Freezing of living cells: mechanisms and implications

MAZUR, PETER. *Freezing of living cells: mechanisms and implications.* Am. J. Physiol. 247 (Cell Physiol. 16): C125–C142, 1984.—Cells can endure storage at low temperatures such as −196°C for centuries. The challenge is to determine how they can survive both the cooling to such temperatures and the subsequent return to physiological conditions. A major factor is whether they freeze intracellularly. They do so if cooling is too rapid, because with rapid cooling insufficient cell water is removed osmotically to eliminate supercooling. Equations have been developed that describe the kinetics of this water loss and permit one to predict the likelihood of intracellular freezing as a function of cooling rate. Such predictions agree well with observations. Although the avoidance of intracellular freezing is usually necessary for survival, it is not sufficient. Slow freezing itself can be injurious. As ice forms outside the cell, the residual unfrozen medium forms channels of decreasing size and increasing solute concentration. The cells lie in the channels and shrink in osmotic response to the rising solute concentration. Prior theories have ascribed slow freezing injury to the concentration of solutes or the cell shrinkage. Recent experiments, however, indicate that the damage is due more to the decrease in the size of the unfrozen channels. This new view of the mechanism of slow freezing injury ought to facilitate the development of procedures for the preservation of complex assemblages of cells of biological, medical, and agricultural significance.

cryobiology; low temperature; cryoprotectants; behavior of cell water; cell membranes and freezing injury; permeability and freezing injury

SINCE LIQUID WATER is considered essential to the structure and function of living cells, it is not surprising that the solidification of water by freezing is usually lethal. Yet paradoxically freezing can also preserve cells for long periods of time in a viable state, and it may someday permit the long-term storage of tissues and organs. It can be used to preserve subcellular constituents and the details of cell ultrastructure, but it is also used to disrupt cells and organelles to isolate constituents. It can slow or stop some biochemical reactions, but it accelerates others. It is a challenge that is successfully met by some organisms in nature but not by others.

Although the uses and consequences of freezing are diverse and even paradoxical, there are fundamental underlying mechanisms that determine how all biological systems respond to the lowering of temperature and the solidification of liquid water. This article is concerned with reviewing some aspects of these underlying mechanisms and with some of the implications of freezing to biology, medicine, and agriculture.

Stopping Biological Time

Contrary to the usual impression, the challenge to cells during freezing is not their ability to endure storage at very low temperatures; rather it is the lethality of an intermediate zone of temperature (∼−15 to −60°C) that a cell must traverse twice—once during cooling and once during warming. No thermally driven reactions occur in aqueous systems at liquid N_2 temperatures (−196°C), the refrigerant commonly used for low temperature storage. One reason is that liquid water does not exist below −130°C. The only physical states that do exist are crystalline or glassy, and in both states the viscosity is so high ($>10^{13}$ poises) that diffusion is insignificant over less than geological time spans. Moreover, at −196°C, there is insufficient thermal energy for chemical reactions (67).

The only reactions that can occur in frozen aqueous systems at −196°C are photophysical events such as the formation of free radicals and the production of breaks in macromolecules as a direct result of "hits" by background ionizing radiation or cosmic rays (96). Over a sufficiently long period of time, these direct ionizations can produce enough breaks or other damage in DNA to become deleterious after rewarming to physiological temperatures, especially since no enzymatic repair can occur at these very low temperatures. The dose of ionizing radiation that kills 63% of representative cultured mammalian cells at room temperature (1/e survival) is 200–400 rads (19). Because terresterial background radiation is some 0.1 rad/yr, it ought to require some 2,000–4,000

yr at −196°C to kill that fraction of a population of typical mammalian cells.

Needless to say, direct experimental confirmation of this prediction is lacking, but there is no confirmed case of cell death ascribable to storage at −196°C for some 2–15 yr and none even when cells are exposed to levels of ionizing radiation some 100 times background for up to 5 yr (48). Furthermore, there is no evidence that storage at −196°C results in the accumulation of chromosomal or genetic changes (6).

Stability for centuries or millenia requires temperatures below −130°C. Many cells stored above ∼−80°C are not stable, probably because traces of unfrozen solution still exist (54). They will die at rates ranging from several percent per hour to several percent per year depending on the temperature, the species and type of cell, and the composition of the medium in which they are frozen (52).

Most implications and applications of freezing to biology arise from the effective stoppage of time at −196°C. But if cells are to be put in suspended animation, they must survive both the initial freezing to −196°C and the subsequent return to ambient temperatures. To understand the problems involved in going to and from such low temperatures, we need first to review the physical chemical events occurring during freezing and thawing and the response of cells to these events.

Fate of Intracellular Water During Freezing

The chief physical events occurring in cells during freezing are depicted schematically in Fig. 1. Down to ∼−5°C, the cells and their surrounding medium remain unfrozen both because of supercooling and because of the depression of the freezing point by the protective solutes that are frequently present. Between −5 and

∼−15°C, ice forms in the external medium (either spontaneously or as a result of seeding the solution with an ice crystal), but the cell contents remain unfrozen and supercooled, presumably because the plasma membrane blocks the growth of ice crystals into the cytoplasm (see below). The supercooled water in the cells has, by definition, a higher chemical potential than that of water in the partly frozen solution outside the cell, and in response to this difference in potential, water flows out of the cell and freezes externally.

The subsequent physical events in the cell depend on cooling velocity. If cooling is sufficiently slow (Fig. 1, upper right), the cell is able to lose water rapidly enough by exosmosis to concentrate the intracellular solutes sufficiently to eliminate supercooling and maintain the chemical potential of intracellular water in equilibrium with that of extracellular water. The result is that the cell dehydrates and does not freeze intracellularly. But if the cell is cooled too rapidly (Fig. 1, bottom and center right) it is not able to lose water fast enough to maintain equilibrium; it becomes increasingly supercooled and eventually attains equilibrium by freezing intracellularly.

Quantitative theory. In 1963 I suggested that these qualitative statements could be described quantitatively (50). More specifically, the proposal was that the rate of exosmosis of water during freezing can be described by four simultaneous equations. The first relates the loss of cytoplasmic water to the chemical potential gradient expressed as a vapor pressure ratio

$$dV/dt = (L_p A R T \ln P_e/P_i)/V_1^o \qquad (1)$$

where V is the volume of cell water, t is time, L_p is permeability coefficient for water (hydraulic conductivity), A is cell surface area, R is gas constant (in $\mu m^3 \cdot atm \cdot mol^{-1} \cdot °K^{-1}$), T is temperature, V_1^o is molar volume of
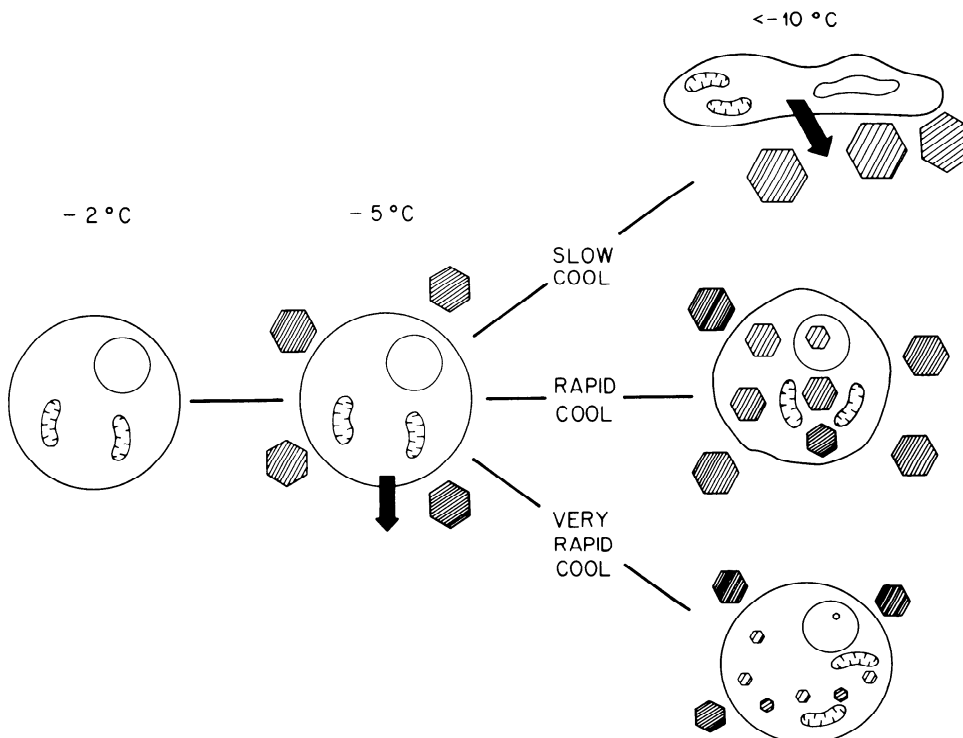

FIG. 1. Schematic of physical events in cells during freezing. *Cross-hatched hexagons*, ice crystals. From Ref. 55.

water, and P_e and P_i are vapor pressure of extracellular and intracellular water. The change in this vapor pressure ratio with temperature can be calculated from the Clausius-Clapeyron equation and Raoult's law

$$d \ln(P_e/P_i)/dT \qquad (2)$$
$$= L_f/RT^2 - [N_2V_i^o/(V + N_2V_i^o)V]dV/dT$$

Here N_2 is osmoles of intracellular solute, and L_f is the latent heat of fusion of ice. Analyses have shown that the temperature difference across the cell membrane rarely exceeds $0.01°K$ (27).

Time and temperature are related by the cooling rate (B), which if linear is given by

$$dT/dt = B \qquad (3)$$

and finally, L_p is related to temperature by the expression

$$L_p = L_p^g \exp[-E/R'(1/T - 1/T_g)] \qquad (4)$$

where L_p^g is the permeability coefficient to water at a known temperature T_g, E is its activation energy, and R' is the gas constant (in $cal \cdot mol^{-1} \cdot K^-$) (62).

To avoid intracellular freezing, the water content of the cell given by Eqs. 1–4 must, before reaching the intracellular ice nucleation temperature ($-5°$ to $-40°C$, see below), have approached the equilibrium water content given by

$$V = V_i^oM_i/[\exp(L_f/RT - L_f/RT_f) - 1] \qquad (5)$$

where M_i is initial osmolality of the extracellular solution and T_f is freezing point of H_2O ($273°K$).

The above quantitative expressions permit one to calculate the extent of supercooling in cells as a function of cooling rate, provided one knows or one can estimate the permeability of the cell to water (L_p), its activation energy (E), the osmoles of solute initially in the cell, and

the ratio of the cell surface area to volume. The results of such calculations are usually expressed as plots of the water content of a cell as a function of temperature relative to the normal or isotonic volume. Two examples are shown in Fig. 2. The calculated extent to which cells become supercooled is the number of degrees that any given curve is displaced to the right of the equilibrium curve at a given subzero temperature. For example, the curve for yeast cooled at $100°C/min$ indicates that the cells are supercooled $14°C$ as the temperature passes through $-15°C$.

