NON-EQUILIBRIUM PHASE TRANSFORMATIONS OF INTRACELLULAR WATER:
APPLICATIONS TO THE CRYOPRESERVATION OF LIVING CELLS

by

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ABSTRACT

A theoretical model for predicting the kinetics of ice crystallization inside cells during cryopreservation was developed by coupling separate models describing (1) cell dehydration by membrane-limited water transport, (2) homogeneous and catalyzed ice nucleation, and (3) diffusion-limited crystal growth. The model can be used with any cell type for which the membrane permeability Arrhenius coefficients and catalyzed-nucleation rate parameters are known, and predicts the effect of cryoprotectant additives (CPAs) given their rheological properties. Phenomenological viscosity models for two CPAs, glycerol and dimethylsulfoxide (DMSO), were developed. The model was extensively tested using literature data on intracellular ice formation (IIF) in mouse oocytes and cultured rat hepatocytes, cell types diverse in their biophysical properties. Using cryomicroscopy, nucleation rate parameters were measured for mouse oocytes in 1.5 M DMSO, yielding a kinetic coefficient $\Omega_{\text{SCN}}=3.92 \times 10^6 \text{ m}^{-2}\text{s}^{-1}$ and a thermodynamic coefficient $\kappa_{\text{SCN}}=3.59 \times 10^3 \text{ K}^5$ for surface-catalyzed nucleation, and $\Omega_{\text{VCN}}=2.26 \times 10^{26} \text{ m}^{-3}\text{s}^{-1}$, $\kappa_{\text{VCN}}=4.38 \times 10^{11} \text{ K}^5$ for volume-catalyzed nucleation.

Using the theoretical model as a tool, a freezing protocol for cryopreserving mouse oocytes was designed and optimized. Because both IIF and prolonged exposure to the extreme physicochemical conditions encountered during cryopreservation have been associated with irreversible cell damage, it was hypothesized that cell survival could be maximized by freezing cells as rapidly as possible while avoiding IIF. A two-step freezing method was chosen, in which oocytes, equilibrated in 1.5 M DMSO, would be cooled slowly at a rate $B$ to a temperature $T_{\text{plunge}}$, at which they would be plunged directly into liquid nitrogen (-196°C). Simultaneously varying $B$ and $T_{\text{plunge}}$ using a simplex optimization algorithm, the calculated protocol duration was minimized subject to the constraint that the predicted probability of IIF remain below 0.05. The optimal protocol parameter combination was predicted to be $B=0.59^\circ\text{C}/\text{min}$ and $T_{\text{plunge}}=-67^\circ\text{C}$. Mouse oocytes were frozen using protocols at and in the neighborhood of the predicted optimum, in order to test the model and determine the sensitivity of cell survival to variations in the protocol parameters. Assays for oocyte survival were membrane integrity (determined using fluorescent dyes) and morphological appearance. Maximum survival (82%) was obtained using a cooling rate of 0.5°C/min with a plunge temperature of -80°C.

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Nomenclature

\( A \)  
plasma membrane surface area

\( a \)  
poro radius

\( a_o \)  
apparent hydrodynamic radius of water molecule

\( B \)  
cooling rate

\( B_{\text{dehydration}} \)  
dehydration cooling rate

\( B_{\text{plunge}} \)  
plunge cooling rate

\( C \)  
Vogel-Fulcher pre-exponential coefficient

\( C_0 \)  
water-DMSO viscosity Arrhenius pre-exponential coefficient for 0 M DMSO

\( C_1 \)  
water-DMSO viscosity Arrhenius pre-exponential coefficient for 8 M DMSO

\( c \)  
cytosol composition

\( c_1 \)  
reference cytosol composition for heterogeneous nucleation rate parameters

\( \bar{c} \)  
non-dimensional supersaturation

\( c^b \)  
water concentration in ice

\( c_{\infty} \)  
water concentration in the bulk cytosol

\( c_a \)  
intracellular CPA concentration

\( c_{oo} \)  
initial intracellular CPA concentration

\( c_{liq} \)  
liquidus water concentration

\( c_s \)  
intracellular NaCl concentration

\( c_{so} \)  
initial intracellular NaCl concentration

\( c_w \)  
intracellular water concentration

\( D \)  
water diffusivity

\( \bar{D} \)  
effective water diffusivity

\( E \)  
Vogel-Fulcher exponential coefficient

\( E_0 \)  
water-DMSO viscosity Arrhenius activation energy for 0 M DMSO

\( E_1 \)  
water-DMSO viscosity Arrhenius activation energy for 8 M DMSO

\( E_{lp} \)  
membrane water permeability Arrhenius activation energy

\( f \)  
heterogeneous nucleator geometric factor

\( f_{\text{HET}} \)  
heterogeneous nucleation rate per unit substrate area

\( f_{\text{HOM}} \)  
homogeneous nucleation rate per unit volume

\( I_o \)  
characteristic nucleation rate

\( i \)  
nucleation instant

\( J \)  
total nucleation rate per cell

\( \bar{J} \)  
average nucleation rate in the interval \( T_g \leq T \leq T_h \)

\( j_{\text{HOM}} \)  
homogeneous nucleation rate per cell

\( j_{\text{SCN}} \)  
surface-catalyzed nucleation rate per cell

\( j_{\text{VCN}} \)  
volume-catalyzed nucleation rate per cell

\( j \)  
sampling instant

\( L_p \)  
plasma membrane water permeability

\( L_{P_{\text{ref}}} \)  
permeability membrane water permeability reference coefficient

\( N \)  
number of ice nuclei in a representative cell

\( \bar{N} \)  
ensemble average of the number of ice nuclei per cell

\( N \)  
number of ice nuclei in a given cell

\( n^* \)  
number of water molecules in stable ice nucleus
\( n_a \) number of moles of CPA in cell
\( n_s \) number of moles of NaCl in cell
\( P \) probability
\( Pe \) Peclet number
\( PIF \) probability that cell will contain one or more ice nuclei
\( p_{rc} \) crystal radius probability density
\( Q \) rheological interaction parameter
\( R \) gas constant
\( r_c \) ice crystal radius
\( S \) Heaviside step function
\( T \) temperature
\( T^* \) pore theory critical temperature
\( T_0 \) initial temperature
\( T_1 \) reference temperature for heterogeneous nucleation rate parameters
\( T_c \) Vogel-Fulcher critical temperature
\( T_g \) glass transition temperature
\( T_h \) homogeneous nucleation temperature
\( T_{ho} \) homogeneous nucleation temperature of water
\( T_m \) equilibrium melting temperature
\( T_{mo} \) equilibrium melting temperature of water
\( T_{plunge} \) plunge temperature
\( T_{refs} \) membrane water permeability reference temperature
\( T_{storage} \) cryogenic storage temperature
\( t \) time
\( t_g \) time at which temperature reaches \( T_g \)
\( t_h \) time at which temperature reaches \( T_h \)
\( U \) average crystal growth velocity
\( V^\beta \) volume of intracellular ice
\( V_b \) osmotically inactive volume
\( V_{CV} \) control volume (cytosol)
\( V_{cell} \) cell volume
\( V_{eq}^{\beta} \) intracellular ice volume thermodynamic equilibrium value
\( V_{wo} \) isotonic intracellular water volume
\( \nu_a \) specific volume of CPA
\( \nu_s \) specific volume of NaCl
\( \nu_w \) specific volume of water
\( X^\beta \) volume fraction of intracellular ice
\( X^\beta' \) volume fraction of intracellular ice, corrected for hard impingement
\( X_{c}^\beta \) critical intracellular ice volume fraction
\( x_0 \) DMSO mole fraction corresponding to 0 M DMSO
\( x_1 \) DMSO mole fraction corresponding to 8 M DMSO
\( x_w \) DMSO mole fraction
\( x_w \) water mole fraction
\( \alpha \) non-dimensional crystal growth parameter
\( \Delta H_f \) specific heat of fusion of water
\( \Delta N_j \) number of nuclei in sampling interval

\( \Delta T \) undercooling

\( \eta \) viscosity

\( \eta_{\text{DMSO}} \) viscosity of water-DMSO

\( \eta_{\text{Glycerol}} \) viscosity of water-glycerol solution

\( \eta_T \) viscosity of water-DMSO at 25\(^\circ\)C

\( \eta_{0} \) viscosity of 0 M DMSO

\( \eta_{8} \) viscosity of 8 M DMSO

\( \kappa_{\text{HET}} \) heterogeneous nucleation rate thermodynamic coefficient

\( \kappa_{I}^{\text{HET}} \) heterogeneous nucleation rate thermodynamic coefficient reference value

\( \kappa_{\text{HOM}}^{\text{HOM}} \) homogeneous nucleation rate thermodynamic coefficient

\( \kappa_{O}^{\text{HOM}} \) homogeneous nucleation thermodynamic coefficient reference value

\( \kappa_{\text{SCN}}^{\text{SCN}} \) surface-catalyzed nucleation rate thermodynamic coefficient

\( \kappa_{\text{ratio}}^{\text{SCN}} \) ratio of surface-catalyzed thermodynamic coefficient to homogeneous thermodynamic coefficient

\( \kappa_{\text{VNC}}^{\text{VNC}} \) volume-catalyzed nucleation rate thermodynamic coefficient

\( \kappa_{\text{ratio}}^{\text{VNC}} \) ratio of surface-catalyzed thermodynamic coefficient to homogeneous thermodynamic coefficient

\( V_i \) dissociation constant of NaCl.

\( \Omega_{\text{HET}}^{\text{HET}} \) heterogeneous nucleation rate kinetic coefficient

\( \Omega_{I}^{\text{HET}} \) heterogeneous nucleation rate kinetic coefficient reference value

\( \Omega_{\text{HOM}}^{\text{HOM}} \) homogeneous nucleation rate kinetic coefficient

\( \Omega_{O}^{\text{HOM}} \) homogeneous nucleation kinetic coefficient reference value

\( \Omega_{\text{SCN}}^{\text{SCN}} \) surface-catalyzed nucleation rate kinetic coefficient

\( \Omega_{\text{ratio}}^{\text{SCN}} \) ratio of surface-catalyzed kinetic coefficient to homogeneous kinetic coefficient

\( \Omega_{\text{VNC}}^{\text{VNC}} \) volume-catalyzed nucleation rate kinetic coefficient

\( \Omega_{\text{ratio}}^{\text{VNC}} \) ratio of volume-catalyzed kinetic coefficient to homogeneous kinetic coefficient

\( \phi \) salt volume fraction

\( \xi \) a given cell in an ensemble

\( \Psi \) cost functional

\( \rho_{c} \) crystal radius number density

\( \sigma \) ice-liquid interfacial free energy

\( \theta \) contact angle between substrate and ice-liquid interface

\( \theta_{l} \) ice nucleus contact angle reference value

\( \tau \) \( T_m^5 \left( T_m - T \right)^2 T^3 \)

\( \tau_{0} \) \( T_{m0}^5 \left( T_{m0} - T_{ho} \right)^2 T_{ho}^{-3} \)

\( \tau_{\text{dehydration}} \) characteristic time scale for cell dehydration
**Key to Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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</thead>
<tbody>
<tr>
<td>CPA</td>
<td>cryoprotectant additive</td>
</tr>
<tr>
<td>CV</td>
<td>control volume</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>HET</td>
<td>heterogeneous nucleation</td>
</tr>
<tr>
<td>HOM</td>
<td>homogeneous nucleation</td>
</tr>
<tr>
<td>IIF</td>
<td>intracellular ice formation</td>
</tr>
<tr>
<td>PIF</td>
<td>probability of intracellular ice formation</td>
</tr>
<tr>
<td>SCN</td>
<td>surface-catalyzed nucleation</td>
</tr>
<tr>
<td>VCN</td>
<td>volume-catalyzed nucleation</td>
</tr>
</tbody>
</table>
Chapter 1

Intracellular Ice Formation

Introduction
It is generally accepted that a primary mode of injury experienced by cells that are subjected to subzero temperatures is associated with the transformation of intracellular water from the liquid to the crystalline state, i.e. intracellular ice formation (IIF). The process of IIF has been found to play a central role in a wide range of applications, which seek either to prevent IIF-related damage, or to control and exploit this mode of injury. In all cases, it is of crucial importance that both the mechanism by which IIF occurs and the factors which are responsible for the damage associated with IIF be understood. In this chapter, an outline is given of the progress that has been made since the early 1960's towards gaining a better understanding of IIF, and areas where further research is required are identified. A review of the significance of IIF to various disciplines of medicine, biotechnology and the biological sciences is followed by an exposé of the major hypotheses that have emerged to explain IIF, the mathematical models that have been used to characterize and predict IIF, and, some of the proposed mechanisms of IIF-related damage. Lastly, the scope of the present work is outlined.

Ramifications of IIF
A better understanding of the causes and effects of IIF would lead to important advances in a number of diverse applications in the fields of medicine, biotechnology and basic
biological sciences (Table 1-1). Two medical fields which stand to benefit significantly from an improved understanding of IIF are transplantation and surgery. Cryopreservation would allow long-term banking of cells and tissues for purposes of transplantation. However, successful cryopreservation appears to be contingent upon prevention of damaging levels of IIF during both freezing and thawing [31]. In contrast, the goal of cryosurgery is to devitalize undesirable tissue (e.g. carcinoma) by *in situ* freezing [18]. In both procedures, freezing causes ice to form first in the extracellular environment, inducing cell dehydration due to the resulting chemical potential difference between the intra- and extracellular water. If the rate of freezing is fast in comparison to the rate of exosmosis of water from the cell, the cytoplasm supercools, and IIF can occur. An additional phenomenon relevant to freezing of tissue is the accumulation of water in the vasculature, which occurs as a result of dehydration of the surrounding cells. The resulting overdistention and rupture of the blood vessels is believed to be an important mechanism of damage in the freezing of healthy tissue [48]. However, there is some evidence that cells in neoplastic tissue resist freeze-induced dehydration, and thus that IIF may become important for cryosurgery of tumors [5].

IIF also has ramifications for biotechnology and basic scientific research. For example, the cryopreservation of *Drosophila melanogaster* (*D. melanogaster*) embryos, for the purpose of banking of mutant strains used in biotechnology research, has become possible only after recent breakthroughs [33,58]. *D. melanogaster* are cryopreserved by complete vitrification, i.e. the formation of an amorphous solid phase by rapid solidification in the presence of high concentrations of cryoprotectants (CPAs), thus preventing deleterious
Table 1-1: Various applications to which IIF is relevant.

| Medical Sciences                      | Banking of Cells and Tissues  |
|                                      |                               |
|                                      | Cryosurgery                    |
| Biotechnology                        | Prevention of Frost Injury in Plants |
|                                      | Pest Control                   |
|                                      | Artificial Insemination and Embryo Transfer |
|                                      | Anti Freeze Glycoproteins      |
| Basic Sciences                       | Electron Microscopy            |
|                                      | Biology of Cold Hardy Animals  |
|                                      | Genetics Research              |
|                                      | Banking of Germplasm of Endangered Species |
changes associated with both IIF and external ice. This process requires the design of vitrification solutions which are non-toxic, yet sufficiently viscous to prevent ice formation during freezing and rewarming. Another field in which the process of IIF is relevant is the study of cold hardy insects, amphibians and reptiles. Animals which must endure prolonged exposures to temperatures below the freezing point of their body fluids have developed two strategies of survival: freeze-avoidance and freeze-tolerance. Freeze-avoidant species achieve deep supercooling of body fluids using various adaptations, including colligative depression of the maximum supercooling point by synthesis of cryoprotectants, and inhibition of ice nucleation by removal of ice nucleating sites within the body, and addition of antifreeze proteins to body fluids [9,59]. Freeze-tolerant species, on the other hand, use regulated freezing of extracellular body fluids as well as synthesis of cryoprotectants to prevent IIF. Ice nucleating proteins induce extracellular freezing at high subzero temperatures (above -10°C), to ensure a controlled rate of external ice formation, and to allow equilibration of the intracellular water by dehydration [59,60]. The study of naturally occurring cold hardiness can provide useful insight into the control of IIF in other applications. For example, antifreeze proteins found in certain Antarctic fishes have been used to partially inhibit damaging IIF in cryopreserved yeast [35]. Ice nucleating bacteria and fungi found in the guts of freeze-tolerant insects and frogs have been used to reduce the cold hardiness of freeze-susceptible grain insect pests, potentially offering a means of biological control of both overwintering (e.g. rusty grain beetle), and stored-product (e.g. Cryptolestes ferrugineus) insect pests [15,22]. Epiphytic ice nucleating bacteria are also responsible for extensive frost-related losses of agricultural
crops. Current methods for reducing frost damage to crops have met with limited success, and better understanding of IIF in plant cells is necessary to further improve winter survival of sensitive crops [25]. Another biotechnological application in agriculture which relies on preventing IIF is the cryopreservation of livestock gametes for artificial insemination and embryo transfer. The freezing of cells without inducing IIF is also an important goal in specimen preparation for electron microscopy, where IIF can disrupt subcellular structures and create undesired artifacts [19]. In summary, significant advances in a wide range of disciplines are predicated upon a fundamental and complete understanding of the processes which govern the formation of intracellular ice and cause freezing damage in cells and tissues.

*Mechanisms of IIF*

Different cells vary considerably in their freezing behavior, undergoing IIF at cooling rates ranging from 1°C/min to 1000°C/min and temperatures between -5°C and -50°C (Fig. 1-1). The wide range of physicochemical conditions under which IIF is observed demonstrates that determining the mechanism of IIF is an exceedingly complex problem. Although the exact mechanism by which IIF occurs remains to be established, there is overwhelming evidence that extracellular ice is involved in initiating IIF. For example, many cells that experience IIF at temperatures above -15°C in the presence of external ice have been observed to undergo IIF at significantly lower temperatures when extracellular ice is absent [28]. Furthermore, liposomes with no intracellular components have been observed to experience IIF (in the presence of external ice) at temperatures above -10°C,
Figure 1-1: Median IIF temperature at the cooling rate yielding 50% IIF, for various cell types (in cases where the experimental data for 50% IIF was not available, the closest cooling rate and the corresponding median IIF temperature were used).
consistent with the IIF temperature range for most biological cells, thus strongly suggesting involvement of the external ice and the cell membrane in the IIF process [6]. The mechanism by which the extracellular ice interacts with the plasma membrane to induce IIF has been subject to speculation, and three major hypotheses of IIF have been proposed: (i) pore theory, (ii) membrane failure, and (iii) surface-catalyzed IIF.

According to pore theory, external ice inoculates the supercooled cytoplasm by growing through aqueous pores in the plasma membrane [28]. In order for the extracellular ice to propagate through the pores, the advancing ice front must have a local radius of curvature on the same order of magnitude as the pore radius (3 - 8 Å). Thus, at temperatures for which ice crystals of the requisite dimensions are thermodynamically unstable, the plasma membrane will be an effective barrier to the external ice, whereas at lower temperatures, the external ice will come in direct contact with the supercooled intracellular solution, causing IIF.

The membrane failure hypothesis assumes that IIF occurs when the cytoplasm is exposed to the external ice as a result of a mechanical breakdown of the plasma membrane. In 1961, Asahina [1] suggested that damage to the cell membrane is the cause of IIF. Subsequently, Dowgert and Steponkus [12] have proposed that IIF in isolated rye protoplasts is a result of destabilization of the plasma membrane, and that the reduced probability of IIF in cold acclimated protoplasts is attributable to an increase in the plasma membrane stability. Physical changes consistent with membrane failure, and occurring immediately prior to IIF, were observed cryomicroscopically in protoplasts by Steponkus
and Dowgert [57], although the total frequency of such observations was low. Exodocytotic vesiculation during osmotic contraction, thermal perturbations, and electrical transients at the advancing extracellular ice front have been suggested as possible causes of plasma membrane breakdown [56]. Recently, Muldrew and McGann [38] have proposed that membrane damage may be caused by a critical gradient in osmotic pressure across the membrane. Specifically, frictional forces on the plasma membrane due to osmotic water efflux were suggested as the cause of membrane rupture [37].

The hypothesis of surface-catalyzed IIF assumes that external ice catalyzes the nucleation of intracellular ice on the internal surface of the plasma membrane. This theory does not presuppose that the extracellular ice comes in contact with the cytoplasm, but rather that the role of the external ice is to promote intracellular nucleation by interacting with the plasma membrane and/or the intracellular water molecules. Toner et al. [63] have proposed a theory of surface-catalyzed heterogeneous nucleation, which assumes that the external ice alters the structure of the plasma membrane in such a way that the internal surface of the membrane becomes an efficient ice nucleator in the temperature range where IIF is typically observed (-5°C to -15°C). Coherent heterogeneous nucleation has been suggested as a possible mechanism of IIF catalysis [61]. An alternative hypothesis of surface-catalyzed IIF, put forward by Bronshteyn [41], is epitaxial nucleation of ice across the plasma membrane. Epitaxy (the nucleation and growth of crystals onto a catalytic substrate) can occur even when there is an intervening layer between the substrate and the supercooled melt, and it is thus possible that the extracellular ice induces epitaxial nucleation on the internal surface of the plasma membrane.
The three hypotheses of IIF propose differing roles for the plasma membrane as a barrier against the external ice. In pore theory, the cell membrane is an effective barrier to extracellular ice crystals only above the temperature at which the ice can propagate through the membrane pores. The membrane failure hypothesis, on the other hand, presupposes a total loss of the barrier properties of the membrane as the cause of IIF. Thus, the membrane failure theory assumes that cell damage is the cause, rather than effect, of IIF. According to the hypothesis of surface-catalyzed IIF, the external ice induces intracellular nucleation without physically penetrating the plasma membrane. In this regard, the surface-catalysis theory differs from the other two hypotheses, which assume that the barrier properties of the cell membrane must be compromised for IIF to occur.

In addition to the mechanisms discussed above, in which IIF is mediated by external ice, supercooling experiments using droplet emulsion techniques have demonstrated that there are other mechanisms of IIF, which are active in the absence of extracellular ice [16,26]. This alternative mode of IIF characteristically occurs at lower temperatures, typically in the range -30°C to -50°C, and homogeneous nucleation or relatively ineffective heterogeneous nucleation (catalyzed by intracellular supramolecular structures or homophase impurities) have been suggested as possible mechanisms. Certain cell types frozen in the presence of external ice also undergo IIF at temperatures below -30°C (see Fig. 1-1), suggesting an IIF mechanism similar to that observed in supercooling experiments [34,40,51,52]. Scheiwe and Körber [52] have shown two apparently different mechanisms of IIF during cooling of granulocytes, and classified them according
to their morphological appearance, namely "darkening" and "twitching" IIF. Given the fact that the twitching IIF occurred only ~7°C above the estimated homogeneous nucleation temperature, they proposed that it might be catalyzed by nucleators present within the cell. On the other hand, the darkening IIF occurred at much higher temperatures, suggesting involvement of the extracellular ice as discussed above. Although these observations and similar results obtained with lymphocytes [50] provide indirect evidence that different IIF mechanisms may be active in a given cell type, it is possible that the two modes of IIF observed represented two subpopulations of cells with distinct IIF characteristics (e.g. related to different phases of the cell cycle). However, further evidence for two-mechanism IIF was provided by quantitative analysis of IIF kinetics [57,63]. For example, in experiments with mouse oocytes cooled at a rapid rate (120°C/min) in hypertonic salt solutions (>800 mOsm), Toner et al. [63] have shown a sharp change in the kinetics of IIF at about -31°C, which was attributed to a change from IIF induced by extracellular ice to heterogeneous nucleation by intracellular supramolecular structures. Although less pronounced, a similar shift in IIF kinetics was observed by Steponkus and Dowgert [57] for acclimated plant protoplasts. In other experiments, distinct mechanisms of IIF were observed in a given cell type depending on the physicochemical environment of the cell [12,46]. For example, Myers et al. [40] reported that dechorionation of D. melanogaster embryos resulted in elevated IIF temperatures, suggesting that dechorionation allowed seeding of IIF by the external ice, while IIF in embryos with intact eggcases was catalyzed by internal nucleators. In addition, Rall et al. [46] observed that the presence of permeating CPAs reduced IIF
temperatures of mouse embryos to values close to the estimated homogeneous nucleation
temperatures, suggesting that IIF catalysis by the external ice was suppressed by CPAs,
and that an alternative mechanism became active. Clearly, then, the prevalent mechanism
of IIF depends on both cell type and environmental factors. However, most biological
cells frozen in the absence of CPAs appear to undergo IIF by a mechanism which is active
in the range -5°C to -15°C, and which probably involves the external ice.

**Mathematical Models of IIF**

While the exact mechanism of IIF remains an unsettled question, there have been some
advances in the ability to predict the incidence of IIF. The development of mathematical
models for the purpose of predicting IIF has been motivated by (i) the potential to guide
the design of cryopreservation protocols which minimize IIF, (ii) the ability to compare
IIF data obtained under different experimental conditions (e.g. inter-species comparison),
and (iii) the desire to gain further insight into the mechanism of IIF. Efforts to predict IIF
using mathematical models were pioneered by Mazur [27,28], who recognized that the
probability of ice formation in the cell was affected by cellular dehydration during freezing.
Mazur [27] developed a physicochemical model of the process of freeze-induced
dehydration, and later coupled this water transport equation to a simple model of IIF [28].
Although methods for determining the probability of IIF have evolved significantly since
Mazur's early models, the approach of coupling the calculation of the likelihood of
intracellular freezing with a water transport model became the basis for all subsequent
efforts to predict IIF.
Phenomenological models

Mazur's initial phenomenological model hypothesized the existence of a critical temperature, at or below which intracellular ice would form if the cytoplasm contained "freezable" water which was sufficiently supercooled [28,30]. These criteria were quantified by defining a supercooling tolerance and a critical water volume below which intracellular freezing would not occur. Given the instantaneous state of the cytoplasm, the probability of IIF can thus be calculated by multiplying three step-functions, corresponding to each criterion, as shown in Table 1-2. The critical temperature is given by pore theory as

\[ T^* = T_{m0} \left(1 + \frac{2 \nu_w \sigma \cos \theta}{a \Delta H_f}\right), \]  

where \( T_{m0} \) is the equilibrium melting point of water; \( \nu_w \), the specific volume of water; \( \sigma \), the ice-water interfacial energy; \( \theta \), the contact angle between the pore wall and the ice-water interface; \( a \), the pore radius; and \( \Delta H_f \), the specific heat of fusion of water. However, because the contact angle \( \theta \) is unknown, and the parameters \( \sigma \) and \( a \) have not been measured directly, the critical temperature cannot be calculated \( a \) priori from the above equation. Mazur thus postulated values for \( T^* \) based on measured temperatures of IIF for the cell type of interest [28,30,32]. The supercooling tolerance was defined arbitrarily to be 2°C, and the critical water volume was assumed to be 10% of the isotonic water content, based on measurements of "unfreezable" water in yeast and E. Coli [32]. Because a significant amount of experimental data must be consulted in order to define the
Table 1-2. Evolution of mathematical models to predict IIF.

