Measurement and Simulation of Water and Methanol Transport in Algal Cells

John R. Walsh¹,²
Kenneth R. Diller¹,⁴
Jerry J. Brand⁴

¹Department of Biomedical Engineering
The University of Texas at Austin
Austin, TX 78712
²Currently at Organ Recovery Systems, Inc.
Charleston, SC 29403, U.S.A.
³Department of Mechanical Engineering
⁴MCD-Biology
The University of Texas at Austin
Austin, TX 78712

Introduction

Cryopreservation is a preferred method for the long-term storage of a broad range of biological materials, including repositories of micro-organisms. Maintenance of frozen cultures at ultralow temperatures over long periods of time is much less expensive and labor-intensive than conventional culturing methods, and virtually eliminates the genetic changes that occur during continuous culturing. Cryopreservation is currently being adapted as a primary storage mechanism for large repositories of microscopic algae that are used in a variety of research and biotechnological applications.

Cryopreservation can be described in terms of a process of coupled heat and mass transfer usually executed under nonequilibrium conditions. It includes several physico-chemical events that may damage or critically injure cells. To achieve maximum viability, protocols must be designed which minimize these damaging effects. Traditional methods of developing cryopreservation protocols have relied heavily upon empirical techniques to determine critical process parameter values necessary to achieve acceptable survival rates. Although efforts to cryopreserve a broad range of microalgae have been somewhat successful, few systematic studies have been conducted to define and quantify the factors that control the response to freezing and thawing [1–5].

Because of their diverse nature, different algae can exhibit vastly different physical responses to identical freezing protocols, necessitating the formulation of unique protocols to optimally cryopreserve the various strains. Although a majority of the algal species in the Culture Collection of Algae at The University of Texas at Austin (UTEX) have been cryopreserved successfully via a standard set of protocols [3,4], there still remain hundreds of species for which it appears that customized protocols will have to be devised. The development of specialized protocols for such a large number of species is a daunting task. A complementary design tool for developing acceptable cryopreservation protocols involves the application of computational biophysical models to determine key freezing parameter values that produce optimal conditions that minimize cellular injury and maximize post-thaw cell viability. An effective model may be constructed using equations of membrane transport and knowledge of membrane parameters governing water and solute permeation. The model can be used to predict the sequence of physico-chemical states through which a cell will pass during the cryopreservation process, and in some cases, the extent of stresses to which the cell will be subjected. By coupling the predictive capability of the model with relevant cytotoxicity and osmotic data, optimal preservation protocols yielding high cell viability may be determined.

One reason for the effectiveness of methanol as a cryoprotectant in algae [6] may be its ability to rapidly penetrate cell membranes [7]. A biophysical model describing methanol and water transport in microalgae has been developed in this study based on measurements of cell membrane transport properties and cell wall biomechanical characteristics. Previous experiments designed to quantify transport properties of water and methanol in algal cells were conducted with large macro-algae using very simple osmotic models of transport. Collander [8–9] and Wartiovaara [10] presented the diffusion rates of several small molecular weight compounds in Chara and Nitella. Although the data presented in these early studies has provided invaluable foundational information, the empirical models used to describe solute permeability did not address potential coupling of water flow with solute diffusion. Principles of irreversible thermodynamics were used by Kedem and Katchalsky [11] to describe the diffusive permeability of membranes to water and nonelectrolytes. Dainty and Ginzburg [12] extended the use of irreversible thermodynamics in Chara and Nitella to characterize transport across the plasma membrane.

Background: Experimental data and a complementary biophysical model are presented to describe the dynamic response of a unicellular microalga to osmotic processes encountered during cryopreservation. Method of Approach: Chlorella texanum (C. texanum) were mounted on a cryoperfusion microscope stage and exposed sequentially to various solutions of sucrose and methanol. Transient volumetric excursions were determined by capturing images of cells in real time and utilizing image analysis software to calculate cell volumes. A biophysical model was applied to the data via inverse analysis in order to determine the plasma membrane permeability to water and to methanol. The data were also used to determine the elastic modulus of the cell wall and its effect on cell volume. A three-parameter (hydrostatic conductivity \( L_p \), solute permeability \( \alpha \), and reflection coefficient \( \sigma \)) membrane transport model was fit to data obtained during methanol perfusion to obtain constitutive property values. These results were compared with the property values obtained for a two coefficient \( (L_p \) and \( \alpha \)) model. Results: The three-parameter model gave a value for \( \sigma \) not consistent with practical physical interpretation. Thus, the two-coefficient model is the preferred approach for describing simultaneous water and methanol transport. The rate of both water and methanol transport were strongly dependent on temperature over the measured temperature range \((25°C \) to \( −5°C)\) and cells were appreciably more permeable to methanol than to water at all measured temperatures. Conclusion: These results may explain in part why methanol is an effective cryoprotective agent for microalgae. [DOI: 10.1115/1.1688775]
Although this model included provision for solute-solvent transport, it did not lead to information about the influence of the elastic cell wall on transport dynamics.

Much more recently Hertel and Steudle [13] have made extensive measurements of the simultaneous transport of both water and rapidly permeating solutes, such as low molecular weight alcohols in Chara. They used a cell pressure probe [14] inserted through the plasma membrane to monitor directly the intracellular pressure during controlled exposure to alcohol solutions in a controlled temperature environment between 10 and 35°C. The temperature variation was used to detect whether separate and independent transport pathways exist across the plasma membrane for water and solutes [15]. The hypothesis was that water was transported across the plasma membrane primarily via dedicated channels [16,17] while alcohol transmembrane flow occurred via a solubility-diffusion mechanism. It was assumed that over a range of temperatures these differing mechanisms would have different activation energies. This proved to be the case, although the data indicated that the separate pathways were not fully selective. They proposed a composite membrane model in which water transport is largely confined to channels that enable its movement via a single-file, no pass mechanism. Small monohydric alcohols are not completely excluded from water channels, indicating the channels are not perfectly selective.

A dependent relationship between diffusive transport and hydrostatic turgor pressure was demonstrated by Zimmerman and Steudle [18], who used an intracellular pressure probe to measure internal turgor pressure during osmotic and hydrostatic treatments. They demonstrated that the bulk elastic modulus of the cell wall was pressure dependent. Wu, et al., [19] showed that the nonlinear behavior of the cell wall could be approximated as a thin-shelled structure composed of cellulose microfibrils embedded in an amorphous matrix of heterogeneous polymers. This model incorporates a representation of the internal stresses in the cell wall that account for the biphasic behavior observed in pressure-volume data. Inclusion of the elastic effects of the cell wall improves the mechanistic basis for describing the transport dynamics in algae. Henzl and Steudle [15] demonstrated that algal membranes can be modeled as composite structures in which water is transported through highly selective aquaporins, while solutes permeate via other routes through the membrane. Yet, the presence of aquaporins raises questions about the use of current methodologies to describe solute and solvent transport.

