LIFE *in the* FROZEN STATE

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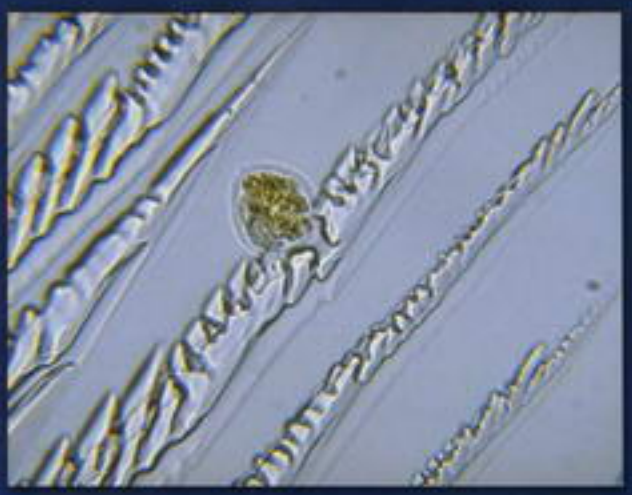

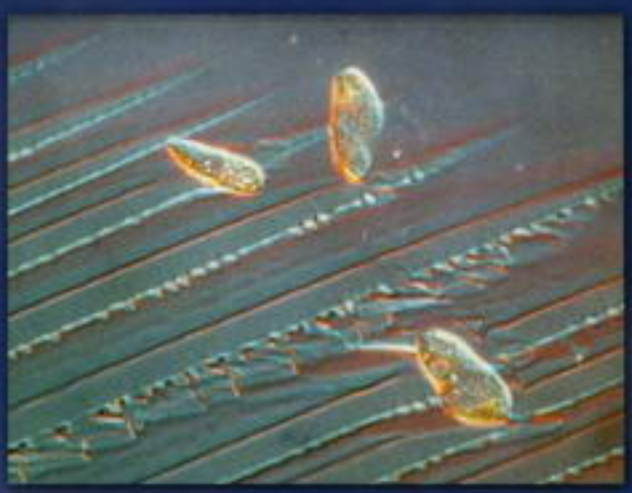

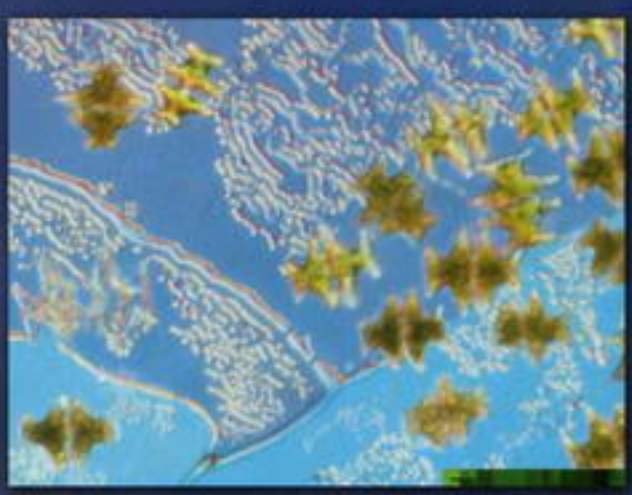

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2 The Water to Ice Transition: Implications for Living Cells

Ken Muldrew, Jason P. Acker, Janet A.W. Elliott, and Locksley E. McGann

CONTENTS

2.1 INTRODUCTION

When liquid water is cooled, a temperature is reached at which the solid phase, ice, becomes stable. That is to say that an ice crystal, when added to the cold water, will not melt. If the liquid is below that fusion temperature, the ice crystal will, in fact, grow; water molecules will leave the jostling, random walk of the liquid phase and join the fixed lattice of the ice crystal. When the pure liquid is cooled below the fusion temperature without any external ice being added, however, the solid phase does not necessarily appear. There is a temperature range in which the liquid is metastable; the presence of a seed crystal (or other nucleating enhancement) is required to initiate the phase transition. In this region there are clusters of water molecules joining together to form networks that extend the correlation length between water molecules beyond the scale of a few molecules. As these networks grow, the number of possible paths for continued growth increases exponentially,

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but the correlation length also decays exponentially because of collisions that break the network. Only the latter phenomenon is dependent on temperature, so there is a critical temperature at which the multiplicity of growth paths can overwhelm the collision-based fractures. Below this temperature a nucleation event can occur whereby the length scale of a particular network exceeds the length scale of the destructive collisions that break these extended clusters. The growing network becomes an ice crystal.

All living organisms primarily consist of water, so their response to freezing is necessarily dramatic. Though humans suffer profound injury when any part of their body freezes, we see an enormous number of organisms that can withstand the harshest winter climate, and therefore we know that life can withstand these conditions. Our investigation of this phenomenon is not merely out of curiosity, either, as there is an enormous practical benefit to being able to store living tissues at low temperatures. Life exists far from equilibrium and will therefore decay quickly without an expensive energy throughput to maintain itself. A steady state that avoids decay can be established, with respect to the timescale that is important for living organisms, by lowering the temperature. The clocks that determine the timescale for life are biochemical reactions, and the rate at which these reactions proceed is controlled by an energy barrier. Though the energy balance favors the forward reaction, the reactants must overcome an intermediate energy barrier before the reaction proceeds. The simplest way to slow the rate with which these reactions occur, and thereby slow biological time, is to lower the free energy of the reactants by lowering the temperature. Indeed, it would be as easy as that were it not for the troublesome tendency of water to form ice when it is cooled. We will look at some of the problems that biological systems encounter that are caused by the water to ice transition. The topic is far too broad to cover in this chapter, and thus we will concentrate on our own investigations that have centered around the direct observation of the freezing process under a microscope, giving only a brief background to set this work in context and to introduce the conceptual tools that have been used to design experiments.

2.2 WATER TO ICE

2.2.1 Aqueous Solutions

Solution thermodynamics describes a variety of processes that occur when a cell suspension is subjected to lowering temperatures. Chemical potential is the thermodynamic property of a solution that governs many of these processes. The chemical potential of a solution is, in general, a function of pressure, temperature, and concentration. If a solution is assumed to be thermodynamically ideal and dilute, then the chemical potential at a given temperature and pressure depends only on the concentration of solute, not on the type of solute. For many biologically relevant solutions, however, both the concentration and types of solutes are important.

At equilibrium, the chemical potential of ice is equal to the chemical potential of the solution with which the ice is in contact. This gives us an equation for the freezing point as a function of solution concentration (freezing point depression). For an ideal, dilute solution the freezing point depression is given by

$$\Delta T = \left(\frac{RT_m^2}{\Delta H_{fus}} \right) X_B \tag{2.1}$$

where ΔT is the freezing point depression, T_m is the melting point of the pure solvent, ΔH_{fus} is the enthalpy of fusion of the solvent, and X_B is the mole fraction of the solute. For a solution that is not ideal and dilute, the relationship between equilibrium temperature and mole fraction will not be linear. As an aqueous solution is cooled, ice will begin to form when the extracellular solution reaches its freezing point (if a nucleation event occurs). As ice forms, the pure crystal excludes

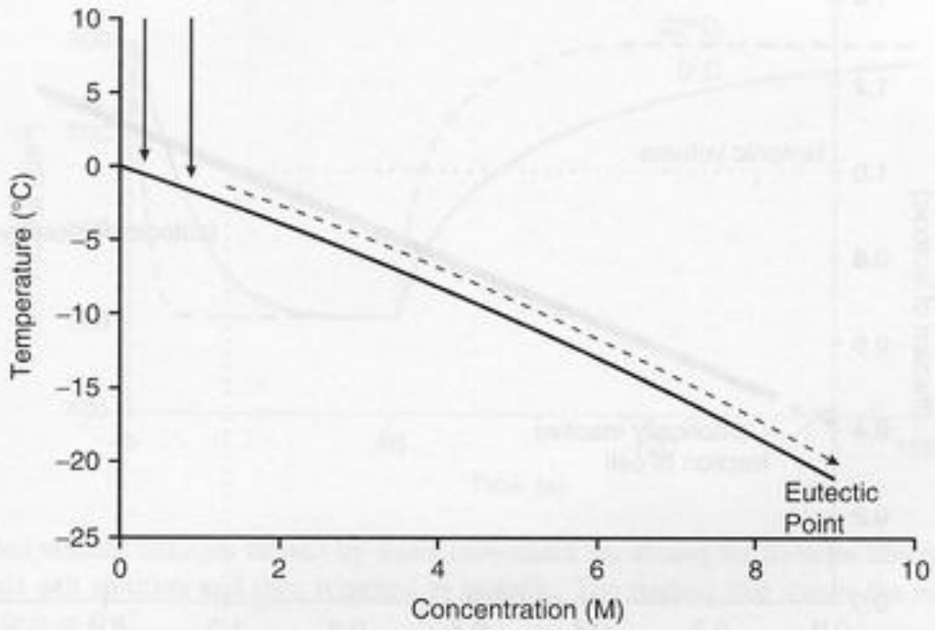

FIGURE 2.1 The liquidus curve for the NaCl–water phase diagram is shown. The arrows indicate starting concentrations of isotonic (0.3 *M*) and 3× isotonic (0.9 *M*). If the temperature of these solutions is dropped, starting at room temperature, the composition of the solution remains the same until the liquidus curve is reached. Any further lowering of the temperature will follow this curve until the eutectic is reached at –21.2°C.

solutes that concentrate in the residual liquid, further lowering the freezing point. The dotted line in Figure 2.1 shows the path of a simple physiological solution (0.15 *M* NaCl) during cooling and warming. Starting at a temperature above freezing, the solution concentration remains constant as the temperature is lowered. Freezing can occur when the sample temperature goes below the melting point of the solution (–0.55°C). As the temperature is further lowered, more ice is formed and the concentration of NaCl in the liquid phase increases. In the presence of ice, the phase diagram in Figure 2.1 shows that the composition of the liquid phase is dependent only on temperature, not on the concentration in the initial sample. Ice continues to form with decreasing temperature until the sample reaches the eutectic point at which the entire system solidifies and there is no further change in composition. During warming, the sample retraces the path, diluting the liquid phase as ice melts. The unfrozen solution in this sample would therefore develop highly concentrated solutions (up to 9 *M*) at subzero temperatures. The nonlinear curve shown in Figure 2.1 indicates that a sodium chloride solution cooled to its eutectic cannot be considered ideal and dilute.

2.2.2 Osmotic Response of Cells

At equilibrium, the chemical potential of the intracellular solution is equal to the chemical potential of the extracellular solution. Because the cell membrane is effectively semipermeable (impermeant to many of the solutes that determine the chemical potential but permeant to water and some added cryoprotectants), water will move in or out of the cell in response to changes in solution concentration outside the cell. With freezing, the extracellular solution becomes more concentrated and water therefore leaves the cell until the gradient in chemical potential is neutralized, establishing "osmotic" equilibrium across the plasma membrane. This new equilibrium results from the concentration of the intracellular solutes. The equilibrium cell volume, *V*, is a function of the extracellular solute concentration and has been traditionally given by the Boyle–van't Hoff equation:

$$V = \pi_e^0\left(V_o - b\right)\frac{1}{\pi_e} + b\,, \tag{2.2}$$

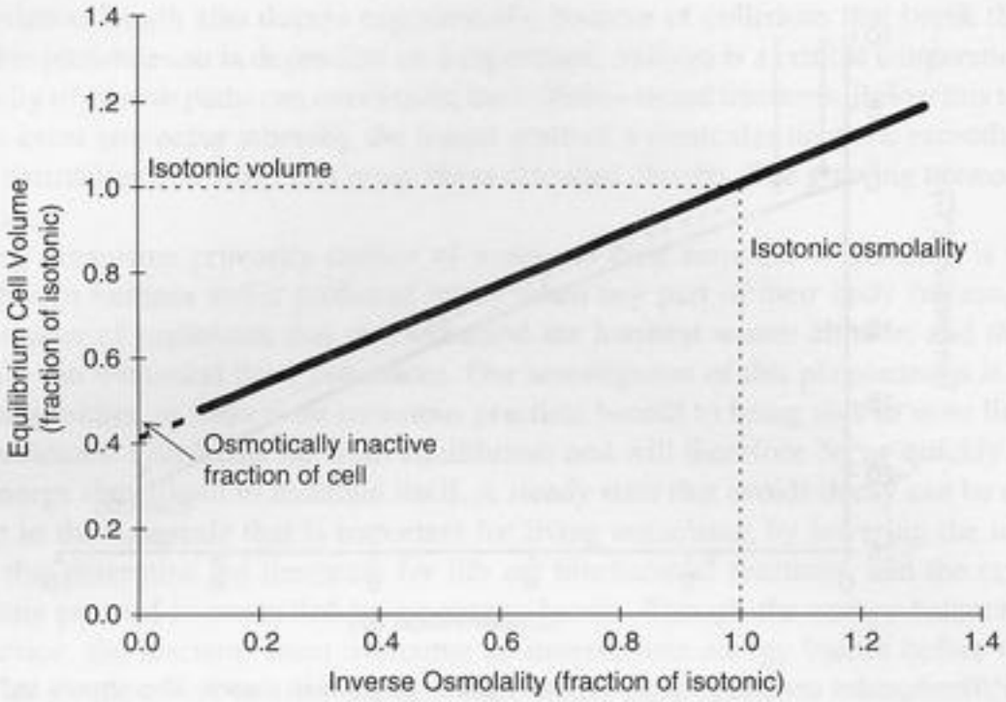

FIGURE 2.2 The Boyle–van't Hoff plot for cell volumes as a function of inverse osmolality. The graph uses an osmotically inactive fraction of 0.41, a value reported for bovine chondrocytes (From McGann et al., 1988. With permission.).

where π_e is the osmolality of the extracellular solution, b is the osmotically inactive volume of the cell and V_o and π_e^0 are the cell volume and the extracellular osmolality, respectively, for the isotonic condition. Note that the Boyle–van't Hoff equation is derived by assuming that the cell contents form a thermodynamically ideal, dilute solution; it is not valid for solutions that are not ideal and dilute. In addition, osmolality in the equation should not be replaced by osmotic pressure if the equation is to be used at a variety of temperatures. The Boyle–van't Hoff plot in Figure 2.2 shows the relative cell volume as a function of inverse osmolality. Extrapolation of this line to infinite osmolality (to zero on the abscissa) has been used to estimate the osmotically inactive fraction of the cell volume. If the cell contents were ideal, dilute solutions, the value of this parameter would represent the proportion of the total cell volume that does not participate in osmotic activity. In real cells, the solutions are not ideal and thus the physical interpretation may be misleading.

When cells are exposed to an increased concentration of an effectively impermeant solute, as occurs when ice forms in a physiological solution, the cell will shrink to a volume defined by the Boyle–van't Hoff plot in Figure 2.2. At the temperatures at which ice forms, the cells do not have the metabolic capacity to alter their intracellular osmotic environment, and thus the cells are essentially ideal osmometers.

The rate of movement of water across a membrane is limited by the permeability properties of the membrane. If there is a chemical potential gradient across the membrane, the rate of change of cell volume will be determined by this gradient and by the permeability of the membrane. Figure 2.3 shows the cell volume as a function of time after exposure to, and dilution from, a solution containing an impermeant solute. The rate at which the cell loses water is a function of the magnitude of the osmotic gradient and the permeability of the plasma membrane (the area of the membrane and its conductivity to water) and is given by:

$$\frac{dV_w}{dt} = L_p ART\left(\pi_i - \pi\right), \tag{2.3}$$

where V_W is the water volume of the cell, t is time, L_p is the hydraulic conductivity, A is the membrane surface area, R is the gas constant, T is the absolute temperature, and π_i is the intracellular osmolality.

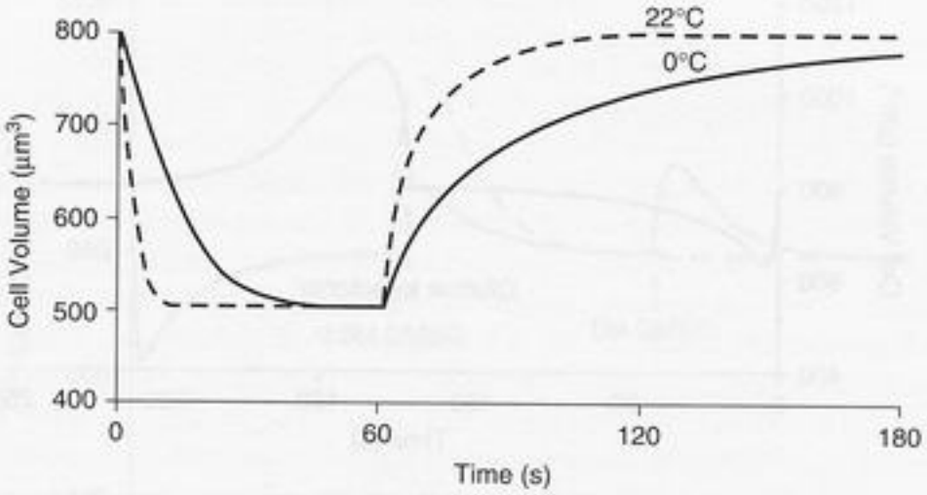

FIGURE 2.3 Cell volume changes caused by water movement are shown for hamster fibroblast cells placed into a 3× isotonic salt solution and then returned to isotonic. The dashed line shows the response at 22°C, and the solid line is at 0°C.

Water movement through the membrane occurs through aqueous pores created by hollow protein cylinders that are embedded in the membrane as well as directly by diffusion through the lipid bilayer (Finkelstein, 1987). There are a variety of water transport pores, known as aquaporins, and the type and quantity of these pores in a given cell type can significantly alter the water permeability (Verkman et al., 1996). Even in cells with aquaporins, though, water will continue to move through the bilayer by a solubility/diffusion mechanism; this permeability will be determined by the lipid composition of the bilayer. In both of these cases permeability will be temperature dependent because of the temperature dependence of water self-diffusion, for both aquaporins and solubility/diffusion, and because of the fluidity of the bilayer lipids in the case of solubility/diffusion (Elmoazzen et al., 2002). Because both processes are caused by diffusion of water, the rate will be proportional to the osmotic gradient across the plasma membrane (but limited by the permeability of the membrane, a property that is different for each cell type). The temperature dependence of the kinetics of water loss is demonstrated in Figure 2.3 by the different curves at 22° and 0°C, though the equilibrium volume depends solely on the solution osmolality. The difference between the two temperatures arises from the explicit temperature dependence of Equation 2.3 and the temperature dependence of L_p (described by an Arrhenius relation). On dilution back to isotonic conditions, the cell volume simply returns to its isotonic value according to the same equation.

In the presence of a permeant solute (such as Me_2SO, glycerol, propylene glycol, or many other cryoprotectants) there is both water and solute movement, and there may be an interaction between these fluxes in the membrane. In a situation in which there is an osmolality difference caused by a solute that cannot permeate the membrane as well as a solute that can permeate the membrane, we need to account for the flow of this penetrating solute as well. The equations to describe such coupled transport have been derived from nonequilibrium thermodynamics by Kedem and Katchalsky (1958). We will introduce a simplified form of the K-K equations given by Johnson and Wilson (1967) so that the interested reader can reproduce the simulations described elsewhere in the chapter.

Johnson and Wilson wrote the water flux equation in terms of both solutes as

$$\frac{dV_W}{dt} = L_p ART\left[\left([I]_i - [I]_e\right) + \sigma\left([S]_i - [S]_e\right)\right], \tag{2.4}$$

where $[I]_i$ is the intracellular concentration of impermeant solute and $[I]_e$ is the extracellular concentration of impermeant solute, $[S]_i$ is the concentration of permeant solute inside the cell, and $[S]_e$ is the

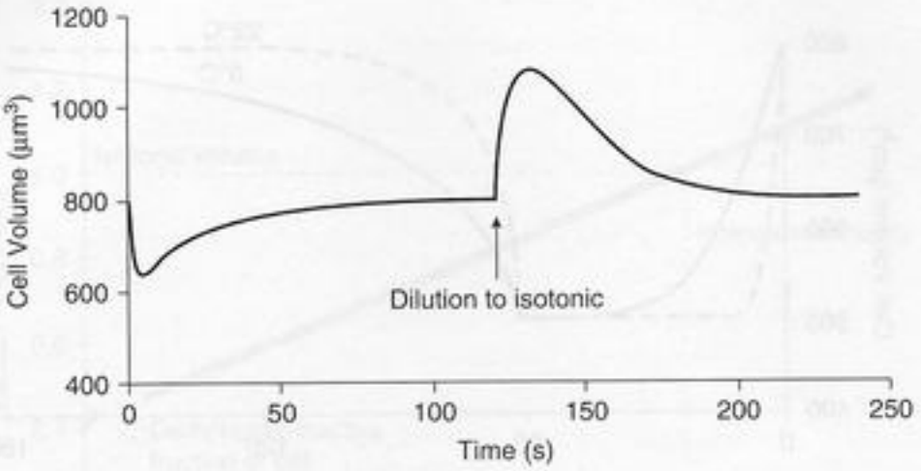

FIGURE 2.4 Cell volume changes caused by water and solute movement are shown for hamster fibroblast cells placed into a 1 *M* Me_2SO solution and then diluted out to isotonic at 22°C.

concentration outside the cell (all concentrations are expressed as osmolalities). The reflection coefficient, σ (on the interval 0–1), was introduced to account for the interaction of solute and solvent.

Johnson and Wilson gave the solute flux equation as

$$\frac{dS}{dt} = P_s A\left([S]_e - [S]_i\right) + (1-\sigma)\overline{[S]}\frac{dV_w}{dt}, \tag{2.5}$$

where S is the number of moles of permeant solute, P_s is the solute permeability of the membrane, and $[S]$ is the average concentration of permeant solute within the membrane:

$$\overline{[S]} = \frac{[S]_e + [S]_i}{2}. \tag{2.6}$$

Because there are a number of assumptions made in using Equation 2.5, it should be recognized that P_s will, in general, be a function of permeant solute concentration; the importance of this concentration dependence needs to be checked for particular circumstances.

