A MICROSCOPIC INVESTIGATION OF INTRACELLULAR ICE FORMATION IN FROZEN HUMAN ERYTHROCYTES

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ABSTRACT

A cryomicroscope capable of effecting controlled freezing and thawing processes in biological cell suspensions has been developed. A miniature freezing and thawing system, adapted to the stage of a light microscope, enables a broad spectrum of cooling and warming rates to be achieved by cooling the specimen at a constant rate with a steady flow of refrigerant fluid through the system and by simultaneously dissipating electrical energy at a variable rate in a resistance heater immersed in the fluid stream and in thermal communication with the specimen. The dual capability for both heating and cooling is utilized in conjunction with an analog control system to provide for precise regulation of the specimen temperature between 100°K and 310°K at time rates of temperature change between zero and several thousand degrees centigrade per minute. A detailed discussion of the design, construction, and calibration of the cryomicroscope is presented in this thesis.

Cell suspensions are prepared for freezing in a thin film between glass coverslips and placed onto the low temperature stage. The freezing and thawing system contains a transparent section through which the dynamics of the cooling and warming processes can be microscopically observed in individual cells.

The cryomicroscope has been employed to study two phenomena associated with the intracellular freezing of human erythrocytes. The presence of intracellular ice within frozen cells was determined by visual examination of the cells in the frozen state on the cryomicroscope. An investigation of the physio-chemical conditions requisite for the formation of intracellular ice indicates that the cooling rate prior to phase change is a parameter of prime importance in regulating internal freezing. There exists a transition band of cooling rates across which the probability of intracellular freezing increases linearily from 0% to 100%. The upper and lower bounds on this band are approximately -17°C/min and -6°C/min. Surface effects of the coverslip were also shown to influence significantly intracellular nucleation in that the magnitude of the transition band of cooling rates is reduced from the value in the free melt.

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To JoAnn

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NOMENCLATURE

SYMBOL	MEANING
A	area
B	rate of temperature change
b	temperature coefficient of membrane permeability
C	electrical capacitance
C	heat capacity rate
c	heat capacity
D	diameter
d	root mean square deviation
e	voltage
G	volume flow rate
G	gain
h	heat transfer coefficient
K	thermal conductivity
$\underline{K}$	thermal diffusivity
k	membrane permeability
L	length
L	molar heat of fusion
l	dimension
$\dot{m}$	mass flow rate

SYMBOL	MEANING
n	number of moles
N_u	Nusselt number
P	power
p	pressure
Q	heat flow
q	heat flow, rate
R	gas constant
R	electrical resistance
R_{ey}	Reynolds number
T	temperature
t	time
U	overall conductance for heat transfer
V	volume
V	voltage
v	molar volume
$\overline{X}$	mean value
x	linear coordinate
α	see equation (1.2)
γ	see equation (1.2)
ϵ	see equation (1.2)
ϵ	thermal efficiency

SYMBOL	MEANING
η	see equation (1.2)
θ	nondimensional temperature
μ	viscosity
ρ	density
τ	inverse temperature
ψ	nondimensional volume
Ω	coefficient

OBJECTIVE

The objective of this thesis is to investigate the occurrence of intracellular ice in frozen human erythrocytes. A two phase program of investigation will be employed to accomplish this objective: first, an experimental apparatus will be developed, capable of detecting the presence of intracellular ice in frozen cells with a high degree of reliability; and second, the conditions requisite for the formation of intracellular ice will be identified.

CHAPTER 1

Definition of the Problem

A. Introduction:

Man has been intrigued by the idea of investigating the effects of subfreezing temperatures on living systems for several centuries. Robert Boyle published the first written account of low temperature biological research in 1683,[1] describing his experiments on the freezing and thawing of various foodstuffs, including meat, eggs, and fruit. He found that fish and frogs could survive short periods of time in the frozen state provided their bodies were not frozen completely through. During the following 250 years an ever increasing number of scientists became concerned with the consequences of low temperatures for living organisms. For the most part, the results of their work were discouraging and few advancements of lasting significance emanated from these efforts. The modern era in cryobiology can be marked from the

publication of two classic monographs, by Belehradek in 1935,[2] and Luyet and Gehenio in 1940,[3] which comprehensively summarized the work of these early investigators and established a basis for the subsequent development of theories describing the lethal effects of ice.

Interest in the preservation of biological systems by freezing was intensified in 1949 by the report of Polge, Smith, and Parkes[4] that the addition of glycerol to a suspension of spermatozoa prior to freezing produced a dramatic increase in the post-thaw viability of the cells. This remarkable and historic discovery directly led to the application of a large body of knowledge, previously of relevance only to laboratory systems, to the practical problems of cell and organ preservation in clinical medicine. The technique of prefreezing addition of a cryophylactic agent was soon adapted to many other cell types, including blood.[5] The first clinical transfusion of frozen red cells was performed by Mollison and Sloviter in 1951.[6] Subsequently, an enormous expenditure of effort by a large number of investigators culminated in the development

of acceptable techniques for the cryopreservation of many biomaterials, including red cells,*[7-10] spermatozoa,[11] cornea,[12] and skin.[13] Endeavors at freezing other biological systems have, however, produced less gratifying results. Platelets,[14] leucocytes,[15] bone marrow,[16] and heart tissue,[17] have been stored with only marginal degrees of success. Due to complexities introduced by the system geometry, organ preservation presents a vastly more complex set of problems which have defied solution to this date.

This discrepancy in the measures of success of low temperature storage techniques in various biological systems can be attributed to a general lack of understanding of the basic physio-chemical events associated with the freezing and thawing of cells and how they are related to the mechanisms of cellular necrosis. The interactions that occur in a cell and its environment during the freezing and thawing processes are

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* A review of human erythrocyte preservation by freezing and a rationale for frozen blood preservation are presented in Appendix A.

highly complex and, in many instances, intimately interrelated, thus rendering attempts at comprehensive analysis very difficult.

A multitude of hypotheses have been postulated to explain the mechanisms of cell injury. Many conflicts and contradictions exist among the proposed theories concerning crucial concepts, and no single theory has proved adequate in explaining freeze-thaw injury by itself. Although most fundamental issues related to freezing injury presently are unresolved, one common point of agreement among the various hypotheses is that the presence of ice plays an important role in determining the consequences of freezing and thawing living cells.

The occurrence of cellular damage can be correlated generally with the physical structure of ice formed during freezing. Two configurations of ice formation are important in biological materials: at slow cooling rates ice forms only extracellularly, whereas at rapid cooling rates ice forms both extracellularly and intracellularly. The study of freezing

processes can be divided accordingly into two categories, one pertaining to slow cooling rates and the other to rapid cooling rates.

A major portion of previous research devoted to the study of freezing processes in biomaterials has been focused upon clarifying the nature of phenomena that occur at slow cooling rates, for which no intracellular ice is formed. This imbalance of effort can be attributed to two factors. First, the range of cooling rates most readily achieved in both research and clinical practice favor the likelihood of extracellular ice formation. The study of intracellular ice has been impeded by the unavailability of experimental hardware capable of effecting this ice structure. Second, greater interest has been exhibited in the consequences ascribed to extracellular ice because of the frequent incidence of this mode of freezing in the present methods of cell preservation. Consequently, to date there has been only a relatively low level of effort devoted to investigation of the effects of intracellular ice attendant to higher cooling rates. These

few studies have demonstrated, however, that intracellular ice can exhibit a profound influence on the survival rate of frozen cells. For this reason, it is of great importance that the event of intracellular ice formation and the physio-chemical conditions that contribute to its occurrence be clarified. The subject of this thesis is directed toward phenomena associated with intracellular ice formation.

B. Prior Research Related to Intracellular Freezing:

The experimental problems of primary concern in the present work are first, determination of the physio-chemical conditions requisite for the formation of intracellular ice, and second, detection of the presence of ice inside of frozen cells. This section will present a synopsis of the current state of knowledge concerning these two areas.

It is generally believed that the major parameter governing the probability of formation of ice inside cells is the rate of cooling prior to the phase transition from liquid to solid. At high cooling rates, the probability of intracellular crystallization is

greater than that associated with low rates of temperature change. Previous experimental evidence suggests the existence of a transition range of cooling rates below which the probability of intracellular ice nucleation is virtually zero.[18] Many investigators have hypothesized that freezing procedures which result in the formation of intracellular ice are usually more damaging to cells than those in which intracellular ice is avoided. If this is actually the case, it is highly important to determine the transitional range of cooling rates above which intracellular ice is likely to occur in order to control most effectively the degree of injury incurred by the cells during freezing and thawing.

It is quite likely that the conditions requisite for intracellular nucleation are also functions of other parameters in addition to the cooling rate. Mazur[19] has developed an analytical model in which the probability of a cell freezing internally is dependent upon the degree of supercooling. Other influential parameters may include surface effects related to the

proximity of solid substrates,[20,21] physio-chemical properties of the plasma membrane prior to freezing,[22,23] the presence of intracellular heterogeneous nucleation sites,[24] cell geometry,[19] increased concentrations of intracellular solutes due to the loss of cell water,[25,26] and the presence of a cryophylactic agent.[27]

Mazur's model describes the relationship between the transition cooling rate for internal ice nucleation, the ratio of the volume of the cell to its surface area, and the permeability of the plasma membrane to water, as expressed in equation (1.1).

$$T e^{b(T_g - T)} \frac{d^2 V}{dT^2} - \left[(bT + 1) e^{b(T_g - T)} - \frac{A R k_g n_2}{B(V + n_2 v_1^\circ)} \frac{T^2}{V} \right] \frac{dV}{dT} = \frac{L_f A k_g}{B v_1^\circ} \qquad (1.1)$$

where b = temperature coefficient of membrane permeability
k_g = membrane permeability at temperature T_g
n_z = osmoles of solute in the cell
t = time
v_1 = molar volume of pure water
A = area of cell protoplast
B = rate of temperature change
L_f = molar heat of fusion of ice
R = gas constant
T = temperature
T_g = temperature at which permeability coefficient is defined
V = volume of water in the cell

The stated assumptions for equation (1.1) include: the protoplasm behaves as an ideal dilute solution, i.e., it obeys Raoult's law; the cell and the external medium constitute an isothermal system; the plasma membrane remains intact and is permeable only to water; the cooling rate is constant; the membrane area remains constant; and, the temperature coefficient of membrane permeability is fixed. Equation (1.1) is cumbersome to handle, both in solution and in application. Ling and Tien[28] have made mathematical modifications to the equation so that it can be expressed in nondimensionalized terms. The nondimensional groups are

$$\theta = \frac{T_f - T}{T_f} , \qquad \psi = \frac{V_i - V}{V_i} ,$$

$$\alpha = b T_f , \qquad \gamma = \frac{\Delta C_p}{R} , \qquad \eta = \frac{n v}{V_i} ,$$

$$\varepsilon = -\left[\frac{A R k_g T_f^2}{B v V_i}\right] e^{b[T_f - T_g]}$$

where C_p = difference between the molar heat capacities at constant pressure of water and ice
T_f = freezing temperature of protoplasm
V_i = initial volume of intracellular water

and the nondimensional form of equation (1.1) is then

$$\frac{d^2\psi}{d\theta^2} + \left[(\alpha+1) + \frac{\varepsilon n}{[(1-\psi)+n](1-\psi)}\right]\frac{d\psi}{d\theta} = \varepsilon\gamma e^{-\alpha\theta} \tag{1.2}$$

for $\theta \ll 1$, equation (1.2) is subject to the following initial conditions

$$\theta = 0, \psi = 0 \quad \text{and} \quad \theta = 0, \frac{d\psi}{d\theta} = 0$$

Analytic solutions may be obtained for equation (1.2) under certain limiting conditions. Ling and Tien consider four such sets of conditions ($\varepsilon \ll 1$, $\epsilon \gg 1$, $\psi \ll 1$, and $\eta = 0$), each of which corresponds to a particular set of physical circumstances. The reader is referred to the original papers[19,28] for a more detailed description of this analytical tool. Also, a further discussion of the approach adopted by Ling and Tien is presented in Appendix B.

The aforementioned model is based on the premise that the contents of the cell are in a supercooled, metastable thermodynamic state at subfreezing temperatures. The initiation of a transformation from a metastable to a more stable phase is termed nucleation. This phenomenon is illustrated in Figure 1.1. The nucleation temperature (T_n) of the system is lower than the melting temperature (T_m), the difference ΔT being defined as the supercooling. The degree of supercooling depends upon several factors, most important of which is the time rate of change of temperature for the cell contents. This fact is demonstrated for red blood cells in Figure 1.2, from Mazur.[19] The percentage

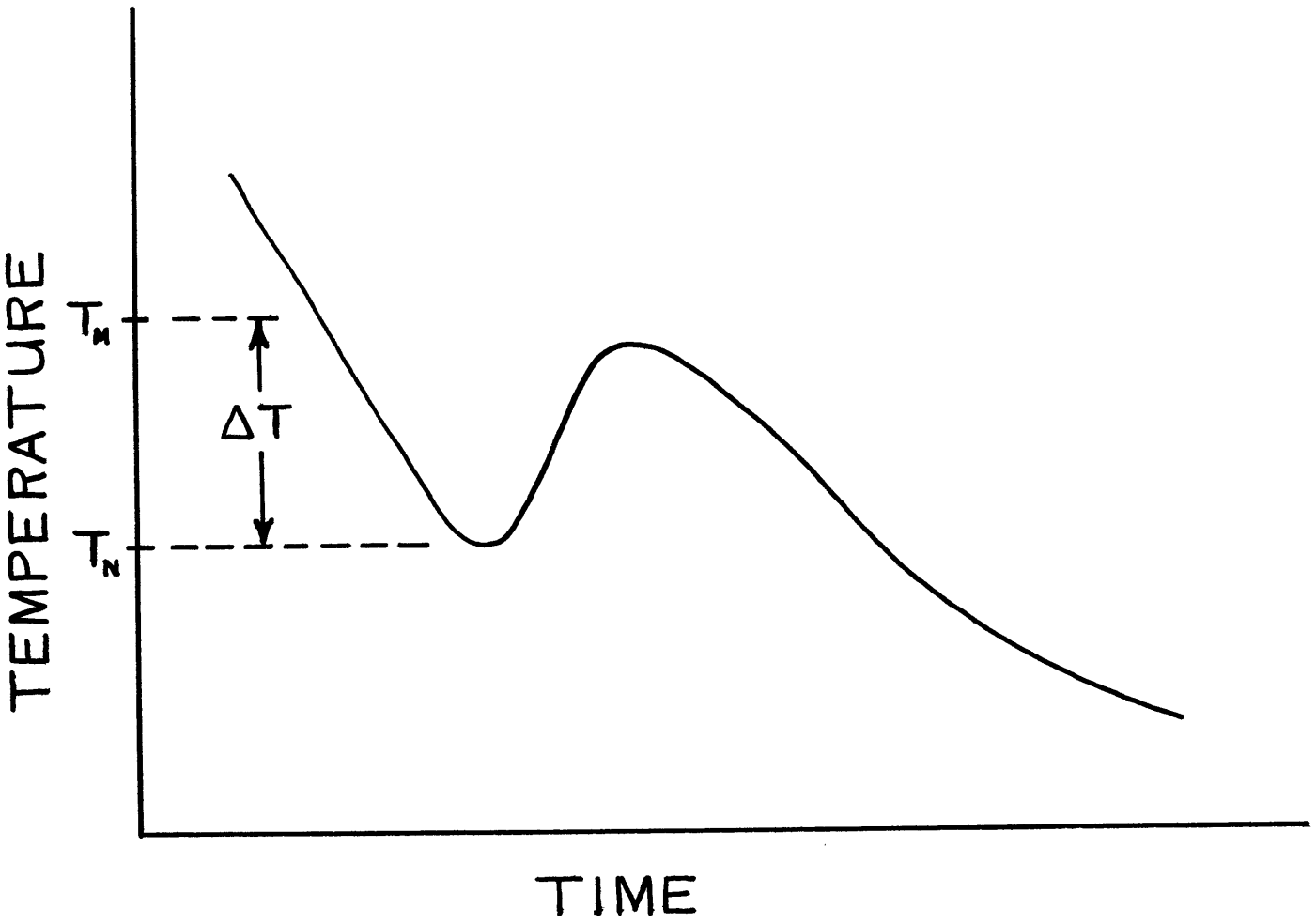

Figure 1.1. Typical Thermal History of Supercooled Water during Freezing

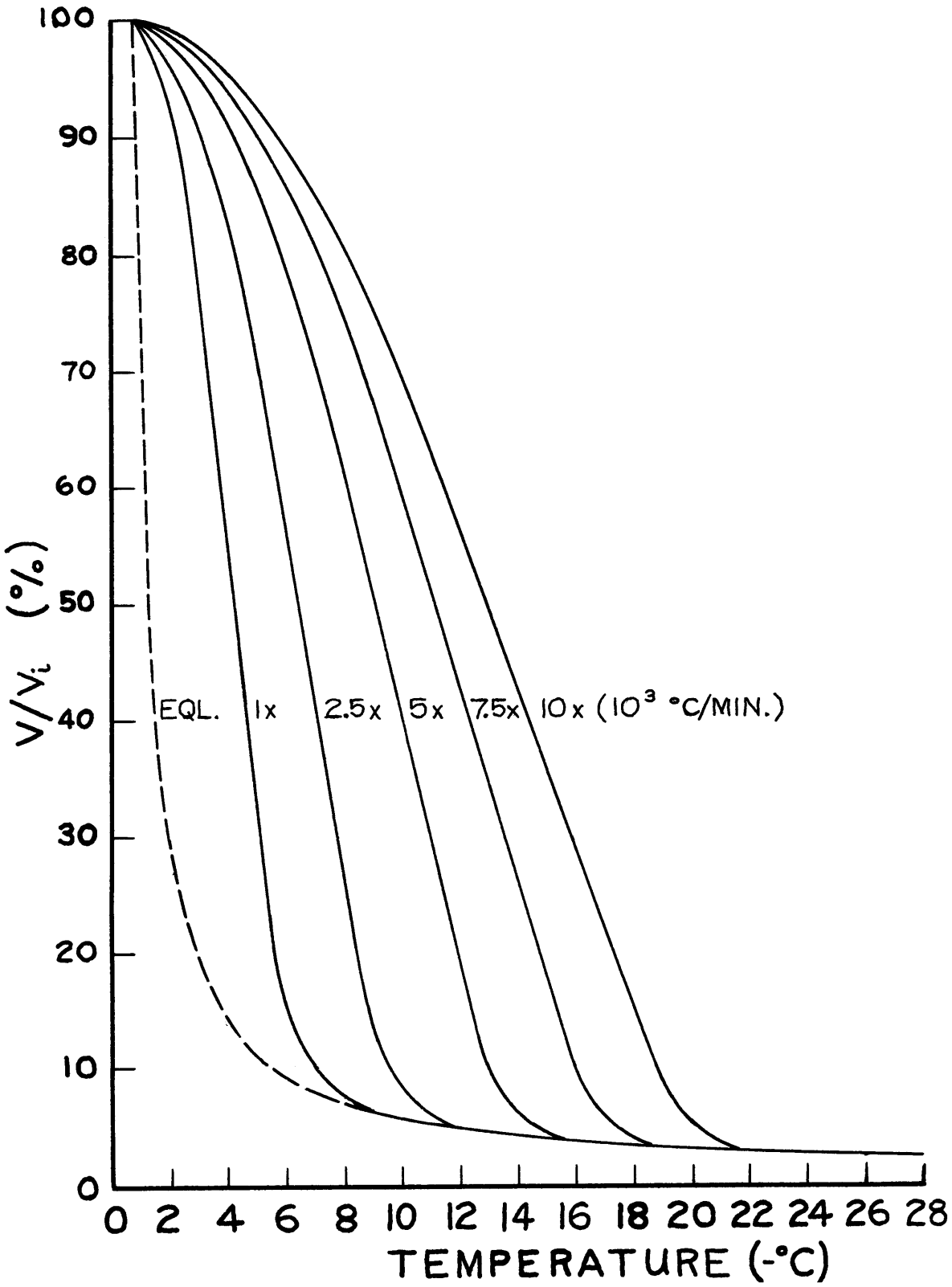

Figure 1.2. Calculated Percentages of Supercooled Intracellular Water Remaining at Various Temperatures in Human Erythrocytes Cooled at the Indicated Rates (after Mazur (19))

of the initial volume of water remaining within the cell at any given temperature is plotted as a function of the cooling rate. The equilibrium curve derived from equation (1.1) for very small values of dT/dt denotes the water content of a cell cooled sufficiently slowly so that chemical equilibrium is continuously maintained with the extracellular medium via the osmotic loss of cell water through the plasma membrane. The degree of supercooling is measured as the horizontal distance at any point on a cooling rate curve to the equilibrium curve for the same cell water content. In attaining the stable equilibrium state, intracellular liquid will tend to equilibrate with extracellular ice, either by flowing through the cell membrane to the surrounding medium or by undergoing a phase change to the solid state. At higher cooling rates, less time is available at any given temperature for the cell to attain chemical equilibrium osmotically, with the result that the degree of supercooling of the intracellular water becomes increasingly greater. Mazur has hypothesized that there exists a critical degree of supercooling between -10°C and -15°C such

that for supercooling in excess of this critical value, the cell water attains stable equilibrium by internal freezing. It is Mazur's conjecture that when the system reaches this thermodynamic state, internal ice is seeded by the penetration of extracellular ice through the cell membrane, and that this seeding is only possible because the cell membrane becomes permeable to the growth of ice crystals at the appropriately high degrees of supercooling. Equation (1.1) may be employed to predict the critical cooling rate above which intracellular ice will be formed. As a consequence, the relative terms, rapid and slow cooling rates, can be quantified. Although analytical solutions to equation (1.1) exist for certain restricted conditions, numerical solutions are not readily obtained for most cell types since quantitative data for the various physical parameters is often difficult to measure. Limited data is available to test this model for sea urchin eggs,[29] yeast,[30] and human red cells.[31] In these particular cases a computer has been employed to obtain numerical solutions which predict the occurrence of intracellular freezing at

cooling rates faster than -1°C/min, -10°C/min, and -5000°C/min respectively for the eggs, yeast, and red cells. This increase in the transition cooling velocity can be attributed in part to similar increases in the permeability of the plasma membrane to water and in the surface to volume ratio of the individual cell types. Very little experimental evidence is available to corroborate the validity of this model. The above predictions have been confirmed under certain, specific experimental conditions,[32,33] whereas under other conditions evidence is available which quantitatively contradicts this model.[34,35] There are several factors which could account for this discrepancy. The physio-chemical assumptions inherent in the analytical model are not always satisfied in the actual system. For example, the assumption of a truly isothermal thermodynamic system is known to be inherently inaccurate, the significance of which may vary according to the particular system under consideration. Other assumptions which are most likely to lead to erroneous results include: the cooling rate is

constant with time; the protoplasm behaves as an ideal solution; the temperature dependence of membrane permeability can be characterized by the equation $k=k_g e^{b(T-T_g)}$; the plasma membrane area remains constant and is permeable to water only; and, the intracellular and extracellular fluids act under the influence of a vapor pressure gradient.

An inherent assumption in this analysis which has not been explicitly stated is that the cell can be modeled as a lumped mass system for which the resistance to mass transfer is far greater across the plasma membrane than in the cytoplasm and extracellular fluid. As is pointed out by Riggs,[36] this assumption requires that the diffusion of any mass component throughout the cell be rapid enough so that the concentration of each component must be uniform both within and without the cell. It does mean that a small increment in the concentration of a component in any part of the cell must quickly permeate the entire cell so that the concentration at all other points will undergo a proportional increase. The high permeability of the erythrocyte membrane to water transport renders the

validity of this assumption questionable for red blood cells.

The proximity of a solid substrate to a frozen cell has been demonstrated to result in the intracellular nucleation of ice at cooling rates more than an order of magnitude less than the transition rate predicted by equation (1.1). The formation of intracellular ice in human erythrocytes frozen in thin films between glass coverslips has been observed at cooling rates as low as -10°C/min to -100°C/min.[35] Two possible explanations can be offered to account for this phenomena. First, since freezing occurs in biomaterials by heterogeneous nucleation, the presence of a glass substrate might provide a nucleation surface such that extracellular freezing would be initiated at a smaller degree of supercooling than would be the case in a free standing melt. Under these conditions, the intracellular fluid might in turn be forced to stable thermodynamic equilibrium by freezing for slower cooling rates (i.e., a smaller degree of supercooling) than normally occurs. A second possibility is that the proximity of a solid substrate

significantly reduces the time of crystal growth in the extracellular medium and consequently decreases the time available at any given temperature for cells to attain equilibrium osmotically. Thus, because of the reduced equilibration period, the cells would be subject to the conditions requisite for intracellular freezing at much lower cooling rates than in a free suspension. Lindenmeyer, et.al.[20] have observed a progressive increase in the rate of growth of ice crystals in supercooled water from a free volume, to a glass substrate, to a brass substrate. This phenomenon is explained in that the rate of growth of ice in water is limited by the rate of conduction of the latent heat of fusion away from the phase interface, both for free growth and for growth on a solid substrate. The faster growth in the latter instance is caused by the proximity of the substrate acting as a heat sink.

Another parameter which could influence the conditions required for intracellular nucleation is the presence of a cryophylactic additive. It has been amply demonstrated (especially for the human erythrocyte)

that the survival rate of cells frozen by rapid cooling can be altered significantly by the addition of a wide variety of compounds, including polyvinylpyrrolidone (PVP),[37] dextran,[38] and glucose.[39] However the mode of action of additive compounds upon frozen cells is not understood. Very few attempts have been made to determine how protective additives influence the formation of intracellular ice. There is a general concurrence that additives act to hinder the nucleation of ice inside of cells, which in effect increases the degree of supercooling that can occur before intracellular ice forms. Smith and Smiles[40] observed that a variety of cell types, suspended in a glycerol solution, did not contain internal ice when frozen at cooling rates rapid enough to produce internal ice in nonglycerolized freezing. An exception to this observation was very large spermatocytes which did freeze internally at the predicted cooling rates. Luyet and Rapatz noted that the nucleation and growth of ice crystals is strongly hindered by additives.[41] However, they also observed by light microscopy[42] and electron microscopy[43] that intracellular ice does occur in frozen erythrocyte

suspensions containing glycerol. Smith et.al.[44] and Diller and Cravalho[45] have verified this finding by light microscopy. In their electron microscopy study, Rapatz and Luyet demonstrated that ice particles, both intracellular and extracellular, become gradually smaller in increasing concentrations of glycerol. Mazur has shown that inorganic salts protect yeast partially against injury from intracellular ice.[46] He hypothesizes that the mode of protection is by cellular dehydration by extracellular salts, thereby reducing the probability of intracellular freezing.

All of this experimental data, taken together, points to the supposition of Lusena[47] that cryophylactic additives do affect the conditions for intracellular ice formation by increasing the degree of supercooling that can be tolerated by a cell before stable equilibrium is attained by internal freezing. It must be emphasized, however, that this concept is entirely hypothetical, and both qualitative and quantitative verification are lacking.

C. Techniques for Detecting the Presence of Intracellular Ice:

A fundamental difficulty associated with the experimental study of intracellular ice is the detection of this ice form. Various methods of approach have been employed to solve this very difficult experimental problem. The technique used most frequently utilizes microscopy in an attempt to "see" ice crystals in frozen cells.

In 1897, Molish[48] became the first person to directly observe, at the microscopic level, the freezing of living material. He froze Amoebae and plant tissue by immersing his light microscope directly into an ice chest, where he could watch the dynamic events of the freezing process and, upon return of the microscope to the room environment, the thawing process. Thus was born the technique of cryomicroscopy. Although the apparatus was inconvenient to use and afforded virtually no control over the specimen temperature and its time rate of change, Molish was able to observe that when filaments of Spirogyra were frozen, a crust of ice formed around the outside

of each filament. He could not detect the presence of any ice within the individual cells, and concluded, therefore, that the water from which the ice was formed leaked out through the cell walls and was frozen extracellularly.

Weigand,[49] in 1906, microscopically investigated the freezing of buds. He chose to effect freezing in the specimen by moving his microscope out of doors during subzero weather. Ice was observed to form inside cells following various degrees of supercooling, ranging between -18°C and -26°C.

Schander and Schaffnit[50] built the first cryomicroscope with a cold chamber mounted on the stage. The chamber and specimen were cooled by a refrigerant stream of cold CO_2 gas, thereby providing a degree of temperature regulation previously unknown. This apparatus was used to observe the formation of a layer of ice on the inside of frozen plant cells.

Although these early investigators realized only limited success in obtaining experimental data, their efforts did illustrate the capability of the cryomicroscope to enhance the scope of certain aspects

of low temperature biological research. The technique of direct microscopic observation of freezing and thawing processes has been subsequently pursued by many investigators, employing a wide variety of approaches to cooling the specimen and regulating its temperature.[51-65] One of the most ingeneous of the early systems was built by Mason and Rochow at Cornell University in 1928.[54] Their apparatus consisted of a light microscope with a modified cold stage employing a circulating refrigerant and devices for controlling very accurately the specimen temperature down to -25°C at moderately rapid cooling rates. More recent systems have attempted to extend the range of specimen temperatures and time rate of temperature change with only limited success. In general, it has been necessary to make sacrifices in the capability for microscopically observing cells during freezing and thawing in order to improve the degree of control over the specimen temperature. Indeed, many systems have not even been capable of achieving the thermal conditions requisite for the formation of intracellular ice. Some investigators have, however, produced very beautiful photomicrographs which appear to illustrate

clearly the presence of ice particles inside forzen cells.[53,55,58,64,65] These observations have produced evidence which strongly supports the contention that ice can be present inside cells under special freezing conditions. The technique of cryomicroscopy has thus proved to be a most useful tool for detecting intracellular ice. The use of the cryomicroscope does have an inherent drawback, though, in that a significant degree of interpretation is required to evaluate the pictorial data obtained by this method. The experience of the investigator is an important factor in the ability to distinguish between actual ice crystals and artifacts created by the experimental technique. Luyet, who can claim the most extensive experience in the world with cryomicroscopy, has questioned recently the identification of intracellular ice by this technique, in that it is difficult to distinguish whether ice crystals are actually within or above the frozen cells.[42] He notes that in some instances it seems quite clear that unfrozen cells are covered by films of ice of criss-crossed structure.

D. Problems Encountered in Measuring and Defining the Thermal Parameters of Freezing and Thawing:

An additional problem which has hindered the correlation of intracellular ice formation with cooling rates is the lack of uniformity among the various investigators in their approach to defining, measuring, and controlling the rate of temperature change during cooling. These three items will be considered individually. Several methods have been used to define cooling rate for any given temperature time curve. First, an averaging technique may be employed in which the cooling rate is defined by the two end point temperatures and the time required to traverse between them. This is the most commonly employed technique, although there is considerable variation in the end point temperatures chosen. A major drawback to this technique is that in using only an average value for the cooling rate, important information describing the freezing process may be lost. For example, the cooling rate at a given temperature, such as at the liquid-solid phase change, may be significantly different from the average value between ambient and storage temperatures and considerably more important

in determining the survival rate of frozen cells. Thus, the value of a potentially significant parameter may go undetected.

Other techniques for measuring the cooling rate include using either the maximum value that is attained or the rate at some given temperature. None of these three techniques when used by itself yields complete data describing the time rate of temperature change, unless it is constant through its complete range. This gives rise to two difficulties in comparing the data of various investigators: the data may not be based on similarily defined cooling rates, and the measured rates may vary in their degrees of revelancy to the phenomena being described.

The measurement of temperature and its time rate of change has been a continuing source of difficulty to cryobiologists. Problems may arise in three aspects of these measurements. The first difficulty concerns the accurate detection of the actual specimen temperature with an appropriate sensor. Typically, thermocouples are used for this purpose. The primary limitation of thermocouples for measuring temperatures of

cell suspensions is that they are capable of detecting only an average temperature of the specimen. It is not possible to sense the spatial temperature gradients that exist intra- and extracellularily and across the cell membrane. Thus a great deal of pertinent information concerning the temperature history of the specimen is lost due to insensitivity of the measuring technique. To date there has been no experimental method devised capable of measuring these small scale temperature gradients within and across individual cells. The challenge of accomplishing this task is very great indeed since it would require the detection of a temperature difference across a space of approximately 1 micron.

A second difficulty in temperature measurement can be attributed to inadequacies in the readout device. This situation is often most critical when large thermal transients are being measured. Recording instrumentation having a very short response time is required to follow the time response of the temperature in these situations.

The third difficulty in accurately sensing temperature can be caused by large scale temperature gradients within the specimen. This effect is particularly pronounced in the freezing of large specimens, such as tissues and organs, in which the surface to volume ratio is small. Since the thermocouple is capable of measuring only a localized bulk temperature, there may be at any given time a large variation in both temperature and time rate of temperature change throughout the specimen. Thus the recorded time-temperature curve can not be assumed to represent that of the entire specimen, and errors will result when experimental results are assessed on the basis of such an averaging assumption. Analytical models of the pertinent heat transfer processes can be developed to describe the spatial and temporal temperature variations in the specimen.[66] The accuracy of these models is limited by the knowledge of the thermal properties of the biomaterial being frozen.

