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FUNDAMENTAL CRYOBIOLOGY OF MOUSE OVA AND EMBRYOS

S. P. Leibo

Biology Division, Oak Ridge National Laboratory,[1]

Oak Ridge, Tennessee 37830

MASTER

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Send proofs to: Dr. S. P. Leibo

Biology Division

Oak Ridge National Laboratory

P. O. Box Y

Oak Ridge, Tennessee 37830

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Abstract

An increasing fraction of mouse ova and embryos are killed as the rate at which they are cooled to -196°C is increased. The survival of these cells depends not only on cooling rate, but also on the minimum subzero temperature to which the cells are cooled. Low temperature microscopy demonstrates that lethal cooling rates are coincident with those that produce intracellular ice formation, and that the lethal temperature appears to be that at which intracellular ice forms. Furthermore, the microscopy shows that ova do not dehydrate when cooled at rates that produce intracellular ice and cell death, but undergo substantial shrinkage when cooled at rates that produce little intracellular ice and high survival.

Measurements of the water permeability of mouse ova and the temperature coefficient of that permeability can be used to test a mathematical model formulated to describe the kinetics of water loss at subzero temperatures from a hypothetical cell. The observed dehydration of ova cooled to subzero temperatures at given rates is approximately predicted by the mathematical model, although there is some quantitative discrepancy between the observed and calculated responses.

I. Introduction

The embryos of six mammalian species can now be successfully preserved by freezing and storing them at -196°C, ultimately yielding live animals when the frozen-thawed embryos are transferred into appropriate foster mothers (Whittingham et al., 1972; Wilmut, 1972; Bank and Maurer, 1974; Whittingham, 1975; Willadsen et al., 1976a,b). One of the purposes of this Ciba Foundation Symposium is to discuss the practical applications and implications of these observations. But another is to consider the freezing of mammalian embryos as an example of fundamental cryobiology. Are the responses of embryos to the stresses of freezing and thawing unique to this specialized cell type or are they typical of mammalian cells in general? Whatever the answer to that question, can a study of the freezing of mammalian embryos contribute to an understanding both of basic cryobiology and of embryonic physiology? I believe that the respective answers to these questions are that the responses of embryos are typical, and, as a result, a study of such responses is already contributing understanding to both cryobiology and embryology.

That the response of embryos to freezing is not unique is suggested by the results shown in Fig. 1. It has been known for some time that, above some critical cooling rate, survival of several different cell types decreases with increasing rate (Mazur, 1970; Leibo and Mazur, 1971; Mazur et al., 1969; Leibo et al., 1970). It has been argued, first on theoretical grounds (Mazur, 1963) and later from experimental observations (Mazur, 1970; Mazur et al., 1972), that decreasing survival with increasing cooling rate is a manifestation of cell injury due to intracellular ice formation. Some direct support for this hypothesis is provided by electron microscopic observations of cells either freeze-fractured or freeze-substituted in the frozen state

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(Bank and Mazur, 1973; Walter et al., 1975). More recently, the ability to observe cells during the actual freezing process has provided additional direct support for this hypothesis (Diller and Cravalho, 1970; Diller et al., 1972; McGrath et al., 1975). The results in Fig. 1 are a summary from Leibo (1977) of the responses of three cell types to cooling rate. They show the percentage survival of each cell type together with the proportion of each cell type containing intracellular ice, both as functions of cooling rate. It is apparent from those data that the numerical values of cooling rate at which those responses occur in each cell type differ by three orders of magnitude. The upper panel of photographs shows that these three cell types differ enormously in size. The respective volumes of mouse ova, HeLa tissue-culture cells, and human erythrocytes are about 2×10^5, 1×10^3, and 1×10^2 μm^3. The respective cooling rates at which 50% of ova, HeLa, and RBC freeze intracellularly are about 2.4°, 72°, and 540°C/min. But basically we see that survival of each cell type decreases over the same range of cooling rates at which an increasing fraction of each type freezes intracellularly. In this respect, then, the response of mouse ova seems not unique. But we must also consider whether ova respond like cleavage-stage embryos, and whether other factors influence the survival of ova and embryos.

II. The Response of Mouse Ova and Embryos to Freezing

The survival of ova, like that of HeLa cells and erythrocytes, depends on cooling rate. The results in Fig. 2 show that the response of cleavage-stage embryos is quite similar to that of fertilized ova. Fifty percent or more of 1-, 2-, and 8-cell embryos survive freezing when cooled to -196°C at a rate of about 0.3°C/min. Practically none of any stage survives when cooled at about 7°C/min or faster. These data suggest that for the purposes

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of this discussion, then, mouse ova and embryos may be considered as a single class. In general terms, what is true of one embryonic stage will also hold for other stages as well.

But cooling rate is not the only experimental variable that determines survival of mouse embryos. Although it had long been recognized that warming rate also affected cell survival, this was usually taken to mean that frozen cells should be warmed as rapidly as possible for maximum survival. The case of mouse embryos was the first apparent exception to this rule for animal cells, since warming frozen embryos at rates of 450°C/min or faster yielded few if any survivors (Whittingham et al., 1972). This phenomenon has now been studied further (Leibo et al., 1974). The results in Fig. 3 (F-3) show that survival of 8-cell embryos frozen in solutions of dimethyl sulfoxide (DMSO) depends as much on warming rate as on cooling rate. The data show that embryos cooled at 1.7°C/min (a rate faster than the optimum; see Fig. 2) survive equally well when warmed at a range of rates from about 1° to 100°C/min, although few survive when warmed at several hundred degrees/min. Embryos cooled more slowly at 0.18°C/min have a more stringent requirement for warming, displaying a distinct optimum warming rate. This means that in the frozen state embryos cooled at about 0.2°C/min differ in some fashion from embryos cooled at about 2°C/min. During the actual cooling process, something occurs that will manifest itself only during warming. Embryos cooled at about 0.2°C/min must be viable. Some 90% survive if warmed at about 2°C/min, but few survive if warmed at about 200°C/min. Embryos cooled at about 2°C/min are also viable. In this case, the same high percentage of embryos survives whether warmed at 2° or 200°C/min. The question is what physiological response might have occurred during freezing that would be so dependent on whether the cooling rate was 0.2°C/min or 2°C/min.

III. Ova and Embryo Volume Changes at Suprazero Temperatures

A clue to the answer to this question is provided by examination of the osmotic response of embryos at temperatures above 0°C. The reason for considering osmotic phenomena in this context is that the properties of solutions undergo drastic, reversible alterations during freezing and during thawing. When a solution is cooled below its freezing point, the following progressive changes occur. First, at some temperature below the freezing point, ice nucleation occurs. The amount of ice increases to produce chemical potential equilibrium for that subzero temperature. As the temperature is lowered, more ice forms, producing an increasingly concentrated solution of dissolved solutes. These correlative changes, increases in the amount of ice and in the concentration of solutes, continue as the temperature is lowered. During cooling, then, the cell is exposed to lowered temperature, ice crystal growth, and, most importantly, increasing concentration of dissolved solutes. During warming, as the ice melts, the cell is exposed to a substantial dilution of the solutes concentrated during freezing.

All cells respond osmotically to maintain chemical potential equilibrium across their membranes. But osmotic responses are time- and temperature-dependent. It is believed that herein lies the answer to the relationship of cell survival and rate-dependent processes that occur during freezing and during thawing.

Experimentally, osmotic phenomena of cells are more easily observed at suprazero, rather than subzero, temperatures. Consider an 8-cell embryo transferred from an isotonic saline solution to an hypertonic solution of DMSO at 0°C (Fig. 4). Shortly after transfer from saline (A) into DMSO (F-4)

(B), the embryo shrinks by losing water. Concurrently, DMSO begins to permeate the embryo, accompanied by an influx of water to maintain chemical potential equilibrium between the intracellular and extracellular water. At 0°C, this process occurs sufficiently slowly so that it can be easily observed. As the DMSO and water enter the cells, the total embryonic volume increases rather obviously (C to F). But even after 90 min in DMSO at 0°C (F), the embryo has not returned to its initial isotonic volume (A). It does so, however, when transferred from DMSO back into isotonic saline (G). That this sequence does not damage the embryo is illustrated by the fact such embryos are capable of normal development in culture (H).

These osmotic phenomena of embryos, manifested by substantial changes in volume, can be quantitated most conveniently with ova. Ova are well suited for such studies because of their large size, relative to other mammalian cells, and their spherical shape. Because they are spheres, their easily measured cross-sectional areas can be used to calculate their volumes. By calculating the volumes of ova as a fraction of their original volumes in isotonic solution, one can quantitate the osmotic response of ova as a function of time in hyperosmotic solutions of permeating solutes. Fig. 5 shows recent (F-5) observations of fertilized mouse ova at 22° and 4°C (Jackowski, 1977). The qualitative observation of an osmotic response shown in Fig. 4 can, in this fashion, be displayed quantitatively. The data show the time- and temperature-dependency of the volume changes exhibited by ova when exposed to 1 M solutions of glycerol. Using appropriate equations (e.g., Mazur et al., 1974) and these quantitative observations of volume change, one can calculate the permeability coefficient for glycerol entry into mouse ova (Jackowski, 1977).

