Mechanisms of intracellular ice formation

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ABSTRACT The phenomenon of intracellular freezing in cells was investigated by designing experiments with cultured mouse fibroblasts on a cryomicroscope to critically assess the current hypotheses describing the genesis of intracellular ice: (*a*) intracellular freezing is a result of critical undercooling; (*b*) the cytoplasm is nucleated through aqueous pores in the plasma membrane; and (*c*) intracellular freezing is a result of membrane damage caused by electrical transients at the ice interface. The experimental data did not support any of these theories, but was consistent with the hypothesis that the plasma membrane is damaged at a critical gradient in osmotic pressure across the membrane, and intracellular freezing occurs as a result of this damage. An implication of this hypothesis is that mathematical models can be used to design protocols to avoid damaging gradients in osmotic pressure, allowing new approaches to the preservation of cells, tissues, and organs by rapid cooling.

INTRODUCTION

Many species of plants and animals are naturally able to survive exposure to low temperatures, and it is now possible to preserve the viability of cells from homeothermic species at low temperatures for indefinite periods of time. However, the techniques for cryopreservation, largely developed using empirical methods, have been remarkably unsuccessful when applied to organized tissues. This has focused recent attention on the mechanisms of damage and protection in living cells and tissues at low temperatures in naturally occurring frost hardiness (1) and in attempts to preserve the viability of tissues (2). Several approaches are being taken to understand and avoid potential damaging conditions during cryopreservation by reducing the total amount of ice formed (3), or by preventing the formation of ice (4). An implicit assumption is that the conditions leading to the formation of ice inside the cell are inevitably lethal, so approaches to preservation must avoid these conditions. This assumption has resulted in a scarcity of information on the mechanisms by which ice forms in living cells and the nature of the concomitant damage to cells.

The formation of ice in the environment of the cell induces changes to which the cell must respond. When a cell suspension is cooled below its freezing point water is removed from the solution in the form of ice, increasing the concentration of solutes which remain in the unfrozen fraction and hence increasing the osmotic pressure of the remaining solution. The resulting gradient in osmotic pressure across the plasma membrane provides the driving force for an efflux of water from cells, with the rate of efflux being limited by the permeability of the plasma membrane to water (5). As the temperature is lowered, more ice is formed and the concentration of solutes in the unfrozen fraction increases. If the cooling rate is sufficiently slow to allow the cell to remain close to osmotic equilibrium, then water efflux will continue to low temperatures (5). At cooling rates high enough to cause significant departure from osmotic equilibrium, where the kinetics of ice formation in the extracellular medium are much faster than the kinetics of osmotic water efflux from the cell, intracellular freezing may result (5). Once ice forms within the cell, osmotic equilibrium with the extracellular solution during further cooling is maintained by in situ freezing of water in the cell rather than movement of water across the plasma membrane.

The mechanisms leading to intracellular freezing are unclear, as indicated by the diversity of the current hypotheses describing the genesis of intracellular ice:

(*a*) *Intracellular freezing as a result of critical undercooling.* As the cooling rate increases, the difference between the intracellular and extracellular osmotic pressures increases, and hence the degree of undercooling (the difference in temperature between the actual temperature of the cytoplasm and its freezing point) in the cytoplasm increases. Levitt proposed that the cytoplasm nucleates spontaneously at a critical degree of undercooling (6).

(*b*) *Cytoplasm is nucleated through aqueous pores in the plasma membrane.* The temperature dependency of the ice nucleation rate and ice crystal growth rate results

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in the formation of smaller crystals at higher cooling rates (7). Mazur (7–9) proposed that, as the cooling rate increases and the radius of the growing tip of ice crystals decreases, propagation of extracellular ice through the plasma membrane occurs when the tip radius of the crystals approximates the radius of aqueous pores in the plasma membrane.

(*c*) *Intracellular freezing as a result of electrical transients at the ice interface.* Electrical transients are created at a growing ice interface in aqueous solutions by the selective exclusion of charged species from ice at the interface (10). It has been suggested (11) that this Workman-Reynolds effect may contribute to the disruption of the plasma membrane when the magnitude of the potential difference across the plasma membrane due to these electrical transients reaches a critical level. Rupture of the plasma membrane would allow extracellular ice to grow rapidly into the undercooled cytoplasm. This hypothesis is consistent with recent observations (12) which show that cell damage precedes the appearance of intracellular ice in isolated protoplasts (13), implying that intracellular ice is a result rather than the cause of injury.