To date, all experimental studies of membrane transport properties in algae have utilized strains of macro-algae with cell volumes greater than $1 \times 10^{-9}$ m$^3$. The unicellular microalgal strain used in this study has a volume typical of the algae maintained in the UTEX culture collection. It is spherical in shape and has a volume of $15 \mu m^3$ (cell diameter=24 pm). Consequently, it is too small for direct handling and requires all experiments required to measure the key biophysical properties.

The algal models described above have been demonstrated to be effective for describing the transport properties of microalgae in the presence of low molecular weight solutes. However, the models have not been successfully applied to cells of similar size in order to quantify membrane transport properties [20]. However many of these require large cell populations, and visual observations of individual cellular response are not possible. For detailed analysis of visual phenomena, the microperfusion stage permits direct observation of single cells exposed to osmotic stress within a temperature-controlled environment during experimentation [21,22]. The perfusion cryostage [21] is particularly adept for making these measurements, whereas the cell pressure probe is expressly unsuitable for use at temperatures below 10°C [13]. The perfusion cryostage developed in our laboratory was therefore used for conducting the present permeability studies.

In the present study, an osmo-mechanical model was developed to simulate the transient behavior of algal cells during the addition and removal of cryoprotective agents (CPAs) at temperatures between ambient and high subzero values. Numerical values for biophysical properties were obtained from osmotic stress experiments conducted with C. texanum in the presence of methanol and sucrose solutions. An inverse solution method was used to compute the membrane transport parameters for both a three-parameter model (hydraulic conductivity: $L_a$, methanol permeability: $\omega_5$, reflection coefficient; $\sigma$) and a two-parameter model ($L_a$ and $\omega_5$). Membrane transport parameter values were also computed for the three-parameter model with a graphical analysis technique. Comparative analyses were used to determine the most appropriate model for subsequent development of optimal cryopreservation protocols. The two-parameter model is preferred based upon its ability to predict the osmotic response of the cell accurately using a minimum number of measurements and calculations. The three-parameter model yields accurate cell volume data, but the model lacks capability for detecting sensitivity in changes in reflection coefficient values, plus it is phenomenologically inconsistent with application for a high permeability solute such as methanol.

The overall goal of this research is to gain a better understanding of the biophysical transport processes that govern the cryopreservation of algae. The purpose of the new model is to simulate the volumetric response of algal cells to osmotic stresses caused by the addition and removal of CPAs. To this end the permeability of a representative algal strain was measured for both water and methanol over a range of temperatures above and below freezing. The resulting data and analysis provide a more substantial basis for the rational design of algal cryopreservation protocols. The model was designed for application to other algal cell types with minimal experiments required to measure the key biophysical properties.

**Theoretical Background**

**Algal Cell Model.** A biophysical model of a microalgal cell was developed, based on morphological and physiological characteristics of C. texanum [23,24]. The total cytosolic volume of C. texanum can be approximated as the sum of the osmotic components of a homogeneous solution containing water, $(V_w = n_w \bar{v}_w)$, CPA, $(V_{cpa} = n_{cpa} \bar{v}_{cpa})$, and impermeable solutes $(V_{imp} = n_{imp} \bar{v}_{imp})$, where $n_j$ represents the number of moles and $\bar{v}_j$, is the partial molar volume for each component, $j$. The cell volume, $V_{cell}$, can be represented by Eq. (1)

$$V_{cell} = n_w \bar{v}_w + n_{cpa} \bar{v}_{cpa} + V_p$$

(1)

where $V_p$ represents the combined volume of impermeable solutes and the osmotically inactive components of the cell. The cytoplasm of an algal cell typically is hypertonic with respect to the surrounding extracellular medium, thus creating a condition in which water would be drawn into the cell. Water influx across the membrane is counteracted by a hydrostatic turgor pressure gradient that develops as cell expansion becomes constrained by the elastic limits of the cell wall. Alterations in the extracellular solute concentration change the chemical potential gradients across the plasma membrane, resulting in an imbalance that causes water and solute fluxes. Active and facilitated mechanisms for electrolyte transport are slow in comparison with the diffusion of water, so over relatively short periods of time electrolyte transport can be neglected.

**Water and Permeable Solute Transport Modeling.** The membrane transport of water and methanol was modeled using three-parameter and two-parameter representations. In the three-parameter methodology, hydraulic conductivity $(L_a)$ and solute permeability $(\omega_5)$ represent permeability of the plasma membrane to water and methanol respectively, and the reflection coefficient, $(\sigma)$, represents the co-influence between solute and solvent during transport [11]. The three-parameter model accommodates an appreciable influence between solute and solvent as they are transported through a common channel across the membrane. The solute-solvent transport dynamics of the membrane were also characterized with a two-parameter model in which the rel-
evant membrane transport parameters were described in terms of independent water and solute permeability [25]. This model is appropriate when solvent and solute do not share a common channel of transport, and solute-solvent interaction can be neglected. Consequently, solution transport across the plasma membrane may be fully characterized in terms of two properties, \( L_p \) and \( \omega \).

Volume changes resulting from the transport of water and permeable solute are driven by chemical potential differences, \( \Delta \mu_j \), between the intracellular and extracellular solutions. The transmembrane chemical potentials of water and permeable solute, \( \Delta \mu_w \) and \( \Delta \mu_s \), can be expressed as

\[
\Delta \mu_j = \mu_j^\text{outside} - \mu_j^\text{inside} = \bar{\mu}_j + \mathcal{F} T \Delta L_j \ln X_j
\]

where \( X_j \) is the mole fraction of species \( j \), \( \mathcal{F} \) is the universal gas constant, \( T \) is the absolute temperature and \( \Delta p \) is the transmembrane pressure difference, which is equal to the turgor pressure of the cell. Fluctuations in turgor pressure can be correlated with membrane pressure difference, which is equal to the turgor pressure of the cell. For small deformations, the elastic modulus is assumed to be constant, and Eq. (2) may be applied to calculate intracellular turgor pressure. However, the wall elasticity is non-linear over the large range of volume changes associated with typical shrinking and swelling incurred during cryopreservation (as much as 40% difference from the volume in normal growth medium). As a result, elasticity must be approximated over a range of turgor pressures using alternative methods [19]. The instantaneous turgor pressure may be estimated by means of a simple expression for the transmembrane osmotic potential difference when the solute concentration differential, \( \Delta c_j \), across the membrane is known:

\[
\Delta p = \mathcal{F} T \Delta c_j
\]

