Nonequilibrium freezing of one-cell mouse embryos

Membrane integrity and developmental potential

Mehmet Toner,* Ernest G. Cravalho,‡ James Stachecki,|| Timothy Fitzgerald,|| Ronald G. Tompkins,* Martin L. Yarmush,*§ and D. Randall Armant||

*Surgical Services, Massachusetts General Hospital, and the Shriners Burns Institute, Boston, Massachusetts 02114; ‡Department of Mechanical Engineering, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139; §Department of Chemical and Biochemical Engineering, Rutgers University, Piscataway, New Jersey 08855; and ||C. S. Mott Center for Human Growth and Development, Department of Obstetrics and Gynecology, Wayne State University School of Medicine, Detroit, Michigan 48202 USA

ABSTRACT A thermodynamic model was used to evaluate and optimize a rapid three-step nonequilibrium freezing protocol for one-cell mouse embryos in the absence of cryoprotectants (CPAs) that avoided lethal intracellular ice formation (IIF). Biophysical parameters of one-cell mouse embryos were determined at subzero temperatures using cryomicroscopic investigations (i.e., the water permeability of the plasma membrane, its temperature dependence, and the parameters for heterogeneous IIF). The parameters were then incorporated into the thermodynamic model, which predicted the likelihood of IIF. Model predictions showed that IIF could be prevented at a cooling rate of 120°C/min when a 5-min holding period was inserted at −10°C to assure cellular dehydration. This predicted freezing protocol, which avoided IIF in the absence of CPAs, was two orders of magnitude faster than conventional embryo cryopreservation cooling rates of between 0.5 and 1°C/min. At slow cooling rates, embryos predominantly follow the equilibrium phase diagram and do not undergo IIF, but mechanisms other than IIF (e.g., high electrolyte concentrations, mechanical effects, and others) cause cellular damage. We tested the predictions of our thermodynamic model using a programmable freezer and confirmed the theoretical predictions. The membrane integrity of one-cell mouse embryos, as assessed by fluorescein diacetate retention, was ~80% after freezing down to −45°C by the rapid nonequilibrium protocol derived from our model. The fact that embryos could be rapidly frozen in the absence of CPAs without damage to the plasma membrane as assessed by fluorescein diacetate retention is a new and exciting finding. Further refinements of this protocol is necessary to retain the developmental competence of the embryos.

INTRODUCTION

The clinical application of embryo and oocyte cryopreservation has become important because of recent advances in human in vitro fertilization (IVF) (Trounson, 1986; Levran et al., 1990). IVF is now a routine clinical procedure for the treatment of infertility with pregnancy rates between 20 and 25% per oocyte collection in many centers (Trounson, 1986; Freidler et al., 1988). The introduction of superovulatory techniques into human IVF has resulted in the production of larger numbers of oocytes and, consequently, more embryos than can be safely transferred. Cryopreservation of excess embryos for subsequent transfer in later cycles has been an important strategy for expanding the procedure (Trounson, 1986; Freidler et al., 1988). Integration of successful cryopreservation techniques into clinical IVF programs will promote the efficient use of excess oocytes and embryos.

The first successful reports of mammalian embryo freezing were published in 1972 (Whittingham et al.; Wilmut). Live mice were obtained after embryo freezing to temperatures of −196°C by a slow cooling procedure in the presence of dimethyl sulfoxide as the cryoprotective agent (CPA). These important studies became the basis for most of the subsequent cryopreservation studies undertaken in various other species (Freidler et al., 1988). Most of these protocols are based on the use of penetrating CPAs at concentrations ranging between 1.5 and 2 M and slow cooling rates between 0.5 and 1°C/min. During "equilibrium" freezing, slow cooling rates after the seeding of external ice allow embryos to dehydrate sufficiently to maintain thermodynamic equilibrium with the partially frozen extracellular solution (Mazur, 1990). Cryopreservation of embryos under "nonequilibrium" conditions have also been attempted in recent years (Rall and Fahy, 1985; Freidler et al., 1988) wherein cells are partially dehydrated in high concentrations of a mixture of penetrating and nonpenetrating CPAs before rapid freezing. Potentially, the CPAs can be lethal because their addition and removal are associated with changes in the cell volume due to the penetrating CPAs (Levin and Miller, 1981; Toupin et al., 1989). Although excessive volumetric changes can be limited by adding and removing the CPA in a stepwise fashion, the prolonged exposure of cells to CPA under nonfrozen conditions can result in direct toxic effects (Van der Elst et al., 1988; Damien et al., 1989). Hence, it is important to develop freezing techniques in which embryos are frozen rapidly under nonequilibrium conditions in the presence of minimal levels of CPAs. Unfortunately, the use of rapid cooling rates to achieve nonequilibrium freezing is usually lethal because of intracellular ice formation (IIF) (Mazur, 1977). To design nonequilibrium freezing protocols while avoiding lethal IIF, the thermodynamic conditions resulting in IIF need to be determined. This could be achieved if the water permeability and ice-nucleation parameters of embryos were known (Toner et al., 1990; 1992). To date the

Address correspondence to Mehmet Toner.

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water permeability parameters of one-cell embryos have been determined only at suprazero temperatures; the more relevant values under freezing conditions are as yet unknown (Leibo, 1980). Similarly, the ice-nucleation parameters have only been determined for mouse oocytes (Toner et al., 1990, 1992), and not embryos.

In the present report, we have used cryomicroscopy and thermodynamic modeling to determine the subzero water permeability and intracellular ice-nucleation parameters of one-cell mouse embryos. Using this information, we were able to optimize a rapid nonequilibrium cooling protocol in the absence of cryoprotectants, and to test the model predictions in experiments using one-cell embryos and a programmable freezer. The nonequilibrium freezing protocol resulted in ~80% membrane integrity as assessed by fluorescein diacetate (FDA) retention following a freeze–thaw protocol in the absence of penetrating CPAs. Experimental results were found to be in reasonable agreement with the model predictions.

MATERIALS AND METHODS

Water permeability model

Water transport across the cell plasma membrane was described using a two-compartment membrane-limited transport model as follows (Mazur, 1963; Toner et al., 1990):

$$\frac{dV}{dT} = L_{pg}\exp\left[\frac{E_{Lp}}{R}\left(\frac{1}{T}-\frac{1}{T_R}\right)\right]\frac{ART}{v_w B} \times\left[\ln\frac{(V-V_b)}{(V-V_b)+v_w(v_s n_s)}-\frac{\Delta H_f}{R}\left(\frac{1}{T_R}-\frac{1}{T}\right)\right], \quad (1)$$

where V is the cell volume; T is the absolute temperature; L_{pg} is the reference water permeability coefficient at 273 K; E_{Lp} is its temperature dependence (i.e., activation energy); R is the universal gas constant; A is the surface area of the cell; B is the cooling rate; v_w is the partial molar volume of water; V_b is the osmotically inactive cell volume (i.e., cell solids and bound water); n_s is the number of moles of solutes in the cell and is constant during dehydration; v_s (=2) is the dissociation constant for NaCl; T_R is the equilibrium freezing temperature of pure water; and ΔH_f is the latent heat of fusion for water. The surface area of the embryos is assumed to vary with dehydration and a two-thirds power dependence of A upon V is used in calculations. Effects of various parameters and assumptions in Eq. 1 have been discussed elsewhere in detail (Mazur, 1963; Shabana and McGrath, 1988; Toner et al., 1990). The solution of Eq. 1 requires knowledge of the water permeability parameters, L_{pg} and E_{Lp}, which were determined from experimental measurements performed at a cooling rate of 2°C/min, and the inactive cell volume, V_b, which was obtained from static osmometric experiments as described in detail below.