The conditions leading to intracellular freezing are almost always associated with lethal injury to cells (14). In the hypotheses where intracellular freezing is the cause of cell injury, it has been proposed that injury is related to the formation (3), recrystallization (7, 9), or melting (15) of intracellular ice.

This study was designed to investigate mechanisms of intracellular freezing in living cells and its relevance to cell damage, to discern between the various hypotheses of cell injury related to intracellular freezing, and to utilize the information in the practical preservation of cells and tissues. The experimental results do not support any of the current hypotheses describing the genesis of intracellular freezing, but point to the conclusions that the plasma membrane is damaged by gradients in osmotic pressure across the membrane, and that intracellular freezing is a result rather than the cause of damage to the plasma membrane.

MATERIALS AND METHODS

Cells

The mouse fibroblast cells (EMT-6) in tissue culture used in these studies were grown in minimal essential medium (Gibco, Grand Island, NY) with Hank's salts, 25 mM Hepes buffer (Gibco) and 10% fetal calf serum (Gibco). Cells, normally adherent to plastic tissue culture dishes (25 cm^2; Corning Glass Works, Corning, NY), were harvested by trypsinization (0.25% trypsin [Gibco] for 10 min at 37°C) and resuspended in tissue culture medium. Cells were maintained in suspension by gently shaking at 4°C before use in the experiments.

Cryomicroscopy

Microscopic observations of cells at subzero temperatures were made using a cryomicroscope stage similar in design to the convection stage described by Diller (16, 17), in which the stage is cooled with cold nitrogen gas and heated through the electrical resistance of a transparent, electrically conductive coating on a glass slide (Corning Glass Works). A copper-constantan thermocouple junction (0.001-in diameter, Omega Engineering, Inc., Stamford, CT) placed at the center of the heated slide was used for temperature measurement through an analog interface (Tecmar, Inc., Cleveland, OH) to a microcomputer (IBM AT). A computer program was developed to monitor the temperature and provide an output for proportional control of the heating element. This stage allowed linear tracking of a predefined thermal protocol (±0.2°C) with cooling rates up to 200°C/min. A 0.2-μl sample of cell suspension under a cover slip was used on the cryomicroscope stage, and all observations were made within 0.5 mm of the thermocouple junction to minimize the effects of any thermal gradients on the stage. Experiments were recorded on video tape for subsequent analysis.

Assays

Intracellular freezing

The formation of small ice crystals in an undercooled cytoplasm scatter light, thus causing the cytoplasm to sharply change from being transparent to dark. This phenomenon, called "flashing," was used as an indicator of intracellular freezing.

Integrity of the plasma membrane

It has previously been shown that the intact plasma membrane is an effective barrier to ice crystals (18, 19). This property of the plasma membrane was used to assess the integrity of the plasma membrane while allowing correlation of membrane integrity with the results of the previous treatment of individual cells. A cell suspension was cooled to and maintained at a subzero temperature (1.0°C below the freezing point of the suspending solution), and ice nucleated at the edge of the sample by placing a copper wire cooled in liquid nitrogen above the edge of the sample chamber. Ice grows through the extracellular solution, but does not pass through the plasma membrane of intact cells, which instead shrink osmotically, displaying semipermeable properties. Extracellular ice passing through the membrane of a cell into the cytoplasm was indicative of damage to the plasma membrane. The ability of the plasma membrane to function as a barrier to extracellular ice crystals was used to assess membrane integrity.

Freezing at constant cooling rates

Cells in tissue culture medium were exposed to solutions of dimethyl sulfoxide (DMSO) using stepwise addition over a 5-min time interval at 22°C, cooled to 0°C, and an aliquot of the suspension placed on the cryomicroscope stage. The stage was cooled to a temperature below the freezing point of the suspending solution (−1°C for 0 M DMSO, −3°C for 0.5 M DMSO, −4°C for 1 M DMSO, and −7°C for 2 M DMSO), and ice formation was nucleated in the extracellular solution using a cooled copper wire. The sample was held at the set temperature for 30 s to allow osmotic equilibration of the cells, then the stage was cooled at one of five predetermined cooling rates (−70, −100, −125, −150, or −200°C/min) to −40°C.