Three-Parameter Model. A linearized version of the Kedem-Katchalsky equations which accounts for non-dilute solution effects [27] was used as the three-parameter model for coupled solute-solvent transport in algal cells. Equations (5) and (6) describe the transient cell volume in terms of the molar flows of water and solute, \( J_w \) and \( J_s \), respectively.

\[
dV/dt = J_w \bar{\nu}_w + J_s \bar{\nu}_s
\]

\[
J_w = L_{11} \Delta \mu_w + L_{12} \Delta \mu_s
\]

\[
J_s = L_{21} \Delta \mu_w + L_{22} \Delta \mu_s
\]

where the phenomenological coefficients \( L_{11} \), \( L_{12} \), \( L_{21} \) and \( L_{22} \) can be written in terms of nonlinear algebraic expressions to define the membrane parameters \( L_p \), \( \omega \), and \( \theta \). As has been shown previously [23], the phenomenological coefficients form an orthogonal matrix where \( L_{11} = L_{22} \) by Onsager’s reciprocity relationship. The membrane coefficients are given by:

\[
L_{11} = \left( \frac{1}{\bar{\nu}_w} \right) \left( \frac{1}{1 + \theta - \omega \theta} \right) \frac{L_p}{1 + \theta - \omega \theta} + \theta \bar{\nu}_w \omega
\]

\[
L_{12} = \left( \frac{C_m}{\bar{\nu}_w} \right) \left( \frac{1}{1 + \theta - \omega \theta} \right) \frac{L_p}{\bar{\nu}_s} \left[ 1 - \frac{1 - \sigma}{1 + \theta - \omega \theta} - \bar{\nu}_s \omega \right]
\]

\[
L_{22} = C_m \left( \frac{1}{1 + \theta - \omega \theta} \right) \frac{L_p (1 - \sigma)}{1 + \theta - \omega \theta} + \theta \omega
\]

where the mean permeable solute concentration across the membrane, \( \theta \), is given by the relation:

\[
\theta = \bar{\nu}_s C_m
\]

\[
C_m = \frac{(C_s - C_i)}{(C_s + C_i)} = \frac{(C_s + C_i)}{2}
\]

Two Parameter Model. The two-parameter model was adapted from a formalism in which the flow of solute and solvent do not occur through co-transporting channels [25]. Cell volume dynamics are expressed in terms of the fluxes of water and solute as functions of their respective chemical potential gradients:

\[
dV_w/dt = -L_p \Delta \mu_w/\bar{\nu}_w
\]

\[
dV_s/dt = -\omega \Delta \mu_s/\bar{\nu}_s
\]

where \( A \) is the surface area of the plasma membrane. Solute permeability is expressed here, as in other places, in terms of solute moles per unit time versus solute volume per unit time as is often used for hydraulic conductivity. Multiplication of \( \omega \) by the partial molar volume of solute converts the expression into terms of solute volume per unit time.

For each model, the temperature dependence of the cell membrane hydraulic conductivity and solute permeability can be described by an Arrhenius function [28,29]:

\[
L_p = L_{pg} \exp \left( -\frac{E_{alp}}{\mathcal{F} T} \left( \frac{1}{T} - \frac{1}{T_{ref}} \right) \right)
\]

\[
\omega = \omega_s \exp \left( -\frac{E_{alw}}{\mathcal{F} T} \left( \frac{1}{T} - \frac{1}{T_{ref}} \right) \right)
\]

where \( E_{alp} \) is the activation energy for transport of molecular species \( j \), while \( L_{pg} \) and \( \omega_s \) are, respectively, the hydraulic conductivity and methanol permeability coefficients of the membrane at a specified reference temperature, \( T_{ref} \).

Methods and Materials

Cell Culture. Algal cell cultures were obtained from the UTEX Collection, which maintains in excess of 2,000 algal strains and of which more than 1300 strains of living algae are under cryopreservation. The UTEX Collection dates from an original collection established by E. G. Pringsheim in the 1930’s, as do several other of the major collections in the world. Initial cell cultures of \( C. \) texanum (UTEX 1788) were collected from a 1.5% (w/v) sterile agar slant containing 2.0 ml of proteose peptone medium [30] at 25°C. New cultures were created by inoculating cells into sterile 50 ml culture flasks containing 15 ml of tris-acetate-phosphate (TAP) medium [31]. Cultures were agitated continuously in an orbital shaker at 22°C with a photoperiod of 16 hours of light using 30W GRO-LUX lights, followed by 8 hours of darkness. C. texanum cells were harvested routinely for this study in late log growth phase 8–11 days after inoculation. Following harvest, cells were prepared for experiments by transferring a 1.0 ml aliquot of suspension from a 50 ml culture flask to a smooth glass homogenizer. Homogenous suspensions were created by gently agitating the mixture with a Teflon coated pestle to break up clumping cells. To ensure consistency of preparation, cells were always harvested immediately prior to sample preparation and experimentation.

Perfusion Cryostage and Cryomicroscopy. Cells of \( C. \) texanum were exposed to solutions of differing osmotic properties on a specially designed perfusion cryostage [21] mounted on a Zeiss Universal microscope and observed via a 40×, 0.65 N.A. objective at a total optical magnification of 800X (see Fig. 1). A 2.0 ml suspension of cells was loaded into the observation area of the cryostage prior to perfusion and allowed to attach to the chamber substrate for a 15-minute settling period. The osmotic challenge caused a transient change in the cell volumes. Video images of the
cells were acquired continuously with a black-and-white CCD camera. Real-time images were stored on video tape and analyzed off-line via digital image processing.

The perfusion cryostage provides simultaneous and independent control of the thermal and chemical environments of a specimen during continuous observation on the microscope. The cryostage design permits rapid changes in the extracellular osmotic environment with a time constant on the order of one second. Temperature of the sample area is measured by a thermocouple laminated between two thin layers of transparent film to isolate it from direct physical contact with the specimen. Cells are placed laterally within 200 μm of the thermocouple to ensure temperature measurements to within ±0.5°C. The cell position within the field of view was maintained during perfusion by a coarse mesh secured between the stage surface and coverslip.

Digital images acquired from videotape recordings were processed to quantify the volumetric response of cells during osmotic challenge experiments. Video frames were digitized at discrete times using the frame grabber integral to a Power Macintosh 8500 computer (Apple Computer, Cupertino, CA). Time-sequenced images were combined to generate a temporal history of volume changes for individual cells throughout an experiment. Cells of C. texanum generally retained a spherical shape and cell volume was therefore not a component of the volume calculation. Volume was determined by plotting the measured volume as a function of the microscope focus and arbitrary scale measurements were extrapolated from the samples and compared to the origin along the inverse concentration axis identifying the cell volume which would occur if the suspension concentration were made infinite.

