The Relationship between Dehydration and Freezing Injury in the Human Erythrocyte*

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Abstract

Analyses have been made of the relationship between hemolysis, temperature, glycerol concentration and the proportion of water converted to ice. This has made it possible to designate a certain proportion of the water in erythrocytes as "vital" and has enabled the formulation of a single hypothesis which explains the survival of red cells following both rapid and slow freezing in the presence of either diffusable or non-diffusable additives.

Introduction

It has been repeatedly observed that temperature reduction *per se* is rarely, if ever, lethal to living systems and that it is the crystallization of water to ice that is responsible for the injury associated with very low temperature. So it is not unreasonable that the emphasis of cryobiology in the past has been primarily concerned with the ice crystal: with its formation, growth, location and its response to variations in cooling rate and the solutions from which it is grown. However, there have always been many examples of the survival of cells despite extensive crystallization, casting doubt on the assumption that injury from freezing is largely mechanical. The fact that the freezing out of water results in a concentration of solutes has also been long appreciated and many investigators have proposed that it is this concentration rather than the physical presence of ice crystals which results in injury. Perhaps the best known and most thoroughly documented is the study by Lovelock (1953) who proposed the concentration of electrolytes as a cause of injury to erythrocytes. Literature reviews which attempt to correlate cell injury with dehydration have not, however, revealed in general a good correlation between the percent of water frozen out and injury to the organism.

Our studies of freezing injury have nevertheless led us to an increasing conviction that dehydration is the primary cause of freezing injury. Since it is well known that, once a potentially injurious freezing temperature has been reached, injury often proceeds at a temperature dependent rate, we felt that the lack of correlation between dehydration and injury might well result from the fact that the ultimate degree of injury will depend not only on the extent of dehydration but on the time-temperature dependent processes

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which subsequently develop. In our calorimetric studies of the relationship between dehydration and injury we have, therefore, chosen as our end point the temperature of freezing just short of that which produces demonstrable injury. We have termed this the temperature of incipient injury.

Our measurements of the degree of dehydration have been made calorimetrically using a continuous recording calorimeter designed and constructed in our laboratory (Williams and Meryman, 1965). This instrument introduces or removes heat at a known rate so that the temperature change of a specimen of known mass enables a calculation of the proportion of energy utilized in phase change and, therefore, the proportion of ice formed. This apparatus was initially applied to a study of two species of mollusks, one of which, *Mytilus edulis* is intertidal with a temperature of incipient injury of −10°C, the other, *Venus mercenaria*, a sand dweller with a temperature of incipient injury of −6°C. The percentage of water frozen out at these temperatures of incipient injury was 66% and 65% respectively for these two species, despite the substantial differences in temperature. A subsequent measurement for human erythrocytes demonstrated that at −3°C, their temperature of incipient injury, 65% of the water of a packed cell preparation was frozen out.

These preliminary experiments lent support to our convictions that freezing injury is associated with dehydration or, put in a different way, that a minimum quantity of unfrozen water is essential for cell survival. However, if this is a valid conclusion, then some minimum quantity of unfrozen liquid should be demonstrable in all cells which survive freezing. In looking for suitable cells other than those of the mollusk in which this hypothesis might be tested, we were attracted to further experimentation with the human erythrocyte since this cell is capable of survival following rapid freezing in the absence of cryoprotective agents although it is hemolyzed by slow freezing and by ultra-rapid freezing. Furthermore, cells which survive rapid freezing to −100°C or below are promptly hemolyzed by subsequent exposure to temperatures above −50°C. Erythrocytes rapidly frozen in liquid nitrogen were therefore compared calorimetrically with an aliquot which had, in addition, been cycled to −20°C. In confirmation of our prediction, some of the freezable water content of the initial preparation was found to have remained unfrozen while, in that which had been exposed to −20°C, no unfrozen water was detected. This evidence has supported us in the development of an hypothesis for the mechanism of freezing injury through dehydration.

Since, in cryobiology, the mammalian erythrocyte has been the most extensively studied of all animal cells and presents the most varied and troublesome contradictions in its response to freezing, our hypothesis is presented here in terms of the human erythrocyte. The following are the principle phenomena which the hypothesis must explain:

1. Slow cooling will produce complete hemolysis in unprotected cells.

2. A compound such as glycerol which will penetrate the cell membrane, colligatively retain water and prevent it from freezing, and which is non-toxic to the cell, will reduce injury from slow freezing. A concentration of about 3 molar will be fully protective at all temperatures.

3. Rapid cooling (around 50°C per second) permits the survival of about 85% of unprotected cells.

4. Non-penetrating agents such as PVP, lactose and sucrose do not protect against slow freezing but will increase the recovery of cells following rapid freezing and render the freezing rate less demanding.

5. Ultra-rapid cooling at rates of several hundred degrees per second will produce complete hemolysis. This freezing rate has also been shown to cause intracellular ice formation.

6. Both penetrating and non-penetrating cryoprotective agents reduce the cooling rate necessary for hemolysis from ultra-rapid freezing.

7. When injury is prevented by rapid freezing, with or without additive, thawing must be rapid. Brief exposure to temperatures above −60°C produce complete hemolysis.