Freezing at constant subzero temperatures

Cells in tissue culture medium were exposed to solutions of glycerol (0, 0.5, 1.0, 2.0 M) using stepwise addition over a 25-min time interval

at 22°C, cooled to 0°C, and an aliquot placed on the cryomicroscope stage. The sample was then cooled at −20°C/min to a predetermined temperature between −3 and −16°C in the absence of ice. A constant temperature was maintained while ice formation was initiated using the cooled copper wire and for an additional 15 s before warming of the sample at 100°C/min. The cooling power of the microscope stage was sufficient to remove the latent heat of fusion from the sample while maintaining the temperature within 1°C of the set value. The percentage of cells which froze intracellularly and the velocity of the ice interface were measured for each experimental condition. Calibrations of distance throughout the optical and video systems were performed using a hemocytometer (American Optical, Buffalo, NY), and interface velocities were determined from distances traversed by the ice interface due to growth of the ice crystals in one video frame (1/30 s).

Membrane damage after freezing and thawing

Cells suspended in medium without cryoprotectant were undercooled on the cryomicroscope to −6.5°C and held for 30 s to achieve thermal equilibrium before ice formation was initiated in the sample. At this temperature, ~50% of the cells showed the presence of intracellular ice. The sample was then warmed at 20°C/min to +2°C to melt the ice, then immediately cooled at −20°C/min to −2°C where the integrity of the plasma membrane was assessed by its ability to act as a barrier to growing ice crystals. The responses of individual cells were followed throughout the entire protocol to assess intracellular freezing after the first ice initiation at −6.5°C and the subsequent ability of the plasma membrane to exclude an advancing ice front on the second freeze at −2°C. This permitted the correlation of observations on intracellular ice formation with damage to the plasma membrane.

Osmotic stresses in the absence of ice

Cells in suspension were held at 0°C and DMSO was added for final concentrations of 3, 4, or 5 M using slow or rapid addition of DMSO. Slow addition of DMSO of either concentration was performed using 12 steps over a 6-min interval. For rapid addition, the concentration was increased in a single, rapid step while the suspension was gently mixed. 2 min after the final step, all suspensions were diluted slowly using 22 steps over an 11-min interval, centrifuged at 500 *g* for 45 s to concentrate the cells, the supernatant removed, and the cells assayed for membrane integrity by the ability to exclude ice.

RESULTS

Intracellular freezing at constant cooling rates

Table 1 presents the data for the experiment in which cells were cooled at various constant rates after equilibrating in several concentrations of DMSO. The total number of cells observed are shown for each case along with the total number of cells which froze intracellularly and the mean temperature at which this event occurred. These data are plotted in Fig. 1 with the temperature at which intracellular freezing occurred shown as a function of cooling rate. The most striking features of the graph are that the temperature at which intracellular freezing occurs is independent of the cooling rate, but strongly dependent on the composition of the solution (i.e., the concentration of DMSO). The greater the concentration of DMSO, the lower the temperature at which intracellular ice forms.

Ice nucleation at undercooled temperatures

Table 2 shows the total number of cells observed and the number of cells in which intracellular freezing occurred when ice formation was initiated at undercooled temperatures in various concentrations of glycerol. These results are shown in Fig. 2 *a*, in which the percentage of cells which froze intracellularly is plotted as a function of the temperature at which ice formation was initiated. The curves are fit using the Bezier method (20) which does not assume any mathematical relationship between the data points. From these curves, the temperatures for intracellular nucleation in 50% of the cells (Ti_{50}) were interpolated, and mapped onto graphs in Fig. 2 *b–d*. Linear regression lines were fitted to the mapped points, with the slopes, intercepts, and standard errors of these coefficients calculated according to Walker and Lev (21).

At low rates of cooling, the magnitude of the electrical

TABLE 1 Intracellular freezing as a function of cooling rate and DMSO concentration

	0 M DMSO			0.5 M DMSO			1 M DMSO			2 M DMSO		
Cooling Rate	No. cells	No. flashes	Flash temp.	No. cells	No. flashes	Flash temp.	No. cells	No. flashes	Flash temp.	No. cells	No. flashes	Flash temp.
°C/min			*mean °C ± SE*			*mean °C ± SE*			*mean °C ± SE*			*mean °C ± SE*
70	55	5	−8.9 ± 1.6	58	2	−18.8 ± 1.2	55	3	−26.0 ± 1.7	55	6	−29.3 ± 1.9
100	49	8	−13.5 ± 1.4	52	5	−20.3 ± 2.6	44	5	−21.5 ± 3.6	59	22	−28.6 ± 1.2
125	54	17	−11.1 ± 1.3	58	3	−17.4 ± 3.6	52	16	−24.7 ± 1.4	54	24	−29.3 ± 1.2
150	55	20	−13.8 ± 1.0	75	16	−18.9 ± 1.9	62	17	−25.1 ± 0.7	51	35	−30.7 ± 1.0
200	51	33	−9.2 ± 0.5	52	25	−17.7 ± 1.3	57	37	−25.3 ± 1.1	51	48	−28.1 ± 0.9

