LIMITS TO LIFE AT LOW TEMPERATURES AND AT REDUCED WATER CONTENTS AND WATER ACTIVITIES*

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Abstract. Liquid water is generally considered an absolute requisite for functional life; consequently, life is expected to function only over the range of temperatures that permit its existence. These limits, however, do not apply to cell survival. Some cells can survive the closest attainable approach to 0 K, and some can survive the loss of over 99% of their water.

1. Cell Survival at Subzero Temperatures

Many cells that can survive the initial trauma of freezing die with time at temperatures above about −80 °C. The rate of death is dependent in a complex way on temperature, on the species and type of cell, and on the composition of the medium in which they are frozen. The killing rate can vary from several percent per hour to several percent per year (Mazur, 1966). Below about −130 °C, on the other hand, no thermally driven reactions can occur in aqueous systems, and there are no confirmed instances of progressive loss in viability (Mazur, 1976).

Liquid water does not exist below −130 °C; the only states of water that do exist are crystalline or glassy, and in both cases the viscosity is so high ($>10^{13}$ poises) that diffusion is not significant over time spans of human interest. In addition to this diffusional barrier, there is insufficient thermal energy for most reactions. A few nonaqueous reactions may occur at −196 °C, but only between highly reactive compounds of O, H, and F in the presence of an exogenous source of energy such as a glow discharge (McGee and Martin, 1962).

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 high-energy particles (Rice, 1960). It is conceivable that, over a sufficiently long period of time, these direct ionizations could 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. One can estimate the death rate at −196 °C from radiation data at physiological temperatures on cells irradiated under anoxic conditions at high dose rates (~100 rad min^{-1}). (At high dose rates, the irradiation is probably completed before significant repair can be initiated, and in that sense it is

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analogous to the long-duration, low-rate irradiation that cells are subjected to at −196 °C. Irradiation under anoxic conditions reduces the biochemical component of ionizing radiation (Wood and Taylor, 1957).) The dose of ionizing radiation that kills 63% of representative cultured mammalian cells is 200 to 400 rad (Elkind and Whitmore, 1967). Since terrestrial background radiation is some 0.1 rad yr^{-1}, it ought to require some 2000 to 4000 yr at −196 °C to kill 63% of a population of typical mammalian cells. The time period would be about 200 yr for the most sensitive known mammalian cells, the early mouse oocyte, and it would be well over a million years for highly radioresistant organisms like *Micrococcus radiodurans*. Analogous arguments apply to mutation rates at −196 °C. They ought to be greatly reduced (see Mazur, 1976, for references).

The fundamental challenge to cell survival, then, is not low temperature *per se*; rather, it is whether cells can survive the initial cooling to the low temperatures and the subsequent return to physiological temperatures.

2. Lethal Events during the Freezing and Thawing of Cells

A. EFFECT OF COOLING RATE

When cells are cooled to −196 °C at various rates, the resulting survivals generally take the form shown in Figure 1. Maximum survival occurs at some intermediate optimal rate.

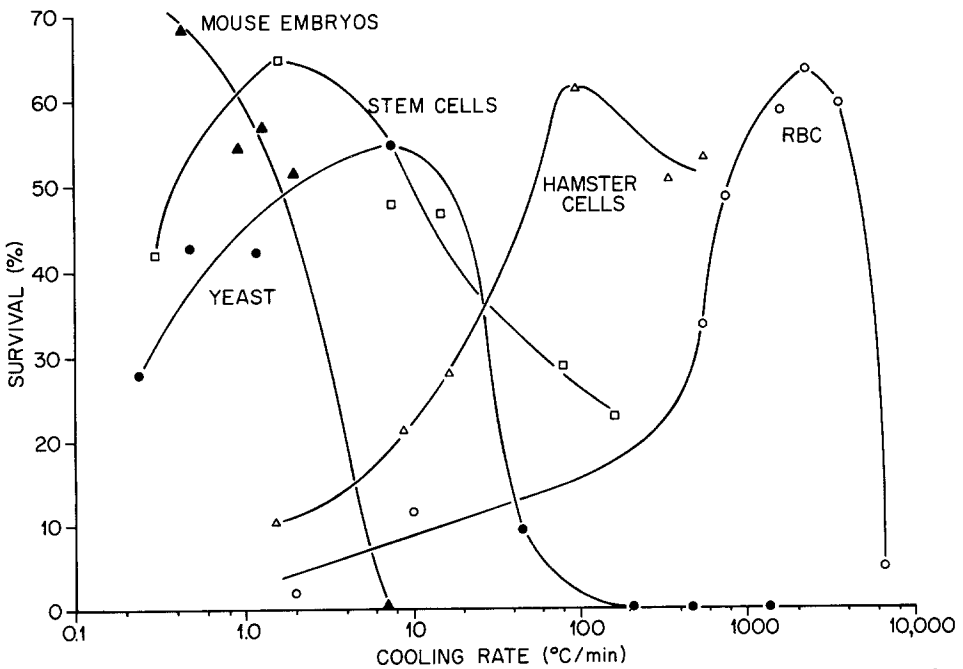

Fig. 1. Comparative effects of cooling velocity on the survival of various cells cooled to −196 °C. The data on mouse embryos, mouse marrow stem cells, yeast, hamster tissue culture cells, and human erythrocytes are from Whittingham *et al.* (1972), Leibo *et al.* (1970), Mazur and Schmidt (1968), Mazur *et al.* (1969), and Rapatz *et al.* (1968).

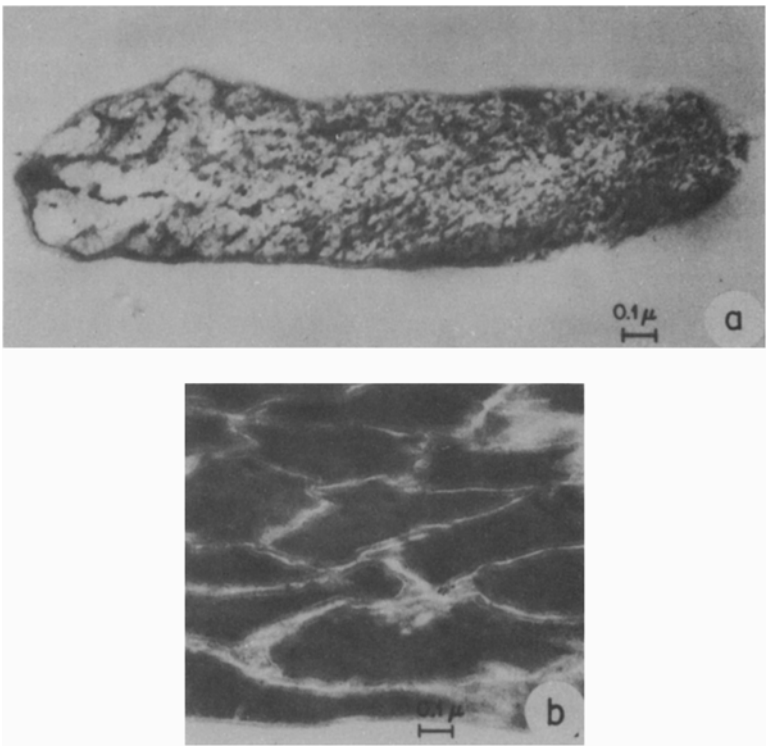

Fig. 2. Microscopic appearance of *Escherichia coli* after rapid freezing (a) or slow freezing at 1 °C min (b). The cells were freeze-substituted at −78 °C with ethanol. (Modified from Rapatz *et al.*, 1966).

Cooling at lower rates is usually significantly more deleterious, and cooling at higher rates is usually dramatically more injurious. However, the numerical value of the cooling rate that produces maximum survival can vary widely among cell types. In the examples shown, it ranges from $<1\ ^{\circ}C\ min^{-1}$ for mouse ova to about $1000\ ^{\circ}C\ min^{-1}$ for human red cells.

Some clues as to the determinants of the inverted-U curves can be obtained from an examination of the morphological appearance of cells frozen at various rates. Figure 2 shows freeze-substituted *Escherichia coli*. Rapidly cooled cells (Figure 2a) are relatively normal in shape and size, but they are pitted with intracellular spaces that represent the former location of ice crystals. Slowly frozen *E. coli* (Figure 2b), on the other hand, are highly shrunken and show no evidence of intracellular ice.