Our hypothesis for injury is based on the assumption that dehydration beyond a certain level is the primary cause of injury. The manner in which different freezing rates and the presence of additives effect dehydration is proposed to be as follows:

Injury from slow freezing. Since an ice crystal nucleus cannot grow unless its radius of curvature exceeds a critical value which is directly proportional to temperature, the probability of a crystal nucleus of adequate radius of curvature existing in a modest volume of solution near its melting point is poor and most aqueous solutions can be supercooled to several degrees below their melting point before the largest nucleus in the solution will become critical and initiate ice crystal growth. It has also been demonstrated that increasing the concentration of a solution not only depresses the melting point but also depresses the supercooling temperature by a comparable amount.

When a tissue or cell suspension is cooled below its melting point, supercooling is thus almost invariably seen. When the temperature is lowered sufficiently, the largest nucleus present becomes critical and crystal growth commences. If the rate of heat withdrawal is modest (slow cooling) this single crystal will produce sufficient latent heat to equal that removed and the specimen temperature will rise to its melting point and

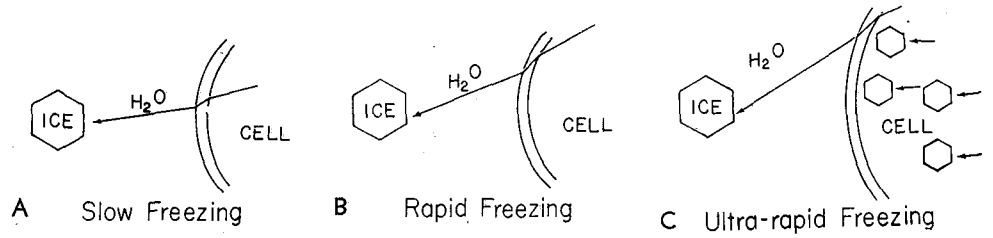

Fig. 1. A. During slow freezing water diffuses from the cell and its suspending medium to add to the ice crystal. The concentration gradient between cell and crystal will be low if the rate of crystal growth is slow. The cell interior will approach vapor pressure equilibrium with the ice and, as the temperature decreases, all freezable water will ultimately be removed leading to cell injury

B. With more rapid growth of the ice crystal, the concentration gradient between ice and cell steepens. Increasing viscosity from concentration and temperature reduction further increases resistance to diffusion. When the cooling rate is fast enough, the system may reach stabilizing temperature before all the freezable water has left the cell, fulfilling the requirements for cell survival

C. At ultra-high cooling velocities, a great deal of freezable water remains in the cell. The supercooling temperature is insufficiently reduced by solute concentration and intracellular nucleation takes place. There is now very little resistance to diffusion between intracellular water and intracullular ice so that all freezable water readily crystallizes, possibly during thawing

freezing will continue without supercooling. Since the initial supercooling demonstrated no nuclei of critical size near the melting point, it follows that there can be no new crystal formation beyond the initial nucleating event. As the melting point is reduced by solute concentration with continued freezing, no new nuclei form since critical size has also been modified by the solute concentration. Thus, a single crystal, branching throughout the specimen, can be responsible for the entire freezing process during slow cooling.

With progressive freezing, the extracellular solution becomes concentrated and water leaves the cell, ultimately to add to the ice crystal; the cell is dehydrated and once its fluid content has been reduced below a critical level it is irreversibly injured (Fig. 1, A).

Effect of penetrating cryoprotective agents. When a penetrating cryoprotective agent (CPA) such as glycerol is added to the suspending solution some water will then be prevented from freezing, remaining available as a solvent. If sufficient glycerol is added the unfrozen water and, to some extent, the glycerol as well will provide sufficient solvent phase to prevent dehydration beyond that incompatible with cell survival.

Survival following rapid freezing without protective agent. With lowering temperature and progressive freezing, increased solute concentration and temperature reduction produce an increased viscosity of the unfrozen solution and a decreased rate of water transport across cell membranes. With more rapid freezing, the concentration gradient which must exist between cell and ice crystal if water is to move from one to the other becomes greater; (Fig. 1, B) that is, more freezable water remains in the cell. If the cooling rate is sufficiently rapid, the preparation may cool to a stabilizing temperature (below -60 to -80°C) before all freezable water has left the cell. If sufficient unfrozen water remains, the minimum fluid phase requirements of the cell will be met and the cell can survive. This unfrozen water does not crystallize *in situ* since its supercooling temperature has been sufficiently reduced by solute concentration to prevent nucleation. Our calorimetric studies of rapidly frozen red cells demonstrate the existence of this unfrozen water.

Enhancement of recovery following rapid freezing with non-penetrating protective agents. Cryoprotective agents, both penetrating and non-penetrating, are notably effective in reducing the velocity of ice crystal growth. Reduction in the rate at which water is crystallized will enhance the tendency for water to remain in the cell, making non-lethal freezing possible at less demanding rates of cooling.

