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Survival of Frozen-Thawed Bovine Red Cells as a Function of the Permeation of Glycerol and Sucrose*

Peter Mazur, R. H. Miller, and S. P. Leibo

Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37830

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Summary. Bovine red cells, like other cells, exhibit maximum survival when frozen at certain optimum rates. Cells cooled more slowly are apparently injured by alterations in the cytoplasm or surrounding medium such as the increased concentration of solutes induced by extracellular ice formation. Additives like glycerol protect against this "slow" freezing injury. It has been generally believed that such protection requires permeation by the additive, but we have found that this supposition is not valid for the bovine red cell.

Cells were suspended in 1, 2 or 3 M glycerol at 20, 15 or 0 °C for 0.7 to 30 min or more and then frozen to −196 °C at 43 or 1.7 °C/min. In nearly all cases, the percentage survival after thawing was as high for cells held in glycerol for 1 min or less prior to freezing as for cells held in glycerol for 30 min, and it was as high for cells held at 0 °C as for cells held at 20 °C. Survivals were the same for these times and temperatures of exposure in spite of the fact that the osmolal ratio of glycerol to salts in the cell after 30 min at 20 °C, for example, was as much as 800 times greater than that in cells held at 0 °C for 0.7 min. In addition, the survival after a contact of 1 or 30 min with 2.3 osmolal sucrose was the same as that after exposure to 2.3 osmolal glycerol even though the bovine red cell is impermeable to sucrose.

Although exposures of 1 and 30 min to glycerol yielded similar survivals, exposures for intermediate times produced a transitory but dramatic decrease in survival. The dip occurred after longer periods of incubation when the concentration of glycerol was increased and when the incubation temperature was decreased. No dip was evident in cells chilled to 0 °C or in cells frozen in sucrose. Thus, the dip seems to be associated in some way with partial permeation of glycerol prior to freezing.

Two major factors appear to be responsible for freezing injury of cells. Cells cooled at high rates are killed by the formation of intracellular ice during cooling and by its growth during slow warming. Cells cooled at low rates are injured by alterations of the intra- and extracellular solutions brought about by ice formation – alterations such as increased solute con-

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centration and decreased water content (Mazur, 1970). The interaction of these two factors produces a maximum in survival at an intermediate optimum rate, a rate high enough to minimize solution effects but low enough to minimize the probability of intracellular freezing.

There are two current major hypotheses of the solution effect injury that accompanies slow freezing: (1) It is due to the concentration of electrolytes produced by freezing (Lovelock, 1953*a*, *b*). (2) It is due to stresses in cell membranes produced by cell dehydration (Meryman, 1968). The theories ascribe the protection by compounds like glycerol to their colligative ability to reduce electrolyte concentration or to reduce the extent of dehydration and freezing of cell water at any temperature (Appendixes A, B). Both theories require that the additives permeate the cell so as to be able to exert their colligative effects intracellularly.

The chief evidence favoring the requirement for permeation of additives is Lovelock's (1953*b*) observation that the survival of human red cells frozen under conditions where little intracellular glycerol is present is lower (67%) than that of cells frozen after equilibration with glycerol (97%). [His experiments were performed in media containing Cu^{++}, an ion that greatly slows glycerol permeation in human red cells (Jacobs, Glassman & Parpart, 1935).]

Nevertheless, survival was still appreciably greater than when no glycerol was present in the medium (3%). Moreover, other evidence is accumulating for other mammalian cells which indicates that permeation of additive may not be necessary, at least for partial protection (Sherman, 1963; Persidsky, Richards & Leef, 1965; Mazur, Farrant, Leibo & Chu, 1969; Mazur, Leibo, Farrant, Chu, Hanna & Smith, 1970; Leibo, Farrant, Mazur, Hanna & Smith, 1970; Morris & Farrant, 1972; Whittingham, Leibo & Mazur, 1972).

The question of the relation between permeation by additives and their ability to protect against freezing injury is a mater of both theoretical and pragmatic importance. Theoretically, if protection can occur without permeation, the cell surface may be the chief target of freezing injury. Practically, if additives protect at the cell surface, then freezing of large complex systems such as organs might be simplified.

The present experiments were designed to determine the relation between the ability of the bovine red cell to survive freezing and thawing and the amount of glycerol in the cell at the onset of freezing. The general procedure was to expose the cells to glycerol solutions for various times at various temperatures before freezing them at controlled suboptimal rates. [Suboptimal rates were used to reduce the possibility of the results being confounded by the formation of intracellular ice (*see* Results).] Cell survival

after thawing was then compared with the internal concentration of glycerol just before freezing, which was estimated by the procedures described in the preceding paper (Mazur, Leibo & Miller, 1974). The results show that protection from freezing injury does not require additive in the cell at the onset of freezing. They also demonstrate the existence of another phenomenon, a transient dip in the survival of cells exposed to glycerol for intermediate lengths of time before freezing.

Materials and Methods

Preparing Suspensions of Blood Cells

The methods for the collection of blood, determination of osmotic fragility and hematocrits, and preparation of suspensions are described in the companion paper (Mazur *et al.*, 1974). For each experiment a 5-ml sample of whole blood was washed by three centrifugations at $1{,}400 \times g$ for 10 min at room temperature and resuspended in 5 ml of 0.301 osmolal phosphate-buffered saline at pH 7.

Preparing Samples for Freezing

The washed cells were thermally equilibrated at 0, 15 or 20 °C, and a 0.5-ml sample was mixed with 9.5 ml of isotonic saline buffer or 1, 2 or 3 M glycerol or 1.4 M sucrose in isotonic saline buffer at those temperatures. Then, after times ranging from a few seconds to more than 2 hr 0.1-ml quantities of the stirred suspension were transferred to temperature-equilibrated freezing tubes (7 × 90 mm Pyrex), which in turn were transferred to an ice bath or to the desired freezing bath 1 to 5 min later. A somewhat modified procedure was used when the time in additive prior to freezing was to be less than 1 min. Washed cells were diluted 1:10 with saline buffer, and 0.05 ml was placed in a freezing tube. At time zero, 0.05 ml of saline buffer containing twice the desired concentration of additive was added to the tube, which was immediately mixed and transferred to the freezing bath.

Procedures for Freezing

Samples held in glycerol for 1 min or longer and frozen at 50 °C/min or less were transferred to an ice bath for 10 sec and then to the freezing bath. Samples to be frozen at higher rates were transferred directly from the equilibrating bath to the freezing bath. Procedures for achieving various freezing rates are detailed elsewhere (Leibo *et al.*, 1970). In brief, they were as follows:

(a) 1.7 °C/min. 500 ml of ethanol were prechilled to −12 °C and transferred to an 85 × 280 mm unevacuated, unsilvered Dewar. When the stirred ethanol had warmed to about −3 °C, the freezing tubes were transferred into it and the Dewar blank was placed in liquid nitrogen.

