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Comparison of actual vs. synthesized ternary phase diagrams for solutes of cryobiological interest ☆

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Abstract

Phase diagrams are of great utility in cryobiology, especially, those consisting of a cryoprotective agent (CPA) dissolved in a physiological salt solution. These ternary phase diagrams consist of plots of the freezing points of increasing concentrations of solutions of cryoprotective agents (CPA) plus NaCl. Because they are time-consuming to generate, ternary diagrams are only available for a small number of CPAs. We wanted to determine whether accurate ternary phase diagrams could be synthesized by adding together the freezing point depressions of binary solutions of CPA/water and NaCl/water which match the corresponding solute molality concentrations in the ternary solution. We begin with a low concentration of a solution of CPA + salt of given R (CPA/salt) weight ratio. Ice formation in that solution is mimicked by withdrawing water from it which increases the concentrations of both the CPA and the NaCl. We compute the individual solute concentrations, determine their freezing points from published binary phase diagrams, and sum the freezing points. These yield the synthesized ternary phase diagram for a solution of given R. They were compared with published experimental ternary phase diagrams for glycerol, dimethyl sulfoxide (DMSO), sucrose, and ethylene glycol (EG) plus NaCl in water. For the first three, the synthesized and experimental phase diagrams agreed closely, with some divergence occurring as wt% concentrations exceeded 30% for DMSO and 55% for glycerol, and sucrose. However, in the case of EG there were substantial differences over nearly the entire range of concentrations which we attribute to systematic errors in the experimental EG data. New experimental EG work will be required to resolve this issue.

Keywords: Ternary phase diagrams; Freezing points; Glycerol; Dimethyl sulfoxide (DMSO); Sucrose; Ethylene glycol

Phase diagrams are of great importance and utility in cryobiology, especially those consisting of a cryoprotective agent (CPA) dissolved in a physiological salt solution (ternary phase diagrams). A ternary phase diagram depicts the freezing point of a solution as a function of the total weight percent (wt%) concentration of solute (CPA + salt) present. Or to put it another way, it depicts the total equilibrium concentration of solutes that will exist in the unfrozen portion of a solution frozen to a given sub-zero temperature. From that concentration, one can also compute the osmotic pressure of the solution which in turn provides one of the essential inputs to calculate the volume response of the cell if it is not in equilibrium with the medium and the rate at which it attains equilibrium. Phase diagrams also permit one to determine the fractions of a solution that are frozen and unfrozen at a given subzero temperature.

A typical ternary phase diagram (glycerol/NaCl/water) is shown in Fig. 1. The curve of freezing point vs. wt% solute is referred to as an isopleth. In ternary phase diagrams the position and shape of isopleths depends on

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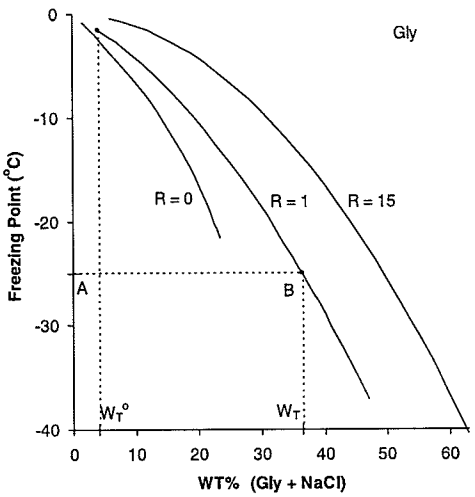

Fig. 1. Representative partial phase diagrams of the ternary system glycerol/NaCl/water for three weight ratios (R) of glycerol to NaCl. The curve for $R = 0$ is the phase diagram for the binary system NaCl/water (see Fig. 2a). The curves for $R = 1$ and 15 are derived from data of Shepard et al. [11] and Pegg [5]. The curves or isopleths depicted are for the freezing portion of the phase diagram; i.e. the portion where ice is in equilibrium with an unfrozen solution of glycerol/NaCl and water. The vertical tie-line W_T^0 represents the freezing of an arbitrary initial solution of glycerol/NaCl/water of that weight percent where $R = 1$. When that solution is frozen to −25 °C (point A), the weight percent composition of the residual unfrozen solution ($W_T = 36.5\%$) is given by the horizontal tie line to point B.