These calculated curves permit one to estimate the probability of intracellular freezing as a function of cooling rate. Cells that have dehydrated close to equilibrium prior to reaching their ice nucleation temperature will have a zero probability of undergoing intracellular freezing. Cells that are still extensively supercooled when cooled to their nucleation temperature, and therefore still hydrated, will have a high probability of undergoing intracellular freezing. Thus if we assume an ice-nucleating temperature of $-12°C$, we would predict from Fig. 2 that the critical cooling rates for yeast would be between 10 and $100°C/min$ and that the critical cooling rates for the human red blood cell would be between 1,000 and $5,000°C/min$, a 50- to 100-fold difference. This difference is chiefly due to differences in the values of L_p^g and E and the ratio of cell surface area to volume for the two cells. An increase in L_p produces the same effect as a comparable decrease in cooling rate. For example, tripling the value of L_p in Fig. 2A would shift the $100°C/min$ curve to the left by the same amount as would decreasing the cooling rate from 100 to $33°C/min$. If the water-loss curves are shifted to the left, the effect is to increase the cooling rate required to produce a given probability of intracellular freezing. The effect of cell size is the opposite. An increase in diameter (D) propor-
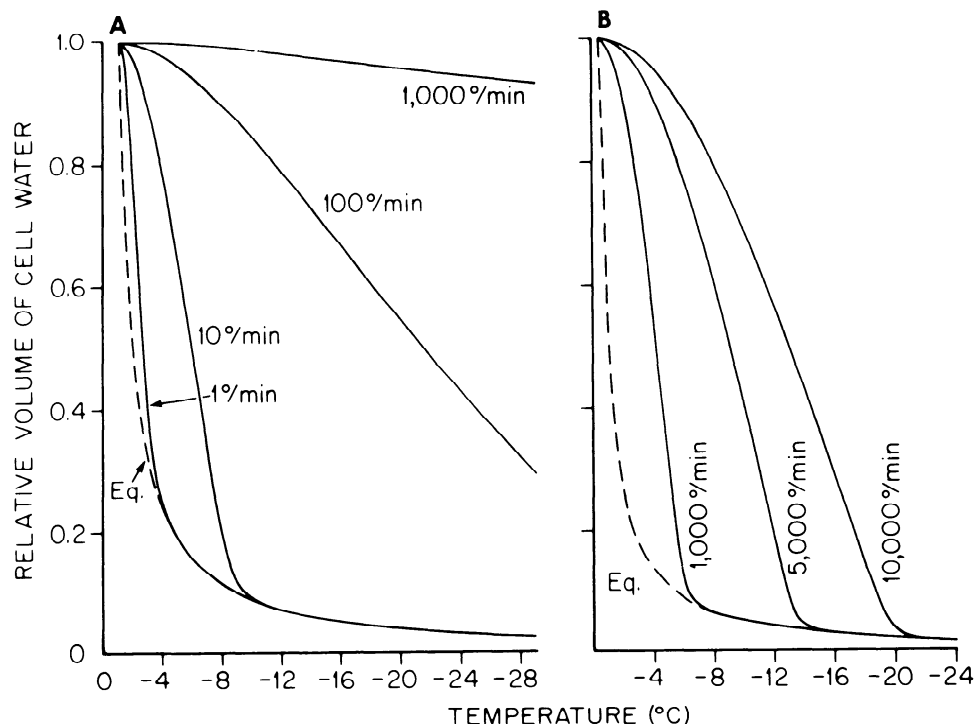

FIG. 2. Calculated fractions of intracellular water remaining in yeast (A) and human red blood cells (B) as they cool to various temperatures at indicated rates. *Curve Eq* represents equilibrium water content. From Ref. 54, copyright 1970 by AAAS.

tionally reduces the cooling rate required to produce a given probability of intracellular freezing because the fractional water loss during cooling is proportional to the ratio of the cell surface (D^2) to cell volume (D^3).

Experimental tests of predicted cooling rates yielding intracellular freezing. The development of cryomicroscopes (optical microscopes with freezing stages provided with sophisticated control of cooling rates) (17, 95) has permitted experimental tests of the presumed relation between cooling rate and the occurrence of intracellular freezing. Figure 3 shows micrographs from Leibo et al. (42) of mouse ova cooled at 1.2 and 32°C/min. Note the strong similarity to the schematic cells in Fig. 1. The ovum cooled at 1.2°C/min dehydrates without evidence of internal freezing, but the ovum cooled at 32°C/min undergoes no discernable shrinkage and freezes intracellularly at −40°C as evidenced by its sudden opacity. The evidence that this abrupt blackening is indeed a manifestation of the crystallization of intracellular water has been summarized by Rall et al. (89).

By making such sequential photomicrographs on ova cooled at various rates, Leibo et al. (42) were able to construct the solid curve in Fig. 4 showing the proportion of ova undergoing intracellular freezing as a function of cooling rate. We see that none of the ova freeze internally at cooling rates of 1°C/min or less but that all of them freeze intracellularly at cooling rates of 5°C/min or more.

To determine how these observations compare with the likelihood of intracellular freezing as predicted by the water-loss equations we must first generate water-
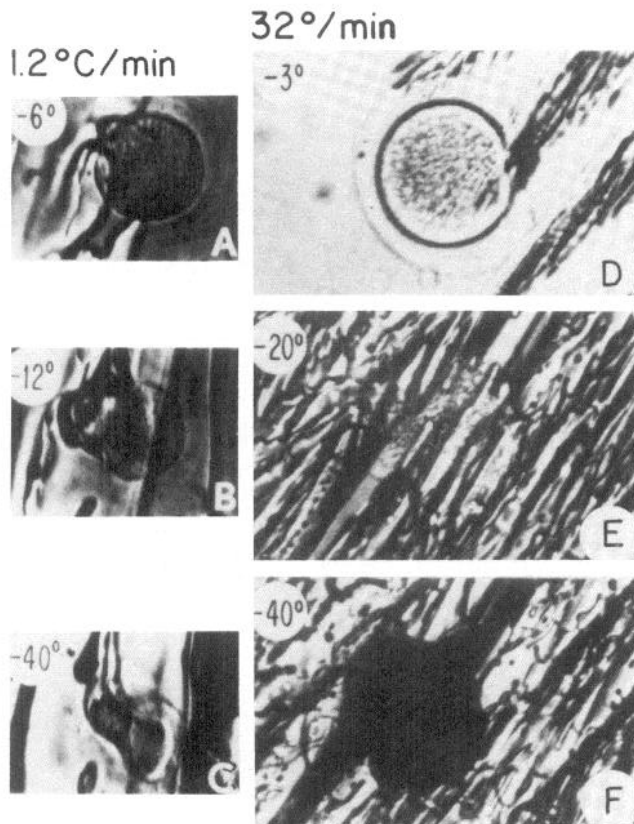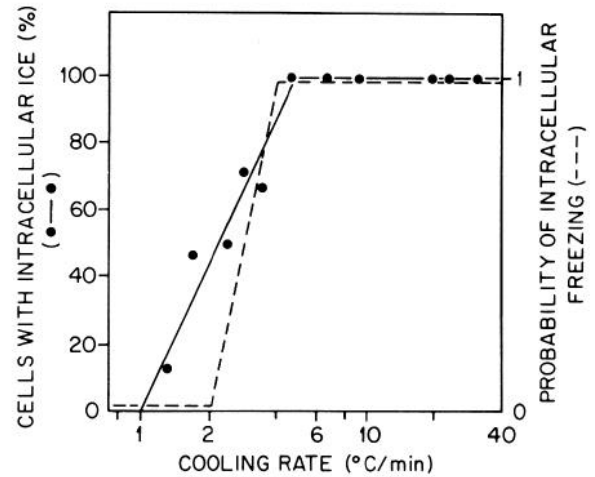

FIG. 4. A comparison between computed and observed likelihood of intracellular freezing in mouse ova frozen in 1 M dimethyl sulfoxide at various rates. Experimental data are from Leibo et al. (42). Computed curve is from Ref. 62.
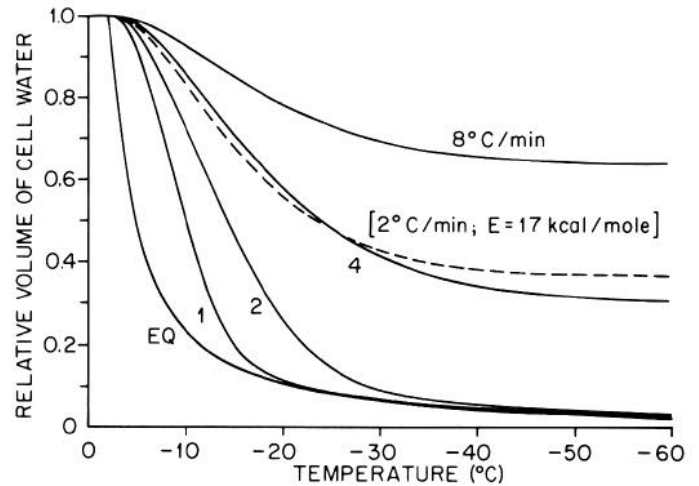

FIG. 5. Computed kinetics of water loss from mouse ova cooled at 1–8°C/min in 1 M dimethyl sulfoxide. *Curve EQ* shows water content that ova have to maintain to remain in equilibrium with extracellular ice. *Other solid curves*, labeled 1–8°C/min, were computed assuming activation energy E of L_P to be 14 kcal/mol. *Dashed curve* shows effect of changing E to 17 kcal/mol.

loss curves for ova. Doing so requires values for the several parameters in *Eqs. 1–4*. Values for the most important of these, L_p and its activation energy E, have recently been published by Leibo (39), and solutions to the water-loss equations for mouse ova in 1 M dimethyl sulfoxide (DMSO) cooled at 1–8°C/min are shown in Fig. 5.

