

to despair may again be mitigated by a job market that, in the outcome, proves to be sufficiently large, even if it is one of changing character.

Disappointment and unmet expectations are a more subtle, but an equally real, problem. They exist on both sides—in some, perhaps many, doctoral recipients who have accepted jobs which utilize their advanced education but which are quite different from those they anticipated, and in employers who find in some new Ph.D.'s narrowness of view and lack of motivation for work outside their specialized research interests. Solution of this complex problem will require

examination of the graduate education process, the informal counseling between professors and graduate students, the expectations of employers, and the values that universities and the larger society attach to graduate education and academic scholarship. National organizations can play a useful role in stimulating a search for solutions that must, in the end, be developed for many different local situations.

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## Cryobiology: The Freezing of Biological Systems

The responses of living cells to ice formation are of theoretical interest and practical concern.

Peter Mazur

Freezing is lethal to most living systems; yet it can also preserve cells and their constituents, and it may some day permit the long-term storage of whole viable organs. It can slow or stop some biochemical reactions, but it accelerates others. It is used both to preserve the fine structure of cells and to disrupt cells. It is a challenge that is successfully met by some organisms in nature but not by others.

These paradoxes illustrate some of the divergent areas in biology in which low temperatures are of interest; other examples are listed in Table 1. But although the reasons for the interest are diverse and even paradoxical, all the areas are concerned with the ways in which biological systems respond to subzero temperatures and to the solidification of liquid water. My purpose in this article is to discuss some general

conclusions that can be drawn about these responses, some of the major questions that remain unresolved, and the way in which some of the various areas of cryobiological interest relate to one another.

### Events during Freezing

Although the freezing point of cytoplasm is usually above  $-1^{\circ}\text{C}$  (corresponding to osmolal concentrations below 0.5), cells generally remain unfrozen, and therefore supercooled, to  $-10^{\circ}$  or  $-15^{\circ}\text{C}$ , even when ice is present in the external medium (1). This indicates that the cell membrane can prevent the growth of external ice into the supercooled interior, and it further indicates that cells neither are, nor contain, effective nucleators of supercooled water. I return to these points toward the end of the article.

Since intracellular freezing is pre-

cluded above about  $-10^{\circ}\text{C}$  and since supercooled water has a higher vapor pressure than ice, cells begin to equilibrate by losing water. The resulting dehydration concentrates their solutes, thus lowering the intracellular aqueous vapor pressure. Subsequent events during cooling depend chiefly on the cooling velocity and on the permeability of the cell to water (2). If cytoplasm obeyed Raoult's law and if water did not flow out of the cell, the ratio of intracellular to extracellular vapor pressure ( $p_i/p_e$ ) would increase with decreasing temperature ( $T$ , in degrees Kelvin) according to the relation

$$\frac{d \ln (p_i/p_e)}{dT} = \frac{nv}{(V + nv)V} \frac{dV}{dT} - \frac{L_t}{RT^2} \quad (1)$$

where  $n$  is the number of osmoles of solute in the cell,  $v$  is the molar volume of water,  $V$  is the volume of water in the cell,  $L_t$  is the heat of fusion, and  $R$  is the gas constant.

But water will flow out of the cell in response to the vapor pressure gradient; and, if one assumes that the cell is permeable only to water, the rate of outflow will be

$$-\frac{dV}{dt} = \frac{L_p A R T}{v} \ln (p_i/p_e) \quad (2)$$

where  $t$  is the time,  $L_p$  is the permeability constant for water, and  $A$  is the area of the cell surface. If the cooling rate and temperature dependence of  $L_p$  are known, one can obtain simultaneous numerical solutions to Eqs. 1 and 2 to give the volume of water in a cell as a function of temperature, cooling rate, and permeability to water. Examples of such numerical solutions are

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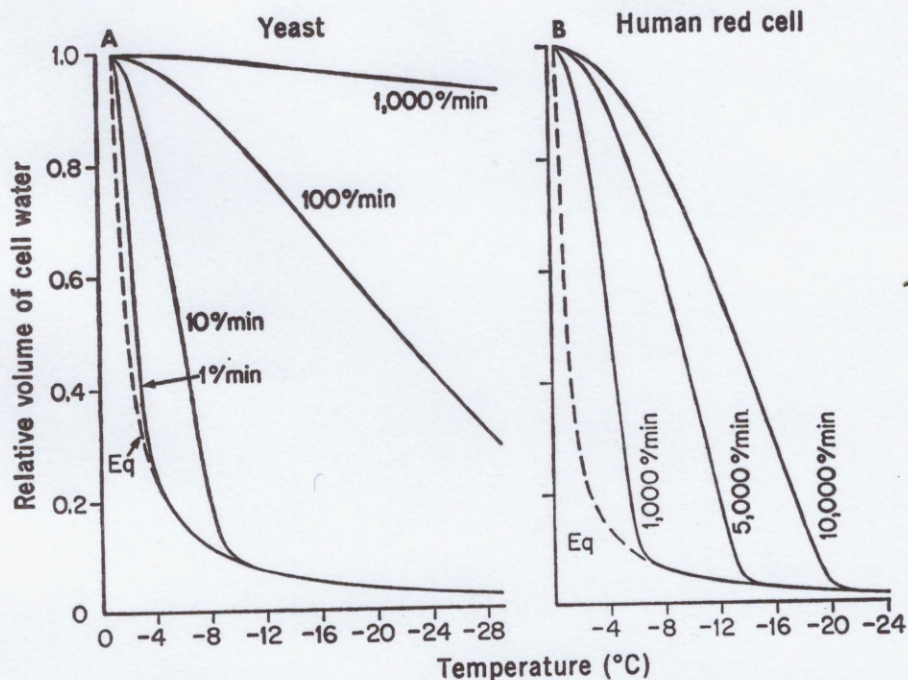


Fig. 1. Calculated fraction of intracellular water remaining in (A) yeast cells and (B) human red cells as they cool to various temperatures at the indicated rates. Curve *Eq* represents the equilibrium water content. [Data from (2) and (4)]

shown in Fig. 1 for yeast and the human red cell. The water content of a cell cooled infinitely slowly would follow curve *Eq*. *Eq* stands for equilibrium, indicating that such a cell would continuously maintain vapor pressure equilibrium with the external ice by dehydration. However, cells cooled at finite rates will contain more than the equilibrium amount of water at certain temperatures and will therefore be

supercooled. For example, according to Fig. 1A, yeast cells cooled at 100°C per minute will contain 70 percent of their original water at  $-15^{\circ}\text{C}$ , and that water would be supercooled 14 degrees. (The amount of supercooling is given by the number of degrees Celsius separating the point on the 100°C-per-minute curve from the point corresponding to the same water content on the equilibrium curve.) But such ex-

treme supercooling cannot occur, for cell membranes apparently lose their ability to block the passage of ice crystals below  $-10^{\circ}$  to  $-15^{\circ}\text{C}$  (1). As a result, cells that are cooled fast enough to contain supercooled water below these temperatures will in fact complete their equilibration by freezing intracellularly.