the wt% ratio (R) of CPA to salt that is used. Fig. 1 depicts isopleths for $R = 0$, 1, and 15. The curve for $R = 0$ is the curve for the binary solution of salt/water. The curve for $R = \infty$ would be the curve for pure CPA/water. It lies close to the $R = 15$ curve. The favored current method of determining freezing points (actually, melting points) is by differential scanning calorimetry. Doing so is not a simple task. It requires access to a $50,000 instrument; it requires a number of runs at many concentrations to determine a single isopleth; and it requires that such runs be repeated for each R value used. The consequence has been that relatively few such determinations have been published: glycerol/NaCl/water [11], DMSO/NaCl/water [3]; sucrose/NaCl/water [1]; and EG/NaCl/water [13]. A much greater number of binary phase diagrams (solute/water) are available and we wished to determine whether usable ternary phase diagrams could be synthesized by combining binary phase data for a CPA/water solution with binary phase data for NaCl/water. Usually, authorities advise against this procedure saying that unknown and incalculable interactions between two solutes and between them and water are likely to introduce large errors. To our knowledge, however, the magnitude of the errors created has never been assessed. The purpose of our study is to make that assessment.

In this paper, we compare the results for constructed synthetic ternary phase diagrams with published ternary phase diagram data for glycerol/NaCl/water; DMSO/NaCl/water; Sucrose/NaCl/water; and ethylene glycol/NaCl/water.

Theory

Our basic approach for constructing the ternary diagrams is as follows: To determine ternary phase diagrams experimentally (here CPA + salt + water), one determines the freezing point (more typically the melting point) of a series of increasing weight percent (wt% or W_T) concentrations of a solution in which the wt% ratio (R) of CPA and salt is held constant. When these freezing points are plotted as a function of the wt% (CPA + salt), the results are the sorts of curves or isopleths shown in Fig. 1. Another way to consider these isopleths is the following: If one cools a solution of initially low wt% concentration of a mixture of CPA and salt (indicated by W_T^0 in Fig. 1), pure water begins to freeze out of the solution at the freezing point (neglecting supercooling), leaving the remaining solution increasingly more concentrated in the solutes. Because no solute is removed, R remains constant while the total weight percent of CPA + salt increases to a value, W_T that is dependent only on temperature. This is exemplified in Fig. 1 by the horizontal and vertical tie lines at −25 °C. Put differently, the freezing point of an $R = 1$ glycerol/NaCl/water solution with a W_T of 36.5% is −25 °C.

Synthesized isopleths for CPA + salt solutions were constructed from published experimental binary phase diagrams for NaCl/water and CPA/water as follows. We begin with a ternary solution of low total weight percent, W_T^0, and given R. We then mimic the effect of progressive ice formation and the resulting increase in total solute concentration (W_T) by progressively reducing the weight percent of water present. Knowing W_T and R, one can readily obtain the corresponding individual weight percents or molalities of CPA and NaCl. We then use published binary phase diagram data to determine the freezing points of these individual molalities of CPA and of salt. Finally, the freezing point of the ternary system CPA/NaCl water corresponding to that W_T is taken to be the sum of the freezing points of the two binary solutions.

Methods

Solution equations

Consider two solutes, A and B dissolved in water, where A is the cryoprotectant (CPA) and B is NaCl. Let W_A and W_B be the weight percents of the solutes in solution. R is defined as:

$$R = W_A/W_B \tag{1}$$

and W_T is defined as:

$$W_T = W_A + W_B \tag{2}$$

Since the wt%'s have to add to 100% for a solution, the wt% of water, W_W, is just:

$$W_W = 100\% - (W_A + W_B) \quad (3)$$

We note, parenthetically, that it is often convenient to think in terms of a 100 g solution. In this case the wt% of each constituent is just its weight in grams in the solution. Then, wt% is often re-expressed in units of (g/100 g). Continuing, W_A and W_B can be expressed in terms of R and W_T as:

$$W_A = R * W_T/(R+1) \quad (4a)$$

$$W_B = W_T/(R+1) \quad (4b)$$

Finally, the molalities of the solutes are given by:

$$m_A = (W_A/MW_A)/(W_W/1000) \quad (5a)$$

$$m_B = (W_B/MW_B)/(W_W/1000) \quad (5b)$$

where MW_A and MW_B are the gram molecular weights of A and B, respectively. We will use experimental binary phase diagram data to determine the freezing points (FP's) of solute A and B from their molalities. Then the freezing point of the synthesized ternary solution corresponding to a given W_T is:

$$FP_T = FP_A + FP_B \quad (6)$$

With this, the synthesized ternary phase diagram isopleths may be drawn.