Destruction by ultra-rapid freezing. When cooling is ultra-rapid, insufficient opportunity is provided for the diffusion of water from the cell to the ice crystal. This in turn results in insufficient concentration of cell fluid to lower the supercooling temperature enough to prevent nucleation, and intracellular crystallization results. When ice forms within the cell, the concentration gradient between cell fluid and ice crystal is virtually eliminated and it is no longer possible to avoid the complete freezing out of water (Fig. 1, C). Even if all freezable water is not crystallized during freezing, it will be during thawing. Since both penetrating and non-penetrating agents tend to reduce crystallization velocity and therefore encourage the retention of intracellular water, they will also facilitate the nucleation of intracellular ice, increasing the hemolysis of cells through ultra-rapid freezing. This hypothesis does not reject the possibility of mechanical

injury from intracellular ice, but rather proposes an alternate or additional mechanism.

The foregoing picture of the freezing process is concerned only with the manner in which the development of ice influences cell dehydration. The next question concerns the manner in which this dehydration causes injury. At the present the various theories found in the literature can be classed into three general categories:

1) *Electrolyte concentration.* This is the most familiar of the hypotheses. It attributes injury to a concentration of electrolytes resulting from the freezing out of water. The precise mechanism by which electrolyte concentration does damage has not been specified. The evidence is circumstantial, based largely on the observation that freezing temperatures which kill produce a concentration of electrolyte which is also injurious in the absence of freezing (Lovelock, 1953). We are inclined to question the salt concentration theory as a specific hypothesis on the basis that it is indistinguishable from dehydration. Cations, such as sodium and potassium are strongly hydrated particularly at low temperature, with a bond strength considerably in excess of the water-water interaction, of those in ice, those between water and cryoprotective agents and, probably, those between protein and water, other than perhaps the first monolayer. Salt can therefore be considered a dehydrating agent. As water is frozen out of a salt solution, the water of hydration of the salt will represent a proportionately larger amount of residual liquid. The water of hydration of the salt may be unavailable for most other solvent functions so that a high concentration of salt can also mean a high degree of effective dehydration. Furthermore, artificially increasing the salt concentration to produce injury does not necessarily constitute evidence that salt concentration is the agent of injury. Since the salt is an osmotically active agent, increasing the concentration in a tissue suspension effectively decreases the amount of available water in the cell. In other words, salt concentration and dehydration are inexorably parallel. A saline solution cannot be dehydrated without increasing the salt concentration, nor can the extracellular salt concentration be increased without intracellular dehydration.

Nevertheless, the general notion of solute concentration cannot be summarily rejected as a possible vehicle by which dehydration effects injury. The concentration of solutes will, of course, be related to the volume of solvent. Both electrolytes and carbohydrates appear to be readily soluble in aqueous solutions of glycerol and DMSO. In solutions of DMSO of 25, 50 and 75 grams percent we were able to dissolve at 27°C, respectively, approximately 24, 18.5 and 13 grams of NaCl to 100 ml of solution and 20.4, 10.2 and 5.7 grams of KCl. Similar solutions of glycerol dissolved approximately 29.8, 27.5 and 22 grams of NaCl and 26, 20.5 and 16.5 grams of KCl. When isotonic salt solutions containing glycerol are frozen, the salt concentration, calculated on the basis of the total solvent volume, does not exceed the solubility of the solution when the starting concentration of glycerol is greater than 1 M. If the entire aqueous solution of cryoprotective agent is assumed to function as solvent, then injury from solute concentration should bear a consistent relationship to the residual liquid phase during freezing.

2) *Physical contact theory.* This theory proposes that the removal of interstitial water from the cell results in the apposition and contact of surfaces which are normally separated by solvent. Irreversible bonds between them result and structures are ruptured or distorted on rehydration. The formation of disulfide bonds from neighboring sulfhydryl

groups or by disulfide interchange has been proposed as a specific mechanism (Levitt, 1962). If it is the maintenance of a minimum distance between opposing structure which is necessary to prevent the formation of undesirable linkages, then nothing more than a space-filling function is required of the intervening liquid phase. Injury from physical contact should therefore also be related to the total volume of residual liquid phase.

3) *Structured water theory.* Many models of protein and membrane propose layers of water which contribute to protein stability. Some investigators feel that there is a condition of mutual support, with the water being stabilized by the protein surface while this water in turn tends to stabilize the configuration of the protein. The removal of such water by freezing would presumably permit denaturation of the protein (Sinanoğlu and Abdulnur, 1965).

Some of the water associated with protein is unfreezable. Literature figures for the water of hydration of proteins place it at from 35 to 50% of the protein dry weight depending on molecular weight. Of the total water in most animal cells, between 5 and 10%, or 20 to 50% of dry weight, is unfreezable. This suggests that the unfreezable water associated with proteins may be monolayer water. Since cell injury must result from the removal of freezable water, if the structured water theory is to be applied, we must presume additional layers of water, presumably of decreasing order, to be important for protein stability. It should be observed, parenthetically, that many purified proteins can be completely dehydrated without being denatured. This does not, however, invalidate the structured water theory, since it is not necessary that all proteins be denatured to create cell injury. Those sensitive to dehydration may well be those that cannot be isolated or purified. In any event, the structured water theory depends not on total solvent phase or liquid volume but on the presence and integrity of a component of the freezable water which is affording structural stability to proteins or perhaps serving some functional purpose in pores and micelles such as those proposed for muscle and membrane.

