Numerical and experimental investigation of transport processes in biological systems

Yimeng He
Louisiana State University and Agricultural and Mechanical College

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NUMERICAL AND EXPERIMENTAL INVESTIGATION OF
TRANSPORT PROCESSES IN BIOLOGICAL SYSTEMS

A Thesis
Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Master of Science in Mechanical Engineering
in
The Department of Mechanical Engineering

by
Yimeng He
B.S., Shanghai Jiao Tong University, 1997
M.S., Shanghai Jiao Tong University, 2001
May, 2004
ACKNOWLEDGEMENTS

I want to give special thanks to my supervisor Dr. Ramachandra Devireddy for offering his extensive knowledge in Bio-engineering and his patience, guidance, encouragements and financial assistance. His academic manner and approach to problems will benefit me in my future study and life. I also want to give my appreciation to my committee members Dr. Sumanta Acharya, Dr. Dimitris E. Nikitopoulos, and Dr. Terrence R. Tiersch for their supports and valuable suggestions.

I thank my colleagues Sreedhar Thirumala and Qiaoxiang Dong for their wonderful friendship, help and valuable suggestion in this study.

The accomplishment of this study should acknowledge support from the project funded by Louisiana Board of Regents (LEQSF 2002-05-RD-A-03) and a Biomedical Engineering Research Grant from Whitaker Foundation to RD.

Finally, I give thanks to my parents and sister for their continuous support, help and encouragement.
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## Subscripts

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<tr>
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ABSTRACT

This thesis focuses on optimization techniques for transport process during the cryopreservation of biological systems.

In the first part of this thesis, a well established shape independent Differential Scanning Calorimeter (DSC) technique was used to measure the dehydration response during freezing of Pacific oyster sperm cells. Volumetric shrinkage during freezing of diploid and tetraploid pacific oyster sperm cell suspensions was obtained at cooling rates of 5 and 20 °C/min in the presence of extracellular ice and CPAs. By fitting a model of water transport to the experimentally obtained volumetric shrinkage data the best fit membrane permeability parameters \((L_{pg} \text{ and } E_{lp})\) were determined. The simulation results were analyzed to predict the amount of water left in the cell after dehydration ceased, in the absence of IIF and the “optimal cooling rates” for diploid and tetraploid pacific oyster sperm cryopreservation. The second part of this thesis, a generic mathematical model based on a 2 parameter Kedem and Katchalsky formulation was developed to simulate the coupled solute and solvent transport in arbitrary tissue sections. Osmotic responses of various tissue cells within the artificial tissue are predicted by the numerical model with three model parameters: permeability of the tissue cell membrane to water \((L_p)\) permeability of the tissue cell membrane to the solute or CPA \((\omega)\) and the diffusion coefficient of the solute or CPA in the vascular space \((D)\). By fitting the model results with published experimental data on solute/water concentrations at various locations within an artificial tissue, we were able to determine the permeability parameters of embedded tissue cells in the presence of Me_2SO. The permeability parameters obtained in the present study represent the first such effort for embedded tissue cells.
CHAPTER 1
INTRODUCTION

1.1 Cryobiology

Generally speaking, cryobiology is the study of living systems at low temperature. One important goal of this field is to preserve or store viable biological material in the frozen state for extended periods of time, commonly denoted as cryopreservation.

The enormous diversity of life on Earth correspondingly results in an enormous diversity of biological tissues which make up living things. These tissues range from plants such as trees, fruits and vegetables to animal tissues such as bone, skin and organs. Each of these has unique micro-structures, which generates different characters.

Modeling the freezing process in this variety of tissues is of importance in cryobiology. Researchers are interested in banking tissues which requires a large amount of the cells within the tissues. These cells can maintain and repair the tissues; therefore, if the cells survive during the freezing process, tissue damage can be repaired after thawing.

The use of low temperature to preserve or store biological material has been applied in a variety of mammalian systems (Mazur 1984; McGrath 1988) including red blood cells, lymphocytes, gametes, embryos, cultured cells, hepatocytes, bone marrow stem cells, cornea, skin cells and pancreatic tissue. Researchers continue to develop cryopreservation protocols to improve the post-thaw viability of biological systems, including human oocytes (Bernard & Fuller 1996), hematopoietic stem cells (Hubel 1997), rat and human liver slices (Day et al. 1999), spermatozoa from variety of species, including mouse (Devireddy et al. 1999c), human (Devireddy et al. 2000), horse (Devireddy et al. 2002a; Devireddy et al. 2002b), canine (Thirumala et al. 2003) and boar (Devireddy et al. 2004). During a typical cryopreservation protocol the cells tolerate five non-physiological conditions: (i) addition of molar concentrations of cryoprotective additives (CPAs), causing “osmotic” injury; (ii) cooling to subzero temperatures, causing “chilling” injury; (iii) removal or conversion of almost all liquid cell water into the solid state, or the freezing process, (iv) warming to room temperature, causing both “recrystallization” and other “thawing” injury; (v) removal of CPAs, causing “osmotic” injury (Devireddy et al. 2003).
In a typical cryopreservation protocol, the cells or tissues to be preserved are first loaded with chemical additives or cryoprotective agents (CPAs) to alleviate cell damage from either by intracellular ice formation (IIF) or dehydration during the subsequent freezing process. The temperature of the biological systems is reduced to the predefined temperature (usually liquid nitrogen temperature) for long term storage. Before the transplantation of the biological systems, the biological systems must be thawed and the CPA removed. To ensure the cell viability and functionality of the biological systems, each step in the above cryopreservation protocol should be handled in a precisely controlled and optimized manner.

1.2 Differential Scanning Calorimeter (DSC) Technique

In this definition of cryopreservation, low temperature, refers to any temperature that is below the temperature at which the specific biological system being studied normally exists. For many organisms, this temperature is based on the essential ingredient present in all life on earth: water. Life exists as a result of the specific properties of water, and in most cases life ceases to exist in the absence of water. In the second part of this thesis, the most interesting result occurs at the temperature below the phase-change temperature of water.

Water is one of the most abundant substances on Earth and also one of the most versatile. Also, liquid water is capable of dissolving a wide variety of chemical solutes, including dimethyl sulphoxide (Me₂SO), propylene glycol (PROH), ethylene glycol (EG), and glycerol (GLY). The water molecule unique ability to form strong hydrogen bonds causes the existence of metastable clusters in the liquid state. These clusters, which are larger and longer lasting at lower temperatures, eventually become the framework for the crystalline lattice structure of ice. Any solute dissolved within water affects this mechanism by which water is transformed to ice; and thus, results in changes in the freezing point of water.

When chemical solutes are dissolved in water, the composite substance is known as aqueous solutions, which have a variety of unique properties such as freezing point and osmotic pressure. These properties are also called colligative properties because they arise as a result of the collection of chemical species that make up a solution. For example, the freezing point varies from the freezing point of pure water as solutes are
added. Generally, as a solute is dissolved in increasing concentration in water, the freezing point the solution will decrease below the usual 0 °C freezing point of pure water to temperatures as low as -60 °C or lower.

In cryobiology, understanding the phase behavior of an arbitrary solution is important because the composition of that solution can be determined from the phase diagram for any particular temperature the solution is subjected to. In biological systems, knowledge of the composition of the chemical solutions present is crucial to predicting the movement of water and solutes within the system. The knowledge is also necessary to understand the effect that these solutions have on living cells.

During freezing of any cell suspension, ice nucleates initially in the extracellular space causing an osmotic gradient to be set up across the intracellular isotonic solution and the freeze-concentrated extracellular solution (Mazur 1970; Meryman 1966). Depending on the cooling rate, the intracellular water permeates across the cell membrane and joins the extracellular ice phase or freezes and forms ice inside of the cell. In most cases, cells undergoing ice formation inside the cells or intracellular ice formation (IIF) are rendered osmotically inactive (lysed), due to the loss of cell membrane integrity (Mazur 1984). Cells which experience a severe loss of intracellular water and the associated long-term exposure to the highly concentrated extracellular salt solutions are also rendered osmotically inactive (Lovelock 1953). Both IIF and long exposures to high solute concentrations are lethal to cells. So cooling rates that are either “too slow” or “too fast” can kill cells, therefore an “optimal” cooling rate should exist between these rates. This has been confirmed experimentally for a variety of cells, and the curve of cell survival plotted as a function of the cooling rate has a characteristic inverted U-shape (Mazur et al. 1972). Whether a prescribed cooling rate is too “slow” or “fast”, is a function of cell membrane permeability to water and the probability that any water remaining trapped within the cell at any given sub-zero temperature will nucleate and turn to ice. Differences in membrane permeability to water and the probability of IIF result in different “optimal” cooling rates for different cells. Therefore, to optimize a cryopreservation protocol it is important to measure the cell membrane permeability to water.
Various methods have been used to investigate water transport during freezing in biological systems. Cryomicroscopy is used to measure water transport in single spherical cells (Cosman et al. 1989; Diller 1982; McGrath 1988), which involves the application of cryogenic temperatures to cellular systems mounted on a light microscope to study the biophysical response to cells freezing. The quantitative measurement of water transport during freezing of a tissue system were efficiently performed by using directional solidification device and two-step freezing technique (Pazhayannur & Bischof 1997). Recently, a differential scanning calorimeter (DSC) technique that dynamically quantifies mass transfer during freezing in a variety of biological systems has been developed and characterized.

The DSC is an ideal device to measure the state of water (especially liquid to solid phase change) in CPA solution and biological systems. The DSC technique has been used to improve our knowledge of water transport response during freezing in several mammalian gametes, including mouse (Devireddy et al. 1999c), human (Devireddy et al. 2000), horse (Devireddy et al. 2002a; Devireddy et al. 2002b), canine (Thirumala et al. 2003) and boar (Devireddy et al. 2004).

In Chapter 2, the DSC technique will also be used, for the first time with an aquatic species, in the present study to improve our understanding of the biophysical response during freezing of sperm cells from diploid and tetraploid Pacific oysters, *Crassostrea gigas*. In the DSC technique, two heat releases from the same cell suspension are measured: i) during freezing of osmotically active (live) cells in media (where the intracellular water is being transported across the membrane to freeze in the extracellular space) and; ii) during freezing of osmotically inactive (dead) cells in media. The difference in heat release measured between the two cooling runs is correlated to water transport (see a recent review by Devireddy and Bischof, 2003) and has been independently verified (Yuan & Diller 2001; Diller 2002). The experimentally determined oyster sperm membrane permeability parameters are used to numerically predict the loss of intracellular water under a variety of cooling rates (5 to 100 °C/min). And finally, the numerical predictions are analyzed to predict the amount of water left in the sperm cell after dehydration ceases, in the absence of IIF and the “optimal cooling rates” for oyster sperm cryopreservation.
1.3 Numerical Model of Water Transport

Kedem and Katchalsky (Kedem & Katchalsky 1958) proposed a model for water and solute transport in response to chemical potential gradients based on irreversible thermodynamics.

\[ J_y = L_p \left[ \Delta p - RT(\Delta C_w + \sigma \Delta C_{cpa}) \right] \]  
(1)

\[ J_{cpa} = \Delta C_{cpaave} \left( 1 - \sigma \right) J_y + RT \omega \Delta C_{cpa} \]  
(2)

In the original K-K formalism, three parameters, the hydraulic permeability \( L_p \), solute permeability \( \omega \) and reflection coefficients \( \sigma \), are used to characterize the membrane. The term \( \Delta p, \Delta c_{w}, \Delta c_{cpa} \) represent, respectively, the difference (extracellular, \( O, \) minus intracellular, \( I \)) in the hydrostatic pressures and the osmolalities of the impermeable and permeable solutes across the cell membrane.

\[ \Delta C_{cpaaverage} \] represents the “log mean” osmolality of the permeable solute

\[ \Delta C_{cpaaverage} = \frac{\Delta C_{cpa}}{\ln(C_{cpa}^0/C_{cpa}^1)} \]  
(3)

If the flux of CPA is negligible in comparison to the water flux (McCaa et al. 1991), then the Kedem-Katchalsky model reduces to a model which assumes only water transport, as proposed by Mazur (Mazur 1963) and later modified by Levin et al. (Levin et al. 1976; Karlsson et al. 1994). The model predicts the change in cell volume with decreasing temperature \((dV/dT)\) after ice has formed outside of an unfrozen cell as,

\[ L_p = L_{pg}[cpa] \cdot \exp \left\{ - \frac{E_{lp}[cap]}{R} \left( \frac{1}{T_R} - \frac{1}{T} \right) \right\} \]  
(4)

with \( L_p \), the sperm cell membrane permeability to water defined by Levin et al. (Levin et al. 1976) as,

\[ \frac{dV}{dT} = - \frac{L_p A_c RT}{B V_w} \ln \left( \frac{V_o - V_h - n_{cpa} \nu_{cpa}}{V_w} / \nu_w + (\varphi_s n_s + n_{cpa}) \right) - \frac{\Delta H_f \nu_s \rho}{R} \left( \frac{1}{T_R} - \frac{1}{T} \right) \]  
(5)

where, \( L_{pg} \) or \( L_{pg}[cpa] \) is the reference membrane permeability at a reference temperature, \( T_R(= 273.15 \text{ K}) \) in the absence and presence of CPA; \( E_{lp} \) or \( E_{lp}[cpa] \) is the apparent activation energy (kJ/mol) or the temperature dependence of the cell membrane permeability in the absence and presence of CPA; \( A_c \) is the effective membrane surface area for water transport, assumed to be constant during the freezing process; \( R \) is the
universal gas constant; B is the constant cooling rate (K/min); finally \(C_i\) and \(C_o\) represent the concentrations of the intracellular and extracellular (unfrozen) solutions. The various assumptions made in the development of the water transport are discussed in detail elsewhere (Devireddy et al. 2003; Gao et al. 1997; Kedem & Katchalsky 1958; McCaa et al. 1991; Mazur 1963; Levin et al. 1976; Karlsson et al. 1994; McGrath 1998; Smith et al. 1998) and are beyond the scope of the present study. The two unknown membrane permeability parameters of the model either \(L_{pg}\) [cpa] and \(E_{lp}\) [cpa] in the presence of CPA or \(L_{pg}\) and \(E_{lp}\) in the absence of CPA, are determined by curve-fitting the water transport model to experimentally obtained volumetric shrinkage data during freezing.