In summary, then, the procedure was: (a) Obtain binary phase diagrams from the literature for NaCl, glycerol, DMSO, sucrose, and EG, each in water. (b) Choose ternary phase data from the literature for each CPA in NaCl/water. (c) Construct synthetic ternary phase diagrams. And (d) compare the synthetic and experimental ternary phase diagrams, i.e. compare some specific isopleths in the range of cryobiological interest. In all cases, we are only interested in the portion of the phase diagrams for which ice is the only solid precipitate.

Binary phase diagrams

Binary phase diagram data (i.e. freezing point depression of a single solute in water as a function of solute concentration, up to the solute solubility limit) were obtained from the sources in Table 1. Other binary phase diagrams have been published, especially for NaCl, EG, and glycerol. The sources in Table 1 were used because of the ranges covered by the data and lack of anomalies. There were differences in freezing points among the various sources, but they were generally small.

The experimental data for the five solutes are plotted in Fig. 2a–e. Each binary phase diagram was fitted with a cubic polynomial to permit the calculation of the freezing point corresponding to any given molality of solute. The data were thinned at low concentrations to yield a more even spread of points across the entire concentration range, Fig. 2a–e. This prevents the low concentration data from being unduly weighted in the fits. Microsoft Excel was used for the fitting and it allows the option of forcing the fit through zero. This was done so that the fits yield zero FP for zero solute. In practice this had little effect on the fits. Eliminating this constraint typically yielded a polynomial constant, C_0, of less than a few tenths degree.

The fits are illustrated in Fig. 2 and the fitting coefficients are in Table 2. In all cases, the fits are smooth and deviate from the published experimental phase curves by 0.3 °C or less, typically much less.

Literature ternary data

Ternary data of cryobiological interest are available for glycerol [11], DMSO [3], sucrose [1], and EG [13]. They were obtained by differential thermal analysis and differential scanning calorimetry. In the case of glycerol, DMSO, and EG, respectively, Pegg [6], Pegg [7], and Woods et al. [13] published general equations that permit one to determine isopleths for any R. For these three cases we chose R values corresponding to solutions of 0.5, 1.0, and 1.5 molar CPA in isotonic saline. In the case of sucrose, no fitting equations are available; i.e. there are data only for specific isopleths; namely, $R = 3/7$, 1, 7/3, 5, 9, and 19. Consequently, for sucrose we chose R values closest to

Table 1
Binary phase diagrams of NaCl, glycerol, DMSO, sucrose, and EG in water

Solute	Data source[a]
NaCl	CRC Handbook of Chemistry and Physics [12]; eutectic from Seidell [10]
Glycerol[b]	CRC Handbook of Chemistry and Physics (low concentrations) [12], Melinder (high concentrations) [5]
Dimethyl sulfoxide[c]	Rasmussen and MacKenzie [9]
Sucrose[c,d]	CRC Handbook of Chemistry and Physics (low concentrations) [12] Gayle et al. (high concentrations) [1]
Ethylene glycol[e]	CRC Handbook of Chemistry and Physics [12]

[a] Older additions of the CRC Handbook of Chemistry and Physics (those published in the 1970s) have extensive tables prepared by AV Wolfe et al. of binary solutions, including their phase behavior, although DMSO is notably absent. Typically these tables do not give the freezing points all the way to the eutectic limit. Thus other sources are necessary to obtain the full phase diagram. One good source is the work of Ake Melinder [5].

[b] For glycerol, the CRC is used from 0 to 10 wt% and Melinder from 19.5 to 63 wt%. In the region of data overlap, they differ by less than 0.2 °C in freezing point.

[c] The Rasmussen/MacKenzie and Gayle et al. data is in graphical form and had to be digitized.

[d] The CRC was used to 24 wt% and Gayle et al. from 30 to 65 wt%. In the region of data overlap, they differ by as much as 0.4 °C in freezing point.

[e] The CRC and Melinder have essentially identical data and Hayes and Pegg [2] have similar data which differ by less than a degree across most of the range.