These two Equations (2.4 and 2.5) are often referred to as the coupled transport equations and can be used to describe the osmotic behavior of a cell with a permeant cryoprotectant present. They are based on the more general coupled transport equations derived from nonequilibrium thermodynamics (Kedem and Katchalsky, 1958).

The advantage of the thermodynamic approach is that the parameters, L_p, P_s, and σ, are not tied to a physical model of membrane transport. For cryobiology, in which extrapolation of osmotic behavior is the dominant concern, rather than membrane biophysics, it is preferable to use the most general formalism. In addition, there are some inconsistencies in the model involving the definition of σ. Thus the physical interpretation of the parameters used in the above model, although pedagogically helpful, should be used with caution unless careful experiments are performed to distinguish physical mechanisms. The interested reader is directed to the review by Kleinhans (1998), in which these issues are discussed, and a two-parameter formalism (i.e., without σ) is reintroduced that may be sufficient for the demands of cryobiological osmotic modeling.

Figure 2.4 shows the typical transient volume changes that will occur in a cell during addition and dilution of a permeant cryoprotectant. The magnitude and duration of the transient volume excursions are dependent on the osmotic properties of the cells, on the concentrations of solutes, and on the temperature. Not only may these transients exceed tolerable limits of cell volume and cell surface increments, particularly during dilution but they also expose cells to high osmotic

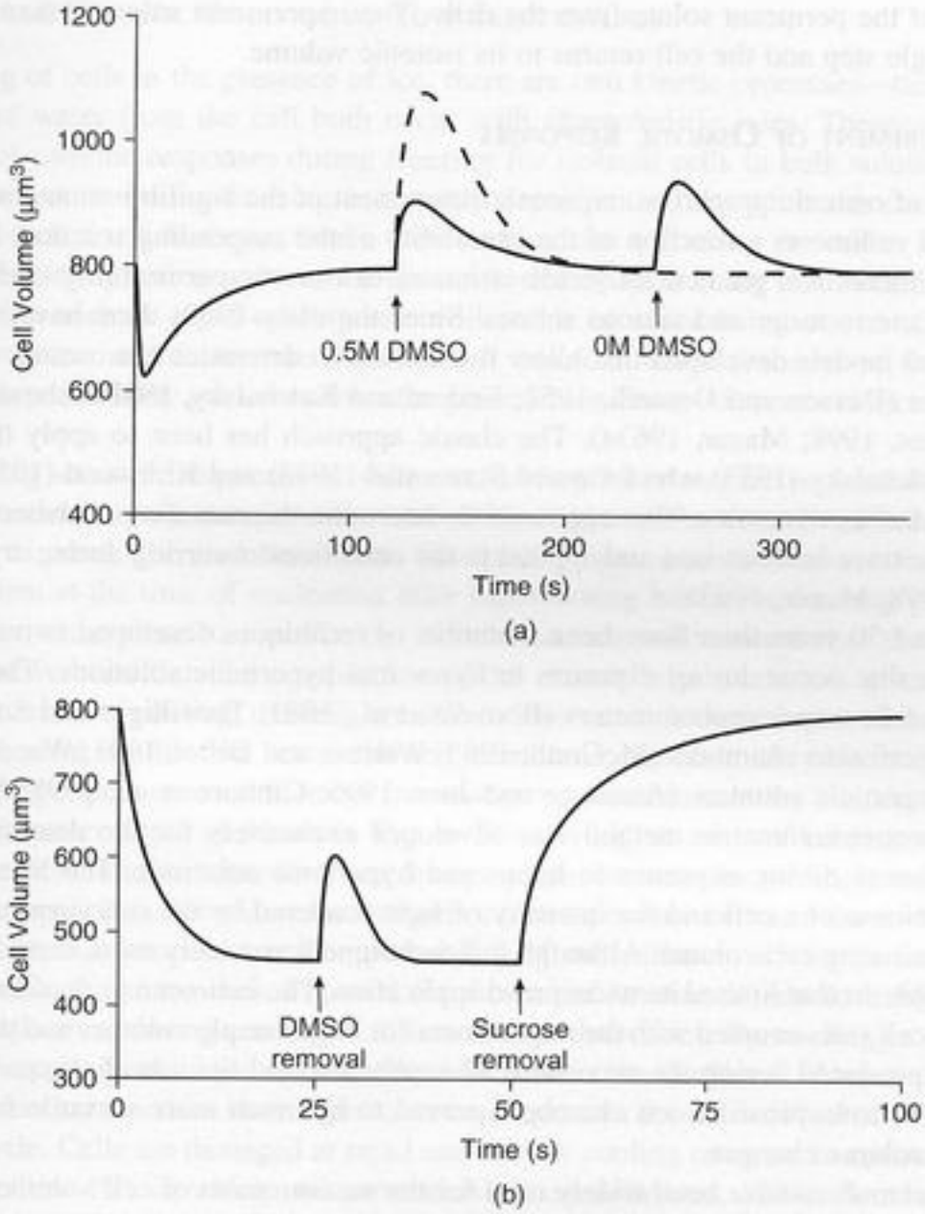

FIGURE 2.5 Cell volume changes during cryoprotectant removal. (a) Cells are initially equilibrated in a 1 *M* Me_2SO solution, then transferred into 0.5 *M* Me_2SO at 120 sec and later into 0 *M* Me_2SO at 240 sec. The dashed line shows the volume change if the cells are placed directly into a 0 *M* Me_2SO solution at 120 sec. (b) Cryoprotectant removal in a sucrose solution. Initially the cells are in equilibrium in a 1 *M* Me_2SO solution and are placed into a solution with 1 *M* sucrose and 1 *M* Me_2SO. At 26 sec, the cells are placed into a solution with 1 *M* sucrose and no Me_2SO. At 52 sec, the cells are placed into an isotonic solution with neither sucrose nor Me_2SO.

gradients across the membrane and high water flux through the membrane, which may also be damaging (Muldrew and McGann, 1990). Strategies to reduce osmotic stresses during removal of cryoprotectants include stepwise dilution and sucrose dilution. In stepwise dilution, the concentration of permeant solute is progressively reduced, allowing enough time between steps for the cell to return to an equilibrium volume to avoid exceeding the osmotic tolerance of the cells. Figure 2.5a compares the dilution of a 1 *M* Me_2SO solution in a single step vs. two steps; the maximum cell volume is substantially lower in the two-step removal. Alternatively, a sucrose dilution (Figure 2.5b) is often used for rapid removal of the cryoprotectant (important if the cryoprotectant exhibits some toxicity) while remaining within the osmotic tolerance of the cells. Sucrose, an impermeant solute, is added to the suspending solution to counteract the water influx that occurs during removal of the permeant solutes from the extracellular solution. The presence of the impermeant solute in the extracellular solution maintains volume excursions and osmotic stresses within tolerable limits

during efflux of the permeant solute from the cells. The impermeant solute is then removed in a subsequent single step and the cell returns to its isotonic volume.

2.2.3 Measurement of Osmotic Responses

Determination of osmotic properties requires measurement of the equilibrium and nonequilibrium changes in cell volume as a function of the osmolality of the suspending solution. Fitting experimental data to theoretical predictions yields estimates of osmotic permeability coefficients of the plasma membrane to water and various solutes. Since the early 1950s there have been a number of mathematical models developed that allow researchers to determine the osmotic parameters of many cell types (Davson and Danielli, 1952; Kedem and Katchalsky, 1958; Johnson and Wilson, 1967; Kleinhans, 1998; Mazur, 1963a). The classic approach has been to apply the methods of Kedem and Katchalsky (1958) who followed Staverman (1948) and Kirkwood (1954) in their use of irreversible thermodynamics. This approach to determine the osmotic parameters of biological membranes has since been revised and applied to the conditions occurring during cryopreservation (Kleinhans, 1998; Mazur, 1963a).

Over the last 50 years there have been a number of techniques developed to measure changes in cell volume that occur during exposure to hypo- and hypertonic solutions. These techniques include stopped-flow spectrophotometers (Boroske et al., 1981; Terwilliger and Solomon, 1981), diffusion and perfusion chambers (McGrath, 1985; Walcerz and Diller, 1991; Woods et al., 1997), and electronic particle counters (Armitage and Juss, 1996; Gilmore et al., 1998; McGann et al., 1982). The spectrophotometric method was developed exclusively for the determination of red blood cell volumes during exposure to hypo- and hypertonic solutions. The linear relationship between the volume of a cell and the intensity of light scattered by the cell suspension is used as a basis for monitoring cell volume. Although this technique was widely used, there were a number of inherent problems that limited its widespread application. The indirect nature of the measurement of the average cell sizes coupled with the requirement for large sample volumes and the considerable shear stresses produced during the procedure adversely affected the use of stopped-flow spectrophotometers. Microscopic diffusion chambers proved to be much more versatile for the determination of cell volume changes.

Diffusion chambers have been widely used for the measurement of cell volume changes since the early 1940s. Many innovative designs have been documented that allow cell suspensions to be viewed using a standard light microscope while the extracellular media is rapidly changed (Aggarwal et al., 1984; Gao et al., 1996; McGrath, 1985; Walcerz and Diller, 1991; Woods et al., 1997). Changes in the physical dimensions of individual cells (typically cross-sectional area) are used to calculate the change in cell volume. By observing and measuring this response, calculations can be made to determine cellular osmotic parameters and critical osmotic limits, though the technique cannot be used to measure solute permeabilities.

Electronic particle counters have also been used to determine cell membrane permeability characteristics (Acker et al., 1999b; Armitage and Juss, 1996; Buckhold et al., 1965; Gao et al., 1998; Gilmore et al., 1998). On the basis of the principle developed by Coulter, cells traversing an aperture in an electronic particle counter displace a volume of conducting fluid proportional to the volume of the cell (Adams et al., 1967; Gregg and Steidley, 1965; Hurley, 1970; Kubitschek, 1958). This displacement results in an electronic pulse that allows for the determination of cell concentration (pulse count) and cell volume (pulse height). Interfacing an electronic particle counter with a computer allows one to track the sequence of pulses and to perform detailed studies on the dynamic osmotic response (McGann et al., 1982). Electronic particle counters provide a rapid, reproducible means to collect the equilibrium and kinetic cell volume data needed to perform osmotic modeling and to extract cell membrane permeability parameters. Although diffusion chambers and electronic particle counters have inherent advantages and disadvantages, both techniques are useful in studies on the osmotic response of cells (Acker et al., 1999b).

2.2.4 Cellular Osmotic Behavior at Low Temperatures

During cooling of cells in the presence of ice, there are two kinetic processes—the growth of ice and the loss of water from the cell both occur with characteristic rates. These are the primary determinants of osmotic responses during freezing for isolated cells in bulk solution. The rate at which cells lose water during freezing is strongly dependent on the particular cooling profile. If cooling proceeds at a constant rate, then a slow cooling rate will allow the cell to remain close to osmotic equilibrium by losing water at the same rate that water is being lost from the extracellular solution (to form ice). A rapid cooling rate will result in water being converted to ice in the extracellular solution at a faster rate than the cell can lose water (because of the permeability barrier); thus the osmolality gradient across the plasma membrane continues to increase as cooling proceeds.

The differing osmotic responses at different cooling rates can be simulated using the cell permeability model. In bulk samples, the rate of freezing must be considered because the latent heat of fusion is often liberated faster than the cooling apparatus can remove the heat. This is mainly a problem at the time of nucleation after supercooling below the freezing point, when the majority of the water turns to ice. For our simulation, we will assume that the latent heat is completely removed and thus the composition of the extracellular solution is given by the phase diagram. At low rates of cooling, the cells are able to lose water through the membrane fast enough to maintain chemical equilibrium across the cell membrane; therefore, they become progressively dehydrated during exposure to solutions of increasing concentrations as the temperature is lowered (Mazur, 1963a); an example is shown in Figure 2.6a. At higher rates of cooling, the rate of ice growth is faster than cellular water loss, and the cytoplasm becomes increasingly supercooled. The amount of supercooling is defined as the difference in temperature between the melting point of a solution and its actual temperature. The probability of nucleation in a solution increases as the amount of supercooling increases. Figure 2.6b shows the amount of supercooling in cells as a function of temperature during cooling at different rates. Increasing the cooling rate increases the amount of supercooling, which correlates with an increase in the likelihood of intracellular freezing.

Cellular osmotic responses have significant consequences for the survival of cells following a freeze–thaw cycle. Cells are damaged at rapid and at slow cooling rates, so an optimal cooling rate exists between these two in which cell survival is maximal (Mazur, 1984). Because "rapid" and "slow" cooling are relative terms, related to the water permeability of a given cell type, it is clear that the optimal cooling rate will also be specific to a given cell type. The current understanding of the cellular response to freezing is that there are two competing mechanisms of injury; one that occurs during slow cooling but that can be avoided by increasing the cooling rate, and another that occurs during rapid cooling but that can be avoided by decreasing the cooling rate (Mazur et al., 1972). Slow-cooling injury has been shown to accumulate with exposure to high concentrations of solutes (though it manifests itself on dilution to isotonic; Lovelock, 1953b) and is often referred to as "solution-effects" injury, whereas rapid cooling injury is associated with the formation of intracellular ice.

That rapid cooling injury should be intimately related to the water permeability of a particular cell type follows from the fact that the cytoplasm has to be supercooled before intracellular ice can form. That solution-effects injury should also be related to a cell's water permeability may be related to the temperature at which a cell is exposed to concentrated solutes; the reduced rate of injury at low temperatures may prevent any accumulation of damage from occurring. Although this explanation provides an understanding of why solution-effects injury would not occur at rapid cooling rates, it does not explain the curious relationship between the cooling rates at which this injury declines and the water permeability of the cell. Indeed, over several orders of magnitude of water permeabilities, the optimal cooling rate for a particular cell is directly related to the cell's water permeability (Mazur, 1984). If the two types of injury were truly independent, then we would expect to see some cells with a broad plateau at the optimum, whereas other cells would show

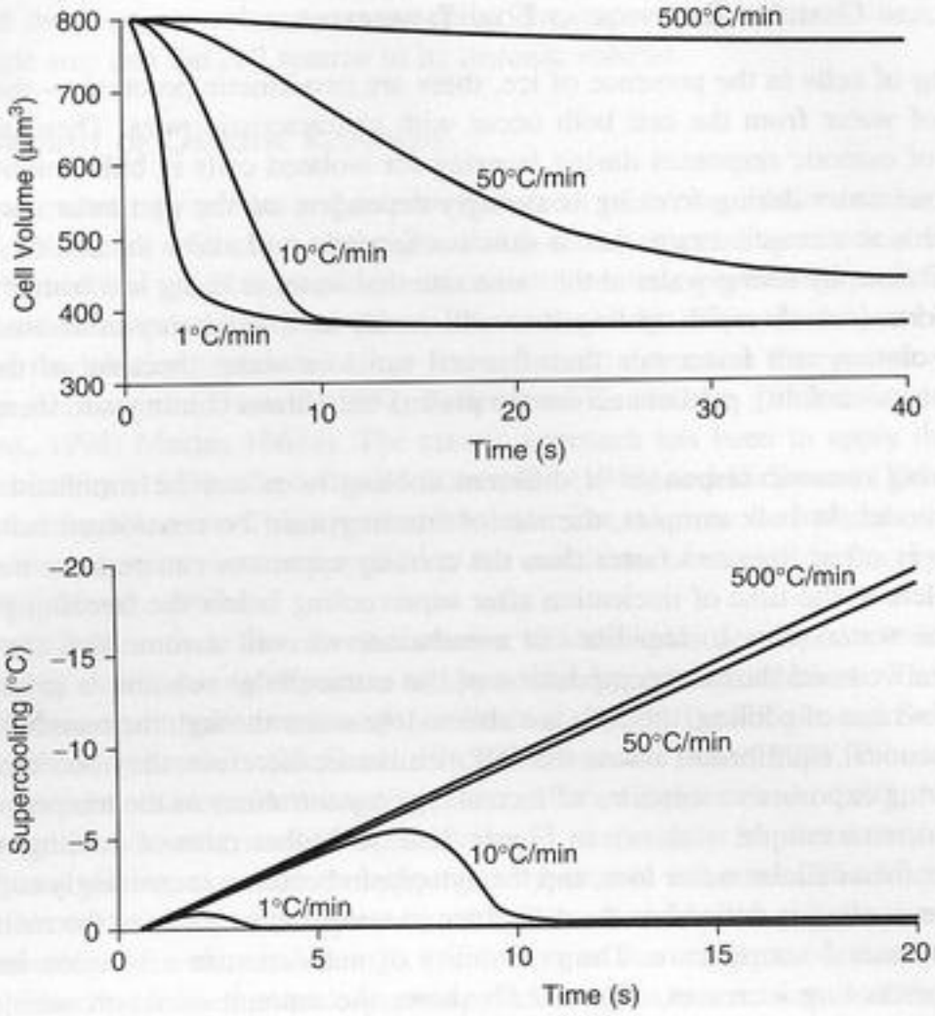

FIGURE 2.6 (a) Cell volume changes are shown for various constant cooling rates. (b) Supercooling of the cytoplasm is shown for the same cooling rates (note that the timescale is changed for clarity).

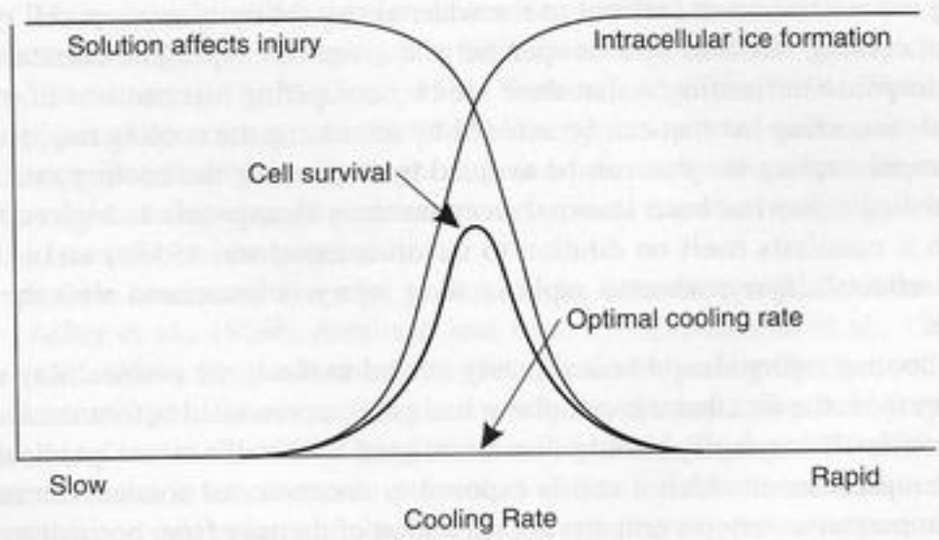

FIGURE 2.7 Overlapping injury mechanisms that occur at slow cooling rates (solution effects injury) and at rapid cooling rates (intracellular ice formation) are shown with the resulting cell survival vs. cooling rate curve.

overlapping injuries with no survival at any cooling rate. Instead we always see an optimal cooling rate that falls away sharply on both sides (Figure 2.7). Thus, both rapid- and slow-cooling injury appear to be related to the presence of an osmotic pressure gradient across the plasma membrane,

although other factors such as temperature are clearly important. Nevertheless, this indicates that a reduction in the osmotic pressure gradient should result in less injury at any given cooling rate.

2.2.5 Two-Step and Nonlinear Protocols

During cryopreservation, an optimal cooling rate is normally selected to avoid intracellular ice formation while minimizing the deleterious effects of concentrated solutes. Constant cooling and warming rates are therefore often mistakenly considered as fundamental variables in cryobiology. An alternate strategy to minimize damage is the "two-step method" for cooling (Farrant et al., 1977; McGann and Farrant, 1976a, 1976b), in which samples are cooled rapidly to a constant subzero temperature and held at that temperature to allow sufficient osmotic water efflux to avoid intracellular freezing on the subsequent rapid cooling to a low storage temperature (–196°C). This approach allows adequate osmotic dehydration at an intermediate temperature (Farrant et al., 1977), which reduces the probability of intracellular nucleation. A subsequent rapid cooling step then minimizes damage caused by exposure to the concentrated solutes at lower temperatures.

2.3 CRYOPROTECTIVE ADDITIVES

Many chemicals have now been identified as having a cryoprotective action; by adding these cryoprotective additives (CPAs) to a cell suspension, the survival following freezing and thawing can be substantially increased. CPAs are broadly classed as penetrating (for chemicals that will diffuse through the plasma membrane and equilibrate in the cytoplasm) and nonpenetrating (chemicals that do not enter the cytoplasm). Penetrating CPAs are small, nonionic molecules that have a high solubility in water at low temperatures and low cellular toxicity. Their activity can be understood by their effect on the freezing point. Most important, the presence of penetrating CPAs lowers the concentration of the salts that are normally found in physiological solutions for a given temperature (below the freezing point, when ice formation causes the concentration of these salts). They do this by lowering the amount of ice present for a given temperature (Figure 2.8a) as well as by acting as a secondary solvent for salt (Pegg, 1984). Thus, a cell that is being frozen in a simple saline solution will see a higher sodium chloride concentration at –10 °C than will the same cell in a saline solution with 1 *M* dimethyl sulfoxide at the same temperature (Figure 2.8b). By lowering the temperature at which the cell is exposed to the increased extracellular solute concentration, the magnitude of injury, and the kinetics at which damage accumulates, is reduced. Penetrating CPAs are able to greatly mitigate slow-cooling injury but provide little protection against rapid-cooling injury.

Nonpenetrating CPAs are generally long-chain polymers that are soluble in water and that have large osmotic coefficients (they increase the osmolality far in excess of their molar concentration). They are thought to act by dehydrating the cell before freezing, thereby reducing the amount of water that the cell needs to lose to remain close to osmotic equilibrium during freezing. The cytoplasm does not supercool to the same extent, and therefore intracellular ice becomes less likely at a given cooling rate. These compounds provide little protection from slow-cooling injury.

