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The temperature of intracellular ice formation in mouse oocytes vs. the unfrozen fraction at that temperature ☆

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Abstract

We have previously reported [Cryobiology 51 (2005) 29–53] that intracellular ice formation (IIF) in mouse oocytes suspended in various concentrations of glycerol and ethylene glycol (EG) occurs at temperatures where the percentage of unfrozen water is about 6% and 12%, respectively, even though the IIF temperatures varied from −14 to −41 °C. However, because of the way the solutions were prepared, the concentrations of salt and glycerol or EG in that unfrozen fraction at IIF were also rather tightly grouped. The experiments reported in the present paper were designed to separate the effects of the unfrozen fraction at IIF from that of the solute concentration in the unfrozen fraction. This separation makes use of two facts. One is that the concentration of solutes in the residual liquid at a given subzero temperature is fixed regardless of their concentration in the initial unfrozen solution. However, second, the fraction unfrozen at a given temperature *is* dependent on the initial solute concentration. Experimentally, oocytes were suspended in solutions of glycerol/buffered saline and EG/buffered saline of varying total solute concentration with the restriction that the mass ratios of glycerol and EG to salts are held constant. The oocytes were then cooled rapidly enough (20 °C/min) to avoid significant osmotic shrinkage, and the temperature at which IIF occurred was noted. When this is done, we find, as previously that the fraction of water remaining unfrozen at the temperature of IIF remains nearly constant at 5–8% for both glycerol and EG even though the IIF temperatures vary from −14 to −50 °C. But unlike the previous results, the salt and CPA concentrations in the unfrozen fraction vary by a factor of three. The present procedure for preparing the solutions produces a potentially complicating factor; namely, the cell volumes vary substantially prior to freezing: substantially greater than isotonic in some solutions; substantially smaller in others. However, the data in toto demonstrate that cell volume is not a determining factor in the IIF temperature.

Keywords: Oocytes; Mouse; Extracellular freezing; Intracellular ice formation; Unfrozen fraction

One vital factor in determining whether cells survive a cryopreservation procedure is whether or not they undergo intracellular ice formation (IIF) during freezing. In two previous publications [11,12], we presented evidence that the temperature at which IIF occurs in mouse oocytes depends to some extent on interactions with the external ice that surrounds them. We found for example that "flashing" (which is the visual manifestation of IIF) occurred at the temperature at which about 90–95% of the water in the external solutions had frozen. However, the freezing of that water also causes the concentrations of cryoprotective agent (CPA) and salts to rise, so there was also a correlation between the flash temperature and the concentration of solutes in the unfrozen fraction.

We need to look at this process in more detail. During freezing, pure water is pulled out of the solution and converted to ice. The ice formation and the progressively lowering temperatures have two consequences (1) they cause a progressive increase in the concentration of solutes in the still liquid portion of the solution, and (2) they cause a

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progressive decrease in the fraction of the external solution that remains unfrozen. The two processes are reciprocally related and ordinarily inseparable. Consequently, when we observe an event like IIF occurring at a specific temperature, and if the event is causally related to the state of the external medium, one cannot ordinarily tell which of the two events (increased solute concentration, or decreased unfrozen fraction) is responsible. That is the case referred to in the first paragraph.

However, as published previously [7–9], it is possible to partially separate the two events; i.e., to keep the unfrozen fraction constant while allowing the concentration of solutes to vary; or, conversely, to hold the concentration constant while allowing the unfrozen fraction to vary.

Theory

The effects of salt concentration and the fraction unfrozen can be partly separated as illustrated in the ternary phase diagram in Fig. 1, the lower curve of which represents the freezing of a solution of 0.5 M glycerol in 0.147 molal NaCl (left vertical dashed line). As the solution is cooled, and in the absence of any supercooling, ice first forms at a temperature that is dependent on the initial total concentration of solute, W_T^0. With further cooling, an increasing amount of liquid solution is converted to ice, and the total solute wt% concentration in the residual unfrozen portion follows the curve or isopleth labeled $R = 5.42$, where R is the ratio of the wt% glycerol to the wt% NaCl. This ratio remains constant until the pseudobinary eutectic is reached (not shown) because the increased concentration of solute is a result of the removal of pure water from the solution and its conversion into ice. Consequently, at any given temperature above the eutectic (−25 °C in the example shown by the horizontal dashed line), the total solute concentration will be fixed at W_T and will be independent of the starting concentration W_T^0.

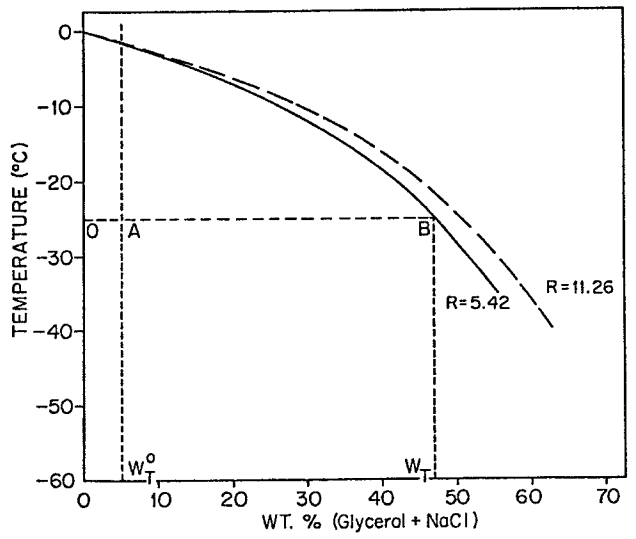

Fig. 1. Equilibrium phase diagrams for the ternary system glycerol/NaCl/water for solutions with weight percent ratios (R) of glycerol to NaCl of 5.42 and 11.26. The vertical and horizontal tie lines are discussed in the text (from [7] by permission of the Biophys. Society).

Although W_T is independent of the starting concentration, the weight fraction of unfrozen liquid (L) at a given temperature is not. The unfrozen fraction is given by the ratio OA/OB($= W_T^0/W_T$) in Fig. 1. If, for example, W_T^0 were doubled, OA/OB would also double and L would double. To clarify this relationship, consider the freezing of 100 g of solution containing 5 g solute (i.e. $W_T^0 = 5$ wt%) to a temperature at which 90% of the solution is frozen ($L = 0.1$). The freezing results from the conversion of water to ice and all solutes are presumed to remain in solution. Consequently, the 5 g of solute are now dissolved in 10 g of solution so that the weight percent of solute in the remaining unfrozen solution(W_T) is $(5 \times 100)/10$, or 50 wt%. In other words, $W_T = W_T^0/L$.

It is possible then, by varying the starting solute concentration, to vary the fraction unfrozen while maintaining a constant solute concentration in that unfrozen fraction. There is one important proviso; namely, in varying the starting concentration, one must maintain a constant R value so as to remain on the isopleth corresponding to that R value. Thus, if we alter the glycerol concentration in an experimental solution, we must alter the salt concentration correspondingly so as to keep, R, the weight ratio, constant.