In summary, both the physical contact and the salt concentration theories require that, for survival, the cell must retain a minimum volume of fluid phase; the former to provide physical separation of structure, the latter to act as a solvent. The structured water theory requires that sufficient structure must remain undisturbed to maintain protein stability. On this basis we felt that an examination of the relationship between residual water, residual total fluid phase (*i.e.* additive-water complex) and cell injury might help to discriminate between these alternatives. Human erythrocytes were selected for the preliminary experiments.

I. Preliminary Experiment

The extent of dehydration from freezing can be modified in two ways. First, as the temperature is lowered below the freezing point, additional water freezes out, increasing the dehydration. Second, the introduction of cryoprotective agents will substantially lower the melting point and reduce the amount of ice formed at any given temperature to an extent proportional to the amount of cryoprotective agent used. The experiments to be reported here consisted initially in determining the percentage of hemolysis of human erythrocytes at a variety of temperatures and cryoprotective agent concentrations.

One preliminary question to be answered concerned the rate at which samples should be frozen and the interval of time that should elapse following arrival at the experimental temperature prior to the measurement of hemolysis. Experiments concerned with the storage stability of erythrocytes at temperatures between −10 and −40°C with less than fully protective concentrations of glycerol (0.5 to 2.5 M) showed a steady increase in hemolysis with storage when the cells were frozen by immersing ampules containing 2 ml aliquots into a liquid bath already at the experimental temperature, producing a cooling rate of between 10 and 30°C/min. On the basis of the hypothesis presented above, we postulated that this relatively rapid freezing might produce a situation in which not all of the freezable cell water had diffused to the ice crystal and that the subsequent continued hemolysis with storage might reflect a slow continuing freezing-out of water with a resulting increase in dehydration and injury. On this basis, freezing at a very slow rate should produce a higher initial hemolysis but with stability on subsequent storage.

Contrary to expectation, erythrocytes frozen by cooling at a rate of 0.3°C/min showed a substantially lower hemolysis after thawing in 37°C water than aliquots which had been frozen by direct immersion into a bath of the same final temperature. (Cooling rates produced by direct immersion were far less than those necessary to produce intracellular ice.) Samples slowly frozen showed no increased hemolysis over a storage period of several hours (Fig. 2).

In searching for an explanation for the improved recovery of cells slowly frozen at low glycerol concentration as compared to those more rapidly frozen, the possibility of

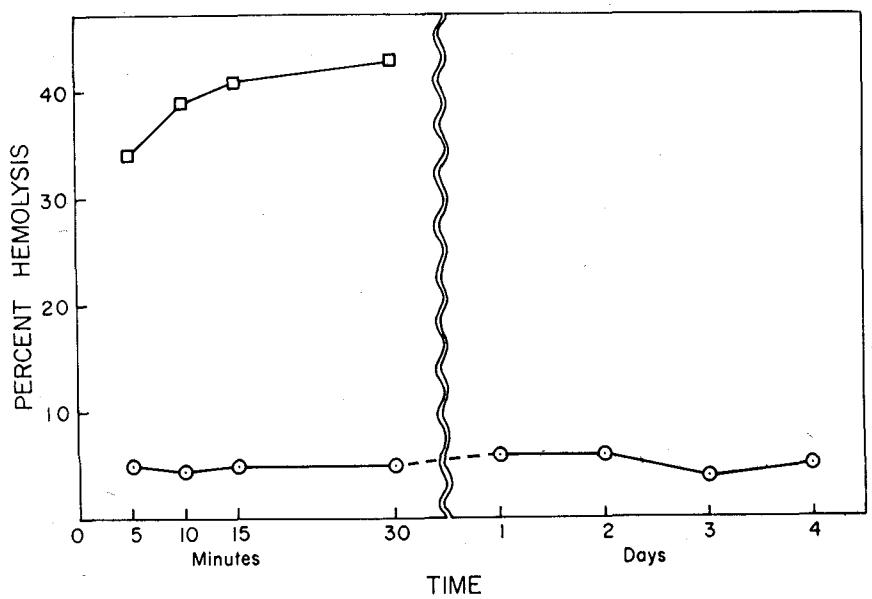

Fig. 2. Human erythrocytes suspened in 1.5 M glycerol in a balanced salt solution containing 0.02 M monopotassium phosphate, 0.02 M dipotassium phosphate and 0.06 M potassium citrate.—□—: Cells frozen by direct immersion of test tube containing 2 ml aliquot into a bath at −26°C. Progressive increase in hemolysis is seen over a 30 min time interval. —○—: Aliquot of the same cells frozen to −26°C at 0.3°C/min after being seeded at −3°C. Much less hemolysis is seen and no appreciable increase over a storage period of 4 days at −26°C

a thermal shock phenomenon was considered. Lovelock (1954) has reported experiments in which cells suspended in 1 M NaCl could be hemolyzed by lowering their temperature at a rate in excess of 6°C/min. Since dehydration from freezing concentrates electrolytes it is clearly possible, as Lovelock proposed, that thermal shock with continued cooling of the partially frozen cells could be responsible for some of the injury.