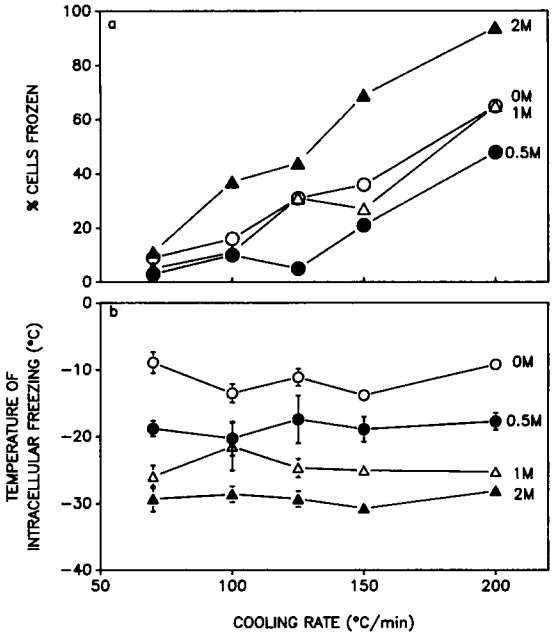

FIGURE 1 Intracellular ice formation in cells equilibrated in various concentrations of DMSO, cooled at constant cooling rates. (*a*) Proportion of cells in which intracellular ice formed is shown. (*b*) Average temperature at which intracellular freezing occurred. Bars represent standard errors of a minimum of 50 replicate samples, unless smaller than the points as plotted.

transients at the interface between ice and the solution increases with increasing velocity of the interface (11). In Fig. 2 *b*, Ti_{50} values are superimposed on the curves showing the velocity of the ice interface as a function of the temperature at which ice formation is initiated for the

TABLE 2 Intracellular freezing upon ice nucleation at undercooled temperatures

	0 M glycerol		0.5 M glycerol		1 M glycerol		2 M glycerol	
Temp. of ice seeding	No. cells	No. flashes	No. cells	No. flashes	No. cells	No. flashes	No. cells	No. flashes
°C								
−3	45	0	—	—	—	—	—	—
−4	49	4	—	—	—	—	—	—
−5	51	7	—	—	—	—	—	—
−6	48	20	49	0	—	—	—	—
−7	42	26	47	3	—	—	—	—
−8	48	45	49	6	48	0	—	—
−9	—	—	49	23	41	6	—	—
−10	—	—	51	25	48	16	48	0
−11	—	—	42	33	48	15	43	1
−12	—	—	—	—	44	30	42	2
−13	—	—	—	—	48	46	51	8
−14	—	—	—	—	—	—	41	18
−15	—	—	—	—	—	—	46	23
−16	—	—	—	—	—	—	45	36