Such time-dependent volume changes are a measure not only of the kinetics of the movement of solute into the cell, they also reflect the movement of

water as well. However, the kinetics of water movement across the cell membrane can be more accurately determined by measuring changes in cell volume when the cell is placed into solutions of nonpermeating solutes. In this case, volume changes result only from the movement of water. Recently, I have conducted such experiments to measure the water permeability of mouse ova. The approach is analogous to that described above to measure glycerol permeability. The difference is that the ova are exposed to hypertonic solutions of nonpermeating salt for accurately measured times ranging from about 5 to 300 seconds at carefully controlled temperatures from 2° to 30°C. Photographs of the spherical ova are used to calculate cell volumes from their cross-sectional areas, and these calculated volumes are expressed as a percentage of the ova volume in isotonic saline. An example of such a sequence at four temperatures is shown in Fig. 6. The (F-6)
results show that ova transferred into 0.5 M NaCl quickly shrink by losing water, and that the rate of shrinkage is temperature-dependent. This approach measures the initial and final volumes, and the intermediate volumes as a function of time at a given temperature. Standard equations (Dick, 1966) are used to calculate the volume of water lost across a unit of membrane surface area within a unit of time for a unit of osmotic pressure difference across the membrane. These calculations yield a water permeability coefficient, Lp, for unfertilized mouse ova at 20°C of 0.27 μ^3/μ^2, min, atm. Preliminary calculations for fertilized ova yield a similar value. Having calculated the Lp at several temperatures, one can express the temperature relationship as an Arrhenius plot (Fig. 7). The activation energy for water (F-7)
permeability calculated from those results is about 12 Kcal/mole. Over the range of 30° to 0°C, this corresponds roughly to a $Q_{10} = 2.2$.

For purposes of comparison, corresponding values of Lp and ΔH for ova and other cell types together with their surface area/volume ratios are shown in Table I. The table also lists the "critical" cooling rates of those cells. This value refers to the rate observed to produce intracellular ice in 50% of ova, fibroblasts, and erythrocytes (see Fig. 1) or to result in 50% survival of lymphocytes relative to that obtained when the cells are cooled at their optimum rate (Thorpe et al., 1976). The water permeability data shown in this table were obtained from several sources. For ova, the data are from Fig. 7. For lymphocytes, the data are from Hempling (1973). The values for fibroblasts are those given in Dick (1966). The activation energy for Lp of this cell type was estimated from Dick's (1959) observations at 38°C and those of Brues and Masters (1936) at room temperature. The data for ascites tumor cells are those of Hempling (1960). The water permeability coefficient of human erythrocytes is that reported by Sidel and Solomon (1957) and the activation energy is that measured by Vieira et al. (1970). Forster (1971) summarizes available data for human erythrocytes, and reports values of Lp ranging from 0.9 to 9.9 μ^3/μ^2, min, atm. (The former value is that of Höber and Orskov, 1932, and the latter is that of Sjölin, 1954, both cited in Forster.) The more recent reports give values close to that shown in Table I. (T-I)

This comparison indicates that cells with lower water permeabilities, or higher activation energies of water permeability, or lower surface to volume ratios have lower critical cooling rates. Or in other words, cell characteristics that tend to increase the rate at which a given cell can lose water tend to increase the rate required to produce intracellular ice formation. For example, the large spherical ova have a low water permeability

that is strongly temperature-dependent. As temperature is lowered, e.g., from +20° to -20°C, the rate at which this cell type can lose water is reduced 25-fold. Fifty percent of these cells freeze intracellularly when cooled at ~ 2°C/min. On the other hand, the small biconcave discoid erythrocytes have a high water permeability that is much less temperature-dependent. As temperature is lowered from +20° to -20°C, the rate at which this cell type can lose water is reduced by about 60%. Fifty percent of these cells freeze intracellularly only when cooled at rates in excess of 500°C/min. But these data relate only to the cooling rate-dependency of cell freezing and not to the temperature-dependency. It is relevant, therefore, to examine the role of temperature in cell freezing as well.

IV. Ova and Embryo Responses at Subzero Temperatures

The data presented above (Fig. 2) show that mouse ova and embryos survive freezing if cooled slowly, but not if cooled rapidly. But rapid cooling *per se* is not necessarily lethal; it depends on the temperature-range over which it occurs. The data in Fig. 8 show that, if 8-cell embryos suspended in DMSO (F-8) are cooled rapidly, 70% or more survive if they have first been cooled slowly to -50°C. If, however, they have first been cooled slowly to only -30°C before being rapidly cooled, none survive. This means that something critical occurs to the embryos between -30° and -50°C. The data imply that slow cooling through that temperature range reduces or prevents that critical event. A corollary to that interpretation follows from the data shown in Fig. 9. (F-9) In this case, mouse ova suspended in saline (PBS), glycerol, or DMSO were cooled only to various minimum subzero temperatures at ~ 15°C/min, a rate fast enough to assure cell death of ova cooled to -196°C. For ova in DMSO,

the data show that virtually all the cells survive rapid cooling to -35°C, but few survive rapid cooling to -45°C or below. Again, these data imply that a critical event occurs in rapidly cooled ova between -35° to -45°C. For cells in PBS and glycerol, this event occurs at higher temperatures.

Identification of this critical event was obtained by direct microscopic observation of mouse ova suspended in DMSO as they were cooled at various rates to subzero temperatures (Leibo et al., 1977). A comparison of ova cooled at about 30°, 3°, and 1°C/min is shown in Fig. 10. These micrographs (F-10) show that as the ova were cooled from 0°C (A) to about -5°C (B) and to -20°C (C), ice first grew to the cells and then completely surrounded them. Subsequent events at lower temperatures depended on the cooling rate. Ova cooled at 30° and 3°C/min suddenly froze intracellularly at about -40°C (D). An ovum cooled at 1°C/min, however, did not display the sudden "blacking out" indicative of intracellular freezing even when cooled to -135°C. The relationship between the temperature at which cells froze intracellularly, the nucleation temperature, and the rate at which they were cooled is shown by the data in Fig. 11. The freezing point of the 1 M DMSO solution in (F-11) which the ova were cooled is -2.6°C, so that the ova were surrounded by ice at temperatures below that. However, the temperature at which ice formed within the cells was, on the average, some 40 degrees below -2.6°C. Despite the variability of those data, they show that over the cooling rate range of about 2° to 40°C/min, none of the ova froze intracellularly above -30°C, and all of those that froze intracellularly did so above -60°C. In other words, one striking event, intracellular ice nucleation, occurred at about -45°C. It is obviously tempting to suggest that this event is the same as the critical event identified from the cell survival measurements shown above

(Figs. 8 and 9).

The low temperature micrographs yield yet another relevant piece of information. They show that the ovum cooled at 30°C/min was virtually the same size when it froze at -40°C as it was at 0°C, but that the ovum cooled at 3°C/min was obviously smaller when it froze at -40°C. Once the cells froze, of course, they were incapable of undergoing further change in volume. The ovum cooled at 1°C/min that did not freeze intracellularly at any temperature was substantially smaller at -135°C than it was at 0°C. In other words, there was apparently little change in the volume of the ovum cooled at the highest rate, but a large volume change of the ovum cooled at the lowest rate.

V. Ova Volume Changes at Subzero Temperatures

Calculating ova volumes from their cross-sectional areas for the purposes of measuring their permeability properties at suprazero temperatures can also be used to calculate their volumes at subzero temperatures as well. Although these latter measurements are less precise because the ova at subzero temperatures assume a highly convoluted shape, and their peripheries are somewhat obscured by the surrounding ice, such estimates are useful. The estimated volumes of ova as a function of cooling rate between 0.5° and 30°C/min are compared to the percentage of cells observed to freeze intracellularly in Fig. 12. The data show that the total cell volume that (F-12) an ovum contains when it freezes intracellularly is substantially less at lower than at higher cooling rates. The comparison between reduction in cell volume and intracellular ice formation indicates that the range of cooling rates over which these two phenomena occur is coincident. That is, ova cooled at rates of 4°C/min or faster do not decrease in volume during

cooling; 100% of ova cooled at those rates freeze intracellularly. Ova cooled at rates of 1°C/min or slower decrease in volume during cooling; few of them freeze intracellularly. An alternative way of examining these same data is shown in Fig. 13. This figure compares the cell volume lost during cooling as a function of rate with the survival of ova cooled at those same rates. The comparison suggests that cooling rates at which ova lose water are the same rates that yield relatively high survival of fertilized ova. Cooling rates at which ova do not lose water are the same rates that yield no survival of fertilized ova. (F-13)

These experimental observations of cell volume changes occurring at subzero temperatures can be used as a test of a quantitative model that describes the response of a cell as a function of subzero temperature and cooling rate (Mazur, 1963; 1965). That model provides a mathematical description of the following proposition. It is known that, when a solution is cooled below its freezing point and ice forms, the concentration of solutes in the remaining unfrozen liquid is increased. As the solution is cooled to lower temperatures, more ice forms and the solute concentration increases still further. It is also known that all cells respond osmotically to maintain chemical potential equilibrium across their membranes. The model describes the rate at which a given cell can respond osmotically to the increasing solute concentration produced by progressive freezing. The rate of volume change of the cell depends primarily on its original volume, its surface area, its permeability to water, and the temperature coefficient of that permeability.