(b) ~10 °C/min. An 85 × 280 mm unevacuated, unsilvered Dewar with a cork support in the bottom was placed in liquid nitrogen, and 5 min later, when the temperature within was about −15 °C, the freezing tubes were transferred into it.

(c) 43 °C/min. A 50×325 mm Pyrex tube was placed in liquid nitrogen, and 30 sec later, when its temperature was about −50 °C, the freezing tubes were lowered into it. When the temperature of the freezing tubes in this and in the preceding procedures dropped below −100 °C, they were transferred to liquid nitrogen.

(d) 200 °C/min. The freezing tubes were immersed directly in a dry ice-ethanol bath at −78 °C, held a few minutes, and transferred to liquid nitrogen.

(e) 650 °C/min. The freezing tubes were immersed directly in liquid nitrogen.

In all cases the frozen sample tubes were held in liquid nitrogen for an hour or so and then thawed rapidly by immersion and agitation for 45 sec in a water bath at 35 °C. The warming rate under these conditions is about 750 °C/min.

Controls

Controls consisted of cells that were placed in additive solution and treated like the experimentals, except that they were held at the equilibration temperature for the duration of the experiment.

Times, Temperatures and Rates

The time that the cells were in additive prior to freezing was defined as the time between the first addition of additive and the transfer of the freezing tubes to the freezing bath. The temperatures during this time (except for the 10 sec in the ice bath) were held to ±0.05 °C, and the times were measured to 0.1 min. Temperatures during freezing were determined for representative samples by thermocouple, and the cooling rates are based on the time required for the temperature to drop from −10 to −65 °C.

Determining Percent Hemolysis

The thawed contents of each freezing tube and the contents of unfrozen control tubes were transferred by Pasteur pipette into 0.9 ml of the same medium in which they were frozen, and the diluted samples were centrifuged at 8,000×g at room temperature for 10 min. Then 0.5 ml of the supernatant was removed by suction and mixed with an equal volume of Drabkin's solution (Wintrobe, 1967). The concentration of hemoglobin in the supernatant was determined colorimetrically as described in the preceding paper (Mazur *et al.*, 1974), and the percent hemolysis was calculated as $100\,A_{exp}/A_{\text{total hemolysis control}}$.

Because the percentages of the total sample hemoglobin released to the supernatant agree closely with the percentages of individual cells that undergo hemolysis, we shall refer to the former values as "percent hemolysis" and to 100% minus the values as "percent unhemolyzed" or "percent survival" (Mazur *et al.*, 1974).

Calculation of Concentrations

The compositions of the various stock glycerol solutions are shown in the preceding paper (Mazur *et al.*, 1974, Table 1). The 1.4 M sucrose (2.40 osmolal, isosmotic with 2 M glycerol) contained sucrose, 1.404 M (2.017 molal); buffer, 0.007 M (0.010 molal); NaCl, 0.104 M (0.149 molal); and water, 0.696 ml/ml of solution.

The concentrations of the experimental mixtures were determined from the known compositions and volumes of the stock solutions and from the hematocrit of the washed

blood. Osmolalities are defined as $\varphi \nu m$, where φ is the osmotic coefficient, ν is the number of species into which the solute dissociates, and m is the molality. Values for φ, ν, and m were obtained as previously described (Mazur *et al.*, 1974).

Results

Concentration of Glycerol in Cells Prior to Freezing

The procedures for estimating the concentration of glycerol in bovine red cells as a function of temperature and exposure time prior to freezing were the subject of the preceding paper (Mazur *et al.*, 1974). The osmolal concentration of intracellular glycerol at time t is $1000\,\varphi_s^i(t)\,s(t)/V(t)$, the symbols being defined in Table 1.

The values of $\varphi_s^i(t)$, $s(t)$, and $V(t)$ were calculated by an iterative solution to four equations:

$$V(t)=[M_{\text{iso}}^i+1000\,\varphi_s^i(t)\,s(t)]/(M_n^e+\varphi_s^e\,m_s^e), \tag{1}$$

Table 1. Definitions of major symbols

Symbol	Description	Units	Value
P_γ	Permeability constant for glycerol	cm/min	parameter
$\varphi_s^i(t)$	Osmotic coefficient of internal glycerol		variable
$\gamma_s^i(t)$	Activity coefficient of internal glycerol		variable
$s(t)$	Moles of glycerol in cell at time t	moles	variable
$V(t)$	Volume of water in cell at time t relative to the volume in an isotonic cell	— or cm^3 [a]	variable
M_{iso}^i	Osmolality of nonpermeating solutes in isotonic cells	osmoles/$liter_{H_2O}$ [b]	0.301
φ_s^e	Osmotic coefficient of external glycerol		parameter
γ_s^e	Activity coefficient of external glycerol		parameter
m_s^e	Molality of external glycerol	moles/$liter_{H_2O}$	parameter
M_n^e	Osmolality of nonpermeating salts in external medium	osmoles/$liter_{H_2O}$	parameter
M_T^e	Total external osmolality	osmoles/$liter_{H_2O}$	parameter
t	Time in contact with glycerol	min	variable
Δt	Small increment in time	min	parameter
A	Total surface area of the number of isotonic cells required to contain 1 cm^3 H_2O	cm^2	2.25×10^4 [a]

[a] *See* Table 2 and the accompanying text in the preceding paper (Mazur *et al.*, 1974).
[b] 1 liter H_2O assumed = 1 kg H_2O.

Table 2. Parameters for calculating osmolality of intracellular glycerol and osmolal ratio of glycerol to other intracellular solutes

Nominal conc. external glycerol [a] (M)	Temp. (°C)	P_γ		$\gamma_s^e m_s^e$	$\varphi_s^e m_s^e$ (osmolal)	M_n^e (osmolal)	M_T^e (osmolal)
		Osmotic shock (cm/min × 10^6)	Time to hemolysis				
1	20	2.22	1.58	1.072	1.059	0.302	1.361
	15	1.18	0.83	1.074	1.060	0.302	1.362
	0	0.19	0.12	1.074	1.060	0.302	1.362
2	20	2.29	2.24	2.374	2.312	0.302	2.614
	15	1.19	1.14	2.376	2.312	0.302	2.614
	0	0.17	–	2.375	2.312	0.302	2.614
3	20	2.71	2.31	3.992	3.816	0.302	4.118
	15	1.42	1.17	3.993	3.817	0.302	4.119

[a] Concentration of glycerol added to cells. The concentrations in the suspensions were 0.97, 1.93 and 2.90 M.

