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Relationship between intracellular ice formation in oocytes of the mouse and *Xenopus* and the physical state of the external medium—a revisit

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Abstract

We have previously reported that intracellular ice formation (IIF) in mouse oocytes suspended in glycerol/PBS solutions or ethylene glycol (EG)/PBS solutions and rapidly cooled to −50°C or below occurs at temperatures where a critical fraction of the external water remains unfrozen (Cryobiology 51, 2005, 29-53; 54, 2007, 223-233). For mouse oocytes in PBS or glycerol/PBS that fraction is 0.06; for oocytes in EG that fraction was calculated to be 0.13, more than double. The fractions unfrozen are computed from ternary phase diagrams. In the previous publication, we used the EG data of Woods et al. (Cryobiology 38,1999, 403-407). Since then, we have determined that ternary phase diagrams for EG/NaCl/water synthesized by summing binary phase data for EG/water NaCl/water gives substantially different curves, which seem more realistic (Cryobiology 54, 2007, 212-222). Unfrozen fractions at the temperatures of IIF computed from these synthesized phase diagrams are about half of those calculated from the Woods et al. data, and are in close agreement with the computations for glycerol; i.e., IIF occurs when about 92-94% of the external water is frozen. A parallel paper was published by Guenther et al. (Cryobiology 52, 2006, 401-416) on IIF in oocytes of the frog *Xenopus*. It too examined whether the temperatures of IIF were related to the unfrozen fractions at those temperatures. It also used the Woods et al. ternary phase data to calculate the unfrozen fractions for EG solutions. As reported here, once again the values of these unfrozen fractions are substantially different from those calculated using synthesized phase diagrams. With the latter, the unfrozen fractions at IIF become very similar for EG and glycerol.

Keywords

Intracellular ice formation; oocytes; mouse; *Xenopus*; unfrozen fraction; phase diagrams

Our laboratory has recently reported on several variables that influence the temperature at which intracellular ice forms in mouse oocytes and in oocytes of the frog *Xenopus*. [5,1] that are cooled rapidly enough to ensure that they undergo intracellular ice formation (IIF); namely 20°C/min and 10°C/min, respectively. One major variable affecting the temperature of IIFin mouse oocytes was the concentration of ethylene glycol (EG) or glycerol present in the

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solutions of Dulbecco's phosphate-buffered saline (PBS) that were used. The higher the EG or glycerol concentration, the lower the temperature, as manifested by "flashing" of the cells.

As part of that study, we compared the "flash" temperatures of mouse oocytes in the various EG and glycerol concentrations with the unfrozen fractions of water and solutions at those temperatures and with the concentration of salt and CPA in those unfrozen fractions. The results of those comparisons are shown in the upper two—thirds of Table 1 and the upper half of Table 2. The values for EG in the right-hand five columns beginning with W_T were obtained from experimental ternary phase data for EG/NaCl published by Woods et al..[8]. Note that the two values for the unfrozen fraction (**L** and **U**) are considerably higher for the EG solutions than for the glycerol solutions and PBS. **L** is the mass fraction of the solution that remained unfrozen at the observed IIF temperature; **U** is the unfrozen mass fraction of water (≅ volume fraction). For PBS alone and glycerol in PBS (Table 1), the values of **U** are identical (0.06). The values of **U** for the six EG/PBS solutions in Tables 1 and 2 are rather tightly grouped but are about double (0.12–0.15) the values for PBS and glycerol/PBS.