It is also possible to express the unfrozen fractions in terms of the mass fractions of unfrozen water (U) using the following equation from Rall et al. [15]:

$$U = (100 - W_T)L/(100 - W_T^0)$$

Fig. 2 is a secondary phase diagram derived from Fig. 1 that plots the values of U as a function of temperature and also shows (upper abscissa) the salt concentrations (m_s) in the unfrozen fraction for five different glycerol/NaCl solutions with a constant weight ratio of glycerol/NaCl ($R = 5.42$) and a constant mole ratio ($R' = 3.44$). They were prepared by dissolving appropriate amounts of glycerol in 0.11, 0.15, 0.31, 0.50, and 0.70 molal NaCl to make the molality of the glycerol equal to 0.37, 0.51, 1.07, 1.70, and 2.42 m. As a shorthand, and to emphasize a point, we refer to the five NaCl concentrations as 0.75×, 1×, 2×, 3×, and 4× the isotonic concentration of NaCl (0.15 molal).

Each of these five solutions represents a different starting W_T^0 in the primary phase diagram in Fig. 1. The unfrozen fraction L at various temperatures is computed as OA/OB and converted to values of U. The value of m_s at a given temperature is computed from the value of W_T at that temperature by the equation $m_s = 1000\, W_s/[58.44(100 - W_T^0)]$, where $W_s = W_T/(1 + R)$, and 58.44 is the molecular weight of NaCl [15]. Note that the values of W_T (and m_s) are determined solely by the temperature and are independent of the starting concentration, W_T^0.

In previous publications [7–9], we have been interested in the effects of slowly freezing cells to temperatures that

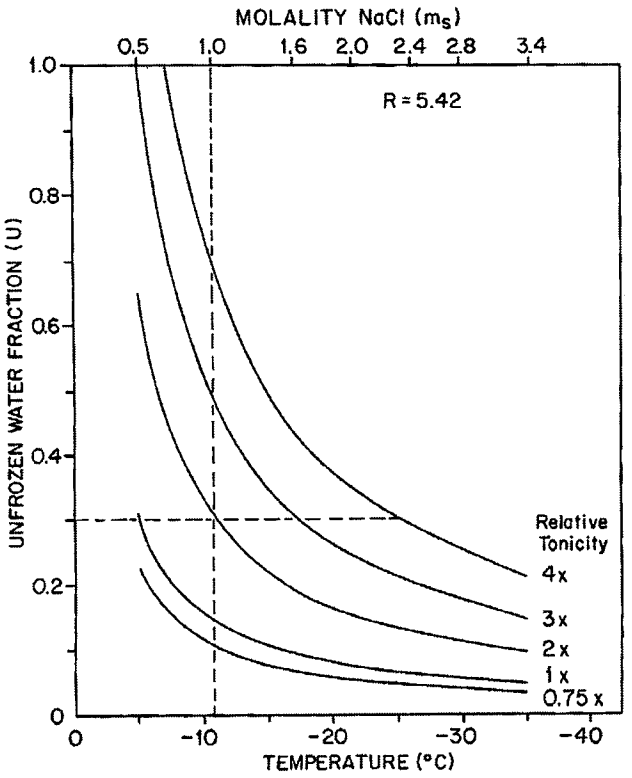

Fig. 2. The unfrozen fraction of water (U) vs. temperature for glycerol/NaCl/water solutions in which $R = 5.42$. The initial concentrations of NaCl range from 0.75 to 4× isotonic. Details of their composition prior to freezing are given in Table 1. The upper abscissa shows the molality of NaCl (m_s) that is present in the unfrozen portions of the solution at the indicated temperatures. The vertical dotted line is an example of conditions yielding constant m_s and variable U. The dotted horizontal line illustrates conditions yielding constant U and variable m_s. (From [8]).

yield a constant m_s but varying U as illustrated by the vertical dashed line in Fig. 2, or have been interested in freezing to temperatures that yield a constant U but varying m_s as illustrated by the horizontal dashed line in Fig. 2. But here the aim is different in two respects. First, here, we wish to use Fig. 2 to determine the values of L, U, and m_s at the observed flash temperatures. For example, for a flash temperature of −25 °C, the value of U in Fig. 2 is 0.05 and the value of m_s is ~2.4 molal. Second, in previous publications, the cells were cooled slowly enough to preclude IIF. Here they are cooled rapidly enough to ensure IIF. In the former case, the cells dehydrate during cooling; in the latter, present case, they undergo little shrinkage during cooling and remain extensively supercooled until IIF occurs.

Methods and materials

Preparation and composition of the initial solutions

Table 1 lists the compositions of the glycerol solutions prepared for these experiments. Solutions with three different R values are shown: 0, 5.42, and 11.26. These correspond to 1× solutions containing 0, 0.5, and 1.0 M glycerol. Within each R value, the solutions are listed in order of increasing glycerol and NaCl concentrations, prepared so as to keep the R value constant or nearly so. We did not use the 3× and 4× solutions experimentally for the reasons given in Results but have included their compositions in the table because they may be useful to others. The top seven lines of Table 1 ($R = 0$) show the composition of the PBS before the addition of glycerol. Their preparation is discussed in the next section. In the calculations and preparation, we have assumed that the molar concentration of the complex of salts (primarily NaCl) in D-PBS (0.1505 M) can be treated as the equivalent molarity of NaCl. With respect to phase diagrams, Pozner et al. [14] have shown that the glycerol/buffered saline/water phase diagram is indistinguishable from the glycerol/NaCl/water phase diagram.

Table 2 gives analogous information on the properties of the experimental EG solutions.

Details of preparation of solutions

First, standard commercial isotonic D-PBS and 10× D-PBS (Gibco) were diluted 2.8% to make the molality of the former equal to 0.147 molal, the value used in previous publications. This permitted us to directly use the detailed compositions and graphs from those earlier publications. Next, 0.6× and 0.75× PBS was prepared by adding 66.51 and 33.25 g of water to 100 ml of the 2.8% diluted isotonic PBS. The 2×, 3×, and 4× PBS solutions were prepared by adding 80.0, 70.0, and 75.0 g water to 20, 30, and 50 ml of diluted 10× PBS. The composition of the solutions labeled 1 × (actual) is that listed in Table 1 of Mazur et al. [11]. These 1× solutions were prepared using full strength D-PBS, which is 0.151 molal. This results in the R values being slightly different in the 1× (actual) solutions than in the others, but this difference is too small to have any effect on subsequent calculations to three significant figures.

The final experimental solutions were prepared by adding appropriate masses of glycerol or EG to 25 ml volumetric flasks, which, when filled to the calibration mark with the appropriate PBS solution, yielded the molarities of glycerol and EG shown in column 10 of Tables 1 and 2. For example, to prepare the R5-0.6×-G3 solution (0.309 M glycerol), 0.711 g glycerol was placed in a 25 ± 0.03 ml volumetric flask which was filled to the mark with 0.6× diluted PBS.

