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Kinetics and activation energy of recrystallization of intracellular ice in mouse oocytes subjected to interrupted rapid cooling✧

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Abstract

Intracellular ice formation (IIF) is almost invariably lethal. In most cases, it results from the too rapid cooling of cells to below -40°C , but in some cases it is manifested, not during cooling, but during warming when cell water that vitrified during cooling first devitrifies and then recrystallizes during warming. Recently, Mazur et al. [*Cryobiol.* 55 (2007) 158] dealt with one such case in mouse oocytes. It involved rapidly cooling the oocytes to -25°C , holding them 10 min, rapidly cooling them to -70°C , and warming them slowly until thawed. No IIF occurred during cooling but intracellular freezing, as evidenced by blackening of the cells, became detectable at -56°C during warming and was complete by -46°C . The present study differs in that the oocytes were warmed rapidly from -70°C to temperatures between -65°C and -50°C and held for 3 to 60 min. This permitted us to determine the rate of blackening as function of temperature. That in turn allowed us to calculate the activation energy (E_a) for the blackening process; namely, 27.5 kcal/mole. This translates to about a quadrupling of the blackening rate for every 5° rise in temperature. These data then allowed us to compute the degree of blackening as a function of temperature for oocytes warmed at rates ranging from 10 to 10,000 $^{\circ}\text{C}/\text{min}$. A 10-fold increase in warming rate increased the temperature at which a given degree of blackening occurred by 8°C . These findings have significant implications both for cryobiology and cryo-electron microscopy.

Keywords

Ice; intracellular; recrystallization; activation energy; oocytes; mouse

The major cause of death in cells subjected to freezing is the formation of intracellular ice (IIF). Or put differently, to survive freezing, a cell must be cryopreserved in ways that avoid or minimize IIF. In a recent study, Mazur et al. [13] investigated intracellular freezing in mouse oocytes subjected to an unorthodox cooling regime referred to as interrupted rapid cooling. In this procedure, cells are cooled rapidly to a temperature above that at which intracellular ice can form, held various times at that temperature, and then cooled rapidly to -70°C or below. The rationale is the following: Since after the initial rapid cool, the cells are unfrozen, they are by definition supercooled, and because their water is supercooled, it will leave the cells osmotically in response to the lower chemical potential outside. If they are held at this intermediate temperature for sufficient time, they will lose most of their freezable water and

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consequently will not undergo IIF when subjected to the second rapid cool to -70°C . The point of the previous study was to determine how IIF in the oocytes responded to the second rapid cool to -70°C when it was preceded by hold times ranging from 5 to 40 min at -25°C .

We divided the responses into five classes.

- ○ Classes 1 and 2 exhibited abrupt black flashing during the cooling steps.
- ○ Class 3 oocytes underwent only limited darkening during cooling, but completed it during warming.
- ○ Class 4 showed no darkening or flashing during cooling, but blackened during warming
- ○ Class 5 showed no darkening during either cooling or warming. They were highly crenated immediately after thawing, but regained normal morphology after return to $+20^{\circ}\text{C}$. Their membranes were osmotically intact.

There was a progressive shift from Classes 1 and 2 to Class 5 as the holding time at -25°C was increased from 5 to 30 min. The percentage that were Class 4 was maximal with a holding time of 10 min, and it was this class that was of interest in the present study. The reason for our interest is that they represent a case where IIF becomes optically manifested only during warming. Presumably, this blackening is either a consequence of the devitrification of a glassy cell solution formed during cooling or it is a consequence of recrystallization of small ice crystals formed during cooling. Recrystallization is the conversion of small ice crystals into large ones as a result of differences in surface free energy.

In our previous study, the oocytes were warmed at $10^{\circ}\text{C}/\text{min}$, and we observed with high reproducibility that blackening of the Class 4 oocytes became first evident at -56°C and was completed by -46°C . However, since warming over that range required 1 min, we could not dissect out the rate of blackening as a function of temperature. The present research was designed to eliminate this confounding between temperature and time. Here, the oocytes were warmed rather rapidly to a given temperature between -65°C and -50°C , held at that specified temperature for times ranging from 3 to 60 min, and then warmed and thawed. In effect, these oocytes were subjected to both interrupted rapid cooling *and* interrupted rapid warming.

These data on the extent of blackening vs. time permitted us to compute the rate of blackening vs. temperature, and that in turn permitted a calculation of the activation energy for the blackening process.