**Biophysical Property Measurements**

**Osmotically Inactive Volume.** The osmolality of the cytoplasm of C. texanum was determined by incipient plasmolysis experiments in which cells were exposed to a series of increasingly hypertonic sucrose solutions. Exposure to hypertonic solutions containing non-permeable solutes results in the exosmosis of intracellular water and a loss of turgor pressure. The point of incipient plasmolysis is marked by the osmolality at which turgor pressure falls to zero, and the intracellular and extracellular osmotic potentials become equal. This condition can be expressed in the relationship:

\[ \pi^o_{\text{plasmolysis}} = \pi^o_{\text{plasmolysis}} \]

where \( \pi = RTc_j \) and \( c_j \) is the impermeable solute concentration.

In practice, incipient plasmolysis can not be determined accurately by simple visual inspection. Since the cell wall is not appreciably deformed by plasmolysis, an approximation of the extracellular osmolality is obtained by measuring the threshold concentration of an extracellular sucrose solution which causes an initial separation of the plasma membrane from the cell wall.

The plasmolytic point was determined under ambient conditions for 50 μl volumes of cell suspension placed in separate 2.0 ml sucrose solutions at concentrations, respectively, of 100, 200, 220, 240, 260, 280, 300, and 400 mOsm. Each cell suspension was gently agitated for 10 minutes followed by a 5 minute settling period. Samples of 20 μl were then mounted on a microscope slide, and the extent of plasmolysis was examined. More than 200 cells were examined in each cell suspension. The extracellular sucrose concentration that produced threshold plasmolysis in over 50% of the cells defined the cell osmolality at the point of incipient plasmolysis.

The osmotically inactive cell volume for C. texanum, \( V_b \), was determined from perfusion data in which cells were sequentially equilibrated with increasingly hypertonic sucrose solutions of 300, 500, 700, and 1250 mOsm. At each perfusion concentration a sufficient time was allowed for osmotic equilibrium to occur, and then the cell volumes were determined by measurement of the microscopic images. At a theoretically infinite solution concentration all available water will be osmotically driven from the cell resulting in a minimum volume defined by nonaqueous intracellular contents and bound water. This osmotically inactive cell volume is determined by plotting the measured volume as a function of the reciprocal volume and fitting a straight line through the data to the origin along the inverse concentration axis (Boyle-Van’t Hoff relationship [29]). The intersection of this line with the volume axis identifies the cell volume which would occur if the suspending solution concentration were made infinite.

The elastic behavior of the cell wall in C. texanum was determined with equilibrium volume data obtained from sucrose perfusion experiments across a broad range of turgor pressures. The hydrostatic pressure within the cell was adjusted from high pressure (no added sucrose) to zero turgor by equilibrating cells with sucrose solutions of 0, 100, 150, 200, 250, and 300 mOsm.
Membrane Transport Properties. The membrane transport properties for water and methanol diffusion across the plasmalemma of C. texanum cells were determined from transient volume data obtained during perfusion experiments. Membrane parameter values are typically obtained from volume data as cells are exposed to penetrating solutes. The flux of water and solute through the membrane typically produces a “shrink-swell” response in cell volume. Preliminary perfusion experiments with methanol solutions showed that volume changes observed during the experiment were smaller than could be accurately detected by our measurement methods. The minute change in cell volume suggested that methanol is transported across the membrane very rapidly, possibly even more rapidly than water. Thus, it was hypothesized that water and solute simultaneously entered the cell and expanded the cell to its maximum size as limited by the constraining cell wall.

To isolate the limiting effects of the cell wall on volume excursions during water and methanol transport studies, two-part perfusion experiments were conducted. In these experiments, cells were initially exposed to a sucrose solution designed to shrink the cell. The cells were then perfused with an equal osmolar methanol-sucrose solution that induced a rapid influx of methanol. With this technique it was possible to obtain volume changes of significant magnitude to enable accurate transient volume analysis.

Experimentally, cells were initially dehydrated with a 500 mOsm sucrose solution causing a efflux of water sufficient to produce plasmolysis. At steady state, turgor pressure in the plasmolysed cell was zero, and the intracellular impermeable solute concentration was equal to 500 mOsm. After reaching this equilibrium state, the extracellular solution was replaced with an equal osmolar solution containing 200 mOsm methanol and 300 mOsm sucrose. The introduction of 200 mOsm methanol into the extracellular solution established a chemical potential gradient across the membrane that produced the impetus for methanol influx into the cell. The uptake of methanol rapidly altered the pre-existing intracellular concentration of water, thereby creating conditions for water influx that caused the cell to swell to a new equilibrium state. It has been demonstrated that 2M methanol is not damaging to these cells, and the concentration used in this study is only 10% of that value [4]. Thus, there was no concern that the cells were damaged by exposure to methanol during the experimental trials.

Numerical Methods. Values of the membrane transport properties were computed using transient volume data recorded during perfusion experiments conducted with sucrose and methanol solutions. Two alternate methods were evaluated to calculate the permeability parameters of the membrane from the experimental data.

Simultaneous Inverse Solution Method. In the inverse solution method, membrane parameters were computed simultaneously with a nonlinear regression technique that solved the constitutive equations with parameter values that best fit the volumetric response data collected during perfusion with 300 mOsm sucrose—200 mOsm methanol solutions. The algorithm computed iterative membrane parameter values until the change in the chi-squared statistic between experimental data and theoretical estimates converged to $\Delta x^2/\chi^2<0.05$. The value of $\chi^2$ for $\omega$ was always $<0.01$.

Zero-time Method. The zero-time method [32] was used to obtain rapid estimates of $L_p$ and $\sigma$ without the use of extensive computer processing. The method uses a simplified form of the volume flux equation developed by Kedem and Katchalsky [11] to calculate the values of $L_p$ and $\sigma$. The volume flux, $J_v$, is given by:

$$J_v = L_p(\Delta p - \Delta \pi_{imp}) - \sigma L_p \Delta \pi_s = \frac{dV}{dt} \frac{1}{A} \quad (12)$$

where $\Delta \pi_{imp}$ and $\Delta \pi_s$ are the transmembrane osmotic pressure gradients of impermeable and permeable solutes, respectively. When cells are perfused with a nonpermeable solute only, the value of $\Delta \pi_s$ becomes zero and Eq. (12) becomes:

$$L_p = \frac{1}{\Delta p - \Delta \pi_{imp}} \frac{dV}{dt} \frac{1}{A} \quad (13)$$

The value of $L_p$ may be determined from the initial slope of the transient volume curve, $(dV/dt)_0$ at time 0, when cells are initially perfused with a nonpermeable solute and the value of $\Delta p_{imp}$ is known accurately from experimental conditions. Once the value of $L_p$ has been calculated, Eq. (12) may be solved in terms of the reflection coefficient, $\sigma$, provided $\Delta \pi_{imp}$ and $\Delta \pi_s$ are known. The permeability of methanol, $\omega$, was computed by performing a nonlinear regression analysis of the theoretical volume predicted by the model with experimental data collected during perfusion with a mixture of impermeable and permeable solutes, and with $L_p$ and $\sigma$ known apriori.