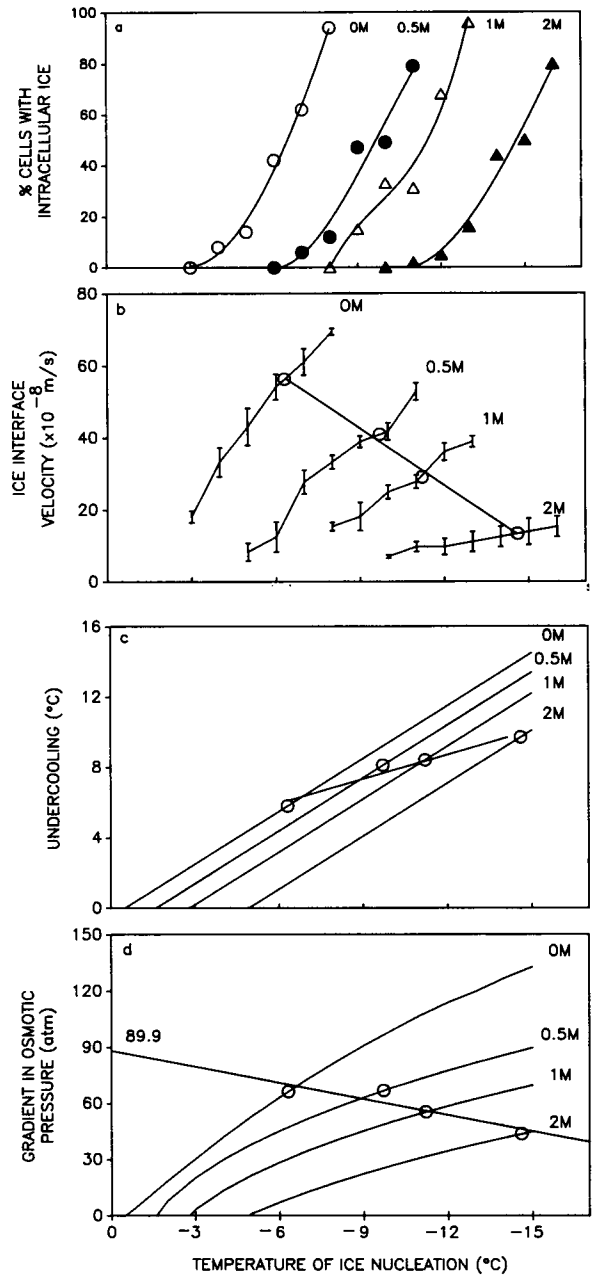

FIGURE 2 (*a*) Proportion of cells with intracellular ice after nucleation at various undercooled temperatures in four concentrations of glycerol. Seeding temperatures at which 50% of the cells freeze intracellularly are mapped onto *b–d*, and used as data points for regression analysis. The measured ice interface velocity for each condiiton is shown in *b*, where bars represent standard errors of three replicate measurements. The extent of undercooling on ice nucleation is shown in *c*, and the osmotic pressure gradient across the cell membrane on ice formation is shown in *d*. Slopes of the regression lines (±SE) are as follows: (*b*) 5.23 ± 0.34; (*c*) −0.46 ± 0.068; (*d*) 2.88 ± 0.95.

four concentrations of glycerol used. The line representing the linear regression of the Ti_{50} points has a positive slope significantly greater than zero (5.23 ± 0.34), showing that the velocity of the ice interface resulting in intracellular freezing was dependent on temperature. Intracellular freezing did not occur at a constant critical velocity of the ice interface.

Fig. 2 *c* shows the relationships between the degree of undercooling and the temperature of ice nucleation. The degree of undercooling is the difference in temperature between the temperature of the solution and its freezing point, the latter being determined from equations developed by Fahy (22) and Pegg (23, 24) to describe the melting points and compositions of several ternary aqueous solutions at low temperatures. The melting points of the tissue culture medium with various concentrations of DMSO and glycerol were measured and found to be the same as predicted from the equations which describe the properties of solutions containing water, NaCl, and either DMSO or glycerol, supporting the use of these equations to describe the compositions of the extracellular solutions after ice nucleation in these experiments. The lines in Fig. 2 *c* are parallel at 45° because both axes represent colligative properties of the solutions. The regression line for Ti_{50} values mapped in Fig. 2 *c* has a negative slope (−0.46 ± 0.068) which is significantly less than zero, indicating that an increased degree of undercooling is required for intracellular nucleation at lower temperatures, as the concentration of glycerol is increased.

Because the cells are in osmotic equilibrium with the suspending solution before ice nucleation in the extracellular solution, the gradient in osmotic pressure across the plasma membrane immediately after nucleation is the difference between the osmotic pressures of the suspending solution before and after ice nucleation. This gradient in osmotic pressure across the plasma membrane is plotted as a function of ice nucleation temperature in Fig. 2 *d*. The regression line fitted to the Ti_{50} values mapped onto these curves has a slope of 2.88 ± 0.95, indicating that the slope of the line is significantly greater than zero. Because the regression line represents the relation between temperature and the magnitude of osmotic pressure gradient which causes damage in 50% of the cells and this relation is causally decoupled from the means by which the osmotic pressure was varied (the concentration of glycerol), then extrapolation of this regression line predicts that, at 0°C, 50% of the cells would freeze intracellularly at an osmotic pressure gradient of 89.9 ± 9.9 atm.

