Available online at www.sciencedirect.com
SCIENCE DIRECT®
CRYOBIOLOGY
Cryobiology 51 (2005) 29–53
www.elsevier.com/locate/ycryo

Extra- and intracellular ice formation in mouse oocytes ☆

Peter Mazur [a,*], Shinsuke Seki [a,b], Irina L. Pinn [a], F.W. Kleinhans [a,1], Keisuke Edashige [a,b]

[a] *Fundamental and Applied Cryobiology Group, Department of Biochemistry and Cellular and Molecular Biology, The University of Tennessee, Knoxville, TN 37932-2575, USA*
[b] *Laboratory of Animal Science, College of Agriculture, Kochi University, Kochi, Japan*

Received 28 January 2005; accepted 14 April 2005
Available online 21 June 2005

Abstract

The occurrence of intracellular ice formation (IIF) during freezing, or the lack there of, is the single most important factor determining whether or not cells survive cryopreservation. One important determinant of IIF is the temperature at which a supercooled cell nucleates. To avoid intracellular ice formation, the cell must be cooled slowly enough so that osmotic dehydration eliminates nearly all cell supercooling before reaching that temperature. This report is concerned with factors that determine the nucleation temperature in mouse oocytes. Chief among these is the concentration of cryoprotective additive (here, glycerol or ethylene glycol). The temperature for IIF decreases from −14 °C in buffered isotonic saline (PBS) to −41 °C in 1 M glycerol/PBS and 1.5 M ethylene glycol/PBS. The latter rapidly permeates the oocyte; the former does not. The initial extracellular freezing at −3.9 to −7.8 °C, depending on the CPA concentration, deforms the cell. In PBS that deformation often leads to IIF; in CPA it does not. The oocytes are surrounded by a zona pellucida. That structure appears to impede the growth of external ice through it, but not to block it. In most cases, IIF is characterized by an abrupt blackening or flashing during cooling. But in some cases, especially with dezonated oocytes, a pale brown veil abruptly forms during cooling followed by slower blackening during warming. Above −30 °C, flashing occurs in a fraction of a second. Below −30 °C, it commonly occurs much more slowly. We have observed instances where flashing is accompanied by the abrupt ejection of cytoplasm. During freezing, cells lie in unfrozen channels between the growing external ice. From phase diagram data, we have computed the fraction of water and solution that remains unfrozen at the observed flash temperatures and the concentrations of salt and CPA in those channels. The results are somewhat ambiguous as to which of these characteristics best correlates with IIF.

Keywords: Oocytes; Mouse; Freezing; Intracellular and extracellular; Plasma membrane; Zona pellucida

☆ This research was supported by NIH Grant R01-RR18470.
* Corresponding author. Fax: +1 865 974 8027
E-mail address: pmazur@utk.edu (P. Mazur).
[1] Present address: Department of Physics, Indiana University-Purdue University at Indianapolis, IN 46202, USA.

0011-2240/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.cryobiol.2005.04.008

The most important single factor determining the success of cryopreservation is whether or not a cell undergoes intracellular ice formation (IIF) during freezing. In "classical" freezing, IIF is avoided by cooling cells sufficiently slowly so that osmotic dehydration results in their water remaining in near chemical potential equilibrium with the outside solution and ice. Fig. 1 illustrates quantitatively what is meant by sufficiently slow cooling. It represents physical–chemical modeling of the kinetics of water loss in mouse oocytes as a function of cooling rate and temperature. The equations on which the curves are based have been published previously [16,20]. The curve labeled EQ depicts the water volume of an oocyte cooled infinitely slowly. The other curves represent the shrinkage at cooling rates of 1, 4, 8, and 32 °C/min. The faster the cooling, the more the cell water volume departs from equilibrium, and the more it departs from equilibrium, the more it is supercooled.

A supercooled cell will eventually freeze intracellularly. The vertical line in the graph at −33 °C represents the median temperature that Rall et al. [30] reported for IIF in 8-cell mouse embryos frozen in 1 M glycerol or DMSO at 20 °C/min. When the water content of embryos or oocytes has returned to equilibrium before reaching −33 °C (e.g., those cooled at 1 °C/min), it will by definition not be supercooled and, therefore, will not freeze when the cells enter the nucleation zone. In contrast, when the internal water content has not returned to equilibrium by −33 °C (e.g., those cooled at 4 °C/min or faster), it will by definition be supercooled, and will be expected to freeze upon entering the nucleation zone.

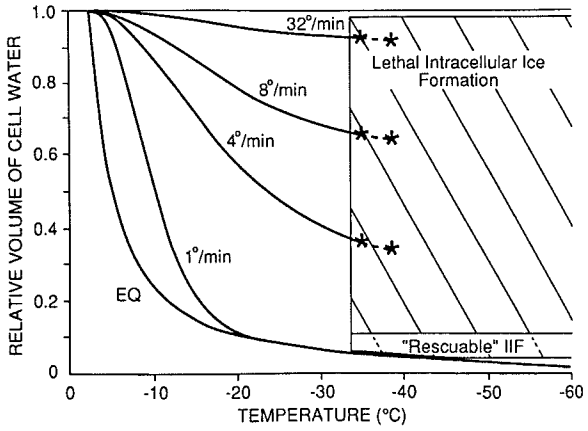

Fig. 1. The computed loss of water from mouse ova as a function of the cooling rate during freezing in 1 M DMSO or glycerol and temperature. The vertical solid line at −33 °C is the median ice nucleation temperature observed by Rall et al. [30] for 8-cell mouse embryos (from Mazur [18]).

Whether or not a cell freezes internally depends on the cooling rate. But it also depends on the boundary temperature of the nucleation zone. In Fig. 1, the boundary is set at −33 °C. If, on the other hand, it were −10 °C, one can readily see that cooling would have to be considerably slower than 1 °C/min to achieve cell water equilibrium prior to −10 °C. If nucleation were to occur appreciably above −10 °C, it would become impossible to achieve sufficient cell dehydration to avoid IIF no matter how low the cooling rate. In other words, it would become impossible to cryopreserve by classical slow freezing. Such appears to be the case in starfish oocytes [9] and zebrafish embryos [8].

Because of its central importance to cryobiology, we have initiated an in-depth study of factors that influence the temperature at which IIF occurs. The model cells chosen for study are mouse oocytes and oocytes of the frog *Xenopus*. This report concerns the former. An important practical reason for selecting the mouse is that results for it may be pertinent to the cryopreservation of human oocytes for clinical use. To date, successes for the latter have been at best sporadic. An important mechanistic reason for selecting the oocytes of these two species is that it is possible to express aquaporin water channels in both. This allows a test of the hypothesis that one route of IIF may be the growth of external ice crystals through existing pores in the plasma membrane [19]. The current paper reports our findings on normal mouse oocytes, which do not express aquaporin water channels.

Methods

As just mentioned, a central aim of our research was to compare the ice nucleation behavior of control mouse oocytes, which lack aquaporin chan-

nels, with that of oocytes in which these channels have been expressed. Obtaining the latter requires special micromanipulation and injection equipment and considerable experience in these techniques and the relevant molecular biology, both of which are available in Dr. Edashige's laboratory in Japan but are lacking in Knoxville. For that reason, it is necessary to ship the oocytes from Japan to Knoxville in the vitrified state. Since the aquaporin-expressing oocytes are to be vitrified in Japan, we considered it essential that the control oocytes be from the identical source and subjected to vitrification in the same laboratory. We briefly mention in this section and in more detail in the Discussion, the evidence that the plasma membrane integrity and the nucleation behavior of the control oocytes used in our experiments is not affected by their having been vitrified.

Obtaining mature (MII) mouse oocytes

The sources of the oocytes were ICR female mice purchased from CLEA JAPAN and Japan SLC. For a given day, about five females in the animal facilities in the College of Agriculture, Kochi University, were injected IP with 0.1 ml of pregnant mare serum gonadotropin (5 IU, Serotrophin, Teikokuzoki, Japan) and 48 h later with 0.1 ml of human chorionic gonadotropin—hCG (5 IU, IP, Puberogen, Sanykyo, Tokyo, Japan) to induce superovulation. Twelve hours after hCG-injection, the animals were euthanized by CO_2 and the mature oocytes were collected from the ampullar region of the oviducts into PB1 medium [27, p. 731]. The cumulus cells of the collected MII oocytes were removed by exposing them to PB1 containing 37 U/ml of hyaluronidase for 15 min.

Vitrifying oocytes and shipment

The vitrification solution (EFAS10/10) consisted of 10% (v/v) ethylene glycol and 10% (w/v) acetamide dissolved in a stock consisting of 30% (w/v) Ficoll 70 and 0.5 M sucrose in PB1 medium. The final concentrations of sucrose and Ficoll are 0.4 M and 24% (w/v). The following solutions were then successively aspirated into 1/4 ml straws: a 60 mm column of 0.5 M sucrose, a 20 mm air bubble, a 5 mm column of EFAS10/10, a 5 mm air bubble, and a 12 mm column of EFAS10/10 to contain the oocytes. The oocytes were first placed for 5 min in PB1 containing 10% (v/v) EG and then washed by transfer to two successive drops of EFAS10/10 at 25 °C. Three (generally) were then transferred by micropipette into the 12 mm column of EFAS10/10 in each straw and the end of the straw opposite the polyvinyl alcohol plug was heat sealed.

Two minutes after introducing the oocytes into the EFAS10/10 in the straw, the straw was placed for 3 min or more in liquid nitrogen (LN_2) vapor at −120 to −150 °C and then into LN_2. For shipment from Japan, the straws were transferred to a Taylor Wharton dry cryogenic shipper (Theodore, AL) and sent to Knoxville by express mail. Upon receipt, the shipping container was checked to make certain it still contained LN_2.

Devitrification and culture in M16

Late in the afternoon of the day before an experiment, 100 μl droplets of M16 medium ([27, p. 390]) are placed into 35 mm tissue plates (Falcon #3005), covered with mineral oil, and transferred to a 5% CO_2/air 37 °C incubator overnight. The morning of the experiment, the droplets are checked to see that the phenol red indicated the proper pH and a watch glass is prepared containing 2 ml of 0.5 M sucrose in PB1. To thaw, a straw is removed from the LN_2 storage dewar, held 10 s in 25 ± 1 °C air (to minimize cracking of the zona from thermal stress), and placed, heat-sealed end down, with stirring, in a beaker of water at 25–30 °C for 7 s. This is sufficient time to melt the contents of the straw.

The thawed contents are then expelled into the watch glass containing 2 ml of 0.5 M sucrose/PB1, timing begun, the oocytes are located and transferred into a second watch glass with 0.5 M sucrose/PB1. Ten to fifteen minutes after thawing, the oocytes are transferred to three successive droplets of PB1 in a #1008 Falcon 35 mm petri dishes without sucrose. (The purpose of the sucrose is to prevent harmful osmotic swelling of the oocytes from their high internal content of EFAS upon transferring to PB1 [14].) Ten minutes is suf-

ficient for the efflux of most of the EFAS so that the eggs can then be returned to PB1. The procedure also provided a test of the osmotic normalcy of the thawed oocytes. They are expected to shrink in the hyperosmotic sucrose and reswell when transferred to the sucrose-free PB1; most did so.

The next step is to transfer the oocytes from PB1 into three successive droplets of the preequilibrated M16 medium. The dishes containing these oocytes are then held in the 5% CO_2/air incubator at 37 °C for 1–3 h. This is an important step to allow latent injury from the vitrification to either develop or be repaired [7]. Some 10–20% of the oocytes that appear normal prior to incubation in M16 degenerate by the end of the incubation. The other 80–90% still appear normal and these are the oocytes used in the subsequent experiments. In the Discussion, we shall review this and other evidences that the vitrified oocytes used in our cryostage experiments are the equivalent of "fresh" oocytes with respect to cell properties relevant to our experiments; namely, intactness of the plasma membrane, normal osmotic response to anisotonic solutes, and intracellular freezing temperatures similar to those reported for fresh oocytes and embryos under conditions some of which overlap with ours.

Experimental media

At the conclusion of the M16 incubation, the oocytes were transferred to 1 ml of the experimental solutions, the composition of which is given in Table 1. The solutions consisted of various concentrations of ethylene glycol (EG) and glycerol (0–1.5 M) made up in Dulbecco's PBS (Gibco 14040). The solvent was PBS rather than the more complex PB1 because we wanted to keep it as physical-chemically simple as possible. Oocytes in EG/PBS media were held at 22–25 °C for 15 min—time enough to permit their complete or nearly complete intracellular equilibration as evidenced by normal or near normal volumes. Oocytes in glycerol media were held 10 min prior to initiating freezing experiments. Mouse oocytes are poorly permeable to glycerol, as evidenced by the fact that they shrink and remain shrunken. Since the exposure time to PBS prior to initiating the freezing phase was only 10–15 min at room temperature, the fact that PBS is somewhat suboptimal relative to PB1 should not have had adverse consequences to our experimental results.

