ON THE MECHANISM OF INJURY TO SLOWLY FROZEN ERYTHROCYTES

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ABSTRACT When cells are frozen slowly in aqueous suspensions, the solutes in the suspending solution concentrate as the amount of ice increases; the cells undergo osmotic dehydration and are sequestered in ever-narrowing liquid-filled channels. Cryoprotective solutes, such as glycerol, reduce the amount of ice that forms at any specified subzero temperature, thereby controlling the buildup in concentration of those other solutes present, as well as increasing the volume of the channels that remain to accommodate the cells. It has generally been thought that freezing injury is mediated by the increase in electrolyte concentration in the milieu surrounding the cells, rather than reduction of temperature or any direct action of ice. In this study we have frozen human erythrocytes in isotonic solutions of sodium chloride and glycerol and have demonstrated a correlation between the extent of damage at specific subzero temperatures, and that caused by the action at 0°C of solutions having the same composition as those produced by freezing. The cell lysis observed increased directly with glycerol concentration, both in the freezing experiments and when the cells were exposed to corresponding solutions at 0°C, showing that the concentration of sodium chloride alone is not sufficient to account quantitatively for the damage observed. We then studied the effect of freezing in anisotonic solutions to break the fixed relationship between solute concentration and the volume of the unfrozen fraction, as described by Mazur, P., W. F. Rall, and N. Rigopoulous (1981. *Biophys. J.* 653–675). We confirmed their experimental findings, but we explain them differently. We ascribe the apparently dominant effect of the unfrozen fraction to the fact that the cells were frozen in, and returned to, anisotonic solutions in which their volume was either less than, or greater than, their physiological volume. When similar cell suspensions were subjected to a similar cycle of increase and then decrease in solution strength, but in the absence of ice (at 20°C), a similar pattern of hemolysis was observed. We conclude that freezing injury to human erythrocytes is due solely to changes that occur in the composition of their surrounding milieu, and is most probably mediated by a temporary leak in the plasma membrane that occurs during the thawing (reexpansion) phase.

INTRODUCTION

In 1953, J. E. Lovelock published two papers (Lovelock, 1953*a* and *b*) that have since become classics. In them he described experiments that led him to the conclusion that the mechanism of freezing injury to red blood cells was not, as had been supposed, a direct mechanical action of ice; rather, it was an indirect effect of freezing mediated by the increase in concentration of the solution which surrounded the cells during freezing. Specifically, when the red cells were suspended in isotonic saline and frozen, damage was ascribed to the increasing concentration of sodium chloride as water was removed to form ice. When glycerol was added, its protective action was explained by the colligative properties of solutions whereby glycerol reduced the concentration of sodium chloride in equilibrium with ice at any given subzero temperature. But in 1981, Mazur and his colleagues published a paper in which they stated that, contrary to the consensus, slow freezing injury to human erythrocytes was caused by the shrinkage of the aqueous channels in which the cells were sequestered and was only secondarily a manifestation of the altered solution composition (Mazur et al., 1981). Consequently, we need to reconsider carefully the evidence that led Lovelock to his conclusion.

First, he showed that red cells frozen in isotonic saline were destroyed within a well-defined zone of relatively high temperatures, extending from −3 to −10°C. When isotonic saline itself was frozen, the structure produced was a meshwork of ice crystals with relatively large and continuous channels, quite sufficient to accommodate the cells over that range of relatively high temperatures. Damage to the cells was not delayed until the much lower temperatures, where actual crushing might occur. Then, when he looked at the structure of the red cell ghosts produced when the cells were lysed by freezing and thawing, Lovelock found them to be characteristic, and remarkably similar to the structures produced by exposure to hypertonic saline followed by redilution. The upper boundary of the damaging zone, −3°C, is the freezing point of 0.8 M sodium chloride solution, and cells suspended in solutions stronger than this were shown to be lysed by reduction in temperature, mechanical stress, or

resuspension in isotonic saline. The damage produced by exposure to hypertonic saline was rapid and, within the limits studied, independent of temperature. Damage produced by freezing occurred on the same timescale. But most convincing of all, Lovelock showed that the amount of lysis produced by freezing to temperatures in the range −2 to −15°C and then thawing was remarkably similar to that produced by exposure to solutions of sodium chloride having the same freezing points as the temperatures used in the freezing experiments, if the cells were then returned to isotonic saline. Lovelock proceeded to show that glycerol reduced the hemolysis seen at any given subzero temperature directly as its concentration increased. If the concentration exceeded 2.5 M then hemolysis was negligible. Whatever the concentration of glycerol, hemolysis started when the mole fraction of sodium chloride was 0.014. If the glycerol concentration was kept constant and the salt concentration was reduced from "isotonic" (0.15 M) to 0.11 M, there was less lysis at any given temperature. If permeation of glycerol into the cells was prevented by incubating the cells with glycerol at 0°C in the presence of 30 μM Cu^{2+}, protection was much reduced. Lovelock emphasised that although Cu^{2+} did not reduce the space available to accommodate the cells in the partially frozen sample, it did reduce their survival.

In total, the evidence seemed very convincing, and was widely accepted as proof of the "salt theory" of slow freezing injury, that is, until Mazur and his colleagues pointed out a fundamental flaw in one of the arguments that had led to acceptance of the theory. This piece of evidence, which had weighed particularly heavily, was that when a wide range of glycerol concentrations was studied, hemolysis was always seen first when the concentration of sodium chloride reached a mole fraction of 0.014, although this happened at widely differing temperatures and glycerol concentrations, of course. Mazur pointed out that in all such experiments, the glycerol solutions in which the cells were suspended for freezing contained the same "isotonic" concentration of sodium chloride, ~0.95% wt/wt. Hence, the production of a given mole fraction of sodium chloride by freezing would necessarily be accompanied by conversion of the same proportion of the total water to ice. Because it is quite obvious that if cells were damaged by mechanical effects of ice, glycerol would protect them by reducing the amount of ice formed, this association between a given concentration of sodium chloride and the onset of hemolysis was of no help in distinguishing between an indirect effect of salt concentration and a direct effect of ice. That distinction could be achieved only if the initial concentration of sodium chloride was varied. Mazur carried out such experiments and, to quote from his paper (Mazur et al., 1981), "The results proved unexpected and even astonishing, for they show that the survival of the slowly frozen human erythrocyte is far more dependent on the fraction of water that remains unfrozen than it is on the concentration of salt (NaCl) in that unfrozen water." The conclusion is astonishing; we recall that the logical problem that Mazur identified effects only one strand of evidence and even without that strand the evidence for the 'salt theory' is remarkably strong. If Mazur's conclusion is correct, then all Lovelock's evidence has to be rejected as mere coincidence.

In this paper we repeat and confirm some of Lovelock's original results, and obtain further data that seems to us to place quite beyond doubt the proposition that freezing injury to human erythrocytes can be quantitatively accounted for by changes in the solution that surrounds them during slow freezing and subsequent thawing. We therefore went on to duplicate and confirm Mazur's basic experimental results. The problem then was to reconcile all this data, and we suggest how this may be done. We conclude that slow freezing injury to human red blood cells is indeed due to changes in the solution in which they are suspended during freezing and thawing, and is unaffected by the fraction of water that remains unfrozen.

METHODS

Red Blood Cells

Human blood was collected into CPD-A.1 anticoagulant mixture by the National Blood Transfusion Service, and was stored at room temperature for 15 h. The blood was then either centrifuged at 250 g for 20 min, the platelet-rich plasma removed, and the remainder refrigerated at +4°C, or the whole blood was refrigerated at +4°C. Storage at +4°C was for no longer than 3 d.

On the day of the experiment, sufficient blood was centrifuged at 1,000 g for 5 min, the supernatant removed, and the cells washed three times in phosphate-buffered saline (PBS; Dulbecco's A solution). The final centrifugation was at 800 or 1,000 g for 20 min, the supernatant was removed, and the hematocrit of the packed cells was measured using a microhematocrit centrifuge (Hawksley and Sons Ltd., Sussex, UK). The final hematocrit was always >80% and the packed cells were kept at +4°C and used within 7 h.

Solutions

All solutions were prepared gravimetrically using analytical grade chemicals and double glass-distilled water. In ternary mixtures of glycerol, sodium chloride and water, the *R* value was defined as the weight ratio of glycerol to sodium chloride.

Equilibration with Glycerol

Where the experiment required cells equilibrated with glycerol solutions, an appropriate volume of packed cells, prepared as described above, was diluted with a sufficient volume of the required solution to produce a hematocrit of 2–4%. The suspension was then left for 30 min at room temperature. If packed cells were needed, the suspension was then centrifuged at 800 g for 20 min, the supernatant removed, and the hematocrit of the packed cells determined as before.

Freezing at Designated Temperatures

Thermostatically controlled, refrigerated baths containing either ethylene glycol/water mixtures or silicone oil (5 cs, Dow Corning Corp., Midland, MI) were set up to provide temperatures in the range −2 to −45°C, with a control accuracy of ±0.1°C for the higher temperatures and no worse than ±0.4°C for the lower temperatures. Cells to be frozen were prepared by dispensing 50 μl of packed cells into glass test tubes measuring 100 × 12 mm diameter; 400 μl of the appropriate suspending solution was added

and the suspension mixed well. In every case, the packed cells had been equilibrated with a solution identical to that which was added. The tubes were then placed in the appropriate refrigerated baths and 30 s later (or 1 min for the −2°C bath) were seeded by touching the side of the tube, at the level of the meniscus, with a brass block that had been cooled in liquid nitrogen. The samples were then allowed to cool to the bath temperature, as indicated by three thermocouples placed in replicate tubes reaching within 0.2°C of the specified bath temperature. The tubes were held for 5 min at the bath temperature and then thawed rapidly (~200°C/min) by gentle agitation in a 40°C water bath. When the last ice had melted, the tubes were held on ice, 8 ml of the original suspending solution was added, and the suspension mixed. Within 2 min, the tubes were then centrifuged at 500 g for 6 min and portions of the supernatant removed for assay of hemoglobin. Duplicate tubes were prepared from each of five separate blood donations for each experiment.

The temperature of each refrigerated bath was set using a calibrated platinum resistance thermometer with an accuracy of ±0.05°C. The thermocouples used to monitor the temperature of the cooled samples were constructed by welding 0.315 mm copper and constantan wires; temperatures were indicated on a 6130 digital thermometer (Comark Electronics Ltd., Sussex, UK), and when required, were recorded on a three-channel pen recorder (Rikadenki Kogyo Co., Tokyo, Japan). The average cooling rate was measured over the central 60% of the temperature range between the freezing point and the final temperature.