Intracellular ice-nucleation model

The nucleation of ice inside mouse embryos was modeled using the heterogeneous nucleation theory as previously described (Toner et al., 1990, 1992). The probability of IIF, PIF, was related to the nucleation rate by assuming sporadic nucleation of identical cells as follows:

$$\mathrm{PIF} = 1-\exp\left[-\frac{1}{B}\int_{T_{seed}}^{T} A\Omega_o(T_f/T_{fo})^{1/2}(\eta_o/\eta) \times (A/A_o)\exp[-\kappa_o(T_f/T_{fo})^4/\Delta T^2 T^3]\,dT\right], \quad (2)$$

where the subscript o refers to isotonic conditions; Ω and κ are, respectively, the kinetic (preexponential) and thermodynamic (exponential) heterogeneous nucleation parameters; $\Delta T = T_f - T$ is the undercooling of the cytoplasm; T_f is the equilibrium freezing temperature of the cytoplasm; η is the viscosity of the intracellular solution; and T_{seed} is the extracellular ice seeding temperature. The effects of various assumptions and parameters in Eq. 2 have been described in detail elsewhere (Toner et al., 1990). The viscosity of the cytoplasm was estimated using decoupled temperature and concentration dependencies (Toner et al., 1990). Cryomicroscopic observations at a cooling rate of 20°C/min were used to determine the two ice-nucleation parameters, Ω and κ, as described below. Eq. 2 was coupled to Eq. 1 to predict the probability of IIF under various physical and chemical conditions. It was also assumed that the probability of IIF was zero if the water content of the embryo was <5% of the initial content (Myers et al., 1989; Mazur, 1990). In the correlation of the predictions of IIF with the actual membrane integrity, it was assumed that IIF during rapid freezing was the primary cause of membrane damage (Mazur, 1977; Rall et al., 1983; Shabana and McGrath, 1988). This assumption is only valid for the rapid cooling conditions used in this study and ignores the injury due to solution effects observed during slow freezing (Lovelock, 1953). Thus the percentage of embryos with membrane integrity was simply expressed as (100 − PIF)% in the model.

Collection, IVF, and culture

The procedures for the collection and fertilization of mouse oocytes were essentially those described previously (Toner et al., 1991). Briefly, follicular activity was stimulated in NSA (Harlan Sprague Dawley, Inc., Indianapolis, IN) or B6/SJL (Jackson Laboratory, Bar Harbor, ME) female mice by intraperitoneal injection of 5 IU pregnant mare serum gonadotrophin (PMSG; Calbiochem Corp., La Jolla, CA) followed 48 h later by injection of human chorionic gonadotrophin (hCG; Serono, Norwell, MA). After the second injection, female mice were paired with B6/SJL males overnight for mating. Cumulus masses were collected from oviducts 12 h after hCG and treated with 120 U/ml hyaluronidase (Irvine Scientific, Santa Ana, CA) for 5 min to remove cumulus cells. Embryos were washed three times in HTF-Hepes (Sigma Chemical Co., St. Louis, MO) medium and incubated in 5% CO_2 at 37°C until freezing was begun. All HTF-Hepes and phosphate-buffered saline (PBS) solutions used in this study contained 0.4% bovine serum albumin (BSA). All embryo culture was done using microdrops of HTF-Hepes medium under a layer of silicone oil (Aldrich Chemical Co., Milwaukee, WI).

Cryomicroscopy

Cryomicroscopy experiments were performed using an upright Zeiss Universal Research microscope (Universal Research; Zeiss, Oberkochen, Germany) with video cameras (model ITC-62; Ikegami, Japan; and model WV-3890B; Panasonic, Japan) that display in black and white on a monitor (model BT-S 1300N; Panasonic,). Video recordings were made on a Panasonic AG-6300 VHS tape machine. The temperature controller and the video system interface with the video system to display the real temperature and time on the video recordings were built by Interface Techniques Company (Cambridge, MA). A specially designed cryostage with a sapphire optical viewing area was used to observe the cells while being frozen under the microscope (Meryman, 1970). Typical lateral and vertical temperature gradients across the cryostage window of 10 mm^2 were <0.1°C, minimizing experimental errors in the determination of water permeability and intracellular ice-nucleation parameters. Cooling rates up to 200°C/min were achieved with negligible error because of the small thermal mass of the sample (Toner et al., 1991).

For permeability experiments, a 16× objective (Plan 16/0.35 Ph2; Zeiss) and a 2× photoocular (Zeiss) were used. Embryos were suspended in a PBS solution containing 4 mg/ml BSA. One to two embryos were visible at one time in the field of view of the microscope. A

cover slip was placed over the embryos on the cryostage and was sealed with silicone grease to generate a sample thickness between 150 and 250 μm. The initiation of sample freezing was achieved at ~ -1°C by manually triggering ice formation by contacting the edge of the sample with a cold needle, followed by cooling at a constant cooling rate of 2°C/min. After each experiment, the videotape was reviewed to measure the volume variation in each embryo during the cooling process. To obtain these measurements, individual frames were converted from videotape to digital format using a hardware/software system (Digi-View; NewTek, Topeka, KS) and a computer (Amiga 1000; Commodore, West Chester, PA). The frames were digitized in a 320 × 200 spatial format in $x - y$ coordinates with four bits (16 discrete values) of gray-level resolution. Normalized volume versus temperature curves were generated from these digitized images. The projected cross sectional area was measured by tracing the cell boundary using a mouse controlled cursor and by electronically counting the number of pixels in the enclosed area. By assuming spherical symmetry, the projected area was converted to volume. To minimize the errors in volume measurements from the projected area, data reduction was only performed for those embryos that shrank uniformly. A typical estimate of error in volume variations is between 5 and 10%. An iterative nonlinear regression analysis was used to obtain values for the permeability coefficients that would produce the best fit between the theoretical volumes predicted by Eq. 1 and the experimental volume measurements (Toner et al., 1990).

For IIF experiments, two to five embryos were placed on the cryomicroscope stage under a coverslip and sealed with silicone grease. After the initiation of extracellular ice, embryos were cooled at a given constant cooling rate to −50°C and IIF was monitored by noting a darkening of the cytoplasm due to the formation of small ice crystals within the embryo. The temperature at which each embryo underwent IIF was then recorded from the video recordings to generate cumulative frequency of IIF versus temperature curves. The ice-nucleation parameters were also determined by curve fitting the experimental observations of IIF at a cooling rate of 20°C/min to the theoretical predictions of the probability of IIF. It was assumed that the experimental cumulative frequency of IIF was equal to the probability of IIF. This analysis was performed at 20°C/min to minimize the volumetric changes during freezing of mouse embryos and to obtain the ice-nucleation parameters.

Bulk freezing technique

Embryos were placed into PBS/BSA containing 0–2400 mosm choline chloride (ChCl) at 5°C for 3 min and loaded into 0.25-cc straws (ZA121; IMV, Minneapolis, MN). In some of the experiments, ChCl was replaced with NaCl. Straws were loaded with 9-mm freezing solution, 4-mm air, 25-mm freezing solution containing embryos, 4-mm air, and 5-mm freezing solution. The straws were then plugged and placed into a programmable freezer (Planer Kryo 10 Series II; TS Scientific, Perkasie, PA) that was precooled to 5°C. The freezing protocol, beginning at 5°C, was as follows: cooling to −6°C at a rate of 20°C/min; pause for ~1 min for seeding of extracellular ice; cooling to an intermediate temperature between −7 and −15°C at a rate of 120°C/min; pause for a given time period between 1 and 20 min at the intermediate holding temperature; cooling to −45°C at a rate of 120°C/min; pause for 4 min at −45°C. After the final pause, the straws were removed to a room temperature water bath for 4 s to quickly melt the ice and then the embryos were unloaded into a petri dish at 0°C. Embryos were removed from the freezing solution by stepwise transfer at 0°C in 3-min intervals through several solutions of decreasing osmolality using a strategy designed to prevent cell volume expansions in excess of 20%. The highest salt concentration used for freezing embryos was 1.2 M ChCl in PBS (2400 mosm). The thawed embryos were transferred through a series of PBS solutions containing 0.9 and 0.7 M ChCl; then through a series of HTF-Hepes medium containing 0.7, 0.5, 0.4, 0.3, 0.25, 0.20, 0.15, 0.10, and 0.05 M sucrose. Embryos frozen in solutions containing intermediate osmolarities entered into the stepout scheme at their respective osmolarities. After equilibrating for 3 min in HTF-Hepes alone, the culture dish was transferred to a CO_2 incubator for warming to 37°C and continued culture.

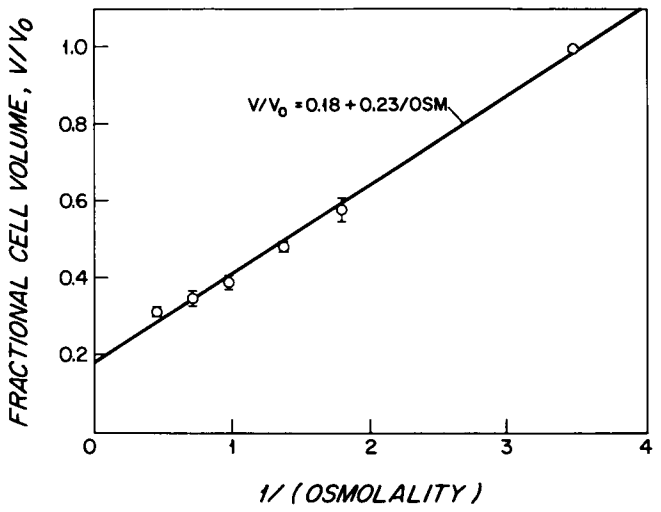


FIGURE 1 Static osmometric behavior of one-cell mouse embryos in various hypertonic PBS/BSA/NaCl solutions at ambient temperature. Six oocytes were used for these experiments. Bars show the standard deviations.