The value of $L_p$ was computed from experimental volume data measured during the initial perfusion with a 500 mOsm sucrose solution. The initial slope of the transient volume curve obtained during cell perfusion with a solution composed of 300 mOsm sucrose—200 mOsm methanol was used to compute the reflection coefficient, $\sigma$. These values of $L_p$ and $\sigma$ were used to compute $\omega$ via a nonlinear regression analysis. The value of $\omega$ was also determined iteratively until the chi-squared statistic between experimental and theoretical estimates converged to a value $\chi^2/\chi^2<0.05$.

Results

Cell Biophysical Properties. The osmotically inactive cell volume, $V_{os}$, was determined from equilibrium volume data collected by perfusion of C. texanum cells with sucrose solutions of 300, 500, 700, and 1250 mOsm. A Boyle-van’t Hoff plot was prepared by plotting normalized cell volume as a function of reciprocal osmolality of the suspending medium (Fig. 2). The cell volume and extracellular solution concentration were both normalized to isotonic conditions, $V_{iso}$ and 58 mOsm [33], respectively. All of the extracellular solutions exceeded the concentration of sucrose (250±20 mOsm) that produced incipient plasmolysis. Thus, the elastic properties of the cell wall did not contribute to the regulation of cell volume, and the cells exhibited typical linear Boyle-van’t Hoff behavior. 

![Fig. 2 Boyle-van’t Hoff plot for C. texanum cells exposed to hypertonic sucroses solutions at ambient temperature and pressure under equilibrium conditions. Cell volume is normalized to the isotonic volume, $V_{iso}$, and expressed as a function of the inverse extracellular solute concentration normalized to the isotonic osmolality of 58 mOsm. The theoretical inactive volume of the cell was 38% as indicated by the intercept of the linear regression. n=16 independent measurements of individual cells. Data represent average ±SEM.](image-url)
Table 1 Summary of average biophysical parameter values obtained for C. texanum

<table>
<thead>
<tr>
<th>Property</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average cell diameter</td>
<td>22.2±4.7 μm</td>
</tr>
<tr>
<td>Cell volume in TAP medium, ( V_{initial} )</td>
<td>5.73×10^{-3} m³</td>
</tr>
<tr>
<td>Cell volume in distilled H₂O, ( V_{full} )</td>
<td>5.90×10^{-3} m³</td>
</tr>
<tr>
<td>Inactive cell volume</td>
<td>0.38 ( V_{ion} )</td>
</tr>
<tr>
<td>Isotonic state of C. texanum</td>
<td>58 mOsm</td>
</tr>
<tr>
<td>Osmolality of TAP medium</td>
<td>131 mOsm</td>
</tr>
<tr>
<td>Cell osmolality at incipient plasmolysis</td>
<td>250±20 mOsm</td>
</tr>
<tr>
<td>Partial molar volume, impermeable solutes</td>
<td>3.56×10^{-5} m³/mol</td>
</tr>
</tbody>
</table>

A linear regression line was fit to the equilibrium volume data. The intercept of the regression line represents the theoretical volume of a cell exposed to an infinitely concentrated solution, defined here as the osmotically inactive volume (\( V_b \)). The value of \( V_b \) for C. texanum cells in late log phase (7–10 days) was 0.38 \( V_{ion} \) (Fig. 1). Thus, under isotonic conditions 62% of the cell volume is assumed to consist of mobile water.

**Cell Osmolalit**. The cells were initially equilibrated in TAP medium at 131 mOsm and then exposed to increasingly concentrated hypertonic sucrose solutions until incipient plasmolysis was detected by microscopic inspection. The number of moles of impermeable solute, \( n_{imp} \), was calculated from the total solute concentration and the cell volume at plasmolysis as

\[
\text{n}_{imp} = \frac{n_{plasmolysis} (V_{plasmolysis} - V_b)}{9\pi} \tag{14}
\]

The partial molar volume of the impermeable solutes, \( v_{imp} \), was estimated for an average partial molar volume of typical solutes found in algae. An \( v_{imp} \) value of 3.56×10^{-5} m³/mol was determined based on intracellular solute data available for Chlamydomonas [23].

The solute concentration of the cytoplasm, \( c_{cell} \), for cells suspended in normal growth medium (TAP) was estimated from the relationship

\[
c_{cell} = \frac{n_{imp}}{\rho_w V_w} \tag{15}
\]

where \( \rho_w \) and \( V_w \) are the density and volume of water, respectively. Equation (15) incorporates the assumption that the value of \( n_{imp} \) remains constant during all osmotic processes. A list of values for biophysical parameters determined for C. texanum is provided in Table 1.

**Osmo-Mechanical Coupling and Cell Wall Elasticity**. Cells of C. texanum were exposed to hypo- and hyper-plasmolytic sucrose solutions during equilibrium perfusion experiments to characterize the elastic behavior of the cell wall and its influence upon volume. Equilibrium volume data over a range of suspending solution concentrations are shown in Fig. 3. Cells behave as perfect osmometers at extracellular concentrations above 250 mOsm, but exhibit nonlinear behavior in more dilute solutions. The departure from Boyle-van’t Hoff behavior indicates an increasing resistance to volume expansion by the cell wall as the turgor pressure increases above the state of plasmolysis induction. The data were normalized to this state since it defines the transition between the linear and nonlinear behavior domains.

Detailed examination of the volumetric data for cells perfused with hypertonic solutions shows a distinctive nonlinear elastic relationship. A graph of expressed cell volume plotted against dilute external solution osmolality is shown in Fig. 4. Equilibrium cell osmolality was calculated using Eqs. (1) and (15). Equation (4) was used to calculate the hydrostatic turgor pressure for each sucrose concentration used in the experiment. Turgor pressure is plotted as a function of extracellular solute concentration in Fig. 4.