E. Summary:

A majority of prior research in cryobiology has not dealt with the formation of intracellular ice and its consequential effects on the survival of frozen biomaterials. As a result very little progress has been made toward understanding the phenomena associated with this type of freezing. Nonetheless, evidence has been produced which conclusively supports two postulates: first, ice does form inside of cells in suspension for certain limited conditions of freezing; second, the presence of ice within a cell in the frozen state almost invariably results in injury upon thawing. Because of the latter phenomenon, it is important to define the conditions requisite for the formation of intracellular ice in order to control the survival rate of frozen biomaterials.

There has been essentially no experimental work directed toward determination of the physio-chemical requirements for intracellular freezing. An analytical model has been proposed that describes the critical processes controlling intracellular nucleation as a competitive balance between heat and mass transfer across

the cell membrane that is regulated by the magnitude of the cooling rate.[19] However, the validity of this analysis has yet to be demonstrated by experimental evidence. Three factors which have contributed heavily to this paucity of data are: difficulty in effecting the conditions necessary for intracellular freezing, lack of reliable methods for detecting the presence of an ice phase within a cell, and uncertainty in measuring and controlling the cell temperature and its time rate of change.

In light of this background, it is proposed to modify the stage of a light microscope with a low temperature freezing and thawing system that incorporates the capability for minimizing the above three restrictions. In this manner it will be possible to accurately measure the conditions for intracellular freezing and to obtain an experimental indication of the validity of the theory that competing heat and mass transfer processes regulate the incidence of intracellular freezing. The design and construction of this apparatus, and experimental data having a direct bearing on the above biological problems are discussed in this thesis.

CHAPTER 2

Experimental Approach

A. Rationale:

As described in the previous chapter, limited prior investigations into the effects of intracellular ice formation indicate that (a) it does occur, (b) its presence has a significant effect on the survival of frozen and thawed cells, and (c) very little detailed information is available concerning the conditions for its formation and the nature of its interaction with biological cells resulting in injury. Two areas of fundamental importance to intracellular freezing which require further experimental clarification are: (a) accurate detection of the presence of ice inside of biological cells, and (b) control of the parameters which govern the conditions requisite for the formation of intracellular ice.

The problem of detecting the presence of intracellular ice has been approached with a number of experimental techniques. The most straight forward method is to try to "see" ice crystals inside of frozen

cells. Several techniques have been employed to look at frozen cells including electron microscopy,[67-70] x-ray analysis,[71-72] and light microscopy, the latter being the most direct method. Light microscopy has important advantages over the other two techniques in that (a) dynamic observations of the freezing and thawing processes can be made as opposed to only a static pictorial record taken at a specific point in time, and (b) the technique of observing the cells is more direct and, as such, less likely to introduce artifacts into the final visual output.

A program of study has been adopted to facilitate the investigation of phenomena associated with the intracellular freezing of biological cells. This program employs a cryomicroscope with the capability for investigating a range of cooling and warming rates significantly larger than has previously been possible by cryomicroscopy, and an automatic temperature control system which can regulate very accurately the specimen temperature and its time rate of change, within the heat transfer limitations of the apparatus. The cryomicroscope is the key component of the experimental

system because it permits direct investigation of the actual dynamics of the physio-chemical processes associated with freezing and thawing.

The design of the total system was dictated in part by the experimental procedure to be employed in the program of investigation. The active role of any individual parameter in governing freezing and thawing processes can be ascertained most effectively when it is isolated from the influence of all other pertinent parameters. This condition can be satisfied by independently controlling the value of each individual parameter, provided no coupling interactions occur. For example, evaluation of the role of the cooling rate in causing intracellular ice formation in a frozen suspension of erythrocytes depends on the minimization of variations in intracellular nucleation attributable to all other influences. This experimental objective can be realized by measuring the frequency of intracellular crystallization as the cooling rate is systematically varied within a selected range of values, with all other relevant parameters held

constant. Thus, to attain the precise degree of regulation of specimen temperature and cooling and warming rates requisite for utilization of the afore mentioned experimental procedure, an automatic temperature control system was adapted to supplement operation of the cryomicroscope.

The design of the cryomicroscope and the ancillary temperature control unit will be described in the following section.

B. Apparatus:

A cryomicroscope and analog temperature control system, shown schematically in Figure 2.1, have been fabricated to incorporate specifically the experimental capabilities set forth in the rationale.* A special thermodynamic system, capable of effecting controlled freezing and thawing processes in biological cells, was made to fit onto the stage of a light microscope. A broad spectrum of cooling and warming rates can be achieved in the test specimen by cooling the system

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* A detailed description of the cryomicroscope design is presented in Appendix C.

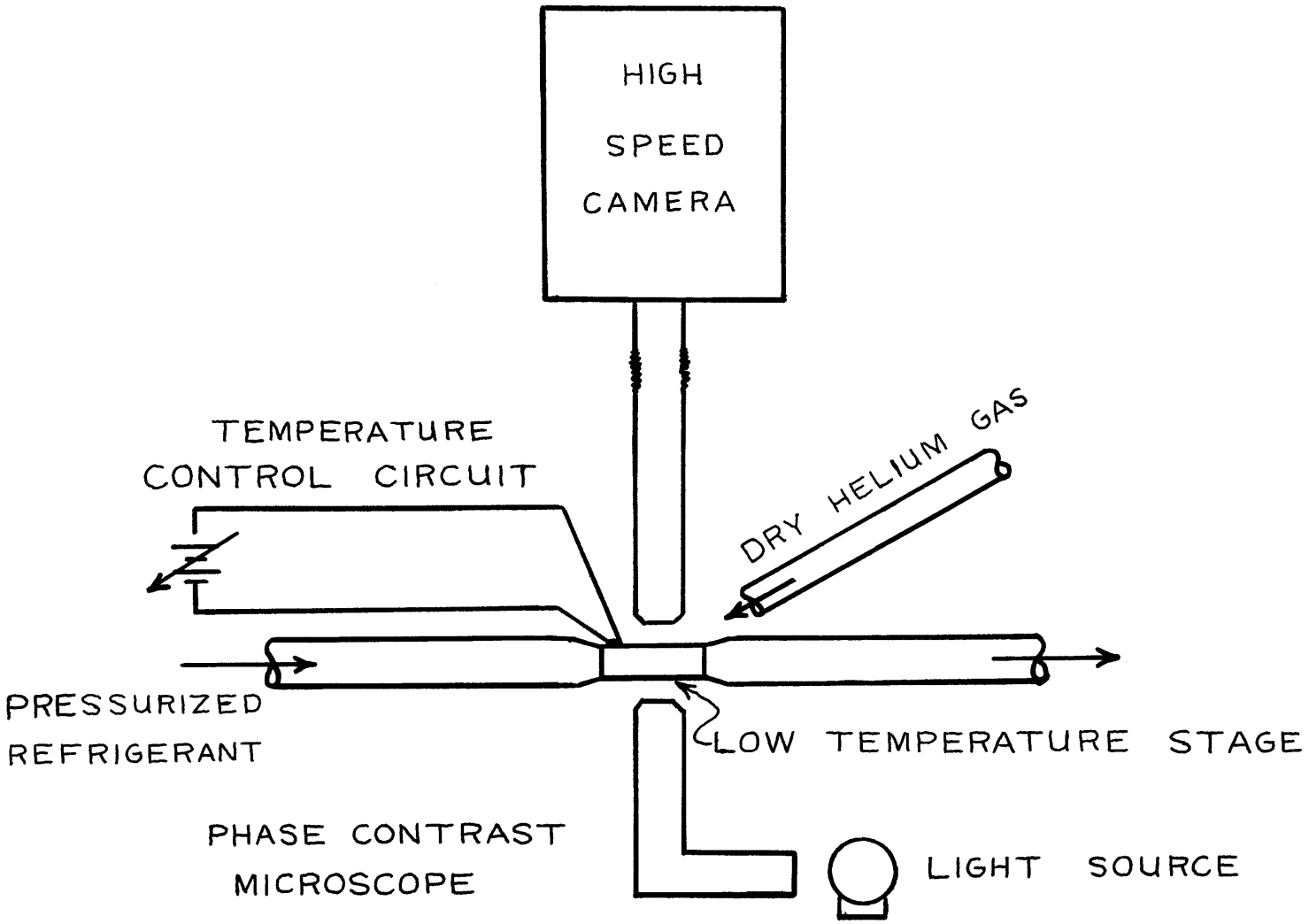

Figure 2.1. Schematic of Cryomicroscope and Temperature Control System

at a constant rate with a steady flow of refrigerant fluid through the device and by simultaneously dissipating electrical energy at a variable rate in a resistance heater immersed in the fluid stream and in thermal communication with the specimen. The maximum cooling rate in the specimen is reached by dissipating essentially no electrical energy in the heater whereas the maximum warming rate is reached by reducing the refrigerant flow rate to zero and dissipating electrical energy at the highest rate consistent with heater integrity. A detailed heat transfer analysis of the thermodynamic system is presented in Appendix D.

The design of the thermodynamic system is illustrated schematically in Figure 2.2. The system consists of a stainless steel chamber, 7/16 inch square by 9/32 inch thick, fabricated from a 0.020 inch thick sheet. Stainless steel refrigerant intake and exhaust tubes, of ¼ inch outside diameter and 0.019 inch wall, are silver soldered to the chamber. Quartz windows 0.0065 inches thick are permanently sealed by epoxy (equal parts of Epon 828 Resin and Epon Curing Agent

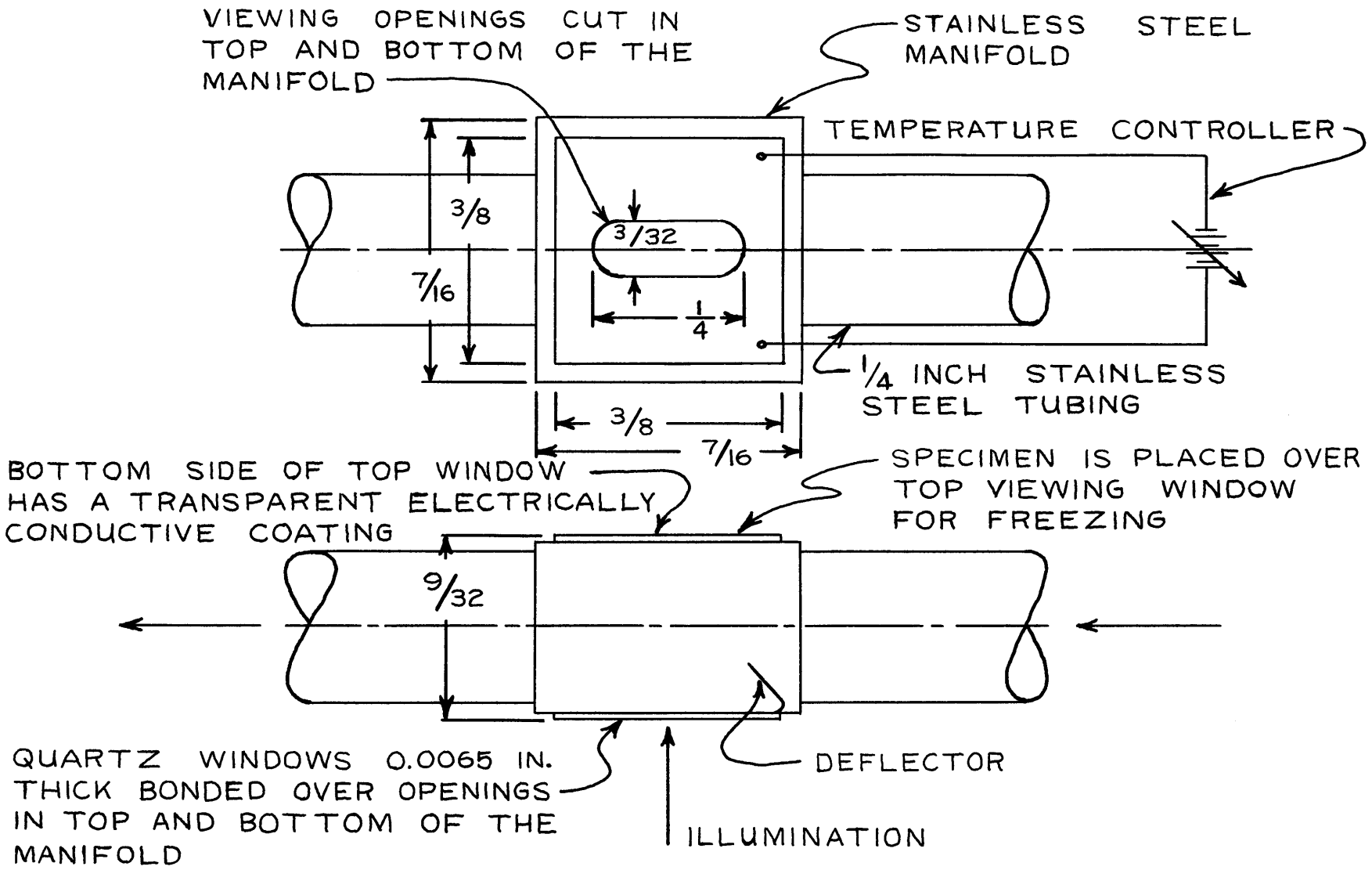

Figure 2.2. Drawing of Low Temperature Chamber

V-25) impregnated with copper powder, over the viewing openings cut into the top and bottom faces of the chamber. Quartz is used as the material for the windows in preference to glass because of its superior optical quality. A deflector mounted inside the chamber at the intake tube directs the impinging refrigerant stream against the bottom side of the top window to enhance the convective component of heat transfer.

The resistance heater consists of a thin, transparent tin oxide coating deposited on the under side of the top quartz window. By varying the thickness of this electrically conductive coating, the desired heater resistance can be achieved. The resistance is essentially independent of temperature between 310°K and 77°K, and is typically fixed in the range of 50-100 ohms in order to best match the other components in the electrical circuit. Precoated glass sheet may be obtained commercially from the Corning Glass Works, Corning, New York. The quartz windows for this sytem were coated in the Cryogenic Engineering Laboratory, M.I.T. The technique for preparing and applying the

conductive coating is described in Appendix E. Copper electrical leads are attached to the coated surface with Dynaloy 350 solderable silver paint* and connected to the temperature control system.

The chamber assembly is mounted in a phenolic block attached to a standard Leitz traversing mechanism with two degrees of motion in the horizontal plane (see Figure 2.3). The low thermal conductivity of the phenolic block serves to isolate thermally the low temperature portion of the stage from the microscope body. The complete unit is bolted to a standard stage base and mounted on a Zeiss Universal light microscope.

The specimen to be viewed can be mounted on the low temperature chamber in two ways. It may be placed directly on the top chamber window and then covered with a coverslip of the appropriate thickness, or it may be placed as a film of cells between two coverslips to form a sandwich which is then placed on the top chamber window. The first method is preferred in that

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* available from Dynaloy, Inc., 408 Adams Street, Newark, N.J., 07114

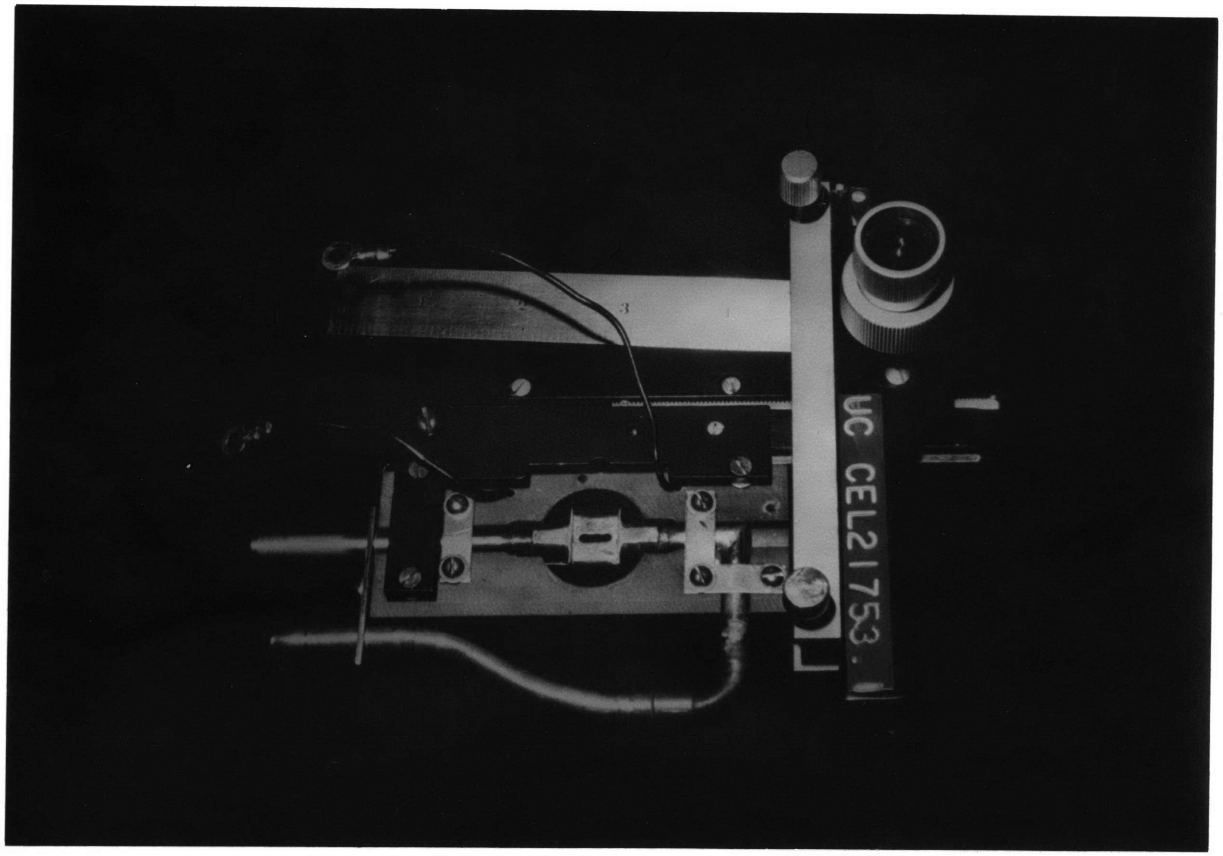

Figure 2.3. Bottom View of Low Temperature Chamber

it minimizes the effective specimen thermal mass and thermal resistance between the refrigerant and the specimen; however, it does have the disdavantage that it is more difficult to prepare than by the latter method. Ease of preparation dictates that the two coverslip technique be most frequently employed. Regardless of which method is used, the top coverslip contains a small pocket etched out with hydroflouric acid and fitted with a copper-constantan thermocouple, 0.002 inch wire diameter. The small diameter of the wires is crucial in order to minimize the thermal mass and hence, the thermal response time of the thermocouple. The output of this thermocouple, which can be displayed on an oscilloscope, oscillograph, or strip chart recorder, serves as a measure of the bulk average temperature of the specimen.

The microscope is fitted with a long working distance (7mm) condenser, Zeiss model IV Z/7, equipped for phase microscopy. The specimen is viewed with a phase contrast 63X, 0.90 NA, Neoflnar Zeiss dry

objective with a working distance of 0.12 mm.* A dry objective is used in preference to oil immersion to avoid the phase change that would occur in the immersion oil at subfreezing temperatures and to minimize the effective thermal mass of the specimen. With this objective the maximum useful total magnification is limited by resolution considerations to approximately 1000X. The objective, condenser, and thermodynamic system are enclosed in a transparent plastic housing in which a slight positive pressure of dry helium gas is maintained in order to prevent the condensation of water vapor on the specimen and to reduce the thermal contact resistance between the specimen coverslip and the low temperature chamber. It is demonstrated analytically in Appendix G that helium functions effectively in this latter capacity.

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* There was initial concern that the close proximity of the objective to the low temperature stage would cause damage to the objective by thermal stresses. A series of tests was performed on an objective to determine the effects of exposure to both subfreezing (0°C)temperatures and rapid rates of temperature change. The objective survived the most strenuous conditions, that being rapid immersion into a stream of pressurized LN_2, without damage. A detailed account of this series of tests and the results are presented in Appendix F.

A photograph of the cryomicroscope as described above is shown in Figure 2.4.

Compressed, dry helium gas, refrigerated by passing through a liquid nitrogen bath heat exchanger, is piped directly to the intake tube of the low temperature chamber. The flow rate of refrigerant is controlled by throttling the helium from a standard compressed gas cylinder through a pressure regulator and a needle valve. The refrigerant temperature can be regulated by both the flow rate of helium gas and the LN_2 level in the heat exchanger. The exchanger was designed to achieve a maximum temperature drop of 190°C at a total refrigerant flow rate of 180ft.^{3}/min.* A photograph of the heat exchanger illustrating the liquid nitrogen bath and the tubing through which the helium gas flows is shown in Figure 2.5.

A temperature feedback control system is employed in conjunction with the cryomicroscope to achieve a broad spectrum of constant cooling and warming rates

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* The design calculations for the heat exchanger are presented in Appendix H.

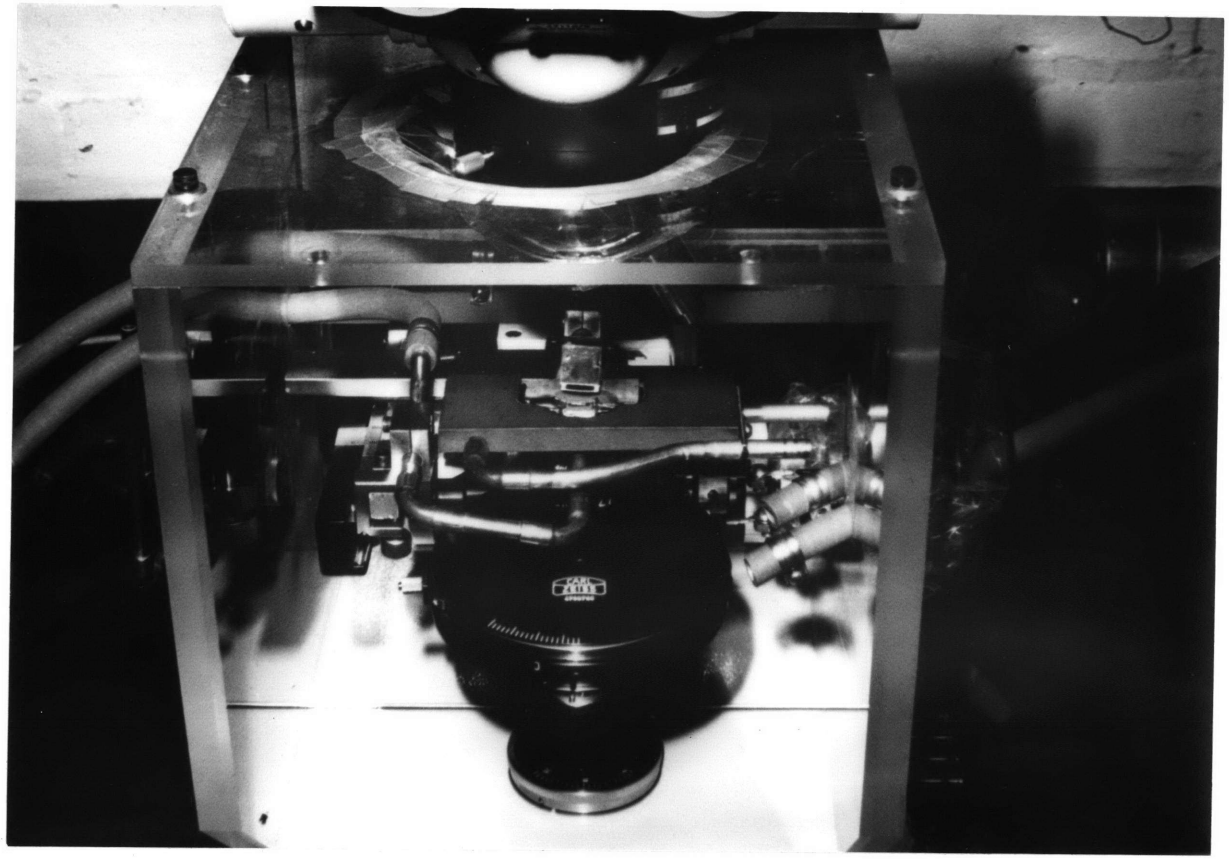


Figure 2.4. Front View of Cryomicroscope

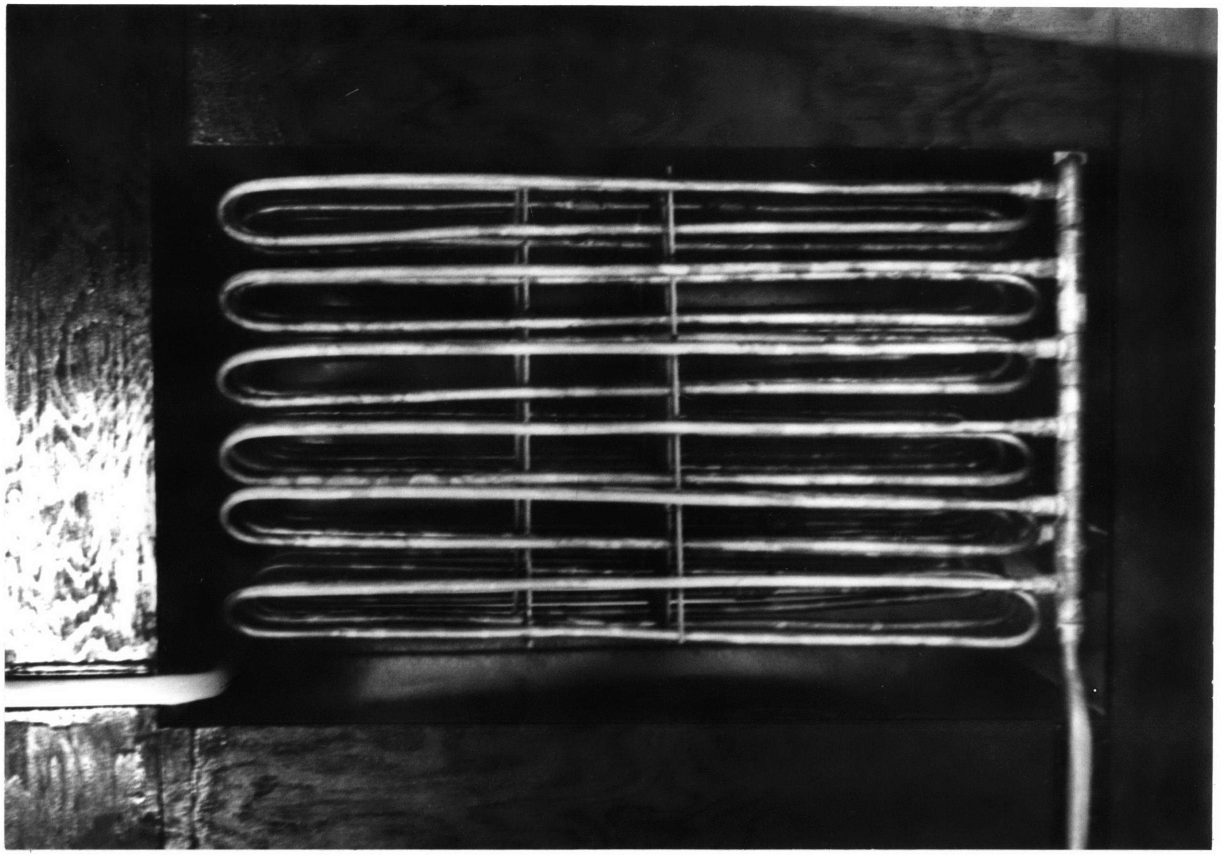


Figure 2.5. Top View of Refrigerant Heat Exchanger

and storage and thawing temperatures in the specimen. Figure 2.6 portrays schematically the functional relationship among the components of the control system. The theory of operation is described as follows.

The average, bulk temperature of the specimen is continuously monitored by a 0.002 inch copper-constantan thermocouple. The voltage signal from the thermocouple is supplied as the input signal to an electronic control circuit, which is shown diagrammatically in Figure 2.7. The control system is of the analog type in which a signal generator, inverter, and integrator are employed to create a voltage representative of the desired linear temperature-time profile, be it for cooling or warming. This profile is continuously compared in a summing unit with the amplified thermocouple voltage, which is representative of the actual specimen temperature. A positive output from the summing unit, indicating that the specimen temperature is lower than the generated reference temperature, requires that heat be supplied to the specimen to raise its temperature to the desired level. If the summing unit output is negative,

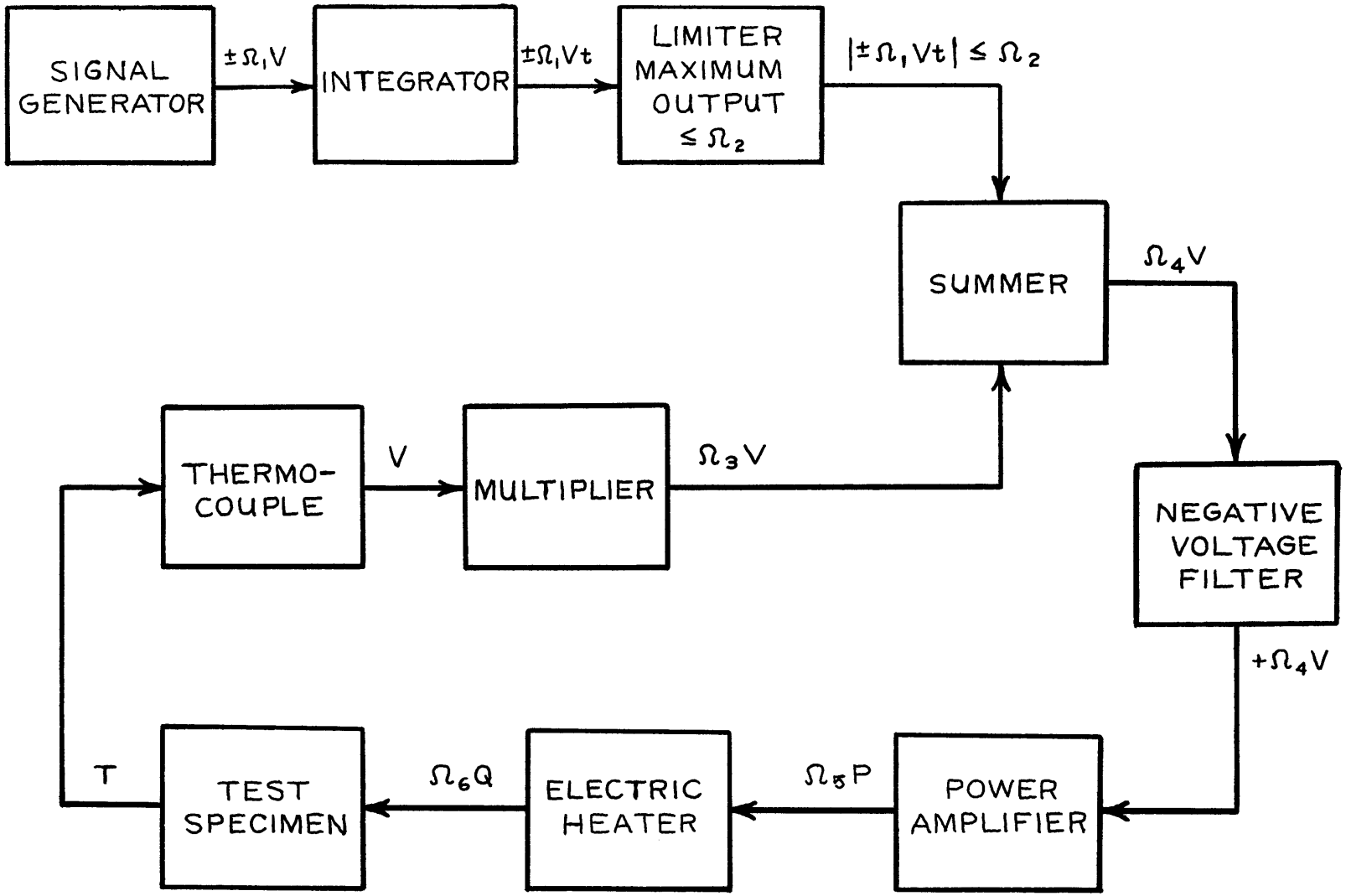
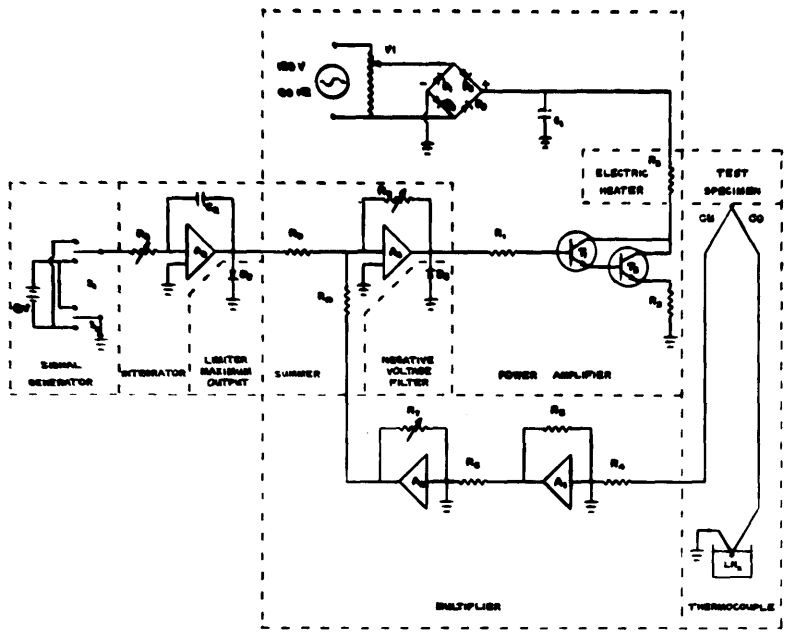


Figure 2.6. Block Diagram of Control System



C_1 - 240 μF, 300 VDC — A_1 - PHILBRICK P65AU — R_{13} - 299 KΩ, 1 WATT — S_2 - SPST SWITCH
C_2 - 5000 μF, 100 VDC — A_2 - PHILBRICK P65AU — R_{14} - 150 KΩ, 1 WATT — T_1 - MOTOROLA MJ423
D_1 - MOTOROLA MR112 — A_3 - PHILBRICK P65AU — R_{17} - 2.53 MΩ or 3.03 MΩ, 1 W. — T_2 - MOTOROLA MJ423
D_2 - MOTOROLA MR112 — A_4 - PHILBRICK P65AU — R_{18} - 650 Ω or 950 KΩ, 1 W. — V_1 - VARIAC
D_3 - MOTOROLA MR112 — R_1 - 710 KΩ, 2 WATT — R_{19} - 230 KΩ, 1 WATT — CO - CONSTANTAN LEAD
D_4 - MOTOROLA MR112 — R_2 - 50 Ω HEATER — R_{20} - 230 KΩ, 1 WATT — CU - COPPER LEAD
D_5 - MOTOROLA MR1049 — R_3 - 40 Ω, 30 WATT — R_{21} - 19 KΩ or 5 MΩ, 1 WATT — LN_2 - LIQUID NITROGEN BATH
D_6 - MOTOROLA MR1049 — R_4 - 10.4 KΩ, 1 WATT — S_1 - DPST SWITCH — 6V - DRY CELL

Figure 2.7. Circuit Diagram of Control System

the heat supplied to the specimen is reduced to zero. The necessary heat input to the specimen is obtained by amplifying the summing unit output signal in a power amplifier having the electrical film resistor, which is coated on the top window of the low temperature chamber, as its load. The power supplied to the heater is proportional to the difference between the actual specimen temperature and the reference temperature. The feedback control loop is closed in that the thermocouple senses changes in the specimen temperature effected by variations in the amount of energy dissipated in the electrical resistance heater, and feeds this signal back into the control circuit.