### 1.4 Numerical Model of Solute and Solvent Transport in Tissue Systems

Although CPAs can alleviate injury during the freezing and thawing process, they can also cause damage to cells by osmotic stress (due to volumetric excursion) and chemical toxicity (due to chemical toxicity and exposure time), denoted as osmotic injury and chemical injury. Typical protocols include loading CPAs stepwise (reducing the volumetric excursions and the consequent osmotic injury) and loading CPAs at a temperature close to 0 ºC (reducing metabolic activity and the consequent chemical injury). Clearly, the rates of introduction and removal of cryoprotective additives (CPAs) affect the successful freeze preservation of biomaterial. Optimization of the cell addition and removal procedures requires knowledge of the tissue cell membrane permeability parameters to both the solvent (intracellular water) and the solute (or the CPA). These parameters are typically obtained by curve-fitting measured variations in the cell volume during a predefined chemical loading procedure to a well defined model of couple water and CPA transport defined as the Kedem-Katchalsky (K-K) formalism (Kedem & Katchalsky 1958).

As stated in last section, KK equations are used to analyze nonequilibrium osmotic flows and net solute movements. Recently, Kleinhans (Kleinhans 1998) demonstrated that a two-parameter model using only \(L_p\) and \(\omega\) yields essentially the same result as the original K-K formulation (when there is no strong evidence or reason to suspect significant levels of solute-solvent co-transport). The reflection coefficient, \(\sigma\) can be expressed as a function of \(L_p\) and \(\omega\) and the original 3 parameter K-K formulation can be reduced to a much simpler two-parameter model that is used in the present study.
On the basis of a non-equilibrium model for the transport of water and permeable solute across cell membranes, Levin and Miller devised an optimum method for the introduction and the removal of permeable cryoprotectants from single, isolated cells so that “osmotic” injury can be minimized. This original work has been extended for other isolated single cells (Kedem & Katchalsky 1958; Levin & Miller 1981); Levin also investigated the osmotic effects of introducing and removing cryoprotectants for perfused tissues and organs; transport of a non-permeating CPA in a liver tissue was studied recently with experimental and theoretical techniques by Bhowmick (Bhowmick et al. 1998).

However, most of the published solute-solvent models are for isolated single cells (Agca et al. 1999; Gao et al. 1995) or in tissue sections for only solvent transport. There are still several challenges to modeling CPA transport in tissue systems. In chapter 3, I will focus on the development of a generic numerical model to simulate the individual tissue cell osmotic response during conditions (i) and (v) of a typical cryopreservation protocol in the presence of both permeable and impermeable solutes. The model accounts for the axial and radial diffusion of the solute as well as axial convection, and a constant or variable vascular surface area (\( A_c \)). In addition, the model also accounts for the radial movement of the solvent (water) to and from the vascular spaces. By fitting published experimental data (Bidault et al. 2001) to our numerical model, seminal tissue membrane permeability parameters are obtained. The predicted tissue cell membrane permeability parameters can now be used to predict optimal chemical loading and unloading procedures under arbitrary CPA concentrations for whole tissue sections.

1.5 Overview of Thesis

This master thesis includes two parts: experiments and numerical simulations. The goal and objectives of the present work are summarized below.

- Obtain the water transport data of Pacific oyster sperm cell in the presence of extracellular ice and cryoprotective agents (CPA) during freezing by using a widely accepted DSC technique at different cooling rates.
• Use the data obtained by the DSC experiment and to predict the membrane permeability parameters by curve fitting the DSC data to a previous transport model.

• Develop a generic numerical model to simulate the coupled solute and solvent transport process during the addition and removal of cryoprotective agents (CPAs) in biological tissue systems.

• Obtain seminal tissue membrane permeability parameters by fitting published experimental data to the numerical model.
CHAPTER 2
CRYOPRESERVATION OF SPERMATOZOA FROM DIPLOID AND TETRAPLOID PACIFIC OYSTERS: VARIATION IN MEMBRANE TRANSPORT PROPERTIES AND PREDICTED RATES OF OPTIMAL FREEZING*

Cryopreservation has been studied in aquatic species for more than 50 years (Tiersch 2000a; Tiersch et al. 2000b; Smith et al. 2001). However, with some notable exceptions (Lin et al. 1995; Zhang et al. 1996; Hagedorn et al. 1996), cryobiology has largely been neglected in these species. Most research in the 200 or so species that have been studied has addressed empirical development of basic cryopreservation procedures (Blaxter 1953; Rana 1995; Tiersch 2001). Similarly, without firm cryobiology understanding, sperm cryopreservation of diploid oysters has been studied in several species, predominantly the Pacific oyster *Crassostrea gigas* (Lannan 1971; Staeger 1973; Usuki et al. 1997; Kurokura et al. 1990; Dong et al. 2002), the eastern oyster, *Crassostrea virginica* (Paniagua-Chavez CG et al. 2001; Hughes 1973; Zell et al. 1979) and others (Yankson et al. 1991). Because reduced gonadal development is associated with improved meat quality and growth (Allen et al. 1991; Akashige et al. 1992), triploid oysters (with three sets of chromosomes), which are functionally sterile, offer advantages over diploid oysters. Commercial application of triploid oysters requires efficient methods for triploidy production such as crossing of diploids with tetraploids (that possess four sets of chromosomes), which in turn requires the availability of tetraploid oysters in the hatchery (Allen et al. 1992). Sperm cryopreservation of tetraploid oysters can be a critical tool for commercial-scale application of tetraploid stocks and the consequent expansion of the world wide triploid oyster market.

Further usage of thawed oyster sperm requires a biophysical understanding of the cryopreservation process. Currently, the bulk of our understanding in the cryopreservation of oyster sperm (as well as in other aquatic and mammalian species) is still empirical in nature. Because the unique size and morphology of sperm cells limits

the applicability of standard cellular cryomicroscopy techniques to measure the biophysical response (water transport and intracellular ice formation) during a freezing process. However, a recent advance in measurement methodology, a differential scanning calorimetry (DSC) based technique (Devireddy et al. 1998), has improved knowledge of the water transport response during freezing in mammalian gametes, including mouse (Devireddy et al. 1999c), human (Devireddy et al. 2000), horse (Devireddy et al. 2002a; Devireddy et al. 2002b), canine (Thirumala et al. 2003) and boar (Devireddy et al. 2004). The DSC technique will also be used, for the first time with an aquatic species, in the present study to improve our understanding of the biophysical response during freezing of sperm cells from diploid and tetraploid Pacific oysters.

As stated in the introduction chapter, an “optimal” cooling rate should exist between the “slow” or “fast” rates. This has been confirmed experimentally for a variety of cells, and the curve of cell survival plotted as a function of the cooling rate has a characteristic inverted U-shape (Mazur et al. 1972). Moreover, the cooling rate is a function of cell membrane permeability to water and the probability that any water remaining trapped within the cell at any given subzero temperature will nucleate and turn to ice. Differences in membrane permeability to water and the probability of IIF result in different “optimal” cooling rates for different cells. Therefore, to optimize a cryopreservation protocol it is important to measure the cell membrane’s permeability to water.

The aim of this project is to establish the membrane permeability parameters of sperm cells of diploid and tetraploid Pacific oysters. In the DSC technique, two heat releases from the same cell suspension are measured: i) during freezing of osmotically active (live) cells in media (where the intracellular water is being transported across the membrane to freeze in the extracellular space) and; ii) during freezing of osmotically inactive (dead) cells in media. The difference in heat release measured between the two cooling is correlated to water transport (Devireddy and Bischof 2003), and has been independently verified (Yuan and Diller 2001; Diller 2002). The experimentally determined parameters for Pacific oyster sperm membrane permeability are used to numerically predict the loss of intracellular water under a variety of cooling rates (5 to 100 °C/min). And finally, the numerical predictions are analyzed to predict the amount of
water left in the sperm cell after dehydration ceases, in the absence of IIF and the “optimal cooling rates” for oyster sperm cryopreservation.

2.1 Differential Scanning Calorimeter (DSC) Technique

Whenever a material undergoes a change in physical state, such as melting or transition from one crystalline form to another, or whenever it reacts chemically, heat is either absorbed or liberated. Many such processes can be initiated simply by raising the temperature of the material. Differential scanning calorimeter is primarily designed to determine the enthalpies of these processes by measuring the differential heat flow required to maintain a sample of the material and inert reference at the same temperature. This temperature is usually programmed to scan a temperature range by increasing linearly at a predetermined rate. The apparatus can also be used to measure heat capacity, thermal emissivity and the purity of solid sample. In addition, it can be used to yield phase diagram information and to provide kinetic data. The DSC technique has been applied, however, to diverse types of compounds, and reviews on the application of DSC to petroleum products, plastics, biological systems and mental complexes have appeared (McNaughton & Mortimer, 1975).

![DSC experimental setup](image)
In this study, the DSC machine, which we used for our aquatic species experiments, is Perkin-Elmer DSC 7, which is a power compensated model and can run from -170 to 725 °C. The DSC measures the amount of power required to keep the sample at the temperature predetermined by the program, while the heat flow to keep a reference (empty sample container) at the same temperature is also measured. Here, the heat reading is the difference between the sample and reference.

The DSC consists of a sample holder and a reference holder, which are insulated from each other (shown in Fig. 2-1). Usually, the reference holder is kept empty or put an empty sample pan inside during the experiments. Individual heaters are provided for sample holder and reference holder, which supply heat power to them. During the experiment, the sample holder and reference holder are always maintained at the same temperature by means of two temperature control loops (average temperature control loop and differential temperature control loop). Finally, a signal, proportional to the difference between the heat input to the sample and that to the reference, dH/dT, is fed into a recorder. In practice, this recorder is also used to register the average temperature of the sample and reference.

![Typical DSC curve](image)

**Fig 2-2 Typical DSC curve**
As the temperature increases at a predetermined rate, the evolution or absorption of heat by a sample and change in enthalpy during a phase change process can be measured by calculating the area under peaks that occur in the power versus time or power versus temperature graphs (shown in Fig. 2-2). Also from peaks generated by a phase transition, we can calculate the melting point fairly accurately by finding the onset temperature. Onset temperature is defined as the intercept between the baseline and a line drawn tangent to leading edge of the curve, near the apex. The area under the peaks is calculated from the baseline created by the heat capacity of the substance. The difference in heat capacities of the sample and reference causes the experimental curve to shift along the ordinate axis by a constant value or one barely changing with temperature and also cause the baseline to shift from the zero level. This problem can be eliminated by using a reference with a heat capacity close to heat capacity of the sample being studied. Generally an empty pan of the same material as the sample pan is used as the reference pan to reduce the heat capacity differences (Gray 1976). The output signal, which is directly proportional to the differential heating power, would directly stand for the heat flow rate into the sample. Also, due to the thermal resistance between the sample and sample pan and similarly, between the sample pan and sample holder, there must be a negligible heat loss. The thermal resistances should be reduced as much as possible by using small masses of the sample and by calibration. A detailed description of the various calibration and control experiment processes are given by previous published paper and will not be given in this thesis.

- **Advantages and Disadvantages of the DSC Technique**

In comparison to standard cryomicroscopy techniques, the DSC technique has several advantages. First, the technique does not depend on the shape or size of the cells being investigated. Based on previous reports, the technique can be used as a generalized method to measure water transport during freezing in spherical and non-spherical cells as well as the whole tissue slices. The technique allows the measurement of the volumetric response of a large number of cells (up to $10^9$/ml) as opposed to single cells by standard cryomicroscopy techniques. In this way the technique is perhaps better able to quickly establish an average cellular response without the difficulties of single cell cryomicroscopic analysis. The technique removes errors due to limitations of image
analysis which have been estimated at as much as 10%. Lastly, the technique can be used to estimate the freezing injury due to multiple freeze-thaw cycles.

The DSC technique has a few notable disadvantages or limitations. The two most important are that the technique requires a prior knowledge of the initial cell volume, surface area and osmotically inactive cell volume. Secondly, the technique requires a prior knowledge that the biophysical response being measured is in fact water transport (and not intracellular ice formation) for the cooling rates investigated. Because of these two limitations, several others naturally follow. The technique is unsuitable for non-homogeneous cell systems; it can not detect the presence of osmotically inactive cells in the initial sample, or the amount of the sample which may be injured during pre-nucleation. In addition, only average cell response between an initial and final volume as measured by cryomicroscopy or some other appropriate technique can be measured. Finally, the technique requires a series of control experiments to be performed on the DSC machine prior to experimentation.

2.2 Materials and Methods

- **Collection and Isolation of Sperm Cells**

  Tetraploid and diploid Pacific oysters were obtained in August, 2003, from the Taylor Resources Quilcene Shellfish Hatchery (Quilcene, Washington) and were shipped chilled by overnight delivery to the Louisiana State University Agricultural Center, Aquaculture Research Station (ARS). Sperm were collected by dry stripping of the gonad (Allen 1992) and suspended in calcium-free Hanks’ balanced salt solution at 1000 mOsmol/kg (Paniagua-Chavez CG et al. 2001). Sperm concentrations were adjusted to 1 to 2 x 10⁹ cells/ml. The sample was transported to the LSU Bioengineering Laboratory for DSC experiments.

