for J_v and J_s, as shown

$$J_v = L_p.A.R.T.[(C_i - C_e) + \sigma(C^p_i - C^p_e)]$$

$$J_s = \omega_s.A.R.T.[(S_e - S_i) + J_v(1 - \sigma)c_s]$$

In the equation for J_v the solutes are partitioned between impermeant solutes (C) and permeating solutes (C^p), the latter interacting with water in the common flow channel. The equation for J_s has an additional term that represents solvent drag on the permeant solute, which is present in the membrane at concentration c_s. Clearly, the K–K is more complex and curve fitting routines can lead to uncertain results because of the lack of independence of the parameter, σ. Kleinhans ***(14)*** has discussed these problems in detail and moreover he has argued that the K–K formalism is often invalid because of the presence of separate channels for water and solute. In practice, the simpler formalism is adequate for the current needs of cryobiologists. The two equations are solved simultaneously by numerical methods and programs to carry out these calculations can be run on an ordinary PC.

Several methods are available for the determination of permeability parameters in cryobiology. If the solute under study can be radiolabeled, the time-course of isotope uptake is easily measured but the calculation of concentration requires the additional measurement of water content at each time-point. Permeating solutes can be extracted after known times of exposure and high-performance liquid chromatography methods are often suitable for their assay. The Karl Fischer method, using a backtitration scheme, is a convenient method for water ***(15)***. If the compound under study has a distinctive nuclear magnetic resonance spectrum, nuclear magnetic resonance can be used to determine the time-course of both solute and water content simultaneously, so this technique yields concentration directly. A commonly used indirect method for isolated cells is to record the time-course of cell volume following exposure to a known concentration of the compound by Coulter counter- or light-scattering methods; the equations described previously are then used to model the experimental data and derive estimates of L_p and P_s. We will now consider in more detail some situations in which these permeability parameters are relevant to cryopreservation.

2.2. Introduction of Cryoprotectants

The exposure of cells to a high concentration of cryoprotectant causes osmotic dehydration. If the cryoprotective compound permeates, the cells then increase in volume, water entering along with the cryoprotectant until the cells

reach their final volume. The extent of shrinkage and the rate of change in cell volume are determined by the permeability parameters. The final equilibrium volume depends on the concentration of impermeant solutes in the solution and is the same as the normal volume only if the concentration of impermeant solutes is isotonic in molar (per liter) terms. This is because of the fact that the cryoprotectant occupies space within the cells and the volume of water must therefore be lower than the physiological water content if the total volume is to be normal ***(16)***. The rate of change of volume, and particularly the equilibrium volume, are both important and must be optimized in cryopreservation procedures.

2.3. Removal of Cryoprotectants

When a permeating cryoprotectant is removed by exposing the cells to a lower concentration of the compound, the osmotic uptake of water causes the cells to swell above their initial volume. They then shrink as the cryoprotectant moves out, accompanied by sufficient water to maintain osmotic equilibrium; they return to physiological volume only if nonpermeating solute has neither been lost nor gained during the process. Because cells are generally more sensitive to swelling than to shrinkage, removal of cryoprotectants tends to be more hazardous than their addition. Again, both the rate of change of volume and the final volume must be considered when designing protocols for the recovery of cryopreserved cells.

2.4. Freezing and Thawing

Freezing causes the solution surrounding the cells to concentrate, and as a consequence the cells shrink at a rate that depends upon the rate of formation of ice, the cell's Lp and its temperature coefficient, and temperature itself. This phenomenon is an extremely important determinant of intracellular freezing. The final extent of shrinkage depends on the cryoprotectant concentration.

2.5. Exposure to Nonpermeating Solutes

Cells immersed in a solution of nonpermeating solute reach an equilibrium volume that is an inverse function of the osmolality of the solution; ideally,

$$V_{rel} = \frac{1}{M_{rel}}$$

where V_{rel} is the volume of intracellular water relative to the physiological water content and M_{rel} is the external osmolality relative to its physiological value.