Slow and rapid cooling produce analogous effects on mouse ova (Figure 3). As noted in Figure 1, the optimum cooling rate for ova is $\leqslant 1\ ^{\circ}C\ min^{-1}$. From Figure 3 we see that those frozen rapidly at $32\ ^{\circ}C\ min^{-1}$ undergo little shrinkage and they freeze intracellularly at about $-40\ ^{\circ}C$, whereas those frozen slowly at close to the optimum rate ($1.2\ ^{\circ}C\ min^{-1}$) become progressively shrunken and show no evidence of intracellular ice.

A close correlation has been found in three types of cells between the cooling rates

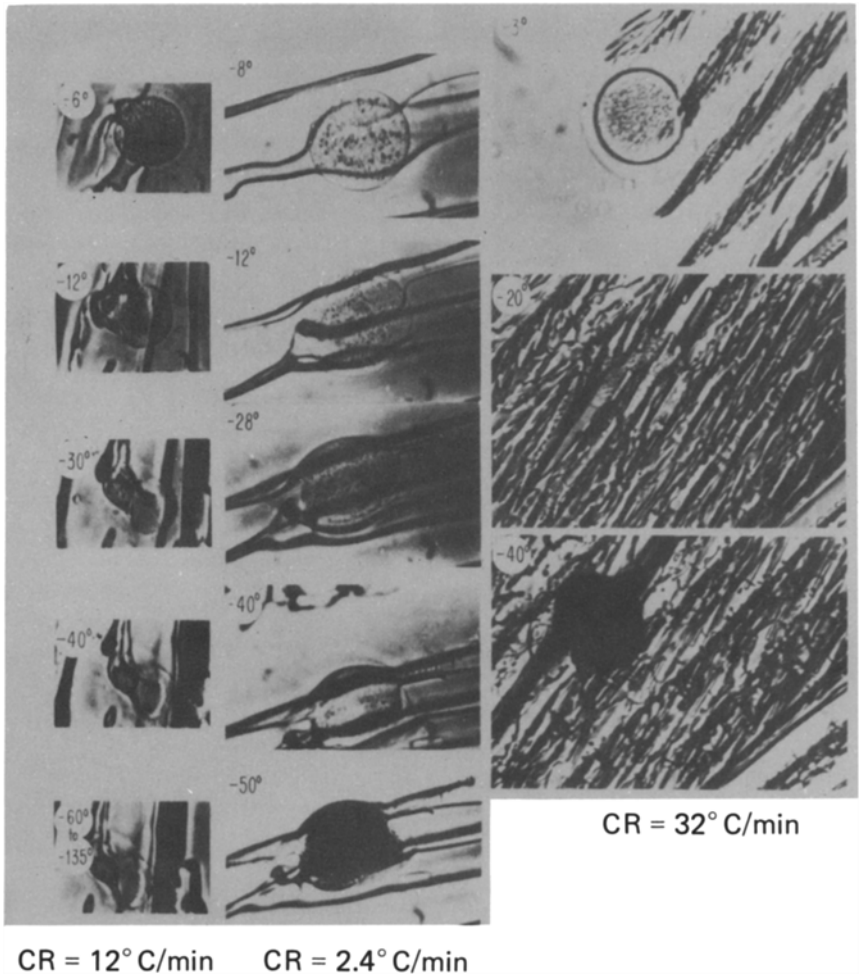

Fig. 3. Effects of cooling rate on the morphology of unfertilized mouse ova during freezing in 1*M* dimethyl sulfoxide. The cells cooled at 1.2 °C min^{-1} dehydrate without intracellular freezing. Those cooled at 2.4 or 32° C min^{-1} undergo little shrinkage during cooling and eventually freeze intracellularly between −40° and −50° C. Intracellular freezing is manifested by the sudden 'blacking out' of the cell as a result of light scattered by numerous small crystals. (Modified from Leibo *et al.*, 1978.)

that are supraoptimal in terms of survival and the cooling rates that produce intracellular ice (Figure 4).

The essential events that occur during freezing are shown in schematic form in Figure 5. Most cells remain unfrozen at −10 to −15 °C even though those temperatures are 9 to 14 degrees below the actual freezing point of their cytoplasm, and even though ice is present in the outside medium. Apparently the cell membrane is able to block the passage of extracellular ice above about −15 °C, and thus is able to prevent the nucleation of the supercooled water within the cell by that ice. Explanations for the ability of membranes to block nucleation have been suggested (Mazur, 1977a).

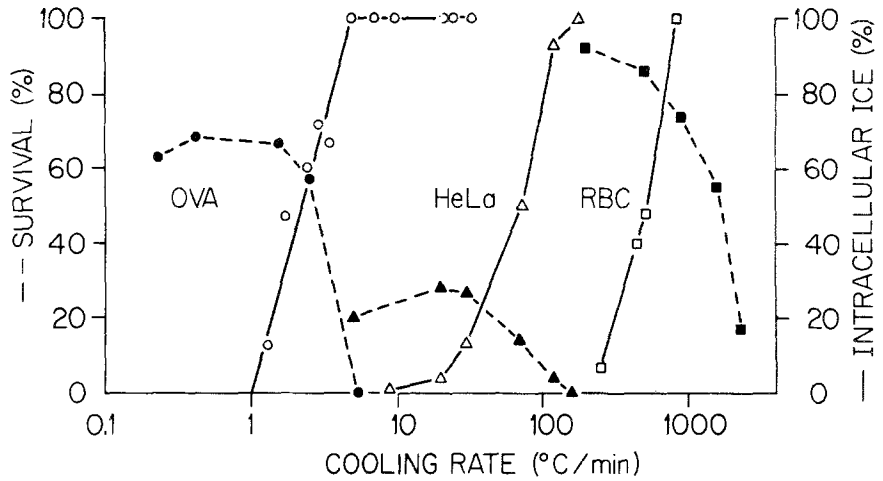

Fig. 4. Percentage survival (– – –) plotted against the percentage of cells undergoing intracellular freezing (—) in three mammalian cells frozen at various rates to –20 °C (HeLa) or –196 °C. (Modified from Leibo, 1977.)

Supercooled water by definition has a higher vapor pressure (activity or chemical potential)* than that of ice outside. Since water will move from regions of high activity to regions of low activity, and since the membrane is permeable to liquid water (although not ice crystals), water will move out of the cell and freeze externally. This efflux concentrates the intracellular solution and lowers its activity (a_w).

The end result depends on the cooling rate. If the cooling rate is sufficiently slow, the cell will be able by progressive dehydration to maintain the intracellular a_w close to the a_w of the external ice and external solution. Under these conditions intracellular freezing is unlikely. But if the cooling is not sufficiently slow, the cell will not be able to lose water fast enough to reduce the intracellular a_w to the equilibrium value. If the cell remains supercooled below −15 °C, it will eventually freeze intracellularly.

The term 'sufficiently slow' can be quantified. Were no water movement to occur, the vapor pressure of ice outside the cell (p_e) would decrease faster than that of the supercooled water inside the cell (p_i), the two vapor pressures being given by

$$\frac{\mathrm{d} \ln p_e}{\mathrm{d}T} = \frac{L_s}{RT^2}, \tag{1}$$

$$\frac{\mathrm{d} \ln p_i}{\mathrm{d}T} = \frac{L_v}{RT^2} + \frac{\mathrm{d} \ln x_i}{\mathrm{d}T}, \tag{2}$$

respectively. (L_s and L_v are the latent heat of sublimation and vaporization, x_i is the mole fraction of intracellular water, R is the gas constant, and T is temperature.) But the very

*Water activity is defined as $a_w = p_{H_2O}$ (solution)/p_{H_2O} (liquid pure) = p_{ice}/p_{H_2O} (liquid pure), where p is vapor pressure. Chemical potential (μ_w) is $\mu_w = \mu_w^\circ + RT \ln a_w$, where μ_w° is the chemical potential of pure water.