Methods and materials

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

Source of oocytes

Mature (MII) oocytes from ICR mice were harvested in Dr. Keisuke Edashige's laboratory at Kochi University, Japan, 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 at 23°C to PB1 lacking sucrose, and then to previously prepared droplets of M16 medium for some two hours. Mazur et al. [11, pp 48–49] give eight points of evidence that the vitrified-thawed-M16-incubated oocytes are normal with respect to plasma membrane integrity and osmotic response. One indication of normality is their morphological appearance. A second is that they shrink or swell in anisotonic solutions of glycerol/PBS or EG/PBS in

quantitative accord with that calculated from fundamental osmotic equations (For example, see Table 7 of Mazur et al.[14]. Thirdly, M. Kasai (personal communication) has found that 84% of ICR oocytes vitrified in the same medium and by the same procedure developed to the 2-cell stage after dezonation and *in vitro* fertilization, a percentage nearly equal to the 89% for untreated controls.

Experimental media and sample preparation

For an experiment, two to three oocytes were transferred from an M16 droplet to 1 ml of Dulbecco's phosphate buffered saline (PBS) containing the desired concentration of cryoprotective agent (here, 1.5 M ethylene glycol [EG]) and Snomax (a commercial preparation of freeze-dried *Pseudomonas syringii*, the ice nucleating bacterium). Snomax is introduced to minimize the supercooling of the suspending medium. Then, 15 min later, a 1.5 μ l droplet of this medium was placed in the center of a 50 μ m or 75 μ m thick spacer in a Linkam quartz sample cuvette, the oocytes pipetted in a minimum volume to that droplet, and a coverglass applied. [The oocytes are 75 μ m in diameter; nevertheless, the 50 μ m spacer was used in most runs because the thinner layer of frozen medium yielded much better optics. With respect to IIF, the response of the oocytes was no different than with the 75 μ m spacer]. 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 $\times$ objective for a displayed magnification of 500 $\times$. The images are displayed at 40 frames/s on a monitor and captured on a computer hard drive at desired intervals as short as 1 image/10 sec. In a few cases where changes occurred rapidly, the computer monitor was videotaped at 40 frames/sec.

The Linkam cryostage, freezing protocols, and ramps

Using liquid nitrogen vapor for cooling and electrical resistors for heating, the Linkam cryostage with its associated Pax-it software allows samples to be subjected to sequential ramps in which cooling rate, limiting temperature, holding time, and warming rate can be specified. The ramps used here are shown in Table 1 and Figure 1. The general procedure was as follows: The oocytes were cooled rapidly to -25°C and held at that temperature for 10 to 12 min (Ramps 1 to 3). We know from previous work [11] that -25°C is some 12 to 15 $^{\circ}\text{C}$ above the temperature at which oocytes in 1.5 M EG normally undergo IIF. We know from our recent study [13] that when they are held for 10 min at -25°C , they undergo sufficient dehydration so that most do not flash during the subsequent rapid cooling to -70°C (Ramp 4). However they do turn black upon warming (Ramps 5 and 6). The purpose here was to determine the rate at which that blackening occurs at various holding temperatures during the warming.

Ramp 1: Rapid cooling ($50^{\circ}\text{C}/\text{min}$) to -8.0°C

Ramp 2: Slow cooling ($5^{\circ}\text{C}/\text{min}$) to -10.5°C . EIF occurs at $-9.63 \pm 0.36^{\circ}\text{C}$, a supercooling of ~ 5 degrees.

Hold 2 min at -10.5°C (This is an important step in maximizing the subsequent supercooling of the cellular water [12], probably because it allows time for the previously supercooled extracellular medium to equilibrate).

Ramp 3: Rapid cool ($50^{\circ}\text{C}/\text{min}$) to -25°C . Hold 12 min. IIF rarely occurs at this temperature. During this hold, the oocytes lose about 50% of their water (In the first few experiments, the hold time was 10 min. In most experiments it was 12 min)

Ramp 4: Rapid cool ($50^{\circ}\text{C}/\text{min}$) to -70°C . If oocytes contain too much water after the hold in Ramp 3, they will undergo flash type IIF in this ramp. But if they are held for the 10 or 12 min indicated for Ramp 3, most of them do not flash in Ramp 4, but darken in Ramp 5

Ramp 5: Rapid warming (20°C/min) to given hold temperatures of -65 , -62.5 , -60 , -57.5 , -55 , -50 °C. Depending on the temperature, the oocytes were held 3 to 60 min to observe and record the kinetics of blackening. Some samples were held at -70 °C for 60 min. The warming rate of 20°C/min was chosen as a compromise between maximizing rapidity and minimizing overshoot. With that warming rate, the overshoot was less than 0.5°C for no more than 2 to 3 sec.

Ramp 6: Moderately rapid warming (10°C/min) to -35 °C. This post-hold warming was kept rather slow to permit observation of whether further blackening occurs.

Ramp 7: Rapid warming (20°C/min) to $+20$ °C. The oocytes become clearly visible at about -1 °C. Any that have blackened during cooling or warming are seen to be severely damaged.

Estimating the degree of blackening

The oocytes of interest were those that Mazur et al. [13] refer to as Class 4; namely, those that show no evidence of darkening during cooling ramps 1–4, but undergo blackening during the hold in Ramp 5. The extent of blackening was visually assigned a score of 0 to 5 based on photomicrographs captured during the hold. Fig. 2 shows an example for oocytes held at -62.5 °C.