Although Lovelock failed to demonstrate thermal shock with a rise in temperature, we felt it advisable to explore the effects of very slow rewarming in comparison with conventional rapid thawing in 37°C water. We were intrigued to find a substantial reduction in hemolysis following warming at 0.3°C/min as compared to thawing at 37°C. This was a consistent observation at all temperatures and at all less than fully protecting concentrations of glycerol. A reduction in hemolysis with very slow thawing was not seen in erythrocytes frozen in the absence of glycerol. Figure 3 illustrates the relationship between two rates of freezing and thawing for erythrocytes equilibrated in 1 M glycerol. A similar relationship was demonstrated for glycerol concentrations from 0.5 to 2.5 M, the highest concentration investigated. On the basis of these observations, all subsequent freezing and thawing procedures were carried out at 0.3°C/min. Slower rates of cooling

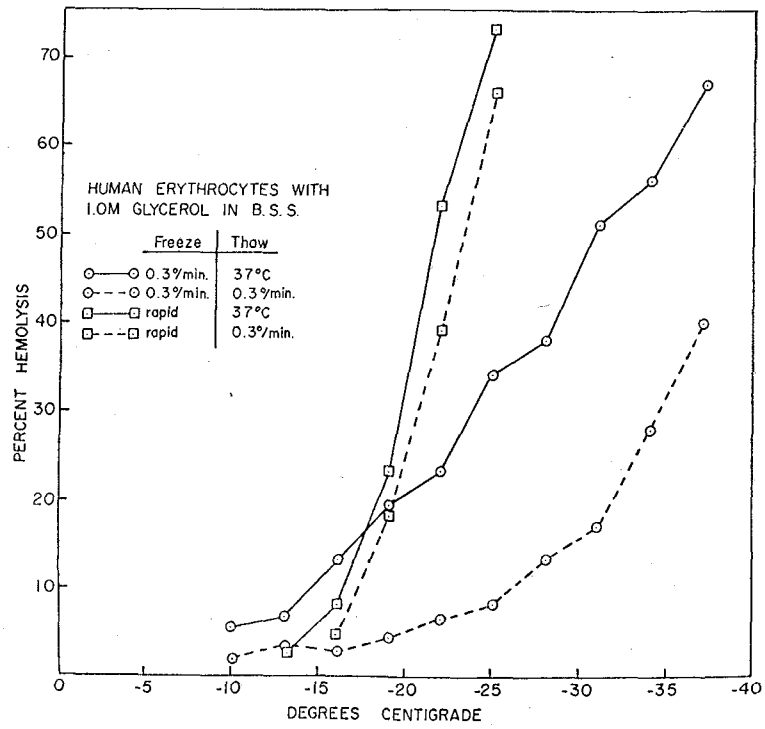

Fig. 3. Hemolysis of erythrocytes suspended in a solution of 1 M glycerol plus balanced salt solution. The freezing and thawing rates are indicated in the illustration. Aliquots were 2 ml contained in 10×75 mm test tubes, hematocrit 20%. Rapid freezing, *i.e.*, immersing the sample into the bath at the indicated temperature, was apparently responsible for increased hemolysis regardless of the thawing rate. Slow cooling at 0.3°C/min caused less hemolysis and thawing at 0.3°C/min further reduced hemolysis as compared with thawing in a 37°C bath. Qualitatively similar results were obtained at glycerol concentrations from 0.5 to 2.5 M

and warming were not found to decrease hemolysis further. We have not yet had an opportunity to explore faster cooling and warming rates to determine the maximum compatible with optimum recovery.

II. Experimental Procedure

Fresh human erythrocytes collected in ACD solution were allowed to sediment spontaneously. The packed cells were withdrawn and resuspended in a balanced salt solution consisting of 0.02 M monopotassium phosphate, 0.02 M dipotassium phosphate and 0.06 M potassium citrate plus glycerol or dimethylsulfoxide to the desired concentration. Cells were not mixed directly with concentrations in excess of 1 molar but were passed sequentially through solutions of gradually increasing strength in order to avoid any injury which might result from sudden osmotic change. On being suspended in the final concentration of cryoprotective agent (CPA) a large excess of CPA solution was used in order that the cells could equilibrate with the solution and thus eliminate the necessity of measuring or calculating final concentration on the basis of the estimated liquid phase of the cells. Following equilibration in the final concentration the cells were transferred again to an excess of CPA solution producing a final hematocrit of approximately 20% in which they were allowed to equilibrate overnight at 4°C.

A number of 2 ml aliquots of each suspension were placed in 20×70 mm tubes and transferred to a liquid bath at 0°C. The bath temperature was reduced in temperature at the rate of 0.3°C/min. At −3°C, suspensions in 1 molar CPA or less were seeded by touching each test tube with dry ice just above the meniscus of the blood suspension. Concentrations up to 2 molar were seeded at −7°C and 2.5 molar at −10°C. As the bath temperature was lowered, an aliquot of each suspension was removed at each 3°C increment and transferred to a plastic beaker immersed in the refrigerated bath and containing liquid at bath temperature. This beaker was then transferred to an insulated foam container in which its spontaneous rate of warming was approximately 0.3°C/min. Following the completion of thawing, the hemolysis was measured by a comparison of the optical density of the supernatant with that of the entire suspension hemolyzed with saponin and corrected for hematocrit.

III. Results

Figure 4 illustrates the results of such an experiment. Quite wide variations in the quantitative results were observed from one sample of blood to another. However, all results were consistently similar on a qualitative basis, with increasing concentrations of glycerol progressively decreasing the percentage of hemolysis at constant temperature or, conversely, permitting the achievement of a lower temperature at a given level of hemolysis.