- **DSC Experiments**

  The DSC dynamic cooling experiments were performed on concentrated oyster sperm samples in standard aluminum sample pans (Perkin-Elmer Corporation, Norwalk, Conn.). The concentration of the sperm suspension used in the DSC experiments ranged from 1 to 2 x 10⁹ cells/ml. Approximately 10 µl of this sperm suspension was loaded in a DSC sample pan. The DSC dynamic cooling protocol used to measure the water transport out of Pacific oyster sperm cells is the same as reported in earlier studies on mammalian
cells (Devireddy et al. 1999c; Devireddy et al.2000; Devireddy et al.2002a, Devireddy et al.2002b; Devireddy et al.2004; Thirumala et al. 2003) and will be briefly stated here.

- **DSC Dynamic Cooling Protocol**

  Step 1: The sperm cell suspension with or without cryoprotective agents (CPAs) initially at room temperature was cooled at 5 °C/min until the extracellular ice nucleated. Step 2: After nucleation the sample was thawed at a warming rate (5 °C/min) such that phase change temperature $T_{ph}$ was reached (but not overshot) and ice remained in the extracellular solution. The phase change temperature can be obtained by using the osmolality relationship ($\text{Osm-1} = 1.858/\Delta T; \Delta T = 273.15 - T$, K); thus for a solution with an osmolality of 1000 mOsm, $\Delta T = 1.0 \times 1.858 = \sim 1.858$ K or $T_{ph} = -1.858$ °C. Step 3: The sample was then cooled to -50 °C at 5 (or 20) °C/min causing the sperm cells to undergo cellular dehydration. The lower curve in Figure 2-1 corresponds to the heat release associated with dehydration and the total area is represented by $q_{initial}$. Step 4: The sample was reequilibrated at $T_{ph}$ by thawing at 20 °C/min. Step 5: To differentiate between the heat released by the media and the intracellular fluid in Step 3, the sample was cooled at a high cooling rate (200 °C/min) down to -150 °C. This causes all the sperm cells to lyse and become osmotically inactive. Step 6: Step 4 was repeated. Step 7: The sample was then cooled to -50 °C at 5 (or 10) °C/min to measure the final heat release due to lysed or osmotically inactive sperm cells mixed with media. The upper curve in Fig. 1 corresponds to this heat release and the total area is represented by $q_{final}$.

- **Translation of Heat Release to Cell Volume Data for Dynamic Cooling**

  The heat release measurements of interest are $\Delta q_{dsc}$ and $\Delta q(T)_{dsc}$ which are the total and fractional difference between the heat releases measured by integration of the heat flows during freezing of osmotically active (live) cells in media and during freezing of osmotically inactive (dead) cells in media. This difference in heat release has been related to cell volume changes in several biological systems (Devireddy et al. 1999c; Devireddy et al.2000; Devireddy et al.2002a, Devireddy et al.2002b; Devireddy et al.2004; Thirumala et al. 2003) as,

  $$ V(T) = V_o - \frac{\Delta q(T)_{dsc}}{\Delta q_{dsc}} \cdot (V_o - V_b) $$
Table 2-1. Measurements, and calculated volumes, surface areas and ratios of surface area to volume (S:V) for heads, tails and combined totals of sperm from diploid and tetraploid pacific oysters.

<table>
<thead>
<tr>
<th>Ploidy</th>
<th>Head Diameter</th>
<th>Volume$^a$</th>
<th>Surface$^b$</th>
<th>S:V</th>
<th>Tail Length</th>
<th>Width</th>
<th>Volume$^c$</th>
<th>Surface$^d$</th>
<th>S:V</th>
<th>Volume$^e$</th>
<th>Surface$^f$</th>
<th>S:V</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diploid</td>
<td>1.66 µm</td>
<td>2.40 µm$^3$</td>
<td>8.66 µm$^2$</td>
<td>3.61</td>
<td>41 µm</td>
<td>0.14 µm</td>
<td>0.63 µm$^3$</td>
<td>18.03 µm$^2$</td>
<td>28.57</td>
<td>3.03 µm$^3$</td>
<td>26.69 µm$^2$</td>
<td>8.81</td>
</tr>
<tr>
<td>Tetraploid</td>
<td>2.14 µm</td>
<td>5.13 µm$^3$</td>
<td>14.39 µm$^2$</td>
<td>2.81</td>
<td>53 µm</td>
<td>0.17 µm</td>
<td>1.20 µm$^3$</td>
<td>28.31 µm$^2$</td>
<td>23.53</td>
<td>6.33 µm$^3$</td>
<td>42.69 µm$^2$</td>
<td>6.74</td>
</tr>
</tbody>
</table>

$^a$Calculated as volume of a sphere ($V_s = \frac{4}{3}\pi R^3$)

$^b$Calculated as surface area of sphere ($S_s = 4\pi R^2$)

$^c$Calculated as volume of a cylinder ($V_c = \pi R^2h$)

$^d$Calculated as surface of a cylinder ($S_c = 2\pi Rh$)

$^e$Calculated as sum of the volumes of the head and tail ($V_t = V_s + V_c$).

$^f$Calculated as sum of the surface areas of the head and tail ($S_t = S_s + S_c$)
Fig. 2-3 Superimposed heat flow thermograms obtained during the initial (live cells; Curve A) and final (dead cells; Curve B) cooling trials for diploid sperm cells (or cells from tetraploid oysters) at a cooling rate of 20 °C/min obtained in the presence of Me₂SO. The negative values on the y-axis for the heat flow implies an exothermic heat release in the DSC sample. The heat flow (mW/mg) is plotted along the y-axis and the sub-zero temperatures (°C) are plotted along the top x-axis and time (sec) is plotted on the bottom x-axis.
Note that the DSC measured heat release readings $\Delta q(T)_{dsc}$ and $\Delta q_{dsc}$ were obtained separately at 5 °C/min (data not shown) and at 20 °C/min (as shown in Fig. 2-3) in the absence and presence of Me$_2$SO. The unknowns needed in Eqn. (1) apart from the DSC heat release reading are $V_o$ (the initial or the isotonic cell volume – in this study, the oyster sperm cell is modeled as a "ball-on-stick" structure with the dimensions shown in Table 2-1 and Figure 2-4) and $V_b$ (the final osmotically inactive cell volume or $0.6V_o$; note that in the presence of 8% Me$_2$SO this value increases to $0.626V_o$). The osmotically inactive cell volume, $V_b$ was assumed to be $0.6V_o$ a value within the range reported for a variety of mammalian sperm (there is no any published values of $V_b$ of spermatozoa from any aquatic species) (Gao et al. 1997) – To further the understanding of the effect of $V_b$ on the predicted oyster membrane permeability parameters and the model simulations, additional calculations were also performed assuming, $V_b = 0.8V_o$ and $0.4V_o$.

2.3 Water Transport Model and Numerical Method

Kedem and Katchalsky (Kedem & Katchalsky 1958) proposed a model for water and solute transport in response to chemical potential gradients based on irreversible thermodynamics. If the flux of CPA is negligible in comparison to the water flux (McCaa et al. 1991), then the Kedem-Katchalsky model reduces to a model which assumes only water transport, as proposed by Mazur (Mazur 1963) and later modified by Levin et al. (Levin et al. 1976) and by Karlsson et al. (Karlsson et al. 1994). The model predicts the change in cell volume with decreasing temperature ($dV/dT$) after ice has formed outside of an unfrozen cell as,

$$L_p = L_{pa}^{[cap]} \cdot \exp \left( - \frac{E_{Lp}[cap]}{R} \left( \frac{1}{T_R} - \frac{1}{T} \right) \right)$$

with $L_p$, the sperm cell membrane permeability to water defined by Levin et al. (Levin et al. 1976) as,

$$\frac{dV}{dT} = - \frac{L_p A_c RT}{Bv_w} \left[ \ln \left( \frac{(V_o - V_b - n_{cpa}v_{cpa})/v_w}{(V_o - V_b - n_{cpa}v_{cpa})/v_w + (\varphi_s n_s + n_{cpa})} \right) - \frac{\Delta H_f v_w\rho}{R} \left( \frac{1}{T_R} - \frac{1}{T} \right) \right]$$
**Fig 2-4** Left: “ball-on-stick” model used to calculate the sperm cell volumes and surface areas from diploid and tetraploid Pacific oysters. Right: representative scanning electron micrographs of sperm from tetraploid Pacific oysters showing relationship of sperm head and tail in alignment with model used to calculate the haploid and diploid sperm cell volumes and surface areas (shown in Table 2-1).
where, $L_{pg}$ or $L_{pg}[cpa]$ is the reference membrane permeability at a reference temperature, $T_R (= 273.15 \text{ K})$ in the absence and presence of CPA; $E_{lp}$ or $E_{lp}[cpa]$ is the apparent activation energy (kJ/mol) or the temperature dependence of the cell membrane permeability in the absence and presence of CPA; $A_c$ is the effective membrane surface area for water transport, assumed to be constant during the freezing process; (see Table 2-1 and Fig. 2-4 for the geometric model of the oyster spermatozoa); $R$ is the universal gas constant; $B$ is the constant cooling rate (K/min); finally $C_i$ and $C_o$ represent the concentrations of the intracellular and extracellular (unfrozen) solutions. The various assumptions made in the development of the water transport are discussed in detail elsewhere (Devireddy et al. 2003; Gao et al. 1997; Kedem & Katchalsky 1958; McCaa et al. 1991; Mazur 1963; Levin et al. 1976; Karlsson et al. 1994; McGrath 1998; Smith et al. 1998) and are beyond the scope of the present study. The two unknown membrane permeability parameters of the model either $L_{pg}[cpa]$ and $E_{lp}[cpa]$ in the presence of CPA or $L_{pg}$ and $E_{lp}$ in the absence of CPA, are determined by curve-fitting the water transport model to experimentally obtained volumetric shrinkage data during freezing.

### Numerical Methods

A nonlinear least squares curve fitting technique was implemented using a custom written computer program to calculate the membrane permeability parameters that best fit the volumetric shrinkage data as previously described (Bevington & Robinson 1992). The optimal fit of Eqn. (2) to the experimental data was obtained by selecting a set of parameters which minimized the residual variance, and maximized a goodness of fit parameter, $R^2$ (McGrath 1998). To predict the membrane permeability parameters that produced a “combined best fit” to the experimental water transport data at two or more cooling rates, the nonlinear curve fitting code was slightly modified such that $R^2$ was maximized by one set of parameters for all cooling rates as previously described (Devireddy et al. 2004; Thirumala et al. 2003; Smith et al. 1998). To simulate the biophysical response of a sperm cell under a variety of cooling rates the best fit parameters were substituted in the water transport equation which was then numerically solved using a 4\textsuperscript{th} order Runge-Kutta method using a FORTRAN code on a Mac Powerbook G4 (Apple 5 Computer Inc, Cupertino, CA) workstation.
2.4 Results and Discussion

- Dynamic Cooling Response and Membrane Permeability Parameters

Fig. 2-5A shows the water transport data and simulation using best fit parameters in Eqn. (3) at cooling rates of 5 and 20 °C/min in the absence of CPAs for sperm cells of diploid Pacific oysters (or haploid sperm cells). The dynamic portion of the cooling curve was between -1.86 °C to -8 °C at these cooling rates. Water transport cessation was observed in the DSC heat release data as an overlap of the thermograms from the heat release signature obtained using osmotically active (initial) and inactive (final) cells, as seen in Fig. 2-3. The best fit of Eqn. (3) to the 5 °C/min diploid water transport data without Me$_2$SO was obtained for membrane permeability parameter values of $L_{pg} = 0.27 \times 10^{-15}$ m$^3$/Ns (0.0015 µm/min-atm) and $E_{Lp} = 61.9$ kJ/mole (14.8 kcal/mole) were with an $R^2$ value of 0.99, while the corresponding values for the 20 °C/min data: $L_{pg} = 0.36 \times 10^{-15}$ m$^3$/Ns (0.0020 µm/min-atm) and $E_{Lp} = 41.8$ kJ/mole (10.0 kcal/mole) were with an $R^2$ value of 0.97 (Table 2-2). Similarly, Fig. 2-5B shows the water transport data and simulation using the best fit parameters in Eqn. (3) at cooling rates of 5 and 20 °C/min in the presence of 8% (v/v) Me$_2$SO for haploid sperm cells. The dynamic portion of the cooling curve at these cooling rates was found to be between -2.9 °C to -11 °C. The best fit of Eqn. (3) to the 5 °C/min water transport data with Me$_2$SO was obtained for membrane permeability parameter values of $L_{pg} = 0.23 \times 10^{-15}$ m$^3$/Ns (0.0013 µm/min-atm) and $E_{Lp} = 51.4$ kJ/mole (12.3 kcal/mole) with an $R^2$ value of 0.99, while the corresponding values for the 20 °C/min data are $L_{pg} = 0.37 \times 10^{-15}$ m$^3$/Ns (0.0021 µm/min-atm) and $E_{Lp} = 45.6$ kJ/mole (10.9 kcal/mole) with an $R^2$ value of 0.97 (Table 2-2).

