

## Determination of the water permeability ( $L_p$ ) of mouse oocytes at $-25\text{ }^\circ\text{C}$ and its activation energy at subzero temperatures $\star, \star\star$

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### ABSTRACT

Typically, subzero permeability measurements are experimentally difficult and infrequently reported. Here we report an approach we have applied to mouse oocytes. Interrupted cooling involves rapidly cooling oocytes ( $50\text{ }^\circ\text{C}/\text{min}$ ) to an intermediate temperature above the intracellular nucleation zone, holding them for up to 40 min while they dehydrate, and then rapidly cooling them to  $-70\text{ }^\circ\text{C}$  or below. If the intermediate holding temperature and holding time are well chosen, high post thaw survival of the oocytes is possible because the freezable water is removed during the hold. The length of time required for the exit of the freezable water allows the water permeability at that temperature to be determined. These experiments used 1.5 M ethylene glycol in PBS and included a transient hold of 2 min for equilibration at  $-10\text{ }^\circ\text{C}$ , just below the extracellular ice formation temperature. We obtain an  $L_p = 1.8 \times 10^{-3}\text{ }\mu\text{m min}^{-1}\text{ atm}^{-1}$  at  $-25\text{ }^\circ\text{C}$  based on a hold time of 30 min yielding 80% survival and the premise that most of the freezable water is removed during the 30 min hold. If we assume that the water permeability is a continuous function of temperature and that its  $E_a$  changes at  $0\text{ }^\circ\text{C}$ , we obtain a subzero  $E_a$  of 21 kcal/mol; higher than the suprazero value of 14 kcal/mol. A number of assumptions are required for these water loss calculations and the resulting value of  $L_p$  can vary by up to a factor of 2, depending on the choices make.

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The success of the classical slow cooling approach to cryopreservation relies on the fact that lethal intracellular ice formation (IIF) can be avoided if cells are cooled slowly enough so that osmotic dehydration can reduce the amount of unfrozen intracellular water to near the equilibrium value, and to do so before the cells reach the temperature at which intracellular nucleation becomes probable. Many cells types can be preserved by this method once “slow enough” has been defined by experiment or modeling [25].

Two critical factors that determine the degree to which cells shrink during cooling at various rates to subzero temperatures are the water permeability or hydraulic conductivity,  $L_p$ , and its temperature coefficient or activation energy,  $E_a$ . Between about  $+30$  and  $0\text{ }^\circ\text{C}$ , their values have generally been estimated by placing cells in hypertonic or hypotonic solutions of non-permeating solutes like NaCl or sucrose and measuring the rate of cell shrinkage or swelling, respectively, at constant temperature. The assumption

has generally been that the water permeability values obtained from above-zero cell volume measurements can be extrapolated to subzero temperatures. That assumption might not be correct. Few direct measurements of cell volume have been made at sub-freezing temperatures because the external ice nearly obscures the cells, and because the cells usually become substantially distorted.

We have recently published data [28] that permit an estimate of  $L_p$  at  $-25\text{ }^\circ\text{C}$  in mouse oocytes, an estimate that is not dependent on knowing the volume of the oocyte at that temperature or the change in cell volume with time. A similar, volume independent method, has previously been reported by Karlsson et al. [17].

The present manuscript reports subzero permeability values and deals with the methods by which they were estimated. It is based on our published data of observations made on intracellular ice formation in mouse oocytes subjected to interrupted rapid cooling using ethylene glycol (EG) as a cryoprotectant. In this procedure, a cell is cooled rapidly to a temperature slightly above the IIF nucleation zone, and is then held at that temperature long enough for it to dehydrate isothermally to near its equilibrium water content before it is cooled rapidly to  $-70\text{ }^\circ\text{C}$  or below. The specific experiments involved rapidly cooling oocytes (8–25 per hold temperature) in 1.5 M EG/PBS to  $-25\text{ }^\circ\text{C}$ , and holding them at  $-25\text{ }^\circ\text{C}$  for 0, 5, 10, 20, 30, or 40 min before subjecting them to a second

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rapid cool to  $-70^{\circ}\text{C}$  and subsequent warming and thawing. The formation of intracellular ice (IIF) is manifested by the cells turning black during the second cool. What we found is that as the holding time was increased from 0 to 30 min, the temporal pattern of IIF changed dramatically. At short holding times (0 and 5 min), nearly all the oocytes flashed during the second rapid cool to  $-70^{\circ}\text{C}$ . At intermediate holding times (10 and 20 min), the visible IIF occurred more and more often during warming and not during cooling. Finally after a hold of 30 or 40 min, no IIF was seen during either cooling or warming, and 80% of the oocytes were viable after thawing based on their morphology and the osmotic integrity of their plasma membranes.

Fig. 1A is a simplified schematic of the experiments. The bottom curve labeled EQ depicts the equilibrium fraction of the water in the external medium that remains unfrozen at the indicated temperatures. The top curve depicts the fraction of the original cell water that remains in a cell subjected to our interrupted rapid cooling procedure. In this simplified version, extracellular ice forms at  $-5^{\circ}\text{C}$ . This causes the water in the cells to be immediately supercooled and have a higher chemical potential than that of the

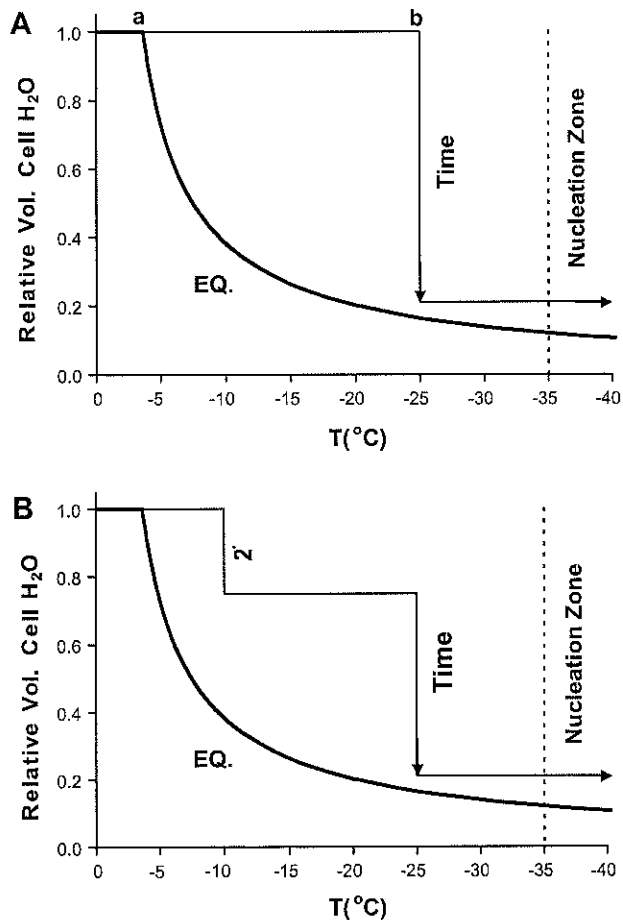
external ice. They are then cooled rapidly to  $-25^{\circ}\text{C}$  at a rate that is too rapid to allow significant osmotic dehydration. Consequently, they arrive at  $-25^{\circ}\text{C}$  still unfrozen and extensively supercooled by an amount corresponding to line a–b. Water that is supercooled is water with a chemical potential higher than that of the external ice, and that in turn sets up an osmotic potential to drive water out of the cell. The rate at which water leaves is proportional to the magnitude of that driving force and the  $L_p$  for the cell at  $-25^{\circ}\text{C}$ . Fig. 1B shows our actual protocol which includes a 2 min hold at  $-10^{\circ}\text{C}$  as discussed later.

Fig. 2 shows the percent of oocytes that manifest no blackening during either cooling or warming, and have osmotically responsive membranes after thawing as a function of the time they were held at  $-25^{\circ}\text{C}$ . We refer to them as survivors. This is based in part on the absence of any IIF, in part on their returning from a shriveled state immediately after thawing to normal volume and morphology upon their warming to  $+20^{\circ}\text{C}$ , and in part because their plasma membranes are intact as evidenced by the fact that in a second rapid cool they remain supercooled to  $<-40^{\circ}\text{C}$  [28]. Oocytes that manifested blackening during either cooling or warming were invariably highly disrupted upon thawing and were unambiguously non-survivors. The maximum percent survivors (80%) occurs with a hold of 30 min. By definition, such cells must have contained little or no freezable water at the end of the hold. The question we posed for ourselves is what value does  $L_p$  have to be at  $-25^{\circ}\text{C}$  in order for the oocytes to have lost nearly all freezable water after a 30 min hold?

