# CRYOPROTECTION OF CHINESE HAMSTER CELLS IN TISSUE CULTURE BY POLYMERS

by

KENNETH WAYNE CONNOR

B.Sc., University of Saskatchewan, 1971

A THESIS SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE
in the Department
of
Biology

ACCEPTED
FACULTY OF GRADUATE STUDIES
[redacted]
DEAN
DATE 7 Sep/73

We accept this thesis as conforming
to the required standard

[redacted]

UNIVERSITY OF VICTORIA

# ABSTRACT

Supervisor: M.J. Ashwood-Smith

The ability of polymers to protect Chinese hamster ovary cells (CHA) from injury due to freezing and thawing was investigated under a variety of conditions. Polymers such as polyvinylpyrrolidone (PVP), hydroxyethyl starch (HES), and dextran all brought about a significant increase in survival, with cryoprotection increasing in the order dextran, PVP, and HES. Slow cooling rates and rapid warming rates were required for the attainment of high survival. Cells frozen in complete medium containing 10% calf serum were found to give higher survival than if they were frozen in a simple salt solution. Inhibition of endocytosis by the use of the drug cytochalasin B had no effect on the survival of PVP-protected cells after freezing and thawing. The addition of PVP to the medium immediately after thawing was found to have no cryoprotective effect.

Micrographs of freeze-substituted CHA cells showed a considerable amount of intracellular ice formation at a cooling rate of 20°C/min. Electron micrographs of cells after thawing showed a general appearance of swelling and rupture of the cytoplasmic organelles and of the cell as a whole. No specific component of the cell appeared to be the target of cryoinjury.

Radioisotope studies on the uptake of $^{14}$C-labelled l-alanine by CHA cells were done and evidence indicated a transport mechanism was involved in this uptake. The presence of PVP in the medium was found to have no effect on the uptake of alanine. The transport mechanism did not show any particular sensitivity to freezing damage since damage to the cells as measured by their decreased ability to incorporate alanine was found to be slightly less extensive than damage as assayed by colony-forming ability.

Several results were obtained which indicated that polymers have an ability to decrease the amount of ice which forms in a solution at any particular temperature and thereby to decrease the concentration of solutes in the unfrozen portion. Direct analysis of the NaCl concentration of the unfrozen portion of solutions by atomic absorption spectrophotometry showed that the amount of increase in salt concentration with decreasing temperature was similar in the presence of 10% PVP K30 or 2.5% dimethyl sulfoxide (DMSO), a low molecular weight cryoprotective agent used for comparison. The freezing point of solutions of PVP was determined with an osmometer. It was found that the freezing point depression was much greater than would be expected on a molar basis, with a dramatic increase in freezing point depression occurring at higher concentrations of polymer. Studies on the liquid water of partially frozen solutions by nuclear magnetic resonance spectroscopy showed that polymers prevented as much water from freezing at -35°C as did the low molecular weight compound DMSO. Although highly structured, the water which remained in the presence of the polymers appeared to be available as a solvent for salt since the presence of salt greatly affected the signal arising from this water.

Results of this study indicate polymers protect cells from injury due to freezing and thawing through the alteration of physical properties of the solution. Evidence obtained suggests these polymers may cryoprotect through their ability to structure water about them and prevent this water from freezing during the cooling process.

[redacted]

## TABLE OF CONTENTS

## LIST OF FIGURES

Figures Page No.

Figures Page No.

# ACKNOWLEDGEMENTS

I wish to express my gratitude to Professor M.J. Ashwood-Smith for his suggestions, criticisms, and continued enthusiasm throughout the course of this work.

I would also like to thank H.F. Dietrich for his advice on various aspects of electron microscopy.

Lastly, I extend my thanks to the Chemistry Department for their co-operation and the free use of their equipment in some areas of this study.

This work was supported by a Medical Research Council of Canada Grant to Professor M.J. Ashwood-Smith.

# INTRODUCTION

Since the process of evolution began millions of years ago, biological systems have been faced with the problem of low temperatures which can cause freezing of the water in these systems. It is difficult to imagine an environmental stress, at least at higher latitudes, more universal than that of being subjected to low temperatures. It is small wonder then that considerable interest has been aroused in the study of the effects of low temperatures on living organisms, the field of study known as cryobiology. Studies in cryobiology yield information as to the nature of freezing damage, the parameters regulating its extent, and possible ways of preventing such damage, as well as information of interest in other fields of biology such as the effect of osmotic changes, pH changes and changes in water structure on cells and cell systems.

Reports of studies concerned with the response of certain organisms to the exposure to freezing conditions date back as far as 1683, when Robert Boyle (16) reported studies on the response of frogs and fish which he allowed to become frozen in ice in a jar. He found they suffered no damage if the length of time in which they were frozen in the ice was relatively short. Reamur (88) found that certain caterpillars survived after being frozen in a salt water mixture at -20°C, but noted that there was always some fluid in these caterpillars if cut open at this temperature, and never found any to recover after freezing throughout.

Scholander *et al* (99) found fish swimming actively in fjords in Labrador in water at a temperature of -1.7°C. The freezing point of the plasmas of these fish was -0.8 to -1.0°C, and Scholander was fascinated by the fact that they could remain active. He found if a crystal of ice was touched to the body of these fish, they rapidly became rigid and died. Supercooling of body fluids seemed tolerable in this and the previously mentioned cases, whereas internal freezing proved fatal.

Several cases of cells and organisms adapting to allow the formation of internal ice are now known. The study of these systems can lead to a much better understanding of the source of freezing damage and methods of preventing it.

A prime example of organisms which undergo periodic freezing is that of certain intertidal mollusks. Kanwisher (41) reports several oysters, mussels, snails and barnacles at Cape Cod were exposed to air temperatures of -15 to -30°C. He found their internal temperatures were essentially the same as that of the air after a few hours, yet they survived upon rewarming. Kanwisher showed that 71% of the body water of the horse mussel, *Modiolus modiolus*, was converted to ice at -22°C. The amazing ability of this mussel, and other intertidal mollusks to withstand these severe conditions is not understood. An explanation of this hardiness could lead to a greater understanding of the process of freeze-thaw damage.

Asahina and Aoki (6) cooled overwintering larvae of the butterfly *Aporia crataegi* adherbal Fruhstorder to -180°C and thawed them without apparent injury. The mechanism which enabled this and other insects to survive freezing in certain stages of their life cycle was unknown until a rash of reports (127, 128, 97) showed a high concentration of glycerol in the stages where the insects passed the winter. A study by Salt (98) showed the concentration of glycerol in the larvae of the wasp *Bracon cephi* rose from negligible in the summer months to 2.8 molal in winter months, and this glycerol apparently allowed them to survive freezing without severe injury.

An example of a plant producing a substance capable of protecting itself from freeze-thaw injury has been extensively investigated by Heber (35). He isolated a protein from frost-hardy spinach plants which was very effective in preventing freeze-thaw damage. Using isolated thylakoid membranes from spinach chloroplasts, Heber found a total loss of the ability of these membranes to produce ATP in the light if they were frozen in salt solution without protective additives. However, in the presence of the protective protein, survival equal to that which can be achieved by glycerol or dimethyl sulfoxide (DMSO) is reached at a concentration of only 0.1% w/v. It is not totally clear, however, that the protein is acting by a mechanism different than that of other non-specific protectors. It has been found (126) that protective agents such as sorbital, polyvinylpyrrolidone (PVP, and hydroxyethyl starch (HES), are also able to protect the membranes, but the concentrations required are greater than those at which spinach protein protects. The mechanism of protection has not yet been determined.

The production of a substance by Chinese hamster ovary cells in tissue culture which aids in survival of the cells after freezing and thawing has been reported by Robinson (92, 93, 94). He found if the cells were frozen and thawed in Hanks balanced salt solution without protective additive, and were subsequently plated into fresh medium there was no cell survival. However, if the cells which had been frozen and thawed were plated into the original medium in which the cells were grown, referred to by Robinson as conditioned medium, one hundred percent survival was attained. Robinson observed that vesicles containing glycoprotein often budded off from the cells following freezing and thawing without protective additives and hypothesized it was this loss of glycoprotein from the cell which caused the damage. He suggested some substance was secreted into the medium during growth which enabled the cells to repair any damage which they had incurred during the process of freezing and thawing.

A further example of freeze-thaw survival being enhanced by placing the cells in a medium in which they can repair the damage they have suffered has been reported by Ray and Speck (86). They found that, following freezing and thawing, about 50% of E. coli NCSM cells were unable to form colonies on Trypticase soy agar containing 0.3% yeast extract, and up to 90% failed to form colonies if the agar contained 0.1% deoxycholate, a surfactant which does not damage normal cells. If, however, the cells were incubated in $K_2HPO_4$ before plating, and increased number of colonies were obtained. The dependence of the increase in colonies on the pH, temperature, and availability of Mg ions led the authors to believe it was an enzymatic repair process. In further studies (87) they showed the repair process was not dependent on protein, nucleic acid, or mucopeptide synthesis. Uncouplers of ATP synthesis did affect the repair process and ATP added to the medium facilitated repair. The authors made no suggestion as to how the ATP brings about the repair process.

Although these approaches to the study of cryobiology can yield an insight into the overall picture of the effect of cold exposure on biological systems, and clues towards the prevention of freezing damage can be obtained from the study of animals which have evolved mechanisms of protection of freezing damage, it seemed the best approach toward gaining an understanding of the freezing process and the mechanism of protection at the cellular level may be to study the effect on survival of varying certain parameters during freezing and thawing under defined laboratory conditions. Using controlled conditions of cooling rate, warming rate, and composition of the suspending medium during the freeze-thaw process, it may be possible to more closely define the changes occurring during freezing, their effect on the cell, and possible ways of altering these effects, and to extrapolate the results to new and more complicated systems.

Applications of the knowledge of the response of cells to freezing and thawing are vast and extremely varied. In the field of cryosurgery regions of tissue are frozen by low-temperature probes with the intent of destroying the tissue rather than removing it by normal surgical means. In blood banks, tissue culture banks, and countless other installations cells are frozen in an attempt to halt all time-dependent alterations which would otherwise occur. Such cells can be preserved in the frozen state for periods up to several years and display their original characteristics upon thawing.

A large part of the work in the field of cryobiology has been towards defining the exact conditions under which the long-term preservation of cells can be achieved, conditions which differ from cell type to cell type. Even though great advancements in cryobiological preservation have been made, most of the discoveries have come through the time-consuming process of the trial of various cryoprotectants, freezing rates, thawing rates, and growth conditions. This is because no universally accepted explanation of the mechanism whereby certain protective agents can protect a cell from freeze-thaw damage has yet been put forward. Without guidelines as to why a particular cryoprotective agent is or is not effective, the search for new and better agents is merely a matter of trial and error. This is especially true of the group of polymeric protective agents, the mechanism of action of which is presently subject to much dispute.

A study was undertaken in an effort to explain the mechanism whereby certain polymers are able to protect nucleated mammalian cells from damage during cooling to liquid nitrogen temperatures. Such information would lead to a more systematic search for new and better cryoprotective polymers.

# REVIEW OF THE LITERATURE

An extensive review of the past advances in cryobiology will not be given at this time since several reviews have already been published (64, 69, 77, 105). Also, a large portion of the studies have been done on aneucleate red cells, which may respond quite differently to a given stress than nucleated mammalian cells. Low molecular weight protective agents have been used in many of the studies which have been published on nucleated cells, the behaviour of which, during the freeze-thaw process, may be quite different than that of polymers. As a result, only some of the major discoveries in the field will be cited in order to give the reader a feeling for the present degree of success in the field of cryobiology.

Reported attempts to preserve spermatozoa date back to 1866 when Mantegazza (62) reported human spermatozoa would survive freezing at -17°C and envisioned the long-term preservation of sperm for future insemination purposes. Jahnel, (39) in 1938, reported the survival of a small proportion of human spermatozoa after freezing and storage at -79, -196, and -269°C. Studies in 1941 by Shaffner *et al.* (101) showed up to 30% survival of fowl spermatozoa after freezing at -76°C if the sample was first partially dehydrated by adding laevulose to the semen. The conditions under which these experiments were done were not always sufficiently well defined and attempts to repeat these results often failed. Rostand (96) reported having successfully preserved the motility of frog spermatozoa after several days at

-4°C to -6°C if glycerol was added to the suspending medium to a concentration of 10% to 20%. The solution he used was only partially frozen and the full significance of his work was not recognized at this time.

With the advent of techniques which allowed the transfusion of blood, there arose a desire to be able to preserve blood for extended periods of time to be used when needed, and work on the preservation of blood in the frozen state began to intensify. Luyet, (59) in 1949, was able to preserve erythrocytes indefinitely of the blood was frozen in thin films at an ultra-rapid rate, a rather impractical technique for the storage of large volumes. Other attempts at freezing erythrocytes up to this time had met with negligible success. Mammalian eggs have also been the object of various attempts at freeze-thaw preservation, especially after the development of techniques for transplanting them into new foster-mothers. These cells were found to be extremely sensitive to thermal changes in the unfertilized state, with a fall of as little as 4°C rendering a portion of them permanently unfertilizable (114). Early attempts to preserve the unfertilized eggs in the frozen state for future fertilization were unsuccessful, and fertilized eggs, although slightly more resistant to thermal changes, invariably died after cooling to -76°C (103).

The vast array of freezing techniques which had been used with erythrocytes, sperm, and eggs, had met with very limited success, and the possibility of preserving these cells in the frozen state with the survival of a sufficient proportion of the cells to allow practical applications seemed remote. It was at this time that Polge, Smith, and Parkes (81) reported the use of glycerol in the medium in which bovine spermatozoa were suspended to bring about the survival of bovine spermatozoa after freezing at -79°C. Although Rostand (96) had previously used glycerol to protect cells in partially frozen solutions, his work had gone largely unnoticed, and this was the first documented report of the use of an additive to enhance survival, and with this discovery the success in freezing preservation mushroomed. Smith (104) found that glycerol would also afford freeze-thaw protection to human and rabbit red blood cells. Lovelock (55) tested several compounds for their ability to prevent freeze-thaw injury in human red cells and found protection against freezing damage at -30°C was given by methanol, acetamide, ethylene glycol, propylene glycol, glycerol, diethylene glycol and monoacetin. Lovelock and Bishop (57) found that dimethyl sulfoxide (DMSO) would protect bovine erythrocytes from damage after freezing and thawing much more efficiently than previously used cryoprotectants. Since that time, a vast array of low molecular weight compounds have been tested for their ability to protect cells from freezing injury in hopes of finding some with superior qualities to those already known. These experiments met with general success, as a large number of the compounds which were non-toxic were able to protect cells from injury incurred during freezing and thawing, although protection with some was more complete than with others. Nash (72) found dimethyl sulfoxide, dimethyl acetamide, dimethyl formamide, methyl acetamide, ethylene glycol and glycerol were able to completely protect red cells from damage incurred after being frozen at -79°C. Doebbler and Rinfret (21) tested the cryoprotection of erythrocytes with twenty-seven low molecular weight compounds and found widespread protection, with a host of monosaccharides and dissacharides being particularly effective. A number of compounds with structures related to glutamic acid have been tested for their effectiveness in protecting bacterial cells during freeze-drying and several good protectors were found (71). Another 26 low molecular weight compounds have been tested by Vos *et al.* (119, 120) for their ability to protect human kidney cells in tissue culture from freeze-thaw injury. They found widespread protection by the chemicals which were not otherwise toxic to the cell line. It can be seen that the number of low molecular weight compounds which can protect cells from freeze-thaw injury is very large and countless other chemicals which have yet to be tried would probably show similar cryoprotection.

Compounds of low molecular weight are not the only ones which have been found to decrease the extent of damage suffered by cells after being subjected to freezing and thawing. Bricka and Bessis (17) found polyvinylpyrrolidone (PVP) and dextran would protect red cells from hemolysis after freezing and thawing. Polymers of ethylene and propylene glycols and Pluronic F-68 were shown to be effective cryoprotectants for red cells by Glauser and Talbot (32). Later studies with red cells showed cryoprotection with human serum albumin, soluble starch, and hydroxyethyl starch (HES) (21) (44).

Doebbler *et al.* (20) showed that 93% of rabbit erythrocytes which were transfused back into the rabbit following freezing and thawing in the presence of 7% PVP were still intact 24 hours later and thereby showed the frozen red cells were suitable for transfusion purposes after thawing. Persidsky and Richards (79) were the first to show that polymers were also effective in protecting nucleated mammalian cells in experiments in which they were able to achieve 30% survival with bone marrow cells when PVP was present in the suspending medium during freezing and thawing. In other studies, PVP has been found effective in protecting bone marrow cells (66) (80) and CHA cells (11, 47, 66) in tissue culture. Dextrans have been shown to have some protective properties in Chinese hamster ovary cells (CHA) (11), and the similar polymer HES has been found to give good protection to CHA cells (11) and bone marrow cells (80). The range of molecules which are good cryoprotective agents thus extends from molecules of small molecular weight such as DMSO (MW 78.13) and glycerol (MW 92.1) to polymers with molecular weights up to the order of hundreds of thousands.

The number of cell systems which have been frozen and thawed with at least partial retention of cell viability has increased as well as the number of protective agents used to protect them. Bacteria are, in general, quite resistant to cryoinjury and show significant survival when frozen in normal saline without any protective additive (33). However, the number of survivors can be increased by the addition of glycerol as shown in a summary by

Smith (106). A review of the survival of protozoans (107) showed they are much more susceptible to freezing damage than are bacteria, but many are protected to some degree if glycerol is present in the suspending medium.

Nucleated mammalian cells are generally more sensitive to freezing damage than cells of the more primitive organisms. Barnes and Loutit (13) first reported the ability of glycerol to protect mouse bone marrow cells after freezing and thawing. DMSO has also been found effective in preventing cryoinjury in this system (7). The successful cryoprotection of other nucleated mammalian cells in the form of cells in tissue culture has been shown in a number of cases (11, 47, 69, 79). Preservation of tissue culture lines in the frozen state is now successful to the extent that large cell culture banks, such as the American Type Culture Collection, store their cultures in the frozen state. In the words of Meryman (68), "there appears to be almost no cell suspension that, with the proper combination of additive and freezing rate, cannot be frozen and thawed with some proportion of its population recovered intact and viable".

Although there have been great advances in the preservation of single cells, the problem of preserving tissues and even whole organs is a much more difficult task. It has been found that embryonic tissues can be frozen with a relatively high degree of success. Whittingham *et al.* (125) have recently demonstrated the successful preservation of mouse embryos at the 1, 2 and 8 cell and blastocyst stage at -196$^{\circ}$C with the use of DMSO or glycerol. These frozen embryos developed normally upon implanting them into foster-mothers. It has recently been found that hearts from 16 day old embryonic mice can be frozen in liquid nitrogen and will continue to beat normally upon thawing, providing DMSO is present in the medium (122).

Preservation of tissues from adult specimens is a more difficult task, but some advances have been made. Billingham and Medawar (15) were able to successfully freeze rabbit ear epithelium in liquid air with the use of a protective additive. In another study with skin using growth in tissue culture as a means of assay, no survival was observed without a protective agent but good protection was afforded by glycerol or ethylene glycol (113). One of the best examples of tissue preservation in the frozen state is in the field of corneal transplantation where lamellar grafts of material frozen with a cryoprotective agent have been shown to be almost as good as unfrozen controls (91). Rapatz (84) has shown adult frog hearts can be frozen at -78$^{\circ}$C and still show some degree of beating in all segments when the heart is perfused with increasing concentrations of ethylene glycol during the cooling process. Little success has been achieved in attempts to preserve organs from mature mammals in the frozen state. The problem arises from the fact that survival of virtually every cell in the organ must be obtained in order for the organ to maintain its function. With larger organs, the problem of achieving penetration of the protective agent becomes critical and better techniques are needed before successes can be expected in the preservation of these organs in the frozen state.

## BASIC PRINCIPLES

Since this study is based on the response of cells to freezing and thawing, it is necessary to consider exactly what is meant by the term frozen, and to describe some of the general physical and chemical changes that occur in a solution during the freezing process. The definition of when a cell is frozen is not a simple matter, and much confusion has resulted and seemingly contradictory results have been obtained in the past simply because the definition of the frozen state was not rigorous enough. For the purposes of this study, the term frozen shall imply the temperature has fallen below that of the lowest eutectic point of the solution and supercooling has not occurred so that no liquid components remain in the solution. It is important to realize that translational movements of water molecules are still possible in a solution, which by this definition is frozen, and ice crystals can grow in size at temperatures far below the freezing point of the solution involved. Even at temperatures as low as -70°C growth of ice crystals has been shown to occur quite rapidly (67).

Let us consider what happens when a solution containing a suspension of cells is cooled to a low temperature, near that of liquid nitrogen, for example. In the first stage of cooling, the temperature falls without there being any phase changes. This stage of cooling lasts from the incubation temperature of the cells down to slightly below 0°C. When the temperature reaches the freezing point of the solution, water will begin to crystallize out as ice. Due to the highly ordered structure of the water molecules in the ice crystals, the solutes present in the original solution will not be able to fit into the crystal lattice of the ice and will remain in the aqueous regions between the ice crystals. As the amount of water which is converted to the crystalline form becomes greater, the concentration of solutes in the region between the ice crystals will increase. The cells in the solution will be in contact with an ever increasing concentration of the solutes which were originally present. Photographs by Rapatz *et al.* (83) and Nei (73) show that the cells in a suspension do end up in the salt channels between the ice crystals. The process of solute concentration will continue until the solutes begin to reach their eutectic points, points of defined solute concentration and temperature. At the eutectic point a particular combination, depending on the solute, of solute and water will crystallize out of solution.

The phenomenon of solutes crystallizing out of solution at their eutectic points is a critical occurrence and deserves closer examination. A typical growth medium contains salts of sodium, potassium, calcium, magnesium and probably traces of many other metals as well as buffers such as $NaHCO_3$, $KH_2PO_4$, and $K_2HPO_4$. Each of these constituents has a particular eutectic point, so that during the cooling process there will be a sequential disappearance of the various components from the solution. This disappearance of electrolyte from solution can be monitored by measuring the electrical conductivity of the solution during the cooling process (89). As each component reaches its eutectic point and crystallizes out of solution, the conductivity of the solution will decrease, ultimately falling virtually to zero after the last eutectic has been reached. The process of crystallizing out of solutes will have profound effects on the remaining solution. The ionic strength and osmolarity of the solution will undergo sharp fluctuations upon passing through each eutectic. Even more critical is the situation where the constituent crystallizing out of solution is the buffer of the system. van den Berg (116) and van den Berg and Rose (117) have shown the fluctuation in pH which occurs during the cooling of systems buffered by combinations of buffers such as mono and di-potassium phasphates and mono and di-sodium phosphates. The difference in eutectics of solutions of these seemingly similar solutes is large, with $NaH_2PO_4$ having a eutectic of -9.7°C whereas that of $Na_2HPO_4$ is -0.5°C. When combinations of these two buffers are present at the same time, $Na_2HPO_4$ will begin to precipitate first and pH changes will result. The variation of the pH is also dependent on the presence of sodium and potassium salts and will undergo fluctuations as these salts reach their eutectic points. The regulation of pH during cooling is a complex phenomenon and large fluctuations can result which, in the case of cell suspensions, can be disastrous. One must keep in mind that cryoprotective agents which are added to a solution also have eutectic points, and if the eutectic point of the cryoprotectant in a solution is reached at relatively high temperatures, the cells will be left unprotected and may be destroyed.

The behaviour of the solutions is greatly altered in the presence of another solute such as glycerol. Rey (89), using Earle's balanced salt solution, showed a sharp increase in conductivity at -21°C during warming due to the melting of $NaCl.2H_2O$ but observed no such jump in the presence of glycerol. The glycerol had clearly allowed the NaCl to remain in solution at temperatures below its normal eutectic point. As cooling proceeds the eutectic point of the last component in the solution will be reached and the remaining liquid water and solute will solidify. At this point, the solution can be considered frozen.

Unfortunately, the point at which water begins to crystallize out of solution as ice is not always at the temperature at which it is thermodynamically advantageous for it to do so. It is known that pure water, which theoretically freezes at 0°C, can be cooled to -38°C without freezing if the system is free of nucleation centers (85). In the case of cell suspensions, it has been suggested by some workers that the cell membrane acts as a barrier for the growth of ice crystals, so the interior of a cell can remain supercooled even in the presence of extracellular ice. Mazur (64), who has summarized the findings to this effect, has suggested the ice crystal growth through the small pores in the external membrane is greatly hampered and is possible only after considerable supercooling has occurred. Thus, the mere attainment of a certain temperature is not enough to conclude that freezing has occurred.

Although it is impossible to say exactly at what temperature a particular biological specimen is completely frozen, it is generally considered that at liquid nitrogen temperature (-196°C) molecular movements in the solution have reached a rate slow enough to be considered negligible even though crystallization may not have occurred, and the solution can be considered frozen. The finding that samples cooled to -196°C remain essentially unaltered over a period of years (61) shows that molecular processes at this temperature proceed very slowly.

In order to understand freezing damage due to changes in the medium during the cooling process, it is imperative to have a knowledge of the parameters which must be controlled in the medium in which a cell is suspended in order for that cell to find it a hospitable environment. Of prime importance is the pH of the solution. Most of the chemical reactions which are carried out in a cell are catalysed by enzymes. These catalysts function by a specialized environment for the reactants at their active sites, the site where the catalytic process will occur, and thereby allowing the reaction to occur at an accelerated rate. They are able to orient the reactant molecules correctly with respect to one another, provide groups which can act as temporary acceptors of donors of electrons or protons, and provide regions of varying degrees of hydrophobicity which may be favourable for a particular reaction. The three dimensional structure of these enzymes is maintained largely by hydrogen bonds, which are quite easily broken. Proteins have many chemical groups, mostly free amino and carboxyl groups, which can take on positive or negative charges, depending on the pH of the medium. If the pH varies too far from the normal value, a net positive or negative charge, depending on the direction of variation, will result on many of the groups of the protein, and the resulting repulsion between similarly charged groups may be strong enough to break the hydrogen bonds maintaining the tertiary structure and the enzyme will lose its catalytic ability. Without the ability to catalyse necessary biochemical reactions, the cell will eventually die. Enzymes are not the only site of damage as a result of incorrect pH. Many organic reactions depend on free protons for catalysis, and if the pH wanders too far from its usual position, some reactions which would normally occur will cease, and other deleterious reactions may take place under the new conditions. The regulation of pH can be seen to be of prime importance in order to maintain normal cell growth, and any factor which causes a change in the pH will cause stress on the cell.

Since the cell is surrounded by a semi-permeable membrane, the osmolarity of the growth medium is another important factor to be controlled if the cell is to be maintained in a normal state. If the osmolarity of the medium is too low, water will flow into the cell until the solutes inside the cell have been diluted to equal the concentration of solutes outside the cell, resulting in swelling and possibly even rupture of the cell or its organelles. A hypertonic medium will cause loss of water from the cell, until the intracellular osmolarity equals that of the medium. Exactly what happens to a cell under conditions of severe loss of water is a matter of much dispute and shall be discussed later. Since a large proportion of the solutes in biological fluids are ions, small decreases in cell volume due to the efflux of water probably affect the cell through the resulting change in the ionic strength of the medium. When ions dissolve in aqueous medium, they form a sphere of water molecules about them, the size of which depends on the electronegativity of the ion. Many biological macromolecules, especially proteins, depend on a small amount of hydration from water molecules to stabilize their structures, and will be denatured if this water is removed (43). If the ionic strength is too high, competition for water molecules will reach the extent where proteins will lose some of their essential water and become denatured, or they may clump together and precipitate out of solution. In either case, the result of incorrect ionic strength of the medium can be disastrous.

It has been shown that the maintenance of the pH, ionic strength, and osmolarity of the medium in which a cell is suspended is of prime importance for the survival of the cell. During freezing, all of these factors undergo large changes as the water freezes out of solution as ice, and damage which results from the process of freezing and thawing is almost certainly due in part to a combination of these changes in the properties of the solution.

There is considerable disagreement amongst present-day workers as to the mechanism whereby protective agents are able to protect cells from freeze-thaw injury, but a few theories have gained considerable support. Generally a distinction is made between the class of compounds which are known to be permeable to the cell membrane and can thus gain access to the cell interior and those which, due to their impermeability, must act extracellularly. Most of the theories use some form of the colligative properties of the protective agent to explain its protective action. Lovelock, in his pioneering papers of 1953 (53, 54) showed a correlation between the mole fraction of NaCl which would bring about hemolysis of red cells and the mole fraction of NaCl which would be present in the unfrozen portion of the suspending medium at the temperature where damage first occurred. He found there was a dramatic increase in the amount of phospholipid extracted from the cell membranes at salt concentrations above 0.8 M. Lovelock suggested that this loss of phospholipid renders the cell membrane permeable to cations, so that a large amount of sodium enters the cell and hemolysis occurs upon thawing.

Lovelock (54) suggested that the protective action of glycerol was due to its ability to lower, through its colligative properties, the amount of ice which freezes out of solution at any particular temperature, and thereby lower the concentration of salts in the medium. If the temperature at which the damaging concentration of NaCl was reached could be lowered far enough, degradative processes would occur too slowly for significant damage to occur. By this mechanism, it was necessary that the protective agent was permeable to the cell membrane in order to protect the interior of the cell as well as the exterior. Experiments with erythrocytes from different animals tended to support the hypothesis that permeability was necessary. Bovine erythrocytes are much less permeable to glycerol than human erythrocytes, and glycerol was found to give little protection to these cells under conditions where human erythrocytes were protected to a substantial degree (57). When Lovelock used DMSO, a non-toxic compound which is highly permeable to bovine erythrocytes, the red cells were not destroyed by the process of freezing and thawing, strengthening the evidence that permeation of the cryoprotective agent was necessary for it to be effective. In order to be an effective cryoprotective agent, according to this hypothesis, a molecule must be able to reduce the amount of water which freezes out as ice, act as a solvent for electrolytes, be freely permeable to the cell membrane, be soluble in aqueous solution, and be non-toxic to the cell system (55).

Other explanations of freezing damage and cryoprotection which shall be discussed in detail later, are similar to Lovelock's in that the cryoprotective properties of a compound are thought to lie in its ability to lower the amount of ice formed at any particular temperature.

The earlier work with cryoprotective agents was done using low molecular weight compounds, of which the molecular weights were known exactly, so that various physical parameters of solutions such as freezing points, osmolarities, and amount of ice formed could be calculated using the laws of physical chemistry. In later studies a new class of protective agents, the high molecular weight polymers, became popular and presented new problems for the description of the solutions in which they were used.

Most of the protective polymers which have been studied are synthetic polymers formed by the addition of monomeric units to the end of a chain. Different molecular weights of the polymers can be obtained by stopping the polymerization reaction at various times. Since the growth of each polymeric molecule depends on the collision with a monomeric group under suitable conditions, the occurrence of which over a given time can only be assigned a probability, the lengths of the molecules in any preparation will vary, making the assigning of exact molecular weights impossible. Molecular weights for polymers are usually given as the number average molecular weight ($\bar{M}_n$), or the weight average molecular weight ($\bar{M}_w$). The number average molecular weight ($\bar{M}_n$) is obtained by adding the number of molecules at each molecular weight, multiplied by that molecular weight, and dividing by the total number of molecules. The weight average molecular weight, $\bar{M}_w$, is obtained by adding the number of grams of material at a particular molecular weight, each multiplied by the molecular weight of the fraction, and dividing by the total number of grams. In a solution containing a polymer with a wide spread of molecular weights, the weight average will be considerably higher than the number average. The molecular weight is often calculated from the light scattering properties of the polymer, a procedure which yields a value for the weight average molecular weight, or from viscosity measurements through the use of a complex equation (11). The molecular weight spread of the molecules is sometimes much greater than that which is indicated by the manufacturer. Plasdone C, which is reported to have a number average molecular weight of 40,000 with a total spread of 20,000 to 80,000 daltons, has been shown to contain an almost continuous spread of molecular weights from 1,000 to 200,000 daltons (9). Such a wide spread of molecular sizes makes discussion of a polymer solution on a molar basis almost meaningless, and calculations of physical parameters of the solution using formulae which depend on the concentration of particles or particle size can be equally meaningless.

Arguments against theories explaining cryoprotection on a colligative basis have come from the demonstration that high molecular weight polymers, such as PVP, dextran, and HES, are able to protect cells from freeze-thaw injury. Colligative properties, by definition, are properties which depend only on the concentration of particles in a solution and not in any way on the nature of these particles. PVP, a typical high molecular weight compound, with a number average molecular weight of 40,000, will have only $1.95 \times 10^{-3}$ as many molecules/ml as the low molecular weight protective agent DMSO, when the two are present at the same w/v concentration, yet protection afforded by the two compounds is often similar. There appear to be three possible explanations of this observation. One, that neither the low molecular weight nor the high molecular weight cryoprotective compounds act on a colligative basis, two, that high molecular weight polymers act on a totally different mechanism than do the lower molecular weight compounds, or three, that polymeric solutions show colligative properties beyond what would be expected on a molar basis.

It has been suggested (79) that the polymers may be able to gain entry to the interior of the cell through the mechanism of endocytosis, and thereby exert their cryoprotective effect intracellularly. Other hypotheses have been put forward to explain how the polymers could protect while remaining extracellular (79). The polymer could absorb electrolytes from the solution so that they were not free to exert a damaging effect on cell constituents. Polymers could coat the cell membrane and cause blocking of pores which could have a protective effect by preventing intracellular ice formation or preventing the loss of electrolytes from the cells. The protection might also be due to binding of the polymer to the cell membrane, resulting in a stabilization of molecules on the surface of that membrane. It has even been suggested that the protection occurs after thawing by preventing breakdown of the membrane (69) or by restoring permeability characteristics of the membrane (79).

It is found that a mixture of two polymeric solutions often results in the formation of a biphasic mixture (1). The existence of a large partition coefficient between the two phases for most large molecules has made the biphasic polymer systems very useful for counter-current separation techniques of large molecules, cellular particles, and even cells. Ashwood-Smith *et al.* (11) have suggested that a suspension of cells in a polymeric solution may be considered as a two-phase system, one phase being the aqueous polymer solution and the second phase being the polymeric surface of the cell. The interface between the solution and the cell surface could act as a barrier for certain molecules which might be lost from the cell in the absence of the polymer and thereby maintain the cell viability. If salts were distributed unevenly between the two polymers the presence of the polymer in solution could protect by altering the salt distribution during cooling. Very little work has been done on the distribution of salts in biphasic polymer mixtures.