<table>
<thead>
<tr>
<th>References</th>
<th>Phenomenological Models</th>
<th>Parameters</th>
<th>References</th>
<th>Mechanistic Models</th>
<th>Parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mazur (1965; 1977)</td>
<td>$P_{IIF}(t) = S(T^* - T) S(\Delta T - \Delta T^<em>) S(\tilde{V} - \tilde{V}^</em>)$</td>
<td>$T^<em>, \Delta T^</em>, \tilde{V}^*$</td>
<td>Toscano et al. (1975)</td>
<td>$\int_0^{IIF} J A dt = N(T_{IIF})$</td>
<td>$R, \theta$</td>
</tr>
<tr>
<td>Pitt and Steponkus (1989)</td>
<td>$P_{IIF}(t) = \int_0^\infty \int_0^T \frac{\partial F_t}{\partial T} \frac{\partial F_{\Delta T}}{\partial y} S(\Delta T - y) dT' dy$</td>
<td>$T_o, \tau$</td>
<td></td>
<td>$J = \Omega(T, c, R, \theta) e^{-\Delta F(T, R, \theta)/RT}$</td>
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<tr>
<td></td>
<td>$F_{T^*}(T') = 1 - e^{-(T'/T_o)^\gamma}$</td>
<td>$\nu_0, \gamma$</td>
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<tr>
<td>Pitt et al. (1991)</td>
<td>$P_{IIF} = P^{TD} + (1 - P^{TD}) P^{TT}$</td>
<td>$T_o, \tau$</td>
<td>Toner et al. (1990)</td>
<td>$P_{IIF} = P^{SCN} + (1 - P^{SCN}) P^{VCN}$</td>
<td>$\Omega^{SCN}, \kappa_o$</td>
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<tr>
<td></td>
<td>$P^{TD}(t) = 1 - e^{-\int_0^t \kappa(T') dt}$</td>
<td>$\tau_o, \rho$</td>
<td></td>
<td>$P^{SCN}(t) = 1 - e^{-\int_0^t J^{SCN}(T, c) A dt}$</td>
<td>$\Omega^{SCN}, \kappa_o$</td>
</tr>
<tr>
<td></td>
<td>$P^{TT}(t) = 1 - e^{-(t/T_o)^\gamma}$</td>
<td>$\Delta T^*$</td>
<td></td>
<td>$P^{VCN}(t) = 1 - e^{-\int_0^t J^{VCN}(T, c) V dt}$</td>
<td>$\Omega^{VCN}, \kappa_o$</td>
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<tr>
<td></td>
<td>$K(T) = \left( \frac{T}{T_o} \right)^\beta$</td>
<td></td>
<td></td>
<td>$J^{SCN}(T, c) = \Omega^{SCN} e^{-\kappa^{SCN} \Delta T - 2T - 3}$</td>
<td>$\alpha, b$</td>
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<tr>
<td></td>
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<td>$J^{VCN}(T, c) = \Omega^{VCN} e^{-\kappa^{VCN} \Delta T - 2T - 3}$</td>
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<td>$\Omega^{SCN}(T, c) = \Omega_o^{SCN} \left( \frac{T}{T_{m_o}} \right)^\frac{1}{\eta} \left( \frac{\eta_o}{\eta} \right) \left( \frac{\Lambda}{\Lambda_o} \right) \left( \frac{f(\theta)}{f(\theta_o)} \right)$</td>
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<td>$\kappa^{SCN}(T, c) = \kappa_o^{SCN} \left( \frac{T}{T_{m_o}} \right)^\frac{4}{\eta} \left( \frac{\eta_o}{\eta} \right) \left( \frac{\Lambda}{\Lambda_o} \right) \left( \frac{f(\theta)}{f(\theta_o)} \right)$</td>
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<td>$\Omega^{VCN}(T, c) = \Omega_o^{VCN} \left( \frac{T}{T_{m_o}} \right)^\frac{1}{\eta} \left( \frac{\eta_o}{\eta} \right) \left( \frac{\Lambda}{\Lambda_o} \right)$</td>
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<td>$\kappa^{VCN}(T, c) = \kappa_o^{VCN} \left( \frac{T}{T_{m_o}} \right)^\frac{4}{\eta} \left( \frac{\eta_o}{\eta} \right) \left( \frac{\Lambda}{\Lambda_o} \right)$</td>
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<td>$\theta(x) = ax + b$</td>
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</tr>
<tr>
<td>Pitt et al. (1992)</td>
<td>$P_{IIF}(t) = 1 - e^{-\int_0^t \kappa(\Delta T) S(\tilde{V} - \tilde{V}^*) dt}$</td>
<td>$\tau_o, \rho$</td>
<td>Toner et al. (1992)</td>
<td>$P_{IIF}(t) = 1 - e^{-\int_0^t J^{SCN}(T, c) A S(\tilde{V} - \tilde{V}^*) dt}$</td>
<td>$\Omega^{SCN}, \kappa_o$</td>
</tr>
<tr>
<td></td>
<td>$K(\Delta T) = \left( \frac{\Delta T}{T_o} \right)^\beta$</td>
<td>$\tilde{V}^*$</td>
<td></td>
<td>$J^{SCN}(T, c) = \Omega^{SCN} e^{-\kappa^{SCN} \Delta T - 2T - 3}$</td>
<td>$\Omega^{SCN}, \kappa_o$</td>
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<td>$\Omega^{SCN}(T, c) = \Omega_o^{SCN} \left( \frac{T}{T_{m_o}} \right)^\frac{1}{\eta} \left( \frac{\eta_o}{\eta} \right) \left( \frac{\Lambda}{\Lambda_o} \right)$</td>
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<td>$\kappa^{SCN}(T, c) = \kappa_o^{SCN} \left( \frac{T}{T_{m_o}} \right)^\frac{4}{\eta} \left( \frac{\eta_o}{\eta} \right) \left( \frac{\Lambda}{\Lambda_o} \right)$</td>
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</table>

$a, b$ linear regression coefficients

$A$ membrane area

$B$ cytoplasm composition

$T$ geometric function

$F$ cumulative probability function

$T$ free energy change

$J$ nucleation rate

$T$ temperature

$y$ critical supercooling

$\eta$ viscosity

$\gamma, \tau$ Weibull shape parameters

$\Omega$ thermodynamic coefficient

$\kappa$ kinetic coefficient

$\eta$ contact angle

$\rho, \tau$ power law coefficients

* critical value

$\Omega$ reference value

$\Omega^{SCN}$ surface catalyzed

$V_{CN}$ volume catalyzed

$TD$ time dependent

$TT$ time independent

Subscripts and Superscripts
necessary model parameters, few extrapolative predictions can be made. Two notable features of Mazur's model are that it contains no information on the kinetics of IIF, and that the predicted probability of IIF cannot assume intermediate values between 0 and 1, in contrast to experimental observations [31].

To be able to predict finite transition regions, Pitt and Steponkus [42] modified Mazur's model to account for the stochastic nature of IIF. The critical temperature and the supercooling tolerance were assumed to be independent random variables with Weibull distributions, and the probability of IIF was calculated as shown in Table 1-2. The amount of intracellular water was assumed always to exceed the critical volume. Because the effect of the supercooling tolerance is negligible at fast cooling rates, Pitt and Steponkus [42] could determine Weibull scale and shape parameter for the critical temperature from IIF data obtained during a rapid-cooling experiment. The parameters describing the supercooling tolerance probability distribution could then be determined from measurements obtained at an intermediate cooling rate (i.e. for which the incidence of IIF lies between 0% and 100%). As a result of the distribution in critical temperatures, transition zones of finite width could be predicted. However, the model assumes that the IIF process is time-independent, and that the IIF temperature is independent of cooling rate at fast rates of cooling. Experimental observations of time-dependent ice formation in D. melanogaster embryos [40] and mouse oocytes [65] could thus not be predicted using this model.
To be able to account for kinetic effects such as those observed in *D. melanogaster* embryos and mouse oocytes, Pitt et al. [43] extended their previous model by incorporating a time-dependent term. The probability of IIF, calculated as shown in Table 1-2, thus combined two separate mechanisms of ice formation, one of which was independent of time, the other of which was time-dependent. The time-dependent term was characterized by a rate function $K(T)$, which was assumed to be a function only of temperature, i.e. independent of the state of the cytoplasm. Time-independent IIF was described by a Weibull distribution in the critical temperature, and was thus invariant to cooling rate. The supercooling tolerance was assumed to be independent of both temperature and cooling rate, and therefore, by implication, invariant to the intracellular solute concentration. The model required measurement of five adjustable parameters. A best-fit power law model for $K(T)$ was determined from isothermal measurements of the time constant for IIF at several temperatures. To account for observed cumulative incidences of IIF less than 100%, a censored-data technique had to be used. The Weibull parameters of the time-independent mechanism could then be determined from a linear-cooling experiment, after subtracting out the time-dependent mechanism from the measured IIF data. The supercooling tolerance was estimated iteratively by comparing model predictions of the maximum incidence of IIF to experimental measurements at several cooling rates in the transition zone. Thus, the predictive abilities of the model were restricted to calculation of IIF temperatures in linear-cooling protocols. At fast cooling rates, the model correctly predicted the cooling rate dependence of the temperature of IIF for *D. melanogaster* embryos in isotonic medium. To predict the
observed IIF behavior at slow rates of cooling, Pitt et al. [43] introduced a log-normal
distribution of the permeability parameter in the water transport model. The model was
also applied to *D. melanogaster* embryos frozen in the presence of 2 M ethylene glycol,
but since all available data were used to determine the five IIF parameters, the accuracy of
the model predictions could not be independently verified.

In response to empirical evidence that the kinetics of IIF are affected by the
thermodynamic state of the cytoplasm [43,63], Pitt et al. [44] modified their stochastic
model (Table 1-2). The time-independent IIF mechanism was removed from the model,
the rate function in the kinetic term was made a function of the intracellular supercooling,
and the supercooling tolerance hypothesis was eliminated. However, the time-independent IIF term was reintroduced into the model when predicting results for *D.
melanogaster* embryos. A critical water volume restriction similar to that of Mazur's
model was introduced to preclude IIF in extensively dehydrated cells. Based on model
predictions for rye protoplasts, 4% of the osmotically active volume was assumed to be
"unfreezable" for all cell types investigated. A two-parameter power law model for the
rate function could be determined either by regression analysis of transient IIF times
during the initial period of isothermal freezing at several temperatures, or by estimating
Weibull parameters during linear cooling at a rate sufficiently fast to preclude cell
dehydration. The model was tested against experimental data obtained on various cell
types by comparing measured IIF behavior during isothermal and constant rate freezing
with corresponding predictions. The model accurately predicted the isothermal IIF
incidence for rye protoplasts at high freezing temperatures, and discrepancies between the
calculated and measured linear cooling behavior were small. In addition, predictions for *D. melanogaster* embryos in isotonic medium agreed reasonably well with experimental data. However, the model predictions for bovine oocytes were "unrealistic" compared with experimental data. By revising the best-fit IIF parameters for this cell type, Pitt et al. [44] obtained improved results. Likewise, the model overestimated IIF incidence in mouse oocytes frozen isothermally and at constant cooling rates. To improve model predictions, the authors adjusted the permeability parameters for mouse oocytes. However, experimental observations of the osmotic behavior of these cells at both suprazero [23] and subzero temperatures [62] suggest that modification of the water permeability parameters may have been unjustified.

Pitt et al. [44] also applied their model to *D. melanogaster* embryos frozen in 2 M ethylene glycol, obtaining for the first time predictions of IIF in the presence of CPA which could be compared to independent experimental data. The model appeared to be less successful in predicting IIF in the presence of CPA than with no CPA present. This may be due to the fact that the rate function $K(\Delta T)$ was assumed to be invariant to changes in the intracellular CPA concentration due to cell dehydration. Experimental observation of nucleation in droplets of aqueous solution demonstrated that the maximum achievable supercooling is strongly dependent on solute concentration, and thus that the kinetics of ice formation cannot be ascribed solely to the degree of supercooling in the solution [47]. This effect is more significant at higher concentrations of solute, and therefore, the resulting errors would be greater in the presence of CPA.
Although the predictive abilities of the phenomenological approach have improved significantly in recent years, the present models of Pitt and associates must be further tested and refined. As discussed above, freezing in the presence of CPAs may provide a critical test for the model of Pitt et al. [44], yet evaluation of model predictions using the limited data currently available is inconclusive. Further experimental investigations of IIF in the presence of CPAs for various cell types and freezing conditions are thus needed. Another important issue is the sensitivity of the model to the water transport predictions [41,44], as a result of which accurate measurements of the plasma membrane water permeability and its subzero temperature dependence are essential.

Mechanistic models

The first mechanistic model of IIF was that of Toscano et al. [67], who applied the heterogeneous nucleation rate theory developed by Turnbull and Fisher [68] to the freezing of human erythrocytes. The rate of nucleation was characterized by a kinetic coefficient, $\Omega$, and a thermodynamic barrier, $\Delta F$, both of which were functions of the state of the intracellular solution, as well as the geometry of the interaction between the ice cluster and a catalytic impurity. Toscano et al. [67] calculated the nucleation temperature as shown in Table 1-2, and undertook a parametric study of their model. Their results indicated that heterogeneous nucleation by internal catalysts was a plausible mechanism of IIF in erythrocytes. However, later experiments demonstrated that such internal nucleators were only active at temperatures close to the homogeneous nucleation
temperature [16,26]. Moreover, the fact that IIF occurs between -5°C and -15°C only in the presence of external ice [28] was not explained by the model of Toscano et al. [67].

To address these limitations, Toner et al. [63] proposed a two-mechanism model, whereby IIF is catalyzed either by a flat surface or by small spherical particles (hypothesized to correspond to the plasma membrane and intracellular heterophase impurities, respectively). Toner et al. [63] furthermore modified the original heterogeneous nucleation rate equations, and calculated the probability of IIF using the method of Carte [7]. As shown in Table 1-2, the nucleation rates for both mechanisms were characterized by a preexponential kinetic coefficient and an exponential thermodynamic coefficient, both of which were functions of the state of the intracellular solution. Moreover, the form of the nucleation rate equations indicated a strong dependence of IIF kinetics on the degree of cytoplasmic supercooling. The nucleation rate parameters were calculated by scaling, and reference values were obtained from linear-cooling experiments at a rate sufficiently rapid to preclude water transport. By freezing the cells in solutions of various osmolarities, the contact angle of the cluster on the substrate could be calculated as a function of intracellular salt concentration, and a linear relationship determined [63]. Toner et al. [65] tested the model against experimental data obtained on mouse oocytes. After determining the model parameters from linear-cooling data at 120°C/min, IIF behavior was predicted for constant cooling rates ranging from ~1°C/min to 100°C/min, and for isothermal freezing at various temperatures between 0°C and -10°C. Agreement between experimental observations and model predictions for constant-rate cooling was excellent, and good agreement between predicted and measured isothermal IIF behavior
was also obtained. IIF kinetics were shown to be dominated by the so-called surface-catalyzed nucleation mechanism, except at high solution osmolarities (>800 mOsm) and fast cooling rates (120°C/min) where nucleation catalysis by internal particles was hypothesized to become the dominant mechanism of IIF at temperatures below -31°C.

In a later paper by Toner et al. [64], their original model was simplified (Table 1-2). Because heterogeneous nucleation by internal catalysts is negligible for most practical purposes, the corresponding mechanism could be eliminated from the model. Furthermore, the change in the cluster-substrate contact angle $\theta$ due to cell dehydration was found to have a small effect on the nucleation parameters; since the technique previously used to measure the concentration-dependence of $\theta$ may have been inappropriate [61], the contact angle factor was not included in the modified model [64]. Thus, the two reference nucleation parameters can be determined from a single linear-cooling experiment at a rapid cooling rate. A threshold water volume similar to that used by Mazur was included in the model, and its value set at ~5%, although model predictions appeared to be fairly insensitive to the exact value of this parameter. Chandrasekaran and Pitt [8] have suggested other improvements to the earlier model of Toner et al. [63]. An improved method for computing the dependence of the pre-exponential factor on the contact angle $\theta$ was not applicable to the modified model by Toner et al. [64], since $\theta$ had been eliminated from the nucleation rate equations. Another improvement proposed by Chandrasekaran and Pitt [8] involved the calculation of the water activity, which would affect the water transport predictions. However, model results were not substantially
altered. The model has been applied to a large number of cell types, including mouse oocytes, one-cell mouse embryos, isolated and cultured hepatocytes, *D. melanogaster* embryos, β-islet cells, and nonacclimated and acclimated rye protoplasts, frozen under a wide range of physical and chemical conditions [61]. Reasonable agreement was obtained between predicted and observed IIF behavior for both linear-cooling and isothermal holding experiments. The model was also successfully used to optimize a multi-step rapid cooling protocol for mouse oocytes in the absence of permeating CPAs [66]. In addition to providing a rigorous test for the model, this study demonstrated the feasibility of using mathematical models of IIF for rational design of cryopreservation protocols.

However, the mechanistic approaches require data on the behavior of the membrane permeability, cytoplasm viscosity, and nucleation rate parameters at low temperatures and in the presence of CPAs. The paucity of experimental measurements of these basic physical and biophysical parameters under conditions relevant to cryobiology thus limits the accuracy of any attempt to model intracellular nucleation and crystal growth, and one concludes that further advances in the ability to predict IIF are contingent upon the availability of better experimental data. Furthermore, the exact nature of the interaction between the external ice and the plasma membrane is not known, and must be further investigated both to improve the predictive abilities of the model and to gain insight into the mechanism of IIF.
Model similarities and differences

Although the phenomenological and mechanistic treatments of IIF described above represent fundamentally different approaches to modeling IIF, there are striking similarities between the models that have emerged from each school of thought. Comparing the model equations of Pitt et al. [44] and Toner et al. [64] in Table 1-2, it is evident that the mathematical forms of the equations are very similar: in both cases, the probability of IIF is exponentially dependent on the integral of a rate function. Furthermore, both models are characterized by two adjustable parameters, which can be determined from a single rapid-cooling experiment. The only difference between the two models lies in the calculation of the corresponding rate functions. In the model of Pitt et al., the rate function $K(\Delta T)$ depends only on the supercooling of the cytoplasm, and is described by a power law. On the other hand, the rate function in the model of Toner et al. is the heterogeneous nucleation rate, which, although a function of the full thermodynamic state of the cytoplasm, is especially sensitive to the extent of cytoplasmic supercooling. In addition, when two nucleation mechanisms are included in the model of Toner and co-workers, the nucleation rate kinetic coefficient $\Omega$ determines the relative sensitivity of each mechanism to time. Comparing the pre-exponential parameters for surface-catalyzed IIF ($\Omega_{SCN} \approx 10^8 \text{ m}^{-2} \text{ s}^{-1}$) and volume-catalyzed IIF ($\Omega_{VCN} \approx 10^{50} \text{ m}^{-3} \text{ s}^{-1}$), it is evident that the latter mechanism will be relatively insensitive to time, while the former mechanism will show stronger time-dependence. Thus, while the two-mechanism model of Pitt et al. [43] contains a time-dependent term and a time-independent term, the two-mechanism form of the model developed by Toner et al. [63] contains a strongly
time-dependent term and a weakly time-dependent term. Thus, the recent phenomenological and mechanistic models of IIF are similar in their basic form, and differ mainly in their mathematical dependence on the state variables of the cytoplasm.

**Mechanisms of IIF-Induced Freezing Damage**

Although the question of whether IIF is a cause or consequence of cell damage is still a matter of some debate, there is significant evidence suggesting that irreversible damage to cells frozen rapidly is due to IIF [31]. These data are not incompatible with the membrane failure hypothesis, considering that the plasma membrane may be repaired after inoculation by the external ice, and that the subsequent IIF may be responsible for lethal injury to the cell. Despite the apparent involvement of IIF in cell damage, observations of innocuous IIF indicate that the presence of intracellular ice *per se* does not cause cell death, especially when the amount of cellular water transformed to ice is limited to a small fraction of the cell water content [3,30,45,53,54]. Thus, in order to be able to predict IIF-related damage, the mechanism by which such injury occurs must be understood. Unfortunately, the mechanism of IIF damage remains unknown. The hypotheses that have been put forward to date are largely speculative in nature, and are corroborated by little or no supportive evidence.

The most widely held view is that IIF-induced cell damage results from mechanical forces due to IIF [2,11,17,30]. Based on electron microscopy studies of frozen human erythrocytes, Fujikawa [17] suggested that the growing intracellular ice crystals exert a physical force on the plasma membrane, and that resulting molecular disorganization of
the lipid bilayer is responsible for post-thaw hemolysis. A different mode of action by the intracellular ice has been proposed by Mazur [29], who suggested that IIF causes rupture of membranes of intracellular organelles. According to one model of this damage mechanism, ice formation inside an organelle results in uptake of water from adjacent unfrozen compartments, causing the organelle to distend as the ice volume within it increases, possibly leading to membrane rupture. In a second model, two adjacent frozen compartments are joined by an ice-filled pore, the diameter of which would increase due to migratory recrystallization during warming, eventually causing damage to the limiting membrane. In addition, Haynes [20] has analyzed mechanical effects of freezing in porous materials, providing some insight into the mechanism of IIF damage. One possibility suggested by Haynes [20] is that ice growth into tapered pores results in increased curvature of the ice interface, leading to increased pressure within the solid and on the pore walls, which may lead to cell damage. Haynes [20] further suggested that disruptive stresses may be caused by the expansion of water upon freezing. Resistance to flow of the displaced liquid would lead to transient pressure build-ups which may be deleterious.

Various non-mechanical modes of damage have also been proposed. Rall et al. [45] showed that cell damage during warming of slowly frozen mouse embryos did not correlate with the visible events associated with IIF, suggesting that the appearance of ice crystals in the cell may be innocuous, and that injury may result from invisible changes in other components of the system. Farrant and Morris [14] suggested that the increased intracellular solute concentration due to IIF may alter intracellular membranes, rendering them susceptible to damage by mechanisms analogous to so-called "solution effects"
occurring during slow cooling of cells. For example, thermal shock has been put forward as a possible explanation for damage during rapid freezing [55]. Farrant [13], on the other hand, has proposed water transport as the damaging mechanism, pointing out that the size difference between intra- and extracellular crystals are indicative of a chemical potential difference between the water on both sides of the membrane, which provides a driving force for water transport. Similarly, preferential melting of intracellular ice crystals during warming may cause exosmosis of water, and concomitant injury [13]. Levitt [24] proposed a different theory of IIF damage, which assumed that intracellular ice crystals pressed proteins against each other, resulting in configurational deformations and allowing the formation of disulfide bridges, thus causing irreversible damage to the cell proteins. Morris and McGrath [36] and Ashwood-Smith et al. [4] observed that IIF induces the formation of intracellular gas bubbles during thawing, and conjectured that such gas bubbles may be the cause of cell injury. Even though a wide range of hypotheses have been put forward to attempt to explain the IIF damage mechanism, none of these model have been conclusively verified. It is possible that the mechanism of damage is multifactorial, and that the specific mode of injury depends on cooling and warming conditions, as well as the sensitivity of the cells to stresses associated with freezing.

Scope and Organization of the Dissertation
Several issues of fundamental importance to cryobiology emerge in the above review; this dissertation attempts to address the following:
• Although the mechanistic approach to modeling IIF has yielded promising results, such models are currently limited to predicting IIF in the absence of CPAs. Because the use of CPAs is ubiquitous in cryopreservation, the state of the art of IIF modeling must be advanced to include the effects of these chemicals. An improved model of IIF, incorporating CPAs, is presented here.

• Current IIF models (phenomenological and mechanistic) predict only the _onset_ of ice formation in cells, assuming that nucleation/seeding is the rate-limiting step for the phase transition of the intracellular water. For conditions where this assumption is not valid (e.g. in the presence of high concentrations of CPAs, or during rewarming of frozen cells), crystal growth kinetics must be taken into account. The IIF model developed in the present work is the first that includes predictions of the rate of growth of intracellular ice crystals.

• Data on physicochemical and biophysical parameter values required for IIF modeling are scarce. In this work, new experimental measurements of heterogeneous nucleation rate parameters in the presence of CPAs are reported, and phenomenological models of cytosol viscosity are constructed.

• The mechanism of IIF-induced cell injury remains a subject of speculation. This is in large part due to the difficulty of making direct observations of the intracellular crystallization and the effect of this process on the cell. Model predictions of intracellular crystal growth are used here in conjunction with experimental data on
cell viability to establish a correlation between intracellular ice volume and irreversible cell damage.

- To date, no systematic approach to designing protocols for the cryopreservation of biological cells exists. Typically, protocols are developed by adapting and experimentally “optimizing” methods previously used with other cell types. Because of the large number of experimental variables affecting the outcome, a true optimization of all factors would be prohibitively difficult — consequently, this approach has led to successful cryopreservation only for a limited number of cell types. In the present work, a rational design approach is used for developing cryopreservation protocols, using a multi-factorial simplex optimization algorithm in conjunction with a theoretical model of IIF.

This dissertation reports the development of a theoretical mechanistic model of ice nucleation and growth inside biological cells in the presence of CPAs, the determination of relevant biophysical parameters, and the application of the model to exploring the relationship between IIF and cell injury, as well as its use in designing cryopreservation protocols. To test the model over a wide range of physicochemical conditions, model verification is performed using two different cryoprotectants, glycerol and dimethylsulfoxide (DMSO), and two different cell types, rat hepatocytes and mouse oocytes: key biophysical parameters which characterize the freezing response of these cell types are known to differ by an order of magnitude.

The dissertation is organized as follows:
Chapter 1: The relevance of IIF to various applications in medicine, biotechnology and the basic biological sciences is reviewed. The current hypotheses regarding the mechanisms by which ice forms in cells and the mechanisms by which IIF results in cell damage are outlined. The history and state of the art of IIF modeling is reviewed and discussed.

Chapter 2: A theoretical model of intracellular ice nucleation and crystal growth in the presence of CPAs is developed, by coupling physicochemical models of membrane-limited water transport, homogeneous nucleation and diffusion-limited crystal growth. The model is applied to mouse oocytes frozen in the presence of glycerol. To estimate the diffusivity of water in the cytosol, a phenomenological model of the viscosity of a ternary H$_2$O-NaCl-glycerol solution is constructed; other model parameters are taken from the literature. The volume and size distribution of intracellular ice crystals during freezing is predicted, and the effect of CPA concentration and cooling rate is investigated. Model predictions are compared with existing data from the literature.

Chapter 3: The model is extended to include heterogeneous nucleation, and applied to cultured rat hepatocytes frozen in the presence of DMSO. The kinetic and thermodynamic coefficients of heterogeneous nucleation in hepatocytes in the presence of DMSO are determined from experimental data on IIF kinetics, and a phenomenological model of the viscosity of a ternary H$_2$O-NaCl-DMSO solution is constructed. Model predictions are verified by comparison with experimental IIF data from the literature. A previously published experimental study on the
experimental optimization of a cryopreservation protocol for cultured hepatocytes is analyzed in depth using the theoretical model, in order to further verify the model and to demonstrate the feasibility of protocol optimization using physicochemical models of intracellular crystallization. Published data on function of frozen-thawed hepatocytes are also correlated with model predictions of intracellular ice volume, in order to shed further light on the relationship between IIF and cell damage.

Chapter 4: The model is used to guide the design of a cryopreservation protocol for mouse oocytes. Heterogeneous nucleation rate parameters for mouse oocytes frozen in the presence of DMSO are measured, and model predictions are verified by a set of independent measurements of IIF kinetics. A simplex optimization technique is used in conjunction with the model to predict the optimal freezing protocol for mouse oocytes. Experimental viability measurements of cells frozen using the predicted optimal protocol are reported. The effect on cell survival of perturbing freezing protocol parameters from their optimal values is experimentally investigated, and compared with model predictions.