For small pressure increments the resulting changes in cell volume are approximately proportional to the bulk elastic modulus, \( e \), as described in Eq. (3). A value for \( e \) may be determined from the data in Fig. 5 by computing the slope between pairs of successive data points on the graph. Although Eq. (3) may be applied successfully to small volume changes, it is not appropriate for large deformations that display nonlinear pressure-volume relationships [19], such as were measured in the present experiments. The development of equations to model the complex changes in elastic modulus within the cell wall during volume changes were beyond the scope of this study. Consequently, a expression for
computing turgor pressure based upon cell volume was generated by performing a nonlinear regression analysis of volumetric data from Fig. 5. The resulting analysis produced a set of expressions for biphasic volume behavior in two distinct regions:

**Linear Region:** $0 < \Delta V/V_{\text{full}} < 0.03$:

\[
p = -4.38 \times 10^6 (\Delta V/V_{\text{full}}) + 3.11 \times 10^5 \quad (16a)
\]

**Non-Linear Region:** $0.03 \leq \Delta V/V_{\text{full}} \leq 0.31$:

\[
p = -1.96 \times 10^5 (\Delta V/V_{\text{full}})^3 + 7.40 \times 10^6 (\Delta V/V_{\text{full}})^2 - 1.09 \\
\times 10^9 \times 10^2 (\Delta V/V_{\text{full}}) + 2.08 \times 10^5 \quad (16b)
\]

where $p$ is the intracellular turgor pressure (Pa), and the volume of water expressed from the cell, $\Delta V = V_{\text{full}} - V$, is normalized to the cell volume at full turgor, $V_{\text{full}}$. This expression can be used to represent the osmo/mechanical coupling of the algal cell.

**Membrane Transport Parameters.** Membrane transport property values were measured for C. texanum from transient volume data collected across a temperature range from 25°C to −3°C during nonequilibrium perfusion experiments with sucrose and methanol solutions. A simultaneous inverse solution technique was implemented as the primary method for computing transport parameter values for the three-parameter ($L_p, \omega, \sigma$) and two-parameter ($L_p, \omega$) models. Permeability coefficients for the three-parameter model were also computed using the zero-time parameter estimation method as an alternative not requiring extensive computational processing. The zero-time method was investigated as a technique for obtaining rapid estimates of $L_p$ and $\sigma$ in solute-solvent systems in which co-transport effects are assumed to be appreciable. For the zero-time method, the value for $\omega$ required computer analysis of the volumetric data after the values of $L_p$ and $\sigma$ were determined. The fitting algorithm used in the simultaneous inverse solution method was also applied to determine the value of $\omega$ from experimental data.

The values of $L_p$, $\omega$, and $\sigma$ computed for the three and two-parameter models at experimental temperatures of 25°C, 15°C, 0°C and −3°C are shown in Table 2. Values in the table were computed with volumetric data obtained in the second part of the two-part experiments in which cells were exposed to a solution containing 300 mOsm sucrose—200 mOsm methanol following exposure to a 500 mOsm sucrose solution. The reflection coefficient computed for the three-parameter model was $0.40 \pm 0.08$ (SEM, n = 12).
Discussion

This study focused on quantifying the parameters that govern water and methanol permeability during cryopreservation. Membrane transport parameters were measured in the presence of a rapidly penetrating solute, methanol, which has been very effective as a CPA for algae. Perfusion experiments were conducted with C. texanum to obtain transient volumetric data for analysis of the membrane permeability parameters and characterization of the effects of cell wall elasticity.

Osmotic Characteristics. C. texanum cells exhibited nonlinear volume behavior with changes in hydrostatic turgor pressure produced by the alteration of extracellular solute concentration within the range 0 to 250 mOsm. Cell volume changes in this range of extracellular osmolality are governed by a combination of elastic and osmotic stresses present in the system. Exposure to non-permeable solute concentrations greater than the plasmolytic concentration of 250 mOsm resulted in volumetric changes that were no longer influenced by the elastic properties of the relaxed cell wall, being governed by osmotic forces only. The cell osmotic properties measured for C. texanum correlate well with studies conducted on other plant and algal cells. A plasmolytic concentration of 270 mOsm obtained for Nitella flexilis via an intracellular pressure probe is comparable to the value of 250 mOsm found in C. texanum.

Cell Wall Elastic Behavior. Osmo-elastic coupling has been observed previously in other plant cells. Melkonian, et al. [35] presented a similar pressure-volume relationship for winter wheat leaves showing a well-defined transition from an elastically dominated region to osmotic dominated behavior as the external pressure on the leaves was increased beyond a plasmolyzing limit. Experimental results presented in this study demonstrate the substantial elastic effect of the cell wall on the volumetric response of algal cells subjected to an imposed osmotic stress. The concept of a bulk elastic modulus, which applies to stress-strain relationships in solid, homogeneous materials, does not describe the elasticity of plant cell walls. Wu, et al. [19], noted the inaccuracies of using a bulk elastic modulus to describe cell wall elasticity and presented a polymer based approach for modeling the pressure-volume properties of thin-shelled plant structures. The nonlinear volume behavior observed in C. texanum is predicted by Wu's model, which illustrates that the extension of the cell wall from zero tension to maximum stiffness produces a biphasic volume response. Wu, et al. [19] describe the plant cell wall as a shell structure composed of cellulose microfibrils embedded in an amorphous matrix of heterogeneous polymers. Initial tension in the cell wall is expressed in an amorphous matrix, and then in the microfibrils as they become taut. Further expansion causes an increase in microfibril stiffness that acts to limit cell expansion. The biphasic response under similar experimental conditions has been observed in other algae [36] and plant cells [37].
Hydraulic Conductivity. The values for $L_p$ obtained with the different modeling techniques in this study, (0.79 to 1.11) × 10^{-14} m^2/N*s at 25°C, fall within the broad range of permeability values measured in other algal cells. Data from this study are close to the value of $L_p$ (1.85 ± 0.27) × 10^{-14} m^2/N*s found for Valonia ventricosa [38]. Values of $L_p$ recorded in other algae span two orders of magnitude, extending from 10^{-15} to 10^{-12} m^2/N*s [12,39]. Widely varying values of $L_p$ are not uncommon and have been recorded in different mammalian cell types that include erythrocytes (1.8 × 10^{-12} m^2/N*s; [40]), islets (2.0 × 10^{-13} m^2/N*s; [41]), oocytes (7.73 × 10^{-14} m^3/N*s; [23]) and keratinocytes (6.17 × 10^{-15} m^3/N*s; [42]).