Another explanation of how polymers could protect a cell is that the polymers do not behave as one would expect them to using the classical rules for describing colligative properties, and that the polymers do, in fact, show colligative properties at higher concentration where the solutions are far from ideal (ideal conditions exist only in solutions in which the solute is present at almost infinite dilution). Farrant (25) has suggested the PVP does, at high concentration, show colligative properties. He showed that the concentration of sodium chloride present various subzero temperatures was decreased by the presence of PVP. Starting out with a solution of 1% NaCl with either 10% glycerol or 30% PVP, and assuming the salt concentration in the unfrozen portion increased the same amount as the PVP, he made up solutions simulating the progression of freezing and measured the freezing points of these solutions. At lower concentrations of salt and protectant, simulating conditions only slightly below the freezing points of the solutions, glycerol was more effective than PVP in preventing ice formation, but at higher concentrations (simulating lower temperatures), the PVP became more effective than the glycerol in preventing ice formation. Measurements on solutions of PVP using much more sophisticated equipment (85) also shows that at higher concentrations of PVP the freezing point falls sharply. It seems that it may be possible for polymers to act on a colligative basis as well as the compounds of low molecular weight.

# OBJECTIVES OF THIS STUDY

A number of problems regarding polymers and their ability to protect cells from damage following freezing and thawing warranted investigation. Some of these problems are:

Polymers have only recently become popular for the preservation of nucleated mammalian cells in the frozen state. It was desirable to determine the effectiveness of various polymers in preventing injury due to freezing and thawing and to quantitate the effect of varying such parameters as cooling rate, warming rate, and concentration of protective additive on the survival of the cells after thawing.

It has been suggested that polymers gain entry to the cell interior by the process of endocytosis and exert their cryoprotective effect intracellularly as well as extracellularly. Experiments to determine whether endocytosis was involved in cryoprotection appeared to be necessary.

Some workers feel (49, 64, 66) that the damage to a cell occurs at the cell membrane. Thought to be contained in this membrane is the mechanism for transporting molecules which otherwise cannot gain entry to the cell. It is possible the damage to the cell was due to the destruction of these transport mechanisms and the investigation of the behaviour of such mechanisms after freezing and thawing as compared to the unfrozen sample could clarify this point.

It has been reported (69) that the polymer PVP can increase the recovery of red cells when it is present in the medium after thawing. Post-thaw protection was a whole new concept and warranted checking in the nucleated cell system.

An excellent example of cellular repair has been reported by Robinson (92, 93, 94), in which cells which were frozen and thawed without the presence of a protective additive and were able to repair the damage which they incurred if they were plated into medium in which cells had been growing for some time, the so-called "conditioned medium". It was desirable to explain the nature of the repair and the components of the conditioned medium which allowed such repair to occur.

Electron microscope techniques allow one to view the cells before freezing, while frozen, and after thawing. By viewing cells at these stages it may be possible to observe the physical alterations occurring in the cells as a result of the freeze-thaw process and to determine at what stage the damage occurs.

There is some controversy as to whether low and high molecular weight cryoprotective agents act on the same or different mechanism. Reports on the failure of dimethyl sulfone to afford cryoprotection (119, 120) suggested that the mode of action of the low molecular weight protective agents was not entirely through their colligative properties since in molecular weight and chemical structure this compound is very similar to the well-known protectant DMSO. An explanation of this seeming failure of the colligative hypothesis warranted investigation.

The class of polymeric protectants may cause an alteration of the physical properties of solutions which could result in protection from freezing damage. An investigation of the behaviour of these solutions, especially during the freezing process, could reveal alterations in the solutions which could be responsible for the protection which the polymers afford.

# MATERIALS AND METHODS

## Tissue Culture Cells

Chinese hamster ovary cells growing in tissue culture were obtained at the 351 st passage level as a gift from D.M. Robinson of the American National Red Cross Blood Research Laboratory at Bethesda, Maryland and are officially designated as Puck's Clone A. These cells, which shall be referred to as CHA cells, have been growing in tissue conditions since 1958, when T.T. Puck isolated them from the Chinese hamster.

## Culture Medium

CHA cells were grown in Eagle's minimal essential medium with Earle's balanced salt solution (24) obtained from Biocult Laboratories Ltd., Glasgow, Scotland. This medium was supplemented with 0.05 M Tricine (N-tris (hydroxymethyl) methyl glycine) obtained from Sigma Chemical Corp., St. Louis, Missouri, adjusted to pH 7.2 with NaOH. 50 μg/ml kanamycin (Biocult Labs) was added as an antibacterial agent and fetal calf serum (Microbiological Associates Inc., Bethesda, Maryland) was added to a concentration of 10%. The osmolarity of this medium, which shall be referred to as CTM, was approximately 290 milliosmoles.

## Cryoprotective Chemicals

DMSO was obtained from Fisher Scientific Company, Fair Lawn, New Jersey. Several different molecular weights of PVP, including Plasdone C-15 (MW 10,000), Plasdone C (MW 40,000) and K90 (MW 360,000) were obtained as gifts from General Aniline and Film Corporation, New York, New York. Dextran T40 (MW 40,000) was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Hydroxyethyl starch (HES) was obtained as a sterile 35% solution from McGaw Laboratories, Glendale, California. This sample had a number average molecular weight of 70,000 and a weight average molecular weight of 450,000.

Dimethyl sulfone was synthesized by the oxidation of DMSO with hydrogen peroxide. 80 ml of DMSO were added to 100 ml of 30% hydrogen peroxide, the solution was mixed thoroughly, left to stand at room temperature for two days, and then concentrated by removal of most of the solvent in a vacuum distillation apparatus. Upon cooling the remaining solution, dimethyl sulfone crystallized out. The sulfone was crystallized twice from chloroform, allowed to dry thoroughly, and used in cryoprotective studies. A sample was later obtained from Matheson, Coleman, and Bell, Cincinnati, Ohio.

To determine whether the product obtained was, in fact, dimethyl sulfone, the melting point of the crystals was determined and the infra-red absorption spectrum was obtained using a Perkin-Elmer Model 337 Grating Infra-Red Spectrophotometer (Hitachi, Ltd., Tokyo, Japan) or a Unicam SP1000 Infra-Red Spectrophotometer (Unicam

Instruments Ltd., Cambridge, England) with the sample dissolved in chloroform and held in NaCl solution cells.

## Cooling and Warming Rate

Samples were routinely frozen by cooling them in liquid nitrogen (-196°C). The variation of the temperature with time was determined with a Honeywell Electronik 16 recorder (Honeywell Controls Limited, Scarborough, Ontario) using a copper-constantan thermocouple immersed in the fluid in a vial under identical conditions to those under which the samples were cooled and warmed. Cooling rates were defined over the range 0°C to -150°C, as it was felt that temperature changes outside this range probably had little effect on the physical changes in the solution. Since fairly rapid temperature changes were used in some cases, there is a large temperature gradient through the sample and the temperature variation recorded by the thermocouple is an indication of the variation at that particular point and may not be an accurate indication for the sample as a whole. It does, however, give a general indication of the rates involved.

## Sterility

Solutions which were heat-resistant were sterilized by autoclaving at 121°C for 20 minutes. Heat-labile solutions were passed through a .22µ pore size Millipore filter (Millipore Corporation, Bedford, Mass.) which had previously been sterilized in an autoclave.

Sterility during the experiments was maintained with the use of an Envirco Laminar Flow Bench (Envirco Division of Belton, Dickinson and Company, Albuquerque, New Mexico).

## Culture Procedure

CHA cells were grown in 30 ml capacity Falcon plastic tissue culture flasks (Falcon Plastics, Oxnard, California) in an incubator set at 36.5°C. It was not necessary to regulate the $CO_2$ content of the atmosphere in order to maintain the correct pH since the organic buffer used is not as sensitive to $CO_2$ as the commonly used bicarbonate buffers. Cultures were transferred to new flasks approximately once a week. The medium was drawn off the cells and a solution of 0.1% trypsin (Bacto-Trypsin 1:250, Difco Laboratories, Detroit, Michigan) in Ca and Mg free balanced salt solution (78) was placed on the cell monolayer. After approximately three minutes the cells became detached from the surface of the flask and floated free in solution. An equal volume of CTM was added to the trypsin solution to halt the tryptic activity and the cells were then separated from the medium by centrifugation at 90 x g for three minutes. The supernatant was drawn off and the cells were resuspended in CTM. Cell counts were made with a hemacytometer and an appropriate number of cells, usually about 20,000, were dispensed into a fresh flask of CTM. The flasks were then returned to the incubator until the next transfer was necessary.

## Freezing Survival Experiments

CHA cells were trypsinized and collected in the same manner as for propagation of the cell line. The number of cells/ml was determined and dilutions of the cell suspension made to result in a final concentration of 20,000 cells/ml. One milliliter of this suspension was then added a 15 ml screw-cap vial containing 1 ml of CTM containing the cryoprotective additive present at two times the concentration desired for the experiment. Vials were capped and cooled to the temperature of liquid nitrogen, remaining at that temperature for at least one-half hour to ensure completion of thermal equilibration. Thawing was accomplished by placing the ampoules into a water bath at 37°C, with constant shaking to bring about as rapid and as uniform a warming as possible. 0.1 ml, corresponding to 1000 cells in the initial suspension, was added to 60 mm petri dishes (Tissue Culture Dish, Kimble Products Division of Owens-Illinois, Toledo, Ohio) containing 5 ml of CTM and incubation at 36.5°C was carried out for 10 days. In order to prevent evaporation of the medium in the petri dishes during incubation, the dishes were kept in air tight plastic boxes (Frig-O-Seal, Modern Plastics Co. Ltd., St. Hyacinthe, Quebec). It is imperative to use a brand of box which shows low toxicity to the cells, as some of the plastic boxes, although airtight, caused complete destruction of the cells incubated within. After incubation for 10 days, the growth medium was drawn off and the colonies of CHA cells were stained by immersion in 0.2% methylene blue in water. After about thirty minutes the dishes were rinsed in water and the number of colonies on the dish were counted through a binocular microscope.

## Robinson Hypothesis

CHA cells were innoculated into several flasks and incubated until the cell monolayer reached confluency. The old medium was drawn off and removed of any living cells by passing it through a Millipore filter (pore size .22μ). This medium shall be referred to as conditioned medium, after the terminology of Robinson (92). The cells in one of the flasks were removed by trypsinization and subjected to freezing and thawing in the usual manner. Cells were dispensed into either fresh CTM or various combinations of CTM and conditioned medium. After an incubation time of 4 1/2 hours the medium was removed from those dishes containing conditioned medium and replaced with fresh medium. Dishes were incubated for 10 days and survival assayed by counting the colonies formed.

## Radioisotope Experiments

Scintillation vials (Beckman low-potassium borosilicate liquid scintillation vials, Beckman Instruments Inc., Fullerton, California) were washed thoroughly in Microsolve tissue culture detergent (Microbiological Associates, Inc., Bethesda, Maryland), rinsed well in distilled water, and sterilized by autoclaving. 3 ml of CTM in which approximately 100,000 cells were suspended were added to the vials. The vials were stoppered with polyethylene liners which had been sterilized by boiling in water, capped, and placed in an incubator to allow cell growth. The cells were incubated until they reached a state of confluency in order to minimize variations in the total number of cells in the vial. Vials were removed from the incubator, medium was removed and fresh CTM containing 0.3 μCi/ml $^{14}C$ labelled l-alanine (Amersham/Searle Corporation, Arlington Heights, Illinois, Specific activity 173 mCi/mmol) was added to the vials. The scintillation vials were returned to the incubator and incubated for varying lengths of time, after which the medium was removed and the cells were washed in two changes of ice cold saline to remove the extracellular counts.

In the experiments involving a freeze-thaw procedure, cells were suspended in CTM after trypsinization, then separated into two separate samples. One sample was subjected to a freeze-thaw regimen, whereas the other served as a control. Approximately 300,000 cells suspended in CTM were placed in each vial and incubated for two hours to allow time for attachment of the cells to the surface of the scintillation vials. The medium was then withdrawn and replaced with medium containing $^{14}C$ labelled l-alanine for studies on the uptake as previously described.

In order to count the number of disintegrations the cells first had to be solubilized. 0.5 ml of a mixture of 50% Protosol tissue solubilizer (New England Nuclear, Pilot Chemicals Division, Boston, Massachusetts) and toluene (scintillation grade, Fisher Scientific Company, Fair Lawn, New Jersey) were added to the vials. The vials were incubated at 60°C for 30 minutes to ensure that the cells were totally dissolved, after which 12 ml of scintillation cocktail containing 5 g/lt of PPO (2, 5-diphenyloxazole, scintillation grade, Amersham/Searle Corporation, Arlington Heights, Illinois) in toluene was added to each vial. Determination of radioactivity was done in a Beckman LS-133 Liquid Scintillation Spectrometer (Beckman Instruments Inc., Fullerton, California).

### Electron Microscopy

### *In Situ* Conditions

Cells were grown to confluency in Falcon culture flasks. The medium was removed and the cells were rinsed in a buffer containing 0.05 M $KH_2PO_4$, 0.15 M NaCl, 0.005% $CaCl_2$, and adjusted to pH 7.2 with NaOH. The cells were then fixed at room temperature with 1.5% glutaraldehyde (Ladd Research Industries, Inc., Burlington, Vermont) dissolved in the same buffer. After 40 minutes, this fixative was removed, the cells were washed in buffer, and post-fixed for 1 hour in 2% $OsO_4$ (BDH Chemicals, Toronto, Ontario) also dissolved in the same buffer. Following this the cells were rinsed in water, stained with 2% uranyl acetate (Fisher Scientific Co.) for 1 hour, and then dehydrated in 30, 50, 70, 95, and 100% ethanol, the cells being left at each stage for 10 minutes. Since propylene oxide dissolves the plastic flask, the usual final dehydration in propylene oxide was omitted. The cells were finally embedded in epon, following the procedure originally developed by Luft (58). Mixture B was omitted, leaving mixture A consisting of 62 ml of Epon 812 (Fisher Scientific Company) and 100 ml of dodecenyl succinic anhydride (National Aniline Division of Allied Chemical and Dye Corporation, New York, New York). Immediately before embedding 10 ml of mixture A was mixed with 0.16 ml of 2, 4, 6-tri(dimethylaminomethyl) phenol (Rohn and Hass Co., Philadelphia, Pa.) which acts as an accelerator. The culture was infiltrated with 1:1 Epon: 100% ethanol, for 30 minutes, 3:1 Epon: ethanol for 1 1/2 hours, and then pure Epon for 8 hours. The Epon was then removed, fresh Epon added to the dishes, and polymerization carried out for 20 hours in an oven set at 60°C.

### Freeze-Substitution

For the freeze-substitution technique, CHA cells were grown to confluency in plastic flasks, the CTM was replaced with CTM with or without protective additive, and the cells were cooled to -196°C at the rate of approximately 20°C/minute. The flasks were left in liquid nitrogen for at least 30 minutes, and then transferred into a Dewar flask containing dry ice and absolute alcohol at -79°C. The ice is gradually replaced by the alcohol so that dehydration takes place without melting of the ice. After three days in this solution, sufficient time to ensure freeze-substitution (118), the flasks were removed, warmed to room temperature, and embedded in the manner previously described.

## Sectioning

A piece of suitable size, containing both plastic and Epon, was cut from the flask and mounted in the chuck so that sections would be cut on a plane perpendicular to the monolayer of cells. The blocks were shaped to give sections containing part of the flask and part Epon. The blocks were positioned so that the knife edge passed from the plastic flask into the Epon-embedded material. It is imperative that the knife edge contacts all regions of the plastic-Epon interface simultaneously, as the two materials have different compression characteristics and wrinkling of the sections at this interface will occur if part of the knife is cutting Epon while part of the knife edge is still cutting the flask. This problem of different cutting characteristics between the flask and embedding medium did not appear in studies by Nelson and Flaxman (74) using Araldite embedding medium. Sections were cut on a Reichert OM U-2 (Reichert, Vienna, Austria) using glass knives cut at a bevel angle of 45° on a LKB Type 7801B glass knife maker (LKB-Produkter AB, Stockholm, Sweden). Thin sections were picked up on Formvar-coated (Monsanto Ltd., St. Louis, Mo.), 100 mesh copper grids (Ernst F. Fullam, Inc., Schenectady, N.Y.) and stained. Cells which had been fixed in glutaraldehyde and osmium were stained for 5 minutes in lead citrate after the method of Reynolds (90). Freeze-substituted cells were stained for 1 1/2 hours in uranyl acetate followed by 5 minutes in lead citrate. Sections were viewed with a Philips EM300 (Philips Electronics Industries Ltd., Holland) operating at 60 KV.

## Electron Microscopy of Frozen Cell Suspensions

A somewhat different procedure, similar to that of Dalen and Nevalainen (18), were subjected to freezing and thawing. The procedure for freezing cell suspensions in vials, as previously described, was used. However, instead of plating the cells after the freeze-thaw procedure, the cell suspension was drawn into a syringe and forced through a Millipore filter. The Millipore filter, containing a layer of collected cells, was washed in 0.05 M $KH_2PO_4$-NaOH, pH 7.2, containing 0.85% NaCl and fixed in 1.5% glutaraldehyde in the same buffer for 40 minutes. After rinsing in saline, the cells were post-fixed for one hour in 2% $OsO_4$ in the same buffer, rinsed in distilled water, and stained with 2% uranyl acetate in water, adjusted to pH 4.5, for one hour. Dehydration and infiltration were carried out in the same manner as for cells embedded directly in the flasks, except that Spurr's embedding medium (109) was used in place of Epon. This medium consists of 10 parts ERL-4206 (Union Carbide Canada Ltd., Belleville, Ont.), 6 parts D.E.R. 736 (Polysciences, Inc., Rydal, Pen.), 26 parts nonenyl succinic anhydride (The Humphrey Chemical Company, North Haven, Conn.) and 0.4 parts dimethylaminoethanol (Pennsalt Chemicals Corporation, Philadelphia, Pa.). After infiltration, the Millipore filters, containing a layer of cells on their surfaces, were placed in BEEM capsules (Better Equipment for Electron Microscopy, Bronx., N.Y.), the capsules were filled with fresh embedding medium, and polymerization at 60°C was carried out for 20 hours. The hardened blocks were shaped to give sections containing Millipore filter and the cell layer contained thereon and sections were cut as previously described. The filter presented no difficulty in sectioning. Sections were picked up on 200 mesh uncoated grids, stained for 20 minutes in lead citrate, and viewed under the electron microscope.

## Determination of NaCl at Subzero Temperatures

Various types and concentrations of protective additives were made up in solution containing 0.16 M NaCl. The solutions were placed in centrifuge tubes, cooled to various subzero temperatures in an alcohol bath controlled by a Haake Model FT Constant Temperature Circulator (Haake Brothers, Berlin), seeded, and allowed to stand for at least one hour to allow for equilibration of temperature in the partially frozen solutions. The samples were then spun for one hour at 12,000 rpm in a Sorvall RC-2B refrigerated centrifuge (Ivan Sorvall Inc., Newton, Conn.) set at the desired temperature. After the run, the liquid portion at the bottom of the centrifuge tube was removed with a plastic syringe carrying a three inch number 17 needle. The sodium content of the solution was analysed with a Varian Tecktron Model AA-4 Atomic Absorption Spectrophotometer (Techtron Pty. Ltd., Melbourne, Australia).

## Nuclear Magnetic Resonance Studies

Aqueous solutions of PVP, HES, dextran, and DMSO were made up in either distilled water or isotonic saline (0.16 M). Samples were placed in glass tubes made from 4 mm Pyrex tubing and frozen by immersion in a dry ice-alcohol bath. The samples were then transferred to an alcohol bath at -40°C and allowed to remain there for at least 20 minutes to ensure temperature equilibration. At the appropriate time, the pyrex tubes were removed from the alcohol bath and placed inside the standard 5 mm NMR tubes which in turn were placed into the low-temperature probe of the Varian HA-60 Nuclear Magnetic Resonance Spectrometer (Varian Associates, Palo Alto, California) which was set at -35°C. In some cases, the spectrum was run on the samples at -25, -15, and -5°C. In these cases after each run the probe was set to the next warmer temperature and allowed a few minutes for the sample to equilibrate to the new temperature before the next spectrum was plotted. The area under the peaks was determined by multiplying the half-width of the peak by the peak height, a method shown to be acceptable under similar circumstances by Kuntz *et al.* (45).

## Freezing Point Depression Studies

Various concentrations of PVP up to 50% were made up in distilled water from a sample of dialysed, freeze-dried pharmaceutical grade Plasdone C. The freezing points of the solutions were determined with a Osmette Precision Osmometer (Precision Systems, Waltham, Massachusetts). This osmometer operates by relating the osmolarity to the freezing point depression of the solution, so that freezing points could be calculated from the osmolarity reading by use of the fact that 1 milliosmol gives a freezing point depression of .00186°C. To compare the behaviour of a polymer solution to a common low-molecular weight compound, various concentrations of DMSO up to 5% v/v were also tested.

### Heats of Solution

Heats of solution of various polymers (PVP, HES, and dextran) and of DMSO were determined by measuring the rise in temperature which occurred when the various substances were dissolved in water in a Dewar flask. The resulting aqueous solution was assumed to have a heat capacity of one calorie per ml, and an attempt to determine the heat capacity of the Dewar flask was made by measuring the temperature drop when a known quantity of hot water was placed into the Dewar flask at room temperature. Five grams of polymer were added to 50 ml of water in the tests with polymers, whereas in the DMSO test 5 ml of DMSO were added to 45 ml of water in an attempt to have a final volume of approximately 50 ml.

### Red Cell Recovery Experiment

Experiments were done to determine the recovery of human red cells after being subjected to freezing and thawing with or without DMSO or dimethyl sulfone. Fresh human erythrocytes were suspended in 0.16 M saline and DMSO or dimethyl sulfone was added to a known concentration. Vials were cooled to -79°C in a dry ice-alcohol bath (cooling rate 20°C per minute) and thawed in a 37°C water bath (warming rate 120°C per minute). The intact cells were removed by centrifugation and the hemoglobin content of the supernatant assayed spectrophotometrically at 410 nm as a measure of hemolysis.

# RESULTS

## Cell Morphology

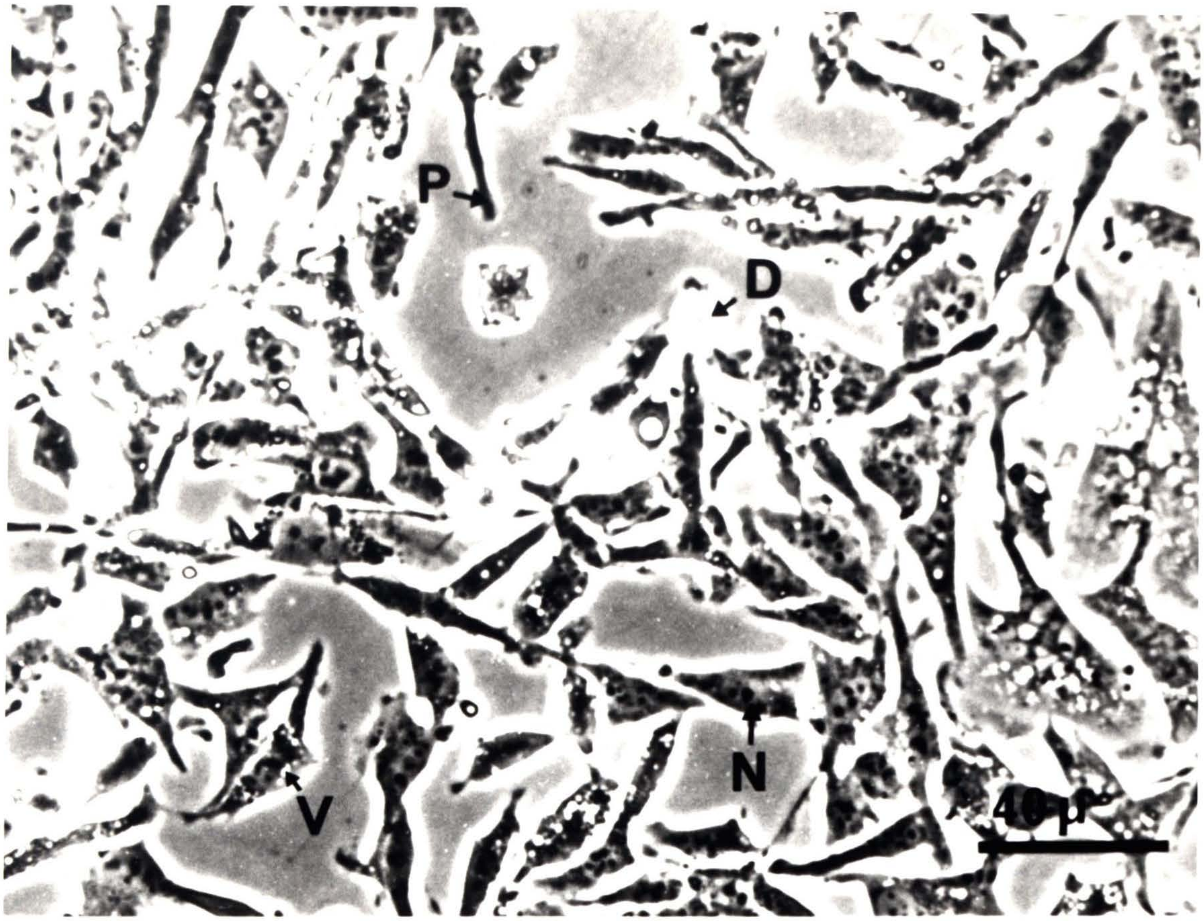
CHA cells grew essentially as a monolayer, (Figure 3), although under highly confluent conditions some piling up of cells is observed (Figure 4, 5). When viewed under phase contrast, the cells appear well spread on the surface of the flask with numerous projections (Figure 1), the ends of which often show regions of membrane ruffling which exhibit endocytotic activity (Becker, unpublished observations). The majority of cells are mononucleate, although the occasional multinucleate cell is observed.

When viewed under the electron microscope, cells were seen to contain the normal mammalian cellular organelles such as mitochondria, lysosomes, golgi, endoplasmic reticulum, and vesicles of various sizes (Figure 3-6). Some micrographs show segments of microtubules (Figure 5, 11), which are thought to be the structural component of the individual cell for the formation of assymetric shapes (23) acting to the cell as the skeleton does to a vertebrate organism. Occasionally they are seen to be of considerable length, although the average length is hard to determine since the entire length of the tubule rarely falls in the plane of the section. Microfilaments, thought to be the driving force for cellular movement (124), are seen in some sections (Figure 13).

Of particular interest is a membranous-appearing layer, referred to by Rosenberg (95) as the microexudate carpet, which is invariably present between the cells and the surface on which they are growing

Figure 1. Phase micrograph of a typical field of CHA cells growing on the surface of a plastic flask. The cells spread well on the surface and are seen to form many long projections (P). Cells which are in the process of dividing (D) round up and detach from the surface of the flask. Several dense-appearing nucleoli appear in each nucleus (N). The cells are seen to contain an abundance of cytoplasmic vesicles (V).
x 590

Figure 2. Freeze-substituted CHA cells as seen through a phase microscope. After cooling at a rate of 20°C/min. in the presence of 10% PVP in CTM, a large number of cells show the presence of intracellular ice (I). Other cells in which ice has not formed appear quite normal (N).
x 590


P
D
N
V
40μ

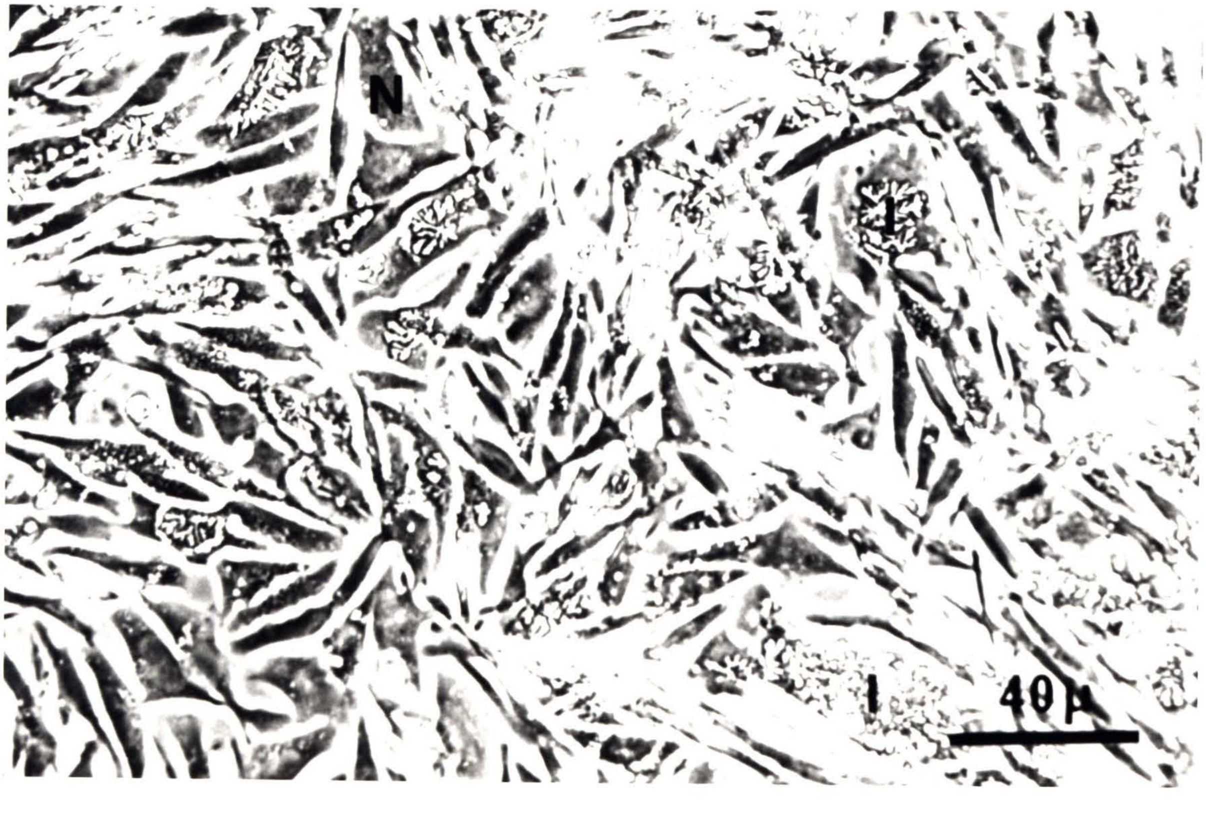
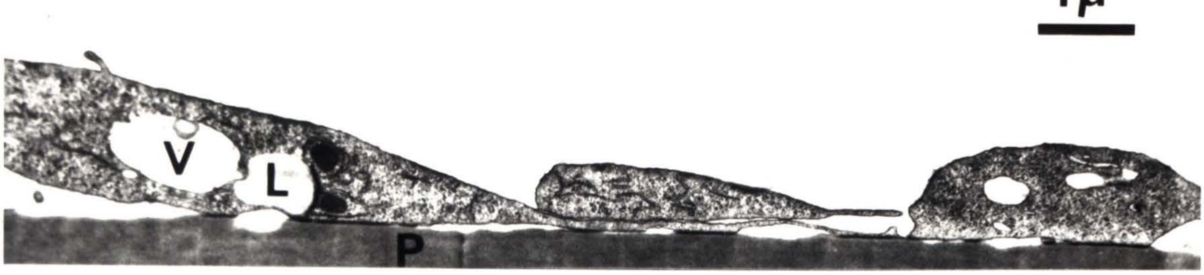
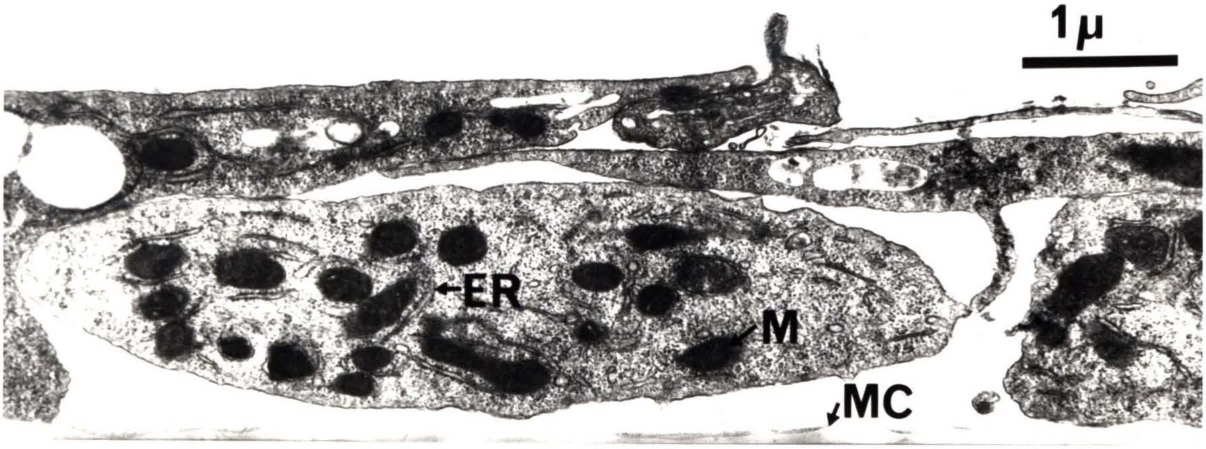

N
I
I
40μ

Figure 3. Electron micrograph of CHA cells growing under normal conditions on the surface of a Falcon plastic tissue culture flask (P). The culture has reached confluency and slight piling up can be seen. Visible are vesicles (V) and a lipid droplet (L). Cells are seen to be slightly over 1µ in depth and several microns in length, depending on the plane of the section.
x 12,000

Figure 4. EM photograph of CHA cells growing on the surface of a flask under confluent conditions, showing considerable piling up of the cells. Note the presence of a membranous film, commonly referred to as the microexudate carpet (MC), between the cells and the surface of the flask. Mitochondria (M) and endoplasmic reticulum (ER) can be seen throughout the cytoplasm.
x 19,300

Figure 5. Vertical section of a CHA cell under normal growth conditions. A typically dense-staining mitochondrion (M) is seen, as is a region containing several microtubules (Mt).
x 41,800


1µ
V
L
P


1µ
ER
M
MC


M
0.5µ
Mt

(Figure 4, 5, 6). This carpet covers the surface of the flask far past the boundary of the cells in cultures which have not reached confluency (Figure 6), and in more confluent conditions is seen to cover the entire surface of the flask. This layer is thought to be formed from a substance which is secreted from the tissue culture cells. Yaoi and Kanaseki (129) have suggested that this carpet is necessary for normal mitosis of chick embryo cells in culture, and use this suggestion as the explanation of why cultures seem to grow more vigorously when the cell density is high than when only a few cells are present in the culture. In the flask containing the dense population the microexudate carpet would be built up rapidly and growth conditions would be more favourable than for the sparse population. Although no direct studies were done to determine the function of this layer in CHA cells, it may well be necessary for cell growth. Throughout the course of this study it was noted that the percentage of cells which will form colonies when plated into a flask decreases as the number of cells plated decreases, indicating that the presence of nearby cells can make growth conditions more favourable. A high density of cells could result in a rapid build-up of the carpet, and thereby enhance the growth of the cells. Further tests would be necessary to clarify this point.