Chapter 5: The major conclusions and implications of this dissertation are reviewed, and directions for future research are discussed.
References


Chapter 2

A Model of Diffusion-Limited Ice Growth inside Biological Cells during Freezing

Introduction
Despite the well-known correlation between cell damage during cryopreservation and the appearance of an intracellular ice phase [28], the exact mechanism of ice formation in the cell, and the role of intracellular crystallization in cell injury during freezing remains unknown. As a prerequisite to the rational design of cryopreservation protocols which maximize cell survival, a fundamental understanding of the physicochemical processes involved in the nucleation and growth of intracellular ice is needed. Specifically, it is of prime importance that one be able to predict the influence of the freezing method on the ice volume and crystal size distribution in the cell.

During freezing of biological cells, the intracellular solution initially assumes thermodynamic states which are not in equilibrium with the external environment due to the formation of extracellular ice. This non-equilibrium state provides a driving force for both dehydration of the cell and for the nucleation and growth of an intracellular ice phase. As a result of this coupling between the competing processes of water transport out of the cell and phase transformation inside the cell, intracellular crystallization occurs in a solution of variable volume and composition. The fate of the intracellular water depends on the rate at which the cell is cooled. At slow cooling rates, water transport is the dominant mechanism of equilibration; thus, cells dehydrate without forming intracellular
ice. At faster rates of cooling, dehydration becomes less significant, and the probability of ice formation in the cell increases. At very rapid cooling rates, it is possible to vitrify the intracellular solution. The freezing behavior of the cell can be modified by the addition of cryoprotective agents (CPAs), which affect the rates of water transport, nucleation and crystal growth. Thus, the final extent of crystallization in the frozen cell is determined by both the cooling rate and the concentration of CPA used.

To date, all theoretical studies of ice formation in biological cells have focused on conditions for which the intracellular phase transformation is complete, and rate-limited by the appearance of a single stable crystal nucleus, i.e. typically conditions where no or limited amounts of CPAs are used. Pitt and co-workers have developed a statistical, phenomenological model which has been successful in predicting the probability of intracellular ice formation for a wide range of cell types and freezing conditions [35]. This approach, however, does not provide information about the physical processes underlying intracellular ice formation. On the other hand, Toner et al. have developed a physicochemical theory of heterogeneous nucleation in cells, coupled with a model of cell dehydration during freezing [46]. Although the model of Toner et al. has been successful in predicting intracellular ice formation in a number of cell types [45], this model is applicable only to cells frozen in the absence of CPAs. Furthermore, all previous models of intracellular ice formation are limited to predicting the onset of crystallization and neglect the growth kinetics of intracellular crystals. However, when slow cooling rates or high CPA concentrations are used, the rate of crystal growth becomes important in predicting the final extent of intracellular crystallization. Although the size and
morphology of ice crystals in frozen cells have been investigated using electron microscopy in a few studies, [43,33,6], the research on ice crystal growth has in general been limited to studies of pure water or aqueous solutions. The distribution of crystal sizes has been measured in specimens of frozen water-glycerol solutions [52,50], and ice crystal growth velocities have been measured in macromolecular solutions and gels [39,7]. Unfortunately, because these studies do not address the effect of time-varying solution compositions relevant to cell freezing, their results cannot be directly applied to studies of crystal growth in cells. Likewise, existing theoretical treatments of ice growth are applicable only to crystallization in solutions of constant composition. MacFarlane and Fragogulis have developed a theory of diffusion-limited crystal growth in binary solutions under non-isothermal conditions, which they used to predict devitrification during warming of a LiCl-H₂O glass [26]. Crystallization in aqueous solutions of CPAs has been studied by Boutron, who developed a semi-empirical crystal growth model for these solutions [8]. These models do not take into account the changing composition which occurs in the intracellular solution during cell freezing.

In the present study, a physicochemical theory of ice growth inside biological cells has been developed, by coupling models of water transport, nucleation and crystal growth. The coupled dehydration-nucleation model of Toner et al. [46] has been extended to include the effect of CPAs, and the diffusion-limited crystal growth model of MacFarlane and Fragogulis [26] has been modified to allow coupling with the water transport equations. The resulting three-part, coupled model has been used to study the freezing of mouse oocytes in the presence of glycerol as a CPA. The volume of intracellular ice and
the size distribution of the ice crystals were predicted as functions of the cooling rate and the initial concentration of CPA. By investigating a wide range of cooling rates and CPA concentrations, a diverse set of freezing behaviors could be predicted using a single model. At low rates of cooling, the addition of low concentrations of CPA was found to increase the extent of intracellular crystallization, while high concentrations of CPA had the opposite effect. At high cooling rates, the amount of intracellular ice formed was predicted to decrease monotonically with increasing CPA concentration, and the conditions necessary for vitrification could be calculated.

**Theoretical Background**

**Cell model**

To accurately predict intracellular crystallization, the equations describing nucleation and subsequent growth of ice must be coupled with a model describing the instantaneous state of the cytosol. The cell model employed in this study is a semipermeable membrane enclosing an aqueous solution. The cytoplasmic volume occupied by proteins and other large molecules ($V_b$), is assumed to be inactive with respect to the physical processes underlying intracellular ice formation. For purposes of this investigation, the relevant control volume $V_{CV}$ in the cell includes the cytosolic solution, but excludes the inactive cell volume $V_b$. The total cell volume is thus $V_{cell} = V_{CV} + V_b$. The cytosol is modeled as consisting of water and salt (e.g. NaCl), and may in addition contain various amounts of glycerol, a cryoprotective additive (CPA). In this study, data for cell-specific biophysical parameters are taken from existing studies of mouse oocytes [46], and are summarized in Table 2-1.
Table 2-1. Parameter values used in model.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Symbol</th>
<th>Value</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial cell volume</td>
<td>$V_{cell}$</td>
<td>$2.62 \times 10^{-13}$</td>
<td>m$^3$</td>
</tr>
<tr>
<td>Osmotically inactive volume</td>
<td>$V_o$</td>
<td>$5.58 \times 10^{-14}$</td>
<td>m$^3$</td>
</tr>
<tr>
<td>Initial salt concentration</td>
<td>$c_{so}$</td>
<td>0.142</td>
<td>M</td>
</tr>
<tr>
<td>Membrane permeability reference value</td>
<td>$L_{ref}$</td>
<td>$7.26 \times 10^{-15}$</td>
<td>m$^2$ s kg$^{-1}$</td>
</tr>
<tr>
<td>Membrane permeability activation energy</td>
<td>$E_{Lp}$</td>
<td>$5.57 \times 10^4$</td>
<td>J mol$^{-1}$</td>
</tr>
<tr>
<td>Membrane permeability reference</td>
<td>$T_{ref}$</td>
<td>273.15</td>
<td>K</td>
</tr>
<tr>
<td>temperature</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nucleation rate kinetic coefficient</td>
<td>$\Omega^{HOM}_o$</td>
<td>$9.7 \times 10^{52}$</td>
<td>s$^{-1}$ m$^{-3}$</td>
</tr>
<tr>
<td>Nucleation rate thermodynamic coefficient</td>
<td>$\kappa^{HOM}_o$</td>
<td>1.00</td>
<td>-</td>
</tr>
</tbody>
</table>
Assuming that the transmembrane flux of CPA and salt is negligible during freezing, and neglecting the effect of intracellular ice formation on the composition of the intracellular solution, the amount of water, salt and CPA in the cell is entirely determined by the transport of water across the plasma membrane. Applying the water transport model originally developed by Mazur [27] to cells containing a ternary water-NaCl-glycerol solution, one obtains the differential equation

\[
\frac{dV_{cv}}{dt} = \frac{L_p A R T}{v_w} \left[ \frac{\Delta H_f}{R} \left( \frac{1}{T_{m_0}} - \frac{1}{T} \right) - \ln \left( \frac{V_{cv} - \left( n_s v_s + n_a v_a \right)}{V_{cv} - \left( n_s v_s + n_a v_a \right) + v_w (v_s n_s + n_a)} \right) \right]
\]

(2-1)

where \( t \) is time; \( L_p \), the water permeability; \( A \), membrane surface area; \( R \), gas constant; \( T \), temperature; \( \Delta H_f \), specific heat of fusion of water; \( T_{m_0} \), equilibrium melting point of water; \( v_w, v_s, \) and \( v_a \), specific volumes of water, NaCl and CPA (i.e., glycerol), respectively; \( n_s \) and \( n_a \), number of moles of NaCl and CPA in the cell, respectively; and \( v_s (=2) \), the dissociation constant of NaCl. For constant-rate cooling protocols, the temperature is given by

\[
T(t) = T_0 - Bt
\]

(2-2)

where \( T_0 \) is the initial temperature, and \( B \), the cooling rate. Integration of Eq. (2-1) together with Eq. (2-2) yields temperature and volume data as functions of time, from which the cytosol composition \( c = [c_w, c_s, c_a] \) can be calculated, where \( c_w, c_s, \) and \( c_a \) are the intracellular concentrations of water, NaCl and CPA (glycerol), respectively.
Model assumptions include membrane-limited transport, with negligible temperature and pressure differentials across the membrane, the external solution in equilibrium with extracellular ice, and an ideal internal solution. To verify the assumption of membrane-limited water transport, the relative importance of transmembrane water flux and intracellular solute redistribution was determined by calculating the Peclet ($Pe$) number [23]. These calculations confirmed that water transport is membrane limited (i.e. $Pe \ll 1$) if cooling is sufficiently rapid ($B > \sim 0.6^\circ C/min$). At a cooling rate of $0.6^\circ C/min$, the Peclet number remains at values well below unity until the temperature falls below $\sim -60^\circ C$, at which point there is a sharp increase in $Pe$, and the assumption of membrane-limited transport breaks down. However, as will be shown, most of the water transport occurs during the early stages of cooling, and at temperatures below $-60^\circ C$, water flux out of the cell is practically negligible. Pushkar et al. have performed a similar analysis, and concluded that transport is membrane-limited in cells with low membrane permeability, but that diffusive transport is significant in highly permeable cells, such as erythrocytes [36].

**Ice nucleation**

$N_\xi(t)$, the number of ice nuclei in a given cell $\xi$ as a function of time, is a stochastic process, the ensemble average of which is given by

$$\overline{N}(t) = \int_0^t J(t)dt ,$$  
(2-3)
where $J$ is the average nucleation rate per cell. However, because intracellular crystal growth is a non-ergodic process, Eq. (2-3) cannot be used to calculate the average extent of crystallization in a cell. A detailed treatment of the stochastic behavior of intracellular crystal growth would be outside the scope of this investigation, and for the purposes of the present study, we define $N_s(t)$ for a "representative" cell as $\bar{N}(t)$ truncated to the nearest integer:

$$N(t) = \text{int}(\bar{N}(t)) .$$ (2-4)

Although the nucleation behavior in an actual cell will always differ somewhat from the behavior of the "representative" cell, it can be shown, assuming Poisson statistics, that the initial nucleation event ($N_s = 1$) will occur within a time interval $\Delta t \sim 1/(J V_C v) \approx 10^{-3}$ s for the system at hand) of the nucleation time predicted by Eq. (2-4), with a probability of at least $\sim 0.35$. It should be pointed out that for the conditions simulated in the present study, the number of ice nuclei appearing in a cell is always much larger than unity ($N \sim 10^2 - 10^{15}$, depending on the cooling rate and CPA concentration), so that $N(t) \approx \bar{N}(t)$.

The average nucleation rate, $J$, is here assumed to arise only from homogeneous nucleation in the cell, and thus

$$J = J_{\text{hom}} = J_{\text{hom}} V_C v ,$$ (2-5)
where $I^{HOM}$ is the homogeneous nucleation rate per unit volume, and is a function of temperature and cytoplasm composition. In homogeneous nucleation theory, the critical ice nucleus is formed by clustering of water molecules in the mother phase, due to statistical fluctuations. By assuming that clusters form by sequential addition of water monomers in a series of bimolecular reactions, and applying Eyring reaction rate theory to this process, the nucleation rate can be determined to be

$$I^{HOM}(c, T) = \Omega^{HOM}(c, T) \exp[-\kappa^{HOM}(c, c, T)]$$

(2-6)

where $\pi(c, T) = T_m^{3} \Delta T^2 T^2$, $\Delta T (= T_m - T)$, the cytoplasmic undercooling; $T_m$, the equilibrium melting point; and $\Omega^{HOM}$ and $\kappa^{HOM}$, the kinetic and thermodynamic coefficients, respectively [46, 48].

Taking into account the effect of a catalytic substrate on the thermodynamics of cluster formation, an equation for heterogeneous nucleation, similar to Eq. (2-6), may be written [46]. However, heterogeneous nucleation mechanisms have been shown to be ineffective in mouse embryos when the CPA concentration is higher than 1.5 M [37]. Since the main focus of the present study is on regimes where crystal growth is slow compared with nucleation, i.e. where CPA concentrations are significant, heterogeneous nucleation mechanisms have been neglected. Nonetheless, for the sake of completeness, some data for low glycerol concentrations will be presented here; these results should be interpreted with the caveat that nucleation may actually occur at higher temperatures than indicated.
Diffusion-limited crystal growth

When the crystallizing phase is of different composition than the bulk solution, such as is the case with ice crystallization from aqueous solutions, the rate of crystal growth is believed to be limited by the diffusion of water molecules to the ice-solution interface. The problem of diffusion limited crystal growth under isothermal conditions has been reviewed by Christian [11].

For a spherically symmetric crystal growing under isothermal conditions, the instantaneous particle radius $r_c$ is given by

$$ r_c = \alpha (\overline{D} t)^{1/2} \quad (2-7) $$

where $\overline{D}$ is the effective diffusivity of water, and the non-dimensional crystal growth parameter $\alpha$ is determined by the equation

$$ \alpha^3 = 2\overline{c} \exp[-\alpha^2/4] \phi(\alpha) \quad (2-8) $$

$$ \phi(\alpha) = \int_a^\infty x^{-2} \exp[-x^2/4] dx \quad (2-9) $$

$$ \overline{c} = \frac{c_a - c_{eq}}{c^* - c_{eq}} \quad (2-10) $$
where $\bar{c}$ is the nondimensional supersaturation, $c_w$ is the concentration of water in the bulk cytosol (far from the crystals), $c_{\text{liq}}$ is the liquidus water concentration at the given temperature, and $c^*$ is the water concentration in ice. The effective diffusivity is given by

$$\bar{D} = \frac{1}{c_w - c_{\text{liq}}} \int D c_w$$

(2-11)

where the diffusion constant $D$ can be calculated using the Stokes-Einstein equation,

$$D(c, T) = \frac{kT}{6\pi \alpha \eta(c, T)}$$

(2-12)

where $\alpha$ is the apparent hydrodynamic radius of a water molecule, and $\eta(c, T)$ is the solution viscosity.

To predict crystal growth under non-isothermal conditions, the approach of MacFarlane and Fragoulis [26] can be used. From their results, the radius at time $t$ of a crystal which nucleated at time $t_i$ is given by

$$r_c(t; t_i) = \left( \int_{\tau(t_i)}^{\tau(t)} \alpha^2 \bar{D} \left( \frac{dT}{B} \right) \right)^{1/2}$$

(2-13)

where $T(t)$ is given by Eq. (2-2).
If the crystals within a given cell are small enough such that neighboring crystals and their depletion regions do not overlap (i.e. no impingement), the total crystallized volume is simply

\[ V^\beta(t) = \sum_{i=1}^{N(t)} \frac{4\pi}{3} r_{c}^3(t; t_i). \]  

(2-14)

The crystallized volume fraction in the absence of impingement is then

\[ X^\beta = \frac{V^\beta}{V_{cv}}. \]  

(2-15)

For the problem being treated here, prediction of damage to biological cells, the crystal dimensions of interest are sufficiently small that impingement may be neglected (e.g. for vitrification \( X^\beta = 10^{-6} \)).

Nonetheless, for conditions under which impingement occurs, the correction due to Avrami [2,3,4] can be used:

\[ X^\beta' = 1 - \exp[-X^\beta]. \]  

(2-16)

Eq. (2-16) describes hard impingement, i.e. the interference of growth caused by physical contact of neighboring crystals. Soft impingement, which occurs when there is an overlap of the depletion regions surrounding neighboring crystals, has been studied by Wert and
Zener [54], whose results for solutions at low undercooling were not significantly different from the Avrami equation. A transformation to correct for soft impingement under conditions of high undercooling is not available, and the Avrami solution is currently viewed as the best available approximation [26,11]. Note, however, that the limiting value of \( X^{g'} \) as predicted by Eq. (2-16) is always unity, i.e. the control volume appears to crystallize completely. While this limit is correct for the case of hard impingement, it inadequately describes soft impingement. The actual limit of \( X^{g'} \) is determined by the thermodynamic equilibrium between the ice crystal and the unfrozen fraction of the control volume, and must take a value less than unity whenever solutes are present. Thus, when using Eq. (2-16) to approximate soft impingement, predicted values of the crystallized volume fraction are not valid in the neighborhood of \( X^{g'}=1 \).

**Numerical simulation**

Eqs. (2-1), (2-3) and (2-13) can be integrated numerically, sampling the respective integrand functions at time points \( t_j \). Noting that direct evaluation of Eq. (2-14) requires prediction of each individual nucleation event, which for \( J^HOM \sim 10^{18} \text{ m}^{-3}\text{s}^{-1} \) would result in prohibitively large computational complexity, a more practical, approximate approach will be used. Considering the number of new nuclei appearing between consecutive sampling instants \( t_{j-1} \) and \( t_j \), i.e.

\[
\Delta N_j = N(t_j) - N(t_{j-1}), \tag{2-17}
\]
one has

\[ V^\beta(t) \approx \sum_j \frac{4\pi}{3} r_c^3(t; t_j) \Delta N_j. \]  

(2-18)

The crystal size distribution can be calculated in a similar manner. For each sampling interval \((t_{j-1}, t_j)\) there will correspond, at a later time \(t\), \(\Delta N_j\) crystals of radius \(r_c(t; t_j)\). The number density \(\rho_c(r) = dN/dr\) can be estimated by constructing a histogram from the computed radius values and frequencies, and the probability density is then obtained by normalization:

\[ p_c(r) = \frac{\rho_c(r)}{\int_0^r \rho_c(r) dr}. \]  

(2-19)

Knowing the probability density function, one can determine the likelihood that the radius of an ice crystal will fall within a given range, say between \(r_1\) and \(r_2\):

\[ P(r_1 \leq r_c \leq r_2) = \int_{r_1}^{r_2} p_c(r) dr. \]  

(2-20)

**Model Parameter Estimation**

Plasma membrane permeability to water

The water permeability, \(L_p\), of the plasma membrane follows an Arrhenius temperature-dependence:
\[ L_p(c, T) = L_{pg}(c) \exp \left[ \frac{E_{lp}}{R} \left( \frac{1}{T} - \frac{1}{T_{ref}} \right) \right], \] (2-21)

where \( L_{pg} \) is the reference permeability at temperature \( T_{ref} \), and \( E_{lp} \) is the activation energy for transport of water across the plasma membrane. Although the dependence of the permeability on solution composition (particularly on the concentration of a permeating CPA) has not been conclusively established, data from several researchers indicate a sharp reduction in \( L_p \) at low CPA concentrations, but no further decrease in permeability as the CPA concentration is increased [41,17,34]. On the other hand, McGrath et al. found that the value of \( L_p \) in mouse oocytes increases by 70% in the presence of 1.5 M dimethyl sulfoxide (DMSO) or 1,2-propanediol [31]. Nonetheless, in the present study, the effect of \( L_p \) on the concentration of a permeating CPA is represented by reducing the value of \( L_{pg} \) by a factor of 0.5 when CPA is present [29]. Based on available evidence, the activation energy \( E_{lp} \) can be assumed independent of solution composition [29]. Two studies of the permeability of human monocytes appear to contradict this assumption. Aggarwal et al., as well as McCaa et al. report an apparent increase in \( E_{lp} \) in the presence of 1 M DMSO, although the results of the latter study may not be statistically significant [1,30]. In the present work, the activation energy is assumed to be concentration-invariant. Model predictions at low cooling rates are sensitive to the values of the water permeability parameters, in particular to the activation energy (see sensitivity analysis in Results), and it is thus important to obtain further experimental data on the effect of CPAs on water transport in cells.
Viscosity

Viscosity data for solutions of interest in cryobiology are scarce: most studies are limited to temperatures above the equilibrium melting point, and relevant viscosity data for ternary water-salt-CPA solutions are virtually non-existent. Eto et al. have obtained viscosity data down to -10°C for aqueous solutions of DMSO [13]. Although they report best-fit Arrhenius parameters describing temperature dependence at several concentrations, there is insufficient data to conclusively establish the concentration-dependence of the Arrhenius coefficients. Kresin and Körber have estimated the viscosity of a 47% (w/w) glycerol solution down to -107°C, by fitting a simple crystal growth model to measured crystallization kinetics [20]. They obtain a temperature dependence which is well described by a Vogel-Fulcher law, but their results are limited to a single concentration of glycerol. In view of the lack of information about the concentration-dependence of the viscosities of water-glycerol and water-glycerol-NaCl at low temperatures, an estimate of the function \( \eta(c, T) \) needs to be constructed by interpolating from available data.

The Vogel-Fulcher form observed by Kresin and Körber at 47% (w/w) glycerol was assumed to describe adequately the temperature dependence of the viscosity for the entire range of glycerol concentrations considered in this study:

\[
\eta_{\text{glycerol}}(c, T) = C(c) \exp \left[ \frac{E(c)}{T - T_c(c)} \right].
\]  (2-22)
To determine the concentration dependence of the Vogel-Fulcher parameters $C$, $E$ and $T_c$, available data for the viscosity of water-glycerol solutions at $T=20^\circ C$ [53], as well as data for the glass transition temperature $T_g$ as a function of glycerol concentration [25] were used. The critical temperature $T_c$, at which the viscosity becomes divergent, was assumed to scale in direct proportion with $T_g$, its value at 47% (w/w) glycerol is given by Kresin and Körber as $T_c = 134$ K, from which $T_c(c)$ can be calculated using $T_g(c)$ data. If one assumes the viscosity at the glass transition point to be $\eta_{glycerol}(c, T_g(c)) = 10^{12}$ Pa s at all $c$, then the Vogel-Fulcher parameters can be determined for any glycerol concentration by writing Eq. (2-22) for both $T=T_g(c)$ and $T=20^\circ C$, and then solving for the two unknowns, $C$ and $E$.

Thus equipped with an estimate of the function $\eta_{glycerol}(c, T)$ for a water-glycerol solution, the viscosity of the ternary solution water-glycerol-NaCl may be estimated by approximating the contribution from the salt particles using a hard-sphere model [51]:

$$\eta(c, T) = \eta_{glycerol}(c, T) \exp \left[ \frac{2.5 \phi_s}{1 - Q\phi_s} \right]$$

(2-23)

where $\phi_s$ is the volume fraction of salt, and $Q=0.609375$ is an interaction parameter. Fig. 2-1 shows the resulting function $\eta(c, T)$ plotted versus $T$, for various glycerol concentrations, and fixed isotonic concentration of NaCl ($c_s = 0.142$ M).
Figure 2-1: Temperature-dependence of the estimated cytosol viscosity in the presence of 0, 4, 6, and 8 M glycerol. Salt concentration is fixed at 0.142 M.
Nucleation rate parameters

The dependence of the nucleation coefficient $\Omega_{\text{HOM}}^n$ on concentration and temperature can be estimated by scaling [46]:

$$\Omega_{\text{HOM}}^n(c, T) = \Omega_{o}^{\text{HOM}} \left( \frac{\eta_{o}}{\eta(c, T)} \right) \left( \frac{T}{T_{ho}} \right)^{1/2} \left( \frac{c}{c_{wo}} \right)$$  \hspace{1cm} (2-24)

where $T_h$ is the homogeneous nucleation temperature, and the subscript zero indicates reference values for pure water at its homogeneous nucleation temperature $T_{ho} = -38.3^\circ C$ [40].

The value of $\kappa_{\text{HOM}}$ was estimated by requiring that Eq. (2-6) correctly predict measured nucleation temperatures. Using the method employed by Rasmussen and MacKenzie [40] and others, the nucleation rate $I_{\text{HOM}}^n$ at the nucleation temperature $T_h$ is assumed to be invariant, with a value

$$I_{\text{HOM}}(c, T_h(c)) = I_o = \Omega_{o}^{\text{HOM}} \exp\left[ -\kappa_{o}^{\text{HOM}} \tau_o \right] = 1.7 \times 10^{18} \text{ m}^{-3} \text{ s}^{-1}$$  \hspace{1cm} (2-25)

where $\tau_o = T_{mo}^{-3} (T_{mo} - T_{ho})^{-2} T_{ho}^{-3}$, and the nucleation coefficient reference values $\Omega_{o}^{\text{HOM}}$ and $\kappa_{o}^{\text{HOM}}$ are given in Table 2-1.

The function $\kappa_{\text{HOM}}(c, T)$ is then obtained by substituting Eq. (2-25) into Eq. (2-6) and solving for $\kappa_{\text{HOM}}$. 

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\[
\kappa^{HOM}(c, T) = \frac{1}{\tau(c, T_h(c))} \left( \ln \Omega^{HOM}(c, T_h(c)) - \ln I_o \right)
\]

(2-26)

where \( T_h(c) \) can be calculated from the depression of the equilibrium melting point [40]:

\[
T_h(c) = T_{h0} + 2(T_m(c) - T_{m0})
\]

(2-27)

Eq. (2-27) is a phenomenological relationship based on experimental measurements in the range \( 0^\circ C > T_m > -20^\circ C \). For melting point depressions outside this range, nucleation temperature data are unavailable, thus limiting the range of concentrations for which the above technique to estimate \( \kappa \) can be used. For concentrations where \( T_m(c) < -20^\circ C \), the above model of \( \kappa^{HOM}(c, T) \) was extended by linear extrapolation along lines of constant ratio \( c_a/c_i \). The accuracy of the model for \( \kappa^{HOM}(c, T) \) was tested by comparing model predictions with experimental measurements of \( \kappa^{HOM} \) for a water-glycerol solution [20]. Reasonable agreement between predicted and measured values of \( \kappa^{HOM} \) was observed (at 40\% w/w glycerol, predicted \( \kappa^{HOM} = 1.49 \), measured \( \kappa^{HOM} = 1.42 \); at 47\% w/w glycerol, predicted \( \kappa^{HOM} = 1.61 \), measured \( \kappa^{HOM} = 1.87 \)), indicating that the model is adequate.