Figs. 2-6A and 2-6B show the water transport data and simulation using best fit parameters in Eqn. (3) at cooling rates of 5 and 20°C/min in the absence and presence of Me$_2$SO for sperm cells of tetraploid Pacific Oysters (or diploid sperm cells). The dynamic portion of the cooling curve at these cooling rates was found to be between -1.86 °C to -8 °C in the absence of CPAs and between –2.9 °C and –11 °C in the presence of 8% (v/v) Me$_2$SO as shown in Figures. 2-6A and 2-6B, respectively. The best fit of Eqn. (3) to the 5 °C/min tetraploid water transport data without Me$_2$SO was obtained for membrane permeability parameter values of $L_{pg} = 0.27 \times 10^{-15}$ m$^3$/Ns (0.0015 µm/min-atm) and $E_{Lp} = 43.1$ kJ/mole (10.3 kcal/mole) with an $R^2$ value of 0.99, while the corresponding values...
Fig. 2-5 Volumetric response of haploid sperm cells (or cells from diploid Pacific oysters) as a function of sub-zero temperatures obtained using the DSC technique in the presence of extracellular ice in the absence of CPAs (Figure A) and in the presence of extracellular ice and Me$_2$SO (Figure B). The open (○) and filled (●) circles represent the DSC water transport (volumetric shrinkage) data at the cooling rate of 5 and 20 °C/min. The dynamic cooling response at 5 and 20 °C/min is shown as a solid line (---) and was obtained by using the “best fit” membrane permeability parameters ($L_{pg}$ and $E_{lp}$ or $L_{pg,cpa}$ and $E_{lp,cpa}$) (Table 2-2) in the water transport equation (Eqns. 3 and 4). The non-dimensional cell volume is plotted along the y-axis and the sub-zero temperatures are shown along the x-axis. The error bars represent the standard deviation for the mean values of 6 DSC experiments.
Fig. 2-6 Volumetric response of diploid sperm cells (or cells from tetraploid Pacific oysters) as a function of sub-zero temperatures obtained using the DSC technique in the presence of extracellular ice but in the absence of CPAs (Figure A) and in the presence of extracellular ice and Me$_2$SO (Figure B). The open (○) and filled (●) circles represent the DSC water transport (volumetric shrinkage) data at the cooling rate of 5 and 20 °C/min. The model simulated dynamic cooling response at 5 and 20 °C/min is shown as a solid line (——) and was obtained by using the “best fit” membrane permeability parameters ($L_{pg}$ and $E_{lp}$ or $L_{pg}[cpa]$ and $E_{lp}[cpa]$) (Table 2-2) in the water transport equation (Eqns. 3 and 4). The non-dimensional volume is plotted along the y-axis and the sub-zero temperatures are shown along the x-axis. The error bars represent the standard deviation for the mean values of 6 DSC experiments.
Table 2-2. Predicted sub-zero membrane permeability parameters for pacific oyster sperm cells in the presence and absence of Me$_2$SO assuming $V_b = 0.6V_o$:

<table>
<thead>
<tr>
<th>Pacific Oyster Type (Sperm Cell)</th>
<th>Conc. of DMSO (v/v)</th>
<th>Cooling Rate (°C/min)</th>
<th>$L_{pg}$ or $L_{pg}[cpa]$ $x 10^{15}$ m$^3$/Ns (µm/min-atm)</th>
<th>$E_{lp}$ or $E_{lp}[cpa]$ kJ/mol (kcal/mole)</th>
<th>$R^2$ Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diploid</td>
<td>0% (No CPA)</td>
<td>5</td>
<td>0.27 (0.0015)</td>
<td>61.9 (14.8)</td>
<td>0.99</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20</td>
<td>0.36 (0.0020)</td>
<td>41.8 (10.0)</td>
<td>0.97</td>
</tr>
<tr>
<td></td>
<td><strong>CBF</strong></td>
<td></td>
<td><strong>0.30 (0.0017)</strong></td>
<td><strong>41.0 (9.8)</strong></td>
<td><strong>0.96</strong></td>
</tr>
<tr>
<td>(Haploid)</td>
<td>8%</td>
<td>5</td>
<td>0.23 (0.0013)</td>
<td>51.4 (12.3)</td>
<td>0.99</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20</td>
<td>0.37 (0.0021)</td>
<td>45.6 (10.9)</td>
<td>0.97</td>
</tr>
<tr>
<td></td>
<td><strong>CBF</strong></td>
<td></td>
<td><strong>0.27 (0.0015)</strong></td>
<td><strong>38.0 (9.1)</strong></td>
<td><strong>0.96</strong></td>
</tr>
<tr>
<td>Tetraploid</td>
<td>0% (No CPA)</td>
<td>5</td>
<td>0.27 (0.0015)</td>
<td>43.1 (10.3)</td>
<td>0.99</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20</td>
<td>0.50 (0.0028)</td>
<td>50.2 (12.0)</td>
<td>0.96</td>
</tr>
<tr>
<td></td>
<td><strong>CBF</strong></td>
<td></td>
<td><strong>0.34 (0.0019)</strong></td>
<td><strong>29.7 (7.1)</strong></td>
<td><strong>0.95</strong></td>
</tr>
<tr>
<td>(Diploid)</td>
<td>8%</td>
<td>5</td>
<td>0.30 (0.0017)</td>
<td>43.1 (10.3)</td>
<td>0.97</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20</td>
<td>0.37 (0.0021)</td>
<td>41.4 (9.9)</td>
<td>0.95</td>
</tr>
<tr>
<td></td>
<td><strong>CBF</strong></td>
<td></td>
<td><strong>0.34 (0.0019)</strong></td>
<td><strong>37.6 (9.0)</strong></td>
<td><strong>0.96</strong></td>
</tr>
</tbody>
</table>

*The combined best fit (CBF) parameters maximized the $R^2$ value, concurrently, at both 5 and 20 °C/min.

for the 20 °C/min data are $L_{pg} = 0.50 \times 10^{-15}$ m$^3$/Ns (0.0028 µm/min-atm) and $E_{lp} = 50.2$kJ/mole (12.0 kcal/mole) with an $R^2$ value of 0.96 (Table 2-2). Similarly, the best fit of Eqn. (3) to the 5 °C/min diploid sperm water transport data with 8% Me$_2$SO was obtained for membrane permeability parameter values of $L_{pg} = 0.30 \times 10^{-15}$ m$^3$/Ns (0.0017
µm/min-atm) and \( E_{Lp} = 43.1 \text{ kJ/mole (10.3 kcal/mole)} \) with an \( R^2 \) value of 0.97, while the corresponding values for the 20 °C/min data are \( L_{pg} = 0.37 \times 10^{-15} \text{ m}^3/\text{Ns (0.0021 µm/min-atm)} \) and \( E_{Lp} = 41.4 \text{ kJ/mole (9.9 kcal/mole)} \) with an \( R^2 \) value of 0.96 (Table 2-2).

- **Statistical Analysis**

All of the curve fitting results presented have an \( R^2 \) value greater than or equal to 0.95 indicating that there was a good agreement between the experimental data points and the fit calculated using the estimated membrane permeability parameters. The difference in the DSC water transport data at 5 and 20 °C/min were found to be statistically significantly from one another with 95% confidence level (in the dynamic part of the cooling curve) in all the combinations investigated. And finally, the differences in the water transport data, when compared across concentrations of CPA (i.e. comparing the water transport data obtained with and without Me\(_2\)SO) were found to be statistically significant with a confidence level, 95% (at both 5 and 20 °C/min).

- **Combined Best Fit Parameters**

As stated earlier, a new set of membrane permeability parameters (\( L_{pg} \) and \( E_{Lp} \) or \( L_{pg}[^{[\text{cpa}]}) \) and \( E_{Lp}[^{[\text{cpa}]}) \)) were obtained that produced a “combined best fit” to the experimentally determined water transport data (see Table 2-2). The “combined best fit” membrane permeability parameters maximized the goodness of fit parameter, \( R^2 \), for the 5 and 20 °C/min water transport data concurrently, as described in earlier studies. Fig. 2-7 shows the contour plots of the goodness of fit parameter, \( R^2 (= 0.95) \) in the \( L_{pg} \) and \( E_{Lp} \) (or \( L_{pg}[^{[\text{cpa}]}) \) and \( E_{Lp}[^{[\text{cpa}]}) \)) space that “fit” the water transport data at 5 and 20 °C/min in the freezing media without CPAs for diploid sperm cells (Fig. 2-7A) and for tetraploid sperm cells (Fig. 2-7B). Similar contours are shown in Figs. 2-7C and 2-7D for diploid and tetraploid oyster sperm cells in the presence of 8% Me\(_2\)SO. Any combination of \( L_{pg} \) and \( E_{Lp} \) (or \( L_{pg}[^{[\text{cpa}]}) \) and \( E_{Lp}[^{[\text{cpa}]}) \)) shown to be within the contour will “fit” the water transport data at that cooling rate with an \( R^2 \) value of > 0.95. Note that the contours for the higher cooling rate of 20 °C/min are smaller than those obtained at the lower cooling rate of 5 °C/min. This suggests that the membrane permeability parameters obtained using the 20 °C/min water transport data could predict the volumetric response of the sperm cell at the lower cooling rate of 5 °C/min accurately, while the converse is not true.
Fig. 2-7 Contour plots of the goodness of fit parameter $R^2 (= 0.95)$ for water transport response in Pacific oyster sperm cells in the presence and absence of Me2SO. The common region corresponds to the range of parameters that “fit” the water transport data at two cooling rates (5 or 20 °C/min) with $R^2 \geq 0.95$. Note that the “combined best fit” parameters are represented by a “*” in all the figures. The membrane permeability at 0 °C, $L_{pg}$ (or $L_{pg}[cpa]$) (m$^3$/Ns) is plotted on the y-axis while the apparent activation energy of the membrane, $E_{Lp}$ (or $E_{Lp}[cpa]$) (kJ/mole) is plotted on the x-axis. (A) Haploid sperm cells without Me2SO: The “combined best fit” parameters were, $L_{pg} = 0.30 \times 10^{-15}$ m$^3$/Ns (0.0017 µm/min-atm) and $E_{Lp} = 41.0$ kJ/mole (9.8 kcal/mole). (B) Diploid sperm cells without Me2SO: The “combined best fit” parameters were, $L_{pg} = 0.34 \times 10^{-15}$ m$^3$/Ns (0.0019 µm/min-atm) and $E_{Lp} = 29.7$ kJ/mole (7.1 kcal/mole). (C) Haploid sperm cells with Me2SO: The “combined best fit” parameters were, $L_{pg} = 0.27 \times 10^{-15}$ m$^3$/Ns (0.0015 µm/min-atm) and $E_{Lp} = 38.0$ kJ/mole (9.1 kcal/mole). (D) Diploid sperm cells with Me2SO: The “combined best fit” parameters were, $L_{pg} = 0.34 \times 10^{-15}$ m$^3$/Ns (0.0019 µm/min-atm) and $E_{Lp} = 37.6$ kJ/mole (9.0 kcal/mole).
• **Water Transport Simulations**

Water transport simulations obtained using the “combined best fit” parameters in Eqn. (3) are shown for a variety of cooling rates (5 - 100 °C/min) in Figure 2-8. In Figures 2-8A and 2-8B, the numerically simulated nondimensional cellular volume (V/V₀) obtained using the “combined best fit” parameters is shown for a variety of cooling rates (at 5, 20, 40, 50 and 100 °C/min) in the absence of CPAs for sperm cells from diploid and tetraploid Pacific oysters. Similar simulations are shown in Figures 2-8C and 2-8D for sperm cells from diploid and tetraploid Pacific oysters in the presence of 8% Me₂SO. The non-dimensional cellular volume (V/V₀), which decreases due to dehydration during freezing, is plotted on the y-axis while the sub-zero temperatures are plotted on the x-axis. From the simulations, the amount of trapped water (or a lower boundary on the intracellular ice) was computed as a ratio of the volume of the water trapped inside the sperm cell at the temperature, T (~-30 °C) where intracellular ice formation can occur by a homogenous or volume-catalyzed nucleation (Toner 1993) to the initial sperm water volume, [(V-V_b)/(V₀-V_b)] as described earlier for a several biological systems.