To avoid intracellular freezing, the ova must be cooled slowly enough to permit their water content to approach the equilibrium value before they have reached their ice-nucleation temperature. How close must the approach to equilibrium be? The available evidence indicates that when ~90% of cell water is removed, the residual ~10% cannot freeze at any temperature (112, 126). The average ice-nucleation temperature of mouse ova and embryos in 1–2 M glycerol or DMSO has recently been found to lie between −33 and −45°C (37, 42, 89). Superimposing this range of nucleation temperatures on the computed water-

FIG. 3. Appearance of unfertilized mouse ova during freezing in 1 M dimethyl sulfoxide at 1.2 (*A–C*) or 32°C/min (*D–F*). From photomicrographs published by Leibo et al. (42).

loss curves in Fig. 5 leads to the prediction that embryos cooled at 2°C/min or less ought not to freeze internally since they will have dehydrated to equilibrium before cooling to the nucleation temperatures. But embryos cooled at 4°C/min or faster ought to freeze internally since they will still contain appreciable supercooled water as they enter the temperature zone for nucleation. These predictions (Fig. 4, dashed curve) agree closely with the solid curve in Fig. 4, which shows Leibo's actual microscopic observations on the proportion of cells undergoing intracellular freezing as a function of cooling rate. Indeed the agreement is better than one has a right to expect in view of the simplifying assumptions underlying *Eqs. 1, 2,* and *4*.

The two most important of these assumptions are that the values of L_p^g and its activation energy E obtained from measurements at 0°C and above are also applicable at −10°C and below (55, 62). In the former case the cells are exposed to rather dilute (≤1 osmolal) solutions of nonpermeating solutes, usually NaCl or sucrose. In the latter case the cells become exposed to multiosmolal solutions both because of the introduction of cryoprotective solutes like glycerol or DMSO and because of the further concentration of solutes by freezing. There is evidence that E is not affected by the rise in osmolality but that L_p^g in nucleated mammalian cells may be about halved in the presence of permeating solutes (62, 83, 97). Accordingly such a reduction in L_p^g was used in the computation of the dashed curve in Fig. 4.

Information necessary to determine whether the water-loss equations can predict the likelihood of intracellular freezing is now available for four cell types: yeast, mouse ova, human lymphocytes, and plant protoplasts. And in these four cases, as shown in Table 1, agreement between the cooling rates predicted to induce intracellular ice and those observed to do so is good. In a fifth case, human red blood cells, the available data permit one to compute the water-loss curves (Fig. 2B), but there is no information on their ice-nucleation temperature. Inspection of Fig. 2B shows that this temperature will affect the conclusions drawn. It shows that red blood cells cooled at 1,000°C/min will dehydrate to equilibrium before reaching the nucleation temperature if that nu-

cleation temperature is −8°C or below, but they will not dehydrate to equilibrium if the nucleation temperature is −5°C or above. Cells in the latter case would be expected to freeze internally, whereas those in the former case would not.

In Table 1 there is a 500-fold range in the numerical values of the cooling rates that produce intracellular freezing (predicted or observed) in the seven types of cells listed. As mentioned, the cause is chiefly differences in L_p^g, E, A/V_i, and the nucleation temperature.

Cell Biological Implications of Ice-Nucleation Temperature of Cells and Ability to Predict Occurrence of Intracellular Freezing

The equations used to predict the cooling rates that yield intracellular freezing make the simplifying assumption that cytoplasm behaves like an ideal dilute aqueous solution that obeys Raoult's law (which relates water vapor pressure to the mole fraction of solute) and the Clausius-Clapeyron equation [which relates water vapor pressure to temperature and phase state (solid or liquid)]. However, others have concluded from nuclear magnetic resonance (NMR) and other observations that the water in cells has special properties, including being "in a state somewhere between the solid state of crystalline ice and the liquid hydrogen-bonded lattice of pure water" (12). But the fact that the predictions from the water-loss equations agree quite well with the several available observations argues that no unusual properties have to be assigned to the water in cytoplasm to account for the response of cells to sub-zero temperatures.

In fact cells as a whole not only respond like sacks of dilute aqueous solution with respect to their osmotic dehydration during freezing at low rates but also with respect to the fact that the water in their cytoplasm crystallizes when the cells are cooled sufficiently rapidly to sufficiently low temperatures. The morphological evidence of this intracellular freezing is dark "flashing" as in Fig. 3F. Quantitative evidence from calorimetric measurements shows that about 90% of cell water becomes converted to ice (112, 126) and, if cooling is rapid, that that conversion takes place within the cell.

TABLE 1. *Calculated and observed effects of cooling rates on intracellular freezing and cell survival*

Cell Type	Additive	Nucleation Temperature, °C	50% Intracellular freezing		50% Survival*	Refs.
			Predicted, °C/min	Observed, °C/min	Observed, °C/min	
Mouse ova and embryos	1 M DMSO	−35 to −45	3	2	4	37, 42, 55, 62, 89
Yeast	None	−12 to −20	30	6–55	20	10, 43, 50, 65, 74, 80, 106
Human lymphocytes	None	−30	40	50	100	102
HeLa	None	>−20		70	70	68
Hamster tissue culture	Various	−35 to −45		~55	40–100	8, 20, 58
Human RBC	None	−5/−15†	700–6,000	845–1,000	5,000	16, 22, 50, 55, 80, 93, 103
	2–3 M glyc			650	500	16, 22, 73, 80, 81
Rye protoplasts (nonacclimated)	None	−15	7‡	5	§	18

DMSO, dimethyl sulfoxide; RBC, red blood cell.   *50% of maximum for cells cooled at optimum rate to below nucleation temperature.
Warming was rapid for yeast and for RBC in saline but was slow (1–30°C/min) in other cases.   †Assumed, see text.   ‡Mazur, unpublished calculations.   §Survival less than 50% at all rates used.

As mentioned in the beginning of this section, cells tend to supercool even in the presence of extracellular ice. There is evidence that cells do not contain substances capable of nucleating supercooled water above ~−25°C. For example, in the absence of external ice, the water in yeast and human red blood cells (21, 94) remains supercooled to between −30 and −40°C. The latter temperature approaches the homogeneous nucleation temperature of water, i.e., the temperature at which water freezes spontaneously in the absence of ice nucleators. Even in the presence of external ice (the best nucleator of all) many higher plant cells supercool to near −40°C (23) and so also do mouse ova and early embryos in 1.5–2.0 M solutions of glycerol or DMSO (37, 89). The absence of effective intracellular ice nucleators puts severe constraints on attributing "ice-like" characteristics to the water in cytoplasm.

When supercooled cells freeze internally well above −40°C, their cytoplasm is probably nucleated heterogeneously by the passage of extracellular ice crystals through the plasma membrane (13, 47, 69, 77). The temperature at which ice nucleation occurs in animal cells in the presence of extracellular ice is typically −10 to −15°C, but it varies from −5 to −30°C or below (51, 89). The nucleation temperature in mouse embryos at least may depend on the extent to which the plasma membrane is protected against changes in the extracellular solution by the presence of cryoprotective additives like glycerol and DMSO (89). Injury from such extracellular changes will be discussed shortly.

Perhaps more interesting than the ability of ice to pass through the plasma membrane below certain temperatures is its inability to pass through the membranes *above* certain subzero temperatures. Liquid water passes easily and readily through biological membranes. Why, therefore, cannot ice? One answer may relate to the fact that an ice crystal is a coordinated hydrogen-bonded assemblage of water molecules, and the assemblage can pass through membranes only if the membranes possess water-filled pores of sufficient size. The narrower the pores, the smaller must be the radius of curvature of the penetrating ice crystal. And, according to the Kelvin equation, the smaller the radius of curvature the lower the melting point of the crystal. For example, an ice crystal with a radius of curvature of 20 Å has a melting point of −15°C. Consequently, above −15°C such a crystal will be unable to grow through a membrane if its pores are smaller than 5–20 Å, the exact value depending on the ability of liquid water to wet the pore walls (52). These values are reasonably consistent with current thoughts on the molecular structure of the plasma membrane.

Effect of Intracellular Freezing on Cell Survival

In the few cases examined those cooling rates that produce intracellular freezing also cause extensive cell death. The close correlation is indicated in columns 5 and 6 of Table 1 and in Fig. 6. There is evidence in these and other cases that intracellular freezing is the cause of death and *not* a consequence of it. The evidence is that a substantially higher percentage of cells often survive
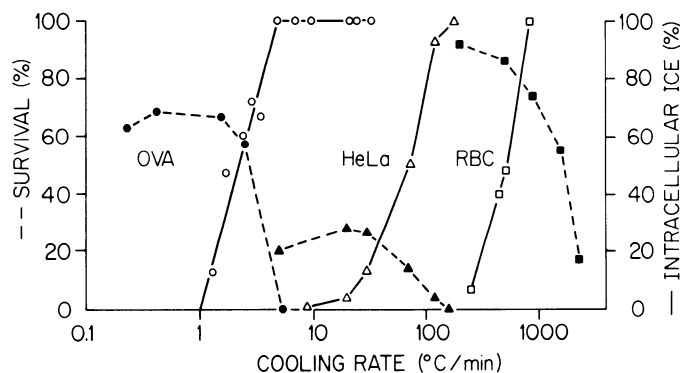

FIG. 6. Percentage survival (*dashed lines*) vs. percentage cells undergoing intracellular freezing (*solid lines*) in 3 mammalian cells frozen at various rates to −20°C (HeLa) or to −78 or −196°C [ova and red blood cell (RBC)]. Modified from Leibo (38). Sources of data for individual curves are given there.

TABLE 2. *Examples of "rescue" of rapidly frozen cells by rapid thawing*

Cell Type	Cooling Rate, °C/min	Survival After Warming		Refs.
		Slowly*	Rapidly†	
Hamster tissue culture cells				
V79	600	5	65	58
CHO	100	30‡	75	24, 25
Mouse and human stem cells	200	0–4	25–30	40, 66
Ascites cells	?	0	54	5
Human red blood cells	600	8	63	73
Mouse and rabbit (8–32 cell embryos)	2 step§	0	80	31, 33, 88, 124
Mulberry cells	100	5	60	100
Cultured carrot	2 step§	5	45	78
Lily pollen	200	0	100	79
Yeast	450	10^{-6}	8	65
Neurospora spores	250	8	95	11, 34

CHO, Chinese hamster ovary. *Slow warming was 1–2°C/min except where noted. †Rapid warming was generally 600–700°C/min except in yeast (10,000°C/min) and embryos (100–200°C/min). ‡Warming was 100°C/min. §0.5°C/min to −40°C, then >100°C/min to −196°C.

observed or inferred intracellular freezing when the rate of subsequent warming and thawing is high rather than low. Table 2 lists some examples, and I have cited a number of others previously (52, 53), in which cells cooled rapidly enough to freeze intracellularly are much more sensitive to slow warming than to rapid, whereas cells cooled slowly enough to preclude intracellular freezing do not show this sensitivity. In yeast for instance, 10 million times as many cells survive intracellular freezing when they are warmed at 40,000°C/min as survive when they are warmed at 1°C/min (65). Obviously, if rapid warming can "rescue" a fraction of intracellularly frozen cells, at least that fraction of cells must have been viable before warming was initiated.