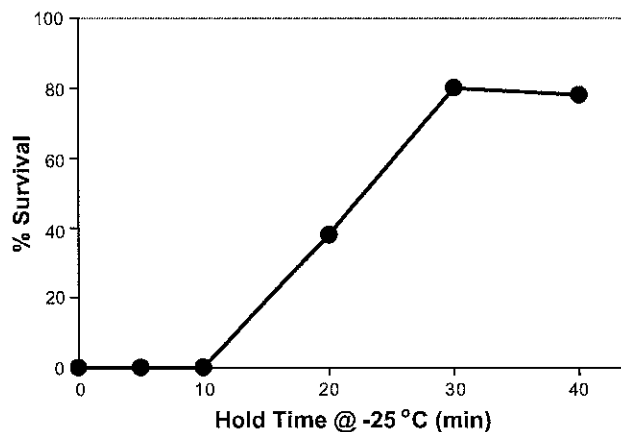
## Methods

### Oocytes

Mature (MII) mouse oocytes were suspended in 1.5 M EG/PBS. Additional details concerning their preparation may be found in Mazur et al. [26]. Fifteen minutes later, the cells were inserted into a Linkam Cryostage (Linkam Scientific Instruments, Surrey, UK) and cooled at  $50^{\circ}\text{C}/\text{min}$  to  $-8^{\circ}\text{C}$  and then at  $5^{\circ}\text{C}/\text{min}$  to  $-10^{\circ}\text{C}$  (EIF occurred at  $-9.08 \pm 0.06^{\circ}\text{C}$ ). Fig. 1B is a schematic of the actual subsequent procedure. It differs from Fig. 1A by showing a 2 min hold at  $-10^{\circ}\text{C}$ . This hold is introduced because we have previously shown [27] that in its absence, IIF occurs at considerably higher temperatures. Some initial cell dehydration occurs during this 2 min hold. Subsequent procedures are as in Fig. 1A. The samples



**Fig. 1.** Cooling protocol. In an idealized version of these experiments (A), oocytes are cooled rapidly ( $50^{\circ}\text{C}/\text{min}$ ) to  $-25^{\circ}\text{C}$  in 1.5 M EG/PBS. Extracellular ice forms at  $\approx -5^{\circ}\text{C}$  and the intracellular water supercools (segment a–b). At  $-25^{\circ}\text{C}$  the cells are held for up to 40 min and then rapid cooling continued to  $-70^{\circ}\text{C}$ , followed by rapid warming back to room temperature. The curve marked EQ shows the water content of the oocytes if they remain in equilibrium with the extracellular water (infinitely slow cooling). In the actual experiments (B), a 2 min hold is introduced at  $-10^{\circ}\text{C}$ , just below the actual extracellular freezing temperature, because this has been found to suppress the ice nucleation temperature of the oocytes. In both figures, the typical temperature below which oocytes in cryoprotectant nucleate is indicated as 'Nucleation zone'.



**Fig. 2.** Survival vs. Holding time at  $-25^{\circ}\text{C}$ . Oocytes are held for 0–40 min at  $-25^{\circ}\text{C}$  using the protocol illustrated in Fig. 1B [28]. Survival is defined as oocytes which did not flash (freeze intracellularly) during either cooling or warming and which had osmotically responsive membranes after thawing. Data from Mazur et al. [28].

were cooled at 50 °C/min to –25 °C and held for 0–40 min. At the end of that hold, they were cooled again at 50 °C/min to –70 °C, and finally warmed to +20 °C at 10 °C/min.

### Permeability analysis

The rate at which water leaves the osmotically shrinking cell (here, at –10 or –25 °C) is proportional to the difference between the total osmolality in the cell and that of the external medium at that temperature; namely,

$$dV_w/dt = L_p * A * R * T * (M_{total}^i - M_{total}^e) * \rho, \quad (1)$$

where  $V_w$  is the volume of cell water ( $\mu\text{m}^3$ ),  $t$  is the time (min), and  $L_p$  is the hydraulic conductivity ( $\mu\text{m min}^{-1} \text{atm}^{-1}$ ) at –10 or –25 °C.  $A$  is the cell surface area ( $17.7 \times 10^3 \mu\text{m}^2$  for the mouse oocyte),  $R$  is the gas constant, and  $T$  is the Kelvin temperature. The subscripts  $i$  and  $e$  refer to the internal and external osmolalities,  $M$ , and “total” means CPA + salt.  $\rho$  is the density of water, taken to be 1 kg/l. It is not normally seen in this equation, but  $\rho$  is required for the equation to be dimensionally correct. This in turn has to do with whether osmolarity or osmolality is used for the driving force. We consider osmolality to be the best measure of the osmotic driving force across the membrane. The value of  $M_{total}^e$  in the partly frozen solution is calculated as  $\Delta T_f/1.86$  where  $\Delta T_f$ , the degrees below 0 °C, is either 10 °C for the 2 min hold at –10 or 25 °C for the variable hold at –25 °C. The value of  $M_{total}^i$  as a function of time is calculated by numerical solution to the following coupled equations (Eqs. (2)–(5)). The starting value of  $V_w$  at –25 °C is taken to be its ending value after the earlier 2 min hold.

The formation of extracellular ice causes an increase in the external concentration of CPA. Here, the CPA is EG and the oocyte is highly permeable to it. Consequently, the increase in its external concentration with freezing produces a driving force for the entrance of EG into the cell. The rate at which it permeates is

$$dn_s/dt = P_s * A * (m_s^e - m_s^i), \quad (2)$$

where  $P_s$  is the permeability coefficient ( $\mu\text{m}/\text{min}$ , converted to  $\text{cm}/\text{min}$  for reporting),  $m_s$  is the CPA molality, and  $m_s^i = n_s^i/(V_w * \rho)$  where  $n_s$  is moles of CPA solute inside the cell and  $\rho$  is the density of water, taken to be 1 kg/l. The value of the external molality of CPA,  $m_s^e$  at –10 and –25 °C is obtained from the ternary phase diagram for EG/NaCl/water (Table 1). The intracellular molality of EG does not remain constant, primarily because of changes in  $V_w$ , but secondarily because of an increase in the number of moles of intracellular CPA from permeation. The precise measure of the driving force for the entry of EG is the difference between the activities of external and internal EG; however, for our purpose the difference in the molalities is an adequate approximation. To link CPA molalities and osmolalities in the equations, we used the simple approximation:

$$M_s = (m_s + c * m_s^2), \quad (3)$$

**Table 1**  
Ethylene glycol test solution.<sup>a</sup>

Temp (°C)	NaCl (molality)	EG (osm)	EG (molality)	R (osm)	R (g_CPA/g_salt)	Total osm (osm)
0 <sup>b</sup>	0.151	0.278	1.636	1.679	11.51	1.957
–10	0.396	0.712	4.289	4.664	11.51	5.376
–25	0.923	1.675	10.007	11.766	11.51	13.441

<sup>a</sup> The subzero solution properties are computed from the phase diagrams in Kleinhans and Mazur [19].

<sup>b</sup> The starting solution is 1.5 M EG in phosphate buffered saline. For computational purposes, the PBS is assumed to be pure NaCl of starting molality 0.151 and osmolality 0.278.

where  $c = 1.76 \times 10^{-2}$  osmoles  $\text{kg mol}^{-2}$ . The constant  $c$  was determined by fitting the molality–osmolality data. The total osmolality is assumed to be the sum of the salt and CPA osmolalities. Table 1 lists the properties of the test solution at 0, –10, and –25 °C. The solute concentrations rise below the freezing point of the solution ( $\sim -3.6$  °C) because of external ice formation.

The values of  $L_p$  and  $P_s$  vary with temperature and we assume they do so in accordance with an Arrhenius relation,  $X(T) = X_{ref} \exp\{-E_a/RT\}$ , which we express in the following way for computational convenience:

$$L_p(T) = L_{p,ref} \exp\{-E_aL/R[(1/T) - (1/T_{ref})]\} \quad (4)$$

$$P_s(T) = P_{s,ref} \exp\{-E_aP/R[(1/T) - (1/T_{ref})]\}, \quad (5)$$

where  $E_aL$  and  $E_aP$  are the activation energy in kcal/mol of  $L_p$  and  $P_s$ , respectively, and  $R$  is the gas constant. The reference temperature is arbitrary.

These five coupled equations are solved numerically [18] to yield values of the water content of the cell as a function of time. Essentially this involves finding the water loss during the 2 min hold at –10 °C following by the water loss at –25 °C as a function of time. The steps from 0 to –10 °C and from –10 to –25 °C are assumed to occur so rapidly that no water loss occurs during them.