The inability of nonpenetrating CPAs to provide protection against solution-effects injury seems to indicate that the intracellular concentration of solutes is at least as important as the extracellular concentration for this type of injury. This indicates that the aforementioned relationship between solution-effects injury and a cell's water permeability is caused by the intracellular solute concentration. During rapid cooling, the intracellular solute concentration remains low because the cell cannot lose water fast enough to remain in equilibrium with the extracellular solution. This delays the effects of having a highly concentrated intracellular solution to lower temperatures (where it has a less damaging effect). Thus it appears that solution-effects injury may be dependent on the solute concentration of both the intracellular and extracellular solutions as well as on the integrated exposure to these solutions as a function of temperature.

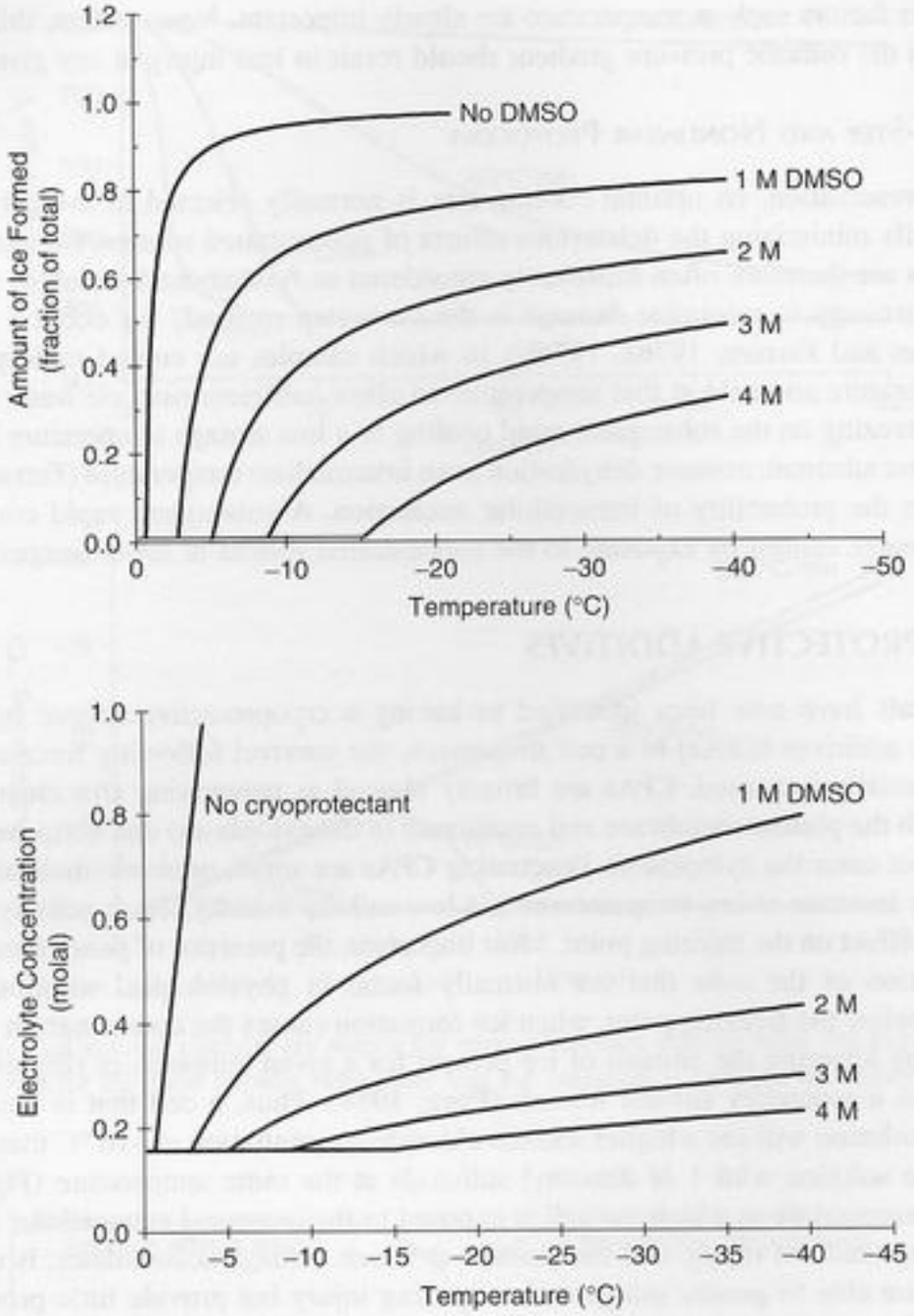

FIGURE 2.8 (a) The amount of ice formed in a simple physiological solution (0.15 *M* NaCl) containing different concentrations of Me_2SO. (b) The concentration of electrolyte in a simple physiological solution (0.15 *M* NaCl) containing different concentrations of Me_2SO. (Data were calculated from Pegg et al., 1987.)

Studies of natural systems that survive extreme environmental stress have shown that one of the adaptive mechanisms used is the overproduction and accumulation of sugars (Crowe and Crowe, 2000). Recent work has shown that these "natural cryoprotectants" are capable of stabilizing and preserving the biological activity of proteins, viruses and bacteria (see Chapter 21, this volume). High concentrations of mono- and disaccharides can act to protect biological structures during dehydration through the formation of a stable glassy matrix (Buitink et al., 1998; Crowe et al., 1998; Wolfe and Bryant, 1999) or through binding to sites previously stabilized by water (Crowe et al., 1993a, 1993b; Gaber et al., 1986). In the absence of more traditional cryoprotectants (e.g., Me_2SO, glycerol), sugars have been shown to be effective protectants in mammalian cell cryopreservation (Eroglu et al., 2000). Although it has been shown that sugars need to be present on both sides of the plasma membrane to be maximally effective (Chen et al., 2001; Eroglu et al., 2000, 2002), the mechanisms by which these "penetrating" agents function to protect cells during cryopreservation are relatively unknown. Further work in this area is warranted.

2.4 MECHANISMS OF CRYOINJURY

2.4.1 Slow-Cooling Injury

During slow cooling, the cell is able to maintain osmotic equilibrium with the extracellular solution through dehydration. Although osmotic equilibrium is maintained and intracellular ice is avoided, damage can still occur. There are two basic mechanisms by which damage is thought to occur during slow cooling: solute toxicity (Lovelock, 1953b; Mazur et al., 1972) and physical changes to the cell induced by excessive cell shrinkage under an osmotic stress (Lovelock, 1953a; Meryman, 1970, 1974; Steponkus and Wiest, 1978). During slow cooling, the increase in concentration of the extracellular and intracellular solutes occurs because of the formation of external ice and the resulting efflux of water from the cells, respectively (Mazur, 1963a). Although the molecular mechanisms by which concentrated solutes damage a cell are unknown, it is foreseeable that alterations in the chemical equilibrium of the cell could result in numerous biophysical and biochemical changes that could lead to cell death. One of the mechanism proposed by Lovelock was a lyotropic effect on the plasma membrane by highly concentrated salt (Lovelock, 1953b); little progress has been made in this area since then.

The second mechanism by which slow cooling is thought to injure cells is by the shrinkage of the cell that results from exposure to hypertonic solutions. Work with red blood cells showed that cells remained intact during exposure to high concentrations of solutes, but then lysed when they were diluted back to isotonic (Lovelock, 1953a). The so-called posthypertonic lysis was thought to be a result of salt loading caused by a limitation on the minimum size that a cell could attain through osmotic shrinkage. Once the cell had reached its minimum volume (the membrane simply could not collapse any further about the physical structure of the cell interior), it could no longer lose water to maintain osmotic equilibrium. The gradient in chemical potential could only be decreased by the movement of salt from the extracellular solution into the cytoplasm. On thawing, the cytoplasm would have a higher than isotonic concentration of salt and would draw water in to reach equilibrium. If the swelling-induced expansion of the cell surpassed the yield strength of the membrane, then the cell would lyse (Zade-Oppen, 1968). Later it was shown that there was indeed an uptake of sodium during hypertonic exposure, but there was no minimum volume that could be surpassed osmotically; the previous result had been an artifact of the measurement technique (Farrant and Woolgar, 1972).

A variant on the minimal volume hypothesis suggested that the osmotic pressure gradient that developed once the minimum volume had been reached led to a mechanical stress on the cell (caused by an induced hydrostatic pressure gradient) that caused injury (Meryman, 1974), though it should be noted that this hypothesis requires a minimum volume below which the cell could not shrink osmotically (something that seems not to occur). Alternatively, it has been proposed that the surface area of the cell is reduced as a result of a loss in membrane material during slow cooling. Shrinkage of the cell leads to membrane fusion events, triggered by the dehydration of the membrane itself, whereby some of the plasma membrane is internalized as vesicles. This reduction in the surface area of the plasma membrane reduces the magnitude of expansion that the cell can tolerate during dilution to isotonic (Steponkus and Wiest, 1978).

Another, more recent hypothesis (Muldrew et al., 2000a) suggests an alternative mechanism for salt loading during hypertonic exposure in which cytoplasmic proteins with salt bridges are brought into solution through the interaction of dissolved ions with fixed charges on the proteins. This sinking of ions by cytoplasmic proteins is the putative mechanism behind the experimentally observed uptake of sodium chloride during hypertonic exposure (Farrant and Woolgar, 1972). On thawing (or dilution to isotonic), the excess ions are released back into solution in the cytoplasm, and the cell may swell beyond its elastic limit.

2.4.2 Intracellular Ice Formation

Intracellular ice formation (IIF) occurs when a cell is unable to maintain equilibrium with the external environment. During rapid cooling, the formation of ice and the concentration of extracellular solutes

occur too quickly for the cell to respond by exosmosis. Thus, the cytoplasm becomes increasingly supercooled, and there is a corresponding increase in the probability of intracellular freezing. Supercooling, however, is not the only prerequisite condition for IIF. Because the cell membrane serves as an effective barrier to ice growth (Chambers and Hale, 1932; Luyet and Gibbs, 1937; Mazur, 1965) and the cytoplasm contains few effective nucleators (Franks et al., 1983; Rasmussen et al., 1975), the mechanism by which the supercooled cytoplasm becomes nucleated has been the subject of much debate.

There is evidence to indicate that extracellular ice and the plasma membrane are involved in the initiation of IIF. Many cells in an isotonic solution will freeze intracellularly between –5° and –15°C (reviewed in Mazur, 1965). However, IIF will only occur under these conditions when extracellular ice is present. Attempts to observe ice formation in a supercooled cytoplasm in the absence of extracellular ice have so far been unsuccessful. In all cases, the cell cytoplasm reaches temperatures very close to the homogeneous nucleation temperature before nucleation occurs (Franks et al., 1983; Rasmussen et al., 1975). Furthermore, investigations with liposomes, membranes with no intracellular components to act as nucleation centers, demonstrated IIF at temperatures above –10°C (Callow and McGrath, 1985).

The cell membrane has been shown to be an effective barrier to ice growth (Mazur, 1965). If extracellular ice is an important element in the nucleation of ice in the cytoplasm, then the plasma membrane must be involved. Work with nonacclimated and acclimated plant protoplasts has shown that compositional alterations of the plasma membrane during cold acclimation change the conditions under which IIF occurs (Dowgert, 1983; Pitt and Steponkus, 1989; Steponkus, 1984). Similarly, work with hydrophilic antifreeze proteins has suggested that by promoting a closer interaction of ice with the plasma membrane, the incidence of IIF can be affected (Ishiguro and Rubinsky, 1994; Larese et al., 1996; Mugnano et al., 1995). In fact, extracellular ice appears to grow right through the plasma membrane when antifreeze proteins are present (Larese et al., 1996; Mugnano et al., 1995). This may be a result of the creation by the amphipathic nature of the antifreeze proteins of a hydrophobic surface on a growing ice crystal that allows the ice to penetrate the hydrophobic region of the membrane.

At present, there are three dominant hypotheses that attempt to explain the mechanism by which extracellular ice interacts with the plasma membrane to initiate intracellular ice formation. The protein-pore theory of Mazur (1965) was motivated by the observations that supercooled cells could freeze internally well above the homogenous nucleation temperature and that the plasma membrane was an effective inhibitor of ice only above a certain temperature. Mazur hypothesized that external ice could seed the supercooled cytoplasm by growing through aqueous pores in the membrane. For this to occur, the tip radius of the growing ice crystal must approximate the radius of the pores in the plasma membrane. The Kelvin equation relates temperature to the smallest stable radius of an ice dendrite through the freezing-point depression because of the curvature of the tip (Acker et al., 2001; Elliott, 2001; Mazur, 1965). For any given pore size, therefore, there is a corresponding temperature below which ice should be able to grow through the pore. The mechanism of injury is thought to be an enlarging of the membrane pore by the process of recrystallization during warming (a reduction in the ratio of surface area to volume by the loss of ice from regions of high curvature and accretion at regions of low curvature). In support of this mechanism of IIF, recent studies have suggested that ice growth through stable proteinaceous membrane pores is responsible for the intercellular propagation of intracellular ice that is observed in confluent monolayers (Acker and McGann, 1998; Acker et al., 2001) and tissues (Berger and Uhrik, 1996), although as we will discuss later, this type of intracellular ice does not necessarily correlate with membrane failure on thawing.

Disruption of the plasma membrane has been proposed as an alternative mechanism by which extracellular ice can nucleate the cytoplasm. Working with unfertilized eggs of the sea urchin *Hemicentrotus pulcherrimus*, Asahina proposed in 1962 that the cause of intracellular ice formation was damage to the plasma membrane, concluding that membrane damage precedes IIF. Similarly,

Steponkus and Dowgert (1984) were able to directly observe ruptures in the membranes of plant protoplasts immediately before the formation of intracellular ice. They later suggested that the disruption occurs as a result of the development of electrical transients created by charge separation at the interface of the growing ice and the aqueous solution (Steponkus et al., 1984). Motivated by the fact that cells can be damaged at 0°C in the absence of ice because of an osmotic pressure gradient remarkably similar to those produced during freezing, Muldrew and McGann proposed that membrane damage may be a result of a critical osmotic pressure gradient across the membrane (Muldrew and McGann, 1990). They noted that the occurrence of IIF correlated with the peak water flux across the plasma membrane and mistakenly postulated that the movement of water could create an outward force on the membrane that could lead to rupture (Muldrew and McGann, 1994). A refined version of this hypothesis will be developed in a later section. In all variants of this basic mechanism, there is damage to the plasma membrane before IIF, and there is a transmembrane ice crystal following IIF; therefore, recrystallization on warming could serve to enhance the preexisting lesion.

Intracellular ice that results from an intracellular nucleation event has been hypothesized, where the membrane of a cell can behave as a nucleation site for internal ice when acted on by extracellular ice. Though the detailed mechanism by which this might occur has yet to be explored, the thermodynamics of the process have been found to agree with experimental data. Toner initially proposed the idea of surface-catalyzed heterogeneous nucleation in 1990 (Toner et al., 1990). The theory attributes the formation of intracellular ice to the ability of external ice to interact with, and alter, the structure of the cell membrane. The nature of this interaction could be chemical, electrical, mechanical, ionic, or thermal, but it appears to make the plasma membrane an effective nucleator. The external ice can therefore induce the formation of intracellular ice without physically disrupting the integrity of the plasma membrane (Toner et al., 1990). A second form of intracellular nucleation, volume catalyzed IIF, was also proposed to account for IIF events that occur at low temperatures when the cell has lost much of its cytoplasmic water (Toner et al., 1990). The mechanism by which intracellular ice leads to failure of the plasma membrane on thawing is presently lacking in this model.

The three mechanisms proposed for the formation of intracellular ice each assume a different role for the plasma membrane. In the protein-pore theory, the cell membrane is an effective inhibitor of external ice only above the temperature at which the ice crystals are too large a size to propagate through permanent transmembrane pores. The membrane failure hypothesis requires that the integrity of the membrane be disrupted and postulates that cell damage precedes the initiation of IIF. Finally, the surface-catalyzed nucleation theory suggests that the barrier properties of the cell membrane do not have to be compromised for the initiation of internal ice. Although each of these theories proposes an alternative means by which ice can enter the cell, each one supports the assertion that it is the extracellular ice interacting with the plasma membrane that is responsible for the formation of intracellular ice.

Although the mechanism of IIF is still controversial, the ability to predict the probability of IIF for a given freezing protocol has been attained using phenomenological curve fitting techniques. This use of mathematical models that can predict the occurrence of IIF has been motivated by the potential utility of these models in the design of cryopreservation protocols and their ability to further the investigation of the mechanisms of IIF. Numerous phenomenological models (Mazur, 1984; Pitt and Steponkus, 1989; Pitt et al., 1991, 1992) have been developed that use statistics and an understanding of the conditions surrounding the cell just before freezing to predict the likelihood of intracellular freezing. Mechanistic hypotheses have also been developed into mathematical models by fitting parameters to IIF data rather than determining their values from more fundamental principles (Muldrew and McGann, 1994; Toner et al., 1990). These two approaches, phenomenological and mechanistic, although fundamentally different in design, have been shown to give relatively similar end results (Karlsson et al., 1993). Each model is able to predict with some degree of certainty the degree of intracellular ice formation in a cellular system under well-defined conditions.

2.5 INVESTIGATION OF THE MECHANISMS OF CRYOINJURY

2.5.1 Introduction

The nature of cellular injury caused by freezing and thawing is undoubtedly complex. Many of the investigations aimed at understanding this injury can be said to fall into the study of first-order mechanisms of injury. This type of injury refers to the catastrophic events that lead to cell death. It is highly likely that there are second-order effects that occur in cells that represent sublethal injury (i.e., the cell is able to repair the damage once metabolism is restored). These second-order effects may be quite independent of the first-order effects that have been studied thus far, adding multiple injuries to the "two factors" that are normally considered. Indeed, the possibility of overlapping modes of first-order damage is also a reasonable proposition. The common behavior of the wide variety of cell types and experimental arrangements might only reflect a common proximal cause (e.g., an osmotic pressure gradient), whereas independent molecular mechanisms could be the detailed causes leading to a common endpoint (e.g., cell lysis). Nevertheless, it is important to identify the primary modes of injury before studying the secondary modes because we need to understand this injury before we can properly run controlled experiments in which we separate out the overlapping effects.

Our own program of investigation into the mechanisms of cryoinjury has exploited two fundamental tools to feed the processes of building theories, generating hypotheses, experimentally testing the hypotheses, and then returning full circle and refining the theories. We are still going around this loop; certainly we are ahead of where we started, but still with many interesting questions and uncertainties remaining. This section will attempt to give an overview of our current thinking along with some of the developmental work that led us to this position. Our primary conceptual tool in this progression has been the mathematical modeling of the osmotic responses of cells. By simulating cellular responses on a computer, we can visualize our idealized model of what the cells are experiencing during freezing and thawing under arbitrary conditions. This tool has allowed us to refine vague notions about how cells might be injured into precise hypotheses that can be tested. Our primary experimental tool has been the cryomicroscope. The computer-controlled cryostage allows an incredibly diverse range of thermal protocols to be generated, and it allows direct visual observation of the cells during the process. Individual cells can be followed during freezing and thawing, and after, allowing direct testing of various cellular parameters. Through the use of these two tools we are beginning to develop a theoretical understanding of first-order cryoinjury, and we have collected a substantial body of experimental work that puts limits on what alternative theories will have to account for. The following descriptions will largely focus on our own developments, leaving different perspectives to be advanced in other chapters.

2.5.2 Osmotic Poration and Intracellular Ice Formation

Hydrophilic pores have been postulated to form spontaneously in the lipid bilayer through thermal fluctuations (Bordi et al., 1998; Glaser et al., 1988; Paula et al., 1996). These pores are small, short lived, and widely spaced, though they are stable enough to substantially raise the permeability of cell membranes to small ions (Bordi et al., 1998; Paula et al., 1996). In fact, the permeability of cell membranes to ions (e.g., sodium) that is predicted from considering the transport of ions in the absence of pores is at least three orders of magnitude lower than the measured value (Paula et al., 1996). If the membrane is subjected to a high potential difference, then these thermal pores can expand through a process of reversible electric breakdown to become relatively stable (Abidor et al., 1979; Glaser et al., 1988). Similarly, the presence of thermal pores during periods of large osmotic water flux will allow the water molecules to preferentially pass through the pore. These water molecules will have collisions with the edge of the pore that tend to enlarge the pore, allowing more water to flow through it if the driving force is sufficient. This positive feedback loop will proceed as long as there is a local osmotic pressure gradient to drive water movement. A corollary

of this hypothesis is that cells that are subject to high osmotic stresses would require dedicated water channels (such as aquaporins) to avoid the potential injury of osmotic poration. This corollary is supported by the high concentration of aquaporins in red blood cells, the cells of the renal collecting ducts, and other cells that are subject to osmotic stresses (Verkman et al., 1996).

Data from osmotic pulse experiments (a technique for loading impermeable solutes into a cell) seem to indicate that osmotic stresses can, in fact, lead to the presence of reasonably long-lived aqueous pores in the membrane (Franco et al., 1986). We have pursued the hypothesis that pores generated by osmotic stresses are responsible for intracellular ice formation during rapid cooling and its concomitant injury. Simply put, the pores form spontaneously because of thermal fluctuations and are then expanded by the interaction of water with the edge of the pore during periods of high osmotically driven water movement. The pore will collapse once the water flux diminishes, but if it opens wide enough when extracellular ice is present (i.e., during freezing), then the supercooled cytoplasm can be seeded by ice growth through the pore. The minimum size of pore that will lead to IIF is given by the relationship between the minimum radius of an ice crystal and the temperature (given by the Kelvin equation; Elliott, 2001). The formation of intracellular ice is not specifically tied to the lysis of the cell on thawing. If the pore through which the ice crystal grows is below the critical size that causes cellular lysis, then the pore will simply reseal when the ice melts. A complicating factor is that ice crystals will undergo recrystallization during warming (the ratio of surface area to volume is reduced). If the transmembrane ice crystal expands during warming, the pore will become enlarged. Rapid warming will tend to lead to resealing (if the pore is below the critical size), whereas slow warming will lead to cell lysis.