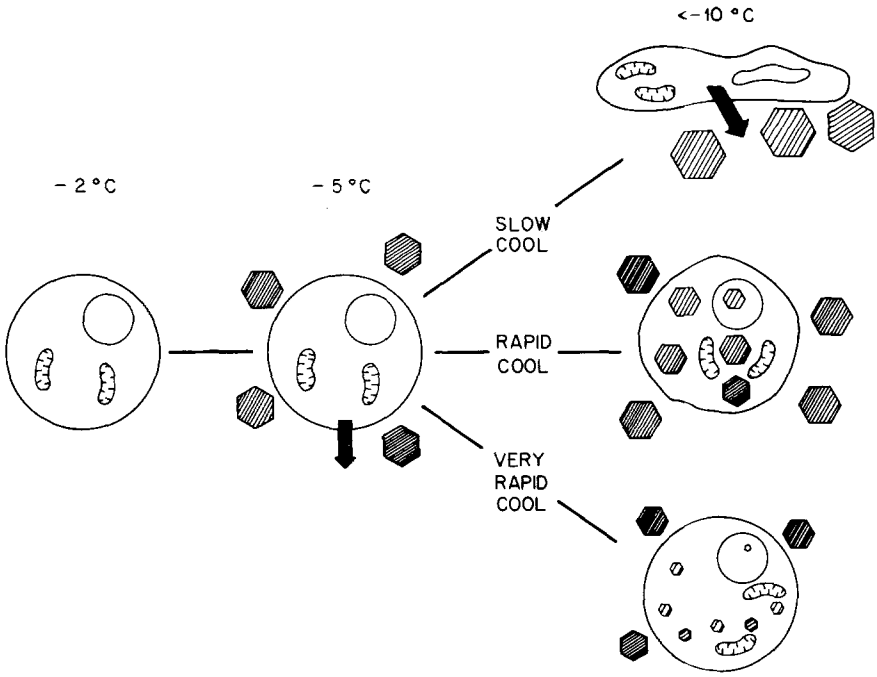

Fig. 5. Schematic of physical events in cells during freezing. The cross-hatched hexagons represent ice crystals. (From Mazur, 1977a.)

development of a difference in vapor pressure produces a proportional force that drives water out of the cell at a rate

$$\frac{dV}{dt} = \frac{KART}{v_1^\circ} \ln p_e/p_i, \tag{3}$$

where V is the volume of cell water, K is the permeability of the cell to water (hydraulic conductivity), and A its surface area. Equations (1), (2), and (3) along with an exponential equation describing the temperature dependence of K, and an equation relating time, t, and temperature, T (i.e., the cooling rate), can be solved numerically to give the volume of cell water remaining in the cell as a function of temperature for given values of cooling rate, permeability, and cell surface area (Mazur, 1963a, 1977a). The equations have been solved for various cells. Calculated solutions for yeast cooled at various rates are shown in Figure 6. Curve 'Eq' expresses the reduction in the relative volume of intracellular water (relative to the normal volume) that is required to maintain equilibrium between the activities of cell water and outside ice. Departures from the equilibrium curve mean by definition that the cell water is supercooled. The calculations indicate that the water contents of yeast cooled at 1 or 10 °C min^{-1} will reach the equilibrium value at −7° or above. We would predict therefore that cells cooled at 1 or 10 °C min^{-1} will not freeze intracellularly. On the other hand, yeast cells cooled at 100 °C min^{-1} or faster are far from equilibrium at −15 °C, the temperature at which most supercooled cells nucleate.

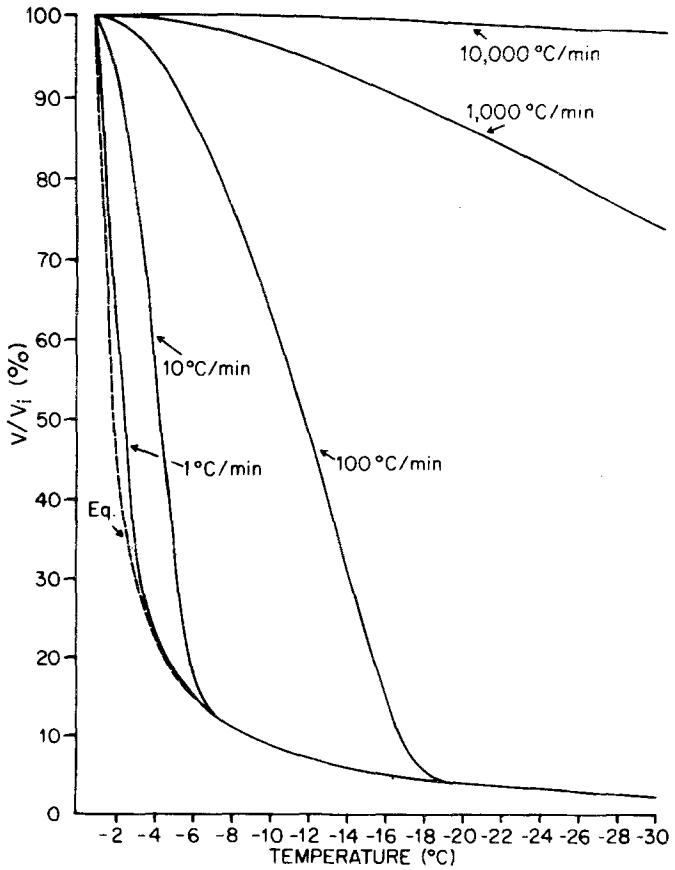

Fig. 6. Calculated percentage of intracellular water remaining in yeast at various subzero temperatures after cooling at the indicated rates. Eq. is the calculated equilibrium curve for cells cooled at infinitesimal rates. (From Mazur, 1963a.)

We would predict, therefore, that yeast cooled at 100 °C min^{-1} or faster will freeze intracellularly. Figure 7 expresses these predictions as a plot of the probability of intracellular freezing versus cooling rate, and it compares the probabilities with the observed survivals. Clearly the cooling rates predicted to produce intracellular ice coincide closely with the cooling rates that cause the abrupt drop on the right-hand side of the survival curve. Considerations of this sort, along with direct microscopic observations on yeast and other cells relating frequency of intracellular freezing to cooling rate (e.g., Figure 4), have provided strong support for the following conclusion: The drop in survival at high cooling rates is a consequence of the formation of intracellular ice (and, as we shall see shortly, of events during slow warming). The numerical value of the critical cooling rate can vary widely (see Figure 1). This variation is chiefly a consequence of differences in the surface area, and in the permeability coefficient for water and its temperature coefficient.

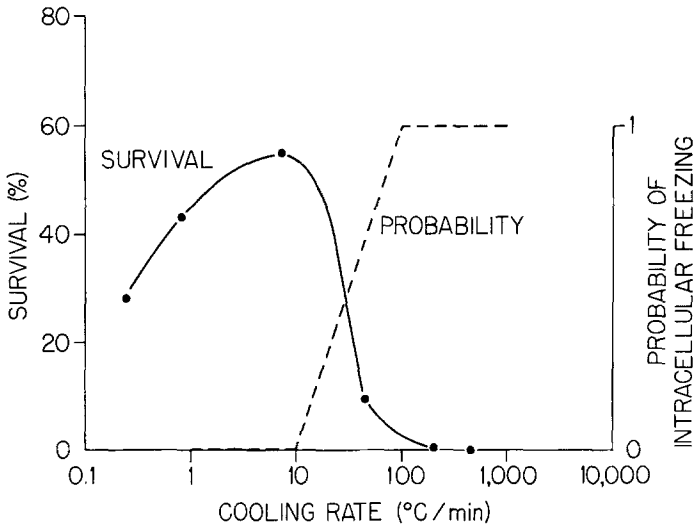

Fig. 7. Comparative effects of cooling rate on the survival and probability of intracellular freezing of yeast. (From Mazur, 1977a.)

B. CONSEQUENCES OF SLOW FREEZING

The data in Figure 7 as well as those in Figure 1 also illustrate that too low a cooling rate is injurious. To what can this slow freezing injury be ascribed? Since slow freezing injury is associated with modifications in the composition and properties of aqueous solutions produced by extracellular ice, it is sometimes referred to as 'solution-effect' injury. On a molar basis, the principal solutes in and around cells are electrolytes, and one consequence of progressive freezing is a dramatic increase in the solute concentration in the residual unfrozen puddles. For example, as shown in the left- hand curve of Figure 8, when a 0.15*M* solution of NaCl is frozen to −10 °C, the concentration of NaCl in the unfrozen portion of the solution rises some 20-fold to 2.8 molal. (It is this high solute concentration that causes the drop in extracellular a_w discussed in the previous section.) Mammalian cells suspended in that solution will generally be killed by about −20 °C (Figure 9). This correlation between cell death and high salt concentration led Lovelock (1953) and succeeding investigators to ascribe a causal relationship between the two.