Recrystallization of Intracellular Ice

Although rapid cooling produces intracellular ice, the crystals tend to be small (80, 108). Indeed, at extremely high cooling rates, the crystals become so small as to be

invisible even at the ultramicroscopic level. This is why high-rate freezing procedures are used by electron microscopists in the freeze-cleaving technique.

Small ice crystals, however, are thermodynamically unstable relative to larger ice crystals for essentially the same reason that small crystals melt at lower temperatures (51, 52). Consequently, there will be a tendency during warming for small crystals to aggregate to form larger crystals, a process referred to as recrystallization. The general view is that slow warming is harmful to frozen cells because it allows time for such recrystallization to occur.

A correlation between the recrystallization of intracellular ice observed under the microscope and cell death has been demonstrated in yeast, higher plant cells (99), ascites tumor cells (109), and hamster tissue culture cells (20). MacKenzie (49) has shown that yeast cells cooled at rates far above optimal will survive if warmed very rapidly, but they do not survive if warmed slowly. The bottom half of Fig. 7 shows his results on warming the yeast slowly from −196°C to various subzero temperatures and then completing the warming and thawing rapidly. Survival begins to drop when slow warming is allowed to progress above −40°C. The upper panel of the figure shows that it is also above −40°C where Bank (7) first began to observe the recrystallization of the intra-

cellular ice that formed during rapid cooling, results that are quite comparable with those reported by Moor (74). It has been suggested that damage from the formation and recrystallization of ice may be related either to crystal size (108) or to the total amount of intracellular ice (20). Shimada (108) indicates that to be innocuous, the size in HeLa cells must remain below about 0.05 μm.

Two groups, however, have reported quantitative discrepancies between the killing temperature during the subsequent slow warming of rapidly cooled cells and the temperatures at which devitrification or recrystallization are observed. MacKenzie (49) has calorimetric evidence that the former begins to occur some 10–20°C below the latter in yeast. And Rall et al. (90) report that devitrification occurs about 10°C below, and recrystallization as evidenced by cell darkening occurs some 5–15°C above, the temperature zone over which slowly warmed mouse embryos are killed (−80 to −60°C). They show in an elegant fashion that the death of rapidly cooled embryos only occurs if intracellular ice crystals form or grow, but because of these temperature discrepancies, they conclude "that slow warming injury is not a direct physical result of the formation [or] recrystallization of intracellular ice," (90) a conclusion with which MacKenzie agrees. Both groups invoke some other, unknown factor as the direct cause of injury. MacKenzie states that "one

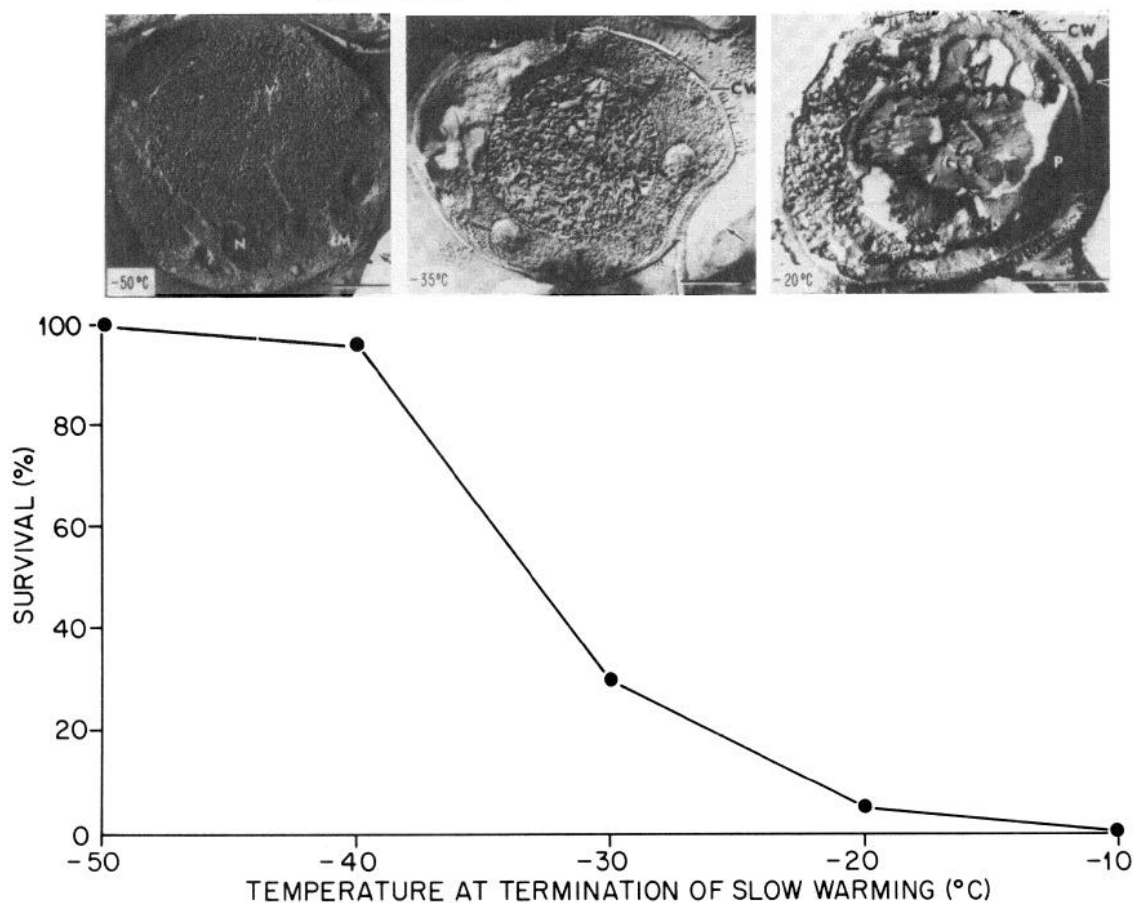## SURVIVAL AND RECRYSTALIZATION IN YEAST

FIG. 7. Effect of warming to various temperatures on survival and on recrystallization of intracellular ice in yeast previously frozen rapidly to −196°C. Data are from MacKenzie (49) and Bank (7), respectively. In MacKenzie's survival studies, cells were warmed slowly to indicated temperatures and then rapidly thawed. In Bank's recrystallization studies, cells were warmed abruptly to −50, −35, and −20°C, held 24 h, 30 min, and 5 min, respectively, recooled to below −100°C, and freeze cleaved. From Ref. 55.

is tempted ... to discount the importance of any gross physical change involving ice and to seek the injury as a more subtle process" (49). And Rall et al. say "that the presence of intracellular ice during warming is innocuous but may lead to a change in another unknown component of the system which then causes injury" (90). But to dismiss a direct cause and effect relationship between killing and ice formation or recrystallization because of these relatively small temperature discrepancies seems unsupportable to me. It could well be, for example, that the crystal size required to produce observed cell darkening or a calorimetric exotherm is not the crystal size required to produce cell death. Or it could be that injury is triggered by the formation or growth of ice crystals in certain critical intracellular sites that occupy only a small percentage of the cell, rather than by the formation and growth of intracellular ice crystals in the cell as a whole.

Changes in Extracellular Medium During Slow Freezing

Although cooling rates low enough to prevent intracellular freezing are often necessary for survival, they are insufficient. Slow freezing itself can be injurious. For purposes of the ensuing discussion I will define a slow cooling rate as one that allows sufficient water to leave the cell to keep the remaining cell water in chemical potential equilibrium with extracellular water and ice throughout cooling, i.e., conditions under which $\mu_w^i \approx \mu_w^e$. Because the only ice that forms under these conditions is extracellular, any observed injury must be a consequence of the direct action of that ice, of alterations in the proportion of ice to extracellular solution, or of changes in the composition of the external solution brought about by the conversion of water into ice.

Concentration of Extracellular Solutes During Freezing

As ice forms outside the cell, the concentration of extracellular solutes in the residual unfrozen medium increases according to the relation

$$\mathbf{M}^e = \phi v m^e = \phi v n_2/V^e = \Delta T/1.86 \qquad (6)$$

where $\mathbf{M}^e$ is by definition external osmolality, ϕ is osmotic coefficient, v is number of species into which the solutes dissociate, m^e is molality, n_2 is moles of solute, V^e is volume of extracellular water, and ΔT is number of degrees below 0°C. The value 1.86 is the molal freezing-point depression constant for water.

In partly frozen solutions, $\mathbf{M}^e$ is independent both of the nature of the solutes and of their total concentration prior to freezing. At constant pressure it is dependent only on temperature. For a solution containing a single given solute, this is also roughly true of the molality m^e. (It is only roughly true because ϕ changes somewhat with concentration.)

A consequence of these considerations is that the *total* osmolal concentration of solutes in the unfrozen portion of a solution at a given temperature is not influenced by the addition of solutes like glycerol or DMSO. (As we shall see such solutes are necessary for the survival of many slowly frozen cells.) For example, the unfrozen

portions of both an isotonic salt solution (0.3 osmolal) and an isotonic salt solution containing 1 M glycerol (1.4 osmol/kg H₂O, total) will have the same total osmolal concentration at −10°C, namely, 10/1.86 or 5.4 osmol/kg.

The presence of additive, however, does reduce the concentrations of salt at the given temperature according to the relation

$$\mathbf{M}^e_{NaCl} = \mathbf{M}^{e'}_{NaCl}/(1 + \mathbf{R}) \qquad (7)$$

where $\mathbf{M}^e_{NaCl}$ and $\mathbf{M}^{e'}_{NaCl}$ are osmolal concentrations of electrolyte in the presence and absence of additive and $\mathbf{R}$ is osmolal ratio of additive to electrolyte prior to freezing (60).

The changes in osmotic coefficients with temperature and concentration make it difficult to solve *Eqs.* 6 and 7 accurately, but accurate determinations of the composition and relative amounts of the concentrated liquid and ice can be made from phase diagrams. Detailed diagrams of the ternary system glycerol-NaCl-H₂O, for example, have been published (107), and from these it is possible to determine the exact NaCl concentration at any temperature. Examples are shown in Fig. 8 for solutions of 0, 0.5, and 1.0 M glycerol in 0.15 M NaCl. This figure illustrates well how the presence of glycerol (or any solute) reduces the concentration of NaCl in the residual unfrozen solution.