A score of 0 means that the appearance of the oocytes was unchanged from that after the end of cooling to -70 °C in Ramp 4 (Fig. 2B and 2C). A score of 5 was assigned to oocytes that had turned as black at the end of the Ramp 5 hold as they became during the warming in Ramp 6 (Figs 2H, 2I). The advantage of this relative method of scoring is that it automatically adjusted for experiment to experiment variation in the opacity of the images of the oocytes and the surrounding medium.

Zero time in Fig. 2 is defined as the time that the hold temperature in Ramp 5 was reached. At hold temperatures of -52.5 °C and below, the blackening index was zero at zero time. However, at a hold temperature of -50 °C, considerable blackening (a score of 3–4) had already occurred before reaching the hold temperature.

In most cases, the oocytes attained a blackening index of 5 before or by the conclusion of the hold. An exception was with the hold temperature of -70 °C. In that case even after holding for 60 min in Ramp 5, the oocytes had only slightly blackened to 1 or 2; blackening to a score of 5 was reached only after warming in Ramp 6 had progressed to about -45 °C.

Results

Degree of blackening vs. time at each holding temperatures

Fig. 3A shows the mean degree of blackening vs. time at each holding temperature. These data were obtained from tabulations of the blackening score of each of the 7 to 12 oocytes vs. the time at each temperature. At a holding temperature -50 °C, the oocytes had already darkened to a score of 3–4 before they reached the hold temperature. At -70 °C, the blackening reached only a score of 2 by the end of the 60-min hold. The maximum score of 5 in that case was not attained until warming in Ramp 6 had progressed to about -45 °C.

Determination of the slope of blackening vs. time at given temperatures

To provide a means of comparing the rates of blackening at the various temperatures, we needed to determine the slopes of the curves in Fig. 3A. For most of the temperatures, the curves became distinctly curvilinear above a blackening score of 3. For that reason, with two exceptions, we restricted our estimates of linear slopes to the region between scores of 1 and

3. The reason for choosing a black score of 1 as the initial value rather than 0 is because at -55°C , a small degree of blackening had already occurred before 0 time. One exception was for -50°C . In that case, because of substantial blackening prior to 0 time, we based the slope on the time required to go from a score of 4 to 5. The second exception was for -70°C , where blackening during the hold did not progress above 2; hence in that case, the rate was based on the time between black levels 1 and 2. The darkening rates were estimated for each oocyte at each holding temperature (68 oocytes in all). Three examples are shown in Fig. 3B. The means of these individual rates of darkening are listed in column 2 of Table 2.

Arrhenius plots

An Arrhenius plot is a graph of the logarithm of the rate of a process as a function of the reciprocal of the absolute temperature. If that plot is linear, the process is assumed to follow Arrhenius kinetics, and the slope of that line is a measure of the activation energy of the process. A commonly used formulation of the Arrhenius relation is

$$\ln(k_2/k_1) = (E_a/R) \cdot (1/T_1 - 1/T_2), \quad (\text{Eq. 1})$$

where k_2 and k_1 are the rates of the process (here, blackening/min) at absolute temperatures (Kelvin) T_2 and T_1 , respectively. The values of these parameters in the present case are given in Table 2. E_a is the activation energy (cal/mole), R is the universal gas constant (1.9872 cal/deg. mole), and $-E_a/R$ is the slope of the line.

The values needed to construct the Arrhenius plot are given in columns 4 and 5 of Table 2. Note that in column 4, the natural log of the darkening rate was computed for each oocyte, and column 4 shows the resulting mean value of the $\log_e$ rate and its standard error. These values differ somewhat from those that are obtained by taking the natural log of the mean values in column 2.

The data in Table 2 are plotted in Arrhenius form in Figure 4. The linearity and degree of fit to the data are excellent. From the slope of the line ($-13,820 \text{ K}$), the computed E_a is 27,463 cal/mole. (In this computation, we have omitted the blackening rate at -50°C . This is because, as already stated, by the time the oocytes have warmed to -50°C , darkening has already progressed into the curvilinear region of the plot.)

Discussion

Recently, Mazur et al. [13] described and defined Class 4 oocytes as those that showed no “flashing” or blackening during rapid cooling to -70°C , but underwent blackening during subsequent warming at $10^{\circ}\text{C}/\text{min}$. That blackening was first detectable at -56°C and was complete 1 min later at -46°C . But in that study, time and temperature were confounded. The present study differs in that Class 4 oocytes were warmed from -70°C to various temperatures between -65°C and -50°C , held at those temperatures for 3 to 60 min, and then further warmed and thawed. In this case we observed that with sufficient holding time, blackening became first detectable at a 10° to 15°C lower temperature than was observed previously, and that the rate of blackening increased dramatically as the hold temperature was raised from -70°C to -50°C . As seen from Table 3, the rate quadruples for every 5° rise in temperature. The consequence is that the time required to blacken to a score of 1 (out of 5) decreased from 65 sec at -60°C to 3.6 sec at -50°C .

