TEMPERATURE DEPENDENCE OF THE SURVIVAL OF HUMAN ERYTHROCYTES FROZEN SLOWLY IN VARIOUS CONCENTRATIONS OF GLYCEROL

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ABSTRACT One widely accepted explanation of injury from slow freezing is that damage results when the concentration of electrolyte reaches a critical level in partly frozen solutions during freezing. We have conducted experiments on human red cells to further test this hypothesis. Cells were suspended in phosphate-buffered saline containing 0–3 M glycerol, held for 30 min at 20°C to permit solute permeation, and frozen at 0.5 or 1.7°C/min to various temperatures between −2 and −100°C. Upon reaching the desired minimum temperature, the samples were warmed at rates ranging from 1 to 550°C/min and the percent hemolysis was determined. The results for a cooling rate of 1.7°C/min indicate the following: (*a*) Between 0.5 and 1.85 M glycerol, the temperature yielding 50% hemolysis (LT_{50}) drops slowly from −18 to −35°C. (*b*) The LT_{50}'s over this range of concentrations are relatively independent of warming rate. (*c*) With glycerol concentrations of 1.95 and 2.0 M, the LT_{50} drops abruptly to −60°C and to below −100°C, respectively, and becomes dependent on warming rate. The LT_{50} is lower with slow warming at 1°C/min than with rapid. With still higher concentrations (2.5 and 3.0 M), there is no LT_{50}, i.e., more than 50% of the cells survive freezing to −100°C. Results for cooling at 0.5°C/min in 2 M glycerol were similar except that the LT_{50}'s were some 10–20°C higher. A companion paper (Rall et al., *Biophys. J.* **23**:101–120, 1978) examines the relation between survival and the concentrations of salts produced during freezing.

INTRODUCTION

Metazoan cells usually survive freezing to below about −20°C only when the suspending medium contains appreciable concentrations of protective solutes. Generally, the most effective solutes are low-molecular weight nonelectrolytes like glycerol and dimethyl sulfoxide. Some years ago Lovelock (1953*b*) reported on the temperature-dependence of the hemolysis of frozen red cells suspended in various concentrations of glycerol. He found that an increase in glycerol concentration lowered both the lethal temperature and the total extent of hemolysis. He then compared the lethal temperature with the composition of the unfrozen portion of the extracellular solution, com-

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positions that he calculated from phase diagrams of glycerol-NaCl-water. From the comparison he reached the important conclusion that hemolysis occurred when the sodium chloride concentration in the external medium reached a certain level during freezing. The higher the glycerol concentration, the lower the temperature at which the critical concentration of electrolyte was attained.

At the time of his experiments, there was no awareness of the important role played by cooling and warming rates on the survival of frozen cells; that is, there was no awareness that the survival of cells, including red cells, exhibits a biphasic dependence on cooling rate (Mazur, 1970). Survivals tend to be low with very slow cooling; they rise with increasing cooling rate to reach a maximum; and then drop off, often abruptly, with still more rapid cooling. Death from rapid cooling has been shown to be due to the formation of intracellular ice during cooling and to ice crystal growth during warming (Mazur, 1970; Bank and Mazur, 1973; Bank, 1973; Diller, 1975; McGrath et al., 1975; Leibo et al., 1978). Cell injury at low cooling rates is associated, not with the location of the ice, but with changes in the cytoplasm and in the surrounding aqueous medium brought about by the progressive transfer of liquid water into ice, changes that have been referred to as "solution effects." These solution effects are the subject of this paper.

Lovelock (1953*b*) performed his experiments by placing samples of suspensions into baths at various subzero temperatures. This procedure produces rather high rates, and it produces a confounding of temperature and rate; i.e., the lower the temperature of the bath, the higher the cooling rate. Rapatz et al. (1968), Morris and Farrant (1972), and Miller and Mazur (1976) have shown that when the confounding is eliminated by cooling samples at constant rates to a fixed final temperature below −75°C, the effect of cooling rate on survival can be appreciable, depending on the concentration of glycerol and the warming rate. Miller and Mazur's (1976) major findings are shown in Fig. 1.