The concentration of solutes during freezing arises because of the conversion of water to ice; i.e., m^e in *Eq.*
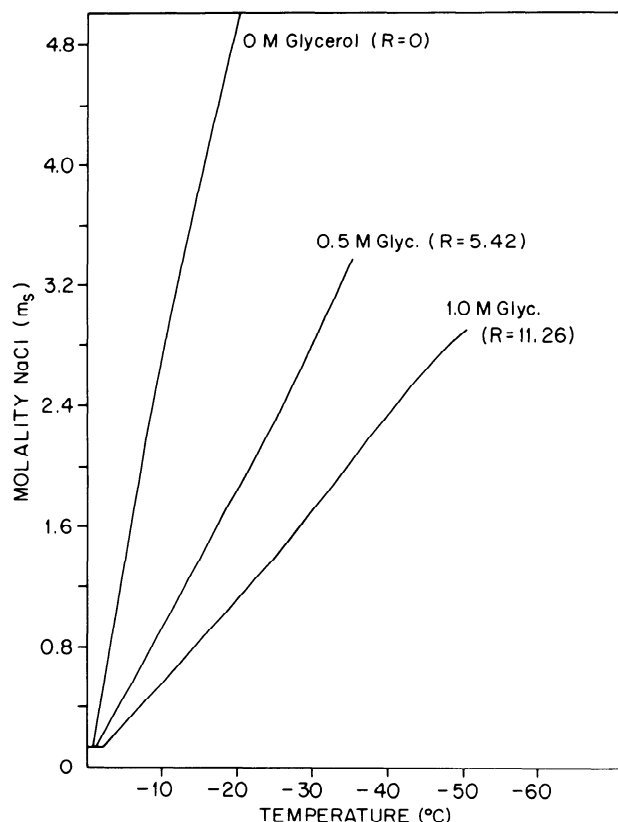

FIG. 8. Molality of NaCl (m_s) in unfrozen portions of glycerol-NaCl-H₂O solutions at various sub-zero temperatures. *Curves* apply to any glycerol-NaCl solution with stated R values, where R is %wt glycerol/%wt NaCl. From Ref. 64.

6 increases because V^e decreases. The value of n_2 remains constant. But if we increase the initial value of n_2 by introducing another solute such as glycerol, the required value of m^e is achieved with less decrease in V^e. That is to say the introduction of glycerol (or any other solute) results in an increase in the unfrozen fraction at any temperature (Fig. 9).

Effect of Slow Freezing on Cell Survival

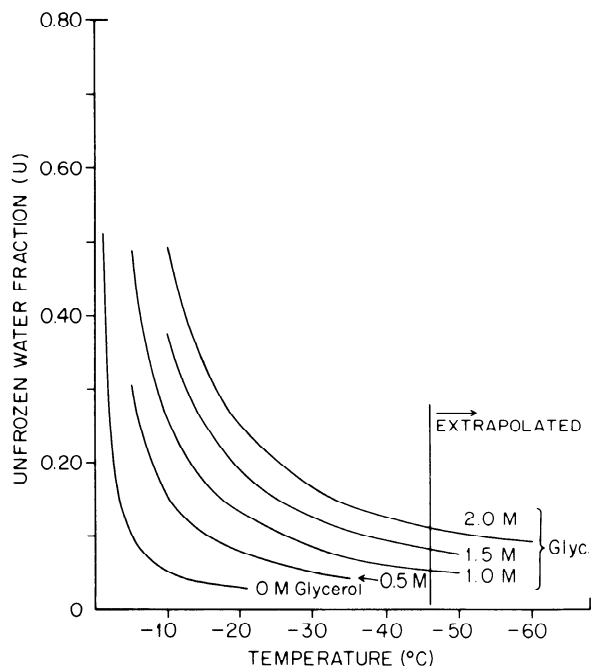The injurious effects of slow freezing are well illus-

FIG. 9. Fractions of water in glycerol-NaCl-H₂O solutions remaining unfrozen at various sub-zero temperatures. Glycerol molarities refer to the initial unfrozen solutions. NaCl concentration in these solutions is fixed at 0.15 M. Reproduced from *Biophys. J.*, 1981, vol. 36, p. 653–675, by copyright permission of the Biophysical Society.

trated by the human red blood cell (Fig. 10). We see first that the survival of slowly frozen cells decreases with decreasing temperature. Second, as the concentration of glycerol is increased from 0 to 1.75 M, the decrease in survival occurs at progressively lower temperatures. Third, the amount of decrease lessens markedly when the glycerol concentration rises above 1.75 M.

If we combined these data on survival vs. temperature with the data on NaCl concentration vs. temperature in Fig. 8, we obtain the results shown in Fig. 11*A* for two of the concentrations of glycerol. The NaCl concentration in the residual unfrozen solution appears to exert major effects: survivals drop from above 80% for salt concentrations of <1 mol/kg H₂O to below 20% for salt concentrations of 2 mol/kg H₂O or more. If, however, we combine the data on survival vs. temperature with the data on the effect of temperature on the unfrozen fraction (Fig. 9), we obtain the results shown in Fig. 11*B*. The curves are mirror images of those in Fig. 11*A*. They show that survival drops from above 80% when the unfrozen water fraction is 0.14 or more to below 20% when the unfrozen fraction is 0.07 or less. The question then is whether the hemolysis of slowly frozen red blood cells is a result of the attainment of high NaCl concentrations as indicated in Fig. 11*A* or a result of the attainment of low unfrozen fractions as shown in Fig. 11*B*.

We have recently shown (63, 64) that the effects of salt concentration and the unfrozen fraction can be separated by appropriate manipulation of the starting glycerol and NaCl concentration and the temperatures to which the cells are frozen. We noted in connection with *Eq. 6* that the total concentration of solute in a partly frozen solution depends on temperature alone and is therefore independent of the starting solute concentration. This also applies to a ternary solution like glycerol-NaCl-H₂O for a given weight ratio of glycerol to NaCl in the initial solution. But it is not the case with the unfrozen fraction. Its magnitude is dependent on both
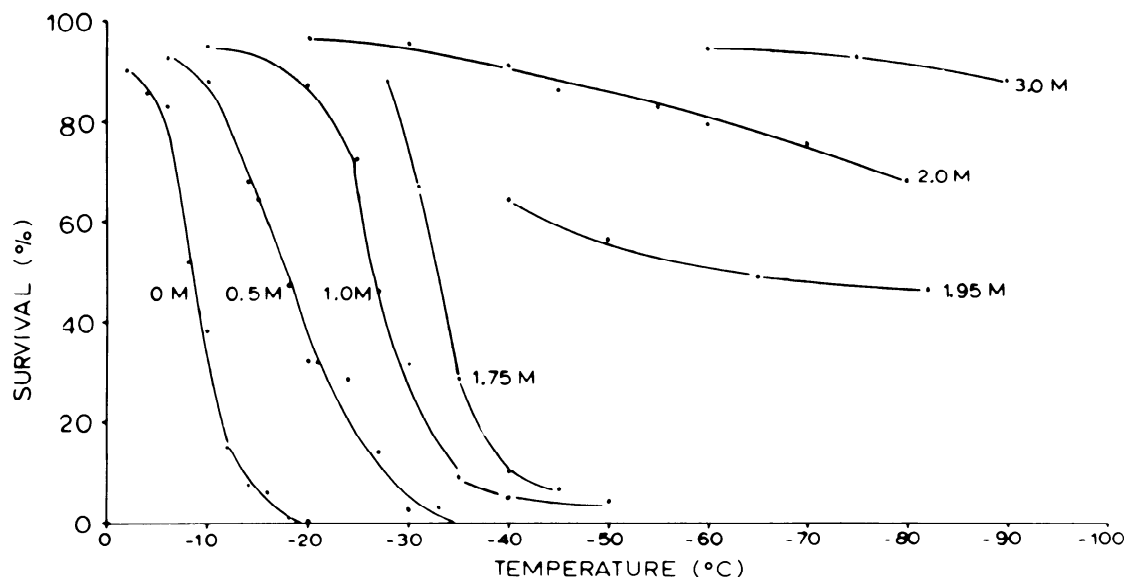

FIG. 10. Survival (% unhemolyzed cells) of frozen-thawed human red blood cells as a function of concentration of glycerol in medium (buffered saline) and as a function of temperature. Freezing was slow (1.7°C/min); thawing was rapid. From Ref. 56 based on data of Souzu and Mazur (111).
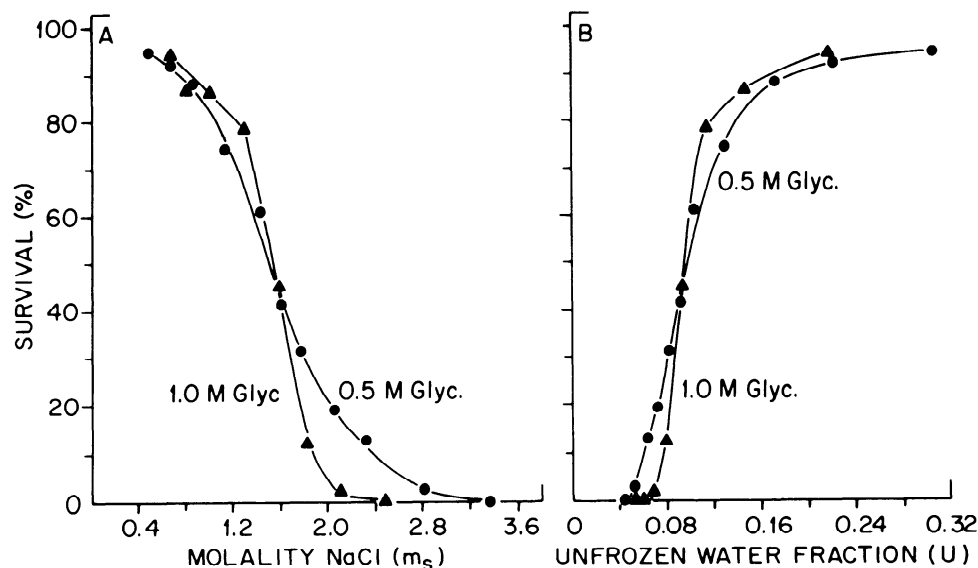
FIG. 11. *A*: survival of human red blood cells as a function of the molality of NaCl (m_s) to which they are exposed after being frozen at 1.7°C/min to various sub-zero temperatures while suspended in solutions of 0.5 or 1.0 M glycerol in isotonic NaCl. Thawing was rapid. *B*: survival as a function of fraction of water that remains unfrozen in solutions. From Ref. 64.
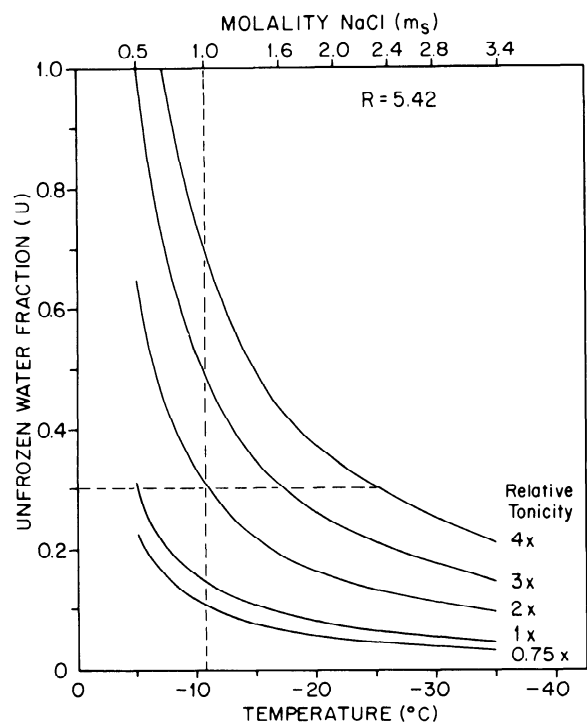

FIG. 12. Unfrozen water fraction (U) vs. temperature for glycerol-NaCl-H$_2$O solutions in which the % weight ratio of glycerol to NaCl (R) is 5.42. Initial concentration of NaCl ranges from 0.75 to 4 × isotonic. *Upper abscissa*, molality of NaCl (m_s) that is present in unfrozen portions of solution at indicated temperatures. *Vertical dashed line*, example of conditions yielding constant m_s and variable U. *Dashed horizontal line*, conditions yielding constant U and variable m_s. From Ref. 64.