Experimental procedures

Most of the methods used here were described in detail in Mazur et al. [11]; consequently here we give details only for those aspects that differed.

Source of oocytes

MII oocytes from ICR mice were harvested in Dr. Keisuke Edashige's laboratory at Kochi University, Japan,

Table 1
Composition and properties of initial glycerol/salt solutions prior to freezing

Solution[a]	w_s^0 (%)	w_g^0 (%)	w_T^0 (%)	R	m_s	m_g	R'	FV-H_2O	M_g	V_c (glyc)	MP (°C)
0.6X	0.512	0	0.512	0	0.088	0	0	0.9985	0	1.58	−0.29
0.75X	0.639	0	0.639	0	0.110	0	0	0.9982	0	1.30	−0.37
1X	0.852	0	0.852	0	0.147	0	0	0.9976	0	1.02	−0.49
1X (Actual)	0.875	0	0.875	0	0.151	0	0	0.9975	0	1.00	−0.50
2X	1.696	0	1.696	0	0.295	0	0	0.9950	0	0.60	−0.99
3X	2.587	0	2.587	0	0.454	0	0	0.9922	0	0.45	−1.52
4X	3.459	0	3.459	0	0.613	0	0	0.9893	0	0.38	−2.06
R5-0.6X-G3	0.520	2.819	3.339	5.421	0.092	0.317	3.440	0.9763	0.309	0.65	−0.93
R5-0.75X-G3	0.610	3.308	3.918	5.423	0.109	0.374	3.441	0.9722	0.363	0.58	−1.10
R5-1X-G4	0.813	4.410	5.223	5.424	0.147	0.505	3.442	0.9629	0.487	0.47	−1.49
R5-1X-G4 (actual)	0.835	4.530	5.365	5.436	0.151	0.520	3.444	0.9619	0.500	0.47	−1.53
R5-2X-G9	1.626	8.819	10.45	5.424	0.311	1.069	3.442	0.9245	0.989	0.32	−3.16
R5-3X-G13	2.439	13.23	15.67	5.424	0.495	1.703	3.442	0.8843	1.506	0.27	−5.07
R5-4X-G18	3.252	17.64	20.89	5.424	0.703	2.421	3.442	0.8427	2.040	0.24	−7.24
R11-0.6X-G6	0.504	5.674	6.178	11.26	0.092	0.657	7.144	0.9539	0.626	0.45	−1.61
R11-075X-G7	0.582	6.552	7.134	11.26	0.107	0.766	7.144	0.9467	0.725	0.41	−1.88
R11-1X-G9	0.776	8.735	9.511	11.26	0.147	1.048	7.143	0.9284	0.973	0.35	−2.57
R11-1X-G9 (actual)	0.796	8.966	9.762	11.26	0.151	1.076	7.144	0.9266	1.000	0.35	−2.65
R11-2X-G18	1.552	17.47	19.02	11.26	0.328	2.343	7.143	0.8524	1.997	0.26	−5.81
R11-3X-G26	2.328	26.21	28.54	11.26	0.557	3.982	7.145	0.7718	3.074	0.22	−10.01
R11-4X-G35	3.104	34.94	38.04	11.26	0.857	6.124	7.143	0.6863	4.203	0.21	−15.62

[a] The column headings have the following meaning: In the *solution* descriptor, R is the weight ratio of glycerol to salt, 0.6X etc. are the weights of salts relative to the weight of salt in an isotonic solution, and G3 etc. refer to the approximate wt.% of glycerol. w_s^0, w_g^0, and w_T^0 are the weight percents (g/100 g solution) of salt, glycerol, and the total of the two, respectively; m_g and m_s are their respective molalities; and R' is their mole ratio. FV-H_2O is the fractional volume of the solution occupied by water. It is obtained by multiplying the fractional volume of water in the given PBS times the fractional volume of water in a solution of that molality of glycerol in water. The values are from the Handbook of Chemistry and Physics [21]. The product of the molality of glycerol and FV yields the molarity of glycerol, M_g. V_c(glyc) is the computed volume of the oocyte relative to the volume of an oocyte in an isotonic medium. It is computed as the volume of intracellular water plus the volume of cell solids. The relative volume of intracellular water (V_w^0) equals $M^0_{iso}/M^0_{gly+salts}$, where M is the osmolality ($M^0_{iso} = 0.278$). The relative volume of the cell (V_c) equals $(V_w^0 + d)/(1 + d)$, where d is the volume of solids relative to the volume of *water* in the isotonic oocyte. Its value is 0.22 [6]. [The volume of solids is more usually expressed as a fraction, b, of the volume of the *whole isotonic cell.*] The value of b is $d/(1 + d)$ and here, $b = 0.18$. The osmolalities were computed as $M = \varphi\nu m$. For glycerol, ν is 1; for NaCl (PBS), $\nu = 2$. The values of φ (the osmotic coefficient) were obtained from Scatchard et al. [18]. MP is the calculated melting point (= thermodynamic freezing point) of the solution. They were obtained from synthesized ternary phase diagrams for glycerol/NaCl/water published by Kleinhans and Mazur [4].

loaded into straws, vitrified in an ethylene glycol-acetamide-ficoll-sucrose mixture, and express shipped to Tennessee. For an experiment, the oocytes in two to four straws were thawed rapidly, and mixed with 0.5 M sucrose. Some 10 min later, the oocytes were transferred to PB1 lacking sucrose, and then to previously prepared droplets of M16 medium for some 2 h. On pages 48–49 of [11] we give eight lines of evidence that the vitrified-thawed-M16 incubated oocytes are normal with respect to plasma membrane integrity and osmotic responses. Table 7 also supports that assertion.

Sample preparation

To initiate an experiment, two to three oocytes were transferred from an M16 droplet to 1 ml of PBS containing the desired CPA/NaCl solution to which had been added 10 mg/l of Snomax (a commercial preparation of freeze-dried *Pseudomanas syringii*, the ice nucleating bacterium). About 15 min later, a 1.5 µl droplet of this medium was placed in the center of a 75 µm thick spacer in a Linkam quartz sample cuvette, the oocytes pipetted in a minimum volume to that droplet, and a coverglass applied. The sample cuvette was then inserted in a Linkam BCS 196 cryostage and the freezing-thawing run initiated. The stage was attached to a Zeiss microscope, and the sample observed with a 20× objective. The images are displayed at 40 frames/s on a monitor and captured on a computer hard drive by Pax-it software at desired intervals as frequent as 1 image/10 s.