The dependence of survival on cooling and warming rates raises questions as to the extent to which the confounding of temperature and rate in Lovelock's (1953*b*) experiments compromised his results and conclusions. We decided, therefore, to reexamine the relation between temperature and survival under conditions in which the confounding is eliminated. The relation was examined for two cooling rates (0.5 and 1.7°C/min), four warming rates (between 1 and 550°C/min), and a series of glycerol concentrations ranging from 0 to 3.0 M. As shown in Fig. 1, the cooling rates used were far below the optimum rate and therefore were far too slow to induce intracellular ice. All experiments were performed on cells fully equilibrated with glycerol before freezing began.

Central to the interpretation of the survival data is accurate information on the composition of the extracellular solutions at various subzero temperatures. Recently, phase diagrams of glycerol-NaCl-water solutions have been determined by differential thermal analysis with greater precision than was obtainable with Lovelock's techniques (Goldston, 1974; Shepard et al., 1976). The question of the relation between

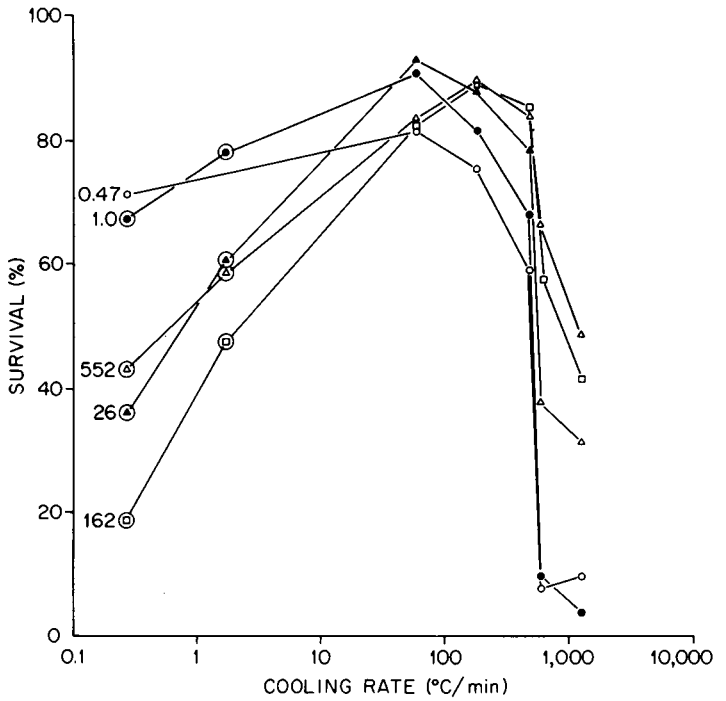

FIGURE 1 Survival of frozen-thawed human red cells as a function of cooling velocity. Cells were equilibrated in 2 M glycerol, cooled at various rates to about −100°C, transferred to −196°C, and then warmed at 0.5°C/min (○), 1.0°C/min (●), 26°C/min (▲), 160°C/min (□), or 550°C/min (△). From Miller and Mazur, 1976. The points circumscribed by a circle refer to the cooling and warming rates used in the present study.

survival and the composition of the extracellular medium during freezing is the subject of the companion paper (Rall et al., 1978).

MATERIALS AND METHODS

Human blood cells collected in heparinized tubes were washed twice by centrifugation with a solution of 0.15 M saline, buffered with 0.01 M phosphate at pH 7.0. The washed cells were then suspended in 0.15 M buffered saline at the same cell concentration as that originally present in the whole blood. The collected blood, stored at 4°C, was used for no longer than 5 days. Washed blood was used for only 1 day.