Subsequently, Kleinhans and Mazur [3] have compared published experimental ternary phase diagrams for the cryoprotective agents (CPA) glycerol, sucrose, DMSO, and EG in NaCl with those that they synthesized by adding the melting points of the binary system CPA/water and the binary system NaCl/water. In the case of glycerol, sucrose, and DMSO, the agreement between the experimental and synthesized ternary phase diagrams is very good. However, in the case of EG, the agreement is not good. In addition, as pointed out in [3] there is a substantive internal discrepency in the Woods et al. experimental data for EG [8]. In the ternary phase diagrams for the other CPAs, the downward curvature of the plots (isopleths) with increasing solute concentration becomes greater as the weight ratio of CPA to salt (**R)** decreases. That is so with the synthesized ternary EG plots in [3]; however, it is not the case, or is much less the case, with the Woods et al. data in [8]. For these two reasons, we concluded that the synthesized phase diagrams are closer to reality than are the Woods et al. data. [The point may be moot. Just after submitting this manuscript, Dr. Woods in a personal communication informed us that he and colleagues are redoing the EG phase diagram determinations in [8] using an updated DSC. The melting points they have obtained in preliminary results for several EG/NaCl/water solutions now differ from those computed from the synthesized phase diagrams in [3] by a mean of only 1.25°C (lower).]

The bottom three lines of Table 1 and the bottom half of Table 2 show the computed values for the right-hand five columns based on the synthesized ternary phase diagrams [3]. The chief conclusion is that now the values for the unfrozen fractions at the flash temperature for the oocytes in the EG solutions are in rather close agreement with the values obtained for oocytes in glycerol and in PBS alone. Consequently, we can now conclude with some definitiveness that whether the oocytes are in PBS, in Glycerol/PBS, or in EG/PBS, they undergo intracellular freezing at a temperature at which about 92 to 94% of the surrounding water has been frozen.

In Tables 1 and 2, **R** is the weight percent ratio of CPA to NaCl. For calculations, the molality of isotonic PBS was treated as the equivalent molality of NaCl. Because of the way the initial solutions were prepared (variable concentration of CPA, fixed (isotonic) concentration of PBS, and therefore variable **R** value), the rather close grouping of the **U** values at flash is accompanied by a reasonably close grouping of the molalities of salt in the unfrozen fractions at flash. This parallel behavior arises because the rise in the molality of the salts is a direct consequence of the decrease in the amount of liquid water available. Consequently, it is not clear whether IIF was correlated with unfrozen fraction or with the NaCl concentration. That confounding was partly uncoupled in a subsequent paper [4] which determined the flash temperatures in EG/PBS or glycerol/PBS solutions in which the CPA and salt concentrations

were both varied so as to hold the **R** value constant. The results with this approach suggest that the unfrozen fraction is the critical factor for IIF.

Table 3 shows analogous data for *Xenopus* Stage I and II oocytes (~ 200-400 μm diameter). As in Table 1 for mouse, the properties of the EG/Ringers solution at the flash temperatures of *Xenopus* derived from the synthesized ternary phase diagram agree much more closely with the values for glycerol than do the values derived from the Woods et al. phase diagrams. The two right hand columns of Table 3 emphasize that point. They show the total osmolalities of the solutions (CPA + ringer salts) computed in two ways. One way is from the equation $\boldsymbol{M} = \mathbf{T_{flash}/1.855}$. The other way is by summing the product of the computed molalities of each solute at the flash temperature times the osmotic coefficients times the number of ions after dissociation. The two methods give closely comparable results for glycerol and for EG based on the synthesized ternary; however, they give results differing by nearly a factor of 2 using the data from Woods et al.[8].

For *Xenopus* in EG, the total osmolalities at flash and the molalities of EG at flash depict another significant point; namely, they are tightly grouped (the bottom-right six values). That is not the case with *Xenopus* oocytes in glycerol or with mouse oocytes in either EG or glycerol. In mouse, the tight grouping occurs with respect to **U** and $\mathbf{m_s}$. A different way to express this is that in mouse oocytes, the flash (IIF) temperature drops substantially with increasing concentrations of glycerol or EG. In *Xenopus*, the flash temperature is essentially unaffected by the concentration of EG.