Most of the figures include a curve (EQ) showing the relative volume of water in an oocyte for the case where it is in chemical potential equilibrium with the water and ice in the outside medium. Those equilibrium values are derived from the ternary phase diagram for 1.5 M EG/isotonic NaCl/water published by Kleinhans and Mazur [19]. The phase diagram gives the total weight percent of solutes ( $W_T$ ) in the unfrozen fraction of the solution as a function of temperature. The mass fraction of the water content of the original medium ( $U$ ) that remains unfrozen at a given subzero temperature is

$$U = [(100 - W_T)(W_T^0/W_T)]/(100 - W_T^0) \quad (6)$$

where  $W_T^0$  is the weight percent solutes before freezing. This equation can also be applied to the interior of the cell if EG has fully permeated the cell prior to freezing and there is no movement of EG across the cell membrane after freezing begins. That means that  $R$  is the same outside and inside the cell; hence, the value of  $W_T$  follows the same isopleth. In the medium,  $W_T$  (the weight fraction of EG + salt in the unfrozen solution) increases with lowered temperature because water is progressively converted into ice. In this work, we track the EG and allow for a flux (Eq. (2)). Our modeling indicates that at the start of freezing intracellular EG is 9% (in moles) below equilibrium and reaches equilibrium after 30 min. Thus Eq. (6) is a good approximation for the cell interior in these experiments, although our reported results do not, in any way, depend on this approximation.

### Analysis overview summary

First, we note that the analysis would be very simple if there were only one hold temperature (–25 °C). Then we would simply adjust the water permeability,  $L_p$  (–25 °C), and CPA permeability,  $P_s$  (–25 °C), until good concordance between the water loss curve and cell survival curve was obtained. However, some water loss occurs at the intermediate hold temperature of –10 °C. Thus, we needed a means of estimating the permeabilities at two subzero temperatures. This is most conveniently and consistently done by linking them together via activation energies,  $E_aL$  and  $E_aP$ , and an Arrhenius temperature dependence. It is instructive to compare these subzero activation energies with the suprazero data in the literature. This matter is considered further in the Discussion.

We began with the literature, suprazero values of water and CPA permeability, extrapolated these to –10 and –25 °C using the suprazero values of  $E_aL$  and  $E_aP$ , and then computed a water loss curve for our experimental cooling protocol. This was

compared with the survival curve and the subzero activation energies were adjusted until there was concordance between the water loss and survival curves.

By concordance we mean the following (see Fig. 2): (i) After 10 min at  $-25\text{ }^{\circ}\text{C}$  the remaining intracellular water should be  $\sim 30\%$  because at this hold time there is no cell survival, (ii) after 20 min at  $-25\text{ }^{\circ}\text{C}$  the cell water content should be several percent higher than the value achieved after 30 min because there is some (40%), but not good (80%) cell survival, and (iii) after 30 min the cell water should be as close as possible to the final equilibrium value of 16% because good survival is achieved after a 30 min hold. These criteria were arrived at empirically after analysis of many water loss simulations and the degree of concordance is judged qualitatively.

We argue that the empirical, qualitative nature of our 'fitting' is appropriate for several reasons. First, the literature data concerning the water loss required to avoid IIF is sparse and of an approximate nature; quantitative rules do not exist. Second, we chose to limit our parameter search to what we consider biologically reasonable values, as discussed in more detail later. Third, the parameter space to be investigated is smaller than may at first be imagined. The value of  $L_p(-25\text{ }^{\circ}\text{C})$ , which is controlled by the choice of  $Ea_L(<0\text{ }^{\circ}\text{C})$ , has by far the greatest impact on the water loss curves. The solute permeability, on the other hand, has only a small impact and, indeed, most investigators assume no solute flux at subzero temperatures. Thus, we are dealing with two adjustable parameters,  $Ea_L(<0\text{ }^{\circ}\text{C})$  and  $Ea_P(<0\text{ }^{\circ}\text{C})$ , with the former dominating. Further, changes in the two permeability coefficients affect the shape of the water loss curve in different ways, allowing for optimization with minimal interaction between the two parameters. Thus our general procedure was to optimize  $L_p(-25\text{ }^{\circ}\text{C})$  first, while holding the other parameters fixed, and then 'fine tune' the results by optimizing  $Ps(-25\text{ }^{\circ}\text{C})$ . We will later illustrate the precision or sensitivity to which the subzero water permeability can be determined with some examples.

## Results

### Water loss using supra zero permeability values

Table 2 summarizes some published permeability data on the mouse oocytes between  $\sim +25$  and  $0\text{ }^{\circ}\text{C}$ . The agreement is remarkably good. The Paynter et al. [31] study is the closest to our experimental conditions with respect to the CPA; i.e. 1.5 M EG in PBS. As a consensus value, we selected an  $L_p$  at  $20\text{ }^{\circ}\text{C}$  of  $0.5\text{ }\mu\text{m min}^{-1}\text{ atm}^{-1}$ , and a suprazero  $Ea_L$  of 14 kcal/mol. For the EG permeability we use

the data of Paynter et al. [31]. They report a  $Ps$  of  $0.09\text{ }\mu\text{m/s}$  at  $19\text{ }^{\circ}\text{C}$  with an  $Ea_P$  of 16 kcal/mol. To provide a 'mental' starting point, these extrapolate to  $L_p = 0.09\text{ }\mu\text{m min}^{-1}\text{ atm}^{-1}$  and  $Ps = 8.0 \times 10^{-5}\text{ cm/min}$  at  $0\text{ }^{\circ}\text{C}$ .

The bold curves in Fig. 3 show the relative volume of cell water of oocytes subjected to the interrupted rapid cooling as a function of time (A) and temperature (B), assuming that the supra zero  $Ea$ 's can be used to extrapolate the permeabilities,  $L_p$  and  $Ps$ , to subzero temperatures. Note that in plots of water volume vs. time, here in Fig. 3a and subsequently, the  $-2$  to  $0$  min on the X axis represents the 2 min hold at  $-10\text{ }^{\circ}\text{C}$ ; the positive values of time are the minutes held at  $-25\text{ }^{\circ}\text{C}$ . In Fig. 3a we have also overlaid the survival data from Fig. 2. When the  $Ea$ 's from above  $0\text{ }^{\circ}\text{C}$  measurements are used, the cell is computed to dehydrate to nearly the equilibrium value after a holding period of only 6 min at  $-25\text{ }^{\circ}\text{C}$  (Fig. 3a). That is 4 min before there is any observed effect of holding time on survival and is 24 min earlier than the 30 min hold required to produce maximum survival (and presumably near maximum dehydration). The conclusion is that the application of the above-zero  $Ea$ 's to subzero temperatures results in too rapid a water loss and specifically, too high an  $L_p$  ( $0.0067\text{ }\mu\text{m min}^{-1}\text{ atm}^{-1}$  at  $-25\text{ }^{\circ}\text{C}$ ); i.e., one that is not consistent with experimental observation.

### Optimized water permeability

To reduce the degree of water loss noted above, we need to decrease  $L_p$  at our holding temperatures of  $-10$  and  $-25\text{ }^{\circ}\text{C}$ . This is accomplished by allowing the value of  $Ea_L$  at subzero temperatures to be an adjustable parameter, while the other parameters are held fixed. We find that the most reasonable accord between model and experiments (Fig. 4) is obtained with an  $Ea_L$  of 21 kcal/mol (vs. 14 kcal/mol from the above zero measurements). That  $Ea_L$  yields a value of  $L_p$  at  $-25\text{ }^{\circ}\text{C}$  of  $0.0018\text{ }\mu\text{m min}^{-1}\text{ atm}^{-1}$  (3.7-fold lower than the value in the previous paragraph). We consider this value of 21 kcal/mol to be the 'best fit' value for the subzero  $Ea_L$ . What one sees in Fig. 4 is that with  $L_p(-25\text{ }^{\circ}\text{C}) = 0.0018\text{ }\mu\text{m min}^{-1}\text{ atm}^{-1}$ , the holding times that produce an increase in survival (10–30 min) also produce sizeable corresponding reductions in the calculated amount of intracellular water.