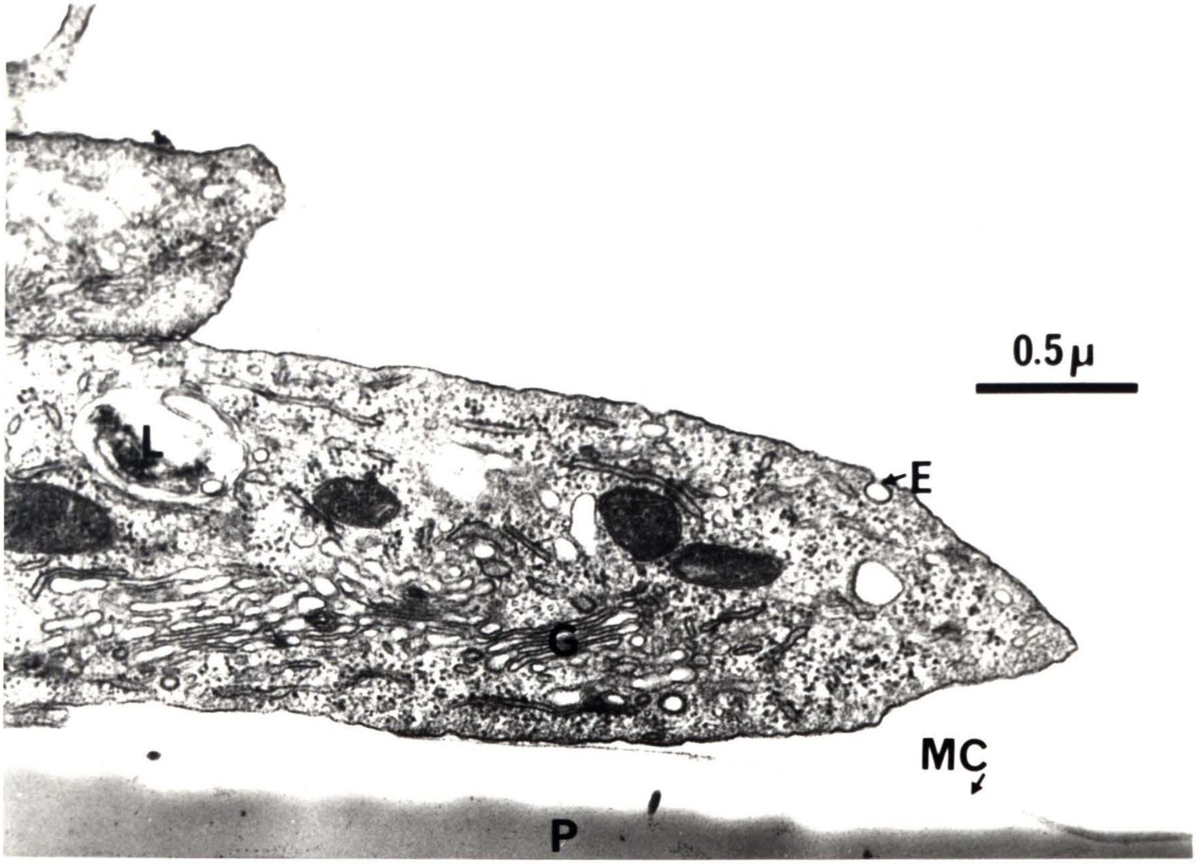
The formation of intracellular ice is considered by many to be lethal to the cell (63, 65), as the ice crystals are thought to disrupt the membranes systems in the cytoplasm, as well as the nuclear membrane. Freeze-substituted CHA cells show that at a rate of 20°C/min., intracellular ice formation is a common occurrence. Figure 9 shows the formation of large crystals of ice both in the cytoplasm and in the nucleus. Approximately half of the freeze-substituted cells viewed showed evidence of intracellular ice, both in sectioned material and in phase micrographs (Figure 2) of a large field of cells. The amount of intracellular ice formed was similar in DMSO protected, polymer protected, or unprotected cells. In micrographs showing very little intracellular ice, the structure of organelles such as mitochondria seem quite normal. Figure 7 shows a section through two freeze-substituted cells showing a multitude of mitochondria with normal-appearing cristae. Although the growth of ice crystals causes massive distortions in some cases (Figure 8, 10), the organelles seem largely intact and there is no evidence of rupture of the cell membrane, although resolution is not great enough to clarify whether breaks have occurred in the cell membrane. The projection of an ice crystal through the cell or nuclear membrane was never noticed in this study.

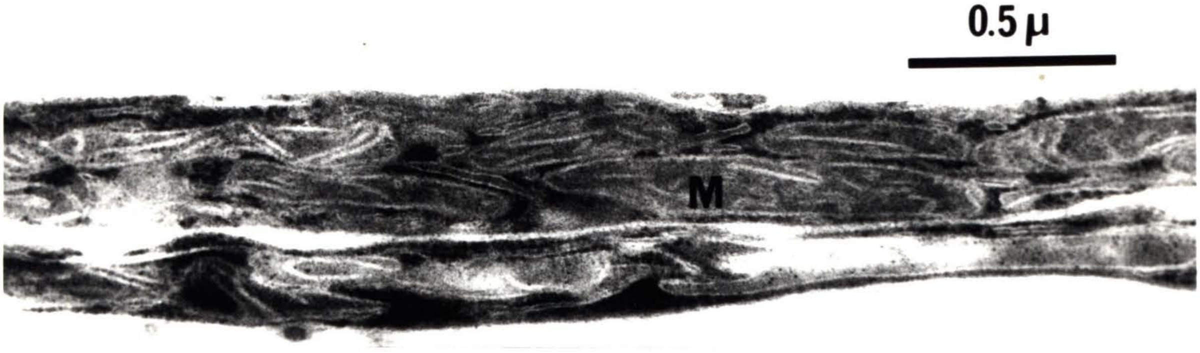
Upon thawing, frozen cells often appear swollen accompanied by the formation of many vesicles in the cytoplasm (Figure 14). A complete range of cells from those appearing perfectly normal to those appearing totally destroyed occurs in each sample, but the most common appearance varies depending on the treatment. Figure 12 shows a CHA cell which has been frozen and thawed in the presence of PVP. The cytoplasmic contents appear normal, with healthy looking Mitochondria, golgi, and lysosomes, although in one region breakdown

Figure 6. Higher power electron micrograph of CHA cells growing on a plastic flask (P). Regions of endocytotic activity (E) can be seen with the cell membrane folding into the cell interior. Cytoplasmic organelles are in abundance, with a golgi apparatus (G) and a lysosome (L) visible. Again the microexudate carpet (MC) is prominent.
x 40,800

Figure 7. Electron micrograph of a freeze-substituted cell in which intracellular ice has not formed. The mitochondria (M) appear intact although the cytoplasmic contents have been highly compressed by dehydration. Freezing was done in 10% DMSO in CTM using a cooling rate of 20°C/min.
x 52,000

Figure 8. Freeze-substituted CHA cell in which very little ice has formed. Over a large portion of the cell ice has formed between the cell and the surface of the flask, but the cell membrane and intracellular organelles appear to be intact. Cooling at a rate of 20°C/min. was done in the presence of 10% PVP in CTM.
x 22,200


0.5µ
L
E
G
MC
P


0.5µ
M

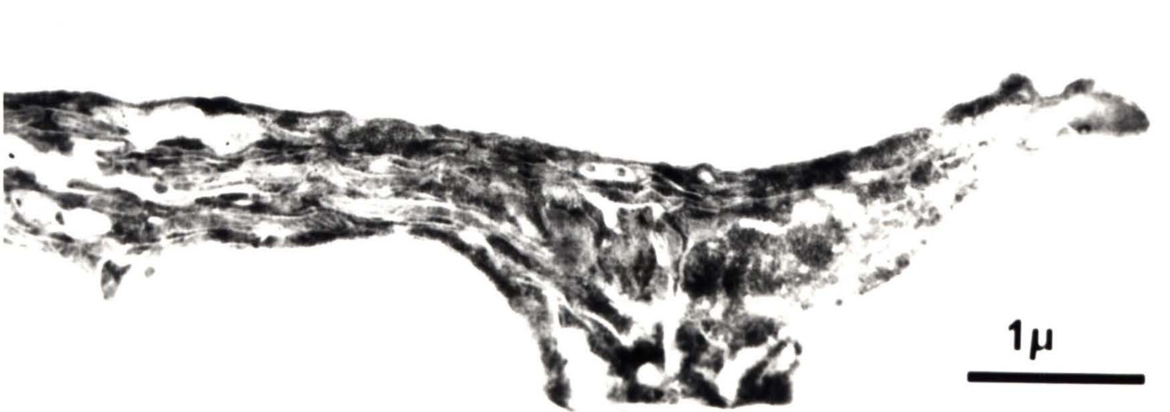
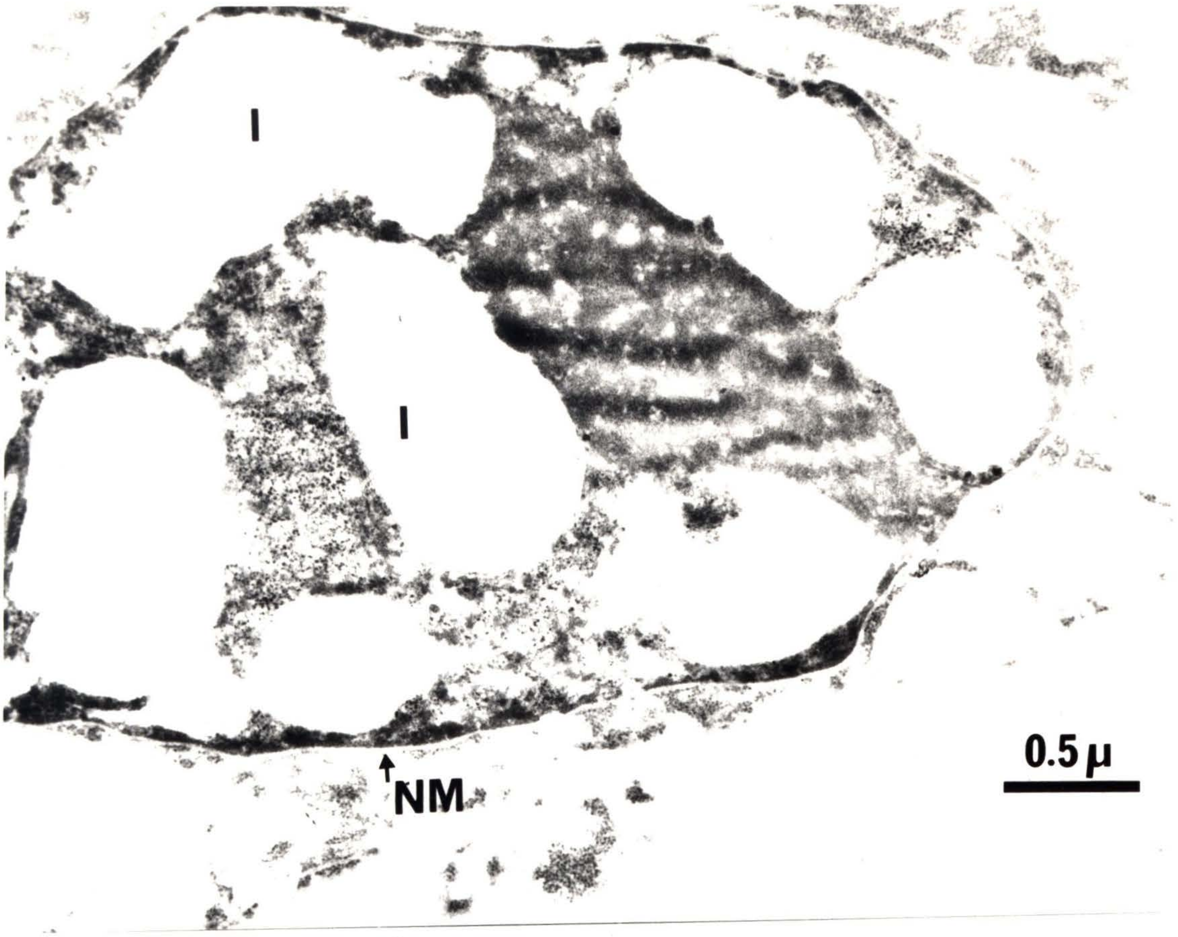

1µ

Figure 9. Freeze-substituted CHA cell as seen under the electron microscope. Several large intracellular ice crystals (I) are seen, both in the cytoplasm and in the nucleus, although none are seen to pass through the nuclear membrane (NM). The cooling rate was 20°C/min. during the freezing in the presence of 10% DMSO in CTM.
x 35,400

Figure 10. Electron micrograph of a CHA cell freeze-substituted after cooling to -196°C at a rate of 20°C/min. in the presence of 10% PVP in CTM. Although there is extensive physical distortion caused by the formation of extracellular ice, several mitochondria (M) with normal appearing cristae are present. Although the nucleus (N) is highly distorted, no obvious breaks have occurred in the nuclear membrane.
x 43,600


I
I
NM
0.5 μ

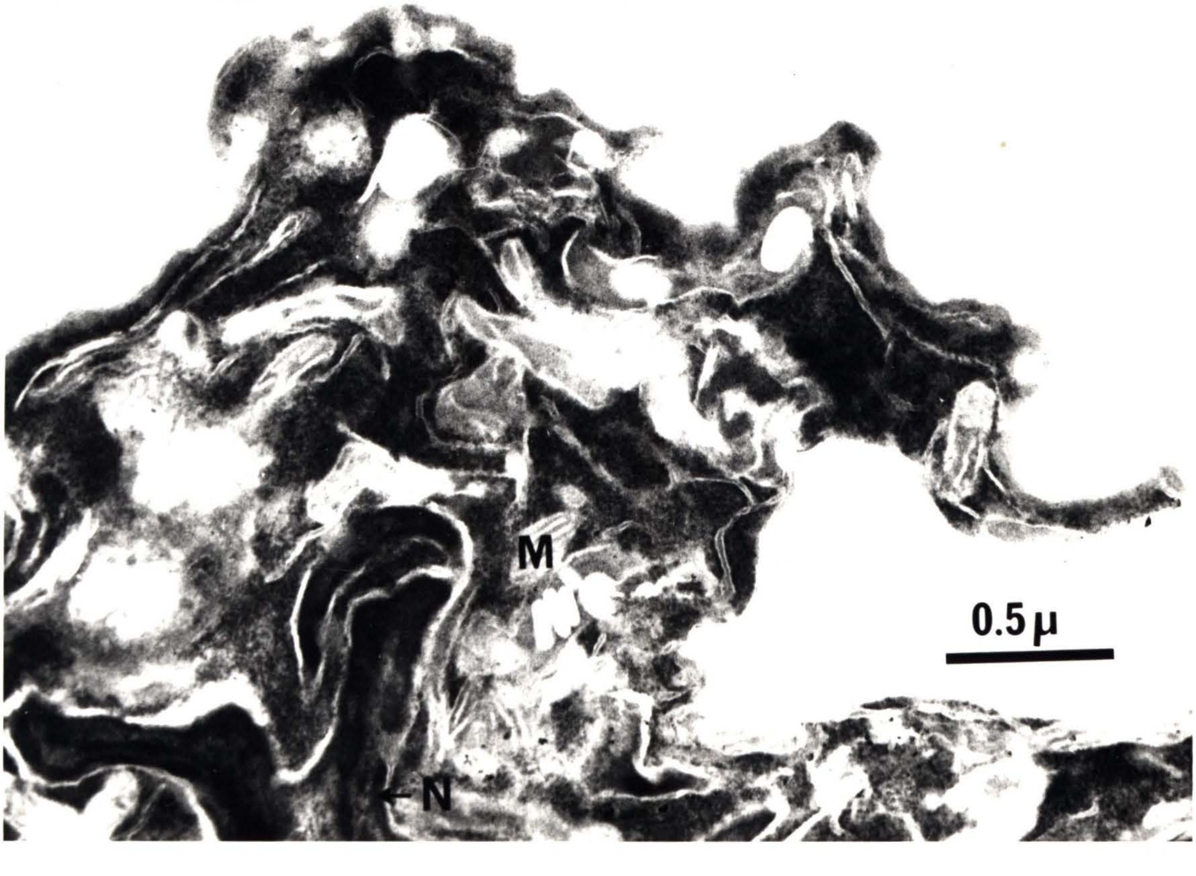

M
0.5 μ
N

Figure 11. Electron micrograph of a CHA cell after trypsinization. Several golgi apparatus are visible (G), as well as regions of endocytotic activity (E). Microtubules are visible in many regions throughout the cytoplasm, and one several microns long is visible (Mt). Mitochondria (M), lysosomes (L), and endoplasmic reticulum (ER) can be seen in the cytoplasm.
x 32,000

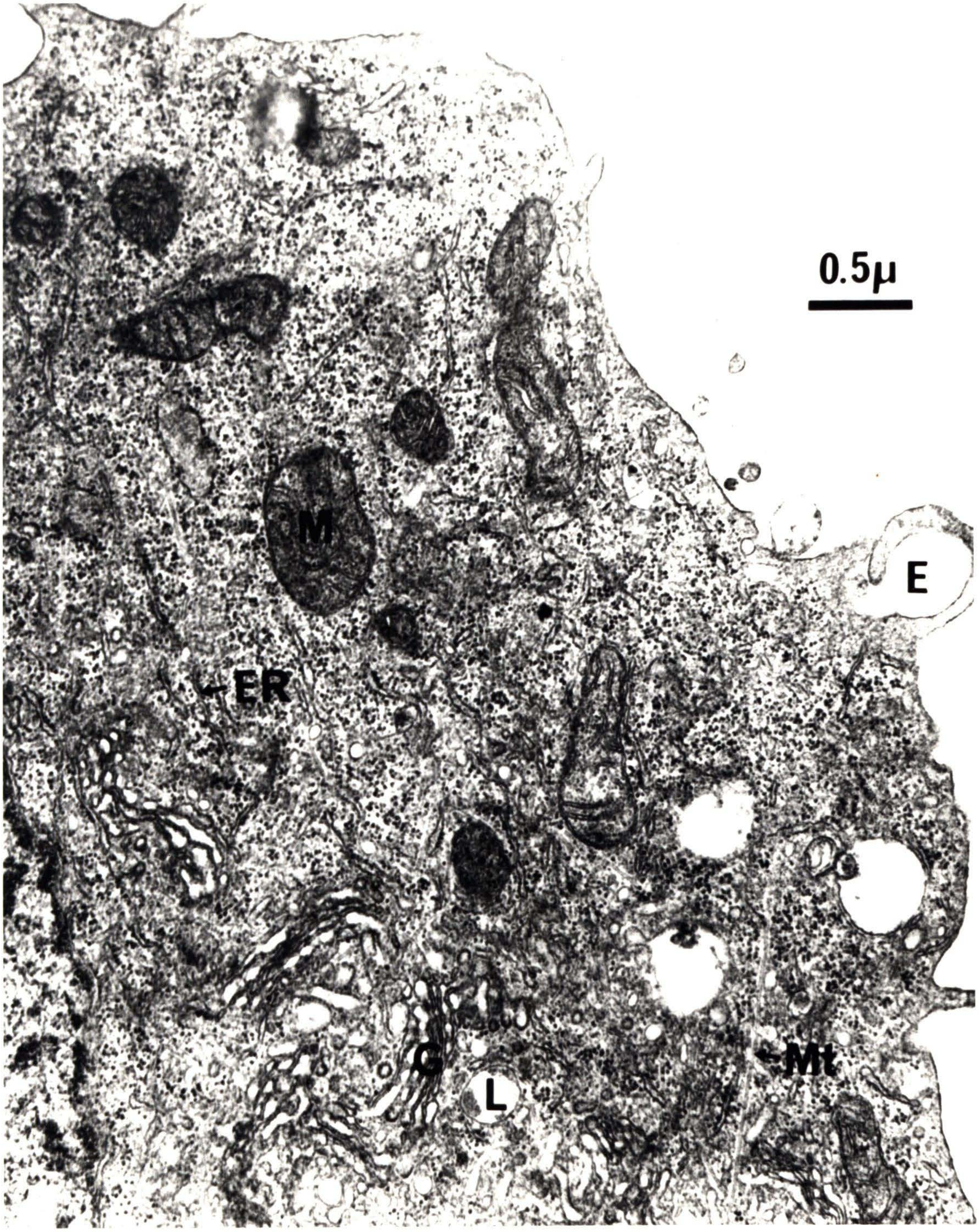

0.5µ
M
E
ER
G
L
Mt

Figure 12. Electron micrograph of a CHA cell after freezing and thawing in the presence of 10% PVP in CTM. Cytoplasmic constituents appear essentially unaltered from normal trypsinized cells, with a lysosome (L) and mitochondria (M) appearing intact. However, in one region the cell membrane has been ruptured (R) and cytoplasmic components can be seen leaking out. This cell appears multinucleate, with segments of three nucleii (N) being visible. It is possible the nucleus is merely highly folded and the plane of the section cut the nucleus in 3 regions.
Cooling rate = 20°C/min.
Warming rate = 115°C/min.
x 24,800

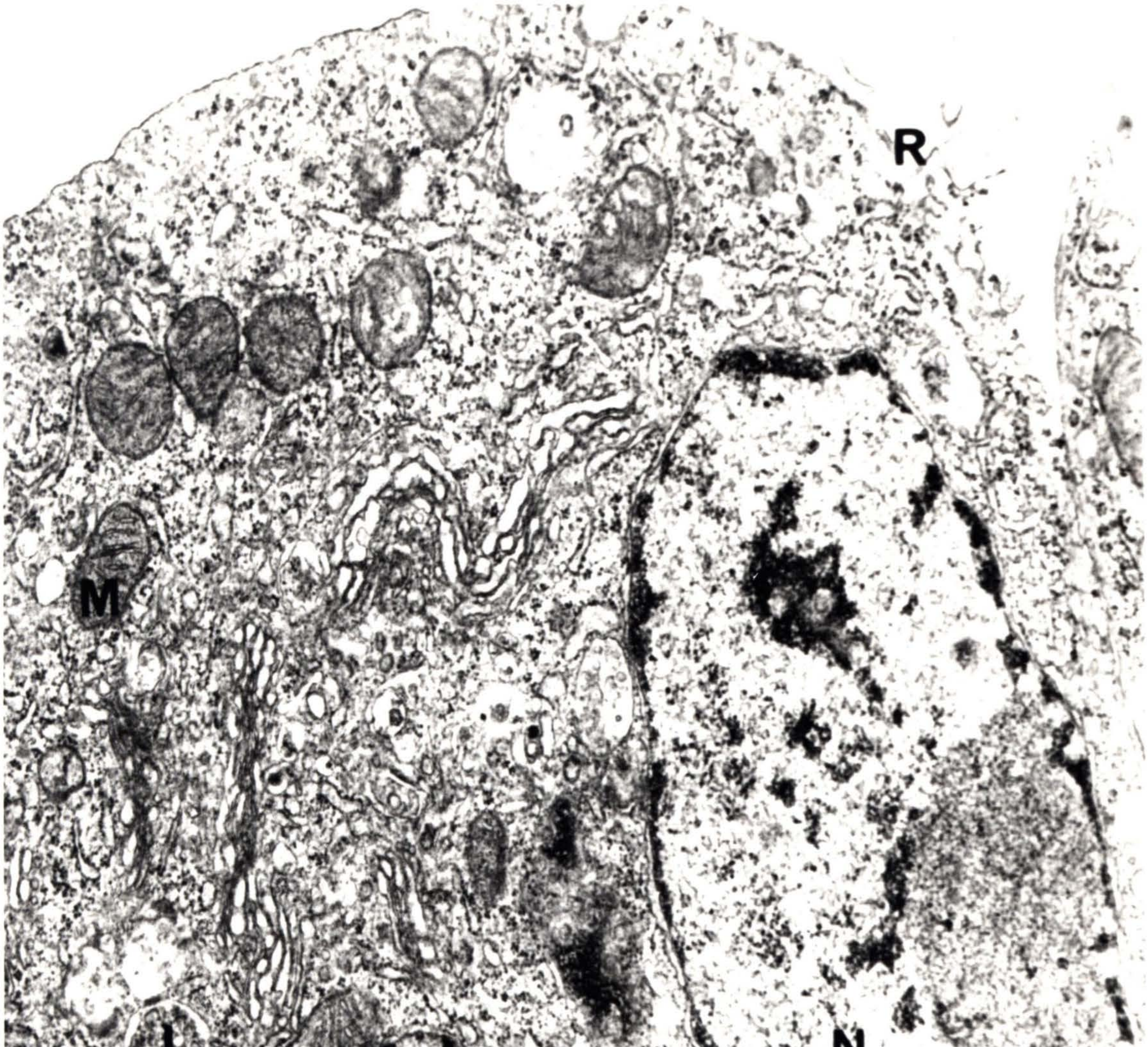

R
M
L
N
N
N
N
1µ

Figure 13. EM photograph of a CHA cell frozen and thawed in CTM without the presence of any cryoprotective agent. Although the golgi systems (G) appear normal, the mitochrondria (M) suffer massive swelling, loss of cristae, and occasional rupture (RM). Rupture of the cell membrane is also seen in several places, with the cell at the top of the photo being completely ruptured, leaving a host of cytoplasmic organelles floating free in the medium. The nucleus (N) appears to have remained intact. Short segments of microfilaments (MF) are visible in the cytoplasm.
Cooling rate = $20^oC$/min.
Warming rate = $115^oC$/min.
x 22,000

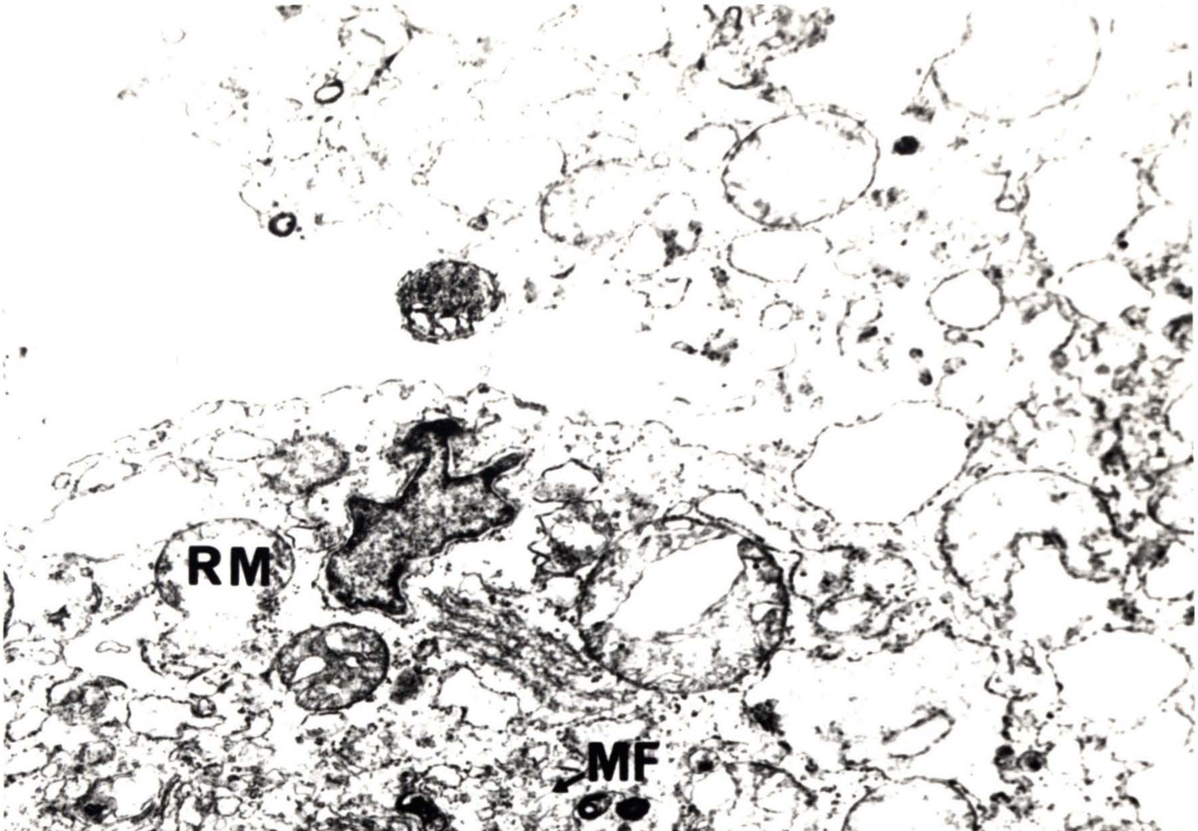
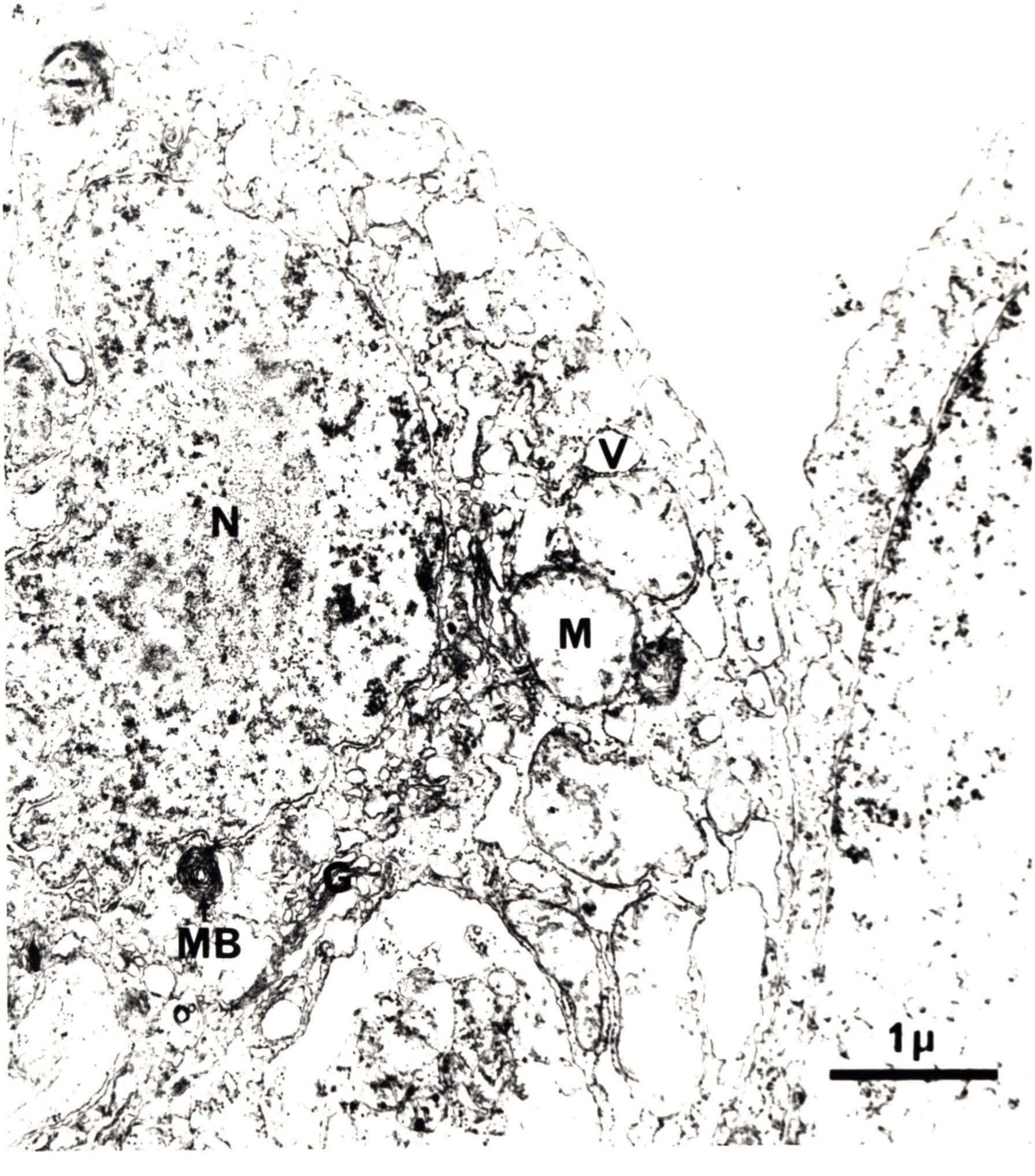

RM
MF
G
M
N
1µ

Figure 14. Electron micrograph of CHA cells frozen and thawed in CTM without any protective agent. Although the external cell membrane appears largely intact, the mitochondria (M) appear swollen and the cytoplasm contains a large number of vesicles (V). Golgi (G) and the nucleus (N) are also visible. A myelin body (MB) which is formed from the breakdown of membrane systems in the cytoplasm, can be seen.
Cooling rate = 20°C/min.
Warming rate = 115°C/min.
x 24,500


V
N
M
G
MB
1μ

of the cell membrane is seen. Typical cells frozen without protective additive appear as in Figure 13, 14. In Figure 13 the cell membrane of one cell has suffered total breakdown, while that of the other cell is largely intact but still possesses regions where breakdown has occurred. The mitochondria of these cells appear swollen, lacking normal cristae, and, in some cases, ruptured. In Figure 14 the cytoplasm is seen filled with vesicles and mitochondria lacking normal cristae or the typical dense matrix. From these observations, it would appear that damage to the cell from freezing and thawing occurs not at any particular site but on the cell as a whole. At this stage, however, it cannot be shown whether the cytoplasm looks abnormal due to the loss of the normal characteristics of the cell membrane or whether the overall appearance of the organelles are poor due to a direct action of freezing and thawing upon them. Upon observation of large numbers of cells which were subjected to a treatment during which they were almost certainly destroyed by freezing and thawing, the most common observation made on these cells was that of an abundance of vesicles forming in the cytoplasm, and the swelling of organelles such as mitochondria, could occur under conditions of altered osmotic balance of the cell.

Experiments were done to determine the survival of CHA cells after having been cooled to -196$^{\circ}$C and warmed to 37$^{\circ}$C under a variety of conditions. Several different polymers, including PVP K30, PVP C15, HES, and dextran 40, all present at a concentration of 10% in the medium, were tried and found to be effective in lessening the extent of damage due to freezing and thawing (Figure 15) HES was found to be the most effective cryoprotective agent, yielding 79% survival of the cells under this particular freeze-thaw procedure. PVP K30 also showed considerable protective ability, with PVP C15 and dextran 40 showing somewhat less protection. Other recent studies (Ashwood-Smith, personal communication) have shown the class of polymers known as Pluronics to be effective in lessening freeze-thaw injury in the CHA cell system. In fact, we have not discovered a water soluble polymer which will not give some degree of cryoprotection to the CHA cells in our test system.

Statistical analysis of these and following results was limited to determining the standard deviation of the values obtained for each treatment. It was felt that this was sufficient since the difference between protected and unprotected samples were usually large. In a few random experiments chosen to determine the significance of the increased survival obtained, a paired t-test gave values of $P<0.01$ in all cases with most samples showing $P<0.001$. Throughout the study differences of only a few percentage points were considered insignificant.