FIG. 13. Survival (% unhemolyzed) vs. unfrozen water fraction of human red blood cells frozen at 0.6°C/min in solutions of glycerol-NaCl-H$_2$O in which % weight ratio of glycerol to NaCl is 5.42. m_s, NaCl concentration. Reproduced from *Biophys. J.*, 1981, vol. 36, p. 653–675, by copyright permission of the Biophysical Society.

the starting solute concentration and the temperature. It is possible then, by varying the starting solute concentrations (holding the solute ratios constant), to vary the fraction unfrozen while maintaining a constant solute concentration at a given temperature in that unfrozen fraction.

The approach then was to establish conditions that produce exposures to various unfrozen fractions (U) at constant NaCl concentration (m_s) and others that produce various values of m_s at constant U. To do this we
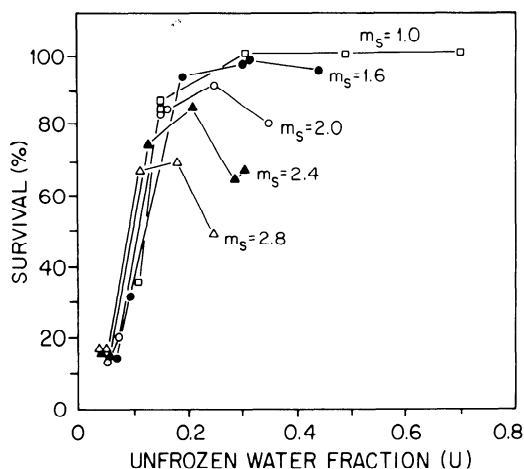
prepared solutions with a fixed ratio of glycerol to NaCl in which the concentrations of glycerol were 0.38, 0.5, 1.0, 1.5, and 2.0 M, and the concentrations of NaCl were 0.75, 1, 2, 3, and 4× isotonic, respectively. Next, from the ternary phase diagram for these solutions, we computed the values of the unfrozen fraction U at various temperatures and plotted them (Fig. 12). The vertical dotted line in Fig. 12 shows, for example, that if the five solutions are frozen to −10.7°C, m_s will remain constant at 1.0, but U will vary from 0.11 to 0.70. Conversely, the dotted horizontal line shows that by freezing the 1, 2, 3, and 4× solutions to −5.1, −10.7, −17.6, and −25.4°C, we obtain conditions where U is a constant 0.3, but m_s will vary from 0.5 to 2.3 mol/kg H$_2$O.

The experiments themselves were straightforward. Human red blood cells were placed in one of five test solutions long enough to allow the glycerol to permeate. They were then frozen slowly at 0.6°C/min to the selected temperatures and thawed rapidly.

The results are shown in Fig. 13 where we plot survival vs. unfrozen fraction for a series of constant m_s values.

We see that in the range of $U = 0.05-0.15$ (i.e., 85–95% of the water is frozen), survival depends entirely on the unfrozen fraction and is independent of the concentration of salt in that fraction. Only at unfrozen fractions above 0.15 is an effect of salt concentration seen. We are finding a similar dependence of survival on unfrozen fraction in preliminary experiments with hamster tissue culture cells and mouse embryos, and others are beginning to find a comparable dependence in other cells. Taylor and Pegg (118), for example, have compared the survival of guinea pig smooth muscle frozen in a DMSO solution to $-21°C$ with the survival of muscle cooled to $-21°C$ in an unfrozen solution having the same composition as the unfrozen fraction of the first solution at $-21°C$. U in the latter case equalled 1, and the survival (contractility) of the muscle was up to fourfold higher than in the partly frozen solution. Santarius and Giersch (101) have also reported a substantial effect of unfrozen fraction on chloroplast thylakoid membranes.

These results at least partly contradict the three current major hypotheses of slow freezing injury. Lovelock (45, 46) proposed that injury during slow freezing is the result of the increase in concentration of extracellular electrolytes, which in turn leads to an increase in the concentration of intracellular electrolytes. He argued that glycerol and other low-molecular-weight additives protect by virtue of their colligative ability to lower the salt concentration in the manner shown in Fig. 8. According to his hypothesis, the curves in Fig. 13 should have been a series of horizontal lines, which is clearly not the case.

Cells also shrink osmotically in response to the increasing concentration of extracellular solutes during freezing, and Meryman (70–72) proposed an alternative hypothesis that slow-freezing injury is a consequence of the shrinkage, or, to be more precise, it is a consequence of the inability of the cells to shrink to the extent required for osmotic equilibrium. More recently, Wiest and Steponkus (125) have proposed a variant of this second hypothesis for plant protoplasts. Their view is that cells that shrink osmotically during freezing can only tolerate a fixed increase in surface area during thawing without being damaged.

All three hypotheses agree on one point, namely, that injury is assumed to be a consequence of the lowered chemical potential of water brought about by the increased solute concentration. This is equivalent to assuming that it is related to the *composition* of the residual unfrozen liquid. Our results oppose that view. As we move down the vertical dotted line in the phase diagram of Fig. 12, U decreases, but the composition of the unfrozen fraction, and hence its chemical potential, remains constant. Figure 13 shows that it is the decrease in U that is the predominant factor in cell injury.

Even though the survival of slowly frozen red blood cells is little affected by changes in the chemical potential of water in the external medium, could it depend on changes in the chemical potential of intracellular water? The answer seems to be no, since the rate of cooling is low enough to equalize the intracellular and extracellular chemical potentials.

Similar considerations argue against the alternative

hypothesis that cell injury is a consequence of cell shrinkage. Figure 14 shows the computed change in the volume of red blood cells during the course of the experiments. Note that during freezing (right side), all cells follow the same shrinkage curve regardless of the experimental solution in which they are suspended. Although their *volume* during freezing is independent of the starting solution, their *survival* is highly dependent on the specific initial solution, for the starting solution influences the value of U.

Why should the survival of cells depend more on the magnitude of the unfrozen fraction of the suspension than on the composition of that unfrozen fraction? Although phase diagrams only give information on the relative proportions of the two phases, ice and unfrozen solution, and say nothing about the microscopic architecture of the two phases, we know from published microscopic observations, such as those on frog erthrocytes in Fig. 15 by Rapatz et al. (92), that cells during slow freezing are sequestered in narrow channels of unfrozen solution between plates of ice and that these channels become progressively narrower as the temperature is lowered. As these red blood cells are cooled from -1.5 to $-5°C$ (Fig. 15, *A* and *B*), the channel width decreaes to about that of the cell diameter, and the value of the unfrozen fraction decreases from 0.23 to 0.07, the range over which U becomes critical for the human red blood cell. Even though frog red blood cells are not human, the correlation between channel width and a critical value of U is suggestive.
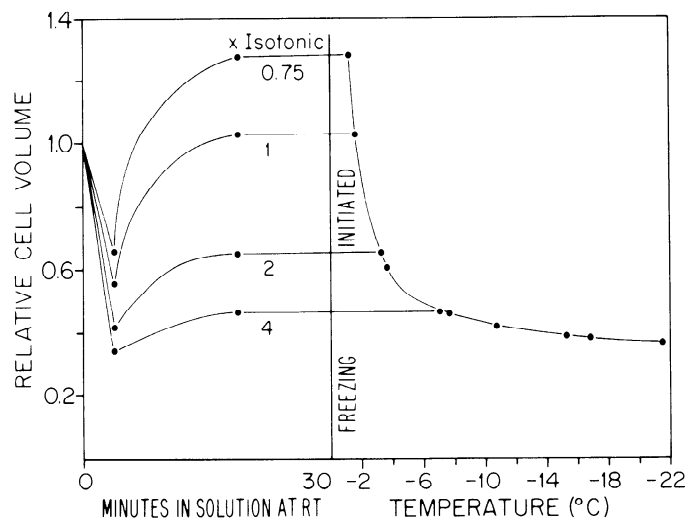
Photomicrographs also often show that cells subjected