Using Mazur's equations with the measured water permeability of an unfertilized mouse ovum of 0.27 μ^3/μ^2, min, atm., the change of cell volume

as a function of subzero temperature has been calculated for three temperature coefficients. The reason for using three coefficients is that Lp has been measured at suprazero temperatures (see Fig. 7), whereas the cell response to freezing occurs at subzero temperatures. Such a large extrapolation of data measured between +30° and +2°C to temperatures of -40°C or below might well introduce an error. The coefficients used are that value calculated by the method of least squares for the observed Lp's at five temperatures ± the 95% confidence limits of that value. The coefficients are 0.032, 0.048, and 0.016, corresponding to activation energies of 12.1, 18.2, and 5.9 Kcal/mole, respectively. The calculations of total cell volume as a function of temperature for cooling rates of 0.5° and 5.8°C/min using temperature coefficients of 0.016 and 0.032 are shown in Fig. 14. The results show (F-14) that with a temperature coefficient of 0.032, the cell volume decreases to 50% of the original by -10°C with a cooling rate of 0.5°C/min, and to 30% of the original by -30°C with a cooling rate of 5.8°C/min. In other words, the calculations indicate that a mouse ovum cooled at 5.8°C/min to -30°C will have lost 70% of its original total cell volume. This calculation is clearly at odds with the observations shown in Figs. 12 and 13. Those results showed that ova cooled to about -45°C at a rate of 4°C/min or faster still contained virtually the same volume that they had at 0°C. The calculations using a temperature coefficient of 0.016 differ even more from the observed volumes of ova cooled at various rates. The calculations of cell volume versus temperature using a temperature coefficient of 0.048 are shown in Fig. 15. (F-15) With this temperature coefficient for Lp, the calculated cell volume changes become strongly dependent on cooling rate. For example, the calculated volumes at -45°C for cooling rates of 1.3°, 3.5°, and 5.8°C/min are about 28%,

58%, and 72%, respectively. These calculated values, using the temperature coefficient corresponding to the upper 95% confidence limit, clearly bear a closer resemblance to the observed volume dependency on cooling rate than those shown in Fig. 14. However, a comparison between the calculated volume of an ovum cooled at 1.3°C/min with the measured volume of an ovum cooled at that rate to various temperatures demonstrates a sizable difference (Fig. 16). [The observed volumes shown in this figure were obtained in the same fashion as described above for Figs. 12 and 13.] The calculation indicates that the cell should exhibit a larger decrease in volume and at a higher temperature than the observations show. The discrepancy between calculation and observation might result from any of several sources. First, the difference might result from one of the assumptions used by Mazur (1963) to formulate his model. [See the original for discussion of those assumptions.] Second, a slight error in one of the measured values for the cell, e.g., the ΔH used, might account for the difference. The activation energy of Lp is calculated from measurements between +30° and +2°C, but the calculation is performed for responses at subzero temperatures. Furthermore, the model assumes that the temperature coefficient of water permeability is constant at both suprazero and subzero temperatures. To my knowledge, the water permeability of no cell has been measured over a range of subzero temperatures. It is quite possible that the temperature coefficient of Lp is different at subzero versus suprazero temperatures. This could result from the phase change itself, or from the effects of viscosity or of solute concentration, both of which increase substantially during progressive freezing. The results in Figs. 14 and 15 show that the calculated volumes are strongly influenced by the value chosen for the temperature coefficient. Therefore,

(F-16)

if Lp does not decrease linearly with the reciprocal of absolute temperature, or if extrapolation of Lp from suprazero to subzero temperatures is slightly in error, a large discrepancy between calculation and observation might result. Finally, the difference might be due simply to an overestimate of the observed cell volumes because of the highly irregular cell shape at subzero temperatures. Despite this quantitative discrepancy, the results in Fig. 16 demonstrate that a mouse ovum cooled at about 1°C/min decreases in volume during cooling in a fashion approximately described by a mathematical model based on classical solution chemistry.

One final comparison between calculation and observation is pertinent in this context. Mazur (1963) formulated his mathematical model to describe the kinetics of water loss at subzero temperatures from cells in general. He was able to provide major circumstantial support for his hypothesis by recognizing that cooling rates calculated from the model to produce intracellular ice formation in several cell types were the same rates that actually destroyed those cells (Mazur, 1963; 1965). He calculated the likelihood of intracellular nucleation for a given cooling rate from the number of degrees that a cell's intracellular contents would be supercooled relative to the partially frozen extracellular solution at some "nucleation" temperature, i.e., the temperature at which the cell contents actually freeze.

I have made a similar analysis for mouse ova cooled at various rates. This calculation of the probability of intracellular ice formation is derived from the difference in degrees between the cell nucleation temperature, at which the cell contains a calculated volume for a given cooling rate, and the freezing temperature of a solution containing that same fraction of unfrozen water. This difference is then expressed as a fraction of the

number of degrees that a solution would be supercooled at the cell nucleation temperature. For example, in Fig. 15 the calculations show that a cell cooled at 3.5°C/min to -40°C would still contain about 57% of its initial total volume, corresponding to a cell water volume of about 42% of that present at 0°C. A solution cooled infinitely slowly would contain about 42% of its water unfrozen at about -6°C. Therefore, the cell contents at -40°C would be supercooled some 34°C, and a solution would be supercooled some 38°C; the probability of the cell freezing intracellularly would be 0.9. The results of such calculations for the probability of intracellular ice formation at -40°C, assuming two different temperature coefficients for water permeability of mouse ova, are shown in Fig. 17. Those calculations are (F-17) compared with the observed incidence of intracellular ice in ova cooled at various rates (see the data for ova in Fig. 1). The calculated probability of intracellular freezing depends on the rate at which a cell is calculated to lose water, and this in turn depends partially on the temperature coefficient chosen. There is an obvious difference between the observed incidence of cell freezing and the calculated probability using a temperature coefficient of 0.032. This temperature coefficient corresponds to that calculated from the water permeability measurements shown in Fig. 7. However, using the temperature coefficient of 0.048, which is within the 95% confidence limits of the observed value, the calculation of the probability of intracellular ice coincides almost precisely with the observed incidence.

VI. Conclusion

A mathematical model exists to describe the kinetics of water loss at subzero temperatures from a hypothetical cell (Mazur, 1963). That model permits one to calculate the probability of intracellular ice formation within

a given cell as a function of cooling rate. A quantitative test of that model requires the following information: (1) The cell's water volume; (2) Its surface area; (3) The cell's permeability to water; (4) The temperature coefficient of that permeability; (5) The nucleation temperature at which the cell contents freeze; and (6) Observation of intracellular ice formation within the cell as a function of cooling rate.

Using values measured for mouse ova, I have attempted to test Mazur's model. The test demonstrates that there is approximately a four-fold discrepancy between the cooling rate calculated to produce 50% intracellular ice formation and that observed to produce 50% ice formation. There are, however, sufficient ambiguities in some of the values used in this test to account for such a discrepancy. The important point is that a model derived to calculate the response of a hypothetical cell to freezing can approximately describe the actual response of mouse ova to freezing. It follows, therefore, that the response of ova and embryos to freezing is not unique. And I believe that the analysis presented demonstrates that the study of such cells can contribute to the understanding of fundamental cryobiology.

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Table I

Water Permeability Coefficients of Mammalian Cells

Cell Type	Perm. Coeff., 20°C (μ^3/μ^2, min, atm)	ΔH (Kcal/mole)	SA/Vol (μ^2/μ^3)	Critical C.R. (°C/min)
Mouse Ova	0.27	12.1	0.08	2.4
Lymphocytes	0.36	16.3	0.6	~ 5
Fibroblasts	~ 0.7	~ 14	0.43	72
Ascites Tumor Cells	6.4	9.6	0.40	-
Human Erythrocytes	5.7	3.3	1.88	540

Figure Legends

Fig. 1. Comparison of survival (dashed lines) and the percentage of cells observed to freeze intracellularly (solid lines) of mouse ova, HeLa tissue culture cells, and human erythrocytes as a function of cooling rate. The ova were frozen in 1 M dimethyl sulfoxide, the HeLa cells in growth medium, and the erythrocytes in about 1.5 M glycerol. The figure is from Leibo (1977) who gives the original sources of the data.

Fig. 2. Survival of fertilized mouse ova and 2-cell and 8-cell embryos as a function of the rate at which they were cooled to -196°C in 1 M DMSO. Survival was based on the percentage of embryos capable of developing normally in culture. The data are those of Whittingham et al. (1972).

Fig. 3. Survival of 8-cell mouse embryos as a function of the rate at which they were warmed following cooling in 1 M DMSO to -196°C at each of the indicated rates. Survival was based on in vitro development to the blastocyst stage. The data are those of Leibo et al. (1974).

Fig. 4. Eight-cell mouse embryos photographed at about 0°C in isotonic saline (A) and after transfer into 1 M DMSO for 2 (B), 6 (C), 15 (D), 35 (E), and 90 min (F). The embryos were then gradually diluted out of the DMSO, transferred into saline (G), and cultured under standard conditions for 16 hr. The observations are those of Leibo et al. (1974).

Fig. 5. The volumes of fertilized mouse ova, relative to their volumes in isotonic saline, as a function of time in 1 M glycerol at 22° and 4°C. The ova were photographed, their cross-sectional areas were measured and used

to calculate their volumes, assuming the areas to be those of spheres. The data are those of Jackowski (1977) and Jackowski and Leibo (1976).

Fig. 6. The relative volumes of unfertilized mouse ova as a function of time in 0.5 M NaCl at each of the indicated temperatures. The volumes were calculated in the same fashion described in Fig. 5.

Fig. 7. Arrhenius plot of water flux, Lp, for unfertilized mouse ova in 0.5 M NaCl. The values of Lp were calculated from replicate measurements like those in Fig. 6 using the equations of Dick (1966). (Unpublished data of Leibo.)

Fig. 8. Survival of 8-cell mouse ova cooled slowly to various temperatures (indicated by the symbols) and then plunged directly into liquid nitrogen before being warmed slowly. The diagram shows the sequences followed. The data are those of Leibo et al. (1974).

Fig. 9. Survival of fertilized mouse ova cooled in each of the indicated solutions at 15°C/min to various temperatures before being warmed slowly. Survival was based on the percentage of ova that cleaved to the 2-cell stage. The data are those of Leibo (1976).

Fig. 10. Unfertilized mouse ova, suspended in 1 M DMSO, were photographed as they were cooled at 30°C/min (left column of photographs), 3°C/min (center column), and 1°C/min (right column) to subzero temperatures. Photographs were taken at 0°C (A), about -5°C (B), -20°C (C), and -40°C (D), except for photograph D in right column taken at -135°C. The observations are those of Leibo et al. (1977) and the figure itself is from Leibo (1977).

Fig. 11. The nucleation temperature at which unfertilized mouse ova suspended in 1 M DMSO were observed to freeze intracellularly when cooled at various rates. The figures refer to the number of ova observed at each rate. The symbols and the bars show the average temperature and the temperature range over which the ova froze. The data are those of Leibo et al. (1977).