**Equilibrium melting point**

The equilibrium melting point \( T_m(c) \) and the liquidus water concentration \( c_{liq}(T) \) are both defined by the phase diagram of the water-glycerol-NaCl solution. In this study, the required phase diagram information was calculated from the Gibbs-Helmholtz equation, which describes the relationship between the activity of water and the equilibrium melting.
point. The water activity in the cytosol can be assumed to be approximately equal to the water mole fraction $x_w$, and thus one can calculate the equilibrium melting temperature as follows:

$$T_* = \left[ \frac{1}{T_{*o}} - \frac{R}{\Delta H_f} \ln x_w \right]^{-1}.$$ \hspace{1cm} (2-28)

Similarly, the equilibrium mole fraction of water can be computed as a function of temperature. However, for a ternary solution, the equilibrium water concentration ($c_{\text{aq}}$) at the solid-liquid interface is not uniquely determined by the water mole fraction: in addition, the ratio of glycerol to salt must be known. While this ratio is constant in the bulk of the cytoplasm, its value at the crystal interface depends on the relative diffusivities of NaCl and glycerol. In this study, the ratio of glycerol to salt was assumed to take the same value at the crystal interface as in the bulk solution. The resulting estimate for $c_{\text{aq}}$ was found to be relatively insensitive to errors in this assumption for CPA concentrations larger than 1 M (error in $c_{\text{aq}}$ was less than 15%). Furthermore, based on the apparent difficulty with which glycerol crystallizes from aqueous solutions [18,42], the glycerol-water eutectic was ignored in the model, i.e. the Gibbs-Helmholtz relation was assumed to describe adequately the water activity at all temperatures and glycerol concentrations.

**Crystal growth parameter**

Calculation of the non-dimensional crystal growth parameter $\alpha$ requires numerical solution of a transcendental equation [Eq. (2-8)]. Because simulation of crystal growth requires
frequent evaluations of $\alpha$ [once at each step in the integral in Eq. (2-13), which in turn must be recomputed for each time step in the summation of Eq. (2-18)]}, the computational cost of the crystal growth simulation can be greatly reduced if the algorithm for calculating $\alpha$ is made more efficient. To that effect, exploiting the fact that the value of $\alpha$ depends only on $\bar{c}$, $\alpha$ was precomputed for values of $\bar{c}$ ranging between 0 and 1, and an estimate of the function $\alpha(\bar{c})$ was constructed by fitting a polynomial to the resulting data:

$$\alpha(\bar{c}) = 89.460\bar{c}^5 - 174.975\bar{c}^4 + 129.602\bar{c}^3 - 41.335\bar{c}^2 + 8.709\bar{c} + 0.029 \ (r=0.9999). (2-29)$$

**Results**

**Water transport**

Eq. (2-1) was integrated using a fourth-order Runge-Kutta scheme, with a fixed step-size of 0.1°C, for various cooling rates and glycerol concentrations. In Fig. 2-2A, the resulting normalized water content in the cell is plotted as a function of temperature, for a mouse oocyte in an isotonic salt solution with no CPA. As can be seen, the extent of dehydration of the cell depends critically on the cooling rate. At very slow cooling rates, the cell dehydrates excessively, e.g. for $B=0.6°C/min$, the intracellular water content drops sharply, converging with the equilibrium value (indicated by the curve $B=0$) at $T \approx -10°C$. In contrast, at fast cooling rates, the water loss from the cell is practically negligible, e.g. at $B=60°C/min$, the cell retains over 95% of its original water content. At intermediate cooling rates, the model predicts an initial decrease in the intracellular water volume, after which the water content curves asymptotically approach a constant value, indicating that water transport out of the cell has effectively ceased, before equilibrium has been attained.
Figure 2-2: Predicted normalized intracellular water volume for mouse oocytes cooled at various rates with no CPA (A) and in the presence of 6 M glycerol (B). Water volumes are relative to the isotonic value, $V_{w0} = 2.06 \times 10^{-13}$ m$^3$. 

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This phenomenon can be attributed to the temperature dependence of the membrane permeability. At -40°C, \( L_p \) has been reduced to approximately 1% of its value at 0°C. As cooling progresses, \( L_p \) decreases faster than the chemical potential difference across the membrane increases, thus effectively stopping water flux out of the cell. As a consequence, the cell volume and the intracellular concentrations of water and solutes remain constant, while the supercooling of the cytoplasm (\( \Delta T = T_m - T \)) increases at a rate approximately equal to \( B \).

Fig. 2-2B shows the normalized water content of a cell which has been equilibrated in a 6 M glycerol solution prior to freezing. Relative to the no-CPA case, the starting point of the curves has been shifted towards lower temperatures. This is due to the melting point depression in the presence of CPA \( (T_m = -17.9^\circ C \text{ for 6 M glycerol}) \), causing a lowering in the temperature at which external ice can be seeded. The curves demonstrate that water efflux is slower in the presence of CPA, as is evidenced by the lower initial slopes of the normalized water content curves. The model also predicts that there is virtually no change in cell water content for \( B=30^\circ C/\text{min} \), whereas cooling at the same rate in 0 M glycerol resulted in approximately 15% dehydration. The levelling out of the water content curves, indicating the termination of water efflux, has a more significant effect in the presence of CPA. For example, at a cooling rate of 0.6°C/min, a cell equilibrated in 6 M glycerol and then cooled retains almost 25% of its initial water content, while the same cell cooled without glycerol treatment dehydrates completely.
The effect of CPA on the intracellular water content is summarized in Fig. 2-3, which shows the final value of the normalized water volume, plotted versus initial glycerol concentration for various cooling rates. For cooling rates as high as 6°C/min, there is a clear increase in the final water content with increased glycerol concentration, as explained above. At high CPA concentrations, this initial increase is followed by a drop in the final water content with further increase in the concentration of glycerol. As the cooling rate is increased, the maximum in the water content curve shifts towards lower glycerol concentrations. The reason for this is that as the cooling rate becomes large with respect to the rate of water transport, the cell volume remains almost constant, and thus the dominating phenomenon which determines the final water content is the initial equilibration with CPA. For example, at 600°C/min, water transport is negligible, and the predicted decrease in final water content is due entirely to displacement of intracellular water by glycerol, prior to cooling. At slow cooling rates, e.g. 1.2°C/min, water efflux is the dominating factor, and the model predicts a continuous increase in the final water content up to a glycerol concentration of 8 M. This increased water retention by the cells in the presence of cryoprotectants, will thus result in a higher probability of intracellular ice formation.

**Crystal growth**

Fig. 2-4 illustrates the effect of cooling rate and CPA concentration on crystal growth kinetics. To determine the crystallized volume fraction, the number of nuclei was first calculated, by numerically integrating Eq. (2-3). Then, for each sampling interval in which new nuclei appeared, as determined using Eqs. (2-4) and (2-17), Eq. (2-13) was
Figure 2-3: Effect of initial CPA concentration on the predicted final intracellular water content in mouse oocytes cooled at various rates. Water volumes are relative to the isotonic value, $V_{w0} = 2.06 \times 10^{-13}$ m$^3$. 
Figure 2-4: Effect of cooling rate on the predicted crystallized volume fraction in mouse oocytes for initial glycerol concentrations of 2 M (A) and 8 M (B).
numerically integrated to compute the resulting crystal sizes. Finally, the volume fraction of ice was determined from Eqs. (2-18), (2-15) and (2-16). The time-dependent terms \( \rho_{\text{HOM}}, V_C, D, \alpha \) were calculated as described above, using data from the water transport simulations to determine the instantaneous state of the intracellular solution.

In Fig. 2-4A, the crystallized volume fraction \( \chi^p \) is plotted versus temperature, for mouse oocytes initially equilibrated with 2 M glycerol and subsequently frozen at various rates. As noted previously, the apparent limit \( \chi^p \rightarrow 1 \) is an artifact of the Avrami transformation. The actual limiting value of the crystallized volume fraction is the thermodynamic equilibrium value, which is determined by the final intracellular water content and temperature. Thus, the portions of the \( \chi^p \)-curves in the vicinity of \( \chi^p = 1 \) are not strictly valid. However, since the initial stages of transformation occur before any impingement, the transition temperatures are correctly predicted. Fig. 2-4A shows that at 2 M glycerol, all intracellular water is transformed into ice, for cooling rates between 1.2 - 6000°C/min. The rate of cooling does, however, affect the temperature at which the cell freezes. For low cooling rates \( (B < 24^\circ \text{C/min}) \), the transition temperature decreases with decreasing cooling rate. For fast cooling rates \( (B > 24^\circ \text{C/min}) \), on the other hand, the transition temperature decreases as the cooling rate is increased. Another point of interest is that for cells in 2 M glycerol cooled at rates larger than 1.2°C/min, the slope of the crystal growth curves does not vary, indicating that the cooling rate has a relatively insignificant effect on the crystallization kinetics, compared with its marked effect on the onset of the phase transition.
Fig. 2-4B shows the crystallized volume fraction as a function of time, for mouse oocytes initially equilibrated with 8 M glycerol. The crystallization curves are significantly different from those obtained at 2 M glycerol. Note that in contrast to the 2 M simulations, in which crystal growth stopped when impingement occurred, the interrupted growth here is due to vitrification of the untransformed solution. This predicted vitrification is a result of the temperature-dependence of the water diffusivity, which becomes vanishingly small as the temperature approaches the Vogel-Fulcher critical temperature $T_c$. The extent of crystallization depends strongly on the cooling rate. Fig. 2-4B illustrates that the final crystallized volume fraction increases with increased cooling rate up to $B = 6^\circ C$/min, while for rates $B \geq 6^\circ C$/min, the model predicts a reduction in the crystallized volume fraction as cooling rate is increased. At the CPA concentration of 8 M used here, the crystallized volume fraction always remains below the vitrification threshold, $10^{-6}$. At less extreme CPA concentrations, the crystallized volume fraction will be larger, but the qualitative behavior will be similar to that shown in Fig. 2-4B. Comparing Figs. 2-4A and 2-4B, one notes that at the higher CPA concentration, the slope of the crystal growth curves vary with cooling rate, while the onset of crystallization appears to be fairly invariant. This indicates that in this regime, the effect of cooling rate on crystal growth is more significant than the effect on nucleation.

Fig. 2-5 shows the calculated transition temperature dependence on cooling rate for various glycerol concentrations. The transitions indicated are the nucleation temperature (open symbols), defined by the formation of the first intracellular crystal nucleus, and the crystallization temperature (filled symbols), defined here as the temperature at which the
Figure 2-5: Effect of cooling rate on predicted nucleation and crystallization temperatures in mouse oocytes for various initial CPA concentrations. The nucleation temperature (open symbols) is defined by the appearance of the first nucleus, and the crystallization temperature (filled symbols) is defined as the temperature for which the crystallized volume fraction reaches $10^{-6}$. 
crystallized volume fraction crosses the threshold of detectability (assumed to be $X_0 \approx 10^{-6}$). One notes, apart from the expected freezing point depression with increased CPA concentration, a very strong cooling rate dependence of the transition temperatures at rates $B < 10^\circ C/min$. Note that for 8 M glycerol, the model predicts nucleation but no subsequent crystallization. This is because at this CPA concentration, crystal growth always ceases before the crystallized volume fraction reaches the threshold value $10^{-6}$, and there are thus no detectable crystals in the final vitrified solution.

Fig. 2-6 summarizes the findings described above, showing how the final ice content in mouse oocytes depends on the CPA concentration and on the rate of cooling. In Fig. 2-6, the final value of the crystallized volume fraction is plotted against the initial glycerol concentration, at $B=1.2^\circ C/min$, $60^\circ C/min$ and $6000^\circ C/min$. For rapid cooling ($B=60^\circ C/min$), the cell water freezes completely at almost all CPA concentrations. More than 6 M glycerol would be required to vitrify the cell at this rate. For very rapid cooling ($B=6000^\circ C/min$), vitrification can be achieved at lower concentrations of CPA, as expected. An interesting behavior is predicted for slow cooling ($B=1.2^\circ C/min$). In the absence of CPA, there is no crystallization, but as glycerol is added there is an increase in the resulting intracellular ice content. At 2 M glycerol, all cell water is frozen. As the CPA concentration is increased further, the crystallized volume fraction again decreases, vanishing completely between 6 M and 8 M.

To describe completely the final state of the frozen cytoplasm, one must know, in addition to the crystallized volume fraction, the size distribution of the intracellular ice crystals.
Figure 2-6: Effect of CPA concentration on the predicted final crystallized volume fraction in mouse oocytes cooled at various rates.
Fig. 2-7 shows theoretical final size distributions of ice crystals in mouse oocytes frozen in 7 M glycerol at various cooling rates. The predicted crystal radii are on the order of $10^{-8}$ m. For cooling rates faster than 3.6°C/min, the radius distribution shifts towards smaller crystal sizes as the cooling rate increases. This effect has been observed experimentally in glycerol solutions [50], and predicted theoretically for crystal growth in pure water [19]. At cooling rates slower than 3.6°C/min, the opposite behavior is predicted: crystal size decreases with decreasing cooling rate. The predicted cooling rate dependence of crystal size is illustrated in Fig. 2-8, which shows the final values of the volume fraction crystallized and the average crystal radius (defined as the radius of a sphere with a volume equal to the total ice volume divided by the number of nuclei), as functions of cooling rate, for oocytes frozen in 8 M glycerol. At this concentration, the average crystal radius peaks at ~3.6°C/min. Note that the maximum in the average crystal radius and the maximum in the total fraction crystallized occur under different freezing conditions.

**Critical cooling rates**

For a given cryopreservation protocol, specified by the initial glycerol concentration ($c_{\infty}$) and the cooling rate ($B$), the model predicts the final state of crystallization in the cell. Specifying a critical ice volume fraction $X_0^c$, one can thus map out the region of ($B$, $c_{\infty}$)-space in which the final intracellular ice fraction will exceed the critical value. The boundary of this region will define the critical cooling rate as a function of the initial glycerol concentration. The predicted critical cooling rates will depend on the value of $X_0^c$. If one chooses $X_0^c \approx 1$, the supercritical region will indicate which protocols result in total
Figure 2-7: Predicted final size distribution of ice crystals inside mouse oocytes frozen in the presence of 7 M glycerol at various cooling rates.
Figure 2-8: Effect of cooling rate on the predicted final crystallized volume fraction and average crystal radius for mouse oocytes frozen in the presence of 8 M glycerol.
crystallization of the cell, and thus cell death. If, on the other hand, one uses $X^0_c = 10^{-6}$, i.e. the conventional criterion for vitrification, protocols outside the critical region should result in fully vitrified, and thus viable cells. Since cells have been observed to survive "innocuous" intracellular ice formation, there presumably exists an intermediate value of $X^0_c$ which better approximates the limits of the lethal region. In Fig. 2-9, $X^0_c = 10^{-3}$ was chosen arbitrarily to define the critical cooling rate.

The upper boundary of the supercritical region in Fig. 2-9 defines the minimum cooling rate necessary to (partially) vitrify the sample, i.e. to achieve a final value of $X^0_r \leq 10^{-3}$. This critical cooling rate decreases from a value of $6 \times 10^{13}$ °C/min when no glycerol is present, to $6 - 60$ °C/min at a glycerol concentration of ~7.5 M. The lower boundary of the supercritical zone represents the maximum permissible cooling rate which avoids intracellular ice formation in conventional slow-freezing protocols. For low initial concentrations of glycerol ($c_{\infty} < 2$ M), the critical cooling rate decreases slightly with increasing glycerol concentration. The critical rate of cooling then remains approximately constant for $2$ M < $c_{\infty}$ < 6 M, and finally increases somewhat as the glycerol concentration increases beyond 6 M. For initial glycerol concentrations of ~7.5 M or higher, the volume fraction of intracellular ice will stay below $X^0_c$ for all cooling rates. Experimental data from the literature have been superimposed on the predicted critical cooling rate curve in Fig. 2-9, indicating reasonable agreement between theoretical and experimental results.
Figure 2-9: Interdependence of predicted critical cooling rate and initial CPA concentration, for mouse oocytes frozen in the presence of glycerol. The shaded region represents the supercritical region, i.e. for which the final crystallized volume fraction exceeds $10^{-3}$, as estimated from predictions of the final crystallized volume fraction at various cooling rates and CPA concentrations. The curves marked $B_I$, $B_{III}$, and $B_{IV}$ represent simplified estimates of the critical cooling rate in regimes I, III and IV, respectively. Other symbols represent experimental and theoretical data from the literature, from references Boutron (1986) [8], Fletcher (1971) [15], Franks (1985) [16], Uhlmann (1972) [49], Bald (1986) [5], Fahy et al. (1984) [14], Leibo et al. (1978) [22], and Toner et al. (1991) [47]. See text for further details.
Sensitivity analysis

The sensitivity of the model to the parameters $L_{pg}$, $E_{lp}$, $\Omega^{HOM}$, $\kappa_{HOM}$ and $\eta$ was tested by studying the effect of perturbations in these parameters. Shown in Fig. 2-10 is the predicted final ice content as a function of cooling rate, for cells initially in 6 M glycerol. In each plot, the solid curve indicates the results obtained the unperturbed values, while the remaining curves demonstrate the effect of varying a particular parameter value.

Figs. 2-10A and B show the sensitivity to the water transport parameters. One notes in Fig. 2-10A that the transition region at high cooling rates, i.e. the glass transition, is unaffected by errors in the permeability coefficient $L_{pg}$. The reason for this is the decoupling of the water transport and crystal growth models which occurs at fast cooling rates. Variations in $L_{pg}$ do, however, affect the crystal growth predictions at lower cooling rates. An error in $L_{pg}$ of 40% in this regime shifts the critical cooling rate almost by a factor of two. As expected, the lower the permeability coefficient is, the slower the cooling rate required to avoid intracellular ice formation. Similar results are obtained in Fig. 2-10B, showing dependence on the activation energy for water transport, $E_{lp}$. However, the model sensitivity to the activation energy is much larger than the sensitivity to $L_{pg}$. This is expected, as $E_{lp}$ appears in the exponential factor of the Arrhenius form. Nonetheless, the effect of $E_{lp}$ on vitrification is negligible.

Fig. 2-10C shows the model sensitivity to the nucleation parameter $\kappa_{HOM}$. Model predictions are relatively insensitive to variations in the pre-exponential factor $\Omega^{HOM}$, the vitrification transition being slightly more affected than the transition in the conventional
Figure 2-10: Sensitivity of the predicted final crystallized volume fraction to variations in the membrane permeability reference coefficient $L_{pg}$ (A) and activation energy $E_{lp}$ (B), nucleation rate thermodynamic coefficient $\kappa$ (C), and cytosol viscosity $\eta$ (D). The initial glycerol concentration is 6 M.
freezing regime (data not shown). By contrast, the parameter $\kappa^HOM$ clearly has a significant effect on the predicted results. When $\kappa^HOM$ is underestimated by 40%, the maximum cooling rate at which intracellular ice formation does not occur is reduced by a factor of approximately three, and vitrification is not predicted, even at cooling rates on the order of $10^3$ °C/min. When $\kappa^HOM$ is overestimated by 40%, no crystallization is predicted at any cooling rate. These results demonstrate that an accurate estimate of $\kappa^HOM$ is crucial for the model.

In Fig. 2-10D, the sensitivity to variations in the viscosity estimate is shown. The most significant effect evident is in the prediction of the critical cooling rate to achieve vitrification. For each increase in $\eta$ by a factor of five, the vitrification cooling rate is approximately halved. The critical rate necessary to avoid intracellular ice formation at slow cooling rates appears to be relatively unaffected by perturbations in the viscosity. In fact, as $\eta$ is decreased, there is no discernible effect on the results. In this regime, the final ice content is determined by the nucleation kinetics, while the crystal growth may be regarded as "instantaneous". Thus, an increase in the the crystal growth rate, due to lower solution viscosity, would not affect the amount of crystallization. When the viscosity is made sufficiently large, the nucleation and crystal growth processes begin to compete, and for increases in $\eta$ by a factor of 5 - 25, a slight effect on the resulting ice content is predicted.
Discussion

Prediction of intracellular ice formation

CPAs were shown to affect intracellular ice formation in different ways, depending on the concentration at which they were initially present in the cell. In conventional slow-cooling cryopreservation protocols, low levels of CPA enhanced intracellular ice formation (Fig. 2-6), causing the critical cooling rate to decrease with increasing CPA concentration (Fig. 2-9). Such a depression of the critical cooling rate has been observed experimentally for different types of cells and CPAs [9,38,44] and an increased incidence of intracellular ice with the addition of CPAs was directly observed by Myers et al. [32]. Mazur [28] postulated that this phenomenon may be explained by the viscosity of the additives, which would retard the exosmosis of water from the cell, thus requiring slower cooling rates to avoid intracellular ice formation. However, pointing out that the ice nucleation temperatures are depressed in the presence of CPA [40], Mazur called the observed lowering of the critical cooling rate a “paradox”. Diller and Lynch [12] attributed the increased occurrence of intracellular ice in the presence of CPAs to an increase in supercooling of the cytoplasm. Because the freezing point of the external solution is depressed by additives, freeze-induced dehydration would be initiated at lower temperatures when CPAs are present. The membrane permeability decreases with decreasing temperature, so the rate of water transport out of the cell would be slower in the presence of CPAs, resulting in increased intracellular supercooling. The theoretical model presented in this study provides an explanation similar to that of Diller and Lynch. As can be seen in Fig. 2-3, the presence of low concentrations of CPA increases the final water content in the cell, thus increasing the extent of intracellular supercooling at any
given temperature, which results in higher rates of nucleation and crystal growth. The presence of CPA promotes the inclusion of increased amounts of water in the cytoplasm by decreasing the water permeability of the plasma membrane, and by lowering the freezing point of the external solution. The freezing temperature depression in the presence of additives has two effects: (1) initiation of cell dehydration at a lower temperature, and thus at a reduced membrane permeability, as explained by Diller and Lynch, and (2) reduction of the temperature interval in which the cell membrane remains permeable to water. This second factor arises from the fact that the permeability activation energy $E_{lp}$ does not depend on the solution composition. Thus there exists an approximately constant temperature below which the cell membrane becomes quasi-impermeable. The lower the freezing point of the external solution, then, the narrower the temperature window within which water transport can occur. Apart from these effects resulting from the freezing point depression by additives, CPAs directly slow the efflux of water from the cell by reducing the membrane permeability coefficient $L_{pe}$.

Although the presence of CPAs tends to inhibit water efflux during freezing, as explained above, permeating additives such as glycerol will reduce the initial water content in the cell, by displacing intracellular water as the cell equilibrates in the CPA solution. As is evident from Fig. 2-3, the former effect dominates at low CPA concentrations, and the latter at high CPA concentrations. The competition between these two effects accounts for the concentration dependence of both the final intracellular ice content (Fig. 2-6, $B=1.2^\circ$C/min) and the critical cooling rate (Fig. 2-9). Note that at intermediate glycerol concentrations ($2 \, M < c_{oa} < 6 \, M$) the predicted critical cooling rate is independent of the
concentration of CPA. Leibo et al. [21] have reported experimental observations of this type of freezing behavior in glycerolized mouse marrow stem cells.

In the rapid-solidification regime of the \((B,c_{ao})\)-space, the boundary of the critical region indicates the minimum cooling rate necessary to (partially) vitrify the cell. Since water flux across the cell membrane becomes negligible at rapid cooling rates, the water transport model can be decoupled from the nucleation and crystal growth models. Thus, the cell behaves as a droplet of water-salt-CPA solution of the same volume and composition as the cytosol. One notes that this critical cooling rate is extremely sensitive to the concentration of CPA, decreasing by approximately one order of magnitude as \(c_{ao}\) is increased by 1 M. For no CPA, the model predicts a critical cooling rate of \(6 \times 10^{13} \, ^\circ C/min\), which coincides with the cooling rate necessary to vitrify water, as estimated by Franks [16] Other estimates of the critical cooling rate for vitrifying water have ranged from \(\sim 10^7 \, ^\circ C/min\) to \(\sim 10^{17} \, ^\circ C/min\) [15,49,5]. As CPA is added, the nucleation temperature is depressed and the solution viscosity is increased, reducing the crystal growth velocity. The model thus predicts a decrease in the cooling rate necessary to achieve vitrification with increasing CPA concentration, in accordance with empirical observations. When the initial glycerol concentration is approximately 7.5 M, vitrification occurs for all cooling rates. This prediction is consistent with experimental data from the literature. Fahy et al. [14] found that a glycerol concentration of 7.1 M was required to vitrify a glycerol solution at low cooling rates. Liehmann et al. [24] report that a minimum concentration of 6.85 M glycerol was necessary for apparent vitrification in their experiments on mouse embryos. Critical cooling rates for vitrification of glycerol-water
solutions, obtained using a semi-empirical crystal growth model proposed by Boutron [8] are shown on the \((B,c_{ao})\)-coordinate system in Fig. 2-9 for comparison with the results of this study. As can be seen, the two critical cooling rate curves are offset by \(\sim 1\ \text{M}\) in \(c_{ao}\). Due to the steepness of the vitrification curves, however, the corresponding cooling rate predictions differ by several orders of magnitude. This discrepancy may in part be due to inaccuracies in the phenomenological estimates of the parameters \(\eta\) and \(\kappa\) used in the present model. As demonstrated by the sensitivity analysis, errors in these two parameters would affect significantly the predictions of the critical cooling rates for vitrification. Better measurements of viscosity and the nucleation rate parameters as functions of CPA concentration and temperature may thus significantly improve the quantitative accuracy of the model.

Strategies for optimization of freezing protocols

The range of possible freezing protocols, mapped into a \((B,c_{ao})\)-space, can be conceptually divided into four quadrants corresponding to which mechanisms are dominant in determining the extent of intracellular ice formation. Consequently, the strategy for optimizing cryopreservation protocols will be different in each quadrant. The major subdivisions of the \((B,c_{ao})\)-space are as follows: regime I, corresponding to slow cooling rates and low initial CPA concentrations; regime II, corresponding to slow cooling rates and high initial CPA concentrations; regime III, corresponding to fast cooling rates and low initial CPA concentrations; and regime IV, corresponding to fast cooling rates and high initial CPA concentrations. The prevalent mechanisms of intracellular crystallization in each regime were determined by analysis of simulation results. From this
information, general strategies for optimization of freezing protocols could be proposed. To verify that the dominant mechanism had been correctly identified, simple estimates for the critical cooling rate were derived based on the proposed strategy, and compared with model predictions in the corresponding regime.