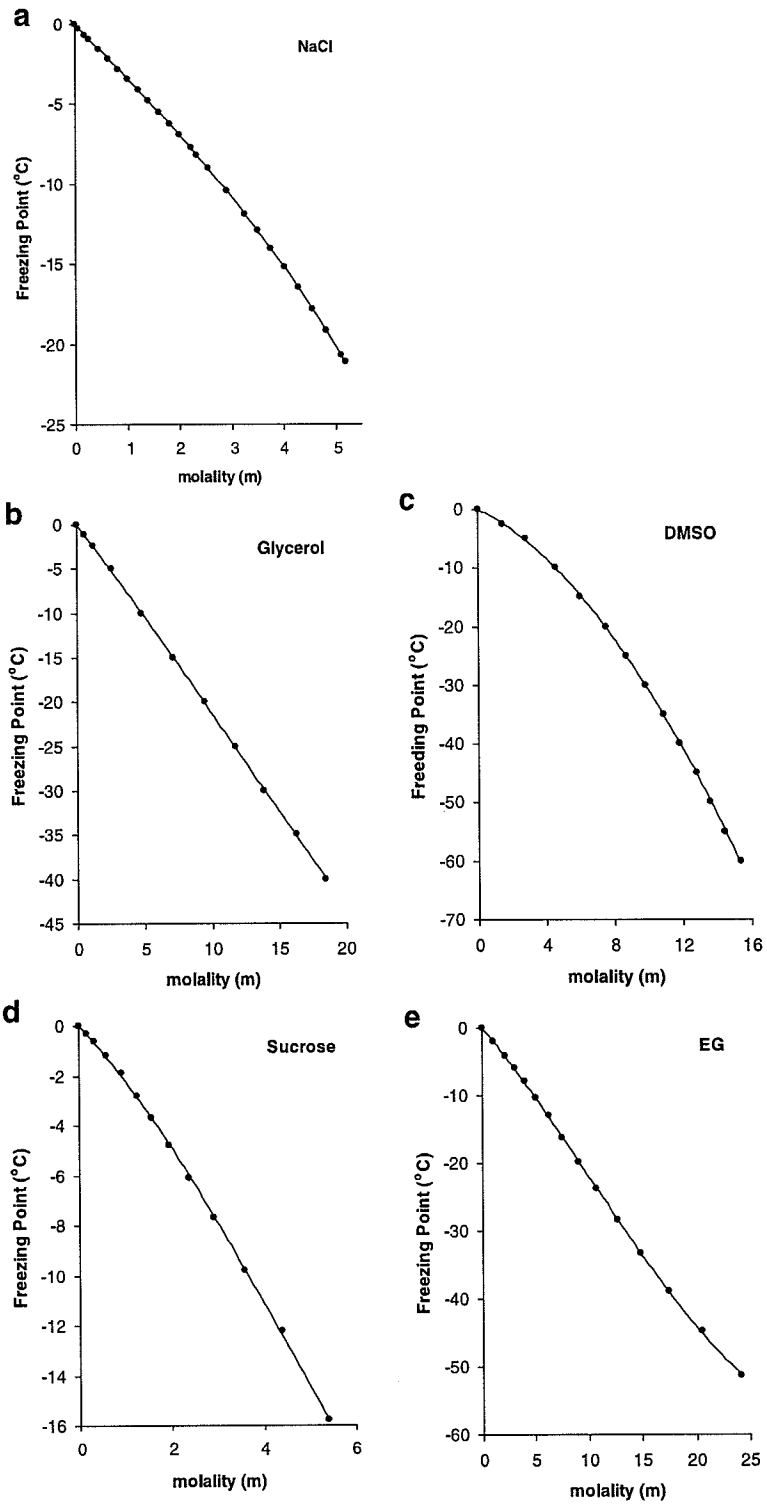

Fig. 2. Partial phase diagrams of the binary systems of NaCl (a), glycerol (b), DMSO (c), sucrose (d), and EG (e), all in water. Solute concentrations are expressed as molality. The sources of the data are given in Table 1. The points depicted are values interpolated from the published fitted curves of experimental data. In the case of sucrose and DMSO, the published data are in graphical form and had to be digitized for use here.

those of cryobiological interest. This latter data set is in graphical form and was digitized for use here. Table 3 lists representative starting compositions including W_T^0 for generating the synthetic ternary data. In that table, the salt concentration is held at isotonic and the weight percent of CPA adjusted to provide the specific R value.