Three years previously, Smith and Polge (1950) had discovered that the addition of about 1*M* glycerol to a suspension of spermatozoa in saline resulted in a high proportion of the cells surviving slow freezing to −80 °C. The marked protective effect of glycerol was subsequently found for many cells, and is illustrated for mouse marrow cells in Figure 10. Inspection of Figure 8 shows that the ability of glycerol to protect viability is paralled by its ability to suppress the concentration of salts at a given temperature. For example, when a solution of 0.15 molal salt in 1.0*M* glycerol is frozen to −10 °C, the salt concentration rises only to 0.6 molal. (As noted, in the absence of glycerol it rises to 2.8 molal.) The suppression of salt concentration by glycerol is not a special property

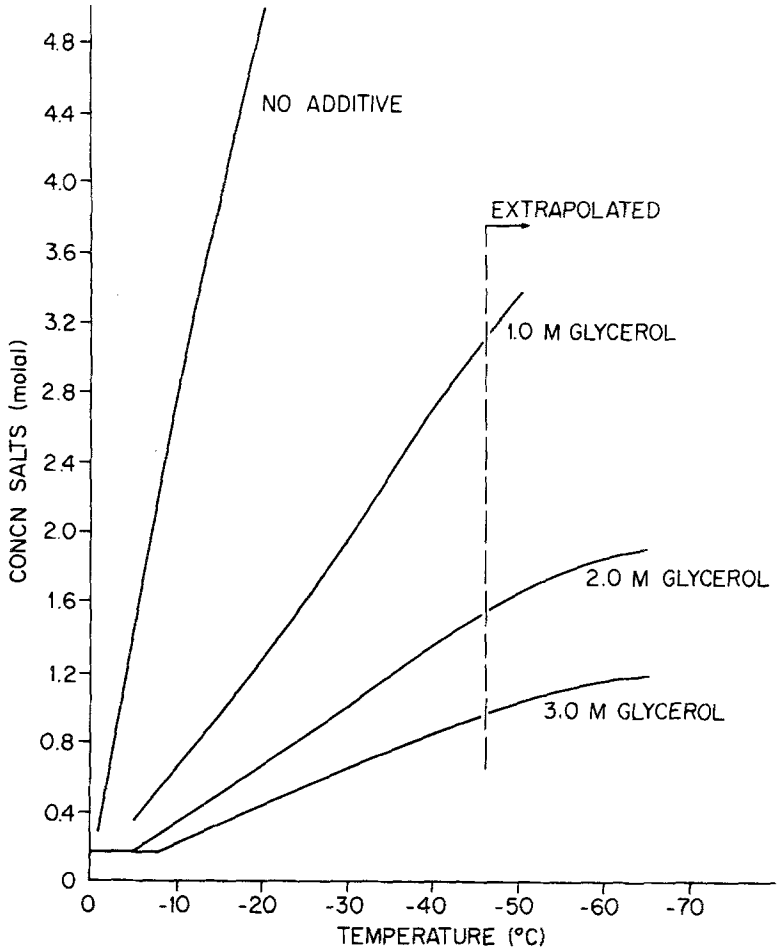

Fig. 8. Effect of the presence of glycerol on the concentration of salt in the unfrozen portions of a solution at various subzero temperatures. (Modified from Rall *et al.*, 1978.)

of glycerol, but is rather a consequence of the phase rule and the colligative properties of solutes. The phase rule dictates that in a two-phase system (here, ice and liquid solution) at constant pressure the *total* solute concentration in the liquid solution is invariant at a given temperature. Since the total solute concentration is fixed, the higher the concentration of glycerol (or any other solute) the lower will be the salt concentration at that temperature. The effect is roughly proportional to the mole ratio of glycerol to salts in the medium. However freezing produces other changes in solutions besides an increase in solute concentration. In fact a current major area of cryobiological research is an attempt to define which changes are responsible for slow-freezing injury and which are prevented or counteracted by additives such as glycerol.

In the absence of protective compounds, mammalian cells are far more sensitive to slow freezing (solution effect injury) than are most microorganisms, although the latter

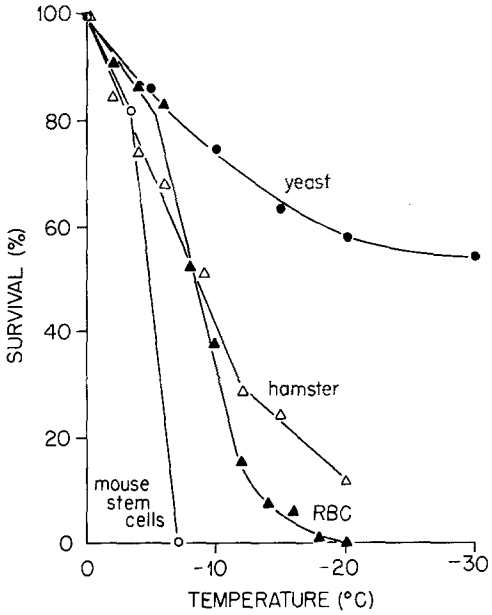

Fig. 9. Comparative sensitivity of four cells to slow freezing to various subzero temperatures in the absence of a protective compound. The data on yeast, hamster issue culture cells, human erythrocytes, and mouse marrow stem cells are from Mazur and Schmidt (1968), Mazur and Leibo (unpublished). Souzu and Mazur (1978), and Leibo *et al.* (1970), respectively.

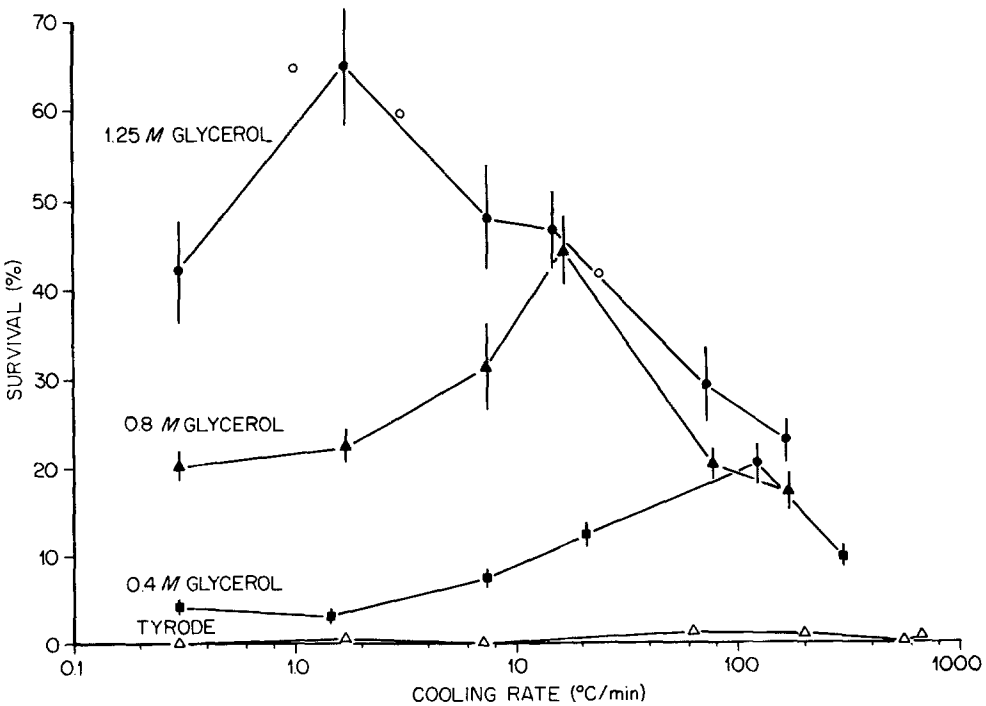

Fig. 10. Survival of mouse marrow stem cells suspended in balanced salt solution containing 0, 0.4, 0.8, or 1.25*M* glycerol after cooling to −196 °C at various rates and rapid warming. (Redrawn from Leibo *et al.*, 1970.)