The Linkam cryostage, freezing protocols, and ramps

Using liquid nitrogen vapor for cooling and electrical resistors for heating, the Linkam cryostage allows samples to be subjected to sequential ramps in which cooling rate, limiting temperature, holding time, and warming rate can be specified. Our protocol involved five ramps during cooling, the limiting temperatures of which differed in the various solutions. These limiting temperatures and the cooling rates are listed in Table 3. Extracellular ice usually formed during Ramp 2. Ramp 3 involved warming to just below the melting point of the solution. As described in Mazur et al. [11] its purpose was to have the oocytes at near equilibrium with respect to the external medium prior to the rapid recool by providing about a 2 min period between the abrupt initiation of EIF and that second cool. IIF (flashing) almost always occurred during Ramp 5 where the cooling rate was 20 °C/min. Warming and thawing were carried out at 10 °C/min.

Table 2
Composition and properties of initial ethylene glycol/salt solutions prior to freezing

Solution[a]	w_s^0 (%)	w_{EG}^0 (%)	R	m_s	m_{EG}	R'	FV-H_2O	M_{EG} (Calc)	M_{EG} (Actual)	V_c (EG)	MP (°C)
R4-0.6X-EG2	0.525	1.920	3.659	0.092	0.317	3.442	0.9815	0.311	0.311	1.544	−0.89
R4-0.75X-EG2	0.619	2.255	3.644	0.109	0.374	3.442	0.9781	0.366	0.366	1.337	−1.05
R4-1X-EG3	0.826	3.014	3.648	0.147	0.505	3.442	0.9707	0.490	0.490	1.044	−1.43
R4-1X-EG3 (Actual)	0.848	3.071	3.620	0.151	0.515	3.410	0.9700	0.500	0.500	1.023	−1.46
R4-2X-EG6	1.676	6.118	3.650	0.311	1.069	3.442	0.9397	1.005	1.004	0.601	−3.05
R4-3X-EG9	2.550	9.316	3.653	0.495	1.703	3.442	0.9073	1.545	1.543	0.453	−4.91
R4-4X-EG13	3.449	12.61	3.657	0.703	2.421	3.442	0.8734	2.114	2.109	0.378	−7.07
R8-0.6X-EG4	0.514	3.898	7.584	0.092	0.657	7.144	0.9637	0.633	0.633	1.569	−1.53
R8-075X-EG5	0.593	4.512	7.602	0.107	0.766	7.144	0.9578	0.734	0.733	1.379	−1.79
R8-1X-EG6	0.800	6.059	7.571	0.147	1.048	7.144	0.9432	0.988	0.988	1.069	−2.46
R8-1X-EG6 (Actual)	0.821	6.117	7.450	0.151	1.059	7.010	0.9426	1.000	1.000	1.047	−2.50
R8-2X-EG12	1.646	12.49	7.586	0.328	2.343	7.144	0.8817	2.066	2.064	0.606	−5.65
R8-3X-EG19	2.544	19.31	7.592	0.557	3.982	7.144	0.8150	3.245	3.240	0.450	−9.87
R8-4X-EG27	3.502	26.58	7.588	0.857	6.124	7.144	0.7420	4.544	4.533	0.371	−15.67

[a] Column headings have the same meaning as in Table 1 except that V_c (EG) was computed differently. Unlike glycerol, the oocyte is highly permeable to EG. The volume of intracellular water depends on the osmolality of the impermeant species; i.e., PBS (NaCl) in the case of EG; i.e., $V_w^0 = M_{iso}^0/M^0{}_{salts}$. $M_{iso}^0 = 0.278$. $M_{salts}^0 = 2\varphi m_s$, where φ is from Scatchard et al. [18]. Another difference from glycerol, is that the intracellular EG occupies space. Consequently, $V_c = [V_w^0 + d + (m_{EG} * 54.5 * V_w^0/1000)]/(1 + d)$, where 54.5 is the mean partial molal volume (cm^3/mole) of EG [4, Appendix] and 1000 has the units cm^3/l. The column M_{EG} (Actual) lists the molarities of EG that were prepared. They were based on multiplying the desired molalities of EG by FV-H_2O. Densities of EG solutions vs molality are needed to calculate FV. They were originally calculated as $D = 0.9995 + 0.656271E{-}02 * m - 0.74257E{-}04 * m^2 - 0.164344E{-}04 * m^3 + 0.959826E{-}06 * m^4 - 0.162208E{-}07 * m^5$. They should have been calculated with a first term of 0.9982, the density of water at 20 °C. The column M_{EG} (Calc) is based on the corrected FV-H_2O. The differences between Actual and Calculated are too small (0.07% for 0.6X to 2X solutions, and 0.15% and 0.25% for the 3X and 4X solutions [which were not used experimentally]) to have any demonstrable effect on the calculations in subsequent tables. Melting points (MP) were obtained from synthesized ternary phase diagrams for EG/NaCl/water published by Kleinhans and Mazur [4].

Results

Table 4 lists the unfrozen fractions at the temperatures at which flashing occurred and lists the molal concentrations of salt and CPA in those unfrozen portions. The values for the W_T of glycerol at a given flash temperature were computed from Pegg's equations [13] fitting the experimental DSC-derived ternary phase diagram data published by Shepard et al. [19]. The values for EG are computed from synthesized ternary phase data; i.e. from ternary phase diagrams synthesized by summing the separate freezing point depressions for the binary system EG/water and the binary system NaCl/water [4].

The most striking results can be seen for the unfrozen fraction of water (U). With the exception of the 2× solutions, all the values lie between a U of 0.04 and 0.08. That is to say that at the temperatures at which flashing occurred (−13.9 to −44.6 °C), 92–96% of the extracellular water was frozen. (We shall return to the 2× solution shortly). That constancy is not observed with the molal concentration of salts (m_s) or of glycerol or EG (m_{CPA}) in those unfrozen fractions. The former varied from 1.26 to 5.49 molal and the latter from 0 to 20.5 molal.

A similar tight grouping , again with the exception of the 2× solutions, is observed for L, the mass fraction of solution that is unfrozen, except that the values are roughly double those of U. This is because L includes the mass of the CPA in addition to the mass of water in the unfrozen fraction.

L_v is the approximate *volume* of residual liquid in the solution at the flash temperature as a fraction of the volume of liquid in the initial unfrozen solution. It was calculated as the sum of the volumes of water, CPA, and salt at −30 to −40 °C divided by the total volume of the sample including the volume of ice. The volumes were obtained by dividing the masses of these components by their densities at ∼−30 °C or by multiplying them by their partial molal volumes at ∼−30 °C. Densities or partial molal volumes depend on concentration and temperature. We used published values for −30 or −40 °C. A numerical example and the relevant references are given in [11].