Since, for each combination of temperature and glycerol concentration, a fixed proportion of water will have been frozen, it should be possible to determine for any sample of blood the percentage of hemolysis as a function, not of temperature and glycerol concentration, but of the proportion of liquid phase remaining.

Experimentally obtained values are currently being acquired but are not yet completed.

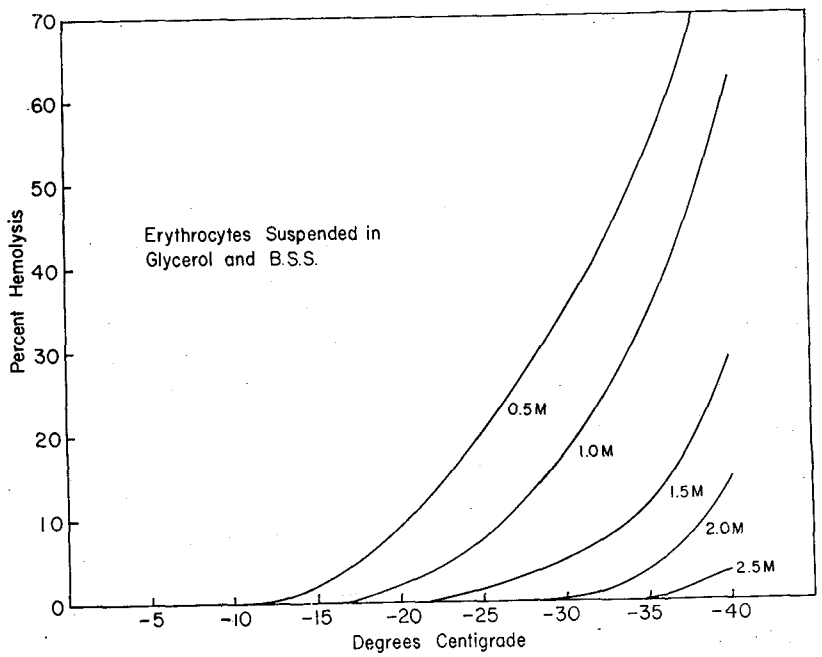

Fig. 4. Hemolysis as a function of final bath temperature for human erythrocytes suspended in various concentrations of glycerol in balanced salt solution. See text for experimental details

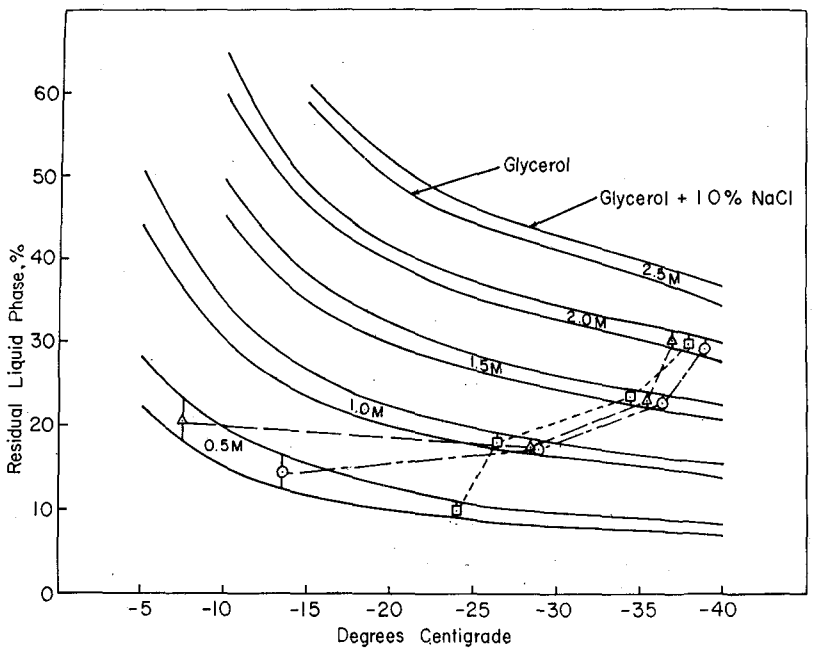

Fig. 5. The calculated total residual liquid phase (see text for details of calculations) when glycerol solutions of from 0.5 to 2.5 M are frozen to temperatures from −5 to −40°C. From three temperature-hemolysis experiments, such as that of Fig. 4, the temperatures at which 10% hemolysis was produced have been transferred to this plot of temperature *vs.* residual liquid. If injury were a constant function of residual liquid, lines joining these points should be parallel to the abscissa

In their absence we have attempted to calculate on the basis of melting point depression the amount of ice which would be formed at a given temperature and initial glycerol concentration. We have assumed initially that the cells are in osmotic equilibrium with their suspending medium and that the cell membrane is permeable to the cryoprotective agent. As water is frozen out, the glycerol solution is concentrated and, at any temperature, the unfrozen solution will have for its melting point the temperature at which it currently exists. On this basis it is a simple matter to calculate the proportions of glycerol and water remaining following freezing to any temperature. The situation is complicated, however, by the presence of other solutes which are also being concentrated and which can also lower the melting point. In the absence of experimental data we have met this obstacle simply by calculating two extreme situations. We have calculated the proportion of water frozen from a simple aqueous solution without other solutes. The results of this calculation should yield estimates which are erroneously high. We have also calculated this value assuming 1% sodium chloride in the initial solution and assuming that as the salt is concentrated in the remaining solution, it will further lower the melting point on a simple additive basis with no interactions between the salt and the glycerol. This calculation should give an erroneously low value. The results of these calculations are illustrated in Fig. 5. The correct values are assumed to lie somewhere between the double lines.