Hydraulic Conductivity Activation Energy. $E_{aL_p}$ ranges from 41.6 to 47.0 kJ/mol, fall within the range of activation energies measured in several alga. Kiyosawa [43] reported a value of 25 kJ/mol for Nitella while Dainty and Ginzburg [12] reported a value of 34 kJ/mol. Alternatively, Andjus recorded a relatively high activation energy value of 75 kJ/mol for Chara [45]. According to Verkman, et al. [44], high activation energy values are indicative of water transport that occurs primarily by diffusion across the lipid bilayer of the membrane and not through water-specific channels, since most aquaporins have activation energies across the lipid bilayer of the membrane and not through water-specific channels, since most aquaporins have activation energies for V erkman, et al. [44] experimental techniques produced comparable values for methanol permeability. C. texanum permeability to methanol falls between the higher and lower values of $E_a$ reported for other algae. In many cases, methanol permeability is greater than or equal to that of water. The data of Collander and Barb and [8] shows the relative permeabilities of several solutes in Chara, demonstrating that methanol is one of few solutes that can permeate into cells more rapidly than water. This phenomenon has also observed in red blood cells which have an $L_p$ of 1.7 × 10^{-13} m^2/N*s at 25°C [48] and an equivalent $E_a$ value of 6.0 × 10^{-13} m^2/N*s [49].

Activation energies determined by the different modeling and computational methods demonstrate an internal consistency. The values from the three- and two-parameter models (64.5 and 67.7 kJ/mol, respectively) are slightly lower than was obtained with the zero-time method (78.5 kJ/mol). Previously published activation energies for methanol permeation in other algae are nonexistent. Although methanol permeability data is readily available, there is a paucity of information at multiple temperatures. Methanol permeability studies conducted by Wartiovaara [10] were performed at two temperatures, but the data were not analyzed for an Arrhenius effect. These measurements for Nitella at 20°C and 0°C have been applied to compute, using linear regression analysis, an estimated activation energy of 60.1 kJ/mol, which is in good agreement with the data presented in this study. Interestingly, the activation energy values for C. texanum and human red cells, 63 kJ/mol [48] are similar. Hertel and Steudle carefully measured the activation energy of Chara to different monohydric alcohols of different molecular size and shape (ethanol, n-propanol, isopropanol, and tert-butanol) between 10°C and 35°C, obtaining values ranging from 37.1 to 51.6 kJ/mol [13], roughly in the same range as the current data. The variation in activation energies across the three models is anticipated given the difference in number of model parameters (three vs. two-parameter models) and variances in estimation methodologies (inverse solution vs. zero-time methods). The discrepancies between the three-and two-parameter models observed in this study were small for both $E_{aL_p}$.

### Table 4 Activation energies for water and methanol computed with the three-parameter, (3-P), and two-parameter, (2-P), models. Parameter values were computed for each model using either the simultaneous inverse solution (Inverse) or with the zero-time method (Zero-Time)

<table>
<thead>
<tr>
<th>Model (Method)</th>
<th>Temperature Range</th>
<th>$E_{aL_p}$</th>
<th>Reference Temperature, $T_{ref}$</th>
<th>$L_{p0}$ (m^2/N*s)</th>
<th>$r^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-P (Inverse)</td>
<td>25°C to -3°C</td>
<td>42.3</td>
<td>25°C</td>
<td>1.04 × 10^{-14}</td>
<td>0.90</td>
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<tr>
<td>2-P (Inverse)</td>
<td>25°C to -3°C</td>
<td>41.6</td>
<td>25°C</td>
<td>7.47 × 10^{-15}</td>
<td>0.98</td>
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<tr>
<td>3-P (Zero-Time)</td>
<td>25°C to -3°C</td>
<td>47.0</td>
<td>25°C</td>
<td>8.21 × 10^{-12}</td>
<td>0.98</td>
</tr>
<tr>
<td>3-P (Zero-Time)</td>
<td>25°C to -3°C</td>
<td>177.2</td>
<td>26°C</td>
<td>1.14 × 10^{-14}</td>
<td>0.99</td>
</tr>
<tr>
<td>3-P (Zero-Time)</td>
<td>23°C to 5°C</td>
<td>40.9</td>
<td>23°C</td>
<td>5.66 × 10^{-13}</td>
<td>0.99</td>
</tr>
</tbody>
</table>
and $E_{aw}$. Similar variations in both $E_{aw}$ and $E_{ap}$ in a three-vs.
two-parameter model study were also reported by Woods, et al. [50].

**Reflection Coefficient.** The reflection coefficient was computed via the simultaneous inverse solution technique and the
zero-time method for a three-parameter model fit to experimental
data. The values obtained with the simultaneous inverse solution
technique (0.40) and the zero-time method (0.52) are both higher
than previously determined reflection coefficients computed for
algae. Dainty and Ginzburg [39] calculated $\sigma$ values of 0.30 and
0.25 for Chara australis and Nitella translucens, respectively, but
subsequent corrections for unstirred layers, when applied to the
Nitella data, yielded a value of 0.50, which is in the range of the
present data. Steudle and Zimmerman [51] likewise applied a cor-
rection for unstirred layers in Nitella flexilis, hypothesizing that
the reflection coefficients for rapidly permeating alcohols mea-
sured in their studies were underestimated by 10–20%. They ap-
plied a worst case underestimate of 20% to the original value of
0.31 to obtain a $\sigma$ value of 0.37. A similar reflection coefficient of

0.38 was found for Chara carollina [14] based on experimental
data for which the solution surrounding the algae was stirred to
minimize boundary layer effects. However, the magnitude of error
cased by residual unstirred layers was not determined or esti-

mated. For the perfusion cryostage used in this study, extracellular
unstirred layers were considered to be negligible since cells were
placed in the middle of a flow field in which the bathing medium
was exchanged with a time constant on the order of one second.
Thus, convective stirring may significantly reduce the magnitude
of the boundary layer on the outside of the cell surface. However,
there still may be a significant unstirred layer on the inner mem-
brane surface. This effect would be most significant subsequent to
plasmolysis when there would be a physical separation between
the cell wall and membrane. The magnitude of this effect would
be very difficult to estimate from the experimental data in this
study.

One model for membrane transport of water and methanol as-
sumes the two species cross the membrane by different pathways
defined as water channels and diffusion across the lipid bilayer,
respectively. However, the relatively high value of $E_a$ suggests that water transport in taxenium is primarily by diffusion across the lipid bilayer [45]. If there is a solvent-solute interaction, water and methanol molecules exert a drag on each other thereby reducing the differential in permeability of each species. The range of values for $\sigma$ are constrained by the relationship [52]

$$\sigma < 1 - \frac{\omega L_p}{L_p} \quad (17)$$

Finkelstein [52] points out that interpretation of the value of $\sigma$ to identify the transport pathway through a membrane can be problematic, but subsequently Hertel and Steudle [13] have used the change in $\sigma$ with temperature to distinguish between the transport pathways for water and alcohols. Their composite model for membrane transport mechanisms is based on water using channels and alcohols using bilayer diffusion to move across the membrane. As noted above, these processes have differing activation energies. Eq. (17) shows that $\sigma$ incorporates the ratio of solute and water permeability. Therefore, as the temperature changes the ratio of $\omega$ and $L_p$ should also change, owing to the differentials in their activation energies. Hertel and Steudle measured an increase in $\sigma$ on the order of 0.5 as the temperature was decreased from $30^\circ C$ to $20^\circ C$. Based on this result, they concluded that the measurement of $\sigma$ did indeed provide definitive information for identifying the transport mechanisms for water and alcohols in Chara.