### Effect of modifying solute permeability parameters

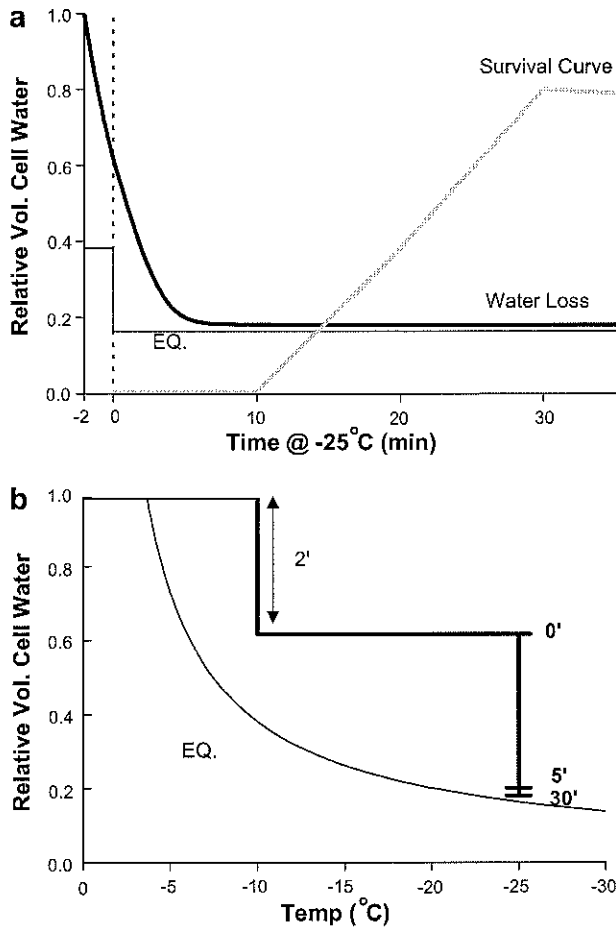
Note that in our best case model in Fig. 4, the water volume of the dehydrating oocytes does not reach equilibrium after 30 min at

**Table 2**  
Mouse oocyte/embryo permeability values and parameters.

$L_p(T)$ ( $\mu\text{m min}^{-1}\text{ atm}^{-1}$ )	CPA	T ( $^{\circ}\text{C}$ )	$Ea$ (kcal/mol)	$\Delta T$ of $Ea^a$ ( $^{\circ}\text{C}$ )	$L_p(0\text{ }^{\circ}\text{C})^b$ ( $\mu\text{m min}^{-1}\text{ atm}^{-1}$ )	$V_b$	Source
0.51	EG	19	14	10 to 30	0.095		Paynter et al. [31]
0.44	PBS	20	14.5	3 to 20	0.071	0.18	Leibo [21]
0.4	DMSO	20	16.4	-3 to 30	0.051		Agca et al. [1]
0.4	PBS	22	11.4	3 to 37	0.084	0.22	Benson et al. [4]
0.7	PB1	25	12.3	15 to 25	0.100	0.15	Edashige et al. [12]
0.044	PBS	0	13.3	-16 to 0	0.044	0.214	Toner et al. [35]
0.08	PBS	0	19.3	-20 to 0	0.080	0.18	Toner et al. [37]
0.83	Gly	25					Edashige et al. [11]
0.47	Buffer	22					Gao et al. [13]
0.65	DMSO	22					Gao et al. [13]
<b>Oocyte working values</b>							
0.5	EG	20	14	Suprazero	0.090	0.18	
D(diameter)=	75	$\mu\text{m}$					
A(area)=	$1.77 \times 10^4$	$\mu\text{m}^2$					
V(volume)=	$2.21 \times 10^5$	$\mu\text{m}^3$					

<sup>a</sup> Range over which  $Ea$  determined.

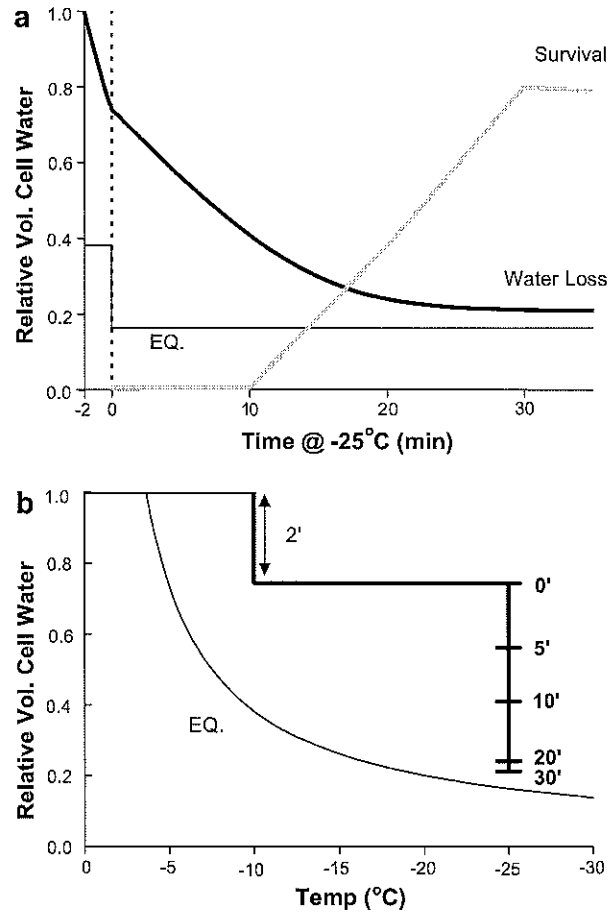
<sup>b</sup> These  $L_p(0\text{ }^{\circ}\text{C})$  values were obtained by extrapolating the  $L_p$ 's in Column 1 to  $0\text{ }^{\circ}\text{C}$  using the  $Ea$ 's in Column 4 and an Arrhenius relationship.



**Fig. 3.** (a): Computed oocyte water loss (bold curve) in 1.5 M EG/PBS as a function of time at  $-25^{\circ}\text{C}$  assuming that the measured values of  $E_a \geq 0^{\circ}\text{C}$  apply below  $0^{\circ}\text{C}$ . The cooling protocol is that in Fig. 1B. The calculations assume the suprazero membrane permeability parameters; namely,  $L_p(0^{\circ}\text{C}) = 0.09 \mu\text{m min}^{-1} \text{atm}^{-1}$ ,  $E_{aL} = 14 \text{ kcal/mol}$ ,  $P_s(0^{\circ}\text{C}) = 8 \times 10^{-5} \text{ cm/min}$ , and  $E_{aP} = 16 \text{ kcal/mol}$ . The survival curve as a function of hold time (see Fig. 2) is superposed (light gray line). The numerical values on the ordinate also indicate the fractional survivals shown in Fig. 2 as %'s. The time from  $-2$  to  $0$  min on the abscissa represents the short hold at  $-10^{\circ}\text{C}$ . The step curve labeled EQ is the equilibrium water loss curve for this cooling protocol. Water loss to near equilibrium (bold curve) is complete in 6 min, however cell survival is zero at this time and only reaches its maximum 24 min later. (b): The relative cell volume is plotted as a function of the cell temperature (step curve) with tick marks showing the time course. The equilibrium water loss curve is the smooth curve underneath, labeled EQ.

$-25^{\circ}\text{C}$  (21.2% vs. 16%)—Nor does it after 40 min (20.9%). In the computations for Fig. 4, the activation energy for the permeation of EG below  $0^{\circ}\text{C}$  ( $E_{aP}$ ) was fixed at 16 kcal/mol. Fig. 5 shows the effect of varying  $E_{aP}$  from 0 to  $\infty$ , which results in corresponding variations in  $P_s(<0^{\circ}\text{C})$ . The curve for  $E_{aP} = 16 \text{ kcal/mol}$  is the second from the bottom. An  $E_{aP}$  of 0 (top curve) means that the  $0^{\circ}\text{C}$  value of  $P_s$  holds constant at subzero temperatures. An  $E_{aP}$  of infinity (bottom curve) means that the oocyte becomes completely impermeable to EG at subzero temperatures. With an infinite value of  $E_{aP}$ , the oocytes reach true equilibrium by 30 min at  $-25^{\circ}\text{C}$ . But the smaller the value of  $E_{aP}$ , the more the curve departs from equilibrium after a 30 min hold.

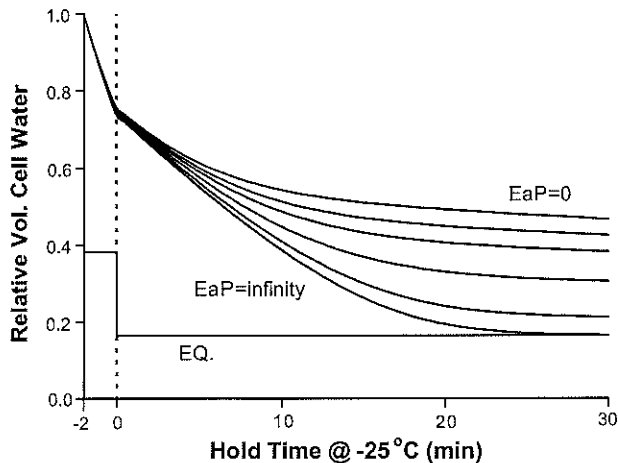
We have selected  $E_{aP} = 24 \text{ kcal/mol}$  as the most reasonable value. If that value is substituted for the value of 16 kcal/mol used in Fig. 4, the results shown in Fig. 6 are obtained. The chief difference between the curves in the two figures is the closer approach of the relative cell water volume to the equilibrium value in the latter.