FIG. 14. Computed relative volume changes in human red blood cells during *1*) equilibration in 4 glycerol-NaCl-H$_2$O solutions and *2*) subsequent freezing. The 4 test solutions contain 0.75, 1, 2, and 4 times the isotonic concentration of salt and corresponding concentrations of glycerol such that weight ratio of glycerol to NaCl remains 5.42 (i.e., ~0.4, 0.5, 1.0, and 2.0 M glycerol). At zero time, cells at relative volume 1.0 are transferred from isotonic NaCl to test solution at room temperature (RT). They initially shrink as a result of exosmosis of H$_2$O and then swell as glycerol permeates. (Time scale during swelling is approximate.) At equilibrium their volume is determined by concentration of impermeant species, NaCl. After some 30 min, slow freezing is initiated. Cells reshrink as a consequence of exposure to progressively increasing concentration of solutes in unfrozen extracellular medium. Modified from Ref. 64.
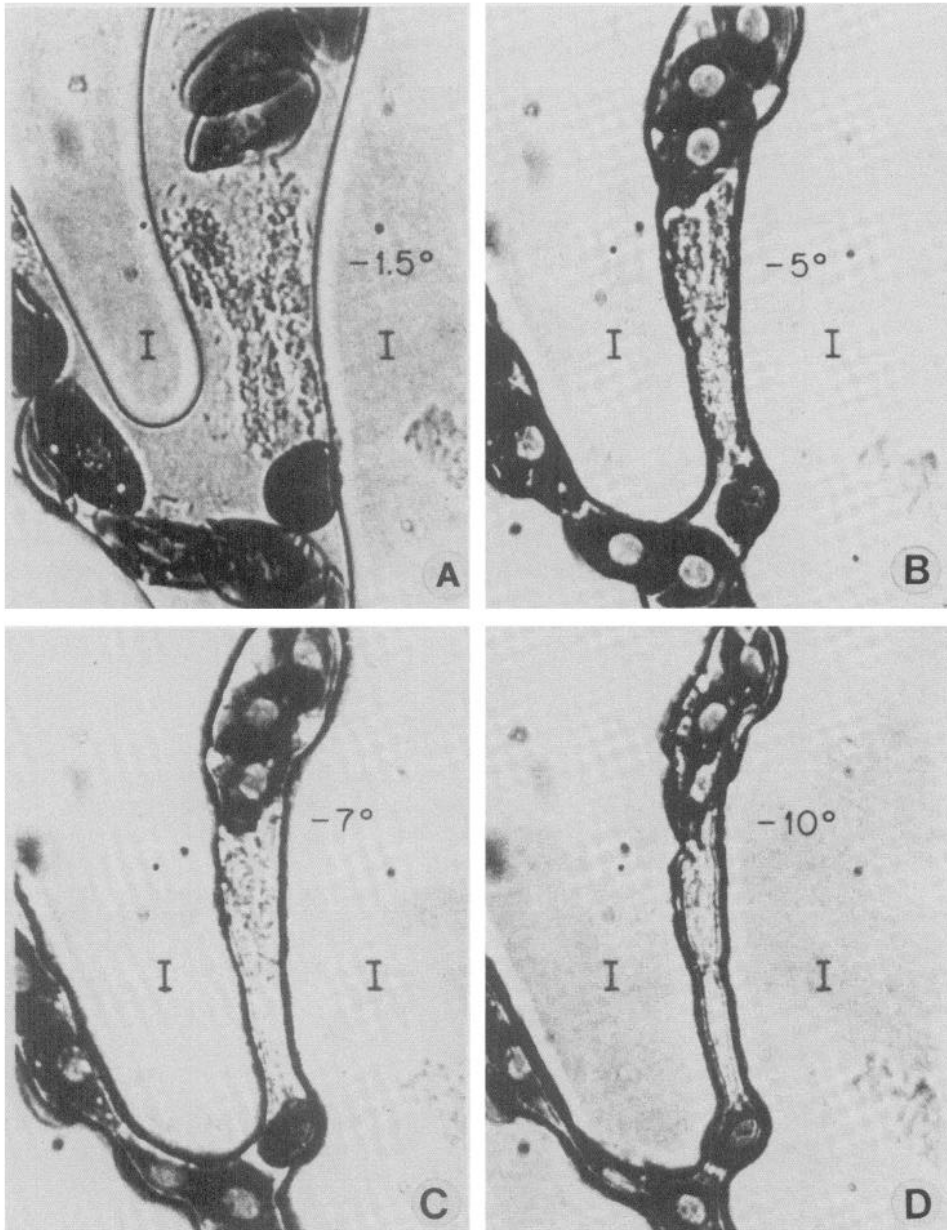
FIG. 15. Photomicrographs of frog erythrocytes in serum during course of slow freezing from −1.5 to −10°C. From Rapatz et al. (92). Note that cells are confined to channels of unfrozen solution between ice crystals (I), that channels decrease in diameter with decreasing temperature, and that cells shrink. Values of unfrozen water fraction are 0.23 at −1.5°C, 0.07 at −5°C, 0.05 at −7°C, and 0.04 at −10°C (64).

to hyperosmotic solutions during slow freezing become distorted and contorted, whereas cells subjected to hyperosmotic solutions in the absence of freezing shrink in an isotropic fashion (28, 35, 42, 110, 116). This distortion suggests that physical forces other than osmotic pressure are present during freezing, and it raises the possibility that cell injury may be a consequence of those forces. One candidate force is that the expanding ice field puts constraints on the shapes that can be assumed by the shrinking cells. This results in their deformation, and deformation at low temperatures may be damaging.

Dependence of Cell Survival on Cooling Rate, Additive Concentration, and Warming Rate

Although lowered temperature per se can cause cell injury in some cells under certain circumstances (75), low temperature injury in most cases is associated with ice formation. The cryobiologist is concerned with the response of cells to three major variables: cooling rate, type and concentration of protective additive, and warming rate. It may be helpful to illustrate these responses and to see the extent to which they are explicable in terms of the mechanisms just discussed. Figure 16 illustrates the general response to cooling rate; namely when cells are frozen to −60°C or below at various rates, a plot of survival vs. cooling rate usually is shaped like an inverted "U." That is, survival is optimum at some intermediate cooling rate, the value of which depends on the particular cell. Figure 16 graphically expresses two of the fundamental questions in cryobiology. *1*) Why is there an optimum cooling rate? Or conversely, why are cells injured when cooled at rates below and above the optimum? *2*) Why does the numerical value of the optimum rate vary so widely in different cell types?

Figure 17 illustrates the second set of responses; namely the left limb of the inverted U is influenced in a dramatic way by the concentration of protective additive.
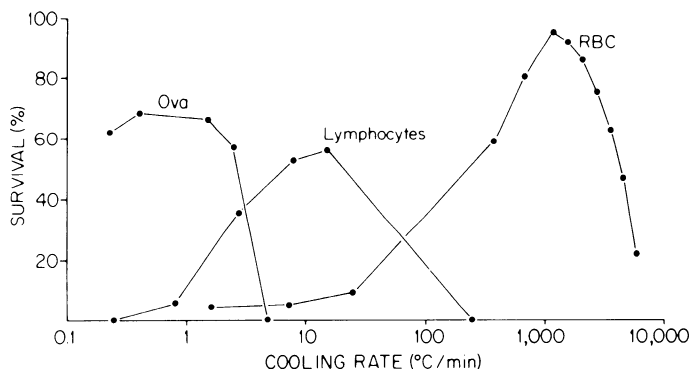
FIG. 16. Survival vs. cooling rate of 3 cell types frozen to −196°C in 0.7–1 M dimethyl sulfoxide. From S. P. Leibo (39a) from data of Leibo et al. (42) on mouse ova, Thorpe et al. (120) on human lymphocytes, and Morris and Farrant (76) and Rapatz and Luyet (91) on human red blood cells (RBC).
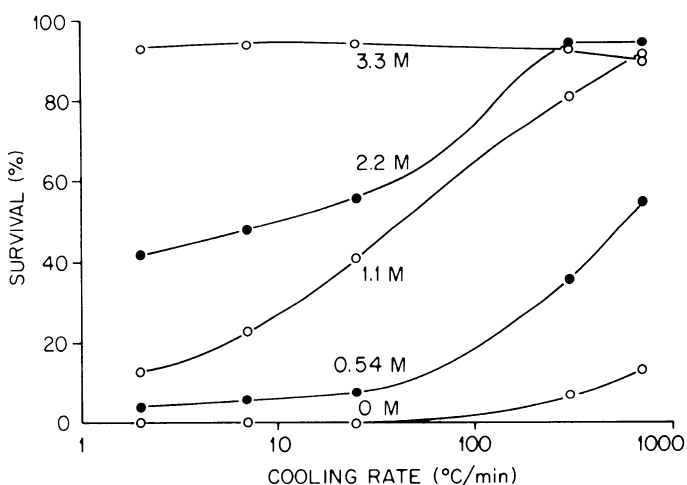

FIG. 17. Effect of various concentrations of glycerol on relationship between survival of human red blood cells and cooling rate. Reprinted from Ref. 57 by courtesy of Marcel Dekker, Inc. based on data of Morris and Farrant (76).

cooling rates that delimit the right limb differ by a factor of 1,000 in the three cells of Fig. 16 because the three cells differ in the ease with which they can dehydrate during cooling. They differ because of wide differences in L_p and its activation energy, in surface-to-volume ratios, and probably in the ice-nucleation temperatures. The limb is steeper when warming is slow because slow warming provides more time for the recrystallization of intracellular ice.

The simplest explanation for the existence of an optimum cooling rate is that survival is a consequence of two factors oppositely dependent on cooling rate. One factor is intracellular freezing, the probability of which increases with increasing cooling rate. The other factor is prolonged exposure to extracellular freezing, the injurious consequences of which decrease with increasing cooling rate. Steponkus (113) has recently argued that injury from intracellular freezing and injury from extracellular freezing are not separate factors but that "they represent a continuum of membrane destabilization, culminating in a common membrane lesion—mechanical breakdown." He bases this conclusion in part on microscopic observations suggesting that intracellular freezing in plant protoplasts is a consequence of a prior membrane defect and not the cause of the defect as argued here and in part on the observation that a high and constant fraction of the protoplasts are killed independent of cooling rate. Cooling rate merely affects the proportion that exhibit intracellular freezing (114). But his conclusion does not seem generally applicable. First, Siminovitch et al. (110) have shown that rye protoplasts cooled slowly in a salt solution to −12°C do not freeze intracellularly, and most survive, whereas those cooled rapidly to −12°C freeze intracellularly and are killed. Second, the survival of many intracellularly frozen cells, even some like yeast frozen in the absence of additives, can be raised by subsequently warming them rapidly (Table

As the concentration of additive (here glycerol) is increased, red blood cells become less and less sensitive to slow cooling. But the dependence of injury on glycerol concentration lessens as one approaches the optimum cooling rate and often disappears. At supraoptimal cooling rates it can even reverse in the sense that increasing concentrations of permeating additives like glycerol can cause cells to undergo lethal intracellular freezing at progressively *lower* cooling rates (16, 55, 91, 98).

The third set of responses, also clearly illustrated in the human red blood cell, is the interaction between cooling rate and warming rate shown in Fig. 18. When red blood cells are cooled faster than optimum, survival is considerably higher with rapid warming than with slow. But when the cells are cooled more slowly than optimum, the response is reversed—survival is considerably higher with slow warming than with rapid. A similar reversal has been found in early mammalian embryos (124). Slow warming is also less injurious to slowly frozen plant cells (44, 121).

The right limb of the inverted U and its dependence on warming rate is readily explainable in terms of the physical-chemical events discussed. It is a consequence of intracellular freezing. The numerical values of the
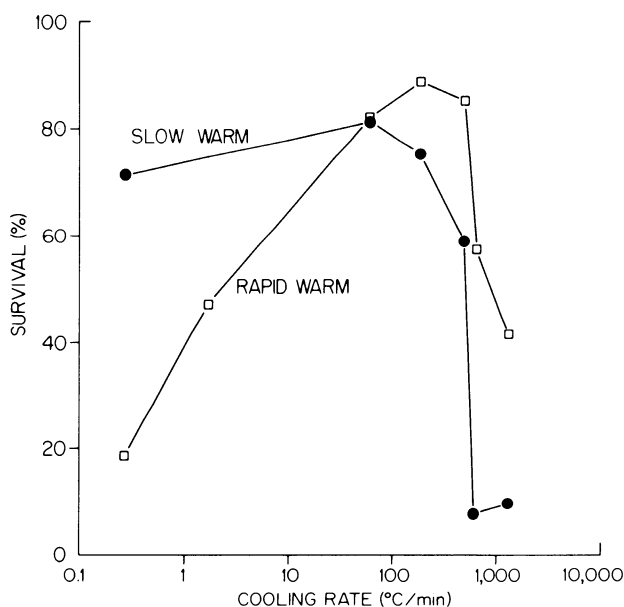

FIG. 18. Survival (% unhemolyzed) of human red blood cells suspended in 2 M glycerol, frozen at various rates to −196°C, and warmed slowly or rapidly. From Ref. 56 based on data of Miller and Mazur (73).