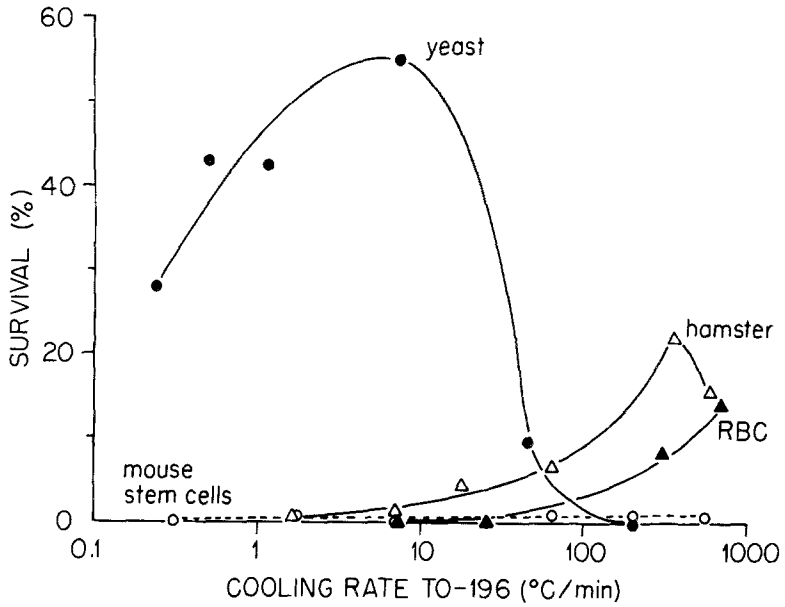

Fig. 11. Comparative sensitivity of four cells to freezing to −196 °C at various rates in the absence of a protective compound. The data on yeast, hamster tissue culture cells, human erythrocytes, and mouse marrow stem cells are from Mazur and Schmidt (1968), Mazur *et al.* (1969), Morris and Farrant (1972), and Leibo *et al.* (1970), respectively.

are by no means immune (Figures 9 and 11). Both mammalian cells and microorganisms are, however, susceptible to the intracellular freezing that results from cooling at supraoptimal rates.

C. EFFECT OF WARMING RATE ON SURVIVAL

The effects of rate of thawing on cell survival are strongly influenced by the prior rate of cooling. In almost all cases examined, cells that are cooled at supraoptimal rates (i.e., rates sufficient to induce intracellular ice) survive better when warming is rapid than when it is slow. The dramatic effect of warming rate on yeast cells is illustrated in Figure 12. There is strong evidence, especially in yeast, that the high sensitivity of rapidly cooled cells to slow thawing results from the growth of intracellular ice crystals to damaging size by recrystallization during warming. This conclusion is based on the sort of evidence presented in Figure 13. In the experiment represented by the bottom graph, yeast were rapidly cooled to −196 °C, warmed slowly to the temperatures indicated on the abscissa, and then thawed rapidly. Slow warming up to −40 °C produced no damage, but continuing the slow warming to temperatures above −40 °C produced increasing injury. By −20 °C, 95% of the cells were killed. The yeast photographed in the upper half of the figure were subjected to somewhat analogous treatments. The cells were first cooled rapidly to −196 °C. They were then warmed abruptly to −50, −35, or −20 °C, held at these temperatures for various times, recooled to −196 °C, and freeze-cleaved below −100 °C. Increasing the intermediate holding temperature from −50 °C to −35 to −20 °C

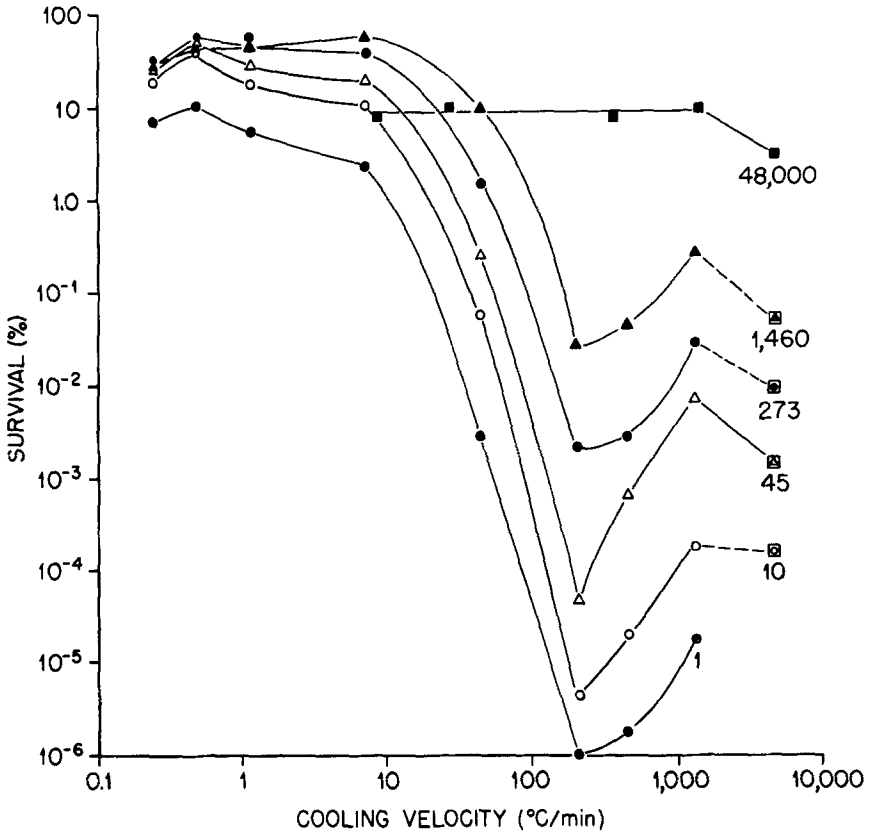

Fig. 12. Interactions of cooling rate and warming rate on the survival of frozen–thawed yeast. The numbers on the individual curves refer to warming rates. (From Mazur and Schmidt, 1968.)

produced progressively larger intracellular ice crystals. This increase in crystal size from recrystallization thus paralleled the decrease in survival shown in the lower half of the figure.

But what about the effect of rate of warming on cells cooled slowly enough to avoid intracellular freezing? In some cases there is little or no effect (e.g., yeast cells, Figure 12). But in other cases there are major effects quite different from those observed in rapidly cooled cells. In human erythrocytes, for example (Figure 14), the sequence of slow freezing (0.5 °C min^{-1}) followed by slow thawing (0.5 to 1 °C min^{-1}) is considerably less damaging than the sequence of slow freezing followed by rapid thawing (160 °C min^{-1}).

Although it is not known whether sensitivity of slowly cooled cells to rapid thawing is a characteristic of all cells, it is characteristic of groups as disparate as erythrocytes, mouse embryos (Leibo *et al.*, 1974), and plant cells (Levitt, 1966). Although its basis is not understood, the response is similar to that seen in osmotic shock. During the progressive thawing that accompanies warming, the highly concentrated extracellular medium is progressively diluted by melting ice. Hence, sensitivity to rapid thawing may be tantamout to sensitivity to rapid dilution, and the latter by definition is osmotic shock. I have

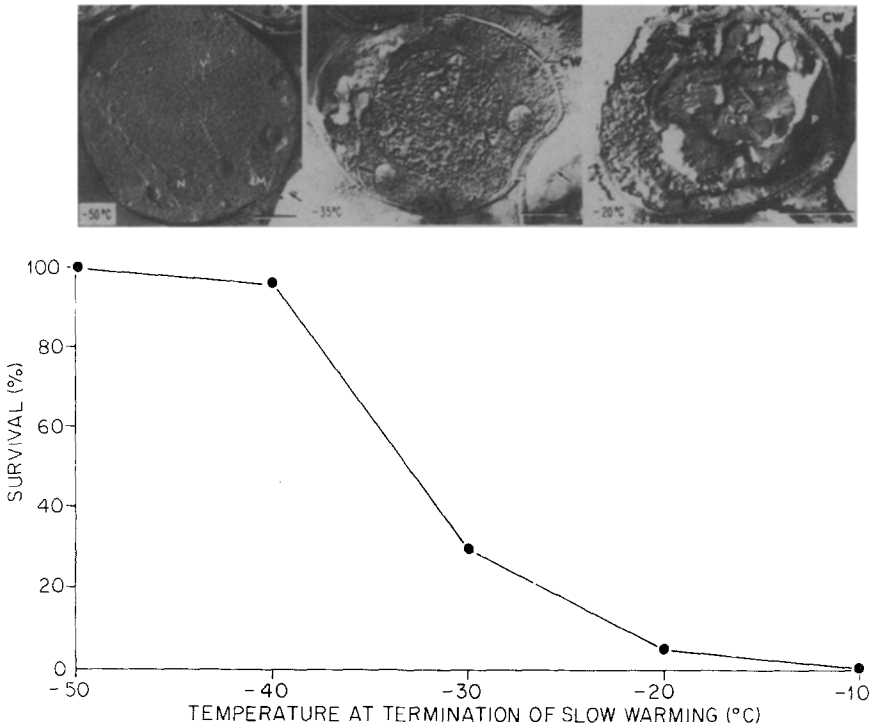

Fig. 13. Effect of warming to various temperatures on survival and on the recrystallization of intracellular ice in yeast rapidly frozen to −196 °C. The data are from MacKenzie (1970) and Bank (1973). In MacKenzie's survival studies the cells were warmed slowly to the indicated temperatures and then thawed rapidly. In Bank's recrystallization studies, the cells were warmed abruptly to −50, −35, and −20°C; held 24 hr, 30 min, and 5 min, respectively; recooled to <−100°C; and freeze-cleaved.