FDA staining procedure

The membrane integrity of embryos after a freeze–thaw cycle was assessed using the vital fluorescent dye, FDA. The assessment of plasma membrane integrity and embryo survival using FDA has been used effectively by other investigators (Damien et al., 1989; Noto et al., 1991). A stock solution of 5 mg/ml FDA was prepared in DMSO and stored frozen at −20°C. The stock solution was diluted 1:200 in freezing solution or HTF-Hepes medium and brought to the same temperature as that of the embryos. Embryos were then transferred into the stain solution for 1 min at ambient temperature, washed in solution or medium without FDA, and observed at 100× using a microscope (model Fluovert FU; Leitz, Weltzlar, Germany) and either epifluorescence or Hoffman modulation contrast optics.

RESULTS

Inactive cell volume for mouse embryos

To integrate Eq. 1 to predict the volumetric response of embryos during freezing, the value of the inactive cell volume, V_b, is needed. The inactive cell volume of one-cell mouse embryos was determined in equilibrium experiments using increasing concentrations of the suspending solution (PBS/BSA/NaCl) as shown in Fig. 1. Regression of the data yields a linear relationship between equilibrium volume and the reciprocal of osmolality. The normalized inactive cell volume, V_b/V_o, for mouse embryos at the one-cell stage was found to be 0.18 by extrapolation of the line to infinite concentration, similar to that determined in previous studies (Leibo, 1980).

Membrane water permeability and activation energy

The formation of extracellular ice increased the solute concentration surrounding the embryos, causing them to dehydrate towards an equilibrium with the external concentration. The permeability of the plasma membrane is related to the rate of dehydration as given by Eq. 1. Cryomicroscopic observations were used to determine the cellular dehydration and hence the water permeability parameters of mouse embryos at the one-cell stage. Fig. 2 shows a series of micrographs during cooling of two, one-cell mouse embryos at a cooling rate of 2°C/min. The volume of embryos was estimated from their projected surface area assuming a spherical shape. Embryos that exhibited large shape deformations because of interactions with extracellular ice were not used in the permeability calculations. Furthermore, for cells that underwent IIF, only the portion of data until IIF was used in permeability calculations. The effects of experimental techniques on the determination of water transport parameters, including the prediction of cellular volume from two-dimensional measurements, are discussed elsewhere (Mazur, 1963; Schwartz and Diller, 1983; Toner et al., 1990). The reference permeability at 0°C, L_{pg}, and the activation energy, E_{Lp}, of mouse embryos were determined simultaneously by fitting theoretically determined volume/temperature curves from Eq. 1 to experimentally measured volumes of embryos (Fig. 3) between 0 and −20°C using nonlinear regression analysis (Toner et al., 1990). A curve fit was performed separately for each individual mouse embryo and then the calculated permeability parameters were averaged. Values of the permeability parameters were adjusted by iteration until the change in the chi-squared statistics χ^2 between successive iterations converged to $\Delta\chi^2/\chi^2 < 0.01$. The χ^2 between the curve fit and experiments was always $<10^{-3}$. The mean value and standard deviation of L_{pg} was found to be 0.08 ± 0.03 $\mu m^2/\mu m^3$ per min per atm for one-cell mouse embryos. The mean value and standard deviation of E_{Lp} was found to be 19.3 ± 4.4 kcal/mol.

Intracellular ice-nucleation parameters

The numerical values of the parameters, Ω_o and κ_o, were estimated from the experimental measurements of the cumulative incidence of IIF, which is assumed to be equivalent to the theoretical probability given in Eq. 2. IIF could be easily observed using the cryomicroscopy system because of the sudden darkening of the cytoplasm. The embryos darkened dramatically in the presence of IIF, presumably because of the formation of very small ice crystals. A cooling rate of 20°C/min was used for these experiments to decouple the effects of dehydration from ice-nucleation, which allows the determination of the nucleation parameters independently from the water permeation process. At this cooling rate, both experimental observations (data not shown) and theoretical predictions (see Fig. 5) showed minimal cellular dehydration (~5%) between 0 and −10°C and the effect of this small volume change on the determination of IIF parameters was negligible. Values of ice-nucleation parameters were obtained by pooling data from five experiments with two to five embryos per experiment (Fig. 4). Correlation of experimental results at a cooling rate of 20°C/min with Eq. 2 using nonlinear regression analysis yields the values 0.8×10^8 ($1/m^2$ per s) and 7.8×10^9 (K^5) for Ω_o and κ_o, respectively. Values of the IIF parameters were adjusted by iteration until the change in the chi-squared statistics χ^2 between successive iterations converged to $\Delta\chi^2/\chi^2 < 0.01$. The χ^2 between the curve fit and experiments was always $<10^{-4}$.

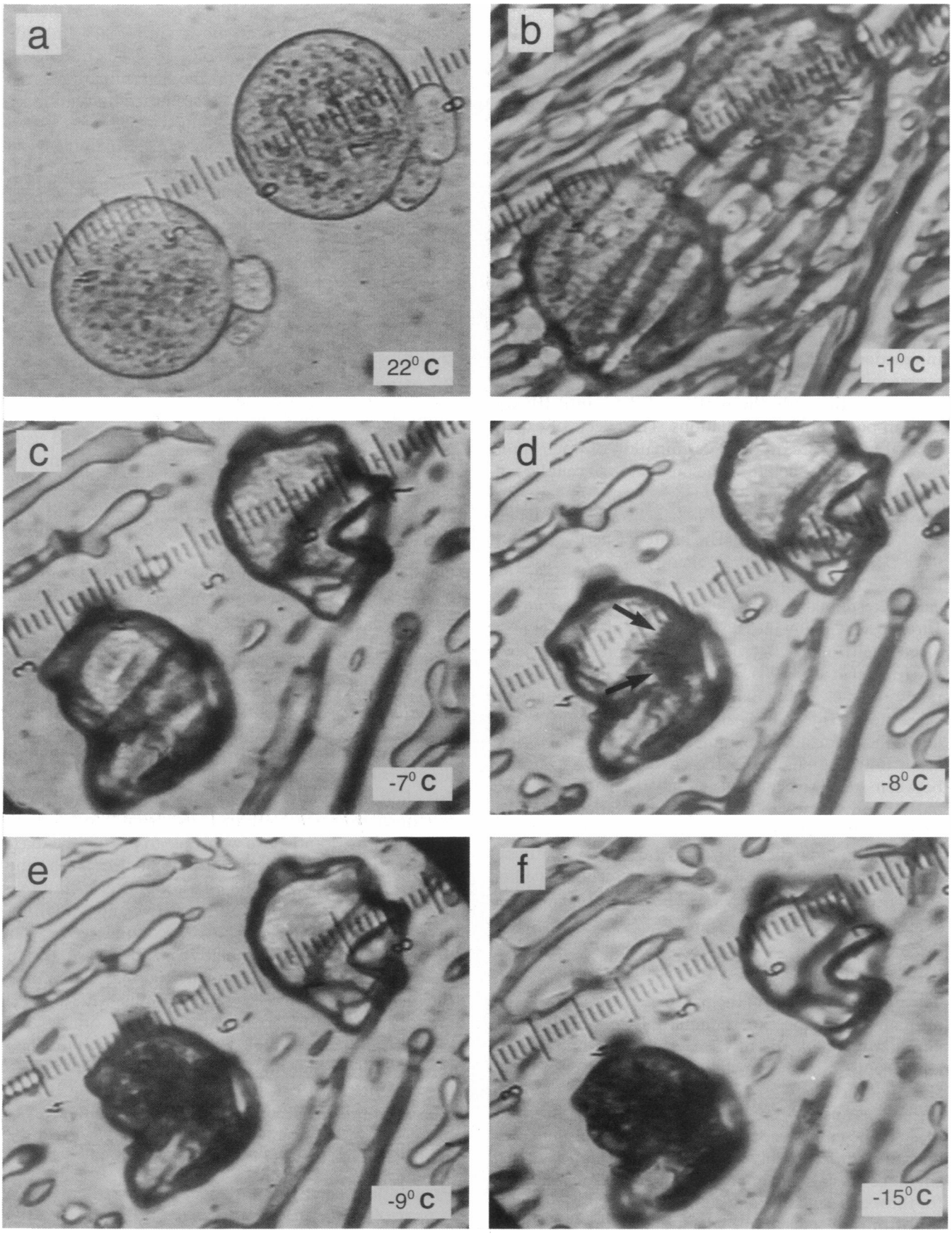
Prediction of cellular dehydration and IIF at constant cooling rates

Simulations of water transport from mouse embryos during cooling at different rates were performed using the water permeability parameters obtained from cryomicroscopic observations and Eq. 1. Fig. 5 shows the strong effect of the cooling rate on the dehydration response of embryos. Even at a cooling rate as slow as 1°C/min, >20% of the water remained in the embryos. On the other hand, the dehydration was almost complete at −8°C for a cooling rate of 0.5°C/min. We also calculated the volumetric response of embryos using the permeability parameters obtained from suprazero measurements (Leibo, 1980). The comparison is shown in Fig. 5 for a cooling rate of 5°C/min. The difference between the two curves were relatively small with a maximal difference of <10% at −18°C.