Specifically,

1. The mass of water/100 g original unfrozen solution at the flash temperature = $W_w^0 * U$. The volume of that water (V_w) = mass/0.984, the density of water at −30 °C.
2. The mass of ice/100 g original solution at the flash temperature = $W_w^0 * (1 - U)$. Its volume (V_{ice}) is the mass/0.921, its density at −30 °C.
3. The mass of CPA/100 g original solution at the flash temperature remains unchanged during freezing and equals w_{CPA}^0. Its volume (V_{CPA}) = moles of CPA * $\overline{V}_{CPA}$, where $\overline{V}_{CPA}$ is the partial molal volume at ∼−30 °C (71.4 cm^3/mole for 2 molal glycerol and 51.5 for 5 to 7 molal EG).
4. Finally, the mass of NaCl/100 g original solution at the flash temperature also remains unchanged during freezing and equals w_s^0. Its volume (V_{salt}) = moles of salt * $\overline{V}_{salt}$, where $\overline{V}_{salt}$ is the partial molal volume at ∼−30 °C (∼10 cm^3/mole).

Table 3
Temperature limits for the ramps in the linkam cryostage

Solution	MP (°C)	~MP (°C)	Diff from 1X (°C)	Estimated EIF (°C)	Observed EIF (°C)	Limit (°C)				
						Ramp 1	Ramp 2	Ramp 3	Ramp 4	Ramp 5
R5-0.6X-G3	−0.93	−0.9	0.6	−5.0	−4.9	−4.0	−6.0	−1.2	−6.0	−50
R5-0.75X-G3	−1.10	−1.1	0.4	−5.3	−5.3	−4.0	−6.3	−1.3	−7.0	−50
R5-1X-G4 (Actual)	−1.53	−1.5	0.0	−6.1	−6.1	−5.0	−6.9	−2.2	−7.0	−50
R5-2X-G9	−3.16	−3.1	−1.7	−9.4	−8.0	−8.0	−9.5	−3.5	−11.0	−65
R5-3X-G13	−5.07	−5.0	−3.5	−13.1	n/a	−11.1	−14.1	−5.2	−15.1	−70
R5-4X-G18	−7.24	−7.1	−5.6	−17.3	n/a	−15.3	−18.3	−7.3	−19.3	−70
R11-0.6X-G6	−1.61	−1.6	0.9	−5.2	−5.3	−4.0	−6.3	−2.3	−7.0	−50
R11-075X-G7	−1.88	−1.8	0.7	−5.8	−5.7	−5.0	−6.8	−2.5	−8.0	−50
R11-1X-G9 (Actual)	−2.65	−2.5	0.0	−7.1	−7.1	−5.0	−8.0	−3.2	−7.0	−50
R11-2X-G18	−5.81	−5.6	−3.1	−13.2	−11.1	−9.0	−11.2	−5.5	−13.2	−65
R11-3X-G26	−10.01	−9.5	−7.0	−21.0	n/a	−19.0	−22.0	−10.2	−23.0	−70
R11-4X-G35	−15.62	−14.5	−12.1	−31.2	n/a	−29.2	−32.2	−15.3	−33.2	−70
R4-0.6X-EG2	−0.89	−0.9	0.6	−4.3	−5.2	−3.0	−5.5	−1.2	−5.0	−50
R4-0.75X-EG2	−1.05	−1.1	0.4	−4.6	−5.1	−4.0	−6.0	−1.3	−6.0	−50
R4-1X-EG3 (Actual)	−1.46	−1.5	0.0	−5.4	−5.4	−5.0	−6.0	−1.7	−7.0	−50
R4-2X-EG6	−3.05	−3.1	−1.7	−8.7	−7.8	−8.0	−9.3	−3.5	−11.0	−65
R4-3X-EG9	−4.91	−5.0	−3.5	−12.4	n/a	−10.4	−13.4	−5.2	−14.4	−70
R4-4X-EG13	−7.07	−7.1	−5.6	−16.6	n/a	−14.6	−17.6	−7.3	−18.6	−70
R8-0.6X-EG4	−1.53	−1.6	0.9	−4.9	−5.4	−4.0	−5.9	−2.3	−6.0	−50
R8-075X-EG5	−1.79	−1.8	0.7	−5.5	−6.0	−4.0	−6.5	−2.5	−6.5	−50
R8-1X-EG6 (Actual)	−2.50	−2.5	0.0	−6.8	−6.8	−5.0	−8.0	−3.2	−7.0	−50
R8-2X-EG12	−5.65	−5.6	−3.1	−12.9	−10.5	−10.0	−14.0	−7.0	−15.0	−65
R8-3X-EG19	−9.87	−9.5	−7.0	−20.7	n/a	−18.7	−21.7	−10.2	−22.7	−70
R8-4X-EG27	−15.67	−14.5	−12.1	−30.9	n/a	−28.9	−31.9	−15.3	−32.9	−70

MP is the true melting point (see Tables 1 and 2).
~MP is the approximate melting point calculated as $1.855 \times (m_{CPA} + 2 \times m_s)$. Diff from 1X is the difference between the calculated melting point of a solution and the melting point of the 1X solutions. Estimated EIF refers to the estimated temperature at which ice will form in the extracellular solution containing the nucleator Snomax. It was calculated as the observed EIF for 1X solutions * 2 * the value in the "Diff from. 1X" column. This was based on the observations of Rasmussen and MacKenzie [17] that the ice nucleation temperature is suppressed approximately twice the depression of the thermodynamic melting point.

Ramp temperature limits and cooling/warming rates. The cooling rates for Ramps 1, 2, 4, 5 were −10, −2, −10, and −20 °C/min. The warming rate for Ramp 3 was +2 °C/min. The limits for Ramp 1 were initially set at the estimated EIF temperature plus 2 °C. The limits for Ramp 2 were initally set 1 °C below the estimated EIF; consequently, EIF occurred during Ramp 2. In Ramp 3, the samples were programmed to warm at 2 °C/min to a temperature below the approximate melting point by the value in the Diff from 1X column. A small amount of external ice was still present at that point. In Ramp 4, the sample was recooled at 10 °C/min to close to the limits for Ramp 2, and finally in Ramp 5 the cooling rate was increased to 20 °C/min to −50 to −65 °C. IIF (flashing) occurred during this ramp.These estimated ramp limit values had to be fine-tuned in some cases, and the limits shown for the 0.6X to 2X solutions are those actually used. The 3X and 4X solutions were not used experimentally.