In mouse oocytes, the fact that IIF occurs within a narrow range of unfrozen fractions leads us to conclude that the two are causally related. In *Xenopus* oocytes the fact that IIF does *not* occur within a narrow range of unfrozen fractions, leads to the conclusion that the two are not causally related. Rather, for *Xenopus* in EG, the correlative factor is that IIF occurs in a narrow range of EG concentrations or in a narrow range of solution osmolalities. Because of the relatively high rate at which the *Xenopus* oocytes were cooled (10°C/min) in [1] and [2], the intracellular concentration of EG and the intracellular osmolality remain effectively what they were prior to cooling (i.e., there is little or no cell dehydration during freezing). But the extracellular molality of EG and the total extracellular osmolality at the flash temperature have risen about 10-fold at the time of flashing. The difference between the total osmolality inside the cell and the total osmolality outside the cell (here a very large value) provides the driving force for water efflux. The greater that difference, the greater will be the number of water molecules traversing a unit area of membrane per unit time. At first thought, this would seem to fit in nicely with the osmotic poration theory of IIF proposed by Muldrew and McGann [6]; namely, that when osmotic water flux across the cell membrane exceeds a critical value, the membrane is damaged in ways that allow the penetration of external ice. But why then doesn't this occur in the case of *Xenopus* in glycerol? In glycerol, the flash temperature does not correlate with the solution osmolality at that temperature. This leads to a tentative conclusion that IIF in *Xenopus* in EG is a result of chemical damage to the membrane occurring when the concentrations of EG rise to 8 or 9 molal. Such chemical damage does not seem to be a major factor in the case of glycerol.

The use of the synthesized ternary phase diagrams to compute unfrozen fractions and solute concentrations in the partly frozen EG solutions results in substantial alterations in the values previously published by Mazur et al. [5] and Guenther et al. [1], but they do not alter the qualitative conclusions drawn in those papers; namely, the occurrence of IIF in the mouse oocyte is closely coupled with the reduction of the unfrozen fraction to a critical value. That is less the case with *Xenopus* oocytes in glycerol and not at all the case for *Xenopus* in EG.

Table 4 has two purposes. First it summarizes the data we have obtained to date on the physical properties of the external solution at the temperatures at which IIF occurs in mouse and *Xenopus* oocytes. All the depicted data for EG are based on the synthesized ternary phase diagrams. Second, the four right-hand columns of the table give the ratios of the high value of a given property in a given set to the low value in that set. The upper third and bottom third of the table summarize the data and ratios from Tables 1–3 in this paper. Note that in these cases the suspending media contained various concentrations of glycerol or EG, all prepared in isotonic PBS. As a consequence, the weight ratios of CPA to salt (R values) varied in each solution, and different isopleths of the ternary phase diagram were applicable. The middle third of Table 4 summarizes the data in [4]. In that case, the external solutions were prepared to hold the R value constant in each set. To meet this restriction, a change in the CPA concentration required a concomitant change in the PBS concentration.

Several comments are in order:

1. The mean **U** value at IIF for mouse oocytes in EG is significantly higher ($p = 0.028$ [two-tailed **t** test]) in EG/PBS (0.08) than in glycerol /PBS or in the absence of any CPA (0.06). The solute concentration values were correspondingly lower. Perhaps this difference is related to the fact that mouse oocytes are highly permeable to EG and highly impermeable to glycerol. As a consequence, oocytes in the latter are considerably more shrunken than those in EG. However, antithetical to this explanation is the fact that in the middle section involving constant R solutions, as the PBS concentration increases from 0.6X to 2 X, the oocytes are increasingly shrunken at the start of an experimental run, even in EG.