Fig. 12. Comparison of the total cell volume of unfertilized mouse ova when they froze intracellularly with the fraction of cells observed to freeze intracellularly, both as functions of cooling rate. Volumes were calculated from the cross-sectional areas of the cells as a fraction of their volumes at 0°C. (The open symbol [△] refers to an ovum that did not freeze intracellularly, but was photographed at -100°C.) The intracellular ice data are those of Leibo et al. (1977) and are the same as those labeled "ova" in Fig. 1.

Fig. 13. Comparison of the volume lost from unfertilized ova during cooling with the survival of fertilized ova, both as functions of cooling rate. The volumes are [100% minus the cell volume at intracellular nucleation (from Fig. 12)]. The survival data are from Whittingham et al. (1972), and are the same as those labeled "ova" in Fig. 1.

Fig. 14. The total cell volume calculated as a function of subzero temperature for cooling rates of 0.5° and 5.8°C/min. The calculations were performed by W. F. Rall using the equations of Mazur (1963), who describes the theory and the method of calculation. The following parameters were used in the calculations: Cell water volume = $1.88 \times 10^5\ \mu^3$; cell surface area = $1.84 \times 10^4\ \mu^2$; number of osmoles of solute in the cell = 2.48×10^{-9} moles; water permeability coefficient at 20°C = $0.27\ \mu^3/\mu^2$, min, atm. The temperature

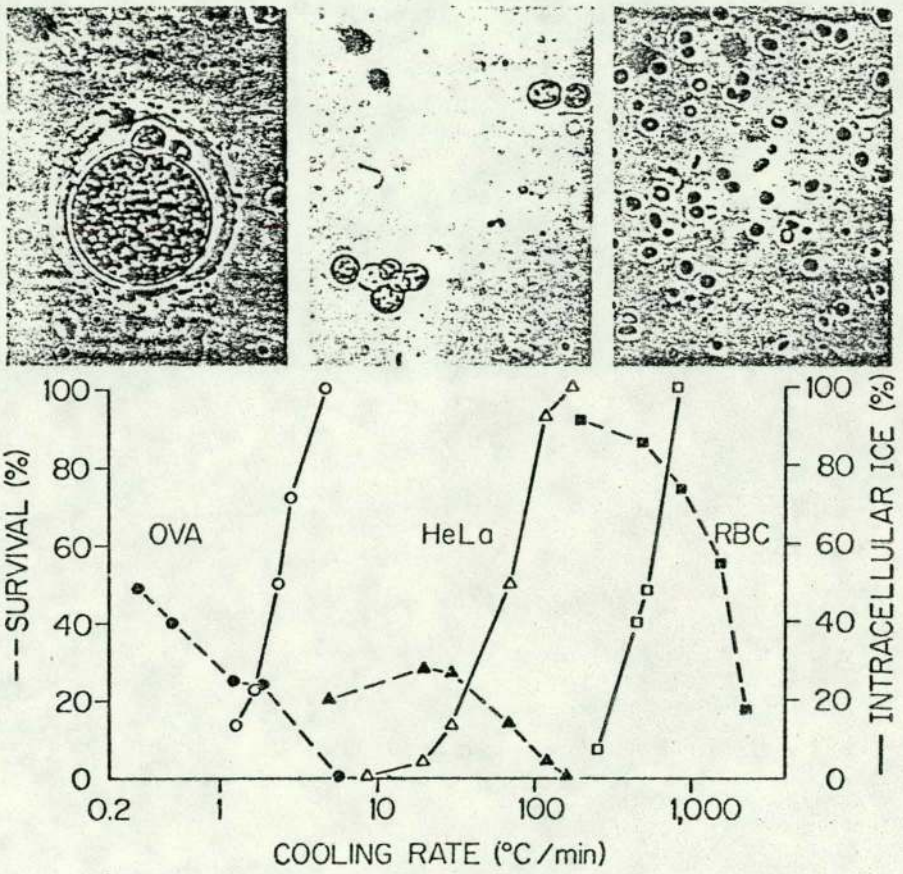
coefficient of water permeability was either 0.016 (solid lines) or 0.032 (dashed lines). The calculations are unpublished data of Leibo, Rall, and Mazur.

Fig. 15. The total cell volume calculated as a function of subzero temperature for the indicated cooling rates (in °C/min). The description of the calculations is the same as in Fig. 14, except that the temperature coefficient for water permeability was 0.048.

Fig. 16. Comparison of the calculated with the observed total cell volumes of an ovum cooled at 1.3°C/min as a function of subzero temperature. The calculated curve is the same as that shown in Fig. 15 for that cooling rate. The observed volumes were calculated from the cross-sectional areas of an unfertilized ovum cooled at 1.3°C/min, using the method described in Fig. 5.

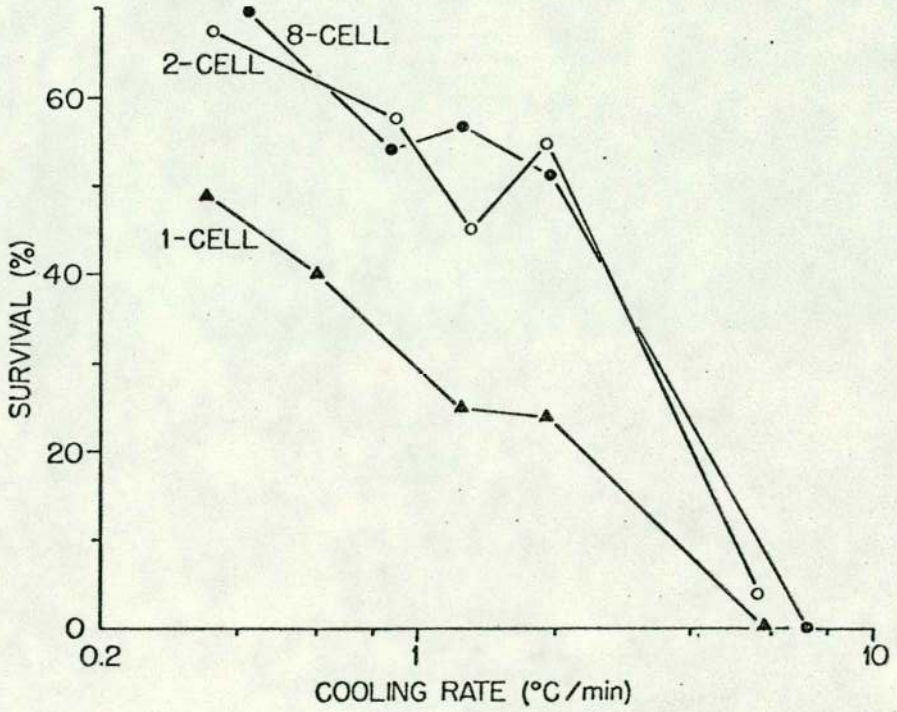
Fig. 17. Comparison of the calculated probability of intracellular nucleation at -40°C with the observed fraction of cells that froze, both as functions of cooling rate. The probabilities were calculated from cell water volume versus temperature graphs similar to those shown for total cell volume in Figs. 14 and 15. The water volume calculations were performed using temperature coefficients of 0.032 (dashed line) or 0.048 (solid line). See text for details.

31417-C

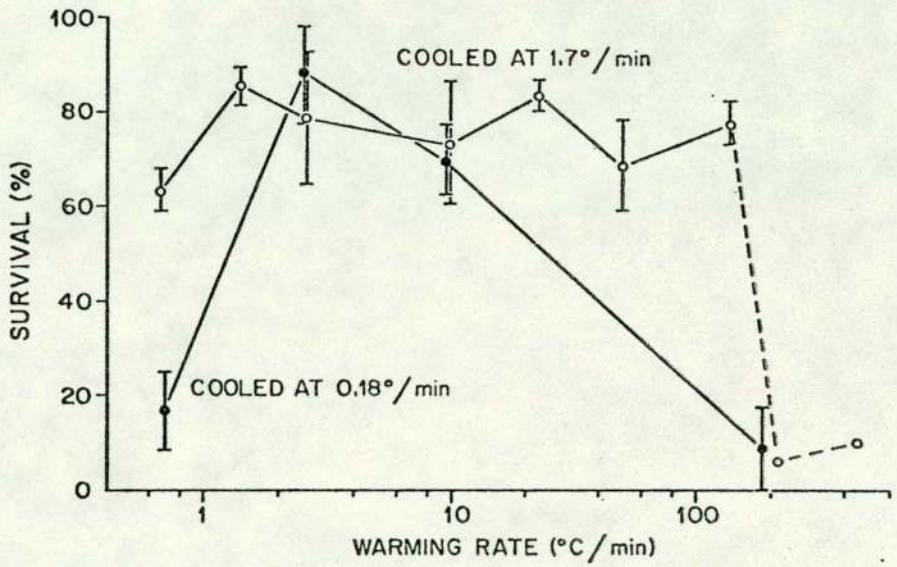

100
80
60
40
20
0
SURVIVAL (%)
OVA
HeLa
RBC
0.2
1
10
100
1,000
COOLING RATE (°C/min)
INTRACELLULAR ICE (%)

(2)