Exposure to Concentrated Solutions

Concentrated solutions of sodium chloride, and of sodium chloride and glycerol, corresponding approximately to the composition of the liquid phase produced in the freezing experiments, were prepared and cooled to 0°C. Packed cells were prepared from suspensions of red cells in solutions of sodium chloride and glycerol containing 0.95 g sodium chloride and either 0, 1.90, 4.75, or 9.50 g glycerol per 100 g of solution. These correspond to R values of 0, 2, 5, and 10. The packed cells were also cooled to 0°C. 50 μl of packed cells were dispensed into 10-ml polycarbonate tubes on ice, and 400 μl of the required concentrated solutions were added. The R value of each concentrated solution was always the same as that of the solution with which the packed cells had been equilibrated. After 5 min, 8 ml of a solution was added, the composition of which had been calculated to bring the final concentration of the suspension back to the concentration of the solution in which the packed cells had been prepared. Thus, for example, in the $R = 0$ experiment, when the 400 μl of concentrated solution contained 6.577 g per 100 g of NaCl, the 8 ml of diluting solution contained 0.669 g per 100 g NaCl, so that the 8.4 ml of resulting solution contained 0.95 g per 100 g NaCl. The tubes were then left for 5 min at 0°C, centrifuged at 500 g for 6 min, and portions of supernatant removed for hemoglobin estimation. Again, duplicate tubes were prepared from each of five blood donations for each experiment.

In these experiments there was some dilution of the concentrated solution by the extracellular fluid trapped between the packed cells, and by the water that was extracted from the cells by osmosis. This dilution was allowed for by calculating the volume of trapped fluid in the 50 μl of packed-cell suspension using the measured hematocrit of that suspension, and by calculating the volume of water withdrawn from the cells as they shrank in accordance with the equations previously published (Pegg, 1984). An iterative method of calculation was used so that the extracted water diluted the surrounding fluid, leading repeatedly to a new volume and new external concentration until no further changes occurred.

Freezing to Designated Unfrozen Liquid Fractions

A procedure is described in the theory section which makes it possible to determine solution compositions such that given unfrozen liquid volume fractions were produced at given subzero temperatures. The density measurements required for these calculations were made by recording the apparent loss of weight of a 50-g brass balance weight immersed in each solution, the volume of the weight being determined by immersion in distilled water. A 1219 MP balance (Sartorius Balances, Westbury, NY) was used. The composition of 25 solutions, with $R = 5$, was calculated so that when each group of five solutions was frozen at −10, −20, −30, −40, or −50°C, the liquid volume was reduced to 0.8, 0.6, 0.4, 0.2, or 0.1 of the original volume. A similar set of 25 solutions was designed for $R = 10$. The equilibrium freezing point of each solution was calculated from Eq. 2 in the Theory section.

100 μl of packed cells was pipetted into two sets of 25 glass test tubes measuring 100 × 12 mm diameter, and 5 ml of the appropriate solution was added and the contents mixed. The tubes were left for 30 min at room temperature and then transferred to an ice tray. One set was left in the ice tray while the other was transferred to a refrigerated silicone oil bath at 0°C (FTS, type MC-4-80). The bath was controlled by a purpose-built temperature programmer such that its temperature could be reduced at a rate of 0.6°C/min. Cooling was initiated. When each tube reached a temperature 2°C lower than its calculated freezing point (temperatures being measured in a duplicate tube containing a thermocouple as previously described), that tube was seeded in the manner already described. When the tubes reached −10°C, one set of five tubes containing solutions calculated to produce unfrozen liquid fractions of 0.1, 0.2, 0.4, 0.6, and 0.8 at that temperature were removed, rapidly thawed in a 40°C water bath, and placed on ice. Cooling of the remaining tubes was continued. Further sets of five tubes were removed at −20, −30, −40, and −50°C, and immediately thawed. A further 5 ml of the original suspending solution was added to each tube, the contents centrifuged, and a portion of supernatant fluid removed for assay of hemoglobin. The corresponding tubes that had been kept on ice were similarly treated at the same time, and the standards, which also contained 100 μl of the packed cells, were lysed with saponin, and hemoglobin assayed. This procedure was repeated with five separate blood donations.

Exposure to Concentrated Solutions Equivalent to Those Produced at the Designated Unfrozen Liquid Fractions

The composition of solutions of R value 5 and 10, equivalent to each of the final temperatures used in the experiment designed to study the effect of unfrozen liquid fraction (−10, −20, −30, −40, and −50°C), were calculated. 100-μl samples of packed cells were equilibrated with 5 ml of each of the 25 solutions used in the freezing experiments, by incubation at room temperature for 30 min. 4 ml of each suspension were then placed in 10-cm lengths of size-3 Cuprophan cellulose dialysis tubing (45 mm wide × 20 μm thick; Medicell International Ltd., London, UK) that had previously been soaked in distilled water and the surplus moisture removed; both ends were clamped and each bag was suspended in 200 ml of a solution equivalent to each final temperature, but at room temperature. Duplicate samples were treated in this manner, while a third sample for each suspension was placed in a polycarbonate test tube that was immersed in the same dialysis beaker. The dialysate was stirred. After 2 h, the dialysis sacs were removed, blotted dry, and placed in 200 ml of the initial suspending solution. The sacs were left to dialyze for 1 h at room temperature in the stirred solution. At the end of this time they were removed, blotted dry, the contents mixed, and then decanted into a polycarbonate test tube. A 1-ml sample was removed and lysed with saponin. The remainder was centrifuged at 500 g for 7 min and an aliquot of the supernatant removed for the assay of hemoglobin. The samples that were kept in test tubes for the duration of the entire dialysis were similarly treated; a portion was lysed to determine total hemoglobin content and the remainder was centrifuged and the supernatant assayed for hemoglobin. This procedure was also repeated with five separate blood donations.

Measurement of Concentrations of Glycerol and Sodium Chloride

The time course of solution composition in selected dialysis experiments was measured as follows. At the desired times, the sodium content of the

solution was measured by emission flame photometry (PFP1 photometer; Petracourt Ltd., Essex, UK). Previously we had established that glycerol, in the range 0–4% wt/wt had no significant effect on the sodium estimation; the maximum concentration present in practice was 0.4%. We then calibrated an Abbé refractometer (Hilger and Watts, London, UK) to measure glycerol concentration in the presence of 0, 1, 2, 4, or 8 g/dl sodium chloride. The calibrations were parallel and it was possible to fit a quadratic equation to each curve, where the intercept was fitted to a second quadratic equation in sodium chloride concentration. Because sodium chloride concentration could be determined independently of glycerol concentration, it was possible to determine the composition of any solution; as will be shown later, the ratio of glycerol to sodium chloride changed during the course of dialysis, which meant that we had no means of knowing the density of the solution throughout the process, and consequently all these measurements had to be made in weight-for-volume terms. The equation for refractive index (RI) was $RI = c + 1.165 \times 10^{-3} G - 4.639 \times 10^{-7} G^2$, where $c = 1.3324 + 1.5158 \times 10^{-3} S - 1.3482 \times 10^{-6} \times S^2$, G = glycerol concentration in grams per deciliter, and S = sodium chloride concentration in grams per deciliter.

Estimation of Hemolysis

In all experiments except the dialysis experiments it was possible to ensure that the volume of blank, standard, and test samples was the same. The hematocrit of the final sample was always <2%, so that corrections for hematocrit were unnecessary (Pegg, 1981). Aliquots of each sample were centrifuged at 16,000 g for 2 min and the supernatant mixed with an equal volume of double-strength Drabkin's reagent (Sigma Chemical Co., St. Louis, MO, or Ortho Pharmaceutical, Raritan, NJ). Optical density was measured at 540 nm using a Gilford 300N spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, OH). Percentage hemolysis was then given by the expression $A_t/A_s \times 100\%$, where A_t = absorbance at 540 nm of the test sample, and A_s = absorbance at 540 nm of the totally lysed standard sample.

In all cases there was some hemolysis in the "blank" samples, and sometimes this was considerable. In some experiments therefore we have presented a measure of hemolysis due to the experimental treatment that removes this background hemolysis. This we call relative hemolysis and define it by $(A_t - A_b)/(A_s - A_b) \times 100\%$, where A_b is the absorbance at 540 nm of the blank sample.

In the dialysis experiments, the volume did not remain constant, and it was therefore necessary to determine a 100% hemolysis absorbance (A_s) separately for each test sample. This was achieved by lysing an aliquot of the mixed sample before the centrifugation to separate the supernatant. Absolute hemolysis of a test sample was then $A_t/A_s \times 100$, as before. However, the calculation of relative hemolysis was now more complex because the blank hemolysis figure had to be corrected for any difference in volume between the blank and the test samples; this was achieved by making a measurement of the hemoglobin concentration of a totally lysed blank sample. If the absorbance of such a lysed blank sample is designated A_{bl}, then relative hemolysis is given by the expression $(A_t - A_s \times A_b)/(A_{bl}/A_s - A_s \times A_b/A_{bl}) \times 100\% = [(A_t \cdot A_{bl} - A_s \cdot A_b)/A_s (A_{bl} - A_b)] \times 100\%$.

THEORY

Procedures were needed that would permit the calculation of equilibrium compositions at subzero temperatures for solutions of sodium chloride and glycerol in water, that is, to identify any point on the liquidus surface for that ternary system. Goldston (1974) has published a study listing the equilibrium melting points of 49 aqueous solutions of glycerol and sodium chloride. It is convenient to designate such solutions by their total solute concentration in weight for weight terms (C in grams per 100 grams in the following analysis) and the weight ratio of glycerol/sodium chloride (R in this analysis). The virtue of such a notation is that the R value remains constant as water is removed from or added to a system (as in freezing and thawing) and consequently, solutions of constant R value can be treated as a binary system, with a single liquidus line. Goldston's data concerns R values of 0–30, and Pegg (1983) has used this data to derive equations that describe the entire liquidus surface. If a is a parameter related to the R value such that

$$a = (-1.6 - 1.27R - 0.25R^2)^{-1}, \tag{1}$$

then the melting point, T_{mp} is given by the expression

$$T_{mp} = a \cdot C - 0.010C^2. \tag{2}$$

Conversely,

$$C = (a + \sqrt{a^2 - 0.04\, T_{mp}})/0.02. \tag{3}$$

Eq. 2 was used to calculate equilibrium freezing points of solutions. When a solution of known composition was frozen to a specified temperature, the composition at that temperature was obtained from Eq. 3. Because solute is conserved the reduction in liquid phase could be calculated thus: if M and C are the mass and the concentration of solution, and the subscript o refers to initial values, while the subscript t refers to values at the selected subzero temperature, and if Q is the mass of solute, then because $C_o = (Q/M_o)$ and $C_t = (Q/M_t)$, it follows that

$$\frac{M_t}{M_o} = \frac{C_o}{C_t}. \tag{4}$$

Thus C_o/C_t, the ratio of the concentrations at the two temperatures, is the inverse of the ratio of the mass of liquid, or L fraction. Mazur's proposal is that the reduction in volume of the liquid fraction is the major damaging influence during freezing. However Mazur has generally expressed the unfrozen fraction as the reduction in the mass of water or U fraction (for perfectly good reasons that are expounded in Mazur et al., 1981). U is derived from L as follows. From Eq. 4,

$$L = \frac{C_o}{C_t}. \tag{5}$$

Hence,

$$U = L\,\frac{100 - C_t}{100 - C_o}. \tag{6}$$

We have prefered to calculate the unfrozen liquid volume, for obvious mechanistic reasons. To do this required data on the density, ρ, of glycerol solutions over a wide range of temperatures. Segur (1953) provided data for temperatures between +25°C and −40°C and for concentrations between 10 and 80% wt/wt glycerol. For each concentration we found that there was a linear dependence of ρ on temperature, and the magnitude of the slope was linearly