The computations in Table 3 assume that all blackening occurs during the hold temperature and none during the warming from -70°C to the hold temperature; i.e., they assume that the warming rate is infinite. Since in fact the warming rate was $20^{\circ}\text{C}/\text{min}$, some relatively small

amount of blackening occurred during the warm. Thus, the times to reach stage 1 blackening vs. holding temperature in Table 3 are upper limit values. Later in the discussion we show how blackening values that incorporate the effects of warming rate can be calculated.

If the rate of blackening vs. temperature is plotted in Arrhenius form (i.e., $\ln k$ vs. $1/T$), a nearly perfect straight line is generated (Fig. 4). The activation energy of the process (E_a) can be calculated from the slope of that line, and is 27.46 kcal/mole—more than double the typical value for reaction rates (10–12 kcal/mole).

Is the cellular blackening a manifestation of the devitrification of a glass or the recrystallization of ice?

An almost universally held presumption is that the blackening of a cell is an optical manifestation of the formation of ice crystals in that cell. The presumption is supported by direct evidence in human lymphocytes and in *Xenopus* oocytes. In these two cases, the observed temperature for flashing on a cryostage coincides closely with the temperature at which an exotherm due to IIF is observed in cells or eggs frozen under identical conditions in a differential scanning calorimeter (DSC) [5,7a]. The exotherm is due to the release of heat when supercooled water is converted to ice. Presumably, the blackening is a consequence of the reflection and refraction of light from crystals and crystal boundaries. If the intracellular crystals are very small, there is too little reflection and refraction to produce visible effects.

Here, we are studying and observing blackening that occurs during the warming of a previously cooled sample. There are two ways that visible ice crystals can form in cells during warming. One is by the devitrification of an intracellular aqueous glass (amorphous solid) formed during cooling. The other is by recrystallization (the conversion of preexisting small ice crystals formed during cooling into thermodynamically favored large crystals during warming).

Luyet and Rasmussen [8] published an extended phase diagram for EG/water showing devitrification (T_D) and recrystallization (T_R) temperatures as a function of the weight percent concentration of EG in the solution. After a 12 min hold during the cooling, the concentration of EG in the oocytes is 22 wt % [13]. For a 25% solution, Luyet and Rasmussen observed a T_D of -125°C and a T_R of -65°C . Hayes and Pegg [3] also report a T_D of -125°C for a 35 wt % solution of EG in water. These values of T_D are 60° to 70°C below the blackening temperature that we observed. The value of T_R reported by Luyet and Rasmussen is almost exactly the same as the blackening temperature observed here. This seems strong evidence that what we are observing during blackening is the recrystallization of intracellular ice— and not devitrification. However, there is a caveat. The Luyet and Rasmussen and Hayes and Pegg phase data are for EG in water. The interior of the oocytes is not just EG/water, and the presence of proteins and other macromolecules and smaller molecules might change T_D and T_R appreciably.

Notwithstanding this caveat, the value of E_a that we observe for the darkening process (27.5 kcal/mole) provides another strong piece of evidence that the mechanism of darkening is recrystallization. In 1960, Kingery [4] placed two 1-mm spheres of ice in contact, and measured the rate at which the neck filled in at temperatures ranging from -2.1 to -25°C . This sintering, as he called it, is a form of recrystallization. When he plotted these rates in Arrhenius form, the result was a straight line, and from the slope of that line, we calculate the activation energy to be 29.0 kcal/mole—a value very close to that we have found here for darkening.

In theory, another way to distinguish between devitrification and recrystallization is by DSC. No heat of fusion is released in passing from supercooled water to glassy water [9]. Consequently, if the glass converts to crystalline ice by devitrification during warming, a process tantamount to freezing, the entire latent heat of fusion (L_f) should be released. (The

heat released would be somewhat less than the 80 cal/g released when freezing occurs just below 0°C because L_f is slightly temperature dependent, but it would be an appreciable fraction of that value.) On the other hand, if the blackening is a consequence of the recrystallization of already existing small ice crystals, only a small quantity of heat would be released because the free energy of the small crystals is only slightly greater than that of the larger crystals. The practical problem with using DSC is that the sample has to contain a minimum of 10 µg of intracellular water to generate an exotherm of visible and useful magnitude. A single mouse ovum contains 0.2 µg water, so at least 50 oocytes (and probably double that) would be required in each DSC run.