The various cooling rate profiles are generated by setting the value of the integrator input resistor, R_8 (Figure 2.7), according to a predetermined calibration. For a constant input voltage, e_i, of 6 volts from the signal generator, the slope of output voltage, $e_{I-o}(t)$, profile for the integrator is determined

according to the following equation(73),

$$e_{I_o}(t) = -\frac{1}{C_2 R_8}\int_o^t e_{I_i}(t)\,dt$$

$$\frac{e_{I_o}(t)}{e_{I_i}} = -\frac{t}{C_2 R_8} \qquad (2.1)$$

The value of the capacitor C_2 is set at $5300\,\mu F$, whereas the resistor R_8 can be varied between 650 ohms and 580×10^3 ohms. These numerical values can be substituted into equation (2.1) to predict that the slope of the output voltage profile can be altered between 0.0574 volts/min and 51.2 volts/min, which covers 3 orders of magnitude. The actual measured performance characteristics of the integrator are discussed in Chapter 4, Section A, Part 1.

The maximum value of $e_{I\text{-}o}(t)$ is set at +2.5 volts by the limiter diode D_5, while the minimum value is 0 volts. The control system is calibrated so that these endpoint voltages represent specimen temperatures of +25°C and -196°C respectively. The function of the integrator is to vary $e_{I\text{-}o}(t)$ with time to simulate cooling

and warming processes through the above range of temperatures. The control system is changed between cooling and warming rates by activating switch S_1, which reverses the polarity of the integrator input voltage, e_{I-i} . Constant temperature processes are simulated by opening switch S_2, which reduces e_{I-i} to zero.

The total voltage gain across the multiplier can be determined from the following equation(73),

$$\frac{e_{M-o}(t)}{e_{M-i}(t)} = \frac{R_5}{R_4}\,\frac{R_7}{R_6} = G_M \qquad (2.2)$$

where resistors R_4, R_5, and R_6 are set respectively at 10.4×10^3 ohms, 258×10^3 ohms, and 180×10^3 ohms and R_7 can be varied between 2.53×10^6 ohms and 3.03×10^6 ohms. $e_{M-i}(t)$, the multiplier input signal, is the specimen thermocouple output voltage. Thus,

according to equation (2.2), the multiplier gain can be adjusted in the range

$$3.55 \times 10^2 \leq G_M \leq 4.26 \times 10^2$$

For a liquid nitrogen reference junction (-196°C), the thermocouple output for a specimen temperature of +25°C is -6.5×10^{-3} volts. Thus, from equation (2.2) the multiplier output voltage lies in the range

$$-2.31 \text{ v.} \leq e_{M-c}(t) \leq -2.77 \text{ v.}$$

The resistor R_7 can be adjusted so that for specimen temperatures between 10°C and 40°C, the two input voltages to the summing unit, $e_{I-c}(t)$ and $e_{M-o}(t)$,

can be made equal in magnitude (2.5 volts) and opposite in sign. The control system is calibrated by appropriately adjusting R_7 before each freezing experiment so that when the specimen is at ambient temperature,

$$-\left[e_{M-O}(t)\right]_{\substack{AMB.\\TEMP.}} = \left[e_{I-O}(t)\right]_{MAX} = 2.50 \text{ volts}$$

The gain across the summing unit is determined by,[73]

$$e_{S-O}(t) = \frac{R_{11}}{R_9} e_{I-O}(t) + \frac{R_{11}}{R_{10}} e_{M-O}(t) \tag{2.3}$$

$$G_S = \frac{e_{SO}}{e_{IO} + \frac{R_9}{R_{10}} e_{MO}} = \frac{R_{11}}{R_9}$$

where $R_9 = R_{10} = 203 \times 10^3$ ohms and 18×10^3 ohms $\leq R_{11} \leq 5 \times 10^6$ ohms. Thus, the upper and lower

bounds on G_s are

$$3.87 \times 10^{-2} \leq G_s \leq 2.46 \times 10^{1}$$

The sensitivity of the control system to correcting differences in the specimen and generated reference temperatures is regulated by adjusting the value of resistor R_{11}.

A photograph of the logic portion of the temperature control circuit is shown in Figure 2.8.

The power amplifier must satisfy the requirement of providing linear voltage gain at frequencies down to D.C. To achieve this criterion, a simple two stage D.C. amplifier was fabricated as shown in Figure 2.7. Two NPN type transistors are connected in cascade formation to provide the necessary amplification. The gain characteristic of the system is plotted in Figure 2.9 for selected values of the output

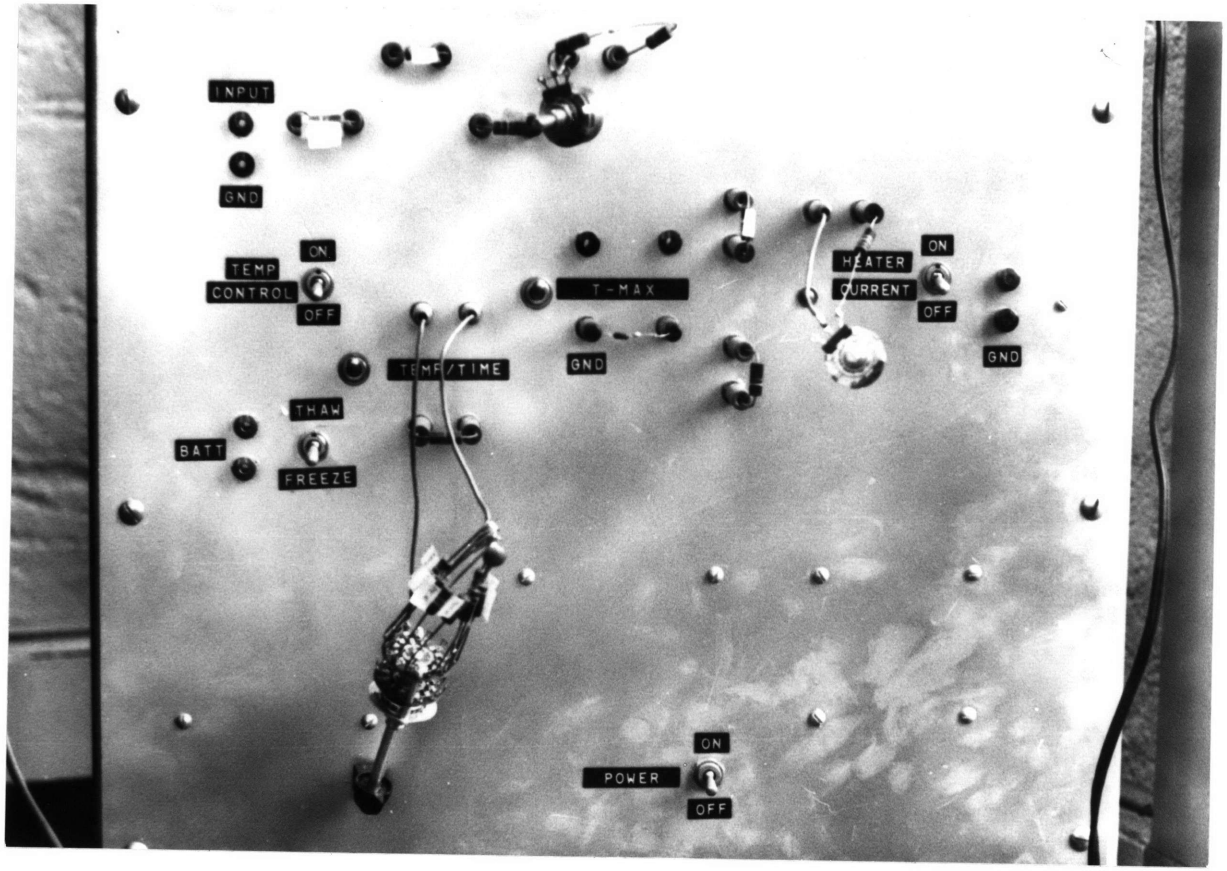


Figure 2.8. Temperature Control System Pannel

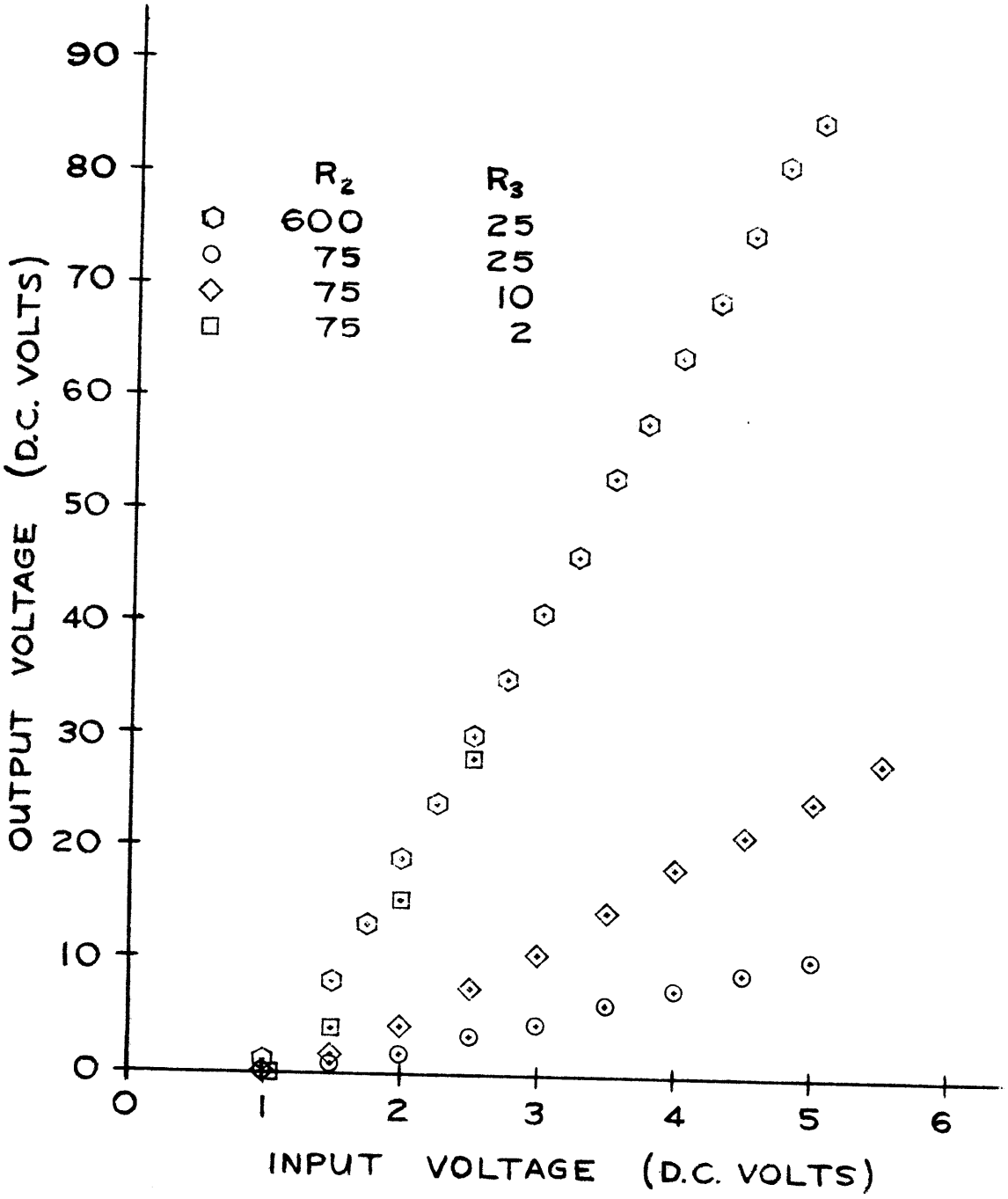


Figure 2.9. D.C. Voltage Gain Characteristic for Power Amplifier

and emitter resistors, R_2 and R_3. A response lag was measured in the amplifier response in that an input of 1.0 volt is required to activate the transistors. At input voltages greater than 1 volt, the gain was measured to be quite linear. The magnitude of the gain is a function of the ratio of R_2/R_3. A high value of gain is desirable so that a high level of sensitivity of the control system to variations in specimen temperature can be maintained. The power amplifier is photographically depicted in Figure 2.10.

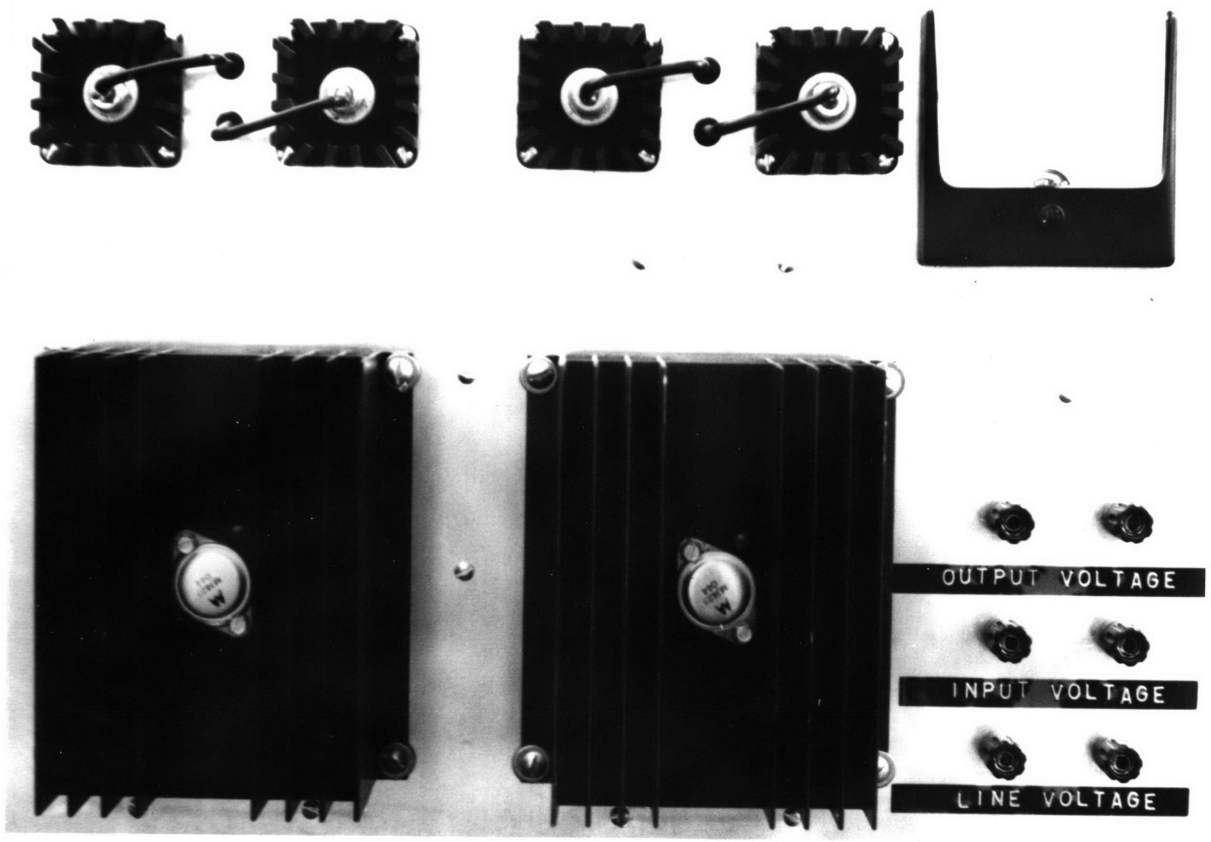


Figure 2.10. Power Amplifier Pannel

CHAPTER 3

Experimental Procedure

A. Specimen Preparation:

Venous blood is collected from donors at the Massachusetts General Hospital Blood Bank in 10 ml glass bottles containing acid citrate dextrose anti-coagulant. The bottles are sealed with rubber stoppers and stored at +4°C for use within 24 hours.

The blood specimens are frozen and thawed on the cryomicroscope in a thin film held between two glass coverslips. It is important that the blood be prepared in a thin film having a thickness of a little more than one cell diameter so that individual cells can be isolated for observation during the freezing and thawing processes. Also, thicker films result in excessive scattering of light from ice grain boundaries in planes not in focus. The phenomenon occurs at the liquid - solid phase change with a consequent uniform white appearance of the field of view. A micro-thermocouple (0.002 inch copper and constantan wires) is placed between the coverslips in contact

with the film of blood. Since the diameter of the thermocouple is greater than the desired thickness of the blood film, it was necessary to modify the coverslips so that the thermocouple would not limit the minimum separation of the slips (and thickness of the film). This was accomplished by etching small grooves into both coverslips with HFl acid. The thermocouple is placed inbetween these grooves so that the coverlips can attain their minimum separation. See Figure 3.1.

The specimens are prepared for viewing by withdrawing a small volume of blood (approximately ½ microliter) from the glass collection bottle into a micropipette and placing it as a drop onto the etched side of one of the coverslips. The thermocouple tip is positioned into the groove of the same coverslip, and the second coverslip placed etched side down onto the first so that both grooves are aligned with the thermocouple. The blood spreads from the droplet into a thin film between the coverslips and into the etched grooves under the action of surface tension. The average thickness of the specimen films is approximately 12 microns. This thickness was directly measured on the

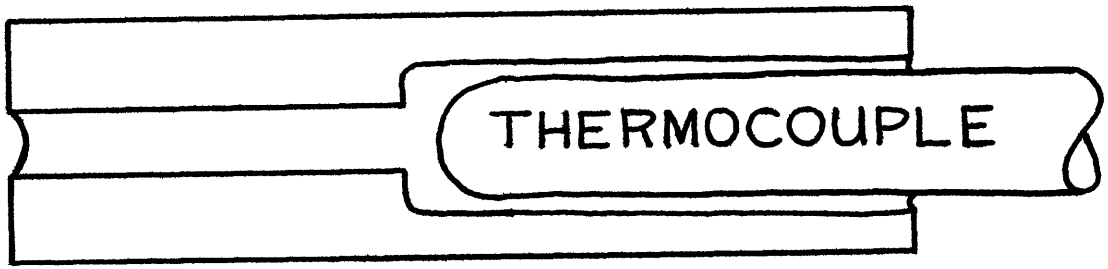


Figure 3.1. Placement of Thermocouple in Contact with Specimen Between Etched Coverslips

microscope by noting the vertical distance traversed by the stage in focusing from the top of the bottom slip to the bottom of the top slip. In a properly prepared specimen the variation in film thickness across the coverslip area is less than one micron.

B. Operation of the Cryomicroscope:

After a film of blood has been prepared between etched coverslips in the above manner, it is placed directly onto the top window of the freezer manifold. The thermocouple leads are attached directly to a millivolt recorder* via a special connector plug mounted on the microscope stage. Next, the plexiglass container, which encloses the low temperature stage and the microscope optics (see Figure 2.4), is purged of air via a stream of dry helium gas to prevent condensation of water vapor on the cold apparatus. The objective is then swung into position over the specimen and the microscope is focused. With the door to

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* Speedomax W, Leeds and Northrup Co., Phila., Pa.

the plexiglass container closed tightly, the system is prepared to initiate the freezing process.

Two modes of control are employed to regulate the specimen temperature and its time rate of change during freezing and thawing: Manual operation and automatic operation. Operating procedure for the cryomicroscope in both of these modes will be described.

1. Manual Operation:

The manual mode of operation, compared to the automatic mode, affords only a very limited degree of control over the specimen temperature and is capable of effecting only a small spectrum of cooling and warming rates in the specimen. These limitations exist because the single parameter of operation in this mode is the flow rate of refrigerant through the low temperature chamber. The entire system, including the specimen, thermocouple, and freezing stage, is initially at ambient temperature. Cooling is instigated by opening valve VI (see Figure 21) to throttle pressurized, dry helium gas through the liquid nitrogen

heat exchanger and into the manifold. The rate of cooling is roughly proportional to both the flow rate of refrigerant through the chamber and the difference in temperature between the specimen and refrigerant. (Refer to Appendix D for a heat transfer analysis of the system.) The cooling rate is affected by refrigerant temperature as it passes through the low temperature manifold and the thermal resistance between the refrigerant and the specimen. The temperature of the refrigerant entering the manifold initially decreases with time as the flow channel between the heat exchanger and the microscope is cooled down from ambient temperature until -196^{o}C is approached. A significant component of the thermal resistance between the refrigerant and the specimen can be attributed to the convective heat transfer between the refrigerant stream and the manifold. The convective film coefficient (and consequently the cooling rate) is increased as the refrigerant flow rate becomes greater. Thus, to increase or decrease the cooling rate, valve V1 is throttled, respectively, further open or further shut, to adjust the refrigerant flow.

A disdavantage associated with manual operation is that it is very difficult to duplicate a predetermined temperature-time history for the specimen flow rate. This situation exists for two reasons: first, the response of cooling rate to variations in refrigerant flow is quite slow, rendering steep temporal thermal gradients unattainable; and, second, only a minimal degree of control can be exerted over the refrigerant temperature, which is related to the rate of heat loss by the specimen. Thus, for a constant flow of refrigerant, the cooling rate decreases as the specimen temperature approaches the refrigerant temperature.

Nonetheless, it is possible to reach very high, but virtually uncontrolled cooling rates by manual operation. Freezing is accomplished by initially filling the tubes of the liquid nitrogen heat exchanger with air, which is subsequently liquified within the exchanger tubes at +79^{o}K. Pressurized, dry helium gas is blown into the exchanger to force the liquid air out of the tubes and through the low temperature stage in two phase flow. The maximum cooling rate attainable by this method is an order of magnitude greater than for

gaseous refrigerant. However, the two phase flow through the manifold stresses the quartz windows very heavily, sometimes resulting in breakage, and affords virtually no control over the specimen temperature history.

Warming of the specimen in manual operation is caused by conduction and convection of heat from the environment (see Appendix D). The maximum warming rate is effected by completely shutting off the refrigerant flow, which produces rates up to approximately +100°C/min. Intermediate warming rates down to 0°C/min are attained by progressively throttling the refrigerant flow to the appropriate rate to achieve the desired balance between heating and cooling effects. As with freezing, it is very difficult to maintain any preselected warming rate by manual operation due to the inability to control the heat flux to the specimen. Also, the maximum warming rate possible using manual operation is about 2 orders of magnitude less than that which can be produced by automatic operation.

The single advantage offered by manual operation is that it is a simpler procedure to perform than that associated with automatic operation.

A slight positive pressure of dry helium gas is maintained within the plexiglass container in order to eliminate the condensation of water vapor and frost formation during the freezing and thawing processes. Access to the focusing knobs is provided through windows cut in the plexiglass wall and sealed with a loose sheet of plastic. The microscope focus must be corrected as the system temperature changes, in order to maintain image sharpness. This requirement necessitates continual visual monitoring of the specimen in order to make the proper focusing adjustments.

2. Automatic Operation:

The temperature feedback control system, illustrated in Figures 2.6 and 2.7 is employed to operate the cryomicroscope in the automatic mode. The entire system, excluding the liquid nitrogen heat exchanger, is initially at room temperature. With switch S_2 (Figure 2.7) on the signal generator unit open, the following preliminary settings are made to ready the

system for automatic freezing: switch S_1 is set for thawing process; resistor R_8 is adjusted to simulate the desired cooling rate during the freezing process; and resistor R_7 is adjusted so that the gain in the thermocouple voltage multiplier is of a magnitude that results in zero input voltage at operational amplifier OA_4. Switch S_2 is then moved to the on position, activating the control system, and the refrigerant flow is turned on. At this setting, the action of the controller-heater combination will maintain the specimen at room temperature. While the refrigerant flow is increased to the desired maximum value, the coolant plumbing and the freezer manifold are cooled down, and the top manifold window and the specimen are maintained at room temperature by the electric heater and controller system. When the freezer system is sufficiently undercooled, switch S_1 is moved from the thaw to the freeze position, and the specimen is cooled at the preselected constant rate. The analog control unit maintains a balance between the warming effect of the electric heater and the cooling effect of the refrigerant flow and the stored thermal capacity of

the freezer manifold so that the desired cooling rate is observed. At the conclusion of the cooling process, the base (or storage) temperature is fixed by moving switch S_2 to the off position, which reduces the voltage input to the integrator to zero. The simulated storage temperature is thus held constant until the voltage input to the integrator is changed from zero. Before the commencement of the warming process, resistor R_8 is adjusted to the value which will produce the desired warming rate. Switch S_1 is then moved to the thaw position and switch S_2 to the on position to activate the integrator and simulate the warming process. For large warming rates, the refrigerant flow rate can be reduced to minimize the load on the resistance heater. The specimen temperature will increase until room temperature is reached, at which point it will be held constant by the control unit.

The time rate of temperature change can be varied at any point in the freeze-thaw sequence simply by manually adjusting the setting of resistor R_5. A limitation to the operation of the control unit is that it can be programmed only to simulate constant rates

of temperature change. Thus, the above procedure of manually adjusting resistor R_5 in process is required to study nonconstant rates of cooling or warming. The response time of the system to alterations in thermal conditions is very rapid because only a very small, localized mass (consisting of the specimen and top manifold window) is subjected to thermal control. The control system is therefore quite sensitive to variations from the simulated temperature-time curve and consequently is able to force the specimen temperature to assume the desired time history.

The range of cooling and warming rates which may be produced using automatic operation is greater than that possible by manual operation. Much larger and more precisely controlled heat fluxes, both to and from the specimen, can be effected by the feedback temperature control system. Heat loss from the specimen during manual cooling is accomplished chiefly by convection to the refrigerant stream. Heat is removed from the specimen during automatic cooling both by convection (as in manual operation) and by conduction to the adjacent freezer manifold, which acts as a heat sink.

Localized heating of the specimen by the resistance heater permits the freezer manifold (which has a much larger mass than the specimen) temperature to be reduced by as much as -100°C below that of the specimen. When the heater input is suddenly decreased, the system experiences the effect of opening a thermal switch, and the conductive component of heat transfer to the heat sink rapidly overrides the convective component to greatly increase the heat flux. Increased heat fluxes during the warming process are readily obtained by the dissipation of energy in the electric resistance heater. This energy source can increase the heat transfer to the specimen, and consequently the rate of temperature change, by orders of mangitude.

C. Data Retrieval:

Both thermal and visual techniques are employed to obtain data describing freezing and thawing processes on the cryomicroscope. The specimen temperature is continually monitored by the copper-constantan thermocouple and plotted on a strip chart recorder.* The

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* Speedomax W, Leeds and Northrup, Inc., Phila., Pa.

physical appearance of the specimen can be visually followed on the microscope during all phases of exposure to subfreezing temperatures and recorded on film, either by still photography or by high speed cinetography.

CHAPTER 4

Experimental Data and Discussion

Experimental results will be presented and discussed in two separate sections, according to the double objective of the program of investigation: the first section will deal with the performance capabilities and characteristics of the cryomicroscope, and the second with the formation of intracellular ice and the detection of its presence in frozen human erythrocytes.

A. Performance of the Cryomicroscope:

As explained in Chapter 2, a primary rationale for building a cryomicroscope is to provide an experimental facility whereby the dynamics of freezing and thawing processes in biomaterials can be studied in microscopic detail at precisely controlled rates of temperature change. In this context, the performance of the cryomicroscope system has been evaluated according to several criteria. These include the following:
(1) capability for microscopically viewing dynamic thermal processes in both real and dilated time;
(2) accuracy of specimen temperature measurement;

(3) precision of control over specimen temperature and its time rate of change.

1. Visual Capability

The cryomicroscope is a unique instrument in that it can provide a dynamic record of changes in cellular ultrastructure that occur during freezing and thawing. The ability to monitor visually the condition of the ultrastructure is limited by the resolution of the microscope.

A record of the transient physical events occurring in biological cells as a consequence of freezing and thawing is made by the techniques of photomicrography and cinemicrography. Photomicrographs of a consistently high quality can be made of frozen cells, provided certain precautions are observed to prevent the formation of frost on the specimen and microscope optics (cf. Chapter 2). The amount of information obtained from photomicrographs is subject to several limitations of varying severity. The two factors of overriding importance are the maximum resolving capability of the microscope and the low contrast of the image to be recorded on film.

Although little can be done to improve the resolution of any given optical system, the photographic image can be enhanced significantly by the use of special developing and printing techniques. The highest quality enlargements are made from very fine grain film, of a low ASA rating. This type of film requires maximum specimen illumination to produce adequate exposure. The longer exposure requirement can result in a blurred image when relatively rapid motions are photographed at slow shutter speeds. In typical applications, satisfactory pictures can be taken on Panatomic X 35 mm film, exposed for ¼ second with phase contrast xenon illumination and a total specimen magnification of 1000X, and developed in Kodak D 11 developer for average contrast.

High speed cinemicrography is a most useful tool for obtaining detailed information which is otherwise inaccessible concerning freezing and thawing processes. This technique is limited in its effectiveness by the maximum framing speed of the motion picture camera consistent with the illumination capacity of the microscope. Presently, filming rates of up to 400 frames

per second can be attained with the high pressure xenon lamp and phase contract illumination on ASA 400 film.

In general, the cryomicroscope is capable of producing visual data over a wide band of specimen temperatures that compare very favorable in optical quality with the images produced by a standard light microscope under isothermal, ambient conditions. Photomicrographs taken on the cryomicroscope at subfreezing temperatures will be presented in later sections of this chapter in the discussion of experimental results.

2. Temperature Measurement

It is essential to the proper functioning of the feedback control system that a high degree of accuracy be maintained in the specimen temperature measurement. Thus, a series of tests were performed to determine the precision of the temperature sensing system employed on the cryomicroscope. Samples of whole blood collected in ACD were frozen and thawed on the microscope, and the indicated melting temperature was carefully noted for each sequence at the instant of melting, as observed

through the cryomicroscope. The data points are compiled in Figure 4.1 and compared with the known melting point of whole blood. The average discrepancy between the actual and measured melting temperatures is less than 1°C.