Data from erythrocyte hemolysis experiments similar to those of Fig. 4 have also been applied to this graph. Three different hemolysis experiments from three different units of blood were used. In this illustration, the temperature at which 10% hemolysis was observed has been transferred to the plot of percent residual liquid for each appropriate glycerol concentration. It can be seen that, for the different glycerol concentrations, hemolysis shows no consistent relationship to the amount of liquid phase remaining. According to this data, the lower the temperature and, accordingly, the higher the initial glycerol concentration, the greater is the volume of residual liquid phase necessary to hold hemolysis at the 10% level. In fact, it is apparent from these curves that it is highly improbable that a linear relationship between hemolysis and residual liquid phase could exist. For this to be true, the line joining points of equal hemolysis should be parallel to the abscissa. However, it is not possible to draw a horizontal line which will intercept both the 2.5 M and the 0.5 M line at temperatures below −5°C. Above −5°C, hemolysis is never seen in the presence of 0.5 M glycerol.

A second method by which the relationship between total solvent phase and hemolysis might be tested, and one which avoids some of the assumptions regarding the solvent capacity of CPA solutions, is to compare the protective capacity of glycerol to that of some other cryoprotective agent such as dimethylsulfoxide. One mole of DMSO binds about four moles of water at 0° and three at −40°C, while glycerol binds about 2.4 and 0.7 moles respectively. On this basis, one would expect DMSO to be more effective than glycerol on a molar basis, presuming free diffusion across the membrane by both compounds. Values for electrolyte solubility in CPA-water solutions have been presented above. When solutions with CPA concentrations of 1.5 molar or greater are frozen, the salt concentration is not increased beyond these levels of solubility. Only at low initial concentration of the order of 0.5 to 1.0 molar do salt concentrations increase beyond

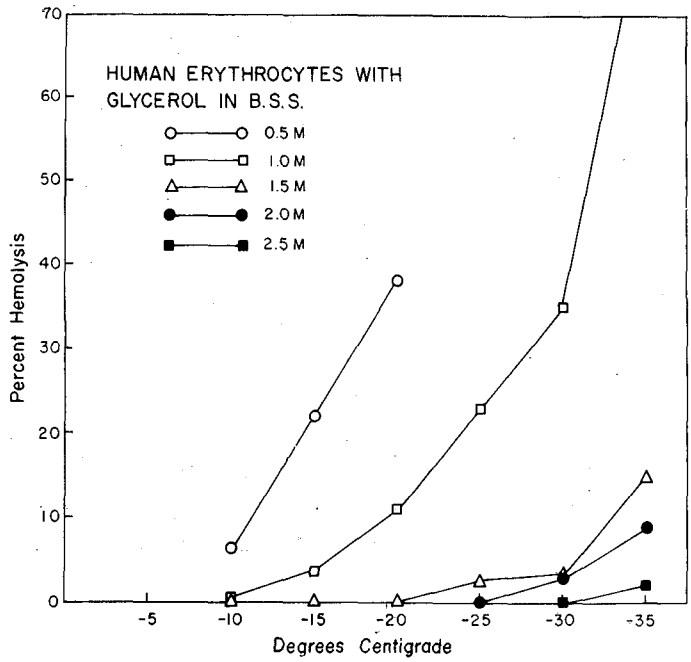

Fig. 6 a. Erythrocytes equilibrated with five concentrations of glycerol in citrate-phosphate solution were frozen and thawed at 0.3°C/min according to the standard procedure described in the text

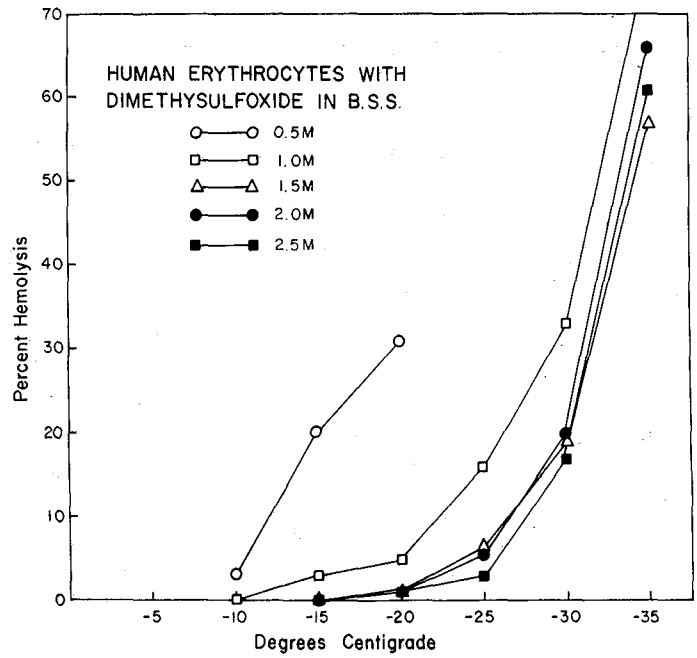

Fig. 6 b. Aliquots of the same unit of blood used in Fig. 6 a were equilibrated with five concentrations of dimethylsulfoxide in citrate-phosphate solution and treated identically to those of Fig. 6 a. The hemolysis of 0.5 and 1.0 M preparations was similar for both the glycerol and DMSO preparations, but the hemolysis curves of higher concentrations were both quantitatively and qualitatively different when the two cryoprotective agents are compared

