PHYSICAL-CHEMICAL BASIS OF THE PROTECTION OF SLOWLY FROZEN HUMAN ERYTHROCYTES BY GLYCEROL

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ABSTRACT One theory of freezing damage suggests that slowly cooled cells are killed by being exposed to increasing concentrations of electrolytes as the suspending medium freezes. A corollary to this view is that protective additives such as glycerol protect cells by acting colligatively to reduce the electrolyte concentration at any subzero temperature. Recently published phase-diagram data for the ternary system glycerol-NaCl-water by M. L. Shepard et al. (*Cryobiology*, **13**:9–23, 1976), in combination with the data on human red cell survival vs. subzero temperature presented here and in the companion study of Souzu and Mazur (*Biophys. J.*, **23**:89–100), permit a precise test of this theory. Appropriate liquidus phase-diagram information for the solutions used in the red cell freezing experiments was obtained by interpolation of the liquidus data of Shepard and his co-workers. The results of phase-diagram analysis of red cell survival indicate that the correlation between the temperature that yields 50% hemolysis (LT_{50}) and the electrolyte concentration attained at that temperature in various concentrations of glycerol is poor. With increasing concentrations of glycerol, the cells were killed at progressively *lower* concentrations of NaCl. For example, the LT_{50} for cells frozen in the absence of glycerol corresponds to a NaCl concentration of 12 weight percent (2.4 molal), while for cells frozen in 1.75 M glycerol in buffered saline the LT_{50} corresponds to 3.0 weight percent NaCl (1.3 molal). The data, in combination with other findings, lead to two conclusions: (*a*) The protection from glycerol is due to its colligative ability to reduce the concentration of sodium chloride in the external medium, but (*b*) the protection is *less* than that expected from colligative effects; apparently glycerol itself can also be a source of damage, probably because it renders the red cells susceptible to osmotic shock during thawing.

INTRODUCTION

Mammalian cells rarely survive slow freezing in the absence of a protective solute like glycerol. Lovelock (1953*a*,*b*) concluded that freezing damage in the unprotected hu-

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man erythrocyte is caused by exposure to high electrolyte concentrations in the cells and in the unfrozen portion of the suspending medium, and that the protective effect of glycerol arises from its colligative ability to reduce the electrolyte concentration at any subzero temperature. He arrived at these conclusions by observing the temperature-dependence of the hemolysis of erythrocytes frozen in saline containing various concentrations of glycerol, and by comparing the temperature-dependence of hemolysis to that of electrolyte concentration, the concentrations being derived from phase diagrams.

Two developments since that time waranted a reexamination of that work: One has been the growing cognizance of the critical role played by cooling and warming rates in cell survival (Mazur, 1970; Miller and Mazur, 1976), variables which Lovelock had not controlled. The second has been the publication of phase-diagram data on solutions of cryobiological importance by Cocks and his colleagues (e.g. Cocks and Brower, 1974, and Shepard et al., 1976), data obtained by techniques more precise than those available to Lovelock.

In the preceding paper (Souzu and Mazur, 1978), the survival of human red cells frozen slowly to various subzero temperatures was related to the concentration of glycerol initially present in the suspending medium. In the present paper, we use phase-diagram data to determine the relation between temperature and the composition of the unfrozen portion of the suspending medium, and then relate survival to that composition, particularly to the concentration of salts.

The comparison leads to two conclusions: (*a*) Glycerol does indeed protect by reducing the concentration of sodium chloride in the external medium, but (*b*) glycerol itself can also be a source of damage, probably by rendering the cells susceptible to osmotic shock during thawing.

MATERIALS AND METHODS

Unless otherwise indicated, the materials and general methods used were the same as those described in the preceding paper (Souzu and Mazur, 1978).

Solutions

Stock suspending solutions consisted of various concentrations of glycerol (0–3 M) in either 0.148 M NaCl buffered with 0.010 M phosphate to pH 7.0 or 0.148 M NaCl alone (physiological saline). The final molarity of salts decreased with increasing glycerol concentration, but the molality of the NaCl and the buffer remained constant at 0.149 and 0.010, respectively (Mazur et al. 1974*a*). The densities of these solutions were measured at 25°C with a Lipkin pycnometer (Lipkin et al., 1944). All calculations concerning the glycerol concentrations have been corrected for dilution (carry-over) by saline when washed red cells are added to the stock glycerol-saline solutions. This correction reduces the nominal glycerol concentration by 3%. Further details are given elsewhere (Mazur et al., 1974*a*; Mazur and Miller, 1976*a*).

Calculation of Weight Percentages of Glycerol and Salt in Unfrozen Solutions

The phase properties of a solution are fixed by the relative amount of glycerol to salt present in the unfrozen solution. The usual way of expressing these relative amounts is the weight ratio of

glycerol to salt. The weight percentage of glycerol (W_g^0) was calculated as

$$W_g^0 = (\mathrm{M} \times 92.09)/(10 \times \rho). \quad (1)$$

where M is the molar concentration of glycerol and ρ is the density of the solution. The water concentration (W_w^0) expressed as a weight percent was calculated as

$$W_w^0 = (100 - W_g^0)f, \quad (2)$$

where f is the weight-fraction water in the saline solution (f = 0.990 for buffered saline, 0.9915 for unbuffered saline). The weight-percent salt is simply 100 minus the sum of the weight percents of water and glycerol.

The densities and composition of the various glycerol solutions used in the red cell freezing experiments (Souzu and Mazur, 1978) on a weight-percent basis are shown in Table I. The quantity R is the weight ratio of glycerol to salts.

RESULTS

Survival vs. Temperature

Fig. 1 shows the percentage of human red cells that survive cooling at 1.7°C/min to various temperatures and rapid warming. (Survival is defined as 100 minus the percent hemolysis.) The cells were suspended in 0.149 molal NaCl containing various

TABLE I

COMPOSITION OF UNFROZEN GLYCEROL-SALINE-WATER SOLUTIONS

Glycerol concentration in:		Characteristics of experimental solutions				
Stock solutions	Experimental solutions*	Density 25°/4°	Percent by weight			Weight ratios
			Glycerol	Salts	Water	
M	*M*	*g/ml*	W_g^o	W_s^o	W_w^o	*R*‡
A. Solutions made with NaCl						
0	0	1.003	0	0.85	99.15	0
0.5	0.48	1.013	4.41	0.81	94.78	5.42
1.0	0.97	1.023	8.73	0.78	90.49	11.26
1.5	1.46	1.032	12.98	0.74	86.28	17.54
2.0	1.94	1.042	17.15	0.70	82.15	24.35
B. Solutions made with buffered saline						
0	0	1.004	0	1.0	99.0	0
0.5	0.48	1.014	4.40	0.96	94.64	4.61
1.0	0.97	1.024	8.72	0.91	90.36	9.56
1.5	1.46	1.033	12.97	0.87	86.16	14.90
1.75	1.70	1.038	15.06	0.85	84.09	17.73
1.85	1.79	1.040	15.89	0.84	83.27	18.89
1.95	1.89	1.042	16.72	0.83	82.45	20.08
2.0	1.94	1.043	17.14	0.83	82.04	20.67
2.5	2.42	1.052	21.22	0.79	77.99	26.94
3.0	2.91	1.062	25.24	0.75	74.01	33.76

*3% dilution by carry over from washed cells.