2. We are interested in determining which property of the external solution seems to correlate best with the flash temperature, and we use the high/low ratios of **U, $\mathbf{m_s}$, $\mathbf{m_{CPA}}$**, and total osmolality in each data set of Table 4 for that purpose. In mouse oocytes with one major exception and one minor one , the high/low ratios are smaller for **U** than for the other properties of the solutions at the temperatures of IIF. The major exception is in the constant R11 glycerol solutions, where the high/low ratio for **U** is substantially higher (2.8) than for **$\mathbf{m_s}$, $\mathbf{m_{CPA}}$**, and total osmolality (1.3-1.4). That is a result of the very high value of 0.114 for the **U** in the R11-2X-G18 solution, a case that represents only a single oocyte. If it is eliminated, the high/low ratio drops to 1.5. The minor exception is in the variable R EG2 to EG9 solutions. There again, if the highest value of **U** (0.105) is omitted, the ratio drops from 1.9 to 1.6, equal to or lower than the ratios for the three concentration properties. As we have pointed out [1,4,5], there is reasoning to believe that IIF at temperatures below −40°C is due to homogeneous nucleation of the intracellular supercooled water rather than due to the attainment of a critically low U value.

3. The **U** values at the flash temperature for *Xenopus* oocytes are 2-3 times higher than the **U** values for mouse oocytes. This supports our growing belief that cell size is an important determinant of the IIF temperature; i.e., larger cells tend to undergo IIF at higher temperatures and higher **U** values than smaller cells. The data in Tables 3 and 4 are for *Xenopus* Stages I and II oocytes (~ 200-400 μm in diameter). Kleinhans et al. [2] have shown by differential scanning calorimetry that the IIF temperature of Stage V *Xenopus* oocytes (~1,000 μm diameter) is far higher.

4. In most cases the flash temperatures do not occur within a narrow range of CPA concentrations or the total osmolalities of the external solution. A dramatic exception to that is in the case of *Xenopus* oocytes in EG solutions where the correlation with **$\mathbf{m_{CPA}}$** or total osmolality is nearly three times better, based on high/low ratios, that that with **U** or **$\mathbf{m_s}$**. We have already discussed the possible significance of that fact.

We increasingly believe that the temperature at which IIF occurs depends on a sequence of events. The first event is the formation of external ice. IIF almost never occurs in its absence. The second event is the reduction of the unfrozen fraction of water in the external solution to a critical value. That critical value in turn depends on the size of the cell that is undergoing freezing. In a large cell like Stage V *Xenopus* oocytes (1 mm diameter), which undergoes IIF at −8.5°C [2], the critical **U** is 0.43. In the smaller Stage I and II *Xenopus* oocytes (diameter ~300 μm), the critical value is 0.145. In the still smaller mouse oocyte (diameter 75 μm), it is 0.06 to 0.08. If the external medium has not attained the critical value of **U** by the time it has cooled below about −40°C, the cell then undergoes IIF by homogeneous nucleation of its internal supercooled water. The CPAs, glycerol and EG, suppress the IIF temperature, at least for mouse oocytes, and the reason they do so is that they suppress the temperature at which the critical value of **U** is attained in the external medium. One can speculate as to why the critical fraction of external unfrozen water might be affected by cell size, but there is currently no experimental information on this point.

Acknowledgements

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Table 1

Physical properties of the external solution at the temperature of IIF in mouse oocytes frozen in PBS, glycerol/PBS, and EG/PBS: Effect of the source of the EG ternary phase diagrams on those properties.

Solution	CPA	M_{CPA}	R	R'	Flash temp. °C	W^o_T	W_T at flash	L at flash	U at flash	m_s at flash	m_{CPA} at flash
R0-1X	none	0	0	0	−13.9	0.88	16.8	0.07	0.06	3.62	0
R5-1X-G4	Glycerol	0.5	5.43	3.44	−30.8	5.37	51.1	0.11	0.06	3.11	10.7
R11-1X-G9		1.0	11.3	7.14	−41.3	9.76	63.0	0.16	0.06	2.44	17.4
								Calculated from Woods et al.[8]			
R4-1X-EG3	EG	0.5	3.62	3.41	−23.4	3.92	26.4	0.16	0.13	1.41	4.80
R8-1X-EG6		1.0	7.45	7.01	−37.2	6.94	39.0	0.18	0.12	1.33	9.06
R12-1X-EG9		1.5	11.51	10.83	−40.8	9.94	42.4	0.24	0.15	1.02	11.1
								Calculated from synthesized phase diagrams- Kleinhans and Mazur [3]			
R4-1X-EG3	EG	0.5			**As above**		35.7	0.12	0.08	2.16	7.35
R8-1X-EG6		1.0					48.5	0.15	0.08	1.95	13.7
R12-1X-EG9		1.5					51.6	0.19	0.10	1.48	16.0