Since it is known that IIF is associated with lethal injury to cells (Mazur, 1963, 1977; Rall et al., 1983), it is especially important to determine the critical cooling rate range over which IIF increases from 0 to 100%. By coupling Eqs. 1 and 2, we were able to predict the maximum cumulative incidence of IIF (i.e., PIF) as a function of the cooling rate for a final cooling temperature of −45°C. This final temperature was low enough for embryos to either completely dehydrate or undergo IIF. The predicted maximum cumulative incidence of IIF increased from 0% at 1°C/min to 100% at 5°C/min is shown in Fig. 6. Experimental results with one-cell mouse embryos under similar conditions are also depicted in Fig. 6, showing good agreement with the theoretical predictions. Although CPA were not used in these experiments, the critical cooling rate inducing IIF was consistent with the conventional equilibrium freezing protocols, which usually use cooling rates between 0.5 and 1°C/min (Freidler et al., 1988).

Prediction of an optimal nonequilibrium freezing protocol

Using the theoretical model with the parameters (L_{pg} and E_{Lp}, and Ω_o and κ_o) described above, we attempted


a
22° C
b
-1° C
c
-7° C
d
-8° C
e
-9° C
f
-15° C

to design an optimal rapid freezing protocol for mouse embryos. The protocol consisted of a rapid cooling rate of 120°C/min down to −45°C with a holding period at an intermediate temperature for a given time to afford cellular dehydration and reduce the likelihood of IIF. The final temperature of −45°C was chosen because the cells were either completely dehydrated or underwent IIF above this temperature. The cooling rate of 120°C/min was about two orders of magnitude faster than the cooling rates used in conventional equilibrium freezing protocols for embryos (Freidler et al., 1988). The model was used to optimize three variables in the freezing protocol: the intermediate holding temperature, the holding time at this intermediate temperature, and the initial ChCl concentration of the suspending solution. In the calculations, it was assumed that IIF resulted in cell death and loss of membrane integrity. Thus, the theoretical survival (i.e., membrane integrity) was estimated to be (100 − PIF)%.

Effect of the Intermediate Pause Temperature

To predict the effect of pausing at various intermediate temperatures, the osmolality of the medium was assumed to be 1100 mosm, which caused an initial dehydration of ~41% as predicted from Fig. 1. In addition, the holding period at the variable intermediate temperature was fixed at 5 min. The predicted behavior shown in Fig. 7 indicates the effect of the intermediate temperature of pause on membrane integrity. The maximum viability was obtained between −6 and −10°C. At temperatures above and below this range, the model predicts that embryos undergo IIF and the membrane integrity decreases accordingly. For holding temperatures > −6°C, the chemical driving force for dehydration is apparently not enough to assure complete dehydration, and thus the prediction is that the embryos undergo IIF during the subsequent cooling process to −45°C. For example, the normalized volume at −4°C after 5 min dehydration was ~30%, which corresponded to ~12% normalized water remaining in the embryo given that the 18% of the total volume was inactive as shown in Fig. 1. This remaining water content crystallized during the subsequent cooling to −45°C yielding cell damage. For holding temperatures < −10°C, IIF occurred during the initial cooling period at 120°C/min. However, the holding temperatures between ~ −6 and −10°C avoided lethal IIF both during the initial cooling and the subsequent 5-min dehydration periods. At the end of 5-min dehydration at −10°C, the normalized total cell volume was ~22%, yielding a normalized water content of <5%. Since we assumed that IIF did not occur for water content <5%, the subsequent cooling to −45°C did not yield IIF and the embryos survived, according to the model predictions.

Effect of the holding time at −10°C

In a second set of calculations, we investigated the effect of the length of pause at −10°C. For these studies, we assumed that the embryos were predehydrated in 1100-mosm ChCl solutions. The results of these predictions displayed a sharp transition zone over wherein the predicted membrane integrity increased from 0% at 3 min to 100% at 4 min as shown in Fig. 8. The cellular dehydration was incomplete for holding times of <3 min, increasing the water content of the cells and promoting IIF during subsequent cooling. For example, the normalized cell volume was ~35% (i.e., ~20% normalized water volume) for a dehydration period of 1 min at −10°C, leaving an adequate amount of water to nucleate ice during the final cooling step of 120°C/min to −45°C. On the other hand, for holding periods of ≥4 min, the normalized cell volume was <22% (i.e., <5% normalized water volume) and thus IIF was avoided in the subsequent cooling step to −45°C.

Effect of the initial osmolality

In a last series of experiments, the role of initial osmolality of the suspending solution was investigated using the theoretical model. The cooling procedure was kept constant using a cooling rate of 120°C/min down to −45°C with a 5-min holding period at −10°C. Fig. 9 depicts the effect of the initial osmolality on the predicted membrane integrity. As can be seen from this figure, the viability decreased dramatically for initial suspending solution osmolalities of <800 mosm. This was mainly because IIF occurred during cooling to −10°C at a rate of 120°C/min in initial concentrations <600 mosm. For concentrations between 600 and 800 mosm, IIF occured mostly during the 5-min holding period at −10°C. For higher concentrations, IIF was completely suppressed and the survival was 100% as predicted by the model.

Experimental verification of the model predictions

To test the model predictions, fertilized one-cell embryos were frozen using the three-step protocol. Briefly, the embryos were dehydrated before the beginning of the freezing protocol by suspending them in hypertonic solution containing ChCl. Subsequently, the effects of the intermediate holding temperature, the holding time at that temperature, and the concentration of the initial suspending media were tested experimentally.

To test the effect of the intermediate holding temperature, one-cell embryos in groups of 15 to 20 were equili-

FIGURE 2 A series of micrograph showing the freezing response of two, one-cell mouse embryos. The extracellular ice was seeded at ~ −1°C with subsequent cooling at a rate of 2°C/min. The suspending solution was isotonic PBS/BSA. The bottom embryo underwent IIF, as evidenced by the blackening of the cytoplasm, at a temperature of −8°C. The darkened area rapidly spread across the whole embryo as temperature declined. On the other hand, the top embryo escaped IIF and dehydrated in response to increased salt concentration in the partially frozen extracellular solution during freezing.