dependent on concentration. Density at constant temperature was also linearly dependent on concentration. Thus, ρ could be expressed in terms of temperature, T, and concentration, C, by an expression of the form

$$\rho = \rho_z + k_1 \cdot C + k_2 \cdot T, \quad (7)$$

where ρ_z is the density of water at 0°C, k_1 is the slope of the line relating ρ to concentration, T is temperature in degrees celsius, and the dependence of k_2 on concentration is given by

$$k_2 = -(2.11355 + 0.0492133 \cdot C) \times 10^{-4}. \quad (8)$$

In Eq. 8, C is constrained [0, 70]. The difference between values for ρ calculated by Eq. 7 and Segur's data is between 10^{-5} and 2×10^{-3}, generally $\sim 5 \times 10^{-4}$ g/cm^3. Our solutions contained sodium chloride in addition to glycerol. We made measurements of the density of solutions containing between 3 and 70 g solute/100 g solution, for R values of 5 and 10, and found that at 20°C a fit within 0.006 g/cm^3 could be obtained if the value of k_1 was 0.00362 for $R = 5$ and 0.0031 for $R = 10$. The complete equation was then

$$\rho = 0.99984 + C \cdot (0.00414 - 1.04R \times 10^{-4}) + k_2 \cdot T, \quad (9)$$

where C is total solute concentration (grams per 100 g), R is the R value, T is temperature in degrees celsius, and k_2 is defined by Eq. 8. (We assumed, in the absence of specific data, that the dependence of ρ on temperature would be the same for solutions of R values 5 and 10 as for Segur's binary solutions). We can now convert the masses of solution in Eq. 4 into volumes of solution, V_o and V_t, as follows.

$$V_0 = \frac{M_o}{\rho_o}$$

$$V_t = \frac{M_t}{\rho_t}$$

$$\therefore \frac{V_t}{V_o} = \frac{C_o}{C_t} \cdot \frac{\rho_o}{\rho_t}. \quad (10)$$

We designated the ratio V_t/V_o the liquid volume fraction, LV. It was now possible to calculate the starting composition of a solution that was required to have a particular LV value at a specified temperature. This was achieved as follows. First the composition at that desired temperature was determined in wt/wt terms, that is C_t, using Eq. 3; next ρ_t was found from Eq. 9, and LV, which is V_t/V_o, is given. Hence, substituting in Eq. 10, we have $(V_t/V_o) \times C_t \cdot \rho_t = C_o \cdot \rho_o$.

The numerical value of the left-hand side is known. The dependence of ρ_o on C_o and temperature is defined by Eq. 9, the right-hand side of which can therefore be substituted for ρ_o, leading to a quadratic equation in C_o, thus

$$(k_t - 0.0492133 \times 10^{-4} \cdot T)C_o^2 + (0.99984 - 2.11355 \times 10^{-4} \cdot T)C_o - \frac{V_t}{V_o} C_t \cdot \rho_t = 0. \quad (11)$$

C_o is the positive root. A program was written to tabulate values of C_o such that LV values of 0.1, 0.2, 0.4, 0.6, and 0.8 would be produced at temperatures of -10, -20, -30, -40, and -50°C.

These equations enabled us to calculate solution composition in weight/weight terms for any required temperature and any selected R value. Conversion to molal and mole fraction units was straightforward. We also needed to calculate equilibrium red cell volumes in any of these solutions, and that was achieved using equations that have previously been published (Pegg, 1984). Briefly, the basis of this calculation is as follows. Where V_n = total volume of a normal cell ($\times 10^{-15}$ liters), W = total water content of a normal cell ($\times 10^{-15}$ liters), N_s = percentage of W that is not osmotically active, V_s = volume of osmotically active intracellular water ($\times 10^{-15}$ liters), V_g = volume of intracellular glycerol ($\times 10^{-15}$ liters), S = concentration of sodium chloride in the suspending medium (grams per 100 g solution), G = concentration of glycerol in the suspending medium (grams per 100 g solution), it is clear that

$$V_s = V_n - \frac{W(100 - N_s)}{100} \times 10^{-15} \text{ liters}. \quad (12)$$

To calculate the volume of intracellular water, V_w, we assume that the normal intracellular osmolality is 300 mosmol/kg, that osmotic equilibrium is maintained and that the external osmolality of sodium chloride can be calculated by multiplying the concentration in moles per kilogram water by 2 for dissociation and by an appropriate osmotic coefficient. The result is that

$$V_w = W(100 - N_s)\frac{(100 - S - G)}{S} \times 0.9478 \times 10^{-4} \times 10^{-15} \text{ liters}, \quad (13)$$

where the constant 0.9478 is the result of combining the assumed normal intracellular osmolality (300 mosmol/kg) the number of species into which NaCl dissociates (2), an osmotic coefficient for NaCl (0.925), and the molecular weight of NaCl (58.45). The complete derivation is given by Pegg (1984).

If we assume that, at equilibrium, the ratio of glycerol to water inside the cells is the same as outside, then assuming that glycerol has a molecular weight of 92.1 and a partial molar volume of 71 ml/mol, then the volume of intracellular glycerol is given by

$$V_g = W(100 - N_s) \times G/S \times 0.7307 \times 10^{-4} \times 10^{-15} \text{ liters}. \quad (14)$$

The final volume of the cell can now be calculated if it is

assumed that the component volumes can be summed; thus

$$V = (V_s + V_w + V_g) \times 10^{-15} \text{ liters.} \quad (15)$$

When volume was calculated at subzero temperatures, the small correction resulting from the change in density of glycerol solutions was made using Eq. 9. It should be noted that the amount of glycerol inside the cell is controlled by the ratio G/S, not simply G, and G/S is the R value which remains constant during equilibrium freezing. Thus, the cells neither gain nor lose glycerol during freezing and thawing, and any change in volume is due only to the movement of water. When, as in the present study, the individual values of V_s, V_w, and V_g are not required, the equations can be combined to yield

$$V = V_n - \frac{W(100 - N_s)}{S} \times 10^{-4} \times (100.95S + 0.217G - 94.77) \times 10^{-15} \text{ liters.} \quad (16)$$

In the present calculations, V_n was assumed to be 87×10^{-15} liters (Altman and Dittmer, 1961), W was assumed to be 65×10^{-15} liters (Farrant and Woolgar, 1972*a*) and N_s was taken to be 23.7% (Wiest and Steponkus, 1979).

Graphical Representation of Data

Many of the experiments described in this paper were designed to study the effect on freezing injury of two variables; thus, the experimental data are three dimensional. We have used contour maps to display such results, because this seems both simpler and more comprehensive than sets of two-dimensional graphs. However, there can be pitfalls in the generation of contour plots. Some programs smooth the data excessively, and the resulting surface may not coincide with the original data points. Our procedure, which is based on that described by Barr et al. (1980*a–c*), avoids this difficulty.

Our experiments provided 25 data points in a rectangular grid, 5×5. (Our contour method requires that the data be in the form of a regular grid; the horizontal and vertical grid lines need not be perpendicular to each other and need not even be straight, but they must not intersect except at the data values and there must be the same number of data values on each horizontal or vertical line, though not necessarily the same number in both directions.) The next step was to generate additional interpolated points to convert the rectangular grid into triangles. Each apex of each triangle was defined by x, y, and z coordinates, the z coordinate in this case being the percentage of cells hemolyzed. The surface describing the dependence of hemolysis on the variables chosen for the x and y axes was therefore a three-dimensional surface consisting of adjacent two-dimensional triangles. The program then examined the z coordinates of each end of each side of each triangle and identified those sides that included, somewhere along their lengths, the z value for the contour that was under construction. The x and y coordinates of each such point were then determined by simple proportion. All the points located for each contour were then plotted and the contour line was drawn through these points. All data were processed on a 380Z microcomputer (Research Machines Ltd., Oxford, UK) equipped with a MP-1000 digital plotter (Graphtec Corp., Tokyo, Japan).

RESULTS AND DISCUSSION

Comparison of Freezing and Thawing with Exposure to Equivalent Solution Compositions in the Absence of Glycerol

The first part of this study was designed to repeat Lovelock's original experiment that provided the basis for his proposition that the degree of hemolysis of red cells frozen to various subzero temperatures could be accounted for quantitatively by the concentration of sodium chloride in the solution to which they were exposed. The experiment was designed to compare the extent of hemolysis produced by freezing and thawing red cells suspended in 0.95% wt/wt sodium chloride with the hemolysis produced by exposing cells to solutions at 0°C that were similar in composition to the liquid phase in equilibrium with ice at the temperature chosen for the freezing experiments. Souzu and Mazur (1978) have pointed out that the technique used by Lovelock confounds cooling rate with final temperature, because the samples were immersed in baths at different temperatures. We agree with that criticism, but because our aim was to duplicate Lovelock's experiment we followed his method as closely as possible. The results are shown in Fig. 1, and the two lines are close, which supports Lovelock's proposition. It may be noted *en passant* that there were difficulties in executing this experiment. The more obvious method of exposing cells to a high

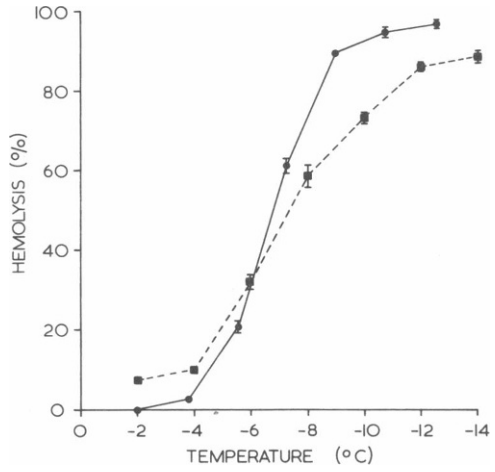

FIGURE 1 Hemolysis observed when human erythrocytes suspended in 0.95% wt/wt sodium chloride solution were frozen to temperatures between −2 and −14°C and thawed (■), compared with exposure to solution compositions similar to those produced in the freezing and thawing experiments, but at 0°C (●).

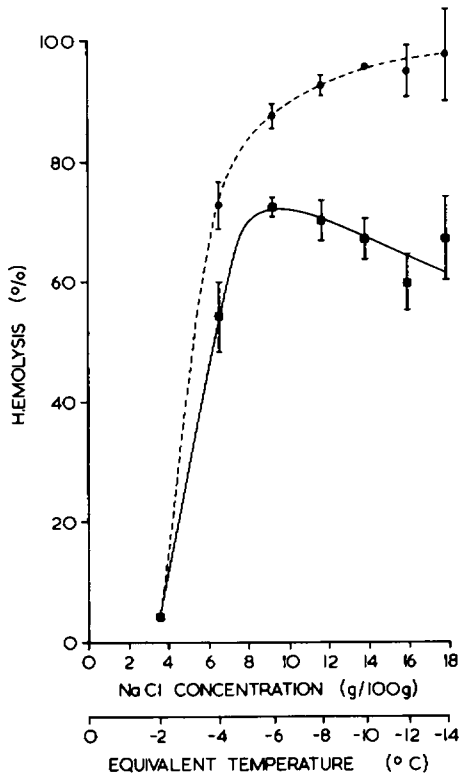

FIGURE 2 Hemolysis observed when erythrocytes that had been exposed to hypertonic solutions of sodium chloride were then centrifuged and resuspended in isotonic saline (●) or the same solution (■). Hemolysis is expressed as a percentage of the cells that survived the hypertonic exposure.

salt concentration and then returning them to their original environment would be to suspend them in a large volume of the desired solution, deposit the cells by centrifugation, remove the supernatant, and resuspend the cells in the original solution. Fig. 2 shows that this was not possible, because cells that were centrifuged in high concentrations of sodium chloride were extensively lysed when they were resuspended, even in the same concentrated solution. Lysis was due mainly to resuspension, not to dilution back to their original environment. It was for this reason that a method avoiding centrifugation and resuspension was designed, even though it meant that the volume of hypertonic saline used had to be smaller than we wished. Consequently there was a significant dilution of the concentrated solution by the original suspending solution, sufficient to require a small correction in the calculation of results.