Equilibration with Glycerol

A 0.25-ml portion of washed cells was mixed with 4.75 ml of phosphate-buffered saline containing various concentrations of glycerol (0, 0.5, 1.0, 1.5, 1.75, 1.85, 1.95, 2.0, 2.5, or 3.0 M). This procedure holds the molal concentration of NaCl and buffer constant at 0.159 mol/kg (Mazur et al., 1974, Table I). Because of the slight dilution by the buffered saline transferred with the cells, the concentrations of glycerol in the resulting cell suspensions were 97% of the above values, which we shall refer to as nominal. The cell suspensions (1/20 of the cell con-

centration in whole blood; hematocrit ~2%) were held at 20°C for 30 min to allow full permeation of the glycerol (Mazur and Miller, 1976*a*). In a few cases, the cells suspended in either glycerolated or nonglycerolated solutions were held at 20°C for as long as 180 min. No effect of longer times was detected.

Procedure for Freezing

Experimental samples were prepared by dispensing 0.1 ml of either glycerolated or nonglycerolated cell suspensions into glass freezing tubes (90 × 7-mm outside diameter, 5-mm inside diameter), secured to Lucite holders. Sets of three replicate tubes were transferred into an ethanol bath cooled to approximately 2–3°C below the freezing point of the suspending solutions. After a 3-min equilibration at that temperature, the suspensions were seeded by being touched with the tip of a Pasteur pipet containing ice. These pipets had also been equilibrated at the bath temperature. The seeding temperatures were −2.0, −2.7, −3.7, −5.0, −7.0, and −10.0°C for 0, 0.5, 1.0, 1.5, 2.0, and 3.0 M glycerol, respectively. Seeded samples were held an additional 4 min at those temperatures to allow equilibration with respect to crystallization, and were then transferred into a cooling bath previously adjusted to be slightly colder than the seeding bath. The temperature of the cooling bath was recorded continuously, with 30-gauge copper-Constantan thermocouples connected to a Leeds and Northrup Speedomax type-W recorder (Leeds & Northrup Co., North Wales, Penn.). (Sample tube and bath temperature agree to within ≤0.5°C). When the bath reached the desired temperatures, samples were usually removed immediately and thawed at one of several rates.

Cooling Rates

Two cooling rates were used (the quoted rates are based on the time required for samples to cool from −10 to −65°C): (*a*) 0.5°C/min: an 85 × 280-mm evacuated, unsilvered Dewar flask containing 500 ml of 95% ethanol, stirred continuously during cooling, was cooled by liquid nitrogen in a 4-liter Dewar flask. (*b*) 1.7°C/min: the procedure was the same as (*a*), but the inner Dewar flask was unevacuated.

Warming Conditions

Two to four different warming procedures were used for each cooling rate. The most rapid warming was obtained by shaking the tubes in a 35°C water bath. Immediately after the ice disappeared, the samples were plunged into an ice bath. The mean warming rate, based on the time for the samples to pass from −65 to −10°C, was about 550°C/min. Moderately rapid warming (160°C/min) was obtained by transferring the freezing tubes into an ice and water mixture. A still lower warming rate (30°C/min) was produced by holding the tubes in room-temperature air. The bath for the lowest warming rate, 1°C/min, consisted of a 1-liter breaker containing 350 ml of ethanol, previously cooled to desired temperatures with Dry Ice (Airco Industrial Gases, Airco Inc., Murray Hill, N.J.). The freezing tubes were transferred into the ethanol bath, and the bath was allowed to warm in air.

Cooling and Warming Rates to and from Various Bath Temperatures

The cooling rates of 0.5 and 1.7°C/min are quite constant between −10 and −65°C (see Miller and Mazur, 1976, Fig. 1). So also is the warming rate of 1°/min. It decreases from 1.0°C/min between −65 and −10°C to 0.9°C/min between −20 and −10°C. However, the higher warming rates are somewhat nonlinear, the rate between −20 and −10°C being about half of that between −65 and −10°C. The rate is lowered by the substantial melting that begins above −20°C. The numerical value of the higher warming rates, therefore, depends somewhat on the concentration of glycerol. For example, warming in a 35°C water bath pro-

duces a rate of 550°C/min for 0.1-ml samples of 2 M glycerol in saline, and a rate of 1,400°C/min for a 0.1-ml sample of water.