Although the present reflection coefficients are in good agreement with data previously published, the values computed using Eq. (17) for both the simultaneous inverse solution and zero-time method are negative, which is unusual. This result occurs as a consequence of the permeability to solute being higher than to water. Negative values of $\sigma$ have been previously reported for algal cells [38] and plant cells [14] exposed to rapidly permeating solutes, including methanol. Negative values of $\sigma$ also have been achieved by blocking water channels by HgCl treatment [15]. In each case, these results occurred in systems for which $\omega$ was large relative to $L_p$. The fact that negative reflection coefficients have been consistently demonstrated in conjunction with rapidly permeating alcohols raises questions regarding the appropriateness of the definition of $\sigma$ and the mechanism(s) of solute-solvent interaction in the membrane. Any justification for redefining these terms without a relevant biological mechanism to explain the reflection coefficient is tenuous. Agreement between experimental data and simulation results from the two-parameter model support the hypothesis that the use of the reflection coefficient as a third transport parameter may be unnecessary within the confines of this study.

**Analysis Models for $L_p$, $\omega$ and $\sigma$.** The membrane parameter values obtained for $L_p$ and $\omega$ are consistent among the different analysis models employed in this study. The closest agreement in values of $L_p$ occurred with data derived from the two-parameter model and the zero-time method. However, a similar comparison for $\omega$ shows a closer match for the three- and two-parameter models.

Despite the shortcomings of the zero-time method, there is intrinsic value in using the quasi-graphical method to approximate permeabilities. The zero-time method provides a first approximation of $L_p$ and $\sigma$ without the need for sophisticated computation schemes. The ability to determine estimates of $L_p$ and $\sigma$ rapidly permits efficient screening of the osmotic properties of cryoprotectants and cell species.

Hydraulic conductivity values were calculated by the zero-time method from osmotic stress data obtained in the absence of methanol, whereas the simultaneous inverse solution method was applied for data measured during the concomitant transport of water and methanol. A higher value of $L_p$ determined by the latter method suggests that the presence of methanol may have altered membrane lipids, thereby increasing permeability of the plasma membrane to water. In contrast, prior studies have shown that the presence of a (nonalcoholic) solute results in a decrease in $L_p$ [29,53] which can be proportional to the solute concentration. Methanol and other alcohols have been demonstrated to interact with phospholipids to destabilize the lipid bilayers of artificial membranes [54]. Short-chain alcohols, such as methanol and ethanol, interact more directly with the polar head group of lipids in bilayers due to their low hydrophobicity [55]. However, the short non-polar region of the alcohol can create voids between the lipid chains in the membrane interior and introduce instability within the bilayer [56]. At methanol concentrations as high as 2.5–3.0 M, the membrane becomes even more unstable resulting in a configuration in which the lipids in the bilayer transition to an interdigitated phase [54,57]. In this configuration, lipid acyl-chains from opposing monolayers are shifted from their normal opposite-facing orientation to an interspersed arrangement that exposes the ends of the hydrophobic tails. An analysis of this type of methanol interaction with the phospholipids in C. texanum has not been performed, although we and others have found that algae are well able to withstand the rigors of cryopreservation with methanol, indicating that the extent of any damage must be limited. It is unknown if the relatively low concentration of methanol used in this study (200 mOsm) modified the lipid orientation significantly to cause the observed increase in hydraulic conductivity. Clearly, there is a need to address the potential of inducing conformational changes in the membrane if methanol is to become a widely adopted CPA.

Simulation tests conducted with the three- and two-parameter models showed that both are sensitive to changes in $L_p$ and $\omega$, and the former is relatively insensitive to $\sigma$. Both models predict essentially identical values when it is assumed that the transport of water and solute are independent. The two-parameter model is preferred for many applications owing to its inherent implementation simplicity and the questionable biological significance to the reflection coefficient [25]. The results of this study support use of the simpler two-parameter model for characterizing membrane transport dynamics of C. texanum in the presence of a rapidly permeating solute.

In view of the primary application of this work, it is well established that microalgae do not survive cryopreservation without the addition of a CPA, usually Me$_2$SO or methanol, to the culture prior to freezing [58]. Empirical observations during the development of methods suitable for cryopreserving hundreds of algal cultures in the Culture Collection of Algae at the University of Texas [J. Brand, unpublished observation] indicate that methanol is a more effective CPA than is Me$_2$SO for many strains because it is less toxic and results in higher viability subsequent to thawing. The results presented here suggest a reason for the higher effectiveness of methanol. Me$_2$SO penetrates membranes much more slowly than does water [5,59], resulting in rapid plasmolysis as water exits cells during its addition to the cells, and swelling when Me$_2$SO is removed from the extracellular medium after thawing. Similar internal osmotic stresses may be experienced when a gradient of Me$_2$SO is experienced between the cytosol and the lumen of intracellular organelles. Volumetric excursions are less pronounced with the addition or removal of methanol to the external medium because methanol penetrates membranes faster, but at rates more closely approaching, the rate of water transport.

**Implications for Cryopreservation.** The cryopreservation of algae is dependent on effective application of a CPA, including introducing the CPA into the cells and ensuring that the CPA composition and concentration do indeed result in a cryoprotective effect for the cells during freezing and thawing. A quantitative understanding of the permeability properties of the cell strain of interest to a candidate CPA at both temperatures, both above and below 0°C, provides a rational basis for the design of these processes for effective implementation. It has been known for many years that methanol renders protection to algal cells during cryopreservation. The present study provides the first extensive data for algal permeability to methanol over a range of temperatures.
relevant to cryopreservation processes. This data can be applied to the quantitative design of these processes to achieve desired performance outcomes.

Acknowledgments
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Nomenclature
Symbols

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<thead>
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<tr>
<td>A</td>
<td>surface area (m²)</td>
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<td>c</td>
<td>concentration (osmol or osmolar, mol/kg H₂O or mol/L solvent or mol/m³)</td>
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<td>e</td>
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Subscripts

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References


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