The numerical value for the critical cooling rate that produces internal ice depends on the ratio of the volume of the cell to its surface area and on its permeability to water. The critical rate should be lower for larger spherical cells and for those less permeable to water than for smaller or more permeable cells (1-3). Figure 2, for example, shows the calculated extent to which cell water could be supercooled at a given temperature in yeast and red cells cooled at various rates. The curves indicate that, at  $-15^{\circ}\text{C}$ , the yeast will contain supercooled water when cooled faster than  $10^{\circ}\text{C}$  per minute and the red cells will contain supercooled water only when cooled faster than  $5000^{\circ}\text{C}$  per minute (4). Since cell water cannot usually remain unfrozen at  $-15^{\circ}\text{C}$ , yeast and red cells should undergo intracellular freezing when cooled faster than  $10^{\circ}\text{C}$  and  $5000^{\circ}\text{C}$  per minute, respectively. These predictions have been confirmed by measurements of the volumes of frozen or freeze-substituted cells cooled at various rates (5). (Freeze-substitution is the process whereby the water in cells is replaced at subzero temperatures by an organic solvent such as ethanol or acetone.) They have also been confirmed by the presence of numerous small holes in red cells that have been cooled faster than  $5000^{\circ}\text{C}$  per minute and freeze-substituted or freeze-dried. The holes presumably represent the prior location of intracellular ice (6). The much higher critical cooling velocity for the red cell ( $5000^{\circ}\text{C}$  per minute as compared to  $10^{\circ}\text{C}$  per minute) is due to its high permeability to water, to the low temperature coefficient of that permeation, and to the high surface-to-volume ratio of the cell (2).

Equations 1 and 2 also successfully predict the occurrence of intracellular freezing in sea urchin eggs cooled faster than  $1^{\circ}\text{C}$  per minute (2, 7), and they are qualitatively consistent with the finding that rapid cooling produces intracellular ice in blood cells of frog and fish, in ascites cells, and in bacteria, whereas slow cooling does not (8, 9, 9a).

Regardless of whether cells equi-

Table 1. Areas of biology concerned with freezing and low temperatures.

Subject	Area of interest	Reference
General	Preservation of cells, tissues, virus, and organelles	(19, 21)
Electron microscopy and histochemistry	Freeze-cleaving and freeze-substitution	(99, 100)
	Scanning microscopy	(101)
Biochemistry and molecular biology	Disruption of cells	(102)
	Preservation and inactivation of enzymes	(97)
	Trapping of unstable intermediates	(103)
	Acceleration of reactions	(104)
	Protein conformation and denaturation	(97, 98)
Biophysics, photobiology, photochemistry, radiation biology, photosynthesis, x-ray diffraction	Separating excitation and ionization phenomena from thermal phenomena; reduction of thermal motion	(105)
	Electronic phenomena in photosynthesis	(79)
	Mechanism of ultraviolet action	(80)
	Absorption spectra	(106)
	Cell membranes and permeability; osmotic forces; cell water	(see text)
Ecology and environmental physiology, plant physiology, agriculture	Frost injury and frost hardiness	(40, 41), (94, 107)
	Ecology and physiology of arctic and subarctic flora and fauna	(86)
	Water movement in plants	(108)
Space sciences	Exobiology and planetary quarantine	(88)
Food sciences	Preservation of food	(109)
	Microbial contamination	(77)
Cryosurgery	Destruction of diseased tissue; formation of scar tissue	(74)

brated by the outflow of water or by intracellular freezing, they are subjected to a second class of physical-chemical events—events associated with the removal of liquid water and its conversion to ice: As the temperature decreases, the amount of cell water decreases, extra- and intracellular solutes concentrate, solutes precipitate as their solubilities are exceeded (thus changing pH), and all solutes precipitate below the eutectic point of the system (8, 10, 11). These events, which I refer to here as “solution effects,” are functions of temperature alone in a given cell suspension at constant pressure, provided, of course, that the cell remains at a given temperature long enough to attain vapor pressure equilibrium (4). For example, in an ideal solution, the osmolal concentration of solutes in a partially frozen solution is

$$m \approx \frac{273 - T}{1.9} \quad (3)$$

and the fraction of water frozen is

$$q \approx \frac{1.9M_0}{273 - T} \quad (4)$$

where  $M_0$  is the osmolal concentration of the solution prior to freezing. Although the rate of cooling does not affect the equilibrium concentration of solutes or the fraction of water frozen at a given temperature, it obviously *does* affect the length of time the cells will be exposed to these solution effects. Slower cooling means longer exposure.

The first and still most complete documentation of these solution effects is that by Lovelock (12, 13). He showed that the hemolysis produced in red cells frozen to a given temperature and then thawed was comparable to that produced in cells at room temperature when they are first exposed to a concentration of sodium chloride equal to that in the frozen sample and are then diluted to isotonic saline. The threshold deleterious concentration of 0.8M occurred at  $-3^\circ\text{C}$ .

One might expect that cells cooled below their eutectic point would not be subjected to further stress. But this is not necessarily so. Reactions can occur in the solid state. One of the more striking of these is recrystallization or grain growth. Small convex ice crystals have higher surface energies than large flat crystals and so tend to reduce their surface energies by growing or by fusing with other small crystals. Recrystallization occurs in pure water at temperatures as low as  $-100^\circ\text{C}$ , and it occurs in gels and concentrated aqueous solu-