When isolated cells are cooled on a cryostage to a temperature just below the freezing point and ice formation is seeded, the ice can be observed to grow around the cells confining them to unfrozen channels between ice dendrites. Unless some insult has been imposed on the cells, it is a universal observation that the ice never grows through the plasma membrane into the cytoplasm, even in the absence of cryoprotective compounds. For this reason, the increased solution concentration in the unfrozen channels imposes an osmotic gradient across the cell membrane and the cell loses water until the osmotic pressure of the cytoplasm equals that of the unfrozen solution. If pores are present in the membrane when extracellular ice is present, then their size must be smaller than the minimum radius of curvature of an ice crystal at that temperature for the membrane to retain this barrier property.

Because the hypothesis of osmotic poration predicts that these pores are not linked to ice growth, except by the osmotic conditions generated by the formation of ice, we postulated that they could be formed through osmotic stresses at temperatures above the freezing point. If large, stable pores could be produced, then we hypothesized the cell membrane would lose its barrier properties to ice growth just below the freezing point. To test this experimentally, we used dimethyl sulfoxide (Me_2SO) to produce an osmotic gradient across the cell membrane at 0°C. Because cells are relatively permeant to Me_2SO, the osmotic gradient developed was transient, and thus we could add it in one dose for a maximal osmotic pressure gradient or in small increments to keep the osmotic gradient low while still developing the same concentration of Me_2SO in the cells. The Me_2SO was then diluted and the cells placed on a cryostage, and ice formation was seeded just below the freezing point. The hypothesis was supported, as the proportion of cells that lost their barrier properties was a function of the originally imposed osmotic pressure gradient (Table 2.1; Muldrew and McGann, 1990).

These data gave us a powerful tool to investigate whether these pores were the cause of intracellular ice, as we could correlate the temperature dependence of IIF and osmotic poration in the absence of ice. If the correlation existed, then we would have additional confidence that the same mechanism was responsible for both phenomena.

Our osmotic modeling work that had first led us to suspect that IIF was caused by excessive osmotic pressure gradients suggested a line of inquiry using cryomicroscopy that would help to answer this question. Cryomicroscopy would allow us to actually measure some of the physical

TABLE 2.1
Membrane Damage Caused by the Osmotic Stress of Rapid Addition of a Permeant Cryoprotectant at 0°C; Toxicity Effects Are Shown to be Negligible by the Low Rate of Injury with Slow Addition

Me_2SO (*M*)	Rapid-Addition Rupture (%)	Slow-Addition Rupture (%)
3	11	2
4	47	0
5	88	2

parameters that had been implicated in the literature on IIF with direct visualization of the formation of IIF in individual cells. In addition, we could also use this data to see whether the osmotic pressure gradients associated with IIF correlated with osmotic poration in the absence of ice.

We chose to combine the modeling of cellular responses to osmotic stresses that we had developed with cryomicroscopic observation of IIF so that we could estimate the physico-chemical environment during freezing and thawing. The two experimental protocols that we focused on were based on freezing cells at constant cooling rates and holding cells at constant temperatures (subfreezing) while seeding ice. The convection cryostage was ideal for these experiments, as it was possible to supercool the sample without ice forming in the extracellular compartment. For the constant cooling experiments, ice had to be seeded just below the freezing point, and then the cells had to be held at that temperature to bring them into osmotic equilibrium (so that our simulations would give reasonable estimates of the conditions). For both of these experimental protocols we used cells that were equilibrated in several different concentrations of cryoprotectant. This helped to prevent any artifacts that might have been caused by cellular injury from exposure to the freezing environment (the solution-effects injury), and it also gave altered conditions for the formation of IIF so that we could look for mechanisms.

The isothermal technique showed, first of all, that IIF was not likely to be caused by electrical transients at the ice interface, as suggested by Steponkus et al. (1984). The magnitude of charge separation at an ice interface is proportional to the interface velocity, but when we measured this, we found that the formation of IIF occurred at lower velocities as the concentration of cryoprotectant was increased (Muldrew and McGann, 1990). With the buffering action of the cryoprotectant, we expected either the opposite trend or an independence from cryoprotectant.

The comparison between the isothermal experiment and the constant cooling experiment led us to initially discount the hypothesis, first postulated by Mazur, that extracellular ice was able to grow through membrane-bound proteinaceous aqueous pores (Mazur, 1960, 1965, 1966). Although this theory is very similar to the osmotic poration theory, the size of the pores is independent of water flux (and is also very small). If the temperature and composition of the solution determine the minimum radius of a growing ice crystal, then there should be a characteristic IIF temperature for a given solution. When we compared the temperature associated with 50% IIF, for each solution, between the isothermal and the constant cooling experiments, we found that this temperature was about a factor of two lower in the constant cooling experiment in each case (Muldrew and McGann, 1990). This conclusion is being reevaluated in light of our recent work on innocuous intracellular ice formation (see following).

This technique was unable to discount intracellular heterogeneous nucleation, as hypothesized by Levitt (Levitt and Scarth, 1936) and others (Pitt and Steponkus, 1989; Toner et al., 1990), as being responsible for IIF. In fact the isothermal experiment supported the hypothesis. The amount of supercooling that correlated with IIF increased as the temperature of IIF decreased (because of

increasing cryoprotectant concentration; Muldrew and McGann, 1990). If the likelihood of nucleation gets smaller as solute is added to a solution, then we would expect the degree of supercooling required for IIF to similarly increase.

These experiments were also consistent with the hypothesis of osmotic poration being responsible for IIF. The osmotic pressure gradient that correlated with IIF decreased as the temperature of IIF decreased (again because of increasing cryoprotectant concentration; Muldrew and McGann, 1990). This is expected because of the relationship between the critical pore size and temperature. We were able to extrapolate the relationship between the osmotic pressure gradient and the temperatures that correlated with 50% IIF to 0°C to compare them with the osmotic pressure gradient that gave 50% osmotic poration from rapid addition of Me_2SO. The values matched exactly (Muldrew and McGann, 1990), giving us confidence in this interpretation. We further tested the osmotic poration hypothesis by looking for the presence of membrane pores immediately after intracellular ice formation. By performing an isothermal experiment in which 50% of the cells underwent IIF, then warming them to just above the freezing point (so that both intracellular and extracellular ice would melt but no metabolic repair could occur), and then cooling to just below the freezing point and seeding ice formation, we were able to correlate IIF with the loss of membrane barrier properties for individual cells. We found that although 7% of cells that did not form IIF had lost their barrier properties (perhaps because of the fact that no cryoprotectant was used), 94% of the cells that had formed IIF were also found to have a pore in the membrane (indicating that some resealing of osmotic pores probably occurred).

Although this evidence strongly supported osmotic poration as the mechanism by which IIF occurred, it also weakly supported a mechanism based on intracellular nucleation. To further test the hypothesis, we developed a physical model of how the water flux might lead to a loss of membrane integrity. The theory that we developed was based on the frictional drag of water moving through the membrane under an osmotic stress (through a solubility/diffusion mechanism rather than through pores). We mistakenly generalized the frictional force to an outward pressure on the membrane (because the membrane moves inward during water efflux, the actual hydrostatic pressure must also be inward) and postulated that this pressure could exceed the yield strength of the membrane (Muldrew and McGann, 1994). When we calculated the magnitude of the "pressure" that correlated to IIF, it matched exactly the hydrostatic pressure required to rupture a lipid membrane. This unlikely coincidence, coupled with the strong experimental support for an osmotically induced membrane defect, led to our inability to see the obvious flaw.

Fortunately, the reduction of the theory to a practical implementation was carried out using statistical arguments and phenomenological parameters and was thus not tied to the interpretation of a water pressure. The equation describing the probability of IIF was based on a critical water flux, which we still hypothesize as the driving force for pore expansion. Unfortunately, our error served to divert attention from this mechanism for IIF despite the experimental support for the general hypothesis of osmotic poration. The phenomenological equation also was found to work very well for describing experimental data involving IIF, as we were able to use it to successfully describe IIF in bovine chondrocytes (Muldrew and McGann, 1994). We measured the permeability parameters for isolated chondrocytes and then performed both constant cooling and isothermal experiments on the cryostage (with various concentrations of cryoprotectant as before). The osmotic response of the cells for both freezing protocols was simulated, and the parameters describing IIF were fit to the experimental data using the simulated water flux. The figures below show that the equation was able to reasonably describe IIF under a wide variety of conditions (it should be noted that the parameters could be adjusted to provide an almost exact fit for either the data in Figure 2.9a or the data in Figure 2.9b; the discrepancies in the fit come from using the same parameters to simulate both experiments).

To further uncouple the effects of temperature (and hence the likelihood of forming IIF through proteinaceous pores) from the effects of water flux, we developed an experimental method that

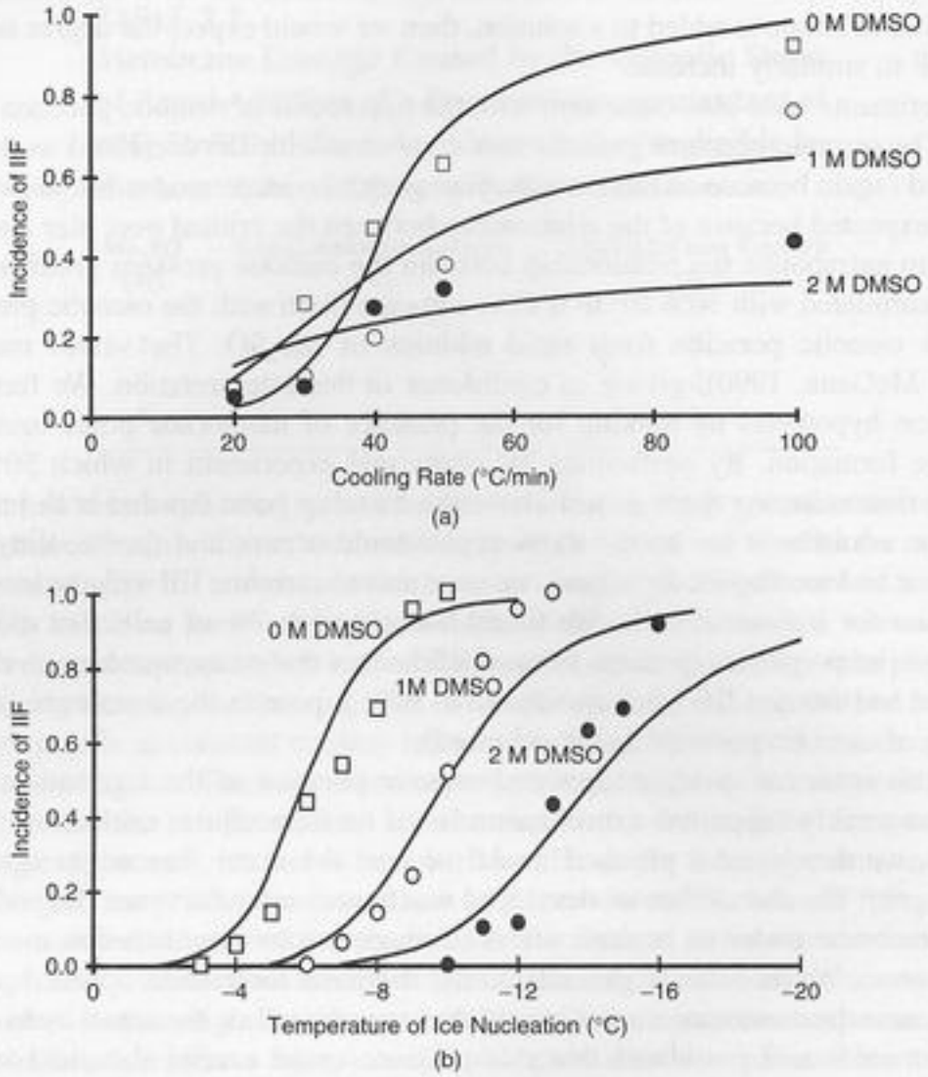

FIGURE 2.9 Intracellular ice formation is shown for hamster fibroblasts in solutions with no Me_2SO, 1 *M* Me_2SO, and 2 *M* Me_2SO. (a) IIF is shown as a function of cooling rate. (b) IIF is shown as a function of holding temperature (the cell suspension is supercooled at the holding temperature and then ice formation is initiated while holding the sample at a constant temperature). The solid lines represent the predictions of the osmotic rupture equation using a single set of parameters for the cell type.

would introduce a lag between the lowest temperature reached and the peak water flux. Even though this would not separate the relative magnitudes of these peak values, by separating them temporally we hoped to be able to correlate IIF with one or the other. The technique relied on cycling the temperature of the cryostage sinusoidally such that the cells would be alternately subjected to hypertonic and "hypotonic" conditions (relative to the instantaneous state of the cytoplasm). The temperature remained below the freezing points of the solutions, so that ice was always present, but the amount of ice was varied so that the osmotic pressure of the unfrozen solution would vary. Figure 2.10 shows the thermal protocol as well as the water flux and the degree of supercooling for a given condition.

Figure 2.10 also shows that the peak water flux occurs just past 180°C into the cycle whereas the minimum temperature occurs at 270°C. The results of the experiment showed that not only did IIF correlate most strongly with the point in the cycle corresponding to the peak water flux, but the magnitude of supercooling of the cytoplasm that corresponded to IIF was inordinately small (Muldrew and McGann, 1994). Indeed, IIF was observed with as little as 2°C of supercooling—a magnitude that would require an astonishingly efficient nucleation site for heterogeneous nucleation to occur. Furthermore, once IIF was observed to occur in a cell, it was also observed in that cell on all subsequent cycles, with the magnitude of water flux (or supercooling) required becoming

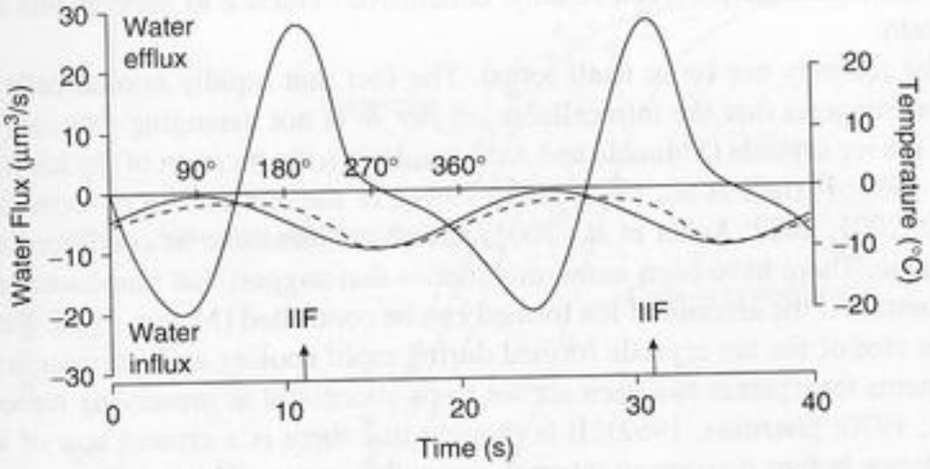

FIGURE 2.10 Water flux is shown during sinusoidal thermal cycling for hamster fibroblasts. The temperature is cycled between –0.5° and –9.5°C. Intracellular ice forms at the point in the cycle corresponding to the peak water flux as well as the peak supercooling. The dashed line shows the freezing point of the cytoplasm; thus, supercooling is given by the difference between this line and the temperature line. In this case, though maximum supercooling is only 3°C, 25% of the cells undergo IIF by the fifth cycle.

less and less with each subsequent cycle (Muldrew and McGann, 1994). In fact, there were some instances in which the hole in the cell membrane was so enlarged by the fifth cycle that we could actually see the ice crystal growing into the cell (the radius of the "pore" was about one-quarter the radius of the cell).

The hypothesis that osmotic poration is the mechanism by which IIF forms remains a viable alternative to nucleation-based theories. It also provides a mechanism of injury (unlike the nucleation theories) and a rationale for avoiding that injury. The substantial base of experimental evidence in support of osmotic poration remains as solid today as ever and should be considered on its own rather than in the context of a previous mechanistic model that was incorrect. The broad range of applicability (explaining poration phenomena above freezing, the type of injury associated with IIF, and the phenomenon of IIF itself) and the very useful extension of the hypothesis toward finding ways to achieve rapid cooling without the lethal injury associated with IIF (discussed below) must surely be taken as strong support for continuing to investigate this phenomenon.

2.5.3 Innocuous Intracellular Ice Formation

A long-held tenet in cryobiology is that intracellular ice is lethal to cells. Because it has been shown that intracellular ice formation in cells in suspension occurs during rapid freezing (Chambers and Hale, 1932; Luyet and Gibbs, 1937), and that rapid freezing causes cell death (Luyet and Gehenio, 1940; Mazur, 1966), it has been assumed that intracellular ice causes cell death (Diller et al., 1972; Karlsson et al., 1993; Mazur, 1966, 1977, 1984; Muldrew and McGann, 1990). However, the degree to which intracellular ice is damaging to cells and the mechanisms by which this injury occurs have been largely speculative. The most widely held view is that IIF damage occurs as a result of mechanical damage caused by a surface-area-to-volume redistribution of the ice crystal (Karlsson, 2001; Mazur et al., 1972; Muldrew and McGann, 1990). Recrystallization of the ice during slow warming will manifest itself as a net increase in the size of an intracellular ice crystal. The observation that cells could be "rescued" from intracellular ice formation as a result of rapid-warming techniques (Fowler and Toner, 1998; Mazur et al., 1972) has provided support for this mechanism of damage. Recrystallization, however, is not the only means by which IIF can be lethal. Various nonmechanical mechanisms have also been proposed including solution effects and thermal shock (Farrant and Morris, 1973), osmotic injury (Farrant et al., 1977), protein denaturation (Levitt, 1962), and gas bubble formation (Ashwood-Smith et al., 1988; Morris and McGrath, 1981; Steponkus and Dowgert, 1981). Although the presence of intracellular ice has been thought to result

in irreversible damage to cells frozen rapidly, conclusive evidence to support this claim has been difficult to obtain.

Intracellular ice may not be in itself lethal. The fact that rapidly cooled cells can survive if rapidly warmed suggests that the intracellular ice *per se* is not damaging. Instead, the amount of ice, the size of the ice crystals (Shimada and Asahina, 1975), the location of the ice formed (Bischof and Rubinsky, 1993; Farrant et al., 1977; Hunt, 1984), or the mechanism of formation (Acker and McGann, 2000, 2001, 2002; Acker et al., 2001) have been identified as conditions that could lead to cellular damage. There have been numerous studies that suggest that innocuous intracellular ice formation is possible if the amount of ice formed can be controlled (Mazur, 1990; Rall et al., 1980). Minimizing the size of the ice crystals formed during rapid cooling and warming to the point that the sample remains transparent has been shown to be successful at preserving tumor cell function (Asahina et al., 1970; Sherman, 1962). It is thought that there is a critical size of ice crystal that the cell can tolerate before damage to internal organelles occurs. Shimada has suggested that this critical size is 0.05 μm (Shimada and Asahina, 1975).

Historical attempts to obtain vitrified tissues using rapid freezing provide an excellent example of the success that researchers have had in minimizing the size of intracellular ice crystals and enhancing postthaw survival (Keeley et al., 1952; Luyet and Hodapp, 1938; Mider and Morton, 1939). Although the initial assumption was that ice formation had been prevented by rapid freezing (Keeley et al., 1952; Mider and Morton, 1939), it was subsequently determined using x-ray diffraction that these cells did indeed form intracellular ice but that the crystals were too small to be resolved using light microscopic techniques (Luyet and Rapatz, 1958). Even today, there are frequent reports of tissues being "vitrified," using rapid-freezing techniques, with superior survival (Day et al., 1999; de Graaf and Koster, 2001; Zieger et al., 1997). Because complete vitrification of bulk systems using traditional freezing protocols is unlikely (Fahy et al., 1990; Meryman, 1957), these results may be the product of "partial vitrification," or the presence of innocuous intracellular ice.

Although it is generally accepted that intracellular ice formation inevitably results in lethal damage to cells in suspension and single attached cells (Acker and McGann, 2000, 2002; Diller et al., 1972; Diller, 1975; Karlsson et al., 1993; Mazur, 1966, 1977; Muldrew and McGann, 1990), the relationship between cell damage and intracellular ice formation has proven to be difficult to experimentally verify (Karlsson et al., 1993; Acker and McGann, 2001). It has not yet been conclusively established whether intracellular ice formation is the cause of (Levitt, 1962; Farrant et al., 1977; Mazur, 1966; Mazur and Koshimoto, 2002; Mazur et al., 1972) or results from (Dowgert, 1983; Muldrew and McGann, 1994; Toner et al., 1990) damage to cellular components. Identifying the temporal sequence of events involved in intracellular ice formation, the role of biology in IIF, and the IIF-related sites of cell injury are critical outstanding issues in the field of cryobiology. Over the last 15 years, we have made some progress toward understanding these issues.