The media also contained 10 mg/L (0.001%) of a commercial freeze-dried preparation (Snomax) of the ice-nucleating bacterium *Pseudomonas syringiae* (York Snow, Victor, NY). This served to ice nucleate the external medium some 8 °C higher than would otherwise be the case.

Cytochalasin B

A few experiments examined the freezing behavior of oocytes exposed to Cytochalasin B, a disrupter of microfilaments. In this case, at the end of the incubation in M16, the oocytes were

Table 1
Composition of solutions

Solution	Wt% salt	Wt% CPA	R	Wt% water	m salt	m CPA	R'	M CPA	MP (°C)
R0-1X	0.875	0	0	99.13	0.151	0	0	0	−0.6
R3-1X-EG2	0.834	2.091	2.51	97.08	0.147	0.347	2.36	0.34	−1.2
R4-1X-EG3	0.848	3.071	3.62	96.08	0.151	0.515	3.41	0.5	−1.5
R5-1X-EG4	0.819	3.863	4.72	95.32	0.147	0.653	4.44	0.63	−1.8
R6-1X-EG5	0.814	4.474	5.50	94.71	0.147	0.761	5.18	0.73	−2
R7-1X-EG6	0.821	6.117	7.45	93.06	0.151	1.059	7.01	1.0	−2.5
R12-1X-EG9	0.795	9.145	11.51	90.06	0.151	1.636	10.83	1.5	−3.6
R5-1X-G4	0.835	4.532	5.43	94.63	0.151	0.52	3.44	0.5	−1.5
R11-1X-G9	0.796	8.866	11.26	90.49	0.151	1.076	7.14	1.0	−2.6

R is the wt% CPA/wt% salt. R' is the mole ratio CPA/salt. R0-1X is Dulbecco's PBS with the concentrations expressed as NaCl equivalents. EG = ethylene glycol; G = glycerol. A slight difference in preparation procedure resulted in the values of the salt molality of the 0.34, 0.63, and 0.73 M EG solutions being 0.147 rather than 0.151. m = molal concentration, M = molar concentration, MP = melting point; all solutions also contained 10 mg/L Snomax.

transferred to PBS or EG/PBS media containing 10.4 μM Cytochalasin B (Sigma C6762) for 15 min.

Dezonated oocytes

Some experiments involved observations on dezonated oocytes. To achieve dezonation, oocytes were transferred singly to acidified tyrode solution [27, p. 726]. As soon as the zona was observed to disappear (20–80 s), the oocyte was washed several times in PBS containing 0.4% PVP 30 (polyvinypyrrolidone, avg. 30 KDa) to reduce stickiness, and then transferred to M16 for the standard 1–2 h incubation. The PVP was also included in the PBS used subsequently.

Preparing the Linkam sample

The sample container for the Linkam cryostage used in our experiments (next section) consists of a shallow quartz dish 14 mm inner diameter and 2.56 mm deep. The bottom is 40 μm thick. A 75 μm thick washer (approximately the diameter of an oocyte) measuring 12.7 mm OD and 7.9 mm ID was punched from plastic shim stock with Ted Pella Inc., disc punches #54743 and 54740, and placed in the quartz dish. Then, (1) a 1.5 μl drop of the test medium was pipetted into the center of the washer, one or two oocytes were quickly transferred to that droplet, and a 12 mm round glass coverslip (Ted Pella No. 26023) was applied with vacuum tweezers. [The purpose of the washer was to prevent distortion of the oocyte by the cover glass.] (2) The quartz dish was then quickly placed in the Linkam sample holder, which was then inserted into the Linkam stage and a freezing run was immediately initiated. Approximately 1–2 min elapsed between initiating step (1) and completing step (2). Separate weighings indicated an evaporation rate of a 1.5 μl drop of 1 M glycerol/PBS under these conditions to be about 1%/min.

The Linkam cryostage

A BCS 196 cryostage (Linkam Scientific Instruments, Waterfield, UK) along with a Paxcam digital CCD camera (800 × 600 resolution) and Pax-it control and capture software (v. 6.1) developed by Midwest Information Services (Franklin Park, IL) for the Linkam, and integrated by McCrone Microscopes and Accessories (Westmont, IL) were used for these experiments. These were attached to a Zeiss bright-field microscope and the oocytes were observed with an Olympus 20× long working distance microscope objective. The Pax-it software permits the setting of multiple ramps in which the variables are cooling rate (up to 50 °C/min), temperature limit, holding time at desired temperatures, and warming rate. Warming and cooling are effected, respectively, by electric heating and nitrogen vapor cooling of a silver control block. The maximum capture rate of 1 image/10 s was used during critical phases of the experiment. Images, however, are observed continuously in real time at 40 frames/s on the monitor.

The quartz dish referred to above rests on the silver cooling block. (Quartz has about twice the thermal conductivity of flint glass.) During a run, the temperature output of a thermocouple imbedded near the top of the cooling block is displayed continuously to 0.1 °C. We have several internal measures indicating that the sample temperature is quite accurately reflected by the displayed temperature. The first and most precise is that 2.5 μl water spread into a thin film between two 12 mm coverslips is observed to melt during warming at 10 °C/min at a displayed temperature of −0.2 to 0 °C. Second, all the experiments involved an initial warming ramp of a seeded sample to a displayed ~1 °C below the melting point of the test solution. As expected, most, but not all, of the ice is observed to have melted at the upper limit. Third, since PBS is primarily NaCl, melting during warming is expected to begin slightly below the eutectic point of pure NaCl, which is −21.1 °C. That in fact was observed. Fourth, when 1.5 μl droplets of oocytes in CPA/PBS undergo their final warming and thawing at 10 °C/min, the last traces of ice vanish at an indicated 3–3.5 °C above the computed melting point. That is in accord with our experience with calibrated thermocouples imbedded in small volumes of solution warmed at comparable rates.

Videotaping

In a number of experiments, the Pax-it acquired images were supplemented with digital video recordings. These DVs permitted the acquisition of images at 0.03 s resolution (the precise scan rate being 29.97 images/s). The images were obtained by photographing a supplementary LCD monitor screen with a Sony DCR-TRV 38 digital video camera recorder. Relevant portions of the resulting tape were captured on computer using U-Lead Video Studio 7 software (www.ulead.com).

Statistics

Plus/minus values in tables are standard errors (standard deviations of the mean). Tests of statistical significance were carried out by a two-tailed Student's t test.

Results

Controls

Oocytes were held in ethylene glycol (EG)/isotonic PBS and glycerol/isotonic PBS for 15 and 10 min, respectively, before initiating the freezing experiments. Their appearance in 0.5 and 1 M concentrations of these CPAs is depicted in Fig. 2. Those in EG undergo an initial shrinkage in the hyperosmotic solutions but after the 15 min exposure, they have returned to or nearly to their original isotonic volume. This return to near normal volume means that sufficient EG has permeated to make the internal concentration nearly equal to the external. For physical reasons, volume restoration is faster in lower concentrations of permeating solutes, and this tended to be the case here. Again for physical reasons, equilibrium

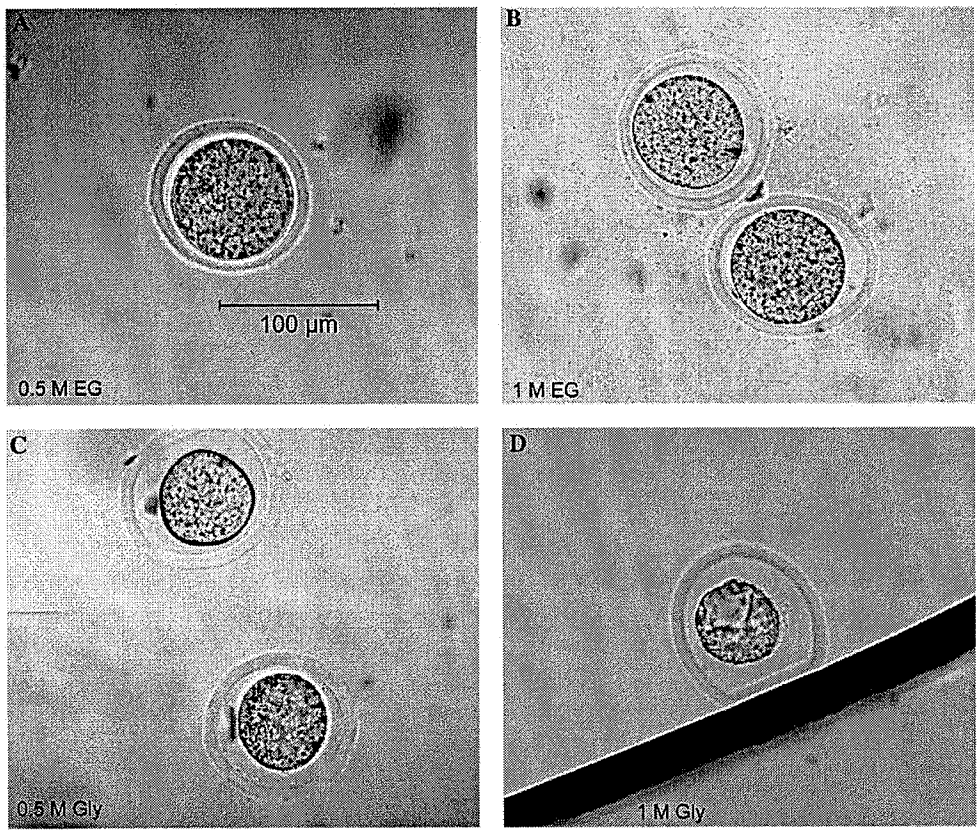

Fig. 2. Unfrozen mouse oocytes in PBS containing (A) 0.5 M EG, (B) 1.0 M EG, (C) 0.5 M glycerol, and (D) 1.0 M glycerol. In (D) note the flattening of the zona at the solution-air interface.

with respect to internal CPA concentration is approached much more rapidly than is equilibrium with respect to cell volume [18, Fig. 7]. In contrast to EG, oocytes in glycerol shrink and remain shrunken, a reflection of their poor permeability to that solute. Oocytes in 1 M glycerol/PBS, as expected, are more shrunken than those in 0.5 M glycerol.

The oocytes are surrounded by a hyaline zona pellucida. It exhibits no osmotic excursions indicating that it is highly permeable to both EG and glycerol. On the other hand, its mechanical rigidity is low as depicted in Fig. 2D, which shows the zona flattened by surface tension forces at a solution–air interface.

Ramps

Table 2 shows the protocol used in most experiments on oocytes frozen in 1 M EG or glycerol. It consists of six ramps. The lower limit for Ramp 2 was set to −8 °C, about a degree below the expected occurrence of extracellular freezing. A unique aspect of our protocols was the incorporation of Ramp 3. It involved the warming of the extracellularly frozen samples to just below the melting point. This had two purposes. First as illustrated in Fig. 3C, most of the external ice melted by the time the upper temperature limit was attained, and the resulting elimination of most of the obscuration by it permitted us to discern the appearance of the oocytes after being subjected to the external freezing. Second, because most of the external ice melted, the chemical potentials of water inside and outside the oocyte were nearly equal at the start of the second and final cool in Ramps 4 and 5. In other words, the oocytes were minimally supercooled at this point. Ramps 1 and 4–6 remained essentially the same for all the concentrations of glycerol and EG studied, but the limits for Ramps 2 and 3 depended on the solution, as summarized in Table 3. This table also depicts the mean observed temperature of extracellular ice formation (EIF) and the calculated thermodynamic melting points of the solutions. The latter were calculated as −1.855× the molality of the CPA minus the 0.6 °C melting point depression of isotonic PBS (1.855 is the so-called molal freezing point constant). The molalities of CPA are given in Table 1.

Most of the experiments used a cooling rate of 20 °C/min in Ramp 5, but some involved lower rates (10 or 5 °C/min), or higher (50 °C/min).

Table 2
Linkam cryostage cooling and warming ramps for oocytes frozen in 1 M ethylene glycol or glycerol

Ramp No.	Rate (°C/min)	Limit (°C)	Capture interval (s)	Comments
1	10	−5.0	30	Cooling
2	2	−8.0	10	Cooling; EIF[a]
3	2	−3.2	10	Warming; partial thawing
4	10	−7.0	10	Cooling
5	20	−50.0	10	Cooling; IIF[a]
6	10	+20.0	30	Warming and thawing

With the exception of the limit temperatures in Ramps 2–3 (see Table 3), most of the other entries were the same or similar in other concentrations of CPA.

[a] EIF and IIF refer to extracellular and intracellular ice formation, respectively.