Conventional cryopreservation protocols fall into regime I. In these regime, ice formation is modulated by the alteration of the thermodynamic properties of the intracellular solution, as a result of water transport during cooling. For example, freeze-induced dehydration causes increased intracellular solute concentrations, thus increasing the cytosol viscosity, depressing the nucleation temperature and reducing the rate of crystal growth. In regime I, crystallization is rate-limited by the nucleation process, and crystal growth velocities are extremely rapid, as can be seen in Figs. 2-4A and 2-5. Thus, within this regime, it is virtually impossible to control the amount of ice that forms in the cell. Consequently, to prevent damaging levels of ice in the cell, nucleation must be totally suppressed. This can be achieved by increasing the intracellular solute concentration, through cell dehydration and addition of CPA. As the concentration of solutes in the cell increases, the nucleation temperature is depressed, while the glass transition temperature increases. At the concentration where the two coincide, the intracellular solution vitrifies before nucleation occurs, and thus no ice is formed in the cell. This strategy for preventing intracellular ice formation is can be expressed by the simple criterion

\[ T_n(c) = T_g(c) \] (2-30)
Eq. (2-30) is satisfied when the glycerol concentration is \(~9\) M. The cooling rate necessary to achieve a final intracellular glycerol concentration of \(9\) M was determined by solving the water transport equation [Eq. (2-1)]; the results are shown in Fig. 2-9. The critical cooling rates estimated using Eq. (2-30) are in reasonable agreement with the predictions obtained from the theoretical model, suggesting that the mechanisms governing intracellular ice formation in regime I have been correctly identified. Moreover, model predictions in regime I were consistent with experimental data obtained by Leibo et al. on mouse oocytes frozen in \(1\) M DMSO [22]. Leibo et al. obtained a mean nucleation temperature of \(-45^\circ\)C, and observed a slight increase in the nucleation temperature as the cooling rate increased from \(~2^\circ\)C/min to \(~30^\circ\)C/min. These results are consistent with the model predictions for oocytes frozen in low concentrations of glycerol (Fig. 2-5). The experimentally determined critical cooling rate was \(2.4^\circ\)C/min [22], in reasonable agreement with the predicted value of \(1.2^\circ\)C/min (Fig. 2-9). The discrepancy is likely a result of the different CPAs used in model and experiments. Toner et al. measured intracellular ice formation in mouse oocytes in the absence of CPAs, and obtained a mean nucleation temperature of \(-12.8^\circ\)C [47], which significantly deviates from the model prediction of \(-38^\circ\)C. This discrepancy is due to the fact that heterogeneous nucleation mechanisms, which are active at low CPA concentrations and low cooling rates, were neglected in the model. Nonetheless, as shown in Fig. 2-9, the predicted critical cooling rate (\(2.4^\circ\)C/min) only slightly overestimates the value of \(1.7^\circ\)C/min measured by Toner et al. [47].
Cryopreservation protocols in the fast-cooling regimes (III and IV) are commonly referred to as "vitrification" protocols. In these regimes, water transport is negligible, and the extent of crystallization is governed by kinetic rather than thermodynamic effects. For example, since the glass transition temperature can be reached in shorter times at faster cooling rates, increasing the rate of cooling will decrease the amount of time in which crystals can grow, thus reducing the amount of ice formed. Because the cytosol volume and composition are constant during rapid cooling, the rates of nucleation and crystal growth become functions of temperature only, i.e. they are independent of cooling rate. Thus, using the transformation $dt = -dT/B$, it is evident from Eqs. (2-3) and (2-13) that the values of $\bar{N}$ and $r_c$ at any given temperature are inversely proportional to $B$.

In regime III, the rate of crystal growth is negligible in comparison with the rate of formation of new ice nuclei, and the dominant mechanism of ice formation is polynuclear nucleation, i.e. a large number of ice nuclei form, but do not undergo appreciable further growth. (see Figs. 2-4A and 2-5). Thus, to avoid damaging amounts of intracellular ice, the cell must be cooled sufficiently rapidly to temperatures below the glass transition that the total volume of nuclei remains at an innocuous level. The critical cooling rate must therefore satisfy the criterion

$$\int_{\tau_e}^{\tau_c} J_n v_w \left( \frac{-dT}{B} \right) \leq X_c^g,$$

(2-31)
where \( n^* \) is the number of water molecules in a stable ice nucleus. For an approximate evaluation of the integral in Eq. (2-31), the nucleation rate can be taken to be negligible for \( T > T_h \), and roughly constant for \( T_g \leq T \leq T_h \). Thus, the estimated critical cooling rate in regime III is given by

\[
B = \frac{J n^* \nu}{X^*_e} (T_h - T_g)
\]  

(2-32)

where \( J \) represents the average nucleation rate in the interval \( T_g \leq T \leq T_h \). The values of \( V_{cv}, T_h \) and \( T_g \) are determined by the initial conditions and remain constant during freezing, since water transport is negligible at rapid cooling rates. As seen in Fig. 2-9, the critical cooling rate estimates calculated using Eq. (2-32) agree well with the theoretical curve at low CPA concentrations. In the vicinity of regime IV, where the rate of crystal growth becomes significant, Eq. (2-32) underestimates the cooling rate necessary for vitrification.

In regime IV, crystal growth kinetics were found to significantly affect the extent of ice formation in the cell. Thus, even if nucleation did occur, the amount of ice formed could be controlled and maintained at innocuous levels (Fig. 2-4B). Consequently, the critical cooling rate can be estimated from the criterion that the total volume fraction crystallized remain below \( X^*_e \), i.e.

\[
\int_{t_0}^{t_f} \frac{4\pi}{3} U (t_g - t)^3 \, dt \leq X^*_e
\]  

(2-33)
where \( t_h \) and \( t_g \) are the times corresponding to the temperatures \( T_h \) and \( T_g \), respectively; and \( U \) is the crystal growth velocity averaged between the instantaneous temperature at time \( t \) and the glass transition temperature, \( T_g \). Assuming that \( U \) is approximately constant, and scales as \( U \sim \alpha D^{1/2} \left[ (T_h - T_g)/B \right]^{1/2} \), the following estimate for the critical cooling rate can be derived from Eq. (2-33):

\[
B = \left[ \frac{\pi \bar{\alpha}^3 D^{3/2}}{3 X_c^\beta} \right]^{2/5} (T_h - T_g). \tag{2-34}
\]

The diffusivity \( D \) was evaluated at the nucleation temperature, since crystal growth is most significant immediately after nucleation [19]. Estimates of the critical cooling rate obtained from Eq. (2-34) are shown in Fig. 2-9. Within regime IV, these critical cooling rate estimates are in reasonable agreement with the theoretical model predictions. The slight discrepancy is mainly due to approximations in the evaluation of Eq. (2-33).

In summary, it has been demonstrated that the relative importance of the coupled processes of water transport, nucleation and crystal growth vary between freezing regimes. In regime I, water transport and nucleation dominate the intracellular ice formation process, and thus the crystal growth equations can be decoupled from the model. In regime IV, water transport is decoupled from the crystallization process, while in regime III, intracellular ice formation is governed only by nucleation, as a result of decoupling of both the water transport and crystal growth processes. In regime II, all three processes are highly coupled, and simplification of the model is not possible. Simplified
expressions for the critical cooling rate in regimes I, III and IV were used to verify the accuracy of the analysis of intracellular crystallization. It should be noted that the equations derived to estimate critical cooling rates can be applied to other cell types and CPAs to obtain approximate data on the freezing behavior of these systems. Another observation of interest is that in each regime, the critical cooling rate is a function of the difference $T_h - T_0$. This difference is a measure of the instability of the solution during freezing, and is analogous to the instability measure $T_m - T_d$ (where $T_d$ is the devitrification temperature) for warming, used by Boutron and co-workers [10].
References


Chapter 3

Model Verification

Analysis of Published Data on Hepatocytes Cryopreserved in DMSO

Introduction
In this chapter, the model of intracellular crystallization developed in Chapter 2 is tested using previously published data on freezing of cultured rat hepatocytes. Hepatocytes were chosen because of the availability in the literature of experimental data both on biophysical parameters required in the model and on cell viability following various cryopreservation protocols. This cell type also affords a particularly rigorous test of the theoretical model, as its freezing behavior is very different from that of oocytes (see Fig. 1-1), thus expanding the range of physicochemical conditions over which the model has been applied. Since the empirical studies on hepatocytes used dimethyl sulfoxide (DMSO) as a CPA, a phenomenological viscosity model for a water-DMSO-NaCl solution is constructed here for use in theoretical predictions. The intracellular crystallization model is further extended to include nucleation by heterogeneous catalysis, as this nucleation mechanism is important in hepatocyte freezing. A recently published study on hepatocyte cryopreservation [2] is here analyzed by theoretically simulating the outcome of the various protocols used, and comparing these predictions with the measured viability data. In general, satisfactory agreement was obtained between the theoretical model predictions and published experimental observations. Furthermore, correlation of predicted intracellular crystal volumes with previous measurements of cell damage allowed
interpretation of the empirical findings in terms of the underlying physical processes, and provided some information about the amount of intracellular ice which causes irreversible damage in hepatocytes.

**Theoretical Background**

**Water transport**

Transport across the cell membrane is driven by chemical potential differences between the intracellular and extracellular solutions, as described in Chapter 2. The transport of water is assumed to be membrane-limited, and the temperature-dependence of the membrane permeability is described by an Arrhenius relationship [Eq. (2-1)], characterized by the parameters $L_{pg}$ and $E_{tp}$. The values of $L_{pg}$ and $E_{tp}$ have been measured for cultured hepatocytes under isotonic conditions in the absence of CPAs [16]. The effect of CPAs on the membrane permeability can be estimated by reducing the value of $L_{pg}$ by a factor of 0.5 for DMSO concentrations in excess of ~1 M [9]. There is still considerable disagreement concerning the magnitude of the effect of CPA on the value of $E_{tp}$. Mazur [9] presents evidence indicating that $E_{tp}$ may be independent of solution composition, while McCaa et al. [10] report an increase in $E_{tp}$ for monocytes in the presence of DMSO. As measurements of the membrane permeability parameters for hepatocytes in the presence of CPAs are not available in the literature, $E_{tp}$ was left as an adjustable parameter in the model. Because the data of Borel-Rinkes et al. [2] suggest that IIF occurs in cultured hepatocytes frozen to -40°C in the presence of DMSO, when using a cooling rate of 16°C/min, $E_{tp}$ was chosen so that the predicted intracellular water content at -40°C for a cell cooled at 16°C/min would be sufficient to cause nucleation at this temperature.
Fig. 3-1 shows the effect of the activation energy on the concentration of DMSO in the cell. DMSO concentrations are plotted as a function of temperature for a cooling rate of 16°C/min, for values of the activation energy ranging between 1.0 and 1.6 times the isotonic value. A factor of 1.55 was predicted to cause intracellular nucleation at -40°C, and this value was used in the rest of this study. This result is in reasonable agreement with the twofold increase in $E_{lp}$ measured by McCa et al. [10] for monocytes in 1 M DMSO. The numeric values of $L_{pg}$ and $E_{lp}$ used in the model of water transport in hepatocytes are given in Table 3-1.

**Heterogeneous nucleation**

A model of the temperature- and concentration-dependence of the rate of homogeneous nucleation in the presence of CPAs was developed in Chapter 2. In hepatocytes, heterogeneous nucleation mechanisms are active even at relatively high concentrations of CPA, and this process must thus be included in the model. Taking into account the effect of a catalytic substrate on the thermodynamics of ice nucleus formation, an equation for the heterogeneous nucleation rate per unit substrate area, $I_{HET}$, may be written as follows:

$$I_{HET}(c, T) = \Omega_{HET}(c, T) \exp[-\kappa_{HET}(c, T) \tau(c, T)]$$  \hspace{1cm} (3-1)

where $\Omega_{HET}$ and $\kappa_{HET}$ are the heterogeneous nucleation rate parameters. The theory of heterogeneous nucleation shows that the coefficients $\Omega_{HET}$ and $\kappa_{HET}$ have the same basic functional forms as the homogeneous nucleation rate parameters ($\Omega_{HOM}$ and $\kappa_{HOM}$), with the effect of the catalytic substrate being described by a single additional parameter $\theta$ [15].
Figure 3-1. Sensitivity of predicted intracellular DMSO concentrations to variations in the activation energy for water transport. Labels indicate the magnitude of the activation energy relative to the isotonic value, $E_{LP} = 76.1$ kJ/mol. The cooling rate was 16°C/min.
Table 3-1: Water transport model parameters for cultured hepatocytes. Values for "DMSO-treated cells" were used in the simulations.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Isotonic Conditions</th>
<th>DMSO-treated cells*</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_{cell}$</td>
<td>5.0x10^{-15} m³</td>
<td>3.4x10^{-15} m³ t</td>
</tr>
<tr>
<td>$V_b$</td>
<td>2.6x10^{-15} m³</td>
<td>2.6x10^{-15} m³</td>
</tr>
<tr>
<td>$A$</td>
<td>2.5x10^{-9} m²</td>
<td>2.5x10^{-9} m²</td>
</tr>
<tr>
<td>$L_{ps}$</td>
<td>2.14x10^{-13} m s^{-1} Pa^{-1}</td>
<td>1.07x10^{-13} m s^{-1} Pa^{-1}</td>
</tr>
<tr>
<td>$E_{Lp}$</td>
<td>7.61x10^{4} J mol^{-1}</td>
<td>1.18x10^{5} J mol^{-1}</td>
</tr>
<tr>
<td>$n_o$</td>
<td>0</td>
<td>3.27x10^{-12} mol</td>
</tr>
<tr>
<td>$n_s$</td>
<td>3.50x10^{-13} mol</td>
<td>3.50x10^{-13} mol</td>
</tr>
</tbody>
</table>

*Cells equilibrated in 1.33 M DMSO.

†Value following isothermal hold at -12°C after seeding of external ice.
Thus, the heterogeneous nucleation parameters can be calculated from the previous models for $\Omega^\text{HOM}(c, T)$ [Eq. (2-24)] and $\kappa^\text{HOM}(c, T)$ [Eq. (2-26)] as follows:

$$\Omega^\text{HET}(c, T) = \Omega^\text{HET}_1 \frac{\Omega^\text{HOM}(c, T)}{\Omega^\text{HOM}(c_1, T_1)} \left[ \frac{f(\theta)}{f(\theta_1)} \right]^{1/6}$$

(3-2)

$$\kappa^\text{HET}(c, T) = \kappa^\text{HET}_1 \frac{\kappa^\text{HOM}(c, T)}{\kappa^\text{HOM}(c_1, T_1)} \frac{f(\theta)}{f(\theta_1)}$$

(3-3)

where $f(\theta)$ is the heterogeneous nucleation factor, $\Omega^\text{HET}_1$ and $\kappa^\text{HET}_1$ are known values of the kinetic and thermodynamic coefficients at a given concentration $c_1$ and temperature $T_1$, and $\theta_1$ is the corresponding value of $\theta$. Reference nucleation rate parameters for heterogeneous nucleation were calculated from measurements of HIF in cultured hepatocytes frozen in 2 M DMSO at a cooling rate of 400°C/min [6]. Using a non-linear regression analysis technique described elsewhere [14], the kinetic and thermodynamic coefficients were determined to be $\Omega^\text{HET}_1 = 4.5 \times 10^9$ m$^{-2}$ s$^{-1}$, $\kappa^\text{HET}_1 = 1.16 \times 10^{-3}$ (Fig. 3-2). The heterogeneous catalysis parameter $f(\theta)$ was assumed to remain approximately constant, thus $f(\theta)/f(\theta_1) \approx 1$. Fig. 3-3 shows the predicted non-equilibrium phase diagram for hepatocytes in 0.142 M NaCl and varying concentrations of DMSO, cooled at a constant rate of 400°C/min. In computing Fig. 3-3, water transport was assumed negligible. Superimposed are nucleation temperature data from Hubel et al. [6] for DMSO concentrations between 0 and 2 M. The predicted heterogeneous nucleation curve is in excellent agreement with the measured values, suggesting that the model assumptions
Figure 3-2. Determination of $\Omega^\text{HET}_f$ and $\kappa^\text{HET}_f$ by curve fitting of predicted HIF-probabilities to measured probabilities of HIF in cultured hepatocytes frozen at 400°C/min in the presence of 2 M DMSO (data from Hubel et al. [6]). The best-fit curve ($\chi^2 = 10^{-4}$) is obtained for $\Omega_f^\text{HET} = 4.5 \times 10^9 \text{ m}^{-3} \text{ s}^{-1}$, $\kappa_f^\text{HET} = 1.16 \times 10^3$. 
Figure 3-3. Predicted non-equilibrium phase diagram for cultured hepatocytes cooled at a rate of 400°C/min. $T_m$ is the equilibrium freezing point, $T_{het}$ the heterogeneous nucleation temperature, and $T_{hom}$ the homogeneous nucleation temperature. The squares represent experimentally measured nucleation temperatures for cultured hepatocytes cooled at 400°C/min, in various concentrations of DMSO [6].
are reasonable. Knowing the heterogeneous nucleation rate $J_{HET}$, one can calculate the total nucleation rate per cell:

$$J = J_{HOM} V_c + J_{HET} A,$$  \hfill (3-4)

which in turn can be used to predict the extent of intracellular ice crystal growth using Eqs. (2-3) and (2-14).

**Crystal growth**

The extent of crystal growth inside hepatocytes was calculated using the diffusion-limited crystal growth model described in Chapter 2. For complete transformation of the intracellular water from liquid to solid ($X^{eq}=1$), the actual volume of ice in the cell was determined by the thermodynamic equilibrium between the intracellular ice and the unfrozen intracellular solution, and was calculated from the temperature and composition of the cytoplasm.

**Viscosity estimation**

Viscosity data for solutions of interest in cryobiology are scarce. Existing studies are typically limited to temperatures well above the equilibrium melting point, and relevant viscosity data for ternary water-salt-CPA solutions are virtually non-existent. Cowie and Toporowski [3] measured the concentration-dependence of the viscosity of a binary water-DMSO system at 25°C. More recently, Eto et al. [4] have obtained viscosity data down to -10°C for aqueous solutions of DMSO. These are the only such measurements
available for subzero temperatures. Although they report best-fit Arrhenius parameters describing temperature dependence at several concentrations, more data are needed to establish conclusively the concentration dependence of the Arrhenius coefficients. For purposes of this study, a crude approximation to the function $\eta(c, T)$ has been constructed from the available data. Cowie and Toporowski's [3] data were first fit with a polynomial

$$\eta_T(x_a) = 0.124x_a^6 - 0.452x_a^5 + 0.627x_a^4 - 0.391x_a^3 + 0.090x_a^2 + 0.004x_a + 0.001,$$

(3-5)

where $\eta_T$ is the viscosity (in Pa s) of a water-DMSO solution at 25°C for a given DMSO mole fraction $x_a$. The viscosity concentration-dependence was assumed to have the same shape at any temperature, and thus the viscosity function $\eta_{DMSO}(x_a, T)$ for water-DMSO was obtained by scaling the curve $\eta_T(x_a)$ to fit the temperature-dependence relationships measured by Eto and Rubinsky [4]. From their data for pure water (DMSO mole fraction $x_0 = 0$) and 8 M DMSO (mole fraction $x_I = 0.25$):

$$x_a(T) = C_o \exp[E_o/RT]$$

(3-6)

$$x_I(T) = C_i \exp[E_i/RT].$$

(3-7)

scaling of $\eta_T(x_a)$ yields the desired function:

$$\eta_{DMSO}(x_a, T) = \left(\eta_T(x_a) - \eta_T(x_0)\right) \frac{\eta_T(x_a) - \eta_T(x_0)}{\eta_T(x_I) - \eta_T(x_0)} + \eta_T(x_0).$$

(3-8)
The viscosity of the ternary solution water-DMSO-NaCl was estimated by approximating the contribution from the salt particles using a hard-sphere model, using Eq. (2-23).

Results

Numerical simulations

In the simulations presented here, the experimental freezing protocol used by Borel Rinkes et al. [2] is reproduced. The hepatocytes were initially equilibrated in a 1.33 M DMSO solution at 22°C and cooled down to -12°C at 10°C/min [1,2]. The cells were then partially dehydrated at -12°C by seeding external ice, and holding the cells at -12°C for 15 minutes [2]. Solution of the water transport equation [Eq. (2-1)] demonstrated that cells equilibrate completely by dehydration in less than ~10 seconds after the seeding of the external ice, and that during the remainder of the isothermal holding period, there is no driving force for transport of either water or DMSO. Assuming that during this initial ~10 second transient, the transport of solutes is negligible in comparison to the water efflux, one obtains a value for the new cell volume ($V_{cell}$) of 3.4x10^{-15} m^{3}, and intracellular solute concentrations of 4.12 M for DMSO and 0.44M for NaCl. These values were used as initial conditions in the water transport simulations (Table 3-1). Simulations were run for the following freezing protocols: (1) freezing from -12°C to -40°C at constant cooling rates $B$ (°C/min) ranging between 1°C/min and 1000°C/min, followed by a 5-minute isothermal holding period at -40°C, and (2) freezing from -12°C to various final temperatures between -40°C and -80°C, at various constant cooling rates. The corresponding temperature histories are shown in Fig. 3-4.
Figure 3-4. Freezing protocol used in simulations: (a) equilibration in 1.33 M DMSO at 22°C, (b) cooling to -12°C at 10°C/min, (c) thermal equilibration at -12°C, (d) seeding of external ice, (e) 15-minute equilibration at -12°C, followed by either (f) cooling at various constant rates to -40°C and (g) 5-minute isothermal holding period at -40°C, or (f’) cooling at various constant rates to various final temperatures between -12°C and -80°C.
State of the cytoplasm

Figs. 3-5 to 3-8 describe the state of the cytoplasm during freezing from -12°C to -80°C at cooling rates ranging between 1°C/min and 1000°C/min. In Fig. 3-5, the intracellular water content, normalized to the value for isotonic conditions, is plotted against temperature. At the onset of constant rate cooling (T=-12°C), the hepatocytes contain only 22.6% of their original water volume, a consequence of the initial DMSO treatment and dehydration prior to freezing. The amount of water loss during cooling to -80°C is strongly dependent on the cooling rate. At slow cooling rates, dehydration is significant. For example, for a cooling rate of 1°C/min, only 4.1% of the intracellular water remains at -80°C. At fast rates, the final temperature of -80°C is reached before significant amount of water can be expressed. The final water content at a cooling rate of 1000°C/min is 21.0%, i.e. a loss of only 1.6% during cooling from -12°C to -80°C.

Figs. 3-6 and 3-7 show the intracellular concentrations of DMSO and NaCl, respectively, plotted versus temperature. Initially, the cells contain 4.12 M DMSO and 0.44 M NaCl; as the cells dehydrate during freezing, both salt and CPA concentrations increase, with slow cooling rates producing high intracellular solute concentrations, as expected. For a cooling rate of 1°C/min, the cytosol contains 9.5 M DMSO and 1.0 M NaCl at the final temperature of -80°C. However, if the same cell had been frozen with no DMSO present, the final intracellular salt concentration would have exceeded 21 M. Thus, the use of CPAs results in significant dilution of intracellular electrolytes. At fast cooling rates (≥400°C/min), the intracellular DMSO and NaCl concentrations do not change appreciably from their respective initial values.
Figure 3-5. Predicted temperature-dependence of the intracellular water content for cultured hepatocytes cooled at various constant rates. The normalized water content is defined as the water volume in the cell relative to the isotonic water volume, $2.5 \times 10^{-15}$ m$^3$. The curve marked 'IIF' indicates the predicted nucleation temperature for each cooling rate.
Figure 3-6. Predicted temperature-dependence of the intracellular DMSO concentration for cultured hepatocytes cooled at various constant rates. The curve marked 'IIF' indicates the predicted nucleation temperature for each cooling rate.
Figure 3-7. Predicted temperature-dependence of the intracellular NaCl concentration for cultured hepatocytes cooled at various constant rates. The curve marked 'IIF' indicates the predicted nucleation temperature for each cooling rate.
Figure 3-8. Predicted temperature-dependence of the degree of intracellular undercooling for cultured hepatocytes cooled at various constant rates.
In Fig. 3-8, the degree of undercooling, $\Delta T$, of the cytoplasm is plotted as a function of temperature. At the onset of cooling at $-12^\circ$C, the cells are in equilibrium with the external ice, and $\Delta T = 0$. As the hepatocytes are cooled in the presence of ice, they express water in order to remain in equilibrium. The rate of water transport thus has a significant effect on the amount of undercooling observed in the cytosol. At a cooling rate of $400^\circ$C/min, the rate of water transport is practically negligible compared to the rate at which the cell temperature drops, and consequently no appreciable equilibration can occur. The resulting undercooling increases approximately linearly as the temperature decreases. At a cooling rate of $1^\circ$C/min, cooling is slow compared with the rate of removal of intracellular water, and the cytosol remains in equilibrium with the external ice (i.e. $\Delta T = 0$) for temperatures down to $\sim 30^\circ$C. However, at lower temperatures, one observes significant intracellular undercooling even at slow cooling rates, as a consequence of the Arrhenius temperature dependence of the membrane permeability [Eq. (2-21)]. The permeability $L_p$ is reduced by about two orders-of-magnitude when the temperature falls from the initial temperature $-12^\circ$C to $-30^\circ$C. Thus, as the cell temperature decreases, the rate of water efflux decreases, and eventually a point is reached where the water transport becomes negligible. This is clearly observed in Fig. 3-5, where the water content in the cell is seen to approach a constant value at temperatures below $\sim 40^\circ$C, even at very slow cooling rates. As a consequence of the drastically reduced membrane permeability, the cytoplasmic undercooling increases linearly as the cell temperature drops.
Nucleation and crystal growth

The number of intracellular ice nuclei $N$ and the crystallized volume fraction $X^B$ were predicted for cells cooled from -12°C to -40°C and then held at -40°C for 5 minutes in order to mimic experimental conditions [2]. Table 3-2 shows the final values of $N$ and $X^B$ for various cooling rates, as well as the corresponding nucleation temperatures. The temperatures at which nucleation would occur if the cells were cooled further at the same rate, with no isothermal hold at -40°C, are also shown in parentheses for comparison purposes. Note that although a cell cooled continuously at a rate of 16°C/min would have a nucleation temperature of -41.6°C, the nucleation rate at -40°C is sufficiently high that a nucleus is formed during the 5-minute isothermal hold at that temperature. For cooling rates less than 10°C/min, no nucleation was observed, and hence no crystal growth occurred in the cells. For cooling rates greater than 16°C/min, the intracellular water was completely transformed ($X^B = 1$) at the end of the freezing protocol at -40°C. The final number of nuclei was $N = 1$ for both 16°C/min and 35°C/min, i.e. the cell contained only a single ice crystal. Thus, for the conditions simulated, only mononuclear nucleation was observed. The final ice volume was calculated from the condition that the fully crystallized intracellular solution be at thermodynamic equilibrium and the assumption that water transport was negligible once nucleation had occurred. The resulting ice volumes, normalized to the total intracellular water volume under isotonic conditions, are shown in Fig. 3-9 as a function of the cooling rate. On the same figure, experimental albumin secretion data from Borel Rinkes et al. [2] are also shown for cultured hepatocytes subjected to the same freezing protocol simulated here, and subsequently thawed rapidly.
Figure 3-9. Effect of cooling rate on predicted intracellular ice content and measured albumin secretion levels for cultured hepatocytes cooled to a final temperature of -40°C, then held isothermally at -40°C for 5 minutes before rewarming. The normalized ice volume (solid line) is the predicted amount of intracellular ice at the end of the isothermal hold, relative to the isotonic water volume, $2.5 \times 10^{15}$ m$^3$. The albumin secretion data (broken line) are from Borel Rinkes et al. [2].
Table 3-2: Predicted nucleation temperature, final number of ice nuclei and final crystallized volume fraction in cultured hepatocytes cooled to -40°C at the given cooling rate, then held isothermally at -40°C for 5 minutes.