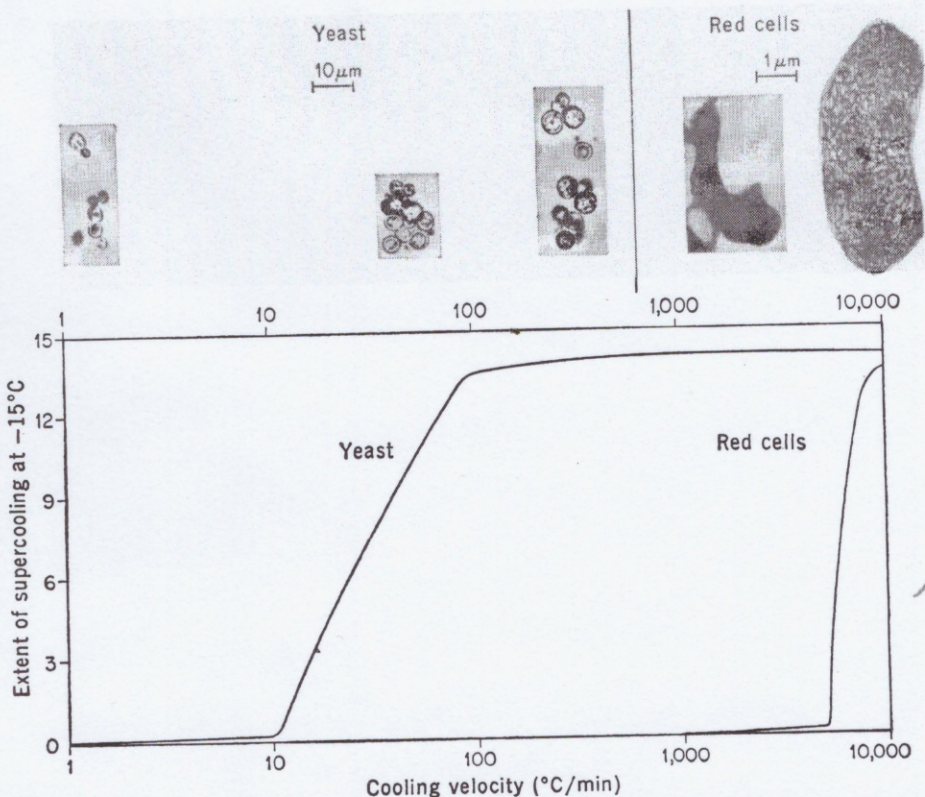


Fig. 2. Effect of cooling velocity on (i) the calculated number of degrees that the water in yeast and red cells would be supercooled at  $-15^\circ\text{C}$  (4) and (ii) the size and morphology of freeze-substituted yeast and freeze-dried rabbit red cells. The photomicrographs are positioned opposite the approximate cooling rates used. [Photographs from Mazur (5) and Nei *et al.* (6)]

tions at higher temperatures (14). Its rate depends on both the size of the crystals formed during cooling and the subsequent temperatures to which they are exposed; the rate is higher in smaller crystals and at higher temperatures (8, 15). As a result, since rapid cooling produces small ice crystals, the crystals that form within rapidly cooled cells will not only be small, they will also tend to recrystallize during warming, especially if warming is slow. An example is shown in Fig. 3.

In summary, when cells are subjected to subzero temperatures, they initially supercool. The manner in which they regain equilibrium depends chiefly on the rate at which they are cooled and on their permeability to water. If they are cooled slowly or if their permeability to water is high, they will equilibrate by the transfer of intracellular water to the external ice—in other words, they will equilibrate by dehydration; but if they are cooled rapidly or if their permeability to water is low, they will equilibrate, at least in part, by intracellular freezing. Rapid cooling not only produces intracellular crystals, it also produces small crystals, which are likely to enlarge during warming because of

their high surface free energies. But, regardless of whether cells equilibrate by dehydration or by intracellular freezing, freezing exposes them to loss of liquid water and to increases in the concentration of intra- and extracellular solutes.

Equations 1 and 2 permit one to quantify the terms “slow” and “rapid” cooling and to estimate how a given cell will equilibrate when cooled at various rates. These estimates are in reasonable agreement with experimental observations on yeast, sea urchin eggs, and red blood cells, but more general statements are precluded by the dearth of information on other cells. Solutions to the equations require numerical values for the permeability of the cell to water and the temperature coefficient of that permeability. Unfortunately, values for the latter are known for few cells above  $0^\circ\text{C}$  (16) and for no cell below  $0^\circ\text{C}$ . Nor is information available on how the permeability of a cell to water will be affected by interactions of the low temperatures and high electrolyte concentrations produced by freezing. Studies on phase transformations in model lipid-water and protein-lipid-water systems suggest that interactions are possible (17). But even if

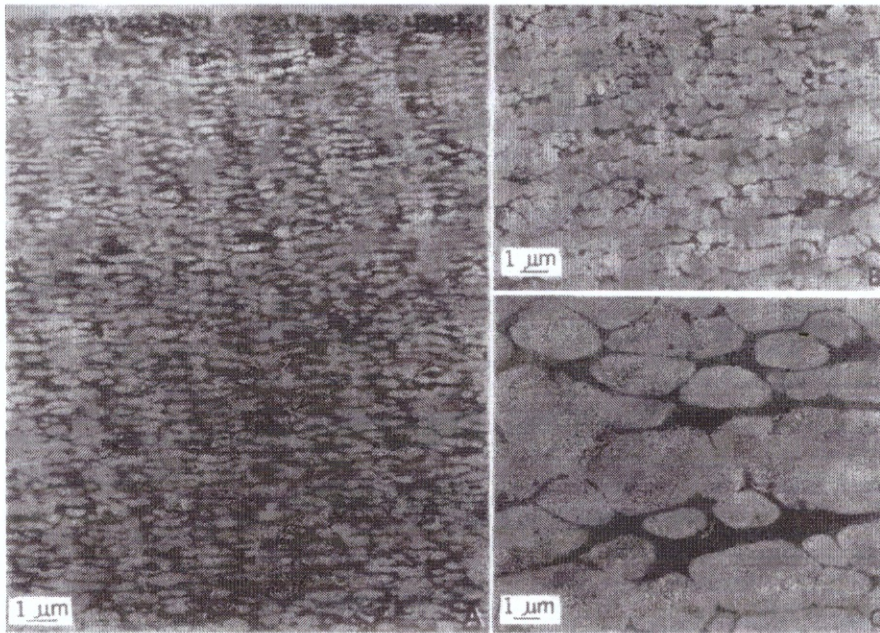


Fig. 3. Electron micrographs of freeze-dried muscle fibers. (A) Fiber frozen rapidly to  $-190^{\circ}\text{C}$  and freeze-dried at  $-60^{\circ}\text{C}$ ; (B) fiber frozen at a moderate rate to  $-60^{\circ}\text{C}$  and freeze-dried at  $-60^{\circ}\text{C}$ ; (C) fiber frozen rapidly to  $-150^{\circ}\text{C}$ , warmed to  $-15^{\circ}\text{C}$  for 1 minute, and then freeze-dried at  $-60^{\circ}\text{C}$ . [From (110)]

numerical values were available to permit estimates of the fate of water in other cells during cooling, there are few precise experimental observations to test the accuracy of such estimates. What are needed are (i) measurements of cell size as a function of cooling rate and temperature, and (ii) electron micro-

scopic observations on the relation between cooling rate and the occurrence of intracellular ice.

The fact that Eqs. 1 and 2 predict the events in yeast and red cells with fair accuracy is rather surprising in view of the several simplifying assumptions that underlie their derivation (2).