There is an innate relationship between intracellular ice formation and the cell plasma membrane (Diller et al., 1972; Karlsson et al., 1993; Mazur, 1965; Muldrew and McGann, 1990; Toner et al., 1990, 1993). The limited permeability of the membrane to water and solutes results in cytoplasmic supercooling during rapid cooling. Because the membrane is a barrier to ice propagation (Chambers and Hale, 1932; Luyet and Gibbs, 1937), and there are few efficient nucleators in a cell (Franks et al., 1983; Rasmussen et al., 1975), nucleation of ice within the supercooled cytoplasm at high subzero temperatures is thought to involve extracellular ice. One of the consistent elements in the study of IIF has been the observation following warming that there is significant damage to the plasma membrane. Although it is not known whether this damage to the plasma membrane occurs during freezing (Asahina, 1962; Muldrew and McGann, 1994; Steponkus et al., 1983) or thawing (Mazur, 1963b, 1965; Fowler and Toner, 1998), or whether IIF is the cause of, or results from, this damage, the fact is that damage to the plasma membrane is almost always observed after IIF (Chambers and Hale, 1932; Dowgert, 1983; Fujikawa, 1980; Mazur, 1965, 1966, 1984; Muldrew and McGann, 1990; Steponkus et al., 1983; Steponkus and Dowgert, 1984). Because a damaged plasma membrane and subsequent loss of semipermeability are strong indicators of lethal injury

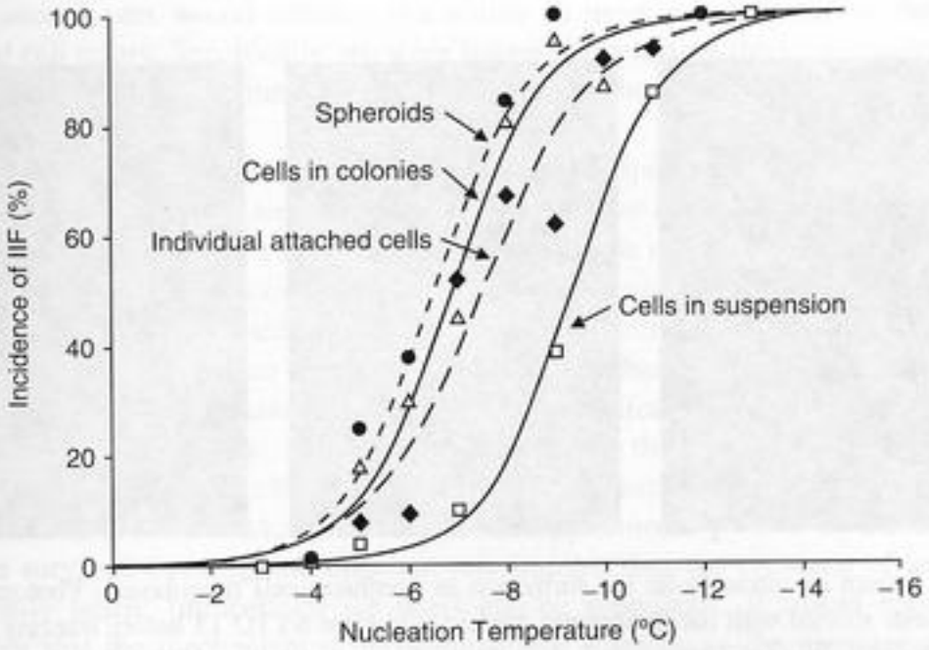

FIGURE 2.11 Cumulative incidence of intracellular ice formation of hamster fibroblasts as a function of temperature of extracellular ice nucleation for four tissue models: cells in suspension (□); individual cells attached to glass (♦); cells in monolayers (△); and cells in a spheroid (•). Lines are logistic curves fitted to the data using a least squares method. (Reprinted from Acker et al., 1999a. With permission.)

to a cell (Acker et al., 1999a; Dankberg and Persidsky, 1976; Mazur, 1965, 1966; Yang et al., 1995), intracellular ice formation has always been correlated with cell death.

In 1997, while studying the effects of cell–cell and cell–matrix interactions on IIF, we were able to dissociate intracellular ice formation and membrane damage. We observed that a majority of cells in confluent monolayers that form intracellular ice at low temperatures did not display a damaged plasma membrane following thawing (Acker and McGann, 2000). Prior work had already shown an increased probability of intracellular ice formation in confluent monolayers and cell spheroids (Acker and McGann, 1998; Acker et al., 1999a), which indicated that cell–cell contact was an important mediator of IIF (Figure 2.11). By directly observing and measuring the diffusion of a membrane impermeable fluorescent stain, ethidium bromide, into frozen cells and correlating the results with the formation of intracellular ice and the postthaw integrity of the plasma membrane, we were able to examine the time course of IIF-related membrane damage (Acker and McGann, 2000, 2001). We found that in monolayers, cells that form intracellular ice before any of their nearest neighbors have a higher probability of having a disrupted cell membrane than cells that freeze next to an already frozen cell. We now had a condition in which intracellular ice formation could occur without damaging the cell membrane, allowing us to begin to understand the causal relationship between IIF and membrane damage.

From this work, it became clear that IIF-related membrane damage was dependent on the location of the frozen cell within a monolayer and that cell–cell contact was an important determinant of both the incidence and kinetics of IIF and membrane damage. Using the cryomicroscope to observe the pattern in which IIF occurred in confluent monolayers of different cell lines gave us important information on the mechanism by which intracellular ice forms in these systems (Figure 2.12). We found that with fibroblast monolayers, the nucleation of ice in one cell was followed, after a brief delay, by the freezing of an adjacent cell. This resulted in well-defined clusters of cells with intracellular ice when the monolayers were frozen at constant subzero temperatures. This effect was amplified in epithelial monolayers in which a wave-like propagation of intracellular ice occurred in the samples, resulting in all cells forming intracellular ice at relatively high subzero temperatures. Using a simple statistical method to test the degree of randomness of IIF in the confluent monolayers, we were able to conclude that the formation of ice in a cell that

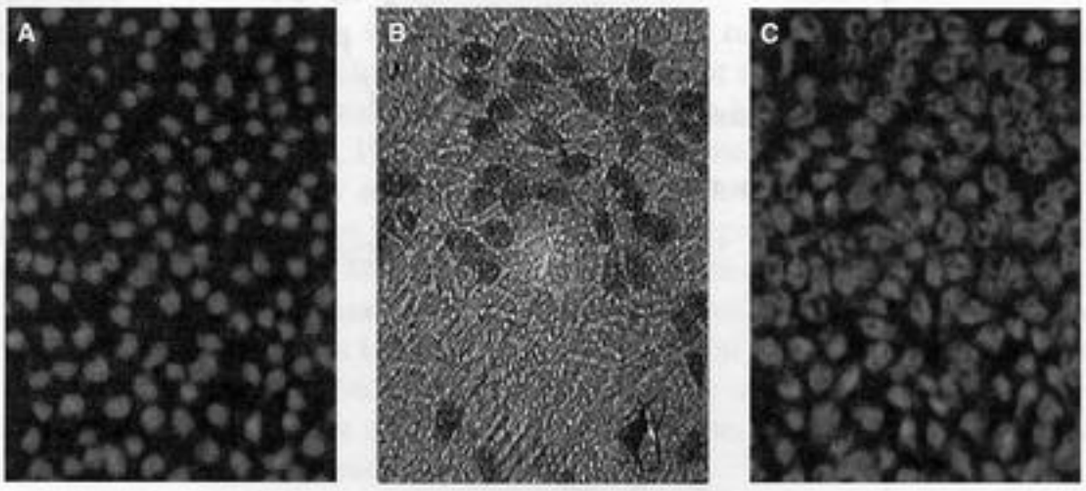

FIGURE 2.12 Pattern of intracellular ice formation in confluent cell monolayers. Photomicrographs of a hamster fibroblasts stained with the fluorescent nucleic acid stain SYTO 13 before freezing on a convection cryomicroscope. (a) Before freezing at –7°C; (b) brightfield photomicrograph of cells frozen at –7°C showing characteristic darkening of the cytoplasm; (c) fluorescent photomicrograph of frozen cell at –7°C.

is part of a monolayer increases the likelihood of intracellular freezing in adjacent cells (Acker and McGann, 1998).

As the propagation of intracellular ice between adjacent cells in monolayers and tissue systems had been previously documented in the literature (Asahina, 1956; Berger and Uhrik, 1996; Brown, 1980; Brown and Reuter, 1974; Chambers and Hale, 1932; Levitt, 1966; Luyet and Gibbs, 1937; McLeester et al., 1969; Molisch, 1982; Stuckey and Curtis, 1938; Tsuruta et al., 1998), we became interested in understanding why this phenomenon occurred and how it affected postthaw cell viability. In 1992, while working with single strands of salivary tissue, Berger and Uhrik demonstrated that the induction of ice between cells could be inhibited using a chemical agent that uncoupled the cells (Berger and Uhrik, 1992, 1996). They concluded that intercellular junctions (gap junctions) were responsible for the propagation of ice between adjacent cells. Critical to their hypothesis was the assumption that ice could grow through small-diameter pores. To test this idea, we examined the theoretical relationship between the equilibrium ice crystal radius and the temperature of extracellular ice nucleation and compared it with experimental data on the temperature of intercellular ice propagation (Acker et al., 2001). By examining cell lines with and without gap junctions, we found a temperature that resulted in a significant deviation in the incidence and pattern of IIF that we attributed to gap junction-facilitated intercellular ice propagation. This temperature agreed (at least qualitatively) with our theoretical predictions. This work provided strong evidence to support the concept that intracellular ice can propagate between adjacent cells via gap junctions. This hypothesis has recently been tested and verified in a much more rigorous manner by Irimia and Karlsson (2002), using micropatterned substrates to control cell–cell interactions.

It is important to note that nonrandom intercellular ice propagation has also been observed in cell lines that do not form pores between cells (Acker and McGann, 1998; Acker et al., 2001; Irimia and Karlsson, 2002,), and therefore, ice propagation via gap junctions cannot be the sole mechanism for ice propagation between adjacent cells. Recent studies have suggested that the induction of ice between adjacent cells can occur as a result of surface catalyzed nucleation in which ice in one cell, using the adhesion between cells, results in the nucleation of ice in adjacent cells (Acker et al., 1999a; Tsuruta et al., 1998). This hypothesis awaits theoretical and experimental validation.

With a better understanding of how intracellular ice formation occurs in confluent monolayers and the role of cell–cell contact on membrane integrity, it became clear to us that intracellular ice formation in confluent monolayers was markedly different from IIF in cell suspensions. As much of our understanding on IIF had come from work with cell suspensions, we believed that studies

with confluent monolayers would enhance our ability to more precisely define the relationship between IIF and cell injury. Specifically, we were interested in testing the long-standing tenets that intracellular ice is lethal and that intracellular ice formation should be avoided during cryopreservation.

We knew that intracellular ice could propagate between adjacent cells and that the majority of the cells that formed intracellular ice as a result of intercellular ice propagation had intact plasma membranes. Because we now had a means to form intracellular ice in cells and thaw them without incurring membrane damage, we could now dissociate IIF from membrane damage and focus on the important question of whether intracellular ice is lethal. We induced all of the cells in confluent monolayers to form intracellular ice by freezing at defined subzero temperatures. We then thawed the cells and assessed the postthaw survival using three different indices: membrane integrity, metabolic activity, and clonogenic function. We found that the postthaw survival of confluent monolayers was dependent more on the presence of an intact plasma membrane than on the presence of intracellular ice (Acker and McGann, 2002). As approximately 80% of the cells that had intracellular ice survived freezing and thawing, this implied that the presence of ice inside cells was not inherently lethal. Intracellular ice itself can be innocuous. It would appear from this preliminary work that the mechanism of intracellular ice formation—the mechanism that results in membrane damage—is the lethal event.

The most surprising results from our work on intracellular ice came when we compared cells frozen to –40°C in 10% Me_2SO to those cells that formed innocuous intracellular ice. By inducing intracellular ice formation in confluent monolayers in the absence of any chemical cryoprotectant, we obtained high cell viability following freezing and thawing (Acker and McGann, 2002). Although the recovery was not as great as that obtained using a standard cryopreservation protocol with 10% Me_2SO, these data would indicate that intracellular ice is not only innocuous but can have a cryoprotective effect. We followed this work with an examination of the effect of innocuous intracellular ice on cell survival following slow cooling (Acker and McGann, 2003). We found that the survival of confluent cell monolayers cooled to –40°C at 1°C/min was highest when the incidence of intracellular ice was close to 100%. Together, these studies support the concept that conditions exist where intracellular ice may confer cryoprotection, a strategy that has been overlooked in our development of protocols for the cryopreservation of cells and tissues.

Recent work has focused on the mechanism by which innocuous intracellular ice functions to protect cells from cryoinjury. We propose that if cryoinjury occurring during cooling results from excessive changes in cell volume during freezing and thawing, then preventing osmotic volume changes would protect cells from this injury. Once intracellular ice forms, there is no longer a driving force for the efflux of water across the plasma membrane during cooling, as osmotic equilibrium is maintained by the formation of more intracellular ice. Innocuous intracellular ice will therefore eliminate osmotic cell shrinkage during cooling. This proposed theory is consistent with our understanding of osmotic poration.

The concepts arising from our work clearly indicate a need to reassess our understanding of intracellular ice formation and the role of intracellular ice in biological systems. We contend that it is not the intracellular ice that is lethal to cells, but the mechanism of formation, and that innocuous intracellular ice is a potentially important means to protect cells from freezing injury. The dramatic differences we have observed in the effect of intracellular ice indicates the need for a better understanding of the differences between the low-temperature response of cells in suspension and the constituent cells of tissues.

2.6 ICE GROWTH IN TISSUES

Although techniques for the successful cryopreservation of isolated cells have been available for over 50 years, there has been little success in scaling these techniques to cryopreserve organized tissues or organs. The ability to model cellular responses to freezing and thawing has certainly not

yet delivered on its promise of facilitating the cryopreservation of tissues. This failure has led us to question the underlying assumptions that are currently being used to develop cryopreservation techniques. The basic method comes from consideration of the two types of cellular cryoinjury mentioned earlier; cooling too quickly leads to intracellular freezing (and cell death), whereas cooling too slowly leads to solution-effects injury (and cell death). The strategy is to cool as rapidly as possible while avoiding intracellular ice so as to minimize the time of exposure to concentrated solutes. Also, chemical cryoprotective additives are added to reduce the concentration of solutes at a given temperature. Under this premise, one need only know the phase diagram for the extracellular solution and the permeability parameters for a particular cell type (to predict the cooling rates that will allow the cell to remain in osmotic equilibrium, as well as the rates that will cause sufficient supercooling of the cytoplasm so that IIF occurs) to plan a cryopreservation protocol. When scaling this program up to tissues, where cells are embedded in an organized three-dimensional structure, it is assumed that the physical and chemical conditions produced by freezing are the same as in bulk solution and that the cellular responses will also be the same.

One of the motivating factors for pursuing this line of inquiry is the curious case of articular cartilage. Articular cartilage is the tissue that is located at the ends of the long bones, distributing load between the bones and providing a near-frictionless surface for articulation. The transplantation of bone is second only to blood in terms of the frequency with which it is used in modern medical practice. Unfortunately, all attempts to store bone with articular cartilage for transplantation have failed to keep the cartilage viable (and the body has no way to repair or regenerate this tissue). Recent work that attempted to scale cryopreservation techniques for isolated chondrocytes (the cells that reside in and maintain the articular cartilage) to the intact tissue met with very poor results (Muldrew et al., 1994, 1996). Follow-up work led to the hypothesis that the structure of the tissue was affecting ice growth and thereby negating all the assumptions that went into designing the cryopreservation protocol (Muldrew et al., 2000b, 2001a, 2001b).

Articular cartilage basically consists of an extracellular matrix with cells embedded within it. The matrix is about 80% water but has a highly organized network of collagenous fibers that constrain the so-called ground substance (a collection of enormous molecules with a high degree of exposed negative charges). Whereas the fibers give the cartilage tensile strength, the ground substance draws water into the matrix and restricts its movement in compression. Nutrients and waste products of the cells must diffuse through the ground substance as well. The structure can be considered as a capillary-porous network in which the water channels exist in small pores that are connected through tortuous paths.

Figure 2.13 is a micrograph of the structure of frozen articular cartilage. The outer surface of the cartilage is visible at the top of the image, whereas the surface on the right side was created with a scalpel blade before freezing. The large holes inside the matrix are lacunae, where the chondrocytes were situated. The two distinct regions visible in the figure (the dense region adjacent to a surface and the spongy region of the interior) are caused by fundamentally different ice morphologies in these regions (the sample was freeze-substituted; thus, vacancies in the image show where ice crystals were). Near the surface of the tissue, ice appears to have grown into the matrix as a single crystal, leaving the tissue without apparent disruptions. Although cartilage is a porous medium, the pore size is on the order of 5 nm (Maroudas, 1970); therefore, we do not expect to see any aqueous vacancies in the tissue except for the lacunae at this magnification. The matrix of the interior region, however, is porous and open, suggesting polycrystalline ice formation and mechanical disruption of the matrix architecture. The growth of ice in this region is of a completely different nature from that near the surface. Since the recovery of cells exactly correlates with these two different ice morphologies (live cells in the outer dense region and dead cells in the spongy interior; Muldrew et al., 2000b), understanding ice growth in this tissue appears to be crucial to understanding cryoinjury.

If this phenomenon were limited to articular cartilage, it would have an important but specialized application to cryobiology. However, all mammalian tissues have connective tissue as part of their

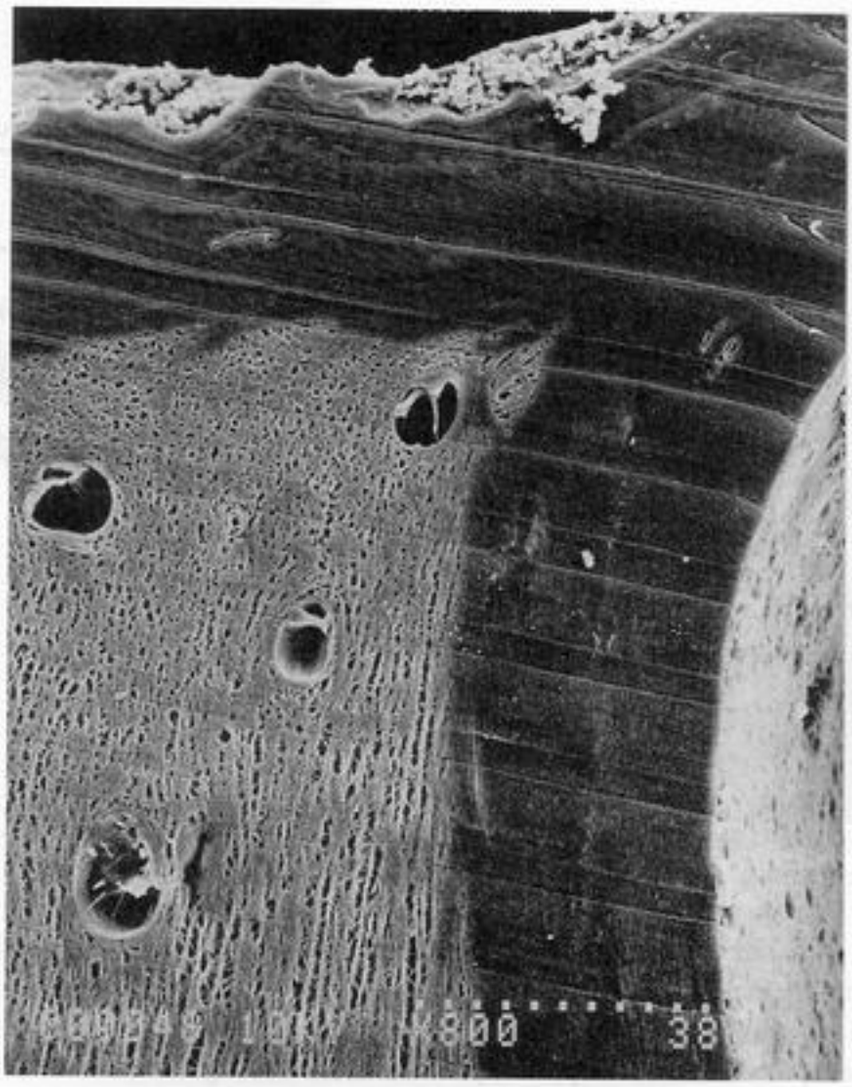

FIGURE 2.13 Scanning electron micrograph showing a cartilage section following freeze substitution (the tissue was cooled at 1°C/min and then freeze substituted without thawing). The top surface is the articular surface and the surface on the right is a scalpel cut that was administered before freezing. The vacant holes are chondrocyte lacunae. The spongy interior is thought to be to the result of mechanical damage to the matrix caused by ice lenses. Bar, 38 μm.

architecture (connective tissue refers to the extracellular matrix, usually collagen based; articular cartilage is a rather specialized form where almost the entire tissue is extracellular matrix). Thus, an understanding of ice growth in connective tissue is relevant to the whole field of medical cryobiology. Before pursuing the specific case of articular cartilage further, we will revisit the process of ice growth in general.