<table>
<thead>
<tr>
<th>B [°C/min]</th>
<th>T_{het}[°C]</th>
<th>N_{final}</th>
<th>\chi_{\text{final}}^B</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-</td>
<td>(-52.9)*</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>-</td>
<td>(-46.8)</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>-</td>
<td>(-43.8)</td>
<td>0</td>
</tr>
<tr>
<td>16</td>
<td>-40.0</td>
<td>(-41.6)</td>
<td>1</td>
</tr>
<tr>
<td>35</td>
<td>-37.2</td>
<td>(-37.2)</td>
<td>1</td>
</tr>
</tbody>
</table>

* Nucleation temperatures for cells cooled continuously at the given rate, with no isothermal hold at -40°C.
The normalized albumin secretion (a measure of the differentiated function of the frozen-thawed hepatocyte cultures) has a maximum between cooling rates of 5°C/min and 10°C/min, corresponding to the optimal cooling rate. At suboptimal cooling rates, albumin secretion decreases with decreasing cooling rate; at supraoptimal cooling rates, the albumin secretion drops with increasing cooling rate. A comparison of these results with the predicted intracellular ice volume as a function of cooling rate shows that at or below the optimal cooling rate, there is no crystal growth in the cells, while for supraoptimal rates the amount of intracellular ice increases with increasing cooling rate. Thus, the level of albumin secretion, and therefore hepatocyte viability, correlates inversely with the intracellular ice volume at supraoptimal rates. The drop in viability at suboptimal rates, where no crystallization occurs, is commonly ascribed to "solution effects" not pertaining to IIIF, i.e. damage related to high intracellular and extracellular solute concentrations, excessive cell shrinkage, mechanical deformation, or other forces [8].

In addition to the dependence of crystal growth and corresponding cell viability on cooling rate, their dependence on the final freezing temperature was also investigated. In Fig. 3-10, the predicted crystallized volume fraction $X^B$ is plotted as a function of temperature, for a cell cooled at 5°C/min down to -80°C. Shown on the same plot are albumin secretion data for hepatocyte cultures frozen at a cooling rate of 5°C/min to various final temperatures between -40°C and -80°C, and rewarmed either rapidly ($\geq$400°C/min) or slowly (5°C/min) following a 5-min isothermal hold at the selected final temperature [2]. As can be seen from the $X^B$ curve, intracellular ice nucleates at -46.8°C, followed by a
Figure 3-10: Effect of the final freezing temperature on the predicted crystallized volume fraction (solid line) and on measured albumin secretion levels (broken lines) for cultured hepatocytes cooled at a rate of 5°C/min. The albumin secretion data are for cells cooled to the given final temperature, held isothermally at the final temperature for 5 minutes, then rewarmed either rapidly (≥400°C/min) or slowly (5°C/min) [2].
very rapid transition from \( X^\beta = 0 \) to \( X^\beta = 1 \). The normalized albumin secretion measured after slow thawing is \( \sim 100\% \) for a final hold temperature of \(-40^\circ C\), and drops to a value of less than 10\% when the final freezing temperature is less than \(-50^\circ C\). This drastic reduction in protein secretion correlates well with the predicted nucleation temperature. For rapid warming rates, the measured albumin secretion remains at approximately normal levels, decreasing only slightly to \( \sim 80\% \) as the final freezing temperature is decreased. Since the model predicts formation of intracellular ice when hepatocytes are frozen to \(-50^\circ C\) or lower, these data suggest that the intracellular crystallization during the specified freezing protocol is innocuous, and that the damage in slowly warmed cells occurs during thawing.

Ice nucleation temperatures were also calculated for freezing at cooling rates other than \( 5^\circ C/min\). These are shown on Fig. 3-5 (curve marked "IIF") superimposed on the corresponding water content curve for each cooling rate. For a given cooling rate, nucleation occurs when the water content curve crosses the IIF curve (i.e., when the nucleation temperature is reached). IIF curves have also been drawn in Figs. 3-6 and 3-7, showing the dependence of the nucleation temperature on the intracellular solute concentrations. At cooling rates below \( 400^\circ C/min\), the intracellular nucleation temperature is depressed as the cooling rate decreases because of the corresponding increase in the intracellular solute concentration. However, the nucleation temperature is also lowered when the cooling rate increases from a cooling rate of \( 400^\circ C/min \) to \( 1000^\circ C/min\). At these high cooling rates, cell dehydration is negligible, and therefore the nucleation rate is independent of the rate of cooling. The observed depression in the
temperature of nucleation is a kinetic effect, i.e. in the time required to form a stable nucleus at a given nucleation rate, a significant temperature drop can be achieved at very fast cooling rates.

**Discussion**

This chapter represents further development of the coupled IIIF model described in Chapter 2 for use with a different cell type and a different CPA, and the verification of the theoretical model using published data on hepatocyte cryopreservation. The model of intracellular nucleation has been extended to incorporate heterogeneous nucleation, including the effect of CPAs on this nucleation process. In addition, a new phenomenological model of cytosol viscosity was developed for simulation of IIIF in the presence of DMSO as a CPA. The intracellular crystallization model has been applied to prior data obtained on the freezing of cultured rat hepatocytes in the presence of DMSO [2], in order to test the theoretical predictions. The model predictions were in good agreement with the experimental data, and provide important physical insight into the processes relevant to cryopreservation of cultured hepatocytes.

**Non-equilibrium phase diagram**

Heterogeneous nucleation temperatures for cultured hepatocytes have been predicted for the first time as a function of DMSO concentration; using these data, a non-equilibrium phase diagram could be constructed (Fig. 3-3). For DMSO concentrations less than ~3 M, the predicted heterogeneous nucleation temperature curve was found to be approximately parallel to the liquidus curve ($\Delta T_{het} \approx 1.3 \Delta T_m$). This finding is consistent
with the empirical results of Reischel and Vali [12]. For hepatocytes, the heterogeneous nucleation curve lies only 7 to 10°C below the liquidus, and thus only limited undercooling is allowed in the cells if IIF is to be avoided. The implication is that to prevent completely the nucleation of ice crystals inside cultured hepatocytes, one must either cool at extremely slow rates, (i.e., much less than 1°C/min; see Fig. 3-5) to maintain near-equilibrium conditions, or use high concentrations of CPA to depress the equilibrium freezing temperature to a point approaching the final storage temperature. In both cases, the cells are exposed to high CPA concentrations, which may be deleterious. An alternative, and more appropriate strategy for cryopreservation of sandwich cultured hepatocytes, might be to permit intracellular nucleation to occur, but to limit the extent of crystal growth in the cell to an innocuous level by expressing enough of the intracellular water during cooling.

At high DMSO concentrations, the predicted heterogeneous nucleation temperature begins to drop sharply as the CPA content increases, and at DMSO concentrations higher than 7.8 M, homogeneous nucleation becomes the dominant mechanism of nucleation. Rall et al. [11] have observed such a transition from a heterogeneous to a homogeneous mechanism of intracellular nucleation in eight-cell mouse embryos frozen in the presence of DMSO or glycerol. Mouse embryos were found to nucleate by a homogenous mechanism when CPAs were present in concentrations of 1.5 M or higher. These results suggest that CPAs have a much more pronounced effect on IIF temperatures for mouse embryos than for cultured hepatocytes. While the shape and location of the homogeneous nucleation curve on the phase diagram depends only on the nature of the freezing solution,
the heterogeneous nucleation curve is highly dependent on cell type and the specific mechanism of catalysis. The shape of the heterogeneous nucleation curve is thus of major importance in selecting strategies for cryopreservation.

**Kinetics of water transport**

The trajectory of the cytosol through its state-space is determined by the kinetics of water transport out of the cell. The main difficulty of accurately predicting water transport is the measurement of the membrane permeability parameters $L_{pg}$ and $E_{lp}$ under conditions relevant to cryopreservation, i.e. at subzero temperatures and in the presence of various concentrations of CPA and other solutes. Although the permeability parameters for cultured hepatocytes under isotonic conditions have recently been measured at subzero temperatures [16], the CPA dependence of these parameters remains unknown. In the present work, the value of the activation energy $E_{lp}$ has been adjusted to yield plausible predictions of intracellular water content. Reasonable results were obtained if $E_{lp}$ increased by a factor of 1.55 in the presence of DMSO. Although this value is in concordance with existing experimental evidence [10], it is clear that direct measurements of the membrane permeability parameters are needed. It should also be noted that the transport of CPA during freezing has been neglected in this study. The assumption that CPA transport is negligible during freezing is justified for sufficiently rapid rates of cooling, but some CPA permeation may occur at very slow cooling rates. However, the main focus of the present work is on cooling rates sufficiently fast to cause IIF (i.e. $>10^\circ C/min$, see Fig. 3-9), at which CPA permeation is probably insignificant.
The water transport simulations reveal that, as a result of the Arrhenius dependence of the membrane permeability, the cell becomes quasi-impermeable at low temperatures, effectively trapping the remaining water inside the cell. Hence, even at very slow cooling rates the cytoplasm will eventually become significantly undercooled, ultimately undergoing IIF. When hepatocytes were cooled to -80°C, intracellular ice nucleated at all simulated cooling rates between 1°C/min and 1000°C/min. Conceivably, extremely slow cooling rates (much less than 1°C/min) could be used to depress the nucleation temperature to below the final storage temperature, thus completely avoiding IIF. However, hepatocytes were found to experience damage due to "solution effects" at cooling rates less than 1°C/min (IIF could be eliminated as a possible cause of the observed cell injury at these slow cooling rates, because cooling was interrupted at -40°C, well above the nucleation temperature). For cooling rates sufficiently fast to avoid damage by solution effects, nucleation always occurred at temperatures higher than -50°C. Thus, when hepatocytes are frozen to temperatures below this value, intracellular crystallization is inevitable. The viability of the cells will therefore be a function of the extent of intracellular crystallization: if the amount of ice formed during freezing is sufficiently low, the cells can be "rescued" by rapid warming. However, if the rate of rewarming is too slow, the intracellular crystals can grow to damaging proportions during warming. Since hepatocytes were predicted to undergo IIF at all cooling rates, rapid rewarming rates were always required to prevent injury during the thawing process.
Intracellular crystal growth

The quantification of the permissible amount of intracellular ice is a problem of critical importance to cryobiology. Progress towards resolving this question has been hampered by the previous lack of a fundamental theoretical treatment of intracellular crystal growth, as well as the paucity of relevant experimental data. Mazur [9] estimates that mouse ova containing ≤16% of their isotonic water volume upon IIF will be "rescuable," but notes that the value of this limit appears to vary for different CPA concentrations. Shimada [13] indicated that ice crystals smaller than ~0.05 μm are innocuous to HeLa cells. To provide a better understanding of damage by intracellular ice in hepatocytes, and to attempt to define what may constitute innocuous crystallization, the crystal growth predictions of the present study have been correlated with existing viability measurements [2]. The intracellular ice volume was predicted to increase with increasing cooling rate, a consequence of the high water content in cells which are frozen rapidly. For cooling rates faster than ~5°C/min, the predicted equilibrium ice volume in the cell was thus found to be positively correlated with the loss of function in hepatocytes, suggesting a link between cell damage and intracellular ice volume for this range of cooling rates.

Quantitative comparison of crystal growth predictions with measurements of cell viability provides useful information regarding the conditions under which intracellular ice can be considered innocuous. Even though IIF is predicted to occur when cells are frozen at a cooling rate of 16°C/min, albumin secretion levels remain at almost 70% of normal values, indicating that the intracellular ice crystals inflicted only partial damage on the hepatocyte culture. At this cooling rate, the cell nucleates with 7.0% of its isotonic water volume
remaining, which in equilibrium at -40°C yields a normalized ice volume of only 2.0% (Fig. 3-9). Hepatocytes, frozen at a cooling rate of 35°C/min, on the other hand, experience total loss of function. The corresponding intracellular water content at the nucleation temperature (-37.2°C) is 8.7%, which when fully frozen at -40°C results in a crystal volume of 3.7% relative to the total isotonic water volume. Therefore, based on the model predictions, the critical ice volume causing irreversible damage in hepatocytes appears to lie in the range of 2.0% to 3.7%.

The results of the above analysis should be interpreted with the caveat that crystal growth velocities may have been overestimated at low temperatures, due to uncertainties in the phenomenological model used to determine the cytosol viscosity. If the rate of crystal growth under the experimental conditions was sufficiently slow to partially vitrify the cell ($\chi^R<1$), then the actual intracellular ice volumes may have been considerably lower than the predicted equilibrium values given above. Nonetheless, 3.7% would still represent an upper bound to the critical intracellular ice volume. Problems with the viscosity model stem from the fact that it was constructed by extrapolation of measurements made above -10°C. Furthermore, the Arrhenius temperature-dependence employed in the viscosity model precludes the typically drastic viscosity increase near the glass transition temperature observed for many glass-forming solutions. Further experimental work to measure the viscosity of water-DMSO solutions at low temperatures is clearly necessary.

The model predictions for cells frozen at a cooling rate of 5°C/min to -80°C (Fig. 3-10) provide further evidence that innocuous crystallization can occur in hepatocytes, and
successfully explain the measured dependence of hepatocyte function on the final freezing temperature and on warming rate. Cells frozen to -40°C at 5°C/min remain above the predicted nucleation temperature of -46.8°C, and are thus not damaged by intracellular ice, either during freezing or subsequent rewarming. Consequently, the survival is independent of warming rate. However, for final freezing temperatures lower than -50°C, total loss of function is observed in hepatocytes which were warmed slowly, while rapidly warmed cells retain protein secretion levels close to normal. Since -50°C lies below the calculated nucleation temperature (-46.8°C), ice should be present in the cell. Because cells which were warmed rapidly appear to be undamaged, these ice crystals must be innocuous. However, if slow warming rates are used, the crystals can grow to sizes which are damaging to the cell; hence the sharp drop-off in survival coincident with the initial appearance of an ice nucleus in the cytoplasm. In contrast, at rapid warming rates, there is no further growth of the intracellular ice crystals which form during freezing, and thus the cells remain viable.

Since ice formation in cultured hepatocytes frozen at 5°C/min to -80°C is innocuous, the corresponding normalized ice volume must be less than 3.7%, the calculated upper bound to the critical ice volume. However, the intracellular water content at the nucleation temperature for cells cooled at 5°C/min is 5.5% of isotonic, which fully frozen at -80°C would yield a normalized ice volume of 4.0%. Hence, one may conclude that hepatocytes frozen at 5°C/min to -80°C partially vitrify, i.e. that the final intracellular ice volume is less than the equilibrium value. Vitrification has been observed experimentally in mouse embryos cooled rapidly in the presence of 4.5 M DMSO and 0.25 M sucrose [7], and
glycerol solutions have been vitrified independent of cooling rate at concentrations of ~7 M [5]. Compared with the predicted intracellular DMSO content of 8.7 M at the nucleation temperature for hepatocytes cooled at a rate of 5°C/min, these data support the conclusion that the hepatocytes vitrify under the given conditions.

In summary, the theoretical model of intracellular crystallization has successfully accounted for the experimentally observed dependence of hepatocyte viability on both cooling rate and final freezing temperature, indicating that the three-part, coupled model developed here is a reasonable representation of the physicochemical processes occurring during cell freezing. The agreement of the data obtained in an experimental protocol optimization with corresponding model predictions further demonstrates the feasibility of using the theoretical model to a priori design and optimize protocols for cryopreservation.
References


Chapter 4

Optimization of a Freezing Protocol for Mouse Oocytes

Introduction
The theoretical model of intracellular crystallization developed and tested in Chapters 2 and 3 is in this chapter used to design and optimize a cryopreservation protocol for mouse oocytes. The ability to cryopreserve mammalian oocytes would represent an important advance for reproductive medicine, especially in treatment of infertility. It has been estimated that infertility affects between 35 and 70 million married couples around the world [22]. Recent studies in the United States have conservatively indicated that there are about 2.3 million infertile couples, which is about 8% of the domestic married couple population base with wives of reproductive age (15 to 44 years). In addition, it has been observed that about 4.9 million U.S. women in this age range display an impaired ability to bear children. Although the cost in personal, familial, and social terms is incalculable, U.S. couples spent over $1 billion on infertility treatments in 1987 [22]. The introduction of superovulatory techniques for in vitro fertilization (IVF), a common treatment modality for infertility, has resulted in the production of larger number of oocytes per cycle and, consequently, more embryos than can be safely transferred. Although cryopreservation of excess embryos has served as an important strategy for allowing embryo transfer in later cycles, the cryopreservation of unfertilized oocytes would avoid the ethical and legal issues associated with banking of fertilized embryos [23].
Unfortunately, compared with the success rates obtained with embryos, the rates of in vitro development of thawed oocytes are still very low [13,30,35]. To date, only five pregnancies have been reported in humans following IVF of more than 900 frozen oocytes [4,7,16]. Current methods for freezing oocytes are typically adaptations of protocols that have been successful with embryos. The effect of various freezing parameters has been investigated experimentally in efforts to develop cryopreservation protocols with maximal yield of viable, fertile oocytes after thawing [7,30], but since there is a large number of protocol variables potentially affecting the cell viability, an experimental search for the optimal combination of these parameters would be prohibitively costly in time and resources.

Recent advances in mathematical modeling of IIF [21,27] have afforded the possibility of rational design of freezing protocols based on model predictions of the effect of a given cryopreservation regimen on the cells. Pitt [21] has demonstrated the power of mathematical models in the optimization of non-linear freezing protocols, using a hypothetical cell type as an example. Toner et al. [29] have optimized a protocol for rapid freezing of one-cell mouse embryos to -45°C in the absence of cryoprotectants, using their IIF model. In Chapter 3, the feasibility of using the current model of intracellular nucleation and crystal growth (Chapter 2) for optimization of a freezing protocol for cultured rat hepatocytes in the presence of DMSO was demonstrated. This chapter reports the design and optimization of a freezing protocol for unfertilized mouse oocytes in the presence of DMSO using the theoretical model, and the successful cryopreservation of cells to -196°C using the optimized protocol.
Theoretical Background

Coupled model of intracellular ice crystallization

The coupled theoretical model of intracellular ice crystallization described in Chapter 2 was used here to predict the probability of IIF and the volume of intracellular ice resulting from various freezing protocols. Whereas the original model of ice formation in oocytes included only nucleation by the homogeneous mechanism, the present application requires a comprehensive model of all nucleation mechanisms active in the cell. Thus, two heterogeneous nucleation mechanisms previously observed in mouse oocytes [28], surface-catalyzed nucleation (SCN) and volume-catalyzed nucleation (VCN), were incorporated into the model as described below. Furthermore, since cryopreservation experiments were done in the presence of DMSO as a CPA, the phenomenological viscosity model for H$_2$O-NaCl-DMSO described in Chapter 3 was used here.

The total nucleation rate per cell, $J$, [see Eq. (2-3)] was calculated by adding together the contributions from homogeneous, surface-catalyzed and volume-catalyzed nucleation, i.e.

$$J = J^{\text{HOM}} + J^{\text{SCN}} + J^{\text{VCN}}$$

where

$$J^{\text{HOM}} = \Omega^{\text{HOM}} \exp \left[ -\kappa^{\text{HOM}} \left( \frac{T}{T_m} \right)^{-3} \left( 1 - \frac{T}{T_m} \right)^{-2} \right] V_{CV} \quad (4-1a)$$

$$J^{\text{SCN}} = \Omega^{\text{SCN}} \exp \left[ -\kappa^{\text{SCN}} \left( \frac{T}{T_m} \right)^{-3} \left( 1 - \frac{T}{T_m} \right)^{-2} \right] A \quad (4-1b)$$
\[ J^{\text{VCN}} = \Omega^{\text{VCN}} \exp \left[ -\kappa^{\text{VCN}} \left( \frac{T}{T_m} \right)^{-3} \left( 1 - \frac{T}{T_m} \right)^{-2} \right] V_{cv} ; \]  \hspace{1cm} (4-1c)

\( \Omega \) and \( \kappa \) are the kinetic and thermodynamic coefficients, respectively, for the nucleation mechanism indicated by the superscript. Homogenous and volume-catalyzed nucleation rates are directly proportional to the cytosol volume \( (V_{cv}) \), while the rate of surface-catalyzed nucleation is proportional to the surface area of the cell membrane \( (A) \) [27].

The calculation of the coefficients \( \Omega^{\text{HOM}} \) and \( \kappa^{\text{HOM}} \) for homogeneous nucleation has been described in detail in Chapter 2. The nucleation rate parameters for heterogeneous nucleation was shown to be directly proportional to the corresponding parameters for homogeneous nucleation (see Chapter 3):

\[ \Omega^{\text{SCN}} = \Omega^{\text{SCN}}_{\text{ratio}} \cdot \Omega^{\text{HOM}} \] \hspace{1cm} (4-2a)

\[ \kappa^{\text{SCN}} = \kappa^{\text{SCN}}_{\text{ratio}} \cdot \kappa^{\text{HOM}} \] \hspace{1cm} (4-2b)

\[ \Omega^{\text{VCN}} = \Omega^{\text{VCN}}_{\text{ratio}} \cdot \Omega^{\text{HOM}} \] \hspace{1cm} (4-3a)

\[ \kappa^{\text{VCN}} = \kappa^{\text{VCN}}_{\text{ratio}} \cdot \kappa^{\text{HOM}}. \] \hspace{1cm} (4-3b)

The calculation of \( J \) thus requires knowledge of the constants \( \Omega^{\text{SCN}}_{\text{ratio}} \), \( \Omega^{\text{VCN}}_{\text{ratio}} \), \( \kappa^{\text{SCN}}_{\text{ratio}} \), and \( \kappa^{\text{VCN}}_{\text{ratio}} \), these were measured experimentally as described below.
Knowing the nucleation rate per cell, the probability of IIF (PIF) can be determined assuming a population of identical cells [27]:

$$\text{PIF}(t) = 1 - \exp\left[-\int_0^t J(t) \, dt \right], \quad (4-4)$$

where PIF is defined as the probability that a given cell will contain at least one ice nucleus. For a large population of cells, PIF(t) will be approximately equal to the fraction of cells which have experienced IIF at time $t$.

The volume of intracellular ice was calculated using the diffusion-limited crystal growth model described in Chapter 2, as follows:

$$V^\rho(t) = X^\rho(t)V_{cv}(t), \quad (4-5)$$

where $X^\rho$, the corrected crystallized volume fraction, was calculated using Eq. (2-16), and $V_{cv}$, the cytosol volume, was determined from Eq. (2-1). Eq. (2-16) includes Avrami's correction for hard impingement (direct contact inhibition of crystal growth) [5]; to correct for soft impingement (i.e., inhibition of crystal growth due to water molecule depletion in the unfrozen fraction), the ice volume was assumed to take on its thermodynamic equilibrium value, $V_{eq}^\rho$, whenever Eq. (4-5) yielded values for $V^\rho$ larger than $V_{eq}^\rho$. The equilibrium ice volume at a temperature $T$ is given by
\[ V_{eq} = V_C - n_s \nu_s - n_a \nu_a - (\nu_s n_s + n_a) \nu_o \left( \exp \left[ \frac{\Delta H_f}{R} \left( \frac{1}{T} - \frac{1}{T_{m,c}} \right) \right] - 1 \right)^{-1}. \]  \hspace{1cm} (4-6)

**Freezing protocol optimization**

To maximize cell survival after a freeze-thaw cycle, rates of injury due to all active mechanisms of damage must be minimized. The injury mechanisms acting during cryopreservation are typically grouped into two broad categories: (1) IIF-induced injury (see Chapter 1), and (2) slow-cooling, or "solution effects" injury. The latter mode of damage is thought to be due to high intracellular and extracellular solute concentrations, excessive cell shrinkage, mechanical deformation, or other forces [15], but has not been characterized as well as IIF. Whatever the exact mechanism underlying solution effects damage, the corresponding rate of injury typically decreases with decreasing temperature, and becomes negligible at cryogenic temperatures. Thus, in order to design optimized freezing protocols, two criteria were used in the present study: (1) the time to reach the final temperature should be minimized, to reduce injury by solution effects, and (2) intracellular ice formation should be avoided. The first criterion was used to define the cost to be minimized, the second criterion specified a constraint to the solution. Thus, the cost associated with a freezing protocol \( T(t) \) was defined as the duration of the protocol, described by the functional

\[ \Psi \{ T(t) \} = \int_0^\infty S(T(t) - T_{storage}) dt , \hspace{1cm} (4-7) \]
where $S(x)$ is the unit step function [$S(x) = 1$ for $x \geq 0$; $S(x) = 0$ for $x < 0$], and $T_{\text{storage}} = -196^\circ C$. The "optimal" protocol was defined as the function $T(t)$ which would minimize $\Psi$, subject to the constraint that the cumulative fraction of cells with IIF remain at all times below 5%. This cumulative probability of IIF during freezing was calculated using Eq. (4-4) coupled with Eq. (2-1) and Eqs. (4-1)-(4-3). The freezing protocol optimized here was a piecewise linear cooling protocol consisting of two steps [see Fig. (4-1)]: first, the sample is cooled at a rate $B_{\text{dehydration}}$ from the seeding temperature $T_{\text{seed}}$ to an intermediate temperature $T_{\text{plunge}}$, at which point the sample is immersed directly into liquid nitrogen, represented in Fig. (4-1) by a cooling step from $T_{\text{plunge}}$ to $T_{\text{storage}}$, at a rate $B_{\text{plunge}}$. The rationale for this freezing strategy is presented in the Results. The cooling rate of the initial step ($B_{\text{dehydration}}$), as well as the plunge temperature ($T_{\text{plunge}}$) were the variable parameters in the optimization. The global minimum of the cost functional was determined using a variable-size simplex algorithm [18] in conjunction with Monte Carlo techniques.

**Materials and Methods**

**Media**

The medium for oocyte handling and culture was prepared essentially following previously published procedures [10]: Earle's balanced salt solution (Sigma, St. Louis, MO) was dissolved in freshly distilled tissue culture quality water and then supplemented with 1.85 g/liter (22 mM) sodium lactate (Sigma), 0.035 mg/liter (0.32 mM) sodium pyruvate (Sigma), 0.08 g/liter penicillin G (Calbiochem), and 0.05 g/liter streptomycin sulfate (Calbiochem); the osmolarity of the modified Earle's medium was adjusted to 295 mOsm.
Figure 4-1. Freezing protocol. Cells are first cooled at a rate $B_{\text{dehydration}}$ from the seeding temperature to the plunge temperature ($T_{\text{plunge}}$), then rapidly cooled to liquid nitrogen temperature (-196°C) at a rate $B_{\text{plunge}}$. 
The medium was sterilized by filtration through a 0.22 μm filter (Nalgene, Fisher Scientific, Boston, MA) and was kept refrigerated up to 4 weeks at 4°C. Prior to use, 4 mg/ml bovine serum albumin (BSA, Sigma) was added to the medium. Dulbecco’s phosphate-buffered saline solution (PBS, Sigma) was prepared in freshly distilled tissue culture quality water and supplemented with 4 mg/ml BSA prior to use. Hyaluronidase (Sigma) for cumulus denudation was prepared at a concentration of 120 units/ml in PBS. Earle's medium for oocyte culture was placed in the center well of organ culture dishes (Falcon 3037; Fisher) andoverlayed with 1 ml of silicone oil (Aldrich Chemical Co., Milwaukee, WI). The oil was treated to remove impurities by shaking it for 1 min with Earle's medium (1:4 Earle's to oil). PBS was placed in the organ culture dish moat for humidification, and the dishes were placed in a CO₂ incubator and kept overnight for equilibration under 5% CO₂ in air at 37°C. Earle’s medium for washes and for cryoprotectant solutions was also equilibrated by overnight incubation in 5% CO₂ at 37°C.