Table 2
Polynomial representation of the binary phase diagrams[a,b]: $FP = 0 + C_1 * m + C_2 * m^2 + C_3 * m^3$

Solute	C_1	C_2	C_3	Maximum molality[c]
NaCl	−3.34	−0.0201	−0.0231	5.2
Glycerol	−1.96	−0.0234	+6.89E−4	18.5
Dimethyl sulfoxide	−1.38	−0.177	+6.92E−4	15.4
Sucrose	−1.93	−0.301	+0.0221	5.4
Ethylene glycol	−1.83	−0.0531	+1.71E−3	24.2

[a] FP, freezing point and 'm', solute molality. The coefficient $C_0 = 0$ because all the fits were forced through zero, yielding zero freezing point depression for zero solute.
[b] The 'goodness of fit', R^2, is greater than 0.9996 for all five fits.
[c] To avoid misuse of these fits, we report the maximum molality for which the fits were determined. In all cases, these are near or at the phase diagram limits for freezing without precipitation of the solute, so extrapolation is meaningless. In the case of sucrose, the solution is supersaturated at freezing points somewhere below ≈−9.5 to −14 °C.

Table 3
Illustrative examples of starting concentrations (i.e. before freezing commences) of ternary solutions of CPA/isotonic NaCl used to generate synthesized ternary phase diagrams[a]

CPA	R	Molarity[b](CPA in isotonic saline)	Wt% NaCl - (g/100 g solution)	Wt% CPA - (g/100 g solution)	Total wt% (NaCl + CPA) (W_T^0)	NaCl (molality[c])	CPA (molality[c])
Glycerol	5.43	0.5	0.835	4.538	5.373	0.151	0.521
	11.28	1.0	0.796	8.985	9.782	0.151	1.082
	17.60	1.5	0.758	13.344	14.102	0.151	1.687
DMSO	4.60	0.5	0.841	3.872	4.713	0.151	0.520
	9.55	1.0	0.808	7.710	8.518	0.151	1.079
	14.87	1.5	0.774	11.514	12.289	0.151	1.680
Sucrose	5	0.125	0.838	4.192	5.030	0.151	0.129
	9	0.220	0.811	7.300	8.111	0.151	0.232
	19	0.442	0.750	14.255	15.005	0.151	0.490
Ethylene glycol	3.63	0.5	0.848	3.079	3.927	0.151	0.516
	7.47	1.0	0.821	6.135	6.656	0.151	1.062
	11.54	1.5	0.795	9.169	9.964	0.151	1.641

[a] These starting concentrations and R values were selected for illustration because they are commonly used in cryobiology; i.e. the salt is isotonic (0.151 molal) and the molar concentrations of CPA fall in the range commonly used. However, as discussed in the text, the choice of starting concentration, W_T^0, to generate an isopleth is arbitrary.
[b] The appendix shows how to convert between wt%'s and molarities.
[c] Molality, m, is given by (wt% solute/gram molecular weight solute) * (1000/wt% water), where the weight% water (W_W) is just ($100 - W_T^0$).

Synthetic ternary phase diagrams

Creating synthetic ternary phase diagrams is straightforward with the information collected above. The specific objective is to create isopleths for the R values shown in Table 3, i.e, we want plots of freezing point vs. total solute concentration (W_T) with R fixed. We will use the solution in the first line of Table 3 to illustrate the process, namely, a glycerol/NaCl/water ternary solution with $R = 5.43$. The starting solution prior to freezing contains 0.835 wt% NaCl (0.151m) and 4.538 wt% glycerol (0.521m) and thus $W_T^0 = 5.373\%$. This corresponds to a 0.5 M solution of glycerol in isotonic NaCl. Considered as (independent) binary solutions and using the polynomial fits in Table 2, we obtain freezing points of −0.50 and −1.03 °C for those starting concentrations of NaCl and glycerol, respectively (Table 4, columns 8 and 9). Summing yields −1.53 °C, our (synthetic) estimate of the freezing point of this initial ternary solution. As this solution is cooled below its FP, pure ice freezes out of the solution while the solutes remain in solution. As a consequence, the fixed mass of solutes is dissolved in a smaller quantity of liquid water. This 'new' solution freezes at a lower temperature and we slide down the curve of FP vs. W_T (Fig. 1). Here, as illustrated by column 5 in Table 4, we mimic this situation by removing increasing quantities of water while holding the masses of the solutes constant. As the percentage of water in solution, W_W, decreases, the solute concentrations, W_A and W_B, increase (columns three and four). Because all the solutes remain in solution, the solute ratio (R) remains fixed, (column two). Note that when the mass percents are expressed as (g/100 g), the 100 g refers to 100 g of solution and not solution plus ice.