Dilution and Determination of Hemolysis

Thawed samples were kept in an ice bath until the samples subjected to the lowest temperatures had been thawed. The 0.1 ml in the thawed suspensions was carefully transferred by Pasteur pipet into the bottom of a centrifuge tube that contained 0.9 ml of the original suspending medium. The freezing tube was back-flushed twice with aliquots of the clear upper portion of the 0.9 ml of medium, and the 1.0 ml in the centrifuge tube was then mixed by vortexing. After centrifugation at 1,600*g* for 10 min, 0.5 ml of the supernatant solution of diluted samples was carefully removed by pipet and mixed with 0.5 ml of Drabkin's solution. After 10 min or more, the absorbance (A) of cyanmethemoglobin was read at 540 nm in Bausch & Lomb Spectronic 20 colorimeter (Bausch & Lomb, Scientific Optical Products Div., Rochester, N.Y.) The absorbance value for 100% hemolysis was obtained by diluting 0.02 ml of washed blood solution with 4 ml of 0.01 M phosphate buffer, pH 7.0. Percent hemolysis was calculated as $100\, A_{exp}/A_{100\%}$. Percent survival is the percentage of unhemolyzed cells. Further details on these procedures have been given elsewhere (Miller and Mazur, 1976).

RESULTS

Cell Survival versus Glycerol Concentration

Fig. 2 shows the percentage of red cells that survived slow cooling to various temperatures when suspended in concentrations of glycerol ranging from 0 to 3.0 M. The cooling rate was 1.7°C/min; the warming rate was 550°C/min. The presence of 0.5–1.75 M glycerol produced a set of similarly shaped survival curves, displaced to progressively lower temperatures. The temperatures producing 50% hemolysis (LT_{50}) were −8.5, −18, −27, −30, and −33°C for cells in 0, 0.5, 1.0, 1.5, and 1.75 M glycerol,

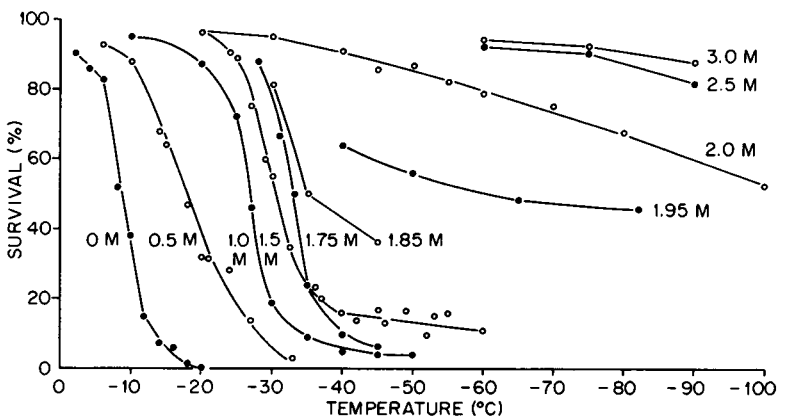

FIGURE 2 Survival of frozen-thawed human red cells as a function of the minimum temperature to which they were cooled. The cooling rate was 1.7°C/min; the warming rate, ~550°C/min. The values on the curves refer to the nominal concentrations of glycerol in the unfrozen suspending medium. The actual values were 3% less (see Methods).