$$s(t+\Delta t)=[V(t)\gamma_s^e m_s^e/1000\gamma_s^i(t)] - [V(t)/\gamma_s^i(t)][\gamma_s^e m_s^e/1000)-\gamma_s^i(t)s(t)/V(t)] \cdot \exp[-\gamma_s^i(t)P_\gamma A\Delta t/V(t)], \quad (2)$$

$$\varphi_s^i(t+\Delta t)=1.00+9.75\, s(t+\Delta t)/V(t), \quad (3)$$

$$\gamma_s^i(t)=1.00+22.3\, s(t)/V(t). \quad (4)$$

P_γ, the permeability coefficient of the bovine cell for glycerol, was estimated by the two procedures described in the preceding paper (Mazur *et al.*, 1974), which involved (a) determining the time for cells suspended in solutions of glycerol plus hypotonic NaCl to undergo hypotonic hemolysis and (b) determining the time for cells in solutions of glycerol plus isotonic NaCl to become susceptible to osmotic shock. The values for P_γ for cells in 1, 2 and 3 M glycerol at 0, 15 and 20 °C are given in Table 2 along with the values for the parameters $\gamma_s^e m_s^e$, $\varphi_s^e m_s^e$, M_n^e, and the total external osmolality M_T^e. The kinetics of permeation of glycerol into cells in 1, 2 and 3 M glycerol at 0, 15 and 20 °C are given in Fig. 1 *A* – *C*.

At 20 °C, the intracellular concentration of glycerol reaches 78 to 89% of the equilibrium (i.e. external) concentration by 30 min, and it is 97% of the equilibrium value after 150 min. However, glycerol permeation in the bovine red cell is strongly temperature-dependent (activation energy 21 kcal/mole), so at 0 °C the concentrations of intracellular glycerol in the first 30 min

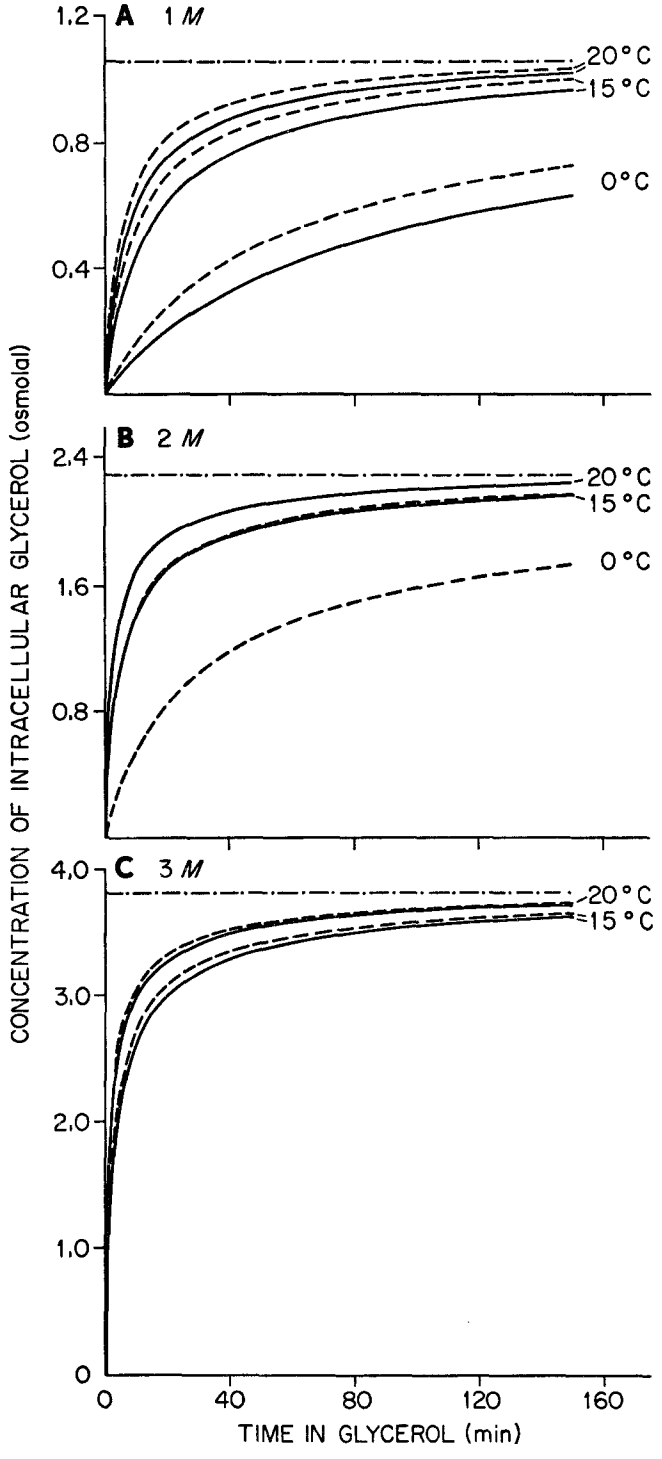

Fig. 1. Kinetics of permeation of glycerol into bovine red cells suspended in isotonic buffered saline, using values of P_g calculated from osmotic shock data (----) or from time to hemolysis data (—). (In 2 M glycerol at 20 °C, the two kinetic curves are indistinguishable.) (*A*) 1 M glycerol, (*B*) 2 M glycerol, (*C*) 3 M glycerol. Concentration of extracellular glycerol (· — · — ·)

are less than half of those at 20 °C. Although the estimates of the concentration of glycerol in the cell are affected to only a minor extent by the set of P_γ values chosen, the values calculated from the osmotic shock procedure were generally higher; and to make our interpretations conservative they will be the ones referred to in the text. Furthermore, the osmotic procedure more closely mimics the freezing conditions in that the cells are suspended in solutions of glycerol in isosmotic saline (as opposed to hypotonic) and in that the end point on which P_γ is based (hemolysis upon dilution) occurs in the early stages of permeation.

For the concentrations of glycerol used in this study the calculations indicate that by appropriate selection of the time and temperature of exposure, the amount of glycerol in the cell prior to freezing can be varied by a factor of about 200, the minimum applying to cells at 0 °C for some 9 sec and the maximum to cells at 20 °C for 30 min or longer.

Effect of Cooling Velocity on Survival of Bovine Red Cells Equilibrated with Glycerol

As mentioned in the Introduction, our concern was to determine the relation between the presence of glycerol in the red cell and the ability of the cell to survive the solution-effect injury associated with slow freezing. This meant that the cells had to be frozen at rates low enough to preclude the formation of intracellular ice; i.e., they had to be frozen at suboptimal rates. Fig. 2 shows the relation between survival and cooling rates for cells equilibrated at 20 °C in buffered saline containing 0, 1, 2 or 3 M glycerol for 145 min before freezing to −196 °C. [By 145 min the concentration of intracellular and extracellular glycerol are nearly equal (*see* Fig. 1).] The optimum cooling rate was about 200 °C/min. Survival was lower at lower cooling velocities, and for a given cooling rate it was higher with higher concentrations of glycerol in the extracellular medium. The dependence of survival on cooling rate and on the concentration of glycerol is closely comparable to that obtained by Morris and Farrant (1972) for human red cells.