Comparison of Freezing and Thawing with Exposure to Equivalent Solutions in the Presence of Glycerol

The experiments shown in Fig. 3 are an extension of the first experiment. Here, cells suspended in solutions containing glycerol plus 0.95 g NaCl/100 g, such that the R values were 2, 5, or 10, were frozen to a range of subzero

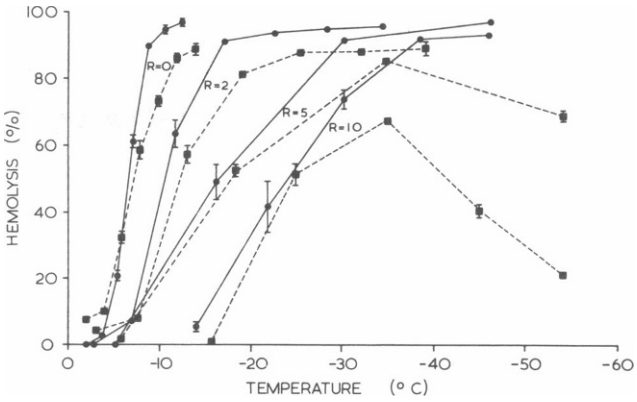

FIGURE 3 Hemolysis observed when erythrocytes were suspended in solutions containing 0.95% NaCl and glycerol in weight ratios (R values) of 0, 2, 5, and 10, and then frozen at and thawed from the indicated temperatures (■) compared with exposure to the equivalent solution composition and return to the original suspending solution (●).

temperatures, thawed, and the hemolysis produced in each case was compared with that recorded after exposing red cells to solutions at 0°C that were similar in composition to those that would have been in equilibrium with ice at the temperatures used. These cells were then returned to their original solutions. The data for $R = 0$, shown in Fig. 1, is included for comparison. When $R = 2$, the agreement between the effect of freezing and of exposure to equivalent solutions was excellent, and when $R = 5$ or 10, the agreement was again good up to 50% hemolysis, but beyond this point there was less damage in the freezing experiments than in the corresponding solution-exposure experiments. It is clear from Fig. 3 that the reduction in hemolysis occurred when the temperature of the bath was −30°C or lower. Table I shows the mean cooling rates that were recorded when samples were immersed in the various baths used in the experiment: the falloff in hemolysis occurred when the cooling rate exceeded ~50°C/min, thus substantiating Souzu and Mazur's criticism (Souzu and Mazur, 1978).

These data show that, at cooling rates <50°C/min, the amount of hemolysis produced by cooling to a specified temperature and rewarming can be mimicked remarkably closely by exposing the cells at 0°C to solutions similar to those produced by freezing in the cooling experiments and returning them to their original solutions. Rapid cooling produced less hemolysis. A given proportional hemolysis, say 50%, was produced at lower temperatures as the R value increased, and because the concentration of sodium chloride produced at a given temperature was inversely related to the R value, such a result is in accord with Lovelock's proposal that glycerol is cryoprotective because it reduces the concentration of sodium chloride in equilibrium with ice at any given subzero temperature. In principle, this result also seems to be compatible with the proposal made by Meryman (1968) that the degree of damage is related to the extent to which the volume of the cells is reduced. It is readily possible, as described in the

TABLE I
AVERAGE COOLING RATES

Bath temperature °C	Cooling rate °C min^{-1}
$R = 0$	
−2	0.16
−4	0.99
−6	4.07
−8	9.32
−10	12.5
−12	12.3
−14	12.5
$R = 2$	
−3.1	0.28
−7.7	4.04
−13.1	12.2
−19.1	25.0
−25.5	35.2
−32.2	67.0
−39.2	44.2
$R = 5$	
−5.9	1.04
−18.4	13.6
−34.8	51.0
−54.1	109.7
$R = 10$	
−15.7	11.6
−25.0	28.3
−35.0	64.3
−45.0	60.6
−54.2	114.3

Theory section, to convert the abscissa in Fig. 3 into cell volume (Fig. 4) or sodium chloride concentration (Fig. 5). It is at once apparent (Fig. 4 and Table II) that the same degree of cell damage is not produced at the same cell volume for different R values. Nor do similar amounts of hemolysis correspond to similar sodium chloride concentrations, although the range of concentration for 50% hemolysis is quite small when concentration is expressed on

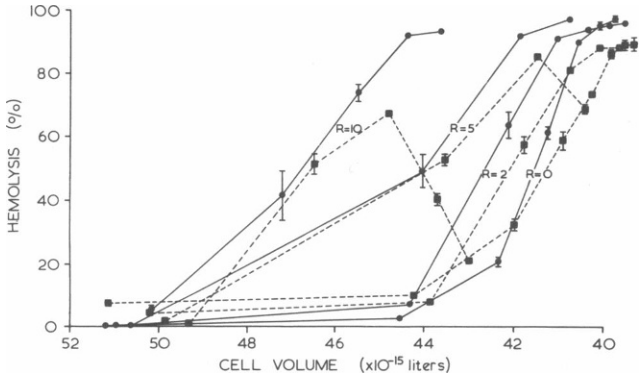

FIGURE 4 Data of Fig. 3 is replotted with calculated cell volume at the minimum temperature (■) or maximum concentration of solution (●) as the abscissa.

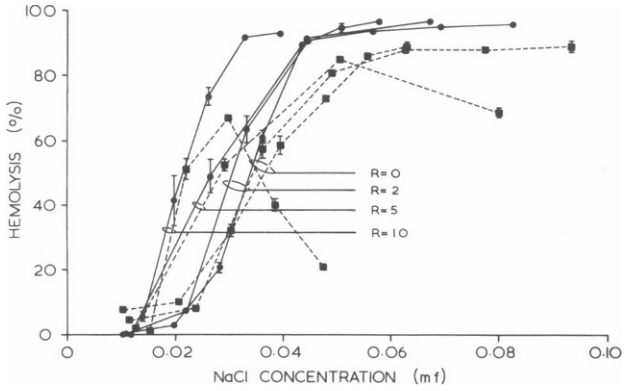

FIGURE 5 Data of Fig. 3 is replotted with the calculated concentration of sodium chloride (mole fraction scale) at the minimum temperature (■) or maximum concentration of solution (●) as the abscissa.

a mole fraction scale. But it is clear that, however the data are presented, there is more damage at a given salt concentration when the glycerol concentration is high than when it is low. This interesting point, to which we shall return, in no way detracts from the central fact established by this experiment: hemolysis correlates quantitatively with the solution composition that is produced by freezing each solution to each temperature. In these experiments, all the solutions used to suspend the cells initially contained 0.95% sodium chloride, and hence, in the freezing experiments, there was a fixed relationship between sodium chloride concentration and unfrozen fraction. If the hemolysis curves agreed for freezing and solution exposure without glycerol, they would be expected to agree when glycerol was also present. However, a given unfrozen fraction (or salt concentration) produced different amounts of hemolysis in the presence of different concentrations of glycerol. Certainly, it is possible to argue, like Rall et al. (1978), that glycerol may have had an effect on the susceptibility of the cells to a given unfrozen fraction, but to accommodate our results one would then have to postulate that glycerol had exactly the same effect on the susceptibility to salt in the solution-exposure experiments as it did to the unfrozen fraction in the freezing experi-

TABLE II
CORRESPONDING VALUES OF TEMPERATURE, CELL VOLUME, AND SODIUM CHLORIDE CONCENTRATION FOR 50% HEMOLYSIS

R-value and condition	Temperature	Cell volume	Sodium chloride
	°C	$\times 10^{15}$ liters	mf
0, frozen	−7.4	41.3	0.037
0, solution	−6.8	41.6	0.034
2, frozen	−12.4	42.1	0.035
2, solution	−10.7	42.7	0.031
5, frozen	−17.8	43.9	0.029
5, solution	−16.6	44.0	0.027
10, frozen	−24.7	46.6	0.022
10, solution	−24.2	46.8	0.021

ments. We considered this vanishingly improbable and consequently we were unable to reconcile this result with Mazur's conclusion that it is the volume of unfrozen solution (or water), rather than the change in solution composition, that is mainly responsible for freezing injury. Therefore we carried out experiments, based on Mazur's experimental approach, to study the effect of the unfrozen fraction.

Effect of Liquid Volume Fraction on Hemolysis during Freezing and Thawing

Fig. 6 shows our experimental data designed to measure the effect of liquid volume fraction (LV) and temperature on hemolysis for R values of 5 and 10. In Fig. 7 we replot the same data in the manner prefered by Mazur, U fraction vs. salt concentration in moles per kilogram water. Our data extend over a greater range of concentration of sodium chloride than Mazur's and the R values differ somewhat, but the general agreement is good. The dominant influence on hemolysis appears to be LV or U fraction, with a secondary effect of temperature or salt concentration when the LV fraction is between 0.2 and 0.5. The fact that the contours for the two R values more closely agree when the abscissa is calibrated in salt concentration rather than temperature suggests that the former is more closely related to the responsible mechanism.

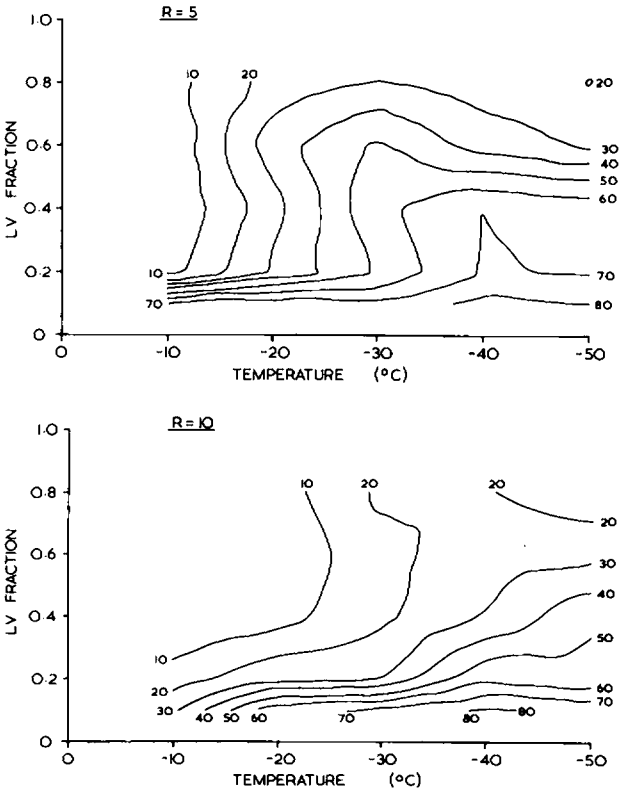

FIGURE 6 Isohemolysis contours for erythrocytes suspended in solutions designed to produce proportional unfrozen liquid volumes (LV) of 0.1–0.8 when frozen to temperatures of −10 to −50°C and thawed. The cells were not resuspended in isotonic saline. Data for R = 5 in the upper panel and for R = 10 in the lower panel.