speculated that shock may occur when excessive additive driven into cells during cooling cannot diffuse out fast enough during warming to prevent osmotic water influx and consequent cell swelling (Mazur, 1977b).

3. Consequences of Cell Dehydration

Many cells in nature undergo periodic or intermittent dehydration, and in the laboratory dehydration is one important method for preserving cultures (Heckly, 1961). The loss of cell water has a number of interrelated but distinguishable physical-chemical consequences. Since water is the only volatile compound present in cells in more than trace amounts, one effect of dehydration is to increase the concentration of intracellular solutes thereby lowering the water activity, a_w. A second effect is to alter the composition of the cytoplasm as various solutes begin to precipitate. Differential precipitation can produce rather major changes in pH. Finally, as more and more water is removed, the structure and properties of the remaining liquid begin to differ sharply from that of normal water.

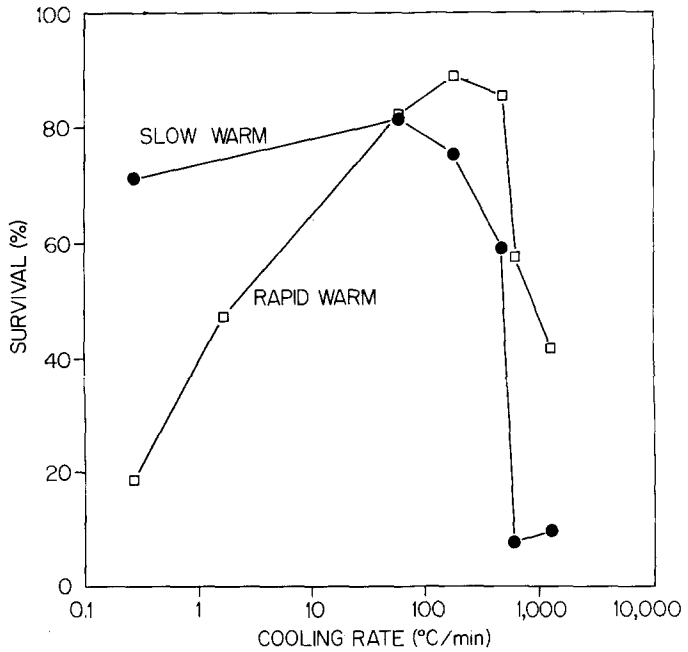

Fig. 14. Survival (percentage unhemolyzed) of human erythrocytes suspended in 2*M* glycerol, frozen at various rates to −196 °C, and warmed slowly (0.5 °C min^{-1}) or rapidly (160 °C min^{-1}). (Modified from Miller and Mazur, 1976.)

As mentioned, these phenomena are to a degree separable. A halophilic bacterium in 4*M* NaCl, for example, is not dehydrated, but it is exposed to low water activity (a_w = 0.84). On the other hand, a cell drifting in the atmosphere on a humid day will be extensively dehydrated even though the relative humidity (≡100 x a_w) may be 97%.

A. FREEZING VERSUS DEHYDRATION AND BOUND WATER

The dehydration produced by slow freezing is self-evident (see Figures 2 and 3). But rapid freezing also produces the equivalent of cell dehydration. The difference is that in rapid freezing liquid cell water is sequestered as ice in the cell's interior, whereas in slow freezing it is sequestered as ice in the external medium.

About 90% of the water of yeast and *E. coli* is frozen by −20 °C. The remaining 10% remains unfrozen no matter how low the temperature (Figure 15). It is by definition 'bound'. These conclusions are based on calorimetric measurements by Wood and Rosenberg (1957), Souzu *et al.* (1961), and Mazur (1963b). A more precise wording would be that 10% of the cell water does not exhibit a latent heat of fusion. Expressed in grams of water per gram of cell solids, the unfreezable fraction is about 0.25 g g^{-1} or about 0.20 g g^{-1} wet weight.

The inability to freeze is not the only unusual characteristic of this bound water fraction. Koga *et al.* (1966) have shown that when the water content of yeast is reduced below these figures, the dielectric constant and NMR signal intensities drop sharply

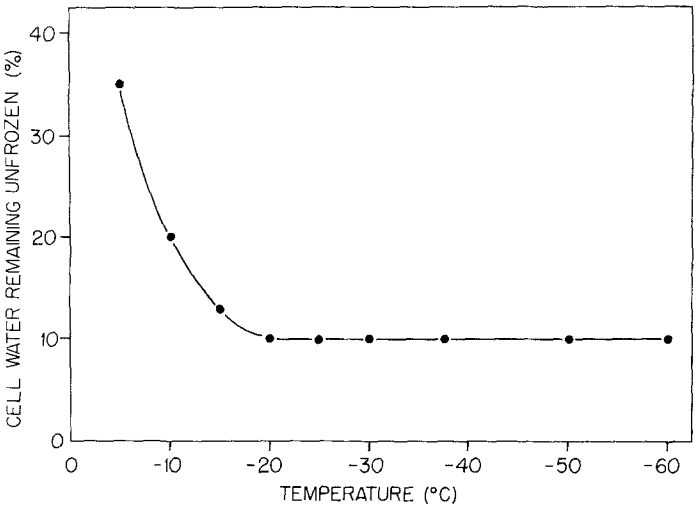

Fig. 15. Calorimetric estimates of the percentage of yeast cell water frozen at various temperatures. (From data of Wood and Rosenberg, 1957.)

(Figure 16). By the time the water content is reduced to about 0.1 g H_2O g^{-1} wet weight, it appears to be irrotationally bound.

Although the bound fraction cannot be converted to ice, it can be removed from cells by exposing them for a sufficiently long time to a sufficiently low a_w. The water content of a system after equilibration with various external a_w is a sorption isotherm, and Koga *et al.* (1966) have also obtained the sorption isotherm of yeast (Figure 17). The sigmoid-shaped curve is typical for cells and for cell constituents like proteins and nucleic acids.

B. EFFECTS OF DEHYDRATION ON CELL SURVIVAL

Much information on the effects of dehydration on cells comes from laboratory studies on freeze-drying. In this procedure, cell suspensions are frozen and placed under high vacuum in communication with a vapor trap. The trap, usually a condenser held at −196 °C, maintains the a_w of trapped water near zero. Water sublimes from the sample and condenses in the trap, so that after some hours the water content of the sample will be reduced to $\leqslant 1$ to 2% of the original. The high vacuum accelerates the sublimation by increasing the mean free path of the water vapor molecules.