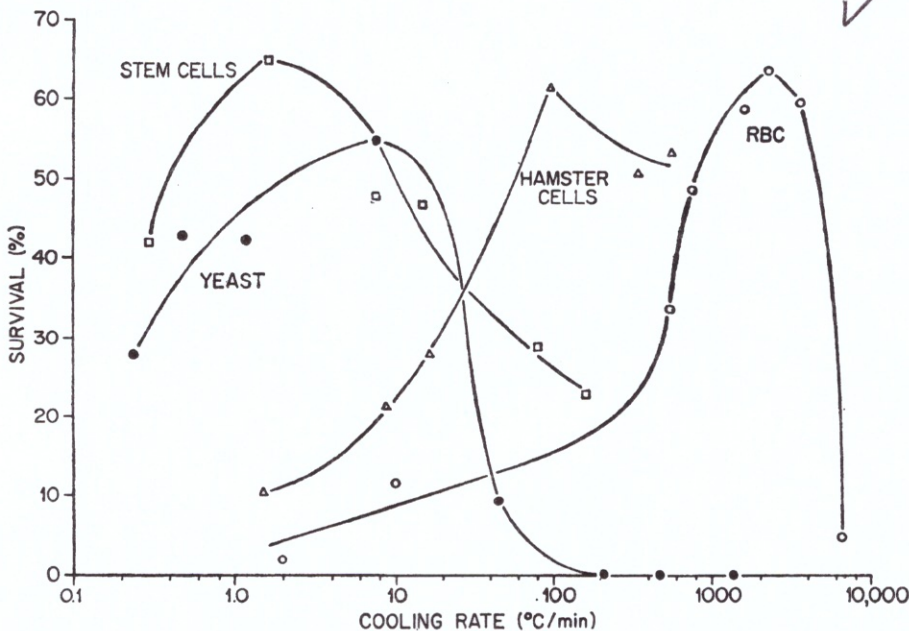


Fig. 4. Comparative effects of cooling velocity on the survival of various cells cooled to  $-196^{\circ}\text{C}$  and thawed rapidly. The yeast (22) and human red cells (RBC) (54) were frozen in distilled water and blood, respectively. The marrow stem cells and hamster cells were suspended in balanced salt solutions containing 1.25M glycerol. [From (45)]

One of these is that cytoplasm behaves like an ideal dilute solution throughout freezing. Another is that all equilibration results from the movement of water alone. Farrant and Woolgar have begun to test the consequences of eliminating the first assumption; that is, they are introducing activity coefficients into Eqs. 1 and 2 (18). But the extent to which the accuracy of the equations would be improved by eliminating the second assumption has not yet been evaluated.

### Events Affecting Cell Survival

Since cooling velocity affects the physical-chemical events in cells during freezing, it is not surprising that it also affects their survival. Figure 4 shows results for four diverse types of cells frozen to  $-196^{\circ}\text{C}$ : yeast, marrow stem cells, hamster tissue-culture cells, and human erythrocytes. In no case is the effect of cooling rate trivial, and in all cases the relation between cooling velocity and survival is similar in that maximum survival occurs at an optimum rate. However, the specific value of the optimum rate varies over a wide range, from  $1.6^{\circ}\text{C}$  per minute for marrow stem cells to about  $3000^{\circ}\text{C}$  per minute for the human erythrocyte.

There has been a tendency among investigators to consider freezing a single stress; but the existence of an optimum refutes this view, for it indicates that survival is affected by at least two factors oppositely dependent on cooling velocity. Another significant feature of the curves of Fig. 4 is that they raise doubts about the general validity of the widely accepted practice of cooling cells at  $1^{\circ}\text{C}$  per minute, a practice that has become the sine qua non of cryobiology, partly for historical reasons [Smith and her colleagues determined that this rate was optimal for bull spermatozoa (19, 20)], partly because it yields high survival of some cells, and partly because it yields sufficient cell survival for the purpose of preserving cultures (19, 21). Sometimes, however, as discussed below, it leads to failure.

The suggestion has been made that an optimum cooling rate arises because of the interaction of the two classes of freezing events just discussed (4). According to this suggestion, solution effects are responsible for injury when cooling is slower than optimal, and intracellular freezing is responsible for injury when cooling is faster than opti-

mal. The optimum rate, then, is a rate that is slow enough to prevent production of intracellular ice and yet is rapid enough to minimize the length of time cells are exposed to solution effects. This hypothesis was originally developed to explain the survival curves of yeast and red cells, but it now appears to be consistent with survival data on nucleated mammalian cells and cells of higher plants as well.

The evidence for the hypothesis is quite persuasive in the case of sea urchin eggs (2, 7), yeast (22–24), and red cells (25). For example, we saw in Fig. 2 that cooling rates above  $10^{\circ}$  and  $5000^{\circ}\text{C}$  per minute ought to produce intracellular freezing in yeast and red cells, respectively; and volume measurements and electron microscopy indicate that they do in fact do so. We now see in Fig. 4 that cooling rates of  $7^{\circ}$  and  $3000^{\circ}\text{C}$  per minute produce maximum survival of yeast and red cells, respectively, and that higher rates result in abrupt killing. Thus, cooling velocities that are above the optimum in terms of survival cause intracellular freezing, and, conversely, cooling rates that are below the optimum cause the cells to dehydrate without the production of intracellular ice. Rapid cooling is also more damaging than slow cooling to many other cells and organisms, but in these others the correlation between damage and the presence of intracellular ice has not been investigated (8, 19, 21).

As mentioned above, the ice formed in cells as a result of rapid cooling is likely to grow by recrystallization during warming, especially if warming is slow. This sequence of events is believed to be the explanation of the curves in Fig. 5, which show that rapidly cooled hamster cells exhibit much lower survival after slow warming than after rapid. Data of this sort have also been published for bacteria, yeast, filamentous fungi, rotifers, mulberry leaves, and marrow stem cells (8, 22, 26–28), and they suggest that the lethal event in rapidly cooled cells is the growth of intracellular ice crystals rather than their initial formation. Microscopic observations on ascites tumor cells (29) and mulberry leaves (30) support this supposition. The damage observed during warming can occur very rapidly. For example, the survival of rapidly cooled yeast cells decreases 40-fold when the time taken to warm them from  $-70^{\circ}$  to  $0^{\circ}\text{C}$  is increased from 0.001 minute to 0.06 minute

(22). Damage is equally rapid in mulberry leaves (28).

Although most cryobiologists agree that extensive formation of large intracellular ice crystals is always lethal (8, 29, 31, 32), there may be exceptions, such as Sherman has reported in organized tissues and ascites cells (33). However, Sherman's observations on ascites cells disagree with Asahina's (9a, 29).

So much for the killing of cells cooled at a rate faster than optimum. The best evidence that the cells cooled at a rate slower than optimum are killed by the other class of events—solution effects—is still that provided by Lovelock's 17-year-old study on the human erythrocyte (12, 13). In addition to finding that the hemolysis produced by freezing was explicable in terms of the concentration of electrolytes, Lovelock also found that the amount of hemolysis increased abruptly when the time between  $-3^{\circ}$  and  $-40^{\circ}\text{C}$  exceeded about 5 seconds during cooling or during warming. The damage from slow warming could not have been a manifestation of recrystallization, for it occurred when cooling was too slow to produce intracellular ice. Slow warming is also damaging to slowly cooled nucleated cells under some conditions (Fig. 5, B and D). However, in other instances it is not (Fig. 5, A and C). The lack of effect is presumably due to the presence of certain protective additives, the role of which is discussed below.