The cooling rate selected for the subsequent studies on the relation between survival and glycerol permeation had to meet two requirements: (a) as mentioned above, it had to be lower than optimum to minimize the possible contribution of intracellular ice, and (b) it had to be high enough to minimize further permeation of glycerol during the actual freezing. The rate selected for most experiments was 43 °C/min. There is direct evidence

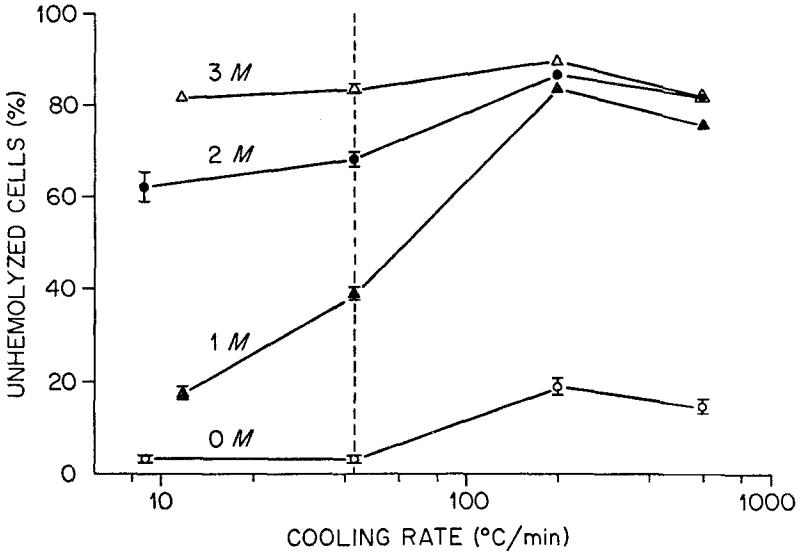

Fig. 2. Survival of bovine red cells frozen to −196 °C at various rates after being suspended in saline-buffer containing 0, 1, 2 or 3 M glycerol for 145 min at 20 °C. The survival of unfrozen controls was 100, 100, 94 and 97% for cells in 0, 1, 2 and 3 M glycerol, respectively. The vertical dashed line indicates the cooling rate (43 °C/min) selected for subsequent experiments. Error bars in this and succeeding figures are standard errors. They have been omitted in crowded regions of the graph and in cases where the standard error is smaller than the symbol

from electron-microscopy studies by Rapatz, Menz and Luyet (1966) and Nei, Kojima and Hanafusa (1964) that intracellular ice does not form in red cells cooled at this rate (Mazur, 1968).

Survival of Bovine Cells as a Function of the Ratio of Glycerol to Other Solutes in the Cell Prior to Freezing

When cells are placed in glycerol they immediately shrink, because water leaves about a million times more rapidly than the glycerol can enter. The loss of water causes a corresponding increase in the concentration of nonpermeating solutes in the cells (mostly ions). But as the glycerol diffuses into the cell, it is accompanied by the inflow of water and, therefore, by a decrease in the concentration of intracellular nonpermeating solutes. Since the total osmolality in the cell at any time is constant and equal to the external osmolality (Mazur *et al.*, 1974), the osmolal concentration of intracellular nonpermeating solutes is simply the total osmolality (Table 2, last column) minus the osmolality of intracellular glycerol at that instant. Thus the curve for the osmolal concentration of intracellular nonpermeating

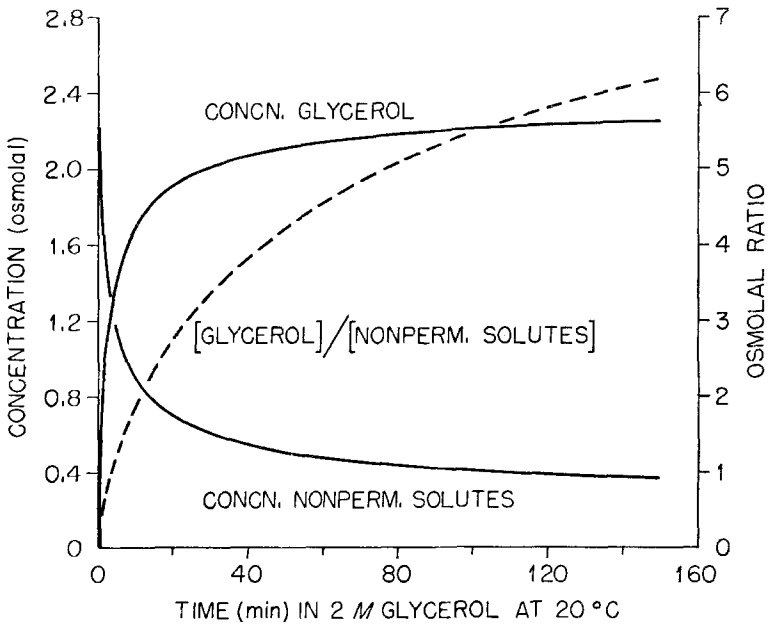

Fig. 3. Changes in the osmolal concentrations of intracellular glycerol and intracellular nonpermeating solutes and in the osmolal ratio of glycerol to nonpermeating solutes as a function of time in 2 M glycerol at 20 °C

solutes (Fig. 3) is the mirror image of the curves in Fig. 1. At $t = 0$, the concentration is high and equal to the total osmolality, but with increasing time it quickly decreases.

Lovelock (1953*b*) showed unequivocally that the protection of human red blood cells that accompanies high concentrations of glycerol is not due to a high concentration of glycerol *per se*, but to the fact that high concentrations of glycerol reduce the concentration of salts during freezing, and reduce the fraction of cell water frozen (Meryman, 1971). As shown in Appendixes A and B, these two phenomena depend not on the concentration of glycerol in the cell but on the osmolal ratio of glycerol to nonpermeating solutes in the cell at the onset of freezing. In reporting the data, therefore, we will show the relation of the ratio to survival after freezing and thawing.

Fig. 4*A* shows the survival of red cells that were frozen and thawed after contact with 2 M glycerol for 0.15 to 120 min. Fig. 4*B* shows the calculated osmolal ratios over the same time span. Three conclusions are evident:

(1) Regardless of equilibration temperature, survivals after 0.15 to 1 min in 2 M glycerol prior to freezing were as high as survivals after 30 min.