Two possible sources of error can be considered to account for this discrepancy. The specimen temperature is sensed by a 0.001" diameter copper-constantan thermocouple placed in physical contact with the film of blood being frozen (see Figure 3.1). This method of measurement is capable of detecting only an average, bulk temperature of the specimen. Thus, the first source of error is the result of the inherent inability of a single thermocouple probe to sense small scale spatial temperature variations within the film of blood. The magnitude of these variations is dependent upon the thermal gradients imposed on the specimen by cooling and warming effects of the automatic control system. The maximum vertical temperature difference across the specimen can be analytically determined as a function of the blood film thickness and the cooling rate. This

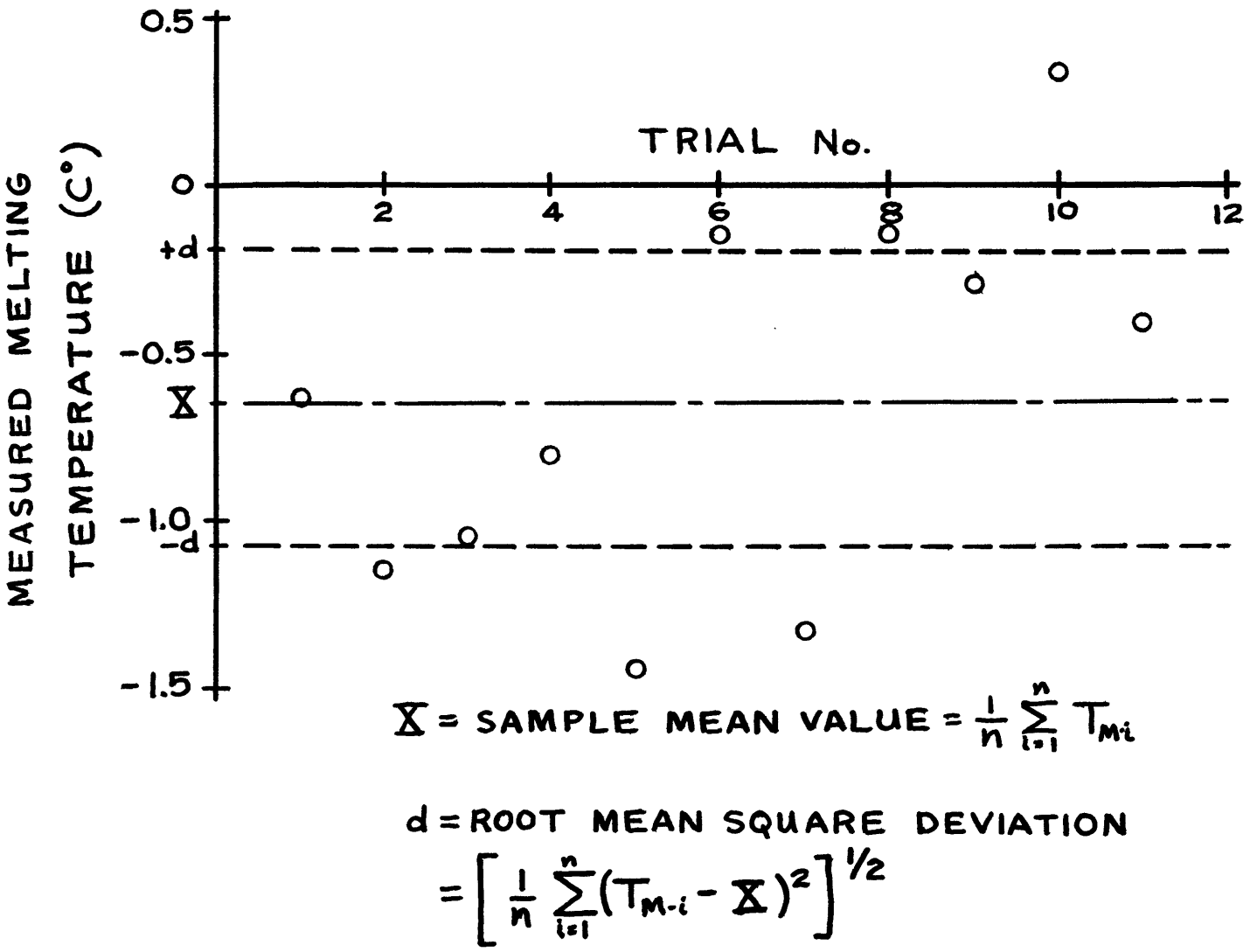

Figure 4.1. Variations in the Measured Thawing Temperature of Human Erythrocytes

analysis, which is shown in Appendix I, predicts that cooling rates in excess of -50,000°C/min would be required to create a vertical temperature differential greater than 1°C across the specimen. Since these high rates can not even be approached at present, it can be concluded that vertical temperature gradients are not a significant source of error in the operational tolerances inherent in the experimental system.

A second source of inaccuracy in temperature measurement is manifested as a deviation of the measured temperature from the bulk temperature of that portion of the specimen in the field of view of the microscope. This error can be attributed to positioning of the thermocouple on the low temperature manifold so that it is not sensing the temperature of the cells being observed. Measurement of the magnitude of this error is discussed in Section 4.1. This temperature variation can be effectively reduced by refinement of the cryomicroscope operating technique.

3. Cooling and Warming Rate Control

a. Electrical Simulation of Thermal History

A limiting factor in the control of the specimen temperature is the precision and range of operation of the electrical temperature simulator in the feedback control system. Therefore, as a measure of one performance parameter of the temperature control system, the controller was programmed to simulate a typical thermal history for frozen and thawed cells. The resulting output of the voltage integrator (point to ground in Figure 2.7) was plotted as a function of time on a continuous chart recorder* for a simulated series of thermal transients. The output voltage was reduced to a measurable level by a 33/1 voltage divider. The transient voltage plot is shown in Figure 4.2. The sequence of processes illustrated was generated in the following manner. The output of the integrator for zero initial voltage input is recorded between points a and b. In actual operation of the microscope this

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* Speedomax W, Leeds and Northrup Co., Phila, Pa.

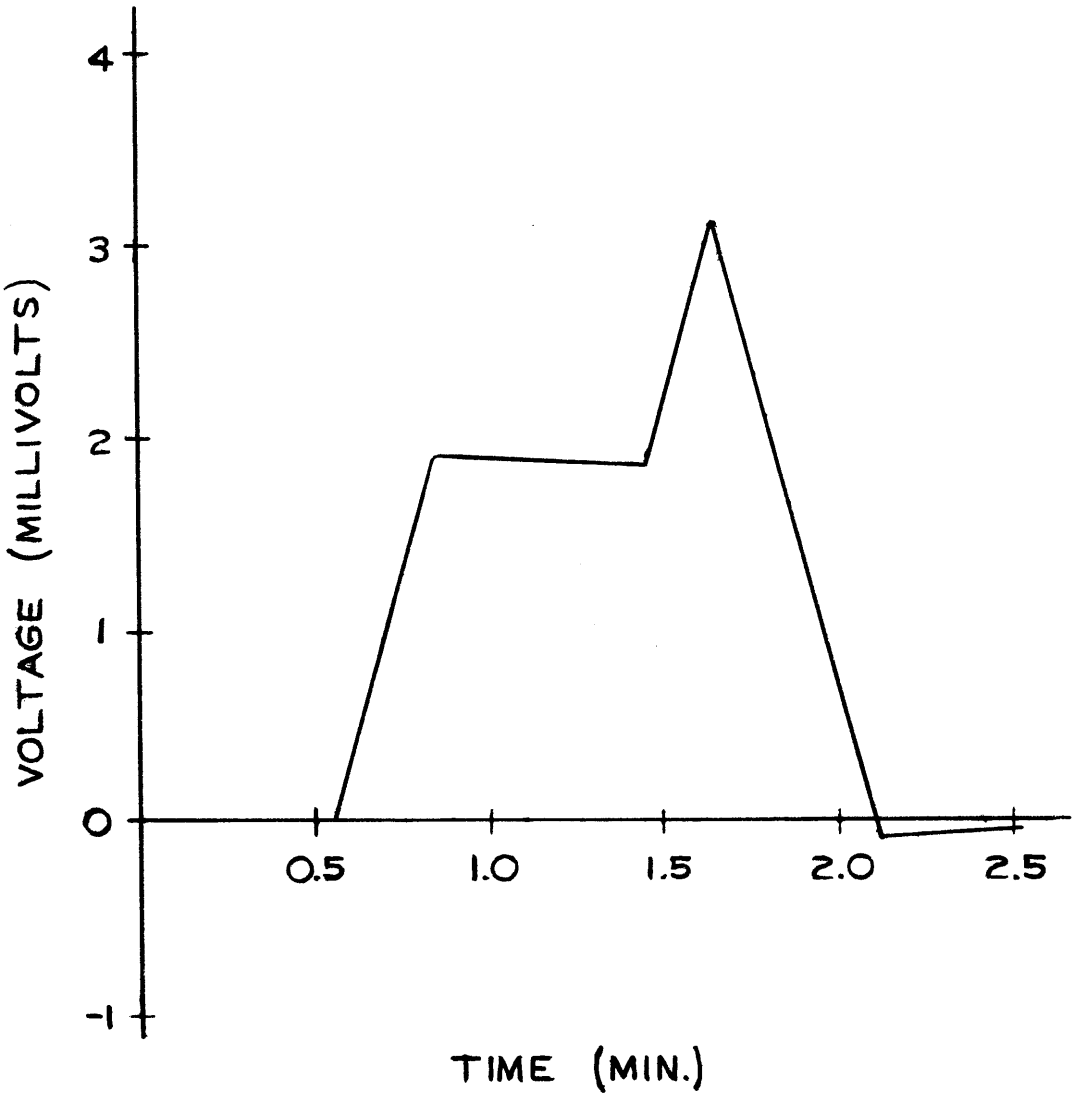

Figure 4.2. Typical Electrically Simulated Specimen Thermal History

output level represents the specimen temperature being held at room temperature. Commencing at point b, a constant positive voltage input is supplied to the integrator, corresponding to a constant cooling rate process. At point c the voltage input is abruptly reduced to zero to simulate maintainence of a constant specimen temperature until point d. The input voltage is then restored to its original value between points d and e. At point e the sign of the input voltage is reversed, to effect a change from cooling to warming. At point f the integrator output voltage passed through zero and its sign was reversed. The integrator input was again reduced to zero at point g to effect a constant specimen temperature. Inspection of the V vs. t curve of Figure 4.2 shows that the simulated cooling and warming rates are constant within the accuracy of the recorder over the range of voltages plotted. The difference in magnitude between the generated cooling and warming rates was 1%. Both rates were generated by an identical integrator input voltage.

The response of the system to changes in the integrator input voltage was more rapid than could be detected by the chart recorder (which has a time constant of 1 second for a full scale step input). The response time was therefore measured on a cathode ray oscilloscope*. The system was set so that a step increment in integrator output voltage resulted when switch S_1 (Figure 2.7) was reversed, thereby changing the sign of the integrator input voltage. The full signal response occurred in approximately 1 millisecond. This measured response represents the period of time required to switch from any one integrator function to another, such as is involved in changing from a simulated cooling rate to a simulated constant temperature process.

One undesirable operating characteristic can be noted from Figure 4.2. Between points c and d and g and h when the system is in the constant temperature mode, there is a drift in the integrator output voltage back to the zero (initial) level. This drift is caused

* Type 502A Dual Beam Oscilloscope, Tektronics, Inc., Portland, Oregon

by the slow discharge of the output capacitor on the integrating operational amplifier (see Figure 2.7). Thus, it is not possible to program the control unit to maintain a constant specimen temperature at a value other than 37°C. The temperature control system has subsequently been modified by M. Ushiyama so that the constant temperature mode of operation can be accurately programmed.

b. Control of the Specimen Temperature and Rate of Temperature Change

Samples of normal saline solution were frozen on the cryomicroscope with automatic control in order to determine the performance capabilities and characteristics of the temperature control system. The specimen temperature history was plotted on the continuous chart recorder, and the rate of temperature change determined from the plotted curve. Figure 4.3 illustrates a series of constant cooling rate, warming rate, and temperature hold processes for a single specimen. The maximum cooling and warming rates available on the cryomicroscope were measured to be -2200°C/min and +6500°C/min, respectively. Both rates were measured

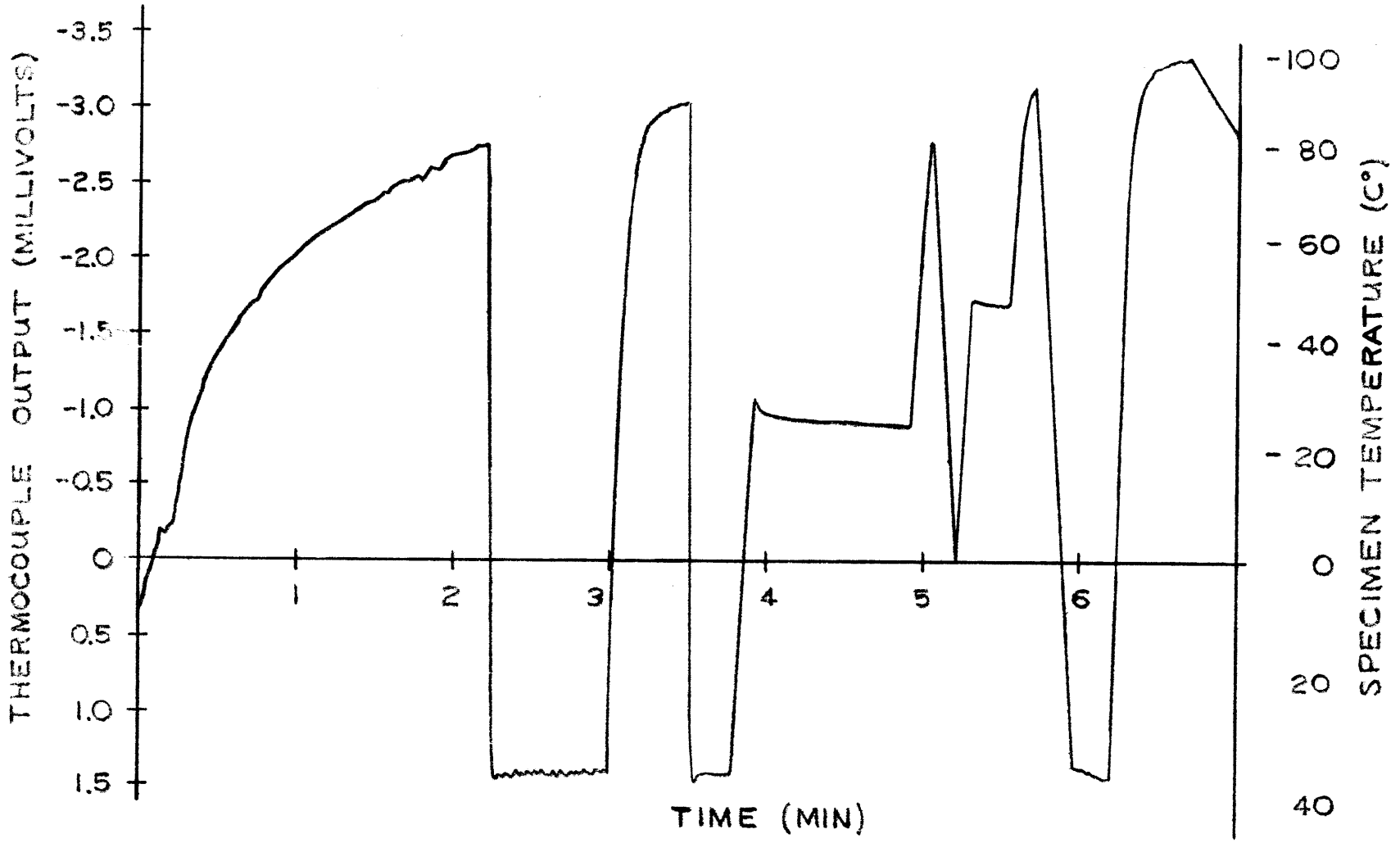

Figure 4.3. Measured Specimen Thermal History for a Series of Constsnt Cooling Rate, Warming Rate, and Temperature Hold Processes in the Automatic Mode of Temperature Control

to be constant throughout the entire temperature range of the test between +37°C and -80°C. The repeatability of programmed cooling and warming rates was determined by successively freezing and thawing a single specimen for identical settings of the temperature control unit. The measured mean values and standard deviations for 8 cooling and warming processes were -230, ± 8°C/min and + 343, ± 9°C/min. The readability of the chart recorder for this magnitude of thermal transients is ± 5°C/min. The control unit was also set to maintain the specimen temperature constant with time. In a series of five tests, variation from a number of initial holding temperatures was consistently less than ½°C/min over a period of five minutes.

B. Formation of Intracellular Ice

Two aspects of intracellular ice formation in frozen erythrocytes were investigated separately: (a) detection of an ice phase inside of frozen cells, and (b) determination of the conditions requisite for the occurrence of intracellular ice. The results of these investigations will be discussed in separate sections.

1. Detection

The most straight forward method of detection is to observe whether intracellular ice appears in a frozen suspension of cells. In some instances it is readily apparent that an ice phase is present inside of cells. As an illustration, Figures 4.4, 4.5, and 4.6 show red blood cells in three different thermodynamic states of interest. Figure 4.4 is a photomicrograph of a blood film between glass coverslips at room temperature as prepared prior to freezing. This picture is particularly useful as a reference for the semblance of cells containing no intracellular ice. Figure 4.5 is a micrograph of cells which have been frozen at a sufficiently slow cooling rate so that ice has formed only in the extracellular medium. As in Figure 4.4, there is no distinguishable solid phase within the cells, whereas an ice phase can be observed outside the cells. This evaluation of the freezing process can be verified much more conclusively by dynamic observations in which the phase change can be directly seen to occur only externally to the cells. Figure 4.6 illustrates the appearance of cells containing intracellular ice. In this specimen the internal grains of ice can be distinguished very clearly.

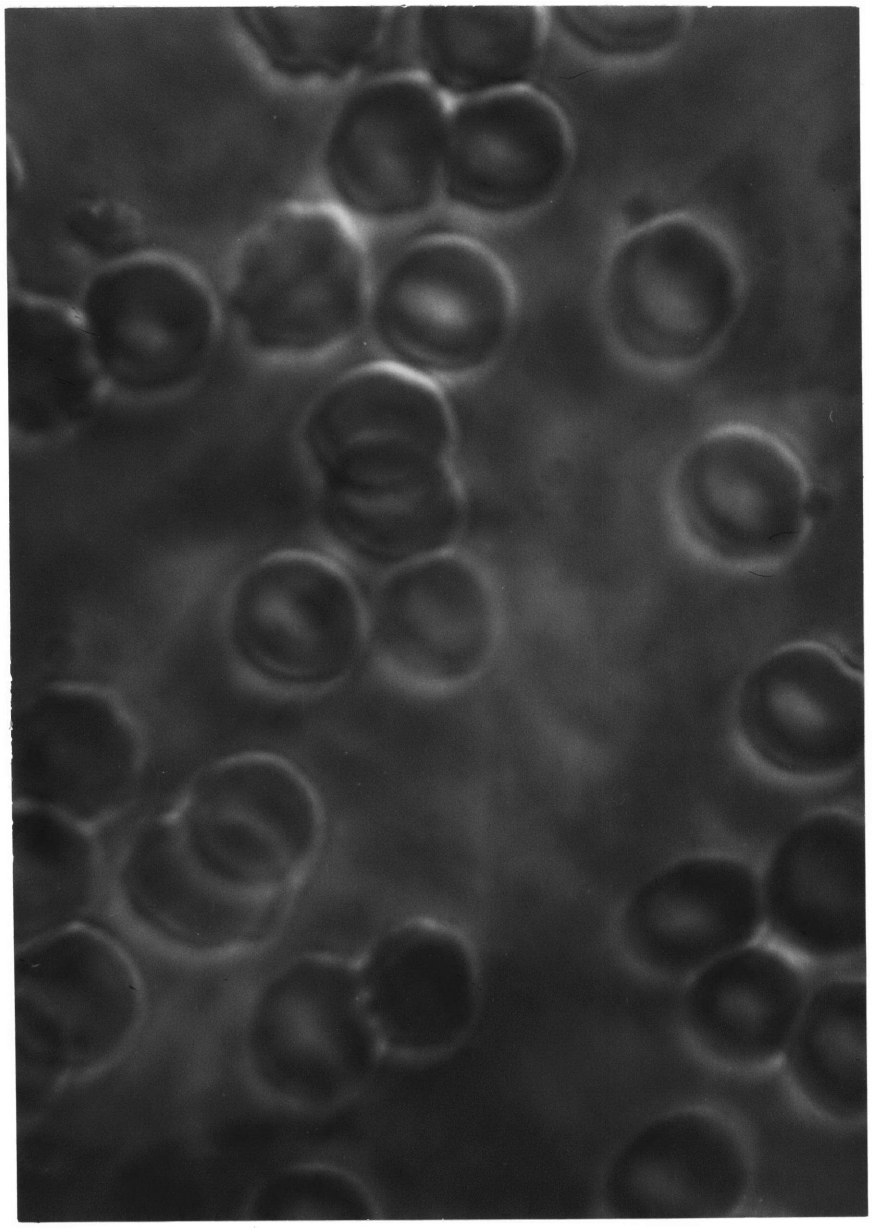

Figure 4.4. Photomicrograph - Erythrocytes at Room Temperature, Prefreezing. 806X

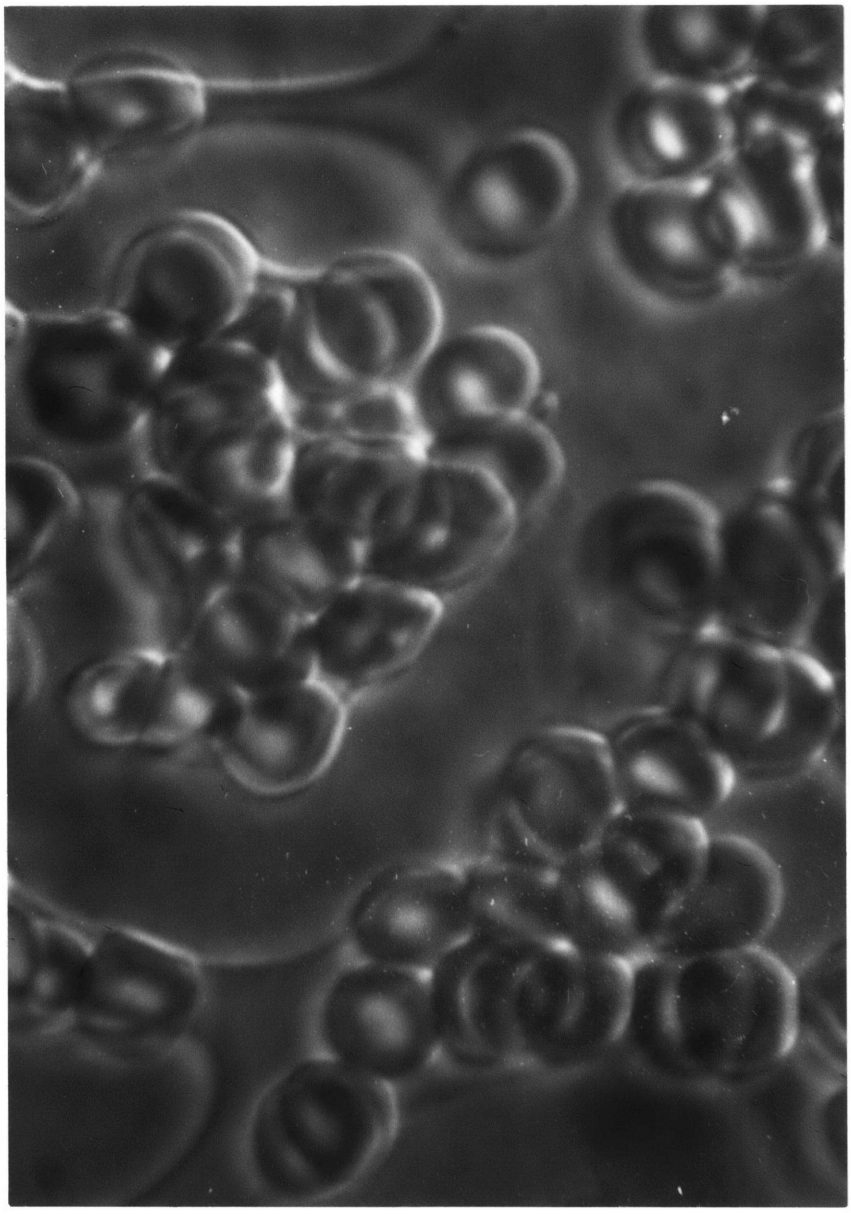

Figure 4.5. Photomicrograph - Frozen Erythrocytes at -20°C, Extracellular Ice Only. 806X

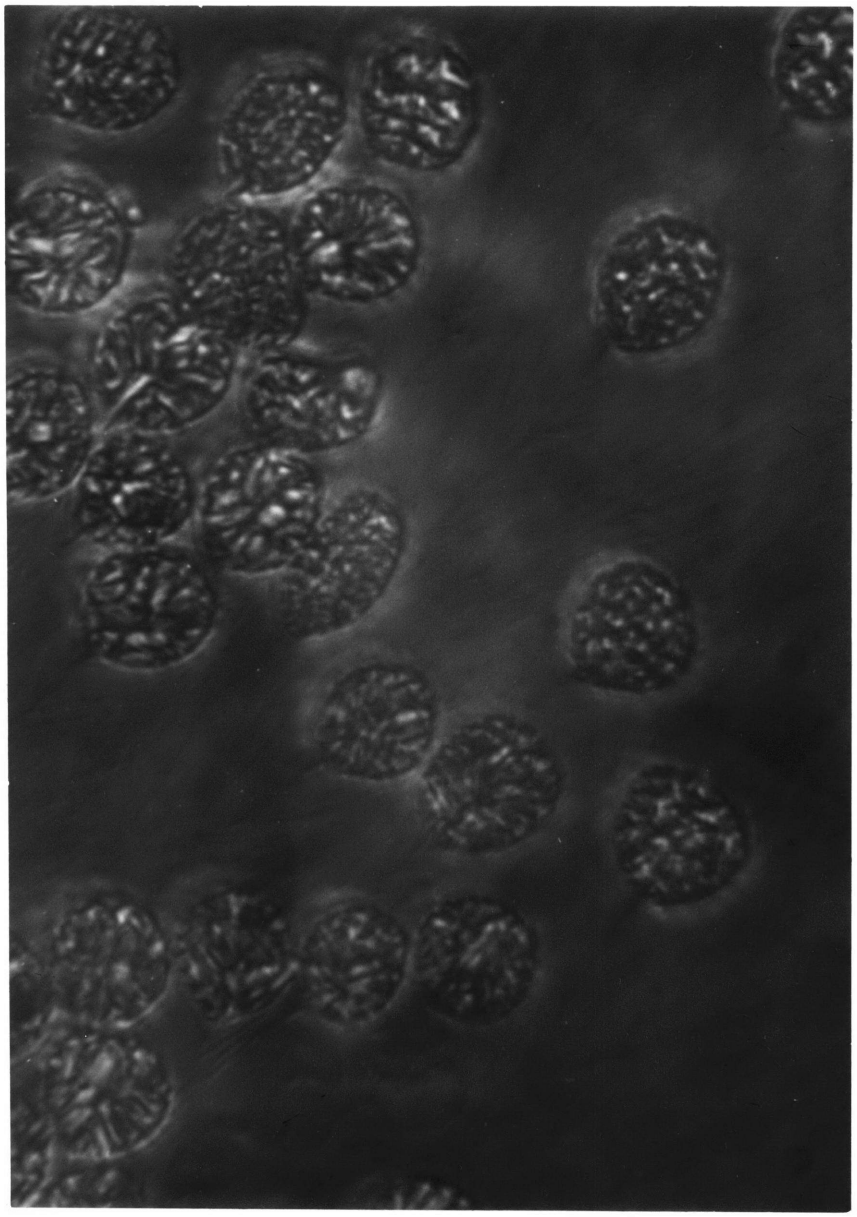

Figure 4.6. Photomicrograph - Frozen Erythrocytes at -20°C, Intracellular Ice. 806X

The boundary of the granular structure within the cell is defined by the plasma membrane, indicating that the ice being seen is contained within the cell rather than in a plane above or below the cell. Further verification exists in that the plasma membrane and ice grains are simultaneously in focus. It would be impossible to simultaneously focus sharply both the membrane and the ice if they were not coplanar, since the depth of focus of the 63x objective is 0.6 microns. It is thus conceivable to distinguish between actual intracellular ice and an artifact created by a sheet of ice above or below the cells. This evidence supports the contention, then, that water has been frozen inside the cell.

The existence of intracellular ice can be demonstrated in an even more convincing manner by a special freezing technique. To create these conditions, the specimen is frozen on the cryomicroscope so that only a portion of the total number of cells are frozen internally. Such a distribution of frozen cells is effected by employing a cooling rate in the transition range for intracellular ice formation (see section B,

part 2) prior to the phase change. The photomicrographs in Figures 4.7 and 4.8 were made of cells frozen in the above manner. The cooling rate prior to the phase change was -10°C/min. Figure 4.7 was made immediately after the specimen was frozen. The appearance of cells containing intracellular ice can be distinguished from that of the unfrozen cells by the internal granular structure. Approximately 37% of the cells contain intracellular ice. Subsequent to freezing, the cooling rate was lowered to -4°C/min. By significantly reducing the cooling rate following the initial phase change, sufficient time is provided for the unfrozen cells to equilibrate osmotically with the extracellular medium by an outflow of cell water rather than by freezing. It is important that the cooling rate be substantially decreased after freezing with respect to the relaxation time for equilibration by mass transport so that the cells initially unfrozen do not have adequate opportunity to subcool sufficiently for intracellular ice to form. The difference in appearance between the frozen and unfrozen cells subsequently becomes more pronounced as the temperature is lowered

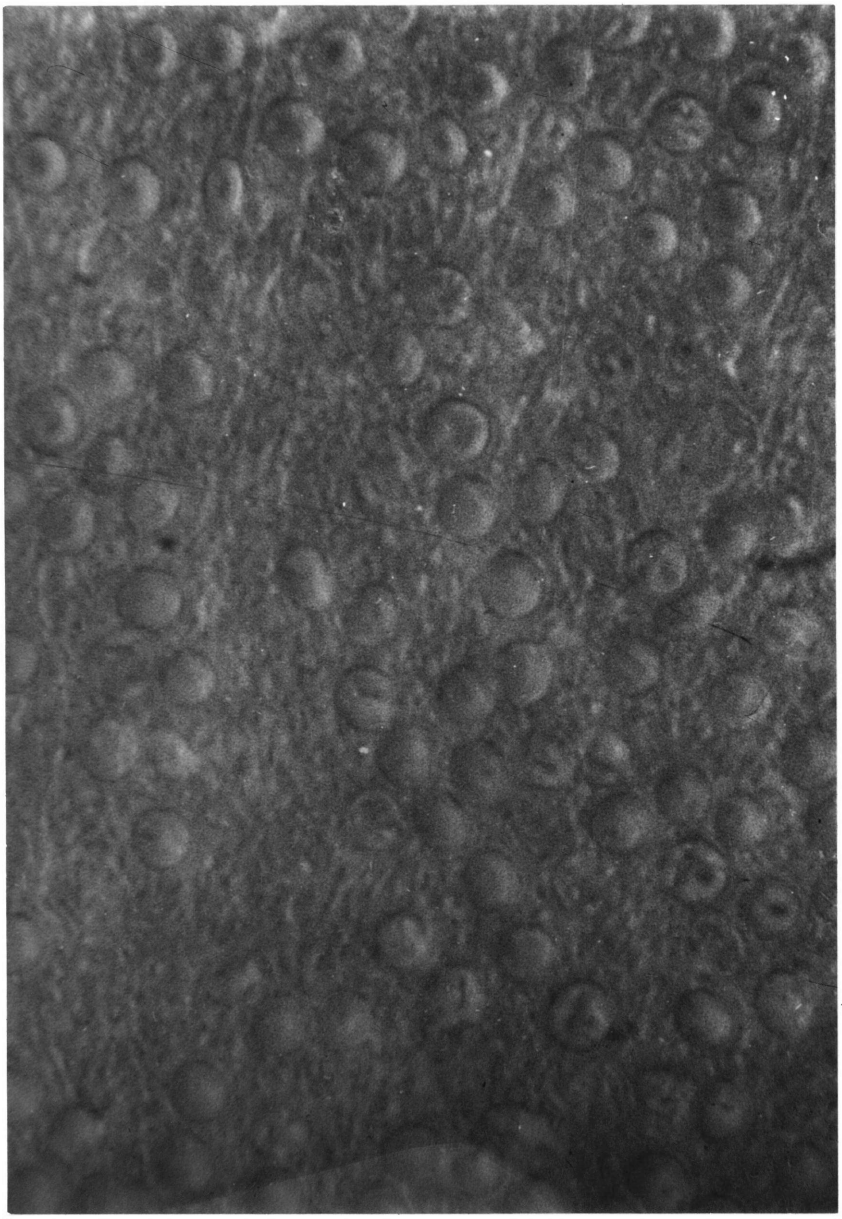

Figure 4.7. Photomicrograph - Frozen Erythrocytes at -2°C Immediately Following Phase Change, Partial Intracellular Ice. 1008X

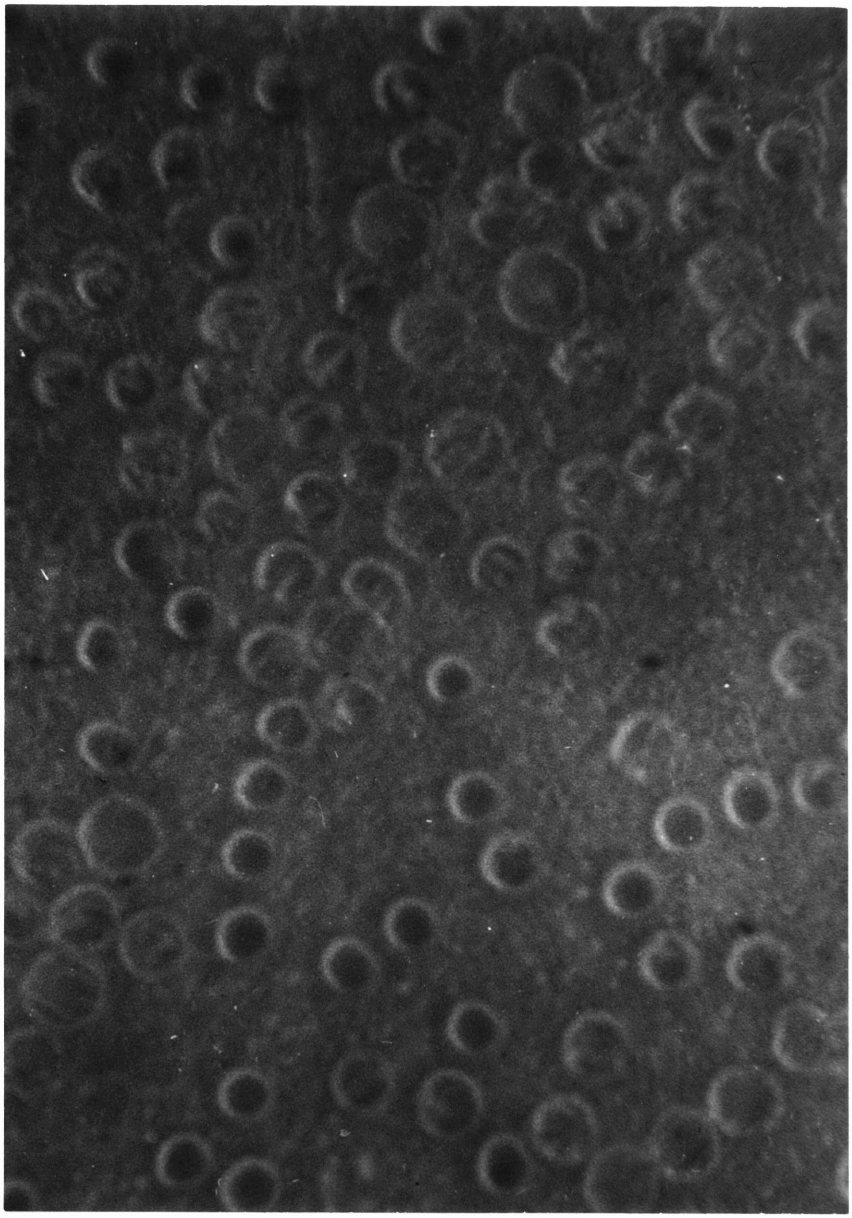

Figure 4.8. Photomicrograph - Frozen Erythrocytes at -15°C, Same Field as Figure (4.7) 1.2 Minutes Following Phase Change, Partial Intracellular Ice. 1008X

following freezing. Cells containing internal ice remain, for the most part, visually unchanged. The granular structure of ice grows larger by crystallization and/or recrystallization, but the volume and optical density of the cell remains unchanged.