‡R = weight percent of glycerol/weight percent of salts.

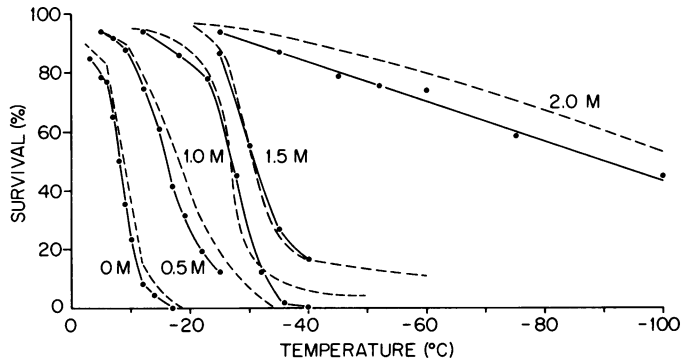

FIGURE 1 Survival of frozen-thawed human red blood cells as a function of the minimum temperature to which they were cooled. The cooling rate was 1.7°C/min and the warming rate ~550°C/min. Cells were frozen in solutions containing the indicated nominal concentrations of glycerol in physiological NaCl (●——●) or in phosphate-buffered physiological NaCl (— - —). The true concentrations of glycerol are 3% lower than nominal (see Methods).

concentrations of glycerol. For comparison we show a portion of the data from the companion paper (Souzu and Mazur, 1978) for cells suspended in buffered saline rather than in NaCl.

Increasing the concentration of glycerol from 0 to 1.5 M lowers the temperature at which hemolysis occurs, but does not affect the final level of survival, which is below 20%. However, increasing the concentration of glycerol to 2 M not only decreases the fraction hemolyzed at a given temperature, but also raises the observed survival at −90°C or below to above 40%. The results for cells in NaCl are similar to those for cells in buffered saline.

Composition of the Liquid Phase of Partially Frozen Glycerol-NaCl Solutions

The information needed to determine the composition of the unfrozen portions of the suspending media as a function of subzero temperature was obtained from phase diagrams. Goldston (1974) and Shepard et al. (1976) have recently reported NaCl-glycerol-water phase diagrams for weight ratios of glycerol to NaCl (R values) ranging from <1 to 30. Fig. 2 summarizes their data for those R values that span the range of interest in the present study, namely, $R = 0$ (no glycerol), 2.2, 4, 9, and ∞ (no salt). In this figure, the melting point is plotted against the total solute concentration (glycerol + NaCl) for solutions with a given value of R. Such curves of constant R (isopleths) can be used to predict the equilibrium composition of a given glycerol-saline solution as it is progressively frozen (Purdon and Slater, 1946). For example, when a solution with $R = 9$ is frozen to −20°C, the concentration of solutes in the unfrozen portion of the solution becomes 44.2 g/100 g. Note that for all solutions with a given value of R the concentration at given temperatures below the freezing point is independent of the initial solute concentration before freezing.

Since Shepard et al. (1976) did not obtain phase diagrams for solutions with the

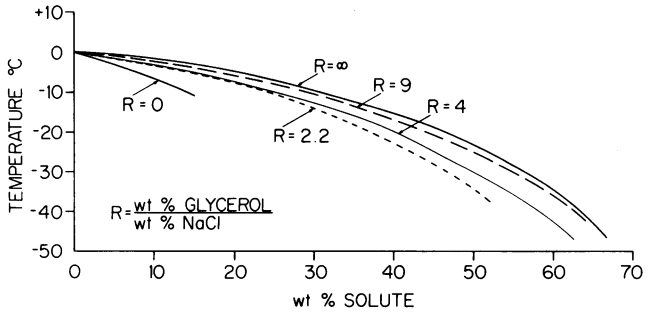

FIGURE 2 Equilibrium phase-diagram for the ternary system NaCl-glycerol-H_2O. The graph shows the freezing point as a function of the total solute concentration (weight percent) for solutions with the indicated R value (glycerol:NaCl weight ratio), plotted from the data of Goldston (1974) and Shepard et al. (1976).

specific R values used in the present study (Table I), it was necessary to interpolate from their data. Isopleths for our solutions with R values between 4 and 9 were obtained by interpolating between the $R = 4$ and $R = 9$ curves shown in Fig. 2. Similarly, isopleths for our solutions with R values >9 were obtained by interpolation between the $R = 9$ and $R = \infty$ curves. Since interpolation between a finite number and infinity is difficult, we used the following procedure. Goldston (1974) measured several freezing points for glycerol-NaCl-water solutions with R values between 20 and 30. Inspection of his data indicates that solutions with R values greater than about 24 are indistinguishable from the $R = \infty$ curve. For the purpose of interpolation then, we considered the $R = 24$ and $R = \infty$ curves to be identical.

Interpolation was performed in two ways. The first was linear. For example, the R value for 0.5 M glycerol in 0.148 M NaCl is 5.4. We assumed that the appropriate solute concentration of this solution at any subzero temperature lay to the right of Shepard et al.'s $R = 4$ curve by 0.28 [that is, $(5.4 - 4)/(9 - 4)$] times the difference in the solution concentrations between their $R = 4$ and $R = 9$ curves at that subzero temperature. The second method of interpolation was logarithmic. Plots of the logarithm of the total solute concentration in weight percent against the logarithm of the subzero temperature approximated straight lines for each isopleth in Fig. 2. Plots were then made of the calculated log-log regression coefficients (slopes) and of the y-axis "intercepts" (log temperature at which solute concentration equals 1%) vs. the R values. The slopes and intercepts corresponding to the R values listed in Table I were read off the plots; and from the slopes and intercepts the appropriate phase diagram was reconstructed for each solution listed in Table I. The isopleths from the two interpolation methods agreed to within 7% and usually to within 3%.

The isopleths based on the linear interpolation are given in Table II in terms of the total solute concentration in the unfrozen portion of the solutions at various temperatures.