In practice, we generate more points than the few in Table 4, and to obtain the full isopleth, some points of

Table 4
Example of the calculation of a synthetic isopleth for glycerol/NaCl/water[a]

W_T	R	W_A	W_B	W_W	m_A	m_B	FP_A	FP_B	FP_T
(Total solute)	(W_A/W_B)	(gly)	(NaCl)	(water)	(gly)	(NaCl)	(gly)	(NaCl)	(total)
(g/100 g)	—	(g/100 g)	(g/100 g)	(g/100 g)	(molality)	(molality)	(°C)	(°C)	(°C)
W_T	R	$=R * W_T/(R+1)$	$=W_T/(R+1)$	$=100 - W_T$	$=(W_A/MW_A) * 1000/W_W$	$=(W_B/MW_B) * 1000/W_W$	$=f(m_A)$	$=g(m_B)$	=sum
5.373	5.433	4.538	0.835	94.63	0.521	0.151	−1.03	−0.50	−1.53
10.0	5.43	8.45	1.55	90.0	1.019	0.296	−2.02	−0.99	−3.01
20.0	5.43	16.89	3.11	80.0	2.293	0.665	−4.61	−2.23	−6.85
35.0	5.43	29.56	5.44	65.0	4.938	1.432	−10.18	−4.89	−15.06
50.0	5.43	42.23	7.77	50.0	9.171	2.660	−19.43	−9.45	−28.88
65.0	5.43	54.90	10.10	35.0	17.032	4.940	−36.80	−19.76	−56.56

[a] The initial, starting solution is defined by the first two values in row one, $W_T = W_T^0 = 5.373$ and $R = 5.433$. All the remaining columns are computed from these first two using the formulas shown. W_A and W_B are the weight percents of solute A (glycerol) and solute B (NaCl). Their total is W_T. MW_A and MW_B are the gram molecular weight of glycerol and NaCl, respectively. FP is the freezing point, and the functions 'f' and 'g' are the polynomial functions in Table 2 for binary solutions of glycerol and NaCl, respectively, which yield the FP of each as a function of molality. For a given total weight % (W_T) and R value (5.43 here), the weight %'s and molalities of glycerol and NaCl can be computed as shown. The synthetic isopleth is constructed by making the assumption that the freezing point of the ternary solution is the sum of the FP's for glycerol and NaCl, each considered independently, i.e. as a binary solution. The first data row gives the composition and FP of an $R = 5.43$ (0.5 molar) solution of glycerol in isotonic NaCl (Table 3). The succeeding rows show the composition of more concentrated solutions of the same R value, where the concentration is increased by the removal of water (column 5). We can provide analogous tables for the other solutions and R values analyzed here.

lower W_T are also included. There is, in fact, nothing sacred about the 'cryobiological' starting solutions contained in Table 3. To generate a full isopleth, we actually started near zero solute weight percent with the desired R value, and increased the wt% to the limit imposed by the experimental phase diagram.

This synthetic isopleth generation procedure is repeated for each R value of each of the CPA solutions in Table 3, yielding Figs. 3–6 for glycerol, DMSO, sucrose, and ethylene glycol, respectively. In no case are synthetic data generated beyond the upper molality limits of the binary phase polynomials in Table 2. On these same graphs, we also plot the experimental, literature, isopleth data. The comparison of the synthetic and experimental isopleths is taken up in the next section.

Results

The ternary system glycerol/NaCl/water

The synthesized data for three R values are compared in Fig. 3a–c with the experimental ternary phase data for glycerol/NaCl/water published by Shepard et al. [11] based on fitting equations published by Pegg [6]. The agreement between the two curves is seen to be very good with substantial differences appearing only at wt% solute above 55% and freezing point depressions of about ≈−30 to −35 °C.

At low values of W_T the freezing points in the Shepard–Pegg curves are seen to be slightly higher than in the synthesized curves. This is probably because Shepard et al. obtained relatively few data points for solutions with R values close to those in Fig. 3 and most of those were at higher values of W_T; consequently, Pegg's fitting equations may be yielding values for the freezing points in the low W_T region that are slightly too high.

Both the experimental isopleths and the synthesized curves are based on solutions containing NaCl. However, Pozner et al. [8] have shown that solutions of glycerol or DMSO in more complex physiological saline exhibit phase behavior that is indistinguishable from that of solutions made with pure NaCl.