**Fig. 4.** (a): Oocyte water loss in 1.5 M EG/PBS as a function of hold time at  $-25^{\circ}\text{C}$  using the 'best' fit  $E_{aL}$  and  $L_p$ . The subzero  $E_{aL}$  were adjusted to give better concordance (than Fig. 3) between cell survival and water loss, yielding  $E_{aL}(\leq 0^{\circ}\text{C}) = 21 \text{ kcal/mol}$  which in turn yields  $L_p(-25^{\circ}\text{C}) = 0.0018 \mu\text{m min}^{-1} \text{atm}^{-1}$ . The other parameters were held fixed; see Fig. 3. 'Concordance' is defined in the text. The water loss curve drops to near equilibrium during the same time frame that cell survival (light gray) rises to its maximum. The equilibrium water loss curve is the step curve labeled EQ. (b): The same data displayed as a function of oocyte temperature with tick marks on the step curve showing the time course.

It is interesting to note that lower values of  $E_{aP}$  at subzero temperatures and as a consequence higher values of  $P_s$ , lead to greater departures from the equilibrium relative volume of cell water after a 30 min hold. When the value of  $E_{aP}$  is 24 kcal/mol, it takes about 6000 min (100 h) for the relative volume of oocyte water (and the intracellular concentrations of EG and salt) to reach true equilibrium at  $-25^{\circ}\text{C}$ , whereas, as mentioned, if  $E_{aP}$  is infinite (no permeation) true equilibrium is reached in 30 min.

A qualitative explanation of this behavior is relatively straight forward. At zero time at  $-25^{\circ}\text{C}$ , the total osmolality (EG + salts) of the medium exceeds that of EG + endogenous cytoplasmic solutes inside the cell. In response to this difference, water leaves the cell at a rate proportional to  $L_p$  and to the difference between the external and internal total osmolalities [i.e., Eq. (1)]. At the same time, the chemical activity of the EG in the medium (closely approximated as the molality) exceeds the molality of EG inside the cell. In response to this difference, EG permeates the cells in amounts per unit time that depend on  $P_s$  and on the difference between the outside and inside molalities [Eq. (2)]. As an incremental volume of EG permeates, two things happen in the cell. First, the total internal osmolality is further raised a small amount. Second, the molality of the internal EG is raised a small amount. The



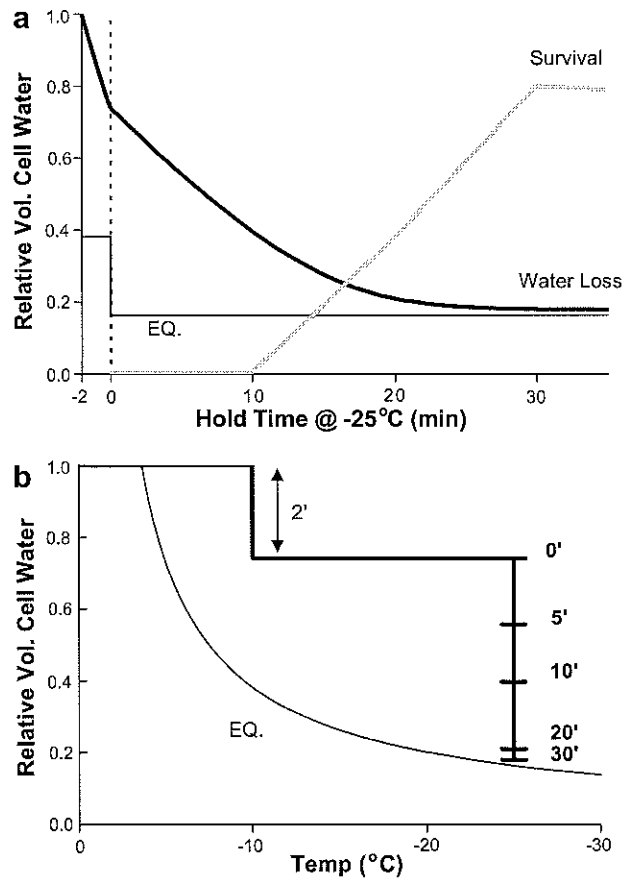
**Fig. 5.** Oocyte water loss in 1.5 M EG/PBS as a function of hold time at  $-25^{\circ}\text{C}$  and as a function of subzero EaP. The final approach of water loss to near equilibrium as a function of hold time depends on the cryoprotectant permeability at  $-25^{\circ}\text{C}$ . This is determined by its assumed subzero activation energy, EaP. The family of curves shown depict the water loss for EaP = 0, 2, 4, 8, 16, and infinite kcal/mol. The subzero EaL and associated  $L_p(-25^{\circ}\text{C})$  used are the 'best' fit values from Fig. 4.  $P_s(0^{\circ}\text{C})$  was held fixed at  $8 \times 10^{-5}$  cm/min. During the time course covered by the survival data (0–40 min), higher values of EaP yield a closer (better) approach to the equilibrium water content at  $-25^{\circ}\text{C}$ .

consequence of the first is a slight decrease in the driving force for the efflux of water from what would be the case if some EG influx had not occurred, and a decrease in driving force results in a small decrease in the rate of water loss. The consequence of the second, a small increase in the molality of the internal EG, is a slight decrease in the driving force for the entry of additional EG, and consequent decrease in the rate of that entry. Overall, then, the consequences of the permeation of that incremental volume of EG into the cell are a decrease in the rate at which subsequent incremental volumes of water leave the cell and a decrease in the rate at which EG enters the cell. If one allows this sequence to occur over and over again in small increments of time [infinitesimal increments in coupled Eqs. (1) and (2)], the end result is that the time required to achieve true equilibrium of the oocyte water volume becomes greatly extended beyond the time required when water is the only molecule that can move between the cell and the medium.

#### *L<sub>p</sub> and EaL sensitivity analysis*

We will address several issues here. First (Fig. 7a), we consider how variations in the 'starting' value of  $L_p(0^{\circ}\text{C}) = 0.09 \mu\text{m min}^{-1} \text{atm}^{-1}$  affect the best fit value of  $L_p(-25^{\circ}\text{C})$ . This starting value is based on experimental measurements made at  $0^{\circ}\text{C}$  or extrapolated from higher temperatures to  $0^{\circ}\text{C}$  using estimates of EaL between  $\sim 30$  and  $5^{\circ}\text{C}$ . But it is certainly possible that  $L_p$  could undergo an abrupt change associated with the initiation of freezing near  $0^{\circ}\text{C}$ . Fig. 7a considers the possibility that  $L_p(0^{\circ}\text{C})$  may be as little as  $\frac{1}{2}\times$  to as much as  $2\times$  our starting value of  $0.09 \mu\text{m min}^{-1} \text{atm}^{-1}$ . Using these values, EaL( $<0^{\circ}\text{C}$ ) was re-optimized to yield water loss curves in concordance with the survival curve. In all three cases,  $L_p(0^{\circ}\text{C}) = \frac{1}{2}\times$ ,  $1\times$ , and  $2\times$ , good concordance with survival was achieved using EaL( $<0^{\circ}\text{C}$ ) values of 16.7, 21.0, and 25.6 kcal/mol, respectively. The corresponding  $L_p(-25^{\circ}\text{C})$  values are  $1.11\times$ ,  $1\times$  and  $0.86\times 0.0018 \mu\text{m min}^{-1} \text{atm}^{-1}$ . Thus, our 'best fit' value for  $L_p(-25^{\circ}\text{C})$  is relatively insensitive to modest variations in the suprazero  $L_p$  starting values.