2). Lethal injury cannot have preceded internal freezing in those cells that can be rescued by the rapid warming. Third, it is unclear how a single continuum cause of injury can generate survival maxima in plots of survival vs. cooling rate such as those in Fig. 16 nor how it can explain the results in Fig. 18, where the dependence of survival on warming rate reverses as one moves from suboptimal cooling rates to supraoptimal cooling rates. Finally, it is unclear how a single continuum can explain the observation that increasing concentrations of protective additive increase the survival of cells cooled at suboptimal rates, have little influence on the survival of cells cooled at optimal rates (Fig. 17), and can actually decrease the survival of cells cooled at a given supraoptimal rate.

I have discussed the evidence that slow freezing injury may be primarily a consequence of the reduction in the volume of liquid surrounding the cells and secondarily a consequence of the increasing solute concentration in that volume. The protective effect of glycerol seen in Figs. 10 and 17 presumably arises because glycerol colligatively increases the volume of extracellular liquid at any temperature and concomitantly decreases the salt concentration in that volume. With sufficient cooling all liquid water must eventually vanish by conversion to either ice or glass. But apparently the reduction of the unfrozen fraction to values below those that are normally critical is relatively innocuous when it occurs below ~−45°C (see Figs. 9 and 10).

A number of unanswered questions remain. Why is cell survival dependent on the residual liquid volume? On the slow-cooling side of the optimum rate, why does survival rise progressively with increasing cooling rate? And why does the effect of warming rate reverse in slowly frozen cells (Fig. 18)?

Osmotic Injury During Thawing and During Return of Cells to Physiological Media

One suggested explanation of the last point, the sensitivity of slowly frozen cells to rapid thawing is that it induces a type of osmotic shock. There is evidence that additional additive may be driven into cells during slow freezing, a process referred to as solute loading (15, 24). If so, there may be insufficient time for the excess additive to diffuse back out when thawing is rapid, so the cells swell and lyse as the medium becomes abruptly diluted by the melting of extracellular ice (56).

The manner in which the plasma membrane accommodates to decreasing cell volume during slow freezing may also be important. As cells shrink, they will require less surface area to enclose the progressively decreasing volume of cytoplasm. Steponkus and his colleagues (114) have presented evidence that the accommodation in plant protoplasts is by the transfer of excess plasma membrane into the protoplast interior or by blebbing. But I am aware of no evidence that this phenomenon occurs during the shrinkage of animal cells. Indeed it seems an unlikely mechanism since, unlike plant protoplasts, the surface of many nucleated animal cells is highly convoluted by folds, pleats, and microvilli. Several measurements indicate that these folds provide 40–100%

more surface area than that required to enclose the volume of the isotonic cell (32, 104). Because the cell surface is so highly folded at isotonic volume, it should be capable of the slight additional folding required to adjust to hyperosmotic conditions like those attained during freezing. Indeed, Albrecht-Buehler and Bushnell (1) have found this to be the case in several animal tissue culture lines.

Still another mechanism whereby spherical cells could match their surface area to a reduction in volume is by undergoing distortion, for gross departures from spherical shape will increase the surface area needed to enclose a given volume. As noted earlier, most cells do in fact become highly distorted during slow freezing.

Osmotic shock can also kill cells when they are returned to isotonic medium. Most cells will contain equilibrium concentrations of cryo-protective additives prior to freezing and therefore subsequent to thawing. In some cases the permeation of additive is required for high survival (87, 119); in other cases, it is not required (59, 60) but is an inadvertent consequence of the time required to manipulate the cell suspensions prior to the onset of freezing. Thawed cells that contain additive will swell osmotically when they are returned to normal physiological media. But although the danger of osmotic damage should be self-evident, it was given little attention (except in human red blood cells) until recently. Now, however, slow removal of additive at appropriate temperatures has been found to be critical to obtaining high survivals of frozen-thawed rabbit, mouse, and cattle embryos (9, 41, 105, 122) and frozen-thawed lymphocytes and hematopoietic cells (14, 117, 120). The rate of additive removal is less critical in other cells, probably because their surface membrane reserve in the form of microvilli permits them to withstand a considerable increase in volume without exceeding the available surface area.

Freezing Injury—A Concatenation of Events

Part of the past difficulty in understanding freezing injury has been the tendency to consider it a single event. But from the time cells face the first extracellular ice to the time they are returned to physiological media they encounter a sequence of physical-chemical phenomena, any one of which is potentially lethal. Survival requires that *all* these potentially lethal events be avoided or nullified.

Freezing and Cell Membranes

I would like to reemphasize that the response of cells to freezing depends critically on the properties of cell membranes: the structure of the surface membrane allows cells to supercool and probably determines their ice-nucleation temperature. The nucleation temperature along with the permeability of membranes to water are the chief determinants of whether cells will equilibrate by dehydration or intracellular freezing. Furthermore, surface and internal membranes seem to be the chief targets of injury with both slow and rapid freezing (26, 115). Soluble enzymes are far more resistant. There is

evidence that changes in cell volume or distortion of cell shape can be damaging at subzero temperatures (36). Very possibly this reflects changes in the physical properties of membranes at low temperatures.

The permeability of the cell to protective solutes is important in two respects. In some cells the additive must be of sufficiently low molecular weight to permeate. And if it does permeate, its presence can introduce osmotic complications both during thawing and during its removal after thawing.

Most of this review has considered the cell to be a sack of dilute aqueous solution devoid of internal membrane-bounded components or other structure. It is surprising that despite this gross oversimplification the predicted responses of cells are in reasonable accord with observation. However, this may not be universally so. Armitage and Mazur (3, 4), for example, have suggested that the inability of human granulocytes to survive even moderately hyperosmotic solutions of both permeating and nonpermeating solutes (glycerol, sucrose, and NaCl) may reflect damage to intracellular organelles rather than the cell surface. Indeed, as cryobiology seeks a deeper understanding of the mechanisms of injury, it is likely to become increasingly concerned with the responses of intracellular components such as mitochondria, lysosomes, microtubules, and microfilaments.

Biological, Medical, and Agricultural
Implications of Frozen Cells

The implications and applications of frozen cells stem chiefly from the fact that storage temperatures below $-130°C$ effectively stop biological time. Perhaps the most far reaching of these implications are those of frozen mammalian embryos, first frozen successfully just over 10 yr ago by Whittingham et al. (123). Several laboratories are now maintaining stocks of mutant strains of mice in the form of frozen embryos, and a new industry has recently emerged in which the techniques of hormonally induced superovulation, freezing, and the transfer of the embryos to foster dams are combined to amplify 10-fold or more the number of calves produced by prize cows.

Embryo freezing could also be extremely valuable in the preservation of rare and endangered species, some of which exist only in zoos. Frozen embryos could be exchanged by zoos world-wide and thus eliminate the serious problem of intensive inbreeding. And on the horizon is the possibility to reincarnate, in a sense, a species that is totally extinct except for its frozen embryos by transferring these embryos to the uterus of a closely related species.

A second class of implications is the value of frozen cells as unchanging "yardsticks" in experimental biology and clinical medicine. For example, in studies on aging, cells and tissue samples that are removed from an animal when it is young and then frozen to $-196°C$ can serve as a precise reference against which to measure changes as the animal ages. The animals of course could include humans. As an extension, frozen embryos can provide precise standards for quantifying subtle processes like genetic drift. Thus, first generation mouse embryos could

be thawed years or decades after their parents had died and allowed to develop to term for direct comparison with the 10th or for that matter the 100th generation offspring.

The last aspect of frozen cells I would like to discuss is the implication of organs in clinical replacement therapy. Freezing offers a logical solution to the establishment of organ banks to facilitate coordinating donor availability and recipient need and to permit closer immunological matching, but unfortunately most whole organs thus far subjected to freezing below $-20°C$ have been nonfunctional after thawing or have quickly become so (30). Still, sufficient progress has been made to be optimistic about eventual success. Frozen-thawed fetal animal pancreases and mature islets of Langerhans, for example, are capable of restoring normality when transplanted into diabetic animals (61). One requirement for the successful freezing of cells is the ability to introduce and remove high concentrations of protective additives like glycerol, and Jacobsen and colleagues (29) have succeeded in perfusing mature rabbit kidneys with up to 3 M glycerol with little or no impairment of function. Unfortunately, however, the perfused organs do not yet survive freezing.

In the cases examined, organs as a whole are far more sensitive to freezing than are their component cells (2, 82). There are several possible reasons (57, 86). *1*) Organs are comprised of several types of cells. Each type may have different cryobiological requirements for the avoidance of injury. *2*) Organs are large and multicompartmented. As noted, the response of single cells to freezing depends importantly on diffusion-limited processes. Water must diffuse or flow out of cells during cooling to avoid intracellular freezing. Protective solutes must diffuse into cells prior to cooling and diffuse out of cells during and after thawing in ways that avoid injurious osmotic swelling. Fortunately, the diffusing unit in organs is far smaller than the whole organ. The unit probably consists of the cluster of cells nourished by neighboring capillaries. The capillary lumens are topologically "outside" of the cluster. But "outside" in this case is obviously far different than is "outside" for a suspension of single cells. For one thing, the length and tortuosity of the capillary system in organs introduces large impedances to the diffusion and flow of water and additives between the capillaries and the larger vessels. *3*) Cells in organs occupy a much higher percentage of the total volume than is generally the case with single cells in suspension. Nei (81) and Pegg and Diaper (84, 85) have shown that injury in frozen human red blood cells increases with increase in the initial cell concentration and becomes marked when hematocrits exceed 40%. This increase in injury may be related to the fact that an increase in cell concentration produces a decrease in the unfrozen fraction of extracellular liquid surrounding a given cell. And as discussed, a decrease in unfrozen fraction seems to be a major factor in slow freezing injury.

As cryobiology attempts to deal with the freezing of increasingly complex and sensitive cellular entities, success will increasingly depend not only on a better understanding of cryobiological fundamentals but on a better understanding of the relevant biology and physiology of

the cells in question. I hope that noncryobiologist readers of this article will become sufficiently intrigued by the implications of frozen cells to contribute to the latter.

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