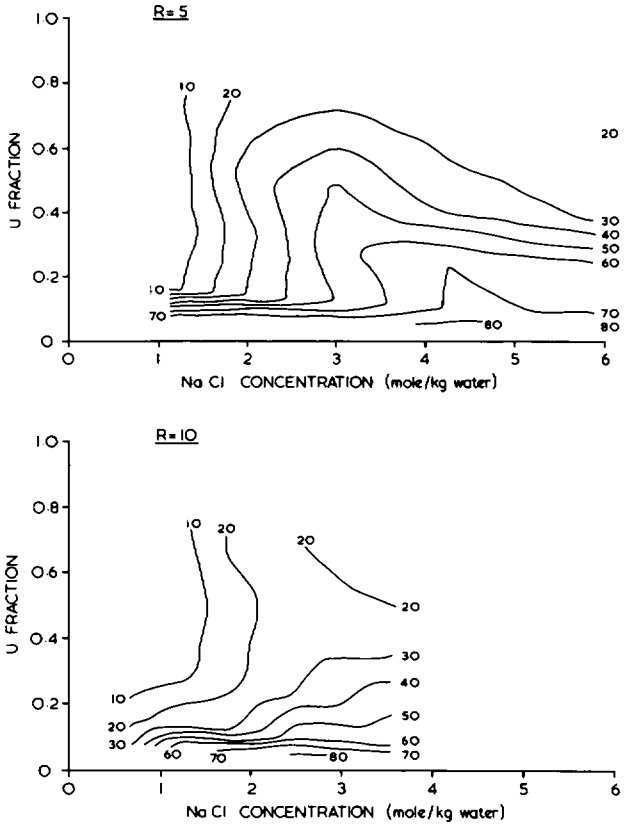

FIGURE 7 The data for Fig. 6 replotted with the unfrozen fraction of water (U) as the ordinate and the concentration of sodium chloride produced by freezing at the designated temperature as the abscissa.

Effect of Solution Compositions Similar to Those Produced in the LV Fraction Experiments

It is at once apparent that these conclusions are discordant with the results of our first two experiments (Figs. 1 and 3). There, the extent of hemolysis for a given R value was closely correlated with the composition of the solution surrounding the cells, whereas here that correlation is poor and the stronger correlation is with the volume of the unfrozen fraction. Our next step was to measure the response of red cells, suspended in solutions similar to those used to study the unfrozen liquid fraction, to changes in solution composition similar to those that occurred in the freezing experiment. We investigated this by exposing cells, at room temperature, to a cycle of concentration and then dilution of their surrounding medium by dialyzing the suspension against solutions of the same composition as that produced by freezing to the selected temperatures, and then against the original suspending solution. It may be noted that, because the R value was kept constant in each set of experiments (either 5 or 10), effectively only water had to traverse the dialysis membrane to achieve the

equilibrium concentrations, so the process was closely analogous to the formation and melting of ice, which also involves the removal and return of water. The results are shown in Fig. 8. The hemolysis response surface was not identical to that produced by freezing but there were many common features; indeed, when $R = 10$, the surfaces were remarkably similar. In all four cases ($R = 5$ and $R = 10$; freezing and dialysis) the high hemolysis contours (>50%) were substantially parallel to the x-axis at low LV or simulated LV values, the greatest hemolysis being produced by the lowest LV. Again in all four experiments, where LV was >0.25, hemolysis increased with decreasing temperature or simulated temperature, although the dialysis contours were generally to the right of the freezing contours. Raising the R value from 5 to 10, in both freezing and dialysis experiments, displaced the contours to the right; that is, the same degree of damage was produced at a lower temperature or simulated temperature. We concluded that the particular dialysis treatment we used was somewhat less damaging than our freezing treatment, a point to which we shall return, but the cells clearly were damaged by the cycle of concentration followed by dilution of their suspending medium, and the pattern of this damage was quite similar to the pattern of damage following freezing and thawing.

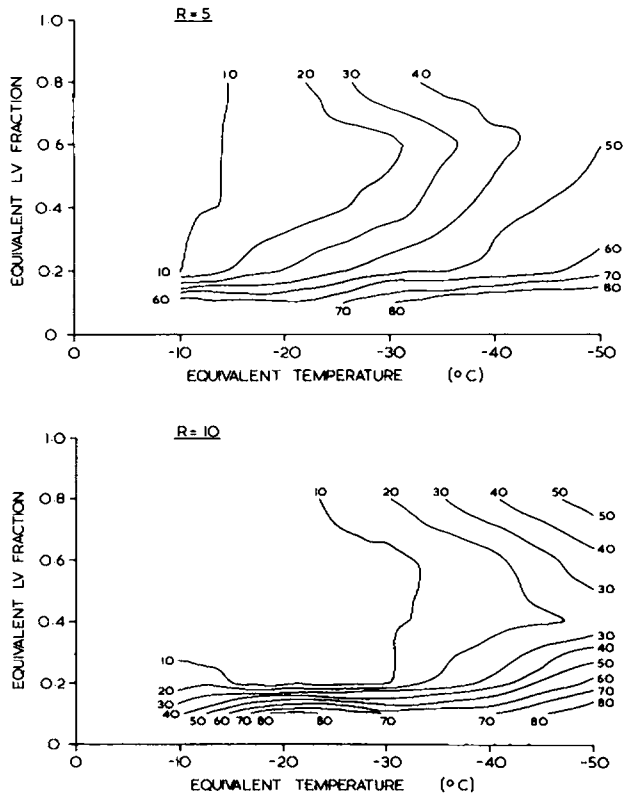

FIGURE 8 Isohemolysis contours for erythrocytes suspended in the same solutions as those used to produce the data in Figs. 6 and 7, but instead of being frozen and thawed, these samples were exposed to solutions of similar composition to those produced in the freezing experiments, but with $LV = 1$ and temperature = +20°C, and were then returned to their original suspending solutions.

Analysis in Terms of Cell Volume

The expressions used to quantitate the unfrozen fraction include two variables, the initial solution concentration, C_o,

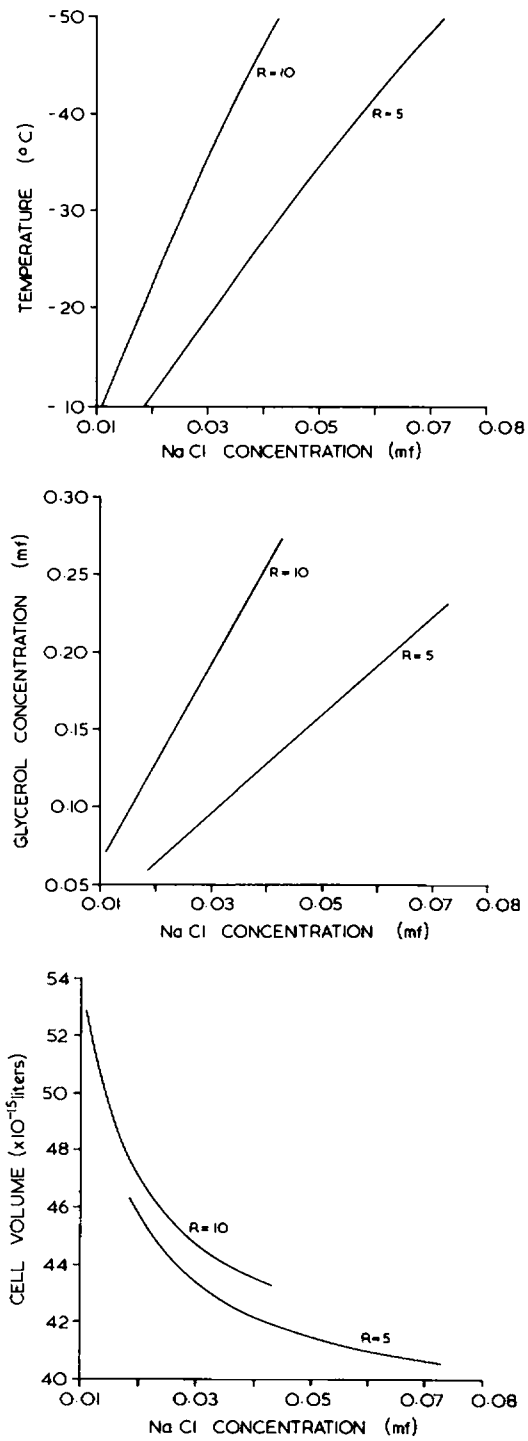

FIGURE 9 Relationship between the concentration of sodium chloride (mole fraction scale) and temperature, glycerol concentration and calculated cell volume during equilibrium freezing of erythrocytes in solutions of sodium chloride plus glycerol, with R values of 5 and 10.

and the concentration at the temperature under study, C_t (see Eqs. 5 and 6), but C_t has a fixed relationship to that temperature (given by Eq. 2) and also to the concentration of sodium chloride ($C_t/(R + 1)$). Thus, the vertical variable, LV or U, is not independent of the horizontal variable, temperature, or salt concentration. If C_t were the only variable controlling LV or U there could be no separation of contours on these plots; hence, the separation seen on Figs. 6 and 7 must be due to the other term in LV and U, namely C_o, the initial solute concentration. Thus, it would be possible to replot the data of Fig. 6 with axis variables of C_o and C_t, or any function of these variables. Fig. 9 illustrates, for both R values considered in this study, the relationship between concentration of sodium chloride, temperature, concentration of glycerol, and equilibrium cell volume. Changes in cell volume have been widely implicated in freezing injury, and because it seems unlikely that the salt concentrations produced in these experiments would have a direct lytic action, we have chosen to present our data primarily in terms of cell volume. Fig. 10 illustrates the initial volumes of the cells suspended in the solutions used in this study. When it was desired to obtain a small liquid volume at a given temperature the initial solution necessarily had a low osmolality and the cells were swollen. When the intention was to produce a large liquid volume at the same temperature, the initial osmolality was high and the cells were reduced in volume. In Fig. 11 we now show the data from our freezing experiments as isohemolysis contours on a grid of minimum cell volume and initial cell volume. Fig. 12 shows the corresponding replot of the dialysis data. In both cases we show, as secondary axes, the salt concentrations that correspond to the cell volumes. It is clear that hemolysis is correlated both with initial cell volume and minimum cell volume, the larger cells appearing more susceptible to shrinkage than the small cells, both in the freezing and in the dialysis experiments.