During progressive freezing at temperatures above the eutectic point, water is the

TABLE II

TOTAL SOLUTE CONCENTRATIONS IN PARTLY FROZEN SOLUTIONS OF GLYCEROL IN 0.149 mol NaCl/kg H_2O

Temperature	Weight percent solute (W_T) in solutions with initial glycerol concentration of*				
	0 M	0.5 M	1.0 M	1.5 M	2.0 M
°C					
Unfrozen	0.85	5.2	9.5	13.7	17.9
−2	3.3				
−5	7.8				
−7	10.6				
−10	14.0	26.3	29.0	29.8	30.6
−12	16.0	30.0	32.7		
−15	18.8	34.8	37.6	38.6	39.7
−18	21.1	38.9	41.9		
−20	22.6	41.3	44.6	45.6	46.6
−25		46.6	50.3	51.0	51.8
−30		51.4	55.1	55.8	56.5
−35		55.8	59.4	59.9	60.4
−40			62.9	63.2	63.5
−45				65.7	65.9
−50‡			67.6	67.7	67.9
−55‡					69.3
−60‡					70.2
−65‡					70.7

*Nominal concentrations. Exact concentrations are 3% less (see Table I and text).
‡Extrapolated.

only substance removed from solution. Accordingly, R remains constant, and this fact permits one to calculate the concentrations of each of the components at given subzero temperatures. Thus the salt concentration in weight percent (W_s) at any temperature (T) is

$$W_S = W_T/(R + 1), \tag{3}$$

where W_T is the total solute concentration (weight percent), at temperature T. One can calculate the weight fraction of the solution remaining unfrozen (L) at any subzero temperature as

$$L = W_T^0/W_T, \tag{4}$$

where W_T^0 is the total solute concentration (weight percent) in the original unfrozen solution ($100 - W_w^0$ in Table I).

The NaCl concentration in the liquid phase, as a function of subzero temperature (Eq. 3), is shown in Fig. 3 A for the several solutions used in the study. Particularly striking is the well-known suppression of the salt concentration when high glycerol concentrations are present. Fig. 4 A shows the weight fraction of the solution remaining unfrozen (L) at subzero temperatures (Eq. 4). Note that the fraction unfrozen at a given temperature increases as the initial glycerol concentration is increased.

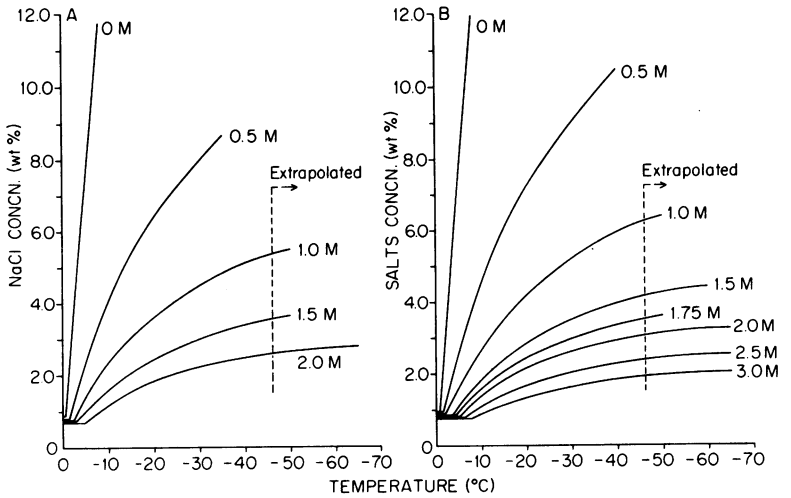

FIGURE 3 Salt concentration in weight percent as a function of subzero temperature. (A) Glycerol in physiological NaCl. (B) Glycerol in phosphate-buffered physiological NaCl. The values on the curves refer to the nominal concentrations of glycerol present in the unfrozen solutions.

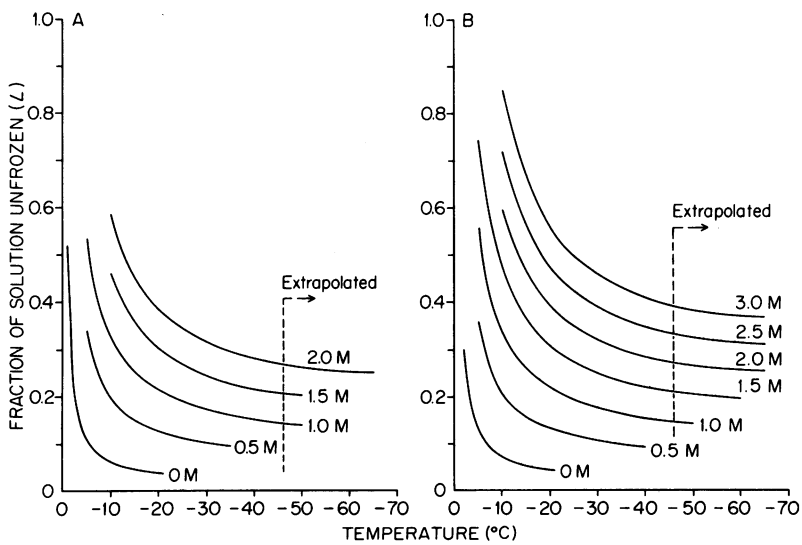

FIGURE 4 Weight fraction of solution remaining unfrozen (L) at various subzero temperatures. (A) Glycerol in physiological NaCl. (B) Glycerol in phosphate-buffered physiological NaCl. The values on the curves refer to the nominal concentration of glycerol in the original unfrozen solution.

Composition of the Liquid Phase of Partially Frozen Solutions of Glycerol in Buffered Saline

Since Souzu and Mazur (1978) had also performed survival experiments with phosphate-buffered NaCl solutions of the composition shown in Table IB, there was need to determine the phase relations in these buffered solutions. Pozner et al. (1977) have shown that the introduction of small quantities of the solutes in Eagle's minimum essential medium (MEM) to glycerol-NaCl solutions does not detectably alter the phase relations at the R values applicable to our study. Therefore, we took the sum of the weights of NaCl and 0.01 M phosphate buffer to be the equivalent weight of sodium chloride alone, assumed that the curves in Fig. 2 applied, and calculated isopleths (Table III) by the interpolation procedures described above.

The weight-percent concentration of salts (NaCl + phosphate buffer) in the unfrozen medium is shown in Fig. 3 B, and the weight fraction that remains unfrozen at given temperatures is shown in Fig. 4 B for the several glycerol concentrations used.