Membrane damage after freezing and thawing

Table 3 shows data on cells that were subjected to two freezing events. The first event (nucleation of an undercooled suspension at −6.5°C) was designed to produce intracellular ice in ~50% of the cells. Table 3 shows the numbers of cells with and without intracellular ice after the first freezing event. The second freezing event was the test for membrane integrity by the ability to act as a barrier for growing ice crystals. The data in Table 3 clearly show that the formation of intracellular ice in a cell is accompanied by loss of ability to block the growing ice crystals from entering the cytoplasm. Cells which are subjected to identical conditions but do not freeze intracellularly retain their barrier properties.

TABLE 3 Correlation of damage to the plasma membrane with intracellular freezing

First freeze: No. cells with intracellular ice		Second freeze: No. cells with barrier properties	
		retained	lacking
Absent	76	71	5
Present	70	4	66

Osmotic stresses in the absence of ice

At 0°C in the absence of ice, cells were exposed to osmotic stresses associated with the slow or rapid addition of a permeant solute (DMSO), and integrity of the plasma membrane assessed by the ability to act as a barrier for extracellular ice. Table 4 shows that, in the slow addition group, there is virtually no damage to the plasma membranes at any concentration of DMSO. However, in the rapid addition group, damage increases with increasing concentrations of DMSO. These results are plotted as a function of osmotic pressure gradient in Fig. 3. A regression line fitted to the data gives an *r* value of 0.996. The osmotic pressure gradient at which 50% of the cells are damaged, interpolated from the graph, is 90.3 atm.

TABLE 4 Membrane damage due to an osmotic pressure gradient at 0°C

Final concentration of DMSO	Rapid addition of DMSO		Slow addition of DMSO	
	No. cells retaining barrier properties	No. cells lacking barrier properties	No. cells retaining barrier properties	No. cells lacking barrier properties
3 M	56	7	47	1
4 M	32	28	48	0
5 M	6	44	57	1

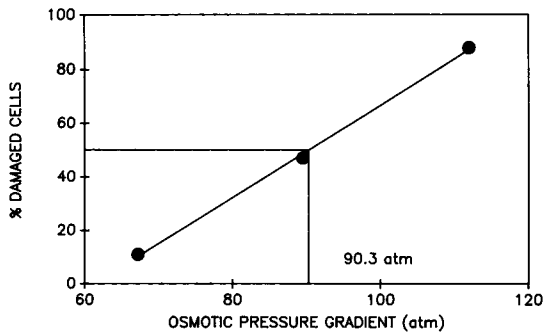

FIGURE 3 Proportion of cells unable to provide a barrier to ice is shown as a function of the osmotic pressure gradient produced by rapid addition of DMSO at 0°C. The osmotic pressure gradient which produced 50% damage is interpolated.

DISCUSSION

Reassessment of current theories of intracellular freezing

Cytoplasm is nucleated through aqueous pores in the plasma membrane

The morphology of ice crystals formed during freezing depends on many factors, including the composition of solution, the cooling rate, and the temperature. As the cooling rate increases, the tip radius of the growing ice crystal decreases (7), and Mazur (9) proposed that extracellular ice crystals will propagate through aqueous pores in the plasma membrane and into the cytoplasm when the tip radius is equal to the radius of aqueous pores in the plasma membrane. Experimental data in Fig. 1 shows that the temperature of intracellular freezing is independent of the cooling rate, and therefore independent of the tip radius of growing ice crystals. The data does not support the hypothesis of intracellular nucleation by extracellular ice through aqueous pores in the plasma membrane.

Intracellular freezing as a result of electrical transients at the ice interface

Electrical transients develop at the advancing ice interface in physiological solutions (25), with the magnitude of the electrical potential difference between the ice phase and liquid phase at the interface proportional to the velocity of the interface (11), leading to the hypothesis (11) that the plasma membrane of cells in this environment will rupture when the magnitude of the electrical transients reaches a critical level. Two lines of experimental evidence refute this hypothesis. The first line of evidence, presented in Fig. 2 *b*, shows that intracellular freezing does not occur at a constant velocity of the ice interface. The composition of the solution influences the magnitude of the electrical transients which occur at the ice interface, with dilute aqueous solutions producing larger electrical transients, and high concentrations of solute reducing the effect due to the presence of mixing currents at the ice-solution interface (11). The data from Fig. 2 *b* show that intracellular freezing occurs at lower velocities in increasing concentrations of solutes, the opposite of what would be expected from the hypothesis of electrical transients as a cause of intracellular freezing. The second line of evidence relates to data presented in Fig. 1, where intracellular freezing was observed at constant temperatures for each solution, independent of the cooling rate. The velocity of the ice interface, for a given temperature and solution, is dependent on the cooling rate. The data in Fig. 1 therefore indicate that intracellular freezing occurs under conditions of different ice interface velocities.