Freeze-drying is almost invariably more damaging than freeze-thawing, a point illustrated in Table I for a number of fungi. It is even harsher to mammalian cells. There is no confirmed instance of any surviving freeze-drying to low water contents. The greater lethality of freeze-drying appears to result from the removal of a portion of the cell's bound water. Sakurada (1958), for example, found that 50% of a population of yeast withstood having their water content lowered to 20% of normal, but only 5% survived the removal of the remaining 20%. Nei *et al.* (1965) report similar findings for *E. coli.* Interestingly, as increasing fractions of the bound water of yeast are removed, the survival of the cells depends increasingly on the temperature of the rehydrating medium. For

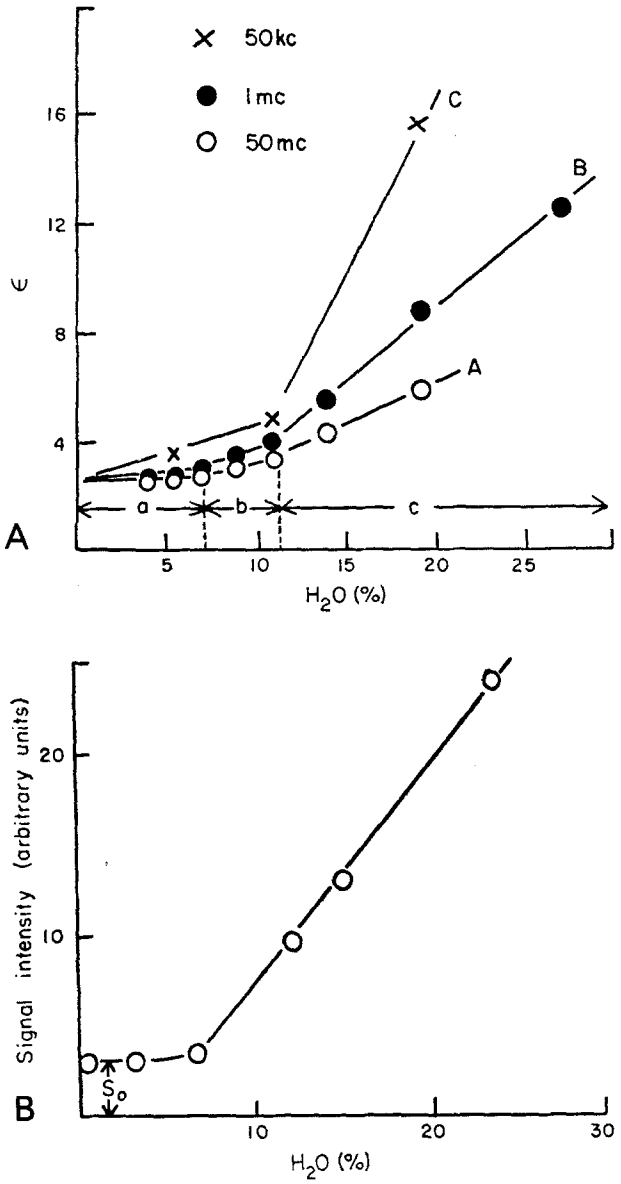

Fig. 16. Dielectric constant (A) and NMR signal intensity (B) of yeast cells as a function of their water content ($100 \times g\ H_2O\ g^{-1}$ wet weight). (From Koga *et al.*, 1966.) (The dielectric constant of pure bulk water and ice at $\leqslant 50$ MHz are about 81 and 3, respectively.)

unknown reasons, rehydration by water at 4 °C kills as many as 95% of the cells that have had some 96% of their water removed (Sant and Peterson, 1958), whereas rehydration at $\geqslant$26 °C does not.

Another approach to examining the effects of dehydration on cells is to equilibrate samples in atmospheres at various a_w's and to determine the subsequent rate of killing.

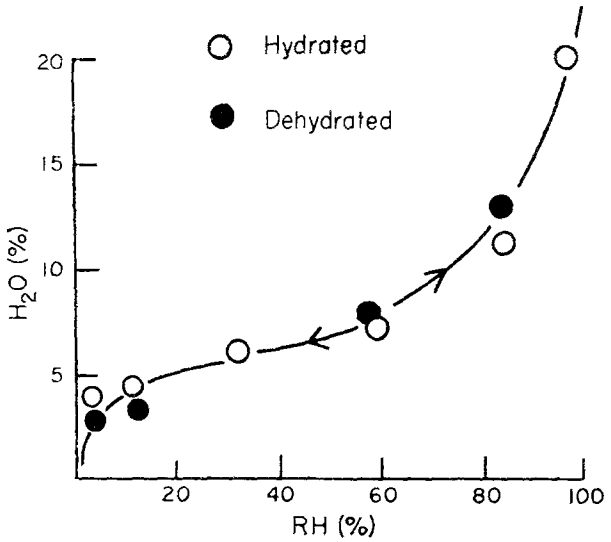

Fig. 17. Sorption isotherms of yeast cells; i.e., the water content of the cells (g H_2O g^{-1} wet weight) versus the relative humidity with which they were equilibrated. RH (%) ≡ 100 (a_w). (Data from Koga *et al.*, 1966.)

Figure 18 summarizes data on the bacterium *Serratia marcescens*. As shown in the right-hand curve, a reduction in water activity from 1.0 to 0.9 reduces the cell water content to 10% of normal. But the consequent rate of killing (left curve) is low, a finding consistent with those for yeast (above), and consistent with the view that little bound water has been removed. However, reducing the a_w from 0.9 to 0.2 drops the cell water content from 10 to 1% and increases the rate of killing by a factor of 10. The general pattern is not unique to Serratia. Other microorganisms also exhibit increased death rates at humidities below 90% (Webb, 1967a; Chapman *et al.*, 1967; Mackenzie, 1971; Cox, 1971;

TABLE I
Comparative effects of freeze-thawing and freeze-drying on the survival of Fungi[a]

Organism	Cell type	Freezing temperature (°C)	% survival[b] after	
			Freezing and thawing	Freeze-drying
Aspergillus flavus	Conidia	−15	65	26
		−65	7	2
A. niger	Conidia	−65	10	9
Fusarium	Conidia	−65	36	0
Gliocladium	Conidia	−65	73	0
Penicillium	Conidia	−65	57	2
Pestalotia	Conidia	−65	27	6
Saccharomyces cerevisia	Veg. Cells	−12	60	1

[a] Data from various authors (see Mazur, 1968).
[b] Survivals of the spores refer to % germination.

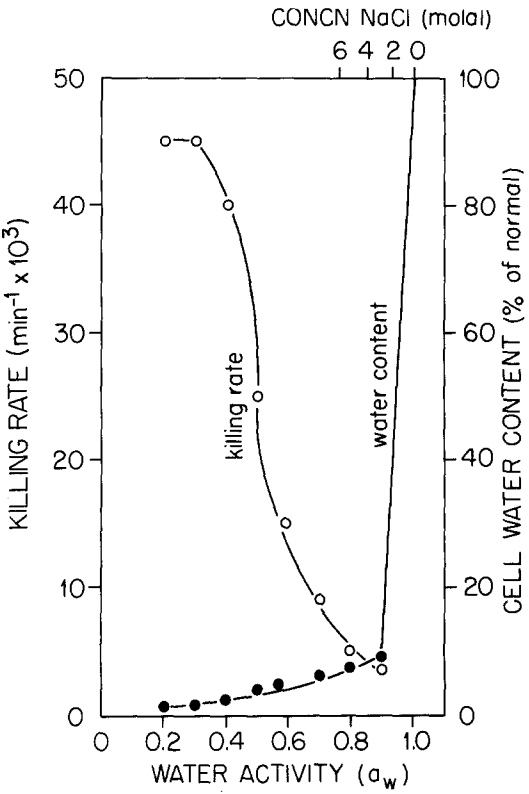

Fig. 18. Effect of water content and water activity on the rate of killing of cells of the bacterium *Serratia marcescens*. Note the concentrations of solute (NaCl) required to achieve various water activities. (From data of Webb, 1960; Bateman *et al.*, 1962.)

Ehresmann and Hatch, 1975). In addition, death rates at lower water activities are influenced rather strongly by the partial pressure of oxygen in the atmosphere. Higher oxygen pressures are usually more damaging (Zentner, 1966; Webb, 1967a). Why the removal of bound water is damaging to cells is by no means clear. The bound water fraction of cells (i.e., ~0.3 g H_2O g^{-1} solids) is comparable to the amount of water in the hydration layer of biological macromolecules (Kuntz *et al.*, 1969); so perhaps the question can be rephrased to ask why is the removal of water of hydration damaging to cells? There is speculation but little in the way of answers.

4. Limits to Survival at Low Temperatures and to Dehydration

Any cell that tolerates freezing to about −70 °C appears capable of withstanding the lowest temperatures attainable. Even complex forms like early mouse embryos survive freezing to −269 °C without difficulty (Whittingham *et al.*, 1972). Nor does there appear to be a fundamental limit to the degree of dehydration that is tolerable by at least some cells in the absence of oxygen. Bacterial forms have been dried to water activities that

approach zero (Portner *et al.*, 1961; Morelli *et al.*, 1962). Still, although there are no absolute limits, organisms exhibit an enormous range in their sensitivity to low temperatures and dehydration, and the sensitivity of a given organism can vary by orders of magnitude depending on how it has been frozen and thawed or how it has been dehydrated.