TABLE III

TOTAL SOLUTE CONCENTRATIONS IN PARTLY FROZEN SOLUTIONS OF GLYCEROL IN BUFFERED SALINE

Temperature	Weight percent solute (W_T) in solutions with initial glycerol concentration of*									
	0 M	0.5 M	1.0 M	1.5 M	1.75 M	1.85 M	1.95 M	2.0 M	2.5 M	3.0 M
°C	g/100 g									
Unfrozen	1.0	5.4	9.6	13.8	15.9	16.7	17.6	18.0	22.0	26.0
−2	3.3									
−5	7.8	14.9	17.3	18.6						
−6	9.2	17.2								
−7	10.6									
−8	11.8	21.6								
−10	14.0	25.7	28.8	29.5	29.8	30.0	30.1	30.2	30.6	30.6
−12	16.0	29.4	32.4							
−15	18.8	34.3	37.2	38.2						
−18	21.1	38.3	41.5							
−20	22.6	40.7	44.2	45.1	45.6	45.8	46.0	46.1	46.6	46.6
−25		45.8	50.0	50.7				51.4		
−30		50.6	54.9	55.5	55.8	56.0	56.1	56.1	56.5	56.5
−35		55.1	59.2	59.7	59.9	60.0	60.1	60.1	60.4	60.4
−40		58.8	62.8	63.1	63.2	63.3	63.3	63.3	63.5	63.5
−45			65.4	65.6	65.7	65.7	65.8	65.8	65.9	65.9
−50‡			67.5	67.6	67.7	67.7	67.8	67.8	67.9	67.9
−55‡							69.3	69.3		
−60‡							70.2		70.2	70.2
−65‡									70.7	70.7

*Nominal concentrations. Exact concentrations are 3% less (see Table I and text).
‡Extrapolated.

Other Measures of Composition vs. Temperature

It is customary to express phase-diagram data in weight percents of the components, but relationships that depend on colligative properties of solutions are best described by expressing solute concentrations as molalities or mole fractions. Accordingly, we show the molality of salts (m_s) in the unfrozen medium vs. temperature in Fig. 5. Molality is given by

$$m_s = \frac{(1000)(W_s)}{[(58.44)(100 - W_T)]}. \tag{5}$$

We have not plotted the data in terms of the mole fraction of salts, but they can be calculated from weight percents as

$$X_s = \frac{W_s/58.44}{[W_s/58.44 + W_g/92.09 + (100 - W_T)/18.02]}, \tag{6}$$

where W_g is weight percent glycerol at temperature $T(\equiv W_T - W_s)$.

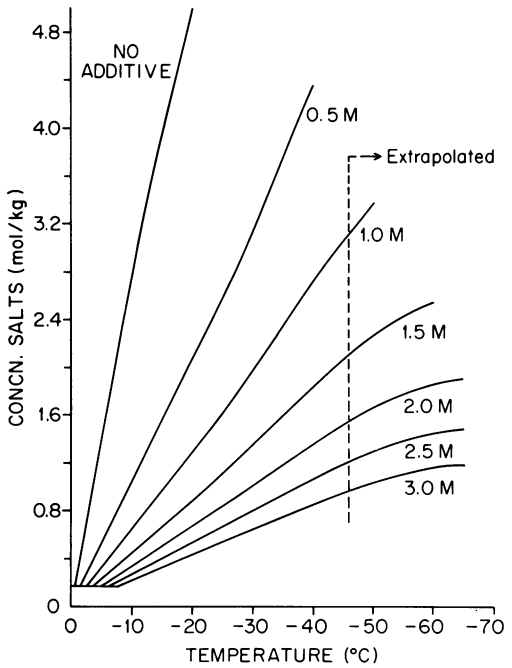

FIGURE 5

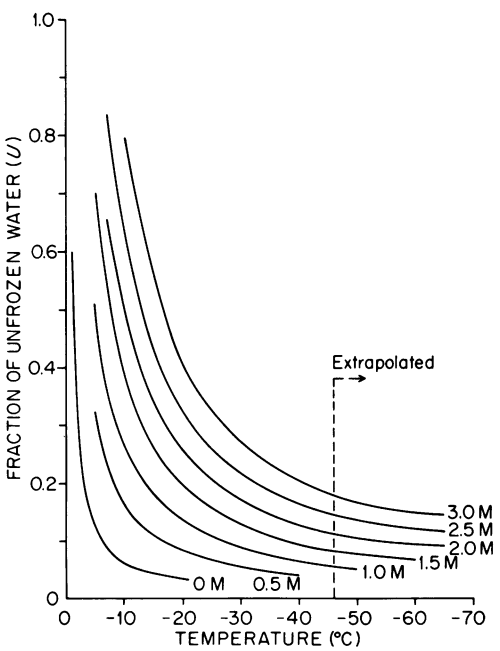

FIGURE 6

FIGURE 5 Molality of NaCl in the unfrozen portions of the solutions at various subzero temperatures. The values on the curves refer to the nominal concentration of glycerol in buffered NaCl before freezing.

FIGURE 6 Fraction of unfrozen water (U) at various subzero temperatures. The values on the curves refer to the nominal concentration of glycerol in buffered NaCl before freezing.

Molality expresses concentration in terms of the mass of water available for solution. Correspondingly, we can calculate the weight fraction of the original water that remains unfrozen at various temperatures (U) as

$$U = (100 - W_T)L/(100 - W_T^0) \quad (7)$$

The values of U vs. temperature are shown in Fig. 6.

Figs. 5 and 6 give the molality of salts and the fraction of unfrozen water for solutions containing buffered saline. The corresponding curves for solutions containing NaCl alone are similar, and can be calculated from the data in Figs. 3 A and 4 A and from Eqs. 5 and 7.

Extrapolation below −46°C

The phase-diagram data of Shepard et al. (1976) extend only to −46°C. But liquid solution will be present down to the ternary eutectic point, which Shepard and his co-workers estimate to be −80°C. In certain of the curves in Fig. 3–6, therefore, we have extrapolated the data to as low as −65°C.

Human Red Cell Survival vs. Salt Concentration in Partially Frozen Glycerol-Saline Solutions

Figs. 3 and 5 give the salt concentrations in glycerol-salt-water solutions as a function of temperature. Fig. 1 here and Fig. 2 in the companion paper (Souzu and Mazur, 1978) give red cell survival as a function of subzero temperature. We can therefore

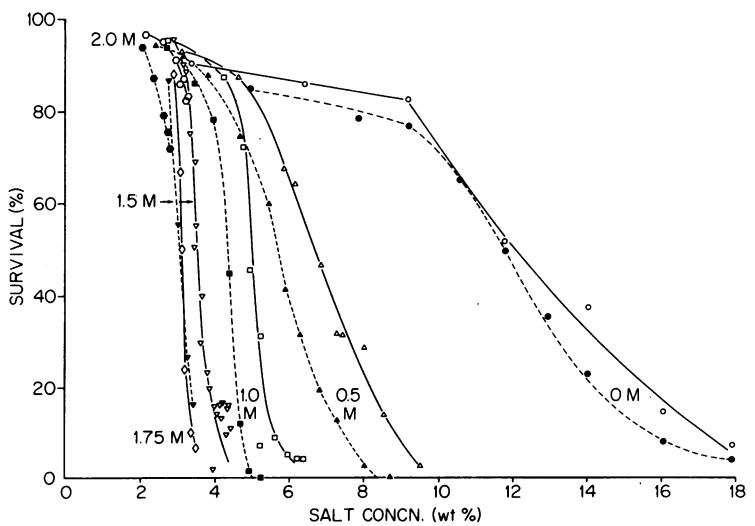

FIGURE 7 Survival of human red blood cells as a function of the maximum salt concentration (weight percent) attained during freezing to various temperatures. Cells were cooled at 1.7°C/min and warmed at ~550°C/min. (------), cells frozen in physiological NaCl. (——), cells frozen in phosphate-buffered physiological NaCl. The values on the curves refer to the nominal concentration of glycerol present before freezing.