Extracellular freezing

Mean EIF occurred when the solutions were supercooled 3.3 °C (PBS), 3.9–4.3 °C (EG), and 4.5 °C (glycerol) below the thermodynamic freezing (melting) points (Table 3). The relatively small amount of supercooling was a consequence of the presence of catalytic quantities of freeze-dried ice-nucleating bacteria (Snomax). (In three runs with PBS lacking Snomax, EIF occurred at −11 to −12 °C; i.e., about 11 °C of supercooling).

EIF was manifested by dendritic spears of ice apparently being projected at high rate (~800 μm/s) across the field of view (Fig. 3B). Projected spears are of course illusory; rather, they are akin to the propagation of a crack in window glass. The initial spears exert no evident force on the oocytes other than a small shift or rotation of the eggs as they are displaced slightly by the developing ice phase.

Immediately thereafter, the thickening extracellular ice in Ramp 2 mostly or totally obscures the oocytes. The obscuration was greater with increasing concentrations of CPA.

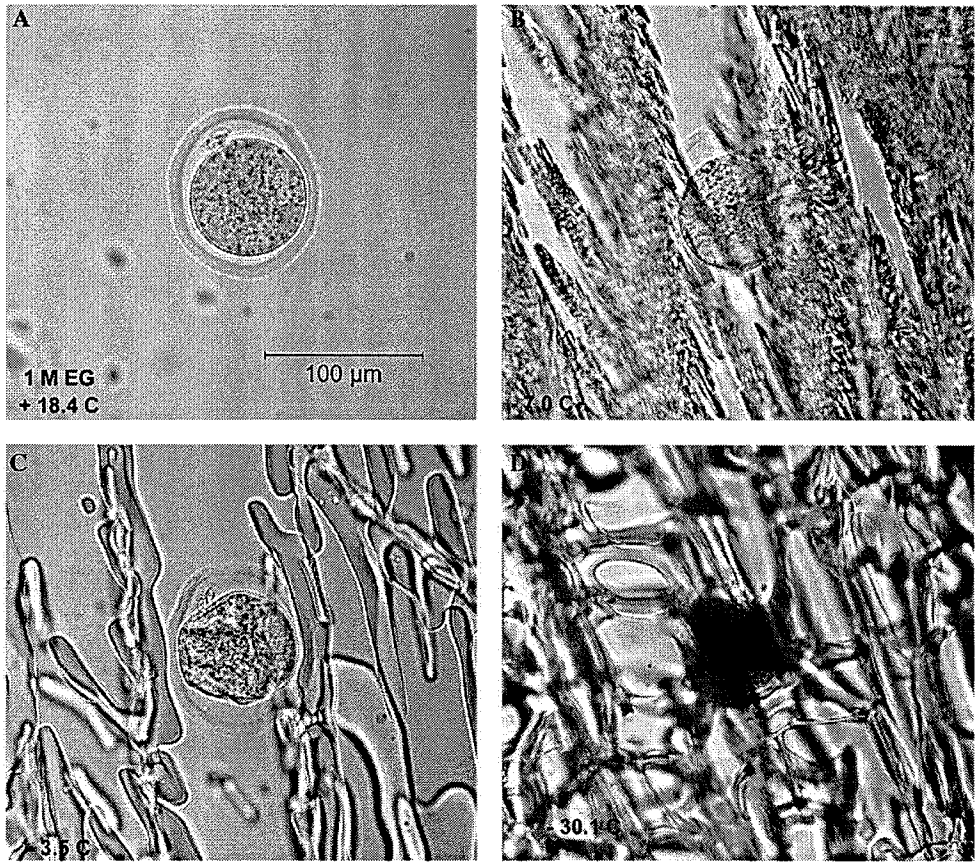

Fig. 3. A sequence of photomicrographs of an oocyte in 1 M EG/PBS subjected to freezing using the ramps described in Table 2. (A) Unfrozen, (B) EIF in Ramp 2, (C) after warming and nearly complete melting of external ice in Ramp 3, and (D) flashing at −30 °C in Ramp 5.

Oocyte distortion from EIF

The oocytes become visible again at the end of the transient warming in Ramp 3 when most of the external ice has melted. At this point, both they and the surrounding zona pellucida are distorted, sometimes severely so. Examples are shown in Figs. 3C and 4. In one run, a bubble about the size of an adjoining oocyte formed at the end of Ramp 3. Fig. 5 shows the flattening and elongation of that bubble with the regrowth of the external ice as the temperature fell from −1 to −5 °C in Ramp 4.

The distortion is prima facie evidence that the growing external ice phase (as opposed to the initial "spears") does exert a physical anisotropic force on both the oocytes and the zona (and the air bubble), presumably as a consequence of the narrowing of the unfrozen channels in which the oocytes lie. Some osmotic shrinkage does occur between the onset of EIF and the end of Ramp 3 (we calculate that they lose about 29% of their water based on Mazur et al.'s [20] equations and published values for mouse oocyte water permeability and its activation energy). The relevant parameters are: oocyte diameter—75 μm; V_b (solids fraction in isotonic cell)—0.2; hydraulic conductivity at 20 °C (L_p)—0.2 μm/min/atm; activation energy of L_p (E_a)—12 kcal/mole (see [19, p. 24]). However, pure osmotic shrinkage, as, for example, that was produced by hyperosmotic glycerol (Figs. 2C and D), does not produce shape distortion in mouse oocytes as discussed here.

In the presence of EG or glycerol, at least, the distortion of the oocyte by the external ice does not appear to affect its plasma membrane integrity,

Table 3
Limits for initial cooling in Linkam cryostage Ramp 2 and warming in Ramp 3, and observed EIF and supercooling in the various concentrations of freezing solutions

Medium (supercooling at EIF (°C)[b])	Ramp 2 limit (°C)	Observed EIF (°C)	Ramp 3 limit (°C)	Calc. MP (°C)
PBS (3.3)	−5.2	−3.9 ± 0.1	−0.2	−0.6
0.34 M EG[a] (4.1)	−6.0	−5.3 ± 0.1	−1.3	−1.2
0.5 M EG (3.9)	−6.0	−5.4 ± 0.2	−1.7	−1.5
0.5 M Gly (4.6)	−7.0	−6.1 ± 0.1	−2.0	−1.5
0.63 M EG (4.0)	−6.2	−5.8 ± 0.1	−2.3	−1.8
0.73 M EG (4.1)	−6.5	−6.1 ± 0.04	−2.5	−2.0
1 M EG (4.3)	−8.0	−6.8 ± 0.1	−3.2	−2.5
1 M Gly (4.5)	−8.0	−7.1 ± 0.1	−3.2	−2.6
1.5 M EG (4.2)	−10.0	−7.8 ± 0.1	−4.2	−3.6

[a] All CPAs in isotonic PBS.
[b] Supercooling was minimized by the presence of Snomax (10 mg/L)).

and it is morphologically reversible. The latter was shown in several cases by allowing oocytes at the end of Ramp 3 to continue warming towards room temperature. After about 5 min of warming and reaching ~+10 °C, all distortions of both oocyte and zona disappeared, and the appearances of the oocytes were indistinguishable from their appearance before EIF. Plasma membrane integrity was manifested by the fact that intracellular ice formation (IIF) as evidenced by "flashing" did not occur until well into Ramp 5 at temperatures far below EIF. In the region between EIF and IIF, the water in the oocytes had to be supercooled and the only way for supercooled water to co-exist with surrounding ice is to be separated from that ice by an intact plasma membrane.

In the case of about half the oocytes frozen in PBS, the EIF and/or the consequent distortion did disrupt or damage the plasma membrane as evidenced by either the total lack of flashing (which we interpret as an inability to supercool) or by flashing at temperatures very close to the EIF temperature. We elaborate on these points in the next section.

Intracellular ice formation

The third column of Table 4 summarizes the temperatures at which the oocytes underwent IIF (flashing) as a function of the CPA present [none (i.e., PBS only), glycerol, and EG] and its concentration (0.34–1.5 M). There is a marked decrease in the mean flashing temperature with increased CPA concentration; namely, from −13.9 °C in PBS alone to −41 °C in 1 M glycerol/PBS and 1.5 M EG/PBS. A given concentration of glycerol was somewhat more effective than an equal molar concentration of EG in depressing the IIF temperature. Thus, in 0.5 M glycerol and EG, the flash temperatures were −30.8 and −23.4 °C; in 1.0 M solutions, the flash temperatures were −41.3 and −37.2 °C, respectively. Perhaps the difference is

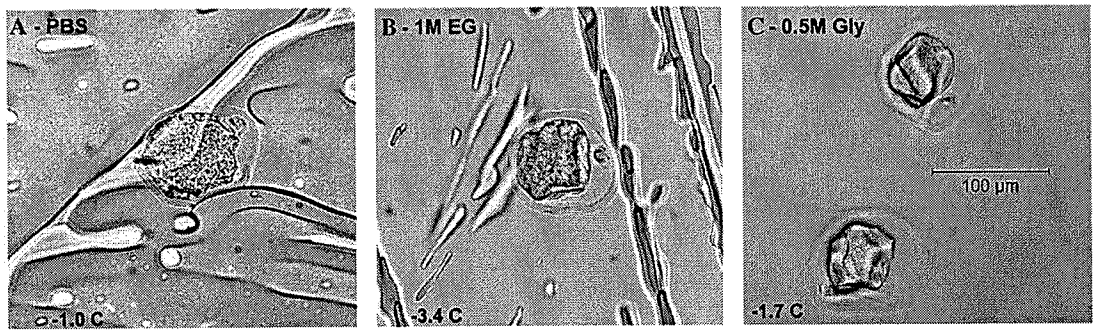

Fig. 4. Further examples of the distortion of oocytes and zonae after EIF and warming in Ramp 3. (A) In PBS (which also contained Cytochalasin B), (B) 1 M EG/PBS, and (C) 0.5 M glycerol/PBS.

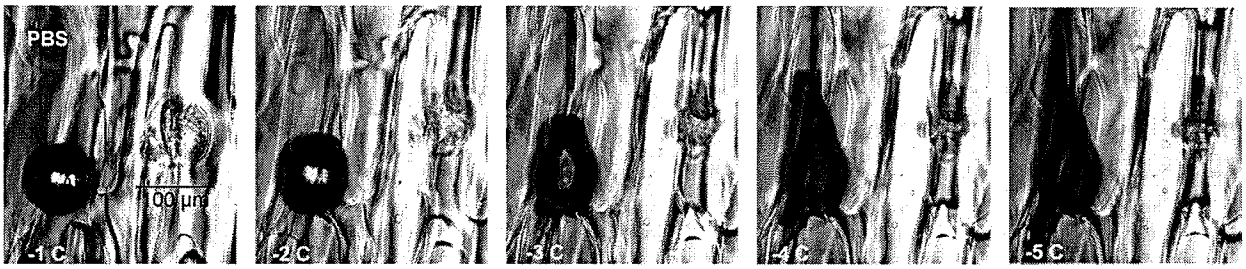

Fig. 5. Compression of a nearby air bubble during the freezing in Ramp 4 of an oocyte in PBS.

causally related to the fact that in glycerol, to which mouse oocytes are effectively impermeable, the cells were substantially shrunken prior to cooling; whereas, in EG, to which the oocytes are highly permeable, the cells were at or near full isotonic volume prior to the initiation of cooling.

The mean value for IIF of oocytes in PBS alone (−13.9 °C) is somewhat misleading. Of the 15 oocytes examined, six underwent no flashing and the mean is based on the nine that did flash. Our presumption is that the plasma membranes of the six that did not flash were sufficiently damaged during the initial extracellular freezing in Ramps 2 and 3 that they were unable to maintain the oocyte protoplasm in the supercooled state; i.e., the extracellular ice grew into the oocyte interior upon initial contact. Even among the nine that did flash, the flashing temperatures did not appear normally distributed. Three flashed between −4 and −5.1 °C, with a time delay after EIF (two actually flashed during the initial warming of Ramp 3).Two flashed at −12.3 and −13.8 °C. And four flashed between −18.6 and −25 °C. With respect to this last group, we note, without necessarily implying causation, that the equilibrium eutectic point of NaCl/water is −21.1 °C; that of PBS will be slightly lower).

This potential skewing and non-normal distribution was much reduced in oocytes frozen in the presence of CPA. In 0.5 and 1.0 M glycerol, the fractions that failed to flash were 2/17 and 1/8, respectively. In 0.34, 0.5, 0.63, 0.73, 1.0, and 1.5 M EG, the fractions that failed to flash were 0/15, 0/8, 0/8, 0/8, 3/37, and 0/14, respectively. Moreover, the distribution of IIF temperatures in the group that flashed is continuous.