## Mechanisms of Damage

The molecular bases of freezing damage are not understood. This is partly because few workers have clearly realized that damage is caused by two independent factors—a failure that can lead to misleading conclusions. For instance, red cells and yeast cells cooled to  $-196^{\circ}\text{C}$  at  $100^{\circ}\text{C}$  per minute both survive poorly (Fig. 4), but for entirely different reasons. The red cells are killed by solution effects, and the yeast, by intracellular freezing. But even in those cases where it is possible to decide which of the two factors is responsible for injury, the mechanism of injury remains obscure. One problem in understanding solution effects arises from the fact that they include at least four discrete events: during freezing, (i) water is removed as ice, (ii) solutes of high and low molecular weight concentrate, (iii) cell volume decreases, and (iv) solutes precipitate. Each of these events has been the basis of hypotheses of injury (12, 13, 31, 34–36), but, unfortunately, all of them, except for the precipitation of solutes, are monotonic functions of temperature and, therefore, all of them occur simultaneously during freezing. For example, the only way to increase solute concentration in a partly frozen solution at constant pressure is to lower the temperature (Eq. 3); but lowering the temperature will simultaneously decrease the amount of residual liquid water

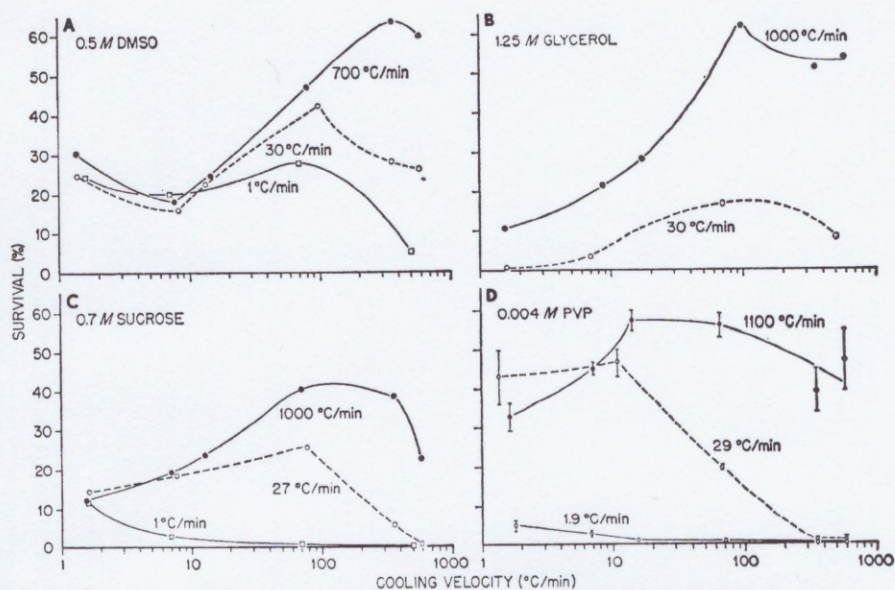


Fig. 5. Survival of Chinese hamster cells as functions of the type of additive, the cooling velocity, and the warming velocity. The cells were cooled to  $-196^{\circ}\text{C}$  prior to warming at the rates shown on the individual curves [see (56) for details]. [Mazur, Leibo, and Chu, unpublished data]

(Eq. 4), the space between macromolecules, and the cell volume.

Lovelock suggested that the high concentrations of electrolyte produced by freezing affect membrane lipids so as to make cells leaky. The cell, as a result, becomes engorged with cations and undergoes osmotic shock because of the inflow of water during thawing (12, 37). One difficulty is that Lovelock gave no mechanism to account for a net increase in the amount of solute entering the leaky cell, a condition that would be required for subsequent osmotic shock. Meryman has recently suggested a mechanism (36). He proposes that damage results not from concentrated electrolytes per se but from the inability of the red cell to shrink below 55 percent of its normal volume, even when the hypertonicity of the surrounding medium reaches levels that would require a perfect osmometer to continue shrinking by water loss. The result, he says, is the establishment of a transient pressure gradient across the membrane, which in turn causes the membrane to become leaky. The cell then equilibrates by the movement of solute into it, and undergoes osmotic lysis during subsequent thawing. The validity of this hypothesis depends on whether a true hydrostatic tension can form across the red cell membrane, for only by this means is it possible to achieve equality of the chemical potentials of intracellular and extracellular water in the face of unequal osmotic pressures (38). The situation would be analogous to that observed when higher plant cells are placed in water, except that the plant cell possesses a rigid wall which can establish the necessary hydrostatic or turgor pressure.

Both Lovelock and Meryman primarily ascribe the ultimate cause of hemolysis to osmotic shock during thawing. If they are correct, hemolysis ought to be reduced by slow thawing, for slow thawing would produce slow dilution, which in turn would permit a thawing cell to equilibrate partly by the outflow of solute rather than only by the osmotic inflow of water. Slow thawing and slow dilution do protect the osmotic-shock-sensitive bacteriophage T4B when it has been exposed to high concentrations of solute at room temperature or during freezing (39), but they do not reduce hemolysis of red cells. In fact, Lovelock found that slow thawing increases hemolysis (12). Nor does slow thawing protect slowly frozen microorganisms and nucleated mammalian cells (8, 22, 26, 27); as in the

Table 2. Survival of Chinese hamster cells frozen slowly in Hanks balanced salt solution containing various additives (thawing was rapid).

Additive	Percentage of survival* after cooling	
	1.6°C/min	8°C/min
0.5M glycerol	2	5
1.25M glycerol	10	20
0.35M sucrose	18	25
0.5M dimethyl sulfoxide	30	18
0.004M polyvinylpyrrolidone	32	45

\* Percentage relation between the number of viable cells in frozen suspensions and the number of viable cells in unfrozen controls. [From (45) and (56)]

case of red cells, it is usually more damaging (see Fig. 5). The only major exception to these findings occurs in higher plants (40), but in this case the protective effect of slow thawing may have more to do with osmotic events taking place between the cell wall and the plasmalemma than with events in the protoplast or at its surface (41). Levitt has suggested that freezing damage in higher plants is primarily due, not to osmotic events, but to the formation of disulfide bonds as the result of compaction of macromolecules produced by dehydration during freezing (34, 40). Although intriguing, the hypothesis at present lacks unequivocal supporting evidence (41).

Our understanding of the basis of injury from the second factor, the formation and recrystallization of intracellular ice, is even more uncertain. I have speculated that the injury occurs because recrystallizing ice crystals exert sufficient force to rupture plasma membranes or the membranes of organelles such as mitochondria (8, 24). Part of the rationale for this suggestion is that recrystallizing ice crystals can disrupt protein gels, and part of it is that cells killed by intracellular freezing have suffered membrane damage and become leaky (1, 42). This latter argument, however, is not persuasive, since membrane damage also occurs in cells and organelles inactivated by extracellular freezing (43, 44).