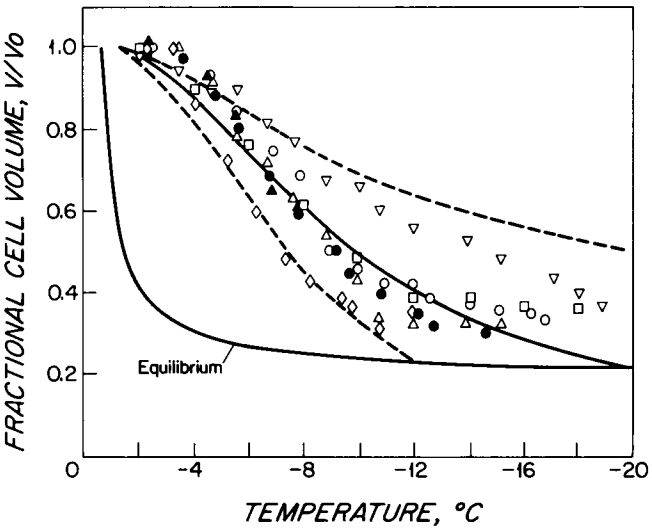


FIGURE 3 Experimental measurements of normalized total volume at various temperatures for one-cell mouse embryos during cooling at a rate of 2°C/min. Each of the seven symbols refers to an individual experiment using a single embryo. The solid line is the theoretical prediction using the mean permeability parameters from each of these individual experimental measurements. The top dashed line is the prediction using the upper limit of the permeability (i.e., L_{pg} + SD and E_{Lp} − SD), and the bottom dashed line is the prediction using the lower limit of permeability (i.e., L_{pg} − SD and E_{Lp} + SD). The equilibrium curve refers to the behavior of embryos cooled at an infinitely slow rate and indicates the deviation from equilibrium conditions for embryos cooled at 2°C/min.

brated at 0°C in PBS/BSA adjusted to 1100 mosm with ChCl, loaded into straws, and cooled to −6°C for extracellular ice seeding. Embryos were brought to various intermediate temperatures at 120°C/min and held there for 5 min before further cooling at 120°C/min to −45°C. After thawing the embryos were stained with FDA to determine whether their cell membranes were intact. The percentage of embryos in each group with intact membranes are shown as the mean of two to four replicate samples (Fig. 7). The experimental results indicated an optimal holding temperature of −10°C, which was in reasonable agreement with the model predictions. The membrane integrity of embryos was ~80% for an intermediate holding temperature of −10°C as assessed by FDA. For temperatures below and above this critical temperature, the membrane integrity of the embryos decreased similarly to model predictions.

We then tested the effect of the holding time at −10°C. For these experiments, one-cell embryos, in groups of 18 to 23, were cooled to −45°C at 120°C/min with a pause at −10°C for 1–20 min. The percentage of embryos with intact membranes, as determined using FDA staining, are shown as the mean of one to three replicate samples in Fig. 8. Experimental observations showed a trend similar to theoretical predictions with maximum membrane integrity being reached at ~5 min. Embryo survival dropped precipitously as the holding time was decreased below 5 min, and it remained approximately constant at 90% for holding periods between 5 and 20 min.

Finally, we verified the model predictions on the effects of suspending solution concentration of ChCl. Embryos in groups of 20 to 180 were cooled to −45°C in PBS/BSA containing 0–2400 mosm ChCl using an isothermal holding step of 5 min at −10°C. Experimental results are shown as the mean percentage of embryos with intact membranes for 1–13 replicate samples (Fig. 9). For initial osmolalities <1000 mosm, the membrane integrity of one-cell embryos was compromised. Theoretical predictions were in reasonable agreement with these experimental observations as shown in Fig. 9. In addition to ChCl, we performed a set of experiments with NaCl as the solute to increase the osmolality of the suspending solution. When one-cell embryos were frozen in NaCl-supplemented PBS/BSA solutions, embryos were completely damaged as assessed by FDA staining for all initial concentrations.

Embryo development after freezing

The protocol derived from the predictions of our thermodynamic model permitted embryo freezing in the absence of CPAs without causing significant damage to the plasma membrane. We next determined the subsequent viability of the frozen–thawed embryos. Embryos following the optimized three-step rapid freezing to −45°C

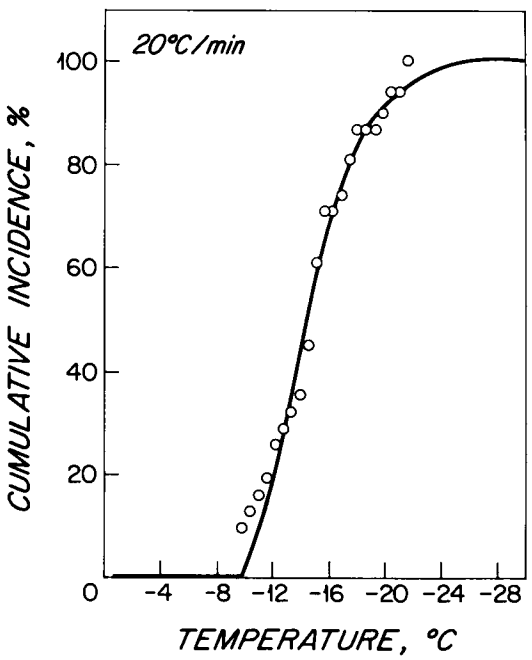


FIGURE 4 Temperature dependence of the cumulative fraction of one-cell mouse embryos with IIF. The cooling rate was 20°C/min in isotonic PBS. This rapid cooling rate was chosed to prevent cellular dehydration and decouple the two complex processes of IIF and exosmosis, permitting the independent analysis of IIF. These experiments were used with Eq. 2 to determine the two unknown heterogeneous nucleation parameters.

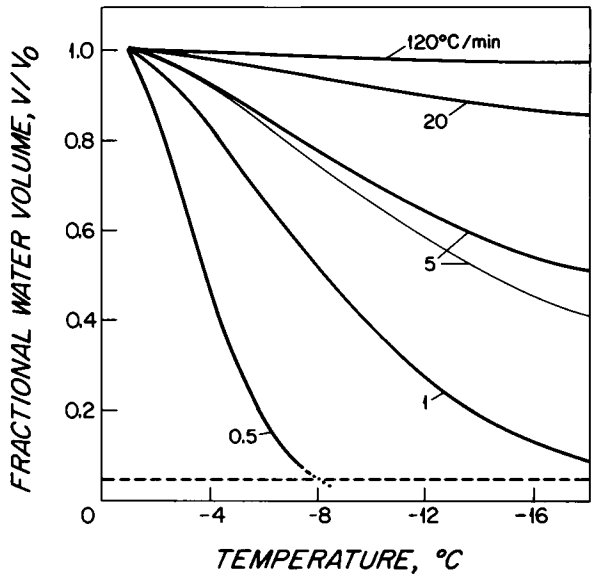


FIGURE 5 Predicted temperature dependence of theoretical normalized water volume for one-cell mouse embryos cooled at various rates. The normalized water content was predicted from the normalized total volume and the normalized inactive cell volume as follows: $V_w/V_{wo} = (V - V_b)/(V_o - V_b)$. The horizontal dashed line refers to 5% water volume where we assumed that the probability of IIF was zero. The thin line for a cooling rate of 5°C/min was obtained using the supra-zero permeability parameters determined by Leibo (1980).

were removed from the freezing solution and cultured. Extensive damage occurred when embryos were transferred directly to medium, most likely because of a rapid swelling of the cells. Therefore, a stepwise procedure was developed to reduce osmotic stress, as described in Materials and Methods. Using this stepwise procedure, membrane damage was minimal, both initially and after step-out, at all concentrations >1200 mosm. After overnight culture, it appeared that embryos frozen in 1200 mosm had better viability than that observed for all other concentrations tested (data not shown). Approximately 50% of the embryos surviving initial thawing in 1200 mosm became damaged after overnight culture. Figs. 10 and 11 show the morphology and FDA staining of embryos immediately after freezing and after removal to HTF-Hepes, ~45 min later. Many embryos remained intact, as evidenced by their staining with FDA, and osmotically readjusted to their original size after the step-out process. Embryos that were damaged could be identified by morphological examination, as well as with FDA staining.

Although many embryos frozen and thawed according to our protocol had intact plasma membrane and displayed no evidence of membrane damage, they were not developmentally competent. We only occasionally obtained two-cell embryos and never later stages. Therefore, we conducted an experiment to determine the temperature at which the damage to development potential was occurring. Groups of 66–134 embryos were cooled in 1200 mosm solution prepared with ChCl, according to the optimized protocol, thawing the embryos after the 5-min pause at −10°C or after further cooling to various temperatures. Embryos exposed to ChCl-supplemented freezing solution for 20 min developed to blastocyst at a frequency of almost 80% (Fig. 12). However, embryos frozen to −10°C and held for 5 min developed normally only to the two-cell stage and thereafter became progressively poorer at reaching the appropriate stage of development (Fig. 12). Development to the two-cell stage was first affected after −25°C, with only 30% of the embryos reaching that stage after exposure to −30°C. Although a few blastocysts were obtained after cooling to −20 and −30°C, damage was clearly occurring to many of the embryos at −10°C.