Throughout the freezing experiments it was noted that considerable variation in the survival values from week to week occurred under seemingly identical conditions. The behaviour within each self-contained experiment was always predictable, which is to say if the experiment shown in Figure 15 was repeated a month later, HES would show slightly better survival that PVP K30 and survival achieved with the use of dextran would be less than the other polymers, but the maximum survival obtained might vary from 80% to 60%. An attempt was made to find and eliminate the sources of such variation.

It has been shown (Becker, unpublished results) that the endocytotic activity of CHA cells varied considerably depending on the age of the culture. An experiment was done to determine whether the age of the culture, which affects the metabolic activities of the cell, could affect the survival values obtained. The results of this experiment (Figure 16), showing the survival obtained after freezing cultures of various age in the presence of PVP, indicates that the age of the culture is not a source of variation in the survival obtained.

It is routine procedure in some labs to seed the sample at slightly below the freezing point of the solution in order to prevent supercooling and thereby create more reproducible conditions during cooling. It was found (Figure 17) that higher survival was obtained in the absence of seeding, both in the case of slow and rapid cooling. This variation may be due to the actual seeding process, or to the increased amount of manipulation and time required to process the vials in which seeding was done. Throughout the experiments it was noted that the longer a particular procedure took, the poorer the recorded survival would be, quite

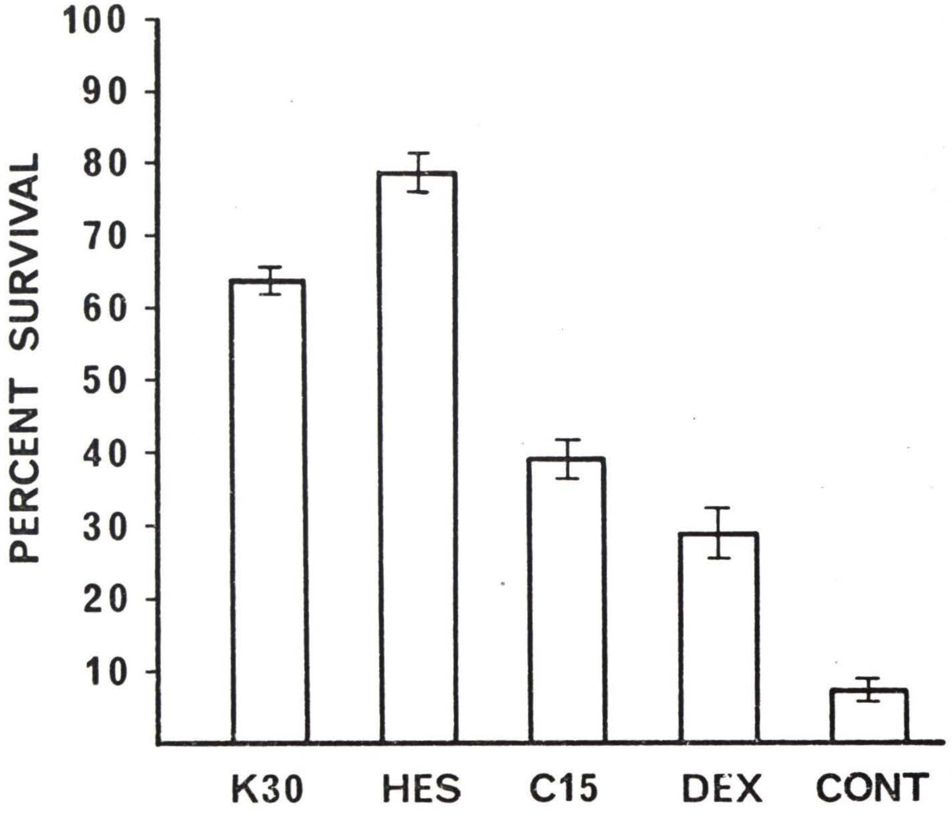
Figure 15. Survival of CHA cells after freezing and thawing in CTM containing various polymeric protective agents. Vertical bar represents 1 standard deviation.

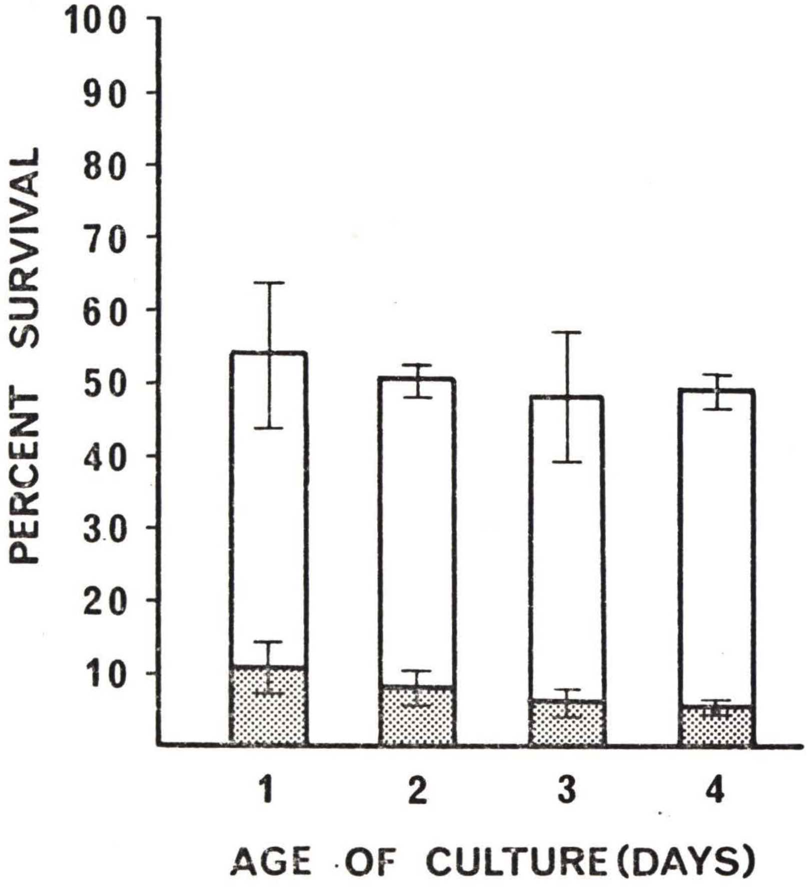
Cooling rate = 20°C/min.
Warming rate = 115°C/min.
K30 = 10% PVP K30, $\bar{M}_n$ = 40,000
HES = 10% HES, $\bar{M}_n$ = 70,000
C15= 10% PVP C15, $\bar{M}_n$ = 10,000
DEX = 10% Dextran 40, $\bar{M}_n$ = 40,000
CONT.= Unprotected control

Figure 16. Effect of age of culture on survival of CHA cells after freezing and thawing in CTM containing 10% PVP K30. Vertical bar represents 1 standard deviation.

▨ = unprotected control

Cooling rate = 20°C/min.
Warming rate = 115°C/min.


PERCENT SURVIVAL
100
90
80
70
60
50
40
30
20
10
K30
HES
C15
DEX
CONT


PERCENT SURVIVAL
100
90
80
70
60
50
40
30
20
10
1
2
3
4
AGE OF CULTURE (DAYS)

likely due to the fact that the cells attach rapidly to the glass vial and therefore do not get plated into the flasks for final counting. At any rate, it was decided that any gain in consistency due to seeding would be lost in the resulting lower counts received for survival, and subsequently seeding was abandoned.

The cooling and warming rates have been shown to have a profound effect on the survival of frozen and thawed cells (66, 65), the optimal rates varying widely depending on the cell system. Experiments were done to find a suitable cooling and warming rate for this system within the limited range attainable without the use of special techniques or equipment. Through the range of cooling rates studies (2.6°C/min. to 260°C/min.) the survival increased as the cooling rate decreased, a relationship which held true both for survival in the presence of PVP (Figure 18, 19) and HES (Figure 20). The reader will note the value quoted for the ultra fast cooling rate (150°C/min.) is considerably lower than that quoted for the fast cooling rate (260°C/min.). The discrepancy arises from the definition of the cooling rate being the average rate betwee 0°C and -150°C. The fast cooling rate is achieved by immersing the vials in liquid nitrogen. At near-zero temperatures of the sample vial, a vapour barrier forms between the vial and the nitrogen which

Figure 17. Effect of seeding on survival of CHA cells after freezing and thawing in CTM containing 10% PVP K30. Vertical bar represents 1 standard deviation.

Warming rate = 115°C/min.

□ = Cooling rate 20°C/min.

▒ = Cooling rate 260°C/min.

Figure 18. Effect of various cooling rates on the survival of CHA cells frozen and thawed in CTM containing 10% PVP K30. Vertical bar represents 1 standard deviation.

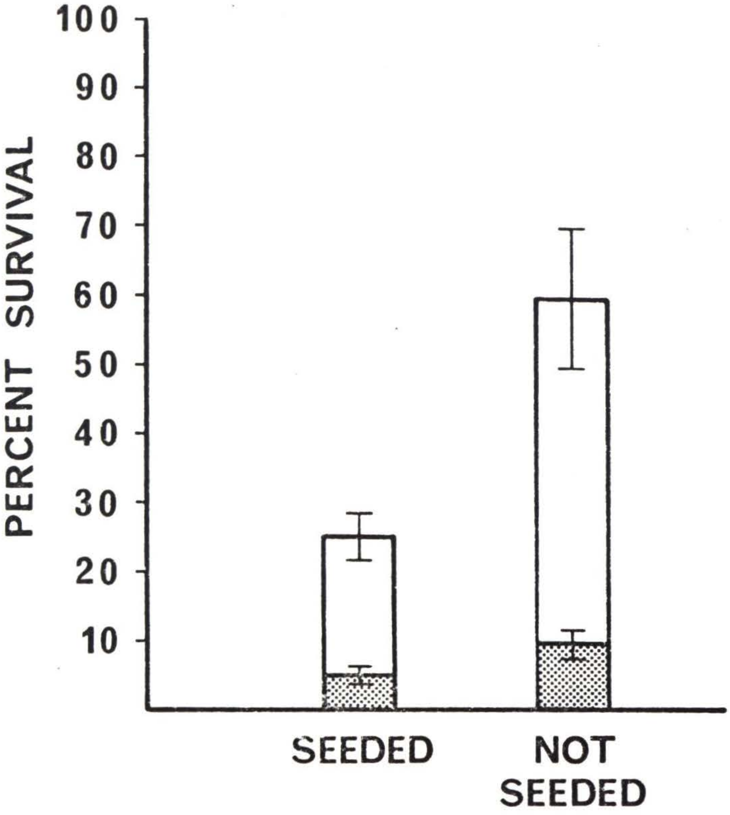
Slow cooling = 20°C/min.

Fast cooling = 260°C/min.

Ultra fast cooling = 150°C/min.

Warming rate = 115°C/min

▒ = unprotected control


PERCENT SURVIVAL
100
90
80
70
60
50
40
30
20
10
SEEDED
NOT SEEDED

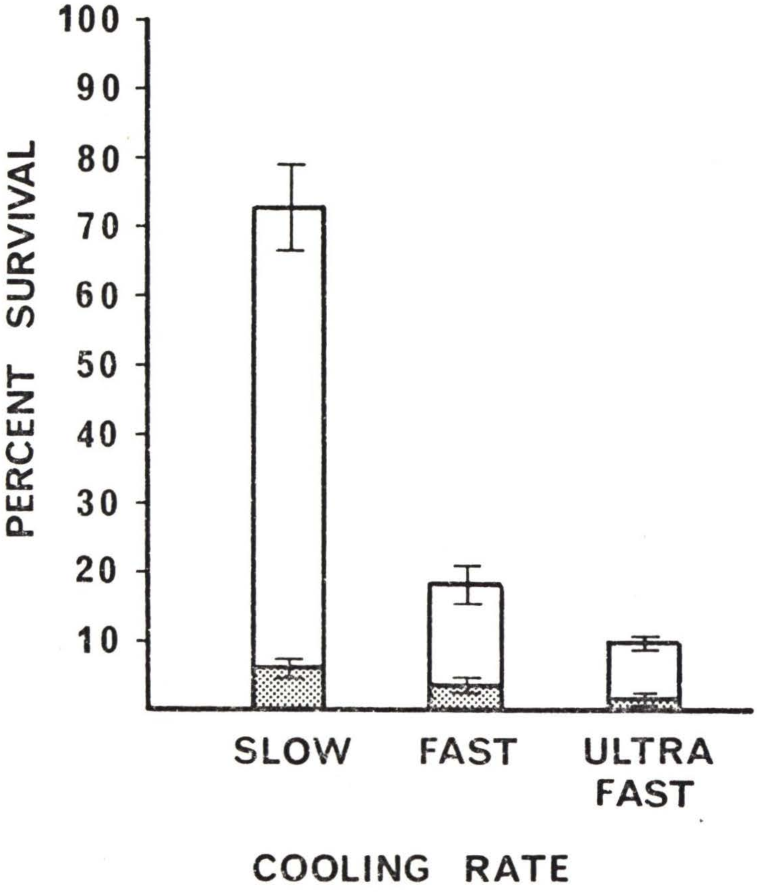
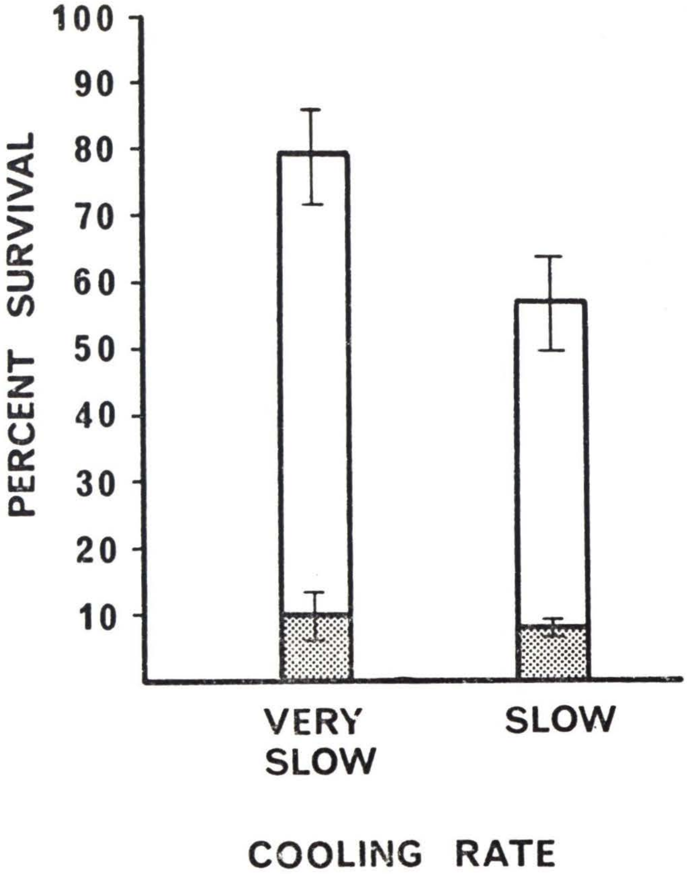
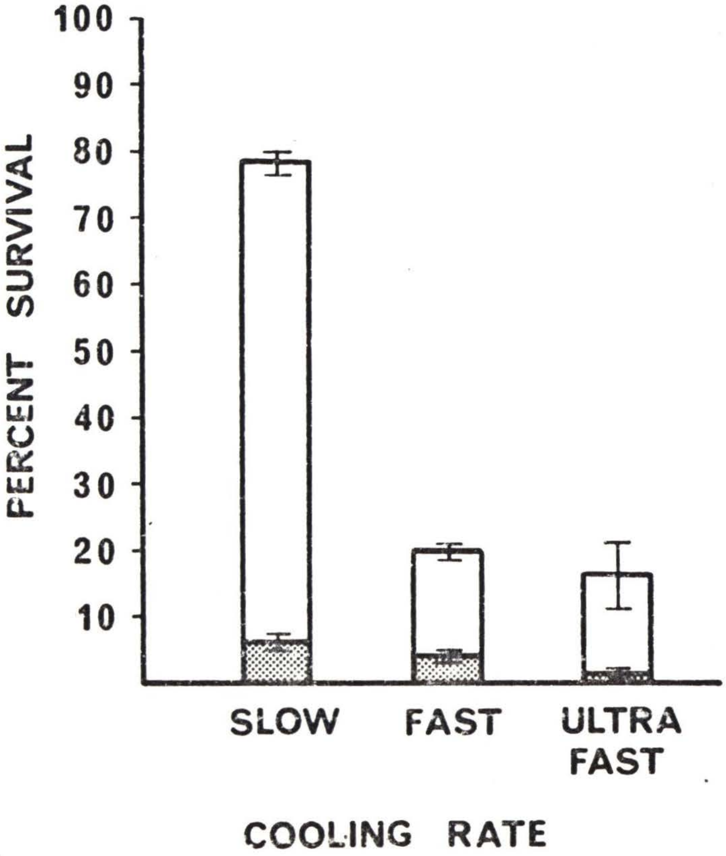

PERCENT SURVIVAL
100
90
80
70
60
50
40
30
20
10
SLOW
FAST
ULTRA FAST
COOLING RATE

Figure 19. Effect of cooling rates on the survival of CHA cells frozen and thawed in CTM containing 10% PVP K30. Vertical bar represents 1 standard deviation.
Very slow cooling = 2.6°C/min.
Slow cooling = 20°C/min.
░ = unprotected controls

Warming rate = 115°C/min.

Figure 20. Effect of various cooling rates on the survival of CHA cells frozen and thawed in CTM containing 10% HES. Vertical bar represents 1 standard deviation.
Slow cooling = 20°C/min.
Fast cooling = 260°C/min.
Ultra fast cooling = 150°C/min.
Warming rate = 115°C/min.
░ = unprotected control


100
90
80
70
60
50
40
30
20
10
PERCENT SURVIVAL
VERY SLOW
SLOW
COOLING RATE


100
90
80
70
60
50
40
30
20
10
PERCENT SURVIVAL
SLOW
FAST
ULTRA FAST
COOLING RATE

hampers heat exchange. The initial cooling rate is thus fairly slow. As the vial cools the vapour barrier breaks down and cooling is very rapid through the range -100 to -150°C. For the ultra fast cooling rate, vials were immersed in isopentane at -160°C. At this temperature, isopentane is a viscous liquid with a high heat capacity. No vapour barrier is formed and initial cooling, through the range where the bulk of the water is frozen, is very rapid. However, through the range -100 to -150°C, the temperature of the fluid in contact with the walls of the vial is much higher than with liquid nitrogen, so that cooling proceeds more slowly, resulting in a overall cooling rate of only 150°C/min. The cooling in isopentane was termed ultra-fast after the terminology commonly used by other workers in the field.

Two techniques were used to warm the vials after freezing. The vials were either left to warm at room temperature (slow warming, 6.5°C/min.) or shaken in a 37°C water bath (fast warming, 115°C/min.). The fast warming was found to give better survival (Figure 21) in agreement with other workers who have found rapid warming to aid cell survival in every case analysed (46, 65). Recent work in our lab using rapid and uniform thawing by the use of a microwave oven has shown potential for still higher survival values (121).

The medium in which the cells are frozen also has a profound effect on the survival after freezing and thawing. The survival of CHA cells frozen in Hanks balanced salt solution (HBSS) with 10% PVP was found to be only 8% or only marginally greater than the survival in CTM containing serum without the addition of PVP, and no survival at all was found in HBSS without protective additive (Figure 22). It may be that the serum present in the CTM is largely responsible for the increase in survival obtained through the use of this medium, since CTM without serum was found to give an effect much like that obtained using HBSS. Serum albumin has been shown to be a good cryoprotective agent in some cases (21, 36), and it is thought that the albumin may be responsible for the increased survival obtained when serum is present in the medium.

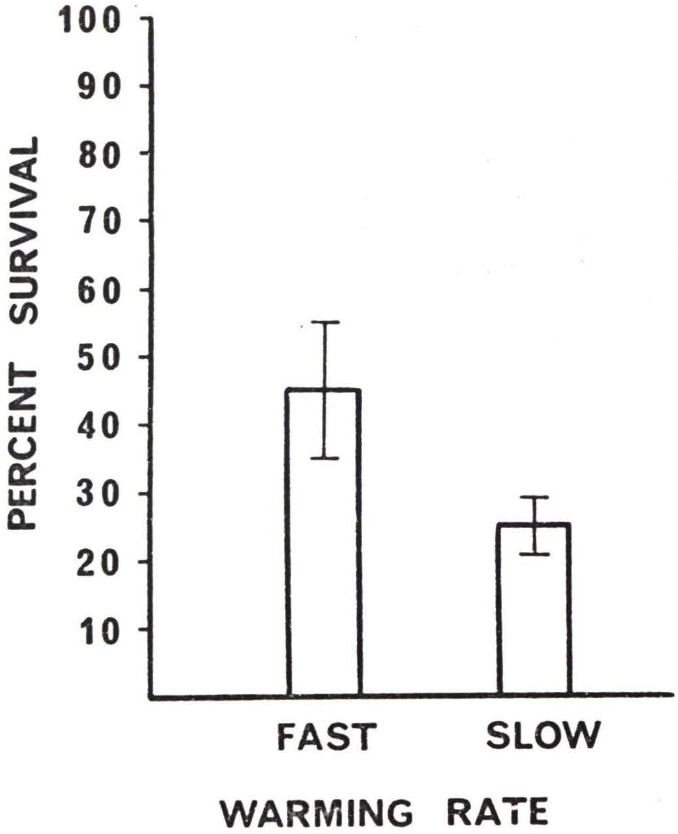
In some cases (8, 10) it has been found that dialysed, freeze-dried PVP acts much differently as a cryophylactic agent than does the non-dialysed PVP. The dialysis membrane used in this study (Seamless Cellulose Dialyser Tubing, Fisher Scientific Co. Ltd.) has a theoretical molecular weight cutoff of about 12,000 daltons, so that any contaminants of low molecular weight components would be removed. The non-dialysed sample was found to give slightly better protection than the dialysed sample in CHA cells, although the difference was very small (Figure 23). Non-dialysed PVP was used throughout the study after it became clear that the dialysed sample was in no way superior to the non-dialysed sample.

The molecular weight of the polymer was found to influence survival values (Figure 24) as well as the type of polymer. PVP C15, with a molecular weight average of 10,000, brought about 37% survival whereas PVP K30 ($M_n$ = 40,000) and K90 ($M_n$ = 360,000) were essentially the same in their cryoprotectivity, showing 57 and 61%

Figure 21. Influence of warming rate on survival of CHA cells frozen and thawed in CTM containing 10% PVP K30. Vertical bar represents 1 standard deviation.
Cooling rate = 20°C/min.
Fast warming = 115°C/min.
Slow warming = 6.5°C/min.

Figure 22. Survival of CHA cells after freezing and thawing in different media containing 10% PVP K30. Vertical bar represents 1 standard deviation.
Cooling rate = 20°C/min.
Warming rate = 115°C/min.
▒ = unprotected control

CTM = complete tissue culture medium
HBSS = Hanks balanced salt solution


PERCENT SURVIVAL
100
90
80
70
60
50
40
30
20
10
FAST
SLOW
WARMING RATE

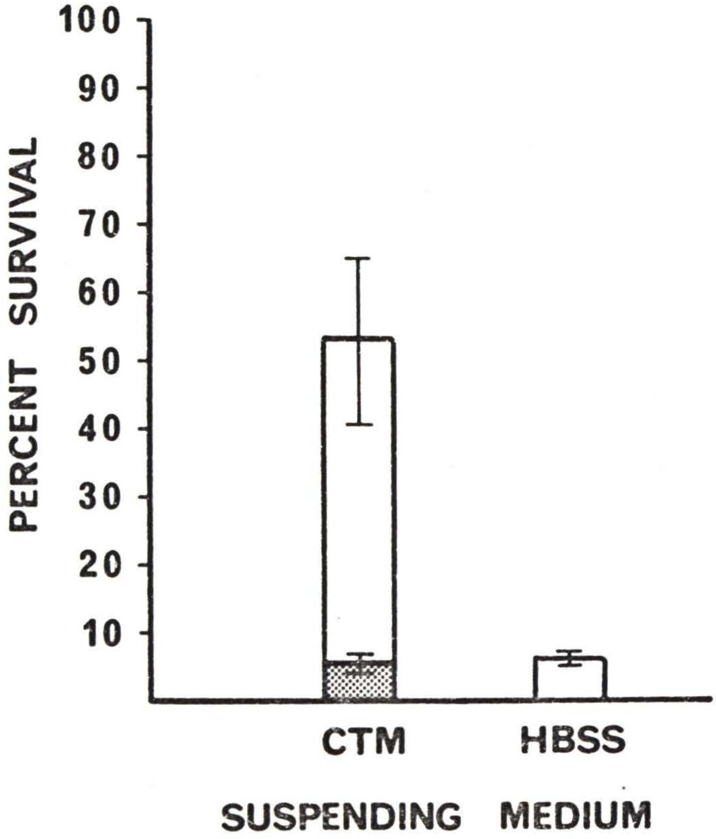

PERCENT SURVIVAL
100
90
80
70
60
50
40
30
20
10
CTM
HBSS
SUSPENDING MEDIUM

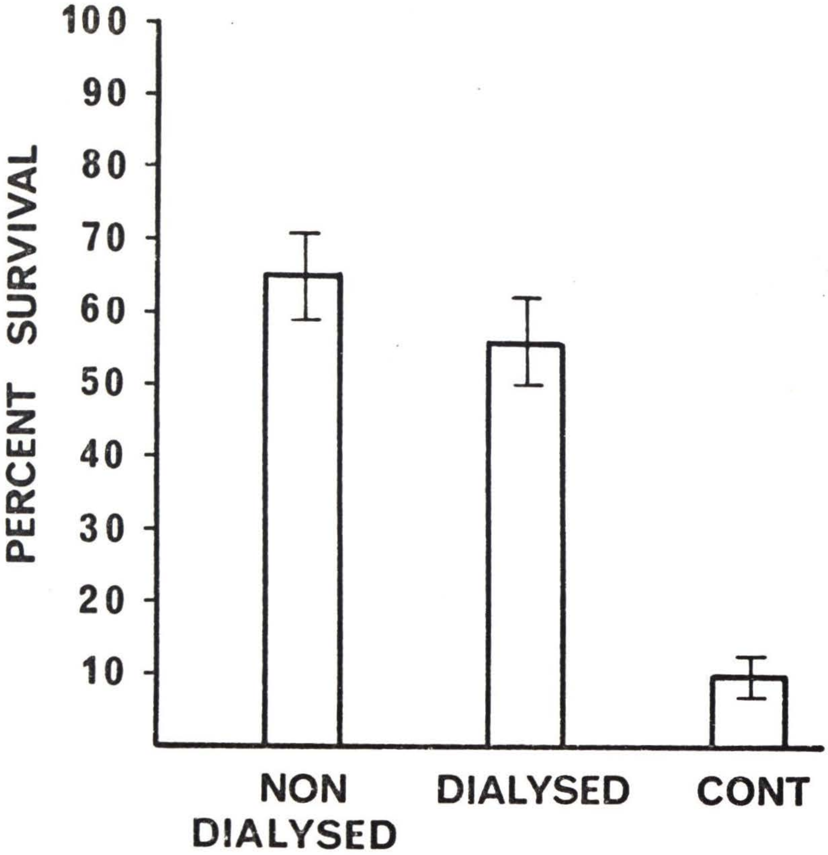
Figure 23. Effect of dialysing on the ability of 10% PVP K30 in CTM to protect CHA cells from freeze-thaw injury. Vertical bar represents 1 standard deviation.
Cooling rate = 20°C/min.
Warming rate = 115°C/min.
CONT. = Unprotected control

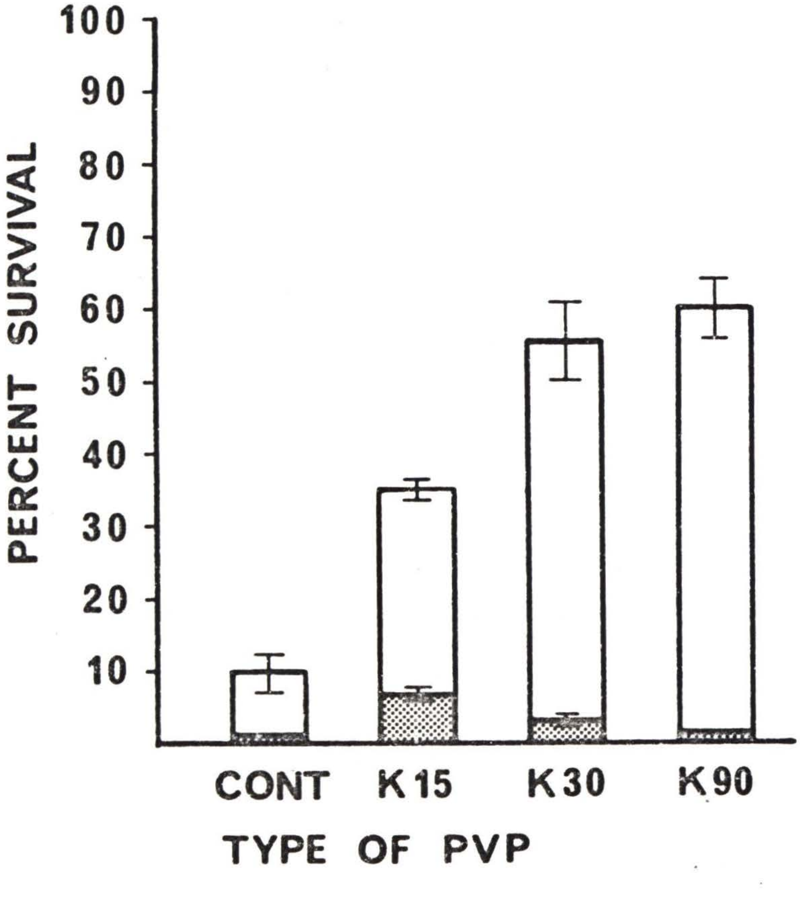
Figure 24. Survival of CHA cells after fast and slow cooling in several different molecular weights of PVP in CTM. Vertical bar represents 1 standard deviation.

▭ = 20°C/min. cooling rate

▒ = 260°C/min. cooling rate

Warming rate = 115°C/min.
K15 = 10% PVP, $\bar{M}_n$ = 10,000
K30 = 10% PVP, $\bar{M}_n$ = 40,000
K90 = 8% PVP, $\bar{M}_n$ = 360,000
CONT. = Unprotected control


PERCENT SURVIVAL
100
90
80
70
60
50
40
30
20
10
NON DIALYSED
DIALYSED
CONT


PERCENT SURVIVAL
100
90
80
70
60
50
40
30
20
10
CONT
K 15
K 30
K 90
TYPE OF PVP

survival, respectively. The nature of the repeating group is clearly not the only factor determining the cryoprotective ability of a polymer.

Most compounds used for cryobiological preservation, although of low toxicity, do have an effect on the growth of the cells if the cells are subjected to the preservation agent for extended periods of time. It is therefore desirable to a use as low a concentration of the cryophylactic agent as possible which will still give acceptable survival values. Accordingly, the variation of the survival with the concentration of PVP present during freezing and thawing was determined (Figure 25). It was found that 1% PVP gave no protection from freeze-thaw damage at all, whereas 5% PVP raised the survival value from 9 to 48% and 10% PVP increased survival still further to 59%. Although PVP was routinely used at a concentration of 10%, in other circumstances where the amount of PVP present in the medium after thawing was critical, a concentration of 5% would give almost as much survival with a 50% decrease in PVP.

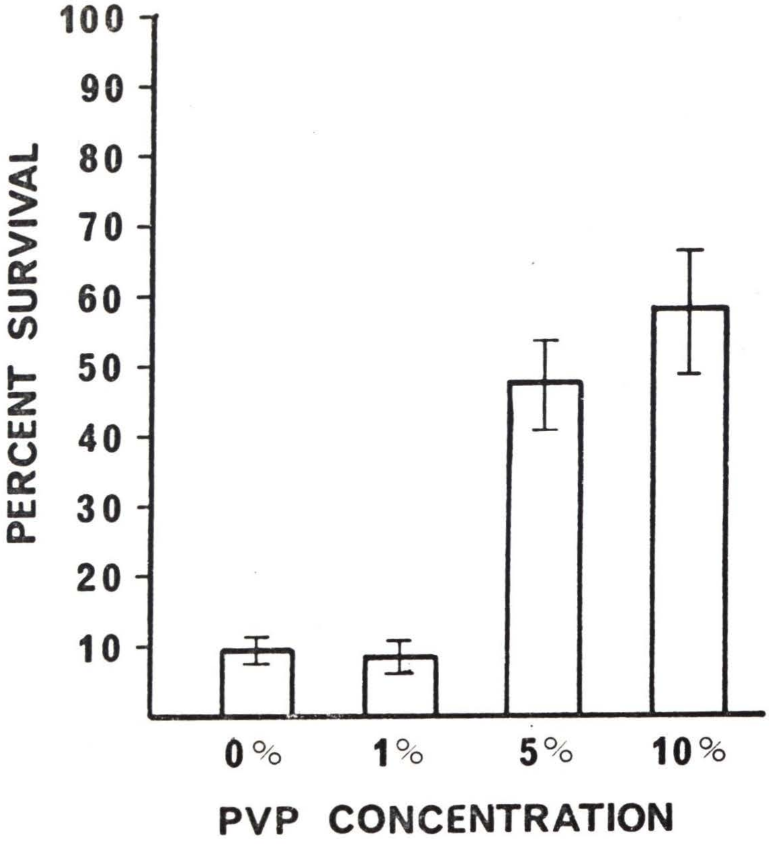
It has been suggested (25) that polymers act in a way similar to low molecular weight compounds, which are thought to protect cells through their colligative properties. For comparitive purposes, a common low molecular weight cryoprotective agent, DMSO, was tested for its protective ability under circumstances similar to those under which the polymers were used. Figure 26 shows the variation in survival with various concentrations of DMSO. 1.25% DMSO was found to raise the survival value from the control level of 9% to

45%, and further increase in concentration of DMSO resulted in increased survival up to a value of 82% for 10% DMSO. In another experiment, an attempt was made to ascertain whether the mechanisms of protection with polymers and with PVP were the same or different by observing the survival when the two protective agents were both present at the same time (Figure 27). Used independently, PVP gave 49% protection and DMSO 71%. When both were present, the survival jumped to 92%. Assuming the two protective mechanisms were completely independent, 85% would have survived under the combined effect of the two agents. These results seem to indicate the two protective agents must be working through different mechanisms. However, knowledge of the variability of results obtained from experiment to experiment makes conclusions based on differences of a few percentages uncertain. In Figure 26 it was seen that 2.5% DMSO brought about survival of 57%. Twice this amount of DMSO brought about 76% survival, whereas if the addition of the second amount of DMSO had acted independently to the first, 81% should have survived. The results clearly do not come from independent cases, yet fall within the experimental error when calculated as if they were independent. It should be pointed out that survival with polymers + DMSO never exceeded that which could be achieved with DMSO alone. A more precise system would be required to clarify the interaction of the two protectants beyond doubt.

Persidsky and Richards (79) have suggested that the polymeric protective agents such as PVP may be able to gain entry to the

Figure 25. Protection of CHA cells afforded by different concentrations of PVP K30 in CTM. Vertical bar represents 1 standard deviation.
Cooling rate = 20°C/min.
Warming rate = 115°C/min.