#### Action of Protective Additives

Certain unprotected cells (for example, red cells, most microorganisms, and a few nucleated mammalian cells) can survive freezing in water or in simple salt solutions if they are cooled at optimum rates. Often, however, a cool-

ing rate low enough to prevent intracellular freezing is also low enough to produce lethal exposure to solution effects. Under these conditions there is no optimum rate (8, 45). This seems to be the case for most nucleated mammalian cells (32). For example, fewer than 2 percent of mouse marrow cells in a balanced salt solution survive freezing at cooling velocities ranging from 0.3° to 600°C per minute (26). On the other hand, they will survive if a protective additive such as glycerol is present. Once again, maximum survival occurs at an optimum rate, but the numerical value of this rate depends on the concentration of glycerol used (Fig. 6A).

The protective effect of glycerol was discovered by Polge, Smith, and Parkes in 1949 (46), and glycerol remained the most effective of additives until Lovelock and Bishop demonstrated protection by dimethyl sulfoxide (DMSO) in 1959 (47). However, other solutes, chiefly sugars and macromolecules such as polyvinylpyrrolidone (PVP), can protect some cells under certain conditions (48).

Most additives appear to protect against solution effects rather than against intracellular freezing. Thus, we see in Fig. 6 that increases in the concentration of glycerol raise the survival of slowly cooled stem cells more than that of rapidly cooled cells. Increases in glycerol concentration also increase the recovery of slowly cooled cow and human red cells. But in this case they actually decrease the recovery of rapidly cooled cells (25, 49, 50).

Lovelock demonstrated that glycerol and dimethyl sulfoxide protect on a molar basis, and he suggested that they act by reducing the electrolyte concentration in the residual unfrozen solution in and around a cell at any given temperature (13, 47, 51). The total mole fraction of solute in a partially frozen solution at constant pressure is determined by temperature alone. If all the solutes are electrolytes, then the required mole fraction will consist entirely of electrolytes. But if a nonelectrolyte such as glycerol is present, the concentration of electrolyte will be reduced, and the extent of reduction at a given temperature will be approximately proportional to the osmolar ratio of glycerol to electrolyte in the initial suspension. Lovelock concluded that only low-molecular-weight hydrophilic solutes with low eutectic points could protect cells, because only such solutes could yield solutions of high molar concentration at

sufficiently low temperatures. He also stated that, to be protective, a solute must permeate a cell, for otherwise it would not prevent a rise in intracellular electrolyte during freezing. Meryman has reached similar conclusions about additives on the basis of his hypothesis ascribing solution-effect damage to osmotic forces (36).

Although protection by glycerol, dimethyl sulfoxide, and most other low-molecular-weight solutes is explicable on the basis of molarity and solubility (51, 52), protection by macromolecules is not; and the number of examples of such protection is accelerating. Doebbler and Rinfret found that red cells suspended in solutions of polyvinylpyrrolidone, polyethylene glycol, serum albumin, and dextran will survive freezing if the cooling rate is 300°C per minute (53). Protection at that cooling rate was correlated, not with the molarity of the additive, but with the molarity of the potential hydrogen-bonding groups on the solutes. Although macromolecules do not protect red cells as effectively as do glycerol and dimethyl sulfoxide when cooling is much slower than 300°C per minute (32, 54), they do protect marrow and spleen cells (26, 55) and hamster tissue-culture cells (45, 56). It can be seen from Table 2 and Fig. 5 that 0.004M polyvinylpyrrolidone (15 percent, weight to volume; molecular weight, 40,000) protects slowly frozen hamster cells as well as does 0.5M dimethyl sulfoxide and far better than does 1.25M glycerol. Peptides isolated from peptone also protect various microorganisms (57), but the most dramatic case of protection by a macromolecule is Heber's finding that a protein with a molecular weight of 10,000 can protect chloroplast membranes from freezing injury in concentrations as low as 0.05 percent (~10<sup>-5</sup>M) (58).

Although these findings argue strongly against ascribing protection to colligative effects, they do not absolutely exclude such effects as a factor. Farant and Woolgar have shown that a 30 percent solution of polyvinylpyrrolidone with a molecular weight of 40,000 has a much greater freezing point depression than one would expect from its gravimetric molarity (59). In fact, the effective molarity is sufficiently high to depress the calculated mole fraction of electrolyte in a partly frozen solution as extensively as do much higher molar concentrations of glycerol. However, there is a possible alternative interpretation—namely, that

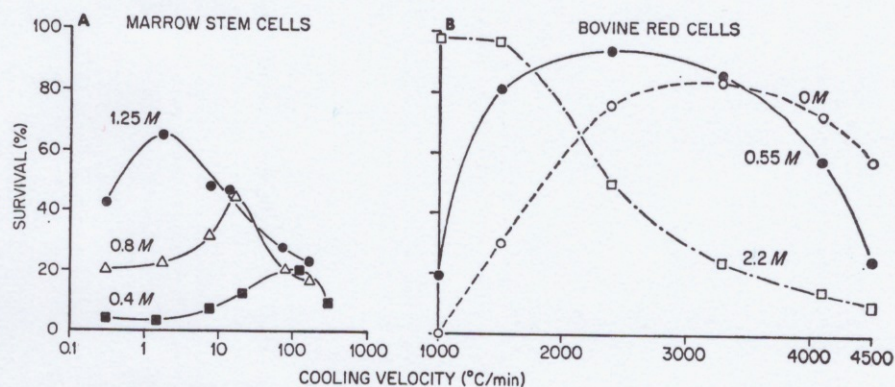


Fig. 6. Effect of cooling velocity on the survival of mouse marrow stem cells and bovine red cells suspended in media containing glycerol in the concentrations indicated. [Curves for the marrow stem cells, from (26); curves for bovine red cells, from (25)]

the anomalously high freezing point depression by polyvinylpyrrolidone results from the preferential binding of water by the macromolecule. Such binding would reduce the amount of free water available for the solution of electrolytes and would actually raise their concentration in the residual unbound water.

Other authors have suggested that the ability of macromolecules to protect is somehow related to their ability to form hydrogen bonds (52) or to stabilize clathrate structures (60), but these suggestions remain speculative, even though infrared spectroscopy suggests that polyvinylpyrrolidone does in fact enhance water structure (61).