combine the two sets of data to obtain plots of survival vs. maximum (i.e. equilibrium) salt concentrations attained at various subzero temperatures. Figs. 7 and 8 give such plots for the survival of red cells frozen at 1.7°C/min to various temperatures and immediately thawed rapidly at ~550°C/min. The two figures express the salt concentrations as weight percent and molality, respectively. In each case, it can be seen that, as the initial glycerol concentration is increased, hemolysis occurs at a progressively lower concentration of salt. The effect is most dramatic in Fig. 7 where, for cells in buffered saline, the salt concentration at the median lethal temperature (LC_{50}) decreases from 12.1 g/100 g to 3.1 g/100 g as the glycerol concentration in the original suspending medium is increased from 0 to 1.75 M. Even when salt concentration is expressed in molal terms (Fig. 8), the LC_{50} decreases from 2.4 mol/kg to 1.3 mol/kg as the glycerol concentration is increased from 0 to 1.75 M. The results for cells in glycerol-NaCl solutions are similar (Fig. 7, dashed curves).

Souzu and Mazur (1978) also obtained survival data for red cells warmed at 1 and 150°C/min as well as at ~550°C/min. In Fig. 9, we plot the LC_{50} (expressed as

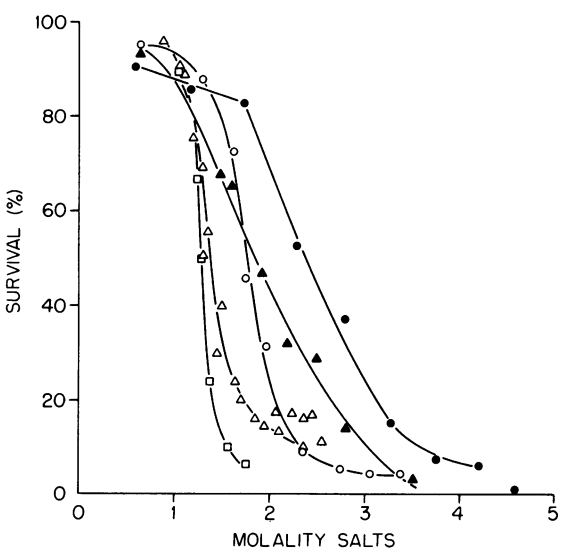

FIGURE 8

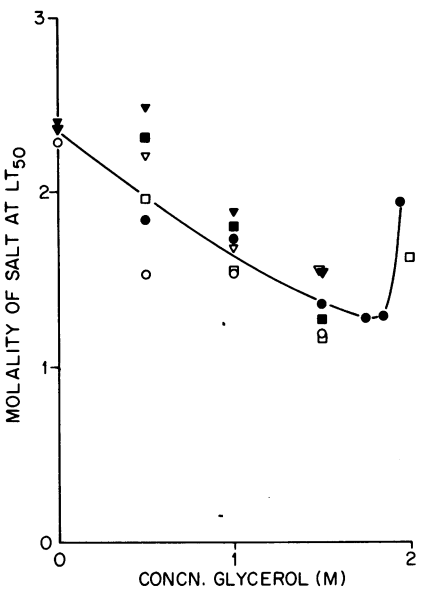

FIGURE 9

FIGURE 8 Survival of human red blood cells as a function of the maximum molal salt concentration attained during freezing to various subzero temperatures. Cells were cooled at 1.7°C/min and warmed at ~550°C/min in phosphate-buffered NaCl with nominal glycerol concentrations of 0 M (●), 0.5 M (▲), 1.0 M (○), 1.5 M (△), and 1.75 M (□).

FIGURE 9 Concentration (molality) of salt at the temperature producing 50% hemolysis of human red blood cells as a function of the concentration of glycerol present before freezing. Cells were cooled at 1.7°C/min in phosphate-buffered physiological NaCl (closed symbols) or physiological NaCl (open symbols), and warmed at rates of about 1°C/min (triangles), 150°C/min (squares) or 550°C/min (circles).

molality) against the molarity of glycerol present initially in the suspending medium for the three warming rates in both buffered saline and NaCl alone. With one minor exception, all cases show a decrease in LC_{50} as the concentration of glycerol increases to 1.85 M. At higher concentrations of glycerol the curves reverse their slope.

Human Red Cell Survival vs. the Fraction of the Solution Frozen and the Fraction of Water Frozen

Fig. 10 plots the percentage survival vs. the weight fraction of solution remaining unfrozen. As the initial concentration of glycerol is increased, hemolysis occurs when a progressively smaller fraction of the solution has been frozen. Fig. 11 is a similar plot for survival vs. the fraction of water remaining unfrozen. The trend is the same as in Fig. 10, but the effect of increasing the glycerol concentration is considerably less.

Fig. 12 A shows, for the various warming rates, the fraction of the solution that remained unfrozen at the median lethal temperature when the cells were frozen in various concentrations of glycerol. Fig. 12 B shows comparable data for the fraction of water remaining unfrozen at the LT_{50}. The latter fraction (LU_{50}) increased from 0.07 in the absence of glycerol to about 0.14 in the presence of 1.85 M glycerol. With higher concentrations of glycerol, the slope of the curve again appears to reverse.