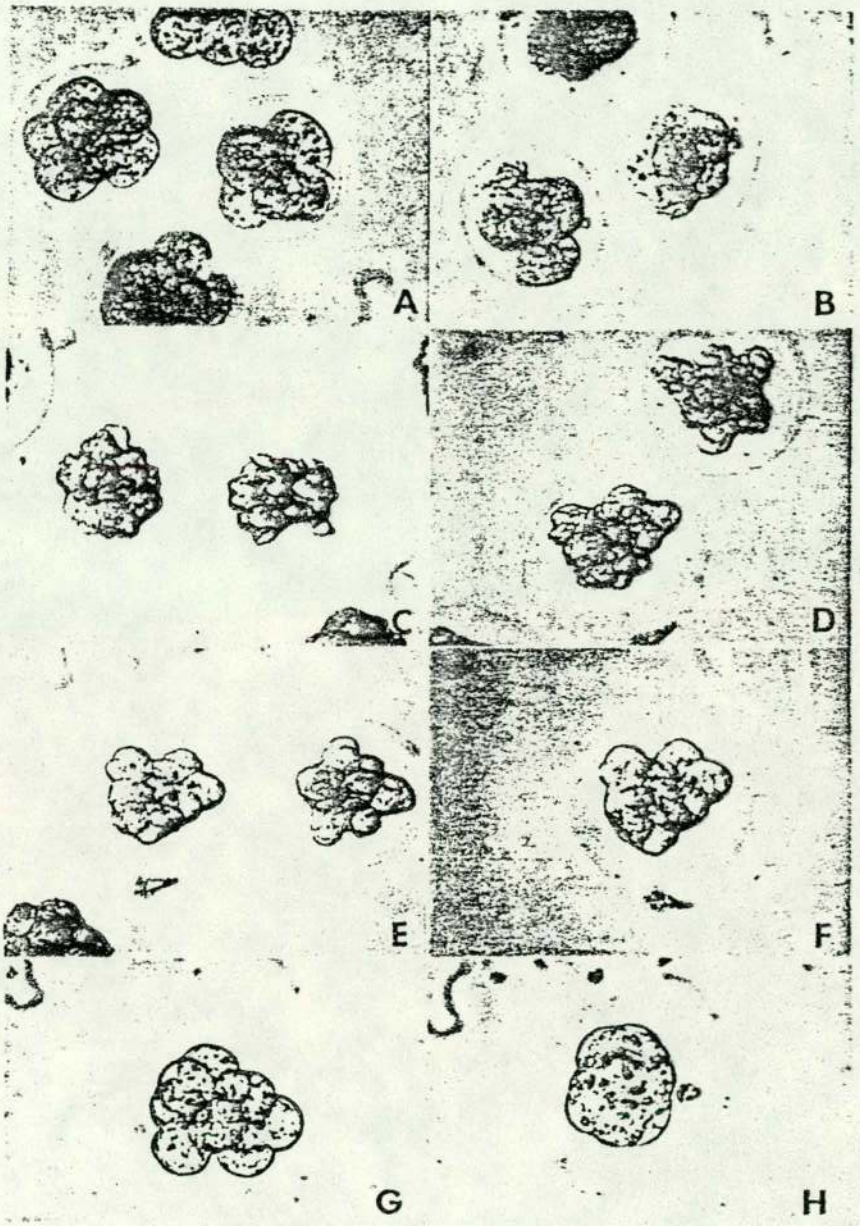
32533


8-CELL
2-CELL
1-CELL
60
40
20
0
SURVIVAL (%)
0.2
1
10
COOLING RATE (°C/min)

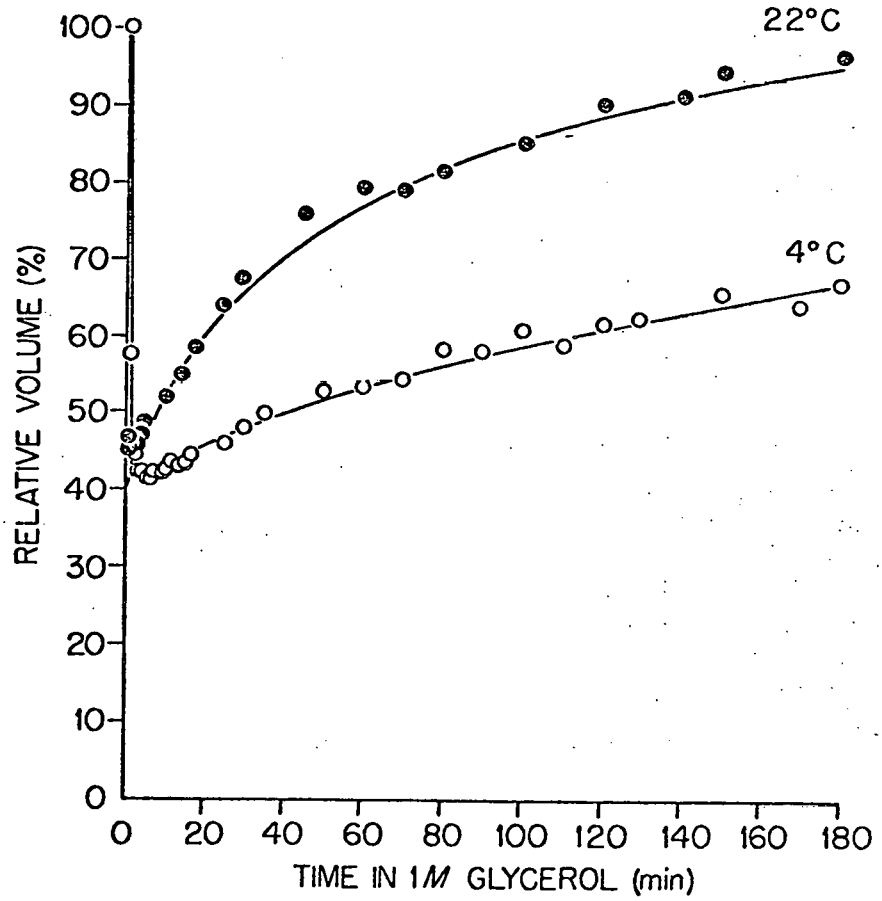
28435-2


100
80
60
40
20
0
SURVIVAL (%)
COOLED AT 1.7°/min
COOLED AT 0.18°/min
1
10
100
WARMING RATE (°C/min)

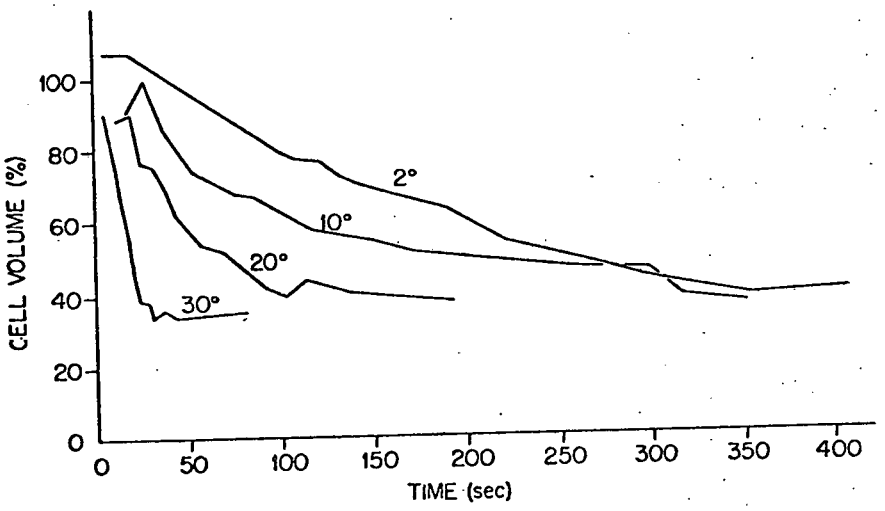
24923



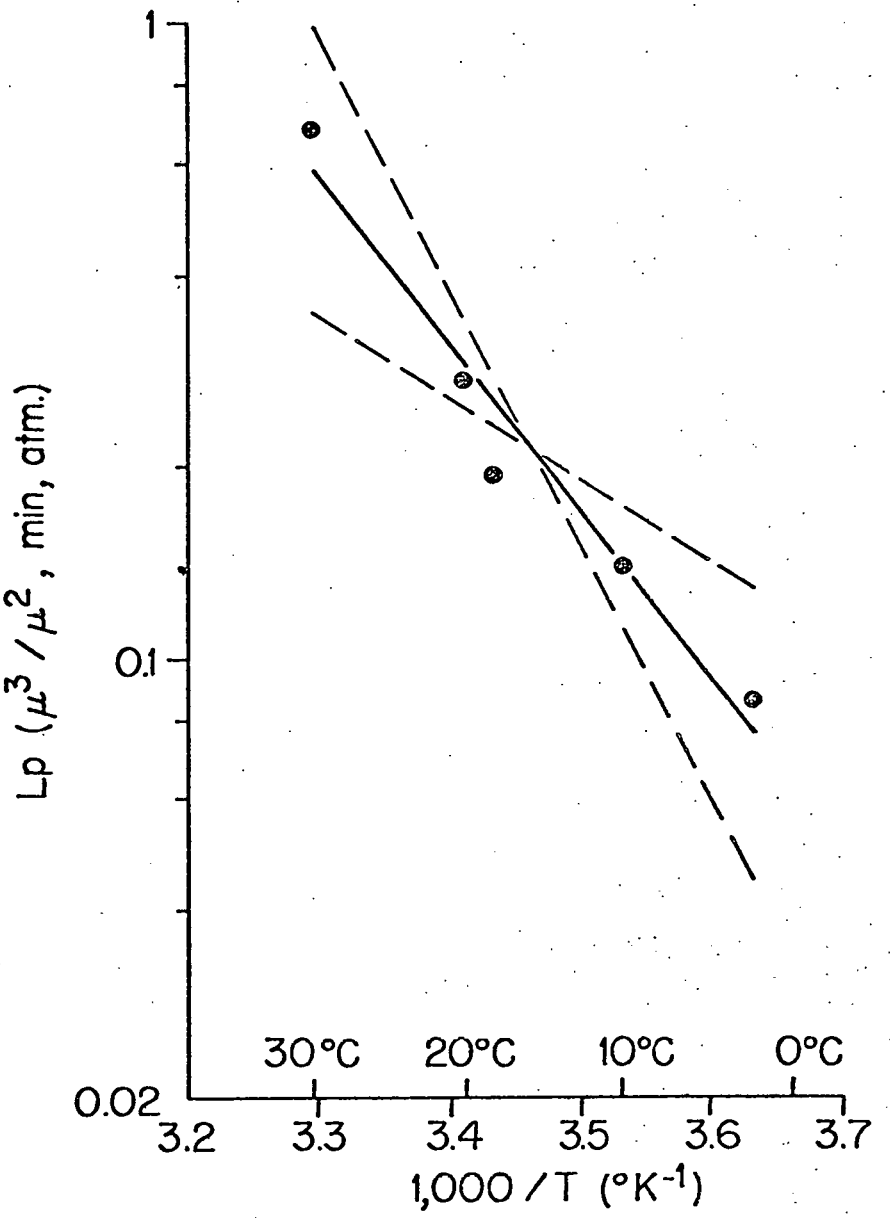
315-10-1


22°C
4°C
RELATIVE VOLUME (%)
100
90
80
70
60
50
40
30
20
10
0
0
20
40
60
80
100
120
140
160
180
TIME IN 1M GLYCEROL (min)