For haploid sperm cells (or cells from diploid Pacific oysters) in the absence of CPAs, for cooling rates of ≤ 40, 50 and 100 °C/min, the trapped water volume was ≤ 1.25%, 1.75% and 47.25% of the initial osmotically active water volume and the end volumes were ≤ 0.605V₀, 0.607V₀ and 0.789V₀ (Figure 2-8A). For haploid sperm cells, in the presence of 8% Me₂SO, for cooling rates of ≤ 20, 40, 50 and 100 °C/min, the trapped water volume was ≤ 1.60%, 2.41%, 11.50% and 52.41% of the initial osmotically active water volume and the end volumes were ≤ 0.633V₀, 0.635V₀, 0.669V₀ and 0.822V₀, (Figure 2-8C). Similarly, for diploid sperm cells (or cells from tetraploid Pacific oysters) in the absence of CPAs, for cooling rates of ≤ 50 and 100 °C/min, the trapped water volume was ≤ 1.25% and 37.5% of the initial osmotically active water volume and the end volumes were ≤ 0.605V₀ and 0.742V₀ (Figure 2-8B). And finally, for diploid sperm cells in the presence of 8% Me₂SO, for cooling rates of ≤ 20, 40, 50 and 100 °C/min, the trapped water volume was ≤ 1.60%, 2.41%, 12.30% and 52.94% of the initial osmotically active water volume while the end volumes were ≤ 0.632V₀, 0.635V₀, 0.672V₀ and 0.824V₀ (Figure 2-8D).
**Fig. 2-8** Volumetric response of Pacific oyster sperm cells at various cooling rates as a function of sub-zero temperature using the “combined best fit” membrane permeability parameters (Table 2-2). **A – D:** the changes in the normalized cell volume ($V/V_0$) are shown as a function of temperature for different cooling rates in haploid sperm cell suspensions without Me2SO (Figure 2-8A), in diploid sperm cell suspensions without Me2SO (Figure 2-8B), in haploid sperm cell suspensions without Me2SO (Figure 2-8C) and in diploid sperm cell suspensions with Me2SO (Figure 2-8D). The water transport curves (________) plotted in Figure 2-8 represent the model simulated response for different cooling rates (from left to right: 5, 20, 40, 50 and 100 °C/min). The sub-zero temperatures are shown along the x-axis while the nondimensional volume is plotted along the y-axis.
As stated in the introduction, the cooling rate which optimizes cell survival after a freeze-thaw of any cellular system can be defined as the fastest cooling rate in a given media without forming damaging intracellular ice formation, IIF (Mazur et al. 1972). Mazur (Mazur 1990) defines IIF as damaging and lethal if > 10-15% of the initial intracellular water is involved. In this study, the “optimal cooling rate” was defined as the cooling rate at which 5% of the initial osmotically active water volume is trapped inside the cells at -30°C. Note, that if intracellular ice formation (IIF) occurs by a heterogeneous or a surface catalyzed nucleation mechanism (Toner 1993) (generally between -5 and -20 °C for a variety of single cells), which present model does not predict, then potentially even more water will be trapped in the sperm cells than predicted by water transport alone (i.e. the lower boundary of intracellular ice discussed above). Thus, the “optimal cooling rates” based on the lower boundary of intracellular ice are probably overestimated. Based on simulations (shown in Figure 2-6) obtained using the combined best fit parameters the “optimal cooling rate” in the absence and presence of a CPA, for haploid sperm cells are ~ 53°C/min and ~44 °C/min. Similarly, the “optimal cooling rates” for in the absence and presence of a CPA, for diploid oyster sperm cells are ~63 °C/min and ~43 °C/min.

- Parameter Sensitivity Analysis - Effect of Varying the Osmotically Inactive Cell Volume (Vb)

The value of the osmotically inactive cell volume of mammalian sperm cells has been reported to range from 0.6Vo (the value used in this and other studies) to as low 0.25Vo and as high as ~0.80 Vo (Devireddy et al. 1999c; Devireddy et al.2000; Devireddy et al.2002a, Devireddy et al.2002b; Devireddy et al.2004; Thirumala et al. 2003; Gao et al. 1997). To study the effect of varying the osmotically inactive cell volume on the predicted membrane permeability parameters (Lpg and E_Lp or Lpg [cpa] and E_Lp [cpa]), the value of Vb was increased to 0.8Vo and decreased to 0.4Vo. The DSC data was correspondingly modified (using Eqn. 1) and the modified data were fitted to the water transport model (Eqns. 2 and 3) using the nonlinear least squares curve fitting technique as previously described. The predicted values of the membrane permeability parameters using a osmotically inactive cell volume of 0.4Vo and 0.8Vo were obtained in the presence and absence of Me2SO (data not shown in the interest of brevity). The predicted
optimal cooling rates obtained using these parameters were in good agreement (± 10%) to the values obtained with $V_b$ of 0.6 $V_o$ described earlier. Thus, the variation in the value of $V_b$ did not significantly alter the model predictions, and as noted earlier depending on the concentration of Me$_2$SO, cooling rates as low as 40 to 70 °C/min can cause a significant volume of intracellular water to be trapped inside the oyster spermatozoa.

- **Parameter Sensitivity Analysis - Effect of Varying the Cell Geometry**

To study the effect of varying the cell membrane surface ($A_c$) and cell volume ($V_o$) on the predicted membrane permeability parameters ($L_{pg}$ and $E_{lp}$ or $L_{pg}$ [cpa] and $E_{lp}$ [cpa]) it was assumed that the oyster sperm cells can be modeled as isolated spherical cells or as heads alone (with diameter of 1.66 µm and 2.14 µm for haploid and diploid sperm cells; see Table 2-1). The corresponding membrane permeability parameters ($L_{pg}$ and $E_{lp}$ or $L_{pg}$ [cpa] and $E_{lp}$ [cpa]) are shown in Table 2-3. Additional numerical simulations were performed assuming a spherical model for the oyster sperm cells and using the “combined best fit” parameters (Table 2-3) in the water transport model (Eqns. 2 and 3). As before, an analysis of these simulations was performed to predict the lower boundary on trapped intracellular ice and the optimal rate of freezing oyster sperm cells. The predicted optimal cooling rates of haploid sperm cells in the absence and presence of 8% Me$_2$SO, assuming a spherical model are ~62°C/min and ~47 °C/min. Similarly, the “optimal cooling rates” in the absence and presence of Me$_2$SO, for diploid oyster sperm cells are ~42 °C/min and ~39 °C/min. These optimal cooling rate values are in the same range as those obtained with the “ball-on-stick” model, presented earlier.

Independent verification of the predicted optimal cooling rates is obtained by comparing the experimentally determined optimal rates of freezing reported in the literature for Pacific oyster sperm cells (Lannan 1971; Staeger 1973; Usuki et al. 1997; Kurokura et al. 1990; Dong et al. 2002) and other oyster sperm cells (Paniagua-Chavez CG et al. 2001; Hughes 1973; Zell et al. 1979; Yankson et al. 1991). In general, Pacific oyster sperm cells are found to exhibit the highest post-thaw function (cell viability and relative larval yield) when cooled at 40 to 80 °C/min in the presence of 8% (v/v) Me$_2$SO (Usuki et al. 1997). However further empirical studies are needed to corroborate the predicted optimal rates of freezing haploid and diploid Pacific oyster sperm cells reported in the present study.
**Table 2-3.** Predicted sub-zero membrane permeability parameters for pacific oyster sperm cells in the presence and absence of Me$_2$SO assuming V$_b$ = 0.4V$_o$:

<table>
<thead>
<tr>
<th>Pacific Oyster Type (Sperm Cell)</th>
<th>Conc. of DMSO (v/v)</th>
<th>Cooling Rate ($^\circ$C/min)</th>
<th>$L_{pg}$ or $L_{pg}$ [cpa] x $10^{15}$ m$^3$/Ns</th>
<th>$E_{lp}$ or $E_{lp}$ [cpa] (μm/min-atm)</th>
<th>$E_{lp}$ or $E_{lp}$ [cpa] (kJ/mol kcal/mole)</th>
<th>$R^2$ Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diploid (Haploid)</td>
<td>0%</td>
<td>5</td>
<td>0.34 (0.0019)</td>
<td>39.3 (9.4)</td>
<td>0.99</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>20</td>
<td>0.53 (0.0030)</td>
<td>42.6 (10.2)</td>
<td>0.97</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CBF$^a$</td>
<td></td>
<td>0.46 (0.0026)</td>
<td>38.0 (9.1)</td>
<td>0.96</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8%</td>
<td>5</td>
<td>0.32 (0.0018)</td>
<td>52.3 (12.5)</td>
<td>0.99</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>20</td>
<td>0.52 (0.0029)</td>
<td>42.3 (10.1)</td>
<td>0.98</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CBF$^a$</td>
<td></td>
<td>0.45 (0.0025)</td>
<td>40.5 (9.7)</td>
<td>0.96</td>
<td></td>
</tr>
<tr>
<td>Tetraploid (Diploid)</td>
<td>0%</td>
<td>5</td>
<td>0.37 (0.0021)</td>
<td>44.3 (10.6)</td>
<td>0.99</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>20</td>
<td>0.68 (0.0038)</td>
<td>41.8 (10.0)</td>
<td>0.97</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CBF$^a$</td>
<td></td>
<td>0.52 (0.0029)</td>
<td>28.8 (6.9)</td>
<td>0.95</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8%</td>
<td>5</td>
<td>0.48 (0.0027)</td>
<td>43.9 (10.5)</td>
<td>0.97</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>20</td>
<td>0.57 (0.0032)</td>
<td>43.5 (10.4)</td>
<td>0.95</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CBF$^a$</td>
<td></td>
<td>0.61 (0.0034)</td>
<td>51.4 (12.3)</td>
<td>0.95</td>
<td></td>
</tr>
</tbody>
</table>

$^a$The combined best fit (CBF) parameters maximized the $R^2$ value, concurrently, at both 5 and 20 $^\circ$C/min.

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**Effect of Varying the Cell Geometry**

The reference membrane permeability ($L_{pg}$ or $L_{pg}$ [cpa]) values obtained using the spherical cell (heads alone) model (Table 2-3) are uniformly higher than those obtained using the “ball-on-stick” (head and tail) model (Table 2-2). This increase in the predicted
membrane permeability between the “ball-on-stick” and the spherical cell model is not surprising, because Eqn. (2) shows that the change in the oyster sperm cell volume as a function of temperature (dV/dT) is proportional to the product of \( L_p \) and \( A_c \). Note that the spherical cell model has lower surface area available for water transport than the “ball-on-stick” model (Table 2-1). Thus, for a given change in the oyster sperm cell volume as a function of temperature (dV/dT), a decrease in Ac will cause a corresponding increase in the predicted value of \( L_{pg} \) and \( L_{pg \ [cpa]} \) where \( L_p = f (L_{pg \ [cpa]}, E_{lp}) \) and \( L_{pg \ [cpa]} = f (L_{pg \ [cpa]}, E_{lp\ [cpa]}) \). Thus any changes to the geometrical model of the oyster sperm cell (specifically membrane surface area, Ac and isotonic cell volume, \( V_o \)) should manifest themselves with corresponding changes to the model predicted membrane permeability parameters (\( L_{pg} \) and \( E_{lp} \) or \( L_{pg \ [cpa]} \) and \( E_{lp\ [cpa]} \)) (Devireddy et al. 1999a).

- **Effect of Cooling Rate on Predicted Membrane Permeability Parameters**

There was good agreement (±20%) in the predicted value of the activation energy, \( E_{lp} \) (or \( E_{lp\ [cpa]} \)) at 5 and 20 °C/min for haploid and diploid sperm cells in the absence and presence of Me\(_2\)SO (with the exception of diploid sperm cells with an assumed \( V_b \) of 0.8 \( V_o \)). This lack of variation in the activation energy values between 5 and 20 °C/min, suggests that the oyster sperm cells cooled at 20 °C/min underwent complete dehydration and the final end volume was comparable to the assumed value of the osmotically inactive cell volume. This assertion is further supported by the observations that: a) the magnitude of the DSC measured difference in heat release, \( \Delta q_{dsc} \), was found to be constant between 5 and 20 °C/min; and b) no secondary heat release was observed at 20 °C/min to suggest incomplete dehydration, as was the case for Esptein Barr Virus Transformed lymphocytes (Devireddy et al. 1998) and liver tissue of a freeze-tolerant wood frog, *Rana sylvatica* (Devireddy et al. 1999b). As stated in earlier studies (Devireddy et al. 1999c; Devireddy et al.2000; Devireddy et al.2002a, Devireddy et al.2002b; Devireddy et al.2004; Thirumala et al. 2003), a major disadvantage of the DSC technique is its inability to distinguish heat released due to water transport and IIF. Based on present results, it is reasonable to presume that oyster sperm cells cooled at 20 °C/min did in fact undergo complete dehydration (where water transport was the dominant biophysical mechanism) and hence the DSC results at the higher cooling rate of 20 °C/min were indicative of the rate of cellular water from oyster sperm cells.
Effect of CPAs on Membrane Transport Parameters

The DSC technique was used to obtain water transport data and water permeability parameters \( (L_{pg\,cpa} \text{ and } E_{lp\,cpa}) \) of oyster sperm cells in the presence of Me\(_2\)SO. Although, the exact mechanism by which the presence of Me\(_2\)SO or other CPAs modifies the water permeability parameters is as yet unknown, several studies have shown that the presence of CPAs tends to reduce the membrane permeability parameters, \( L_{pg\,cpa} \) and \( E_{lp\,cpa} \) (Devireddy et al. 1999c; Devireddy et al. 2000; Devireddy et al. 2002a; Devireddy et al. 2002b; Devireddy et al. 2004; Thirumala et al. 2003; Smith et al. 1998; Gilmore et al. 1995). This is at least partially the case in present study, where an increase in the concentration of solutes in the extracellular medium was shown to reduce, although not significantly, the predicted value of membrane permeability parameters for Pacific oyster sperm cells. However, the addition of Me\(_2\)SO actually resulted in an increase in the predicted values of the “combined best fit” membrane permeability parameters for tetraploid sperm cells. Further studies are clearly needed to elucidate the mechanism by which CPAs modify membrane permeability parameters.