The structure of ice is such that it does not allow the inclusion of impurities, except within defects in the crystal structure. Thus, when an ice nucleus begins to grow, any solutes that are present in the liquid will be excluded from this growing ice front. If the rate of crystal growth is faster than the rate at which diffusion of the particular solutes can carry them away from the ice front, then a concentration gradient will very quickly form in the liquid that surrounds the ice crystal. The concentrated solute in the liquid phase, just in advance of the ice front, will then lower the freezing point of the solution (Figure 2.14). When a sufficient amount of ice has formed, then the solution at the interface will have a freezing point equal to the temperature of the interface; at this point, if the ice interface is planar (and stable), then continued growth will be limited by diffusion of the solute away from the crystal. If this situation arises when the solution well away from the ice crystal (in which the solute is not concentrated) is supercooled (at a temperature below the melting point), then we have constitutional supercooling of the solution in this region. Eventually

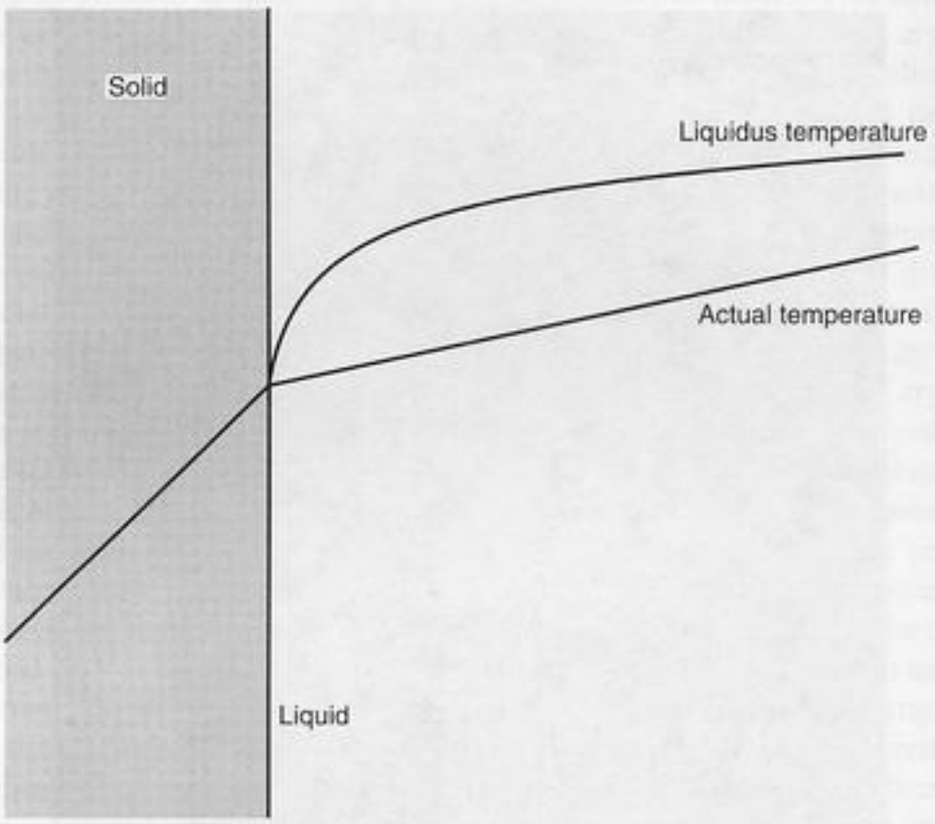

FIGURE 2.14 Constitutional supercooling occurs in advance of the ice front because of a high concentration of excluded solutes immediately adjacent to the interface.

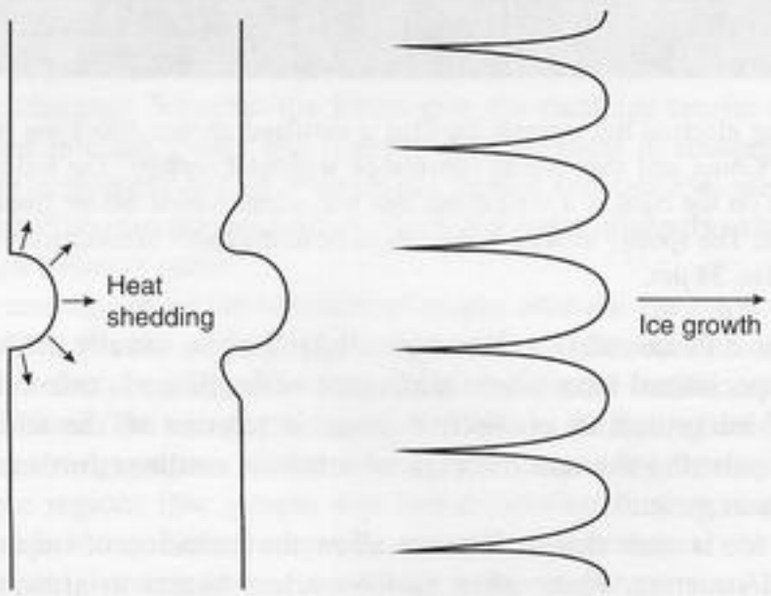

FIGURE 2.15 Ice growing into supercooled solution has an unstable interface because of the advantage that a protruding region has for shedding its latent heat into a colder region of liquid.

diffusion will ensure that the system goes to equilibrium; however, an unstable situation is created when this occurs.

The growth of ice in bulk solution is only planar for the case when heat is removed through the solid phase (i.e., ice is growing on the surface of a heat sink). When nucleation occurs in the solution (such that the crystal is completely surrounded by liquid, so-called equiaxed growth), then the interface is unstable because of the release of heat from crystallization. The heat is created at the crystal surface and must either be shed through the crystal or through the liquid (Figure 2.15).

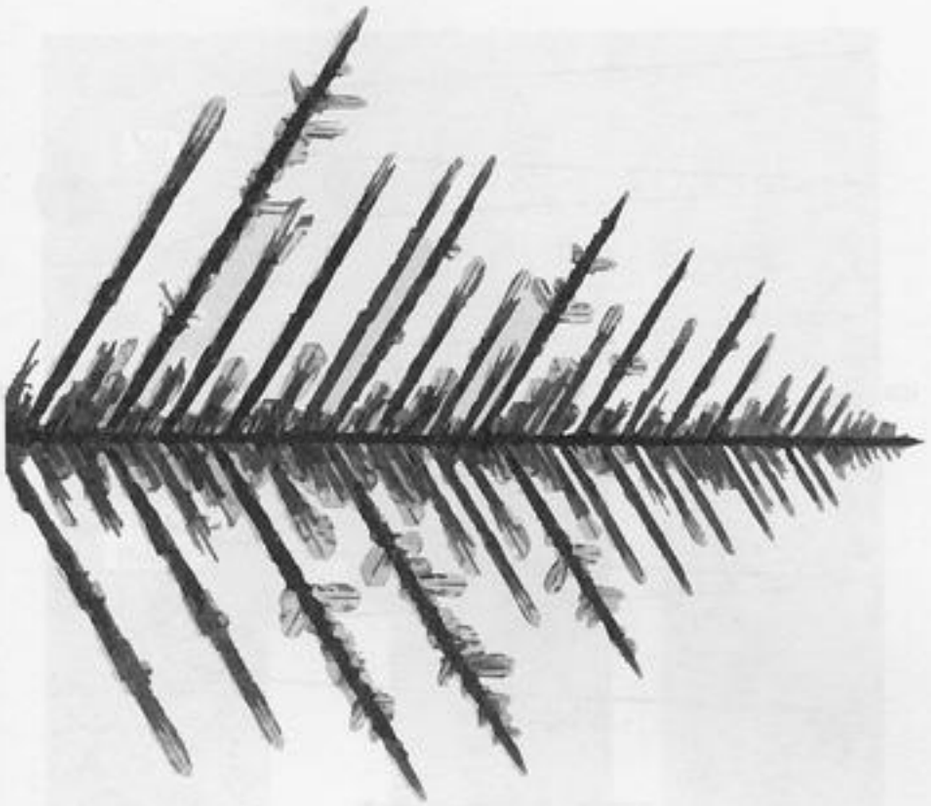

FIGURE 2.16 Growth of ice into supercooled solution proceeds as dendritic growth. The branches of ice have smaller branches growing off them in turn. Solute is encapsulated between the dendrites.

The removal of this heat occurs by conduction and can only occur through the liquid if it is supercooled. If the latent heat of fusion is conducted away through the ice (e.g., if the crystal is in connection with a heat sink), then the growing crystal will remain essentially smooth, as any part of the interface that grows beyond the planar front will not be able to lose its heat as quickly as the ice on either side of it; thus, these instabilities quickly die out. If the heat is conducted away through the liquid, however, then there are several aspects in which crystal growth is altered. For instance, growth occurs preferentially along the a-axis compared with growth along the c-axis: This occurs because of the rise in temperature of the liquid surrounding the crystal. As the molecules become more energetic, they are less likely to join a planar surface where they can only hydrogen bond with a single neighbor. On an a-axis face, they can bond with at least two neighbors, losing more of their kinetic energy to potential energy of bond formation. Thus, we see a symmetry about a hexagonal disk in ice crystals growing under such circumstances.

The existence of constitutional supercooling in advance of a growing ice crystal results in an unstable situation. Because there is a gradient in the degree of supercooling, which is maximal a little way out in front of the interface, a planar ice front will be susceptible to small perturbations. If a local region of the interface advances just slightly ahead of the plane, then its growth rate will increase as well. This is because of the fact that it will now be able to shed its latent heat of fusion to a greater volume of liquid, as well as to liquid that is more supercooled. Such an instability will grow through the supercooled region until the supercooling is reduced to the level at which the rate of growth is limited by the conduction of latent heat once again. In fact, an entire planar interface will form an array of these "cells" when constitutional supercooling occurs.

Further to the conduction of latent heat, there is also the destabilizing effect of solute exclusion. The (ice) cells will exclude solute to the sides as well as in front; therefore, the regions between cells will contain concentrated pockets of solute. If the conditions leading to cellular growth are particularly pronounced, then the cells may turn to dendrites—protuberances that start to grow side branches (Figure 2.16). The sides of the cells become plane fronts in themselves—also subject to the same destabilizing effects of heat conduction and solute exclusion. Because the ice crystal is built on a hexagonal symmetry, these side branches will follow that symmetry.

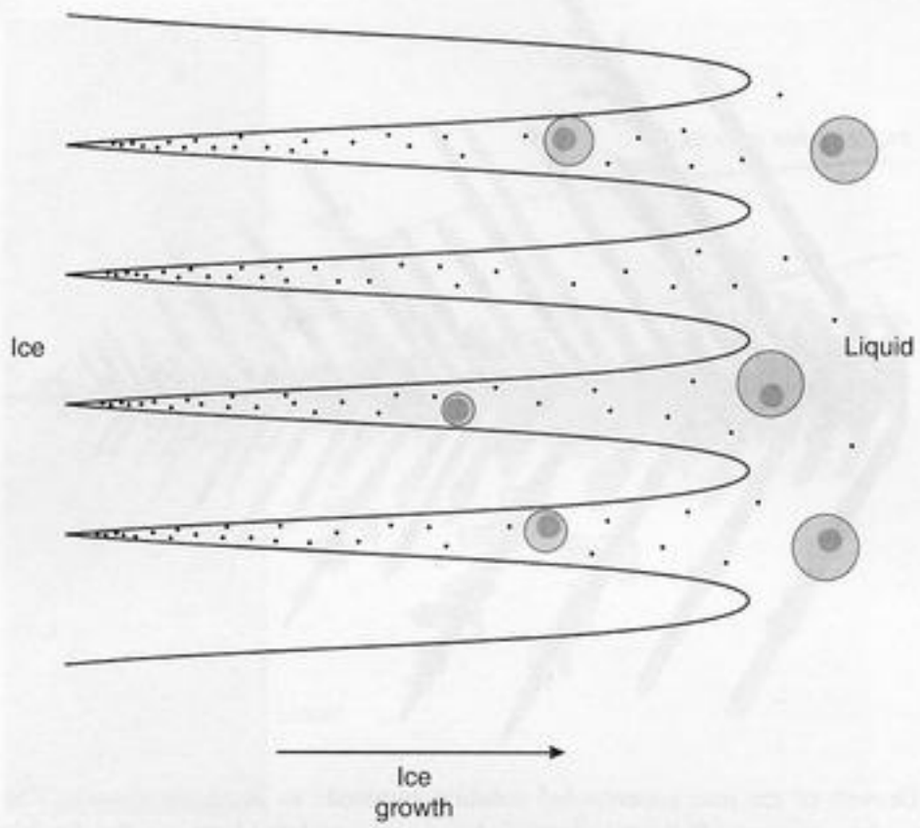

FIGURE 2.17 Dendritic ice growth encapsulates solute and biological cells between the dendrites; the unfrozen solution becomes progressively more concentrated causing the cells to lose water.

Once dendritic breakdown (the formation of growing dendrites from a planar interface) occurs, then the excluded solute will be encapsulated in channels that are sandwiched between the growing dendrites. If the freezing is initiated by a single nucleation event, then these channels will all be connected. Any (biological) cells that are present in the solution will be encapsulated in these channels along with the solute that was originally present in the solution (Figure 2.17).

Ice growth in bulk physiological solution proceeds dendritically, allowing the ice crystal to extend throughout the solution and encapsulate solute in unfrozen channels. No region of the solution is shielded from the ice crystal; thus, significant supercooling does not occur unless the sample is small and the cooling very efficient. In contrast, however, ice growth in a capillary-porous medium can proceed very differently, as the solid regions of the medium impose additional constraints on ice morphology. Ice growth in a narrow capillary proceeds as a confined crystal with a hemispherical interface with liquid water (Figure 2.18). The radius of the capillary, as well as the contact angle between ice and the capillary wall, imposes a curvature on the interface. The temperature at which a crystal of a given radius will be thermodynamically stable is given by the Kelvin equation, which shows that as capillaries get to the submicron size, the curvature effects can be appreciable. It is also possible for a capillary-porous medium to have capillaries that are too small to allow cellular or dendritic ice growth, and all solute will be excluded at the ice front. The rate of ice growth will then be limited by the diffusion of solutes away from the ice interface (diffusion also has to proceed through the same channels), as the solutes cannot be encapsulated between dendrites.

Ice growing into such a medium from one side could appear macroscopically planar in cross-section because of the growth of many ice dendrites through the tortuous paths defined by the medium. Though a real capillary-porous medium will have a complicated topology (it is not simply connected) and nonuniform porosity, we will consider the simpler model in which the aqueous channels are parallel and separate from each other (Figure 2.19).

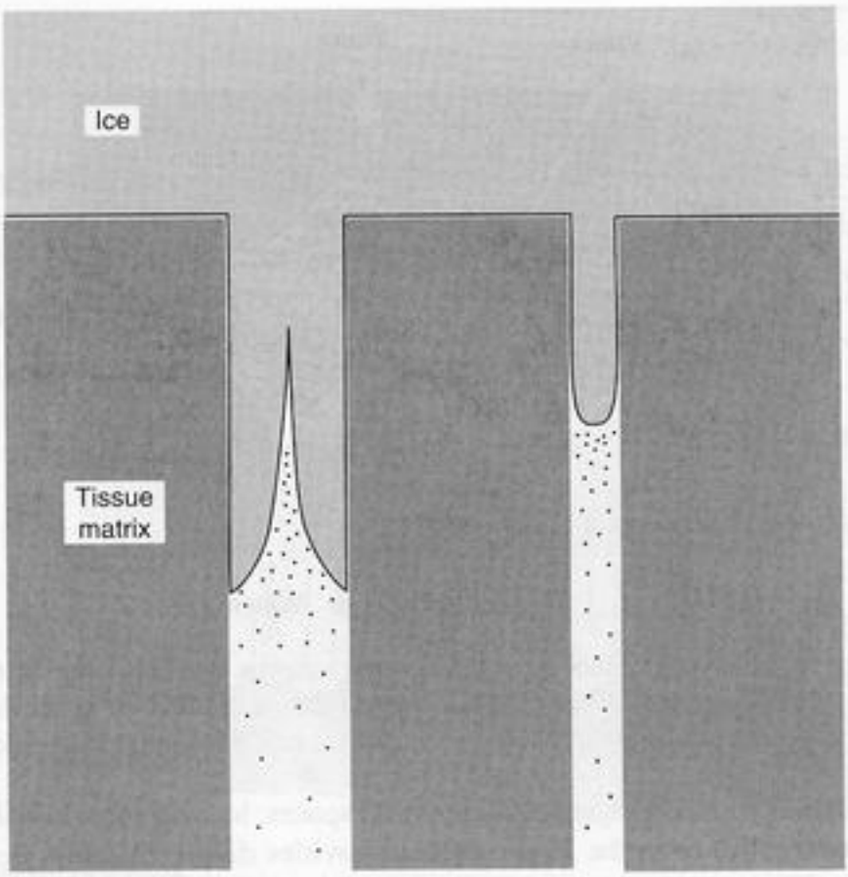

FIGURE 2.18 Ice dendrites have a minimum radius for a given temperature; therefore, capillaries that are below some critical radius will not allow the excluded solutes to be encapsulated in the unfrozen channels between dendrites. Ice growth in capillaries below this radius will then be rate-limited by diffusion of the solutes away from the interface because diffusion will have essentially one dimension, rather than two.

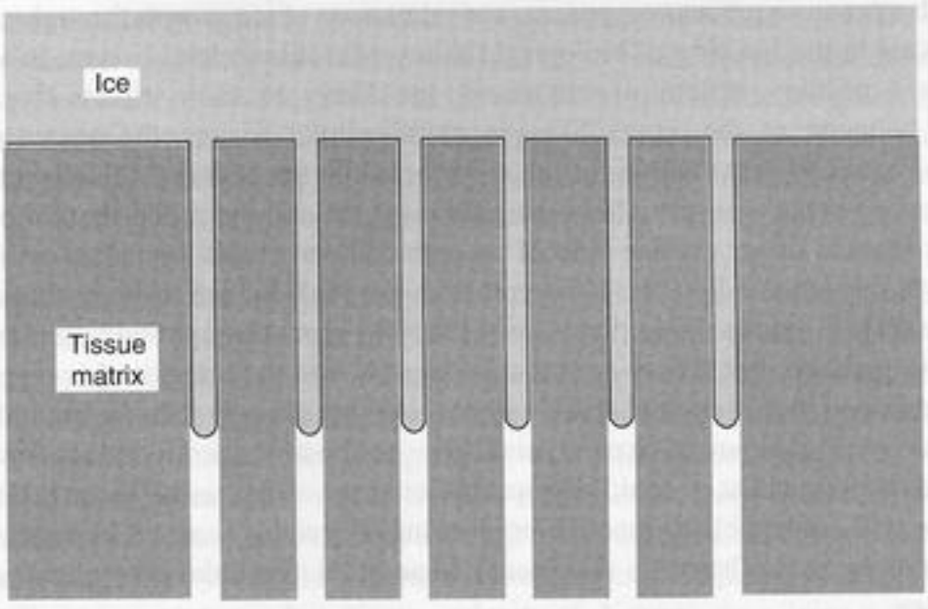

FIGURE 2.19 Schematic of a capillary-porous medium in which ice growth appears to be planar macroscopically because of growth along porous channels from the outer surface. The rate of ice growth in these channels (and hence through the medium) is diffusion limited rather than temperature limited.

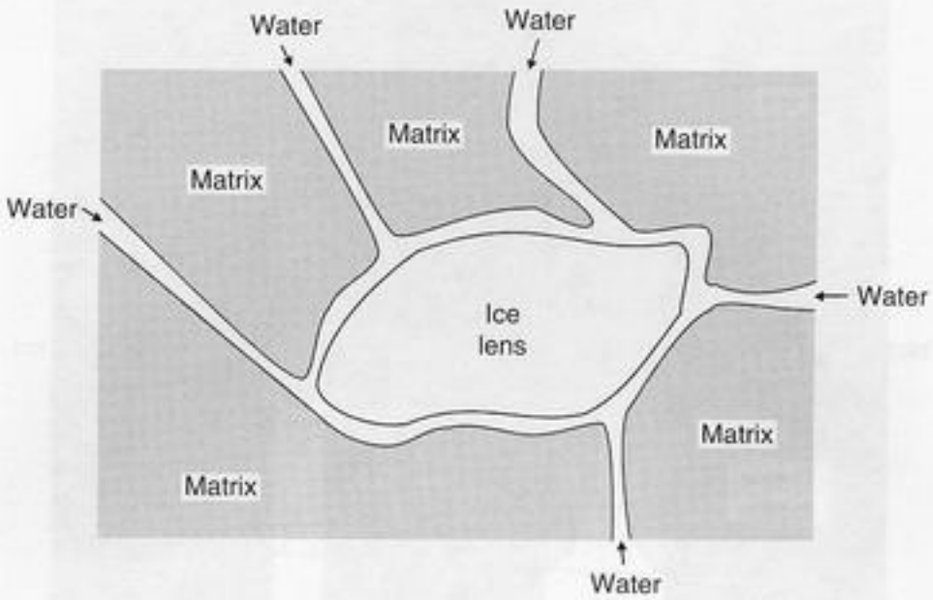

FIGURE 2.20 When an ice crystal cannot grow through the aqueous channels adjacent to it because of the necessity of a highly curved interface in the channel, water is drawn through the channels to the ice crystal. Equiaxed growth in this situation forms an ice lens that can produce substantial mechanical forces.

In a porous medium with heterogeneous interstitial spaces, ice will form initially at the surfaces (if ice is present externally) or in the larger aqueous cavities during freezing. For example, if ice is growing into a capillary-porous tissue, then the growth of ice will be diffusion-limited. Continued cooling of this tissue will lead to substantial constitutional supercooling of the interior, making nucleation of ice likely. Once an ice crystal forms in a cavity in such a tissue, if it is connected to water sources through capillaries that are small enough so that freezing cannot occur within them, then water will flow to the ice crystal, leaving part of the sample in a desiccated state. The ice crystal in the cavity continues to grow, generating mechanical forces that are responsible for enlarging the cavity. In soils, where this phenomenon occurs frequently, the ice cavities are usually shaped like a convex lens and are thus called "ice lenses" (Figure 2.20).

Because all biological tissues contain an extracellular matrix that consists of a macromolecular framework with a porous aqueous component, considerations of ice growth through capillary porous media are relevant to the freezing of biological tissues. Musculoskeletal tissues, in which extracellular matrix is a primary structural component, are likely to show effects that dominate the cryobiological behavior of the tissue. Even in more cellular tissues, the presence of basement membranes and blood vessels (with tight junctions between the endothelial cells and a connective tissue basal lamina) could severely affect the growth of ice and the redistribution of water during freezing. The presence of ice on one side of an extracellular matrix boundary will preferentially draw water from the other side if the temperature is too high for ice to grow through the porous structure of the matrix. For example, ice is very likely to grow through the vascular channels of a tissue without impedance, but it may not be able to grow into the interstitial compartment. As the temperature is dropped, the interstitial space will become increasingly dehydrated and the ice crystal in the vasculature will continue to expand, analogous to the aforementioned ice lens in soil. Both mechanical and osmotic stresses could disrupt the integrity of the tissue architecture to an extent where the tissue will not be able to function on thawing. Cryoinjury caused by tissue structure (and cryodestruction of the tissue) has to be considered, in addition to cellular cryoinjury, when organized tissues are frozen.