R is the wt. ratio of CPA/NaCl; **R'** is the mole ratio; $\mathbf{W^o_T}$ is the total weight % of solute prior to freezing;$\mathbf{W_T}$ is the total weight % of solutes in the unfrozen solution at the temperature of IIF; **L** is the unfrozen mass fraction of liquid solution at IIF; **U** is the unfrozen fraction of water at the IIF temperarture; $\mathbf{m_S}$ and $\mathbf{m_{CPA}}$ are the molalities of salt and CPA in the unfrozen fraction at IIF.

L is calculated as $\mathbf{W^o_T}$/ $\mathbf{W_T}$; $\mathbf{W_T}$ for PBS and glycerol/PBS are calculated from the equations of Pegg [7] [See Mazur et al.[5, p.39]; $\mathbf{W_T}$ for EG in the middle section is calculated from the equations of Woods et al. [8] (see [5, p. 39];$\mathbf{W_T}$ for the bottom three rows is from Fig 6 of Kleinhans and Mazur [3];formulas for U, m_S, and m_{Cpa} are given on p. 40 of [5].

The values in columns 3, 4, 5, and 7 are from Table 1 of [5]; the values of the flash temperatures (column 6) are from Table 4 of that paper.

The means in the five right hand columns (here and in succeeding tables) were calculated by averaging the individual computed values; e.g. $L_{mean} = \Sigma\,(L_i))/N = \Sigma(W^o_T/W_{Ti})/N$ [Eq 1]. Because of this, inserting the mean values into the formulas in some instances gives slightly different results from those shown; e.g. $L_{mean} = W^o_T/(\Sigma\, W_{Ti}/N)$ [Eq 2] can differ from $L_{mean} = \Sigma(W^o_T/W_{Ti})/N$.

Standard errors have been omitted for clarity. Those in the upper two-thirds are given in Table 4 of [5]. Those in the bottom third are nearly identical.

Table 2

Same as Table 1 except the data are for 0.34, 0.63, and 0.73 M EG*

M_{EG}	R	R'	Flash Temp (°C)	W^o_T	W_T at flash	L at flash	U at flash	m_s at flash	m_{EG} at flash
						Calculated from Woods et al. [8]			
0.34	2.51	2.36	−25.3	2.92	28.3	0.11	0.09	1.99	4.69
0.63	4.72	4.44	−25.4	4.68	28.3	0.19	0.15	1.25	5.54
0.73	5.50	5.18	−30.0	5.29	33.0	0.16	0.12	1.30	6.76
						Calculated from synthesized phase data [3]			
0.34					36.8	0.08	0.06	2.92	6.90
0.63			**Same as data above**		37.9	0.13	0.09	1.93	8.56
0.73					43.2	0.12	0.07	2.02	10.45

*The values for these molarities are tabulated separately from Table 1 to facilitate visual comparison between Table 1 (mouse) and Table 3 (*Xenopus*).

Table 3

Physical properties of the external solution at the temperature of IIF in *Xenopus* oocytes frozen in Ringers, glycerol/Ringers, and EG/Ringers: Effect of the source of the EG ternary phase diagrams on those properties.