One interesting feature is that cells with initial volumes less than $\sim 50 \times 10^{-15}$ liters often showed an increased susceptibility to shrinkage. In other words, there appeared to be an optimal cell volume where the cells had maximum resistance to freezing and thawing or to high solute concentrations, and this was at ~60% of normal volume. It also seems that, for a given minimum cell volume and initial cell volume there tended to be more hemolysis when R = 10 than when R = 5. Obviously, for a given minimum cell volume there will be a higher glycerol concentration as the R value increases. This interpretation then is entirely compatible with the original dta of Lovelock, and with our results reported earlier in this paper. Cells of a given initial

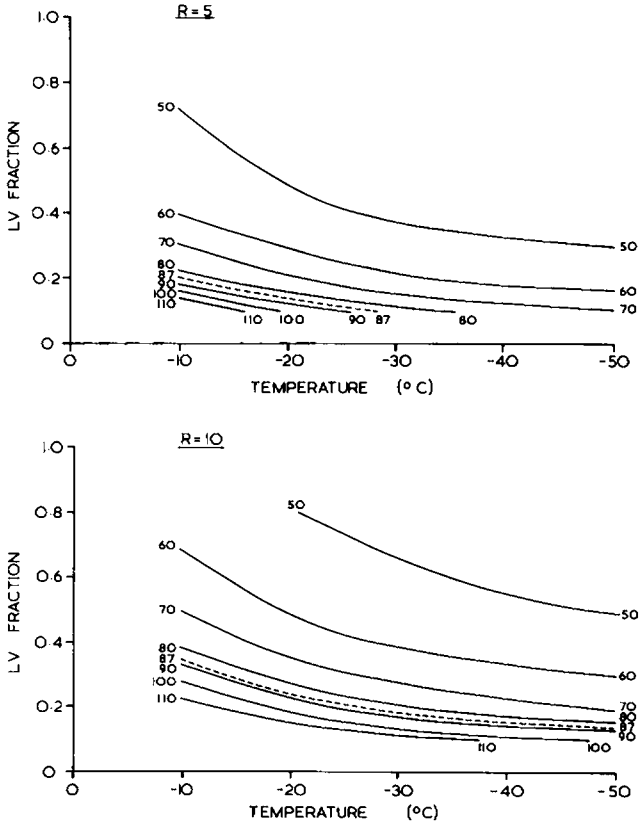

FIGURE 10 Contour plots to indicate the initial volume of erythrocytes suspended in the solutions used to produce the data of Fig. 6. The normal physiological volume (----) of human erythrocytes is 87 × 10^{-15} liters.

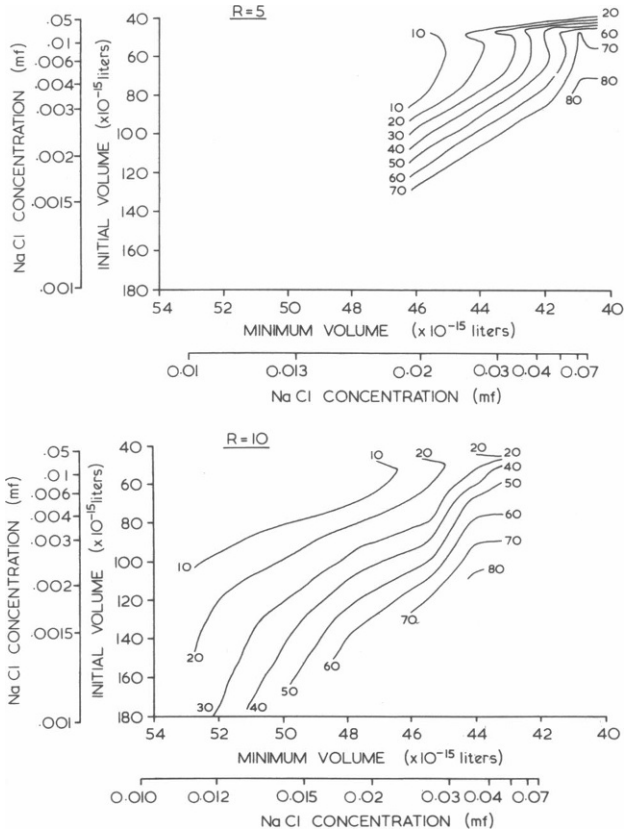

FIGURE 11 Isohemolysis contours from the freezing and thawing experiments used to produce the data of Fig. 6, replotted on a grid of initial cell volume and calculated cell volume at the freezing temperature. The concentrations of sodium chloride corresponding to the cell volume scales for each R value are also shown.

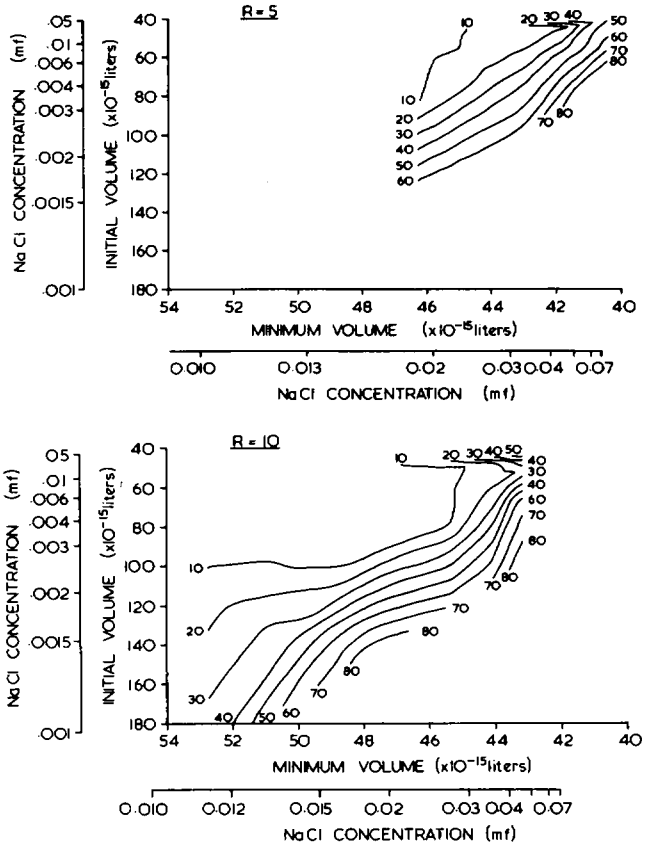

FIGURE 12 Isohemolysis contours from the experiment in which erythrocytes were exposed to solutions equivalent to those occurring at the temperatures used in the freezing and thawing experiments, replotted with cell volume and sodium chloride concentration axes, as in Fig. 11. The experimental data are the same as those used to construct Fig. 8.

volume are hemolyzed by exposure to high solute concentrations and return to initial conditions, and their sensitivity is increased as the glycerol concentration is increased.

GENERAL DISCUSSION

Role of Salt and Glycerol Concentration in Freeze-Thaw Injury

As we stated in the introduction, Lovelock's original experimental data strongly supported his "salt damage" theory of freezing injury, but it has to be conceded that his evidence was circumstantial; he showed only correlations. Nevertheless, it was difficult to believe that the quite precise correlation between the extent of hemolysis and salt concentration produced by freezing did not arise from a causal relationship. It would be surprising indeed if the zone of temperatures in which the mechanical action of ice destroyed the cells was precisely the same as that in which salt concentration reached levels that were shown to damage red cells in the absence of ice. Moreover, this result has been confirmed by Takahashi and Williams (1983) and is now also confirmed by us, so there can be no doubt about the existence of this correlation. But logically, it is possible for the correlation to be a seductive but irrelevant and misleading coincidence.

Lovelock had stated (Lovelock, 1953*b*) that hemolysis was first seen whenever the mole fraction of sodium chloride reached 0.014, whatever the glycerol concentration. There are two problems with this statement. One is that pointed out by Mazur; if the initial concentration of sodium chloride is the same in all the solutions used, it follows that any given concentration of sodium chloride will be associated with the same proportion of water being converted to ice. Hence the correlation of hemolysis with salt concentration is no evidence for the salt damage theory. However, there is another problem, which is that it is very difficult to identify from the experimental data the point at which hemolysis starts; it is much more precise to determine the salt concentration at which finite amounts of hemolysis are recorded. Fahy and Karow (1977) made this determination using Lovelock's own data and they were unable to support the conclusion that the proportion of cells hemolyzed is quantitatively related to salt concentration, whatever the glycerol concentration. In Fig. 13, we reproduce Fahy and Karow's illustration, which shows that damage is greater for a given salt concentration in the presence of glycerol than in its absence. Rall et al. (1978) carried out experiments in which cells were cooled at a constant rate to various subzero temperatures in the pres-

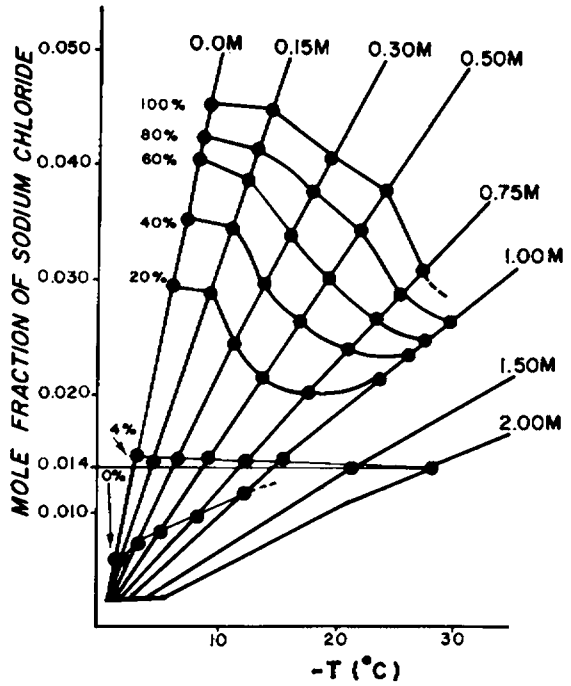

FIGURE 13 The figure published by Fahy and Karow (1977) demonstrating curves relating mole fraction of sodium chloride to temperature for solutions containing 0.95% sodium chloride and the indicated molarity (0–2.00 M) of glycerol. The temperatures at which given percentages of hemolysis (0–100%) were obtained are marked on each curve. (Reproduced with permission of the authors and Cryobiology and Academic Press).

ence of a range of glycerol concentrations (thus eliminating the confounding of final temperature with cooling rate, inherent in Lovelock's method) and they also confirmed that damage is greater for a given salt concentration in the presence of glycerol. In this paper, we also find that the damage associated with a given salt concentration during freezing is dependent on the glycerol concentration. However all these results can equally be claimed to show that the damaging effect of ice is dependent on the glycerol concentration in the remaining liquid because, again, here is a fixed linkage between salt concentration and the amount of ice formed due to the fact that the initial salt concentration was constant. However, our studies in which cells were exposed at 0°C to the same solution compositions as those produced by freezing in the presence of glycerol permit an examination of the damaging effect of the liquid phase in toto, not just its salt concentration, with the unfrozen fraction held constant at a value of 1. The close agreement is striking, and it was this result which led us to examine more closely Masur's proposal that the amount of unfrozen solution is more important than the composition of that solution in controlling the extent of hemolysis.