(2) Survival after 0.2 min in 2 M glycerol at 0 °C was as high as survival after 30 min at 20 °C, even though the corresponding osmolal ratios differed

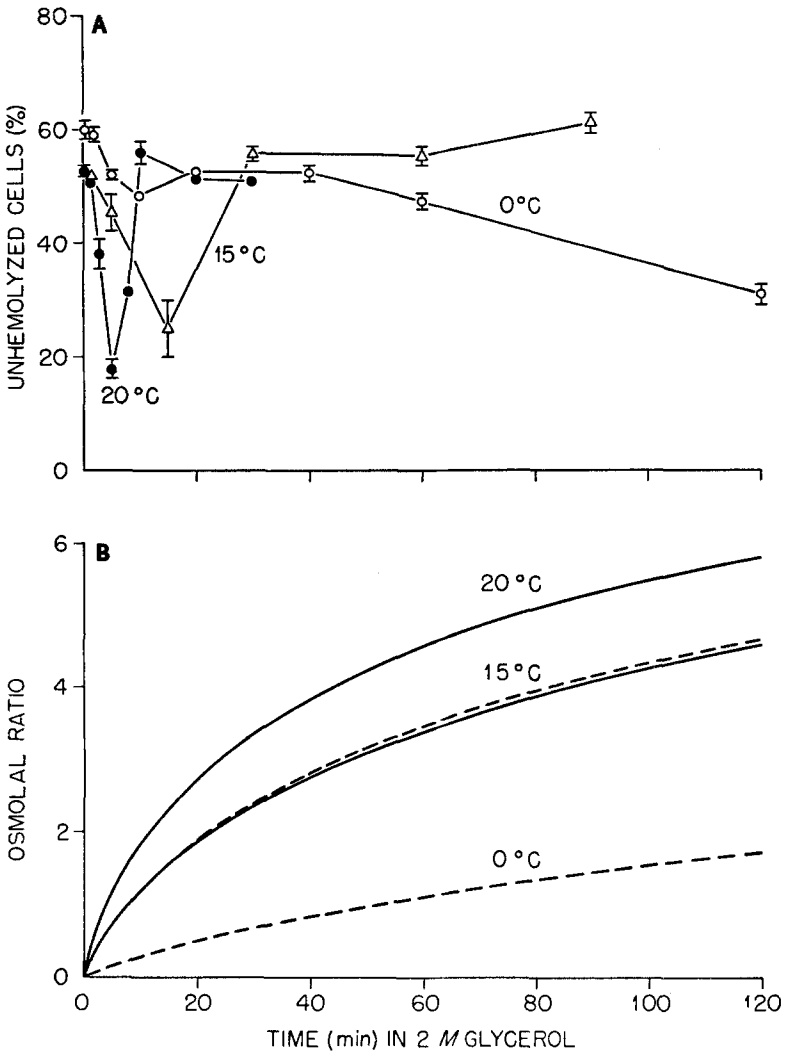

Fig. 4. Bovine red cells in 2 M glycerol. A comparison between survival after freezing to −196 °C at 43 °C/min and the intracellular osmolal ratio of glycerol to nonpermeating solutes at the onset of freezing. (*A*) Survival after freezing *vs.* time in 2 M glycerol at various temperatures. (The survivals of unfrozen controls at 0, 15 and 20 °C were 92, 97 and 94%, respectively.) (*B*) Intracellular osmolal ratio of glycerol to nonpermeating solutes *vs.* time in glycerol. P_γ was calculated from osmotic shock data (----) or from time to hemolysis data (—). (At 20 °C, the two curves are indistinguishable)

by a factor of 560 (0.0061 *vs.* 3.36), and the corresponding osmolal concentrations of intracellular glycerol differed by a factor of 125 (0.016 *vs.* 2.01). These findings also apply to cells cooled more slowly than 43 °C/min. Although cells cooled at 1.7 °C/min showed lower survivals than cells cooled

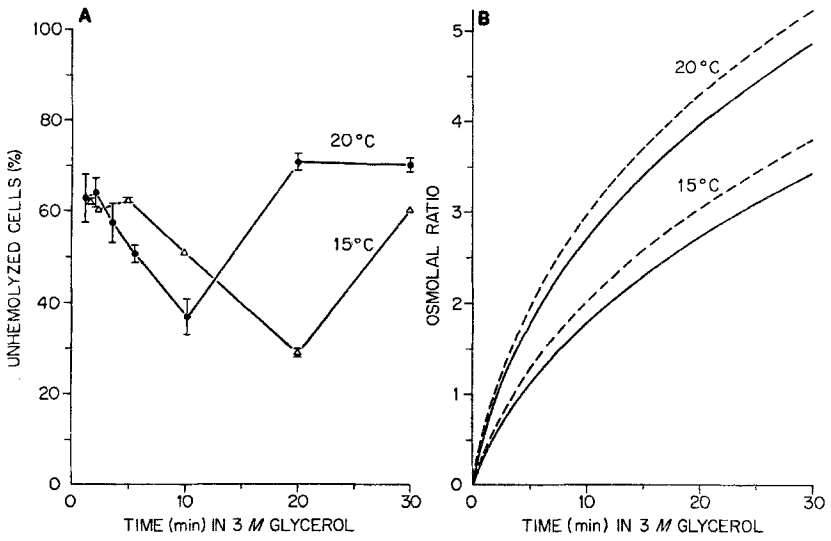

Fig. 5. Bovine red cells in 3 M glycerol. A comparison between survival after freezing to −196 °C at 43 °C/min and the intracellular osmolal ratio of glycerol to nonpermeating solutes at the onset of freezing. (*A*) Survival after freezing *vs.* time in 3 M glycerol at various temperatures. (The survivals of unfrozen controls at 15 and 20 °C were 92 and 97%, respectively.) (*B*) Intracellular osmolal ratio of glycerol to nonpermeating solutes *vs.* time in glycerol. P_γ was calculated from osmotic shock data (----) or from time to hemolysis data (—)

at higher rates (as expected from the results in Fig. 2), survival after exposure to 2 M glycerol for 1.5 min at 0 °C was about the same as that after 145 min at 20 °C (38.0% *vs.* 41.0%).

(3) A transient but marked dip in survival after freezing and thawing appeared with intermediate times in 2 M glycerol prior to freezing. The lower the temperature, the later the dip. Thus, at 20, 15 and 0 °C, the minimum in survival occurred at 5, 15 and 120 min or later, respectively.

The results for cells in 3 M glycerol were similar (Fig. 5). Again, survivals after 1.2 min in glycerol were essentially as high as after 30 min, and survivals were as high at 15 °C as at 20 °C. This was true in spite of the fact that the osmolal ratios in cells held at 20 °C for 30 min were about 10 times higher than those in cells held at 15 °C for 1.7 min (5.2 *vs.* 0.56) (Fig. 5*B*). Again there was a pronounced dip in survival at intermediate holding time, and again the dip occurred later at lower temperatures.