Solution	CPA	MCPA	R	R'	Flash temp. °C	W^{o}_{T}	W_T at flash	L at flash	U at flash	m_s at flash	m_{CPA} at flash	Total Osm from molal	Osm from $T_{flash}/1.855$
XR0-1X	none	0	0	0	−11.4	0.60	14.6	0.04	0.04	3.03	0	6.1	6.3
XR8-1X-G5	Glycerol	0.5	7.88	5.00	−12.2	5.12	33.1	0.16	0.11	0.95	4.76	6.7	6.6
XR16-1X-G9		1	16.4	10.4	−15.3	9.53	37.9	0.25	0.18	0.64	6.60	7.8	8.2
XR25-1X-G13		1.5	25.5	16.2	−29.4	13.87	53.4	0.27	0.15	0.78	12.57	14.0	15.6
							Calculated from Woods et al. [8]						
XR5-1X-EG3	EG	0.5	5.26	4.95	−24.6	3.66	28.3	0.14	0.10	1.09	5.40	7.6	13.3
XR11-1X-EG6		1	10.8	10.2	−18.6	6.70	22.4	0.34	0.29	0.45	4.54	5.4	10.0
XR17-1X-EG9		1.5	16.7	15.7	−21.1	9.72	25.9	0.39	0.33	0.34	5.40	6.1	11.4
								Calculated from synthesized phase diagrams- Kleinhans and Mazur [3]					
XR5-1X-EG3	EG	0.5			**As above**		38.3	0.10	0.06	1.74	8.60	12.1	13.3
XR11-1X-EG6		1					32.3	0.22	0.17	0.74	7.53	9.0	10.0
XR17-1X-EG9		1.5					36.3	0.27	0.20	0.56	8.82	9.9	11.4

Column headings have the same meanings as in Table 1, except that two more columns are added. The values in columns 3, 4, 5, and 7 are from Table 1 of Guenther et al. [1]; the values of the flash temperature (column 6) are from Table 5 of [1].

The two new columns are the calculated total osmolality of the external medium at IIF. In column 13 it is approximated as $\boldsymbol{M} = \Sigma\varphi\nu m$, where φ (the osmotic coefficient is assumed = 1 for both CPA and salt, and ν (the number of species of molecules) is 1 for CPA and 2 for NaCl. In column 14, 1.855 is the molal freezing point constant for water.

* The values in rows 5-8 of columns 8–12 differ significantly from those reported for EG in Table 5 of [1]. That is because in preparing the present manuscript, we found that a set of parantheses in the Excel formula used in the Guenther et al. paper to calculate $\mathbf{W_T}$ had been misplaced and yielded erroneous values.

Table 4

Physical properties of the external solution at the temperature of IIF in mouse and *Xenopus* oocytes: Ratios of high to low values