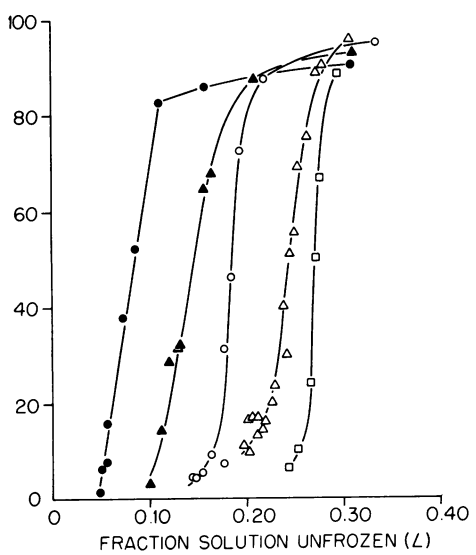

FIGURE 10

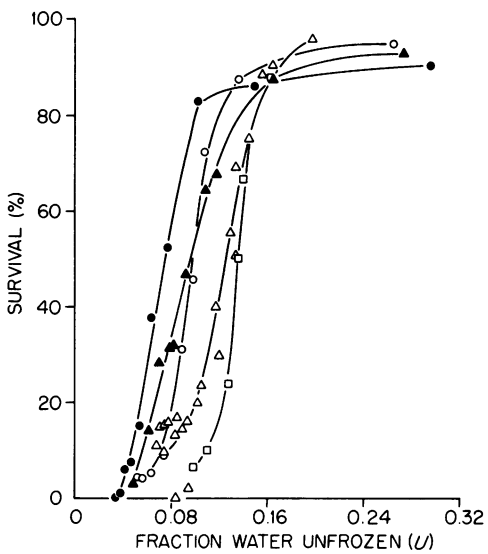

FIGURE 11

FIGURE 10 Survival of human red blood cells as a function of the fraction of solution remaining unfrozen (L) after freezing to a given subzero temperature. Cells suspended in buffered saline were cooled at 1.7°C/min and warmed at ~550°C/min. Cells were suspended in glycerol concentrations of 0 M (●), 0.5 M (▲), 1.0 M (○), 1.5 M (△), or 1.75 M (□). The results for cells frozen in glycerol-NaCl solutions are similar.

FIGURE 11 Survival of human red blood cells as a function of the fraction of water remaining unfrozen (U) after freezing to a given subzero temperature. Cells were suspended in phosphate-buffered NaCl with glycerol concentrations of 0 M (●), 0.5 M (▲), 1.0 M (○), 1.5 M (△), or 1.75 M (□). Cells were cooled at 1.7°C/min and warmed at ~550°C/min.

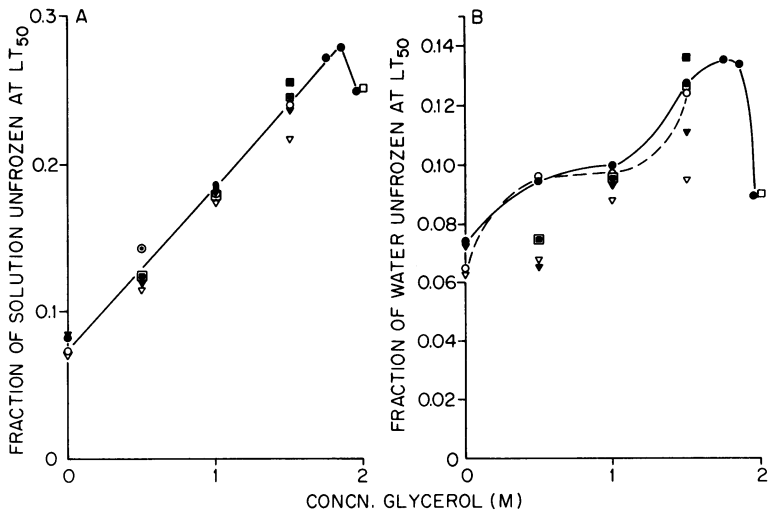

FIGURE 12 Relation between: (A) the fraction of solution remaining unfrozen (L); or (B) the fraction of water remaining unfrozen (U) at the LT_{50} and the concentration of glycerol present in the suspending solution. Cells were cooled at 1.7°C/min in physiological NaCl (open symbols) or phosphate-buffered NaCl (closed symbols) and warmed at 1°C/min (○, ●), 150°C/min (□, ■), or 550°C/min (▽, ▼).

Temperatures Corresponding to the Median Lethal Concentration

It should be reemphasized that the subzero temperatures (LT_{50}) that correspond to the LC_{50} (and LU_{50}) differ widely in the different concentrations of glycerol. The relation between LC_{50} and LT_{50} is shown in Table IV.

TABLE IV

CONCENTRATION OF SALT (LC_{50}) AT THE MEDIAN LETHAL TEMPERATURE (LT_{50}) FOR RED CELLS FROZEN AT 1.7°C/MIN IN GLYCEROL SALINE SOLUTIONS*

Concentration‡ of glycerol in unfrozen solution	Salt present			
	NaCl		Phosphate-buffered NaCl	
	LC_{50}	LT_{50}	LC_{50}	LT_{50}
M	*mol/kg*	°C	*mol/kg*	°C
0	2.28	−8	2.36	−8.5
0.5	1.53	−16	1.84	−18
1.0	1.54	−27	1.73	−27
1.5	1.19	−31	1.36	−30
1.75			1.28	−33
1.85			1.29	−35
1.95			1.94	−60

*Warmed at ~550°C/min.

‡Nominal. Exact concentrations are 3% less (see Table I).

DISCUSSION

Survival vs. Salt Concentration

Glycerol has long been known to protect cells from the deleterious effects of freezing and thawing (Smith and Polge, 1950). Its ability to protect slowly frozen human red cells is dramatic. As illustrated in Fig. 2 of the companion paper (Souzu and Mazur, 1978) and in Table IV of the present paper, the median lethal temperature drops from −8°C in the absence of glycerol to −35°C in 1.85 M glycerol, and to −60°C in 1.95 M glycerol. When the glycerol concentration is increased to ≥2 M, there is usually no LT_{50}—more than 50% of the cells survive even when frozen to −90°C or below.

Lovelock (1953*a*, *b*) carried out the first systematic investigation into the protective action of glycerol during red cell freezing and thawing. He concluded from his experiments that hemolysis is first detected when freezing concentrates the electrolyte in the solution to a mole fraction of 0.014 (0.8–1.0 mol/kg H_2O, depending on the glycerol concentration). Because of colligative properties, the higher the concentration of glycerol initially present, the lower is the temperature required to produce that mole fraction or molality. But even in the presence of glycerol, he found that the critical concentration of NaCl for the onset of hemolysis remained at about a mole fraction of 0.014. Lovelock and succeeding workers have tended to conclude from these findings that a constant critical concentration of NaCl applies over the entire range of hemolysis. Put differently, it has been assumed that plots of red cell survival vs. salt concentration at subzero temperatures are superimposable for all initial glycerol concentrations.

Although our data show that the correlation between survival and the concentration of salts produced during freezing in various glycerol solutions is closer than that between survival and temperature (e.g., compare Figs. 8 and 1), the former curves clearly do not superimpose. Although we find, in agreement with Lovelock (1953*b*) that the onset of hemolysis occurs at a salt concentration of approximately 1.0 mol/kg H_2O (Fig. 8), we also find that as the cells are cooled to progressively lower and more deleterious temperatures, the critical salt concentrations begin to differ for the various initial glycerol concentrations. The higher the initial glycerol concentration, the lower becomes the concentration of sodium chloride that produces a given percentage of survival. The divergence becomes major. For example, for cells frozen in the absence of glycerol and in 1.75 M glycerol, 50% hemolysis occurs at mole fractions of NaCl of 0.041 and 0.018 (2.36 and 1.28 mol/kg), respectively (Fig. 8), and 90% hemolysis occurs at mole fractions of 0.06 and 0.02 (3.6 and 1.6 mol/kg), respectively. The critical salt concentration thus decreases by as much as a factor of two to three as the concentration of glycerol is increased.[1]

[1] Just before the submission of the present paper, Fahy and Karow (1977) reported that full inspection of the entire range of hemolysis data given by Lovelock (1953*b*) shows a qualitatively similar effect; namely, increases in the concentration of glycerol produced decreases in the salt concentration at which given levels of hemolysis were attained.