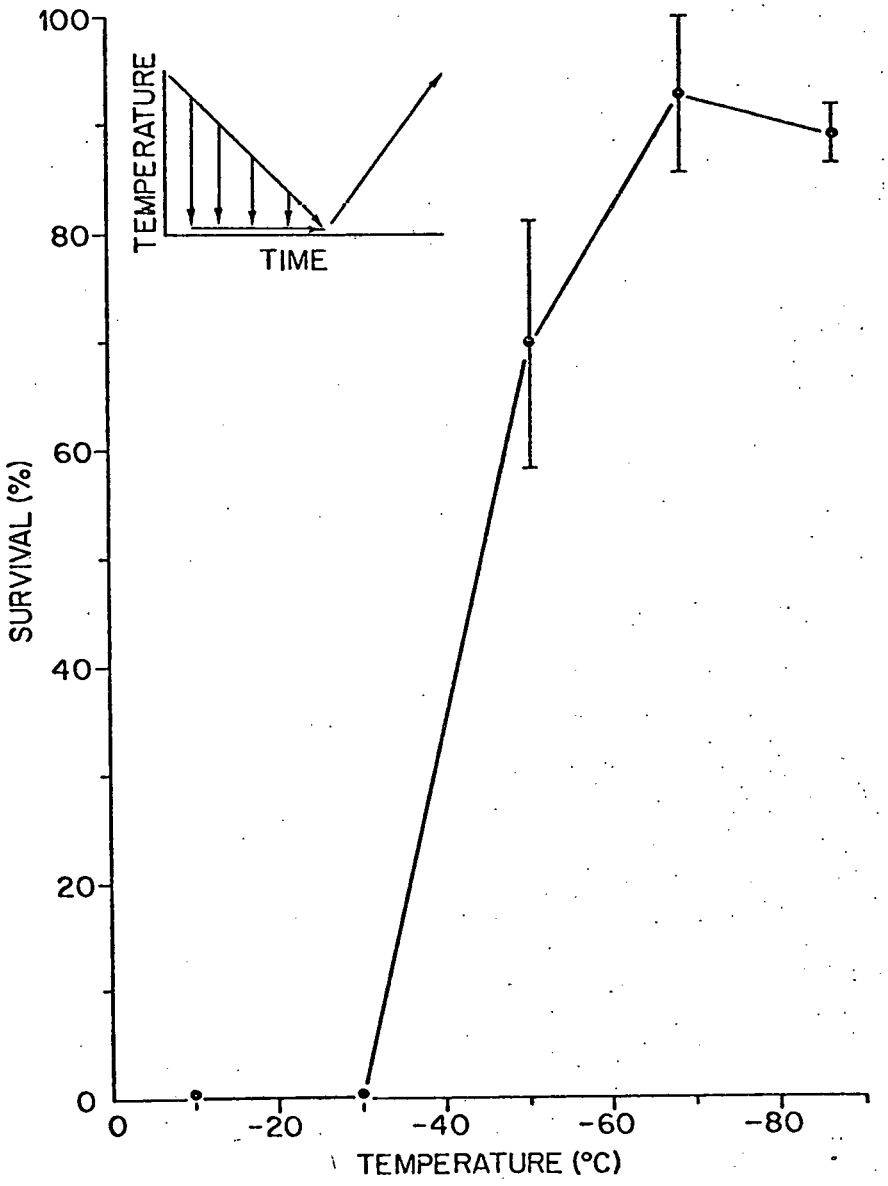
325-30


CELL VOLUME (%)
100
80
60
40
20
0
0
50
100
150
200
250
300
350
400
TIME (sec)
2°
10°
20°
30°

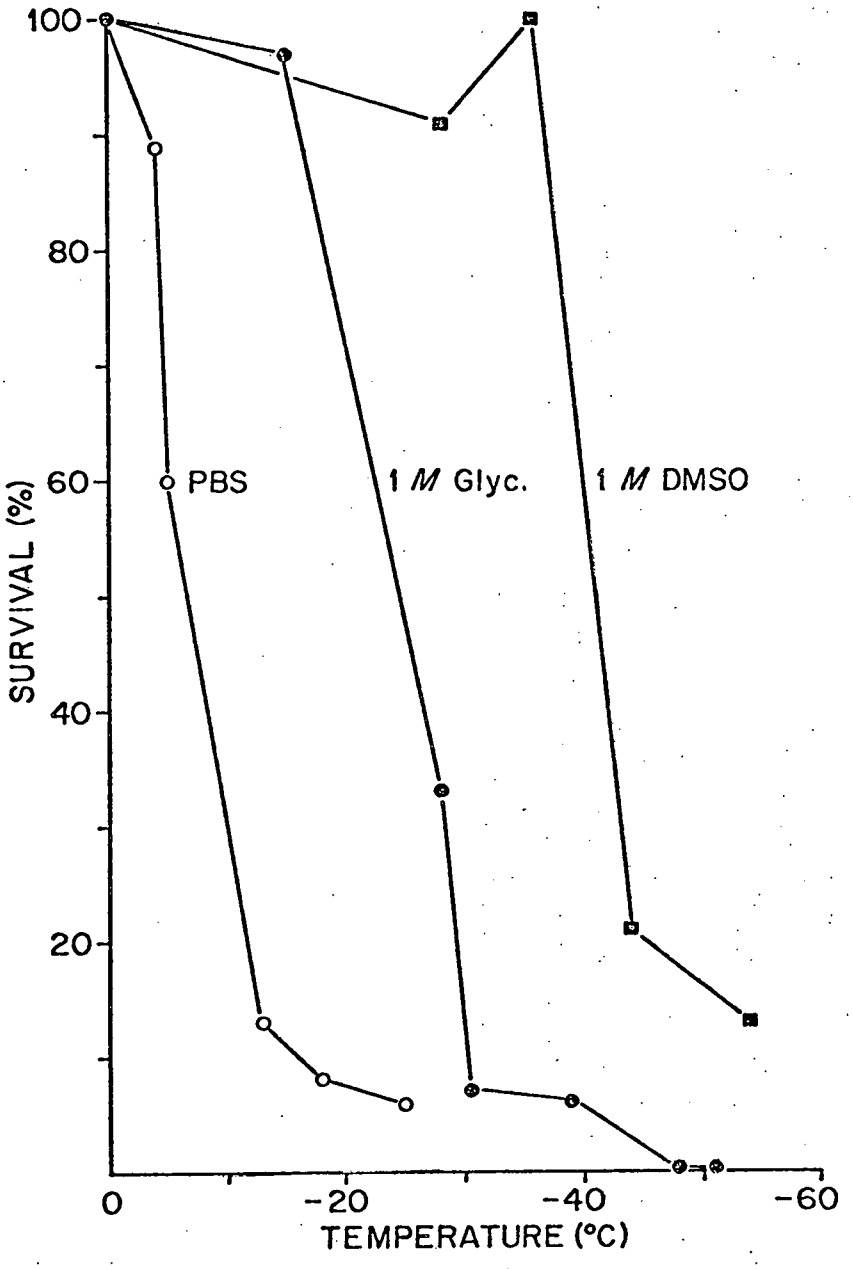
32524


1
0.1
0.02
Lp (μ^3 / μ^2, min, atm.)
30°C
20°C
10°C
0°C
3.2
3.3
3.4
3.5
3.6
3.7
1,000 / T (°K^{-1})

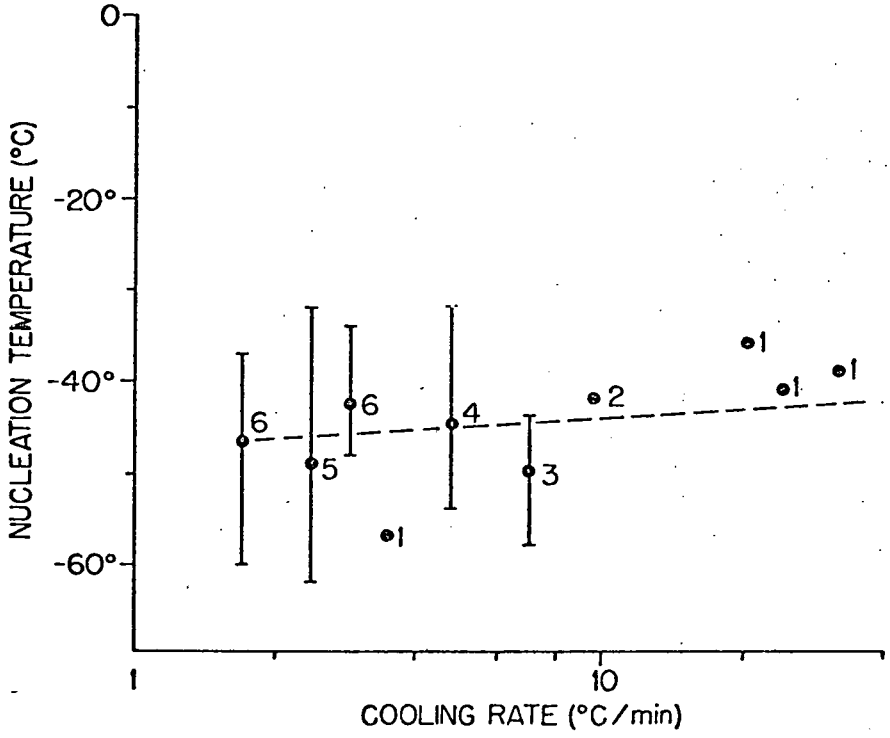
28938


100
80
60
40
20
0
SURVIVAL (%)
TEMPERATURE
TIME
0
-20
-40
-60
-80
TEMPERATURE (°C)