DISCUSSION

The low temperature behavior of one-cell mouse embryos was investigated using cryomicroscopy and thermodynamic modeling to determine their membrane permeability and ice-nucleation characteristics. Reference water permeability of the plasma membrane at 0°C and its temperature dependence were determined to be 0.08 $\mu m^2/\mu m^3$ per min per atm and 19.3 kcal/mol at a cooling rate of 2°C/min between 0 and −10°C, respectively. The ice-nucleation parameters were estimated from rapid cooling experiments at a rate of 20°C/min in the absence of water efflux to decouple the two processes of

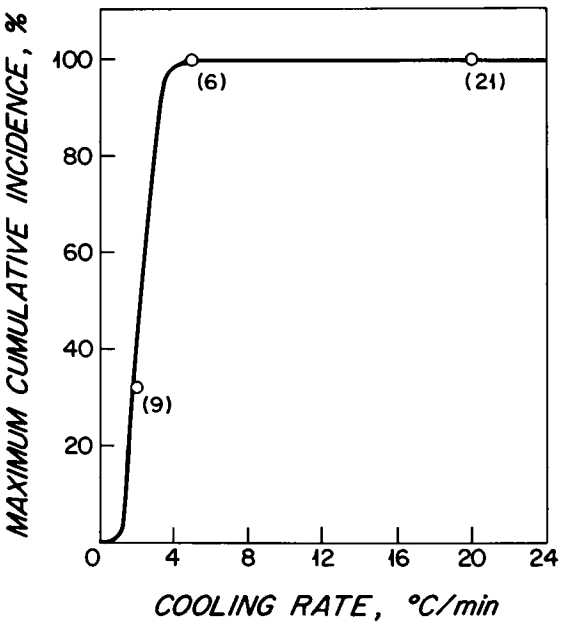


FIGURE 6 Prediction of maximum cumulative incidence of IIF for one-cell mouse embryos cooled down to −45°C as a function of the cooling rate. Circles refer to cryomicroscopic observations at three different cooling rates to verify the model predictions. The numbers in parenthesis are the numbers of embryos used for each experimental condition.

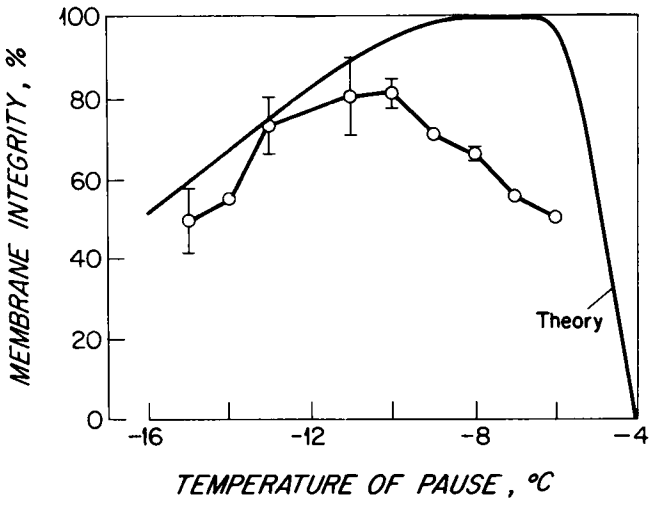


FIGURE 7 Effect of isothermal holding temperature on predicted and measured embryo membrane integrity during freezing in 1,100 mosm PBS/ChCl solution. The cooling rate was 120°C/min from −6 to −45°C with an intermediate holding period of 5 min at a variable temperature. The predicted membrane integrity of embryos, (100 − PIF)%, for various holding temperatures is shown by the solid line. Open circles denote our experimental results under similar conditions. Embryos were frozen–thawed under those conditions, and the membrane integrity was assessed by staining with FDA. The percentage of embryos in each group with intact membranes are shown as the mean of one to four replicate samples. Error bars representing the standard error are shown for samples containing replicates.

IIF and dehydration. The kinetic (or preexponential) parameter was 0.8×10^8 ($1/m^2$ per s) and the thermodynamic (or exponential) parameter was 7.8×10^9 (K^5). We then used the model to design a nonequilibrium rapid freezing protocol for one-cell mouse embryos. The protocol consisted of an initial rapid cooling at 120°C/min from −6 to −10°C, 5-min holding period at −10°C, followed by a subsequent rapid cooling at 120°C/min down to −45°C in an initial suspending ChCl solution of 1,100 mosm. Our model predictions were then verified using bulk freezing techniques. Theoretical predictions were in good agreement with the experimental measurements, suggesting that mouse embryos could be frozen in the absence of CPAs without damaging their membranes.

Biophysical parameter evaluation

The biophysical parameters obtained in this study are crucial for understanding the cellular response to a given freezing stress. Leibo (1980) reported a value of 0.43 $\mu m^2/\mu m^3$-min-atm for reference permeability at 20°C (L_{pg}) and a value of 13 kcal/mol for activation energy (E_{Lp}) of one-cell mouse embryos at suprazero temperatures. By extrapolating Leibo's value of L_p to 0°C, the reference temperature used in this study, one finds L_{pg} to be ~0.08 $\mu m^2/\mu m^3$-min-atm, which is similar to our subzero measurement of 0.08 $\mu m^2/\mu m^3$ per min per atm.

On the other hand, the activation energy of the water permeability (19.3 kcal/mol) obtained from subzero measurements in this study is higher than the value of 13 kcal/mol obtained by Leibo (1980) from suprazero observations. Fig. 5 shows the predicted response of mouse embryos cooled at 5°C/min using the suprazero permeability parameters from Leibo (1980). As can be seen from this figure, the discrepancy in the volumetric response of mouse embryos predicted using the supra- and subzero permeability parameters is <10% between 0 and −18°C. This study assumed that the surface area of an embryo was proportional to its volume throughout its dehydration. Leibo assumed that as the embryo shrinks the surface area remains constant (Leibo, 1980). Given the differences in the experimental conditions and techniques, we believe that it is not appropriate to conclude that there are real differences between suprazero and subzero permeability parameters of one-cell mouse embryos. Similar results have also been reported for unfertilized metaphase II mouse oocytes (Leibo, 1980; Toner et al., 1990). On the other hand, several other cellular systems including *Drosophila melanogaster* embryos (Lin et al., 1989; Pitt et al., 1991) and granulocytes (Schwartz and Diller, 1983) showed a three- to fivefold increase in E_{Lp} at subzero temperatures. Further studies are warranted with respect to the exact interaction of the external ice with the plasma membrane and its role in the transport of water molecules across the plasma membrane.

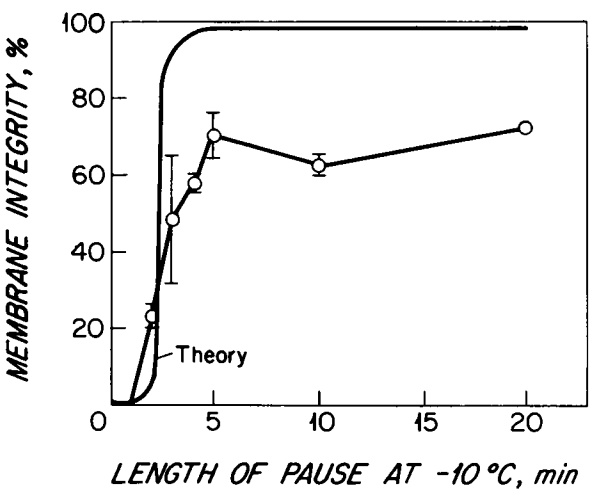


FIGURE 8 Effect of length of time at an isothermal holding temperature of −10°C on embryo membrane integrity during freezing in 1,100 mosm PBS/ChCl solution. The model predictions are shown by the solid line. The cooling rate between −6 and −45°C was 120°C/min with an intermediate holding temperature of −10°C. The embryo membrane integrity was predicted from (100 − PIF)%. Open circles denote the experimental results under similar conditions. One-cell embryos in groups of 18 to 23 were cooled to −45°C with a pause at −10°C for 1–20 min. The percentage of embryos with intact membranes, as determined using FDA staining, is shown as the mean of one to three replicate samples. Error bars representing the standard error are shown for samples containing replicates.