Figure 26. Protection of CHA cells afforded by different concentrations of DMSO in CTM. Vertical bar represents 1 standard deviation.
Cooling rate = 20°C/min.
Warming rate = 115°C/min.


PERCENT SURVIVAL
100
90
80
70
60
50
40
30
20
10
0%
1%
5%
10%
PVP CONCENTRATION

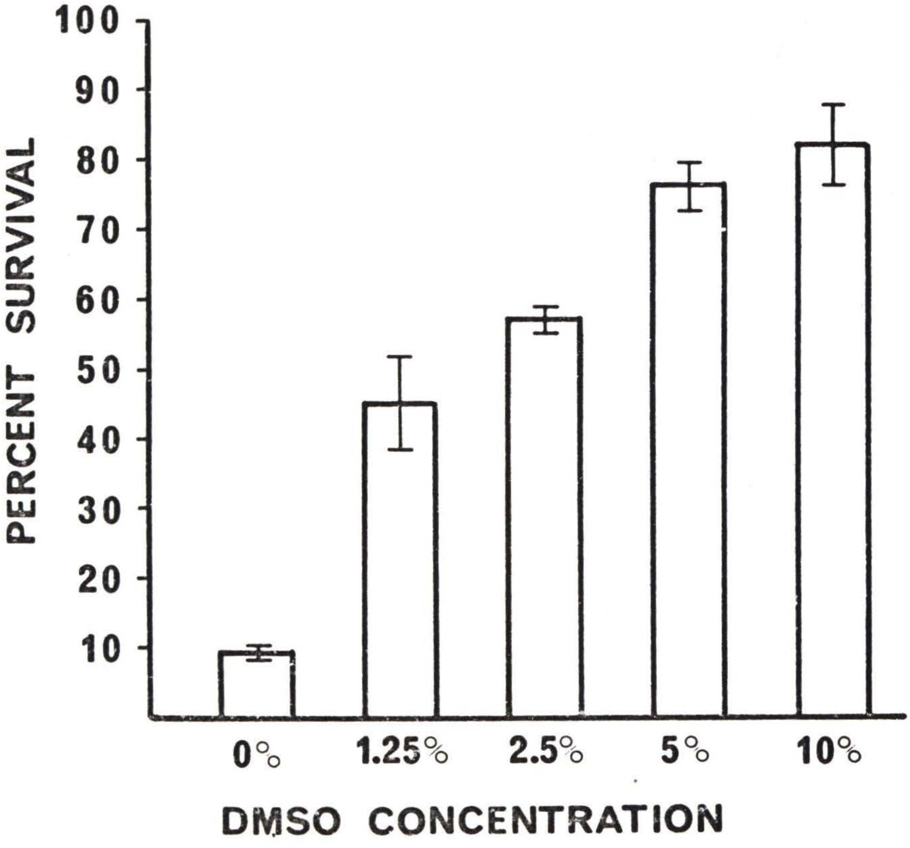
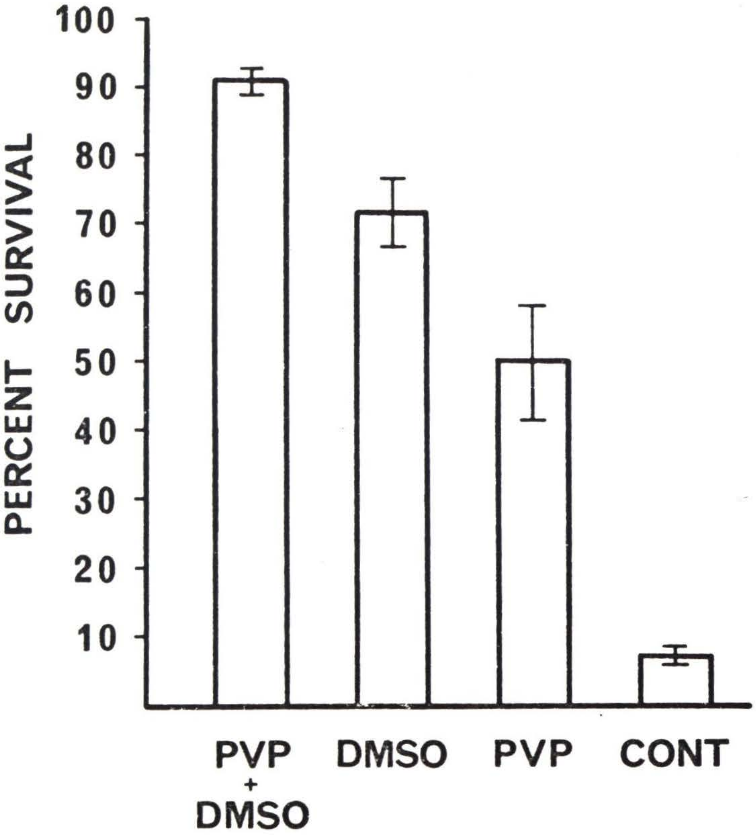
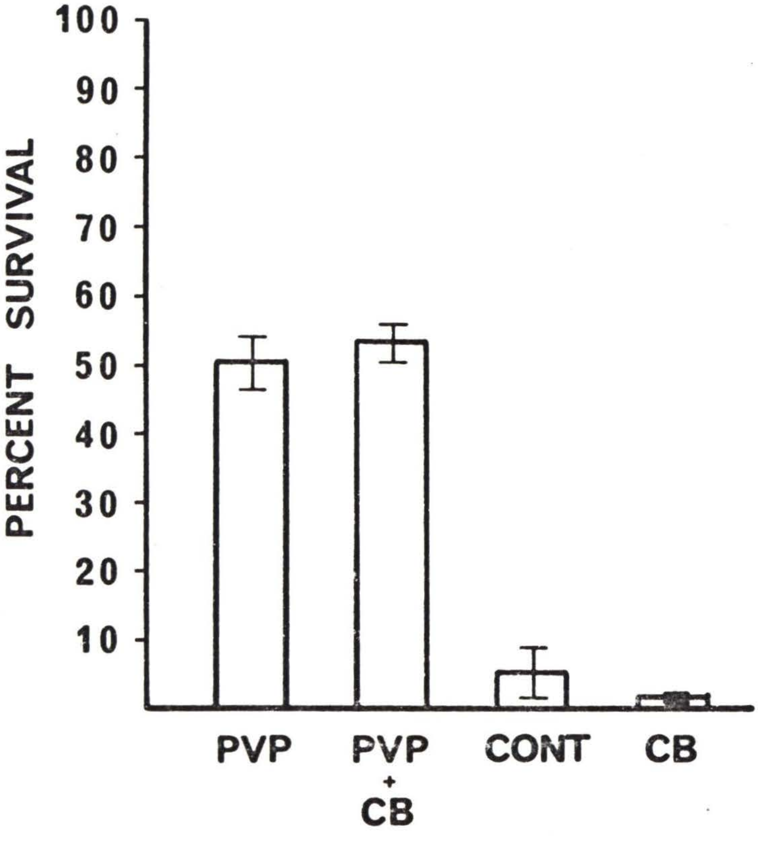

PERCENT SURVIVAL
100
90
80
70
60
50
40
30
20
10
0%
1.25%
2.5%
5%
10%
DMSO CONCENTRATION

Figure 27. Interaction of PVP and DMSO in protecting CHA cells after freezing and thawing in CTM with the indicated additives. Vertical bar represents 1 standard deviation.
Cooling rate = 20°C/min.
Warming rate = 115°C/min.
PVP + DMSO = 10% PVP K30 + 2.5% DMSO
DMSO = 2.5% DMSO
PVP = 10% PVP K30
CONT. = Unprotected control

Figure 28. Effect of cytochalasin B on protection of CHA cells in CTM in the presence of 10% PVP K30. Vertical bar represents 1 standard deviation.
Cooling rate = 20°C/min.
Warming rate = 115°C/min.
PVP = 10% PVP K30
PVP + CB = 10% PVP K30 + 20 μg/ml cytochalasin B
CONT. = Unprotected control
CB = Survival in CTM with 20 μg/ml cytochalasin B


100
90
80
70
60
50
40
30
20
10
PERCENT SURVIVAL
PVP + DMSO
DMSO
PVP
CONT

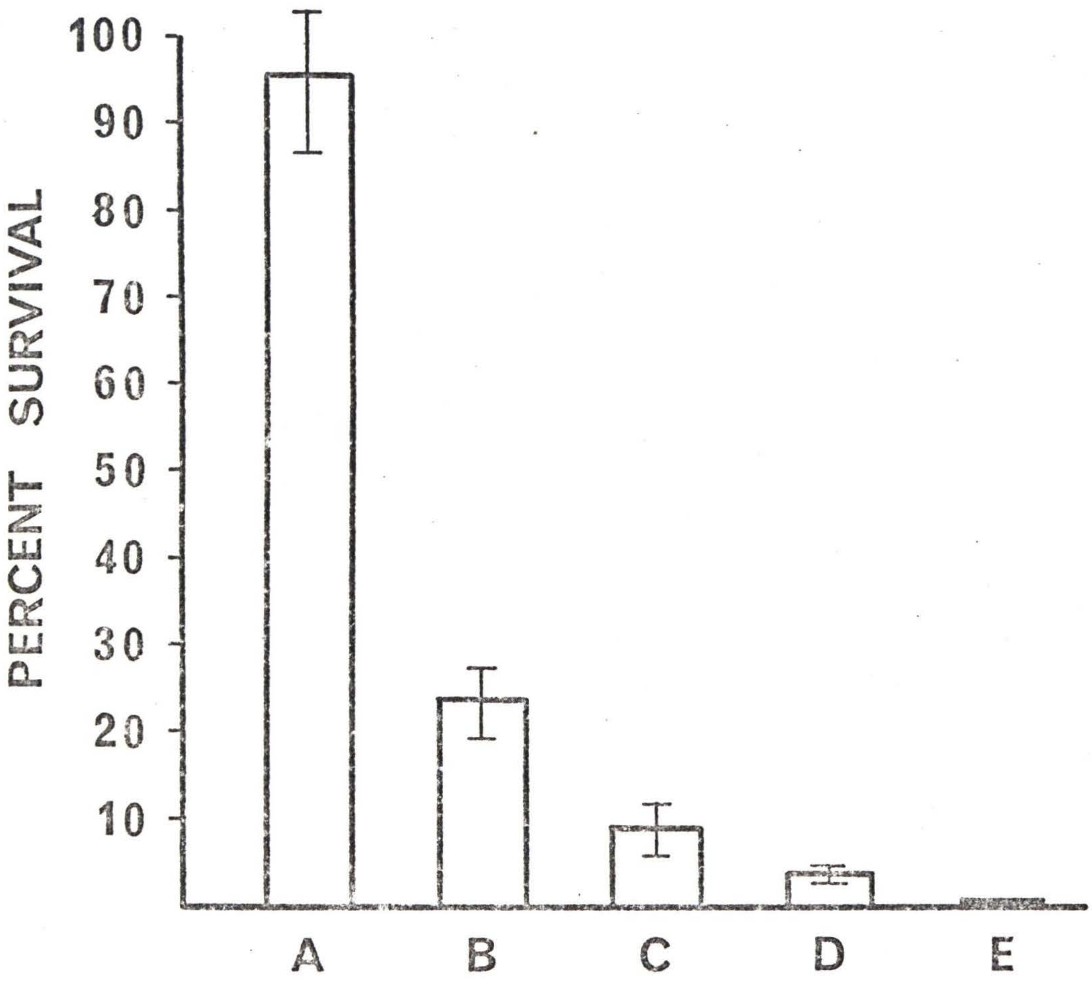

100
90
80
70
60
50
40
30
20
10
PERCENT SURVIVAL
PVP
PVP + CB
CONT
CB

interior of the cell and thereby exert their effect intracellularly. To test the possibility of PVP being taken into the cell interior during the short time in which the cells are suspended in the PVP-CTM solution before freezing, the effect of the endocytotic inhibitor cytochalasin B (I.C.I. Research Laboratories, Cheshire, England) on cell survival following freezing and thawing was determined (Figure 28). Cytochalasin B is known to disrupt the microfilaments of the cell, and since microfilaments are probably the contractile elements driving membrane invagination (124), endocytosis is inhibited (4). No change in survival was noted after freezing in CTM with 10% PVP with or without cytochalasin B, indicating that endocytosis is not important as a factor in the protection of cells by polymers.

There has been a brief report on the ability of PVP to decrease the amount of freeze-thaw injury if unprotected cells are placed in a solution of PVP immediately upon thawing (69). Several attempts were made to achieve some degree of post-thaw protection under a variety of circumstances, one of which is shown in Figure 29. Cells were frozen in CTM with or without PVP and then plated into petri dishes containing CTM with or without PVP. The PVP in the petri dishes did not appear to impair cell attachment since 96% of the unfrozen cells plated into CTM containing PVP were able to form colonies. Cells frozen in the presence of PVP showed decreased survival when plated into CTM containing PVP, as did cells frozen with no protective agent. Time lapse photography showed the rate of

Figure 29. Effect of post-thaw incubation in PVP on survival of CHA cells frozen in CTM with or without protective additives. Vertical bar represents 1 standard deviation.

A = unfrozen control incubated for two hours in PVP before final incubation
B = sample frozen in 10% PVP K30 and plated into CTM
C = sample frozen in 10% PVP K30 and plated into 10% PVP K30 in CTM for two hours before final incubation.
D = sample frozen with no protective agent and plated directly into CTM
E = sample frozen with no protective agent and plated into 10% PVP K30 in CTM for two hours before final incubation
Cooling rate = 20°C/min.
Warming rate = 115°C/min.


PERCENT SURVIVAL
100
90
80
70
60
50
40
30
20
10
A
B
C
D
E

attachment of frozen cells was as rapid as unfrozen controls so the cells in PVP supplemented medium should have had plenty of time to attach. In this case the PVP must be present during the freeze-thaw process to afford protection. An attempt to achieve faster post-thaw contact with the PVP was made by freezing the cells by direct injection from a syringe into liquid nitrogen and then thawing the frozen drops obtained by immersion in media containing PVP. Unfortunately, under the high freezing rates present in this technique, no cells survived the freeze-thaw procedure even if PVP was present during the freezing, and subsequently this technique was abandoned. Other variations in technique confirmed the conclusion that PVP protects hamster cells only when present during the actual freezing and thawing.

The semi-permeable membrane surrounding a typical cell is freely permeable to only a small number of relatively non-polar low molecular weight compounds. Several metabolites which are essential for cell survival are sufficiently impermeable to simple diffusion through the membrane that the cell would be unable to sustain growth if diffusion was the only way the substance could gain entry to the cell interior. To overcome this problem, and to allow a cell to regulate the metabolite level in its interior, specialized transport mechanisms have been evolved in which a special transport molecule is thought to combine with the molecule to be transported at one side of the membrane and release it at the other side, thus allowing it to pass through the barrier imposed by the membrane. This transport can be either passive (following the electrochemical gradient) or active, in which the transport process is energy requiring. The transporting molecule is thought to be a protein, at least in some cases (110), due to the high specificity it is able to show for the molecules to be transported, and also from the correlations between protein synthesis and permease systems in bacteria.

It has been suggested (49, 64, 66) that the cell membrane is highly sensitive to damage from freezing and thawing. It is possible, if the cell membrane is the site of damage to the cell, that the damage is due to destruction of the transport molecules in the membrane, leaving the cell unable to acquire necessary metabolites and resulting in cell death. Amino acids are found to cross the cell membrane by a specific transport process in a variety of cells (109) and it appeared that they might serve as a good marker for the behaviour of a transport-mediated uptake process. After tests on the uptake of several radioactive amino acids into CHA cells, alanine was chosen as one whose rate of uptake was in a range suitable for study.

It was first necessary to ensure that the increase in counts obtained was due to uptake by a transport mechanism rather than merely a non-specific adsorption to the vial and its contents or a simple diffusion into the cell interior. Most transport mechanisms are known to be stereospecific, meaning they will take up one optical isomer but not the other. Since mammalian cells can utilize only l-amino acids, it would be expected l-alanine would be the chosen isomer for transport. Since the d-isomer of $^{14}C$-alanine was not available, the stereospecificity was determined by diluting the $^{14}C$-labelled l-alanine, with either cold l-alanine or dl-alanine. If the transporting molecule is able to interact with only l-alanine, the competition for sites on the molecule will be greater with the l-alanine and the percentage of l-alanine molecules transported into the cell interior which are radioactive will be proportionately less. It was found that the uptake in the presence of l-alanine was, in fact, slower than in the presence of dl-alanine (Figure 30) in accordance with that which is expected if it is a true transport process.

Biological processes are highly temperature-dependent, in general much more so than purely physical processes such as diffusion or absorption. If a large difference in counts recorded is obtained at two temperatures which are quite close together on the absolute temperature scale but far apart on the temperature range over which biological specimens can function, it is a strong indication the phenomenon is a biological one. The increase in counts with time was recorded at 0°C and 37°C in the presence of $^{14}C$-alanine and $^{14}C$-ethanol. Ethanol is known to pass through the membrane by a simple diffusion process (42) and was used in order to compare the uptake of alanine with the uptake of a process operating by diffusion. The results in Figure 31 show the uptake of alanine was greatly reduced at 0°C. The uptake of ethanol, however, was totally inhibited at 0°C and was very low at 37°C. Increasing the amount of labelled ethanol in the medium did not increase the amount of uptake at 37°C. It seemed as though the movement of ethanol across the membrane was so rapid the only radioactivity which remained after the usual rinsing was the radioactivity arising from ethanol which had been converted into some other less permeable compound. This conclusion is strengthened by the observation that CHA cells plunged into 30% ethanol show no signs of shrinkage, indicating the ethanol concentration on the two sides of the membrane equilibrates very rapidly.

One further piece of information indicated the process was a true transport process rather than simple diffusion or absorption. The uptake in the presence of 100 mM cold l-alanine (Figure 30) was less than 0.02 times as rapid as the uptake in the presence of only the labelled alanine (Figure 32), indicating there were specific sites which the cold alanine was competing for. The sum of these observations indicated a specific transport mechanism for l-alanine existed in the membrane of the CHA cells.

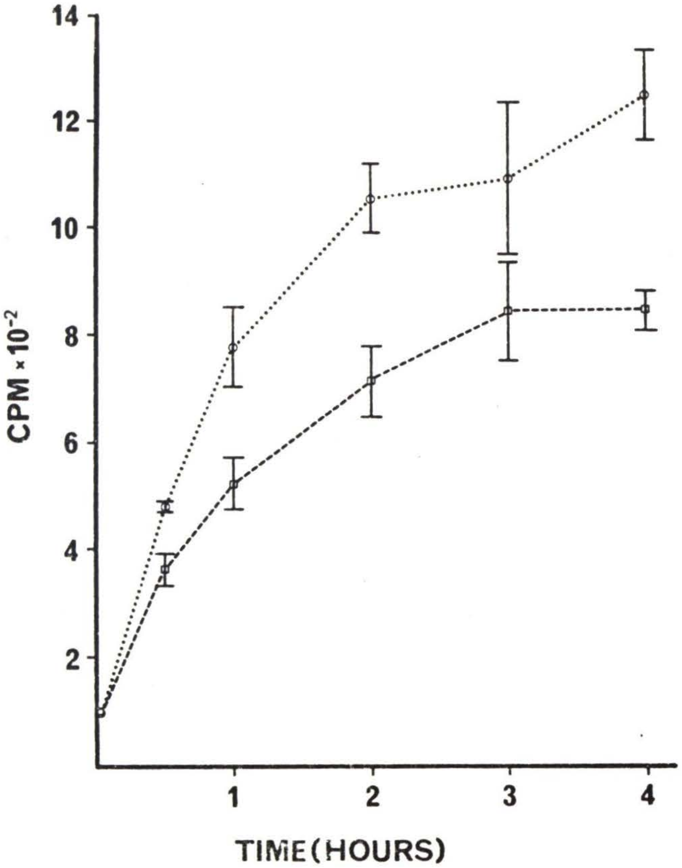
The effect of PVP on the rate of uptake in idential samples of unfrozen cells was determined. It was found (Figure 33) that PVP did not alter the uptake of alanine in the unfrozen samples, so that a specific binding of the PVP to this membrane component in order to stabilize it seems unlikely. Similar results have been obtained by Bican and Dobry (14) in the case of red cells using transport of sugars.

Figure 30. Uptake of $^{14}$C-labelled l-alanine by CHA cells from CTM containing 0.3 μCi/ml $^{14}$C-alanine plus 100 mM cold alanine. Vertical bar represents 1 standard deviation.

..... = uptake in the presence of dl-alanine
----- = uptake in the presence of l-alanine

Figure 31. Uptake of $^{14}$C-labelled l-alanine and $^{14}$C-ethanol at 0°C and 37°C by CHA cells. Vertical bar represents 1 standard deviation.

—△— = uptake of $^{14}$C-alanine at 37°C from medium containing 0.3 μCi/ml $^{14}$C-alanine plus 100 mM cold dl-alanine
----- = uptake of $^{14}$C-alanine at 0°C from medium containing 0.3 μCi/ml $^{14}$C-alanine plus 100 mM cold dl-alanine
..... = uptake of $^{14}$C-ethanol at 37°C from medium containing 0.5 μCi/ml $^{14}$C-ethanol
—◇— = uptake of ethanol at 0°C from medium containing 0.5 μCi/ml $^{14}$C-ethanol


14
12
10
8
6
4
2
CPM × 10$^{-2}$
1
2
3
4
TIME(HOURS)

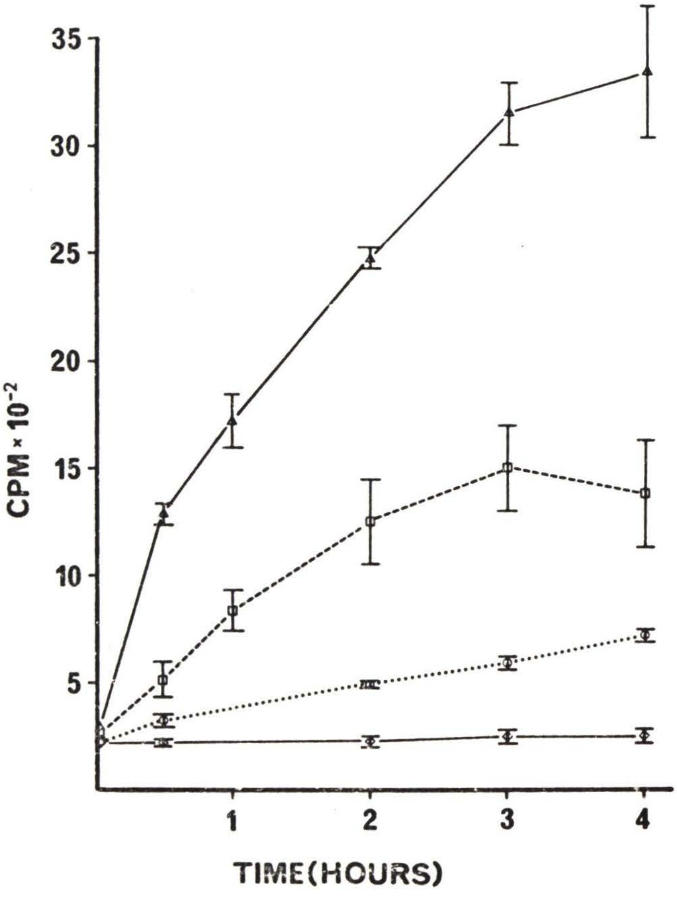

35
30
25
20
15
10
5
CPM × 10$^{-2}$
1
2
3
4
TIME(HOURS)

Figure 32. Uptake of $^{14}$C-alanine by CHA cells from CTM containing 0.3 µCi/ml $^{14}$C-labelled l-alanine. Vertical bar represents 1 standard deviation.

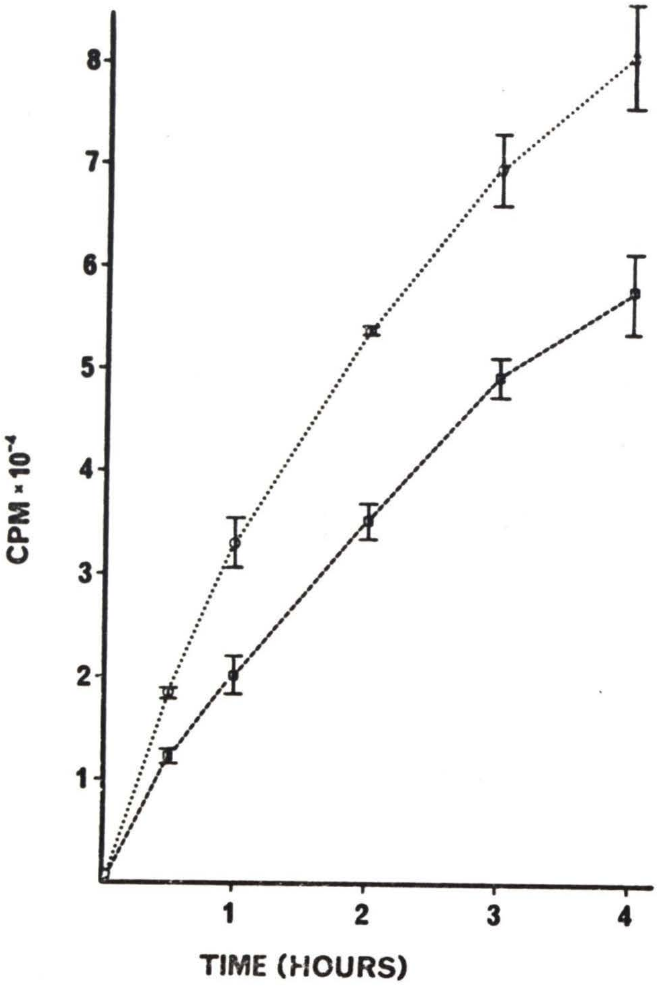
..... = unfrozen control

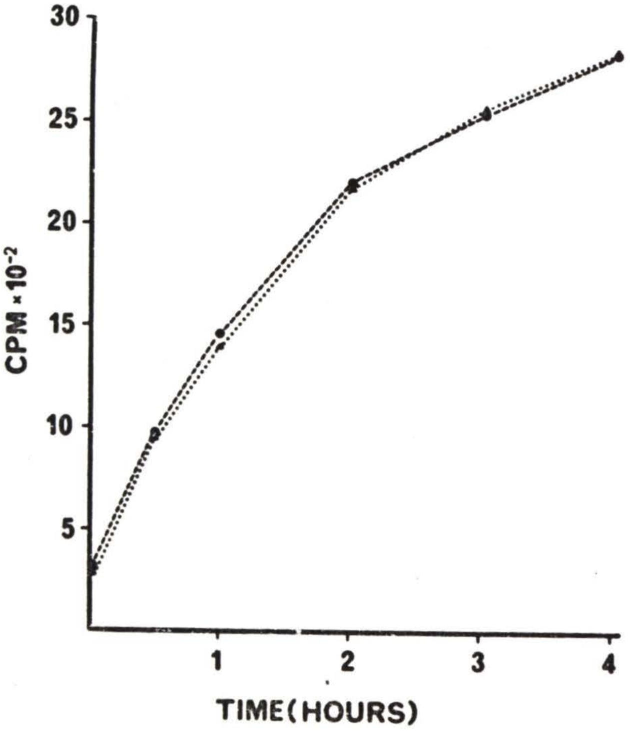
----- = uptake of cells frozen in 10% PVP in CTM in a manner in which approximately 50% survival would be expected (cooling rate 20°C/min., warming rate 115°C/min.)

Figure 33. Uptake of $^{14}$C-labelled l-alanine by CHA cells from CTM containing 0.3 µCi/ml $^{14}$C-alanine plus 100 mM cold dl-alanine in the presence or absence of PVP K30.

..... = uptake without PVP

----- = uptake in the presence of 10% PVP


CPM × 10⁻⁴
8
7
6
5
4
3
2
1
1
2
3
4
TIME (HOURS)

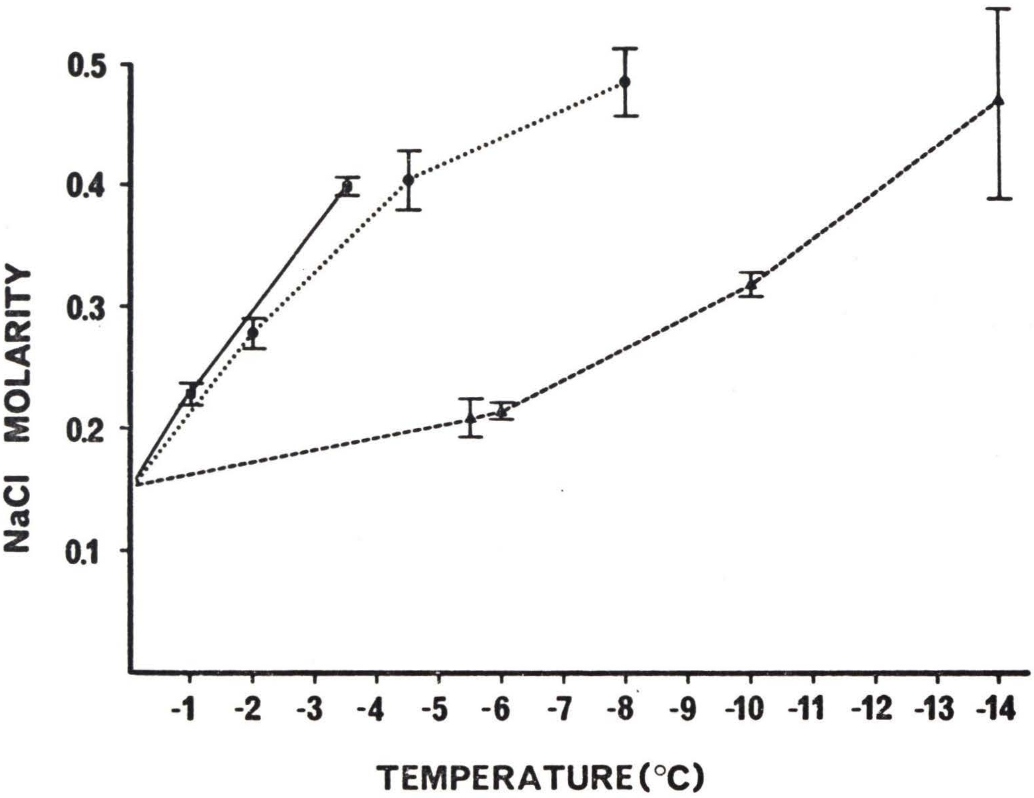

CPM × 10⁻²
30
25
20
15
10
5
1
2
3
4
TIME(HOURS)

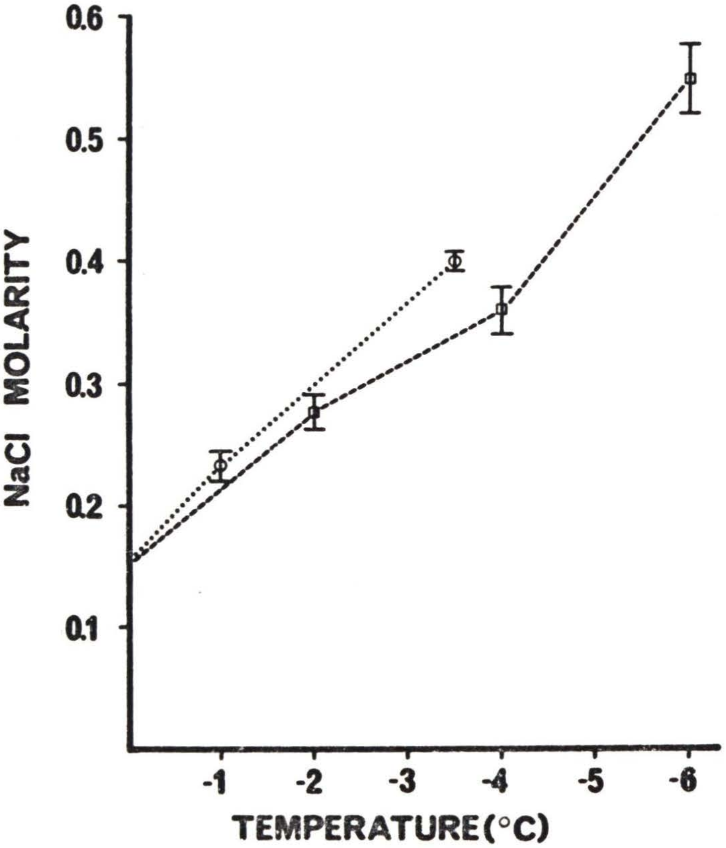
If the death of the cells is due to specific damage to transport systems such as the one for alanine, these systems should show a disproportionate decrease in activity following freezing and thawing, whereas if damage was to some other cell component or to the cell as a whole, the uptake activity would be expected to decrease approximately in parallel with the decrease in survival. In an experiment measuring the rate of uptake of alanine after freezing the cells in the presence of 10% PVP as compared to the uptake in an unfrozen control (Figure 32), it was found that the uptake of the frozen sample was approximately 70% as great as in the control. Cell survival as measured by the ability to form colonies would have been approximately 50% under these conditions, so that cell damage from a specific destruction of this particular transport mechanism does not seem to occur. There are a large number of transport mechanisms in any one cell, and it may be that destruction occurred to a transport mechanism other than the one for alanine. Other discoveries, such as the failure of the polymers to bind to the membrane, and the structural damage seen in electron micrographs after freezing and thawing, made the concept of protection by the stabilization of transport molecules by direct interaction with the polymeric protector seem highly unlikely.

One of the most attractive explanations of the mechanism of freezing protection by polymers was that proposed by Farrant (25, 26), in which he suggested that at higher concentrations polymeric solutions began to behave in a very non-ideal manner, and did, in fact, behave as if they possessed extensive colligative properties despite their extremely low molarity. Several techniques were employed in an attempt to determine whether polymers could decrease the amount of ice formed at subzero temperatures as the low molecular weight compounds do through their colligative properties. The sodium chloride concentration in the unfrozen portion of salt solutions at various subzero temperatures was determined by atomic absorption spectrophotometry. The rise in concentration in the salt solution with no protectant was so rapid with decreasing temperature that determinations could not be made by this technique. The rise in salt concentration in the unfrozen portion of the medium was lowered dramatically by the presence of 10% DMSO (Figure 34). 30% PVP was similar to 10% PVP over early stages of cooling, but as freezing progressed and the concentration of PVP in the unfrozen portion increased the ability of the PVP to prevent water from freezing out as ice began to increase. Unfortunately, problems in accurately controlling the temperature and in separating the liquid and frozen portion made it impossible to investigate the behaviour of the system at more advanced stages of freezing. It was earlier shown (Figure 27) that the addition of 2.5% DMSO could often result in as much survival as the addition of 10% PVP. The rise in salt concentration during the cooling of 2.5% DMSO and 10% PVP was found to be similar, within the experimental error (Figure 35). These results indicate that PVP may be able to act colligatively as Farrant suggested.