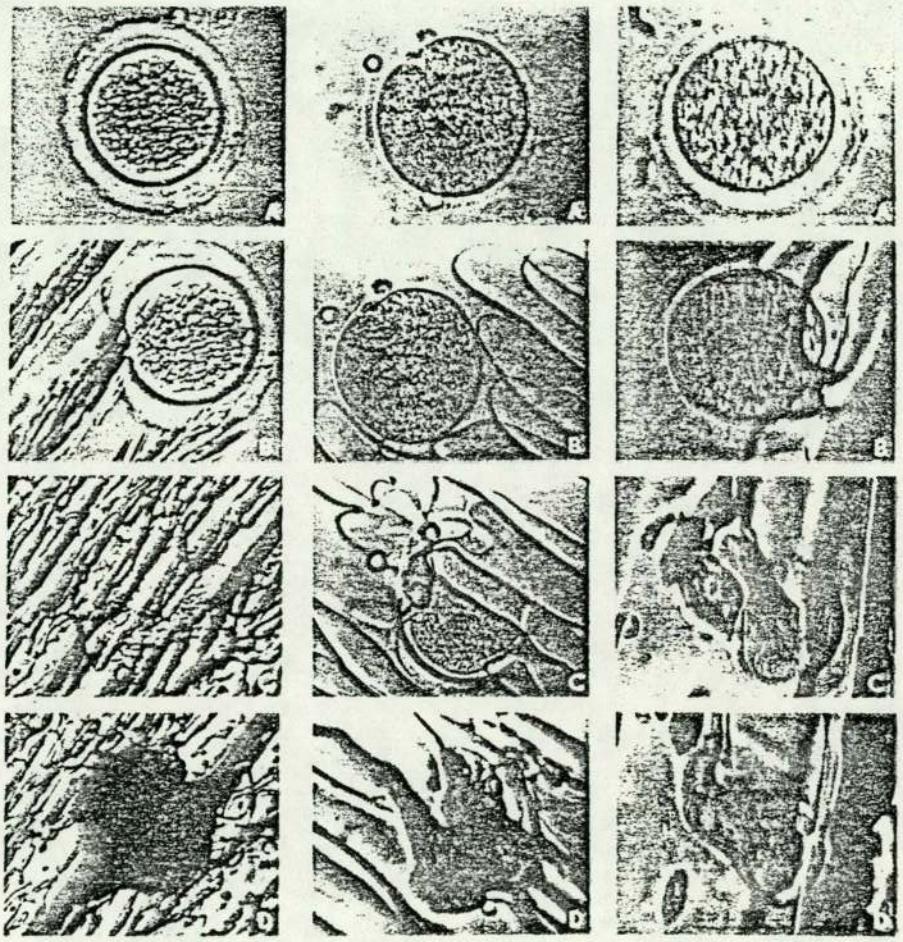
31947


100
80
60
40
20
SURVIVAL (%)
PBS
1 M Glyc.
1 M DMSO
0
-20
-40
-60
TEMPERATURE (°C)

31354

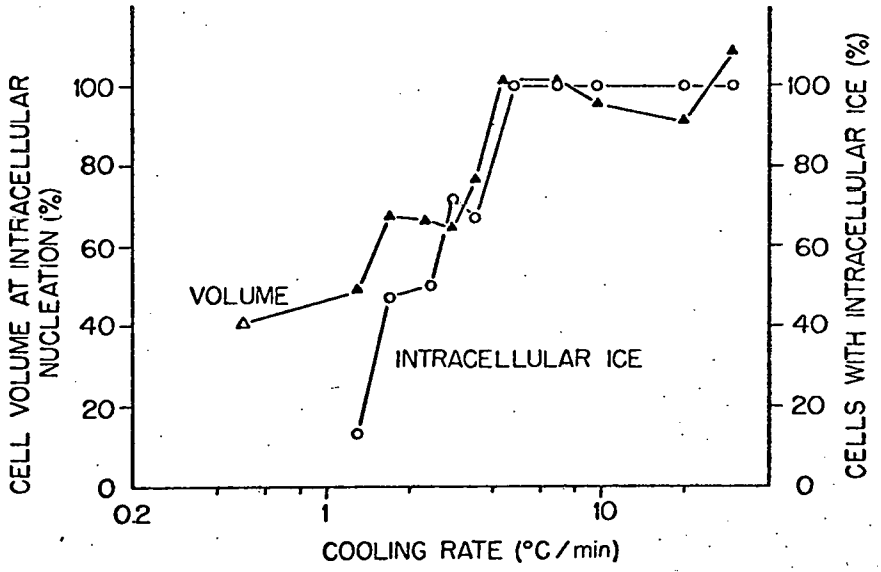

0
-20°
-40°
-60°
NUCLEATION TEMPERATURE (°C)
1
10
COOLING RATE (°C/min)
6
5
6
1
4
3
2
1
1
1

32136

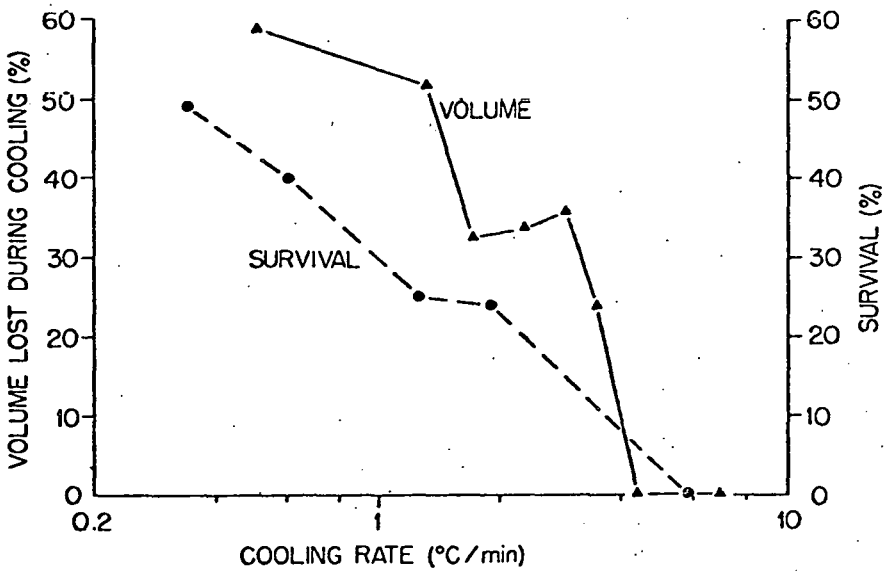


(13)

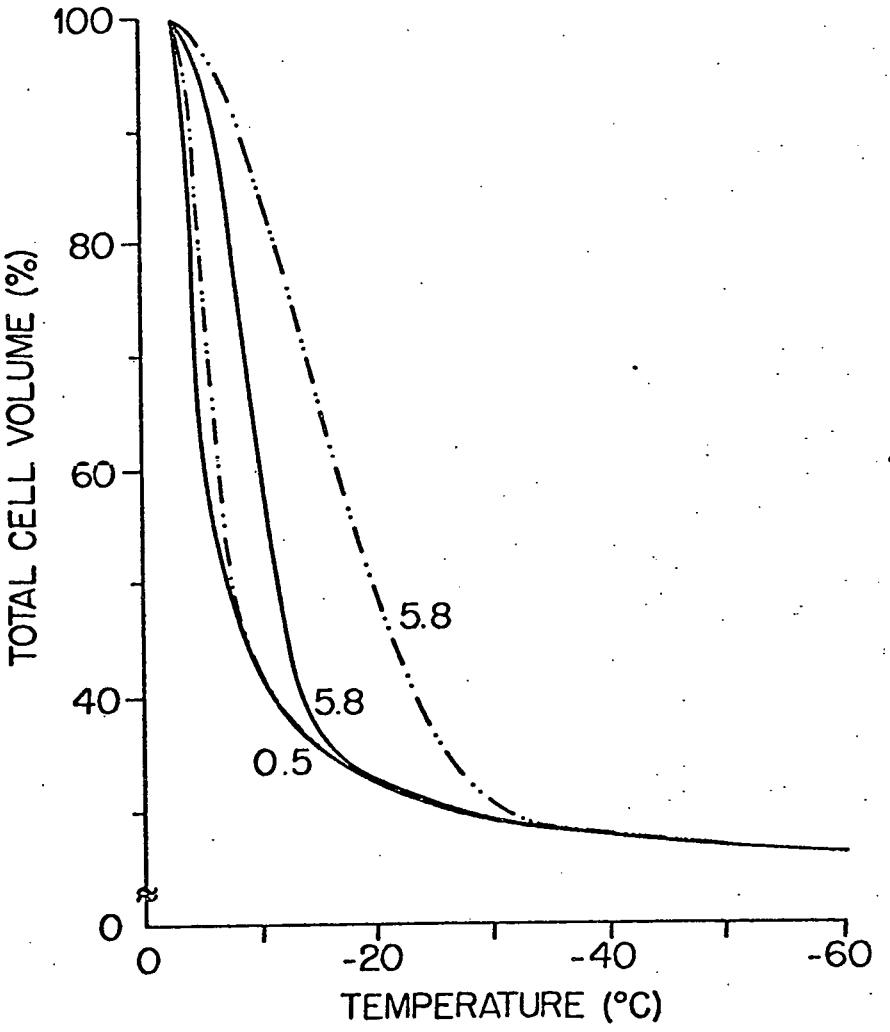
32522


CELL VOLUME AT INTRACELLULAR NUCLEATION (%)
CELLS WITH INTRACELLULAR ICE (%)
100
80
60
40
20
0
0.2
1
10
VOLUME
INTRACELLULAR ICE
COOLING RATE (°C / min)