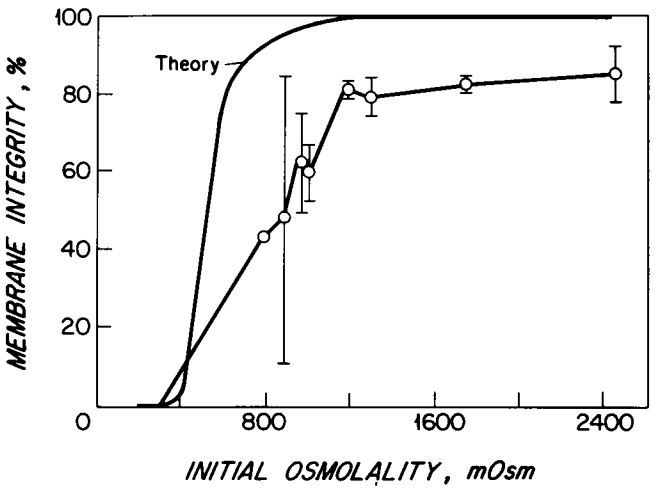


FIGURE 9 Effect of osmolarity of the suspending medium on embryo membrane integrity during freezing. The solid line represents the model predictions, (100 − PIF)%, for various initial suspending solution osmolarities. A cooling rate of 120°C/min from −6 to −45°C with a 5-min pause at −10°C. Open circles represent the experimental results under similar conditions. Embryos in groups of 20–180 were cooled to −45°C in PBS/BSA containing 0–2,400 mosm ChCl. The percentage of embryos with intact membranes, as determined using FDA staining, is shown as the mean of 1 to 13 replicate samples. Error bars representing the standard error are shown for samples containing replicates.

We also investigated the IIF characteristics of one-cell mouse embryos using the cryomicroscopy system. The values of both the preexponential and exponential terms were in the same range as the previous results with mouse oocytes (Toner et al., 1990). The preexponential kinetic term that basically describes the addition of a water molecule to the critical size cluster is 0.8×10^8 ($1/m^2$ per s) for one-cell mouse embryos as opposed to 3.6×10^8 ($1/m^2$ per s) for mouse oocytes. And, the exponential term that describes the activation barrier to ice-nucleation is 7.8×10^9 (K^5) for fertilized mouse embryos compared with 4.6×10^9 (K^5) for mouse oocytes. The mean temperature of IIF was determined from the cumulative frequency of IIF given in Fig. 4 to be ~ -15°C for one-cell mouse embryos cooled at 20°C/min. This value was slightly lower than the mean temperature of IIF for mouse oocytes, which was determined to be ~ -12°C (Toner et al., 1992).

Using the water permeability and ice-nucleation parameters, the threshold cooling rate range over which the probability of IIF increases from 0% at ~1°C/min to 100% at ~4°C/min was determined to be similar for both fertilized and unfertilized mouse ova. These results suggest that the overall cellular response to freezing is independent of the fertilization process. Thus the relatively poor attempts in cryopreserving unfertilized eggs are most likely due to their sensitivity to damaging freezing stresses and not due to the changes in the plasma membrane water transport and/or ice-nucleation parameters. Some recent studies investigating the sensitivity of the spindle to exposure to CPAs and to cold suggest that the spindle may be one of the sites of freezing injury in oocytes (Johnson and Pickering, 1987; Van der Elst, 1988).

Design of freezing protocols

It was shown in the mid-1950s and in some later studies that cells can survive rapid freezing to ≤ -70°C when the rapid freezing was interrupted by a short holding period at an intermediate temperature (Luyet and Keane, 1955; Taylor, 1972; Farrant et al., 1974; Walter et al., 1975; McGann and Farrant, 1976). However, the design of these protocols requires optimization of a large matrix of variables. Models to predict IIF are needed to help design these complex multistep freezing protocols (Toner et al., 1990; Pitt, 1992). In this study, we used the heterogeneous intracellular ice-nucleation model to design a three-step rapid-freezing protocol. The designed and experimentally tested protocol used a cooling rate of 120°C/min with a 5-min holding period at −10°C and requires <10 min, reducing drastically the time of exposure to damaging freezing stresses. This protocol was referred as nonequilibrium because the rapid cooling at 120°C/min prevents embryos from reaching thermodynamic equilibrium with the partially frozen extracellular solution during the freezing protocol, including the period of dehydration at −10°C, with the only exception at the end of 5 min of dehydration at −10°C. On the other hand, typical conventional slow equilibrium freezing protocols for embryos use a cooling rate of 0.5°C/min down to ~ −60°C and require ≥2 h for completion. Furthermore, the results obtained were encouraging given the fact that this is the first study to suggest that embryos can be cooled at 120°C/min in the absence of CPAs to subzero temperatures and recovered with 80% membrane integrity. This study clearly demonstrates the power of the quantitative integrated theoretical and experimental approach in optimizing cryopreservation protocols. In a recent study, Pitt (1992) published an elegant analysis underlying the advantages of using nonlinear cooling protocols to maximize the survival for a given cell type. Although the application of nonlinear and/or nonequilibrium freezing protocols require precise control of the thermal history of the sample, the benefits gained by increasing the survival easily justify the use of these new approaches to cryopreservation.

Although the embryos were viable after a freeze–thaw cycle as assessed by FDA, the further development of embryos was impaired, most likely because of the complete absence of CPAs, which are known to stabilize cellular components and membranes. The rationale behind omitting the use of CPAs in this study was threefold. First, we wanted to test the model predictions for the simplest possible case. The presence of CPAs is known to alter both the water permeability and the ice-nucleation characteristics of cells (Rall et al., 1983; Mazur, 1990).

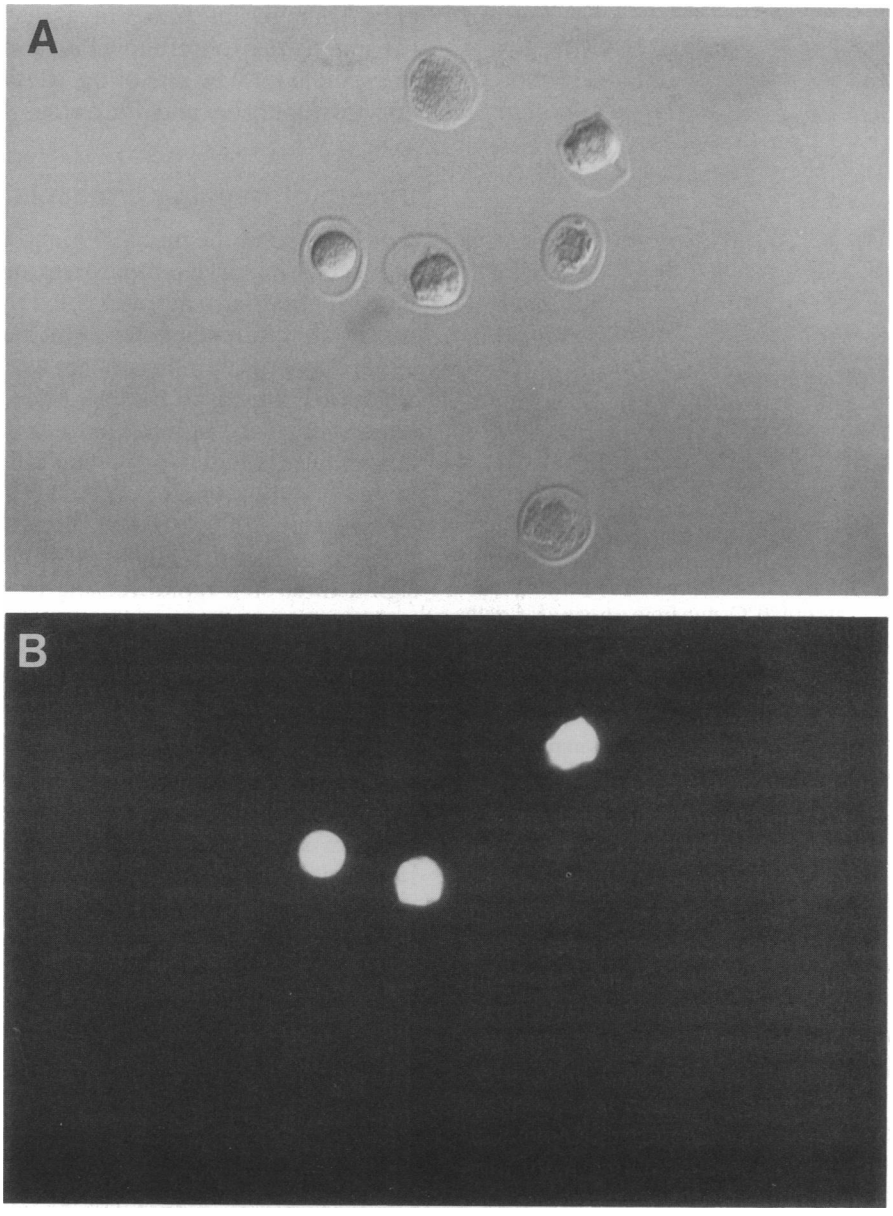


FIGURE 10 Six, one-cell mouse embryos after rapid nonequilibrium freezing in the absence of CPAs immediately after thawing: (*A*) morphological appearance and (*B*) fluorescent staining with FDA. Embryos were transferred into the FDA stain solution for 1 min at ambient temperature, washed in solution without FDA, and observed with the fluorescent microscope.