The results for cells in 1 M glycerol (Fig. 6) are similar to the findings with higher concentrations in that survival after 0.17 min at 0 °C was as high as after 30 min at 20 °C, even though the osmolal ratios differed by a factor of 850 (0.0022 *vs.* 1.85), and the intracellular concentrations of

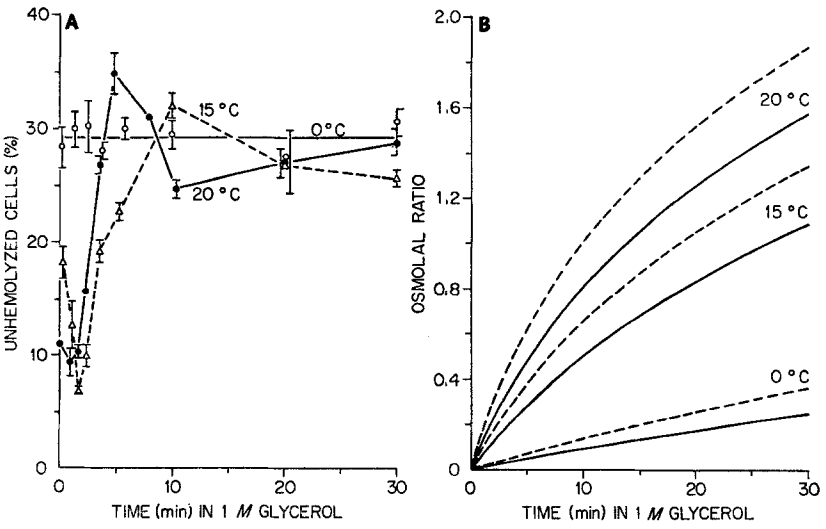

Fig. 6. Bovine red cells in 1 M glycerol. A comparison between survival after freezing to −196 °C at 43 °C/min and the intracellular osmolal ratio of glycerol to nonpermeating solutes at the onset of freezing. (*A*) Survival after freezing *vs.* time in 1 M glycerol at various temperatures. (The survivals of unfrozen controls at 0, 15 and 20 °C were 93, 94 and 96%, respectively.) (*B*) Intracellular osmolal ratio of glycerol to nonpermeating solutes *vs.* time in glycerol. P_γ was calculated from osmotic shock data (----) or from time to hemolysis data (—)

glycerol by a factor of 300 (0.88 *vs.* 0.0034 osmolal). And the results are also similar in that survivals at 0 °C were independent of time between 0.17 and 30 min and survivals at 15 and 20 °C were nearly constant with time between 10 and 30 min.

The chief difference between the results for 1 M and those for 2 and 3 M glycerol is in the occurrence of the dip. In 1 M at 15 and 20 °C, the dip occurred after such brief contact with glycerol that survivals were low even at the earliest times measured. (The alternative explanation, that the low survivals after short times at 15 and 20 °C reflect the absence of significant permeation of glycerol, is ruled out by the high survivals after only a few seconds in 1 M glycerol at 0 °C.)

The time of occurrence of the dip was not only temperature-dependent but also dependent on the external concentration of glycerol. As shown in Fig. 7, the higher the concentration of extracellular glycerol the later the dip. However, the magnitude of the dip, as measured by the difference in minimum and maximum survivals, was relatively unaffected by the glycerol concentration.

The dip is associated with the freezing and thawing of cells, and not with chilling. Thus, as shown at the top of Fig. 7, rapid chilling of cells to

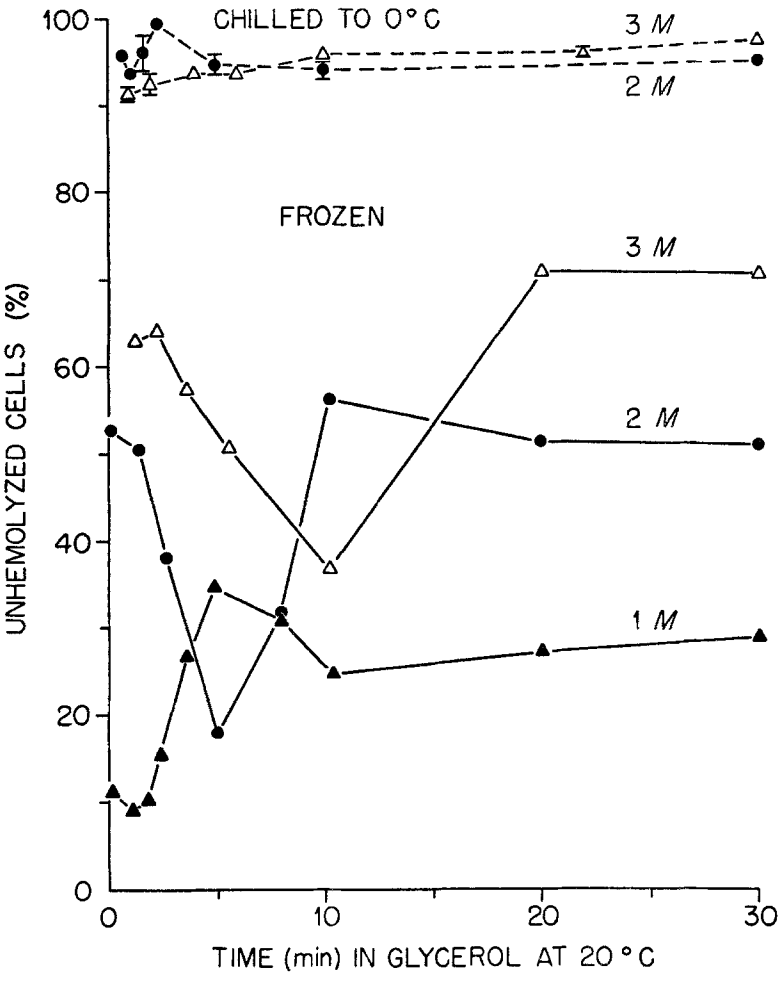

Fig. 7. Survival of bovine red cells chilled rapidly from 20 to 0 °C after various times in glycerol *vs.* survival after freezing at 43 °C/min and rapid thawing. The chilled cells were held for 0.5 or 10 min at 0 °C and then placed in a 35 °C bath for 15 sec

0 °C after various times in 2 or 3 M glycerol at 20 °C produced essentially no drop in viability.

Unlike human red cells (Lovelock, 1954; Meryman, 1971), the bovine red cell also appears relatively insensitive to rapid chilling (thermal shock) when suspended in concentrated NaCl. The survivals of bovine cells held in 1.33 M (2.60 osmolal) NaCl plus 0.01 M buffer (isosmotic with a solution of 2 M glycerol in 0.30 osmolal saline buffer) at 20 °C for 1, 2, 5, 10 and 30 min and then chilled rapidly to 0 °C for 10 min were 50, 50, 50, 57 and 50%, respectively. Survival of unchilled controls was 72% after 65 min.