Figure 34. Concentration of sodium chloride in the unfrozen portion at various sub-zero temperatures in solutions containing cryoprotective agents. Vertical bar represents 1 standard deviation.
----- = 10% DMSO in saline
..... = 30% PVP K30 in saline
_____ = 10% PVP K30 in saline

Figure 35. Concentration of sodium chloride in the unfrozen portion at various sub-zero temperatures in solutions containing either PVP or DMSO. Vertical bar represents 1 standard deviation.
..... = 10% PVP K30
----- = 2.5% DMSO


0.5
0.4
0.3
0.2
0.1
NaCl MOLARITY
-1
-2
-3
-4
-5
-6
-7
-8
-9
-10
-11
-12
-13
-14
TEMPERATURE(°C)

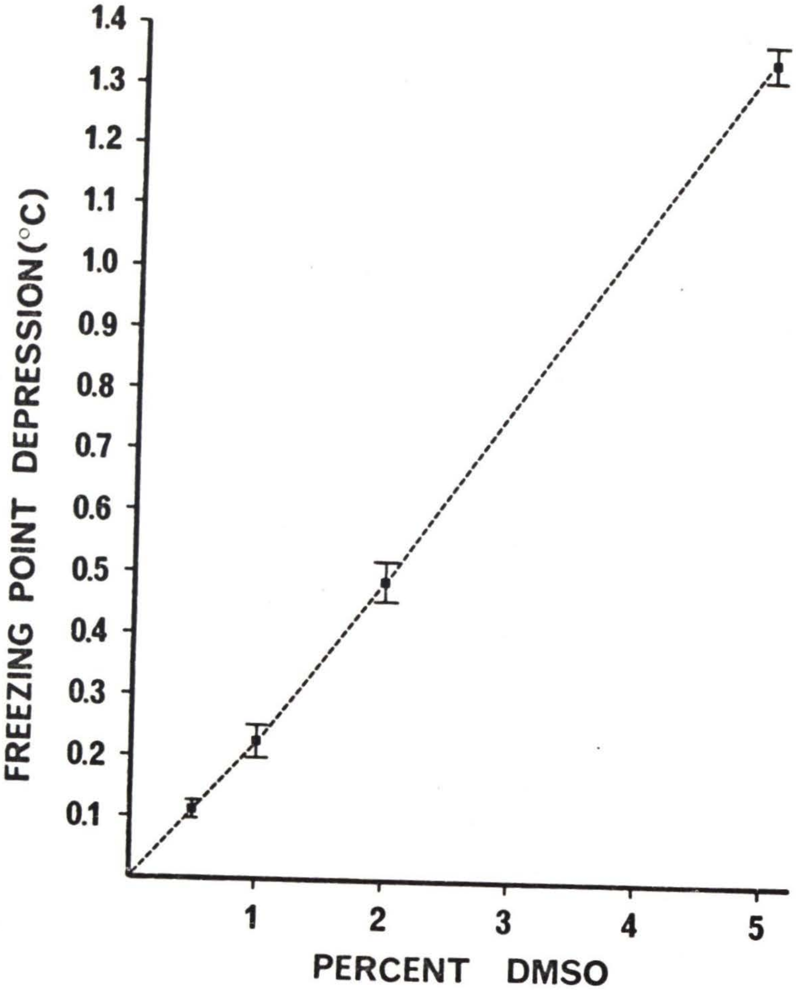

0.6
0.5
0.4
0.3
0.2
0.1
NaCl MOLARITY
-1
-2
-3
-4
-5
-6
TEMPERATURE(°C)

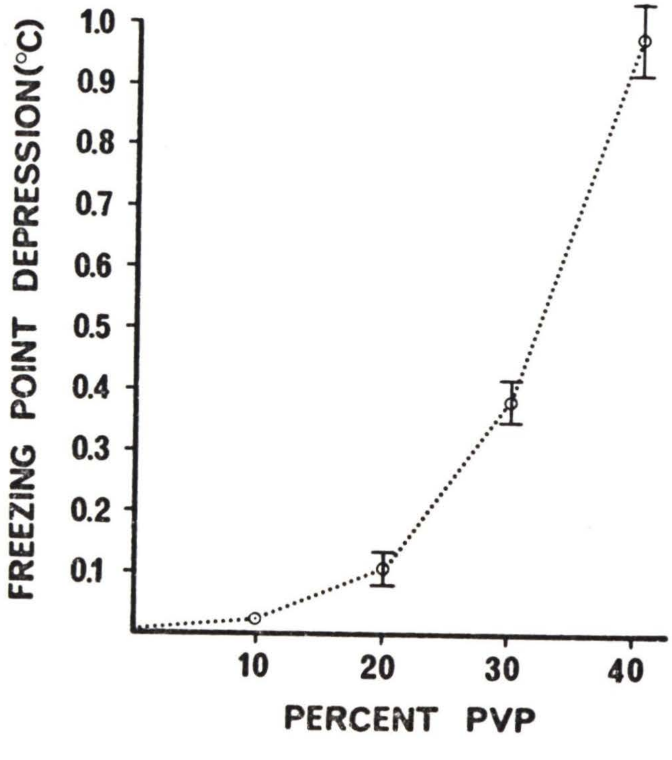
Freezing-point depressions were determined for solutions of DMSO and PVP at various concentrations. DMSO behaved in a manner which would be expected for an ideal solution, the freezing-point varying essentially linearly with DMSO concentrations from 0 to 5% (Figure 36). The behaviour with PVP, however, was seen to be a marked deviation from ideal behaviour (Figure 37). At low concentrations the PVP solution behaves as would be expected from the number of solute particles in solution and as a result the freezing-point depression is very small. At higher concentrations the freezing-point depression begins to rise sharply, so that at a concentration of 40% the freezing-point depressions of the PVP solution was similar to that of a 3.7% solution of DMSO. Measurements on a 50% solution of PVP were attempted but the freezing point was below -1.86°C and off the scale of the osmometer. It appeared from these results that PVP could act in a colligative manner when present at high concentrations.

Nuclear magnetic resonance (NMR) studies were done on the water fraction of several solutions since this technique allowed analysis of the water content of the solutions at temperatures far below those possible by the other methods. The protons from the polymers added only very slightly to the absorption by the water protons, presumably because the polymers at these temperatures behaved almost like miniature crystals, giving very short relaxation times and very broad energy spreads for the absorption lines so that they remained undetected by the high resolution instrument used in this study.

Figure 36. Freezing point depressions of various concentrations of DMSO in distilled water. Vertical bar represents 1 standard deviation.

Figure 37. Freezing point depressions of various concentrations of PVP K30 in distilled water. Vertical bar represents 1 standard deviation.


FREEZING POINT DEPRESSION(°C)
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
1
2
3
4
5
PERCENT DMSO


FREEZING POINT DEPRESSION(°C)
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
10
20
30
40
PERCENT PVP

For similar reasons the protons associated with the ice were undetected because their absorption curves were so broad. Deuterium has a nuclear spin of 1 compared to 1/2 for hydrogen and precesses at a different frequency than hydrogen under the influence of the magnetic field. It will therefore remain undetected in an instrument set to record signals from hydrogen. A sample run with PVP dissolved in $D_2O$ showed negligible absorption as did a sample of water with no protective additive. The absorption signal was arising from unfrozen water in the solution.

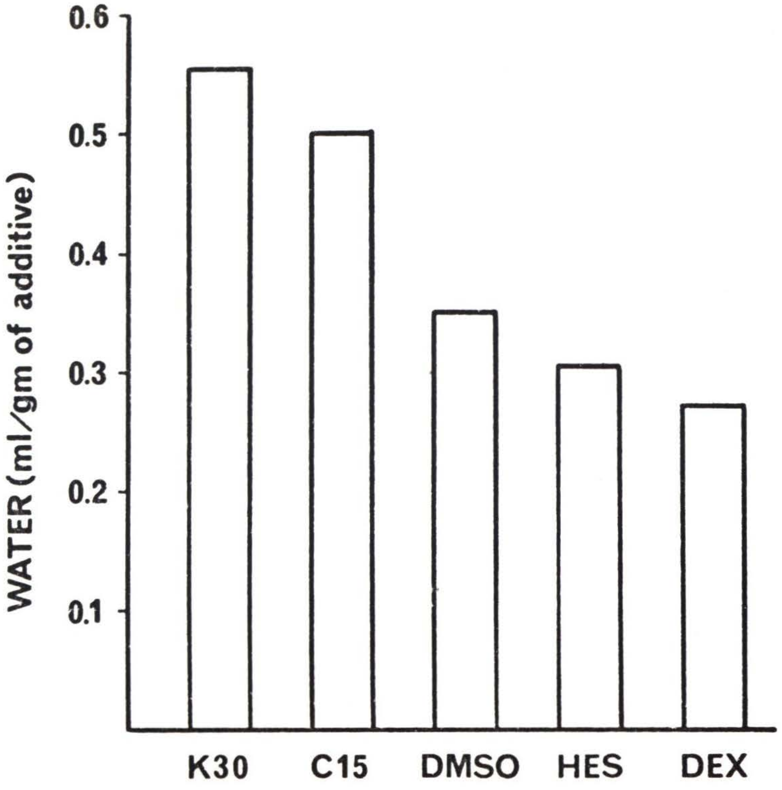
All the polymers previously used in survival studies (PVP, HES, and dextran) showed an ability to prevent a significant amount of water from freezing out as ice at -35°C (Figure 38). PVP K30 retained the largest amount of unfrozen water, 0.56 ml/gm of polymer, with dextran T40 being the poorest agent for the prevention of freezing, with only 0.27 ml water/gm dextran being present at -35°C. At this temperature the amount of unfrozen water in the presence of 2% DMSO was 0.35 ml/gm of DMSO, approximately mid-way between the largest and smallest value received for the polymers. No correlation was observed between a polymers ability to protect cells during freezing and thawing and the amount of water which that polymer was able to prevent from freezing at -35°C. The samples containing PVP K30 and DMSO were allowed to warm and the amount of liquid water was determined at various temperatures during the warming process (Figure 39). The DMSO sample showed a steady increase in the amount of liquid water as the temperature was raised.

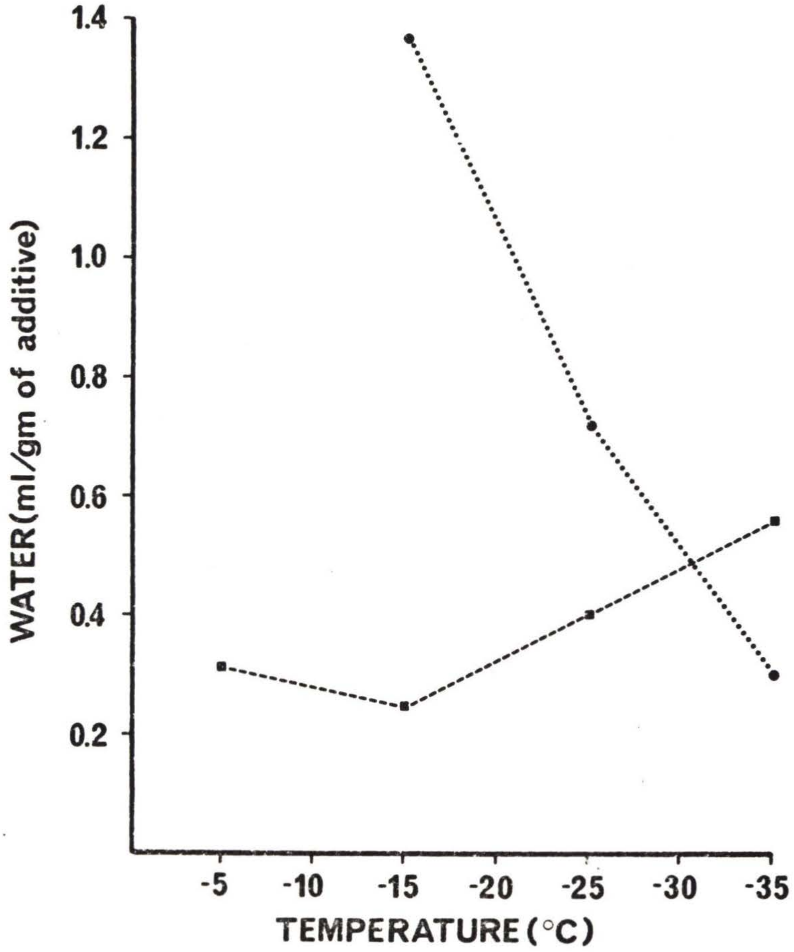
Figure 38. Amount of water remaining unfrozen at -35 $^{o}$C in the presence of several cryoprotective compounds. Solutions were originally made up in distilled water.

K30 = 10% PVP K30
C15 = 10% PVP C15
DMSO = 2% DMSO
HES = 10% HES
DEX = 10% Dextran T40

Figure 39. Amount of water remaining unfrozen at several sub-zero temperatures in the presence of cryoprotective compounds. Solutions were made up in distilled water.

..... = 10% DMSO
----- = 10% PVP K30


0.6
0.5
0.4
0.3
0.2
0.1
WATER (ml/gm of additive)
K30
C15
DMSO
HES
DEX


1.4
1.2
1.0
0.8
0.6
0.4
0.2
WATER(ml/gm of additive)
-5
-10
-15
-20
-25
-30
-35
TEMPERATURE (°C)

The PVP sample, however, showed no increase in water content in warming from $-35^{0}$C to $-5^{0}$C. In fact, a slight decrease was observed, which probably resulted from the rather large errors introduced during the integration of the broad peaks in the PVP sample. More studies would have to be done to clarify the exact relationship through this temperature range, but the important fact is that no significant increase in water resulted as it did with DMSO. It appears as though the polymers were able to structure a certain volume of water about them such that this water was prevented from forming ice. Over a fairly wide temperature range the volume of water in this region remains fairly constant. The water outside this structured region appears to be largely unaffected, so that it freezes out at fairly high temperatures. The sharp increase in apparent colligative properties at high concentrations shown by polymeric solutions may be due to the fact that at higher concentrations the majority of the water in the solutions exists in the structured regions about the polymers.

As well as an indication of the volume of water remaining in the unfrozen state, the line shapes obtained give an indication of the degree of structuring of that water. The more structured the water becomes, the wider the absorption peak will become due to decreased relaxation times of the excited state. Figures 40, 41, 42, show the absorption peaks obtained at $-35^{0}$C for Dextran T40, HES, and PVP K30. Since the settings of the rf attenuator and the gain were set at different positions for different runs, the heights of the peaks are not directly comparible from graph to graph.

The half-widths are, however, independent of the gain settings so can be directly compared. PVP has the smallest half-width, (227 Hz) indicating the water associted with PVP is quite loosely structured. Dextran showed a much larger half-width (785 Hz) with HES showing the most highly structured water of all the polymers with a half-width of 885 Hz. Loosely structured water, such as was found in the sample containing DMSO at -15°C (Figure 46) shows a half-width of approximately 20 Hz. The PVP C15 sample (Figure 45) showed a half-width of 214 Hz, similar to that obtained for the PVP K30. The water remaining in the samples containing DMSO (Figure 43, 44) is seen to be much less structured than in any of the polymer samples. Note the increase in volume of water remaining unfrozen in the 10% DMSO solution over the 2% solution, although the amount of water/ml DMSO differed by less than 10%. The smaller peak to the right of the water peak is due to the protons from the DMSO.

It was of interest to see if the relatively highly structured water present at low temperatures in the presence of polymers could act as a solvent for solutes such as NaCl. The peaks were examined in samples containing 10% PVP K30 dissolved in distilled water or in isotonic saline (Figure 47). If NaCl was totally excluded from the structured water, no change would be expected in the signal arising from this water. If the NaCl could enter the space occupied by the structured water, the water structure might be altered and this could show up as a change in the half-width of the absorption line. Such a change did occur, with the saline sample showing a half-width of 170 Hz as compared to 368 Hz for the sample in

Figure 40. NMR plot of water protons which are unfrozen at-35$^{o}$C in the prsence of 10% Dextran T40. Note the very broad linewidth indicative of highly structured water.

Figure 41. NMR plot of the absorption of 60 MHz radiation by unfrozen water at -35$^{o}$C in the presence of 10% HES. Again the absorption peak is very broad.

Figure 42. NMR signal from water in the presence of PVP K30. At -35$^{o}$C the peak is much narrower than that obtained with dextran or HES, although the total volume of unfrozen water is greater than with HES or dextran.

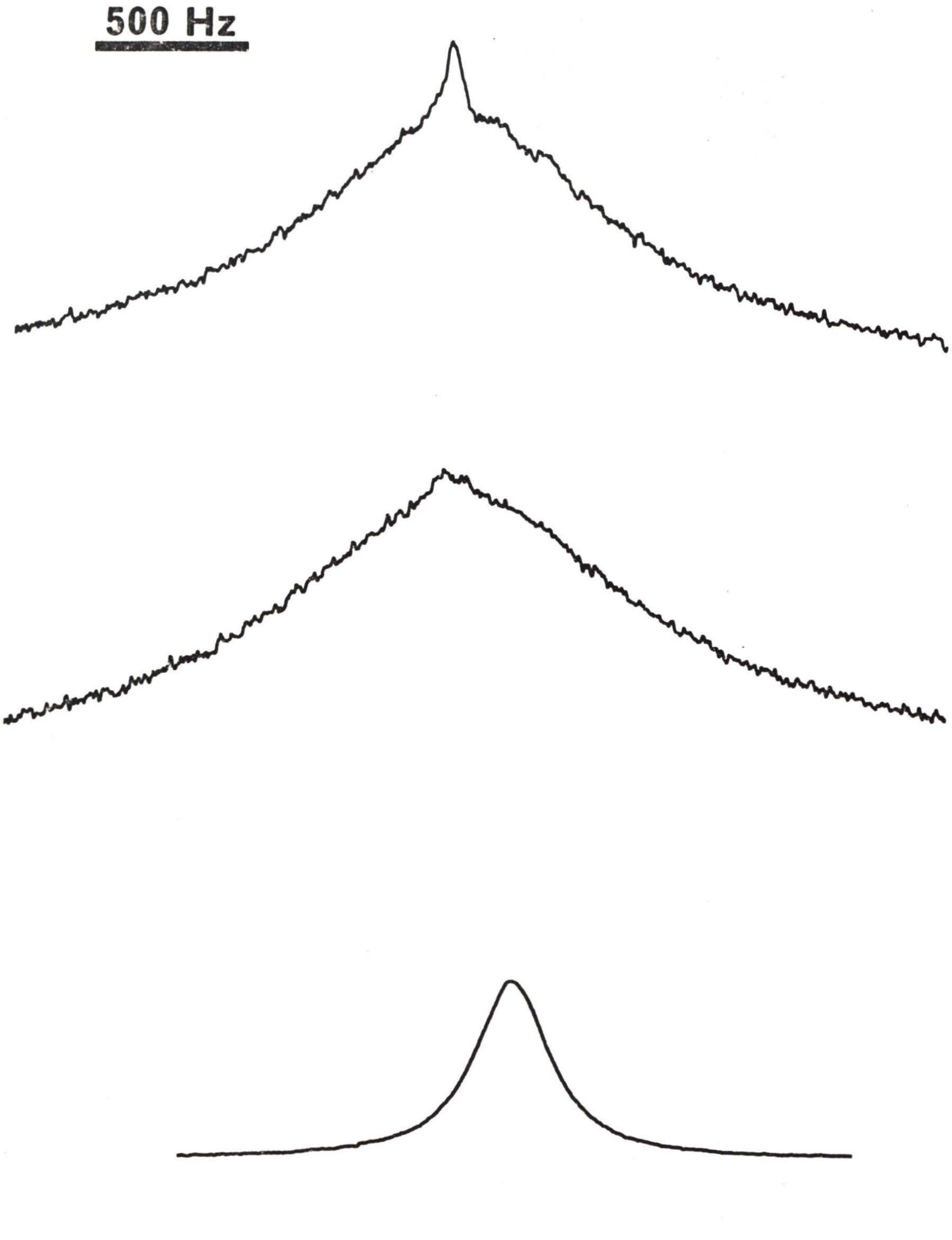

500 Hz

Figure 43. NMR spectrum of a 10% DMSO sample at $-35^{\circ}$C. A sharp absorption peak due to the water is visible, as is a smaller peak due to the DMSO protons. The halfwidth is seen to be much smaller than in the polymeric samples.

Figure 44. Signal received with 2% DMSO under identical conditions to those seen in Figure 43. The volume of unfrozen water is approximately 1/5 of that which was detected in the presence of 10% DMSO.

Figure 45. Plot of water proton absorption in the presence of PVP C15 at $-35^{\circ}$C. The shape is very similar to the plot found with PVP K30.

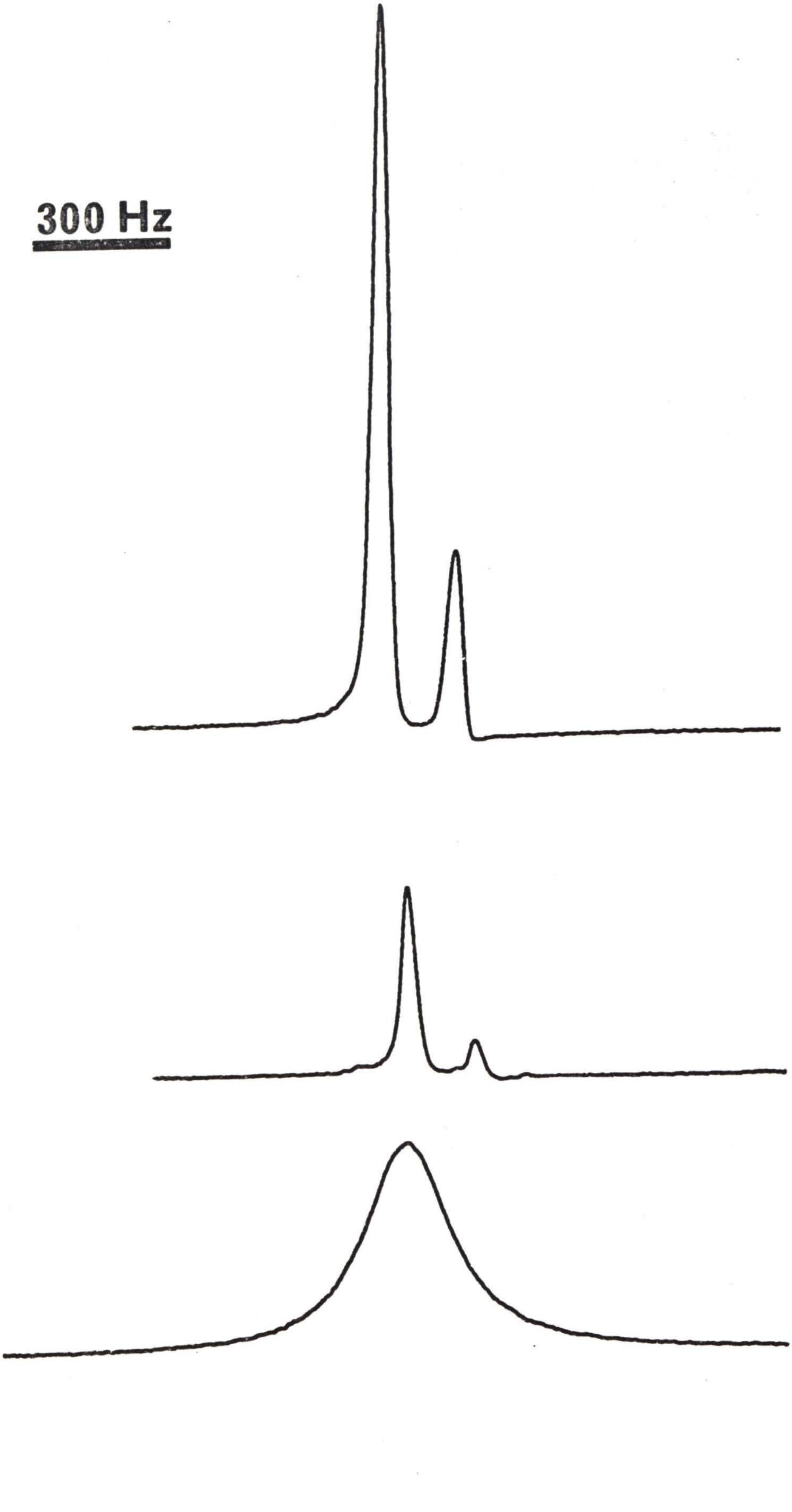

300 Hz

Figure 46. NMR spectra of 10% DMSO in distilled water at various temperatures. Note the progressive narrowing of the peaks, indicative of more mobile water, as the temperature rises. The volume of liquid water is also seen to increase with increasing temperature.

A = -35°C
B = -25°C
C = -15°C

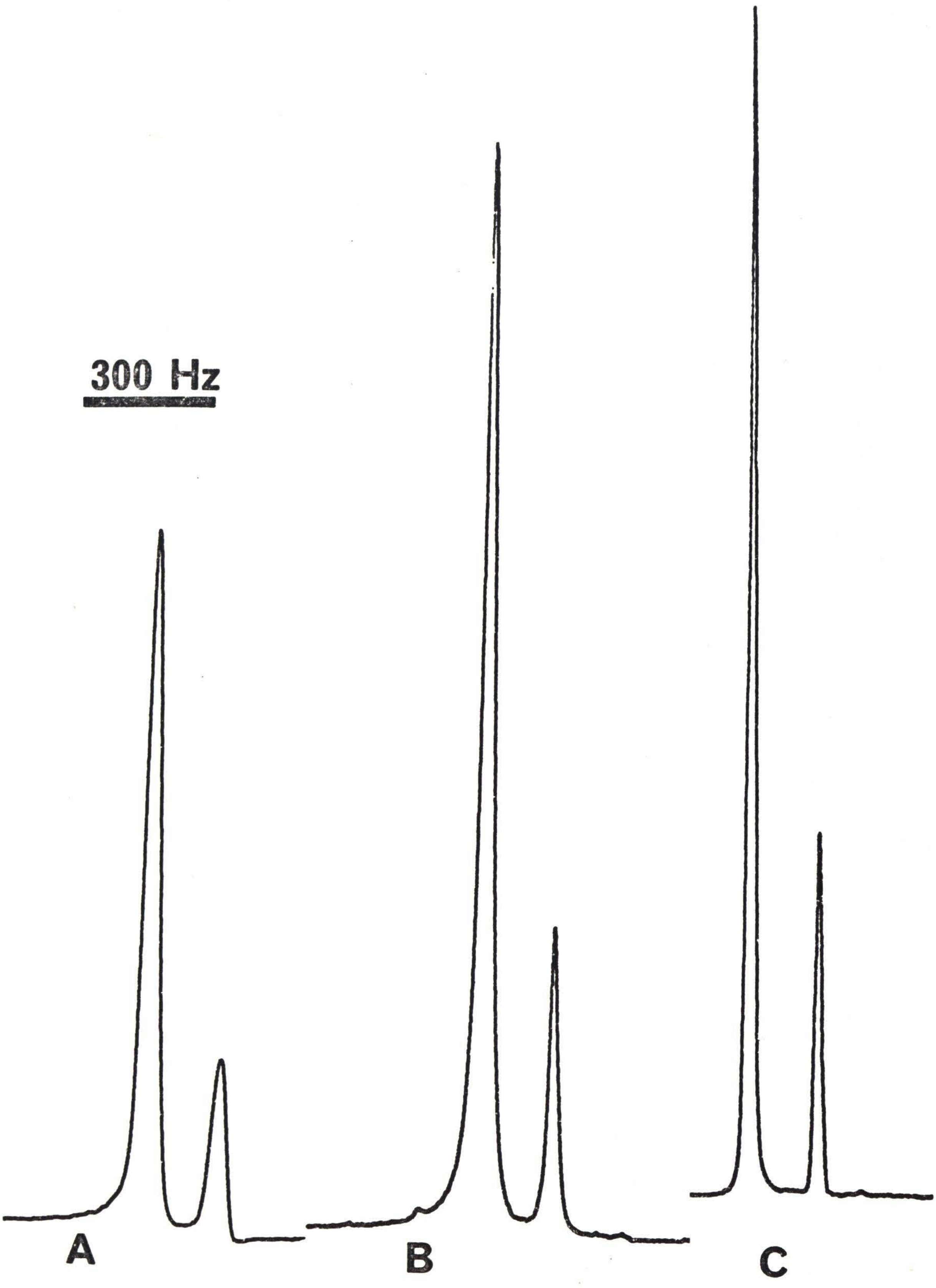

300 Hz
A
B
C

Figure 47. Variation in the water absorption signal with different temperatures or with the addition of NaCl to the solution. All samples contained 10% PVP K30.

A = plot at -25°C in the presence of isotonic saline
B = plot at -25°C in distilled water. Note the much larger halfwidth than in the sample containing saline
C = plot at -15°C in distilled water. Note the destructuring of the water which has occurred in warming from -25°C
D = plot at -5°C in distilled water. The water is becoming much more mobile resulting in a sharp absorption peak

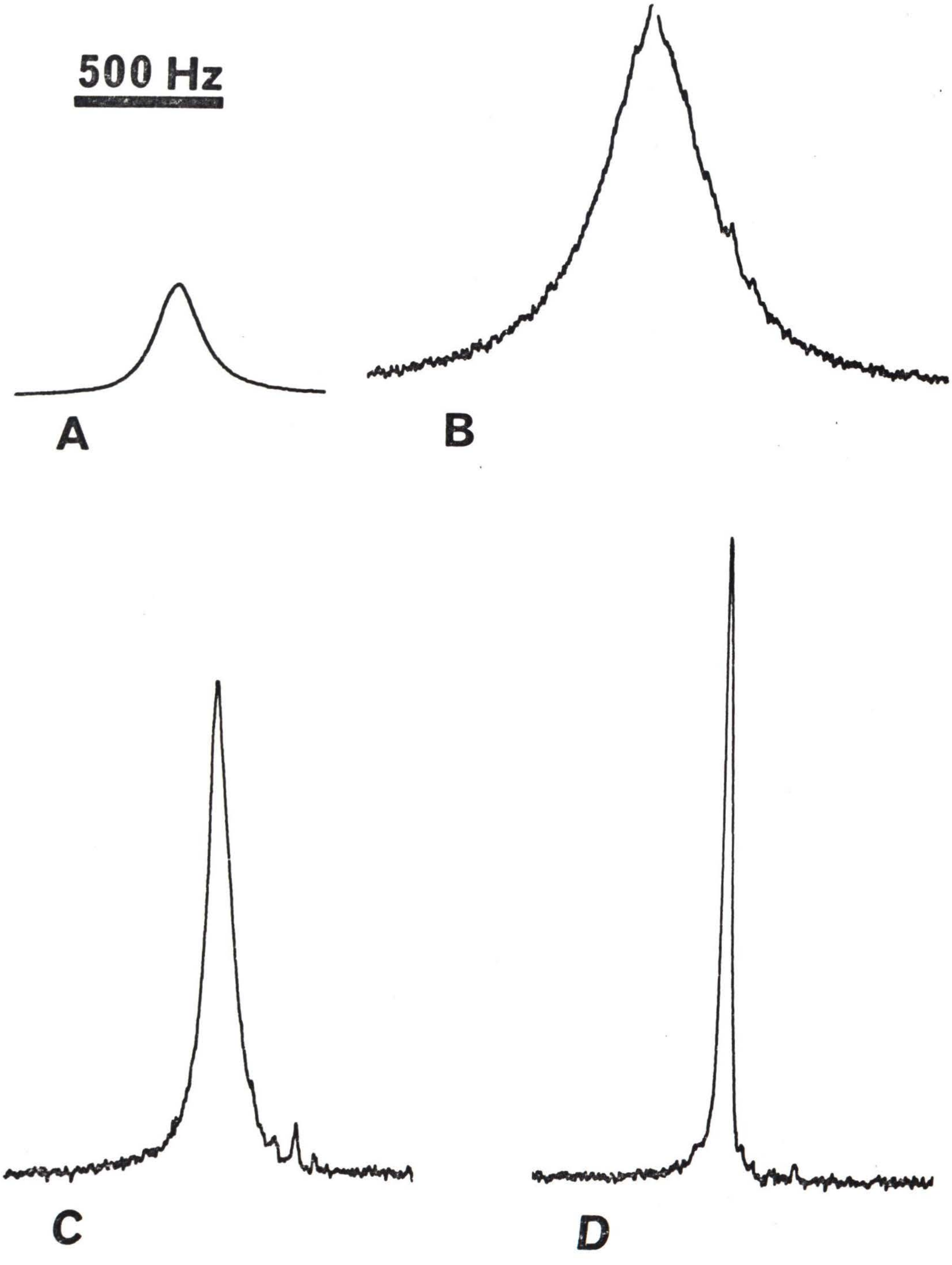
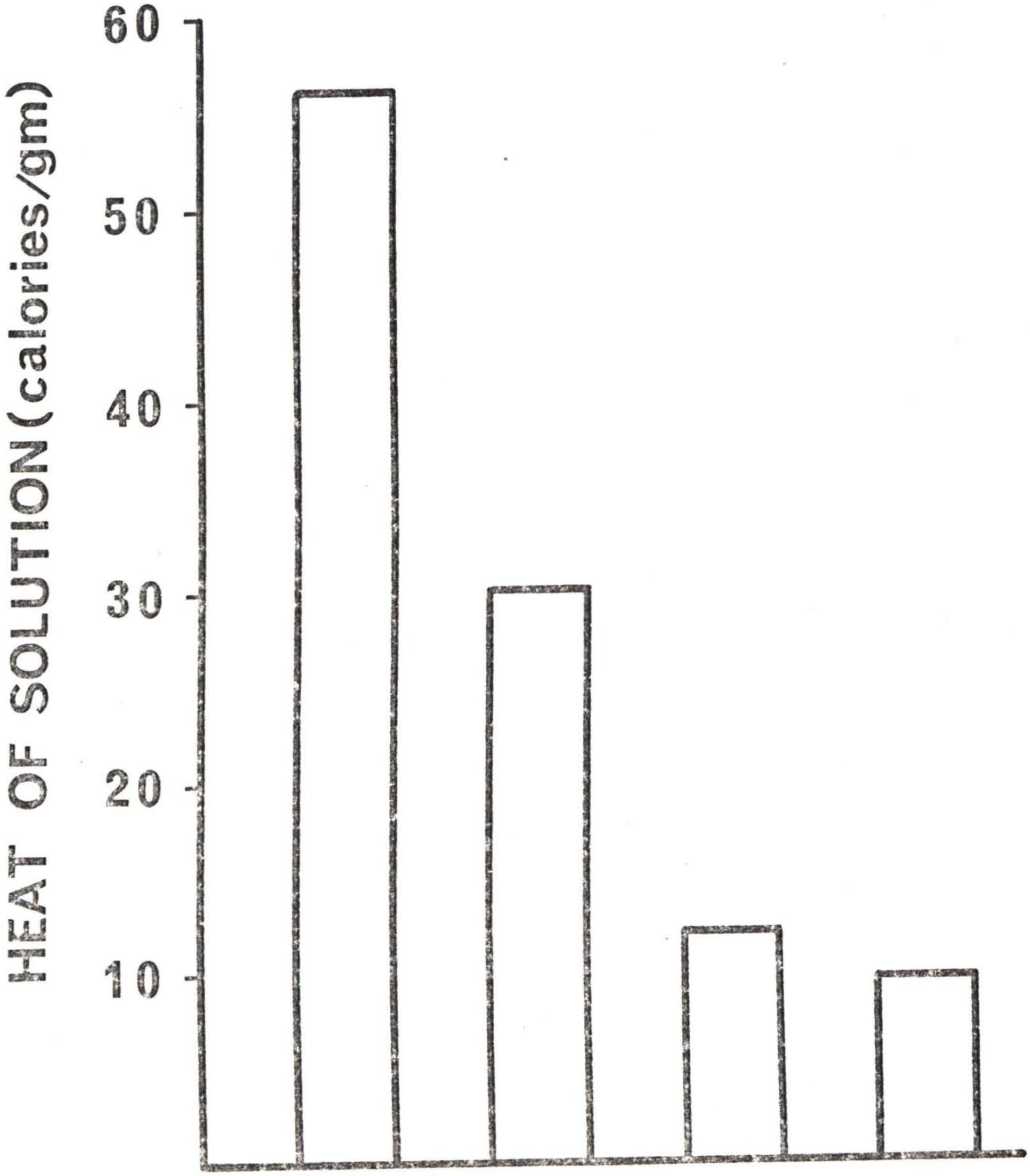

500 Hz
A
B
C
D

Figure 48. Heats of solution of some cryoprotective compounds observed upon dissolving them in distilled water to a concentration of 10%.
DMSO = reagent grade DMSO
PVP = PVP K30, dialysed, freeze-dried
DEX = Dextran T40
HES = HES, dialysed, freeze-dried


HEAT OF SOLUTION (calories/gm)
60
50
40
30
20
10
DMSO
PVP
DEX
HES

distilled water, in good agreement with the effect of salt on the half-widths reported by Kuntz *et al.* (45).