Cells, too, may have additional modes of cryoinjury when ice growth is altered by extracellular matrix. If ice growth is rate-limited by the diffusion of solutes away from the ice front, the consequence of further lowering of the temperature will be an increased supercooling of the interior of the tissue and, eventually, spontaneous nucleation at many sites within the matrix and the

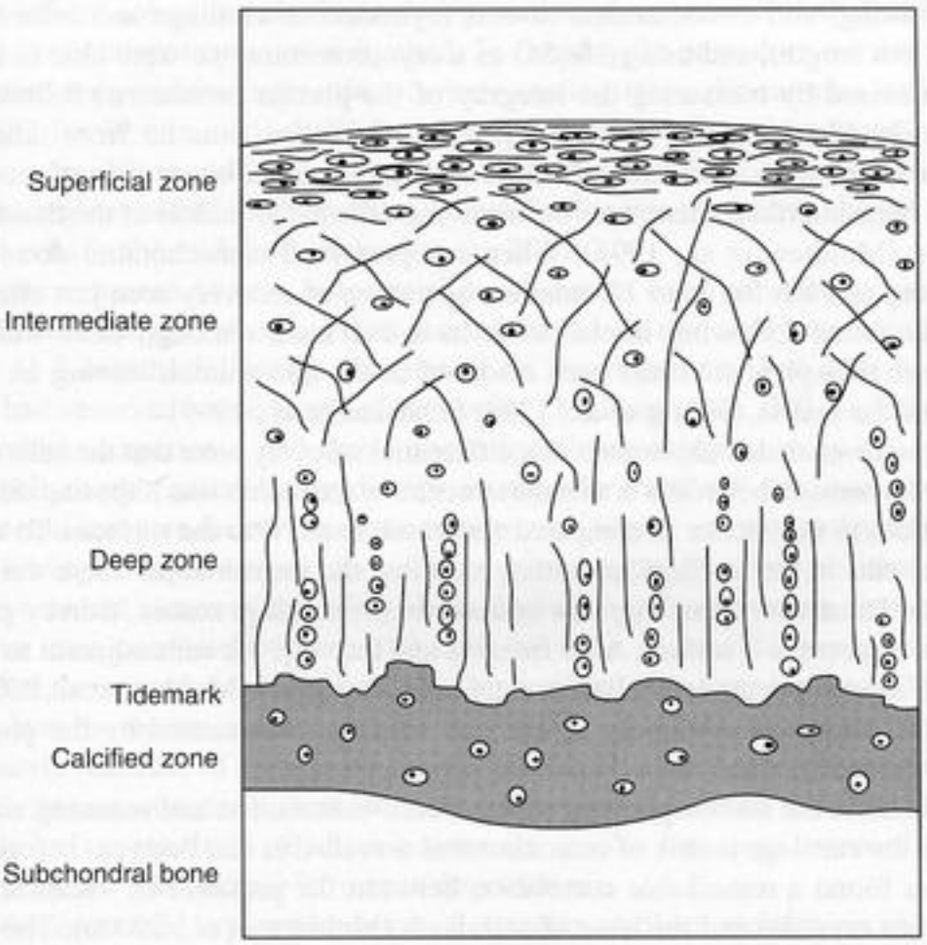

FIGURE 2.21 A cross-sectional view of articular cartilage showing the various morphological zones. Cell survival was restricted to the top portion of the superficial zone and most of the deep zone following cryopreservation. (Reprinted from Muldrew et al., 1994. With permission.)

formation of ice lenses. Because ice lenses draw water to them from every direction, they can expand against a resisting force and create mechanical effects. Cells adjacent to an ice lens may be disrupted by mechanical forces resulting from ice growth. As a further consequence of ice lens formation, the distribution of water is altered. Although water and solute would be drawn through the capillaries to an ice lens, only pure water will join the crystal. Because the crystal can enlarge without forming a network of aqueous channels (as occurs with dendritic ice growth), on warming, the potential exists for cells to be subject to transient hypotonic stresses. Thus, the formation of ice lenses could produce mechanical forces and osmotic stresses that lead to cellular injury and the disruption of tissue architecture, acting in parallel with the conventional mechanisms of cryoinjury.

We have used articular cartilage as a model to study ice growth in biological tissues because of its relative simplicity. The tissue has neither nerves nor blood vessels, and only a single cell type (the chondrocyte), yet there is still a great deal of complex biology that occurs in the tissue. The cells in adult articular cartilage are immobile, and they are not active in the cell cycle (Figure 2.21). They do, however, monitor the integrity of the extracellular matrix in which they are embedded (the functional component of the tissue—it distributes loads between bones and allows smooth articulation of the joints) and remodel it to suit the biomechanical environment. This process involves the trafficking of enzymes and structural molecules extracellularly to effect the remodeling process; as yet, these processes are but poorly understood. Nevertheless, from a structural point of view, we will consider the tissue to be composed of cells embedded in a homogeneous capillary-porous medium.

Our initial attempts to cryopreserve articular cartilage were based on a rationale that treated the cartilage matrix as bulk solution (i.e., we assumed that ice would grow dendritically throughout

the matrix). Working with osteochondral dowels (cylinders of cartilage and bone of about 1 cm diameter and 1 cm length), and using Me_2SO as a cryoprotectant, we were able to achieve a 40% cell survival (assessed by measuring the integrity of the plasma membrane) following storage in liquid nitrogen (Muldrew et al., 1994). The pattern of recovery was far from random, however, with a thin zone of recovery adjacent to the articular surface and a larger zone of recovery adjacent to the cartilage-bone interface; there were no surviving cells in the middle of the tissue when viewed in cross section (Muldrew et al., 1994). When cryopreserved osteochondral dowels were transplanted into sheep and left for up to 12 months, the pattern of recovery seen just after thawing was identical to that found following implantation in a live host, although cells without an intact membrane before transplantation had been resorbed in the live animal, leaving an acellular zone in the interior of the matrix (Hurtig et al., 1998; Schachar et al., 1999).

The two hypotheses that could explain this differential recovery were that the cells of the different zones had different osmotic behaviors or that the structure of the matrix was imposing different physico-chemical conditions in the interior as compared with those found near the surfaces. To test these ideas, we made scalpel cuts in the cartilage, extending well into the interior zone where the cells had been killed previously. These cuts opened up new surfaces in the cartilage matrix, thereby putting some of the interior cells adjacent to a surface. After freezing and thawing, the cells adjacent to the cut surface showed identical recovery to the cells adjacent to the articular surface (Muldrew et al., 2000b), providing evidence that the pattern of cryoinjury in articular cartilage was caused by the physico-chemical environment in the matrix rather than by a difference in cell types.

Figure 2.13 shows the ice morphology (using freeze-substitution and scanning electron microscopy [SEM]) in the cartilage matrix of osteochondral dowels that had been cut before being frozen. In this study we found a remarkable correlation between the presence of vacancies in the tissue (left behind by ice crystals) and the zone of cell death (Muldrew et al., 2000b). The interior of the cartilage matrix (where the cells had died) had a spongy appearance with large vacancies on the order of 1 μm across, at least 100 times the average pore size in normal cartilage. Ice growth in this region had clearly caused a mechanical disruption to the tissue structure, whereas the region adjacent to the surfaces (where the cells survived) had no visible vacancies, as would be expected if the pore size remained on the order of 10 nm (Muldrew et al., 2000b). The boundary between these two regions, as with the boundary between live and dead cells, was a sharp line, exactly parallel to the outer surfaces.

We hypothesized that ice was growing into the cartilage matrix in a planar fashion rather than dendritically, at least macroscopically. On a microscopic level, the ice would grow through the aqueous channels as dendrites, but within each channel, the curvature imposed on the ice interface would be too high to allow more than one dendrite to form in a channel. The effect of such a mode of ice growth would be to impose a diffusion-limited growth rate on each ice dendrite, and hence on the macroscopic ice front as a whole. The rate that ice could grow into the matrix from the surface would then be limited by diffusion of solutes away from the interface (toward the interior of the cartilage), even in the presence of a steep temperature gradient. A consequence is that the interior of the tissue would become constitutionally supercooled, making nucleation events more likely. Any ice crystal that formed in the interior of the matrix because of a nucleation event would thus become an ice lens, drawing water from the nearby matrix and expanding the porosity of the matrix. This would explain the spongy morphology of the matrix seen with freeze substitution and also give a plausible reason for cell death. The chondrocytes in this region may have been injured by mechanical effects of ice lenses, or the redistribution of water that accompanies the formation of ice lenses may have imposed an osmotic stress that led to cell injury.

To test this hypothesis, we decided to look at the rate of cooling. If the growth of ice into the matrix was truly diffusion-limited, then we could allow ice growth to proceed further into the tissue—and reduce the likelihood of interior nucleation events—by slowing the cooling rate. Because this would also have the effect of increasing the solution-effects injury to the cells that were encapsulated in the ice growing into the tissue, we attempted to keep the temperature high,

where solution-effects injury accumulates slowly, and to then proceed with freezing at a much faster rate once lower temperatures were reached. To simplify the procedure, we used holding times at various subzero temperatures rather than various cooling rates; the samples were held at –4°C for 60 min, –8°C for 30 min, and –40°C for 10 min and then plunged into liquid nitrogen. Using this method, we were able to see the overall cell recovery almost double, compared with the 1°C/min protocol, and the regions of cell recovery extend much further into the matrix, though there remained a zone of dead cells in the middle (Muldrew et al., 2001b).

Although this appeared to be a substantial improvement over previous techniques for cryopreserving articular cartilage, when we transplanted this tissue into sheep we found that it was far from optimal. At 3 months following transplantation, the number of live cells was dramatically lower than we had observed immediately after thawing (Muldrew et al., 2001a). This was a surprise because our previous experience had shown that a membrane integrity assay had given a reliable indication of cell death caused by cryoinjury in articular cartilage (because chondrocytes are immobile, we could compare the results of the membrane integrity assay with the cells that remained in the host following transplantation). As a further surprise, the chondrocytes that remained after 3 months in the host had reentered the cell cycle, and by 12 months there were chondrocyte clusters, clonal in origin, that consisted of over 100 cells in some cases (Muldrew et al., 2001a). We hypothesized that the extended holding period at high subfreezing temperatures, although facilitating ice growth into the tissue, was responsible for the loss of cellular processes that extended into the cartilage matrix (because of hypertonic shrinkage). Because the normal chondrocyte phenotype is dependent on these connections, their loss may have induced the cells to either initiate apoptosis or to reenter the cell cycle (both of which actions are seen in late-stage osteoarthritis when cell-matrix connections are lost because of matrix degradation).

Although the successful cryopreservation of articular cartilage remains an elusive goal, there are lessons that may be applied to tissue cryopreservation in general. The most important are that the tissue structure can alter the growth of ice, which can lead to osmotic conditions that are far from the phase diagram; that ice lenses can cause mechanical and osmotic stresses; and that even novel modes of cryoinjury may occur (because of altered cell-matrix interactions), when compared with the cryopreservation of cells in bulk solution. Any attempt to optimize cell recovery within a tissue using mathematical models should not assume that the cells are on the liquidus curve of the phase diagram or that simple mechanisms of cellular injury are dominant. Articular cartilage is unique in that it has almost no capacity to repair any injury (and thus cryopreservation must be almost perfect for successful transplantation), but it has been precisely this property that has shown us that the problem is much more complex than the mere cryopreservation of cells. Although empirical methods have been very successful in developing cryopreservation techniques for different cells, a sound understanding of ice growth, and the cellular responses to that growth, will be more likely to lead to success with tissues.

2.7 MELTING OF ICE

During rewarming at temperatures above the eutectic temperature, as ice melts, the concentration of solutes in the residual phase decreases. For cells equilibrated by dehydration during slow cooling, there is a progressive rehydration during warming (Figure 2.22). Most practical protocols for cryopreservation use the highest cooling rate that avoids intracellular freezing to minimize cryoinjury. These cells are, therefore, not in osmotic equilibrium during cooling, so there will be further dehydration occurring during the warming phase (Figure 2.22) as cells respond to the lower extracellular chemical potential. However, as more ice melts, diluting the extracellular solution, the water flux is reversed, and the cell becomes rehydrated.

For cells containing intracellular ice, recrystallization is a thermodynamic phenomenon that occurs during thawing and that is thought to add further to cryoinjury. Small ice crystals will have a higher internal pressure than larger ice crystals because of their increased interfacial curvature

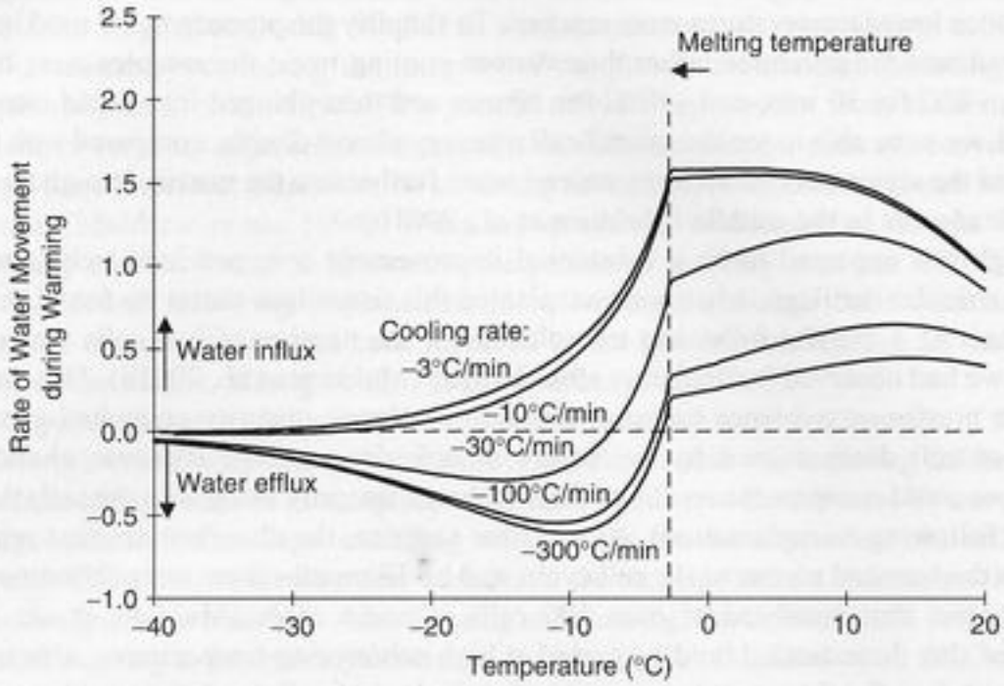

FIGURE 2.22 The water movement across the plasma membrane of cells during warming at 100°C/min after cooling at different rates to –40°C in the presence of 1 *M* Me_2SO.

and the existence of ice–solution interfacial tension. Because of the dependence of chemical potential on pressure, large ice crystals may grow while small ice crystals are melting at a given temperature. These growing ice crystals may cause damage to cells (Mazur, 1984).

2.8 CONCLUSIONS AND FUTURE DIRECTIONS

The ability to cryopreserve many cellular systems with high postthaw viability has led to many important developments in agriculture, animal husbandry, and medicine. Concomitant increases in understanding the behavior of cells at the ice–water interface have refined these processes to the point at which many cellular systems are routinely preserved with high recovery. Despite these advances, new challenges for cellular preservation are emerging as the requirements for even higher cell survival is becoming important, as in the areas of stem cell preservation, where a limited number of valuable cells—natural or engineered—are available for specific applications. There also are still some important cell types for which effective methods for cryopreservation are not available, such as sperm cells and oocytes from several species and some blood cells (platelets and granulocytes). The detailed mechanisms of injury need to be explored and understood, both to provide a rational basis for extrapolating cryopreservation techniques and for discovering novel techniques that may provide unforeseen benefits.

The cellular studies have provided a starting point for modeling low-temperature responses of the cells within a tissue matrix, but the extrapolation is not trivial. At the very least, tissues are three-dimensional structures, so heat and mass transfer within the tissue are important factors. Tissues generally contain several cell types, with specific characteristics that affect low-temperature responses. Cell–cell and cell–matrix adhesions strongly influence low-temperature responses, and these interactions appear to be the factors limiting cryopreservation of tissues. Therefore, the limits to our understanding on low-temperature responses of biological systems are most evident at the ice–water interface in tissue systems. Like natural tissues, engineered tissues are constructed in a variety of configurations—encapsulated beads, monolayers on flat or spherical surfaces, embedded sheets, and three-dimensional structures with different morphologies.

Cryopreservation is often the only method for preserving physiological structure, viability, and function in living tissues for extended periods of time, so considerable research efforts around the world are now exploring methods for cryopreservation of tissue systems, motivated by the fact that, in medicine, tissue transplantation is increasingly being used in the treatment of a variety of disorders, and engineered tissues are emerging for many biomedical applications. Our ability to preserve these natural or engineered constructs for distribution has lagged significantly behind their development and use. The pending use of engineered tissues (corneal, skin, liver) for toxicology testing has increased the imperative for effective methods for routine preservation. Current indications are that low-temperature responses of engineered tissues are similar to those of natural tissues.

Experience over the last few decades has demonstrated that low-temperature responses of tissues are too complex for purely empirical development of effective cryopreservation protocols, so conceptual developments at the ice–water interface for tissues will require increased understanding to guide empirical experimentation.

REFERENCES

Abidor, I.G., Arakelyan, V.B., Chernomordik, L.V., Chizmadzhev, Y.A., Pastushenko, V.F., and Tarasevich, M.R. (1979) Electric breakdown of bilayer lipid membranes I. The main experimental facts and their qualitative discussion, *J. Electroanal. Chem.*, 104, 37–52.

Acker, J.P., Elliott, J.A.W., and McGann, L.E. (2001) Intercellular ice propagation: Experimental evidence for ice growth through membrane pores, *Biophys. J.*, 81, 1389–1397.

Acker, J.P., Larese, A., Yang, H., Petrenko, A., and McGann, L.E. (1999a) Intracellular ice formation is affected by cell interactions, *Cryobiology*, 38, 363–371.

Acker, J.P. and McGann, L.E. (1998) The role of cell-cell contact on intracellular ice formation, *Cryo-Letters*, 19, 367–374.

Acker, J.P. and McGann, L.E. (2000) Cell–cell contact affects membrane integrity after intracellular freezing, *Cryobiology*, 40, 54–63.

Acker, J.P. and McGann, L.E. (2001) Membrane damage occurs during the formation of intracellular ice, *Cryo-Letters*, 22, 241–254.

Acker, J.P. and McGann, L.E. (2002) Innocuous intracellular ice improves survival of frozen cells, *Cell Transplant.*, 11, 563–571.

Acker, J.P. and McGann, L.E. (2003) Protective effect of intracellular ice during freezing? *Cryobiology*, 46, 197–202.

Acker, J.P., Pasch, J., Heschel, I., Rau, G., and McGann, L.E. (1999b) Comparison of optical measurement and electrical measurement techniques for the study of osmotic responses of cell suspensions, *Cryo-Letters*, 20, 315–324.

Adams, R.B., Voelker, W.H., and Gregg, E.C. (1967) Electrical counting and sizing of mammalian cells in suspension: An experimental evaluation, *Phys. Med. Biol.*, 12, 79–92.

Aggarwal, S.J., Diller, K.R., and Baxter, C.R. (1984) Membrane water permeability of isolated skin cells at subzero temperatures, *Cryo-Letters*, 5, 17–26.

Armitage, W.J. and Juss, B.K. (1996) Osmotic response of mammalian cells: Effects of permeating cryoprotectants on nonsolvent volume, *J. Cell Physiol*, 168, 532–538.

Asahina, E. (1956) The freezing process of plant cell, *Contrib. Inst. Low Temp. Sci. Ser. B*, 10, 83–126.

Asahina, E. (1962) Frost injury in living cells, *Nature*, 196, 445–446.

Asahina, E., Shimada, K., and Hisada, Y. (1970) A stable state of frozen protoplasm with invisible intracellular ice crystals obtained by rapid cooling, *Exp. Cell Res.*, 59, 349–358.

Ashwood-Smith, M.J., Morris, G.W., Fowler, R., Appleton, T.C., and Ashorn, R. (1988) Physical factors are involved in the destruction of embryos and oocytes during freezing and thawing procedures, *Hum. Reprod.*, 3, 795–802.

Berger, W.K. and Uhrik, B. (1992) Dehydration and intracellular ice formation during freezing in single cells and cell strands from salivary glands, *Cryobiology*, 29, 715–716.

Berger, W.K. and Uhrik, B. (1996) Freeze-induced shrinkage of individual cells and cell-to-cell propagation of intracellular ice in cell chains from salivary glands, *Experientia*, 52, 843–850.

Bischof, J.C. and Rubinsky, B. (1993) Large ice crystals in the nucleus of rapidly frozen liver cells, *Cryobiology*, 30, 597–603.

Bordi, F., Cametti, C., and Naglieri, A. (1998) Ionic transport in lipid bilayer membranes, *Biophys. J.*, 74, 1358–1370.

Boroske, E., Elwenspoek, M., and Helfrich, W. (1981) Osmotic shrinkage of giant egg-lecithin vesicles, *Biophys. J.*, 34, 95–109.

Brown, M.S. (1980) Freezing of nonwoody plant tissues. IV. Nucleation sites for freezing and refreezing of onion bulb epidermal cells, *Cryobiology*, 17, 184–186.

Brown, M.S. and Reuter, F.W. (1974) Freezing of nonwoody plant tissues. III. Videotaped micrography and the correlation between individual cellular freezing events and temperature changes in the surrounding tissue, *Cryobiology*, 11, 185–191.

Buckhold, B., Adams, R.B., and Gregg, E.C. (1965) Osmotic adaptation of mouse lymphoblasts, *Biochim. Biophys. Acta*, 102, 600–608.

Buitink, J., Claessens, M.M.A.E., Hemminga, M.A., and Hoekstra, F.A. (1998) Influence of water content and temperature on molecular mobility and intracellular glasses in seeds and pollen, *Plant Physiol.*, 118, 531–541.

Callow, R.A. and McGrath, J.J. (1985) Thermodynamic modeling and cryomicroscopy of cell-size, unilamellar and paucilamellar liposomes, *Cryobiology*, 22, 251–267.