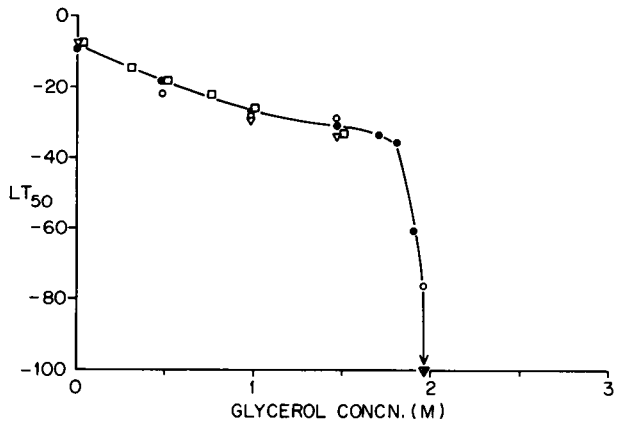

FIGURE 3 Relation between the temperature producing 50% hemolysis (LT_{50}) in frozen-thawed human red cells and the concentration of glycerol in the medium. The cooling rate was 1.7°C/min. The warming rate was 550°C/min (●), 160°C/min (○), or 1.0°C/min (▽). The points indicated by □ are from Lovelock (1953*b*, Fig. 1). The cooling rates in his experiments were some 10–50°C/min and warming was rapid (probably ~100°C/min). The arrow indicates that no LT_{50} is observed, even at −196°C.

respectively. Cooling the suspensions 10°C below the LT_{50} usually resulted in less than 10% survival after thawing.

Increasing the concentration of glycerol to above 1.75 M produced dramatic effects. First, the LT_{50} dropped from −35°C in 1.85 M glycerol to −60°C in 1.95 M, and to < −100°C in 2.0 M glycerol (Fig. 3). Second, survivals at the minimum temperatures rose from 10% or less in lower concentrations to over 50% in 2 M glycerol and to over 80% in 2.5 and 3.0 M glycerol (Fig. 2).

Effects of Warming Rate on Survival

The data just discussed were for cells warmed at 550°C/min. The effect of other warming rates (1–160°C/min) is shown in Fig. 4. No effect was observed for cells in 0 and 1.0 M glycerol (Fig. 4 A), but clear effects were observed for cells in 1.5 and 2 M glycerol (Fig. 4 B), namely, warming at 550°C/min was more deleterious than warming at 1°C/min, but was less deleterious than warming at 160°C/min. Warming rate affects both the LT_{50} and the percent survival at the minimum temperature. Thus, for cells in 1.5 M glycerol, changes in the warming rate from 160 to 550 to 1°C/min lowered the LT_{50} from −28.5 to −30.5 to −34°C, respectively, and raised the final survival from 0 to 10 to 30%. For cells in 2 M glycerol, the final survivals were raised from 35% to 53% and 82%, respectively.

Effect of Cooling Rate on Survival

Table I compares the results of cooling cells in 2 M glycerol at 0.5°C/min with those obtained at 1.7°C/min. The findings at the two cooling rates are similar except that the lower rate produced slightly higher LT_{50}'s and slightly lower final survivals. The relative effects of different warming rates were similar.

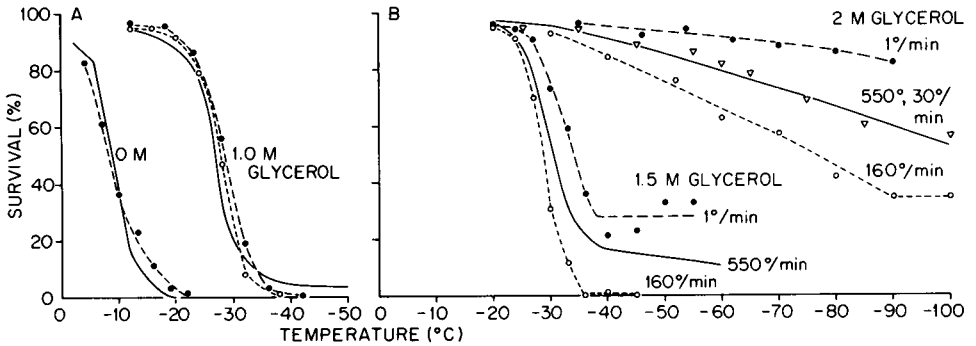

FIGURE 4 Effect of warming rate on the temperature dependence of red cell survival. Cells in indicated nominal concentrations of glycerol were cooled at 1.7°C/min. Upon reaching the indicated temperatures, they were warmed at 1°C/min (●), 30°C/min (▽), 160°C/min (○), or 550°C/min (——). The 550°C/min curve is from Fig. 2.