These findings contrast with those recently reported by Meryman et al. (1977). They report that plots of red cell survival vs. the osmolal concentration of sodium chloride attained during freezing are superimposable for suspending media containing glycerol in concentrations ranging from 0 to ~2 M. When we replot our data in osmolal units (results not shown),[2] we obtain a series of curves very similar to Fig. 8; namely, a series which continues to show that an increase in glycerol concentration shifts the survival curves to lower concentrations of salt.

Whether the differences between our data and those of Meryman et al. (1977) are due to differences in the estimates of red cell survival versus temperature or to differences in the calculation of osmolal salt concentration at various temperatures is not certain, because they did not give data for the two independent sets of correlations. But we suspect that the latter is more likely. Small differences in freezing procedures appear to make little difference in the survivals after freezing. For example, as shown in Figs. 5 and 6 of Souzu and Mazur (1978), the survivals in the present study are quite similar to those obtained by Lovelock (1953*b*) and Morris and Farrant (1972), in spite of differences in the volumes frozen (0.1 vs. 1.0 ml) or in the composition of the freezing tubes (glass vs. polyethylene).

On the other hand, the use of osmolal concentration units requires several assumptions not required when concentrations are expressed in gravimetric terms (Figs. 7–8). We obtained these gravimetric values from published phase diagrams of Shepard et al. (1976), who in turn obtained their phase data by first freezing solutions to $<-100°C$ and then estimating equilibrium melting points by differential thermal analysis during warming at 5 to 10°C/min. We have assumed that the solutions also remain close to equilibrium during the slow cooling (1.7°C/min) used here in the freezing of the red cells. We know from thermocouple measurements that temperature differences throughout the sample at these cooling rates are <0.5°C. This fact means that ice crystal growth cannot lag far behind that required to maintain the solution in equilibrium. Furthermore, Lusena (1955, 1960) has shown that the rate of growth of ice crystals even in 4 M glycerol at −79°C is high enough for ice to traverse the micrometer-wide unfrozen channels in a matter of seconds. Still, some lagging and supercooling could occur, especially as the solution becomes highly viscous and even glassy at very low temperatures. But even if significant lags occur, the consequence of the resulting supercooling or glass formation would be to make the solute concentration at a given temperature *lower* than that predicted from the phase diagram. Accordingly, if major lags were occurring, one might expect that increases in glycerol concentration would shift the curves of survival vs. assumed salt concentration to the right, since the cells would actually be exposed to lower salt concentrations than those calculated from the phase diagrams. The experimental results were the opposite.

Another conceivable source of error could be the presence of red cells in our solu-

[2]Space did not permit publishing these plots and others such as survival vs. mole fraction, but we can furnish them to interested readers.

tions. But they were too few (hematocrit ~2%) to have influenced the phase relations significantly. Their contribution to the total solutes in the system is unlikely to be any more than the contribution of the many minor solutes in balanced salt solutions like MEM; Pozner et al. (1977) have shown that the introduction of MEM solutes into glycerol-NaCl-water solutions does not detectably alter the phase relations at the R values of concern here.

Damage Associated with the Presence of Glycerol

We observed a decrease in the critical salt concentration with increasing concentration of glycerol. To what may the decrease be ascribed? The simplest interpretation is that glycerol has two effects on the red cells, one beneficial and one deleterious. The beneficial effect is probably, as Lovelock proposed, its colligative ability to suppress the concentration of sodium chloride at a given temperature (Figs. 3 and 5). But this beneficial effect is partly counteracted by deleterious factors that cause overall survival after freezing to a given temperature to be somewhat lower than what would be predicted if the sole action of glycerol were the colligative suppression of electrolyte concentration. What might these deleterious factors be?

One of them may be time. We see from Table IV that the higher the initial glycerol concentration, the lower the LT_{50}. Since the cells were cooled at a nearly constant rate (Miller and Mazur, 1976), lower final temperatures meant longer exposure times to unfrozen solution. For example, cells in saline alone required about 4 min to reach their LT_{50} (−8°C), whereas cells in 1.85 M glycerol required about 20 min to reach their LT_{50} (−35°C). Although the longer exposure time could conceivably increase the damaging effects of given salt concentrations, the system is unfortunately too complex to assess the role of time definitively. For instance, opposing the potential detrimental effects of longer exposure would be the protective effect arising from the fact that a given salt concentration is not produced until a lower temperature is reached (Lovelock, 1953*b*). The time effects at the two extremes, cells in saline alone vs. cells in saline with 2 M glycerol, are fairly clear. The former tend to be susceptible to time (Lovelock, 1953*a*); the latter are almost totally insensitive to time (Miller and Mazur, 1976).

One might think that the role of time could be assessed by comparing the effects of two cooling rates to given temperatures with and without subsequent holding periods. This comparison was made on cells in 2 M glycerol for cooling rates of 0.5 and 1.7°C/min in the companion paper (Souzu and Mazur, 1978, Table II). The lower rate was slightly more harmful at every temperature, but this has been shown to be due not to longer exposure time but to the fact that slower cooling makes red cells in 2 M glycerol increasingly susceptible to rapid thawing (Miller and Mazur, 1976). Indeed, for cells in 2 M glycerol, the longer exposure times associated with slower warming produce less injury in slowly cooled red cells than that produced during the short exposure in rapid thawing (Figs. 4 B and Table I of the preceding paper). Similarly, we see from Fig. 9 of the present study that slowing the warming rate from 550° to 1°C/min decreases the sensitivity of the cells to salt even at concentrations of glycerol below 2 M.

Miller and Mazur (1976) have hypothesized that injury from rapid thawing is the result of osmotic shock. The osmotic shock would occur when excess intracellular glycerol is unable to leave the cells rapidly enough during warming to prevent the cells from swelling excessively.

Our conclusion, then, is that the decrease in the numerical value of the salt concentration associated with increasing glycerol concentration results from (*a*) the lower temperatures and consequent longer exposure times required to reach given salt concentrations, and (*b*) the increased susceptibility to osmotic shock during warming that results from the presence of excess intracellular glycerol. We do not know the relative contributions of these two factors, but suspect that the latter is the more important. Experiments could be designed to help resolve the situation, but they will not be simple.