Effect of cytochalasin B

In the previous section, we described the deformation of oocytes that occurs as a result of EIF. One possibility is that this deformation is responsi-

Table 4
Effect of CPA type and concentration on the flash temperature of mouse oocytes and the characteristics of the unfrozen channels at the flash temperature

CPA	Molarity	Flash temperature (°C)	n	L at flash	U at flash	$\sim L_v$	R	R'	m_s at flash	m_{CPA} at flash
None	0	−13.9 ± 2.4	9	0.07 ± 0.014	0.06 ± 0.014	0.06	0	0	3.6 ± 0.6	0
Glycerol	0.5	−30.8 ± 3.4	15	0.11 ± 0.009	0.06 ± 0.009	0.08	5.43	3.44	3.1 ± 0.4	10.7
	1.0	−41.3 ± 2.5	7	0.16 ± 0.005	0.06 ± 0.006	0.12	11.3	7.14	2.4 ± 0.2	17.4
EG	0.34	−25.3 ± 2.0	15	0.11 ± 0.01	0.09 ± 0.01	0.093	2.51	2.36	2.0 ± 0.2	4.6
	0.5	−23.4 ± 3.7	8	0.16 ± 0.019	0.13 ± 0.019	0.13	3.62	3.41	1.4 ± 0.25	4.8
	0.63	−25.4 ± 3.8	8	0.20 ± 0.03	0.16 ± 0.03	0.14	4.72	4.44	1.2 ± 0.2	5.4
	0.73	−30.0 ± 1.5	8	0.16 ± 0.01	0.11 ± 0.01	0.14	5.50	5.18	1.3 ± 0.1	6.7
	1	−37.2 ± 1.4	34	0.18 ± 0.01	0.12 ± 0.01	0.16	7.45	7.01	1.3 ± 0.06	9.2
	1.5	−40.8 ± 1.7	14	0.24 ± 0.01	0.15 ± 0.01	0.21	11.51	10.83	1.0 ± 0.05	10.9

L and L_v are the fractional mass and volume of the solutions that remain unfrozen; U is the fractional mass of unfrozen water L, U, and m_s values were calculated for each flash temperature, averaged, and the standard deviations of the mean calculated. R is wt% CPA/wt% salts. R' is mole CPA/mole salts.

ble for the large non-flashing population in oocytes frozen in PBS alone, and that deformation or its injurious consequences may be related to rigidity of the cytoskeleton. If so, then cytochalasin B, which disrupts microfilaments, might reduce the deformation or its injurious consequences. That appears at best marginally so. The flashing temperatures of five cytochalasin-treated oocytes frozen in PBS (-15.3 ± 5.9 °C) were lower but not statistically different ($p = 0.8$) from those not so treated (-13.9 °C). The corresponding flash temperatures for treated ($n = 9$) and untreated oocytes frozen in 0.5 M EG were -27.5 ± 2.6 and -23.4 °C ($p = 0.4$).

Effect of cooling rate on IIF temperature

Several investigators have reported that the IIF temperatures of mammalian oocytes and embryos rise with increasing cooling rate [15,13,26,35,34]. However, as shown in Table 5, we find that not to be the case in mouse oocytes frozen in 1 M EG/PBS over a 10-fold range of cooling rates (5–50 °C/min). The differences in flash temperature are small and there is no trend. [In addition to the 16 runs on control oocytes cooled at 20 °C/min, we have made another 12 runs using oocytes injected with 4 pl water prior to vitrification (these serve as controls for aquaporin studies to be reported on subsequently). The mean flash temperature of these 12 water-injected controls was -40.4 °C.) Since cooling rate did not significantly affect the flash temperature, the results for these four cooling rates have been incorporated into the mean value in Table 4 for oocytes in 1 M EG.

Table 5
Intracellular flashing temperature versus cooling rate

Solution	Cooling rate[b] (°C/min)	Flash temperature (°C)	n
1 M EG[a]	5	-42.3 ± 4.0	4
	10	-37.2 ± 2.2	11
	20	-35.4 ± 2.2	16
		(-37.5 ± 1.5)[c]	(28)
	50	-40.6 ± 1.0	3

[a] Ethylene glycol dissolved in isotonic PBS.
[b] Cooling rate for Ramp 5.
[c] Including 12 runs with water-injected controls.

Composition and physical characteristics of the unfrozen medium at the instant of flashing

As extracellular ice develops during cooling, water is progressively removed from the solution and transformed into ice. Cells are located in the unfrozen channels between the growing ice crystals. With lowered temperature, these channels diminish in size and the concentration of solutes in them increases. Both the fractions that remain unfrozen at the flash temperature and the concentration of salts and CPA in those channels can be calculated from phase diagrams. Columns 5–11 of Table 4 summarize these quantities. The data for glycerol/NaCl/water and EG/NaCl/water are calculated from equilibrium ternary phase relations published by Pegg [28] and Woods et al. [41], respectively. Pozner et al. [29] have shown that the phase relations of glycerol/physiological saline/water are essentially indistinguishable from those of glycerol/NaCl/water.

The procedure is as follows: the phase diagrams depict the freezing or melting points as a function of the total weight percent (W_T) of CPA + salt. The position of the curves (or isopleths) depends on R, the weight ratio of CPA/salt. Equations published by the two groups permit one to calculate W_T for a specified R and subzero temperature. For glycerol/NaCl/water solutions, Pegg's equation is

$$W_T = (a + (a^2 - 0.04T_{fl})^{1/2})/0.02,$$

where $1/a = -1.6 - 1.27R - 0.25R^2$ and T_{fl} is the flash temperature.

For EG, rearrangement of Woods et al.'s Eq. (3) yields

$$W_T = (-b - (b^2 - 4ac)^{1/2})/2a,$$

where $b = -0.676 + (4.77\text{E}{-}03)R$, $a = (-7.64\text{E}{-}03) + (-2.75\text{E}{-}05)R$, $c = -T_{fl}$.

From knowledge of R, W_T, and W_T^o, the total weight percent of CPA and salt in the solution prior to freezing (see Table 1), one can compute the values shown in Table 4. L is the weight fraction of the original solution that remains unfrozen at the specified flash temperature. It is calculated as W_T^o/W_T. U is the weight fraction of the water in the unfrozen solution that remains unfrozen at a

specified temperature. It is calculated from Eq. (7) of Rall et al. [31]; namely,

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

We see from Table 4 that L and U, the unfrozen fractions of solution and of water, respectively, at the flash temperature, fall within a fairly narrow range of low values even though the flash temperatures range from −13.9 to −41.3 °C. The values of L exceed those of U because the former includes the mass or volume occupied by the CPA and salt molecules. Consequently, the differences between the two measures increase as the concentration of CPA increases. The U value at flashing is identical for oocytes in PBS alone and oocytes in PBS containing glycerol; i.e., 0.06. Put differently, flashing occurs when 94% of the water is converted to ice. Most of the U value for oocytes in EG are about twice that for oocytes in glycerol; i.e., 0.09–0.16.

The quantity L is a mass fraction derived directly from the ternary phase diagrams. What is probably more relevant is L_v, the volume fraction that is unfrozen; i.e., the ratio of the volume of the unfrozen solution to the total volume of the sample. The volumes of given masses of the components depend on their partial molal volumes or densities, and these in turn are functions of temperature and the concentrations of the components. Exact calculations are formidable, but approximations sufficient for our purposes can be obtained as illustrated by the following numerical example for the freezing of 1 M glycerol in isotonic salt. Columns 2, 3, and 5 of Table 1 show that 100 g of that solution (R11-1X-G9) contains 8.966, 0.796, and 90.49 g of glycerol, salt, and water, respectively. At the mean flash temperature of −41.3 °C, U equals 0.063. The fractional mass of liquid water at the flash temperature is (initial wt% water) × U, or 5.70 g/100 g. The fractional mass of ice is (initial wt% water) × (1 − U), or 84.8 g/100 g. The masses of glycerol and salts in solution do not change during freezing and they correspond to 0.0974 and 0.0136 moles of glycerol and salts, respectively, per 100 g solution.

Converting these masses to volumes requires values of their partial molal volumes or densities. The density of water decreases from 0.9998 at 0 °C to 0.9839 at −30 °C (the lowest temperature available), or 0.0067%/°C [40]. Assuming the latter value, the volume of external water at IIF is 5.83 cm^3. The density of ice at −40 °C is 0.9228 ([6], Table 201). Consequently the volume occupied by the external ice at IIF is 91.9 cm^3.

The partial molal volume of glycerol varies with both concentration and temperature. At room temperature, it increases slightly from 71.35 cm^3/mole for 1 molal glycerol to 71.43 for 2 molal to 72.9 for pure glycerol. That for a 66.7 wt% (~20 molal) solution decreases by only 1.5% upon cooling from −5 to −40 °C [22]. Considering both factors we used a value of 71.4 cm^3/mole, on the basis of which, the glycerol occupies 6.95 cm^3 at the flash temperature.

The partial molal volume of NaCl in water increases appreciably with concentration and decreases appreciably with decreasing temperature. Akinfiev et al. [3] give data for concentrations up to 6 molal and for temperatures down to −15 °C. For a 3 molal solution, the partial molal volume is 15 cm^3/mole at −15 °C versus 19.7 at 20 °C. Assuming that it continues to decrease at still lower temperatures, we selected a value of 10. Assuming that value, the volume occupied by salt is 0.14 cm^3. This is such a small percentage of the total volume of the sample, that even large errors in the assumed value of the partial molal volume will have trivial consequences.

The total volume of the 100 g solution at −40 °C is the sum of the above, or 104.8 cm^3. And $L_v = (5.83 + 6.95 + 0.14)/104.8$ or 0.123.

The L_v values in EG/PBS solutions were obtained in a similar fashion. Data on the partial molar volume of EG as a function of concentration and temperature are given by Conde corporation [5]. For the temperatures and concentrations relevant here, they range from 51.3 to 51.7 cm^3/mole. We used a value of 51.5 cm^3/mole.

The quantities m_s and m_{CPA} are the molalities of salt and CPA in the unfrozen solution at the flash temperature. The former is calculated from Rall et al.'s [31] Eq. (5); namely

$$m_s = 1000W_s/[58.44(100 - W_T)],$$

where W_s, the weight of salts in solution at the flash temperature, equals $W_T/(1 + R)$.

The m_{CPA} is $R' \times m_s$, where R' is the mole ratio of CPA/salt. That mole ratio is assumed to remain unchanged as freezing progresses. The salts and CPA concentrate simultaneously because pure water is being pulled out of the solution and converted to ice. The molality of salts at the flash temperature varies nearly fourfold from 1.0 to 3.6 molal. The molality of CPA at the flash temperature varies nearly fourfold from 4.6 to 17.4 molal.

All the flash temperatures occur well above the eutectic points for the ternary system glycerol/NaCl/water. From Eqs. (5) and (6) of Pegg [28], the eutectic temperatures are −62.0 and −67.3 °C for 0.5 M glycerol and 1.0 M glycerol in isotonic saline. Theoretically, there is no liquid present below those temperatures. The eutectic point of EG/water is −51 °C [21]. We know of no data for EG/NaCl/water.

These findings and conclusions with respect to unfrozen fractions relate to the overall fraction of liquid in the sample as derived from the phase diagrams. We have no information on the microstructural distribution of that unfrozen fraction, and in particular what microstructural environment of ice and unfrozen medium a given oocyte "sees" at the instant of its flashing. On the other hand, the microenvironment that a given oocyte sees with respect to the concentrations of CPA and salt should be close to the global values derived from the phase diagrams.