A very different phenomenon occurs in the unfrozen cells. As water moves across the plasma membrane from within the cell to the extracellular medium, the cell volume decreases quite noticeably and the intracellular fluid becomes much darker due to the concentration of hemoglobin inside the cell. Figure 4.8 illustrates the appearance of the same cells after 3 min of cooling at -4°C/min following the initial phase change. Here it can be noted that the physical difference between frozen and unfrozen cells is very convincingly apparent. The two categories of cells can be distinguished by differences in granularity and darkness of the intracellular medium and in the volume (radius) of the cells. This evidence amounts to a triple check on the presence of intracellular ice. Analysis of the photomicrograph in Figure 4.8 reveals the following information concerning the distribution of intracellular ice: by

granularity, 44%; by density of intracellular medium, 38%; and, by volume, 44%. The consistency among these three methods of measurement verifies the capability of the cryomicroscope as an effective instrument for determining the presence of intracellular ice in frozen cells.

Visual techniques for detecting intracellular ice are beset with a major drawback, however, in that the degree of accuracy and rigor is significantly dependent upon the interpretive ability and experience of the operator. This fact is illustrated in Figure 4.7 in which it is not immediately obvious, for each individual cell in the field of view, whether or not intracellular ice is present. This difficulty is most serious when the ice structure is not well defined or is so small as to render resolution by light microscopy impossible. These conditions are associated, respectively, with very low and very high cooling rates, as compared to the transition rate, and, as such, are not commonly manifested in the situations in which the presence of intracellular ice is least certain.

As a means of offsetting the consequences of this inherent limitation, the basic capability of the cryomicroscope for detecting intracellular ice can be enhanced by two supplementary techniques: polarized light illumination and high speed cinemicrography. Polarized light is commonly employed to increase the level of contrast of the granular or crystaline structure of solids in photomicrography. High speed cinemicrography enables the time scale of the freezing process to be greatly dilated to facilitate detailed visual examination of the sequential occurrence of events during freezing. Cooling rates high enough to effect the formation of intracellular ice normally produce such rapid phase changes that they appear instantaneous to the human eye in real time. However, the capability for observing in slow motion the physical phenomena associated with freezing provides an opportunity to follow the dynamics of the freezing process in great detail and to discern any physical changes within individual cells associated with the phase change. It is possible to observe the seeding of ice crystals within individual cells as the phase boundary moves

through the film of blood. Even when the structure of the ice is too minuscule to be resolved by light microscopy, the change from liquid to solid can be detected by a darkened appearance of the specimen following freezing due to increased scattering of light by the ice particles. High speed motion pictures illustrating the freezing process for human erythrocytes may be obtained from the Cryogenic Engineering Laboratory. Figures 4.9 and 4.10 were enlarged from selected frames of motion picture records of intracellular and extracellular freezing, respectively. Growth of the phase front through the field of view and the interaction with the suspended erythrocytes can be noted in both of these sequences.

In summary, a prime rationale for building the cryomicroscope was to create a facility which could be employed to accurately and reliably detect the formation of intracellular ice. The foregoing experimental results are indicative that, in this capacity, the microscope satisfactorily performs to expectation.

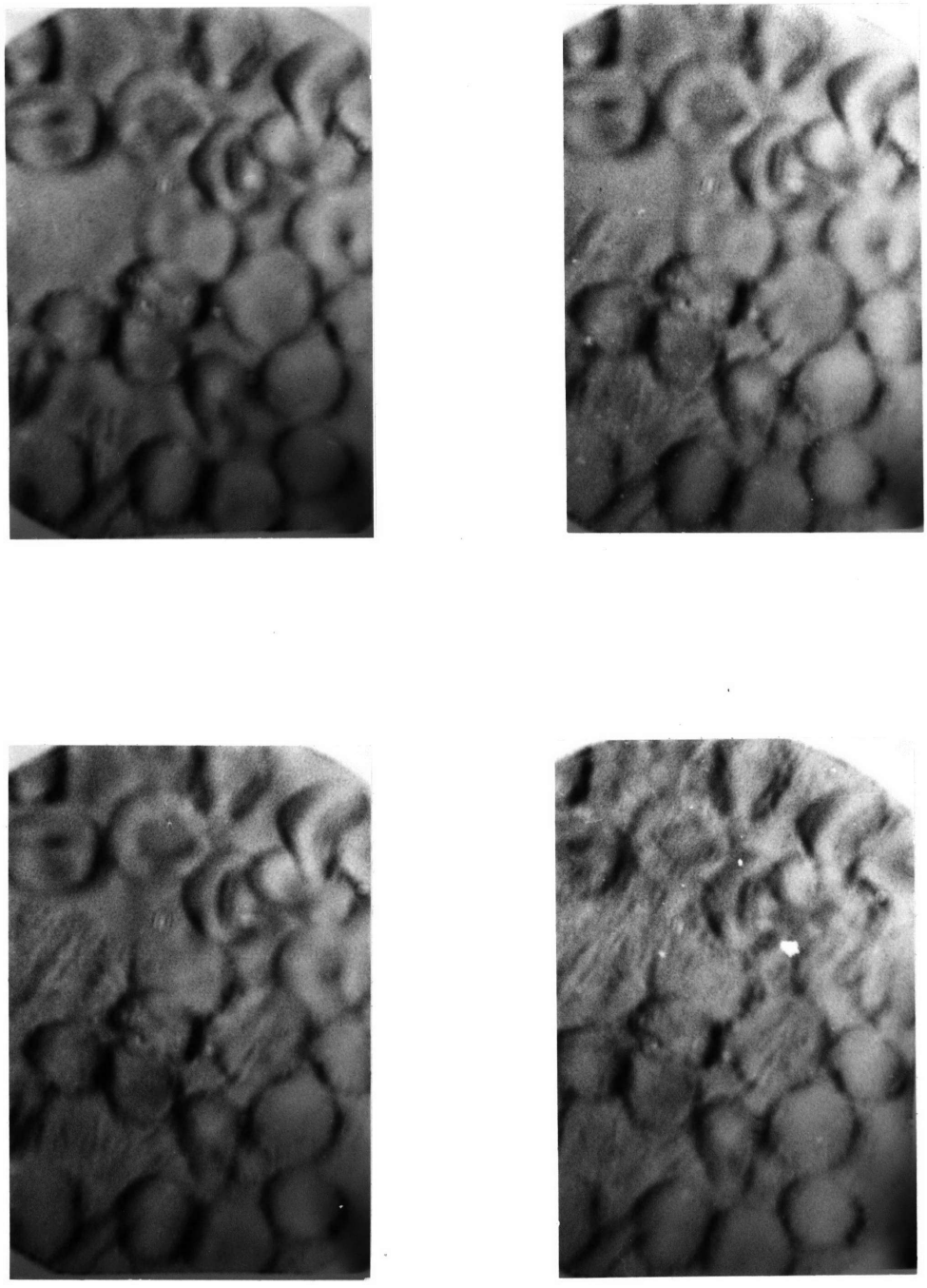

Figure 4.9. Cinephotomicrograph - Motion Picture Sequence Depicting Intracellular Freezing of Erythrocytes. 788X

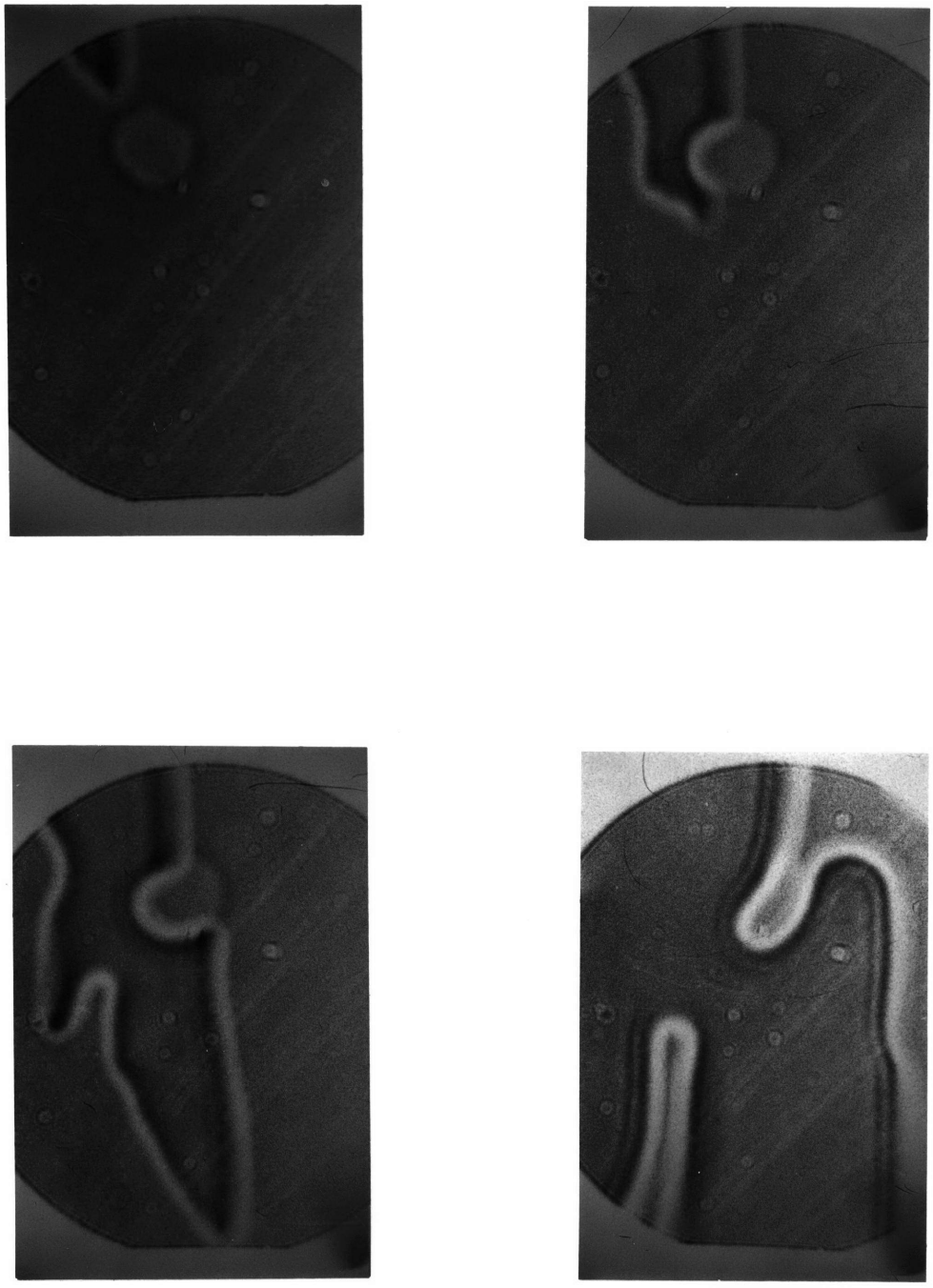

Figure 4.10. Cinephotomicrograph - Motion Picture Sequence Depicting Extracellular Freezing of Erythrocytes. 788X

2. Conditions Requisite for Intracellular Ice Formation

As outlined in Chapter 1, it has been demonstrated both analytically and experimentally that the magnitude of the cooling rate prior to freezing is a parameter of prime importance in determining whether or not intracellular ice will be formed in a frozen suspension of cells. In general, the probability of intracellular nucleation increases with more rapid cooling rates. The objective of this portion of the investigation is to determine the functional relationship between the cooling rate and the probability of intracellular nucleation; of specific interest is the range of cooling rates above which this probability is close to 100% and below which it is virtually 0%.

A series of experiments was conducted in which the incidence of intracellular ice was measured in thin films of fresh, human erythrocytes frozen at constant cooling rates between glass coverslips on the cryomicroscope. The data from these tests is shown in Figure 4.11 in which the percentage of cells containing intracellular ice is plotted as a function of cooling rate. It is apparent that there exists a transition

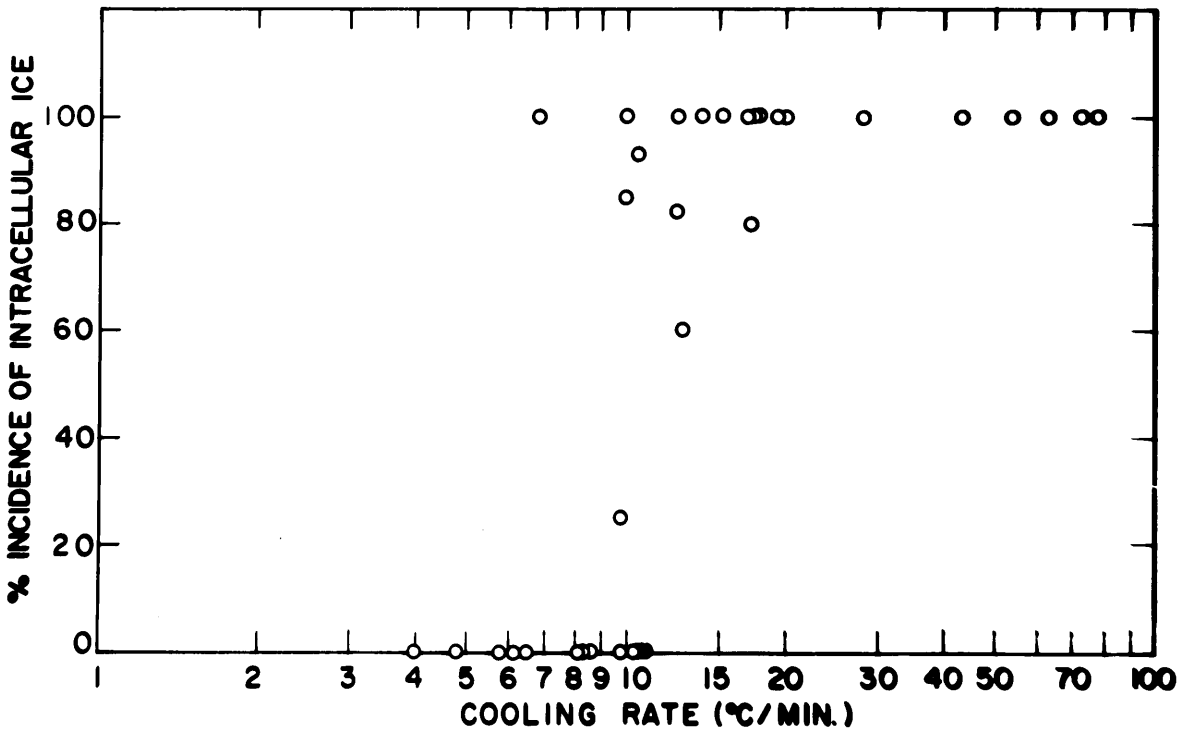

Figure 4.11. Incidence of Intracellular Ice in Frozen Human Erythrocytes as a Function of the Cooling Rate Prior to Freezing

band of cooling rates above and below which the incidence of intracellular ice is virtually 100% and 0%. The upper and lower bounds on this band are approximately -17°C/min and -6°C/min, respectively.

The analytical model proposed by Mazur[19] for the freezing of biomaterials predicts the existence of a transition cooling rate which would define a boundary between the regimes of intracellular and extracellular freezing. The experimental evidence presented here supports this hypothesis in that such a transition range does indeed exist as shown in Figure 4.11. This transition range has one important characteristic not predicted by the analytical model: the transition occurs over a finite band of cooling rates, rather than being defined by a single rate. There are several factors which could contribute to this phenomenon. Knight[74] has noted that a nucleation temperature may not be precisely defined for any given material. Repetitive freezing of water has shown variations in the degree of supercooling within a temperature range of 10°C. It is to be expected that the same type of variation would occur during the freezing of erythrocyte

suspensions. Since the probability of intracellular ice formation depends on the degree of supercooling, the spread of data in the transition range of cooling rates can be attributed in part to random variation in the nucleation temperature.

The susceptability of cells to internal freezing is also a function of the plasma membrane permeability to water. Marks and Johnson[75] have reported a relationship between the age of human erythrocytes and their resistance to osmotic stress. They found that young erythrocytes less than 30 days old are significantly more resistant to hemolysis in hypotonic media than are cells older than 65 days. Thus, in any frozen specimen, there may be a distribution in membrane permeability among the cells according to age with a corresponding variation in the osmotic properties of the total specimen population. The measured osmotic response of individual cells to variations in cooling rate could be different from the average response of a large population of whole blood. The significance of this osmotic factor in contributing to the data

scatter of Figure 4.11 in the transition cooling range can not be evaluated because the relationship between membrane permeability and cell age has not been quantified. However, until proved otherwise, it can be considered as a possible influencing parameter. The magnitude of this effect could be ascertained by repeating the measurements of intracellular ice formation as a function of cooling rate on erythrocyte specimens prepared from selected age groups, such as all cells younger than 30 days or older than 65 days.

A second source of data scatter associated with membrane permeability effects arises from the technique employed for preparing the cells for freezing between glass coverslips. The resulting surface forces certainly subjects the cell membranes to some degree of trauma, which might in turn alter the permeability from its normal value. The preparation trauma is quite difficult to hold constant from experiment to experiment. It is thus probable that the consequences of varying degrees of increased resistance to intracellular freezing associated with higher membrane permeability

occurred in a random pattern in the experimental program, i.e., the magnitude of their effects is not correlated with the sequence of experiments in any manner.

The same data is replotted in Figure 4.12 to yield a statistically more significant indication of the effect of cooling rate on the frequency of intracellular freezing. Specifically, each plotted point represents the average value of the cooling rate and the corresponding percentage of intracellular ice formation for five separate experimental tests of thirty to seventy cells. The RMS deviation for each point is indicated by the bracketed lines. The curve drawn through the data points can be approximated mathematically, as in Equation (4.1), which in turn represents a measure of the probability of internal freezing for human erythrocytes at any given cooling rate.

$$\begin{aligned} &0 \leq B \leq -6\ ^{\circ}C/MIN, && FREQ = 0 \\ &-6\ ^{\circ}C/MIN \leq B \leq -17\ ^{\circ}C/MIN, && FREQ = \frac{B+6}{-17+6} \\ &-17\ ^{\circ}C/MIN \leq B, && FREQ = 1.0 \end{aligned} \qquad (4.1)$$

The curve in Figure 4.12 may be compared with that in Figure 4.13, which was hypothesized by Mazur[76]

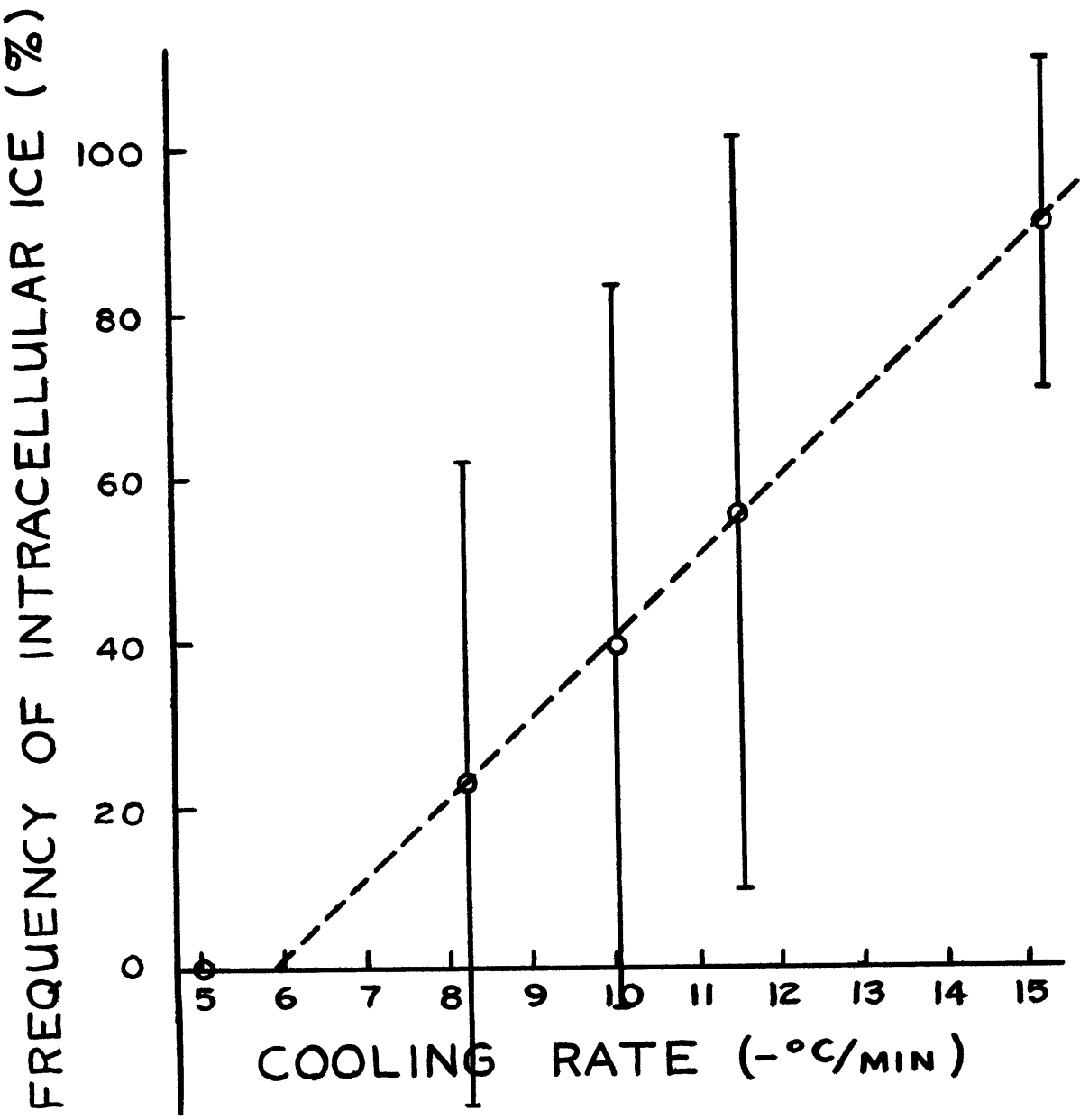

Figure 4.12. Relation Between Intracellular Freezing and Cooling Rate

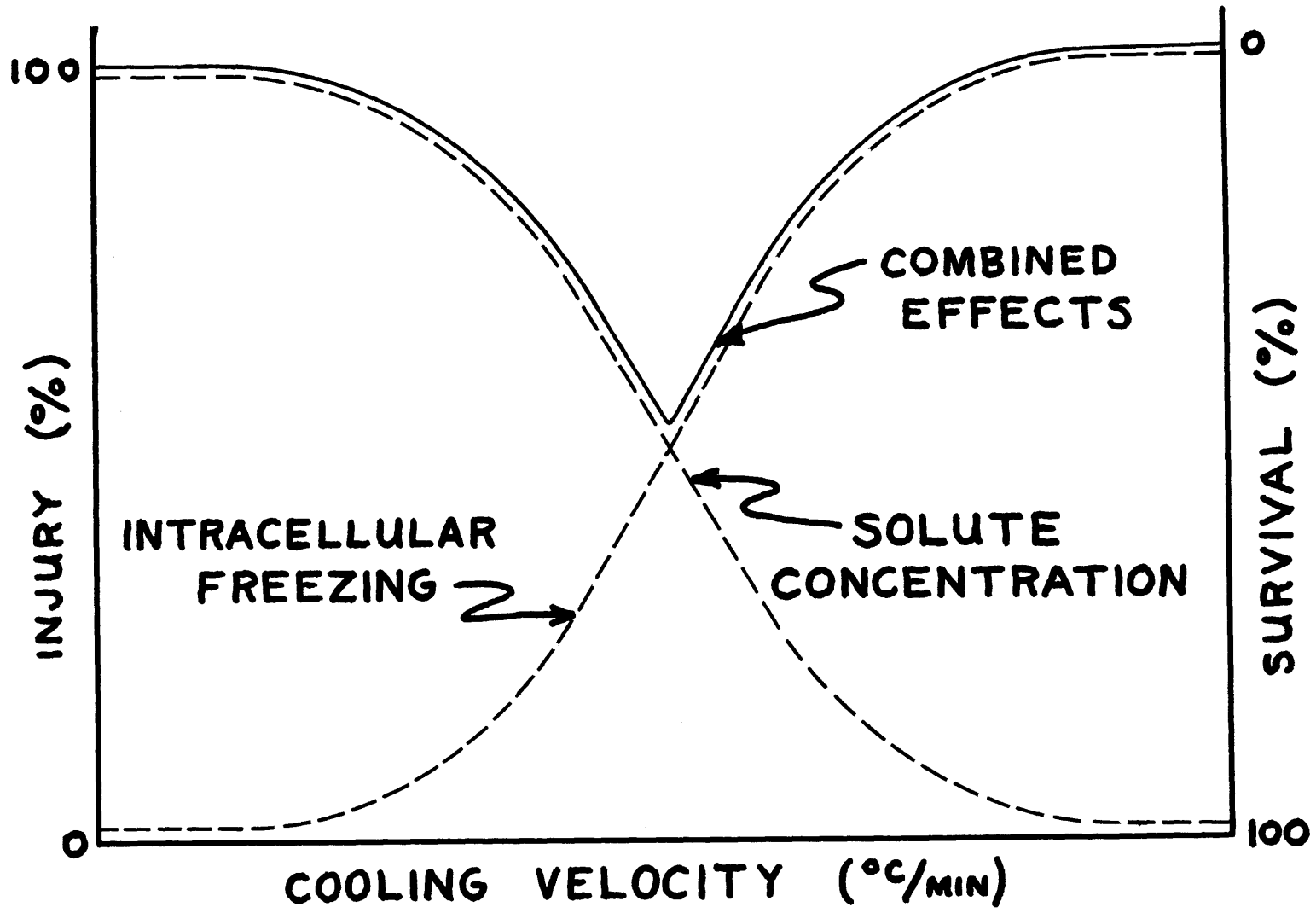

Figure 4.13. Hypothetical Relation Between Cooling Velocity and Survival, Representing Effects of Intracellular Freezing and Solute Concentration (after Mazur (76))

from experimental and analytical evidence. The important similarities to note between the two curves are as follows: (a) a transition range of cooling rates exists across which effects associated with intracellular ice change from 0% to 100% influence; (b) this transition occurs in such a manner as can be described by a simple probability function; (c) the cooling rate prior to the phase change is a parameter of primary importance in controlling the incidence of intracellular ice. Thus, the close degree of qualitative agreement between the hypothetical and experimental results gives credance to existing theories concerning the mechanism of intracellular freezing, that is, that competing rate processes for heat transfer and mass transfer control the intracellular nucleation process.

Quantitative comparison of the data in Figure 4.12 with previous analytical and experimental results is less favorable. The minimum cooling rate for which intracellular ice formed (-6°C/min) is much lower than that predicted by Mazur's analytical model[19] (-5000°C/min) and the experimental data of Luyet and Rapatz[77] (several thousand °C/min). The survival signatures for several

cell types, including unprotected erythrocytes, are shown in Figures 4.14. Several explanations can be offered to describe this discrepancy. First, freezing was effected in the cell specimens at controlled cooling rates which were measured very accurately. As is shown in Figure 4.1, the degree of uncertainty in temperature measurement is limited to $\pm$ 1/2 °C. Previous experimental techniques for studying intracellular freezing have been restricted in that cooling rates were effected at the mercy of the variable heat flux to the specimen associated with quenching in a cold refrigerant bath. Cooling rates are commonly determined as an average value from the end point temperatures and the elapsed time for the process. This average value of the cooling rate is larger than the value for the lowest end point temperature, at which the phase change occurs. Thus, there has been a trend to report more rapid cooling rates than are actually associated with the phase change process. This trend likely accounts in part for the discrepancy between previous experimental results and the data from this work, which was obtained by carefully controlling and measuring the cooling rate prior to freezing. Second, the assumptions inherent in the analytical

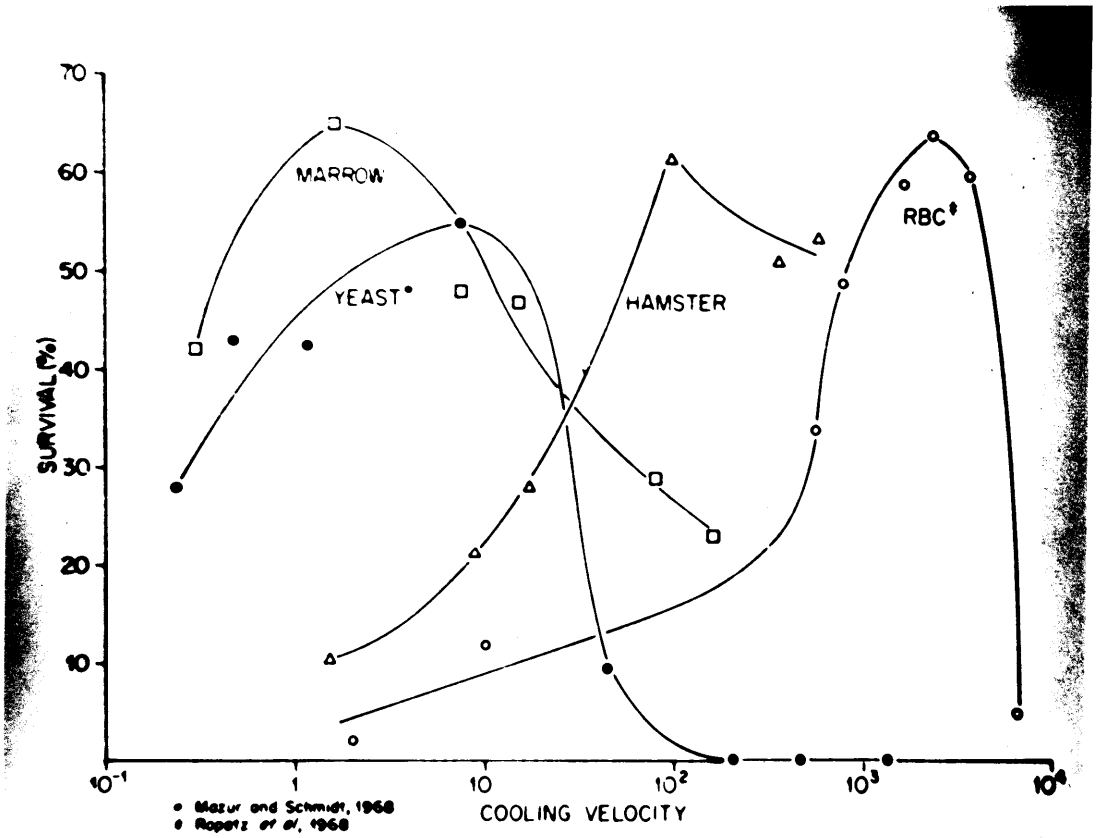

Figure 4.14 Comparative Effects of Cooling Velocity on the Survival of Various Cells Cooled to -196°C and Thawed Rapidly

model may result in an overly high prediction of the critical cooling rate. In particular, if the temperature coefficient of permeability is not linear as assumed, but instead drops significantly at subzero temperatures, then the probability of intracellular ice formation would be high at cooling rates much slower than -5000°C/min. Permeability data obtained by Jacobs[78] indicate a trend to decreased coefficients at low temperatures. Third, preparation of the specimen between coverslips results in a relatively large ratio of the suspension-solid substrate interfacial area to the suspension volume as compared to bulk frozen samples of larger free volumes. It has been demonstrated previously that the rate of crystal growth in frozen aqueous solutions increases markedly with larger ratios of substrate interface to melt volume.[20,21,79-81] This pehnomena could directly contribute to higher probability of intracellular ice formation in that the competitive rate process for equilibration of intracellular fluid with the frozen extracellular medium by osmotic dehydration of the cells would be relatively less effective in the face of a higher rate of phase change.