Source of oocytes

Freshly ovulated oocytes were obtained from a hybrid-inbred strain (C57B1/6 × DBA) of virgin female B₄D₂F₁ mice, 4 to 10 weeks of age (Charles River, Boston, MA), maintained on a 7 am/7 pm light/dark cycle. Female mice were induced to superovulate by intraperitoneal injection of 7.5 IU of pregnant mare serum gonadotropin (Sigma) in the late afternoon (approximately 5 pm). Approximately 49 to 50 hours later, mice were injected intraperitoneally with 7.5 IU of human chorionic gonadotropin (hCG, Sigma). These females were killed by cervical dislocation 12 to 15 hr after hCG injection for collection of oocytes. Oviducts were excised and placed into Petri dishes (Falcon 3001)
containing a large drop of PBS+BSA at ambient temperature. The cumulus mass was released from each oviduct by puncturing the ampulla with a 27 gauge needle. The cumulus was then enzymatically removed by treatment in hyaluronidase for 5 min at room temperature. The oocytes were washed three times in equilibrated Earle's medium before being transferred into the culture dishes. Any degenerate oocytes (typically 0-10% of the total yield) were discarded.

**Oocyte freezing and viability assay**

**Cryoprotectant loading**

Oocytes were loaded with 1.5 M DMSO by a two-step procedure. Cells were transferred to a drop of 0.75 M DMSO (Aldrich) in Earle's medium and allowed to equilibrate 5 min at room temperature, and then washed in a 1.5 M DMSO solution before being transferred to a drop 1.5 M DMSO and equilibrated for 10 min at room temperature. The drops of cryoprotectant solution were overlaid with silicone oil to reduce changes in the medium pH during equilibration. Some experiments were performed using 0.5 M DMSO; in those cases, oocytes were washed in 0.5 M DMSO, then transferred directly to a drop of 0.5 M DMSO and allowed to equilibrate 5 min at room temperature.

**Freezing**

Oocytes were counted under a Nikon SMZ-2B stereo dissection microscope before being loaded into 0.25 ml plastic straws (TS Scientific, Perkasie, PA) by aspiration with a syringe. Each straw was loaded with 15 mm cryoprotectant solution, 5 mm air, 20 mm cryoprotectant solution, 5-15 oocytes, and an additional 20 mm of cryoprotectant
solution, so that the oocytes were approximately in the center of a contiguous 40 mm length of cryoprotectant solution. After successive aspirations were completed, the straws were rapidly sealed and placed horizontally in a Planer Kryo 10 Series II programmable freezer (TS Scientific) which had been precooled to 0°C. Straws were cooled from 0°C to -5°C at a rate of 10°C/min, and held at -5°C for 5 min to allow samples to equilibrate thermally before seeding of extracellular ice by touching the straws with metal forceps precooled in liquid N₂. Oocytes frozen in 0.5 M DMSO were seeded at -4°C. At 15 min after seeding, straws were cooled at a rate between 0.05°C/min and 3°C/min to a given plunge temperature between -30°C and -150°C; upon reaching its designated plunge temperature, each straw was immediately removed from the freezer and plunged directly into a dewar containing liquid nitrogen. Straws were kept in liquid nitrogen for at least 5 min before thawing.

**Thawing and cryoprotectant dilution**

Oocytes were thawed by reintroducing the straws into the freezer preset to a temperature of -80°C and then warming to +4°C at 8°C/min. At +4°C, straws were removed from the freezer, and their contents expelled into drops of room temperature 1.5 M DMSO under oil. DMSO was then removed from the oocytes by a two-step dilution: oocytes were first transferred to drops of 0.75 M DMSO under oil and equilibrated for 10 min at room temperature, and then washed once in Earle’s medium at room temperature before transfer to a culture dish with 1 ml Earle’s medium under oil and incubation for one hour under 5% CO₂ in air at 37°C. The isotonic medium used in this final wash and equilibration step contained a fluorescent dye used for assaying cell viability as described below.
Viability assay

The viability of frozen-thawed oocytes was evaluated by assessing membrane integrity and morphological appearance at one hour and 24 hours after thawing and cryoprotectant removal. Membrane integrity was assessed using two fluorescent dyes, calcein AM (Molecular Probes, Eugene, OR), a fluorogenic esterase substrate which stains the cytoplasm of cells with intact membranes, but leaks rapidly from cells with compromised membrane integrity, and ethidium homodimer 1 (Molecular Probes), which stains DNA in cells with damaged membranes, but is impermeant to intact cell membranes. A staining medium was prepared from equilibrated Earle's medium, with calcein AM at a concentration of 0.3 μM, and ethidium homodimer 1 at a concentration of 2 μM. Oocytes were washed once in the staining medium and then incubated at 37°C in a 5% CO₂ incubator for one hour in 1 ml of staining medium under oil. This stain loading procedure also served as the last dilution step for cryoprotectant removal, in order to minimize oocyte handling and thus reduce the number of oocytes lost or damaged during transfers. After one hour incubation, oocytes were counted and examined for fluorescent staining and normal morphological appearance under a microscope with epifluorescence and phase contrast optics, respectively. The culture dishes were then returned to the CO₂ incubator, and reexamined at 24 hours post cryoprotectant removal. In some damaged oocytes, large fragments of the cytoplasm had leaked out of the zona pellucida; to avoid double-counting these when determining the number of cells recovered, only zonae were counted, whether or not they contained cytoplasmic material. Vitelline membranes were scored as intact if the cytoplasm exhibited yellow-green fluorescence (calcein AM emission wavelength) and did not exhibit fluorescent emission in the red wavelengths.
(ethidium homodimer 1); oocytes which either stained red or did not stain at all (i.e., zona "ghosts" devoid of protoplasm) were scored as non-intact. Oocytes were considered morphologically abnormal if they exhibited a broken zona pellucida, ruptured vitelline membrane, unusual polar body, or a shrunken, expanded or granular vitellus.

Cryomicroscopy
The cryomicroscope system used in this study has been described in detail elsewhere [6]. This experimental apparatus allows video-microscopic observation of samples cooled and warmed at controlled rates. In the present system, the Thermascope (Interface Techniques, Cambridge, MA) programmable thermal microscope stage was used in conjunction with a Nikon Optiphot-2 microscope. Video-microscopic images were obtained using a Hamamatsu C2400 video camera with a Sony SVO-9500MD video recorder and a Sony PVM-122 black and white video monitor. Video images were automatically annotated with time and temperature data using the Datavideo system (Interface Techniques).

For experiments to measure IIF kinetics, 10 to 20 oocytes which had been loaded with 1.5 M DMSO were placed on the cryomicroscope stage under a coverslip and sealed with silicone grease. The initiation of sample freezing was achieved by slightly supercooling the solution and manually triggering ice growth by contacting the edge of the sample with the tip of a chilled forceps. Controlled cooling, at a rate of 50°C/min or 120°C/min, was initiated as soon as the growing ice front had engulfed each oocyte in the field of view. Depending on the distribution of the oocytes on the freezing stage, 10 to 20 oocytes could
be observed at a time with a 4x objective. Brightfield illumination was used to allow for an easier visual determination of IIF. IIF is manifested by a sudden darkening of the cytoplasm, believed to be caused by light scattering due to microscopic ice crystals and/or air bubbles in the cells. Examination of video recordings of the freezing experiments permitted the determination of the IIF temperature for each cell. At the end of each freezing experiment, the thermal gradients across the sample window were determined by recording the location of the ice front at several temperatures close to the melting point of the solution. Using these data, the instrument temperature reading could be corrected to estimate the actual temperature at the location of each individual oocyte.

In one set of cryomicroscopy experiments, cooling at 50°C/min was interrupted at -10°C and the sample held for 0, 5, 10, or 30 min at that temperature before continuing cooling to -60°C at 50°C/min, in order to investigate the effect of dehydration on the nucleation kinetics.

**Determination of nucleation rate parameters**

To determine the heterogenous nucleation rate parameters $\Omega_{ratio}^{SCN}$, $\Omega_{ratio}^{VCN}$, $\kappa_{ratio}^{SCN}$, and $\kappa_{ratio}^{VCN}$ for mouse oocytes in 1.5 M DMSO, IIF kinetics were measured during cooling to -60°C at a rate of 120°C/min, using the cryomicroscopy techniques described above. The cumulative fraction of cells with intracellular ice at a given temperature provided a measure of the probability of IIF for cooling to that temperature. The nucleation rate parameters were determined from the experimental data and Eqs. (4-1)–(4-4) by fitting Eq. (4-4) to the measured probability of IIF. Briefly, a simplex algorithm was used to
determine the combination of values for the nucleation rate coefficients which minimized
the variance ($\chi^2$) between the experimental and predicted PIF data. Precision in the
determination of nucleation rate parameters is maximized if the effect of water transport
on the intracellular ice nucleation is minimized. This was achieved by cooling the oocytes
at a rate sufficiently rapid (120°C/min) to impede water efflux, effectively decoupling the
water transport equation [Eq. (2-1)] from the ice nucleation equations [Eqs. (4-1)–(4-4)].

Results

IIF kinetics

Fig. 4-2 shows the cumulative fraction of cells with IIF as a function of temperature for
mouse oocytes cooled at a constant cooling rate of 120°C/min, in the presence of 1.5 M
DMSO. Under these conditions, IIF was observed at temperatures as high as $\sim$13°C;
50% of the oocytes had undergone IIF at $\sim$38°C, and all cells were frozen internally at
-42°C. The sharp breakpoint in the IIF kinetics observed at $\sim$36°C indicates the
involvement of two distinct mechanisms of nucleation [17,28]; these have been attributed
to SCN (active at high temperatures) and VCN (active at low temperatures) [27]. To
determine the nucleation rate parameters for the SCN mechanism, IIF data for
temperatures higher than -34°C only were used (open symbols in Fig. 4-2), obtaining the
best fit between model predictions and experimental data for $\Omega_{\text{ratio}}^{\text{SCN}} = 3.75 \times 10^{-47} \text{ m}$ and
$\kappa_{\text{ratio}}^{\text{SCN}} = 2.36 \times 10^{-3}$, with a goodness of fit $\chi^2 = 1.1 \times 10^{-4}$. The VCN nucleation
parameters were then determined using IIF data at temperatures below -34°C (closed
symbols in Fig. 4-2), and subtracting the predicted contribution from the SCN mechanism
to the cumulative incidence of IIF at lower temperatures (dashed line in Fig. 4-2). The
Figure 4-2. Nonlinear curve-fit to determine the heterogeneous nucleation rate parameters for mouse oocytes in 1.5 M DMSO. The cumulative incidence of IIF was measured at a cooling rate of 120°C/min. Open symbols indicate IIF by the SCN mechanism; closed symbols represent VCN. The dashed curve shows model predictions for SCN only; the solid line is the theoretical prediction including both SCN and VCN.
resulting best fit parameters were $\Omega_{\text{ratio}}^{\text{VCN}} = 2.16 \times 10^{-27}$ and $\kappa_{\text{ratio}}^{\text{VCN}} = 0.288$, with $\chi^2 = 1.5 \times 10^{-4}$. The heterogeneous nucleation parameters determined from this experiment were used in all subsequent model simulations.

Fig. 4-3 shows the effect of interrupting rapid cooling (50°C/min) by inserting an isothermal holding step at -10°C on the cumulative fraction of IIF in mouse oocytes cooled in the presence of 1.5 M DMSO. Oocytes were held at -10°C for 0, 5, 10, or 30 min. Also shown in Fig. 4-3 are theoretical predictions corresponding to each experimental condition tested. Both experiments and model predictions showed a depression of the onset of VCN as the isothermal hold time was increased; however, IIF kinetics were not altered appreciably for dehydration times beyond 10 min. The SCN mechanism also became less effective as the hold time was increased from 5 min to 10 or 30 min. Curiously, when cooling was allowed to progress at 50°C/min without holding at -10°C, the SCN mechanism appeared to be almost totally absent. While correspondence between model predictions and experimental observations was satisfactory for SCN when the hold time was 5 min or longer, theoretical predictions of VCN were in excellent agreement with experimental data. This is illustrated in Fig. 4-4, which shows the experimental and theoretical median IIF temperatures for VCN. The theoretical curve was obtained by setting $\mathcal{J}_{\text{SCN}} = 0$ when solving the nucleation equations, and the experimental median temperatures for VCN were determined from Fig. 4-3, by assuming that all cells undergoing IIF after the breakpoint temperature nucleate by VCN (SCN kinetics can be neglected because they are much slower than VCN kinetics in this regime). As is evident
Figure 4-3. Effect of an isothermal hold step at $-10^\circ$C on the cumulative incidence of IIF during subsequent rapid cooling. The cooling rate to and from $-10^\circ$C was $50^\circ$C/min; cells were held isothermally at $-10^\circ$C for 0 min (open squares), 5 min (closed squares), 10 min (open circles), or 30 min (closed circles). Lines represent model predictions for each condition.
Figure 4-4. Effect of an isothermal hold step at -10°C on the median temperature of IIF (VCN only) during subsequent rapid cooling. The cooling rate to and from -10°C was 50°C/min. Closed circles are experimental data; the solid line represents model predictions.
from Fig. 4-4, agreement between model predictions and experimental measurements was excellent for the VCN mechanism.

**Design and optimization of freezing protocol**

As demonstrated in Chapter 2, the probability of IIF and the final volume of ice in a cell can be reduced significantly by using slow cooling rates to dehydrate the cell and increase the intracellular solute concentrations. Fig. 4-5 shows the effect of the dehydration cooling rate on the predicted trajectory of the thermodynamic state of the cytosol in its state-space; shown on the same graph is the non-equilibrium phase diagram of the intracellular solution. A common feature of the state-space trajectories for all cooling rates shown in Fig. 4-5 is the cessation of dehydration at low temperatures (indicated by the invariance in intracellular DMSO concentration). This is due to the decreased membrane permeability to water at low temperatures, which is also illustrated in Fig. 4-6, showing the characteristic time for cell dehydration as a function of temperature. Because water transport effectively ceases at low temperatures, there is a finite range of temperatures in which the state of dehydration of the cell can be controlled; e.g., only at temperatures above \(-52^\circ\text{C}\) is the characteristic dehydration time-scale is less than 1 hr (line marked ‘\(t_{\text{dehydration}}=1\text{ hr}\)’ in Fig. 4-5). For lower temperatures, dehydration times become prohibitively long, and the concentration of the cytosol becomes increasingly more difficult to alter. Thus, there is no benefit in employing slow cooling rates as the final storage temperature is approached; on the contrary, the faster the final cooling rate, the lower the cost [Eq. (4-7)] and the the less intracellular crystallization occurs (see Chapter 2). The curve marked ‘\(T_{\text{sec}}\)’ is a representative temperature at which nucleation
Figure 4-5. Thermodynamic state space for the intracellular solution, with non-equilibrium phase diagram. ‘T_m’ is the liquidus; ‘T_{nuc}’ is the intracellular nucleation temperature. Dashed lines represent predicted state space trajectories for mouse oocytes cooled at rates of 0.05, 0.5, 5, and 50°C/min. The critical concentration c_{crit} is the minimum DMSO concentration at which the intracellular solution can be vitrified. The line labelled ‘τ_{dehydration}=1 hr’ represents the temperature at which the characteristic timescale for water transport is 1 hour.
Figure 4-6. Predicted temperature-dependence of the characteristic time-scale for water transport in mouse oocytes. The dehydration time is the time necessary for the cell to shrink 50% at the given temperature.
occurs in cells (predicted median IIF temperature for cells cooled at a rate of 300°C/min); state-space trajectories which cross this curve result in IIF. Thus, oocytes cooled at 50°C/min and 5°C/min undergo IIF, while those cooled at 0.5°C/min and 0.05°C/min do not; only cells sufficiently dehydrated to reach an intracellular DMSO concentration greater than the critical DMSO concentration, at which the nucleation temperature is depressed to below the glass transition temperature \( c_{\text{crit}} \approx 10 \text{ M} \) in Fig. 4-5), can be cooled to liquid nitrogen temperature without undergoing IIF. Thus, the cryopreservation strategy employed here was to use a two-step protocol (see Fig. 4-1), consisting of a dehydration step (cooling rate \( B_{\text{dehydration}} \)) to achieve the critical intracellular DMSO concentration which will prevent IIF, followed by rapid cooling from \( T_{\text{plunge}} \) to \( T_{\text{storage}} \) at the highest experimentally attainable rate \( B_{\text{plunge}} \).

Figs. 4-7 through 4-9 show the sensitivity of the final cumulative fraction of cells with IIF and the final intracellular ice volume to the value of the plunge cooling rate and to the extent of cell dehydration before plunging. In Fig. 4-7, the final fraction of oocytes with internal ice is graphed as a function of the intracellular DMSO concentration at \( T_{\text{plunge}} \) for two plunge cooling rates, \( B_{\text{plunge}} = 10^2 \text{ °C/min} \) and \( B_{\text{plunge}} = 10^4 \text{ °C/min} \). At low DMSO concentrations, all cells contain intracellular ice nuclei; as the intracellular DMSO concentration at \( T_{\text{plunge}} \) increases beyond a critical value \( c_{\text{crit}} \), the fraction of cells with IIF drops rapidly to 0%. The critical DMSO concentration decreases with increasing plunge cooling rate. Similarly, Fig. 4-8 shows the final intracellular ice volume (normalized to the water volume in an oocyte under isotonic conditions) as a function of the DMSO concentration at \( T_{\text{plunge}} \) for the same two plunge cooling rates; also plotted is the volume of
Figure 4-7. Effect of the intracellular DMSO concentration and cooling rate during plunge on the predicted final fraction of oocytes with internal ice.
Figure 4-8. Effect of the intracellular DMSO concentration and cooling rate during plunge on the predicted final volume of intracellular ice (normalized to the isotonic water content, $V_{\text{iso}} = 2.06 \times 10^{-13} \text{ m}^3$). The dotted line indicates the maximum volume of water freezeable at each DMSO concentration, i.e., the ice volume that would be in thermodynamic equilibrium with the unfrozen cytosol fraction at -196°C.
Figure 4-9. Effect of the intracellular DMSO concentration and cooling rate during plunge on the predicted final fraction of oocytes with internal ice (PIF) and the final normalized intracellular ice volume ($V_i$). Shown are contour lines of constant PIF (solid lines) and constant ice volume (dotted lines). The non-shaded region indicates the order of magnitude of the plunge cooling rate for the present experimental system.
freezeable water in the cell for each concentration. For low DMSO concentrations, all freezeable water in the cells is transformed to ice (~10% of the ismic water volume), while at higher DMSO concentrations there is a sharp reduction in the amount of intracellular ice. When the volume fraction of ice in the cytosol decreases to below $10^{-6}$, the intracellular solution is vitrified; as the plunge cooling rate is increased, a lower DMSO concentration is necessary for vitrification. Fig. 4-9 summarizes the data in Figs. 4-7 and 4-8, showing the final fraction of cells with intracellular ice nuclei (contour lines marked 'PIF') and the final normalized ice volume (contour lines marked $V^q$) as functions of the intracellular DMSO concentration and cooling rate during the plunge step. As can be seen, the higher the plunge cooling rate, the lower the DMSO concentration required to achieve low probabilities of PIF and small intracellular ice volumes. However, for any given plunge cooling rate, the transition in PIF from 100% to 0% occurs at higher DMSO concentrations than the transition in $V^q$ from 1 to 0. Thus, inhibition of crystal growth (vitrification) requires lesser levels of cell dehydration than suppression of intracellular nucleation. Intracellular ice nuclei are undesirable even if the extent of crystal growth during freezing is negligible, because these growth centers can cause damaging levels of intracellular crystallization as devitrification occurs during warming. Thus, a threshold value of PIF≤5% was used in the freezing protocol optimization. The value of $B_{\text{plunge}}$ for the present experimental system is estimated to be on the order of 100 to 1000°C/min (non-shaded region in Fig. 4-9), and was assumed to be $B_{\text{plunge}} = 300°C/min$ for purposes
of calculating the probability of IIF during freezing. Fig. 4-9 shows that the final fraction of IIF is relatively insensitive to the exact value of $B_{\text{plunge}}$.

Given the plunge cooling rate, both the final extent of IIF and the protocol duration are determined by the choice of dehydration cooling rate ($B_{\text{dehydration}}$) and plunge temperature ($T_{\text{plunge}}$). Fig. 4-10 shows the effect of the dehydration cooling rate on the final fraction of oocytes with IIF for two plunge temperatures, $T_{\text{plunge}} = -60^\circ\text{C}$ and $T_{\text{plunge}} = -80^\circ\text{C}$. When plunging at $-60^\circ\text{C}$, a dehydration cooling rate of 0.8°C/min results in 50% cumulative incidence of IIF. For a plunge temperature of $-80^\circ\text{C}$, the probability of IIF is 50% for a dehydration cooling rate of 1.1°C/min. For a dehydration cooling rate of 0.9°C/min, plunging at $-60^\circ\text{C}$ results in ~80% IIF, while plunging at $-80^\circ\text{C}$ results in ~20% IIF. In the theoretical protocol optimization, the criterion that the final fraction of cells with IIF remain below a 5% threshold level defined the domain of permissible combinations of $B_{\text{dehydration}}$ and $T_{\text{plunge}}$. Within this domain, the parameter combination which minimized the cost functional $\Psi$ [Eq. (4-7)] was determined using a simplex algorithm [18]. The optimal combination of freezing protocol parameters for mouse oocytes frozen in the presence of 1.5 M DMSO was thus predicted to be a dehydration cooling rate of 0.59°C/min with a plunge temperature of $-67^\circ\text{C}$.

**Viability measurements**

**Test of predicted optimal protocol**

Cell survival was experimentally measured in the neighborhood of the predicted optimum in order to verify model predictions and to establish the sensitivity of the freezing outcome
Figure 4-10. Effect of the dehydration cooling rate and the plunge temperature on the predicted final fraction of oocytes with internal ice. The plunge cooling rate was 300°C/min.
to the protocol parameters cooling rate and plunge temperature. Due to limitations of the experimental system, the set of freezing parameters closest to the predicted optimum that could be reliably achieved was a cooling rate of 0.5°C/min and a plunge temperature of -70°C. Of 30 oocytes frozen under those conditions, all were recovered after thawing and cryoprotectant removal, and 47±20% were intact and morphologically normal after incubation for one hour. After incubation for 24 hours, 43±17% were morphologically normal. To further test the model in the vicinity of this set of protocol parameters, $T_{\text{plunge}}$ and $B_{\text{dehydration}}$ were varied independently and the resulting cell viabilities were compared with predictions of cell damage due to IIF.

Effect of plunge temperature

The effect of varying the plunge temperature with a constant dehydration cooling rate of 0.5°C/min is shown in Table 4-1. For each plunge temperature, data from 2 experiments were pooled. Cell survival was found to decrease with increasing plunge temperature; no intact cells were recovered for plunge temperatures higher than -60°C. The maximum morphological survival rate at 1 hour post-thaw (82% survival of the total number of oocytes frozen) was achieved with a plunge temperature of -80°C. There was little or no variation between the morphological and membrane integrity assays for cell survival, or between evaluations at 1 hour and 24 hours post-thaw. The morphological survival rate at 1 hour post-thaw is graphed versus plunge temperature in Fig. 4-11, together with theoretical predictions for the fraction of cells containing no ice nuclei, i.e., $1 \cdot \text{PIF}$ [Eq. (4-4)]. The model predicts a sharp increase in the fraction of IIF-free cells (and hence, presumably, survival) as the plunge temperature is lowered from -50°C to -60°C,
Table 4-1. Effect of varying plunge temperature on oocyte viability. Dehydration cooling rate was constant at 0.5°C/min.

<table>
<thead>
<tr>
<th>$T_{plunge}$ (°C)</th>
<th>Repeats</th>
<th>Frozen (%)</th>
<th>Recovered (%)</th>
<th>Intact at 1 h (%)</th>
<th>Normal Morphology at 1 h (%)</th>
<th>Intact at 24 h (%)</th>
<th>Normal Morphology at 24 h (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-40</td>
<td>2</td>
<td>21</td>
<td>100.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>-50</td>
<td>2</td>
<td>29</td>
<td>96.4 ± 3.6</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>-60</td>
<td>2</td>
<td>30</td>
<td>80.0 ± 6.7</td>
<td>13.3 ± 0.0</td>
<td>13.3 ± 0.0</td>
<td>13.3 ± 0.0</td>
<td>13.3 ± 0.0</td>
</tr>
<tr>
<td>-70</td>
<td>2</td>
<td>30</td>
<td>100.0 ± 0.0</td>
<td>46.7 ± 20.0</td>
<td>46.7 ± 20.0</td>
<td>46.7 ± 20.0</td>
<td>43.3 ± 16.7</td>
</tr>
<tr>
<td>-80</td>
<td>2</td>
<td>22</td>
<td>100.0 ± 0.0</td>
<td>81.8 ± 0.0</td>
<td>81.8 ± 0.0</td>
<td>81.8 ± 0.0</td>
<td>77.3 ± 4.5</td>
</tr>
<tr>
<td>-120</td>
<td>2</td>
<td>22</td>
<td>100.0 ± 0.0</td>
<td>77.3 ± 4.5</td>
<td>77.3 ± 4.5</td>
<td>77.3 ± 4.5</td>
<td>77.3 ± 4.5</td>
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</tbody>
</table>

153
Figure 4-11. Effect of plunge temperature on viability of mouse oocytes frozen at a dehydration cooling rate of 0.5°C/min. Experimental data (dashed line) is morphological survival rate 1 hour post-thaw, mean and standard error. Theoretical data (solid line) is fraction of cells with no intracellular ice.
then a "plateau" region in which the predicted fraction of cells without IIF remains above 90%, decreasing only slightly with decreasing plunge temperature; as the plunge temperature is lowered beyond \(\sim -110^\circ\text{C}\), a sharp drop-off in survival is predicted, due to an increase in PIF. Although the predicted transition region at high plunge temperatures was observed experimentally at a somewhat lower temperature (\(\sim -70^\circ\text{C}\)) than that predicted by the model (\(\sim -55^\circ\text{C}\)), the overall model predictions are in good qualitative agreement with the corresponding experimental data.

**Effect of cooling rate**

The effect of varying the dehydration cooling rate with the plunge temperature fixed at the experimental optimum value \(T_{\text{plunge}} = -80^\circ\text{C}\) was investigated next. Table 4-2 shows the results pooled from 2-3 experiments at each condition. Maximum survival (86% morphologically normal 1 hour post-thaw) was obtained with a cooling rate of 0.3°C/min, although the difference between this value and the 82% morphological survival rate at 0.5°C/min was not statistically significant \(p > 0.70\). At higher dehydration cooling rates, cell viability decreased; no cells survived when cooled at a rate of 2°C/min. Also, a statistically significant reduction in the 1-hour post-thaw morphological survival rate was observed when the dehydration cooling rate was decreased from 0.3°C/min to 0.1°C/min \(p < 0.20\). In Fig. 4-12, the experimental viability data (morphological survival 1 hour post-thaw) is compared with theoretical predictions of the probability of avoiding IIF. The predicted fraction of cells without intracellular ice remains above 90% for cooling rates less than \(\sim -0.8^\circ\text{C/min}\), decreasing only slightly with increasing cooling rates. However, at a dehydration cooling rate of \(\sim 1^\circ\text{C/min}\), 50% of all oocytes are predicted to
Table 4-2. Effect of varying dehydration cooling rate on oocyte viability. Plunge temperature was constant at 0.5°C/min.