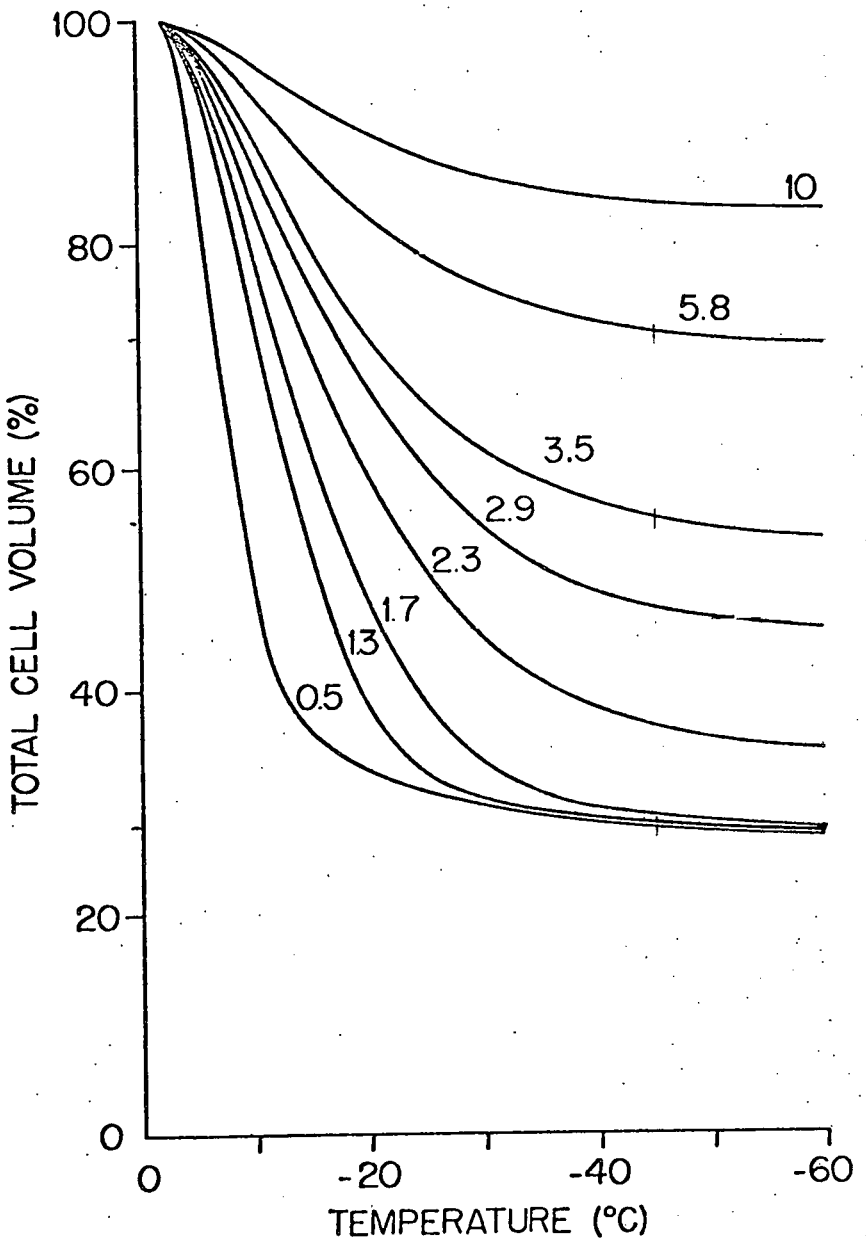
32521


VOLUME LOST DURING COOLING (%)
SURVIVAL (%)
VOLUME
SURVIVAL
COOLING RATE (°C/min)
0
10
20
30
40
50
60
0.2
1
10

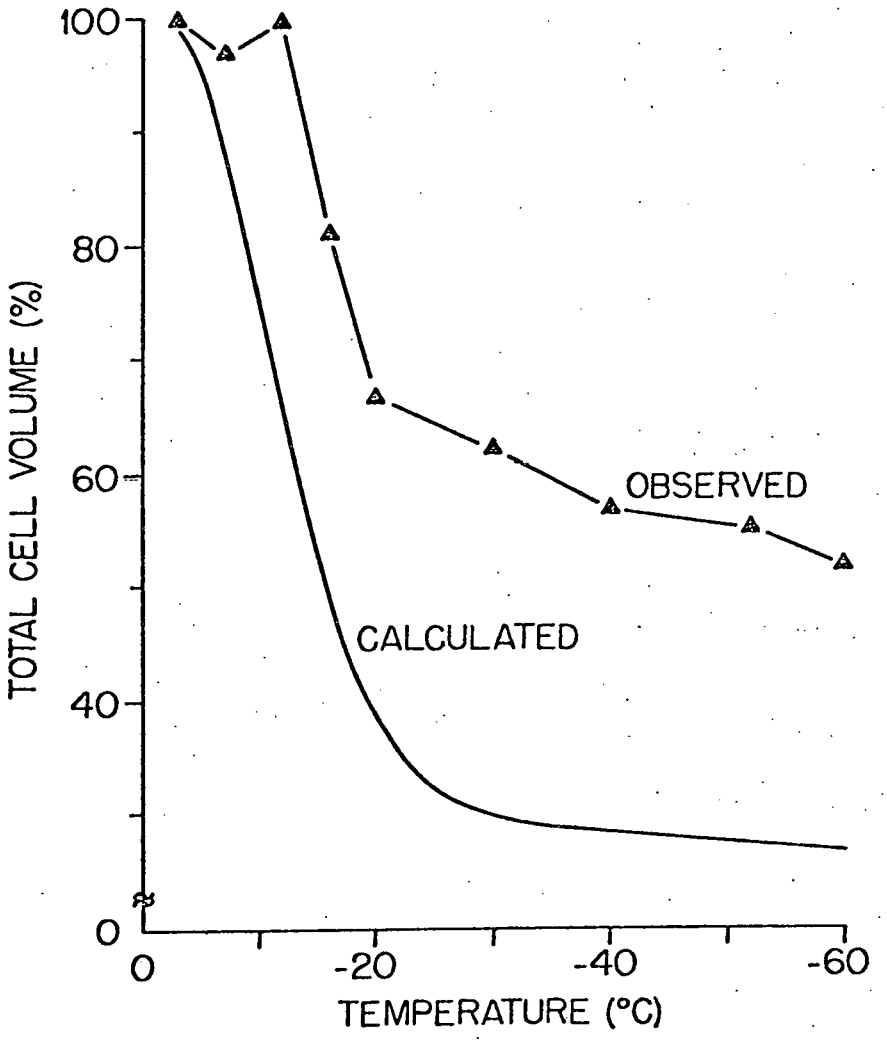
325-13


100
80
60
40
0
TOTAL CELL VOLUME (%)
5.8
5.8
0.5
0
-20
-40
-60
TEMPERATURE (°C)

32529

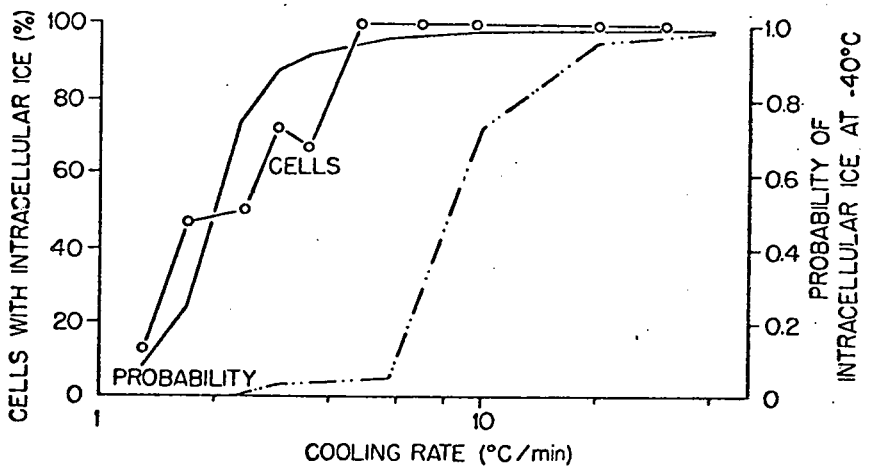

100
80
60
40
20
0
TOTAL CELL VOLUME (%)
10
5.8
3.5
2.9
2.3
1.7
1.3
0.5
0
-20
-40
-60
TEMPERATURE (°C)

31524


100
80
60
40
0
0
-20
-40
-60
TOTAL CELL VOLUME (%)
TEMPERATURE (°C)
OBSERVED
CALCULATED

(17)

32528


CELLS WITH INTRACELLULAR ICE (%)
100
80
60
40
20
0
CELLS
PROBABILITY
1
10
COOLING RATE (°C/min)
1.0
0.8
0.6
0.4
0.2
0
PROBABILITY OF INTRACELLULAR ICE AT -40°C