Second (Fig. 7b), we demonstrate the degree to which concordance is lost when our 'best fit'  $L_p(-25^{\circ}\text{C})$  is varied from  $\frac{1}{2}\times$  to  $2\times$

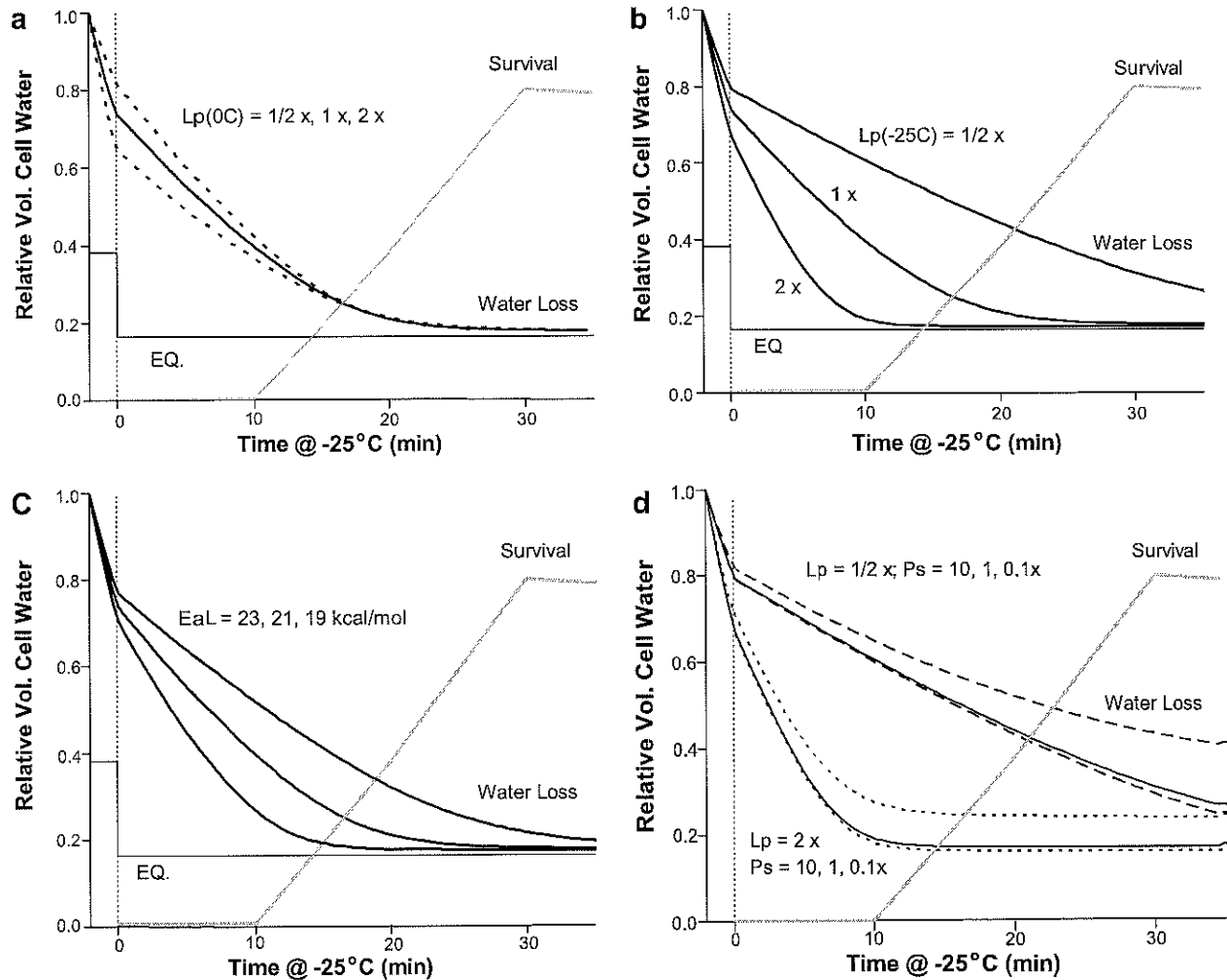


**Fig. 6.** Oocyte water loss in 1.5 M EG/PBS as a function of hold time at  $-25^{\circ}\text{C}$  using 'best' fit permeability values. The permeability values which yield the best concordance between water loss (smooth curve) and oocyte survival fraction (light gray) as a function of hold time at  $-25^{\circ}\text{C}$  is depicted in (a). These values are  $L_p(-25^{\circ}\text{C}) = 0.0018 \mu\text{m min}^{-1} \text{atm}^{-1}$ ,  $EaL(\leq 0^{\circ}\text{C}) = 21$  kcal/mol,  $P_s(-25^{\circ}\text{C}) = 0.09 \times 10^{-5}$  cm/min, and  $EaP(\leq 0^{\circ}\text{C}) = 24$  kcal/mol. Note that  $L_p(-25^{\circ}\text{C})$  and  $P_s(-25^{\circ}\text{C})$  are completely determined by their respective  $0^{\circ}\text{C}$  values and subzero Ea's. They are not independent parameters. By concordance, we mean the oocyte water loss approaches equilibrium at the same time that survival is rising to 80%. The equilibrium water loss curve is the step curve labeled EQ. in (b), the same data are displayed as a function of oocyte temperature with tick marks on the step curve showing the time course.

$0.0018 \mu\text{m min}^{-1} \text{atm}^{-1}$ . When  $L_p(-25^{\circ}\text{C})$  is reduced to  $\frac{1}{2}\times$ , the intracellular water only drops to 31% after 30 min which is too high to yield the observed high survival. On the other hand, when  $L_p(-25^{\circ}\text{C})$  is doubled, water loss is rapid, and the intracellular water content is nearly at its equilibrium value after only 10 min, while cell survival is still negligible. Thus, changes of a factor of two in  $L_p(-25^{\circ}\text{C})$  produce obvious, substantial changes in the water loss curve and loss of concordance with the survival curve.

Third, Fig. 7c demonstrates the sensitivity of the simulated water loss curves to the selected value of subzero EaL. Increasing its value from the 'best fit' of 21 to a value of 23 kcal/mol causes them to retain significantly more water after given times, especially after 20 min and is probably not consistent with the observed cell survival. Decreasing the value of EaL to 19 kcal/mol causes the simulated oocytes to dehydrate to equilibrium too early. These values of EaL are predicated for a discontinuous change at  $0^{\circ}\text{C}$ . Later we discuss the consequences if EaL were to undergo the discontinuous change at lower temperatures.

The fourth point which we wish to demonstrate (Fig. 7d) is that readjusting  $P_s(-25^{\circ}\text{C})$  does not improve the poor concordance ob-



**Fig. 7.** Sensitivity analysis. Several scenarios are considered which demonstrate the sensitivity of the fitted subzero permeability parameters and water loss curves to changes in the starting assumptions. The permeability parameters are the same as in Fig. 6, except as noted. The step curve labeled EQ is the equilibrium water loss curve; it is omitted from the last panel to reduce clutter. The light gray curve is the experimentally observed oocyte survival. In (a) the initial value of  $L_p(0^\circ\text{C})$  is halved (upper dotted line) and doubled (lower dotted line) relative to our best value of  $0.09 \mu\text{m min}^{-1} \text{atm}^{-1}$  (solid line). In each case  $EaL(<0^\circ\text{C})$  is readjusted for best concordance with survival (light gray). The adjusted values are 16.7, 21.0, and 25.6 kcal/mol for  $L_p(0^\circ\text{C}) = 1/2 \times, 1 \times,$  and  $2 \times,$  respectively. Good concordance with survival is obtained in all three cases. The corresponding values of  $L_p(-25^\circ\text{C})$  only vary between  $1.11 \times$  and  $0.86 \times$  our best value of  $0.0018 \mu\text{m min}^{-1} \text{atm}^{-1}$ . In (b),  $L_p(0^\circ\text{C})$  is held fixed at  $0.09 \mu\text{m min}^{-1} \text{atm}^{-1}$  and  $EaL(<0^\circ\text{C})$  is adjusted to yield  $L_p(-25^\circ\text{C})$  values of  $1/2 \times, 1 \times,$  and  $2 \times$  our best fit of  $0.0018 \mu\text{m min}^{-1} \text{atm}^{-1}$ . The  $L_p(-25^\circ\text{C})$  value of  $1/2 \times$  yields too slow a water loss at  $-25^\circ\text{C}$  and the  $2 \times$  value yields too rapid a water loss at  $-25^\circ\text{C}$  for concordance with the survival curve. (c) Shows the sensitivity of our 'best fit' concordance to small changes in  $EaL(<0^\circ\text{C})$ . Water loss curves for  $EaL(<0^\circ\text{C}) = 19, 21,$  and  $23$  kcal/mol are plotted. Increasing  $EaL$  from the 'best fit' of 21 to a value of 23 kcal/mol causes the water loss curve to retain significantly more water after given times, especially after 20 min and is probably not consistent with the observed cell survival. Decreasing the value of  $EaL$  to 19 kcal/mol causes the simulated oocytes to dehydrate to equilibrium too early. Thus, our analysis is sensitive to small changes in  $EaL$  of only a few kcal/mol. In (d) the effect of varying  $P_s$  in an effort to improve the too slow and too fast cases in (b) is illustrated. The upper solid curve reproduces the  $L_p(-25^\circ\text{C}) = 1/2 \times$  case. Above and below it, the dashed lines show the effect of increasing and decreasing  $P_s$  by  $10 \times,$  respectively ( $P_s(0^\circ\text{C}) = 8 \times 10^{-5} \text{cm/min}$ ). The lower solid curve is the  $L_p(-25^\circ\text{C}) = 2 \times$  case. Above and below it, the dotted lines show the effect of increasing and decreasing  $P_s$  by  $10 \times,$  respectively. In no case is good concordance with survival (light gray) obtained. Thus, (d) demonstrates that too slow or too rapid water loss cannot be corrected by adjusting  $P_s$ . In this panel the value of  $P_s(0^\circ\text{C})$  was adjusted to change the subzero values of  $P_s,$  while  $EaP(<0^\circ\text{C})$  was held constant at 24 kcal/mol. Similar results are obtained if  $P_s(0^\circ\text{C})$  is held constant and  $EaP(<0^\circ\text{C})$  varied.