Survival vs. Time in Sucrose

The bovine red cell, like the human red cell, is impermeable to sucrose (Bishop, 1964; Farrant & Woolgar, 1972*b*; Mazur *et al.*, 1974). Fig. 8 compares the survival of cells that were frozen and thawed after various times in 1.4 M (2.27 osmolal) sucrose at 20 °C with that after various times in 2 M (2.31 osmolal) glycerol. Survivals after 1, 10, 20 and 30 min were similar in the two isosmotic solutions. The chief difference is that the abrupt transient dip in survival in glycerol does not occur with cells in sucrose. In other words, the dip is probably associated in some way with the permeation of solute. Long exposure to hyperosmotic sucrose prior to freezing is detrimental. After 145 min, survival after freezing in sucrose dropped to 32.5% *vs.* 68% for cells in glycerol.

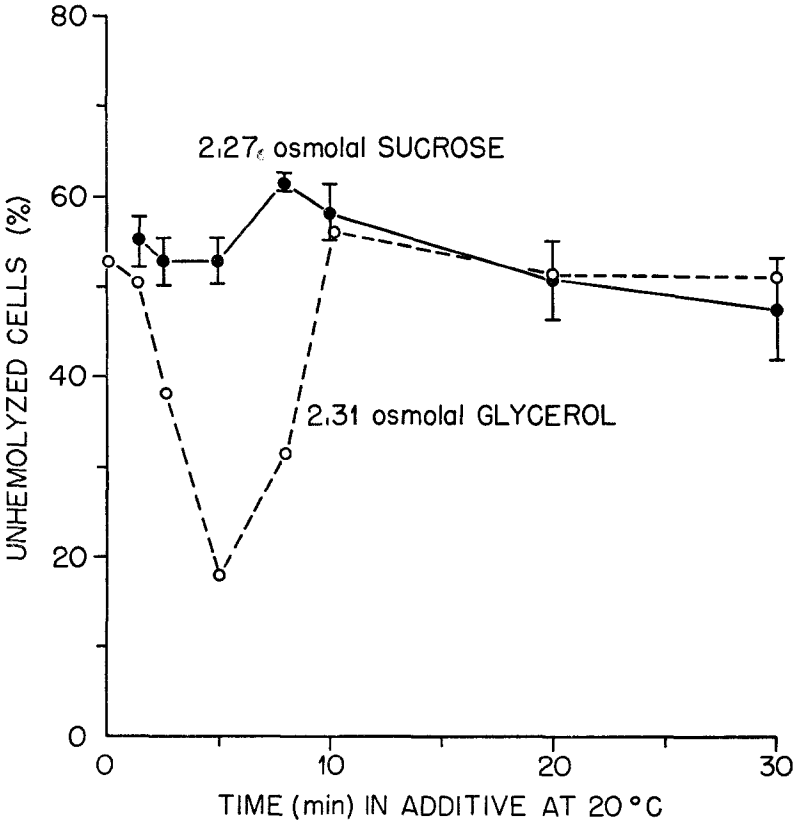

Fig. 8. Survival of bovine red cells frozen and thawed after various times in 1.4 M sucrose at 20 °C *vs.* survival after various times in an isosmolal solution of glycerol. The cooling rate was 43 °C/min. Survival of unfrozen controls in sucrose was 96%

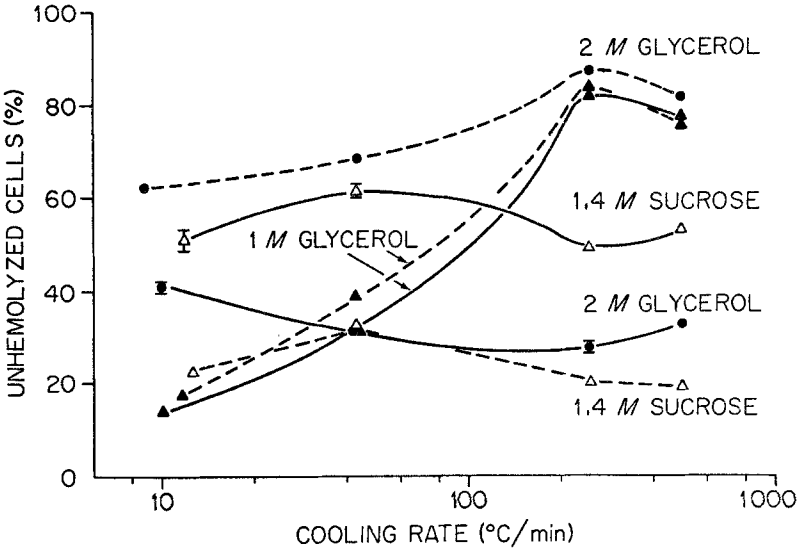

Fig. 9. Survival of bovine red cells frozen to −196 °C at various rates after being suspended in 1 M glycerol, 2 M glycerol, or 1.4 M sucrose for 8 min (—) or 145 min (----). Survivals of unfrozen controls in 1 M glycerol, 2 M glycerol, and 1.4 M sucrose were 100, 95 and 95%, respectively

Interaction between Time in Additive Prior to Freezing and Survival at Various Cooling Rates

As shown in Fig. 9, the time bovine cells are in additive prior to freezing strongly influences the response of the cells to cooling rate, responses that would be inexplicable without the knowledge of the transient dip. Although cells held in 1 M glycerol for 8 and for 145 min prior to freezing respond very similarly, cells held in 2 M glycerol for 8 and for 145 min respond very differently. The reason, of course, is that in 1 M glycerol the dip has disappeared by 8 min (Fig. 6*A*), whereas in 2 M glycerol (Fig. 4*A*) it is about maximum. In 2 M glycerol survival after 8 min is lower than after 145 min, but in sucrose the situation is reversed. Apparently, the longer the cells are held in concentrated sucrose the more susceptible they become to freezing at any rate. This confirms the indications in Fig. 8.

Discussion

The chief purpose of our experiments was to determine the relation between the permeation of glycerol and the ability of the bovine red cell to

survive slow freezing. The conclusion is clear. Although high survivals require high concentrations of glycerol in the extracellular medium, they do not require significant amounts of glycerol in the cell at the onset of freezing. The evidence for the first part of the statement comes from Fig. 2 and from previously published findings for bovine red cells (Rapatz & Luyet, 1963, 1965). The evidence for the latter conclusion about protection in the absence of permeation is as follows: (1) Survivals of red cells held in glycerol for less than 1 min at 0 °C were as high as survivals of cells held for 30 min at 20 °C. The concentrations of intracellular glycerol in the cells under these two conditions and the osmolal ratio of intracellular glycerol to non-permeating solutes differed by as much as factors of 300 and 800, respectively. (2) Survivals in the nonpermeating solute, sucrose, were as high as in an equiosmolal solution of glycerol. This shows both that permeation prior to freezing is not required for survival and that sucrose protects bovine red cells as effectively as does glycerol. Morris and Farrant (1972) have reported similar results for human red cells.