Kinetics of IIF

Frame-by-frame analysis of the captured digital videotapes permitted an assessment of the kinetics of the flashing. Fig. 6A shows the elapsed time in seconds from the first indication of flashing to its midpoint as a function of the temperature at which IIF initiated. Fig. 6B shows the elapsed time to the completion of flashing versus temperature. The initiation was taken as the first frame in which an intracellular change was noted. The midpoint was taken as the time at which approximately half the oocyte had darkened or, more commonly, as the approximate time at which the entire oocyte has reached about half its ultimate darkness. Usually, as flashing reached its final stages, small chocolate-brown vacuole-like spaces filled in with

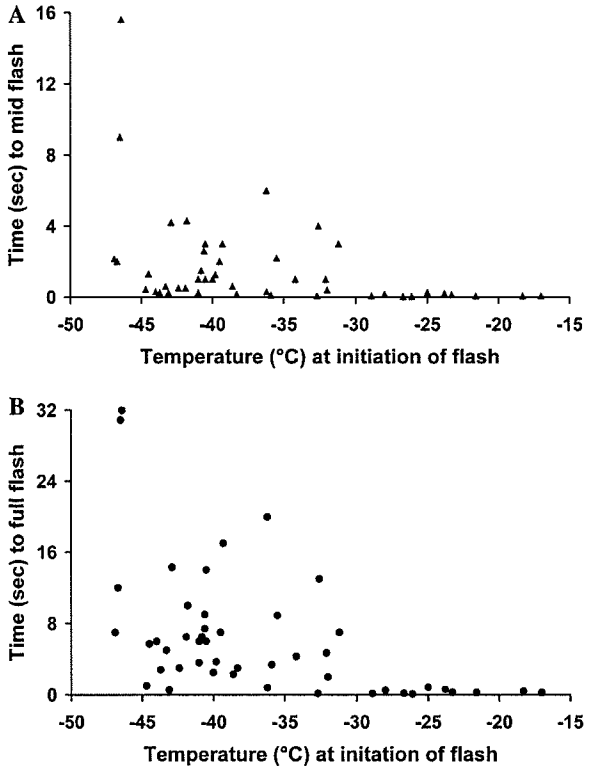

Fig. 6. The time taken for oocytes to darken during flashing as a function of the temperature at the initiation of the flash. (A) Seconds to mid-darkening, (B) Seconds to complete darkening.

black. The completion of flashing was taken to be the time at which that filling in was complete, and no further change in extension or degree of blackness could be observed. Generally, the midpoint was attained quickly relative to the time for completion of flashing.

The figure shows a dramatic change in the kinetics of flashing at ∼−30 °C. Above that temperature, the mean time to midpoint darkening (Fig. 6A) was 0.10 ± 0.03 s [range 0.03–0.2 s (note that 0.03 s represents 1 frame of video)]. Below that temperature, the mean time was 2.1 ± 0.5 s, about 20-fold longer, with a wide scatter (range 0.07–16 s). The times to the completion of flashing (Fig. 6B) showed a similar pattern. Above −30 °C, the mean time was 0.34 ± 0.10 s; below −30 °C, it was 7.6 ± 1.2 s, again a 20-fold difference and again a wide scatter.

In the majority of cases, initial IIF temperatures above −30 °C occurred in oocytes in 0.5 M EG,

and initial IIF temperatures below −30 °C occurred in oocytes in 1 or 1.5 M EG. But there were exceptions. In the 11 eggs in the >−30 °C group, 4 were in 1 or 1.5 M EG, and their times to midflashing were 0.03, 0.06, 0.06, and 0.16 s, as rapid as the other seven in 0.5 M EG. In the 39 oocytes in the <−30 °C group, eight were in 0.5 M EG, and their times to midflash ranged from 0.1 to 6 s, with a mean of 2.5 s, almost identical with the overall mean for that group (2.1 s). In other words, the striking dichotomy between the IIF kinetics above and below −30 °C appears to be a matter of the temperature at which IIF was initiated and not the concentration of EG inside or outside the oocytes.

Influence of the zona pellucida

The mouse oocyte, like those of all mammals, is surrounded by a non-living layer—the zona pellucida—a layer that is separated from the oocyte proper by the perivitelline space. All published cryobiological work on IIF to date has been on oocytes and embryos with zona pellucidas. An important question is whether the zona influences the process of extracellular and intracellular freezing, and, if so, how.

The first specific question is when EIF occurs, does the extracellular ice pass through the zona into the perivitelline space and into direct contact with the oocyte plasma membrane? While we can not answer the question by direct microscope observation because the oocyte is obscured by the EIF in Ramp 2, there is indirect evidence that it does penetrate. However, if external ice crystals do grow through the zona, they do not do so unimpeded. As mentioned previously, at the conclusion of the transient warming in Ramp 3, both the zona and the oocyte are distorted. The distortion of the zona means that a physical force is exerted on it by the external ice, and the only way that a physical force can be generated is for the zona to exert a resistance to the passage of ice.

The question still remains as to whether the zona absolutely blocks the passage of ice or merely impedes it transiently. If external ice were absolutely blocked by the zona, the solution in the perivitelline space, like that in the oocyte proper, would become increasingly supercooled with a progressive fall in temperature. And when the supercooling eventually terminated, both the perivitelline space and the oocyte ought to flash. This supposition was tested by measuring the diameter of the flashed image of oocytes frozen in glycerol and comparing it to the diameter of the unfrozen oocyte + perivitelline space.

The measurements were made on oocytes in glycerol because they are poorly permeable to glycerol and consequently, they are osmotically shrunken and exhibit a wide perivitelline space (Figs. 2C and D). Measurements on the flashed images of oocytes in 1 M glycerol/PBS show their diameters to be close to that of the unfrozen oocyte and *not* equal to that of the oocyte + perivitelline space; namely, the mean diameter of the oocytes prior to freezing was 54.0 ± 0.9 μm ($n = 10$) and that of the oocytes plus perivitelline space was 82.0 ± 1.2 μm. The mean diameter of the flashed images was 49.6 ± 4.4 μm, not significantly different from that of the oocytes proper prior to freezing ($p = 0.34$). In other words, the perivitelline space did not flash, indicating that it did not supercool. Lack of supercooling means that ice had to have penetrated the space.

IIF in dezonated oocytes

Another way to assess the role of the zona in IIF is to examine the nucleation behavior of dezonated oocytes. Dezonation was achieved by 20–80 s exposure to acidified Tyrode's solution (see Methods). The mean nucleation temperature of those in 1 M EG/PBS was the same as that for the oocytes with zona in that medium (-38.5 ± 1.4 °C vs −37.2 °C). But the nucleation behavior in 0.5 M EG/PBS and in PBS alone was decidedly different in two respects from that of their zonated counterparts. First the mean nucleation temperature was lower in the dezonated oocytes; namely, -35.2 ± 1.9 °C ($n = 8$) versus −23.4 °C in 0.5 M EG/PBS and -19.3 ± 2.9 °C ($n = 10$) versus −14 °C in PBS. The difference in the first pair is significant ($p = 0.04$); the difference in the second pair is not ($p = 0.17$). Second, the type of IIF was dramatically different between zonated and dezonated oocytes in these two med-

Table 6
Flash temperatures (IIF) and type of flashing in dezonated oocytes frozen at 20 °C/min in 1 M EG

Solution[a]	Fraction flashing		IIF w/o zona		IIF w/zona[b]	Warming range
	Black	Brown	Black (°C)	Brown (°C)	Black (°C)	Brown to black (°C)
PBS	3/10	7/10	−9.5	23.5	−13.9	−23 to −17
0.5 MEG	1/8	7/8	−22.4	35.2	−23.4	−34 to −30
1 M EG	8/8	0/8	−38.5	—	−37.2	—

[a] The EG was dissolved in PBS.
[b] From Table 4.

ia. In oocytes with zona, the conversion to deep black with only a few exceptions, always occurred during cooling and appeared homogeneous. No changes were observed during warming. That was also true of dezonated oocytes frozen in 1 M EG/PBS (Table 6). But it was not the case in most

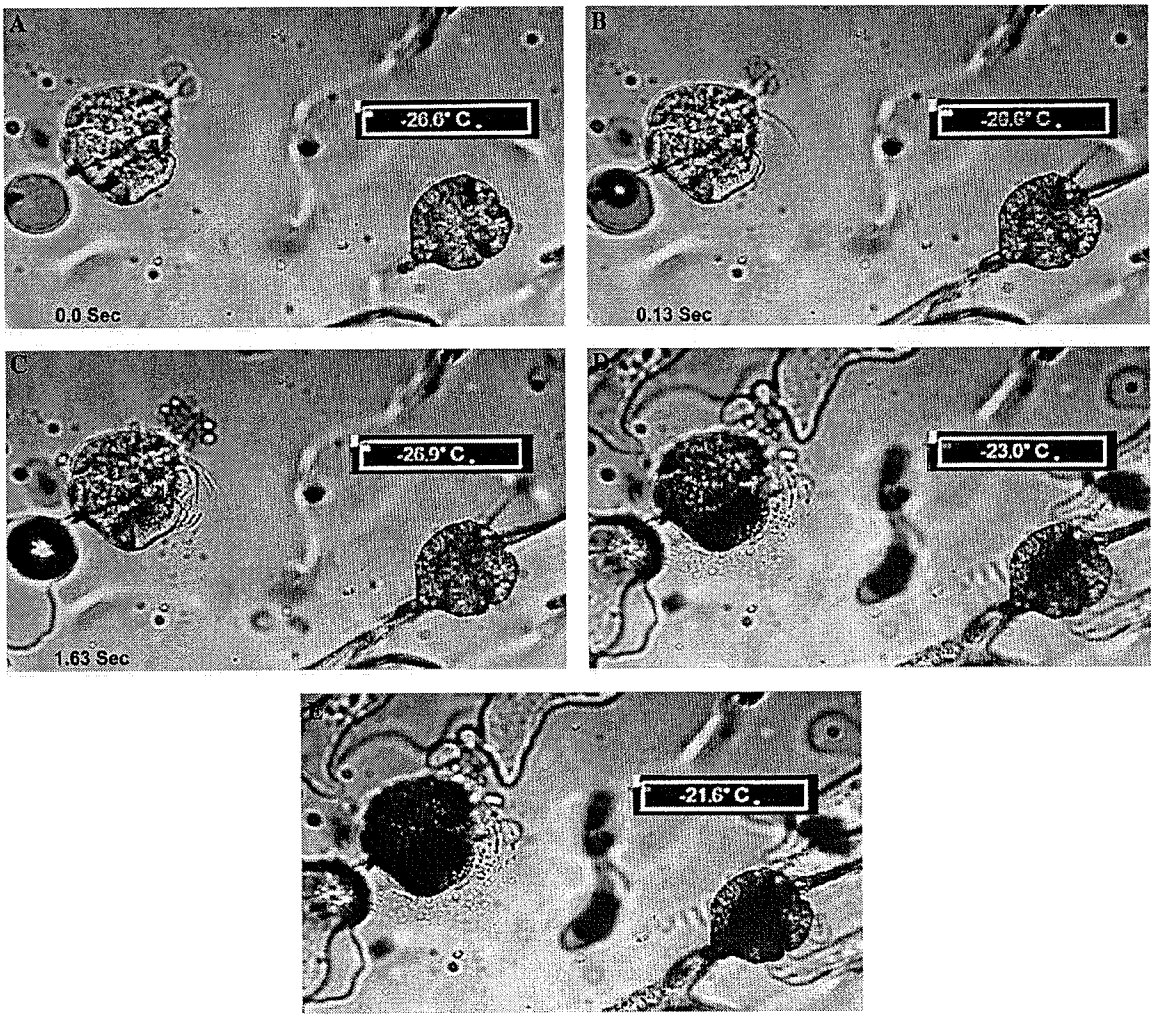

Fig. 7. A series of digital video micrographs during the freezing of two dezonated oocytes in PBS demonstrating (A) an initial pale chocolate brown flash, (B and C) expulsion of cytoplasm during cooling in Ramp 5, and (D) and (E) the oocyte becoming black during warming in Ramp 6. See text for further details.

of the dezonated oocytes frozen in PBS or 0.5 M EG/PBS. In most of those, intracellular nucleation was manifested by an abrupt (0.03 s) slight reduction in transparency followed swiftly by a pale brown granular sugary appearance. There was no further change during the remainder of the cooling, but during warming in Ramp 6, at close to the same temperature at which "browning" had occurred during cooling, the oocytes underwent a slow progressive blackening over an approximate 3–6 °C span. In PBS and 0.5 M EG, the temperature at which the oocytes flashed "brown" was 14 °C lower than the IIF temperature of those that flashed black. The specifics are noted in Table 6.

Fig. 7 is a digital video sequence of two dezonated oocytes during freezing in PBS. Fig. 7A shows their appearance at −26.6 °C during cooling Ramp 5. A light brownish "veil" had previously formed in the right-hand one at −14 °C. Fig. 7B was taken 0.13 s later than 7A, also at −26.6 °C. A light veil has formed in the left oocyte. The external medium is now traversed by a small crack above or below the z-axis of the right oocyte, and simultaneously the oocyte has become slightly browner. In Fig. 7C, 45 frames or 1.5 s later than 7B, the temperature has dropped to −26.9 °C. Both oocytes have become distinctly browner. In addition, a flower-like cluster has appeared at 2 O'clock on the left oocyte and plume-like extensions appear at 3 O'clock. Presumably they represent the abrupt expulsion of cytoplasmic material. No further change occurred as cooling continued to −50 °C, but during warming in Ramp 6, the left and right oocytes began to darken at ∼−23 °C (Fig. 7D) and turned black by −21.6 °C. (Fig. 7E).