tained in Fig. 7b when  $L_p(-25^\circ\text{C})$  is halved or doubled. In Fig. 7d we show the effect of varying  $P_s(0^\circ\text{C})$ , and thus  $P_s(-25^\circ\text{C})$ , by a factor of ten. In neither case is the concordance improved, as detailed in the figure caption. This last example illustrates our point, made previously, that  $L_p$  and  $P_s$  can be optimized relatively independently of each other in this particular experimental situation. In this fourth point,  $P_s(0^\circ\text{C})$  was varied to change  $P_s(-25^\circ\text{C})$  while  $EaP(<0^\circ\text{C})$  was held constant at 24 kcal/mol. Similar results to those discussed here were obtained if  $EaP(<0^\circ\text{C})$  was used to adjust  $P_s(-25^\circ\text{C})$  (Results not shown.)

Finally, we address the effect on  $L_p(-25^\circ\text{C})$  and  $EaL$  of varying the temperature at which the 'suprazero' permeability values

change to their 'subzero' values. In the above analysis, we used a temperature of  $0^\circ\text{C}$ . If instead, we assume the change in  $EaL$  and  $EaP$  occurs at  $-10^\circ\text{C}$ , the best concordance between the survival and water loss curve occurs with an  $EaL(<-10^\circ\text{C})$  of 27 kcal/mol (29% higher than our 'best' value). This in turn yields an  $L_p(-25^\circ\text{C})$  of  $0.0015 \mu\text{m min}^{-1} \text{atm}^{-1}$ , 17% less than our 'best' value of 0.0018. Repeating these calculations assuming a transition at  $-20^\circ\text{C}$  yields an  $L_p(-25^\circ\text{C})$  of  $0.0009 \mu\text{m min}^{-1} \text{atm}^{-1}$  and an  $EaL$  of 65 kcal/mol. In both cases, the suprazero permeability values were extrapolated to  $-10$  or  $-20^\circ\text{C}$ , as needed, to connect with the low temperature values and  $EaP(< \text{transition } T)$  was held fixed at 24 kcal/mol.

## Discussion

First, we need to discuss issues relating to the temperature dependence of the water and CPA permeability. We repeat that if there were only one subzero hold temperature ( $-25\text{ }^{\circ}\text{C}$ ), this entire exercise would reduce to simply finding the values of  $L_p(-25\text{ }^{\circ}\text{C})$  and  $P_s(-25\text{ }^{\circ}\text{C})$  that give the best concordance between the water loss and survival curves. However, since there are two subzero hold temperatures ( $-10$  and  $-25\text{ }^{\circ}\text{C}$ ), we need a logical way to include an estimate of the permeabilities and consequent water loss at  $-10\text{ }^{\circ}\text{C}$ . Using our 'best fit' parameters, about 30% of the total water loss after 30 min at  $-25\text{ }^{\circ}\text{C}$  occurs during the initial 2 min hold at  $-10\text{ }^{\circ}\text{C}$  (Fig. 4). This is too large to ignore, but not so large that it need be known precisely to get a good estimate of  $L_p(-25\text{ }^{\circ}\text{C})$ . The most logical and consistent way to link the  $L_p(-10\text{ }^{\circ}\text{C})$  and  $P_s(-10\text{ }^{\circ}\text{C})$  values to the  $-25\text{ }^{\circ}\text{C}$  values is via an activation energy for each. A corollary issue is that of connecting the subzero permeability values to the suprazero permeability values. Do we expect/wish to make  $L_p(T)$  and  $P_s(T)$  continuous functions of temperature or should discontinuities be permitted?

In our analysis, we began by assuming the permeabilities to be continuous, with an abrupt change in activation energies occurring at  $0\text{ }^{\circ}\text{C}$  to determine our 'best' value permeability parameters:  $L_p(-25\text{ }^{\circ}\text{C}) = 0.0018\text{ }\mu\text{m min}^{-1}\text{ atm}^{-1}$  and  $E_{aL} = 21\text{ kcal/mol}$ . As shown in the Sensitivity analysis Section, relaxing these assumptions yields an  $L_p(-25\text{ }^{\circ}\text{C})$  which differs by no more than a factor of two from our 'best' value and an  $E_{aL}$  as high as  $3\times$  our 'best' value.

We now consider some of the biological implications of and reasons for the mathematical modeling choices made. As indicated, we began our analysis by assuming that at  $0\text{ }^{\circ}\text{C}$  the  $E_{aL}$  changes abruptly and that the new value remains constant at lower temperatures. The reason for choosing  $0\text{ }^{\circ}\text{C}$  for the transition temperature is that, with a few exceptions, it is the lowest temperature at which the  $L_p$ 's of cells in an ice-free environment have been determined. However, the transition could occur at a temperature below  $0\text{ }^{\circ}\text{C}$  with constant  $E_{aL}$  below that temperature, or the  $E_{aL}$  below  $0\text{ }^{\circ}\text{C}$  could be curvilinear—not constant.

We have analyzed the former possibility. If the transition occurs at  $-10\text{ }^{\circ}\text{C}$ , the best fit value of  $E_{aL}$  below that would increase from 21 to 27 kcal/mol and  $L_p(-25\text{ }^{\circ}\text{C})$  would drop from 0.0018 to 0.0015  $\mu\text{m min}^{-1}\text{ atm}^{-1}$ . If the transition occurs at  $-20\text{ }^{\circ}\text{C}$ , the  $E_{aL}$  would increase markedly to 65 kcal/mol and the  $L_p(-25\text{ }^{\circ}\text{C})$  would decrease to 0.0009  $\mu\text{m min}^{-1}\text{ atm}^{-1}$ . Thus, one can see that a lowering of the transition temperature could have a large effect on  $E_{aL}$  but a much smaller effect on  $L_p(-25\text{ }^{\circ}\text{C})$ .

The very high  $E_{aL}$  associated with a transition at  $-20\text{ }^{\circ}\text{C}$  is not consistent with published data on IIF vs. cooling rate or survival vs. cooling rate. If an  $E_{aL}$  of 65 kcal/mol were operating below  $-20\text{ }^{\circ}\text{C}$ , the efflux of intracellular water would effectively cease a few degrees below  $-25\text{ }^{\circ}\text{C}$ , and if sufficient cell water were 'locked in', it would not be possible to avoid IIF and consequent death unless one cooled the cells at a rate that was far-far below the standard of  $0.5\text{ }^{\circ}\text{C/min}$  (for oocytes).

Our analysis is predicated on the existence of a discontinuity of  $E_{aL}$  at  $0\text{ }^{\circ}\text{C}$  (or possibly below  $0\text{ }^{\circ}\text{C}$ ). If that predicate is valid, the question is to what might the discontinuity be ascribed? Using an electrical analogy, the hydraulic conductivity can be considered to be the reciprocal of three resistances in series. R1 would be the resistance to water movement in the external medium; R2, the resistance in the membrane; and R3 the resistance to water movement in the cytoplasm. Ordinarily, R2 is considered dominant (but see Dick, [10]). Published Arrhenius plots of  $L_p$  at  $\geq 0\text{ }^{\circ}\text{C}$  show no discontinuity or change in  $E_{aL}$  with temperature, but most do not contain sufficient data to exclude the possibility. A discontinuity at  $0\text{ }^{\circ}\text{C}$  or below, which we propose, could be solely a tempera-

ture-driven reorganization of the membrane or it could be associated with the formation of external ice. If a temperature effect, one possible agent would be phase changes in the lipids of the membrane. The problem here is that observed lipid phase changes occur over a range of temperatures from well above to below  $0\text{ }^{\circ}\text{C}$ . The other possibility is that a discontinuous change in  $E_{aL}$  is a consequence of ice formation and the resulting large increases in solute concentration. These higher concentrations might affect the properties of the membrane and they would produce an increase in the viscosity of the external medium and the cytoplasm. That in turn would decrease the diffusion of water since Fick's law contains a viscosity term, and it would increase R1 and R3 and possibly R2.