Several hypotheses have been offered to explain the mechanisms of a solid-fluid interaction in affecting the growth rate of ice, including the following: (1) the substrate acts as a heat sink to enhance the rate of heat flow from the freezing liquid,[20] (2) the contact angle made between the melt liquid and the substrate greatly influences the morphology of the advancing phase front and consequently the heat flow conditions and the kinetics of crystal growth,[82] (3) after nucleation has occurred in the subcooled liquid at the solid interface, further heterogeneous nucleation rapidly spreads along the interface, thus providing innumerable more loci for the growth of crystals out into the liquid melt,[21,83] (4) the electrically insulating surfaces of the glass coverslips carry small areas of positive and negative electrical charge, which function to order water molecules in the cell suspension and reduce the reduce the amount of energy removal necessary to effect the phase change.[83] However, none of these hypotheses provides a satisfactory basis for explaining qualitatively or quantitatively the reduction in the transition cooling rate of intracellular ice formation in human erythrocytes frozen between glass coverslips.

CHAPTER 5

Conclusions

The physio-chemical conditions requisite for intracellular ice formation in unprotected human erythrocytes have been defined experimentally. The incidence of intracellular freezing was measured visually as a function of the cooling rate prior to phase change. The data indicates that the probability of intracellular freezing increases linearly with the cooling rate from 0% at -6°C/min to 100% at -17°C/min. No cells cooled more slowly than -6°C/min were frozen, whereas all cells cooled more rapidly than -17°C/min experienced internal freezing. This data supports the dual factor theory of cell freezing injury as proposed by Mazur[18] in that the presence of intracellular ice becomes increasingly more frequent across a transition band of cooling rates until a constant maximum value is reached. In a similar manner one of the two hypothesized cell injury factors becomes influential across a band of increasingly higher cooling rates until a level of total predominance is attained. This injury factor has been construed to be caused by intracellular freezing. The experimental evidence presented here demonstrates that the formation of intracellular ice is dependent upon the cooling

rate in a like manner.

Data has also been obtained which indicates that the thermodynamic state of cells in a frozen saline matrix can be dictated either by heat transfer considerations resulting in internal freezing or by mass transfer considerations resulting in dehydration by osmotic loss of cell water. The implication of this data is that competing heat and mass transfer processes exist simultaneously, the relative magnitude of which determines the ultimate mode of freezing, be it extracellular or intracellular. An analytic model of cell freezing has previously been proposed by Mazur[19] in which it is hypothesized that the mode of cell freezing is governed by the two competing rate processes of heat and mass transfer, and that the experimentally controllable parameter which regulates the relative magnitudes of these two processes is the cooling rate. The direct correlation of intracellular freezing with cooling rate that was demonstrated in this thesis strongly affirms the above hypothesis.

Data on the incidence of intracellular ice formation was obtained visually on a light microscope uniquely modified to incorporate the capability of effecting controlled freezing and thawing processes in biological cell suspensions.

A broad spectrum of cooling and warming rates achieved by cooling the specimen at a constant rate with a steady flow of refrigerant fluid through the system and by simultaneously dissipating electrical energy at a variable rate in a resistance heater immersed in the fluid stream and in thermal communication with the specimen. The dual capability for both heating and cooling is utilized in conjunction with an analog control system to provide for precise automactic regulation of the specimen temperature between 100°K and 310°K at time rates of temperature change between zero and several thousand degrees centigrade per minute.

CHAPTER 6

Suggested Subsequent Investigations

The cryomicroscope is particularly well adapted to the pursuit of investigations in cryobiology at a fundamental level due to its unique capability for gathering information concerning the dynamics of freezing and thawing processes and to its precision temperature control system. There are many significant, unresolved problems in cryobiology which can be effectively studied on the cryomicroscope. Several such studies will be described in this chapter.

The investigation of the formation of intracellular ice in biological cells can be expanded in two dimensions from the studies on human erythrocytes which were the subject of this thesis: first, by including other cell species in the program of study, and second, by freezing cells in the presence of cryophylactic agents of various chemical compositions and concentrations. The rational for expanding the scope of the investigation to include these two areas can

be explained by referring to the two composite graphs shown in Figures 4.13 and 4.14 both of which were originally developed by Mazur.[18,76] Figure 4.13 illustrates a typical survival signature (i.e., a plot of the percentage of surviving cells vs. the cooling rate) for freeze-thawed cells. It is assumed that the warming rate is fixed for all freeze-thaw processes represented on any given survival signature. Several characteristics of a survival signature can be described. First, the survival rate reaches a maximum value at some particular optimum cooling rate. Second, the survival rate falls off from the maximum value as the cooling rate is varied either larger or smaller than the optimum value. Third, an area is defined under the survival signature for any given minimum survival rate. This area can be considered as a measure of the sensitivity of the percentage of surviving cells to variations in the cooling rate. As has been hypothesised by Mazur,[76] the survival signature can be characterized as a composite of two survival curves, one resulting from an injury factor occuring at cooling rates less than optimum and the other from a factor

occurring at rates higher than optimum. These two survival curves are denoted by the dashed lines in Figure 4.13. Mazur's hypothesis states that intracellular ice is the factor responsible for cellular injury at high cooling rates.[76] Fifth, there is a transition band of cooling rates across which the survival curve of the rapid cooling factor decreases from its maximum to its minimum value.

In Figure 4.14 the survival signatures for various cell species, frozen and thawed in different compositions and concentrations of cryophylactic agents, are compared. It is readily apparent that the characteristics of the individual signatures are strongly influenced by the cell species and cryophylactic agent as evidenced by differences in maximum magnitude, optimum cooling rate, and area under the signature. The program of investigation for this thesis has considered only one such signature, that of human erythrocytes in no cryophylactic agent. Thus, there remain many more signatures to be explored. Of particular interest is the determination of the combination of cryophylactic agent, its concentration, and the optimum cooling rate

which maximize the survival rate for certain types of cells, such as blood platelets and granulocytes and bone marrow stem cells.

All of the survival signatures shown in Figures 4.13 and 4.14 were obtained for the same warming rate. Thus, an obvious extension of this program of investigation would be to explore the effects of the warming rate on the survival of frozen-thawed cells. The temperature control system employed with the cryomicroscope is uniquely suited to isolating the influence of the warming rate on cellular injury for systematic study.

One inherent drawback of the cryomicroscope is that it is capable of freezing only very small specimen volumes, on the order of ½ microliter. This small volume severily restricts the choice of viability tests which may be employed to assay the cell survival rates. Thus, it is desirable to verify experimental results obtained on the cryomicroscope for a much larger specimen volume, in order to be able to apply more rigorous measures of cell viability at the

expense of sacrificing the capability for directly viewing the freezing and thawing processes. A freezer having a specimen capacity of approximately 30 milliliters has been designed to satisfy this need and is currently being fabricated in the Cryogenic Engineering Laboratory. It will operate with the same automatic temperature control system as is employed with the cryomicroscope.

The temperature control system used with the cryomicroscope affords the opportunity for the first time to measure experimentally the validity of Mazur's analytical model for freezing biological cells.[19] The control system has been built so that one of the primary assumptions incorporated into the model, namely that the rate of cooling is constant, can be experimentally duplicated. Thus, the cell volume can be measured as a function of temperature and rate of temperature change. This data can then be compared with the analytical predictions of Mazur to evaluate the applicability of the model, and to indicate possible refinements which would result in superior experimental correlation.

A final suggested study is to employ the cryomicroscope to investigate hypertonic stress in red cells as a function of temperature. This study would be of value as it is related to the mechanism of cellular injury occuring at cooling rates less than the optimum rates in which cells are subjected to hypertonic stress. The use of the cryomicroscope would allow the study of the response of cells to a concentrated extracellular salt solution in a closely controlled experimental environment having a variable temperature. In this manner pertinent thermodynamic data, including cell volume and temperature, could be obtained with the objective of more accurately describing the relevant injury mechanisms experienced by cells frozen in similar circumstances.

APPENDIX A

A Review of Human Erythrocyte Preservation by Freezing

One of the most significant events in the history of low temperature biological research was the empirical discovery by Polge, Smith, and Parkes,[4] of the protective effect of glycerol on frozen bovine spermatozoa. Smith subsequently demonstrated that human erythrocytes equilibrated with 10%-15% glycerol solution could also withstand the trauma of slow freezing and thawing.[5] This finding immediately catalyzed considerable investigation into both the basic mechanisms of freezing injury in blood and the development of clinically applicable techniques for the preservation of blood for transfusion. Glycerolized, frozen red cells were first used successfully for human transfusions by Mollison and Sloviter.[6] Since then, extensive research efforts by many investigators have resulted in the development of different freezing procedures. Cryopreservation of blood has now reached the point of clinical applicability.

The interactions that occur in a cell and its surrounding medium during freezing and thawing are very complex, and it is a difficult task to isolate the effects which may occur almost simultaneously. Imprecise knowledge of the actual events occurring during the freezing and thawing of cells has led to conflict and contradiction among the various theories

of freezing injury. One common point of agreement is that the mechanisms of ice formation are intimately involved in causing cellular damage. Ice formation occurs in two different configurations in biological material: at slow cooling rates ice forms only extracellularly, whereas at rapid cooling rates ice forms both intracellularly and extracellularly. The study of the freezing of blood has accordingly been divided into two catagories, one dealing with slow cooling rates and the other with rapid cooling rates.

Extensive research by many investigators has been directed toward determining how the type and extent of cellular injury can be controlled by the physical parameters associated with freezing and thawing. The following five parameters have been studied quite extensively as they relate to the survival of frozen red blood cells:

(1) the time rate of temperature decrease during cooling

(2) the time rate of temperature increase during warming

(3) the storage temperature

(4) the physical and chemical composition of cryoprotective additive

(5) the concentration of cryoprotective additive in the specimen.

Although these parameters are all interdependent in their action on cells and are therefore difficult to study individually, significant trends have been observed and are the basis for current theories of freezing injury.

The influence of cooling rates on the formation of ice in biological materials has led to the categorizing of freezing processes into two distinct classifications: one for which the cooling rate is less than the transition value for the formation of intracellular ice, and one for which the cooling rate is greater than the transition value. Accordingly, practical processes for the cryopreservation of blood have been developed based upon relatively slow cooling rates requiring the presence of an additive which penetrates the cell membrane, or on relatively rapid cooling rates in the presence of a nonpenetrating protective additive.

An important contribution was made by Lovelock,[84] in 1953 in his investigations of the protective action of glycerol in red blood cells frozen at slow cooling rates. He suggested that a major cause of cellular injury could be attributed to an increased concentration of intracellular electrolytes resulting from the removal of cellular water by an osmotic pressure gradient across the membrane. The difference in chemical potential between intracellular and extracellular fluids is the cause of the osmotic action. The concentrated solutes, particularly sodium chloride, cause chemical hemolysis in the blood cells. The protective action of glycerol was thought to be related to its ability to form hydrogen bonds with intracellular water and prevent it from diffusing out of the cell. The glycerol-water solution thus provides an effective solvent in which the electrolyte

concentration remains below the lethal level. Consequently, in order to exhibit this protective ability, it is essential that the additive penetrate the cell membrane to dilute the intracellular salts. The presence of a cryoprotective additive inside the cell has caused some of the greatest difficulties in adapting blood frozen at slow cooling rates to clinical use. For this type of freezing process, the cells must be subjected to a post-thaw washing process to remove the additive before transfusion. Although post-thaw washing has been very helpful in purifying blood prior to infusion, improved understanding of basic problems might lead to simplification, or possibly elimination of this step.

Lovelock's theory of salt-concentration injury has received considerable support since its inception, and a vast amount of experimental evidence has been accumulated to substantiate his assertion. However there also exists sufficient contradictory evidence to indicate that salt injury is not the only mechanism of cellular damage caused by freezing.[24,63,76,85-88] Many investigators have proposed alternate theories substantiated by varying amounts of supportive experimental work.[45,76,86,89]

Smith, Polge, and Smiles,[44] conducted the first systematic investigation of the protective action of glycerol on red blood cells. By adapting a special freezing stage to a light microscope they were able to directly observe a suspension of glycerolized red cells being frozen and thawed.

They commented that the formation and dissolution of ice crystals in the medium caused less mechanical stress to cells in the presence of glycerol than in its absence. Their observations have been verified by later investigators using similar experimental procedures.[45,65] Huggins,[88] and Nei,[63] have supported the contention that hemolysis can be caused by physical as well as chemical action. Huggins' hypothesis states that the readjustment of the structured water on either or both sides of the cell membrane during thawing can create physical stresses in the membrane sufficitnely large to result in cell hemolysis. This effect is especially pronounced when the cells are suspended in a hypotonic solution prior to freezing. Under these conditions, the cell volume increases with attendant increases in membrane stress. Further support for this hypothesis is evidenced by the diverse chemical nature of the many substances such as serum,[90] egg yolk,[91] citrate,[91] glucose,[92] and other sugars,[93] which are known to exhibit protective action for frozen cells. Thus it would seem probable that the effectiveness of these additives is due to their colligative action as an external source of osmotic pressure to reduce the size of swollen cells. This renders the membranes less susceptible to mechanical damage from recrystallizing ice during the storage period and thawing.

On the basis of his work with plant tissues, Levitt,[89] has proposed a theory in which cell dehydration is the primary mechanism of freezing injury. The osmotic removal of solvent water from the cells might cause proteins to cross-link via the formation of disulfide bonds. The phenomenon of protein denaturation would appear to offer an explanation for the combined roles of ice formation and salt concentration in that both contribute to the mechanism of cell dehydration.

A hypothesis recently proposed by Meryman,[87] states that the hemolysis of erythrocytes by freezing at slow cooling rates is the result of damage to the cell membrane caused by a greater osmotic pressure gradient across the membrane than can be compensated for by changes in cell volume. The protective additive then acts on a purely colligative basis to prevent the concentration of extracellular solutes, with no specific chemical action involved.

Other investigators have related the function of cryoprotective agents to more complex mechanisms than simple colligative action and are therefore more closely aligned with Lovelock's theory. Doebbler and Rinfret,[94] have correlated protective activity with the concentration of potential hydrogen bonding groups provided by an additive. Nash,[95] asserts that the most important single characteristic of an additive which protects living cells at temperatures below freezing is very probably its affinity for water.

It should be apparent that after twenty years of research there is still a great deal of conjecture and misunderstanding concerning the basic mechanisms of injury experienced by erythrocytes frozen at slow rates of cooling. Extensive experimental documentation has been accumulated to support both the physical and chemical theories of injury, making it difficult to dismiss the significance of either one. It is almost certain that both mechanisms are involved in freezing injury, and the real problem that remains is to determine their respective spheres of influence as a function of the physical parameters controlling the freezing process.

A second approach to the freezing of blood involves the use of rapid cooling rates to produce intracellular ice. It was first demonstrated by Luyet,[96] in 1949 that human erythrocytes could be frozen and thawed without causing hemolysis. He attained a rapid rate of cooling by sandwiching unprotected whole blood between two thin glass coverslips and quenching the specimen in a liquid nitrogen bath. After rapid warming by immersion in saline, intact red cells were recovered from the solution. Luvet's observations were pursued further in 1955 when Meryman and Kafig,[39] reported a technique for the rapid cooling of whole blood by spraying it through a fine capillary tube as droplets onto a surface of liquid nitrogen. The droplets required only a few seconds to reach -196°C at the surface after which they sank to the bottom of the container and could be removed for long term

storage. Recovery rates high in the 90% range were obtained when small quantities of glucose were added as a cryoprotective agent. In an attempt to develop this technique on a clinical level, Rinfret and Doebbler,[97] built an elaborate apparatus to process transfusion size quantities of blood by droplet freezing. Concurrently, Strumia,[98] et.al., designed a rapid cooling system in which red cells mixed with 5% glucose (membrane permeable) and 7.5% lactose (non permeable), were frozen in thin-walled, envelope-shaped, closed metal containers by quenching in liquid nitrogen. Again, very high in vitro recovery rates were attained.

Although clinically accpetable levels of hemolysis are not consistently attainable with rapid freezing, these techniques do offer the advantage that lengthy, elaborate washing procedures are not required after thawing. The protective additive and the products of hemolysis are infused along with the red blood cells. Red cells frozen by rapid cooling are protected from injury by various nontoxic additives at relatively low concentrations which would hopefully be tolerated by the circulatory system. The practical utility of freezing blood protected by glucose and lactose was later questioned by Pert, et. al.,[9] who showed that when blood cells suspended in these sugar solutions are suddenly introduced into a isotonic medium as during transfusion, they experience rapid and extensive osmotic hemolysis.

A wide variety of other compounds have been examined for their protective effect during rapid cooling. Polyvinyl pyrrolidone (PVP) was found to exhibit an unusually high ability to protect red cells from freezing injury. Maximum protection to blood is afforded at an overall concentration of about 7% (w/v) for which viability rates greater than 90% have been attained.[99]

Although rapid cooling rates have been successfully used to preserve red cells, the pertinent mechanisms of freezing injury and cryoprotective activity have not been elucidated. It has been postulated that intracellular ice crystals mechanically damage the ultrastructure and macromolecular system of the cell or cell membrane,[100,101] but a definite correlation has not been demonstrated.[85] There is no evidence that indicates whether or not the injury mechanisms generally thought to be responsible for slow cooling damage do indeed play a significant role in rapid cooling injury. Improved understanding of the course of action of cryoprotective agents would facilitate the search for, or synthesis of more effective agents than are presently recognized. Additionally, the mechanisms of freezing injury might be clarified by a more thorough knowledge of cryoprotective activity.

The effects of the warming rate on frozen cell survival have not been investigated as thoroughly as have the effects of the cooling rate. It is well known that the rate of warming

during the thawing process plays an important role in controlling injury to cells. The experimental study of warming rates is more formidable than that of cooling rates because of the difficulty in designing a device to thaw the frozen cells in such a manner that spatial temperature gradients are not large enough for some of the cells to become warmer than 45°C., at which temperature death occurs. However, the exact involvement of the warming rate with the mechanisms of cellular injury is not understood, aside from the simple observation that the more rapidly the cells are warmed, the less time they are exposed to the damaging actions of physical and chemical factors. In general, the extent of damage to cells varies inversely with the magnitude of the warming rate. Recent work by Diller and Cravalho,[45] indicated that the thawing process may be more intimately involved in the actual occurrence of injury than was previously believed and therefore merits special attention in future research activity.

Investigations to determine the effect of storage temperature on freezing injury have centered around establishing a "safe" temperature below which biological materials can be kept with no deterioration in viability with time. Although many frozen materials appear to be insensitive to the period of storage at -79° (dry ice), others, such as erythrocytes, are not.[24,102] Huggins,[88] has demonstrated that depending upon the experimental conditions, the viability of

human erythrocytes stored at -85°C can seriously deteriorate over a period of several days. A storage temperature of -130°C has been generally accepted as the limit below which all biological material can be safely stored.[103] However, it has been reported more recently that temperatures as low as -196°C (LN_2) are required for acceptable long term preservation.[104] At storage temperatures above the safe value, cellular injury accumulates in proportion to the storage time and to the magnitude of the increment of the storage temperature above the "safe" value.[98]

Recently some effort has been devoted to the use of pressure as an additional parameter to control hemolysis in freeze-preservation of red blood cells.[105,106] Preliminary data of this early work indicate that pressure can be successfully used to minimize hemolysis, but that the precise nature of its effect is not known. Clearly, more work needs to be done in this area before pressure might be used for this purpose in clinical situations.

Although blood freezing research since 1949 has been successful in developing clinically acceptable procedures, the empirical orientation of this work has left unanswered many fundamental questions regarding the mechanisms of freezing injury and additive protection. Consequently, although there is still not a clear understanding of the events experienced by cells when they are exposed to the freeze,

store, thaw and wash cycle, red cells are currently being stored in the frozen state for clinical use by many different techniques, one* of which is licensed by the Division of Biologics Standards, National Institutes of Health.

In each of these methods, blood is modified by the addition of cryoprotective agents prior to freezing. Two processes using glycerol to protect red cells during slow cooling have been developed. The most difficult problem encountered in the use of these procedures involves the addition and removal of the protective agent from the freezing medium. These cryoprotective procedures require elaborate processing equipment, large quantities of consumable washing solutions and lengthy processing times over and above those already needed for the collection, storage, and transfusion of blood. In an attempt to simplify the procedures, Huggins,(7) devised a technique for agglomerating red cells suspended in glycerol that requires no centrifugation. Upon the addition of non-electrolyte solutions to the freezing medium, the red cells clump together and sink to the bottom of the container. Here they may be readily separated from the supernatant solution and resuspended for transfusion. This method of cryopreservation has seen application in many parts of the world.

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*Massachusetts General Hospital System

Other methods involve either continuous centrifugation in an Arthur D. Little Cohn Fractionator or batchwise washing to add and remove the cryoprotective agent.[107] These methods have achieved excellent results.

The slow cooling techniques require a relatively high concentration of cryoprotectant (30% or more) but have the advantage that the survival rate of red cells is relatively insensitive to variations in the freeze-store-thaw conditions. The washing processes have also proved useful for removing unwanted components from the blood prior to transfusion.

Storage methods involving freezing at rapid cooling rates are also in use. Several employ rapid cooling by liquid nitrogen in the presence of an extracellular (non penetrating) additive such as PVP[108] or hydroxyethyl-starch (HES).[109] Unique aspects of these methods are that they involve freezing whole blood rather than only separated blood products and that they require no post-thaw washing process. The cryoprotective additive (7.5% PVP and 6% HES) and the products of hemolysis are transfused directly to the patient after thawing. Viability rates on the order of better than 80% are currently attainable; thus these methods have potential value for clinical use.

A method of cryopreservation which has experienced extensive application combines certain aspects of both the slow and rapid cooling approaches:[8,9] an intracellular cryoprotective additive, glycerol, is used at a much lower concentration (14%) than for slow cooling rates, together with rapid cooling in liquid nitrogen. The glycerol is washed from the thawed blood by centrifugation before transfusion. The cryopreservation of blood by this technique has proved to be clinically quite acceptable.

The various approaches to freezing blood have been submitted to extensive comparative evaluation and clinical testing at the Chelsea Naval Hospital.[110] These studies have demonstrated the practical potential for the freeze preservation of blood, but they also illustrate the necessity for advances both in basic understanding and in the capability of present methods.

Prospects for the future application of frozen blood in routine transfusion service appear to be very good. Although unit frozen storage costs are nominally higher than in liquid storage and processing rates are slower and more expensive, several important advantages may be cited for frozen preservation which should ultimately offset

the above drawbacks in many circumstances. These advantages are discussed as follows. First, the rate of metabolic deterioration is decreased by orders of magnitude in frozen cells. As a result the oxygen transport capability of the cells undergoes minimal diminution during storage and is near its maximum value upon infusion. Second, the incidence of infectious disease transmission via transfusion is reduced to practically zero. As an example, there has been only one detected case of hepatitis transmission associated with more than 23,000 frozen red cell transfusions at the Massachusetts General Hospital. This statistic can be compared with the personal and financial hardships incurred with the .3% incidence of transfusion hepatitis for liquid stored blood. Third, due to the decreased deterioration rates, freezing enables the long term stockpiling of blood to maintain a ready supply of rare types and to smooth out seasonal and regional variations in donor shortages. An important aspect of this long term storage is that no outdating occurrs, which in turn reduces the relative unit cost of infused cells because of the 20% discard rate associated with refrigerated blood due to excessive aging. Fourth, the processes of freezing, thawing, and washing whole blood eliminate many components of the blood which

can cause undesirable side effects in the transfusion. This factor is especially significant in light of the increasing frequency of organ transplantations, for which frozen blood has been demonstrated to have superior therapeutic value.

APPENDIX B

Discussion of: "Analysis of Cell Freezing and Dehydration", ASME Paper No. 69-WA/HT-31 by G. R. Ling and C. L. Tien

by

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One of the major contributions to the field of cryobiology is the theoretical model of cell freezing proposed by Mazur.[19] Unfortunately the analytical expression describing the behavior of the model is rather complicated and difficult to handle mathematically. By establishing various regiemes of the freezing process, the present effort by Ling and Tien greatly simplifies the expression formulated by Mazur. On the basis of this new analysis, it is now possible to establish in a simple manner the effect of the various parameters on the freezing process. Thus the freezing process in cells is now better understood than it has been heretofore.

The authors have addressed themselves to the problem of cell preservation by freezing, but their analysis is just as relevant to the problem of cell destruction by freezing. That is, this analysis will aid not only in establishing the optimum cooling rates and storage temperatures for cell preservation, but also in establishing the optimum cooling rates and cryoprobe temperatures for cryosurgical procedures.

There are a few specific points in the analysis of Ling and Tien that merit additional comment. These comments are primarily concerned with the limitations imposed by the assumptions inherent in equation (1), (equation 1.2 in thesis text), and by the assumptions necessary to reduce this equation to a form suitable for solution in closed form.

First, the derivation of equation (1) is based on the assumption that the intra-cellular and extra-cellular materials are in equilibrium with a vapor phase (See reference (19)). However, there is no vapor phase present in either a real cell system or or in the thermodynamic system as defined by Figure 1.

Therefore, from this point of view the validity of equation (1) must be held in some degree of question.

Second, it is assumed that the system is at a uniform temperature and experiences a constant cooling rate. Unfortunately, these conditions are not satisfied in many experimental cases. That is, the cooling rate is usually a function of the temperature of the cell boundary, which may or may not be the same temperature as the central portions of the cell. Clearly these conditions severely limit the applicability of the present analysis.

Third, the expression for the temperature dependence of the latent heat L (See discussion following equation (4).) is incorrect. The correct expression can assume several forms, all of which originate with the Clapeyron equation

$$L = T \frac{dP}{dT} \left(v_f - v_s \right) \qquad (B.1)$$

where the subscript f refers to the liquid phase and the subscript s refers to the solid phase. Similarly,

$$\frac{dL}{dT} = (c_p)_f - (c_p)_s + \frac{dP}{dT}\left[\left(\frac{\partial(v\tau)}{\partial\tau}\right)_{pf} - \left(\frac{\partial(v\tau)}{\partial\tau}\right)_{ps}\right] \qquad (B.2)$$

or

$$-\tau\left[\frac{d(L\tau)}{d\tau}\right] = \frac{dL}{dT} - \frac{L}{T}$$

$$= (c_{sat})_f - (c_{sat})_s \qquad (B.3)$$

where $\tau = (1/T)$ and the subscript sat denotes conditions obtaining at saturation, i.e., for the liquid and solid phase in equilibrium. There are a number of assumptions that can be made to simplify the analytical form of the above equations, but none of these will yield the precise form given by Ling and Tien.

One reasonable assumption is that the third term on the right-hand side of equation (B.2) above is negligible. Then equation (B.2) can be integrated to give

$$(L - L_o) = (T - T_o)\left[(c_p)_f - (c_p)_s\right] \qquad (B.4)$$

where the subscript o denotes some reference state such as the triple point. This expression is in good agreement with the available experimental data.

Finally, it appears that in applying the analysis to the yeast cells Saccharomyces cerevisiae the authors have incorrectly computed some of the constants, particularly γ. A value of 4.60 would appear to be more appropriate for γ. It is interesting to note that the application of the analysis has been limited to yeast cells which are certainly not typical of mammalian cell systems, the class of cells of greatest practical interest. This limitation is a matter of necessity rather than convenience since at the present time experimental data is available only for yeast cells.

In this regard we would like to point out that at the MIT Cryogenic Engineering Laboratory, we have developed a cinephotomicrographic system which will give continuous experimental values of the parameters Θ, Ψ, and ε (as defined by Ling and Tien) as Θ is varied. The apparatus provides for direct observation of a biological specimen during the actual freezing and thawing processes by incorporating a transparent freezing chamber in the stage of a high-resolution light microscope. Cooling rates up to -10,000°C/min have been obtained. The critical value of Θ and the corresponding value of Ψ can thus be determined for a wide variety of freezing experiments, which will give a direct experimental check on the validity of the analysis of Ling and Tien. Experimental data will be forthcoming shortly.

In conclusion, we would like to point out that the present analysis is devoted entirely to the freezing process of cell preservation. However, cell preservation by this method is really a two-step process, freezing followed by subsequent thawing, and recent

evidence indicates that the thawing process may be just as significant, perhaps more so, for cell survival. It is our hope that a theoretical model of cell freezing and thawing capable of resolving the aforementioned conceptual discrepancies will be forthcoming. Since freezing and thawing are often non-equilibrium processes, an analysis based upon the theory of irreversible thermodynamics might prove fruitful. Certainly the analysis of Ling and Tien represents a significant first step in this direction that hopefully others will follow.

APPENDIX C

A Cryomicroscope for the Study of Freezing and Thawing Processes in Biological Cells

(The material in this appendix was published as a paper by K. R. Diller and E. G. Cravalho in Cryobiology, Vol. 7, No. 4-6, 1970, pp. 191-199)

A. Introduction:

The potential clinical importance of selectively freezing and thawing biological material either reversibly or irreversibly has been recognized for many years. Reversible freezing techniques have been developed and are currently being employed in the long term preservation of certain biological materials such as red blood cells and spermatazoa, so that essentially full living activity is regained upon the return of these materials to normal temperatures. Hopefully in the not too distant future, these techniques will be extended to more complex biological materials such as whole organs. Conversely, certain surgical procedures use localized irreversible freezing to cause necrosis in the treated area while producing no adverse effects in the surrounding tissue. Here again it is just a question of time until these procedures find more widespread application.

Stated rather simply, the basic problem in each of these applications is to effect heat transfer, at the proper rate and proper temperature, within a biological system composed, in general, of a wide variety of cells arranged in a complex

geometry. Although this problem appears straightforward, there are really two very different aspects to it. First, the various cell types in a given biological system will respond in different ways to the cooling and warming processes, and second, as in the case of an organ, the structure and geometry of the biological system can have a significant effect on the response of the total system to the cooling and warming processes. That is, the problems associated with freezing and thawing biological materials exist on both microscopic and macroscopic levels, and these two levels are sufficiently different that they merit their own particular methods of approach. It is the purpose of the present paper to describe one method relevant to the microscopic level.

There exist in the literature reference works,[24,111] sufficiently current that it is hardly necessary to review here previous research at the microscopic (cellular) level. Suffice it to say that this previous research has concentrated on the mechanisms of freezing injury. Although several vastly different theories have been proposed to explain irreversible freezing damage, all of them agree that the mechanisms of ice formation play a major role. Two different types of ice formation have been observed in cell suspensions: at relatively low cooling rates, i.e., when the time rate of temperature decrease is small,

ice crystals form only extracellularly, while at relatively high cooling rates they are formed both intracellularly and extracellularly. The magnitude of the governing cooling rate and its effect are dependent upon many parameters, the more important of which are the species of cell being frozen and whether a cryoprotective agent has been added to the specimen.

It is also known that the temperature-time history during the thawing process plays an important part in determining the final condition of the specimen, but again, the exact mechanism is not well understood. Curiously enough, the freezing process has received the lion's share of the research effort with little effort devoted to the thawing process. Whether or not such a division of effort is justified remains to be seen.

B. Objective:

It is interesting to note that in the case of both freezing and thawing processes, hypotheses regarding injury mechanisms are formed on the basis of observations made on the specimen prior to and subsequent to, but never during, these processes. There is virtually no information available about the dynamics of these processes. On the basis of such limited information, it is not surprising,

then, that conflicts and contradictions exist among the various investigators regarding the mechanisms of injury.

Light microscopy offers a very effective means for resolving these differences, but its application to this problem is not a trivial matter. Nei,[63] Luyet and Pribor,[100] and Rinfret,[62] have used this method previously, but their tests covered only a small range of temperatures and cooling rates. These limitations were due, in part, to the difficulties associated with the control of the heat transfer rates and with the instrumentation of the specimen to obtain accurate measurements of rapidly changing temperatures. In spite of these limitations, the results of these early experiments are impressive and indicate that more extensive optical studies are warranted.

Any system that attempts to overcome the limitations of these early efforts should satisfy the following design requirements. In order to fully test the analytical, kinetic freezing models proposed by Mazur [19] and Ling and Tien,[28] the system should be capable of cooling rates in the range 1°C/min. to 10,000°C/min. and temperatures in the range 310°K to 77°K. Because of the assumptions inherent in these models, the system should be equipped with a control system capable of maintaining these cooling rates constant over the entire range of temperatures.

The control system should also be capable of controlling the final temperature of the cooling process to any desired value in this range. Although no meaningful analytical models exist at present for the thawing process, the system capability for warming rates should be comparable with that for cooling rates. Since these cooling and warming rates will involve events with time scales on the order of milliseconds, the microscope should be fitted with a cinephotographic system capable of several thousand pictures per second. Finally, the microscope itself should be capable of operating in the phase contrast mode so that transparent objects can be more easily identified.