Effect of Oyster Cell Type on Membrane Transport Parameters

As stated earlier, the differences in the measured water transport data between haploid and diploid oyster sperm cells were not statistically significant. It is possible that the DSC technique cannot distinguish the “subtle” differences in water transport between these cells. This possibility is small given a recent study on equine sperm that demonstrated differences in volumetric shrinkage among samples that were collected and cooled to 4 °C under different conditions, including osmotic shock and cold shock (Devireddy et al. 2002b). Thus it is likely that the ability of oyster sperm cells to shrink in the presence of extracellular ice during a thermal insult is independent of the ploidy level. However, differences in the size of the sperm cells did result in different membrane permeability parameters for haploid and diploid oyster sperm cells. Cursory evaluation of the predicted membrane permeability parameters didn’t determine any consistent effect of cell type on the predicted parameters \( (L_{pg} \text{ and } E_{lp} \text{ or } L_{pg\,cpa} \text{ and } E_{lp\,cpa}) \). Also, an examination of the contour plots shown in Figure 2-7 suggested that the parametric space which would “fit” the haploid sperm cell water transport data is significantly larger than it is for diploid sperm cells (comparing the contours of Figure 2-
7A with Figure 2-7B, especially at 5 °C/min in the absence of Me$_2$SO; and the contours of Figure 2-7C with that of Figure 2-7D, especially at 20 °C/min in the presence of Me$_2$SO). This suggests that the membrane permeability parameters obtained for the diploid sperm cells would predict the volumetric response of the haploid sperm cell quite accurately, while the converse was not true. Further illustration of this observation was obtained by comparing the predicted optimal cooling rates with diploid sperm cell parameters and the haploid sperm cell volume and surface area with those obtained earlier with haploid cell parameters and haploid cell dimensions (41 vs. 53 °C/min in the absence of Me$_2$SO and 34 vs. 44 °C/min with Me$_2$SO or ±22% of each other). Conversely, the predicted optimal cooling rates with haploid sperm cell parameters and the diploid cell volume and surface area with those obtained earlier with diploid cell parameters and diploid cell dimensions were significantly different (83 vs. 63 °C/min in the absence of Me$_2$SO and 57 vs. 43 °C/min with Me$_2$SO or ±33% of each other). It is as yet unclear if this a general effect related to the ploidy level of the sperm cells or an unclear if this a general effect is related to the ploidy of the sperm cells or an unique result for diploid and tetraploid oyster sperm cells from *Crassostrea gigas*. 
CHAPTER 3
DETERMINATION OF SOLUTE AND SOLVENT PERMEABILITY PARAMETERS IN ARTIFICIAL OR NATIVE TISSUE SECTIONS: AN INVERSE APPROACH

3.1 Introduction

As stated earlier, for single cells, the use of low temperature to store them has been successfully applied to a variety of mammalian systems (Mazur 1984; McGrath 1988). In biomedical research, the most extensive use of animal and human tissue slices is associated with in vitro metabolism studies of candidate drug, safety evaluation of food additives, pesticides and other chemicals. A reliable technique for cryopreservation of tissue slices will help researchers make better use of tissues when they become available.

In a typical cryopreservation protocol, the tissue to be preserved is first loaded with chemical additives or cryoprotective agents (CPAs) to alleviate cell damage from either dehydration or intracellular ice formation (IIF) during the subsequent freezing process. The tissue is reduced to a predefined temperature (liquid nitrogen temperature) for long term storage. Before the tissue is transplanted, it must be thawed and the added CPAs must be removed. To ensure the viability and functionality of the tissue, each step in the above cryopreservation protocol should be handled in a precisely controlled and optimized manner.

When CPAs are added to a tissue section, due to the concentration difference between the vascular space and intracellular space, intracellular water (solvent) goes out, at the same time, permeable CPAs (solute) moves into the intracellular space. The addition (and removal) of CPAs causes the osmotic injury (due to volumetric changes) and chemical injury (due to chemical toxicity and exposure time) to the tissue cells. The mass exchange between the vascular space and intracellular space results in the osmotic injury. While a prolonged CPA loading time will result in chemical injury and negatively impair their viability. Typical loading procedures include loading CPAs stepwise (reducing the volumetric excursions and the consequent osmotic injury) and loading CPAs at temperature close to 0 °C (reducing metabolic activity and consequently the chemical toxicity). Clearly, the rates of addition and removal of cryoprotective additives
affect the outcome of a freeze preservation protocol. Optimization of the cell addition
and removal procedures requires knowledge of the tissue cell membrane permeability
parameters to both the solvent (intracellular water) and the solute (or the CPA). These
parameters are typically obtained by curve-fitting measured variations in the cell volume
during a predefined chemical loading procedure to a well defined model of couple water
and CPA transport defined as the Kedem-Katchalsky (K-K) formalism (Kedem &
Katchalsky 1958). In the original K-K formalism, three parameters, the hydraulic
permeability \( L_p \), solute permeability \( \omega \) and reflection coefficients \( \sigma \), are used to
characterize the membrane. Recently, it was (Kleinhans 1998) demonstrated that a two-
parameter model using only \( L_p \) and \( \omega \) yields essentially the same result as the original K-
K formulation (when there is no strong evidence or reason to suspect significant levels of
solute-solvent co-transport). The reflection coefficient, \( \sigma \) can be expressed as a function
of \( L_p \) and \( \omega \) and the original 3 parameter K-K formulation can be reduced to a much
simpler two-parameter model that is used in the present study.

On the basis of a non-equilibrium model based on the K-K formalism, Levin and
Miller (Levin & Miller 1981) and Levin (Levin 1981) devised an optimum method for
the introduction and the removal of permeable cryoprotectants from single, isolated cells
and perfused organs so that “osmotic” injury can be minimized. Recently, Bhowmick and
his colleagues (Bhowmick et al. 1998) presented an experimental and numerical study of
a non-permeating CPA (sucrose) in liver tissue sections. However, most of the published
solute-solvent models are for isolated single cells (McGrach et al. 1992; Gao et al. 1995)
or in tissue sections for only solvent transport (Bhowmick et al. 1998). In the present
study, a generic numerical model is developed to simulate the coupled solute and solvent
transport in arbitrary tissue sections. The model accounts for the axial and radial diffusion
of the solute as well as axial convection, and a constant or variable vascular surface area
\( A_c \). In addition, the model also accounts for the radial movement of the solvent (water)
to and from the vascular spaces. By fitting published experimental data (Me2SO
permeation data in artificial tissues) recently obtained by Bidault et al. (Bidault et al.
2001) to our numerical model, seminal tissue membrane permeability parameters are
obtained. The predicted tissue cell membrane permeability parameters can now be used
to predict optimal chemical loading and unloading procedures under arbitrary CPA concentrations for whole tissue sections.

3.2 Mathematical Model

The two main components being transported in the tissue systems are solvent (water) and solute (Me$_2$SO). Several assumptions were made in the development of our numerical model, presented here: 1) the salt equilibration is much faster than that of CPAs (Levin 1981; Bhowmick et al. 1998); 2) the boundary condition at the edge of the tissue is equal to the overall concentration of CPAs solution and that there is no unmixed layer of CPAs and water at the boundary (Bhowmick et al. 1998) and 3) the artificial tissue section could be approximated by a series of Krogh cylinders (Bhowmick et al. 1998; Krogh 1919).

As stated earlier, the K-K formalism has been extensively used in the literature to analyze non-equilibrium osmotic flows and net solute movements in biological systems (Bidault et al. 2001; Katchalsky et al. 1965; Diller et al. 1983) and is generally presented as,

\[ J_V = L_p \Delta p - RT \Delta C_w - \sigma \Delta C_{CPA} \]  
\[ J_{CPA} = \Delta C_{CPAAVE}(1 - \sigma)J_V + RT\omega \Delta C_{CPA} \]

where $J_V$ represents the total flux across the cell membrane, $J_{CPA}$ represents the solute (CPA) flux across the cell membrane, $R$ is the universal gas constant, $T$ is the temperature in K, $L_p$ is the cell water permeability, $\sigma$ is the solute reflection coefficient ($\sigma \leq 1$), $\omega$ is the solute permeability coefficient, $\Delta p$ represents the hydrostatic pressure difference between the extracellular and intracellular space (generally assumed to be zero in mammalian cells), $\Delta C_w$ and $\Delta C_{CPA}$ represent the concentration difference between the intracellular and extracellular space for water and CPA, respectively. And finally, the $\Delta C_{CPAAVE}$ represents the “log mean” osmolality of the permeable solute as,

\[ \Delta C_{CPA} = \frac{\Delta C_{CPA}}{\ln(C_{CPA}^O/C_{CPA}^I)} \]

At equilibrium the fluxes of both permeable solute and water into and out of a cell will be zero. Consequently, from the K-K equations it follows that for mammalian cells where $\Delta p = 0$, the osmolality difference of the permeable and impermeable solutes will also be zero at equilibrium (4). A cursory
examination of Eqn. [2] reveals that $J_{\text{CPA}}$ will be zero (or the cell membrane will be impermeable to the solute) when $\omega = 0.0$ and $\sigma = 1.0$. As stated earlier, the solute reflection coefficient, $\sigma$, will be modeled as a function of $L_p$ and $\omega$ as $\sigma = 1 - \frac{\bar{v}_{\text{CPA}}}{L_p}$, where $\bar{v}_{\text{CPA}}$ is the partial mole volume of the CPA (Kleinhans 1998). Thus, reducing the 3 parameter K-K formalism to a simpler 2 parameter formulation.

The transport of solute and solvent in the vascular spaces is governed by the coupled convection - diffusion equation, given as (Bhowmick et al. 1998),

$$\frac{\partial C_I}{\partial t} + \frac{\partial (vC_I)}{\partial x} = D \frac{\partial^2 C_I}{\partial x^2}$$

(3)

where $\frac{\partial C_I}{\partial t}$ represents the local unsteady CPA concentration term; $\frac{\partial (vC_I)}{\partial x}$ represents the convection transport term, $v$ represents the local convective velocity; $D \frac{\partial^2 C_I}{\partial x^2}$ is the diffusion term, assuming a constant diffusivity. The subscript I denotes the species being conserved (I represents the corresponding CPA). Accounting for conservation of mass, it can be found that for each Krogh cylinder (tissue cell unit) $(\rho Av)_{\text{in}} = (\rho Av)_{\text{out}}$

where $\rho$ is the density of the solution, $A$ is the cross sectional area of the vascular space, $v$ is the convective velocity of the solute/solvent flux. Using Eqns. 1 to 3, along with a control volume analysis, a numerical solution was developed using a fully implicit finite element method, following the procedure detailed by Bhowmick et al. (Bhowmick et al. 1998). The finite element code was validated by comparing the predicted volumetric excursions with published simulations obtained earlier by Levin and Miller (Levin & Miller 1981) and other investigators (Levin 1981; McGrach et al. 1992; Gao et al. 1995) for single cells and tissues (Bhowmick et al. 1998). Under similar conditions, the predictions from the current formulation are within ±0.1% to those obtained earlier by other investigators.
Fig. 3-1 A series of Krogh cylinders are used to represent the artificial tissue section. The characteristic dimensions are deduced from published experimental data: $X = 182.8 \, \mu m$, $R_v = 88.7 \, \mu m$ and $L = 30 \, \mu m$. The horizontal arrows in the Fig 3-1B represent convective solute (CPA) flow in the axial direction while the vertical arrows represent solvent (water) movement into the vascular space. Arrows in Figure 3-1C represent the solute movement into the intracellular space.
Artificial Tissue Unit (Krogh Model Dimensions)

Fig. 3-1A shows series of Krogh cylinders that are used to represent the artificial tissue section (Bidault et al. 2001). A single tissue cell is represented using a Krogh cylinder geometry (Krogh 1919; Pazhayannur et al. 1997). In the Krogh cylinder geometry, the cylinder represents the vascular space (vascular volume = $\pi R_V^2 L$) while the area surrounding the cylinder represents the cellular space (cell volume or $V_{IN} = L \Delta X^2 - \pi r_v^2 L$). Additionally, $\Delta X$ represents the distance between two adjacent cells, while $L$ is the length of the Krogh cylinder, an adjustable parameter, to match the is adjusted such that the total cell represents. In the Fig. 3-1B, the horizontal arrows depict the convective flow of the solute/solvent mixture in the axial direction while the vertical arrows depict the intracellular water entering the vascular area due to the osmotic gradient. Similarly, the arrows in the Fig. 3-1C depict the transport of CPA into the intracellular space.

The coupled solvent-solute transport model developed in the present study is capable of simulating either a constant or variable vascular surface area during the transport process. For simulating a constant vascular surface area, it can be assumed that all the water leaving the cellular space due to the osmotic gradient created by CPA will be convected into the adjacent Krogh cylinder or into the vascular space of the adjacent cell. For simulating a variable vascular surface area, we calculated the change in the radius of the vascular space based on the amount of cellular water entering and the amount of CPA leaving the vasculature at each time point for each Krogh cylinder. Or the change in the vascular radius, $R_V$ can be correlated to the model predicted change in the cellular volume, $\Delta V$ as, $\Delta V = \pi \cdot L \cdot \Delta R_V^2$ at each time step for each tissue cell unit. To facilitate an easy comparison of our model predictions with the experimentally measured data (Bidault et al. 2001), the model was modified to generate the normalized water concentration at various locations within the tissue as a function of time.

A nonlinear least squares curve fitting technique was implemented using a custom written computer program to calculate the membrane permeability parameters that best fit the normalized water concentration data as previously described (Bevington & Robinson 1992). The optimal fit of Eqns. (1&2) to the experimental data was obtained by selecting
a set of parameters which minimized the residual variance, $\chi^2$, and maximized a goodness of fit parameter, $R^2$ (Pazhayannur et al. 1997; Bevington & Robinson 1992; Montgomery et al. 1994). To predict the membrane permeability parameters that produced a “combined best fit” to the experimental water transport data at three different locations in the tissue section, the nonlinear curve fitting code was slightly modified such that $R^2$ was maximized by one set of parameters for all locations (Devireddy et al. 1999; Thirumala et al. 2003). All the curve fitting results presented have an $R^2$ value greater than or equal to 0.89 indicating that there was a reasonably good agreement between the experimental data and the fit calculated using the predicted membrane permeability parameters.

- **Model Verification & Parameter Study**

As stated in the introduction, the addition and removal of CPA from single cells is fairly well understood and can be measured with microscope techniques and modeled with irreversible thermodynamics as expressed in the equations of K-K; the model is extended to tissue in this study.