Role of Unfrozen Fraction in Freeze-Thaw Injury

Our data from the unfrozen fraction experiments were consistent with Mazur's, and with his conclusion that the dominant influence is the unfrozen fraction, with a secondary effect, most apparent at relatively low hemolysis levels, of sodium chloride concentration. However, we found that exposure of cells at constant temperature and in the absence of ice to a similar cycle of concentration and dilution of their surrounding medium, produced rather similar effects. These solution effects must also have occurred during the freezing and thawing experiments, and we therefore argue that there is no need to invoke additional mechanisms to explain these results. We can see two grounds on which this position might be attacked; the dialysis method caused the hematocrit of the cell suspension to increase, and the dialysis results did not exactly duplicate the results of our freezing and thawing experiments. We would respond as follows.

During dialysis, LV and U remained constant at 1.0 in the sense that no ice was formed, but the total volume within the sac decreased during simulated freezing and increased again during simulated thawing, causing cell packing first to increase and then to decrease. Measurement of the change in weight of sacs containing the same volumes and compositions of solution as those used in six of our experiments gave the results shown in Table III. In each case, the reduction in volume was considerably less than that occurring in the freezing and thawing experiments; in the worst case, the nominal hematocrit would have increased from ~2 to ~6%. It should be noted however, that because the cells shrank during the simulated freezing, as also indicated in Table III, the maximum real hematocrit was only ~3.6%. We know from our own experiments to investigate the effect of hematocrit on the survival of red cells suspended in an isotonic solution containing 2.5 M glycerol that such a small increase in initial hematocrit has no detectable effect on hemolysis (Pegg, 1981), and Mazur and Cole (1985) also found that small increases in hematocrit had a negligible effect in their U fraction experiments. It can be argued that any increase in hematocrit will increase the probability of the cell-to-cell interactions that Mazur discusses, but in view of the evidence we quote above, it is difficult to accept that an increase in hematocrit to <5% would significantly affect the level of hemolysis. Mazur also discusses possible cell-to-ice interactions, which are absent in our dialysis system, but our experiments do introduce the possibility of cell-to–cellulose membrane interaction. The ratio of the area of cellulose membrane to the volume of fluid to which

TABLE III

REDUCTION IN VOLUME OF SOLUTION IN CUPROPHAN DIALYSIS SACS DURING SIMULATED FREEZING TO −50°C, AND THE CORRESPONDING CALCULATED CHANGES IN ERYTHROCYTE VOLUME AND HEMATROCIT

	Simulated LV value (R = 5)			Simulated LV value (R = 10)		
	0.1	0.4	0.8	0.1	0.4	0.8
Relative volume in sac at equilibrium	0.32	0.53	0.87	0.34	0.53	0.87
Calculated initial cell volume ($\times 10^{15}$ liters)	71.4	45.7	41.4	97.8	52.3	44.6
Calculated cell volume at equilibrium ($\times 10^{15}$ liters)	40.6	40.6	40.6	43.3	43.3	43.3
Calculated nominal hematocrit at equilibrium (%)	6.3	3.8	2.3	5.9	3.8	2.3
Calculated real hematocrit at equilibrium (%)	3.6	3.4	2.3	2.6	3.1	2.2

Initial nominal hematocrit was 2% in all cases.

it was exposed increased somewhat during dialysis, by ~40% in the worst case, but erythrocytes do not lyse during prolonged exposure to the membrane used in these experiments. This is illustrated by the low levels of hemolysis obtained when the equivalent *LV* fraction and simulated minimum temperature were high.

The second criticism is that our dialysis results did not exactly duplicate those of the freeze-thaw experiment, but nor did the dialysis experiments exactly duplicate, either qualitatively or quantitatively, the time course of the freezing and thawing experiments; the actual measured concentrations of glycerol and sodium chloride in a typical experiment are plotted against time in Fig. 14. Osmotic equilibrium occurred before chemical potential equilibrium for glycerol and sodium chloride because the permeability of the cellulose membrane was greater to sodium chloride than to glycerol; the concentration of sodium chloride changed more rapidly than that of glycerol, and there was therefore a transient overshoot of salt concentration, between 30 and 90 min in the example illustrated. Hence the *R* value was not constant in the dialysis experiments, whereas it must have been in the freezing experiments.

Next, we would point out that the freezing experiments were carried out using a constant cooling rate of 0.6°C/min and warming at ~200°C/min, whereas the equivalent "cooling rate" in the dialysis experiments varied, typically between 0.25 and 1.4°C/min, depending upon the particular combination of initial and final solution composition that the experiment required, whereas the "warming rate" was typically between 0.6 and 3.3°C/min. It will be obvious that the rates were slowest when the initial and final solution were closest in composition, that is, when the simulated unfrozen fraction was high. Mazur has studied a cooling rate of 1.8°C/min as well as 0.6°C/min in his experiments to study the effect of the unfrozen fraction (Mazur et al., 1981); he has also compared a warming rate of 550°C/min with 8–20°C/min (Mazur and Rigopoulos, 1983), and his results do show differences that could be due to these factors. In general, slow cooling and warming gave less hemolysis, but the situation is complex in that cells that were initially swollen appeared to be relatively less susceptible to rapid cooling, whereas cells that were initially shrunken were less susceptible to rapid warming. We cannot draw precise conclusions, but there is clear evidence that the extent of hemolysis is influenced by the rate of change of temperature, and this could therefore contribute to the observed differences between our dialysis and our freezing and thawing experiments.

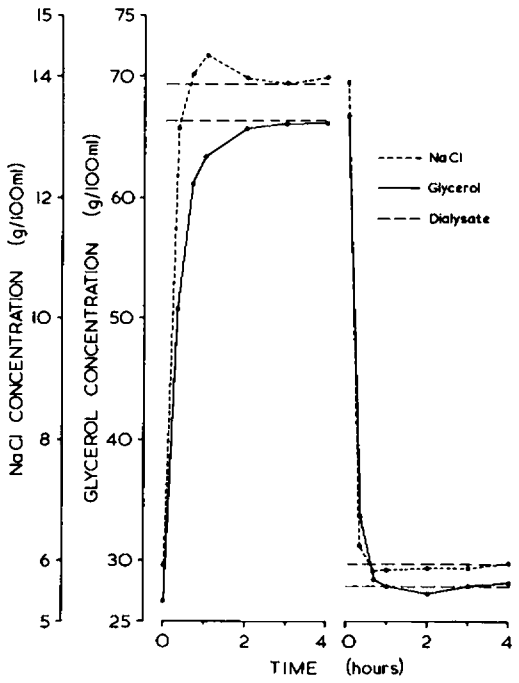

FIGURE 14 Time course of change in concentration of glycerol and sodium chloride in a typical dialysis experiment used to produce the data shown in Figs. 8 and 12. The initial and final solution composition was that used to produce $LV = 0.4$ at -50°C for $R = 5$, and the dialysate composition for the first stage (simulated freezing) was the equilibrium composition for $R = 5$ at -50°C.

Finally, and perhaps most obviously, it should be pointed out that whereas the dialysis experiments were carried out at a relatively high and constant temperature (+20°C), the freezing and thawing experiments involved cooling to subzero temperatures, followed by warming. The work of Lovelock, and the initial part of this paper, suggest that reduction in temperature has little direct effect, and we believe this to be so for cells that are initially suspended in isotonic media. In these experiments however, the cells that were to be frozen with a high unfrozen fraction were suspended in solutions that contained high concentrations of solute, including sodium chloride, and these are precisely the sort of conditions that induce susceptibility to thermal shock. However, the induction of thermal shock is very dependent on the nature of the solutes present, and different solutes produce a different dependence on cooling rate (Morris and Farrant, 1973). Again, it is difficult to be sure whether thermal shock played a part in the unfrozen fraction experiments, but we suggest that it may have.

Our conclusion, taking account of the changes in *R* value during the dialysis experiments, the differences in rate of change of concentration in the two experiments and the fact that only one involved changes in temperature, is that it would actually be rather surprising if the results of the dialysis and freezing and thawing experiment were identical. We believe that the similarity of response is actually quite striking, and we find no reason to invoke a direct effect of extracellular ice.

A Unifying Theory of Freeze-Thaw Injury

Our first inclination was to ascribe the damage we observed in both experiments to the cycle of contraction followed by reexpansion that the cells experienced, their "volume excursion" (Pegg, 1987), but in that case it was necessary to invoke a second damaging factor, the final salt

concentration. However, like *LV* or *U* fraction, volume excursion is partially determined by the final salt concentration, so the two variables are not independent, as we have already discussed. For these reasons we now prefer the more general statement that cells of differing initial volume show different responses to shrinkage as the external mole fraction of sodium chloride is increased. Swollen cells are more sensitive and contracted cells are less sensitive to exposure to high solute concentrations and return to their original media. Even this statement is somewhat tentative. We do not know that it is the initial volume of the cells that controls their susceptibility to subsequent decrease and increase in volume; any other property that is dependent on tonicity could be responsible. In addition, we have not examined the effect of returning the cells to isotonic saline. The cells that were initially in a hypertonic solution were still surrounded by a hypertonic medium at the end of the experiment, and the glycerol was not removed. Interestingly, Woolgar has shown that red cells frozen and thawed in 0.75 M sodium chloride solution are less damaged than cells frozen to the same temperature in 0.15 M sodium chloride (Woolgar, 1972 and 1974). Clearly this result cannot be due to differing salt concentrations during freezing because they will be identical, but in the same papers, Woolgar also showed that posthypertonic hemolysis (which we discuss below) is reduced as the osmolality of the sodium chloride solutions to which the cells are returned, is increased. We cannot assess the relevance of this phenomenon to our results until further experiments have been completed, but it must be stressed that neither did Mazur return his cells to isotonic saline in the experiments that provided the basis for the unfrozen fraction hypothesis. We remain convinced that freezing injury to red cells, under the conditions we are considering here, can be explained completely by changes that occur in the surrounding solution.