### Subzero $L_p$ and $E_{aL}$ in other cell types

Values for  $E_{aL}$  of other cells in partly frozen solutions at subzero temperatures have been reported. They are summarized in Table 3 and, in the few cases available, compared with published values of  $E_{aL}$  obtained from measurements of  $L_p$  at  $0\text{ }^{\circ}\text{C}$  and above. In the four cases listed, the  $E_{aL}$  above  $0\text{ }^{\circ}\text{C}$  lies between 8 and 19 kcal/mol. In seven cases, the  $E_{aL}$  ( $<0\text{ }^{\circ}\text{C}$ ) lies in the same range. However, in six cases it has much higher values ranging above 80 kcal/mole. Five of the seven occur with cells frozen in isotonic saline. The consequence of a very high  $E_{aL}$  is that the cells cease to shrink below a high subzero temperature, which means that freezable water in the cell becomes locked in. We strongly suspect that in some cases at least the failure to continue shrinking is not due to a very high  $E_{aL}$  but is due to the fact that cell membranes are being damaged by being exposed to high concentrations of salt during progressive freezing. This damage converts the system from one with two compartments (cell and medium) to a single compartment. That is, the cell becomes osmotically non-responsive. We emphasize, as shown in Table 2, that this problem is not occurring in mammalian oocytes. There, the values of  $E_{aL}$  fall in the typical range and there is little or no difference between the values above and below  $0\text{ }^{\circ}\text{C}$ .

### Subzero $L_p$ and its $E_{aL}$ in mammalian spermatozoa

We first mentioned in connection with Table 2 that there is good agreement among various studies for the values of  $L_p$  and  $E_{aL}$  at  $\geq 0\text{ }^{\circ}\text{C}$  in mouse oocytes and zygotes. Second, we conclude from our study and those of others that there is also rather good agreement for the values at subzero temperatures. And third, there appears to be, at most, only a moderate increase in  $E_{aL}$  upon freezing. Table 4 gives analogous information for mammalian sperm, and one can see that the above conclusions for oocytes do not apply to sperm. The values for sperm show the sorts of variability listed in Table 3 for various other cell types. In sperm, the only values for subzero  $L_p$  and its  $E_{aL}$  have come from the DSC technique of Devireddy et al. [5], and the values of  $L_p$  extrapolated to  $0\text{ }^{\circ}\text{C}$  from this technique are considerably lower and the  $E_{aL}$  considerably higher than those extrapolated to  $0\text{ }^{\circ}\text{C}$  from measurements made above  $0\text{ }^{\circ}\text{C}$ . Unfortunately, practical considerations rule out applying the DSC procedure to oocytes. It would take hundreds of them in each run to generate the necessary thermal signatures in a single run. Similarly, Linkam cryomicroscopy cannot be used for sperm. They are too small to permit observation of IIF or osmotic shrinkage.

One advantage of the inverse analyses developed by Karlsson et al. [17], by Yarmush et al. [38] for attached hepatocytes, and by us here for mouse oocytes is that it does not require knowing the volume of the cell and the rate at which that volume decreases during freezing. One disadvantage is that the computed best-fit value of  $E_{aL}$   $<0\text{ }^{\circ}\text{C}$  depends on a number of assumptions. An important



**Table 3**  
Published activation energies (Ea) for water permeability (Lp) both above and below 0 °C.

Cell type	Medium	Ea > 0 °C (kcal/mol)	Source	Ea (frozen) (kcal/mol)	Source	Subzero method <sup>a</sup>
Yeast	Water			11–16	Levin [22]	I
Monocytes	Isotonic saline			14.6	McCaa et al. [29]	II
	DMSO			39.7	McCaa et al. [29]	II
Lymphocytes	Isotonic saline	13	Hempling and White [16]	15.5	Devireddy et al. [5]	I, III
Granulocytes	Isotonic saline	14–18	Hempling and White [16]	52	Schwartz and Diller [34]	I
Keratinocytes	Isotonic saline			10.7	Aggarwal et al. [2]	II
Dermal fibroblasts	Isotonic saline			39–43	Balasubramanian et al. [3]	II
Hepatocytes	Isotonic saline			19.6	Korniski et al. [20]	II
				18–81	Yarmush et al. [38]	IV
Drosophila eggs	Isotonic saline			18–102	Toner et al. [36]	II
				18	Karlsson et al. [17]	V
				28	Karlsson et al. [17]	V
				39	Pitt et al. [33]	I
				11.6–19	Table 2	
Mouse oocytes	EG		Table 2	13.3–19.3	Table 2	
				21	Present work	V

<sup>a</sup> Methods: (I) Cells are cooled at a constant rate in a cryostage after external freezing occurs and their volumes determined as a function of time and subzero temperature. Theoretical curves of cell volume vs. subzero temperature and time, based on equations such as those published by Mazur [24] and Levin [22] are fitted to the experimental data using Lp and Ea as adjustable parameters. (II) Cells in media are supercooled to given subzero temperatures in a cryostage, the medium ice nucleated, and the sample then held at the nucleation temperature for selected durations. Cell volumes are measured during this isothermal hold and the theoretical shrinkage curve (Eq. (1)) fitted to the experimental curve with Lp as an adjustable parameter. (III) A calorimetric method developed by Devireddy et al. [5]. (IV) An inverse method. Cells were cooled at rates high enough to prevent water loss and a determination made of the cumulative percent that underwent IIF. These data permitted the authors to calculate the kinetic and thermodynamic parameters that describe ice nucleation. Cells were then cooled at rates low enough to allow the efflux of water during cooling. The values of Lp and Ea were then determined by adjusting them to provide the best fit for the coupled IIF and water transport models. (V) The inverse method developed in this paper. A somewhat similar approach was used by Karlsson et al. [17].

**Table 4**  
Water permeability (Lp) of mammalian sperm and its activation energy (Ea) above and below 0 °C.

Species	CPA	Ea > 0 °C (kcal/mol)	Ea < 0 °C (kcal/mol)	Lp (0 °C) ( $\mu\text{m min}^{-1} \text{atm}^{-1}$ )	Method	Source
Mouse	PBS	13	–	0.33	Time to lysis	Noiles et al. [30]
	Various	8	–	0.09	Coulter	Phelps et al. [32]
	PBS	–	22	0.01	DSC	Devireddy et al. [6]
Human	PBS	3.5	–	1.14	Coulter	Gilmore et al. [14]
	Saline	–	86	0.14	DSC	Devireddy et al. [7]
	Glycerol	–	33	0.04	DSC	Devireddy et al. [7]
Equine	Kenney extender	–	33	0.02	DSC	Devireddy et al. [8]
Boar	Glycerol	7.8	–	0.052	Coulter	Gilmore et al. [15]
	Modena extender	–	29	0.02	DSC	Devireddy et al. [9]
	Glycerol	–	18	0.005	DSC	Devireddy et al. [9]

one in our case is the subzero temperature at which the transition in Ea occurs, for the lower the temperature of that transition, the higher the subsequent Ea has to be to yield results consistent with the experimental data at –25 °C. However, although a rather large range in best-fit values for Ea can be obtained mathematically, other considerations put limits on what the maximum subzero EaL can be. For example, if Ea rises too high (probably above 30 kcal/mol), residual unfrozen intracellular water will effectively be locked into the oocytes and it will undergo IIF at temperatures below –25 °C.

## Conclusion

The ability to model the water loss during the freezing of cells as a function of temperature and time has proved a powerful tool in estimating the likelihood of lethal intracellular ice formation during cooling and analyzing the osmotic properties of cells at subzero temperatures. These modeling computations require values for the hydraulic conductivity of water (Lp) and the activation energy (Ea) or temperature coefficient of that permeability below 0 °C in the partly frozen state. Because of difficulties in directly obtaining the required measurements (usually cell volume) in the presence of ice, the values of Ea obtained from measurements

at 0 °C and above have commonly been assumed to apply below 0 °C. In the case of mouse oocytes in EG, our analysis indicates that this assumption is not correct, for it leads to the shrinkage of oocytes at a much higher rate than occurs experimentally. The Ea below 0 °C is higher than that above 0 °C. Our best-fit value is 50% higher.

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