With a system that satisfies these design requirements, it would be possible to expand the time scale of freezing and thawing processes to the extent that phenomena such as the movement of a solid-liquid interface through the test specimen, the nucleation of intracellular and extracellular ice crystals, the threshold cooling rate for the formation of intracellular ice, morphological alterations to the cells and their structural components, and volume changes in the cells would be readily observable. This information is invaluable in the testing of the various freezing models and in the formation of models for the thawing process.

C. Design:

To meet the foregoing design requirements, a cryomicroscope shown schematically in Fig.2.1, has been built. Specifically, a thermodynamic system has been fabricated to fit on the stage of a light microscope. The wide variety of cooling and warming rates are achieved by cooling the system at a constant rate with a steady flow of refrigerant fluid through the device and by simultaneously dissipating electrical energy at a variable rate in a resistance heater immersed in the fluid stream and in thermal communication with the specimen. The maximum cooling rate is achieved essentially by dissipating no electrical energy in the heater while the maximum warming rate is achieved essentially by turning off the refrigerant flow and dissipating electrical energy at a maximum rate consistent with heater integrity.

The specific design of the thermodynamic system is shown schematically in Fig.2.2. The system consists of a stainless steel chamber, 0.4375 in. square, fabricated from sheet 0.020 in. thick, with refrigerant inlet and discharge tubes, 0.250 in. O.D. x 0.019 in. wall, silver soldered in place. The openings in the top and bottom faces of this chamber are fitted with quartz windows 0.007 inches thick and sealed with epoxy. These windows are

actually quartz cover slips cut to fit the chamber. Ordinary glass cover slips were originally used, but their optical quality was found unsatisfactory. A small deflector mounted on the bottom face of the chamber at the inlet tube directs the inlet refrigerant stream against the bottom side of the top window to enhance the convective component of heat transfer. The height of the chamber must be such that the working distance of the light microscope condenser is not exceeded.

The resistance heater consists of a thin, transparent tin oxide coating deposited on the underside of the top quartz window. By varying the thickness of this electrically conductive coating, any desired heater resistance can be achieved. (Pre-coated glass sheet is available for this purpose from Corning Glass Works, Corning, N. Y., but the present material was coated in our laboratory.) The electrical resistance of the heater is typically on the order of one hundred ohms and remains essentially independent of temperature between 310°K and 77°K. Copper electrical leads are attached to the heater with Dynaloy 350 solderable silver paint and connected to a D. C. power source.

The chamber assembly is mounted in a phenolic block attached to a standard Leitz traversing mechanism with two

degrees of freedom in the horizontal plane. (See Fig. 2.3) The low thermal conductivity of the phenolic block serves to thermally isolate the chamber assembly from the body of the microscope. The complete unit is mounted on a standard stage base and attached to a light microscope, Zeiss Universal Model. The microscope is fitted with a long working distance condenser (7 mm), Zeiss Model IV Z/7, equipped for phase contrast microscopy.

The specimen to be viewed can be mounted on the chamber in two ways. It may be placed directly on the top chamber window and then covered with a cover slip of the appropriate thickness, or it may be placed between two cover slips to form a sandwich which is then placed on the top chamber window. The first method is preferred since it minimizes the thermal mass and thermal resistance between the refrigerant and the specimen; however, it does have the disadvantage that it is more difficult to assemble than the latter method. Regardless of which method is used, the top cover slip contains a small pocket etched out with hydroflouric acid and fitted with a copper-constantan thermocouple, 0.001 in. wire diameter. The small diameter of the wires is crucial in order to minimize the

thermal mass and hence, the thermal response time of the thermocouple. The output of this thermocouple, which can be displayed on an oscilloscope, oscillograph, or strip chart recorder, serves as a measure of the bulk average temperature of the specimen.

The specimen is viewed with a phase contrast 63x, 0.90 NA, Neoflaur Zeiss dry objective with a working distance of 0.12 mm. A dry objective was selected in preference to an oil immersion objective in order to minimize the thermal mass of the specimen and to avoid the phase change that would occur in the immersion oil at the lowest temperatures. With this objective, maximum useful total magnification is limited by resolution considerations to approximately 1000x. The objective, chamber assembly, and phase contrast condenser are enclosed in transparent plastic housing in which a slight positive pressure of dry helium gas is maintained in order to prevent the condensation of water vapor on the specimen and to reduce the thermal contact resistance between the specimen and the refrigerant chamber.

The viewing end of the microscope is fitted with an 8x Ramsden type eyepiece and camera such as a 35 mm or 4 in. x 5 in. camera for static studies or a 16 mm motion picture camera for dynamic studies. Two motion picture

cameras have been used on this system:

(1) A Beaulieu Model R16 fitted with 17 mm or 35 mm lenses focused on infinity and

(2) A Wollensak Fastax Model W163269 fitted with a 35 mm lens also focused on infinity.

The Beaulieu camera is used for slow speed work, 8-64 pictures/sec., while the Fastax camera is used for high speed work, 400-2000 pictures/sec.

A magnetic probe is positioned adjacent to the drive sprocket of the Fastax camera so that it produces a voltage pulse as each tooth of the sprocket passes.[112] This voltage pulse is amplified and fed into a General Radio type 1191 counter to monitor the number of picture frames exposed during a given filming period. The magnetic probe output can also serve to correlate the events recorded on film with their temperature-time history as obtained from the thermocouple output. To this end, the voltage outputs of the probe and thermocouple can be simultaneously printed with the same time base on a two-channel strip chart recorder. Thus, the temperature of the specimen for a particular frame of the film can be directly determined from the thermocouple output printed in time between the two voltage pulses that identify that frame. An additional time check of the filming speed is provided by a flash lamp located within the Fastax camera housing. The

lamp, which flashes at a rate of 60 Hz, exposes a small portion of the film margin at regular time intervals, thereby producing an accurate reference for measuring the elapsed time between events recorded on the film. Finally, the voltage output of the thermocouple can be used to trigger either movie camera on or off so that predetermined portions of the freezing and thawing processes may be selectively filmed.

In order to meet the demanding illumination requirements of high speed cinephotography, the microscope is fitted with a high pressure Xenon illumination source, Zeiss Model XBO 150. This light source has a mean luminance of 15,000 stilbs and a color temperature of 6,000°K making it ideally suited for high speed photography both in color and in black and white.

A specially designed feedback control system is employed in conjunction with the cryomicroscope to achieve a wide variety of constant cooling and warming rates and storage and thawing temperatures in the cell specimen. (See Fig.2.6) As shown in Fig.2.7, the control system is of the analog type in which a signal generator, inverter, and integrator are used to generate a voltage representative of the desired linear temperature-time profile, be it cooling or warming. This profile is continuously

compared in a summing unit with the amplified thermocouple output, representative of the specimen temperature. If the output from the summing unit is positive, indicating that the specimen temperature is lower than the generated reference temperature, heat input to the specimen is required. If the output is negative, the heat supplied to specimen is reduced to zero. The necessary heater power is obtained by amplifying the summing unit output signal in a power amplifier, (Fig.2.6) with the heater resistance as the load. The various cooling rate profiles are generated by switching to different integrator resistance, R_5, (Fig.2.6) according to a predetermined calibration. The control system is changed from cooling rates to warming rates by activating the temperature rate inverter. The end temperature for warming is set by the value of diode D_1 in the limiter, whereas the final temperature for cooling is determined by reducing the voltage input to the integrator to zero. The magnitude of the input signal to the power amplifier can be changed by adjusting the value of resistor R_8 to produce an effect synonymous with varying the power amplifier gain. The sensitivity of the control system to differences in the specimen and generated reference temperatures is regulated by varying the value of resistor R_6.

A photograph of the cryomicroscope as described above is shown in Fig. 2.4.

D. Operation:

To operate the cryomicroscope, the specimen is mounted on the freezing and thawing stage by means of one of the two previously indicated methods. The analog controller is set to a desired initial temperature, e.g. 37°C, by switching to the appropriate diode combination in the limiter. The controller is set for a thaw process while the refrigerant flow is increased to the desired maximum value. The action of the controller-heater combination will maintain the specimen at the desired initial temperature. The cooling rate is preprogrammed by selecting the proper value for resistor R_5 in the integrator. The temperature rate inverter is then activated and the specimen will cool at the selected rate. The base temperature for the cooling process is determined by switching off the voltage input to the integrator when the desired minimum specimen temperature is reached. The specimen is thawed simply by setting the upper limit on the temperature and the warming rate as before and activating the temperature rate inverter.

For both the freezing and thawing processes, film records and temperature-time histories are obtained simply by activating the necessary apparatus at the proper time.

E. Applications:

This unique apparatus now enables the cryobiologist to study the dynamics of the freezing and thawing processes, not only in real time but in dilated time as well. From the film records of these processes, the propagation velocity of the liquid-solid interface through the specimen can be evaluated, volume changes and rates of volume change can be measured, and morphological changes associated with these thermodynamic processes can be determined. With the aid of polarized light, intercellular and extracellular ice crystals can be detected. Finally, since cooling and warming rates can be precisely controlled in this apparatus, it is now possible to experimentally determine the effect these parameters play in freezing and thawing injury.

F. Limitations:

While it is clear that the cryomicroscope has opened a new avenue of research for the cryobiologist, it would be naive to presume that such an apparatus is without its limitations.

For example, the thermocouple output is not linear with temperature. Thus when this output is read into the analog control system in its present form, the net result is a temperature-time profile which is non-linear in temperature but linear in thermocouple c.m.f. The magnitude of this non-linearity is approximately 5 per cent over the temperature range 310°K to 77°K, but can be reduced to zero by using a preprogramed temperature-time profile that compensates for the non-linearity. Such a record could be generated on an analog computer and recorded on magnetic tape for subsequent input to the control system. The thermocouple is an additional source of error in that it does not detect the temperature of an individual cell but rather the average temperature of a cell suspension. This situation is further complicated by the fact that the method of cooling and warming the specimen in this system results in a temperature gradient in the specimen. The magnitude of this gradient depends upon the heat transfer rate and can be quite large; however, the specimen is so thin (10-50μ) that the actual temperature differences across the specimen are essentially zero. (See Appendix I) In its present state of development, it is not possible to measure intracellular temperature gradients

with this system; however, this limitation is not unique to this apparatus. In addition, the supercooling that occurs during freezing can be both substantial and random, but there is no method for controlling it. Whether this effect results in misleading data remains to be seen.

The experimental procedure required by the cryomicroscope places rather severe restrictions on the types of viability tests that can be employed. Any test requiring a significant volume of cells is clearly unsuitable due to the size of the specimen. Also, the specimen is often destroyed during the retrieval process, prohibiting the use of post-thaw viability tests. For this reason, it has been necessary to depend heavily upon visual indications of cell viability. It is quite possible that the method of specimen preparation between cover slips subjects the cells to strong surface forces that might cause results to be misleading. The thickness of the cell suspensions is such that individual cells might also experience strong interactions with solid surfaces.

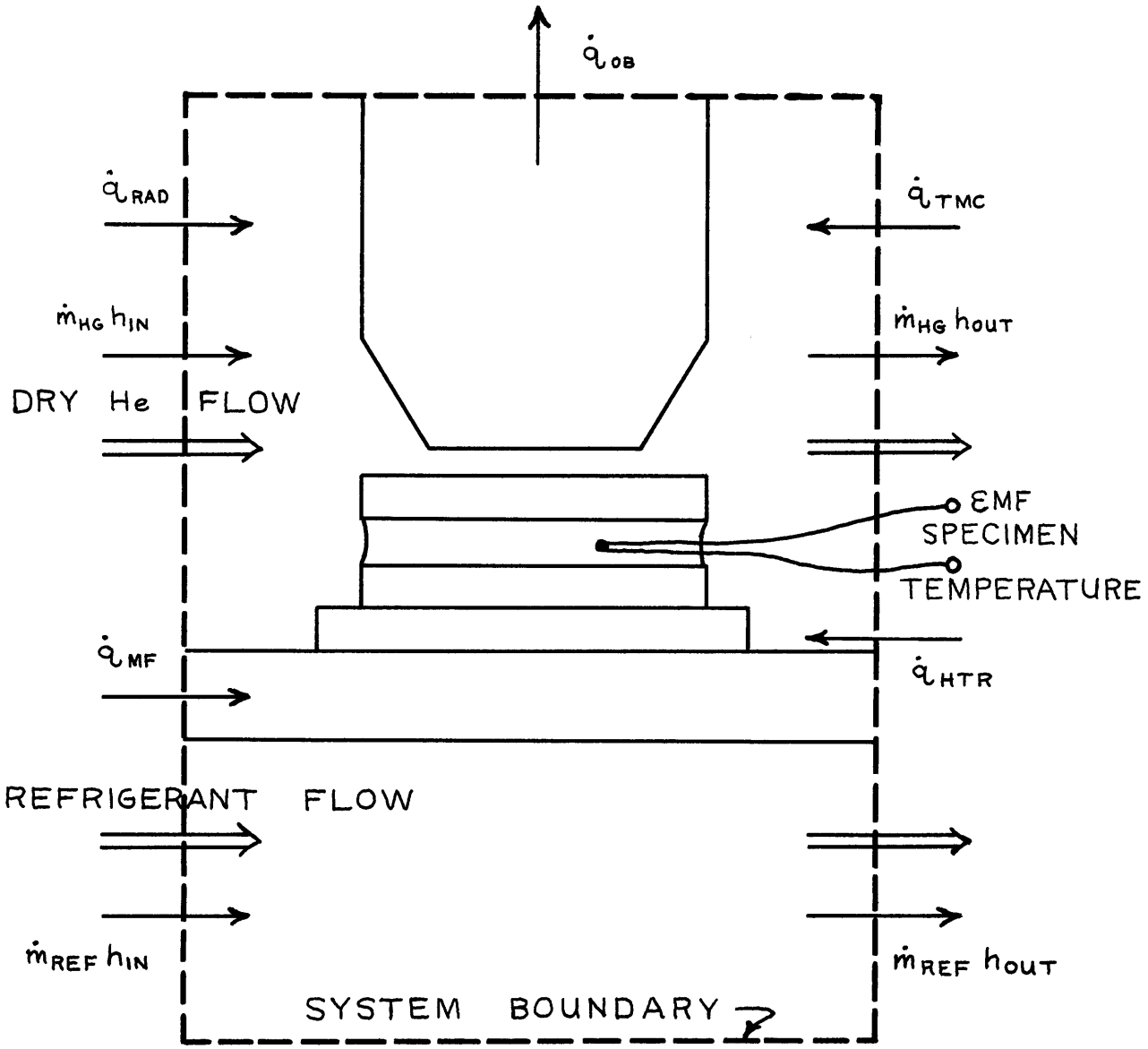
The ability to visually monitor the condition of cellular ultrastructure during freezing and thawing is limited by the resolution of the microscope. However, this limitation is not unique to the cryomicroscope since at present there exists no other instrument which can provide a dynamic record of changes in the ultrastructure.

Finally, meaningful quantitative data can be obtained with the cryomicroscope only at the expense of considerable care on the part of the experimenter; however, this situation improves with the refinement of technique and improvements in apparatus design.

APPENDIX D

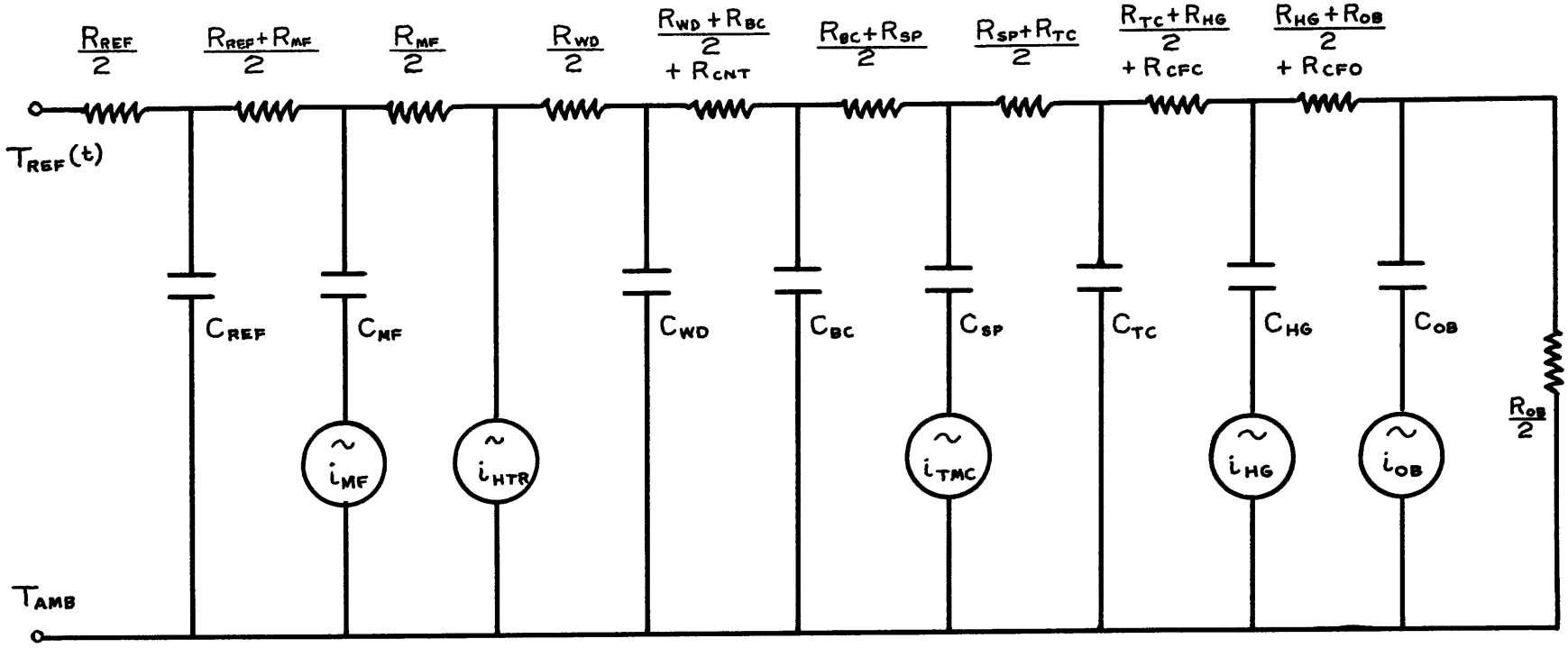
Heat Transfer Analysis of Low Temperature Stage

The low temperature system has been modeled by the method of electrical analogy. In Figure D.1 the boundary of the thermodynamic system is defined and the appropriate heat transfer and mass transfer fluxes across the boundary are identified. The analogous electrical network is presented in Figure D.2 in which temperature corresponds to voltage potential, thermal conductivity to the recipricol of electrical resistance, thermal heat capacity to electrical capacitance, and a heat source to a current source. It is assumed that initially the entire system is at a uniform temperature T_o, analogous to electrical grounding to a potential V_o, that all heat fluxes are one dimensional, that radiation heat transfer is negligible, that the distributed parameter thermal system can be modeled as a lumped parameter electrical system, and that all properties are independent of temperature. Numerical values can be calculated for each of the components of the model by employing standard methods of heat transfer analysis in conjunction with known performance parameters of the system. After extensive calculations, the values shown in



SUBSCRIPTS ARE DEFINED IN FIGURE D.3

Figure D.1. Definition of the Thermodynamic System for the Low Temperature Stage



SUBSCRIPTS ARE DEFINED IN FIGURE D.3

Figure D.2. Analogous Electrical Circuit for Heat Transfer Model of the Low Temperature Stage

Figure D.3 were determined for the resistances and capacitances defined in Figure D.2. The electrical network can now be simplified by neglecting relatively small values of the R's and C's and lumping together resistances connected directly in series. This revised model is shown in Figure D.4 in which the newly defined components are identified.

Numerical solutions for this model will be obtained by analysis on the digital computer in order to evaluate the system design and to determine optimum thermal performance characteristics. A preliminary solution has been obtained by Mr. John McGrath for the case in which no heat is dissipated in the electrical resistance heater and the influence of the microscope objective is removed. The specimen thermal history predicted by the model is compared in Figure D.5 with the experimentally measured thermal history for a given refrigerant flow rate. Based on these early indications, it appears that this method of analysis will provide a valid technique for determining modifications to improve the system design.

COMPONENT	CAPACITANCE (BTU/ft².C°)		HEAT FLUX (BTU/hr)		RESISTANCE (hr.ft².C°/BTU)	
Bottom Coverslip	C_{BC}	1.2×10^{-2}			R_{BC}	8.15×10^{-4}
Convection Film Coverslip					R_{CFC}	1.45×10^{-2}
Convection Film Objective					R_{CFO}	
Surface Contact					R_{CNT}	
Helium Gas	C_{HG}	2.24×10^{-2}	i_{HG}		R_{HG}	
Electric Heater			i_{HTR}	VARIABLE		
Manifold	C_{MF}		i_{MF}		R_{MF}	
Objective	C_{OB}		i_{OB}		R_{OB}	
Quartz Window	C_{QW}	1.2×10^{-2}			R_{QW}	8.15×10^{-4}
Refrigerant	C_{REF}	5.76×10^{-4}			R_{REF}	1.07×10^{-2}
Specimen	C_{SP}	9.51×10^{-3}			R_{SP}	7.3×10^{-5}
Top Coverslip	C_{TC}	1.2×10^{-2}			R_{TC}	8.15×10^{-4}
Thermocouple	C_{TMC}		i_{TMC}	NEGLECT		

Figure D.3. Compilation of Numerical Values of Components in the Analogous Electrical Circuit

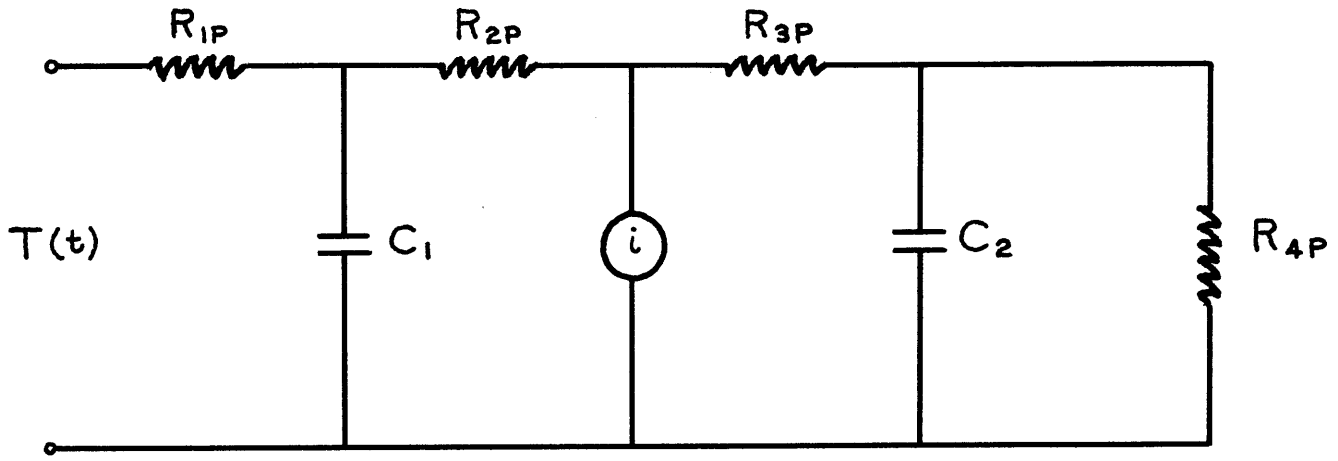


Figure D.4. Simplified Network for Analogous Electrical Circuit

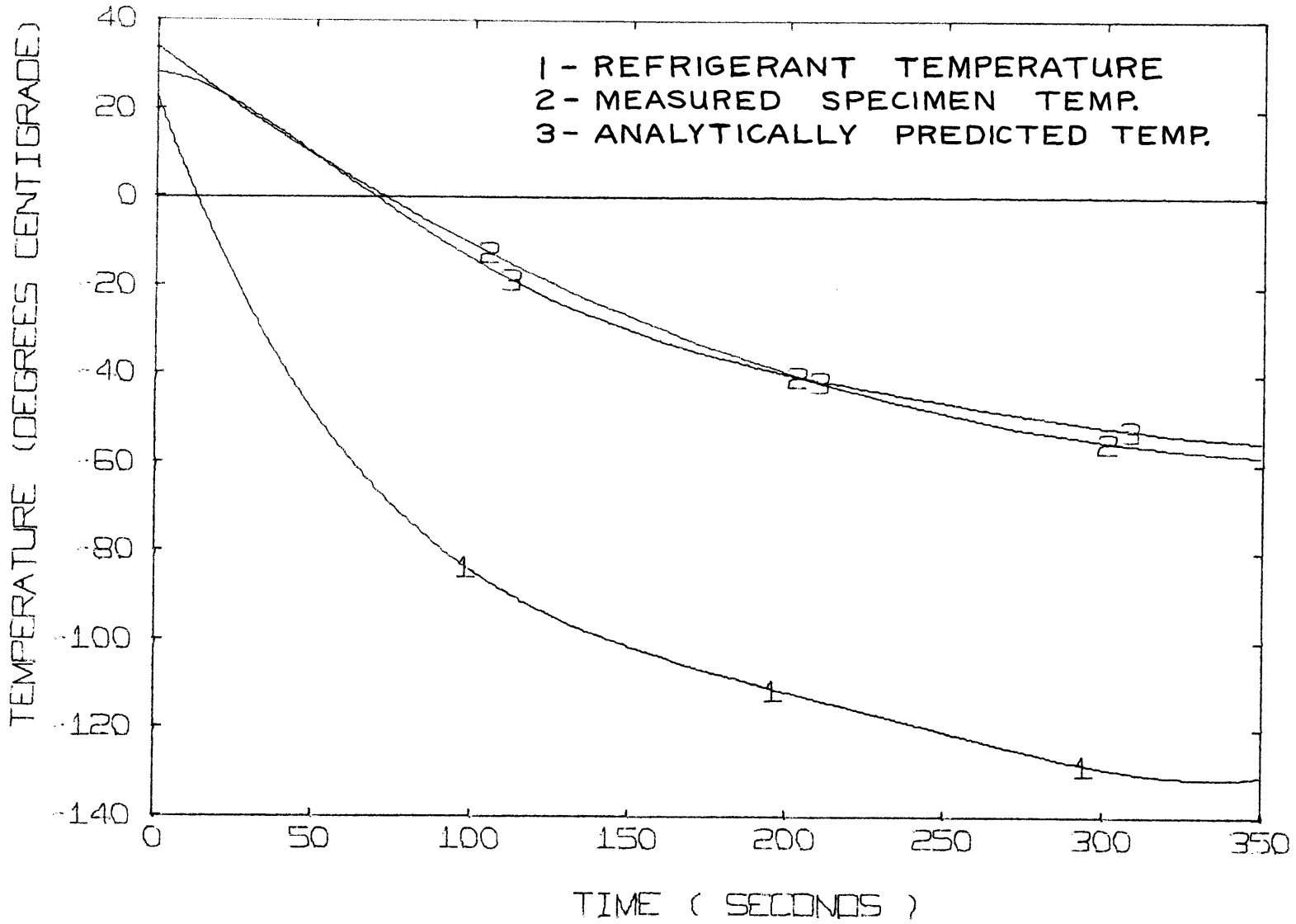


Figure D.5. Numerical Solution to Heat Transfer Model of Specimen Thermal History

APPENDIX E

Process for Depositing an Electrically Conductive Tin Oxide Film on Glass

1. Spray mixture preparation: dissolve 17 gm Stannic Chloride Pentadihydrate and 0.5 Antimony Trichloride in 34 gm Acetone.

2. Heat glass in an oven to 1100°F.

3. A Paasche H "3 in 1" airbrush* under 20 P.S.I. dry nitrogen is used to spray the mixture onto the glass in the oven for approximately 10 seconds to produce a resistance of about 500 ohms per square.

4. After spraying, glass can be immediately cooled to room temperature.

NOTE: (a) The spray mixture can be kept in a glass container after preparation until the mixture turns yellow, at which time it becomes explosive. This condition is reached after about 1 week. After

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* Paasche Airbrush Company, Chicago 14, Ill.

the mixture turns yellow, it should be highly diluted with water and washed down a drain.

(b) All contact of the spray mixture with metal should be avoided because it is very corrosive.

(c) The airbrush should be washed thoroughly with soapy water immediately after spraying.

(d) The glass temperature must be kept close to 1100°F during the spraying process for best results. This may require that the spraying be interrupted several times so that the glass can be reheated.

(e) Suggested product for making contacts: Dynalloy 350 Solderable Silver Epoxy, 3 oz. can @ $10.00, Dynalloy, Inc., 7 Great Meadow Lane, Hanover, New Jersey.

APPENDIX F

Consequences of Rapid Changes in Temperature on the Performance of a Microscope Objective

Summary

A Bausch and Lomb 40x, 0.65 n.a. microscope objective (weighing 53.3 gm.) was subjected to rapid cooling rates to determine whether the resulting thermal stresses affected the performance capability of the objective. In the most severe test performed, a high velocity stream of liquid nitrogen (at -196°C) was sprayed directly onto the objective. Experimental results indicate that an objective can be satisfactorily employed with no damage under such rigerous thermal conditions, as in the examination of a specimen frozen to -196°C on a cryomicroscope.

Experimental Methods and Equipment

The microscope objective was thermally stressed by two separate techniques. For the first method the objective was rapidly lowered to a preset working distance from a liquid nitrogen cooled plate and the

initial temperature-time curve and the final equilibrium temperature of the objective were simultaneously measured and recorded (see FigureF.1). The temperature of the objective was measured by a copper-constantan thermocouple held to the tip of the objective by silver base paint. The thermocouple voltage was measured on a Leeds and Northrup potentiometer at slow rates of temperature change and on a cathode ray oscilloscope for rapid rates ot temperature change. A gaseous helium environment was provided for the cold plate and objective to prevent the accumulation of condensate from air onto the apparatus.

The objective was cooled in the second method by directing a stream of liquid nitrogen from a pressurized dewar vessel onto the objective. Maximum cooling rates were obtained by rapidly opening the valve on the dewar from closed to wide open as quickly as possible. The liquid nitrogen was aimed directly onto the bottom of the objective (see FigureF.2). The transient temperature was measured by a copper-constantan thermocouple and recorded on a cathode ray oscilloscope. The objective was exposed to the liquid nitrogen stream for approximately 3 seconds.

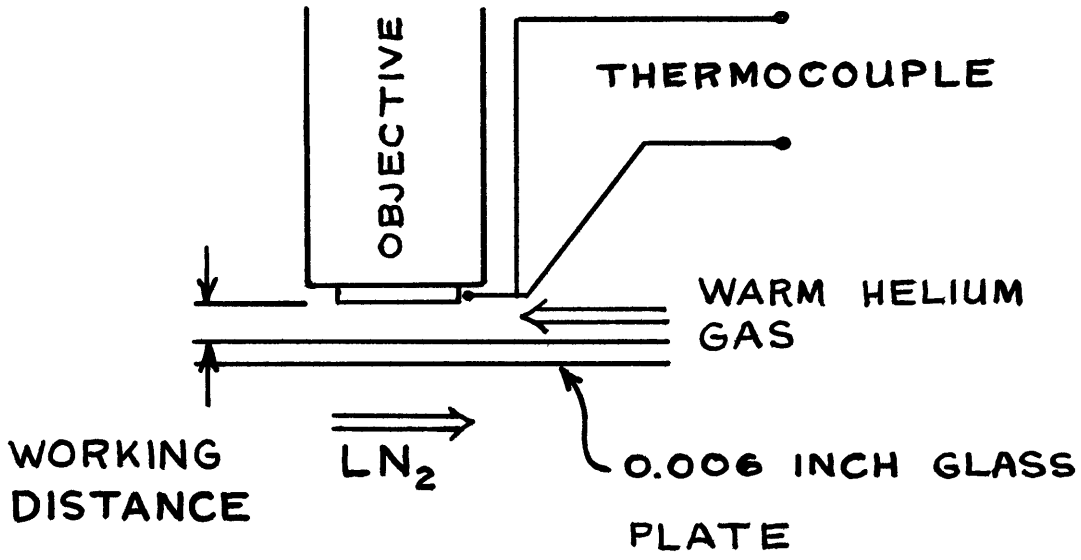

Figure F.1. Apparatus for Measuring Cooling Effects Across a Working Distance on a Microscope Objective

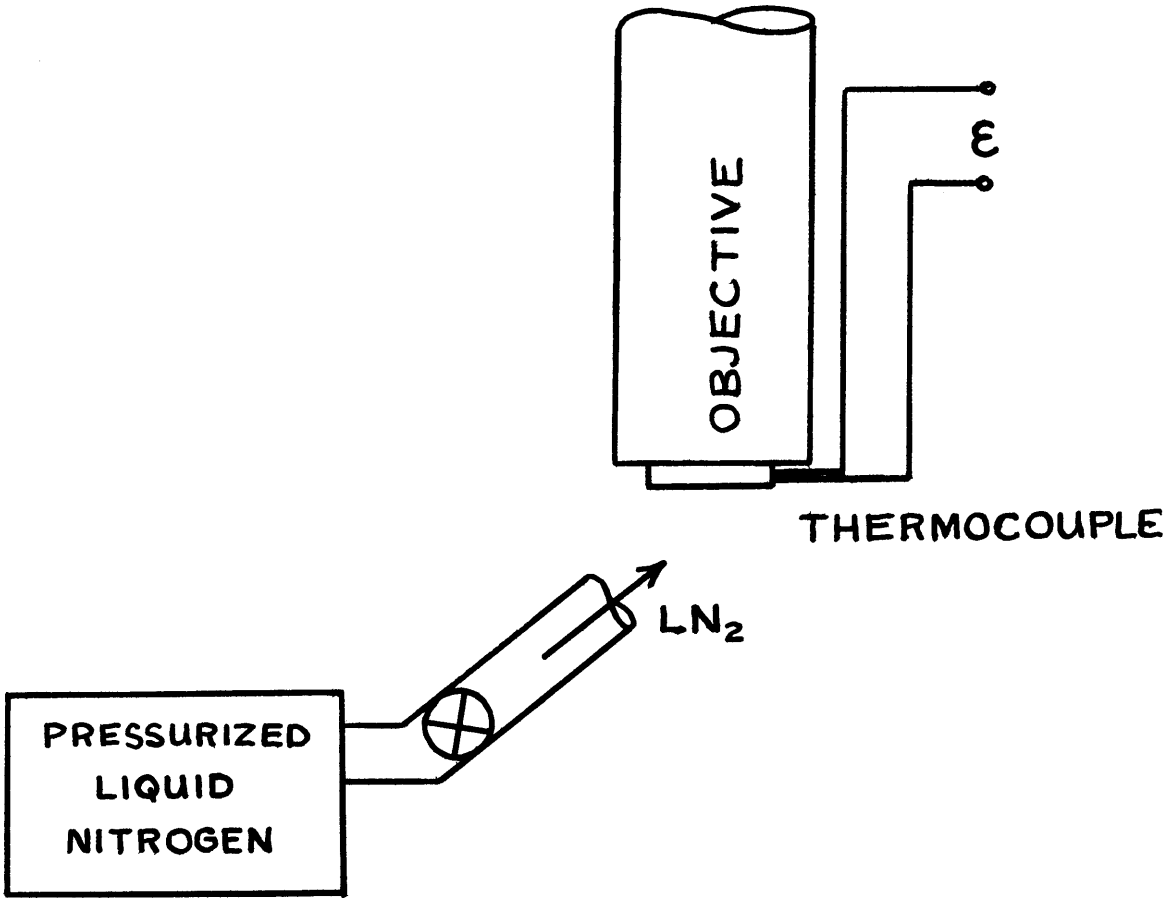

Figure F.2 Apparatus for Quenching a Microscope Objective in a Liquid Nitrogen Stream

Results

The temperature-time curve obtained for a spacing between the cold plate and the objective of 0.005 in. (0.128 m.m.) is shown in Figure 3. The cooling rate was highest when the objective was first moved close to the cold plate, after which it decreased and the temperature approached an equilibrium value. This equilibrium temperature is a function of both the working distance and the flow velocity of helium gas over the objective. As the working distance is made smaller, the equilibrium temperature decreases. When the working distance is held constant an optimum helium velocity exists that maximizes the equilibrium temperature. If the velocity is either raised or lowered from the optimum value the equilibrium temperature will drop.