It was felt that the heat of solution of a polymer in water may be a further measure of the ability of that polymer to interact with the water molecules, and may be correlated with the polymer's cryoprotectivity. PVP K30 had a much higher heat of solution than either Dextran T40 or HES (Figure 48). DMSO, used for comparison, had a higher heat of solution than any of the polymers. The apparently greater interaction with water molecules seen with PVP than with HES or dextran is in accordance with the volumes of water these polymers were able to retain in the liquid state at lower temperatures. Again there appeared to be no correlation between the degree of polymer-water interaction and cryoprotectivity.

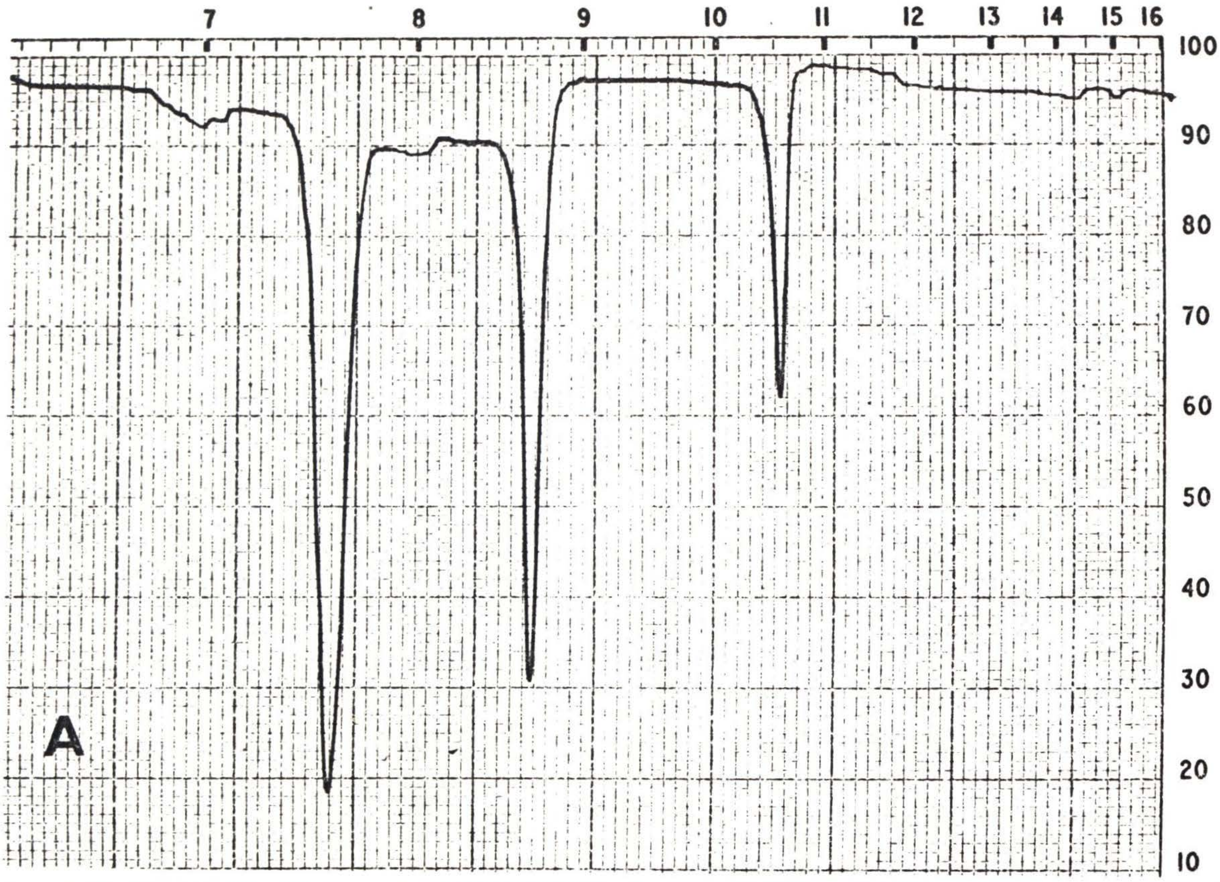
## Dimethyl Sulfone Experiments

In order for a compound to be able to protect on a colligative basis, they must be non-toxic, permeable to the cell membrane, and soluble at low temperatures (54). DMSO is known to be a good protective agent, whereas its close analogue, dimethyl sulfone, had been reported (119, 120) to be ineffective in preventing freeze-thaw injury. Dimethyl sulfone has been shown to be relatively non-toxic (119) and would be expected to be relatively permeable to the membrane. Water solubility of solutes involves successful competition with water molecules for hydrogen bonds, and dimethyl sulfone would be expected to hydrogen bond at least as strongly as DMSO. The reason for the lack of cryoprotectivity of dimethyl sulfone was not obvious from these considerations.

Dimethyl sulfone was tested to determine its ability to protect human red cells from damage during freezing and thawing. Two samples of dimethyl sulfone were used, one synthesized by the oxidation of DMSO with hydrogen peroxide, and the other a commercial product, were first analysed to determine their purity. Infra-red spectra were taken to detect any traces of DMSO in the sample since DMSO is a powerful cryoprotective agent and its presence would cause ambiguity as to the origin of the cryoprotection. Neither the commercial sample (Figure 49) nor the sample synthesized in our lab (Figure 50) showed any trace of the strong S=0 stretching frequency absorption at 1050 $cm^{-1}$ (9.5μ) indicative of the sulfoxide (12). Melting points of both samples were 108-110°C, very close to the theoretical value of 109°C quoted in the Handbook of Chemistry and Physics (34). Schreiber (100) has reported $-SO_2$ groups show absorption peaks near 1150 and 1310 $cm^{-1}$ (8.7μ and 7.6μ) peaks which were found in the test samples. It was concluded both samples contained dimethyl sulfone of acceptable purity.

Red cell recovery after freezing and thawing in the presence of 10% dimethyl sulfone never exceeded 5%. Tests with DMSO under identical conditions yielded a recovery of 71%. The explanation of the poor protective properties of the sulfone presented itself

Figure 49. Infrared spectrum of a sample of a commercial preparation of dimethyl sulfone (A) as compared to DMSO (B). No trace of the strong S=O stretching frequency at 9.5 microns indicative of the sulfoxide is seen with the sulfone.
ordinate = percent transmittance
abscissa = wavelength (microns)


7
8
9
10
11
12
13
14
15
16
100
90
80
70
60
50
40
30
20
10
A

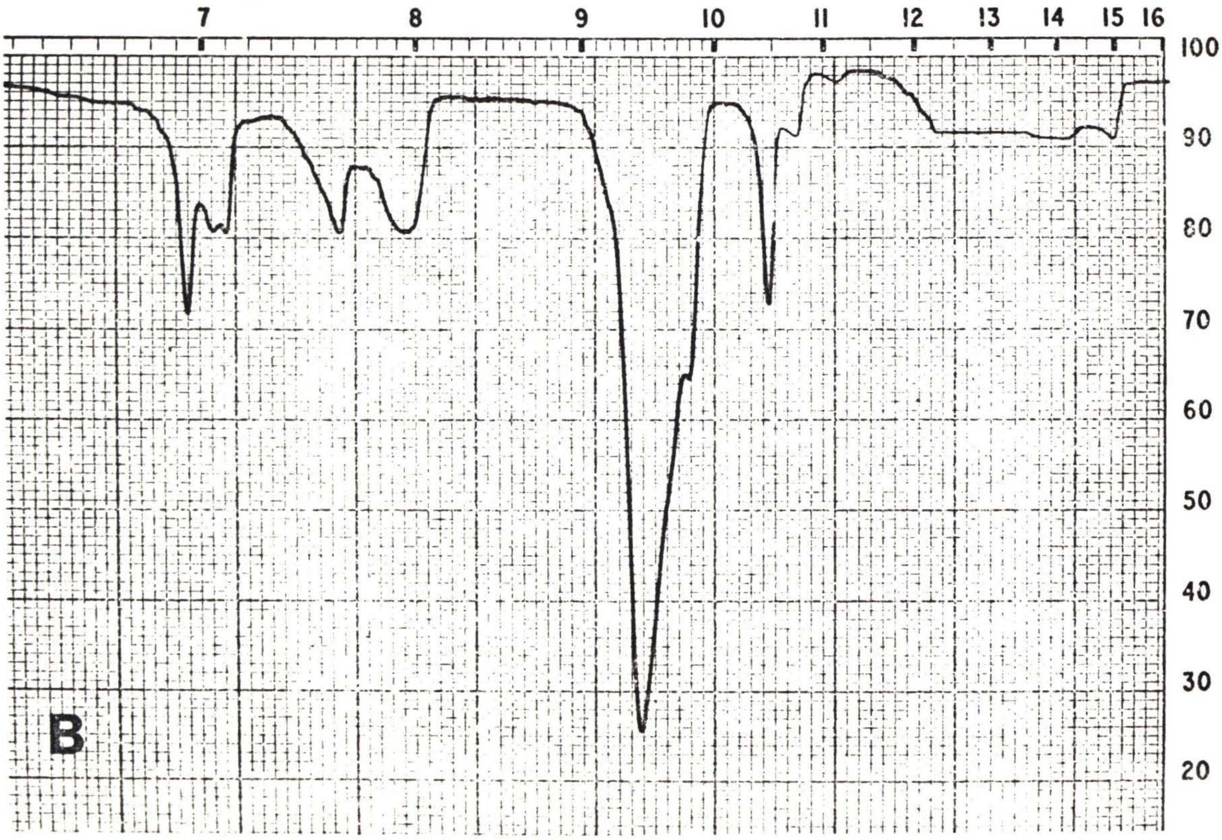
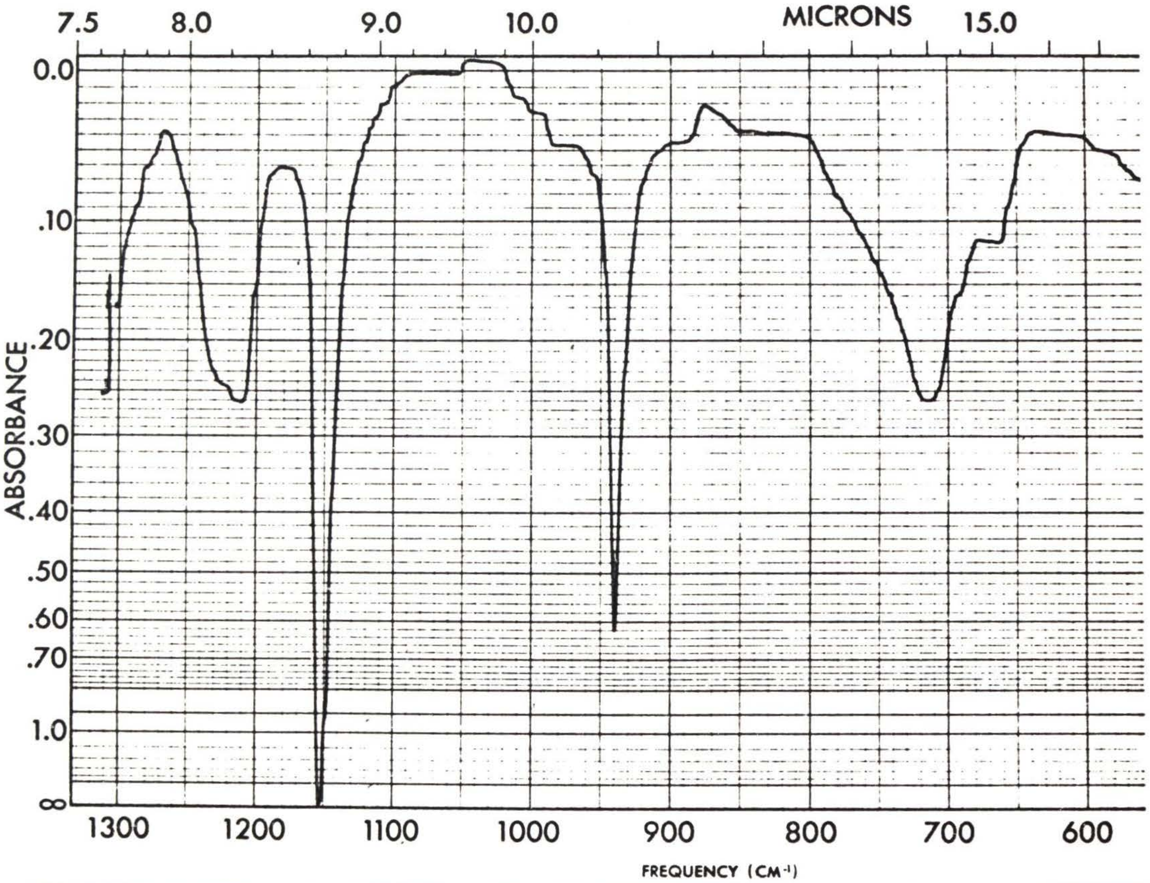
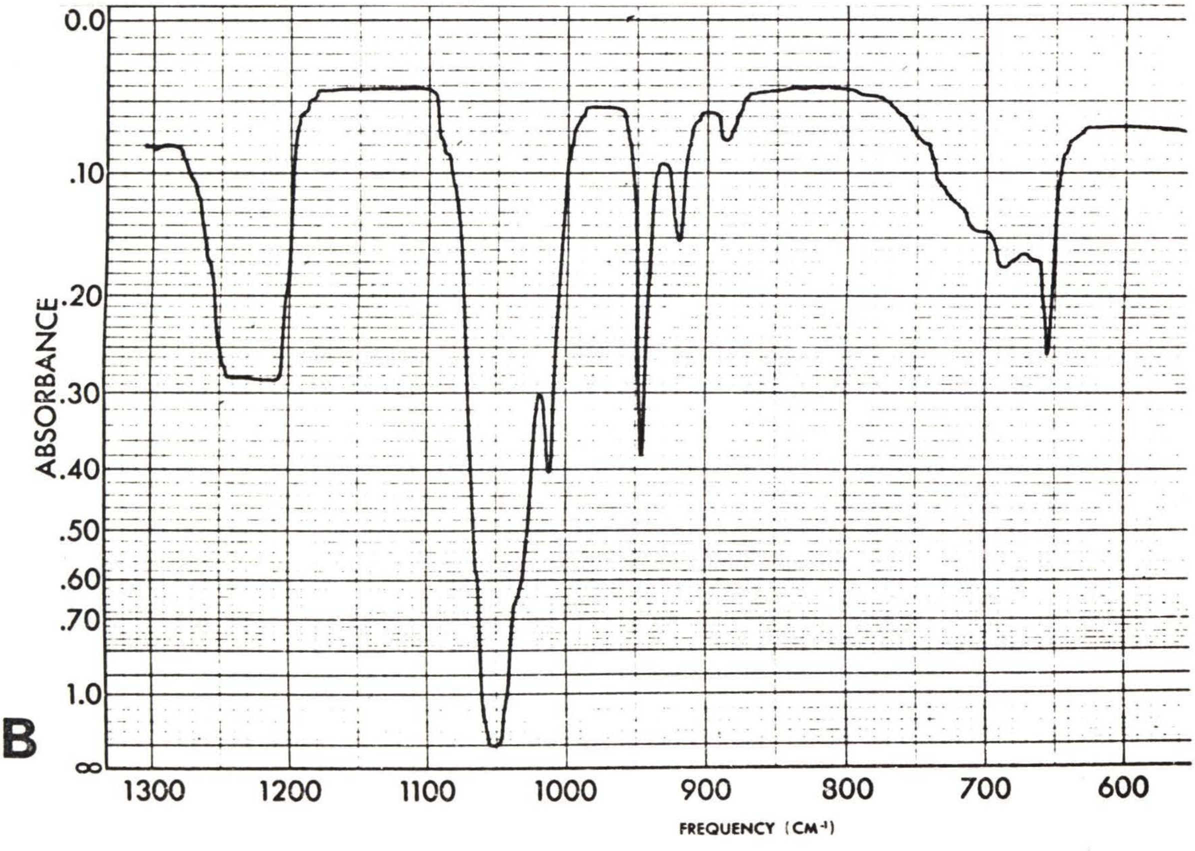

7
8
9
10
11
12
13
14
15
16
100
90
80
70
60
50
40
30
20
B

Figure 50. Infrared spectrum of dimethyl sulfone (A) synthesized by the oxidation of DMSO with $H_2O_2$ as compared to a sample of DMSO (B). The strong absorption peak at 9.5 microns, caused by the S=O stretching frequency of the sulfoxide is completely absent in the sulfone. The appearance of the large peak at 8.6μ is due to one of the new vibrational modes of the O=S=O group.


7.5
8.0
9.0
10.0
MICRONS
15.0
0.0
.10
.20
.30
.40
.50
.60
.70
1.0
∞
ABSORBANCE
1300
1200
1100
1000
900
800
700
600
FREQUENCY (CM⁻¹)

**A**


0.0
.10
.20
.30
.40
.50
.60
.70
1.0
∞
ABSORBANCE
1300
1200
1100
1000
900
800
700
600
FREQUENCY (CM⁻¹)

**B**

when it was noted that the sulfone began to crystallize out of a solution of 20% dimethyl sulfone when the temperature was lowered to 0°C. DMSO has a melting point of 18.45°C as compared to 109°C for dimethyl sulfone, which suggests the crystalline packing is much more stable for the sulfone. The high stability of the solid state causes the sulfone to crystallize out of solution at relatively high temperatures. The red cells, left without a protective agent, are hemolysed.

### Robinson Hypothesis

Experiments were done to determine the ability of conditioned medium to allow cells which had been frozen and thawed with no protective agent to repair the damage which they had incurred. The presence of conditioned medium after the freeze-thaw process was found to have no cryoprotective effect under the conditions employed. This observation, coupled with the fact that Robinson has yet to publish a full report of these experiments in a refereed journal, makes cryoprotection by conditioned medium unlikely.

# DISCUSSION

Chinese hamster ovary cells frozen and thawed in normal growth media without any protective additive generally show less than 10% survival. If frozen in a simpler medium such as HBSS, survival figures fall virtually to zero. In the presence of the low molecular weight compound DMSO or high molecular weight polymers, including PVP, HES, and dextran, this survival figure is markedly increased. Before an attempt can be made upon explaining how these compounds are able to prevent freeze-thaw injury, one must know how the freeze-thaw process is causing injury to the cells. Knowing that, postulates can be made as to how the additive can reduce the extent of that injury.

Damage to a cell from the physical spearing action of extracellular or intracellular ice is difficult to quantitate since it is impossible to know whether a cell with a certain appearance in the frozen state would have remained viable upon thawing. A general indication of the action of ice formation can be gained by viewing large numbers of freeze-substituted cells which have been frozen under conditions where the approximate percentage of survivors is known.

Extracellular crystal formation can cause massive distortion of the cell being frozen as seen in Figure 10 and it would seem that membrane systems could be stretched beyond their limit under such circumstances. Massive distortion due to the formation of extracellular ice was seen to be the exception rather than the rule in CHA cells and is not considered to be a prime factor in cell damage.

In the case of red cells it has been found (82) that intracellular ice forms only at cooling rates of several hundred degrees centigrade per minute, and the damaging effects of very high cooling rates are thought to be due to the formation of intracellular ice which otherwise does not occur (63, 65). The cooling rate at which ice will form intracellularly is directly dependent upon the rate at which the water can escape from the cell interior during dehydration, a rate which, in the case of CHA cells, is apparently quite slow. Freeze-substituted cells which had been cooled at a rate of $20^{o}C$/minute often show the presence of large intracellular ice crystals, some of which are seen even within the nucleus. Although it is difficult to imagine how such large crystals could form without having some damaging effect on the membrane systems of the cell, they do not appear to puncture holes in organelles by a simple spearing action. Often mitochondria and other organelles are seen clustered along the periphery of the ice crystal and, although they often appear compressed and somewhat distorted, they generally appear intact. Other cells in which no ice crystals can be seen generally show organelles which appear intact although shrunken from the dehydration of the freezing process. The finding that the amount of intracellular ice formed is not enhanced by the absence of a protective additive indicates ice itself is not the primary damaging factor at slower cooling rates.

The amount of intracellular ice which will form will increase as the cooling rate increases. The formation of excessive amounts of intracellular ice does seem to be damaging, since survival was found to decrease steadily with increasing cooling rate over the range of cooling rates studied.

Some workers (102, 111) have suggested that damage to the cell occurs as a result of injury to a particular constituent of the cell rather than to the components of the cell in general. Sherman (102) has suggested that the mitochondria are particularly sensitive to freezing injury and that cell death occurs due to loss of function of the mitochondria of the frozen specimen. Stowell *et al.* (111) directed their attention to damage suffered by the nucleus after freezing and thawing. A very extensive study on cytoplasmic structures in general by Trump *et al.* (115) showed damage to all cytoplasmic structures after the cell had been subjected to freezing and thawing. Changes in the mitochondria and nucleus were found, as well as changes in the other organelles of the cell such as the golgi, microbodies, and endoplasmic reticulum. These results are in agreement with those found in this study, in which general alteration of cytoplasmic structures was observed.

When viewing the micrographs of CHA cells after various treatments, it is important to realize that the picture represents a typical cell of the population. In any preparation of cells, there was always some which looked normal and some which appeared totally destroyed. An unfrozen preparation contained a high proportion of healthy appearing cells, whereas a sample frozen with no additive contained very few cells with intact membrane systems. Electron micrographs of freeze-thaw damaged cells typically showed regions of destruction of the cell membrane where cytoplasmic contents could be seen escaping into the medium. Mitochondria became swollen with the loss of the densely staining matrix and disappearance of cristae, and in severe cases mitochondria were seen to be ruptured. Even when fixation was done at 0°C so that autolysis could not occur the cytoplasmic contents appeared swollen with the appearance of many vesicles, as could result from altered membrane permeabilities causing large ionic imbalances. Staining of the cytoplasmic matrix after freezing and thawing seemed to be much lighter than in unfrozen preparations, indicating an overall destruction, extraction, or dilution of strongly staining components had occurred. In view of these findings, it seems that cellular damage occurs due to an alteration in the properties of the solution which causes general destruction of the constituents of the entire cell rather than due to action upon a particular component.

Meryman (70) found that red cells suspended in concentrated solutions of NaCl lost their impermeability to the sodium ions when the NaCl concentration of the medium had reached 1300 mOs. When these red cells were resuspended in isotonic saline they underwent swelling and hemolysis. A similar influx of Na in CHA cells during freezing could conceivably result in an unusually large amount of water flowing into the cell upon thawing, resulting in swelling and even rupture of the cell and its organelles. The appearance of an abundance of large vesicles in the cytoplasm in unprotected cells may be due to swelling of small vesicles and membrane-bound compartments in the cytoplasm.

Of the several mechanisms which have been postulated to explain how freezing and thawing results in injury due to changes in the suspending medium, the one which has gained the most acceptance is that proposed by Lovelock (53). He found that the temperature at which damage to red cells first occurred corresponded to the temperature at which the salt concentration of the medium was 0.8 M, irrespective of the concentration of glycerol present at the same time. He also found that decreasing the salt concentration of the suspending medium to 0.11 M from its original value of 0.16 M brought about a marked increase in recovery of red cells after freezing, results which led him to believe the damage was due to a direct effect of NaCl on the cell constituents. In later studies (56) he found that there was a sudden appearance of phospholipids in the suspending medium when the salt concentration of the medium reached 0.8 M. Lovelock concluded the phospholipids were extracted from the cell membrane upon exposure to concentrated salt solutions and this extraction altered the membrane to the extent that hemolysis occurred.

According to the Lovelock hypothesis, cryoprotective compounds act solely through their colligative properties by lowering the salt concentration attained at any temperature (54). This hypothesis predicts that, in order to cryoprotect, a molecule must be permeable to the membrane of the cell, non-toxic, and soluble at sufficiently high concentrations.

Meryman (70) has questioned the suggestion that the damage is due to a direct interaction of the salt, and has proposed an alternate model, which he has termed the minimum volume hypothesis. He considers freeze-thaw damage to be caused by the high osmolality of the medium during freezing rather than the specific action of the NaCl. Meryman found if a sugar was substituted for NaCl to give a similar osmotic effect, hemolysis of red cells occurred at approximately the same osmolar concentration as when NaCl was used, indicating the electrolyte concentration in the suspending medium is not the factor causing hemolysis in red cells. He proposed that, during freezing, the cell shrinks from loss of intracellular water in response to the increasing osmolarity of the fluid until a point is reached where the contents of the cell are compressed to the point where they begin to exert resistance on the plasma membrane. An osmotic gradient is set up across the membrane, which can lead to destruction of the external membrane in two ways. Meryman has suggested the constituents of the cells may not exert an even pressure on the cell membrane and, in fact, the membrane can be thought of as a thin film stretched over a wire mesh. In this case mechanical rupture can be envisioned. Meryman has also suggested that the semi-permeability characteristics of the membrane may be located at the outer surface of the cell membrane, and under severe osmotic stress the membrane is compressed by the force of the osmotic pressure acting at the outer surface of the membrane and the resistance of intracellular structures pressing on the inner membrane surface, which may create excessive dehydration in the membrane. Water content of membranes has been shown by Hechter (37) and Ling (52) to be necessary for normal membrane structure, so that the loss of this water could result in destruction of the membrane.

Since, according to this model, damage only occurs when the cell reaches a minimum volume, a protective agent must have the ability to raise the osmolarity of the cell interior in order to prevent loss of intracellular water. Non-penetrating protectants could accomplish this by allowing the influx of other extracellular solutes such as sodium. In all cases, however, Meryman found that resuspension of red cells which had apparently lost their impermeability to NaCl, as evidenced by cellular swelling below 1100 mOs NaCl, in isotonic saline resulted in rupture of the red cells, so that it would appear that the inward flux of NaCl could hardly be considered a cryoprotective measure. A satisfactory explanation of how an extracellular cryoprotective agent could prevent damage as suggested in this model has yet to be put forward.

Levitt and Dear (51) have hypothesized a different mechanism of injury from freezing and thawing. Levitt noted (50) that some proteins, free in solution, which are known to have many SH groups and which are normally inactivated by freezing can be protected in the frozen state if thiols are present in the medium during freezing. This would indicate the damage may be due to the formation of abnormal disulfide bonds. In cell systems, it has also been found that there is an increase in disulfide bonds as a result of freezing, drought and heat injury (48). Gaff (31) has shown that this increase occurs in the structural proteins and not in the soluble proteins.

Levitt and Dear (51) suggested that freezing may cause massive stretching of the plasma membrane due to the dehydration effects of the freezing out of water. As the plasma membrane is stretched upon pulling away from the rigid cell wall, it is hypothesized that holes may be formed in the lipid layer of the cell membrane since the cohesive forces holding this layer together are not as strong as those in the protein layers. The break in the lipid layer will allow the two protein layers of the membrane to come into contact and it is at this point the abnormal disulfide bonds are thought to be formed between the two layers. Upon returning to isotonic conditions, the strong disulfide covalent bonds will not be broken and regions of the membrane will exist which have lost their central layer of lipid. It is hard to imagine how such an alteration would not drastically affect the permeability characteristics of the membrane, and it is hypothesized that essential contents of the cell are lost through these holes, resulting ultimately in cell death. By this model of cellular cryoinjury, a protective compound would reduce the amount of dehydration and thereby reduce the degree of cell shrinkage and hole formation.

There appear to be several weaknesses to Levitt's model. Protective agents prevent damage by decreasing cell shrinkage in a manner similar to that in Meryman's model, and extracellular protection is difficult to account for. According to this model, the damage arises from a stretching of the cell membrane, and since animal cells are highly flexible, effects of freezing will be one of compression rather than stretching. Disulfide bonds may form from the close association of other protein constituents of the cell under conditions of severe dehydration, but there is insufficient evidence at this time to consider this as a prime source of injury.

Although the models of cryoinjury discussed to this point disagree as to the actual cause of injury, they all agree that the source of the injury lies in the conditions of elevated salt concentrations and severe dehydration which result during the freezing process. Cryoprotection in all these models is achieved by reducing the amount of water which freezes and thereby decreasing the extent of dehydration and electrolyte concentration.

Some of the suggestions which have been made regarding how a polymer could afford cryoprotection were reviewed in the introduction. These included mechanisms involving endocytosis, absorption of electrolytes, coating of the cell membrane, blocking of pores in the cell membrane, lowering of the freezing point, and protection after thawing by preventing membrane breakdown or by restoring membrane permeability. Results from this study and from investigations done in other labs have added weight to some of these showed that this polymer did not bind to the cell membrane. Tests which were done to determine the effect of freezing and thawing on the membrane transport mechanism for alanine indicated the protection afforded by PVP was due to a maintenance of the integrity of the entire cell rather than a direct stabilization of this membrane component. Results shown in Figure 15 show that several polymers with different molecular structures can protect CHA cells from freeze-thaw injury. If the protection was the result of the specific binding and stabilization of membrane components, only a few polymers with the correct molecular structure would be expected to bind to the membrane resulting in protection. If the protection was by some less direct mechanism, the general ability of water soluble polymers to protect would seem more acceptable. On these grounds, explanations of protection by direct interaction between the membrane molecules and the cryoprotectant seem untenable.

If PVP cannot gain entry to the cell interior and does not bind to the cell membrane, it must exert its cryoprotective effect by modification of some property of the solution as a whole. The fact that all the polymers tested were able to increase the cell survival to some degree indicated that it may be a property of polymeric solutions in general to modify some property of the solution in order to lessen the damage from freezing and thawing.

Polymer solutions could protect cells by simulating a two-phase system, the solution and the cell comprising the two phases, and altering the distribution of molecules between the two phases

(11). Tests on the NaCl distribution in a PVP-dextran biphasic mixture revealed no difference in the concentration of NaCl in the two phases. No reports were found indicating biphasic polymer systems could alter ionic distributions, and protection by altering the electolyte distribution seems unlikely.

Some biological molecules are known to show large partition coefficients between polyermic phases (1). Little work has been done with smaller molecules such as amino acids in such systems, but it seems unlikely that an alteration of the distribution of smaller molecules such as amino acids and vitamins could account for the protective effect of polymers. Electron micrographs of cells after freezing and thawing indicate death is a consequence of physical breakdown of cellular components as a whole rather than the alteration of available metabolites. Lovelock (56) has linked damage to the loss of lipoproteins from the cell membrane, and these polymers could very well be prevented from going into solution by the presence of a polymer in the suspending medium. This possibility cannot be excluded at the present time, although a cell whose membrane is altered to the extent that the constituents would float free into solution without the prevention of this by the suspending medium would probably lose its normal characteristics and damage due to other factors such as abnormal electrolyte fluxes could easily occur.

The lowering of the freezing-point of a solution by the addition of a solute is one of several phenomena which are due to the colligative properties of a solute, that is, properties which depend solely on the concentrations of particles in a solution and in no way on the nature of these particles. Since the concentration of particles in a solution of high molecular weight polymers is exceedingly low, there has been considerable reluctance amongst cryobiologists to believe Farrant's (25) suggestion that polymeric solutions could behave as though they had appreciable colligative properties when the concentration of the polymer was high.

An excellent example of a polymer which shows an unusually high colligative effect has been demonstrated by de Vries *et al.* (19). The polymer was a glycoprotein isolated from the serum of the antarctic fish *Trematomus borchgrevinki*, a fish which lives in seawater at a temperature of -1.9°C. The glycoprotein was found to be responsible for approximately 1/3 of the freezing-point depression of the serum of these fish, and upon purification, was found to depress the freezing point of a solution as much as NaCl on a weight basis. Since the glycoprotein had a molecular weight of approximately 20,000, the molarity of solutes in the polymer solution was approximately $1.5 \times 10^{-3}$ as great as in the salt solution. The special ability of the polymer structure to lower the freezing point was demonstrated by digesting the protein with trypsin. Although this would clearly increase the number of solute particles, the freezing point depression ability was almost entirely lost. It is clear that properties of polymeric solutions can deviate drastically from those predicted from the ideal laws of physical chemistry and can show colligative properties under certain conditions which are very much greater than would be predicted from the concentration of particles in solution.

Several pieces of information were obtained from this study which support Farrant's findings. Experiments in which the unfrozen portion of saline solutions were removed from partially frozen samples by centrifugation showed that the presence of PVP in the solution resulted in a decrease in the concentration of NaCl reached at any temperature similar to that produced by DMSO at a concentration which would give similar cryoprotection. In studies on the freezing points of solutions of PVP, it was found that the freezing point depression was extremely low at lower concentrations of PVP, as would be expected if the solution was behaving ideally in this region. However, as the concentration rose, the freezing point began to decrease sharply, so that at 40% PVP the freezing point depression was increasing very rapidly for a relatively small increase in concentration. In a solution undergoing cooling, a solution originally at 10% PVP would very rapidly reach high concentrations due to the freezing out of a large portion of the water in early stages. At the higher concentrations the polymer will begin to behave as if it had an unusually large colligative effect and will prevent water from freezing out as ice and reduce the corresponding increase in salt concentration of the unfrozen portion. Even at -35°C, the protective polymers used in this study showed the ability to prevent 0.2 to 0.5 grams water/gm polymer from freezing out as ice, a quantity of water similar to that which was retained in the liquid state by DMSO at this temperature. -35°C has been shown by Lovelock to be the temperature at which damage to red cells is most rapid, although damage does occur throughout the range -4°C to -40°C. At this temperature, approximately equal volumes of water are retained in the liquid form by either the low molecular weight compound DMSO or a polymer, and cryoprotection could be due to the ability of both these compounds to lower the salt concentration and degree of dehydration experienced by the cells at this temperature.