Relationship between the water contents of oocytes after freezing and the occurrence of recrystallization during warming

Class 4 oocytes were the subject of this study. These are oocytes that exhibit no darkening during the initial interrupted rapid cooling, but darken during subsequent warming. In our previous study [13], their occurrence was maximal (88%) with a holding time of 10 min at -25°C during the interrupted rapid cool. In the present study with a 12-min hold the percentage was 83%. The calculated water contents of the oocytes after these holds are 41% and 36% respectively of the value in an isotonic cell (Fig. 6 in [13]). In [13], we found that if the hold was only 5 min, the cell water content was 56% and only 10% were Class 4. An increase in the holding time to 20 min decreased the cell water content to a calculated 23% and it reduced the percentage of Class 4 oocytes to 38% (with a concomitant increase in Class 5 oocytes; i.e., those showing no darkening during cooling or warming). Finally, increasing the holding time to 30 min reduced the water content to 19.7%, reduced the percentage of Class 4 oocytes to 12%, and increased the fraction of Class 5 oocytes to 80%. In other words, there was a large increase in the percentage of oocytes undergoing recrystallization as their water content was reduced from 56 to 36%, and then a large decrease in the percentage as the water content was further reduced from 36% to 20%. In the latter case, the shift was to Class 5 oocytes.

These water contents vs. holding time were estimated from the integrated form of the standard water permeability equation using the value of the water permeability (L_p) at -25°C determined by Kleinhans and Mazur [7]); namely, 0.018 µm/min atm. That equation is

$$dV/dt = L_p A N R T (1/V - 1/V_{eq}),$$

where V is the volume of cell water, A is the cell surface area, N is osmoles of solute in the cell, R is the gas constant, and T the absolute temperature.

How rapidly do oocytes have to be warmed to “outrun” the recrystallization process?

Table 3 shows the estimated time that is required for an oocyte to darken to Stage 1 at various subzero temperatures. At -35°C and -30°C, the times to reach black stage 1 are 72 and 22 msec, respectively. There are two unknowns in trying to convert those times into the warming rates that would be required to avoid damaging recrystallization. One is that we do not know the relationship between degree of darkening and lethality. Second, we do not know to how high a temperature the rate of darkening will continue to accelerate in accordance with our measured E_a . A solution begins to melt when warmed above its eutectic point. For EG/water that eutectic point is somewhat indeterminate, but appears to be about -60°C [3,8]. We have also noted from our photomicrographs that blackened oocytes begin to become paler as the temperature rises above -35°C, presumably because a significant fraction of the internal ice has melted by that temperature. From the ternary phase diagram for 1.5 M EG/PBS/water in Fig. 6 C of Kleinhans and Mazur [6], the total weight percent of EG + salt (W_T) at -35°C is 48.1%. The fraction of the cell solution (L) that is unfrozen at -35°C is W^0_T/W_T , where

W^0_T is the total weight percent of solutes before freezing (9.94 wt %); consequently, $L = 0.207$. The fraction of the ice that has melted is equal to $(100 - W_T) * L / (100 - W^0_T)$ which equals 0.119. That is to say, after warming to -35°C , 12% of the ice in the cell has melted.

To incorporate the effect of warming rate on the degree of blackening at various subzero temperatures we make use of a modification of the algorithm given on p. 53 of Mazur et al [10].

1. The initial temperature is taken to be -70°C and the initial degree of blackening is 0.
2. We instantaneously increment the temperature 0.1°C
3. We compute the rate of blackening (k) at that higher temperature from the experimentally derived Arrhenius relation (Eq. 1 and footnote to Table 3)
4. We compute the time (t) for the temperature to increase 0.1°C when warmed at a specified rate.
5. We assume that the oocyte remains at that temperature for t min and compute the degree of blackening in that interval as $k * t$.
6. We loop back to step 2 with the starting degree of blackening being that in step 5. The looping continues until the degree of blackening exceeds 3.

The program to execute this algorithm was written in Microsoft QuickBASIC, and the results are plotted in Fig. 5 as the degree of blackening vs. temperature for warming rates ranging from 10 to 10,000 $^\circ\text{C}/\text{min}$. A 10-fold increase in warming rate raises the temperature to attain a given black level (e.g. Black 1) about 8°C .

If we assume that recrystallization ceases above -35°C because of melting, the curves in Fig. 5 indicate that the oocytes would have to be warmed at 4,000 $^\circ\text{C}/\text{min}$ between -70°C and -35°C to avoid undergoing recrystallizing to Black 1 by -35°C . When a cell sample in a standard $\frac{1}{4}$ ml artificial insemination straw is transferred with mild stirring from liquid nitrogen (LN_2) to room temperature water, we estimate that it warms at some 3,000 $^\circ\text{C}/\text{min}$ between -70°C and -35°C .