Table 4
Unfrozen fraction of water and solution and concentration of CPA and salt in that fraction vs. the flash temperature

CPA	Solution	Relative tonic	Molarity	R	R'	n	Flash temperature (°C)	W_T	L	U	$\sim L_v$	m_s	m_{CPA}
		PBS	CPA					at flash	at flash	at flash	at flash	at flash	at flash
None	R0-1X	1X	0	0	0	10	-13.9 ± 2.4	17.4	0.05	0.04	0.04	3.60	0
Glycerol	R5-0.6X-G3	0.6X	0.31	5.42	3.44	9	-26.9 ± 1.9	48.8	0.07	0.04	0.05	2.54	8.7
	R5-0.75X-G3	0.75X	0.36	5.42	3.44	6	-23.7 ± 2.4	45.6	0.09	0.05	0.07	2.23	7.7
	R5-1X-G4	1X	0.5	5.44	3.44	15	-30.8 ± 3.4	52.5	0.10	0.05	0.08	2.94	10.1
	R5-2X-G9	2X	0.99	5.42	3.44	10	-49.5 ± 2.9	67.3	0.16	0.06	0.12	5.49	18.9
Glycerol	R11-0.6X-G6	0.6X	0.63	11.26	7.14	7	-36.1 ± 2.1	59.0	0.11	0.05	0.08	2.00	14.3
	R11-0.75X-G7	0.75X	0.72	11.26	7.14	8	-44.6 ± 1.9	65.7	0.11	0.04	0.09	2.67	19.1
	R11-1X-G9	1X	1.00	11.26	7.14	7	-41.3 ± 2.5	63.2	0.16	0.06	0.12	2.40	17.1
	R11-2X-G18	2X	2.00	11.26	7.14	1	-46.8	67.3	0.28	0.11	0.23	2.87	20.5
EG	R4-0.6X-EG2	0.6X	0.31	3.66	3.44	10	-14.6 ± 2.7	27.7	0.09	0.07	0.08	1.41	4.8
	R4-.75X-EG2	0.75X	0.37	3.64	3.44	7	-19.6 ± 2.6	33.4	0.09	0.06	0.07	1.85	6.4
	R4-1X-EG3	1X	0.50	3.62	3.41	9	-23.4 ± 3.7	37.1	0.11	0.07	0.09	2.18	7.4
	R4-2X-EG6	2X	1.00	3.65	3.44	8	-42.9 ± 1.2	50.7	0.15	0.08	0.12	3.79	13.0
EG	R8-0.6X-EG4	0.6X	0.63	7.58	7.14	6	-23.7 ± 2.6	38.7	0.11	0.07	0.10	1.26	9.0
	R8-0.75X-EG5	0.75X	0.73	7.60	7.14	9	-27.2 ± 3.3	41.7	0.12	0.08	0.11	1.42	10.2
	R8-1X-EG6	1X	1.00	7.45	7.01	34	-37.2 ± 1.4	48.9	0.14	0.08	0.12	1.94	13.6
	R8-2X-EG12	2X	2.06	7.59	7.14	7	-50.5 ± 0.9	56.6	0.25	0.13	0.21	2.60	18.6

The values for the 1X solutions are from Mazur et al. [11], Table 4.
The molarity for the CPA in the initial solutions (column 4) and the R and R' values are from Tables 1 and 2.
W_T is the weight percent of solute (CPA + salt) in the residual unfrozen solution at the observed flash temperature. For glycerol, it is calculated by the equation of Pegg [13]; namely, $W_T = (a + (a^2) - 0.04T_{fl})^{1/2}/0.02$, where $1/a = -1.6 - 1.27R - 0.25R^2$ and T_{fl} is the flash temperature. The values of W_T for EG are from the synthesized ternary phase diagrams of Kleinhans and Mazur [4].
L, U, and L_v are three measures of the unfrozen fraction at the flash temperature. L is the weight fraction of unfrozen solution; U is the weight or volume fraction of unfrozen water; and L_v is the volume fraction of unfrozen solution. The equations for obtaining them are in the text.
m_s and m_{CPA} are the molalities of salt and CPA in the unfrozen fraction. The equation for computing the value of m_s is given in the Theory section. The molality of the CPA is $R' \times m_s$. The $\pm$ values in this and succeeding tables are standard errors.

Thus, $L_v = (V_w + V_{CPA} + V_{salt})/(V_w + V_{CPA} + V_{salt} + V_{ice})$. For glycerol and EG, the L_v values are seen to lie about half-way between L and U.

Woods et al. [22] have published ternary phase diagrams of EG/NaCl/water of various R values that they derived experimentally by differential scanning calorimetry and fitting equations to the data. Calculations based on their experimental data and fitting equations are shown in Table 5. We did not include them in Table 4 because Kleinhans and Mazur [4] have presented evidence that there are systematic errors of an unknown source in their experimental determinations. There are two sorts of concerns. One is that there are sizeable differences between their experimental phase data for EG and the Kleinhans and Mazur synthesized ternary diagrams. Such differences are not present in the other solutes analyzed by Kleinhans and Mazur; namely, glycerol, DMSO, and sucrose. If one calculates U and L at the flash temperatures based on the WT's calculated from the Woods et al. fitting equations (Table 5), one sees a similar tight grouping of U and L values (except for the 2× solutions) but the values in Table 5 are considerably higher than those in Table 4 for a given solution. There are similar large differences for the calculated molalities of NaCl and of EG at the flash temperatures, with the molalities based on the Woods et al.'s data being considerably lower than those based on the synthesized data. A second concern is that there are internal discrepancies in their experimental isopleths, discrepancies that are discussed by Kleinhans and Mazur [4].

Table 6
Comparison between the flash temperature of oocytes and their computed homogeneous nucleation temperature

CPA	Solution	M.P.	Homogeneous nucleation temperature (°C)	Flash temperature (°C)
Water		0	−36	—
PBS	R0-1X	−0.50	−37	−13.9
Glycerol	R5-0.6X-G3	−0.93	−38	−26.9
	R5-0.75X-G3	−1.10	−38	−23.7
	R5-1X-G4	−1.53	−39	−30.8
	R5-2X-G9	−3.16	−42	−49.5
Glycerol	R11-0.6X-G6	−1.61	−39	−36.1
	R11-0.75X-G7	−1.88	−40	−44.6
	R11-1X-G9	−2.65	−41	−41.3
	R11-2X-G18	−5.81	−48	−46.8
EG	R4-0.6X-EG2	−0.89	−38	−14.6
	R4-.75X-EG2	−1.05	−38	−19.6
	R4-1X-EG3	−1.46	−39	−23.4
	R4-2X-EG6	−3.05	−42	−42.9
EG	R8-0.6X-EG4	−1.53	−39	−23.7
	R8-0.75X-EG5	−1.79	−40	−27.2
	R8-1X-EG6	−2.50	−41	−37.2
	R8-2X-EG12	−5.65	−47	−50.5

The homogeneous nucleation temperature is calculated as −36 °C + 2 * M.P. The value of −36 °C is the homogeneous nucleation temperature of a droplet of water with a volume equal to that of the water in an oocyte [1]. That homogeneous nucleation temperature is suppressed by approximately 2-times the thermodynamic freezing point depression (or M.P.) of the solution [17].

The exceptions for the 2× solutions

As noted in Table 4, the unfrozen fractions at the flash temperature for the 2× EG and glycerol solutions were significantly higher than those for the 0.6×, 0.75×, and 1× solutions. From Table 6, we see that the flash temperatures of the former (the 2×) were all well below −40 °C whereas the flash temperatures of 11/13 of the latter were above −40 °C. Table 6 also lists the computed homogeneous ice nucleation temperatures (T_h) for oocytes in the various solutions. These are the temperatures at which a volume of supercooled water equal to that in an isotonic oocyte is expected to freeze spontaneously. Note that in nearly all the 2× solutions, there is fairly close correspondence between T_h and the flash temperature. This suggests that for these solutions, IIF occurred primarily as a consequence of homogeneous nucleation. We shall return to this point in the Discussion.