Conditions in nature reflect the close coupling between freezing and dehydration in that forms that tolerate the latter will generally tolerate the former (Levitt, 1966). Examples are fungous conidia, bacterial spores, and Artemia cysts. But the reverse is not necessarily true. Numerous forms that in nature or the laboratory are highly sensitive to dehydration will survive freezing under appropriate conditions. Examples include many protozoa and apparently all mammalian cells. Finally, there are forms in nature (higher plants and insects) that undergo remarkable seasonal increases in their ability to tolerate freezing (Levitt, 1966; Li and Sakai, 1978).

The fraction of cells that survives freezing is generally no more resistant to subsequent freezing and thawing than the original population. This fact plus other evidence indicates the survivors of freezing are not a genetic subset of the original population. In other words, freezing is neither selective nor mutagenic. On the other hand, there is evidence that freeze- and air-drying can be mutagenic (Webb, 1967; Servin-Massieu and Cruz-Camarillo, 1969; Hieda and Ito, 1973; Ashwood-Smith and Grant, 1976). However, its mutagenicity is apparently not severe enough to cause obvious problems for those who use the procedure to preserve cultures of microorganisms (Brown, 1963).

5. Minimum Temperatures for Cell Growth and Function

A. EFFECT OF SUBZERO TEMPERATURES *PER SE*

Some information on the effects on cells of subzero temperature *per se* (i.e., in the absence of freezing) can be obtained by taking advantage of the ability of dilute aqueous solutions to supercool to about -10° and occasionally to as low as $-20\ ^{\circ}C$. Most (although not all) cells survive supercooling to such temperatures, but relatively little is known about their ability to grow and function for extended periods of time. One fascinating exception is the report of Scholander *et al.* (1957) and Scholander and Maggert (1971) who discuss certain arctic fish that live permanently supercooled a degree or so. Presumably, cell responses a few degrees below $0\ ^{\circ}C$ ought not to be qualitatively different from their responses at $0\ ^{\circ}C$ or a few degrees above, and the responses to the latter been reviewed extensively (Baross and Morita, 1978; Inniss and Ingraham, 1978).

B. CELL GROWTH IN THE PARTLY FROZEN STATE

Unlike cell survival, there is a definite lower temperature limit for cell growth in the frozen state. Michener and Elliott (1964) thoroughly reviewed the situation for microorganisms and found that the number of reported instances of cell growth diminished rapidly a few degrees below $0\ ^{\circ}C$ (Figure 19). In fact, they report no confirmed cases of growth below $-12\ ^{\circ}C$. The inability of organisms to grow below $-12\ ^{\circ}C$ is consistent with the known physical state of aqueous solutions at these temperatures. As Table II shows, when

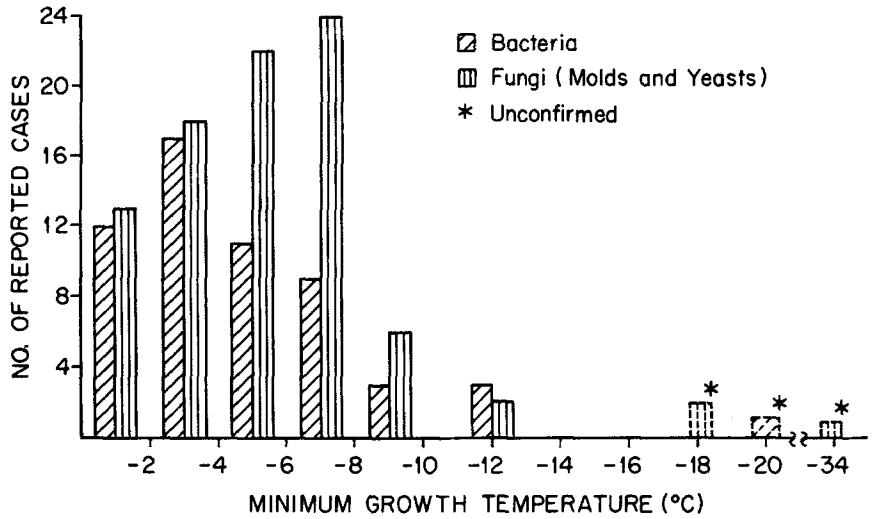

Fig. 19. Reported cases of microbial growth below 0 °C. Adapted from Michener and Elliott (1964) by Mazur *et al.* (1978).

solutions of sodium chloride in water, for example, are equilibrated at various subzero temperatures, the concentrations in the unfrozen portions reach 3.7 molal (3.5 molar) by −14 °C. For solutes in general, the concentrations of solutes in the unfrozen portions of solutions are given by $\phi \nu m = \Delta T/1.86$, where ϕ is the osmotic coefficient, ν the number of species into which the solute dissociates, and m is the molality. Aside from the toxic effects to nearly all microorganisms of such high concentrations of electrolytes, the high concentrations also depress the water activity (a_w) below the value permitting the growth even at optimal temperatures of nearly all microorganisms save halophilic and

TABLE II
Solute concentrations and water activities in NaCl solutions at various temperatures

Temperature (°C)	Concentration NaCl[a] (molal)	a_w [b]
−5	1.45	0.95
−10	2.79	0.91
−14	3.73	0.87
−16	4.17	0.85
−18	4.58	0.84
−20	4.99	0.82

[a] In the unfrozen portion of the solution. From International Critical tables (1926).

[b] Calculated from data of Dorsey (1940). The activities depend only on temperature (and total hydrostatic pressure); they are independent of the nature of the solute.

osmophilic forms (see below). As shown in Table II, the value of a_w at −16 °C has dropped to 0.85.

7. Cell Growth at Low Water Activities

The minimum water activities (a_w) for cell growth at normal temperatures have recently been reviewed by Rose (1976). Table III is a portion of his data. Most bacteria require an a_w above 0.9; most fungi an a_w above 0.85. The lowest limit is 0.61 for the fungus *Xeromyces bisporus*. The general lower limit of ~0.9 is not surprising. As just mentioned, a sodium chloride solution in equilibrium with an a_w of 0.9 has a concentration of about 3 molal (Table II).

I pointed out in the beginning of this chapter that cells can be suspended in *liquid* water at low a_w (lowered by high solute concentration), or they can be equilibrated with water *vapour* at any desired a_w between 0 and 0.99. There have been arguments over the years as to whether cell function and growth demand the presence of extracellular liquid water or whether high a_w water vapour would suffice. The preponderant opinion favors the former view. However, a careful study by Lange *et al.* (1970) indicates that lichens in the Negev Desert, which have become extremely dehydrated during the day,

TABLE III
Minimum water activities for growth of representative microorganisms
(From A. H. Rose, 1976)

Organism	Min a_w for growth	Equiv. conc. of NaCl at 25 °C[a] (molal)
Bacteria		
Aerobacter aerogenes	0.94	1.8
Bacillus cereus	0.99	0.3
Salmonella typhimurium	0.92	2.3
Micrococcus sp.	0.83	4.4
Halobacterium	0.76[b, c]	6.0
Yeasts		
Saccharomyces cerevisiae	0.92	2.3
Hansenula suaveolens	0.97	0.9
Saccharomyces rouxii	0.85[b]	4.0
Molds		
Rhizopus nigricans	0.93	2.0
Aspergillus niger	0.84	4.2
Aspergillus ruber	0.70	>6[d]
Xeromyces bisporus	0.61[b]	>6[e]

[a]From Robinson and Stokes (1959).
[b]Lowest value recorded for the indicated category of microorganism.
[c]The activity for a saturated solution of sodium chloride.
[d]Equivalent to 3.4 m $CaCl_2$.
[e]Equivalent to 4.0 m $CaCl_2$.

are capable at night of absorbing enough water vapor, even in the absence of the condensation of dew, to permit a short pulse of photosynthetic activity early in the morning. However the major portion of photosynthetic activity occurs when the lichen thalli are wet by condensed dew. Even in this remarkable case, then it appears that the organism may exhibit its full complement of functions only in the presence of liquid water.

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