Prior to this analysis, we subjected six Stage 4 oocytes to warming at 100 $^\circ\text{C}/\text{min}$ (about the upper limit for the Linkam). The blackening values vs. temperature as observed in video recordings are shown by the open circles in Fig. 5 (Black 1, 2, and 3 were attained at $-49.2 \pm 0.6^\circ$, $-46.5 \pm 0.4^\circ$, and $-45.2 \pm 0.4^\circ\text{C}$, respectively). As seen, the observed temperatures fall very close to those predicted by the 100 $^\circ\text{C}/\text{min}$ curve. The photomicrographs in Fig. 1 in Mazur et al, [13] depict the blackening vs. temperature of three Class 4 oocytes warmed at 10 $^\circ\text{C}/\text{min}$. The observed degree of blackening in this set and an additional set of nine not published is shown by the solid circles in Fig. 5 (Black 1, 2, and 3 were attained at $-57.5 \pm 0.4^\circ$, $-54.8 \pm 0.4^\circ$, and $-52.1 \pm 0.5^\circ\text{C}$, respectively). These temperatures fall almost exactly on the computed curve for 10 $^\circ\text{C}/\text{min}$. In other words, the degree of blackening predicted from the Arrhenius relationship agrees closely with the experimentally derived values. (The two exceptions are for the data points lying at 0 blackening on the two curves. The computed curves predict a blackening of about 0.1 at those temperatures, but we cannot distinguish 0.1 from 0 visually.)

Rall et al. [16] and Rall and Polge [15] reported that 8-cell mouse embryos cooled at 0.5 $^\circ\text{C}/\text{min}$ to -42°C and then cooled rapidly to -150°C underwent blackening at $\sim -55^\circ\text{C}$. when warmed at 2 $^\circ$ and 10 $^\circ\text{C}/\text{min}$ but not if warmed at 25 $^\circ$, 50 $^\circ$, 100 $^\circ$, or 250 $^\circ\text{C}/\text{min}$. They ascribe the darkening to the recrystallization during warming of intracellular ice formed during cooling, an interpretation with which we would agree since it occurs at the same temperature range that we observe. However, there is a substantive difference, since they report that

blackening is absent in embryos warmed at 25°C/min or faster, whereas we still see it occurring in oocytes warmed at 100°C/min. Perhaps the difference is that their interrupted *slow* cool procedure probably reduced the water content of the embryos to a lower water content prior to the plunge than did our interrupted *rapid* cooling procedure.

The 4,000°C/min warming rate, which we predict is needed to prevent recrystallization, is a very high rate, but it is still much lower than the computed warming rates required to prevent the devitrification of a vitrified cell. Fahy [1] has reported the upper limit of the latter rate to be 1.4×10^8 °C/min for a 45 wt% EG/H₂O solution. Hayes and Pegg [3] have computed a much lower but still high critical warming rate of 30,000°C/min for that EG concentration. The critical warming rate to preclude the devitrification of a 22 wt% solution of EG (the calculated internal concentration of EG in an oocyte after the 12 min hold in interrupted cooling) would be many fold higher.

Implications for cryopreservation and cryo-electronmicroscopy

Classical (equilibrium) slow cooling—In this procedure, cells in cryoprotective agent (CPA) are cooled to –60°C or below at a rate (commonly 1°C/min) shown or believed to preclude IIF. It is called “equilibrium” cooling because during cooling the cell water content is progressively reduced so that its chemical potential is maintained in near equilibrium with that of the water in the external medium. The presumption is that after reaching –60°C, the cells contain little or no freezable water, and consequently IIF can not occur during a subsequent plunge to –196°C. If there is no IIF during cooling, there can be no recrystallization during warming. With this procedure one expects little or no effect from the rate of warming, a prediction fulfilled with many cell types.

Interrupted slow freezing—This is a procedure being used increasingly. In it, cells are cooled slowly (typically 1°C/min, as above) to an intermediate temperature (usually –30°C to –35°C) and then plunged into LN₂. The rationale is that during the initial slow cool, the cells lose enough of their intracellular water to preclude their undergoing IIF during the plunge to –196°C. The procedure is somewhat analogous to the interrupted rapid cool used here. The difference is that in interrupted slow cool, the cells dehydrate progressively over a range of temperatures during the initial cool whereas in interrupted rapid cooling, the dehydration occurs isothermally during the holding period. Interrupted slow cooling first became prominent when Willadsen [17] applied it to the cryopreservation of sheep embryos. High survivals with this procedure usually require that the cells be warmed rapidly. The reason for this is that after the initial slow cool to the plunge temperature, the cells likely still contain a small amount of freezable water and that water undergoes damaging recrystallization unless the subsequent warming is rapid.

Vitrification procedure—The intent of vitrification is to avoid IIF by converting supercooled intracellular water into a non-crystalline glass. This requires high cooling and warming rates and/or requires high concentrations of permeating glass-inducing solutes. The two are reciprocally related in that the higher the concentration of solutes, the lower can be the required cooling and warming rates. If the cooling rate is insufficient for a given solute concentration, the supercooled intracellular water will freeze rather than vitrify. If the warming rate is insufficient, the vitrified cell contents will first devitrify (i.e. freeze) and then recrystallize. As noted, in EG/water solutions the former occurs about 60°C below the latter. It is not clear which event contributes the more to lethality, devitrification or recrystallization. However, Fahy [2] suggests that recrystallization may be the more damaging.