Fig. 8 depicts another example of the abrupt formation of a pale brown flash in dezonated oo-

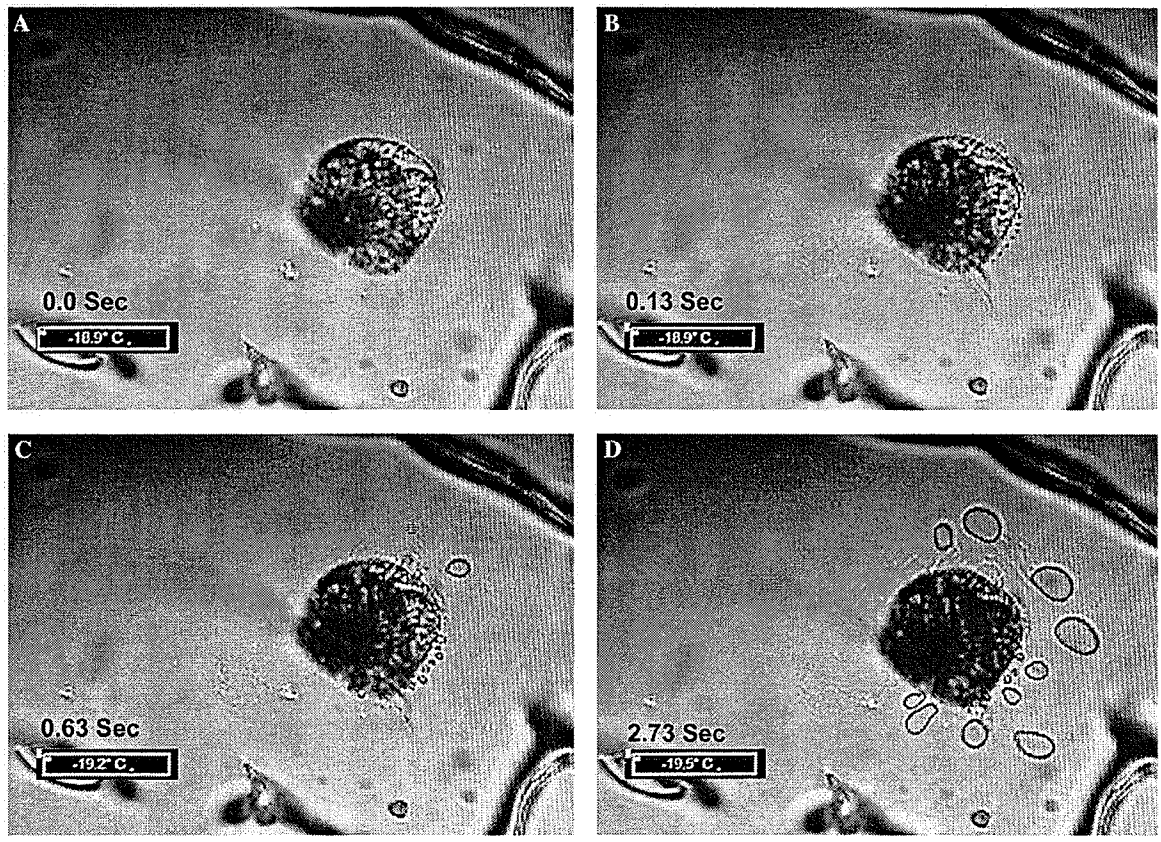

Fig. 8. A series of digital video micrographs during the freezing of a dezonated oocyte in PBS demonstrating a directional wave of browning and the expulsion of cytoplasm during cooling in Ramp 5.

cytes frozen in PBS. Fig. 8A shows its appearance at 0 time at −18.9 °C. Figs. 8B–D were captured 0.13, 0.6, and 2.7 s later at temperatures of −18.9, −19.2, and −19.5 °C and show the spread of brown through the cytoplasm. As in Fig. 7, this oocyte underwent no further change during cooling to −50 °C but turned black during warming in Ramp 6 between −21 and −16 °C. While not so in the great majority of zonated oocytes, we did observe a slow browning during cooling and blackening during warming in seven zonated oocytes, three frozen in 0.5 M EG and four in 1 M EG.

Homogeneous versus directional intracellular ice formation

Video recordings of flashing were made on half the runs on dezonated oocytes. In five recordings of those frozen in 1 M EG, all flashed homogeneously in the sense that no specific intracellular locus for the origin of IIF could be discerned. That was also the case for all of the 50 videotaped zonated oocytes. But that was not the case for dezonated oocytes frozen in PBS or 0.5 M EG. In 11 of these whose flashing was videotaped, five exhibited directional flashing between −14 and −35 °C of the "brown" type or exhibited jet-like extrusions of cytoplasm during flashing. For example, we have already noted the protuberances suddenly appearing at 2–3 O'clock on the outer surface of the left oocyte in Fig. 7. In Fig. 8, the intraoocyte browning occurs as a rapid wave from 8 O'clock to 2 O'clock and tiny bubbles appear at 1–5 O'clock (Fig. 8B), which rapidly enlarge (Figs. 8C and D). We presume they are bubbles of cytoplasm.

Double flashing

Two oocytes out of the many runs exhibited an interesting phenomenon; namely, they underwent a clear flashing in Ramp 3 at temperatures close to the EIF temperature, blackening that disappeared at the end of Ramp 3, and then underwent a second typical black flashing at much lower temperatures in Ramp 5 (namely, −17.3 °C for an oocyte in 0.5 M glycerol and −33.3 °C for the second one in 1 M glycerol). Since both were in glycerol, they were osmotically shrunken prior to the run and exhibited a wide perivitelline space. Two explanations come to mind. One is that the initial blackening represented IIF, in which case the fact that they flashed a second time would have to mean that the first IIF caused no damage to the plasma membrane or that the damage was reversed in Ramp 3. An alternative explanation is that the initial flashing represented ice formation in the wide perivitelline space, not in the oocyte proper.

Discussion

Effects of extracellular ice

Extracellular ice produces a physical distortion of both the oocyte proper and the surrounding zona. The distortion presumably results from the oocytes being forced to accommodate their shape to fit within the narrowing channels of liquid solution between the growing ice crystals. The exertion of a force on the zona is prima facie evidence that the growth of the ice through it is impeded. However, we have presented inferential evidence that the impedance is transient; namely, the fact that the perivitelline space (the space between the oocyte proper and the zona) does not supercool. We will further discuss this point later.

The distortion produced by EIF is reversible as evidenced by the fact that if the surrounding ice is allowed to melt, both the oocyte proper and the zona return to normal shape in 3 or 4 min. The distortion is not only reversible, but it appears innocuous to the great majority of oocytes frozen in media containing CPA. The evidence for this latter statement is, first, the cytological appearance of oocytes allowed to regain normal shape is normal, and, second, when they undergo the second cool in Ramps 4 and 5, they supercool extensively. The ability to supercool is evidence of an intact plasma membrane. And third, when they are osmotically shrunken in hyperosmotic concentrations of glycerol, they remain shrunken at the conclusion of warming in Ramp 3, again indicating an intact membrane impermeable to glycerol.

These statements apply to oocytes frozen in PBS containing EG or glycerol. However, in PBS alone, the EIF and the resulting distortion apparently causes enough membrane damage in many of the oocytes to preclude their flashing or to undergo flashing at temperatures very close to the EIF temperature. We interpret a lack of flashing to mean a lack of intracellular supercooling and lack of supercooling means that the plasma membrane has been sufficiently damaged to lose its barrier properties.

The question is what is responsible for the damage from EIF in PBS alone? One possibility is that damage results from the increase in external salt concentration accompanying EIF. In three oocytes in PBS that flashed at −4, −4.9, and −5.1 °C, the salt concentration (m_s) at flash was 1.1, 1.3, and 1.4 m, respectively. That seems too low to have caused membrane disruption chemically in view of the fact that many others in PBS and some in CPA remained unfrozen down to temperatures at which the external salt concentration rose to 2–3.6 molal. (Lower temperatures, however, may suppress their toxicity.)

An alternative possibility is that severe deformation itself caused sufficient damage to the plasma membranes to preclude subsequent deep supercooling. Our subjective impression is that oocytes frozen in PBS alone are more distorted at the end of Ramp 3 than those frozen in PBS + CPA. It is possible that sharp bends in the plasmalemma can produce weakened sites or defects that are more subject to penetration by external ice. Some support for this idea comes from Acker et al. [2] who have shown that IIF in mammalian cells flattened on glass occurs at a significantly higher temperature than it does when the same cells are in suspension and are spherical.

IIF

Our criterion of intracellular ice formation is the "flashing" or blackening of the oocyte. The strongest internal evidence that the blackened material is in fact intracellular ice is that the blackness diminishes and then vanishes as the sample temperature rises above ~−10 °C in the final warming phase of Ramp 6. In parallel studies that we are conducting with early *Xenopus* oocytes, we are able to correlate visual flashing observed by cryomicroscopy with the release of latent heat of fusion detected by differential scanning calorimetry. Presumably, the blackening results from the incident microscope light being reflected and refracted by many small highly branched crystals. Note, however, that the converse is not necessarily true; that is, a lack of flashing does not necessarily mean the absence of intracellular ice. It does mean, as indicated above, that internal ice in that case has formed with little or no supercooling of the cell.

The most obvious effect we have seen is a dramatic fall in flash temperature with increasing concentration of CPA. The mean flash temperature drops from −14 °C in PBS alone to −41 °C in 1 and 1.5 M EG. Glycerol appears somewhat more effective than EG in that respect; i.e., 0.5 M glycerol suppresses the flash temperature to about the same extent as 0.7 M EG (−30 °C) and 1 M glycerol suppresses it to the same extent as 1.5 M EG (−41 °C). One possible explanation for the difference is that oocytes in glycerol are considerably shrunken prior to and during cooling whereas those in EG are at or near isotonic volume. As indicated, the difference in volume arises from the difference in the permeability of the eggs to the two solutes.

We see no dependence of the IIF temperature on cooling rate over a 10-fold range from 5 to 50 °C/min. This disagrees somewhat with Leibo et al. [15] who report a slight decline in nucleation temperature with decreasing cooling rate over that approximate range. However, there could be a small effect of cooling rate which we could not discern because of the small number of replicates we used to test the matter.

Table 7 compares the flash temperatures versus CPA concentration that we found with the findings on mouse oocytes and 8-cell embryos reported by others. The agreement for cells in PBS and in PBS with 1 or 1.5 M CPA is good. The agreement in 0.5 M CPA is somewhat less good.

Rall et al. [30] have calculated the homogeneous ice nucleation temperature in objects the size of oocytes suspended in PBS containing 0, 0.5, 1.0, and 1.5 M CPA to be −37, −39, −41, and −43 °C (Zachariassan et al. [42] give temperatures

Table 7
Flash temperatures of mouse embryos and oocytes in various studies

CPA	Mean or median flash temperature (°C)			
	Rall et al. [30] (8-cell)	Leibo et al. [15] (oocytes)	Toner et al. [37] (oocytes)	This study (oocytes)
PBS	−12	—	−12.5	−13.9
0.5 M Gly	−14	—	—	−30.8
1.0 M Gly	−32	—	—	−41.3
1.5 M Gly	−30	—	—	—
0.5 M DMSO	−22	—	—	—
1.0 M DMSO	−33	−39	—	—
1.5 M DMSO	−34	—	—	—
0.5 M EG	—	—	—	−23.4
1.0 M EG	—	—	—	−37.2
1.5 M EG	—	—	—	−40.8

This study and the Rall et al. study used a cooling rate of 20 °C/min; Leibo et al. used 32 °C/min, and Toner et al. used 120 °C/min.

that are 3 °C higher). The low flash temperatures we observe for oocytes in 1 M glycerol and 1.5 M EG (−41 °C) are at their expected homogeneous nucleation temperature. The homogeneous nucleation temperature is the temperature at which enough water molecules aggregate spontaneously in a short time into an ice nucleus of sufficient size to be stable and grow.

Composition and physical characteristics of the medium at the flash temperature

There are two central questions with respect to IIF: (a) Is cell damage a consequence of IIF or is IIF a consequence of prior damage to the cell membrane? (b) If the latter, what is the source of the damage? If the former, why does IIF occur only when temperatures drop to low values?

Relevant to both questions may be the physical characteristics and chemical composition of the external medium at the flash temperature shown in columns 5–11 of Table 4. Columns 5–7 give three measures of the fraction of the solution that is unfrozen at the mean flash temperature. Columns 5 and 7 are the mass and volume fractions of the solution that are unfrozen (L and L_v); column 6 is the mass fraction of water that is unfrozen (U). For oocytes in PBS and glycerol/PBS, the U value is 0.06; i.e., it occurs when 94% of the water is frozen. In four of the six EG/PBS solutions, the U is equally tightly grouped, but has a value that is twice as high (0.12); i.e., IIF occurs when 88% of the water is frozen. The unfrozen fractions of the solutions (L and L_v) are larger than U because they include the mass and volume occupied by the CPA and PBS salts. Nevertheless, they are also tightly grouped. The L values in EG are still somewhat larger than those in glycerol, but by a considerably smaller margin.