<table>
<thead>
<tr>
<th>$B_{\text{dehydration}}$ ($^\circ$C/min)</th>
<th>Repeats</th>
<th>Frozen (%)</th>
<th>Recovered (%)</th>
<th>Intact at 1 h (%)</th>
<th>Normal Morphology at 1 h (%)</th>
<th>Intact at 24 h (%)</th>
<th>Normal Morphology at 24 h (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>3</td>
<td>60</td>
<td>86.7 ± 6.0</td>
<td>55.0 ± 16.1</td>
<td>55.0 ± 16.1</td>
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<td>53.3 ± 15.9</td>
</tr>
<tr>
<td>0.3</td>
<td>3</td>
<td>36</td>
<td>100.0 ± 0.0</td>
<td>88.9 ± 11.1</td>
<td>66.1 ± 10.0</td>
<td>88.9 ± 11.1</td>
<td>86.1 ± 10.0</td>
</tr>
<tr>
<td>0.5</td>
<td>2</td>
<td>22</td>
<td>100.0 ± 0.0</td>
<td>81.8 ± 0.0</td>
<td>81.8 ± 0.0</td>
<td>81.8 ± 0.0</td>
<td>77.3 ± 4.5</td>
</tr>
<tr>
<td>1.0</td>
<td>2</td>
<td>21</td>
<td>95.5 ± 4.5</td>
<td>20.0 ± 20.0</td>
<td>20.0 ± 20.0</td>
<td>15.0 ± 15.0</td>
<td>15.0 ± 15.0</td>
</tr>
<tr>
<td>2.0</td>
<td>3</td>
<td>29</td>
<td>100.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
</tbody>
</table>
Figure 4-12. Effect of dehydration cooling rate on viability of mouse oocytes plunged into liquid nitrogen at -80°C. Experimental data (dashed line) is morphological survival rate 1 hour post-thaw, mean and standard error. Theoretical data (solid line) is fraction of cells with no intracellular ice.
contain intracellular ice nuclei, and at ~1.3°C/min, the model predicts that no cells are ice-
free. This predicted transition corresponds well with the experimentally observed drop-off
in survival at high cooling rates, even though the cooling rates at which IIF becomes
significant are slightly overestimated by the theoretical model. The experimentally
observed reduction in survival at 0.1°C/min is not predicted by the IIF model, as cell injury
in at this cooling rate is presumably due to solution effects, and not intracellular
crystallization.

Fig. 4-13 summarizes both the theoretical optimization and the experimental viability data,
showing the protocol parameter space \((B_{\text{dehydration}}, T_{\text{plunge}})\). Graphed are contours of
constant cost \((\Psi=3, 4, 5, \text{ and 15 hours are marked})\), indicating the shape of the cost
surface. The point on the cost surface corresponding to the theoretical optimum is
marked. The curve labelled ‘PIF=5%’ marks the constraint used to restrict the permissible
solution space in the theoretical optimization. Protocols falling outside this demarcation
result in predicted levels of IIF greater than 5%. Although the true experimental optimum
was not established in this study, local optima for fixed \(B_{\text{dehydration}}\) or fixed \(T_{\text{plunge}}\) were
determined (Figs. 4-11 and 4-12); these should correspond to locations on the “ridge” of
the cost surface (i.e., the line ‘PIF=5%’ on the theoretical surface). The optima obtained
at fixed \(B_{\text{dehydration}}=0.5°C/\text{min}\) and \(T_{\text{plunge}}=-80°C\) coincide, and are indicated in Fig. 4-13 by
the point marked ‘82%’ (the experimentally obtained survival rate). Also plotted is a
point marked ‘69%’, representing experiments at fixed \(T_{\text{plunge}}=-60°C\) (not reported above).
As can be inferred from the locations of the two experimental points on the cost surface,
Figure 4-13. Shape of the cost surface in protocol parameter space. Thin lines are contours of constant cost (protocol duration); heavy line is a predicted contour of constant PIF (=5%), representing the constraint on the solution space for optimization. Open diamond symbol is the theoretical cost optimum. Closed squares are experimental local optima, see text for details.
the lesser survival rate at the optimum for \( T_{\text{plunge}} = -60^\circ \text{C} \) correlates with the higher cost (i.e., longer duration) of that protocol.

Fig. 4-14 further demonstrates the correlation between measured oocyte viability (morphological survival 1 hour post-thaw) and the freezing protocol duration (i.e., the cost \( \Psi \)), a putative measure of solution effects injury. Fig. 4-14 is a summary of all experiments reported here for oocyte freezing in 1.5 M DMSO, and also includes data obtained in experiments using 0.5 M DMSO. There is a drop in survival for rapid freezing protocols (duration < \( \sim 200 \) min), which is expected due to increased likelihood of IIF in this regime (data not shown). For longer freezing protocols, a strong negative correlation between measured cell viability and protocol duration was observed; for these data, less than 5% IIF was predicted to occur, thus suggesting that cell injury in this regime was due to solution effects. Solution effects were observed to be more pronounced when the cryoprotectant concentration was reduced to 0.5 M DMSO; at this concentration the maximum survival rate obtained was \( \sim 20\% \), and no viable cells were recovered for a 5-hour protocol. When using 1.5 M DMSO, cell survival would be significant (\( \sim 80\% \)) for a 5-hour protocol; for total loss of cell viability, a 24-hour protocol would be required (by extrapolation of the trend in Fig. 4-14).

**Discussion**

This study represents the first time that mechanistic models have been successfully employed to design and optimize a cryopreservation protocol for reversibly freezing cells to \( -196^\circ \text{C} \). The technique allowed rapid identification of the optimal protocol using a
Figure 4-14. Correlation of measured oocyte viability (morphological survival rate, 1 hour post-thaw) with duration of freezing protocol. Circles represent oocytes frozen in the presence of 1.5 M DMSO; squares represent oocytes frozen in the presence of 0.5 M DMSO. Open symbols represent experiments in which the predicted cumulative incidence of IIF was greater than 5%.
small number of experiments, a significant improvement in efficiency over the experimental optimization methods currently used. The theoretical optimization approach used in the present work should be applicable to any cell type for which the relevant biophysical parameters have been measured.

The nucleation rate coefficients obtained in the present study are the first such measurements reported for mouse oocytes frozen in the presence of a cryoprotectant. Converting the values of $\Omega_{\text{ratio}}^{\text{SCN}}$, $\Omega_{\text{ratio}}^{\text{VCN}}$, $\kappa_{\text{ratio}}^{\text{SCN}}$, and $\kappa_{\text{ratio}}^{\text{VCN}}$ determined here to the units used by Toner et al. [27] for purposes of comparison, one obtains for the SCN mechanism $\Omega_{\omega}^{\text{SCN}} = 3.92 \times 10^6 \text{ m}^2 \text{s}^{-1}$, and $\kappa_{\omega}^{\text{SCN}} = 3.59 \times 10^9 \text{ K}^3$, and for VCN $\Omega_{\omega}^{\text{VCN}} = 2.26 \times 10^{26} \text{ m}^3 \text{s}^{-1}$, and $\kappa_{\omega}^{\text{VCN}} = 4.38 \times 10^{11} \text{ K}^3$. Toner et al. [27] measured values of $\Omega_{\omega}^{\text{SCN}} = 3.56 \times 10^8 \text{ m}^2 \text{s}^{-1}$ and $\kappa_{\omega}^{\text{SCN}} = 4.60 \times 10^9 \text{ K}^3$ for mouse oocytes frozen in an isotonic solution, i.e. the thermodynamic coefficient ($\kappa$) was only slightly affected by the presence of 1.5 M DMSO, while the kinetic coefficient ($\Omega$) was reduced by two orders of magnitude when the cryoprotectant was used. Nucleation rate parameters for VCN were determined by Toner et al. [27] for oocytes frozen in a hypertonic (1035 mOsm) solution; they obtained $\Omega_{\omega}^{\text{VCN}} = 1.84 \times 10^{40} \text{ m}^3 \text{s}^{-1}$ and $\kappa_{\omega}^{\text{VCN}} = 1.08 \times 10^{12} \text{ K}^3$. Compared with the values measured here for oocytes in 1.5 M DMSO, the kinetic coefficient was significantly reduced (by a factor of $10^{24}$), while the thermodynamic coefficient was reduced by a factor of $\approx 2$ in the presence of DMSO. Although the effect on the thermodynamic coefficient was not as large in magnitude as the reduction in the pre-exponential parameter, II F kinetics are very sensitive to the coefficient $\kappa$ (see Chapter 2); and the increased value of
this parameter for oocytes in 1.5 M DMSO results in a depression of the median IIF temperature of ~5°C compared to experiments using 1035 mOsm PBS.

Direct measurement of IIF kinetics during rapid cooling with an intervening isothermal hold step allowed verification of the theoretical model predictions. Using a constant isothermal hold temperature (-10°C), and varying only the duration of holding, the extent of dehydration of the cells could be precisely regulated. By choosing a long holding time (30 min), cells could be completely equilibrated with the external environment at -10°C before rapid cooling was resumed, i.e., the final cytosol volume and intracellular solute concentrations were determined only by the temperature of the isothermal hold, effectively decoupling the water transport equation [Eq. (2-1)] from the model. Thus, equilibrium dehydration allowed an independent test of the heterogeneous nucleation model under conditions different from those used for nucleation rate parameter determination (the DMSO concentration after equilibration at -10°C is 4 M). Then, the water transport model and the coupling between water transport and IIF were tested by using shorter isothermal holding times (0, 5, 10 min), which would result in non-equilibrium conditions at the end of the hold. The excellent agreement obtained between experimental observations and theoretical predictions of VCN for all dehydration conditions (Figs. 4-3 and 4-4) indicates that both the water transport model and the VCN nucleation model are accurate. Reasonable agreement was also obtained between measured and predicted kinetics of IIF by the SCN mechanism for isothermal holding times ≥5 min. Because SCN kinetics during rapid cooling are sensitive to the exact temperature of the preceding isothermal hold (data not shown), deviations in the actual (corrected) temperatures
experienced by the oocytes from the nominal value of -10°C would contribute to the discrepancy between the measured and predicted PIF data (standard deviations in the corrected hold temperatures ranged between 1.0 and 1.7°C). Also, the data used to determine the nucleation rate parameters (Fig. 4-2) were dominated by the VCN mechanism (~65% of the cells nucleated by VCN), thus favoring a better measurement of the VCN parameters than the SCN parameters. However, comparing the experimental IIF kinetics for oocytes cooled at 120°C/min with no isothermal hold (Fig. 4-2) with those for oocytes cooled at 50°C/min with no isothermal hold (Fig. 4-3), it is evident that the SCN mechanism is less pronounced at the slower cooling rate: at 120°C/min, approximately 35% of the oocytes experienced IIF with kinetics consistent with SCN, while at 50°C/min, only ~10% of all oocytes were observed to experience SCN. Because both of these cooling rates are sufficiently rapid to impede water transport, thus precluding depression of IIF kinetics due to cell dehydration, and whereas SCN kinetics are sensitive to the rate of cooling (see Chapter 1), an increase in the incidence of SCN would be expected at the lower cooling rate, in conflict with the experimental observation. Similarly, the data for cooling at 50°C/min with no hold do not conform to the experimentally observed and theoretically predicted trend of increased probability of SCN with decreasing duration of the isothermal hold (Fig. 4-3). Both of these observations were unexpected, and merit further investigation. One possible explanation lies in the fact that SCN, which is hypothesized to arise from interactions of the external ice with the cell membrane, is dependent not only on the thermodynamic state of the intracellular solution (as is the case for VCN), but also the extracellular environment. Thus, effects of the cooling rate on the
extracellular environment (e.g. the morphology of the external ice crystals, or their distance from the cell membrane), may account for the anomalous experimental observations. Nonetheless, nucleation by the SCN mechanism was observed to become ineffective when oocytes were dehydrated, and thus, for the purpose of designing a freezing protocol for oocytes, VCN is the more important mechanism.

The mechanistic model of intracellular ice crystallization developed in Chapter 2 was used here to design and theoretically optimize a simple two-step freezing protocol for mouse oocytes. Using the theoretical predictions as a guide for experiments, it was possible to rapidly find a combination of protocol parameters which resulted in recovering over 80% morphologically normal oocytes post-thaw. These cells remained intact and with a normal morphological appearance for at least 24 hours, indicating that no deleterious metabolic lesions had been incurred. This result is comparable to the highest survival rates reported for mouse oocytes in the literature (see Table 4-3). Direct comparison of the success of freezing protocols from different studies is often difficult or impossible due to confounding variables such as differing culture techniques, oocyte source and isolation techniques, freezing apparatus, pre- and post-preservation processing, and viability assays. Often, oocytes are frozen as cumulus-oophorus complexes; inasmuch as it is usually not possible to establish the number of oocytes in such cumulus masses, the yield of cells (i.e., the percentage of cells frozen which were recovered) post-thaw cannot be determined, and all measured survival rates must be expressed as fractions of the number of cells recovered rather than the total number of cells frozen. Table 4-3 is a literature review of conventional freezing protocols for metaphase II mouse oocytes, including only studies
Table 4-3. Review of literature on conventional cryopreservation of mouse metaphase II oocytes.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Year</th>
<th>$T_{	ext{storage}}$ (°C)</th>
<th>Protocol Duration (min)</th>
<th>Normal Morphology*</th>
<th>Fertilization Rate*</th>
<th>Blastocyst Formation*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sherman and Lin [25]</td>
<td>1958</td>
<td>-20</td>
<td>14</td>
<td>90%</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Parkening et al. [20]</td>
<td>1976</td>
<td>-75</td>
<td>154</td>
<td>72%</td>
<td>46%</td>
<td>17%</td>
</tr>
<tr>
<td>Parkening and Chang [19]</td>
<td>1977</td>
<td>-75</td>
<td>154</td>
<td>66%</td>
<td>38%</td>
<td>13%</td>
</tr>
<tr>
<td>Tsunoda et al. [32]</td>
<td>1976</td>
<td>-196</td>
<td>210</td>
<td>12%</td>
<td>0.4%</td>
<td>-</td>
</tr>
<tr>
<td>Whittingham [36]</td>
<td>1977</td>
<td>-196</td>
<td>195</td>
<td>58%</td>
<td>36%</td>
<td>-</td>
</tr>
<tr>
<td>Leibo et al. [12]</td>
<td>1978</td>
<td>-196</td>
<td>129</td>
<td>65%</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Fuller and Bernard [8]</td>
<td>1984</td>
<td>-196</td>
<td>100</td>
<td>-</td>
<td>-</td>
<td>54%</td>
</tr>
<tr>
<td>Chen [4]</td>
<td>1986</td>
<td>-196</td>
<td>58</td>
<td>80%</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Glenister et al. [9]</td>
<td>1987</td>
<td>-196</td>
<td>148</td>
<td>56%</td>
<td>28%</td>
<td>-</td>
</tr>
<tr>
<td>Ko and Threlfall [11]</td>
<td>1988</td>
<td>-196</td>
<td>140</td>
<td>57%</td>
<td>11%</td>
<td>-</td>
</tr>
<tr>
<td>Carroll et al. [2]</td>
<td>1989</td>
<td>-196</td>
<td>125</td>
<td>61%</td>
<td>28%</td>
<td>21%</td>
</tr>
<tr>
<td>Trounson and Kirby [31]</td>
<td>1989</td>
<td>-196</td>
<td>146</td>
<td>5%</td>
<td>1%</td>
<td>-</td>
</tr>
<tr>
<td>Carroll et al. [1]</td>
<td>1990</td>
<td>-196</td>
<td>125</td>
<td>65%</td>
<td>57%</td>
<td>40%</td>
</tr>
<tr>
<td>Schroeder et al. [24]</td>
<td>1990</td>
<td>-196</td>
<td>148</td>
<td>84%</td>
<td>74%</td>
<td>50%</td>
</tr>
<tr>
<td>Carroll et al. [3]</td>
<td>1993</td>
<td>-196</td>
<td>121</td>
<td>95%</td>
<td>78%</td>
<td>-</td>
</tr>
<tr>
<td>Van Blerkom and Davis [34]</td>
<td>1994</td>
<td>-196</td>
<td>209</td>
<td>55%</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*Expressed as percentages of the total number of cells frozen.
which report the total number of cells frozen, and thus permit absolute survival rates to be calculated. Three groups obtained morphological survival rates greater than 80%: as early as 1986, Chen [4] reported that ~80% of oocytes frozen in 1.5 M DMSO at a rate of 0.5°C/min and plunged into liquid nitrogen at -36°C appeared morphologically normal after thawing; however, these results were not subsequently reproduced by other researchers [7]. Schroeder et al. [24] obtained a morphological survival rate of 84% after freezing oocytes in 1 M DMSO at 0.5°C/min and plunging at -80°C. Recently, Carroll et al. [3] have reported morphological survival rates of 80-95% for oocytes frozen in 1.5 M DMSO using a three-step protocol: cells were first cooled to -40°C at a rate of 0.3°C/min, then at a rate of 10°C/min to -150°C before being plunged into liquid nitrogen. The highest survival rates were obtained by treating oocytes with polyvinyl alcohol (PVA) and fetal calf serum prior to freezing, and diluting the cryoprotectant in the presence of PVA [3]. Also noteworthy is the study by Trounson and Kirby [31], as the DMSO concentration, dehydration cooling rate and plunge temperature used by that group were ostensibly the same as those determined to be optimal in the present work (i.e., 1.5 M DMSO, \( B_{\text{dehydration}} = 0.5°C/\text{min}, \ T_{\text{plunge}} = -80°C \)). However, in the experiments by Trounson and Kirby, only 5% of all oocytes frozen were recovered intact, illustrating that survival rates are sensitive to factors other than the freezing protocol parameters. Trounson and Kirby reported a higher survival rate (72% of the number of cells recovered) when oocytes were frozen with their cumulus [31].

The general approach to avoiding IIF in the present study was to induce a depression in the nucleation temperature to below the glass transition temperature by dehydrating the
cell (see also Discussion in Chapter 2). Although the median IIF temperature could be depressed to $-49^\circ C$ by dehydrating the oocytes for 10 min at $-10^\circ C$, it is thermodynamically impossible to dehydrate oocytes to an extent sufficient for vitrification (i.e., achieving intracellular DMSO concentrations above $c_{\text{crit}} \approx 10 \text{ M}$) at temperatures above the liquidus temperature for the critical concentration, $T_m(c_{\text{crit}}) \approx -50^\circ C$. However, because the characteristic time constant for water transport at this temperature is approximately 2 hours (Fig. 4-6), one may conclude that freezing protocols for mouse oocytes will by necessity have durations on this order of magnitude if IIF is to be avoided. In effect, a “bottle-neck” is created due to the high activation energy for water transport and the high DMSO concentrations required for vitrification. Thus, to able to freeze mouse oocytes to liquid nitrogen temperature using rapid or ultra-rapid protocols, one must widen the bottle-neck either by lowering the activation energy $E_{\text{lp}}$ (i.e., permeabilizing the plasma membrane), or by decreasing the critical concentration $c_{\text{crit}}$, e.g. by increasing the plunge cooling rate (see Fig. 4-9) or by employing cryoprotectants or cryoprotectant mixes which are better glass-formers than DMSO. A different approach would be to increase the final storage temperature to above the nucleation temperature. For example, the non-equilibrium phase diagram (Fig. 4-5) indicates that the nucleation temperature for oocytes with 8 M intracellular DMSO would be depressed to $\sim -80^\circ C$, and that this level of dehydration could be achieved at a temperature as high as $-30^\circ C$, with a corresponding time-scale for water transport on the order of 15 min (Fig. 4-6). Toner et al. [29] have recently developed a rapid ($\sim 5$ min) freezing protocol for fertilized mouse oocytes, using a storage temperature of $-45^\circ C$. Published freezing protocols for
unfertilized mouse oocytes in which the final temperature was -196°C have typically had durations longer than 2 hours (Table 4-3), supporting the theory that the bottle-neck effect described here is important for oocyte freezing.

Apart from IIIF, the main biophysical phenomenon governing the success of cryopreservation is injury due to solution effects. Because the mechanism of solution effects damage is unknown, and no effective phenomenological models for this mode of injury are available, the simple strategy of minimizing solution effects injury by minimizing protocol duration was employed in the present study. Two assumptions about the nature of solution effects are implicit to this approach: (1) solution effects damage is a stochastic process, i.e., a random occurrence with an underlying average rate of injury, implying that the probability of damage under constant conditions is directly proportional to the time of exposure to those conditions; and (2) the rate of injury is negligible at the cryogenic storage temperature. In fact, inspection of Eq. (4-7) reveals that the rate of injury is assumed to be constant for all temperatures above $T_{\text{storage}}$, and zero at or below $T_{\text{storage}}$. Although this is not a realistic assumption, the approximation is reasonable and justified considering the lack of relevant experimental data. Pitt [21] hypothesized a more specific form for the non-IIIF injury rate, assuming it to be directly proportional to the extent of osmotic dehydration and having an Arrhenius type temperature dependence. However, due to the lack of data concerning the constant of proportionality and the Arrhenius activation energy, Pitt’s predictions were limited to a parametric analysis using hypothesized values for the biophysical model parameters. The experimental results
obtained in the present study indicate that under the freezing conditions used here, the protocol duration is a reasonable predictor of non-IIF cell injury (Fig. 4-14).

The cost functional as defined by Eq. (4-7) does not have any stationary points in the parameter space \((B_{\text{dehydration}}, T_{\text{plunge}})\), and thus the intermediate value theorem posits that the minimum cost will occur at the boundary defined by the constraint PIF=5% (see Fig. 4-13). Theoretical simulations demonstrated that the probability of IIIF was very sensitive to the protocol parameters in the neighborhood of the constraint, as evidenced by the sharp transitions in PIF seen in Figs. 4-11 and 4-12. Consequently, then, cell survival is also sensitive to the exact values of \(B_{\text{dehydration}}\) and \(T_{\text{plunge}}\) for near-optimal freezing protocols. This may partly explain the degree of variability in the success of freezing protocols for mouse oocytes reported in the literature (see Table 4-3), or the discrepancy between the current results and those obtained by Trounson and Kirby using a similar procedure [31]. Furthermore, the high sensitivity of cell survival on the protocol parameter values underscores the need to narrow down the search for the experimental optimum to as narrow a range as possible. Cell survival drops off rapidly in the vicinity of the optimum, and outside of the immediate neighborhood of the optimal protocol, cell survival rates are uniformly low, making it difficult or impossible to identify improved protocols based on experimental viability data alone. Thus, even though the predicted optimal protocol yielded only 47% viable oocytes, the theoretical model identified the neighborhood of the optimum in parameter space, allowing a rapid experimental determination of freezing protocols yielding cell survivals in excess of 80%.
In summary, a successful cryopreservation protocol for unfertilized mouse oocytes was designed and optimized using a theoretical model of intracellular nucleation and crystal growth. By suitably assigning values for the physicochemical and biophysical parameters of this model, cryopreservation protocols for any cell type and cryoprotectant can in principle be developed using the techniques reported here.
References


Chapter 5

Conclusions and Future Work

Conclusions
The preceding four chapters detail the development of a mechanistic mathematical model of intracellular ice nucleation and crystal growth, verification of this model using published as well as new experimental data, and application of the model as a tool for the rational design and optimization of an experimental protocol for the cryopreservation of mouse oocytes. The major conclusions are as follows:

- The coupled model of intracellular crystallization developed in the present study represents a significant improvement in the state of the art of IIF modeling. For the first time, the effect of cryoprotectants have been incorporated in a mechanistic model of IIF, broadening the range of physicochemical conditions over which predictions can be obtained. Also, by incorporating crystal growth equations into the IIF model, the volume and size distribution of intracellular ice crystals can now be predicted. This is significant for two reasons: (1) it enables exploration of the relationship between the extent of intracellular crystallization and the resulting level of damage to the cell (see Chapter 3), possibly leading to clues as to the mechanism of IIF-induced cell injury, and (2) the exact state of crystallization of the cytosol (i.e., the number of ice nuclei, and the sizes of the corresponding crystals) must be known in order to be able to predict devitrification and
recrystallization, two potentially damaging processes during rewarming of frozen cells.

- IIF-related freezing injury correlates with the volume of intracellular ice. While this is not a new finding, the model of intracellular crystallization permitted a quantitative correlation between predicted crystal size and measured cell viability (Chapter 3), a potentially powerful tool for the elucidation of mechanisms of IIF-induced damage. The nature of intracellular crystallization was shown to be fundamentally different in different regimes of protocol parameter space (Chapter 2), which suggests that different mechanisms of cell injury may be operational depending on the exact freezing conditions.

- Non-IIF ("solution effects") injury correlates with protocol duration. Although this is expected assuming that the solution effects injury is a stochastic process, the duration of the freezing protocol was found to be a remarkably good predictor of non-IIF cell damage. This indicates that within the subspace of thermodynamic states for the cell explored in the present study (determined by the class of freezing protocols used), the rate of injury due to solution effects was only weakly temperature-dependent.

- The methods used in Chapter 4 for rational design and optimization of a freezing protocol using the theoretical model represent a significant improvement over the currently used experimental approach to developing cryopreservation protocols. The advantage of using mechanistic models in evaluating protocols is that these models make available estimates of important variables (e.g. the thermodynamic
properties of the cytosol, and the number and size distribution of intracellular ice crystals) which are not experimentally accessible, but are nonetheless essential in understanding the freezing response of a cell. In addition to the increased amount of information afforded by theoretical models, these have the advantage that iterative searches for optimal combinations of protocol parameters are significantly less costly in time and laboratory resources than corresponding experimental iterations. Thus, as was seen in Chapter 4, the theoretical models developed here enabled the identification of successful cryopreservation protocols for mouse oocytes with a minimum number of experiments. The same techniques can now be used for efficiently developing freezing protocols for other cell types.

**Future Work**

A number of issues which merit further research became apparent during the course of this work:

- The predictive abilities of a model are limited by the quality of the measurements of the model parameters; unfortunately, very little data exist on several biophysical and physicochemical parameters relevant to IIF modeling. The required parameters for the coupled model developed in this work are: $L_{pg}$ and $E_{Lp}$, the water transport parameters; $\Omega$ and $\kappa$, the nucleation rate coefficients (for all active nucleation mechanisms); and $\eta$, the cytosol viscosity. In the present work, phenomenological models of the viscosity of H$_2$O-NaCl-glycerol (Chapter 2) and H$_2$O-NaCl-DMSO (Chapter 3) were constructed from a sparse set of available data. Sensitivity analysis indicated that model predictions are sensitive to $E_{Lp}$ and
κ, as well as η. These and other model parameters must be measured directly and more accurately, if model predictions are to improve.

- Whereas the state of intracellular ice crystallization can be predicted with a reasonable degree of accuracy using present modeling techniques, the rate-limiting step in predicting the effect of a given cryopreservation protocol on cell survival has become the lack of understanding of non-IIF cell injury. Solution effects must be explored experimentally and predictive models for this phenomenon must be developed in order to improve the ability to define the optimal protocols which simultaneously minimize both modes of injury.

- The intracellular crystallization model developed here must be extended to predict crystal growth during warming from cryogenic to normothermic temperatures, since cell damage may occur due to devitrification and/or recrystallization during warming (see Chapter 3).
och då var isen smält och nya släktled
upplevde nya vårars gunst i Gond.

FINIS