Second, the consensus in the field of cryobiology is that embryos cannot be frozen in the absence of CPAs. However, in this study we obtained reasonable viability after a freeze–thaw cycle by using rapid nonequilibrium freezing protocols. Obviously, more studies needed to ameliorate the viability results; however, this study clearly demonstrates the power of using quantitative approaches to the design of nonequilibrium freezing protocols, even in the absence of CPAs. Third, the absence of CPAs make the freezing procedure much easier. Given all these we decided to optimize a freezing protocol in the absence of CPAs and verify experimentally the model predictions. The methodology developed in this study can now be extended to other cells and to the presence of minimal quantitaties of CPAs.

Another interesting observation from this study was the complete membrane damage that occurred when ChCl was replaced with NaCl. High concentrations of electrolytes have been shown to correlate with cellular damage (Lovelock, 1953). In this study, we showed that this may be true for rapid-freezing protocols as well. Since ChCl has the same colligative properties as NaCl (i.e., the same freezing point depression, data not shown), these experiments directly demonstrate the possible damaging effects of NaCl on embryos. The exact nature by which the damage occurs is not clear. It is

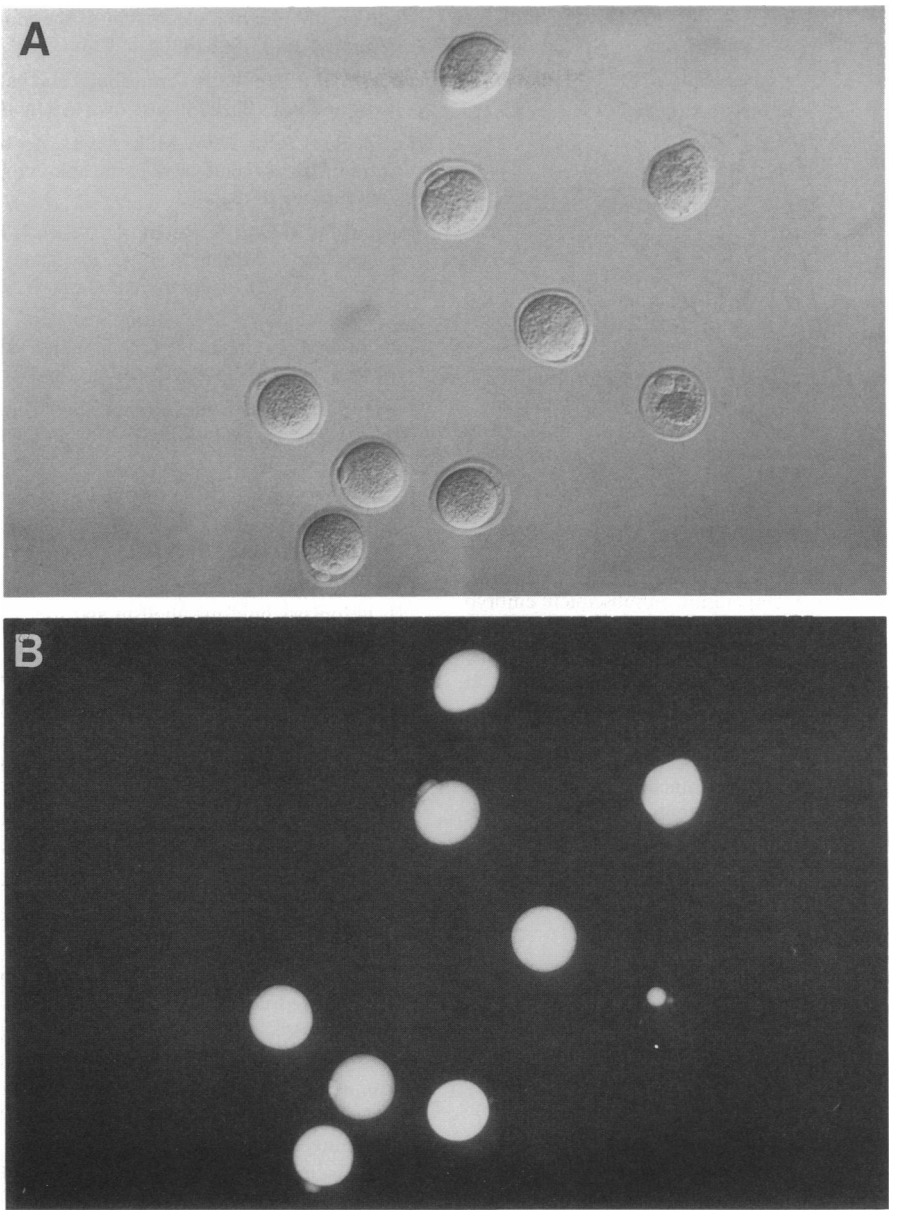


FIGURE 11 Nine, one-cell mouse embryos after rapid nonequilibrium freezing in the absence of CPAs after step-out transfer to HTF-Hepes medium: (*A*) morphological appearance and (*B*) fluorescent staining with FDA. Embryos were first stepped-out of the freezing solution into HTF-Hepes medium as described in detail in the text. Embryos were then transferred into the FDA stain HTF-Hepes medium for 1 min at ambient temperature, washed in medium without FDA, and observed with the fluorescent microscope.

plausible that NaCl may cause irreversible damage to membranes, such as desorption of membrane proteins at low temperatures (McGrath, 1977). It is also very likely that there may be some overload of NaCl during freezing which may in turn lead to cellular damage subsequent to a freeze–thaw cycle (Meryman, 1970). Since there are no channels for the transport of ChCl across the plasma membrane, the penetration of ChCl to the intracellular milieu is less likely. Although there may be other explanations for the protective action afforded by ChCl such as direct interactions of ChCl with the plasma membrane lipids (Lynch et al., 1979), the approach developed in this study offers a reproducible freezing technique in the absence of CPAs to investigate various mechanisms of freezing damage at a fundamental level.

In summary, we developed a rapid nonequilibrium freezing protocol for embryos on the basis of their dehydration and IIF characteristics and tested this protocol with bulk freezing experiments. Results showed that embryos can be frozen and thawed with ~80% membrane integrity in the absence of CPAs. Further refinement of this protocol is necessary to retain the developmental competence of the embryos. This study clearly demonstrated the advantages of nonequilibrium rapid cooling

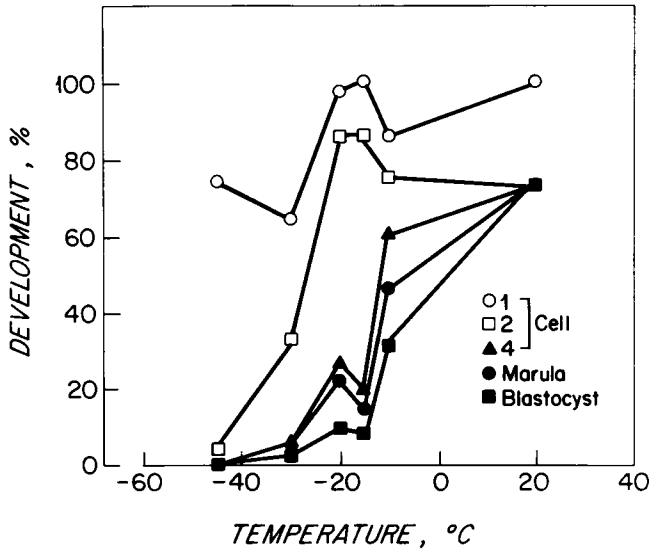


FIGURE 12 Effect of final freezing temperature on subsequent embryo development. Groups of 66–134 one-cell mouse embryos were cooled in 1,200 mosm freezing solution prepared with ChCl, according to the optimized protocol, thawing the embryos after the 5-min pause at −10°C or after subsequent cooling to various temperatures. The embryos were stepped into HTF-Hepes and cultured for 4 d. Each day (day 1 = day of the freezing) the percentage of embryos reaching the appropriate stage of development were determined. Shown here are the percentage of embryos completing development to the one-cell (survived freezing), two-cell (day 2), four-cell (day 3), morula (day 4), or blastocyst (day 5) stage after cooling to various final temperatures.

protocols for the freezing of mouse embryos and indicated that the refinement of these techniques in the future may help to minimize freezing-associated damage mechanisms.

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