Table I also compares the survivals of cells cooled to −90 or −100°C with those reported previously by Miller and Mazur (1976) for cells cooled to −196°C. There were no additional deleterious effects as a result of lowering the temperature an additional 100°C.

Slower cooling produces longer exposure times. To examine the question of rate vs. time, experiments were performed in which cells in 2 M glycerol were cooled at 1.7°C/min to various temperatures and then, before thawing, were held at these temperatures for the times required for suspensions cooled at 0.5°C/min to reach those same temperatures. Table II compares the effects of cooling at 1.7 and 0.5°C/min, without holding, with the effects of cooling at 1.7°C/min with holding. The results seem to fall into three categories: (*a*) Cooling cells at 1.7°/min followed by holding at −35°C was

TABLE I

EFFECT OF COOLING RATE AND WARMING RATE ON THE LT_{50} AND FINAL SURVIVAL LEVEL OF HUMAN RED CELLS FROZEN IN 2 M GLYCEROL

Cooling rate	Warming rate	LT_{50}	Survival at −90 to −100°C	Survival at −196°C*
°C/min	°C/min	°C	%	%
0.5	1	<−90	55	71 (67)‡
	30	−71	33	44 (36)
	160	−50	26	28 (19)
	550	<−90	52	48 (43)
1.7	1	<−100	82	78
	30	<−100	57	60
	160	−75	35	47
	550	<−100	53	59

*From Miller and Mazur (1976).

‡The values in parentheses are observed values for a cooling rate of 0.3°C/min. The accompanying values are interpolated from Fig. 1 for a cooling rate of 0.5°C/min.

TABLE II

COMPARISON OF THE SURVIVAL OF RED CELLS COOLED AT 1.7°C/MIN TO VARIOUS TEMPERATURES AND HELD BEFORE THAWING WITH THAT OF CELLS COOLED AT 1.7 OR 0.5°C/MIN TO THE SAME TEMPERATURES AND IMMEDIATELY THAWED

Cooling rate	Warming rate	Survival at					
		−35°C	−40°C	−45°C	−50°C	−55°C	−60°C
°C/min	*°C/min*				%		
1.7	550	90	89	83	81	—	77
1.7 + hold	550	73	71	65	85	—	76
0.5	550	83	75	68	67	—	60
1.7	160	92	83	—	76	73	63
1.7 + hold	160	61	66	54	87	55	60
0.5	160	82	57	58	45	37	32

In the first case cells were held at a given temperature for the length of time required for cells cooled at 0.5° C/min to reach that temperature. Cells were suspended in 2 M glycerol in buffered saline or in saline alone.

more harmful than was cooling cells to −35°C at 0.5°C/min. (*b*) At −40 and −45°C, the combination of cooling at 1.7°C/min and holding gave results comparable to those for cells cooled at 0.5°C/min without holding. (*c*) At lower temperatures, the deleterious effect of holding tended to disappear and cooling at 0.5°C/min became more harmful than cooling at 1.7°C/min.

DISCUSSION

The factors affecting the survival of frozen-thawed cells are multivariant, and depend chiefly on interactions of the type and concentration of additive, cooling rate, final temperature, and warming rate. In a recent study, Miller and Mazur (1976) examined the interactive effects of cooling and warming rates on the survival of human red cells equilibrated in 2 M glycerol and cooled to −196°C. A portion of their results is depicted in Fig. 1. The present study restricted itself to the combinations of cooling and warming rates circled in Fig. 1, and proceeded to examine the events transpiring between −2 and −100°C for cells suspended in concentrations of glycerol ranging from 0 to 3 M.