The criticality of the storage temperature—No degradation of cryopreserved samples should occur in liquid nitrogen (–196°C), liquid nitrogen vapor (–120 to –140°C), or ultra-

low temperature mechanical freezers ($\sim -120^{\circ}\text{C}$), for all these maintain temperatures that are equal to or below T_D and far below T_R , at least for EG/water solutions. But our computations predict that substantial or total cell injury or death will occur if cells submitted to other than equilibrium slow cooling are purposefully or accidentally held relatively short times above -70°C . Based on the Arrhenius relation in the footnote of Table 3, recrystallization to Black 1 will be attained in 26 min at -70°C ; and at -79°C (Dry Ice temperature), it will be attained in 10 hours. Exposure to temperatures above -70°C is much less serious in cells preserved by slow equilibrium freezing since their lack of freezeable water should preclude recrystallization.

Implications for cryo-electron microscopy—Many electron microscope studies involve determining the structures and morphology of cells at cryogenic temperatures. In some, cells are suspended in glass-inducing solutes and slammed onto cold polished metal surfaces to produce cooling rates that are so rapid that IIF can not occur. In other techniques, after being cooled to ultra-low temperatures, the water and/or ice in the cells is removed at temperatures of around -75°C by substitution with an organic liquid like ethanol. In still others, cells are examined by cleaving them at similar temperatures, often supplemented by etching. The aim in all these techniques is to eliminate or minimize morphological artifacts that might result from chemical fixation or internal ice crystal formation.

The caveats just stated above with respect to cell viability apply here as well. That is, even if the intracellular solution has become vitrified during cooling or contains only minute ice crystals, the cytoplasmic glass may devitrify and internal crystals may recrystallize sufficiently to produce artifacts if the cells are held for relatively short periods at -70°C or above.

Extending these results to other cell types and concentrations of CPA

The experiments reported here were carried out using one cell type (the mature mouse oocyte) and one type and concentration of CPA (1.5M EG in PBS). To what extent can one generalize to other cells and other concentrations of other CPAs? A reduction in the CPA concentration to, say, 1 M should have little effect. The driving force for the efflux of cell water at -25°C , the initial hold temperature, depends on the difference between the external and internal osmolalities. The external osmolality will remain nearly unchanged. The internal osmolality will be somewhat lower in 1 M EG than in 1.5 M, and consequently one would expect the cell water content to be reduced to a given value in a somewhat shorter time. But, once the cells have dehydrated to a given value, they are essentially in an identical state to those initially suspended in 1.5 M EG, and they should recrystallize at the same rates. Similar reasoning applies to oocytes suspended in higher concentrations of CPA; e.g. 2 M. In this case, however, there will be less difference between the external and internal osmolalities at the start of the hold at -25°C than for oocytes in 1.5 M EG; consequently, it will take a longer time for the oocyte to dehydrate to a given water content.

The relation between cell blackening and the structure of intracellular ice crystals/the nature of damage from intracellular ice

This ending paragraph is essentially a statement of unknowns. The first unknown is what is the relation between the cell blackening we observe and the nature of the underlying ice crystal structure responsible for it? Is the degree of blackening a function of the mass of ice? Is it a function of the crystalline structure of that ice? The second unknown is what aspects of IIF and recrystallization are actually the cause of lethality? Is it the mass of internal ice? Is it the number of ice crystals? Is it the fine structure of the crystals; e.g., the extent to which they are dendritic? Or is it the specific intracellular location of the ice? And finally there remains the long lingering question of whether IIF and its recrystallization is the cause of lethality or whether prior injury or prior defects in the cell membrane lead to IIF.

Karlsson [3a] addressed some of these questions in a theoretical and analytical fashion. That is, he presented plots showing the computed fraction of the water in human keratinocytes undergoing devitrification as a function of temperature, and the number and size of ice nuclei formed during warming at different rates. The problem is that our experiments provide no data against which to compare these computed values. Furthermore his computations are based on the volumes, surface-to-volume ratios, and water permeabilities of keratinocytes—not the much larger mouse oocytes used here. Finally, his computations are based on glycerol as the CPA. We used EG.

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Fig. 1.

Experimental temperature/time protocols for mouse oocytes in 1.5 M EG/PBS. The numbers 1, 2, 3, 4, 5, 6, and 7 refer to the Linkam cryostage temperature/time ramps described in Table 1. This schematic illustrates a hold for 10 min at -60°C .

Fig. 2.

