An experimental study of freezing in erythrocytes*

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Abstract—A unique experimental apparatus (termed cryomicroscope) has been developed for the study of freezing and thawing processes in living cells and has been used specifically to determine the conditions requisite for intracellular freezing in human erythrocytes. Careful measurements of the incidence of intracellular ice formation have been correlated directly with the magnitude of the cooling rate during the freezing process and degree of extracellular supercooling prior to nucleation of ice. The probability for intracellular freezing increases with the magnitudes of both the cooling rate and the extracellular supercooling. A 3-dimensional contour has been defined experimentally by which the frozen state of human erythrocytes can be predicted as a function of these two parameters.

The influence of cooling rate and extracellular supercooling on the mode of ice formation may be explained in terms of physiochemical phenomena which are controlled by the balance between the rates of heat transfer and mass transfer across the cell membrane during freezing.

Keywords—Freezing of erythrocytes

Introduction

The concept of creating a state of suspended animation in living systems has intrigued man for centuries. Countless scientific and nonscientific approaches have been taken toward solving this problem with the nearly uniform result of not being able to reinitiate the life-giving biochemical processes once they had been stopped. One approach which has held great potential for providing a technique of reversible suspended animation is to depress the temperature below a threshold level necessary to reduce the rates of the appropriate chemical reactions to zero. Unfortunately, the temperature range requisite to halt the chemical processes is below the point at which living systems experience a phase change from the liquid to solid state.

The heat-transfer and mass-transfer processes associated with the phase changes to and from the solid state were thought always to produce irreversibly lethal effects in living cells. However, in 1949, Polge, Smith and Parks discovered that the pre-freezing addition of glycerol to a cell suspension afforded protection during the freezing and thawing processes and enabled the attainment of post-thaw survival rates near 100% (Polge, et al., 1949). Subsequent to that discovery, the technique of adding solutions of nonelectrolytes to cells prior to freezing has been employed to develop long term methods of storage for several simple cell systems including human erythrocytes (Huggins, 1973) and spermatozoa (Sadler, 1966). These materials are now preserved in the frozen state for periods of several years with very little loss of viability upon thawing and return to an in vivo environment.

Extrapolation of the techniques of frozen preservation from these simple cell suspensions to more complex biological systems such as organised tissues and organs has not proved successful, however (Pepp, 1970). Problems related to controlling the heat transfer and mass transfer processes in such biological systems have not been resolved at this time. This paper describes the results of an experimental effort to quantify the effects of heat and mass transfer which determine the physiochemical state of a frozen cell as it relates to the post-thaw viability.

Definition of the problem

In order to clarify the nature of the rate processes which control cellular response to freezing and thawing, it is beneficial to consider a model thermodynamic system consisting of a single cell having a

Fig. 1 Thermodynamic system for cell freezing model
semipermeable membrane in an environment of extracellular solution. (See Fig. 1). The membrane is assumed impermeable to the electrolytes in the solution but capable of transporting water at experimentally determined rates. When this model system is frozen, ice will form first in the extracellular region while the intracellular medium is still liquid, due to a thermal gradient in the system associated with heat transfer away from the cell, and due to the effect of the plasma membrane acting as a barrier to intracellular nucleation. As extracellular ice forms, the extracellular solution becomes concentrated with electrolytes and an osmotic pressure gradient is set up across the cell membrane. Intracellular water is driven across the cell membrane into the extracellular ice phase resulting in dehydration and shrinkage of the cell. As the extracellular ice mass becomes larger with decreasing temperature, the electrolyte concentration in the remaining extracellular solution becomes increasingly higher. Consequently, the cell is exposed to hypertonic solutions on both the inside and outside of the plasma membrane. This process, for which ice forms only outside the cell until the eutectic temperature is reached, is termed extracellular freezing.

The criterion for thermodynamic equilibrium in this system is that the chemical potential of intracellular liquid water must be equal to the chemical potential of extracellular ice. This condition requires that the mass transfer of water across the cell membrane, which is a rate process that varies inversely with the system temperature, be completed to the equilibrium state at any given temperature. However, the system temperature may be lowered sufficiently rapidly so that the rate of water transport across the cell membrane will not be great enough to maintain the conditions of thermodynamic equilibrium. As a consequence, shrinkage of the cell will be reduced and the intracellular concentration of water will be higher than the equilibrium value. Intracellular water is said to be supercooled in this non-equilibrium state. When the degree of supercooling becomes sufficient, intracellular water will equilibrate with extracellular ice by undergoing a phase change into the solid state. This process is termed intracellular freezing. Both phenomena of intracellular freezing and extracellular freezing are damaging to cells, although the injury mechanisms are separate and distinct. The cells experience chemical damage as a consequence of extracellular freezing due to the exposure to concentrated solutes. Injury may be caused by subsequent denaturation of intracellular proteins and/or by conformational changes in the plasma membrane resulting in leaks. The addition of cryoprotective agents, such as glycerol, which are able to permeate the cell membrane affords protection to cells from extracellular freezing damage by diluting the concentrated solutions of electrolytes. Intracellular freezing is lethal to cells through some unspecified mechanisms of mechanical damage. Permeating cryoprotectants appear to exhibit very little protective effect against intracellular freezing injury. Thus, in order to achieve a high survival rate in the frozen preservation of bio-materials, it is necessary both to add a cryoprotective agent to the cell system and to avoid freezing protocols which result in the formation of intracellular ice. This paper presents experimental evidence which defines clearly those protocols which result in intracellular freezing for human erythrocytes.

**Experimental procedure**

An experimental technique has been developed which utilises a light microscope with a modified cooling stage to observe the dynamics of freezing and thawing processes in cell suspensions in real time. The apparatus, termed a cryomicroscope, permits the direct observation of heat transfer and mass transfer controlled phenomena in individual cells. A top view of the cooling stage, which can be mounted directly to the traversing mechanism of a Zeiss Universal light microscope, is shown in Fig. 2. The stage consists of inlet and outlet manifolds which open into a hollow central chamber having windows on top and bottom. The cell suspension to be observed is placed directly on to the top window and covered with a small coverslip. Refrigerant is pumped through the manifold and under the top chamber window thus cooling the cells to effect the freezing process. The bottom side of the top chamber window has a transparent tin-oxide coating, which is electrically resistive, deposited on it. This coating serves as a resistance heater to provide a warming effect to the cells. The system thus provides the capability for simultaneously cooling and warming the cells while under direct observation with the light microscope. It is possible, therefore, to create the desired freezing, storage, and thawing protocol on