'Minimum Volume' Hypothesis

Meryman (1968) pointed out that there is no evidence for a denaturing action of sodium chloride at the relatively low concentrations at which lysis is observed, and he suggested that cells are damaged by dehydration below a critical limiting volume. Because sodium chloride is an impermeant solute, its concentration by freezing will cause the cells to lose water and suffer damage at this critical "minimum volume." The protective effect of glycerol and all other nontoxic, penetrating solutes was due to their ability to prevent this shrinkage. Meryman made hematocrit measurements of red cells in solutions of sodium chloride up to 10× isotonic, and found a minimum value at 4.0–4.5× isotonic (that is a mole fraction of 0.012–0.014), and showed that at this concentration the cells started to gain sodium ions and lose potassium. He attributed this membrane leak to the development of a mechanical strain in the membrane as a result of attaining the minimum volume (Meryman, 1970). Subsequently, he listed evidence from a wide range of plant and animal material to support the proposition that cells in general are damaged by reduction to 40–50% of their original volume (Meryman et al., 1977). There can be no doubt that this correlation exists in the material that Meryman compiled, but there is extensive evidence against the mechanism that he proposed. First, red cells continue to follow classical osmotic (Boyle van't Hoff) behavior up to much greater osmolalities than 4.5× isotonic: at least to 12× isotonic (Farrant and Woolgar, 1972*a*; Weist and Steponkus, 1979). There is in fact no minimum volume when precise measurements are made. Then, if penetration of glycerol is prevented by the use of Cu^{2+} and incubation of human red cells at 0°C (Lovelock, 1953*b*), or by allowing only a brief period of incubation at 0°C (Mazur and Miller, 1976), or by using bovine erythrocytes which are less permeable to glycerol (Mazur et al., 1974), cryoprotection is not abolished, yet the cell volume will be much smaller at a given temperature than it is when glycerol penetrates fully. Similarly, nontoxic, nonpenetrating solutes like sucrose (Farrant and Woolgar, 1972*b*; Mazur and Miller, 1976) and PVP (Farrant, 1969) are protective even though they do not reduce cell shrinkage. Equally, substitution of other salts for sodium chloride alters freezing injury although the cell volume will be similar at the same temperature (osmolality). Sodium iodide is more damaging and sodium acetate less damaging than sodium chloride (Farrant and Woolgar, 1969). Finally, in this paper we have shown that the same degree of hemolysis occurs at differing cell volumes in different concentrations of glycerol. Our conclusion is that cell shrinkage must occur during progressive freezing, and because damage also occurs, there must be a correlation within each experimental system between shrinkage and damage, but when the conditions of the experiment are changed a given degree of damage is observed at a different volume. Consequently, reduction of volume to a single critical limiting value is not the fundamental cause of freezing injury. This does not mean however that shrinkage and reexpansion are not damaging mechanisms; but if they are, sensitivity to these stresses must be influenced by other factors.

"Thermal Shock" Hypothesis

Farrant and Morris (1973) argued that the dominant effect of increasing salt concentration was to make the cell membrane sensitive to damage by further cooling ("thermal shock") perhaps by differential elution of lipids as proposed by Lovelock (1954, 1955). Although shrinkage accompanied the induction of thermal shock during freezing, it was not the cause of the sensitivity to cooling; it was the reduction in temperature that actually caused the membrane to fail (Morris and Farrant, 1973). Susceptibility to thermal shock can be initiated in human red cells by exposure to a variety of differing conditions some, but not all of which, involve dehydration (Lovelock, 1954, 1955; Morris and Farrant, 1973). This sensitization is almost

certainly not due to differential elution of membrane lipids from otherwise intact cells, because Morris (1975) has shown that the lipids released into the supernatant fluid during sensitization are derived from the small percentage of cells that are lysed by the treatment, not from the remaining sensitized cells. Moreover, thermal shock is actually far too damaging to be responsible for freezing injury. It can be seen from Lovelock's original paper that cells that were exposed to sensitizing concentrations of sodium chloride at 20–37°C and then cooled were much more severely injured than cells that were frozen to temperatures that produced the same concentrations. In fact, Lovelock did not consider thermal shock to be an important factor in freezing injury. Finally, and quite conclusively, Takahashi and Williams (1983) have shown that sensitivity to thermal shock is induced by hypertonic sodium chloride solution only if the exposure is at temperatures above 13°C. During normal freezing, the cells are exposed to hypertonic conditions only below 0°C. It was also shown directly that when cells were cooled and simultaneously exposed to a progressive increase in sodium chloride concentration, similar to that produced by freezing isotonic sodium chloride, there was no lysis. We conclude from these results that thermal shock can be eliminated as a cause of freezing injury in red cells frozen in initially isotonic solutions. We have already mentioned the possibility that it may occur when cells are frozen in the hypertonic solutions used to produce high unfrozen fractions.

Central Role of Osmotic Phenomena in Freeze-Thaw Injury

Lovelock's original proposal was that freezing injury to red cells was due to exposure to external sodium chloride concentrations >0.014 mole fraction and was independent of temperature. The high salt concentration so damaged the cell membranes as to make them permeable to sodium; the cells acquired a "burden of sodium ions" from the hypertonic, partially-frozen solution and therefore they lysed when they were returned to an isotonic environment by thawing (Lovelock, 1953*a*). High concentrations of sodium chloride also caused them to become sensitive to damage by further cooling or mechanical stresses (Lovelock, 1954 and 1955). Hence, the protective effect of glycerol was due to its colligative action in modulating the rise in salt concentration, and the fact that partial inhibition of glycerol penetration (by incubating at 0°C in the presence of Cu^{2+} ions) reduced protection was because the concentration of internal potassium chloride was just as damaging as the external sodium chloride (Lovelock, 1953*b*). A number of experimental observations support the proposal that the principle cause of damage was loading of the cells with sodium ions, leading to osmotic lysis during thawing. First, lysis was seen to occur primarily during thawing of erythrocytes that had been frozen on the cryomicroscope (Smith, 1961) and on return to isotonic conditions in the solution experiments (Lovelock, 1953*a*). Then, it has been shown, first by Meryman (1968) and subsequently in a series of meticulous measurements by Farrant and Woolgar (1972*a* and *b*), that an inward leak of sodium ions and an outward leak of potassium does occur when human red cells are exposed to osmolalities >1.8 osmolal (~0.017 mole fraction of sodium chloride). Thus, loading with sodium ions would seem to be possible. If it does occur then it should be more severe with slow cooling than rapid, because slow cooling provides more time for solute movement at relatively high temperatures, and in that case slow warming should be preferable because it would afford more time for the leaky cells to lose excess sodium during warming; slow warming does in fact give higher survival of slowly cooled red cells (Miller and Mazur, 1976; Pegg et al., 1984).

It should be stressed that it is the entry of normally impermeant solutes that we are considering, sodium and nonpenetrating cryoprotectants like sucrose for example, and not any penetrating solutes, such as glycerol, that might be added. Providing that full equilibration of any such additive occurred before freezing started, and contrary to some statements in the literature (e.g., Myers and Steponkus, 1986), there will be no driving force for more solute to enter during the freezing process as long as the cell membrane remains impermeant to those solutes to which it was originally impermeant. This arises because loss of water from the cell concentrates the internal penetrating solute sufficiently to maintain equilibrium without solute movement (Pegg, 1984). Next, we should recall that red cells remain quite precisely on a linear Boyle van't Hoff plot right up to 3.6 osmolal sodium chloride at room temperature. Therefore, they cannot gain significant amounts of solute because this would cause a departure from linearity. Careful examination of Farrant and Woolgar's data (Farrant and Woolgar, 1972*a*) shows that the sodium uptake is at least partially counterbalanced by the potassium leak, and the errors in estimating sodium content in the presence of high external sodium concentrations make the conclusion that there is a net cation uptake somewhat uncertain. Moreover, there was no net solute uptake in sucrose/sodium chloride solutions (Farrant and Woolgar, 1972*b*), yet cells that were recovered after being frozen and thawed in 40% sucrose/0.95% sodium chloride were loaded with sucrose and sodium and had twice their original water content (Daw et al., 1973). If solute loading was not a simple consequence of exposure to hypertonic conditions then it must have been induced either by exposure to low temperatures simultaneously with the hypertonic environment, or it must have occurred during the dilution (warming) phase. If low-temperature exposure was involved to a significant degree, one would not expect the solution-exposure experiments to duplicate the freezing and thawing experiments so well. We therefore suggest that the cell membranes are damaged during the shrinkage that is produced by freezing, but that they do not become

leaky until the reexpansion phase. The argument is as follows. Up to the moment that the leak starts, osmotic equilibrium is maintained by water movement; during thawing the environment is becoming more dilute, so water is entering the cells, and there is no concentration gradient to cause solute to enter. As the cell reexpands its membrane becomes leaky, but the leak is selective; the cells contain some solutes to which the membrane still remains impermeable (colloids), and these solutes exert an osmotic gradient causing solvent to enter the cells. This solvent is in fact a solution containing all penetrating solutes, including sodium, penetrating cryoprotectants and, if the membrane should be sufficiently damaged, normally nonpenetrating cryoprotectants like sucrose. Farrant and Woolgar (1972*b*) and Woolgar (1972, 1974) have reported that sucrose does not enter red cells during hypertonic exposure, but it does during freezing and thawing (Daw et al., 1973) which is the result one would expect if loading were to occur during dilution. Daw's result indicates that the membranes are able to reseal with an excess of internal solute and therefore stabilize at a greater volume than normal; up to 170% of normal should be possible because only 50% of human red cells lyse at this volume at room temperature (Pegg, 1984). This would explain the high volume of the cells recovered after freezing in sucrose/saline, and if the time taken to reseal is temperature dependent, it would also explain why the percentage hemolysis observed when cells exposed to 2.65 osmolal sucrose/sodium chloride are resuspended in isotonic sodium chloride, is 12% when the solution is at 35°C, but rises to 93% at 0°C (Woolgar and Morris, 1973). Mazur and Miller (1976) have contrasted the fact that the amount of posthypertonic hemolysis is similar whether the cells are exposed to hypertonic sodium chloride solution or to the same osmolality of sucrose/sodium chloride, whereas with cells frozen and thawed in 1 M sucrose, even after resuspension in saline, survival is higher (25%) than it is when they are frozen in the same osmolality of sodium chloride (0.75 M; 0% survival). The difference may be due to the fact that the posthypertonic hemolysis experiments involved resuspension in isotonic saline, whereas thawing of cells that had been frozen in sucrose/sodium chloride caused them to be resuspended in sucrose/sodium chloride. As Woolgar (1972, 1974) has shown, if sucrose is present during the posthypertonic phase, hemolysis is reduced, presumably because even in the leaking cell the reflection coefficient for sucrose is higher than that for sodium chloride.

CONCLUSIONS

On present evidence we conclude that damage to red cells during freezing and thawing is caused by damage to the cell membrane, brought about by shrinkage in the specific solution compositions occurring in the particular system, that this damage causes the cells to become permeable during thawing and immediately afterwards to normally impermeant solutes, that the osmotic pressure of those intracellular solutes to which the cell membrane remains impermeant causes an uptake of water and permeant solutes, that the cell membrane of surviving cells reseals at a temperature dependent rate, and that the final cell volume may be larger than normal, depending upon the excess solute inside the cell when resealing occurs.

We have no specific explanation for the effect of glycerol in potentiating the damaging action of shrinkage in high concentrations of sodium chloride. Rall et al. (1978) suggested two possible mechanisms. The first was that the effect was due to the longer time of exposure, during constant-rate cooling, required to reach the temperature giving any specified sodium chloride concentration. This mechanism could have applied to their experiments but it does not apply to ours or Lovelock's because our cooling rates were more rapid when lower temperatures were used and exposure times were relatively constant. Their other proposal was that excess glycerol entered the cells during cooling, and was unable to leave the cells sufficiently rapidly during thawing. For reasons we have already discussed, we do not believe that solute loading occurs during freezing, although it certainly is possible that glycerol enters by a "colloid osmotic" mechanism during thawing (or dilution).

The apparent optimum initial volume, at ~60% of normal volume for cells that are to be frozen slowly, requires further study. The observation recalls the fact that many techniques of red cell preservation include the use of impermeant solutes in the medium, and these will reduce the initial volume of the cells (Huntsman et al., 1960; Rowe and Allen, 1965; Pegg et al., 1982). However, the precise relevance of this observation to the colloid osmotic mechanism of cell lysis that we have discussed is obscure.

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