A striking feature of the curves in Fig. 1 is the abrupt drop in survival at cooling rates above 500°C/min. There is convincing evidence that this results from the formation and recrystallization of intracellular ice (Rapatz and Luyet, 1961; Diller, 1975; Mazur, 1977). The cooling rates used in the present study were much lower than 500°C/min and hence were far too slow to induce intracellular ice. Consequently, whatever damage was observed must be ascribable to alterations in the intracellular and extracellular solutions brought about by the conversion of intra- and extracellular water into extracellular ice—i.e., the damage must be ascribable to what has been referred to as solution effects (Mazur, 1970).

Effects of Cooling Rate, Warming Rate, and Time

From Table I we see that cooling at 0.5°C/min to −90°C was somewhat more deleterious than cooling at 1.7°C/min, a finding that confirms the data of Miller and Mazur (1976) shown in Fig. 1. The somewhat more detrimental effects of slower cooling are also reflected in a higher LT_{50}. With a warming rate of 160°C/min, for example, the LT_{50} shifts upward from −75 to −50°C when cooling is slowed from 1.7 to 0.5°C/min.

One of the unexpected findings in the Miller and Mazur (1976) study was the response of slowly cooled cells to warming rate. Damage increased in the order of 1 < 30, 550 < 160°C/min. The same sequence of damage was observed in the present study for cells in 1.5 and 2 M glycerol (Fig. 4 B and Table I). There was, however, little or no effect of warming rate on cells suspended in 0 or 1 M glycerol (Fig. 4 A).

The warming rate affects both the final level of survival and the temperature at which 50% of the cells are hemolyzed (LT_{50}). It has little effect, however, on the temperature at which survival falls halfway between the highest and lowest values observed. For example, for cells cooled at 1.7°C/min (Fig. 4 B), we find the temperature of midpoint survival to be −68, −67, −62, and −67°C for cells suspended in 2 M glycerol and warmed at 1, 30, 160, and 550°C/min, respectively. For cells in 1.5 M glycerol, the midpoint survivals occur at −32, −29, and −31°C for cells warmed at 1, 160, and 550°C/min.

Effect of Glycerol Concentration

Morris and Farrant (1972), and to a lesser extent Mazur and Miller (1976*b* and unpublished), have examined the interaction between cooling rate and glycerol concentration on the survival of human red cells cooled to −196°C and warmed rapidly.

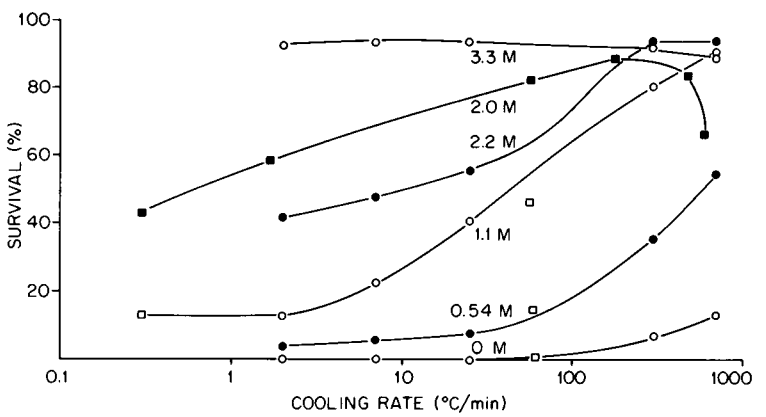

FIGURE 5 Survival as a function of cooling rate for human red cells suspended in physiological saline containing the indicated concentrations of glycerol, cooled to −196°C, and warmed rapidly. Data indicated by squares are from Mazur and Miller (1976*b* and unpublished). The other data are from Morris and Farrant (1972).

Their data are summarized in Fig. 5. At low cooling rates (e.g., 1.7°C/min) survival is strongly dependent on glycerol concentration. At high cooling rates survivals become high, and the dependence on glycerol concentration diminishes or disappears.