Chambers, R. and Hale, H.P. (1932) The formation of ice in protoplasm, *Proc. R. Soc.*, 110, 336–352.

Chen, T., Acker, J.P., Eroglu, A., Cheley, S., Bayley, H., Fowler, A., and Toner, M. (2001) Beneficial effect of intracellular trehalose on the membrane integrity of dried mammalian cells, *Cryobiology*, 43, 168–181.

Crowe, J.H., Carpenter, J.F., and Crowe, L.M. (1998) The role of vitrification in anhydrobiosis, *Annu. Rev. Physiol.*, 60, 73–103.

Crowe, J.H. and Crowe, L.M. (2000) Preservation of mammalian cells: Learning nature's tricks, *Nature Biotechnol.*, 18, 145–146.

Crowe, J.H., Crowe, L.M., and Carpenter, J.F. (1993a) Preserving dry biomaterials: The water replacement hypothesis, Part 1, *Biopharm*, 4, 28–33.

Crowe, J.H., Crowe, L.M., and Carpenter, J.F. (1993b) Preserving dry biomaterials: The water replacement hypothesis, Part 2, *Biopharm*, 5, 40–43.

Dankberg, F. and Persidsky, M.D. (1976) A test of granulocyte membrane integrity and phagocytic function, *Cryobiology*, 13, 430–432.

Davson, H. and Danielli, J.F. (1952) *The Permeability of Natural Membranes*, The University Press, Cambridge, U.K.

Day, S.H., Nicoll-Griffith, D.A., and Silva, J.M. (1999) Cryopreservation of rat and human liver slices by rapid freezing, *Cryobiology*, 38, 154–159.

de Graaf, I.A.M. and Koster, H.J. (2001) Water crystallization within rat precision-cut liver slices in relation to their viability, *Cryobiology*, 43, 224–237.

Diller, K.R. (1975) Intracellular freezing: Effect of extracellular supercooling, *Cryobiology*, 12, 480–485.

Diller, K.R., Cravalho, E.G., and Huggins, C.E. (1972) Intracellular freezing in biomaterials, *Cryobiology*, 9, 429–440.

Dowgert, M.F. (1983) Effect of cold acclimation on intracellular ice formation in isolated protoplasts, *Plant Physiol.*, 72, 978–988.

Elliott, J.A.W. (2001) On the complete Kelvin equation, *Chem. Eng. Educ.*, 35, 274–278.

Elmoazzen, H.Y., Elliott, J.A.W., and McGann, L.E. (2002) The effect of temperature on membrane hydraulic conductivity, *Cryobiology*, 45, 68–79.

Eroglu, A., Russo, M.J., Bieganski, R., Fowler, A., Cheley, S., Bayley, H., and Toner, M. (2000) Intracellular trehalose improves the survival of cryopreserved mammalian cells, *Nat. Biotechnol.*, 18, 163–167.

Eroglu, A., Toner, M., and Toth, T.L. (2002) Beneficial effect of microinjected trehalose on the cryosurvival of human oocytes, *Fertil Steril*, 77, 152–158.

Fahy, G.M., Saur, J., and Williams, R.J. (1990) Physical problems with the vitrification of large biological systems, *Cryobiology*, 27, 492–510.

Farrant, J. and Morris, G.J. (1973) Thermal shock and dilution shock as the causes of freezing injury, *Cryobiology*, 10, 134–140.

Farrant, J., Walter, C.A., Lee, H., and McGann, L.E. (1977) Use of two-step cooling procedures to examine factors influencing cell survival following freezing and thawing, *Cryobiology*, 14, 273–286.

Farrant, J. and Woolgar, A.E. (1972) Human red cells under hypertonic conditions: A model system for investigating freezing damage, *Cryobiology*, 9, 9–15.

Finkelstein, A. (1987) *Water Movement Through Lipid Bilayers, Pores and Plasma Membrane*, Wiley, New York.

Fowler, R. and Toner, M. (1998) Prevention of hemolysis in rapidly frozen erythrocytes by using a laser pulse, *Ann. N.Y. Acad. Sci.*, 858, 245–252.

Franco, R.S., Barker, R., Novick, S., Weiner, M., and Martelo, O.J. (1986) Effect of inositol hexaphosphate on the transient behavior of red cells following a DMSO-induced osmotic pulse, *J. Cell. Physiol.*, 129, 221–229.

Franks, F., Mathias, S.F., Galfre, P., Webster, S.D., and Brown, D. (1983) Ice nucleation and freezing in undercooled cells, *Cryobiology*, 20, 298–309.

Fujikawa, S. (1980) Freeze-fracture and etching studies on membrane damage on human erythrocytes caused by formation of intracellular ice, *Cryobiology*, 17, 351–362.

Gaber, B.P., Chandrasekhar, I., and Pattabiraman, N. (1986) The interaction of trehalose with the phospholipid bilayer: A molecular modeling study, in *Membranes, Metabolism and Dry Organisms*, Leopold, A.C., Ed., Cornell University Press, Ithaca, NY, pp. 231–241.

Gao, D.Y., Benson, C.T., Liu, C., McGrath, J.J., Critser, E.S., and Critser, J.K. (1996) Development of a novel microperfusion chamber for determination of cell membrane transport properties, *Biophys. J.*, 71, 443–450.

Gao, D.Y., Chang, Q., Liu, C., Farris, K., Harvey, K., McGann, L.E., English, D., Jansen, J., and Critser, J.K. (1998) Fundamental cryobiology of human hematopoietic progenitor cells I: Osmotic characteristics and volume distribution, *Cryobiology*, 36, 40–48.

Gilmore, J.A., McGann, L.E., Ashworth, E., Acker, J.P., Raath, C., Bush, M., and Critser, J.K. (1998) Fundamental cryobiology of a selected African mammalian spermatozoa and its role in biodiversity preservation through development of genome resource banking, *Anim. Reprod. Sci.*, 53, 277–297.

Glaser, R., Leikin, S., Chernomordik, L.V., Pastushenko, V.F., and Sokirko, A. (1988) Reversible electrical breakdown of lipid bilayers: Formation and evolution of pores, *Biochim. Biophys. Acta*, 940, 275–287.

Gregg, E.C. and Steidley, K.D. (1965) Electrical counting and sizing of mammalian cells in suspension, *Biophys. J.*, 5, 393–405.

Hunt, C.J. (1984) Studies on cellular structure and ice location in frozen organs and tissues: The use of freeze-substitution and related techniques, *Cryobiology*, 21, 385–402.

Hurley, J. (1970) Sizing particles with a coulter counter, *Biophys. J.*, 10, 74–79.

Hurtig, M., Novak, K., McPherson, R., McFadden, S., McGann, L.E., Muldrew, K., and Schachar, N.S. (1998) Osteochondral dowel transplantation for the repair of focal defects in the knee: An outcome study using an ovine model, *Vet. Sur.*, 27, 5–16.

Irimia, D. and Karlsson, J.O.M. (2002) Kinetics and mechanism of intercellular ice propagation in a micro-patterned tissue construct, *Biophys. J.*, 82, 1858–1868.

Ishiguro, H. and Rubinsky, B. (1994) Mechanical interactions between ice crystals and red blood cells during directional solidification, *Cryobiology*, 31, 483–500.

Johnson, J. and Wilson, T. (1967) Osmotic volume changes induced by a permable solute, *J. Theor. Biol.*, 17, 304–311.

Karlsson, J.O.M. (2001) A theoretical model of intracellular devitrification, *Cryobiology*, 42, 154–169.

Karlsson, J.O.M., Cravalho, E.G., and Toner, M. (1993) Intracellular ice formation: Causes and consequences, *Cryo-Letters*, 14, 323–334.

Kedem, O. and Katchalsky, A. (1958) Thermodynamic analysis of the permeability of biological membranes to non-electrolytes, *Biochim. Biophys. Acta*, 27, 229–246.

Keeley, R.L.A., Gomez, A.C., and Brown, I.W. (1952) An experimental study of the effects of freezing, partial dehydration and ultra-rapid cooling on the survival of dog skin grafts, *Plast. Reconstr. Sur.*, 9, 330–344.

Kirkwood, J.G. (1954) Transport of ions through biological membranes from the standpoint of irreversible thermodynamics, in *Ion Transport Across Membranes*, Clarke, H.T., Ed., Academic Press, New York, pp. 119–127.

Kleinhans, F.W. (1998) Membrane permeability modeling: Kedem-Katchalsky vs. a two parameter formalism, *Cryobiology*.

Kubitschek, H.E. (1958) Electronic counting and sizing of bacteria, *Nature*, 182, 234–235.
Larese, A., Acker, J.P., Muldrew, K., Yang, H., and McGann, L.E. (1996) Antifreeze proteins induce intracellular nucleation, *Cryo-Letters*, 17, 175–182.
Levitt, J. (1962) A sulfhydryl-disulfide hypothesis of frost injury and resistance in plants, *J. Theor. Biol.*, 3, 355–391.
Levitt, J. (1966) Winter hardiness in plants, in *Cryobiology*, Meryman, H.T., Ed., Academic Press, London, pp. 495–563.
Levitt, J. and Scarth, G.W. (1936) Frost hardening studies with living cells. II. Permeability in relation to frost resistance and the seasonal cycle, *Can. J. Res.*, 14, 285–305.
Lovelock, J.E. (1953a) The haemolysis of human red blood cells by freezing and thawing, *Biochim. Biophys. Acta*, 10, 414–426.
Lovelock, J.E. (1953b) The mechanism of the protective action of glycerol against haemolysis by freezing and thawing, *Biochim. Biophys. Acta*, 11, 28–36.
Luyet, B.J. and Gehenio, P.M. (1940) The mechanism of injury and death by low temperature, *Biodynamica*, 3, 33–99.
Luyet, B.J. and Gibbs, M.C. (1937) On the mechanism of congelation and of death in the rapid freezing of epidermal plant cells, *Biodynamica*, 1–18.
Luyet, B.J. and Hodapp, E. (1938) Revival of frog's spermatozoa vitrified in liquid air, *Proc. Soc. Exp. Biol.*, 39, 433–444.
Luyet, B.J. and Rapatz, G. (1958) Patterns of ice formation in some aqueous solutions, *Biodynamica*, 8, 1–68.
Maroudas, A. (1970) Distribution and diffusion of solutes in articular cartilage, *Biophys. J.*, 10, 365–379.
Mazur, P. (1960) Physical factors implicated in the death of microorganisms at subzero temperatures, *Ann. N.Y. Acad. Sci.*, 85, 610–629.
Mazur, P. (1963a) Kinetics of water loss from cells at subzero temperatures and the likelihood of intracellular freezing, *J. Gen. Physiol.*, 47, 347–369.
Mazur, P. (1963b) Studies on rapidly frozen suspensions of yeast cells by differential thermal analysis and conductometry, *Biophys. J.*, 3, 353.
Mazur, P. (1965) The role of cell membranes in the freezing of yeast and other single cells, *Ann. N.Y. Acad. Sci.*, 125, 658–676.
Mazur, P. (1966) Physical and chemical basis of injury in single-celled micro-organisms subjected to freezing and thawing, in *Cryobiology*, Meryman, H.T., Ed., Academic Press, London, pp. 213–315.
Mazur, P. (1977) The role of intracellular freezing in the death of cells cooled at supraoptimal rates, *Cryobiology*, 14, 251–272.
Mazur, P. (1984) Freezing of living cells: Mechanisms and implications, *Am. J. Physiol.*, 247, C125–C142.
Mazur, P. (1990) Equilibrium, quasi-equilibrium, and nonequilibrium freezing of mammalian embryos, *Cell Biophys.*, 17, 53–92.
Mazur, P. and Koshimoto, C. (2002) Is intracellular ice formation the cause of death of mouse sperm frozen at high cooling rates? *Biol. Reprod.*, 66, 1485–1490.
Mazur, P., Leibo, S.P., and Chu, E.H.Y. (1972) A two-factor hypothesis of freezing injury—evidence from Chinese hamster tissue-culture cells, *Exp. Cell Res.*, 71, 345–355.
McGann, L.E. and Farrant, J. (1976a) Survival of tissue culture cells frozen by a two-step procedure to –196°C. I. Holding temperature and time, *Cryobiology*, 13, 261–268.
McGann, L.E. and Farrant, J. (1976b) Survival of tissue culture cells frozen by a two-step procedure to –196°C. II. Warming rate and concentration of dimethyl sulphoxide, *Cryobiology*, 13, 269–273.
McGann, L.E., Stevenson, M., Muldrew, K., and Schachar, N.S. (1988) Kinetics of osmotic water movement in chondrocytes isolated from articular cartilage and applications to cryopreservation, *J. Orthopaed. Res.*, 6, 109–115.
McGann, L.E., Turner, A.R., and Turc, J.M. (1982) Microcomputer interface for rapid measurement of average volume using an electronic particle counter, *Med. Biol. Eng. Comput.*, 20, 117–120.
McGrath, J.J. (1985) A microscope diffusion chamber for the determination of the equilibrium and non-equilibrium osmotic response of individual cells, *J. Microscopy*, 139, 249–263.
McLeester, R.C., Weiser, C.J., and Hall, T.C. (1969) Multiple freezing points as a test for viability of plant stems in the determination of frost hardiness, *Plant Physiol.*, 44, 37–44.
Meryman, H.T. (1957) Physical limitations of the rapid freezing method, *Proc. R. Soc.*, 147, 452–459.

Meryman, H.T. (1970) The exceeding of a minimum tolerable cell volume in hypertonic suspension as a cause of freezing injury, in *The Frozen Cell*, Wolstenholme, G.E. and O'Connor, M., Eds., Churchill, London, pp. 51–64.

Meryman, H.T. (1974) Freezing injury and its prevention in living cells, *Annu. Rev. Biophys.*, 3, 341–363.

Mider, G.B. and Morton, J.J. (1939) The effect of freezing *in vitro* on some transplantable mammalian tumors and on normal rat skin, *Am. J. Cancer*, 35, 502–509.

Molisch, H. (1982) Investigation into the freezing of plants, *Cryo-Letters*, 3, 331–390.

Morris, G.J. and McGrath, J.J. (1981) Intracellular ice nucleation and gas bubble formation in spirogyra, *Cryo-Letters*, 2, 341–352.

Mugnano, J.A., Wang, T., Layne, J.R., DeVries, A.L., and Lee, R.E. (1995) Antifreeze glycoproteins promote intracellular freezing of rat cardiomyocytes at high subzero temperatures, *Am. J. Physiol.*, 269, R474–R479.

Muldrew, K., Acker, J.P., and Wan, R. (2000a) Investigations into quantitative post-hypertonic lysis theory using cultured fibroblasts, *Cryobiology*, 41, 337.

Muldrew, K., Chung, M., Novak, K., Schachar, N.S., Rattner, J.B., and Matyas, J. (2001a) Chondrocyte regeneration in adult ovine articular cartilage following cryoinjury and long-term transplantation, *Osteoarthritis Cartilage*, 9, 432–439.

Muldrew, K., Hurtig, M., Novak, K., Schachar, N.S., and McGann, L.E. (1994) Localization of freezing injury in articular cartilage, *Cryobiology*, 31, 31–38.

Muldrew, K. and McGann, L.E. (1990) Mechanisms of intracellular ice formation, *Biophys. J.*, 57, 525–532.

Muldrew, K. and McGann, L.E. (1994) The osmotic rupture hypothesis of intracellular freezing injury, *Biophys. J.*, 66, 532–541.

Muldrew, K., Novak, K., Studholme, C., Wohl, G., Zernicke, R., Schachar, N.S., and McGann, L.E. (2001b) Transplantation of articular cartilage following a step-cooling cryopreservation protocol, *Cryobiology*, 43, 260–267.

Muldrew, K., Novak, K., Yang, H., Zernicke, R., Schachar, N.S., and McGann, L.E. (2000b) Cryobiology of articular cartilage: Ice morphology and recovery of chondrocytes, *Cryobiology*, 40, 102–109.

Muldrew, K., Schachar, N.S., and McGann, L.E. (1996) Permeation kinetics of dimethyl sulfoxide in articular cartilage, *Cryo-Letters*, 17, 331–340.

Paula, S., Volkov, A.G., Van Hoek, A.N., Haines, T.H., and Deamer, D.W. (1996) Permeation of protons, potassium ions, and small polar molecules through phospholipid bilayers as a function of membrane thickness, *Biophys. J.*, 70, 339–348.

Pegg, D.E. (1984) Red cell volume in glycerol/sodium chloride/water mixtures, *Cryobiology*, 21, 234–239.

Pegg, D.E., Hunt, C.J., and Fong, L.P. (1987) Osmotic properties of the rabbit corneal endothelium and their relevance to cryopreservation, *Cell Biophys.*, 10, 169–189.

Pitt, R.E., Chandrasekaran, M., and Parks, J.E. (1992) Performance of a kinetic model for intracellular ice formation based on the extent of supercooling, *Cryobiology*, 29, 359–373.

Pitt, R.E., Myers, S.P., Lin, T., and Steponkus, P.L. (1991) Subfreezing volumetric behavior and stochastic modeling of intracellular ice formation in *Drosophila melanogaster* embryos, *Cryobiology*, 28, 72–86.

Pitt, R.E. and Steponkus, P.L. (1989) Quantitative analysis of the probability of intracellular ice formation during freezing of isolated protoplasts, *Cryobiology*, 26, 44–63.

Rall, W.F., Reid, D.S., and Farrant, J. (1980) Innocuous biological freezing during warming, *Nature*, 286, 511–514.

Rasmussen, D.H., Macaulay, M.N., and MacKenzie, A.P. (1975) Supercooling and nucleation of ice in single cells, *Cryobiology*, 12, 328–339.

Schachar, N.S., Novak, K., Hurtig, M., Muldrew, K., McPherson, R., Wohl, G., Zernicke, R., and McGann, L.E. (1999) Transplantation of cryopreserved osteochondral dowel allografts for repair of focal articular defects in an ovine model, *J. Orthopaedic Res.*, 17, 909–920.

Sherman, J.K. (1962) Survival of higher animal cells after the formation and dissolution of intracellular ice, *Anatomical Rec.*, 144, 171–177.

Shimada, K. and Asahina, E. (1975) Visualization of intracellular ice crystals formed in very rapidly frozen cells at –27°C, *Cryobiology*, 12, 209–218.

Staverman, A.J. (1948) Non-equilibrium thermodynamics of membrane processes, *Trans. Faraday Soc.*, 48, 176–185.

Steponkus, P.L. (1984) Role of the plasma membrane in freezing injury and cold acclimation, *Annu. Rev. Plant Physiol.*, 35, 543–584.

Steponkus, P.L. and Dowgert, M.F. (1981) Gas bubble formation during intracellular ice formation, *Cryo-Letters*, 2, 42–47.

Steponkus, P.L. and Dowgert, M.F. (1984) Phenomenology of intracellular ice nucleation in isolated protoplasts, *Plant Physiol.*, 67, S58.

Steponkus, P.L., Dowgert, M.F., and Gordon-Kamm, W.J. (1983) Destabilization of the plasma membrane of isolated plant protoplasts during a freeze-thaw cycle: The influence of cold acclimation, *Cryobiology*, 20, 448–465.

Steponkus, P.L., Stout, D., Wolfe, J., and Lovelace, R. (1984) Freeze-induced electrical transients and cryoinjury, *Cryo-Letters*, 5, 343–348.

Steponkus, P.L. and Wiest, S.C. (1978) Plasma membrane alterations following cold acclimation and freezing, in *Plant Cold Hardiness and Freeze Stress—Mechanisms and Crop Implications*, Li, P.H. and Sakai, A., Eds., Academic Press, New York, pp. 75–91.

Stuckey, I.H. and Curtis, O.F. (1938) Ice formation and the death of plant cells by freezing, *Plant Physiol.*, 13, 815–833.

Terwilliger, T.C. and Solomon, A.K. (1981) Osmotic water permeability of human red cells, *J. Gen. Physiol.*, 77, 549–570.

Toner, M., Cravalho, E.G., and Karel, M. (1990) Thermodynamics and kinetics of intracellular ice formation during freezing of biological cells, *J. Appl. Phys.*, 67, 1582–1593.

Toner, M., Cravalho, E.G., Stachecki, J., Fitzgerald, T., Tompkins, R.G., Yarmush, M.L., and Armant, D.R. (1993) Nonequilibrium freezing of one-cell mouse embryos: Membrane integrity and developmental potential, *Biophys. J.*, 64, 1908–1921.

Tsuruta, T., Ishimoto, Y., and Masuoka, T. (1998) Effects of glycerol on intracellular ice formation and dehydration of onion epidermis, *Ann. N.Y. Acad. Sci.*, 858, 217–226.

Verkman, A.S., Van Hoek, A.N., Ma, T., Frigeri, A., Skach, W.R., Mitra, A., Tamarappoo, B.K., and Farinas, J. (1996) Water transport across mammalian cell membranes, *Am. J. Physiol.*, 270, C12–C30.

Walcerz, D.B. and Diller, K.R. (1991) Quantitative light microscopy of combined perfusion and freezing processes, *J. Microscopy*, 161, 297–311.

Wolfe, J. and Bryant, G. (1999) Freezing, drying, and/or vitrification of membrane–solute–water systems, *Cryobiology*, 39, 103–129.

Woods, E.J., Zieger, M.A.J., Lakey, J.R.T., Liu, J., and Critser, J.K. (1997) Osmotic characteristics of isolated human and canine pancreatic islets, *Cryobiology*, 35, 106–113.

Yang, H., Chen, A., Muldrew, K., Novak, K., Zernicke, R., Schachar, N.S., and McGann, L.E. (1995) *In situ* assessment of cell viability in tissues, *Cryobiology*, 32, 582–583.

Zade-Oppen, A.M.M. (1968) Posthypertonic hemolysis in sodium chloride systems, *Acta Physiol. Scand.*, 73, 341–364.

Zieger, M.A.J., Tredget, E.E., Sykes, B.D., and McGann, L.E. (1997) Injury and protection in split-thickness skin after very rapid cooling and warming, *Cryobiology*, 35, 53–69.