Intracellular freezing as a result of critical undercooling

During cooling at rates where water efflux from the cytoplasm, limited by the hydraulic conductivity of the plasma membrane, is insufficient to maintain osmotic equilibrium with the extracellular environment, the cytoplasm becomes increasingly undercooled. As the cooling rate increases the degree of undercooling increases, and Levitt (6) proposed that intracellular nucleation will occur at a critical level of undercooling. Fig. 2 *c* shows that intracellular freezing does not occur at a constant degree of undercooling.

A new theory of intracellular freezing

The three existing hypotheses on the genesis of intracellular freezing are not supported by the experimental evidence. The data, however, support the hypothesis that the plasma membrane is damaged at a critical gradient in osmotic pressure across the membrane (26), and intracellular freezing occurs as a result of this damage. After intracellular freezing, the plasma membrane loses its ability to act as a barrier for extracellular ice (Table 3), indicating damage to the plasma membrane is concomitant with intracellular freezing. Exposure of cells to osmotic stresses (Table 4) showed conclusively that similar damage to the plasma membrane can be induced at 0°C in the absence of ice at a magnitude of osmotic pressure gradients (90.3 atm) remarkably similar to those producing damage during freezing (extrapolated to 89.9 atm at 0°C, in Fig. 2 *d*). The magnitude of critical osmotic pressure gradient decreases with decreasing temperature (Fig. 2 *d*), consistent with the interpretation that the tensile strength of any material is temperature dependent. The membrane becomes more brittle as the temper-

ature decreases, thereby requiring less force for rupture. During freezing of cell suspensions, it is not possible to isolate the osmotic pressure gradients across the plasma membrane from the degree of undercooling. However, the observation that it is possible to induce damage to the plasma membrane in the absence of ice using an osmotic pressure gradient with magnitude almost identical to the gradients causing damage during freezing and thawing supports the hypothesis of damage due to critical osmotic pressure gradients. The mechanism by which osmotic pressure gradients may be related to membrane damage have not been explored in this study, but it is possible that the damaging agent may not be the osmotic pressure itself. It could, for example, be related to the flux of water through the membrane which is driven by the gradient in osmotic pressure.

The phenomenon of recrystallization in frozen samples occurs as smaller ice crystals with higher surface energy dissolve and larger crystals grow; the rate of recrystallization increases with increasing temperature (27). Recrystallization of both intracellular and extracellular ice occurs during warming although the total amount of ice decreases as the temperature increases. It has been suggested (9, 28) that cells cooled under conditions resulting in little intracellular ice formation may be recovered undamaged if the warming rate is rapid, but would be irreparably damaged by the recrystallization of the intracellular ice on slow warming. This suggests that membrane damage caused by the osmotic pressure gradient may be resealed if the warming conditions prevent the recrystallization of ice crystals traversing the membrane. A similar resealing repair mechanism for freeze-thaw damaged cells was proposed by Law et al. (29) which could also explain the 6% of cells that retained their barrier properties after forming intracellular ice as shown in Table 3.

It should be stressed that this theory addresses injury related to the formation of intracellular ice, and does not explain all freezing-related damage to cells in suspension of which there is unlikely to be a single mechanism of injury.

An implication of this hypothesis is that mathematical models can be used to design protocols to avoid damaging gradients in osmotic pressure, allowing new approaches to the preservation of cells, tissues, and organs by rapid cooling.