Although we prepared 3× and 4× solutions of glycerol and EG, we did not use them experimentally because it was clear from the observations on oocytes in the 2× solutions that we were very unlikely to see any flashing of oocytes in these more concentrated solutions. Indeed in R11-2× glycerol, IIF was observed in only 1 of 6 oocytes.

Table 5
Unfrozen fractions of EG solutions and molalities of EG and salt in those fractions based on the ternary phase diagrams of Woods et al. [22]

Solution	Flash temperature (°C)	W_T (%)	L at flash	U at flash	$\sim L_v$ at flash	m_s at flash	m_{CPA} at flash
R4-0.6X-EG2	−14.6	18.3	0.13	0.11	0.12	0.82	2.82
R4-.75X-EG2	−19.6	23.3	0.12	0.10	0.11	1.12	3.85
R4-1X-EG3	−23.4	27.0	0.14	0.11	0.13	1.36	4.64
R4-2X-EG6	−42.9	43.2	0.18	0.11	0.15	2.79	9.60
R8-0.6X-EG	−23.7	27.6	0.16	0.12	0.14	0.76	5.43
R8-0.75X-EG5	−27.2	30.9	0.17	0.12	0.15	0.89	6.35
R8-1X-EG6	−37.2	39.3	0.18	0.12	0.16	1.31	9.19
R8-2X-EG12	−50.5	49.2	0.29	0.17	0.22	1.93	13.8

W_T was computed using the fitting equations of Woods et al. [22]; namely, $W_T = (-b - (b^2 - 4ac)^{1/2})/2a$, where $b = -0.676 + (4.77E{-}03)R$, $a = (-7.64E{-}03) + (-2.75E{-}05)R$, and $c = -T_{fl}$. The values of unfrozen fraction and molality were computed as in Table 4.

Do osmotically induced changes in oocyte volumes confound the results?

Our procedure for experimentally separating the magnitude of the unfrozen fraction at a given flash temperature from the concentrations of salt and CPA in that unfrozen portion involved placing the oocytes in solutions containing a range of concentrations of CPA and NaCl in which the weight percent ratio of the two (R) was held constant. An unavoidable consequence of this is that the volumes of oocytes varied considerably prior to the initiation of freezing. Since oocytes behave as ideal osmometers, after they have equilibrated in the solutions, the volume of water in them would be reciprocally related to the osmolality of the impermeant species in the medium. In the case of EG (to which they are highly permeable), their water volume and cell volume relative to their isotonic volume would be reciprocally related to the ratio of the NaCl osmolality to that of the isotonic salt. The result is that the relative cell volumes of oocytes in EG solutions where the salt concentration was 0.6× or 0.75× of isotonic will be 55% and ~35% *above* the isotonic volume, respectively (Table 2, column 11). In contrast, when they were placed in the 2× medium, their volumes will be only 60% of isotonic. The volumes they exhibited prior to freezing are effectively maintained during subsequent cooling and freezing because the cooling rate was high enough (20 °C/min) to preclude significant osmotic shrinkage.

In glycerol the principle is the same but the results are different because in this case, both the salts and the glycerol are non-permeating. Consequently, in glycerol/NaCl, the oocytes are shrunken in all the media used but to different extents (Table 7). From Tables 1 and 7, we see that in the $R = 5.42$ solutions, their relative volumes will range from 65% of isotonic in the R5-0.6× solution to 32% of isotonic in the R5-2× solution. In the $R = 11.26$ glycerol solutions, the corresponding relative cell volumes are 45% and 26% of isotonic. These relative cell volumes in the initial solutions also apply to their volumes at IIF. This is because the cooling rate in Ramp 5 is high enough to preclude significant loss of cell water during cooling to IIF.

In neither glycerol or EG, however, is there any correlation between the cell volume before the initiation of freezing and the flash temperature. For example, oocytes in the R5-0.75×-G3 and the R8-0.6×-EG4 solutions both flashed at −23.7 °C (Table 4), but the values of V_c are 58% and 157%, respectively (Tables 1 and 2).

Discussion

Ordinarily, as one freezes cells in a medium made of CPA in isotonic salt, the concentration of solutes in the medium rises and the fraction of the external medium that is unfrozen drops. Indeed, the former is a consequence of the latter. One further consequence is that if an event (here, flashing) occurs at certain temperatures, one can not tell whether it is associated with solute concentration or unfrozen fraction. However, as discussed in *Theory*, one can separate the two to a certain extent by freezing cells in solutions where the total solute concentration is varied while holding the weight ratio of the CPA and salt constant. When this was done, we see (Table 4) that, with one exception, IIF (flashing) occurred when the percentage of frozen water in the solution ($1 - U$) was 92–96%, and when the percentage of the solution that was frozen ($1 - L$), was 85–95%. The exception was the 2× solutions, where the percentage of the solution that was frozen dropped to 72–84%. We shall return to this exception shortly.

Note that no such close correlation occurs between the flash temperature and the concentration of salt or CPA in the unfrozen fraction at that temperature (columns 13 and 14 of Table 4).

We have hypothesized previously [11] that the two major determinants of IIF in mouse oocytes and in other cells are (1) heterogeneous nucleation of supercooled intracellular water by external ice at temperatures above ~−40 °C and (2) spontaneous homogeneous nucleation of intracellular supercooled water at −40 °C and below. We believe that the data in Tables 4 and 6 strongly support this view. When flashing occurred above −40 °C, it occurred at temperatures at which some 94% of the external water and ~90% of the external solution were frozen. The very narrow range of U and L and their very low values are evidence for the view that IIF occurs as a consequence of close contact between the external ice and the cell plasma membrane.

In the 2× solutions where IIF occurred below −40 °C, it occurred at somewhat lower frozen fractions. This we would argue is because it now occurred as a consequence

Table 7
Measured and calculated volumes of oocytes in various glycerol/PBS solutions

Solution	Osmolality	Diameter (μm)	Volume (μm^3)	n	Relative volume	Calculated relative volume
Isotonic PBS	0.278	75	2.21×10^5	—	1	1
R5-0.6X-G3	0.488	64.3 ± 0.4	$1.39 \pm 0.02 \times 10^5$	10	0.63 ± 0.02	0.65
R5-0.75X-G3	0.577	61.6 ± 0.7	$1.23 \pm 0.01 \times 10^5$	10	0.56 ± 0.01	0.57
R11-0.6X-G6	0.833	58.0 ± 0.2	$1.02 \pm 0.01 \times 10^5$	4	0.46 ± 0.01	0.45
R11-075X-G7	0.972	55.3 ± 0.8	$0.89 \pm 0.04 \times 10^5$	8	0.40 ± 0.02	0.42
R11-1X-G9	1.333	53.1 ± 0.7	$0.79 \pm 0.03 \times 10^5$	10	0.36 ± 0.01	0.35

The diameters of the oocytes proper (i.e., excluding the zona pellucida) were measured using the Pax-it measuring module on images collected in the experiments. Their volumes were computed assuming them to be spheres.
The procedure for calculating the relative volumes of the oocytes is given in the footnote to Table 1.

of homogeneous nucleation, in which case contact with external ice would be irrelevant. Homogeneous nucleation is the spontaneous nucleation of supercooled water in the absence of a foreign nucleating agent.