A sequence of photomicrographs of Class 4 mouse oocytes cooled to -70°C and then warmed to -62.5°C and held for 24 min (C–H). They were then warmed and thawed (I [taken at -46°C], J [taken at $+20^{\circ}\text{C}$]). The oocytes were suspended in 1.5 M EG/PBS and subjected to the ramps described in Table 1 and Fig. 1. The normal oocyte (A) is 75 μm in diameter.

Fig. 3.

(A) The mean degree of blackening of mouse oocytes vs the time they were held at the indicated temperatures after warming from -70°C . Table 2 gives the numbers of oocytes in each curve. (B) An example of three individual runs at holding temperatures of -60 , -62.5 , and -65°C illustrating how the slopes of the curves (darkening rates) were calculated.

Fig. 4.

Arrhenius plot of the logarithm of the mean rate of darkening of mouse oocytes vs. the reciprocal of the absolute temperature of the hold. The hold temperatures from left to right were -55 , -57.5 , -60 , -62.5 , -65 , and -70°C . The error bars are standard errors (standard deviations of the mean). The darkening rate for -50°C ($1/T = 0.00448$) has not been plotted for reasons given in the text. The $\log_e$ rate for it was 1.618.

Fig. 5.

Degree of blackening (from 0 to 3) as a function of temperature for oocytes warmed at indicated rates from -70°C . The lines are values computed from the measured rates of blackening at various temperatures, the computed activation energy for blackening, and the algorithm described in the text. The closed circles are data obtained in [13] for twelve Class 4 oocytes warmed at $10^{\circ}\text{C}/\text{min}$. The open circles are data obtained in the present study for six Class 4 oocytes warmed at $100^{\circ}\text{C}/\text{min}$. The experimental oocytes were suspended in 1.5 M EG/PBS, cooled rapidly to -25°C , held 10 or 12 min, cooled rapidly to -70°C , and then warmed at 10° or $100^{\circ}\text{C}/\text{min}$ to $+20^{\circ}\text{C}$.

The plots were computed using a temperature increment of 0.1°C in our BASIC program (See text). These plots are indistinguishable from those using a temperature increment of 0.01°C .

Table 1

Linkam cryostage cooling and warming ramps for oocytes frozen in 1.5 M ethylene glycol

Ramp	Rate (°C/min) ^a	Limit (°C)	Hold (min)	Comment
1	-50	-8	0	
2	-5	-10.5	2	EIF ^b occurs above -10.5° C
3	-50	-25	12	
4	-50	-70	0	
5	+20	-70, -65, -62.5, -60, -57.5, -55, -50	3-60	Recrystallization occurs in this ramp
6	+10	-35	0	
7	+20	+20	1	

^aCooling is indicated by negative rates; warming by positive rates.^bEIF refers to extracellular ice formation.

Table 2

Rate of darkening at each holding temperature

Holding temp. (°C)	Rate $\pm$ S.E. (min ⁻¹)	n	Ln Rate $\pm$ S.E.	Inverse Hold temp. (1/T, K ⁻¹)
-50	5.143 $\pm$ 0.404	7 ^a	1.618 $\pm$ 0.082	0.00448
-55	5.455 $\pm$ 1.354	11	1.370 $\pm$ 0.257	0.00458
-57.5	2.918 $\pm$ 0.788	10	0.758 $\pm$ 0.260	0.00464
-60	1.078 $\pm$ 0.127	12	-0.018 $\pm$ 0.139	0.00469
-62.5	0.513 $\pm$ 0.155	11 ^b	-0.995 $\pm$ 0.226	0.00475
-65	0.208 $\pm$ 0.032	10 ^b	-1.703 $\pm$ 0.185	0.00480
-70	0.059 $\pm$ 0.024	7	-3.206 $\pm$ 0.332	0.00492

^a 3 additional oocytes turned maximally black (5) by the time they had warmed to -50°C

^b An additional oocyte did not blacken. Upon thawing it proved to be a Class 5 survivor.

Table 3

Time for mouse oocytes to blacken to stage 1 as a function of temperature

Temperature	1/T	k	Time to reach	Black Stage 1
(°C)	(K ⁻¹)	(1/min)	(min)	(sec)
-79	0.00515	0.002	620	37200
-70	0.00492	0.038	26.5	1590
-60	0.00469	0.918	1.090	65.4
-57.5	0.00464	1.946	0.514	30.8
-55	0.00458	4.055	0.247	14.8
-50	0.00448	16.770	0.060	3.580
-45	0.00438	65.140	0.015	0.921
-40	0.00429	238.8	0.004	0.251
-35	0.00420	828.6	0.001	0.072
-30	0.00411	2732	0.0004	0.022

The values of the rate of blackening (k) were calculated from the exponential form of the equation of the Arrhenius plot in Fig. 4; namely, $k = \exp [(-E_a/R)(1/T) + 64.748]$. The times to Black 1 in min. were simply $1/k$.