At the conclusion of the first series of tests the objective was tested for the quality of the image produced. There was no observable change in optical characteristics at this time.

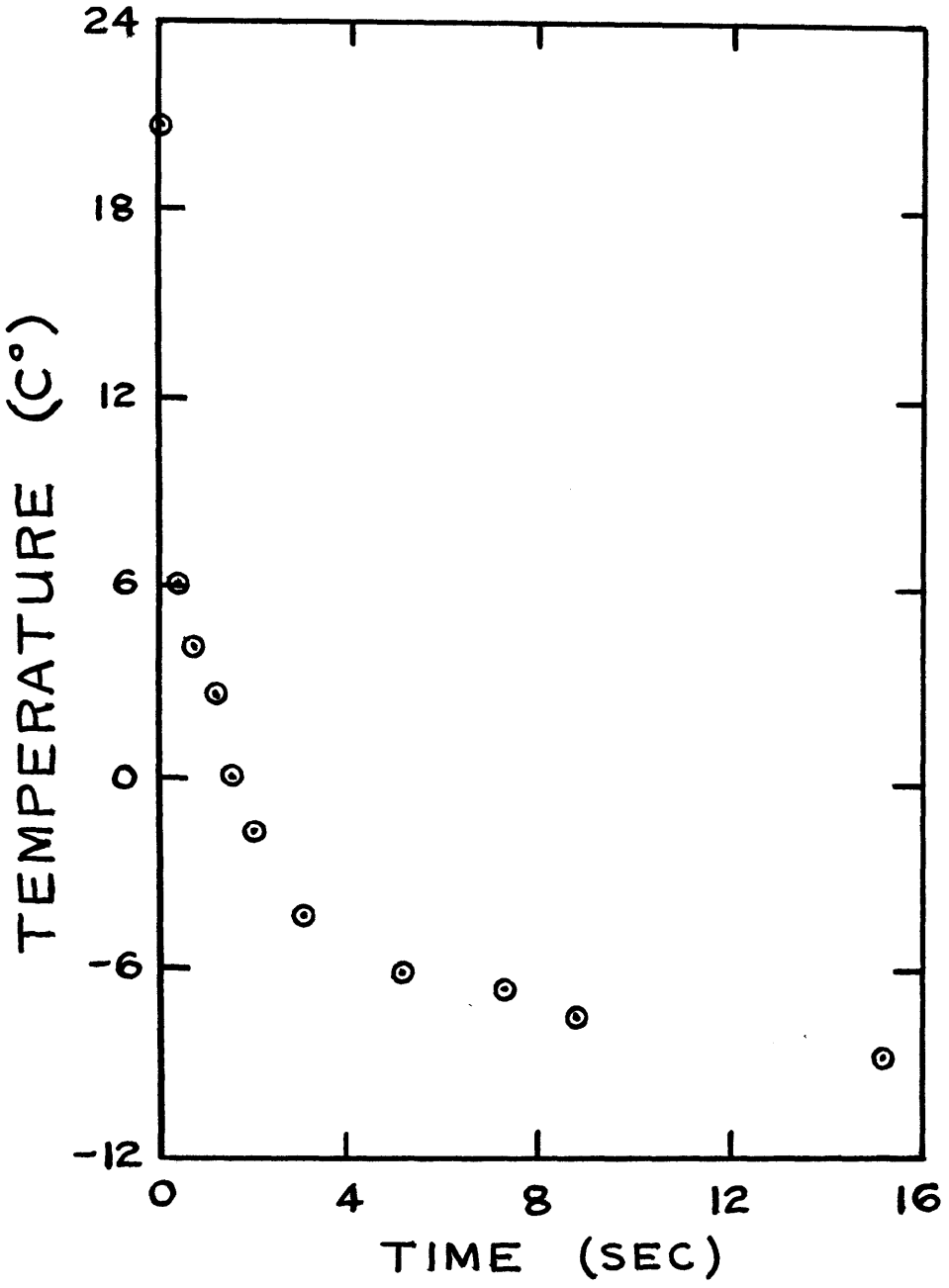

Figure F.3. Thermal History for Microscope Objective Cooled Across a 0.005 inch Working Distance

The temperature-time curve from the second test for the sudden exposure of the objective to a liquid nitrogen stream is shown in Figure F4. A second examination of the objective after this test revealed that no damage had occurred.

Discussion

The purpose of the tests was to determine whether an objective with a 0.12 mm working distance would be damaged during examination of a frozen specimen at 77°K on a cryomicroscope. The first series of tests duplicates the actual operating conditions for an objective on a cryomicroscope. The results indicate that an objective can be safely used in this application provided it is continually warmed by a stream of helium gas.

The second test was conducted to determine what would happen if the low temperature freezing stage (see Figure 2.3) should rupture causing a pressurized stream of liquid nitrogen to be suddenly sprayed directly onto the objective. This test simulated the most

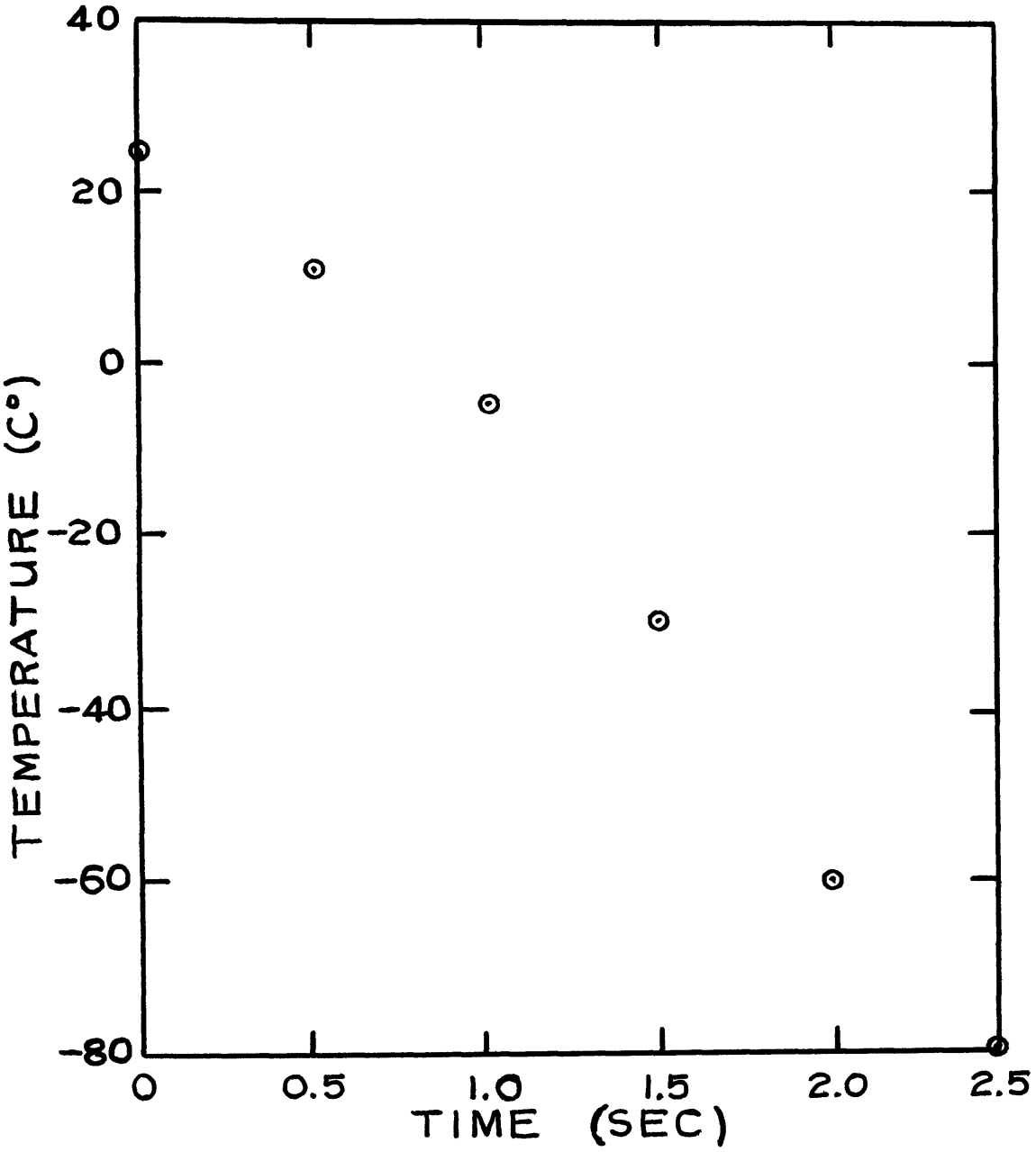

Figure F.4 Thermal History for an Objective Quenched in a Liquid Nitrogen Stream

severe condition of thermal stress that the objective might be required to withstand. The objective was not damaged during this test, and therefore should be suitable for use on a cryomicroscope.

APPENDIX G

An Analytical Model for Thermal Resistance Between the Low Temperature Chamber and the Specimen Coverslip

A resistance to heat flow exists at the interface between the top of the low temperature chamber and the adjacent specimen coverslip. An illustration of the microscopic geometry representative of the substrate surfaces at this interface is shown in Figure G.1. Materials a and c represent respectively the low temperature chamber window and the coverslip, whereas b is the interstitial medium between substrates a and c. Figure G.1 illustrates that the macroscopically smooth surface of a and c are actually quite rough and randomly shaped when viewed at high magnification. Consequently, when the two surfaces are pressed together, contact is made at only a few discrete points, which compose a very small percentage of the total surface area. Nonetheless, a significant portion of the heat flow between a and c is directed through these small contact areas because the thermal resistance of the solid substrates is in general much lower than that of the interstitial fluid. It will be assumed that the value of the parameters governing heat flow

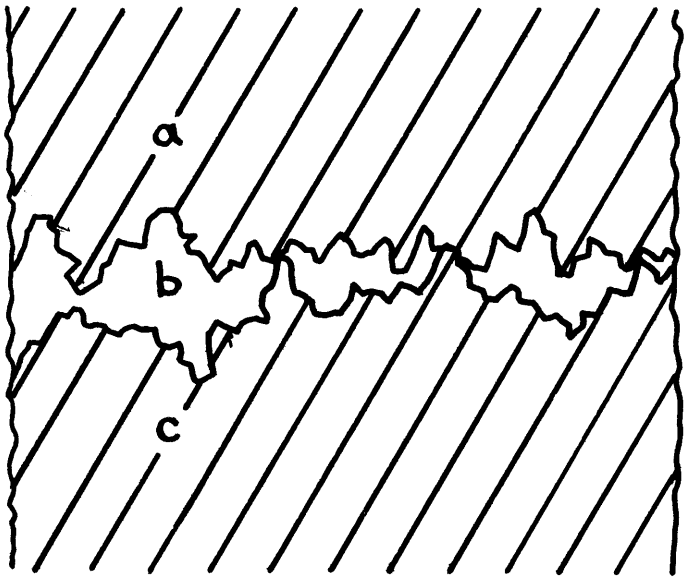

Figure G.1. Microscopic Representation of the Interface Between Two Adjacent Solid Surfaces

through the areas of solid to solid contact are constrained by the design of the system, and that the heat transfer can be enhanced only by varying properties of the interstitial medium b. The achievement of optimum system performance requires that the thermal relaxation time of b for changes in substrate temperature be minimized. Alternatively stated, it is desirable to have the specimen temperature respond as rapidly to variations in the rate of heat input or removal from the low temperature chamber as is dictated by the temperature control system. It is assumed that there is no relative motion between a, b, and c, that the medium b can be modeled as a one dimensional system along the x coordinate, and that heat transfer by natural convection and radial conduction can be neglected. An electrical analogy can be drawn to represent the transmission of heat between the substrates a and c. The circuit diagram is shown in Figure G.2. The thermal equivalents in this circuit are as follows: R_1 and R_3 are the interfacial resistances to heat transfer between the two substrates and the interstitial fluid, R_2 the resistance to heat conduction through b, R_4 the resistance to heat conduction through the areas

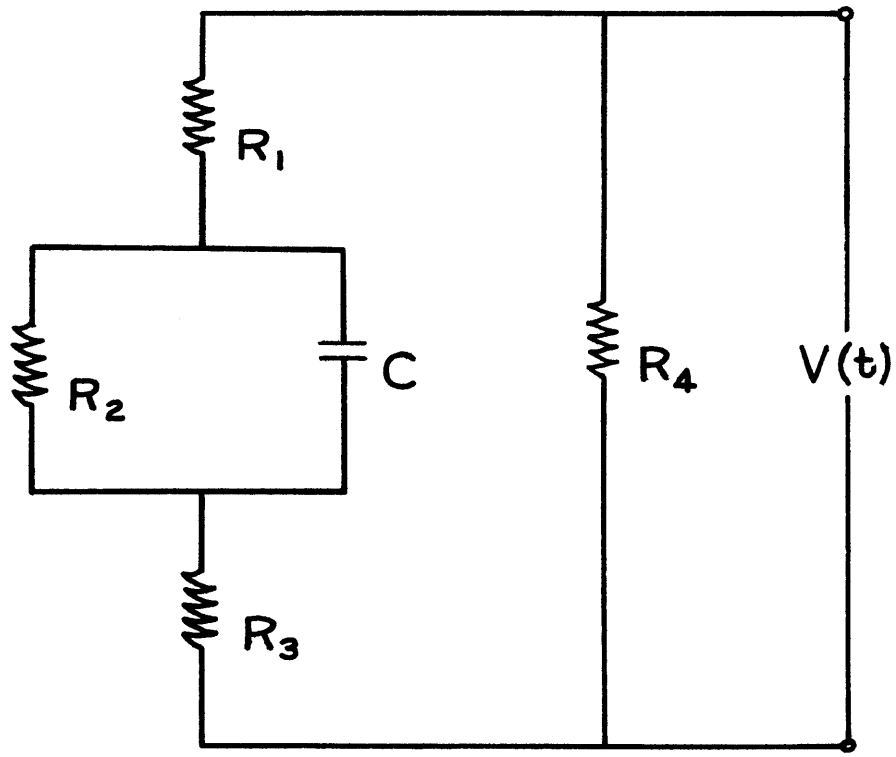

Figure G.2. Analogous Electrical Circuit for Thermal Contact Resistance

of direct contact between a and c, capacity of b, and V(t) the temperature difference between the substrates, which varies as a function of time. R_4 has been assumed to be fixed, and therefore only the parallel heat flux through the interstitial R-C circuit will be considered. The thermal function of R_2 and C is described by the one dimensional differential equation for the transient conduction of heat,

$$\frac{\partial^2 T}{\partial x^2} = \frac{1}{\underline{K}} \frac{\partial T}{\partial t} \qquad \text{(G.1)}$$

where $\underline{K} = \frac{K}{\rho c_p}$ = thermal diffusivity. By inspection of equation (G.1), it is seen that for any given alteration in the boundary temperatures, the transient response time of medium b is reduced as the thermal diffusivity becomes larger. A list of the thermal diffusivity at $0^{\circ}F$ for a number of possible interstitial media is presented in Figure G.3. It is apparent that helium gas is the most effective of these materials for transient heat conduction at $0^{\circ}F$.

MATERIAL	K (ft^2/hr)
GASES -	
CO_2	0.358
CO	0.707
O_2	0.708
N_2	0.719
AIR	0.720
H_2	5.21
He	5.91
LIQUIDS -	
$FREON_{12}$	2.16
H_2O	5.07
NH_3	7.03

Figure G.3. Thermal Diffusivity at $0^{\circ}C$ (from Kreith,F.,"Principles of Heat Transfer", International Textbook Company, Scranton, Pa., 1958.)

Next, the effects of resistances R_1 and R_3 will be considered. For any given substrate temperatures, the magnitude of R_1 and R_3 effectively determine the boundary temperatures for equation (G.1). Jakob[114] has described the phenomena of thermal interfacial resistance between liquids and gases and metal substrates. Very little numerical data is available describing this complicated phenomenon. In general the resistance of gases is much greater than that of liquids, although this effect has not been quantified as it relates to the present problem. A conclusion that can be drawn from Jakob's discussion is that the surface resistance is heavily dependent upon the thermal conductivity of the fluid immediately adjacent to the solid substrate. However, this effect has been included in resistance R_2 in the definition of this model. It will therefore be assumed that the surface contact resistance does not significantly alter the boundary temperatures for equation (G.1) as a function of the medium between the solid substrates. Thus, based upon the preceding analysis, gaseous helium may be effectively employed as a heat

transfer medium between the low temperature chamber and the specimen coverslips for the temperature range of application.

APPENDIX H

Analysis of Refrigerant Heat Exchanger Design

Cool, dry helium gas is employed as the refrigerant for the low temperature stage of the cryomicroscope. The source of helium is a pressure regulated, compressed gas cylinder at room temperature. Refrigeration is effected by forcing gas from this cylinder through a liquid nitrogen bath heat exchanger, from which it is introduced directly into the low temperature stage. This appendix will present the specifications and operation criteria for the heat exchanger.

A photograph of the refrigerant heat exchanger is shown in Figure 2.5. It consists of a folded array of copper tubing placed within an insulated stainless steel container. Six identical copper tubes, having 1/8 inch inside diameter and 1/16 inch wall thickness, are connected in parallel between the inlet and outlet manifolds such that each tube has 15 straight sections, 11 inches long, and 14 180^{o} bends of ½ inch radius (for a total length L=187 inches). The exterior dimensions of the liquid nitrogen container are 8 inches width, 13½

inches length, and 11½ inches height. This entire unit is surrounded by a 2 inch layer of expanded silica insulation. The boil off time for a full container of liquid nitrogen with no helium gas flow is 25 hours.

The number of transfer units (NTU) can be calculated to determine the effective heat transfer size of the exchanger, according to the definition presented by Kays and London[115]

$$NTU = \frac{A U_{AVG}}{C_{MIN}} = \frac{1}{C_{MIN}} \int_0^A U \, dA \qquad (H.1)$$

where U = overall conductance for heat transfer
A = the transfer area used in the definition of U
C = $\dot{m}c_p$ = minimum capacity rate of the two exchanger fluids (helium gas in this instance)

U will be calculated for heat transfer at the helium-tube interface, and A is therefore the internal surface area of the copper tubing. The overall heat conductance between the liquid nitrogen and the gaseous helium is assumed to be composed of three terms, due to the film coefficients at the inner and outer surfaces of the tube and conduction through the tube wall

$$\frac{1}{U} = \frac{1}{h_{He}} + \frac{1}{\frac{A_{LN_2}}{A_{He}} h_{LN_2}} + \frac{a}{\frac{A_{Cu}}{A_{He}} K_{Cu}} \qquad (H.2)$$

where a is the wall thickness. It is additionally assumed that U is constant across the heat transfer area of the exchanger. Numerical values for the terms in equation (H.1) are obtained as follows.

The maximum volumetric flow of helium through the system was measured to be G = 180 ft^3/hr, corresponding to a gas bottle regulator pressure of 15 psi. The density of helium gas at 70°F and 1 atmosphere of pressure is $\rho = 1.01 \times 10^{-2} lbm/ft^3$. The corresponding mass flow rate of helium is then

$$\dot{m} = \rho G = 1.01 \times 10^{-2} lbm/ft^3 \times 180\ ft^3/hr = 3.03\ lbm/hr$$

The specific heat of helium gas at 70°F and 1 atmosphere is c_p = 1.24 BTU/lbm - R°, and is nearly invarriant with temperature. The capacity rate for helium gas is determined to be

$$C = \dot{m} c_p = 3.03\ lbm/hr \times 1.24\ BTU/lbm - R^o = 3.76\ \frac{BTU}{hr\text{-}R^o}$$

The heat transfer area on the inside of the exchanger tubes is given by

$$A = \pi DL = \frac{\pi \times 1.25 \times 10^{-1}\ in \times 6 \times 1.87 \times 10^{2}\ in}{1.44 \times 10^{2}\ in^2/ft^2} = 3.06\ ft^2$$

The film coefficient for heat transfer on the liquid nitrogen side of the tubing can be evaluated if q/A is known. The measured outlet temperature of the helium gas at 180 ft^3/hr flow rate is $T_{He\text{-}OUT}=-160^{\circ}C$. Thus, q/A is determined according to the relation

$$q/A = C(T_{He\text{-}IN} - T_{He\text{-}OUT})/A$$

$$= 3.76 \frac{BTU}{hr\text{-}R^{\circ}} (530^{\circ}R - 203^{\circ}R)/3.06\, ft^2 = 400 \frac{BTU}{hr\text{-}ft^2}$$

Next, the temperature difference between the boiling liquid nitrogen and the outer tube wall is noted to be $3.25^{\circ}R$ from the curve of heat flux (q/A) versus temperature difference for steady state pool boiling.[116] Thus, the film coefficient is given by

$$h_{LN_2} = \frac{4.0 \times 10^2\ BTU/ft^2 \cdot hr}{3.25^{\circ}R} = 1.23 \times 10^2 \frac{BTU}{ft^2 \cdot hr \cdot R^{\circ}}$$

The Reynolds number for helium gas flow through the tubing must be determined in order to evaluate h_{He}.

$$Re_y = \frac{\rho V D}{\mu} = \frac{4 \rho G}{\pi D \mu}$$

Rey = 7.61×10^2, which is in the laminar flow regime. Rohsenow[117] gives a Nusselt number correlation for laminar flow of gas inside of tubes having uniform wall temperature.

$$Nu = \frac{hD}{K} = 3.66$$

It follows that

$$h_{He} = \frac{3.66K}{D} = 3.08 \times 10^1 \frac{BTU}{hr\text{-}ft^2\text{-}R^\circ}$$

An average value for the thermal conductivity of copper is evaluated at temperature $T = \frac{T_{LN_2} + T_{He\text{-}IN}}{2}$ such that

$$Kcu = 2.39 \times 10^2 \text{ BTU/hr - ft - } R^\circ$$

The effective heat transfer areas for Kcu and h_{LN_2} are taken as 1.5 A_{He} and 2.0 A_{He}, respectively. Substitution of the appropriate terms into equation (H.2) yields a numerical value for U.

$$U = \frac{1}{3.08\times10^{1}} + \frac{1}{2\times1.23\times10^{2}} + \frac{6.25\times10^{-2}}{1.5\times1.2\times10^{1}\times2.39\times10^{2}}$$

$$= 2.74\times10^{1}\ \frac{BTU}{hr\cdot ft^{2}\cdot R^{\circ}}$$

The NTU rating of the exchanger is now determined from equation (H.1)

$$NTU = \frac{AU}{C} = 22.3$$

The heat transfer effectiveness of an exchanger is defined by Kays and London[115] as

$$\varepsilon = \frac{C_h}{C_{MIN}} \frac{(T_{h\text{-}IN} - T_{h\text{-}OUT})}{(T_{h\text{-}IN} - T_{c\text{-}OUT})} \qquad (H.3)$$

In this application equation (H.3) becomes

$$\varepsilon = \frac{T_{He\text{-}IN} - T_{He\text{-}OUT}}{T_{He\text{-}IN} - T_{LN_2}} = \frac{530-203}{530-139} = 0.836$$

APPENDIX I

Analysis of the Vertical Temperature Gradient Across the Specimen

The maximum vertical temperature difference across the specimen can be analytically determined as a function of the blood film thickness and the cooling rate. A model of the thermodynamic system under consideration is illustrated in Figure I.1. ℓ is the specimen thickness, which is assumed to be (1) uniform across the entire area, A, and (2) much smaller in magnitude than A ($\ell \ll A$). It follows that only one dimensional variations (as a function of position along the x coordinate) in the system thermodynamic properties need be considered. The convective and radiative components of heat transmission may be disregarded in this system, so that the temperature distribution in the specimen is described by the diffusion equation,

$$\frac{\partial \bar{T}}{\partial t} = K \frac{\partial^2 \bar{T}}{\partial x^2}, \quad (0 < x < \ell) \qquad (I.1)$$

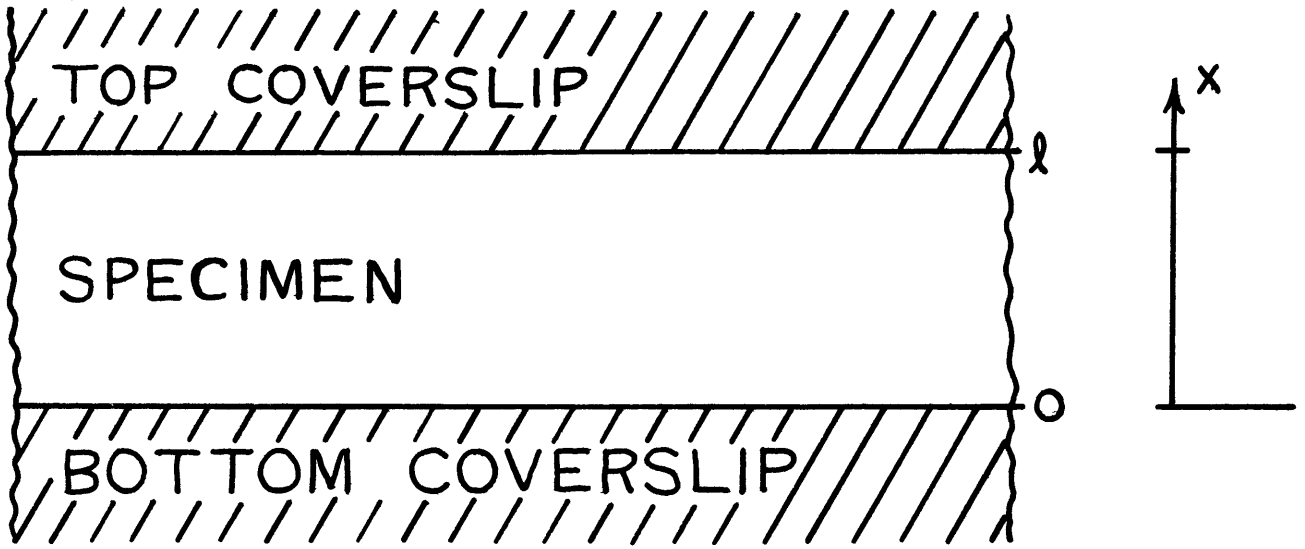

Figure I.1. Film of Blood Prepared Between Coverslips

where $\bar{K} = \frac{K}{\rho c_p}$ is the thermal diffusivity and $\bar{T}$ is defined by

$$\bar{T} = T - T_i$$

and T_i is initial, uniform temperature of the specimen. It is also assumed that the temperature at surface $x = \ell$ is changing at a constant rate and surface $x = o$ is insulated. Thus, the boundary and initial conditions which describe this problem are,

$$\begin{aligned} \bar{T} &= 0 , & t &= 0 \\ \bar{T} &= Bt , & x &= \ell \\ \frac{\partial \bar{T}}{\partial x} &= 0 , & x &= 0 \end{aligned} \qquad \text{(I.2)}$$

where B is the rate of temperature change. The general solution to this problem is given in Carslaw and Jaeger[118] as

$$\bar{T} = Bt + \frac{B(x^2 - \ell^2)}{2K} + \frac{16B\ell^2}{K\pi^3} \sum_{n=0}^{\infty} \frac{(-1)^n}{(2n+1)^3} e^{-\frac{K(2n+1)^2\pi^2 t}{4\ell^2}} \cdot \cos \frac{(2n+1)\pi x}{2\ell} \qquad \text{(I.3)}$$

The temperature difference across the specimen can be expressed in the form of equation (I.3) as

$$\Delta\bar{T} = \bar{T}_{x=\ell} - \bar{T}_{x=0}$$

$$= Bt - Bt + \frac{B\ell^2}{2K}$$

$$- \frac{16B\ell^2}{K\pi^3}\sum_{n=0}^{\infty}\frac{(-1)^n}{(2n+1)^3}\, e^{-\frac{K(2n+1)^2\pi^2 t}{4\ell^2}} \qquad \text{(I.4)}$$

$$= \frac{B\ell^2}{K}\left[\frac{1}{2} - \frac{16}{\pi^3}\left(e^{-\frac{K\pi^2 t}{4\ell^2}} - \frac{e^{-\frac{9K\pi^2 t}{4\ell^2}}}{27}\right.\right.$$

$$\left.\left. + \frac{e^{-\frac{25K\pi^2 t}{4\ell^2}}}{125} - \frac{e^{-\frac{49K\pi^2 t}{4\ell^2}}}{343} + \cdots\right)\right]$$

$$\doteq \frac{B\ell^2}{K}\left[\frac{1}{2} - \frac{16}{\pi^3}\, e^{-\frac{K\pi^2 t}{4\ell^2}}\right]$$

$\Delta\bar{T}$ can be determined from equation (I.4) as a function of the cooling rate (B) and the average specimen temperature (which sets the values of the physical parameter $\bar{K}$). The dependence of $\Delta\bar{T}$ upon these two parameters is illustrated in Figure I.2 which is a compilation of solutions to equation (I.4) for $T_i = 25^{\circ}C$ and $\ell = 20\mu$.

T_{AVG} (C^o)	$\bar{K}$ (ft^2/hr)	B ($^oC/min$)	t (min)	$\Delta\bar{T}$ (C^o)
O_{LIQ}	5.07×10^{-3}	10	2.5	2.5×10^{-4}
		10^4	2.5×10^{-3}	2.5×10^{-1}
		10^6	2.5×10^{-5}	2.0×10^{1}
O_{ICE}	4.80×10^{-2}	10	2.5	2.7×10^{-5}
		10^4	2.5×10^{-3}	2.7×10^{-2}
		10^6	2.5×10^{-5}	2.2

Figure I.2

Vertical Temperature Gradient Across Specimen for

T_i = 25°C and ℓ = 20 μ

Calculations of $\Delta\bar{T}$ based on this model indicate that cooling rates in excess of -50,000°C/min would be required to create a temperature drop of greater than 1°C through the system. It can be concluded that vertical temperature gradients are not a significant source of error for the operation tolerances inherent in the experimental system.

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Let us come into his presence with thanksgiving;
Ps.95:1

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BIOGRAPHICAL SKETCH

The author was born in Wooster, Ohio on November 20, 1942, and attended primary and secondary schools in the neighboring community of Orrville. After graduation from Orrville Senior High School in June, 1960, he attended Oberlin College for two years. In September, 1962, he entered Ohio State University and was awarded the Bachelor of Mechanical Engineering degree, cum laude, in June, 1966 and the Master of Science degree in September, 1967. The title of his thesis was "Design and Calibration of a Low Speed Wind Tunnel". The author's publications include the following:

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