Although flashing occurred at tightly grouped unfrozen fractions, that was not the case for the molal concentrations of either salt or CPA. They varied considerably.

Do oocyte volume changes confound the interpretation?

As noted towards the end of Results, an unavoidable consequence of the procedures we used to separate effects of unfrozen fraction from effects of solute concentrations is that the volumes of oocytes varied considerably in the various solutions prior to the initiation of freezing. In some cases the oocytes were swollen well above their isotonic volume; in other cases they were shrunken well below their isotonic volume. A question is whether these differences in starting volumes could have had a significant influence on the subsequent flash temperatures. We believe the answer is no. In both our previous publication [11] and here, a comparison of the results in glycerol and EG shows that the flash temperature in both media correlated closely with the fraction of the external medium that was frozen at that temperature and not with the volume of the oocytes at the time of the flash or their volume before the initiation of freezing. The initial volumes and the volume at flash were actually similar, since the cooling rate in Ramp 5 was sufficiently high to prevent much shrinkage during cooling. As a numerical example, consider the R5-0.75× glycerol solution and the R4-0.75× EG solutions in Table 4. Both flashed at identical L values (0.09) and closely similar U values (0.05 and 0.06), but their computed initial volumes differed greatly. The oocyte volumes were 58% of isotonic in glycerol (Table 1) and 134% of isotonic in EG (Table 2).

CPA concentration and the IIF temperature

In 1983, Rall et al. reported that the flash temperature of 8-cell mouse embryos decreased progressively from −12 °C to −30 °C or −35 °C as the concentrations of glycerol or dimethyl sulfoxide increased from 0 to 1.5 M [16]. They hypothesized that the lowering of the IIF temperature was due to the fact that the higher the CPA concentration, the greater was the unfrozen fraction at a given temperature, or to put it differently, in the presence of CPA, the samples have to be cooled to lower temperatures to attain the same unfrozen fraction. Mazur et al. [11] extended these findings to mouse oocytes and to EG as the CPA. They further showed that the IIF temperature did indeed correlate closely with the attainment of a given unfrozen fraction, but could not distinguish that correlation from the mirror-image effects of salt and CPA concentrations. The present study eliminates that confounding and provides strong quantitative evidence that the suppression of the IIF temperature by increasing concentrations of CPA is strongly correlated with the fact that CPAs reduce the temperature at which a very low and critical unfrozen fraction is attained. A very high correlation does not prove causality, but it strongly implies it.

Heterogeneous and homogeneous nucleation

There is increasingly persuasive evidence that the heterogeneous nucleation of supercooled cell cytoplasm requires the presence of ice in the suspending medium, and there is accumulating evidence that the external ice itself is the nucleating agent. (See [10, pp. 31–32] for a discussion). The findings in the present study make a significant contribution to that evidence. If external ice is to nucleate intracellular water, the ice has to come in close contact with the outer surface of the plasma membrane of the cell. The less liquid in the external medium, the greater the likelihood that such close contact will occur. Here, we see that when flashing in mouse oocytes occurs above T_h, it occurs only when the external liquid is reduced to about 10% of that originally present. That fraction is constant in different starting concentrations of CPA, and in two different CPAs (glycerol and EG). It is constant even though at the flash temperature, the concentrations of the CPA and NaCl in the outside medium differ widely, and the flash temperatures differ widely, ranging from −14 °C to −50 °C. We can not yet answer the question of why close contact between external ice and the cell surface should trigger IIF. Perhaps the close contact enhances the probability that an external ice crystal will come in contact with a previously existing pore or defect in the plasma membrane. Perhaps close contact with ice causes severe distortion of the cell surface as the cell attempts to conform its shape to the small extracellular liquid space, and that this distortion causes membrane defects that now permit the passage of an external ice crystal into the supercooled cell interior.

The degree to which these results and interpretations for mouse oocytes are applicable to embryos of different species and to different cell types is currently unknown, Guenther et al. [2] found no correlation between the flash temperatures in Stage I and II oocytes of the frog *Xenopus* and the unfrozen fraction (their Figs. 8c and d). But they also reported no correlation with the solute concentration at the flash temperature (their Table 5). The question of why the response of these amphibian eggs is so different from mouse oocytes could be important but is currently unanswered. One possibility is that the size of the cell is a factor. Large eggs and embryos tend to undergo IIF at higher temperatures than mouse oocytes; e.g., zebrafish [3] and starfish [5].

Two distinct roles for low molecular weight cryoprotectants

The results of this study demonstrate that low molecular weight cryoprotectants like glycerol and EG play two sharply distinct roles in protecting mouse oocytes (and presumably, mouse early embryos, and oocytes and embryos of other mammalian species) from freezing injury. One role is the classical one of protecting cells that are cooled slowly

enough to preclude IIF. This protection is either a consequence of the action of the CPA to reduce electrolyte concentration during slow freezing (the majority view) or a consequence of CPA to reduce the likelihood of cell–cell interactions or cell–ice interactions during slow freezing (the minority view), or both [10]. The other distinct role of CPA is to greatly suppress the temperature at which the cell can undergo IIF. The evidence presented here indicates that this suppression arises from the suppression by CPA of the subzero temperature at which a critical unfrozen fraction is attained. If it were not for this suppression, it would be very difficult to successfully cryopreserve mouse, and presumably other mammalian oocytes and embryos. Further, it would almost certainly be impossible to cryopreserve them by the commonly used modified procedure that involves slow cooling to ~−35 °C followed by a plunge into liquid nitrogen.

The conclusion reached here for mouse oocytes still does not permit one to distinguish between the two chief hypotheses of nucleation by external ice. One is the hypothesis of Toner and colleagues [20] that contact of external ice with the outer surface of the plasma membrane induces configurational changes on the inner surface that make it an effective ice nucleator. The other is Mazur's hypothesis [10] that contact by external ice leads to the growth of that ice through the plasma membrane; in other words, external ice itself is the direct nucleating agent. Our findings here also do not permit distinguishing between the view that the induction of intracellular ice by external ice takes place in a normal plasma membrane (Toner and Mazur) and the view that the act of extracellular freezing produces defects in the plasma membrane which allow passage of the external ice into the supercooled interior of the cell.

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