There are two sources of uncertainty in these conclusions. One concerns the accuracy of our estimates of the concentration of glycerol in the cell prior to chilling and freezing. This was discussed in some detail in the preceding paper. The chief points are that (1) the estimates of P_γ are based on experimentally defined end points, i.e. time to hypotonic hemolysis and time to susceptibility to osmotic shock, and where comparisons are possible they agree well with published data; and (2) although the two estimates of P_γ differ somewhat, the choice of P_γ does not affect the results sufficiently to influence the conclusions drawn.

A second source of uncertainty is the question of whether significant quantities of glycerol enter the cell during freezing. Favoring entry would be the large increase in the concentration of external glycerol brought about by the progressive freezing of the solution. Countering this is the high temperature coefficient for glycerol permeation (lowering the temperature from 20 to 0 °C reduces P_γ by a factor of about 12) and the short time the cells were exposed to liquid during freezing. That time for cells cooled at ~50 °C/min is 2 min at most, for it takes less than 2 min for the cells to cool below −70 °C by which temperature all detectable liquid water has disappeared (Sussman & Chin, 1966; Wikefeldt, 1971). These two factors combined ought to prevent the diffusion of much glycerol into the cell during freezing, provided that freezing produces no major alterations in the permeability characteristics of the cell membrane.

Our criterion of survival (absence of hemolysis) means of course that the membranes of surviving cells have not undergone alterations sufficient to allow the leakage of hemoglobin; but more subtle changes are possible, and there are some indications that they occur in human red cells. Human red cells in hyperosmotic solutions of NaCl or sucrose become leaky to cations (Meryman, 1971; Farrant & Woolgar, 1972*a*, *b*), but this cation leak does not occur with the permeating additive DMSO (Farrant, 1972). Although cells in hyperosmotic sucrose leak cations, they do not become sufficiently leaky to allow surcose to permeate, if held at 20 or 0 °C (Farrant & Woolgar, 1972*b*). On the other hand, after freezing and thawing they contain appreciable sucrose (Daw, Farrant & Morris, 1973). But whether the sucrose entered during freezing or after thawing is not known. Meryman and Hornblower (1972) have reported altered cation contents in human red cells after rapid freezing and thawing, but in this case much of the alteration occurred after thawing rather than during freezing.

The finding that intermediate lengths of time in glycerol prior to freezing produce a dramatic decrease in survival remains unexplained. The "dip" is not a result of thermal shock, for it does not occur in cells chilled rapidly from 20 to 0 °C. It is apparently related to the permeation of solute, for it did not occur in cells suspended in the nonpermeating solute sucrose. The chief quantities that change during the permeation of glycerol are the amount and concentration of glycerol, the concentration of intracellular salts, the amount of intracellular water, and the volume of the cell. Presumably, the explanation of the dip and its temperature and concentration dependence is associated with one or more of these. Studies are in progress to determine which (Leibo & Mazur, 1972).

There have been two other reports on the freezing of bovine cells at about 100 °C/min after various periods of equilibration with glycerol. Blackshaw (1954) actually found better survival after short times in glycerol at 5 °C (<1 min) than after 1 hr. Dalgliesh (1969) found, as we did, that survivals were high after short exposures at 5 °C, low after intermediate times, and high again after exposure times of 100 min or more.

Until recently, protection against freezing injury by nonpermeating solutes was considered to be a special case that applied to just a few cell types cooled at rather high rates (Meryman, 1966; Meryman & Hornblower, 1972). But it appears more and more that the requirement for permeation prior to freezing may be the special case, and that most additives may protect most cells chiefly by acting at the outer cell surface. What remains unknown is the mechanism of that protection.

Appendix A

Relation between the Osmolal Ratio of Glycerol to Nonpermeating Solutes in the Cell and the Osmolality of Intracellular Nonpermeating Solutes During Freezing

The total osmolality of liquid solution in a cell at a given freezing temperature (Mazur, 1965) is

$$M^i_{sf}+M^i_{nf}\approx(273-T)/1.86, \tag{A.1}$$

where M^i_{sf} and M^i_{nf} are the osmolalities of intracellular glycerol and intracellular nonpermeating solutes, respectively, and T is the temperature in °K.

Let R be the osmolal ratio of intracellular glycerol to nonpermeating salts; i.e.,

$$R=M^i_s(t)/M^i_n(t)=M^i_{sf}/M^i_{nf}, \tag{A.2}$$

where $M^i_s(t)$ and $M^i_n(t)$ are osmolalities of glycerol and nonpermeating solutes in the cell at time t just prior to freezing. (The second equality assumes there is no solute transfer during freezing.) Solving Eq. (A.2) for M^i_{sf} and substituting for M^i_{sf} in Eq. (A.1), we obtain

$$M^i_{nf}=\Delta T/1.86(1+R).$$

Let $M^{i'}_{nf}$ be the osmolal concentration of nonpermeating solutes in the cell when there is no glycerol in the cell (i.e., $R=0$); then

$$M^{i'}_{nf}/M^i_{nf}=1+R.$$

In other words, during freezing, glycerol reduces the intracellular osmolality of nonpermeating solutes at a given freezing temperature to $1/(1+R)$ of the value it would have in the absence of glycerol. It exerts a comparable effect on the concentration of electrolytes in the extracellular medium.

Appendix B

Relation between the Fraction of Cell Water Remaining Unfrozen (q) at Subzero Temperatures and the Osmolal Ratio of Glycerol to Nonpermeating Solutes in the Cell Prior to Freezing

The fraction of cell water that remains unfrozen (q) (Mazur, 1965) at a given subzero temperature is

$$q=1.86\,[M^i_s(t)+M^i_n(t)]/(273-T). \tag{B.1}$$

Again, let R be the osmolal ratio of intracellular glycerol to nonpermeating solutes just prior to freezing; i.e.,

$$R = M_s^i(t)/M_n^i(t). \tag{B.2}$$

Substituting Eq. (B.2) in (B.1), we obtain:

$$q = 1.86[M_n^i(t)(R+1)]/(273-T).$$

Let q' be the fraction of cell water that would remain unfrozen in the absence of intracellular glycerol (i.e., when $R=0$). Then

$$q/q' = 1+R.$$

In other words, during freezing in the presence of intracellular glycerol, the fraction of cell water remaining unfrozen at a given temperature will be $1+R$ times the fraction that would remain unfrozen at that same temperature in the absence of glycerol.

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