Although the volumes of water detected using the two types of protectant were the same, the water present in the polymers was highly structured, as seen by the large width of the NMR absorption lines. If the retention of this water in the liquid state is to be effective in lowering the salt concentration, this structured water must be available as a solvent for the NaCl in the solution. Although no quantitative results were obtained on the availability of this water for solvent purposes, the marked decrease in structuring resulting from the presence of NaCl in the medium indicated that the NaCl had entered the water space of the structured water and had caused a disruption of the order of that water. The water in the low molecular weight sample (DMSO) and the polymer may be equally effective as a solvent for concentrated salts.

It has been argued that an extracellular agent could not protect on a colligative basis since the intracellular ion concentration would be unaltered from the situation without a protective agent and destruction of the cell interior would result. Closer examination of the situation shows that, since polymers seem to be able to act colligatively at higher concentrations, the intracellular ion concentration does not increase in parallel with the extracellular ion concentration under conditions where no protective agent is present. The cytoplasm of a cell contains an abundance of polymers, mostly proteins, which are free in solution. These proteins will become more concentrated during the process of freezing, and will begin to exert an osmotic effect. Farrant and Woolgar (26) found that the osmotic coefficient of hemoglobin in red cells rose sharply from a value of 2.7 at a hemoglobin concentration of 31.5% to 12 at a concentration of 54%, results which agree with those found by McConaghey and Maizels (60). Because of this sharp increase in the osmotic coefficient of hemoglobin, the shrinkage of red cells with increasing osmolarity of the medium over this range is very slight, and it has been suggested (26) that it is this increase in osmotic effect of hemoglobin which causes cells to reach the minimum volume observed by Meryman (70). Therefore, in a cell undergoing dehydration, the osmolarity of the intracellular polymers will account for a greater and greater proportion of the total osmolarity of the cell interior. The concentration of ions in the interior will be much less than the external concentration and damage may occur to the cell membrane. This would explain the findings of some workers (49, 64, 66) that damage to a cell occurs primarily to the external membrane. An extracellular protective agent may protect a cell by lowering the concentration of extracellular ions and thereby preventing membrane destruction. The interior will be protected by the colligative effect of the intracellular polymers.

Other considerations of the cell after freezing and thawing suggest alternative ways in which protection of the outer cell membrane could result in survival of the entire cell. If the permeability characteristics of the limiting membrane are maintained, the cell may be able to carry on metabolism and resynthesize the components of the interior which have been damaged. Some components may be dissociated but not completely denatured, and since the components would not be completely lost from the cell, they may be able to reassemble themselves.

Although the lowering of the salt concentration by the retention of water in the liquid state is almost certainly beneficial to the cell, this factor alone is insufficient to explain the cryoprotective properties of the compounds used. It was noted that the protective properties of the polymers were not related to the volume of water which they retained in the liquid state, although the range of values for volume retained was not very great. Similarly, with low molecular weight compounds, it has been shown that compounds which should behave identically according to the

Lovelock hypothesis exhibit varying degrees of effectiveness (21, 71, 72). The explanation of other factors involved in freezing damage may lie in a closer examination of what an aqueous solution is like at the molecular level. The concept of water structure and the effect of solutes upon that structure is a highly disputed matter at this time and further studies on the physical chemistry of solutions, and in particular polymeric solutions, at the molecular level are needed to enable a clearcut explanation of the behaviour of these solutions during freezing and the cause of freezing damage. Recent studies on the interaction of water and macromolecules make it possible to propose an explanation of present observations of systems subjected to freezing and thawing.

Bulk water is no longer believed to be composed of a homogeneous mixture of independent water molecules, but is thought to be composed of groupings of water molecules which are held together by hydrogen bonds (112). The details on the size of clusters, orientation of molecules within the clusters, and mean lifetime of a cluster are points of much conjecture at the present time. A model consisting of hexagonally arranged three-dimensional crystals of water molecules in the liquid, the so-called polywater, was postulated (2) and gained considerable acceptance but has recently been questioned by the original authors (3). Several possible configurations ranging from bulky ice-like arrangements to more densely packed and less rigidly structured arrangements have recently been summarized (29). A concise description of how the molecules in bulk water are actually arranged is impossible at this time, and it is merely noted here that some degree of structuring does occur.

Polymers are thought to have the ability of structuring relatively large volumes of water about them (22, 30 45). The relatively high heats of solution observed for the polymers in this study support the fact that they cause a structuring of water molecules in the solution. Dole and Faller (22) found PVP exposed to air at a relative humidity of 90% at 25°C absorbed 3.77 moles of water/100 gm PVP. NMR studies by Fuller and Brey (30) on water absorbed on serum albumin have shown the existence of three different states of structured water about the protein and calculated the three groups, identifiable by the width of their absorption signal, were due to water molecules held between two polar groups, those held between one polar group, and those which were relatively mobile but still more structured than molecules in bulk water. The large half-widths obtained for the absorption of water in polymeric solutions noted in this study also indicates a structuring of the water occurs.

A possible mechanism whereby a polymer could affect the freezing point of a solution can be formalated from these observations. The polymer in solution can be though of as being surrounded by successive layers of progressively less tightly bound water molecules. Due to the small number of polymer molecules in a solution of low concentration on a weight basis, the bulk of the water is only slightly altered and little change in the freezing point is seen. At higher concentrations of polymer, the situation is approached where most of the water being close to a polymer molecule is highly structured. The stability of the water in this structured state is such that even at -50$^{o}$C, it can be prevented from freezing (45). The amount of water which is available to freeze out as ice during cooling is reduced much as it is using a low molecular weight compound, although with the polymer the effect only becomes significant at higher concentrations. In this way polymers may be able to act on a colligative basis. It is commonly accepted that solutes affect the structure of water (30, 38, 40, 45), but the converse, that the structure of water has a determing effect on macromolecular structures, is sometimes overlooked. Studies have indicated water is necessary for the maintenance of proteins (5, 43, 123) and membranes (27), and changes in the availability or structure of the water can alter the molecular structure.

Water structure has a large influence on molecular configurations through its role in the maintenance of hydrophobic bonds (112). Non-polar regions of molecules are known to cause a large amount of structuring of surrounding molecules (28), a process which involves a large increase in free energy (76). As a result, non-polar regions of macromolecules prefer to reside in the interior of globular molecules so they are not exposed to an aqueous environment. In a situation where water is already highly structured, such as at low temperatures (75), the exposure of these nonpolar regions to the water will cause no net structuring so that no increase in free energy will result from the breakage of these bonds. This decrease in strength of hydrophobic bonds at decreased temperatures could explain the phenomenon of thermal shock noted by Meryman (70) and Lovelock (56), in which rapid cooling without the formation of ice resulted in hemolysis. At more advanced stages of cooling severe dehydration may result in the physical removal of water from cellular components, a process which may be magnified by the presence of ions in the solution. Ions in solution form a sphere of water molecules about them by dipole attraction, and a high concentration of salts may result in the free water being bound to the salts, thus making it unavailable for the stabilization of cellular macromolecules.

Both the structure and the quantity of the remaining water are seen to be important for the maintenance of the structure of biological macromolecules. Solutes are known to alter the water structure of a solution (40), and it may be that the damaging effects of high salt concentrations are mediated through their effect on water structure in the system. Whether the prime factor in cryo-injury is the alteration in the structure of water associated with macromolecules or an actual removal of that water remains to be shown.

In summary, the freezing process appears to proceed in the following manner. As the temperature drops below the freezing point, water begins to crystallize out as ice, leaving behind a solution containing the original solutes of the solution at an elevated concentration. The water in the system becomes progressively less abundant as freezing continues, and the structure of the remaining water is altered both by the changing temperature and the presence of solutes. If the abundance or structure of the water deviate too far from normality, components of the cell, in particular the cell membrane, which depend upon water to stabilize their structures may be altered. The loss of Na impermeability would be a likely consequence of such alterations, and could result in swelling upon thawing by remaining in the cell interior at an elevated concentration, or by remaining permeable, in which case there would be no extracellular solute to balance the intracellular osmotic effect of the proteins. The effects of alteration of cellular components, swelling, and even possible rupture could account for the freeze-thaw damage incurred.

Polymers, which can increase the abundance of water during freezing and also alter the structure of the remaining water, may prevent conditions from becoming severe enough to result in alteration of the membrane. If the temperature at which a particular level of dehydration and solute concentration is reached can be lowered sufficiently, the thermal energy of the macromolecules involved may not be sufficient to bring about the denaturation which would result under similar conditions at higher temperatures. Polymers may be bringing about freeze-thaw protection in the same manner as do the low molecular weight compounds, although the large difference in the behaviour of polymeric solutions and more ideal solutions of low molecular weight compounds in some temperature regions during cooling can cause large differences in the damage resulting from the procedure.

Although the results obtained in this and other studies fit this model of freeze-thaw damage and cryoprotection, several points need to be investigated further to verify that this is indeed what is happening. Several experiments which could lead to greater understanding of the mechanism of freeze-thaw damage and cryoprotection by polymers shall be listed.

It would be highly instructive to monitor the intra and extracellular concentration of several of the constituents of the medium during the freezing process, in an attempt to correlate the point where a certain change occurred to a point where cellular damage began to become apparent. In particular, the flux of NaCl into the cell which has been suggested to cause the swollen appearance after thawing would be a logical parameter to investigate. The technique developed for removing the unfrozen portion of partially frozen solutions by centrifugation could also be used with cell suspensions. The cells and medium could be collected separately after partial freezing and assayed. The study of pH changes occurring during freezing also lends itself readily to this technique.

The osmolarity of the cell interior is due to the osmotic effects of both the low molecular weight components and the polymers, and, according to Farrant (25), the proportion of the total osmotic effect which can be accounted for by the polymers will increase with increasing concentration. Studies to determine the osmotic effect of the high molecular weight components in the cytoplasm at various degrees of dehydration could clarify whether the rise in ionic concentration of the cell interior was significantly less than that of the exterior.

A major obstacle to the understanding of the mechanism of action of polymers in cryoprotection has been the lack of knowledge of the physical chemistry of polymeric solutions, a field of research which has been somewhat neglected. Assumptions made in deriving the laws for the calculation of such parameters as osmolarity and freezing point depression are invalid in concentrated polymeric solutions, so that a whole new approach to these parameters are needed for this situation. Knowledge of the behaviour of such solutions could bring about the rationalization of several observations which intuitively seemed impossible under presently accepted laws.

Finally, complete knowledge of the interaction between the solutes, the water, and the molecules of the cell can only be achieved by the knowledge of solutions at the molecular level. Progress toward defining the structure of water, the effect on that structure of such things as solutes and changes in temperature, and the effect of the resulting structure on the molecules of the cell has been made over the last few years, but much is still to be explained. Until such time as breakthroughs are made in this field, biologists will have to be content to observe macroscopic phenomenon and postulate what is happening at the molecular level. Perhaps through such observations they will be able to aid the chemists in arriving at an explanation of the nature of solutions at the molecular level.

As well as questions relating directly to the field of cryobiology, a few of more general interest to biologists arose in this study. The microexudate carpet which was observed in several electron micrographs could be an important factor in the behaviour of cells in tissue culture, and the knowledge of the kinetics of the buildup of this carpet and the interaction between it and the cell could be very useful. Its presence has now been detected in several cell cultures (95, 129) and it would be interesting to see if this carpet is a characteristic of tissue cultures in general, and to determine if the ability to form this carpet bore any relation to the ability of a particular cell line to survive in tissue culture.

Under confluent conditions piling up of cells was seen, which indicates the cells may have lost their contact inhibition. Loss of contact inhibition has been related to cancer since some cancer cells migrate freely through the tissues of normal cells. It would be interesting to inject these cells into a Chinese hamster and watch for the appearance of cancerous growth.

## REFERENCES

1. Albertsson, P. 1971. Partition of cell particles and macromolecules. John Wiley & Sons, Inc., New York.

2. Allen, L. and P. Kollman. 1970. A theory of anomalous water. Science 167, 1443.

3. -----. and P. Kollman. 1971. Theoretical evidence against the existence of polywater. Nature 233, 550-551.

4. Allison, A.C., P. Davies, and S. de Petris. 1971. Role of contractile microfilaments in macrophage movement and endocytosis. Nature New Biology 232, 153-155.

5. Anderegg, J.W., W.W. Beemer, S. Shulman and P. Kaesberg. 1955. An investigation of the size, shape and hydration of serum albumin by small-angle X-ray scattering. J. Amer. Chem. Soc. 77, 2927-2937.

6. Asahina, E. and K. Aoki. 1958. Survival of intact insects immersed in liquid oxygen without any antifreeze agent. Nature 182, 327-328.

7. Ashwood-Smith, M.J. 1964. Low temperature of mouse lymphocytes with dimethyl sulfoxide. Blood 23, 494-501.

8. -----, and C. Warby. 1971. A species of Pseudomonas, a most useful bacterium for cryobiological studies. Cryobiology 8, 208-210.

9. -----, and C Warby. 1971. Studies on the molecular weight and cryoprotective properties of polyvinylpyrrolidone and dextran with bacteria and erythrocytes. Cryobiology 8, 453-464.

10. -----, and C. Warby. 1972. Protective effect of low and high molecular weight compounds on the stability of catalase subjected to freezing and thawing. Cryobiology 9, 137-140.

11. -----, C. Warby, K.W. Connor and G. Becker. 1972. Low-temperature preservation of mammalian cells in tissue culture with polyvinylpyrrolidone (PVP), Dextrans, and hydroxyethyl starch (HES). Cryobiology 9, 441-449.

12. Barnard, D., J. Fabian, and H. Koch. 1949. Valence vibration frequencies and hydrogen bond formation of sulphoxide and sulphone groups. (Absorption spectra and structure of organic sulphur compounds Part V). J. Chem. Soc. 2442-2454.

13. Barnes, D. and J. Loutit. 1955. The radiation recovery factor. J. Nat. Cancer Inst. 15, 901-905.

14. Bican, P. and E. Dobry. 1969. Transport of some sugars into red blood cells treated with cryoprotective agents. Cryobiology 6, 133-135.

15. Billingham, R.E. and P.B. Medawar. 1952. The freezing, drying, and storage of mammalian skin. J. Exp. Biol. 29, 454-468.

16. Boyle, R. 1683. New experiments and observations touching cold. London.

17. Bricka, M. and M. Bessis. 1955. On the preservation of erythrocytes by freezing at low temperatures in the presence of polyvinylpyrrolidone and dextran. C.R. Soc. Biol. 149, 875.

18. Dalen, H. and T. Nevalainin. 1968. Direct epoxy embedding for vertical sectioning of cells grown as a monolayer on millipore filter. Stain Technology 43, 217-220.

19. DeVries, A., S. Komatsu, and R. Feeney. 1970. Chemical and physical properties of freezing point depressing glycoproteins from Antarctic fishes. J.B.C. 245, 2901-1908.

20. Doebbler, G.F., R.G. Buckhect, and A.P. Rinfret. 1961. Recovery and *in vivo* survival of rabbit erythrocytes. Nature 191, 1405.

21. -----, and A.P. Rinfret. 1962. The influence of protective compounds and cooling and warming conditions on hemolysis of erythrocytes by freezing and thawing. Biochimica et Biphysica Acta, 58, 449-458.

22. Dole, M. and I.L. Faller. 1950. Water sorption by synthetic high polymers. J. Am. Chem. Soc. 72, 414-419.

23. DuPraw, E. 1969. Cell and molecular biology. Academic Press, New York. p. 212.

24. Eagle, H. 1959. Amino acid metabolism in mammalian cell cultures. Science 130, 432-437.

25. Farrant, J. 1969. Is there a common mechanism of protection of living cells by polyvinylpyrrolidone and glycerol during freezing. Nature 222, 1175-1176.

26. -----, and A.E. Woolgar. 1970. Possible relationships between the physical properties of solutions and cell damage during freezing. In: The Frozen Cell, Ciba Found. Symp. (G.E.W., Wolstenholme and M. O'Connor, eds.), Churchill, London. p. 97-114.

27. Ferrandez-Moran, H. 1959. Fine structure of biological lamellar systems. In: Biophysical Science - A study program. J.L. Oncley, ed., John Wiley and Sons, Inc., New York. p. 319-330.

28. Frank, H.S. and M.W. Evans. 1945. Free volume and entropy in condensed systems. III. Entropy in binary liquid mixtures, partial molal entropy in dilute solutions: structure and thermodynamics in aqueous electrolytes. J. Chem. Phys., 13, 507-532.

29. -----. 1972. Water structure. In: Water. A comprehensive treatise. Edited by F. Franks, Plenum Press, N.Y. Vol. 1. p. 515-545.

30. Fuller, M., II and Wallace Brey, Jr. 1968. Nuclear magnetic resonance study of water sorbed on serum albumin. J.B.C. 243, 274-280.

31. Gaff, D.F. 1966. The sulphydryl-disulfide hypothesis in relation to dessication injury of cabbage leaves. Aust, J. Biol. Sci., 19, 291-299.

32. Glauser, S.C. and T.R. Talbot. 1956. Some studies on freezing and thawing human erythrocytes. The American Journal of the Medical Sciences 231, 75-81.

33. Haines, R.B. 1938. The effect of freezing on bacteria. Proc. Roy. Soc., 124, 451-463.

34. Handbook of Chemistry and Physics. 1963, 44th edition, The Chemical Rubber Publishing Co., Cleveland, Ohio. p. 1102.

35. Heber, U. 1970. Proteins capable of protecting chloroplast membranes against freezing. In: The Frozen Cell, Ciba. Found. Symp. (G.E.W. Wolstenholme and M. O'Connor, eds.). Churchill, London. p. 175-186.

36. -----, and K. Santarius. 1964. Loss of adenosine triphosphate synthesis caused by freezing and its relationship to frost hardiness problems. Pl. Physiol. 39, 712-719.

37. Hechter, O. 1965. Role of water structure in the molecular organization of cell membranes. Fed. Proc., 24, S-91 - S-102.

38. Holtzer, A., and M. Emerson. 1969. On the utility of the concept of water structure in the rationalization of the aqueous solution of proteins and small molecules. J. Phys. Chem., 73, 26-33.

39. Jahnel, F. 1938. Über die Widerstandsfähigkeit von menschlichen Spermatozoen gegenüber starker Kälte. Widerauftreteten der Beweglichkut nach Abkühlung auf -196°C (flüssiger stickstoff) und -269.5°C, etwa 3.7° vom absoluten Nullpunkt entfernt (flüssiges Helium) Klin. Wschr. 17, 1273-1274.

40. Jencks, W.P. 1965. Water structure and protein denaturation. Fed. Proc., 24, Supp. 15, S-50 - S-54.

41. Kanwisher, J.W. 1955. Freezing in intertidal animals. Biol. Bull., Woods Hole, 109, 56-63.

42. Kissin, B., and H. Begleiter, editors. 1971. The Biology of Alcoholism. Plenum Press, New York, Volume 1: Biochemistry. p. 1-12.

43. Klotz, I.M. 1958. Protein hydration and behaviour. Science, 128, 815-822.

44. Knorpp, C.T., W.R. Merchant, P.W. Gikas, H.H. Spencer, and N.W. Thompson. 1967. Hydroxyethyl starch, extracellular cryophylactic agent for erythrocytes. Science, 157, 1312-1313.

45. Kuntz, I.D., Jr., T.S. Brassfield, G.D. Law, and G.V. Purcell. 1969. Hydration of macromolecules. Science, 163, 1329-1331.

46. Leibo, S., J. Farrant, P. Mazur, M. Hanna, Jr., and L. Smith. 1970. Effects of freezing on marrow stem cell suspensions: interactions of cooling and warming rates in the presence of PVP, sucrose, or glycerol. Cryobiology 6, 315-332.

47. -----, and P. Mazur. 1971. The role of cooling rates in low-temperature preservation. Cryobiology 8, 447-452.

48. Levitt, J. 1962. A sulfhydryl-disulfide hypothesis of frost injury and resistance in plants. J. Theor. Biol., 3, 355-391.

49. -----. 1966. Winter hardiness in plants. In: Cryobiology, H.T. Meryman, ed. Academic Press, New York. p. 495-563.

50. -----. 1966. Cryochemistry of plant tissue. Protein interactions. Cryobiology 3, 243-251.

51. -----, and J. Dear. 1970. The role of membrane proteins in freezing injury and resistance. In: The Frozen Cell, Ciba Found. Symp. (G.E.W. Wolstenholme and M. O'Connor, eds.). Churchill, London. p. 149-173.

52. Ling, Gilbert. 1965. Physiology and anatomy of the cell membrane: the physical state of water in the living cell. Fed. Proc., 24, S-103 - S-114.

53. Loveloch, J.E. 1953. The haemolysis of human red blood cells by freezing and thawing. Bioch. Biophys. Acta 10, 414-426.

54. -----. 1953. HET mechanism of the protective action of glycerol against haemolysis by freezing and thawing. Bioch. Biophys. Acta 11, 28-36.

55. -----. 1954. The protective action of neutral solutes against haemolysis by freezing and thawing. Biochem. Journal 56, 265-270.

56. -----. 1954. Physical instability and thermal shock in red cells. Nature 173, 659-661.

57. -----, and M. Bishop. 1959. Prevention of freezing damage to living cells by dimethyl sulfoxide. Nature 183, 1394-1395.

58. Luft, J.H. 1961. Improvements in epoxy resin embedding methods. J. Biophys. Biochem. Cytol., 9, 409-414.

59. Luyet, B.J. 1949. Ultra-rapid freezing as a possible method of blood preservation. In: Preservation of the formed elements and of the proteins of the blood. Washington American Red Cross. p. 141-146.

60. McConaghey, P. and M. Maizels. 1961. The osmotic coefficients of haemoglobin in red cells under varying conditions. J. Physiol., 155, 28-45.

61. Malinin, T., D. Pegg, V. Perry and C. Brodine. 1970. Long-term storage of bone marrow cells at liquid nitrogen and dry ice temperatures. Cryobiology 7, 65-69.

62. Mantegazza, P. 1866. Sulla sperma umano. R.C. 1st. lombardo, 3, 183-196.

63. Mazur, P. 1963. Kinetics of water loss from cells at subzero temperatures and the likelihood of intracellular freezing. J. Gen. Physiology 47, 347-369.

64. -----. 1966. Physical and chemical basis of injury in single-celled micro-organisms subjected to freezing and thawing. In: Cryobiology, H.T. Meryman, ed. Academic Press, New York. p. 220-234.

65. -----, and J. Schmidt. 1968. Interaction of cooling velocity temperature, and warming velocity on the survival of frozen and thawed yeast. Cryobiology 5, 1-17.

66. -----, S. Leibo, J. Farrant, E.H.Y. Chu, H.O. Hanna and L.H. Smith. 1970. Interactions of cooling rate, warming rate and protective additive on the survival of frozen mammalian cells. In: The Frozen Cell, Ciba Found. Symp. (G.E.W. Wolstenholme and M. O'Connor, eds.) Churchill, London. p. 69-85.

67. Meryman, H.T. 1957. Physical limitations of the rapid freezing methods. Proc. Roy. Soc. 147, 452-459.

68. -----. 1966.
Cryobiology, Academic Press, New York, page IX.

69. -----. 1966. Review of biological freezing. In: Cryobiology, Academic Press, New York. p. 1-114.

70. -----. 1971. Osmotic stress as a mechanism of freezing injury. Cryobiology 8, 489-500.

71. Morichi, T., R. Irie, N. Yano and H. Kembo. 1963. Protective effect of glutanic acid and related compounds on bacterial cells subjected to freeze-drying. J. Gen. Appl. Microbiol., 9, 149-161.

72. Nash, T. 1962. The chemical constitution of compounds which protect erythrocytes against freezing damage. J. of Gen. Physiol., 46, 167-175.

73. Nei, T. 1970. Mechanism of haemolysis of erythrocytes by freezing, with special reference to freezing at near-zero temperatures. In: The Frozen Cell, Ciba Found. Symp. (G.E.W. Wolstenholme and M. O'Connor, eds.). Churchill, London. p. 135.

74. Nelson, B. and B. Flaxman. 1972. *In situ* embedding and vertical sectioning for electron microscopy of tissue cultures grown on plastic petri dishes. Stain Technology 47, 261-265.

75. Nemethy, G. and H.A. Scheraga. 1962. Structure of water and hydrophobic bonding in proteins. I. A model for the thermodynamic properties of liquid water. J. Chem. Phys., 36, 3382-3400.

76. -----, and H.A. Scheraga. 1962. Structure of water and hydrophobic bonding in proteins. II. Model for the thermodynamic properties of aqueous solutions of hydrocarbons. J. Chem. Phys., 36, 3401-3417.

77. Parkes, A.S. and A.U. Smith. 1960. Recent research in freezing and drying. Blackwell Scientific Publications, Oxford.

78. Paul, J. 1959. Cell and tissue culture. E. & S. Livingstone Ltd., London. p. 173.

79. Persidsky, M. and V. Richards. 1962. Mode of protection with polyvinylpyrrolidone in freezing of bone marrow. Nature 196, 585-586.

80. -----, and M. Ellett. 1971. Hydroxyethyl starch as a cryopreservative for nucleated mammalian cells. Cryobiology 8, 586-588.

81. Polge, C., A.U. Smith and A.S. Parkes. 1949. Revival of spermatozoa after vitrification and dehydration at low temperatures. Nature 164, 666-667.

82. Rapatz, G. and B. Luyet. 1960. Microscopic observations on the development of the ice phase in the freezing of blood. Biodynamica 8, 181-239.

83. -----, L.J. Menz and B.J. Luyet. 1966. Anatomy of the freezing process in biological materials. In: Cryobiology, H.T. Meryman, ed. Academic Press, New York. p. 143.

84. -----. 1970. Resumption of activity of frog hearts after freezing to low temperatures. Biodynamica 11, 1-12.

85. Rasmussen, D.H. and A.P. Mackenzie. 1972. Effect of solute on ice-solution interfacial free energy: calculation from measured homogeneous nucleation temperatures. In: Water structure at the water-polymer interface. Edited by H. Jellinek, Plenum Press, New York. p. 126-145.

86. Ray, B. and M. Speck. 1972. Repair of injury induced by freezing *Escherichia coli* as influenced by recovery medium. Applied Microbiology 24, 258-263.

87. -----, and M. Speck. 1972. Metabolic process during the repair of freezing injury in *Escherichia coli*. Applied Microbiology 24, 585-590.

88. Réaumur, R.A.F. de. 1736. Memoires pour servir a l'histoire des insects. Tome II, 141-147, Paris de l'Impremerie Royale.

89. Rey, L.R. 1960. Study of the freezing and drying of tissues at very low temperatures. In: Recent research in freezing and drying. Edited by A.S. Parkes and A.U. Smith. Blackwell Scientific Publications, Oxford. p. 40-62.

90. Reynolds, E.S. 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J. Cell Biol., 17, 208-212.

91. Rob, C. and H. Eastcott. 1954. The preservation of arteries and other tissues for chemical use. In: Preservation and transplantation of normal tissues. Ed. G. Wostenholme and M. Cameron, Ciba Foundation Symposium. Churchill, London. p. 190-195.

92. Robinson, D.M. 1970. Mammalian cells can repair freeze-thaw-induced damage. Cryobiology 6, 573.

93. -----. 1971. Repair of freeze-thaw-induced damage in mammalian cells: Possible role of membrane glycoproteins. Cryobiology 8, 384.

94. -----. 1972. The repair of freezing injury in mammalian cells recovered from liquid nitrogen. John Hopkins Med. J. 1, Suppl. 1, 104-107.

95. Rosenberg, M.D. 1960. Microexudates from cells grown in tissue culture. Biophysical Journal 1, 137-159.

96. Rostand, J. 1946. Glycérine et résistance du sperme aux basses temperatures. C.R. Acad. Sci., Paris, 222, 1524-1525.

97. Salt, R.W. 1958. Role of glycerol in producing abnormally low supercooling and freezing points in an insect *Bracon cephi* (Gahan). Nature 181, 1281.

98. -----. 1959. Role of glycerol in the cold-hardening of *Bracon cephi* (Gahan). Canad. J. Zool., 37, 59-69.

99. Scholander, P.F., L. van dam, J.W. Kanwisher, H.T. Hammel, and M.S. Gordon. 1957. Supercooling and osmoregulation in arctic fish. J. Cell Comp. Physiol., 49, 5-24.

100. Schreiber, K. 1949. Infrared spectra of sulfones and related compounds. Analyt. Chem., 21, 1168-1172.

101. Schaffner, C.S., E.W. Henderson and C.G. Card. 1941. Viability of spermatozoa of the chicken under various environmental conditions. Poultry Sci., 20, 259-265.

102. Sherman, J.K. 1972. Comparison of *in vitro* and *in situ* ultrastructural cryoinjury and cryoprotection of mitochondria. Cryobiology 9, 112-122.

103. Smith, A.U. 1949. Cultivation of rabbit eggs and cumuli for phase-contrast microscopy. Nature 164, 1136-1137.

104. -----. 1950. Prevention of haemolysis during the freezing and thawing of red blood-cells. Lancet 2, 910-911.

105. -----. 1961. Biological effects of freezing and supercooling. Edward Arnold Ltd., London.

106. Smith, A.U. 1961. Biological effects of freezing and supercooling. Edward Arnold Ltd., London. p. 79-93.

107. -----. 1961. Biological effects of freezing and supercooling. Edward Arnold Ltd., London. p. 110-137.

108. Spurr, A. 1969. A low-viscosity epoxy resin embedding medium for electron microscopy. J. Ult. Res.. 26, 31-43.

109. Stein, W.D. 1967. The movement of molecules across cell membranes. Academic Press, New York. p. 130-133.

110. -----. 1967. The movement of molecules across cell membranes. Academic Press, New York. p. 266-308.

111. Stowell, R.E., D. Young, E. Arnold and B. Trump. 1965. Structural, chemical, physical and functional alterations in mammalian nucleus following different conditions of freezing, storage and thawing. Fed. Proc., 24, S-115 - S-141.

112. Tait, M.J. and E. Franks. 1971. Water in biological systems. Nature 230, 91-94.

113. Taylor, A.C. and R. Gerstner. 1955. Tissue survival after exposure to low temperatures and the effectiveness of protective pretreatments. J. Cell. Comp. Physiol., 46, 477-502.

114. Thibault, C. 1947. La parthenogenèse expérimentale chez le lapin. C.R. Acad. Sci., Paris, 224, 297-299.

115. Trump, B.F., D. Young, E. Arnold and R. Stowell. 1965. Effects of freezing and thawing on the structure, chemical constitution, and function of cytoplasmic structures. Fed. Proc., 24, S-144 - S-172.

116. Van den Berg, L. 1959. The effect of addition of sodium and potassium chloride to the reciprocal system: $KH_2PO_4$ - $Na_2HPO_4$ - $H_2O$ on pH and composition during freezing. Arch. Biochem. Biophys., 84, 305-315.

117. -----, and D. Rose. 1959. Effect of freezing on the pH and composition of sodium and potassium phosphate solutions: The reciprocal system $KH_2PO_4$ - $Na_2HPO_4$ - $H_2O$. Arch. Biochem. Biophys., 81, 319-329.

118. van Harreveld, A., J. Crowell and S.K. Malhotra. 1965. A study of extracellular space in central nervous tissue by freeze substitution. J. Cell. Biol., 25, 117-137.

119. Vos, O., M.C.A.C. Kaalen and L. Budke. 1964. Radiation protection by a number of substances preventing freezing damage. I. Protection of mammalian cells *in vitro*. Int. J. Rad. Biol., 9, 133-142.

120. -----, and M.C.A.C. Kaalen. 1965. Prevention of freezing damage to proliferating cells in tissue culture. Cryobiology 1, 249-260.

121. Voss, W.A.G., C. Warby, R. Rajotte and M.J. Ashwood-Smith. 1972. Microwave thawing of tissue culture cells. Cryobiology 9, 562-564.

122. -----. 1973. Personal communication.

123. Wang, J.H. 1954. Theory of the self-diffusion of water in protein solutions: A new method for studying the hydration and shape of protein-molecules. J. Amer. Chem. Soc., 76, 4755-4763.

124. Wessells, N., B. Spooner, J. Ash, M. Bradley, M. Luduena, E. Taylor, J. Wrenn and K. Yamada. 1971. Microfilaments in cellular and developmental processes. Science 171, 135-143.

125. Whittingham, D.G., S.P. Leibo and P. Mazur. 1972. Survival of mouse embryos frozen to -196 and -269°C. Science 178, 411-414.

126. Williams, R. and H.J. Meryman. 1970. Freezing injury and resistance in spinach chloroplast grana. Plant Physiol., 45, 752-755.

127. Wyatt, G.R. and G.F. Kalf. 1957. The chemistry of insect hemolymph. II. Trehalose and other carbohydrates. J. Gen. Physiol., 40, 833-847.

128. -----, and W.L. Meyer. 1959. The chemistry of insect hemolymph. III. Glycerol. J. Gen. Physiol., 42, 1005-1011.

129. Yaoi, Y. and T. Kanaseki. 1972. Role of microexudate carpet in cell division. Nature 237, 283-285.

# VITA

Surname: CONNOR Given Names: KENNETH WAYNE

Place of Birth: KINDERSLEY, SASKATCHEWAN Date of Birth: July 24, 1950

Educational Institutions Attended, with Dates of Entering and Leaving:

UNIVERSITY OF SASKATCHEWAN, SASKATOON 1967 to 1971

Degrees, Diplomas, Etc., Awarded, with Dates and Names of Institutions:

B.Sc. 1971 University of Saskatchewan, Saskatoon

Honors and Awards:

Saskatchewan Government Scholarship, 1967

University of Saskatchewan Undergraduate Scholarship, 1968

University of Saskatchewan Undergraduate Scholarship, 1969

Doucie Scholarship for Physics, 1969

Honors Scholarship, 1970

N.R.C. Postgraduate Scholarship, 1971

N.R.C. Postgraduate Scholarship, 1972

Publications:

1. Ashwood-Smith, M.J., C. Warby, K.W. Connor and G. Becker. 1972. Low-temperature preservation of mammalian cells in tissue culture with polyvinylpyrrolidone (PVP), Dextrans, and hydroxyethyl starch (HES). Cryobiology 9, 441-449.

2. Connor, K.W., and M.J. Ashwood-Smith. in press. Ineffectiveness of dimethyl sulfone as a cryoprotective agent.

PARTIAL COPYRIGHT LICENSE

Title of Thesis/Dissertation:

"Cryoprotection of Chinese Hamster Cells in Tissue Culture by Polymers"

Author:

[redacted]

Signature

KENNETH WAYNE CONNOR

Name

SEPT. 11, 1973

Date