This relationship requires that a plot of V_{rel} against M_{rel}, which is known as a Boyle van't Hoff plot, is a straight line of slope = 1, which must pass through the origin because water content is zero at infinite osmolality ***(17)***. In reality there is always an intercept on the *y*-axis—the so-called nonosmotic water

volume or V_{inf}. This probably represents a physically distinct portion of the cell water that is so structured that it does not participate in solution phenomena. (Alternatively, it could reflect the nonideal behavior of the intracellular solutes such that osmolality increases with concentration more than in linear proportion.) The value of V_{inf}, determined by a Boyle van't Hoff plot, is needed to interpret volume/time data for the cells in question and to calculate intracellular concentrations of permeating solutes. Experimentally, the collection of such data can usefully be combined with determining the upper and lower volume limits that the cells will tolerate without damage.

3. Preservation of Cells and Tissues

3.1. Preservation of Cells

The basic cryobiological knowledge reviewed here has made it possible to develop effective methods for the preservation of a very wide range of cells, and these have found widespread applications in biology and medicine. Examples include the long-term preservation of spermatozoa of many species, including cattle, laboratory animals, and man, very early embryos and ova, red and white blood cells, hemopoietic stem cells, tissue culture cells, and so on (*see* following chapters in this volume). For each type of cell there is a set of conditions that is optimal for preservation, determined by the interaction of the particular properties of the cell in question with the cryobiological factors that have been discussed. If the characteristics of the cell are known, it is usually possible to predict with reasonable precision the conditions that will provide effective cryopreservation.

3.2. Preservation of Multicellular Systems

The situation becomes much more difficult when we move from single cells to complex multicellular systems. Cell survival is still required, of course, but tissues and organs contain a heterogeneous collection of cells, which may have quite different optimum requirements for preservation, unlike the situation in cell preservation where one is usually dealing with a single type of cell. Yet it is necessary to find a method that will secure adequate survival of all the cells that are important for the function of that tissue. Fortunately, the use of high concentrations of cryoprotectant results in a flattening of the bell-shaped survival curve and a broadening of its peak: with sufficiently high concentrations of cryoprotectant it is possible to secure overlapping survival curves for many different cells. Another problem is that it is not sufficient to obtain high levels of survival for the various types of cell that are present in tissues and organs; it is also imperative to avoid damage to important extracellular structures and to retain normal interconnections between the cells and their attachments to basement membranes ***(18)***. Ice that forms outside the cells when a cell suspension

is frozen is outside the system that it is desired to preserve, and it can damage the cells only by indirect means (solution effects) or by exerting a shear or compressive force on them externally. The situation is quite different for organized tissues; here, extracellular ice is still within the system that is to be preserved and can disrupt the structure of the tissue directly. The first evidence of such an effect was provided by Taylor and Pegg ***(17)*** when they showed that smooth muscle, frozen to –21°C by cooling at 2°C/min in the presence of 2.56 *M* dimethyl sulfoxide was functionally damaged, whereas exposure to the solution conditions produced by freezing that solution to that temperature, at the same temperature, was innocuous. Structural studies using freeze substitution showed that ice formed within the muscle bundles ***(20)***. If cooling was slowed to 0.3°C/min, freezing produced less damage and ice was shown to form only between the muscle bundles. This showed that extracellular ice damaged this tissue, but the extent of such damage was dependent on the site at which the ice formed. Damaging effects of extracellular ice have also been demonstrated in kidneys and livers, where it has been shown to cause rupture of the capillaries. Rubinsky and Pegg ***(12)*** have proposed a mechanism for this effect; ice forms within the vessel lumens, drawing in water from the surrounding tissue until the volume of intraluminal ice exceeds the elastic capacity of vessel and rupture ensues. In organs and tissues that require an intact vasculature for function, vascular rupture is lethal, even if many cells survive, and this mechanism provides the major barrier to effective cryopreservation of such systems. The avoidance of freezing, or at least limitation of the amount of ice to very small quantities in the least susceptible locations, seems to be the only way to avoid this problem. Attempts to cryopreserve complex multicellular systems simply by adapting techniques from single-cell systems have generally been unrewarding. In the medical field, the situation may be more favorable with tissues that can be transplanted without revascularization; it all depends on the precise requirements for surgical acceptability. For example, the primary requirement for heart valve grafts is that the collagen structure is intact, and it is unclear whether the survival of donor fibroblasts has any useful effect. Similarly, human skin can be cryopreserved by methods similar to those used for cell suspensions and will then retain significant numbers of viable keratinocytes, although it is questionable whether these influence the clinical results when skin grafts are used as a temporary covering on seriously burned patients. For other tissues, such as small elastic arteries, satisfactory methods have only been developed relatively recently ***(21)***. For corneas and cartilage and for whole vascularized organs there are no effective methods.