**Table 3-1** Cryoprotectant permeation characteristics for different relationship between solute and solvent

<table>
<thead>
<tr>
<th>Case Number</th>
<th>$R_{vin}$ [µm]</th>
<th>$L_p$ ($\times 10^{14}$) [m²/(N·sec)]</th>
<th>$\omega$ ($\times 10^{9}$) [mol/(N·sec)]</th>
<th>$\sigma$</th>
<th>Condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.8</td>
<td>31</td>
<td>4.35</td>
<td>0</td>
<td>solute as permeable as water</td>
</tr>
<tr>
<td>2</td>
<td>3.8</td>
<td>31</td>
<td>1.09</td>
<td>0.75</td>
<td>solute less permeable than water</td>
</tr>
<tr>
<td>3</td>
<td>3.8</td>
<td>31</td>
<td>0</td>
<td>1.0</td>
<td>solute impermeable</td>
</tr>
</tbody>
</table>

In previous published paper, Levin simulated a single, isolated cell response to CPA loading (Levin & Miller 1981). It should be similar to the cells at the edge of the tissue. Fig 3-2 shows that cell volume excursion in the time scale at the edge of the tissue. Table 3-1 shows the corresponding permeability parameters used in the Fig 3-2.
Fig. 3-2 Representative variations with time of the volume of a single cell of the tissue (at the edge) suddenly exposed to a hypertonic permeable solute solution.

Case 1, 2, 3 are the same condition with the case 1, 2, 3 in Figure 2-2.

Fig. 3-3 Representative variations with time of the vascular radius of a single cell of the tissue (at the edge) suddenly exposed to a hypertonic permeable solute solution.
Fig 3-2 shows the cells at the edge respond due to the concentration difference in 3 different ways: when the solute is as permeable as water (case 1), the cells will monotonically swell to a new equilibrium volume; when solute is less permeable than water (case 2), the cells will initially shrink, after reaching an excursion point, then swell to a new equilibrium volume; when the cell membranes are completely impermeable to the CPA (case 3), then the cells will monotonically shrink to a new equilibrium volume.

The normalized vascular radius is shown in Fig 3-3. When the solute is as permeable as water (case 1), vascular radius always goes down, until it reaches to equilibrium and it will be decreased by 36.8%; when solute is less permeable than water (case 2), vascular radius will increase first, after it reaches to an extreme value (the ratio of vascular radius to initial vascular radius is 145%), it decreases to an equilibrium state (the ratio of vascular radius to initial vascular radius is 31.8%). The equilibrium state is restricted by the concentration difference between the extracellular and vascular fluid. So the vascular radius value should be the same when it reaches to equilibrium. But, for case 3, because

![Fig. 3-4](image.png)

**Fig. 3-4** Representative variations with time of the volume of a single cell of the tissue (at the different position of the tissue) exposed to a hypertonic permeable solute solution.
solute is impermeable, solute won’t get in, so the equilibrium is not the same with the former two. When it reaches an equilibrium state, the ratio of vascular radius to initial vascular radius is 242%.

In this study, the model was extended to the whole tissue by introducing the axial convection of the solute. It is evident from Fig 3-4 that at the different positions within the tissue, the volumetric shrinkage response are different from each other; at the edge, it response fastest, and the minimum value of the normalized cell volume can be 0.804; the second is the middle point, but the minimum value of the normalized cell volume is 0.977; the position of line of the symmetry responses most slowly, and the minimum value is 0.984.

The result shown here also means that the axial solute convection into the inner part of the tissue is a time-consume process. For example, at the edge, the normalized cell volume reaches the extreme value (0.804) at 10 sec; at the middle point, the normalized cell volume reaches the extreme value (0.977) at 220 sec; it takes about 500 sec for cell volume to reach its excursion point at the line of symmetry.

![Fig. 3-5](image)

**Fig. 3-5** Representative variations with time of the volume (at the edge) by different CPA concentration.
The equilibrium state is restricted by the concentration difference between the extracellular and vascular space, so the concentration of CPA is a key factor to the coupled solute and solvent transport. Fig 3-5 shows the volumetric shrinkage response with different concentration difference at the edge of the tissue.

In case 1, it takes 10 seconds to reach the excursion point \((V/V_{in}=0.804)\); in case 2, it takes 6 seconds to reach the excursion point \((V/V_{in}=0.706)\); in case 3, it takes 4 seconds to reach the excursion point \((V/V_{in}=0.648)\). When it reaches the equilibrium (after 600 seconds), the values of normalized volume are 1.04, 1.07, 1.09.

The diffusion coefficient of CPA is also a crucial factor. With our model, we can verify the change the value of the diffusion coefficient doesn’t change the transport at the edge; it only affects the transport of the inner part of the tissue. In the Fig 3-6, it can be easily found that the tissue reaches the equilibrium faster with bigger diffusion coefficient. When the value of the diffusion coefficient is 0.028E-8m²/sec, it takes about 1500 sec to reach the equilibrium state; while when the value of the diffusion coefficient is 0.028E-9m²/sec, it takes more than 2400 seconds to reach the equilibrium state. These results mean that the value of the diffusion coefficient only affects the axial diffusion of CPA in present model.

![Fig. 3-6](image.png)

**Fig. 3-6** Representative variations with time of the volume (at the line of symmetry) by different diffusion coefficient. [unit: m²/sec]
The results of our numerical simulation indicate that the relationship of the membrane hydraulic permeability, membrane permeability to CPA and reflection coefficient affect the cells volume change. For tissues, the axial solute diffusion and axial solute convection must be accounted for. The diffusion coefficient of the solute is also a crucial factor to the process.

3.3 Results and Discussion

- Krogh Model Dimensions

Based on the information (shown in Fig. 3-7) provided in the earlier experimental study (Bidault et al. 2001), the characteristic dimensions of the Krogh cylinder model were found to be $\Delta X = 182.8 \mu m$, $R_V = 88.7 \mu m$, and $L = 30 \mu m$. The osmotically inactive cell volume of the embedded tissue cells was assumed to $0.4 \cdot V_{IN}$, where $V_{IN}$ is the initial isotonic cell volume. This assumption was found to have a minimal effect on the model predicted parameters, described below; varying the assumed osmotically inactive cell volume by a factor of 2, i.e. from $0.4 \cdot V_{IN}$ to either $0.8 \cdot V_{IN}$ or $0.2 \cdot V_{IN}$ only impacted the predicted membrane permeability parameters by $\sim \pm 20\%$. And finally to complete this model, the diffusivity of Me$_2$SO in the vascular space was assumed to be $1 \times 10^{-9} \text{ m}^2/\text{s}$ (McGrath 1985; Mudrew et al. 1996).

![Normalized water concentration as a function of time for a dermal replacement containing viable cells. The samples were perfused with a solution containing 10%v/v Me$_2$SO/D$_2$O solutions at 19°C.](image)
Membrane Permeability Parameters

Normalized water concentration simulations obtained using the “best fit” parameter model parameters are shown for different locations in Fig. 3-8A, assuming a constant vascular surface during the transport process. The normalized water concentration, which decreases due to the increasing concentration of CPA within the tissue, was plotted on the y-axis while the time (sec) is plotted on the x-axis. As shown in the simulations, the normalized water concentrations reach an equilibrium state at time periods ranging from 100 to 800 secs depending on the location within the artificial tissue. The “best fit” permeability values to the experimentally determined normalized water concentration data were obtained for membrane permeability parameters values of $L_p = 4.22 \times 10^{-14}$ m$^3$/N-sec and $\omega = 0$ mol/N-sec at the edge of the tissue exposed to the CPA ($x = 120 \mu$m); $L_p = 1.08 \times 10^{-14}$ m$^3$/N-sec and $\omega = 4.8 \times 10^{-13}$ mol/N-sec at a distance of 570 μm into the tissue section; and finally, $L_p = 0.59 \times 10^{-14}$ m$^3$/N-sec and $\omega = 6.0 \times 10^{-13}$ mol/N-sec at a distance of 1200 μm into the tissue (the corresponding solute reflection coefficient, $\sigma$ values along with the goodness of fit parameter, $R^2$ values are shown in Table 3-2). As

<table>
<thead>
<tr>
<th>Position [µm]</th>
<th>$L_p \times 10^{14}$ [m$^3$/(N-sec)]</th>
<th>$\omega \times 10^{13}$ [mol/(N-sec)]</th>
<th>$\sigma = 1 - \frac{\omega \sigma_{CPA}}{L_p}$</th>
<th>Goodness of fit $[R^2]$</th>
</tr>
</thead>
<tbody>
<tr>
<td>120</td>
<td>4.22</td>
<td>0</td>
<td>1.0</td>
<td>0.9892</td>
</tr>
<tr>
<td>570</td>
<td>1.08</td>
<td>4.8</td>
<td>0.9969</td>
<td>0.9529</td>
</tr>
<tr>
<td>1200</td>
<td>0.59</td>
<td>6.0</td>
<td>0.9930</td>
<td>0.9949</td>
</tr>
<tr>
<td>CBF*</td>
<td>1.6</td>
<td>0</td>
<td>1.0</td>
<td>0.8881</td>
</tr>
</tbody>
</table>

*The combined best fit (CBF) parameter maximized the $R^2$ value, concurrently, at 3 different locations.
Fig. 3-8 Experimental and numerical simulations of the normalized water concentration at various locations within the artificial tissue. The symbols (the open triangles, the open squares and the open diamonds) represent experimental data at different locations (120 µm, 570µm and 1200 µm) within the artificial tissue. The numerically simulated non-dimensional water concentration with the “best fit” ($L_p$ and $\omega$) parameters at different locations with constant vascular surface area (parameters in Table 3-1) are shown in Figure 3-8A (———); Figure 3-8B shows the numerically simulated non-dimensional water concentration plotted using the “combined best fit” parameters shown in Table 3-2. Similarly, the numerically simulated non-dimensional water concentration with the “best fit” and the “combined best fit” parameters assuming a variable vascular surface area (parameters in Table 3-3) are shown in Figures 3-8C and 3-8D (—).
stated earlier, a new of membrane permeability parameters \((L_p\) and \(\omega\)) were also obtained that produced a “combined best fit” to the experimental data at all the three locations, i.e., the “combined best fit” parameters maximized the goodness of fit parameter, \(R^2\), for the three locations concurrently. Fig. 3-8B is plotted using the “combined best fit” membrane permeability parameters, of \(L_p = 1.6 \times 10^{-14}\) m\(^3\)/N-sec and \(\omega = 0 \times 10^{-13}\) mol/N-sec with an \(R^2\) value of 0.89.

As stated earlier, due to the transport of the solvent and solute, the cellular and the vascular volumes are not “fixed” during the CPAs addition and removal process. The model was appropriately modified, to account for this behavior. The modified model predictions were “fit” to the experimental data and the best fit membrane permeability parameters were: \(L_p = 4.0 \times 10^{-14}\) m\(^3\)/N-sec and \(\omega = 0\) mol/N-sec at the edge of the tissue exposed to the CPA (\(x = 120\ \mu\)m); \(L_p = 1.088 \times 10^{-14}\) m\(^3\)/N-sec and \(\omega = 4.4 \times 10^{-13}\) mol/N-sec at a distance of 570 \(\mu\)m into the tissue section; and finally, \(L_p = 0.59 \times 10^{-14}\) m\(^3\)/N-sec and \(\omega = 6.6 \times 10^{-13}\) mol/N-sec at a distance of 1200 \(\mu\)m into the tissue (the corresponding solute reflection coefficient, \(\sigma\) values along with the goodness of fit parameter, \(R^2\) values are shown in Table 3-3). The numerically simulated non-dimensional water concentration excursion obtained using these “best fit” parameters at different locations are shown in Fig. 3-8C. Fig. 3-8D is plotted using the “combined best fit” membrane permeability parameters obtained assuming a variable vascular surface area, of \(L_p = 1.6 \times 10^{-14}\) m\(^3\)/N-sec and \(\omega = 0 \times 10^{-13}\) mol/N-sec with an \(R^2\) value of 0.90. It is interesting to note that the combined best fit parameters are exactly the same as those obtained earlier assuming a constant vascular surface area during the transport process.

**Statistical Analysis**

To analyze the sensitivity of the model to variations in the value of \(L_p\) and \(\omega\), a contour plot was generated. Fig. 3-9A shows the contour plots of the goodness of fit parameter, \(R^2\) (= 0.95) in the \(L_p\) and \(\omega\) space that “fit” the normalized water concentration excursion data at different locations within the artificial tissue (assuming a constant vascular surface area). Similar data obtained assuming a variable vascular surface area during the transport process are shown in Fig. 3-9B. Any combination of \(L_p\) and \(\omega\) shown to be within the contours (in Fig. 3-9A and 3-9B) will “fit” the normalized
Fig. 3-9 Contour plots of the goodness of fit parameter, $R^2 (=0.95)$. The region within each contour corresponds to the combination of parameters ($L_p$ and $\omega$) that “best fit” the normalized water concentration excursion data at three different locations with $R^2 \geq 0.95$. Figures 3-9A and 3-9B are plotted assuming a constant and variable vascular surface area, respectively. Note that the “best fit” parameters at each location are represented by a “*” and the “combined best fit” parameters are represented by a “+” (the numerical values are shown in Tables 3-2 and 3-3); The water permeability $L_p$ (m$^3$/N-sec) is plotted on the x-axis while the solute (CPA) permeability $\omega$ (mol/N-sec) is plotted on the y-axis.
water concentration excursion data at the location shown with an $R^2$ value > 0.95. It is interesting to note that, in the Figures. 3-9A and 3-9B, the contour space that best fits the experimental data obtained at the distance of 540 µm into the artificial tissue was significantly smaller than the corresponding contours at the inner and the outer most locations of the tissue section exposed to CPAs. Additionally, there was a progression of the contour space from high values of $L_p$ ($> 1.6 \times 10^{-14} \text{ m}^3/\text{N-sec}$) and low values of $\omega$ ($< 0.25 \times 10^{-12} \text{ mol/N-sec}$) at the edge of the tissue ($x = 120 \mu m$) to increasingly lower values of $L_p$ ($< 1.5 \times 10^{-14} \text{ m}^3/\text{N-sec}$) and higher values of $\omega$ (0 to $3.0 \times 10^{-12} \text{ mol/N-sec}$) as the location progresses into the tissue, (i.e. as $x$ increases from 120 µm to 1200 µm). And finally, the contour spaces obtained assuming a constant vascular surface area (Fig. 3-9A) are smaller (~10%) than the corresponding spaces obtained assuming a variable vascular surface area (Fig. 3-9B), at all locations. This result suggests that the permeability parameters obtained assuming a constant vascular surface area can predict reasonably accurately the behavior of the tissue cells with varying vascular surface area, while the converse might not be true.