The conclusion that the overall protection by glycerol is less than that ascribable to its ability to suppress electrolyte concentration differs from that of most previous investigators. Lovelock (1953*b*) and Meryman et al. (1977) concluded that protection by glycerol is completely accounted for by its colligative suppression of electrolyte concentration. Others have suggested that additives confer additional protection beyond that ascribable to their colligative effects (Mironescu, 1977; Steponkus et al., 1977). Fahy and Karow (1977), however, have reached conclusions for rat hearts similar to ours for human red cells; namely, the presence of protective additive (dimethyl sulfoxide in their study) leads to freezing damage beyond that ascribable to the concentration of electrolytes.

Responses to Glycerol Concentrations above 1.95 M Glycerol

The decrease in the LC_{50} with increasing initial concentration of glycerol ceases at a glycerol concentration of 1.85 M, reverses at 1.95 M, and becomes immeasurable at 2 M and above (Fig. 9). The reversal in LC_{50} is correlated with the fact that the LT_{50} drops abruptly at glycerol concentrations above 1.85 M (Table IV, and Souzu and Mazur, 1978, Fig. 2). In fact, in 2 M glycerol and above there usually is no LT_{50}. Thus, for cells in 2 M glycerol in buffered saline, survival drops slowly and progressively from 95% at −30°C to 70% at −80°C (Fig. 1), and over this same temperature range the salt concentration rises slowly from 1 to perhaps 2 mol/kg (Fig. 5). Actually, much of the drop in survival in 2 M glycerol occurs below the temperature (−46°C) for which phase data are available. As a result, in the plots of survival vs. salt concentration, the curves for 2 M glycerol are truncated, and the significance of their precise position relative to the curves for lower concentrations of glycerol becomes uncertain. Presumably, though, the protection occurs, because when the concentration of glycerol is ≥ 2 M, the electrolyte concentration reaches potentially dangerous levels only at temperatures low enough to prevent them from being especially damaging.

Salt Concentration vs. the Fraction of Solution Frozen

So far we have discussed the relation between survival and the concentration of salts in the residual unfrozen medium. But since the progressive concentration of salts is a

result of the progressive freezing of the water in the solution, there are also corresponding correlations between survival and the fractions of solution and of water remaining unfrozen. The correlation is best between survival and the fraction of unfrozen water (Fig. 11). As shown in Figs. 11 and 12 B, survival drops to 50% when that fraction drops to between 0.07 and 0.13. The fraction of extracellular water that the cells tolerate being frozen decreases with both increasing glycerol concentration and increasing warming rate.

Since salt concentration and the fraction frozen are intrinsically coupled, we cannot determine from the facts so far presented which has more influence on cell survival. The value of ~ 10% unfrozen water corresponds closely to the value of 10% "bound" or unfreezable water observed in yeast and *Escherichia coli* (Wood and Rosenberg, 1957; Souzu et al., 1961); hence it might seem attractive to speculate that it is significant to red cells. The chemical potentials of intracellular and extracellular water ought to remain close to equilibrium at the low cooling rates used here (Mazur, 1963). Accordingly, one might argue that when freezing of the external solution has progressed to the point where only 10% of the water remains unfrozen, it has reached the point where all the freezable water in the red cell is frozen, and that further cooling and extracellular freezing then begin to influence the bound water fraction of the cell.

The problem with this speculation is twofold. First, the question of the existence of bound water in the red cell is uncertain. Some measures indicate it is present; others indicate it is not (see Cook, 1967; Sacks et al., 1975). But second, and even more important, if the critical factor were the fraction unfrozen and not the concentration of salts, then survival should depend on the total initial osmolality of the suspending medium rather than on its composition. (The higher that initial osmolality, the greater would be the fraction unfrozen at a given temperature, and the lower would be the temperature required to freeze a given fraction [Eqs. 4 and 7].) Limited data do not support this view. Miller and Mazur (1976) and Mazur and Miller (1976*b*) compared the survivals after freezing and thawing of human red cells suspended in three equiosmolal solutions: 2 M glycerol, 1.40 M sucrose, and 1.23 M NaCl, all made up in isotonic saline. The cells in glycerol were frozen after minimum or maximum permeation by glycerol. (The sucrose and NaCl do not permeate unfrozen red cells at significant rates.) The survivals of cells frozen in sucrose and glycerol were 65–94%, depending in part on the cooling and warming rates used. The survival of cells frozen in an equiosmolal concentration of NaCl was 0–5% regardless of the cooling rate used. Data from Lovelock (1953*b*, Fig. 2 [Note: Symbols in figure legend inadvertently reversed.]) provide additional evidence that red cell survival after freezing in equiosmolal solutions is not equal, but depends on the ratio of nonelectrolyte to sodium chloride initially present in the solution. A solution of 2 M glycerol in 0.08 M NaCl and a solution of 1 M glycerol in 0.6 M NaCl have comparable osmolalities (about 2.5 and 2.2, respectively); yet, after freezing cells to −30°C, Lovelock observed survivals of >90% and <10%, respectively.

In summary, we draw the following conclusions:

We concur with Lovelock (1953*a*, *b*) that injury in slowly frozen, unprotected

human red cells is associated with the concentration of salts produced by freezing and their dilution during thawing. Specifically, the injury appears to be a consequence of the increase in the concentration of sodium chloride in the external medium. Our data suggest that injury is not to any major extent a consequence of the freezing of a critical fraction of the external medium or of the cell's water. Other work (Mazur et al., 1974*a*, *b*; Mazur and Miller, 1976*a*, *b*) suggests that injury to unprotected red cells is not, contrary to Lovelock's view, primarily a consequence of the increase in the concentration of intracellular salts and is not, as Meryman has proposed (Meryman, 1974; Meryman et al., 1977), primarily a consequence of the cell's dehydrating to a critical volume. The basis of these last two conclusions is that hyperosmotic glycerol and sucrose can partially or fully protect human and bovine red cells in the complete or near absence of permeation. In the near absence of permeation, the protection is occurring even though the cells are extensively dehydrated.

Correlations between killing and salt concentration are best when concentration is expressed in terms of the water available for solution (i.e., molality). Perhaps this means that the damage from sodium chloride arises either from its effects on the structure or properties of water or its effects on the conformation and stability of the hydrophilic portions of the plasma membrane.

The protection conferred by glycerol results chiefly from its ability to suppress the sodium chloride concentration in the external medium during freezing. To a minor extent in human red cells (Mazur and Miller, 1976*b*), and to a greater extent in other cells (Jackowski and Leibo, 1976; Rajotte and Mazur, 1977); the protection may also be due to the suppression of the concentration of intracellular salts by intracellular glycerol.

Although it protects against salt damage by colligatively suppressing the concentration of salts, glycerol also introduces other potentially injurious consequences (Leibo, 1976). One of these is that it appears to make red cells susceptible to osmotic shock during thawing.

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