3.3. Vitrification Methods

Much of the very early work in cryobiology, notably by Luyet ***(22)***, had been based on the assumption that freezing damaged cells directly and, consequently,

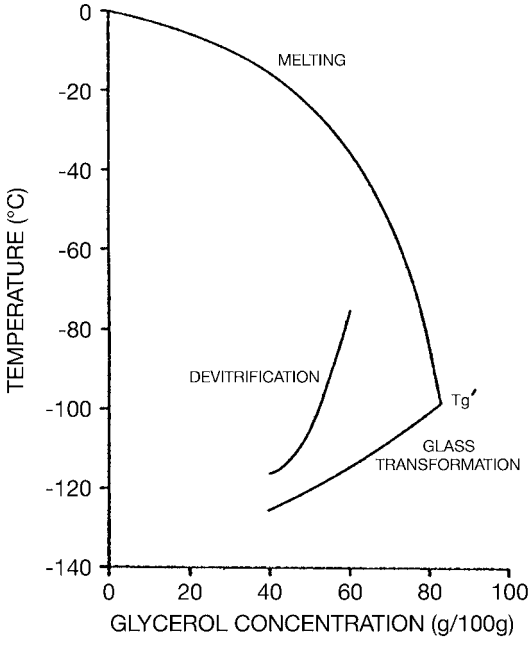

Fig. 9. Supplemented phase diagram for glycerol/water. The intersection of the melting curve and the glass transition curve at Tg´ indicates the lowest concentration of glycerol that, in theory, will vitrify. In practice, the lower temperatures on the melting curve are unlikely to be reached owing to the high viscosity preventing the crystallization of ice. (Reproduced with permission from **ref. *23***.)

that effective preservation would require a technique that completely prevented the crystallization of ice. Luyet devoted a great deal of effort to the search for conditions that would produce a vitreous or glassy state with biological systems and that living cells could survive. Vitrification is defined by the viscosity of the solution reaching a sufficiently high value (~10^{13} poises) to behave like a solid but without crystallization. In conventional cryopreservation, the concentration of solute in the remaining liquid increases during progressive freezing, and a temperature (Tg) is eventually reached with many systems where the residual liquid vitrifies in the presence of ice (**Fig. 9**). Cells can survive this situation, they do so in conventional cryopreservation, but they will not tolerate exposure to the necessary concentration for vitrification without freezing (~ 80g% [w/w]) at temperatures above 0°C. Some other solutes will vitrify at lower concentrations, for example butane-2,3-diol at around 35% (w/w), but unfortunately this compound is more toxic than glycerol. Luyet knew that it was possible to vitrify solutions that are less concentrated than this if sufficiently rapid cooling