Table 3-3 Predicted Kedem-Katchalsky model parameters at various locations with Me$_2$SO as the CPA, D=$1.0 \times 10^{-9} \text{ m}^2/\text{sec}$ at 292K, with variable vascular surface area in an artificial tissue.

<table>
<thead>
<tr>
<th>Position [µm]</th>
<th>$L_p \times 10^{14}$ [m$^3/(\text{N-sec})$]</th>
<th>$\omega \times 10^{13}$ [mol/(N-sec)]</th>
<th>$\sigma = 1 - \frac{\omega \bar{v}_{CPA}}{L_p}$</th>
<th>Goodness of fit $[R^2]$</th>
</tr>
</thead>
<tbody>
<tr>
<td>120</td>
<td>4.0</td>
<td>0</td>
<td>1</td>
<td>0.9839</td>
</tr>
<tr>
<td>570</td>
<td>1.088</td>
<td>4.4</td>
<td>0.9972</td>
<td>0.9551</td>
</tr>
<tr>
<td>1200</td>
<td>0.59</td>
<td>6.6</td>
<td>0.9923</td>
<td>0.9945</td>
</tr>
<tr>
<td>CBF*</td>
<td>1.6</td>
<td>0</td>
<td>1</td>
<td>0.8962</td>
</tr>
</tbody>
</table>

*The combined best fit (CBF) parameter maximized the $R^2$ value, concurrently, at 3 different locations.
- **Alternate Modeling Approach**

The results shown in Tables 3-2 and 3-3 and Fig. 3-8 and 3-9 were obtained assuming a constant value for the CPA (Me\textsubscript{2}SO) diffusivity into the vascular spaces, i.e. \(D = 1.0 \times 10^{-9} \text{ m}^2/\text{s}\). To investigate this assumption further and recognizing that in Tables 3-2 and 3-3 \(\omega \approx 0\) and \(\sigma \approx 1.0\) (which suggests that the artificial tissue cell membrane is impermeable to Me\textsubscript{2}SO), the model can be modified to predict the best fit values of \(L_p\) and \(D\), as described earlier. Using the modified model, a new set of membrane permeability parameters (\(L_p\) and \(D\)) were obtained that produced a “best fit” to the experimentally determined normalized water concentration excursion data. Tables 3-4 and 3-5 show the corresponding best fit values at various locations within the artificial tissue obtained assuming a constant and variable vascular surface area, respectively. The model simulated normalized water concentration data using the best fit and combined best fit values of \(L_p\) and \(D\) are shown in Fig. 3-10A to 3-10D. In Fig. 3-10A and 3-10C, the model predictions obtained using the best fit parameters at that location are plotted while in Fig. 3-10B and 3-10D, the model predictions obtained using the combined best parameters are shown. Fig. 3-11A and 3-11B delineate the contour space that best fit the experimental data at various locations in the artificial tissue using the alternate modeling approach. As before, any combination of \(L_p\) and \(D\) shown to be within the contours (in Fig. 3-11A and 3-11B) will “fit” the normalized water concentration excursion data at the location shown with an \(R^2\) value > 0.95. Although, the contour spaces were more “jagged” than those obtained with the earlier modeling effort, the contour space at \(x = 540 \mu\text{m}\) was smaller than the contour spaces at the other two locations. Intriguingly, with the alternate modeling approach the contour spaces obtained assuming a variable vascular area are somewhat smaller (~10%) than that obtained using constant vascular area. This suggests that, with the alternate modeling approach, the permeability parameters obtained assuming a variable vascular surface area can predict the behavior of the tissue cells with constant vascular surface area, while the converse might not be true. (This is in direct contrast to the results obtained with the earlier modeling efforts and shown in Fig. 3-9A and 3-9B).
Fig. 3-10 Experimental and numerical simulations (alternate modeling approach) of the normalized water concentration at various locations within the artificial tissue. The symbols (the open triangles, the open squares and the open diamonds) represent experimental data at different locations (120 µm, 570µm and 1200 µm) within the artificial tissue. The numerically simulated non-dimensional water concentration with the “best fit” (L_p and D) parameters at different locations with constant vascular surface area (parameters in Table 3-3) are shown in Figure 3-10A (————); Figure 3-10B shows the numerically simulated non-dimensional water concentration plotted using the “combined best fit” parameters shown in Table 3-3. Similarly, the numerically simulated non-dimensional water concentration with the “best fit” and the “combined best fit” parameters assuming a variable vascular surface area (parameters in Table 3-4) are shown in Figures 3-10C and 3-10D.
As shown in Tables 3-4 and 3-5, the predicted value of D varies from $1.52 \times 10^{-9}$ m$^2$/s to $0.63 \times 10^{-9}$ m$^2$/s (or approximately ±50% from the assumed value of D in the earlier modeling efforts). Note that the value of $L_p$ obtained using the alternate modeling approach shows the same trend with increasing value of x as that obtained earlier (i.e. comparing the values of $L_p$ in Tables 3-2 and 3-4 and in Tables 3-3 and 3-5). However, there is a huge increase (by a factor of 100) in the value of $L_p$ at $x = 120$ µm between both the modeling approaches. It is unclear if this is an artifact of the model or an intrinsic error in the measurement methodology. Future studies are warranted.

**Table 3-4** Predicted Kedem-Katchalsky model parameters at various locations for Me$_2$SO at 292K, with constant vascular surface area in an artificial tissue by alternate modeling approach, where $\omega = 0$ mol/(N-sec) and $\sigma = 1.0$.

<table>
<thead>
<tr>
<th>Position [µm]</th>
<th>$L_p$ (x10$^{14}$) [m$^3$/(N-sec)]</th>
<th>D (x10$^9$) [m$^2$/sec]</th>
<th>Goodness of fit $[R^2]$</th>
</tr>
</thead>
<tbody>
<tr>
<td>120</td>
<td>440</td>
<td>1.52</td>
<td>0.9885</td>
</tr>
<tr>
<td>570</td>
<td>3.8</td>
<td>0.70</td>
<td>0.9697</td>
</tr>
<tr>
<td>1200</td>
<td>3.8</td>
<td>0.63</td>
<td>0.9923</td>
</tr>
<tr>
<td>CBF*</td>
<td>4.0</td>
<td>0.70</td>
<td>0.9774</td>
</tr>
</tbody>
</table>

*The combined best fit (CBF) parameter maximized the $R^2$ value, concurrently, at 3 different locations.

Finally, the membrane permeability parameters obtained in the present study are comparable to previously published values for single isolated cells (McGrath 1985; Mudrew et al. 1996; McGann 1979; Wusteman et al. 2002; Walcerz et al. 1995; Fedorwo et al. 2001; Leibo 1979; Gilmore et al. 1997; Xu et al. 2003; Paynter et al. 1997; Shabana et al. 1988; LeGal et al. 1994). The published values of membrane permeability to water ($L_p$) range from $8.91 \times 10^{-16}$ m$^3$/N-sec (Wusteman et al. 2002) to $1.7 \times 10^{-13}$ m$^3$/N-sec (Walcerz et al. 1995). And the published values of single cell permeability to Me$_2$SO ($\omega$) ranges from $2.287 \times 10^{-13}$ mol/N-s (Walcerz et al. 1995) to $1.181 \times 10^{-10}$ mol/N-s.
(Fedorwo et al. 2001). Moreover, the published values of the diffusivity coefficient ($D$) range from $2.97 \times 10^{-11}$ to $1.32 \times 10^{-9}$ m$^2$/s (Bhowmick et al. 1998; McGrath 1985; Mudrew et al. 1996).

**Table 3-5** Predicted Kedem-Katchalsky model parameters at various locations for Me$_2$SO at 292K, with variable vascular surface area in an artificial tissue by alternate modeling approach, where $\omega = 0$ mol/(N-sec) and $\sigma = 1.0$.

<table>
<thead>
<tr>
<th>Position [\mu m]</th>
<th>$L_F \times 10^{14}$ [m$^3$/N-sec]</th>
<th>$D \times 10^9$ [m$^2$/sec]</th>
<th>Goodness of fit $R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>120</td>
<td>640</td>
<td>1.104</td>
<td>0.9879</td>
</tr>
<tr>
<td>570</td>
<td>3.2</td>
<td>0.74</td>
<td>0.9716</td>
</tr>
<tr>
<td>1200</td>
<td>2.1</td>
<td>0.76</td>
<td>0.9925</td>
</tr>
<tr>
<td>CBF*</td>
<td>3.0</td>
<td>0.75</td>
<td>0.9720</td>
</tr>
</tbody>
</table>

*The combined best fit (CBF) parameter maximized the $R^2$ value, concurrently, at 3 different locations.*
Fig. 3-11 Contour plots of the goodness of fit parameter, $R^2 (= 0.95)$ using the alternate modeling approach. The region within each contour corresponds to the combination of parameters ($L_p$ and $D$) that “best fit” the normalized water concentration excursion data at three different locations with $R^2 \geq 0.95$. Figures 3-11A and 3-11B are plotted assuming a constant and variable vascular surface area, respectively. Note that the “best fit” parameters at each location are represented by a “*” and the “combined best fit” parameters are represented by a “+” (the numerical values are shown in Tables 3-3 and 3-4). The water permeability $L_p$ (m$^3$/N·sec) is plotted on the x-axis while the solute (CPA) permeability $D$ (m$^2$/sec) is plotted on the y-axis.
CHAPTER 4
CONCLUSION

The success of low temperature preservation of biological systems requires a firm biophysical understanding of the cryopreservation process. Currently, the bulk of our knowledge in the cryopreservation of biological systems is still empirical in the nature. The intent of this study was to utilize experimental method and numerical simulation to investigate the transport processes during the cryopreservation of the biological systems.

In the first part of the thesis, water transport (volumetric shrinkage) was evaluated for haploid and diploid oyster sperm cells in the presence of extracellular ice and a CPA (Me₂SO) during freezing using the DSC technique at two different cooling rates (5 and 20 °C/min). This represents the first such effort for sperm cells from any aquatic species. The predicted “combined best fit” permeability parameters ranged from, \( L_{pg} \) or \( L_{pg} [cpa] = 0.23 \times 10^{-15} \) to \( 0.50 \times 10^{-15} \) m³/Ns (0.0013 to 0.0028 μm/min-atm) and \( E_{lp} \) or \( E_{lp} [cpa] = 37.6 \) to \( 61.9 \) kJ/mole (9.0 to 14.8 kcal/mole) while the predicted optimal rates of freezing ranged from 40 to 70 °C/min. It is hoped that the water permeability parameters presented in this study will help to establish cryopreservation of Pacific oyster sperm cells on a firm biophysical basis. Future studies should make direct comparisons of the optimal cooling rates predicted using the water transport models developed here with empirical values for spermatozoa of Pacific oysters, *Crassostrea gigas*.

In the second part of this thesis, a generic mathematical model based on a 2 parameter Kedem and Katchalsky formulation was developed to simulate the coupled solute and solvent transport in arbitrary tissue sections. The model accounts for the axial and radial diffusion of the solute as well as axial convection, and a constant or variable vascular surface area (\( A_v \)). In addition, the model also accounts for the radial movement of the solvent (water) to and from the vascular spaces. Osmotic responses of various tissue cells within the artificial tissue are predicted by the numerical model with three model parameters: permeability of the tissue cell membrane to water (\( L_p \)) permeability of the tissue cell membrane to the solute or CPA (\( \omega \)) and the diffusion coefficient of the solute or CPA in the vascular space (\( D \)). By fitting the model results with published experimental data on solute/water concentrations at various locations within an artificial
tissue, we were able to determine the permeability parameters of embedded tissue cells in the presence of Me₂SO. The permeability parameters obtained in the present study represent the first such effort for embedded tissue cells. It is hoped that the seminal tissue cell parameters reported here will help to optimize chemical loading and unloading procedures for whole tissues and consequently, tissue cryopreservation procedures.

4.1 Future Work

The DSC technique does have some limitations as stated in the thesis. Meanwhile, another new technique, Fourier Transform Infrared Spectroscope, has shown the potential to measure important biological changes including protein denaturation, membrane lipid transformations and accumulation of thermal damage (Bischof et al. 2002). Clearly, future improvements in calorimetric methods in conjunction with correlative methods such as FTIR will further elucidate important changes at the level of both water/ice biophysical changes as well as protein and lipid phase changes in cryobiology. Future work will also focus on determining the effect of different CPAs and CPA combinations on the water transport characteristics of different aquatic species sperm membrane during freezing.

The present model has successfully been used to simulate the coupled solute and solvent transport in arbitrary tissue sections. It will be used to simulate the coupled solute and solvent transport in other types of tissue sections, such as human ovarian tissue and rat live tissue. This model will be improved by accounting for more geometric (interstitial) changes and inclusions.
REFERENCES


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