The present study was concerned with the temperature dependence of the survival of cells cooled at the two lowest rates shown in Fig. 5. It showed (Figs. 2 and 4) that with increasing concentration of glycerol there is a progressive decrease in the temperature that hemolyzes half the cells (LT_{50}). A major reduction in the susceptibility of the cells occurs between glycerol concentrations of 1.75 and 2.0 M. This decreased susceptibility is reflected both in a decrease in the LT_{50} and in an increase in survival at the minimum temperature. The relationship between LT_{50} and glycerol concentration found in the present study (Fig. 3) agrees closely with the relationship first reported by Lovelock (1953*b*). The agreement is good although Lovelock's procedures produced increasing cooling rates with decreasing temperature, and rates some 10–30 times higher than ours. On the other hand, he held samples at the minimum temperatures for 10 min before thawing, whereas we generally initiated the warming of the samples immediately upon reaching a desired temperature.

Although the LT_{50}'s in our work and Lovelock's agree closely, the overall curves for survival vs. temperature are very different (Fig. 6). Ours shows a progressive decline with temperature; his is U-shaped. An inspection of the data in Figs. 1, 2, and 5 shows that the U-shape results from the confounding of temperature and cooling rate. Similar U-shaped survival curves have been reported by others (e.g., Rapatz and Luyet, 1968; Rapatz et al., 1975), and undoubtedly have the same genesis.

It would appear from the close correspondence between our data and the left arm of the U in his data and from the sharp divergence below about −30°C, that down to −30°C the deleterious effect of lowered temperature overwhelms any protective effect of progressively higher cooling rates, but that below −30°C the situation re-

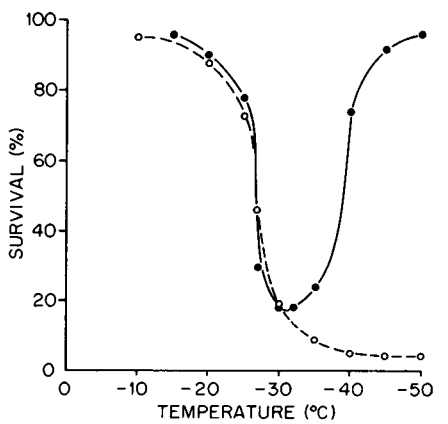

FIGURE 6 Survival of frozen-thawed human red cells in 1 M glycerol as a function of the minimum temperature attained. Data from Lovelock [1953*b*, Fig. 1 (●)] and from the present study (○).

verses and the protective effect of higher cooling rates more than compensates for the additional damage incurred at lower temperatures. This interpretation is consistent with our findings (Table II) that at −35°C survival is more affected by holding time than by cooling rate, but at temperatures below −45°C survival is more affected by cooling rate than by holding time.

The effect of time depends not only on temperature, but also on the concentration of glycerol. Lovelock (1953*a*) showed that, in the absence of protective additive, survival is inversely dependent on exposure time. Increased exposure time between −3 and −40°C produced increased hemolysis regardless of whether most of the time elapsed during cooling or during warming. On the other hand, when cells are in 2 M or higher concentrations of glycerol, the detrimental effect of time per se diminishes, and the effects of the time spent cooling and the time spent warming cease to be additive. The basis for these statements is the finding that the sequence of slow cooling and slow warming, which produces the longest exposure time, is not more damaging than the shorter exposures produced by higher rates (Figs. 1 and 4 B, and Table I). In fact, in 2 M glycerol the shorter exposure times produced by the rapid warming of slowly cooled cells become markedly deleterious.

In summary, the situation appears to be this: The damage suffered by red cells frozen in saline is time- and temperature-dependent. Glycerol in 2 M concentrations or higher protects against *this* damage and permits cells to be frozen to lower temperatures. But, as we shall see in the companion paper, the presence of glycerol and the attainment of lower temperatures introduce other factors that may cause damage. Miller and Mazur (1976) have suggested these other factors may be osmotic in nature.

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