The protection by macromolecules also raises questions about Lovelock's and Meryman's conclusion that protection requires permeation. Penetration of more than trace quantities of molecules with molecular weights greater than 10,000 into cells seems unlikely. Uptake by pinocytosis is a possibility, but Persidsky and Richards claim that marrow cells at 0°C take in little polyvinylpyrrolidone (62). The matter of permeation also arises in the case of protection by sucrose. Table 2 shows that 0.35M sucrose protects slowly frozen hamster cells better than does 0.5M glycerol, and yet measurements of the volumes of cells exposed to a hyperosmotic concentration of sucrose indicate that sucrose does not penetrate (56). Sucrose also completely protects slowly frozen chloroplast membranes (43, 58), and partially protects red cells (50, 53, 54), from solution effects in spite of its inability to permeate (63), and it protects marrow stem cells even when the cells have been in contact with it for only 2 or 3 minutes prior to freezing (26).

There is even some doubt whether glycerol has to permeate in order to

protect. It is true that Lovelock found that the recovery of frozen human red cells was poorer when glycerol permeation was blocked by copper ions than when it was not (63 percent recovery, as compared to 97 percent), but it was far better than the 2 percent recovery obtained in the absence of glycerol (13). Moreover, bovine red cells exposed to a 10-percent solution of glycerol for 30 seconds survived freezing just as well as did human red cells, even though the former are about 30-fold less permeable to glycerol (47, 63, 64). And, finally, frozen mouse eggs undergo less structural damage when they have been equilibrated with glycerol at 5°C rather than at 37°C, even though measurements of the volumes of the eggs indicate little or no glycerol permeation at 5°C (65). These results suggest that the ability of a cell to survive freezing may depend more on protection of the cell surface than on protection of the cell interior. Perhaps the cell interior is protected by the high concentrations of macromolecules normally within it.

I turn now from a general analysis of freezing events and mechanisms to some brief comments on some specific biological problems involving freezing, and their implications.

### The Freezing of Organs

The transplantation of unpaired organs such as hearts will remain a surgical and medical tour de force until satisfactory methods of avoiding immunological rejection are developed. But solving that problem will not reduce the logistic problem of providing a donor organ of the correct antigenicity at the time and place needed. In fact, it may only intensify the logistic problem.

Freezing offers a logical solution to

the difficulty of coordinating donor availability and recipient need, but, unfortunately, all whole organs thus far subjected to freezing below  $-20^{\circ}\text{C}$  have been nonfunctional after thawing, or have quickly become so (66). The successful freezing of organs will undoubtedly require more than the ability to freeze the component cells successfully, but even this more limited objective may be difficult to achieve. Abbott, for example, assayed the viability of frozen and thawed kidney slices by subjecting them to a battery of enzymatic and histochemical tests. The results indicate that slices frozen under conditions that are usually considered optimal fared little better than those frozen in the absence of protective additives, a condition that probably yielded few viable cells (67). Isolated myocardial cells can also be difficult to freeze. For example, those from chick embryos survive poorly (68), although those from newborn rats survive well (69).

Most attempts to freeze organs have involved procedures similar to those presumed to be optimum for cell suspensions; namely, the organ is perfused with a protective additive (usually dimethyl sulfoxide) and frozen at a cooling rate of about  $1^{\circ}\text{C}$  per minute. The procedure is probably doomed to failure. One problem is the matter of scale-up. Solutions to Eqs. 1 and 2 indicate that larger spherical cells or cell aggregates will freeze intracellularly at lower cooling velocities than smaller cells. They indicate, for example, that if a cell 6 micrometers in diameter, such as yeast, freezes when cooled faster than  $10^{\circ}\text{C}$  per minute (Figs. 1A and 2), similar cells in an aggregate 1 millimeter in diameter would undergo intracellular freezing when cooled faster than  $0.03^{\circ}\text{C}$  per minute (3). Thus, one question in attempts to freeze organs is the size and geometry of the cellular aggregates that have to equilibrate during cooling by the transfer of cell water to the ice in the surrounding blood vessels and extracellular spaces. A second question is the permeability of this equilibrating unit to water. There are indications that it is either much larger than a single cell or else has a much lower permeability to water. Farrant and Pegg report that perfused dimethyl sulfoxide takes 20 and 100 minutes at  $37^{\circ}\text{C}$  to equilibrate in guinea pig uteri and rabbit kidneys respectively (70). These times contrast sharply with equilibration times for dimethyl sulfoxide of less than 2 minutes in suspensions

of hamster cells at  $5^{\circ}\text{C}$  (45) and ascites cells at  $37^{\circ}\text{C}$  (71). If the differences in permeability to water are as large, then organs cooled at  $1^{\circ}\text{C}$  per minute are very likely to undergo intracellular freezing, as is the case in the freezing of some tissues (33). Most workers, although not all (33), agree that this event will be lethal. Another problem is that organs are composed of several cell types. As seen in Fig. 4, a cooling rate that is optimal for one cell type may not necessarily be optimal for all others. In fact, it may be difficult or impossible to find a single cooling procedure that is optimal for all types (45, 72).

What, if any, are the solutions to these problems? One prerequisite to solutions is to determine just what physical events are occurring in organs subjected to freezing and thawing. What are the sizes and locations of ice crystals as a function of cooling velocity? Does freezing affect cell-cell interactions, and, if so, how? If, as I suspect, avoidance of lethal intracellular freezing will require cooling much slower than  $1^{\circ}\text{C}$  per minute, how will one avoid lethal solution effects? One approach being pursued by Farrant and Pegg is to avoid solution effects by avoiding ice formation (73). They propose to increase the concentration of protective additive, as cooling progresses, to keep the freezing point of the organ below the temperature at that instant. Although feasible, the procedure will not succeed if the cooling rate required to permit additives such as dimethyl sulfoxide to equilibrate intracellularly is so low that it produces an intolerable exposure to the additive. The problem would be much less serious if protection could be conferred by extracellular additives, for the required equilibration times would be much shorter.

The other approach to preventing injury from solution effects is to find protective additives that are more effective than any now known. To do this, however, will require far better understanding of the mechanisms by which solution effects kill, and the mechanisms by which additives protect.

### Cryosurgery

Cryosurgery is the antithesis of organ freezing in that the usual intent is to kill all cells in a diseased target area. Although this goal may be as difficult

to achieve as 100-percent survival, cryosurgery has nevertheless been an effective, and a sometimes dramatically effective, procedure in the treatment of certain neurological disorders, the destruction of some neoplasms, the attachment of retinas, and the removal of organs such as prostates and tonsils, especially in patients with blood diseases (74).

Although freezing totally destroys some tumors, it does not prevent the regrowth of others, even when the entire tumor mass appears to have been enveloped in ice. Some cells apparently survive, and the reason they survive probably relates to the fact that cryosurgical freezing, unlike the freezing discussed so far, involves steady states and large temperature gradients rather than true equilibrium. The initial step of the procedure is to place a probe in the target tissue (74). The probe is then cooled with liquid nitrogen to produce a sphere of frozen tissue which expands until the heat flow into the probe is balanced by heat flow from the unfrozen tissue into the sphere of ice. Near the probe the cooling rate is high and the temperature is as low as  $-100^{\circ}\text{C}$ ; but at the edge of the ice sphere the temperature must be  $-0.6^{\circ}\text{C}$ , the freezing point of mammalian tissue (75). Hence, temperatures just inside the edge will be above the values of  $-3^{\circ}$  to  $-10^{\circ}\text{C}$  required to kill cells in the absence of any protective additive and even farther above the much lower temperatures required to kill cells in the presence of additives (13, 26). A possible source of protective additive might be proteins released from damaged cells near the probe and pushed outward by the advancing ice front.