A hypothesis to which we ascribe is that IIF requires intimate contact between external ice and the cell membrane. In that light, we would interpret the data to mean that intimate contact is achieved when about 90% of the surrounding water is frozen. The fact that the critical unfrozen fraction in glycerol is lower than in EG could be explained by the fact that the cells are smaller in the former. Others like Mugnano et al. [25], Berger and Uhrik [4], Larese et al. [11], and Köseoglu et al. [9] have also postulated (from microscopy observations) that IIF depends on intimate contact with external ice.

The decrease in unfrozen fraction with decreasing temperature comes about, of course, from the progressive transformation of water into ice. A consequence of that transfer is that the concentration of salts and CPA in the unfrozen fraction increases progressively. Indeed, there is a mirror-image relation between the molal salt concentration and U (see for example, Mazur [19, Fig. 1.11]). Thus, as shown in column 10 of Table 4, the low values of U at the instant of flashing are accompanied by increased salt molalities (m_s) in the external medium. The values of m_s at the flash temperature, however, are not as tightly constrained as the values of U; they range from

1.0 molal in 1.5 M EG/PBS to 3.6 molal in PBS alone.

As mentioned, U (or L) is a global quantity derived from the phase diagram. If IIF is related to intimacy of contact with extracellular ice, the intimacy of that contact at a given U will also depend on the microcrystalline ice structure that surrounds a given oocyte in a given run. We thus might expect a considerable range in the observed values of U for oocytes frozen in a given CPA/PBS solution. That is in fact the case. For example, in 0.5 M EG/PBS, the flashing in individual oocytes occurs at U ranging from 0.06 to 0.21. In 0.5 M glycerol/PBS, it occurs at U ranging from 0.03 to 0.12. Because of the mirror-image aspects, the observed m_s at flash also varies widely in individual runs from 0.73 to 2.6 molal in 0.5 M EG and from 1.2 to 4.9 molal in 0.5 M glycerol. However, the molalities of salt experienced by individual oocytes should not be affected by the microcrystalline structure of ice surrounding that oocyte; that is, the global values of m_s for a given CPA/PBS solution should apply. To us, it seems unlikely that the membranes of individual oocytes would differ that much in the concentration dependence of damage from salt concentration. Studies are in progress which will permit a degree of separation between U and m_s. At this point, we merely say that they also suggest that the former is more important to IIF than is the latter.

For the sake of completeness, the last column of Table 4 lists the molality of glycerol or EG at the mean flashing temperature. It is computed as $R' \cdot m_s$. It seems not to be playing a role in intracellular ice nucleation in that it varies from 0 (in PBS alone) to 17.4 molal at the observed mean IIF temperatures.

Evidence that the membranes and nucleation behavior of the vitrified oocytes are normal

As indicated in Methods, the oocytes used in this study were shipped to us from Dr. Edashige's laboratory in Japan in the vitrified state. Here, we summarize the evidence that the oocytes used in our cryomicroscopy experiments were normal with respect to plasma membrane intactness, osmotic properties, and IIF responses.

1. Upon thawing the vitrified samples, the oocytes are mixed with 0.5 M sucrose to act as an osmotic buffer during the efflux of the CPA mixture used in vitrification. The oocytes are expected to dehydrate during the sucrose exposure and then return to normal volume when subsequently placed in M16 medium. Most oocytes passed this test; those that did not were discarded.
2. Edashige et al. [7] have shown that a 1–2 h incubation of vitrified 2-cell mouse embryos in M16 medium following thawing results in the restoration of the ability of some 90% to withstand hypotonic challenge. Our procedure included this ~2-h incubation in M16 post-thaw. It distinguished the oocytes in a binary fashion: some 80–90% exhibited completely normal morphology; the other ~10–20% were clearly damaged and were discarded.
3. The unfrozen oocytes responded osmotically to permeating (EG) and non-permeating (glycerol) CPA qualitatively as expected; i.e., they exhibit normal volume in EG and are substantially shrunken in glycerol.
4. As has been mentioned, oocytes at the end of the warming in Ramp 3 are distorted. However, when they are allowed to warm further to above 0 °C, they recover normal shape and morphology in a few minutes. This reversibility again demonstrates normalcy of the plasma membrane.
5. In other experiments not reported here, oocytes were placed in EG containing 0.75 and 0.6× concentrations of PBS. Since cell volume depends on the concentration of the non-permeating solutes (here, PBS), the cells are expected to swell osmotically. They do so.
6. The fact that both zonated and dezonated oocytes supercool well below the EIF temperature before flashing indicates that the membrane is sufficiently intact to be a barrier to external ice. In dezonated oocytes, the membrane must be the barrier since the external ice has to come in close contact with it.
7. As just mentioned, the flash temperatures observed here for oocytes in glycerol and EG agree reasonably closely with the flash temperatures observed by Leibo et al. [15] in "fresh" mouse oocytes and by Rall et al. [30] in "fresh" 8-cell mouse embryos.

8. M. Kasai, Edashige's colleague at Kochi University has unpublished data showing that ICR mouse oocytes vitrified by the procedures used here undergo fertilization (as evidenced by development to 2-cell embryos) at the same rate as fresh oocytes. Lane and Gardner [10] made a detailed comparison of the functional viability of vitrified mouse oocytes and found that they were nearly identical with those of unfrozen oocytes. Their criteria of functional viability included percentage in vitro fertilization, percentage developing to blastocyst, number of cells in the blastocyst, percentage implanting, and percentage developing to fetuses. High in vitro fertilization rates in both cases required the removal or formation of a small hole in the zona. Others have reported similar findings for cryopreserved oocytes. Either the CPA or the cryopreservation procedures appear to cause zona hardening as a consequence of the release of cortical granules. While we do not know for certain that such hardening does not affect the subsequent intracellular ice nucleation, we note again the point made in [7] above that the IIF temperatures that we observe for zonated oocytes agree reasonably well with those reported by others.

Morphology and kinetics of flashing

In oocytes with zona we saw no instances of asymmetric flashing, by which we mean flashing that progressed out from a localized region of the oocyte. In the case of dezonated oocytes, however, we did see several such instances. Furthermore, in three instances we observed what appeared to be jets or bubbles of cytoplasm abruptly and forcefully expelled asymmetrically from the oocyte. Our inability to observe flashing asymmetry does not necessarily mean that it did not occur. First, the optics in the frozen sample are poor because of obscuration by the external ice. Consequently, a point origin of the ice may not have been visible. Second, we are essentially examining an optical midsection of a spheroid in the XY plane. Events could occur asymmetrically in the z-axis without our ability to distinguish them.

One aspect we could measure unambiguously is the time taken for flashing to progress. Above −30 °C, the midpoint of darkening was attained in ~0.1 s. Below that temperature it took about 20× longer on average, but with high variability. At the cooling rate of 20 °C/min in Ramp 5 used in the majority of experiments, the oocytes should have undergone very little shrinkage in that ramp (Fig. 1). This means that the intracellular concentration of EG at the instant of flash should have been fairly close to the intracellular concentration prior to the initiation of freezing. Consequently, as we indicated in Results, the time required for the progression of the flash was more a matter of temperature than a matter of the initial intracellular EG concentration. The simplest possibility is that the speed of flashing drops abruptly below −30 °C because the viscosity of EG/cytoplasm rises abruptly below that temperature. But that factor itself does not explain the high variability in flashing kinetics below −30 °C.

The influence of the zona pellucida on IIF

In Results and earlier in this discussion, we presented indirect evidence that the zona is a transient barrier to the passage of external ice. The evidence that it is a barrier is that the extracellular ice forming in Ramp 2 exerts a force on the zona sufficient to distort it, and such a force would not be exerted if the external ice were able to grow through it unimpeded. The evidence that it is a transient, not an absolute, barrier is that the perivitelline space (the space between the oocyte proper and the zona) does not flash which means that it does not supercool.

One would expect the zona not to be an absolute barrier to the passage of ice. It is perforated by large well-defined more-or-less cylindrical pores that are the remnants of cytoplasmic extensions from the surrounding cumulus cells. In the bovine oocyte, these pores have an average diameter of 182 nm on the outer surface of the zona and taper to slightly smaller diameters on the inner surface. There are some 1500 such pores per 5000 μm^2 of surface area [39]. We have found no comparable direct measurements for the pores in the zona of the mouse oocyte, but Vanroose et al. [39] report

that the mouse zona allows passage of an encephalitis virus with a diameter of 28 nm. Another measure of pore size is the relation between the size of a probing molecule and its ability to pass through the zona. Legge [12] reports that the zonae of mouse oocytes allow the ready permeation of fluorescently conjugated dextrans and lectins of up to 100 kDa. Still another more qualitative indication is that hyperosmotic solutions of sucrose (342 Da), for example, do not produce even transient osmotic shrinkage of the zona indicating that it is freely permeable to that solute. These pores almost certainly represent the channels through which grew cytoplasmic extensions of the surrounding cumulus cells, extensions that made contact and formed gap junctions with the oocyte plasma membrane. When the cumulus cells are removed, either purposefully as was the case here, or spontaneously as occurs later in normal development, these cytoplasmic extensions disappear or are withdrawn, leaving the empty pores.

Pore diameter can affect the ability of ice to transit them. If sufficiently narrow, above certain subzero temperatures, they can thermodynamically prevent the passage of ice. More precisely, if ice crystals attempt to grow through a pore, they have to assume a finite radius of curvature, and the smaller that radius, the higher is the surface energy of the crystal and lower is its thermodynamic melting point. The relation is given by the Kelvin equation [17,1]. However, such thermodynamic melting point suppression only becomes significant for pores of <20 Å in diameter. Here, where the zona pores are $\geqslant$1000 Å, the suppression should be at most a small fraction of a degree.

The role of the zona in IIF can also be assessed by examining flashing of dezonated oocytes. The mean flash temperature and flash kinetics of dezonated oocytes in 1 M EG/PBS was the same as that of their zonated counterparts, but that of most of the dezonated oocytes in 0.5 M EG/PBS and in PBS alone was quite different. The biggest difference was that in this majority, the flashing during cooling in Ramp 5 was observed as a slight but extremely abrupt (0.03 s) increase in oocyte opacity or as an abrupt pale sugary brown appearance. No further change occurred during the remainder of the cooling, but during warming in Ramp 6, the pale brown oocytes darkened to black over an interval of some 3–5 °C, beginning roughly at the temperature at which the pale flashing occurred during the prior cooling in Ramp 5.

This raises the following questions: (1) What does the initial abrupt pale brown flash signify? (2) What causes the blackening during warming? (3) Why do (1) and (2) occur only rarely in dezonated oocytes in 1 M EG and in zonated oocytes at all CPA concentrations? In these latter cases, flashing is manifested by blackening during the cooling phase of Ramp 5. We can only speculate. The speculation is that the pale brown flash is a manifestation of the conversion of the water in the protoplasm to a glass or to ice crystals sufficiently small to minimally reflect and refract light. Pursuing that thought, the blackening during warming in Ramp 6 would then be a manifestation either of the devitrification of a prior glassy state or the recrystallization of pre-exiting small intracellular crystals. (Recrystallization is the process by which small ice crystals, because of their higher surface energies, become converted to larger crystals.) Rall et al. [32] also observed cell blackening at $\sim$−55 °C during the slow warming of 8-cell mouse embryos that had been previously rapidly cooled from −42 to −150 °C. They, too, attribute this blackening to recrystallization. Although our interpretation (and theirs) seems to be a reasonable explanation for the sequence of pale brown flash during cooling followed by blackening during subsequent warming, it does not explain why the two phenomena occur at about the same temperature and it does not explain why they do not occur in zonated oocytes or in dezonated oocytes in 1 M EG.

Exogenous nucleation versus endogenous and homogeneous versus heterogeneous

In examining several hundred oocytes in this study, with one exception, we observed no case where IIF occurred in the absence of EIF. In other words, in all cases but one, the presence of external ice was a prerequisite for the occurrence of IIF. This is the conclusion reached as well by others (e.g., Berger and Uhrik [4]; Toner et al. [37]), In the one exception, the ice appeared to originate

within the oocyte and radiate out. But it is quite possible that it actually originated externally just above or just below the oocyte.

In the absence of foreign-nucleating agents, water in bulk volume nucleates at −35 °C. This is the homogeneous nucleation temperature, T_h. T_h decreases with decreasing volume of the water sample and it decreases with a lowering of the thermodynamic melting/freezing point of the solution by the presence of solutes. The rule-of-thumb is that the nucleation temperature is decreased by ~2× the suppression of the melting point [33]. When nucleation occurs above the homogeneous temperature, it is believed to be induced by foreign-nucleating agents and is referred to as heterogeneous nucleation.