Solution	CPA	Molarity CPA	R	Flash Temp (°C)	U at flash	m_s at flash	m_{CPA} at flash	Osm_K at flash	High-Low Ratios U	m_s	m_{CPA}	Osm_K
Mouse oocytes-variable R-fixed PBS (isotonic)												
R0-1X	None	0	0	−13.9	**0.062**	**3.62**	**0**	**7.49**	—	—	—	—
R5-1X-G4	Glycerol	0.5	5.43	−30.8	0.063	3.11	10.7	16.6				
R11-1X-G9		1.0	11.3	−41.3	0.065	2.44	17.4	22.3				
			Mean (Glycerol)		**0.064**	**2.78**	**14.1**	**19.4**	**1.0**	**1.3**	**1.6**	**1.3**
R3-1X-EG2	EG	0.34	2.51	−25.3	0.056	2.92	6.9	13.6				
R4-1X-EG3		0.5	3.62	−23.4	0.080	2.16	7.4	12.6				
R5-1X-EG4		0.63	4.72	−25.4	0.089	1.93	8.6	13.7				
R6-1X-EG5		0.73	5.5	−30.0	0.074	2.02	10.5	16.2				
R8-1X-EG6		1.0	7.45	−37.2	0.082	1.95	13.7	20.1				
R12-1X-EG9		1.5	11.51	−40.8	0.105	1.48	16.0	22.0				
			Mean (EG)		**0.081**	**2.08**	**10.5**	**16.4**	**1.9**	**1.4**	**1.9**	**1.6**
Mouse oocytes-constant R-variable PBS												
R5-0.6X-G3	Glycerol	0.31	5.42	−26.9	0.037	2.57	8.7	14.5				
R5-0.75X-G3		0.36	5.42	−23.7	0.052	2.27	7.7	12.8	**1.7**	**2.5**	**2.5**	**2.1**
R5-1X-G4		0.50	5.44	−30.8	0.063	3.11	10.7	16.6				
R5-2X-G9		0.99	5.42	−49.5	0.059	5.68	18.9	26.7				
R11-0.6X-G6	Glycerol	0.63	11.26	−36.1	0.045	2.11	14.3	19.5				
R11-0.75X-G7		0.72	11.26	−44.6	0.041	2.71	19.1	24.0	**2.8**	**1.4**	**1.4**	**1.3**
R11-1X-G9		1.00	11.26	−41.3	0.065	2.44	17.4	22.3				
R11-2X-G18		2.00	11.26	−46.8	0.114	2.87	20.5	25.2				
			Mean (Glycerol)		**0.060**	**2.97**	**14.6**	**20.2**				
R4-0.6X-EG2	EG	0.31	3.66	−14.6	0.098	1.17	4.8	7.9				
R4-0.75X-EG2		0.37	3.64	−19.6	0.073	1.71	6.4	10.6	**1.3**	**3.2**	**2.7**	**2.9**
R4-1X-EG3		0.50	3.62	−23.4	0.080	2.16	7.4	12.6				
R4-2X-EG6		1.00	3.65	−42.9	0.083	3.76	13.0	23.1				
R8-0.6X-EG4	EG	0.63	7.58	−23.7	0.077	1.25	9.0	12.8				
R8-0.75X-EG5		0.73	7.60	−27.2	0.080	1.42	10.2	14.7	**1.6**	**2.1**	**2.1**	**2.1**

Solution	CPA	Molarity CPA	R	Flash Temp (°C)	U at flash	m_s at flash	m_{CPA} at flash	Osm_K at flash	High-Low Ratios U	m_s	m_{CPA}	Osm_K
R8-1X-EG6		1.00	7.45	−37.2	0.082	1.95	13.7	20.1				
R8-2X-EG12		2.06	7.59	−50.5	0.126	2.60	18.6	27.2				
			Mean (EG)		**0.087**	**2.00**	**10.4**	**16.1**				
Xenopus oocytes-variable R-fixed Ringer's (isotonic)												
XR0-1X	None	0	0	−11.4	0.037	3.03	0.0	6.1	—	—	—	—
XR-8-1X-G5	Glycerol	0.5	7.88	−12.2	0.113	0.95	4.8	6.6				
XR-16-1X-G9		1.0	16.35	−15.3	0.177	0.64	6.6	8.2				
XR-25-1X-G13		1.5	25.48	−29.4	0.148	0.78	12.6	15.6				
			Mean (Glycerol)		**0.146**	**0.79**	**8.0**	**10.1**	**1.6**	**1.5**	**2.6**	**2.4**
XR5-1X-EG3	EG	0.5	5.25	−24.6	0.063	1.74	8.6	13.3				
XR-11-1X-EG6		1.0	10.81	−18.6	0.167	0.74	7.5	10.0				
XR17-1X-EG9		1.5	16.7	−21.1	0.196	0.56	8.8	11.4				
			Mean (EG)		**0.142**	**1.00**	**8.3**	**11.6**	**3.1**	**3.1**	**1.2**	**1.3**

Osm_K is the total osmolality of the external solution at the flash temperature calculated as **T_{flash}/1.855**.

The values for EG have been computed using the synthesized ternary phase diagram for EG/salt [3].

Some of the 1X data in the Mouse Oocytes-Variable R section also appear in the Constant R section. All the data in the Constant R section come from Table 4 of [4]; however, some of the values for **U**, **m_S**, and **m_{CPA}**, here differ slightly from those in [4]. This is because, here, the means were calculated by Eq 1 in the footnote of Table 1, whereas in [4], they were calculated by Eq 2.