One way to produce maximum injury in the target area, therefore, would be to extend the freezing area beyond the target to ensure that all the diseased cells will be cooled to sufficiently low temperatures (75). Another possible approach would be to introduce an "anti-additive"—that is, a compound that, when concentrated by freezing, will be highly toxic to the cells at the periphery of the ice.

### Ecology and Exobiology

I have given several examples of cells surviving to  $-196^{\circ}\text{C}$  ( $77^{\circ}\text{K}$ ), and there are even instances of organisms surviving exposure to  $2^{\circ}\text{K}$  (76). Thus,



one cannot rule out the *existence* of living forms on the basis of extremely low temperature. On the other hand, of course, there are temperature limits below which growth and reproduction do not occur, but even these limits are surprisingly low (about  $-30^{\circ}\text{C}$ ) (77). Moreover, biochemical reactions can occur at still lower temperatures. One indication of this is the fact that many cell types lose viability with storage at  $-78^{\circ}\text{C}$  (8, 19, 21, 32), probably because liquid water is still present (78). Although photobiological excitations can occur at  $77^{\circ}\text{K}$  (79, 80) and even at  $1^{\circ}\text{K}$  (81), the lower limit for thermally driven biochemical reactions in *aqueous* systems is probably the glass transition point of water at about  $-140^{\circ}\text{C}$  (82). Below this, the viscosity exceeds  $10^{13}$  poises (83). The lower limit in nonaqueous systems could, however, be even lower (84), an interesting possibility in view of the report by Siegel *et al.* on the ability of *Penicillium* spores to germinate and incorporate nucleotides and amino acids in a liquid ammonia-glycerol medium at  $-40^{\circ}\text{C}$  (85).

In view of these laboratory findings, it is not surprising that living organisms can survive the coldest of terrestrial environments (86, 87). Moreover, nothing that we know about the freezing of terrestrial forms either precludes the survival of earth organisms on the surface of Mars and the outer planets or excludes the possibility that Mars possesses indigenous forms of life (88).

### Freezing, Membranes, and Cell Water

By way of summary I would like to reemphasize the close relation between the responses of cells to freezing and the properties of cell membranes. It is the surface membrane that prevents ice from nucleating cells at temperatures above about  $-10^{\circ}$  to  $-15^{\circ}\text{C}$ , and it is the permeability of that membrane to water that is the chief determinant of whether cells will equilibrate by dehydration or by intracellular freezing at lower temperatures. Furthermore, cells that are injured either by solution effects or by intracellular freezing undergo damage to surface and internal membranes, and the damage from the solution effects can be reduced by the use of certain additives that appear to act at the cell surface. Finally, the process of frost hardening in higher plants

is accompanied by increases in the permeability of the cells to water (40, 41).

One explanation of the ability of membranes to block nucleation above about  $-15^{\circ}\text{C}$ , but not below, involves the assumption that membranes contain water-filled pores similar to those proposed by Solomon and others (89). The barrier properties would arise because ice crystals small enough to pass through such pores cannot exist above about  $-10^{\circ}$  to  $-20^{\circ}\text{C}$ , because of the high water activity produced by a small radius of curvature (1, 90).

Perhaps the clearest evidence that membranes are the chief targets of freezing damage comes from studies on mitochondria and chloroplasts (43, 58, 91). Damage is primarily manifested by loss of the ability to carry out the membrane-mediated processes of oxidative and photosynthetic phosphorylation, respectively, rather than by loss of the function of soluble enzymes. In fact, Heber has evidence that the damage in chloroplasts is to the membranes themselves (43); they lose the ability to discriminate against protons. The protective macromolecule he discovered is interesting in this regard (58). The extremely low concentrations (0.05 percent) that suffice to protect suggest that protection may involve interactions between the protein and specific membrane sites rather than some generalized effect. If so, it would be extremely interesting to determine the nature of the sites and the interactions, both from the point of view of membrane structure and from the point of view of designing more effective additives.

Discussions on the permeability of cells to water generally imply that cell water is normal water, but there has been a recent resurgence in the idea that much of the water in cells is anomalous and "ice-like" (92). This may be true, but the responses of cells to freezing set a limit on just how much is "much," and on just how "ice-like" the water is. In the first place, the physical-chemical model based on Eqs. 1 and 2 essentially pictures a cell as a membranous bag of dilute solution, the solvent being water of normal properties. To date, the model satisfactorily predicts which physical events will occur in cells during freezing. Second, the amount of heat absorbed by frozen cell suspensions during warming, as measured by differential thermal analysis, is in semiquantitative agreement with the view that the bulk of frozen cyto-

plasm behaves like an ordinary frozen dilute solution (11).

This may not be true of the 10 percent of cell water that is incapable of freezing and is therefore bound (11, 93). But the degree to which even this water is "ice-like" has limits. In the absence of external ice, insects, yeast, and other cells can supercool to  $-20^{\circ}\text{C}$  or below (1, 94, 95). Furthermore, samples of pure water which supercool to  $-21^{\circ}\text{C}$  also do so in the presence of macromolecules such as DNA, soluble collagen, trypsin, and collagenase (96). Since the water in cells and around macromolecules thus shows no ability to nucleate supercooled water, to this extent it fails to be "ice-like."

Since liquid water is considered mandatory for the functioning of living systems, it is interesting to observe the response of cells and their constituents to the perturbations brought about by freezing. We have seen that the physical response ranges from osmotic dehydration to intracellular freezing, and that the biological response ranges from innocuousness to irreversible damage, depending on the type of cell, the cooling and warming velocity, the minimum temperature attained, and the type and concentration of protective additive present. We have seen, further, that the chief target of injury is membranes. They appear much more susceptible than do most soluble enzymes (58, 97). And yet most proteins ought to denature at subzero temperatures because of the heavy contribution of hydrophobic interactions to the stability of the native structure (98), and some in fact do denature.

How, then, is one to interpret the fact that the denaturation of soluble enzymes does not appear to be a major factor in freezing injury? Does this mean that denaturation does not occur in the frozen cell, or does it mean that the denaturation is reversible, or the damage repairable? Our inability to explain this lack of damage is matched by our inability to explain the cause of the observable damage. How do intracellular freezing, recrystallization, and solution effects injure and how do additives protect? How are damage and protection interpretable in terms of present views of membrane structure, or, alternatively, what information do they provide about membrane structure? Although our inability to answer these questions and the others implied in Table 1 is vexing, our ability to at least pose the questions is promising.

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