We find that IIF in oocytes in 1 M glycerol and 1.5 M EG occurs at −41 °C. That means it is occurring by homogeneous nucleation and not as a result of a heterogeneous nucleator. A corollary conclusion would have to be that IIF at this temperature is not related to the presence of external ice. If it were, flashing should occur above the homogeneous nucleation temperature since ice is the most effective heterogeneous nucleator of all.

The converse argument also holds. When we see IIF occurring well above T_h as in PBS (IIF at −13.9 °C, T_h −37 °C), 0.5 M glycerol (IIF at −31 °C, T_h −39 °C), and 0.5 M EG (IIF at −23 °C, T_h −39 °C), we have to conclude that intracellular freezing occurs heterogeneously. The question remains, what is the heterogeneous nucleator? We submit that it is the external ice.

Relevance of present findings to theories of intracellular ice nucleation

Theories of nucleation fall into two major groups. One group postulates that intracellular ice forms as a consequence of damage or defects developing in the plasma membrane, defects that allow external ice to pass through the membrane. The other group postulates that ice external to a normal intact plasma membrane causes internal ice to form, and the plasma membrane and cell contents are damaged as a consequence of the ice formation. There are two versions of the latter concept. Mazur [17,19] has proposed that external ice grows through preexisting pores in the membrane. Toner et al. [36,38] have proposed that external ice causes a conformational change in the membrane which transforms it into an effective heterogeneous nucleator of the supercooled cell contents. Both hypotheses require that for IIF to occur, external ice crystals must be in intimate contact with the outer surface of the membrane.

The chief proponent of the first group (membrane damage precedes IIF) is Muldrew and McGann's [23,24] osmotic flux theory. As cells become increasingly supercooled during cooling, the driving force for water efflux from the cells increases. They propose that if that flux reaches a critical value, the high flux itself causes membrane defects. These defects, they postulate, then permit the passage of external ice and the initiation of intracellular freezing. We find it difficult to fit the findings in this study to their hypothesis. The driving force for water efflux depends on the difference between the chemical potential of supercooled water in the cell and the chemical potential of the external ice. Since in our study, the oocytes are cooled sufficiently rapidly to keep their internal composition close to constant, the difference in chemical potential is primarily a function of temperature. The rate of water efflux is a function of the chemical potential driving force and the hydraulic conductivity of the cell, which itself is temperature dependent. The type and concentration of CPA in which the oocytes are suspended should have only a relatively small effect on the kinetics of efflux (chiefly through a halving of L_p). Yet as we have seen, the IIF temperatures vary from −14 to −41 °C depending on the concentration of CPA. Obversely, the IIF temperature does correlate closely with the unfrozen fraction and possibly with the salt concentration in that unfrozen fraction; yet these factors are totally unrelated to the kinetics of water efflux.

Our findings do offer some support for one underlying postulate of the second group of theories; namely, that intimate contact between external ice and cell surface leads directly (Mazur) or indirectly (Toner) to the nucleation of intracellular supercooled water. The chief factual support is that IIF tends only to occur when >88% of the external water has been converted to ice. But the data shed no light on the validity of the specifics

of the two hypotheses; i.e., nucleation through pre-existing pores (Mazur) or nucleation via conformational changes in the plasma membrane brought about by contact with ice (Toner).

We see hints that IIF may be related to the deformation of the cell surface. Clearly, the cells become moderately to severely deformed when they are forced into the relatively large channels between external ice crystals forming at rather high subzero temperatures in Ramp 2. We can not see the cell surface during the rapid cooling in Ramp 5 where IIF occurs, but almost certainly the deforming forces must increase as the fraction unfrozen drops towards 10%. Conceivably, such deforming forces could produce structural changes in pre-existing pores in the plasma membrane or induce localized defects. This and other questions raised in this report remain to be resolved.

Acknowledgments

We appreciate the assistance of the following undergraduate and graduate students of Drs. Edashige and Kasai in collecting and vitrifying the oocytes sent to Knoxville: D. Valdez, M. Tanaka, T. Kuwano, K. Yazawa, M Futiwaki, S. Oota, Y. Hara, S. Takahashi, T. Kouya, K. Yamasaki, M. Ueda, and J. Bo.

References

[1] J.P. Acker, J.A.W. Elliot, L.E. McGann, Intracellular ice propagation: experimental evidence for ice growth through membrane pores, Biophys. J. 81 (2001) 1389–1397.

[2] J.P. Acker, A. Larese, H. Yang, A. Petrenko, L.E. McGann, Intracellular ice formation is affected by cell interactions, Cryobiology 38 (1999) 363–371.

[3] N.N. Akinfiev, M.V. Mironenko, S.A. Grant, Thermodynamic properties of NaCl solutions at subzero temperatures, J. Solution Chem. 30 (2001) 1065–1080.

[4] W.K. Berger, B. Uhrik, Freeze-induced shrinkage of individual cells and cell-to-cell propagation of intracellular ice in cell chains from salivary glands, Experientia 52 (1996) 843–850.

[5] M. Conde Engineering Thermophysical Properties of Brines (http://www.mrc-eng.com/Downloads/BrineProperties.pdf). Zurich, Switzerland, 2002

[6] N.E. Dorsey, Properties of ordinary water-substance, Reinhold Publishing Co., New York, 1940.

[7] K. Edashige, A. Asano, T.Z. An, M. Kasai, Restoration of resistance to osmotic swelling of vitrified mouse embryos by short-term culture, Cryobiology 38 (1999) 273–280.

[8] M. Hagedorn, A. Peterson, P. Mazur, F.W. Kleinhans, High ice-nucleation temperature of zebrafish embryos: slow-freezing is not an option, Cryobiology 49 (2004) 181–189.

[9] M. Köseoglu, A. Eroglu, M. Toner, K.C. Sadler, Starfish oocytes form intracellular ice at unusually high temperatures, Cryobiology 43 (2001) 248–259.

[10] M. Lane, D.K. Gardner, Vitrification of mouse oocytes using a nylon loop, Mol. Reprod. Dev. 58 (2001) 342–347.

[11] A. Larese, J. Acker, K. Muldrew, H. Yang, L. McGann, Antifreeze proteins induce intracellular nucleation, Cryo-Letters 17 (1996) 175–182.

[12] M. Legge, Oocyte and zygote zona pellucida permeability to macromolecules, J. Exp. Zool. 271 (1995) 145–150.

[13] S.P. Leibo, Cryobiology: preservation of mammalian embryos, in: J.W. Evans, A. Hollaender (Eds.), Genetic engineering of animals, Plenum Publishing Corp., NY, 1986, pp. 251–272.

[14] S.P. Leibo, P. Mazur, Methods for the preservation of mammalian embryos by freezing, in: J.J.C. Daniel (Ed.), Methods in mammalian reproduction, Academic Press, NY, 1978, pp. 179–201.

[15] S.P. Leibo, J.J. McGrath, E.G. Cravalho, Microscopic observations of intracellular ice formation in unfertilized mouse ova as a function of cooling rate, Cryobiology 15 (1978) 257–271.

[16] P. Mazur, Kinetics of water loss from cells at subzero temperatures and the likelihood of intracellular freezing, J. Gen. Physiol. 47 (1963) 347–369.

[17] P. Mazur, Physical and chemical basis of injury in single-celled micro-organisms subjected to freezing and thawing, in: H.T. Meryman (Ed.), Cryobiology, Academic Press, London, 1966, pp. 231–315.

[18] P. Mazur, Equilibrium, quasi-equilibrium, and non-equilibrium freezing of mammalian embryos, Cell Biophys. 17 (1990) 53–92.

[19] P. Mazur, Principles of cryobiology, in: N. Lane, B.J. Fuller, E.E. Benson (Eds.), Life in the frozen state, CRC Press, Boca Raton, 2004, pp. 3–65.

[20] P. Mazur, W.F. Rall, S.P. Leibo, Kinetics of water loss and the likelihood of intracellular freezing in mouse ova:Influence of the method of calculating the temperature dependence of water permeability, Cell Biophys. 6 (1984) 197–214.

[21] A. Melinder, Thermophysical Properties of Liquid Secondary Refrigerants IIR, 1997

[22] C.S. Miner, N.N. Dalton, Glycerol, Reinhold, New York, 1953.

[23] K. Muldrew, L.E. McGann, Mechanisms of intracellular ice formation, Biophys. J. 57 (1990) 525–532.

[24] K. Muldrew, L.E. McGann, The osmotic rupture hypothesis of intracellular freezing injury, Biophys. J. 66 (1994) 532–541.

[25] J.A. Mugnano, T. Wang, J.R. Layne Jr., A.L. DeVries, R.E. Lee, Antifreeze glycoproteins promote intracellular

freezing of cardiomyocytes at high subzero temperatures, Am. J. Physiol. 269 (1995) R474–R479.
[26] S.P. Myers, T.-T. Lin, R.E. Pitt, P.L. Steponkus, Cryobehavior of immature bovine oocytes, Cryo-Letters 8 (1987) 260–275.
[27] A. Nagy, M. Gertsenstein, K. Vintersten, R. Behringer, Manipulating the mouse embryo: a laboratory manual, third ed., Cold Spring Harbor Laboratory Press, Woodbury, NY, 2003.
[28] D.E. Pegg, Simple equations for obtaining melting points and eutectic temperatures for the ternary system glycerol/sodium chloride/water, Cryo-Letters 4 (1983) 259–268.
[29] R.I. Pozner, M.L. Shepard, F.H. Cocks, The equilibrium and non-equilibrium thermal behavior of aqueous ternary solutions based on complex physiological support media, containing NaCl, and dimethyl sulfoxide or glycerol, J. Mater. Sci. 12 (1977) 299–304.
[30] W.F. Rall, P. Mazur, J.J. McGrath, Depression of the ice-nucleation temperature of rapidly cooled mouse embryos by glycerol and dimethyl sulfoxide, Biophys. J. 41 (1983) 1–12.
[31] W.F. Rall, P. Mazur, H. Souzu, Physical-chemical basis of the protection of slowly frozen human erythrocytes by glycerol, Biophys. J. 23 (1978) 101–120.
[32] W.F. Rall, D.S. Reid, C. Polge, Analysis of slow-freezing injury of mouse embryos by cryomicroscopical and physiochemical methods, Cryobiology 21 (1984) 106–121.
[33] D. Rasmussen, A.P. MacKenzie, Effects of solute on the ice-solution interfacial free energy; calculation from measured homogeneous nucleation temperatures, in: H.H.G. Jellinek (Ed.), Water structure at the water–polymer interface, Plenum Press, NY, 1972, pp. 126–145.
[34] N.A. Ruffing, P.L. Steponkus, R.E. Pitt, J.E. Parks, Osmometric behavior, hydraulic conductivity, and incidence of intracellular ice formation in bovine oocytes at different developmental stages, Cryobiology (1993) 562–580.
[35] M. Shabana, J.J. McGrath, Cryomicroscope investigation and thermodynamic modeling of the freezing of unfertilized hamster ova, Cryobiology 25 (1988) 338–354.
[36] M. Toner, E.G. Cravalho, M. Karel, Thermodynamics and kinetics of intracellular ice formation during freezing of biological cells, J. Appl. Phys. 67 (1990) 1582–1593.
[37] M. Toner, E.G. Cravalho, M. Karel, D.R. Armanti, Cryomicroscopic analysis of intracellular ice formation during freezing of mouse oocytes with cryoadditives, Cryobiology 28 (1991) 55–71.
[38] M. Toner, E.G. Cravalho, J. Stacheki, T. Fitzgerald, R.G. Tomkins, Nonequilibrium freezing of one-cell mouse embryos. Membrane integrity and developmental potential, Biophys. J. 64 (1993) 1908–1920.
[39] G. Vanroose, H. Nauwynck, A.V. Soom, M.-T. Ysebaert, G. Charlier, P.V. Oostveldt, A.D. Kruif, Structural aspects of the zona pellucida in invitro-produced bovine embryos: a scanning electron and confocal laser scanning microscopic study, Biol. Reprod. 62 (2000) 463–469.
[40] R.C. Weast, Handbook of chemistry and physics, 55th ed., CRC Press, Cleveland, OH, 1974.
[41] E.J. Woods, M.A.J. Zieger, D.Y. Gao, J.K. Critser, Equations for obtaining melting points for the ternary system ethylene glycol/sodium chloride/water and their application to cryopreservation, Cryobiology 38 (1999) 403–407.
[42] K.E. Zachariassen, E. Kristiansen, S.A. Pedersen, H.T. Hammel, Ice nucleation in solutions and freeze-avoiding insects—homogeneous or heterogeneous? Cryobiology 48 (2004) 309–321.