10%. Since these are the concentrations of additive at which glycerol and DMSO perform equally well (Fig. 6), it would appear that limitations in solubility are probably not involved in freezing hemolysis.

Figure 6 illustrates the relationship between hemolysis and temperature for varying concentrations of DMSO as compared to glycerol. The protection afforded by 0.5 and 1.0 molar solutions is about the same for both compounds, while at higher concentrations glycerol is substantially more effective.

IV. Discussion and Conclusion

On the assumption that both solute concentration and space-filling should be related to the total solvent or the total liquid phase, freezing injury from these causes should show a relationship to the proportion of liquid remaining when some of the water is frozen out as ice. We have presented two pieces of evidence that this relationship does not exist during the freezing of human erythrocytes. First, measurements of the hemolysis of erythrocytes following freezing in glycerol solutions of 0.5 to 2.5 M to temperatures between 0 and −40°C, failed to demonstrate a consistent relationship between hemolysis and the calculated residual liquid phase present at the final temperature reached. Second, a comparison between glycerol and dimethylsulfoxide under these same circumstances failed to demonstrate either a quantitative or qualitative relationship between the protective capacities of the two, suggesting that their protective abilities are not based on a non-specific solvent or water-binding capability but on some more specific quality of the CPA molecule itself.

In considering the structured water theory, we have found useful a grossly oversimplified model, in which cell water is present in three general categories. The first is unfreezable water of hydration, such as that associated with cations. Also included in this category in the context of the present discussion would be the unfreezable water associated with proteins. A second category of water is that freezable component which, for the purposes of a discussion of the structured water theory, we will assume is essential to protein stability. One might postulate that this is composed of less ordered layers of water with insufficient bond strength to compete successfully with ice. The third category, having the lowest bond energy, is the remaining water which is not engaged in hydration but which constitutes the "bulk" or "free" water of the cell.

When freezing occurs, a fourth form of water, ice, is introduced into the system. Since both the bulk water and the protein stabilizing water can be frozen out, the bond strength of ice is presumed to be exceeded only by that of the water of hydration. According to this picture, freezing initially removes bulk water without injury to the cell. However, as dehydration begins to involve the stabilizing water, the more labile proteins become unstable and denatured.

Now to this simplified system consider the addition of CPA, the cryoprotective agent. The CPA will retain additional water on a colligative basis. However, at any given temperature, a solution in equillibrium with ice must have a fixed water activity, or effective mole fraction of water, regardless of the quantity of solute.

This line of reasoning, however, leads us into something of a dilemma. If the water activity of a solution at constant temperature is unchanged by the addition of

CPA, then the driving force leading to protein dehydration is unchanged and no protection is conferred on the structured water by the addition of the cryoprotective agent. That this is not generally true is obvious from the fact that the presence of cryoprotective agent is tolerated in high concentration by many cells and does, in fact, prevent injury from freezing. There is no obvious way in which the presence of a cryoprotective agent, successfully competing for water in the presence of ice, could at the same time prevent the removal of all structured water which is freezable in the absence of CPA.

If the water essential to protein is indeed stripped away by the superior competition of both ice and CPA, as is inevitable in this simple model, the only alternative left is that the CPA must either substitute for or stabilize water and thus maintain the stability of the protein. Were this the case, one would expect the effectiveness of cryoprotective agents to be related to some characteristic of structure. Webb (1965) has reached a similar conclusion in studies on the aerosol dehydration of bacteria and, in his system, has reported protective capacity to be associated with the "water-likeness" of the protective compound. We have not yet had an opportunity to explore a wide variety of cryoprotective agents. However, the data of Fig. 6 illustrates that there is indeed a qualitative as well as a quantitative difference in the protective capacity of glycerol as compared with dimethylsulfoxide at intermediate concentrations.

In summary we have concluded that injury from freezing is the result of dehydration and have presented a model whereby the varying effects of slow and rapid freezing, penetrating and non-penetrating cryoprotective agents, and intracellular crystallization can be explained on the basis of their effects on dehydration.

We have also presented evidence that the hemolysis of erythrocytes with freezing does not appear to be related to the total residual liquid phase as would be expected if solute concentration or the simple filling of interstitial spaces were the primary factor in survival following freezing. We find the concept of water essential to protein stability to be incompatible with the protective effect of cryoprotective agents unless they are presumed to substitute for or stabilize water in which case the effectiveness of a cryoprotective agent will depend on its ability to make this substitution rather than simple "antifreeze" properties.

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