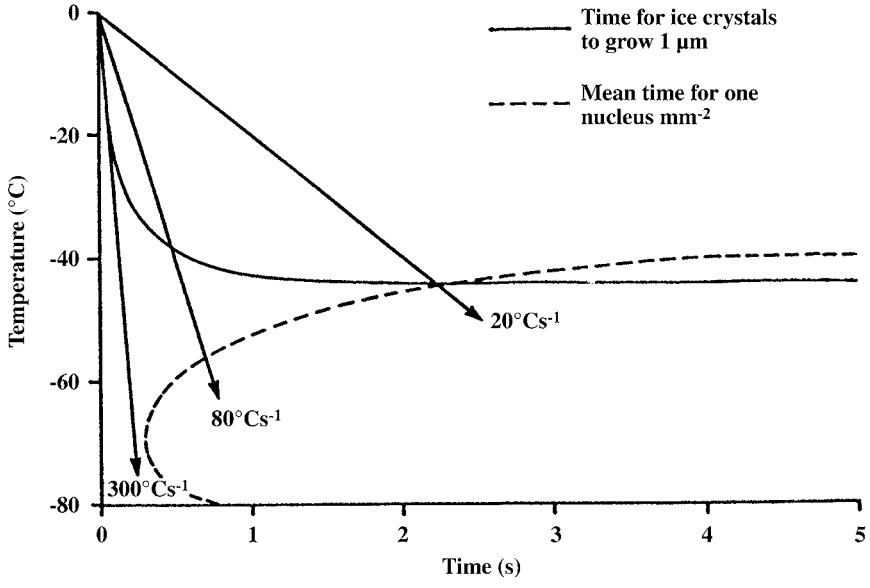

Fig. 10. Diagram constructed from data by Luyet showing the time- and temperature dependence of nucleation and ice crystal growth in a thin film of a 50% (w/v) solution of polyvinylpyrrolidone. The arrows indicate cooling trajectories that avoid nucleation (300°C/s), nucleate without crystal growth (80°C/s) and produce ice crystals (20°C/s). (Reprinted with permission from **ref. *8***.)

was employed; the reason is that ice crystals form by the accretion of water molecules onto a nucleus. Both the formation of the nuclei and the subsequent growth of ice crystals are temperature dependent. Nucleation is unlikely just below the equilibrium freezing point (hence the phenomenon of supercooling), but it becomes more probable as the temperature falls, reaches a maximum rate, and then decreases as the movement of water is limited by viscosity. However, the growth of ice crystals is maximal just below the freezing point and is progressively slowed, and eventually arrested, by cooling. The interaction of these two processes creates three possibilities for a cooled sample (**Fig. 10**); if it cools rapidly it may escape both nucleation and freezing; if it cools sufficiently slowly it will nucleate and then freeze; and at an intermediate cooling rate it will nucleate but not freeze. Upon warming, however, there are only two possibilities; if heated sufficiently rapidly it will escape both nucleation and freezing during warming; the alternative is that the trajectory passes through both the nucleation and the ice crystal growth zones and, therefore, it will nucleate (if it is not already nucleated) and the ice crystals will then grow before eventually melting. Therefore, unless a sufficient concentration of cryoprotectant has been used to ensure that no ice can form under any circumstances, there is

a risk that freezing will occur during warming. The problem is that bulky tissues and organs cannot be cooled much more rapidly than a few degrees per minute in practice. For small samples it is more feasible to cool rapidly, as was demonstrated by the successful vitrification of *Drosophila melanogaster* embryos ***(24)***. These were complex organisms comprising some 50,000 cells with advanced differentiation into organ systems, and they cannot be preserved by conventional freezing methods. The successful method required careful permeablization of the waxy vitelline membrane to allow penetration of the cryoprotectant, exposure to 8.5 *M* ethanediol, cooling at 100,000°C/min., storage at approx –200°C, and warming at 100,000°C/min. The extremely high rate of warming was far more critical than the rate of cooling, which is consistent with the crucial importance of maintaining the vitreous state.

The demonstration that ice forming in tissues produces so much damage has created renewed interest in the possibility of using vitrification with very high concentrations of appropriate cryoprotectants to avoid the formation of ice completely. Current research aims to identify materials that will inhibit the formation of ice crystals during warming ***(25,26)***, and one interesting possibility is the antifreeze proteins that some polar fish and overwintering insects have evolved to avoid freezing in nature. One effect of such compounds is to reduce the warming rate required to prevent ice crystallization to more manageable rates. This approach is being used in conjunction with electromagnetic heating ***(27,28)*** to achieve more rapid and more uniform heating. However, despite progress in the design of vitrification cocktails with reduced toxicity, the major problem remains cryoprotectant toxicity. One approach to this problem is to increase the concentration of cryoprotectant progressively during cooling so that the tissue concentration follows the liquidus curve: ice does not form but the cells do not experience any greater concentration of cryoprotectant than occurs during freezing. This has recently proved to be practical and very effective for the cryopreservation of articular cartilage, an otherwise recalcitrant tissue ***(29)***. The same method may potentially be effective for other resistant tissues and perhaps even for organs.

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