REFERENCES

1. Steponkus, P. L., and S. C. Wiest. 1978. Plasma membrane alterations following cold acclimation and freezing. *In* Plant Cold Hardiness and Freezing Stress: Mechanisms and Crop Implications. (P. H. Li and A. Sakai, editors. Academic Press, Inc., New York. 75–91.
2. Karow, A., and D. Pegg, editors. Organ Preservation for Transplantation. 2nd ed.
3. Farrant, J., C. Walter, H. Lee, and L. McGann. 1977. Use of two-step cooling procedures to examine factors influencing cell survival following freezing and thawing. *Cryobiology.* 14:273–286.
4. Farrant, J. 1977. Water transport and cell survival in cryobiological procedures. *Phil. Trans. R. Soc. Lond. B Biol. Sci.* 278:191–205.
5. Mazur, P. 1963. Kinetics of water loss from cells at subzero temperatures and the likelihood of intracellular freezing. *J. Gen. Physiol.* 47:347–369.
6. Levitt, J., and G. W. Scarth. 1936. Frost hardening studies with living cells. II. Permeability in relation to frost resistance and the seasonal cycle. *Can. J. Res. Sect. C Bot. Sci.* 14:285–305.
7. Mazur, P. 1961. Manifestations of injury in yeast cells exposed to subzero temperatures. *J. Bacteriol.* 82:662.
8. Mazur, P. 1960. Physical factors implicated in the death of microorganisms at subzero temperatures. *Ann. NY Acad. Sci.* 85:610–629.
9. Mazur, P., S. P. Leibo, and E. H. Y. Chu. 1972. A two factor hypothesis of freezing injury. *Exp. Cell Res.* 71:345–355.
10. Workman, E., and S. Reynolds. 1950. Electrical phenomena occurring during the freezing of dilute aqueous solutions and their possible relationship to thunderstorm electricity. *Phys. Rev.* 78:254–259.
11. Steponkus, P., D. Stout, J. Wolfe, and R. Lovelace. 1984. Freeze-induced electrical transients and cryoinjury. *Cryo Lett.* 5: 343–348.
12. Dowgert, M. G., and P. L. Steponkus. 1983. Effect of cold acclimation on intracellular ice formation in isolated protoplasts. *Plant Physiol. (Bethesda).* 72:978–988.
13. Steponkus, P., and M. Dowgert. 1981. Phenomenology of intracellular ice nucleation in isolated protoplasts. *Plant Physiol. (Bethesda).* 67:S-58.
14. Leibo, S. 1977. Preservation of mammalian cells and embryos by freezing. *Cryoimmunology.* 62:311–334.
15. Farrant, J. and G. J. Morris. 1973. Thermal shock and dilution shock as the causes of freezing injury. *Cryobiology.* 10:134–140.
16. Diller, K. R., and E. G. Cravalho. 1971. A cryomicroscope for the study of freezing and thawing processes in biological cells. *Cryobiology.* 7:191–199.
17. Schwartz, G. J., and K. R. Diller. 1982. Design and fabrication of a simple, versatile cryomicroscope stage. *Cryobiology.* 19:529–538.
18. Chambers, R., and H. Hale. 1932. The formation of ice in protoplasm. *Proc. R. Soc. Lond. B Biol. Sci.*, 110:336–352.
19. Luyet, B. J., and M. C. Gibbs. 1937. On the mechanism of congelation and of death in the rapid freezing of epidermal plant cells. *Biodynamica.* 1: 1–18.
20. Newman, W., and R. Sproull. 1979. Principles of Interactive Computer Graphics. McGraw-Hill Book Co., New York. 315–319.
21. Walker, H., and J. Lev. 1953. Statistical Inference. Holt, Rinehart and Winston, New York. 239–241.
22. Fahy, G. M. 1980. Analysis of "solution effects" injury: equations for calculating phase diagram information for the ternary systems NaCl-dimethylsulfoxide-water and NaCl-glycerol-water. *Biophys. J.* 32:837–850.
23. Pegg, D. E. 1983. Simple equations for obtaining melting points and

eutectic temperatures for the ternary system glycerol/sodium chloride/water. *Cryo Lett.* 4:259–268.

24. Pegg, D. E. 1986. Equations for obtaining melting points and eutectic temperatures for the ternary system dimethyl sulphoxide/sodium chloride/water. *Cryo Lett.* 7:387–394.
25. Lodge, J., M. Baker, and J. Pierrard. 1956. Observations on ion separation in dilute solutions by freezing. *J. Chem. Phys.* 24:716–719.
26. Muldrew, K., and L. E. McGann. 1988. Mechanisms of intracellular ice formation. *Cryobioiogy.* 25:554.
27. Mazur, P., and J. Schmidt. 1968. Interactions of cooling velocity, temperature, and warming velocity on the survival of frozen and thawed yeast. *Cryobiology.* 5:1–17.
28. McGann, L., and J. Farrant. 1976. Survival of tissue culture cells frozen by a two-step procedure to – 196°C. II. Warming rate and concentration of dimethylsulfoxide. *Cryobiology.* 13:269–273.
29. Law, P., D. Sprott, J. Lepock, and J. Kruuv. 1980. Post-thaw lysing and osmotic reactivation of frozen-thawed cells. *Cryo Lett.* 1:173–180.