The ternary system DMSO/NaCl/water

The synthesized ternary data for DMSO/NaCl/water are plotted in Fig. 4a–c for three R values. These are compared with experimental phase data given by the ternary phase equations of Pegg [7] based on the published data of Hildebrandt et al. [3]. For $R = 4.60$, the two curves slowly diverge with increasing wt% up to 42% where the synthetic curve has a freezing point that is 6.9 °C higher. For $R = 9.55$ and 14.87, the synthesized curves follow the experimental curves up to 20 wt% and then lie slightly above the experimental curves up to the limit of 56 wt% solute.

The ternary system sucrose/NaCl/water

Fig. 5a–c compare the synthesized ternary phase data with the experimental data for sucrose, corresponding to three R values published by Gayle et al. [1]. The agreement between the two is extremely close up to near the limit of the binary sucrose/water phase data (Table 2) used to generate the synthetic curves. A small divergence begins to appear at about 55 wt% total solute.

The ternary system ethylene glycol/NaCl/water

The synthesized ternary data for EG/NaCl/water are plotted in Fig. 6a–c for three R values and compared with phase data computed from the experimental phase measurements of Woods et al. [13].

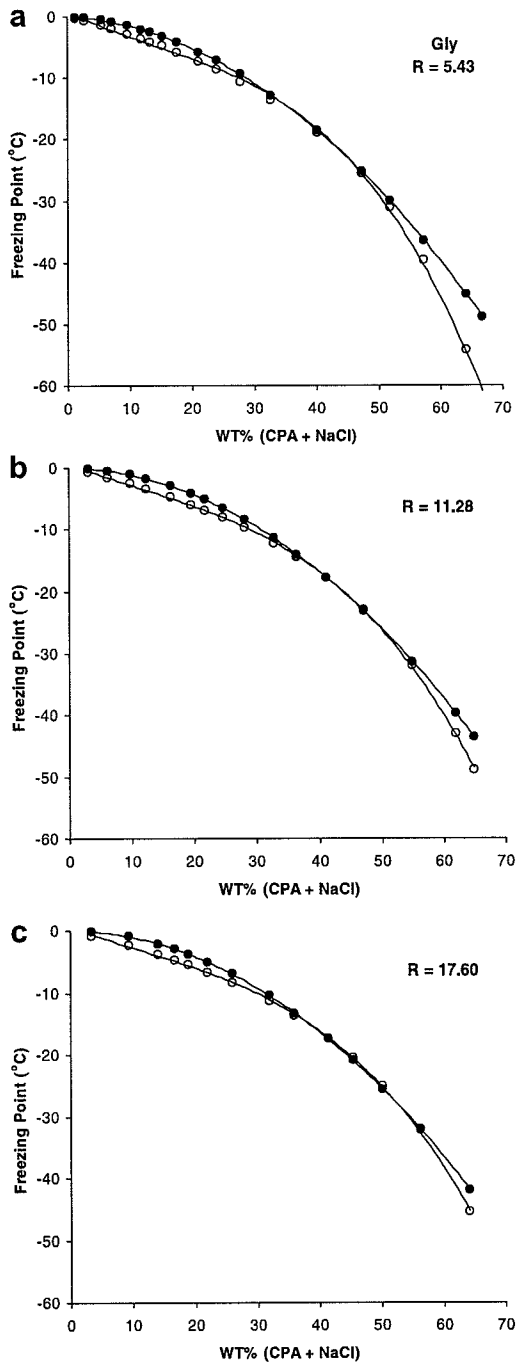

Fig. 3. A comparison of published experimental partial phase diagrams for the ternary system glycerol/NaCl/water (closed circles) with synthesized ternary phase diagrams (open circles) for three different R values (5.43, 11.28, 17.60). These R values apply to 0.5, 1.0, and 1.5 molar glycerol, respectively, in isotonic saline. The experimental data are from Sheppard et al. [11], with the freezing points for the solutions with the indicated values of R (isopleths) calculated with the fitting equation published by Pegg [6]; namely, $FP_T = W_T(-1.6 - 1.27R - 0.25R^2)^{-1} - 0.010W_T^2$, where FP_T = freezing point, R is the CPA/NaCl ratio, and W_T = weight percent solute (both salt and CPA). The points indicate concentrations for which freezing points were calculated.

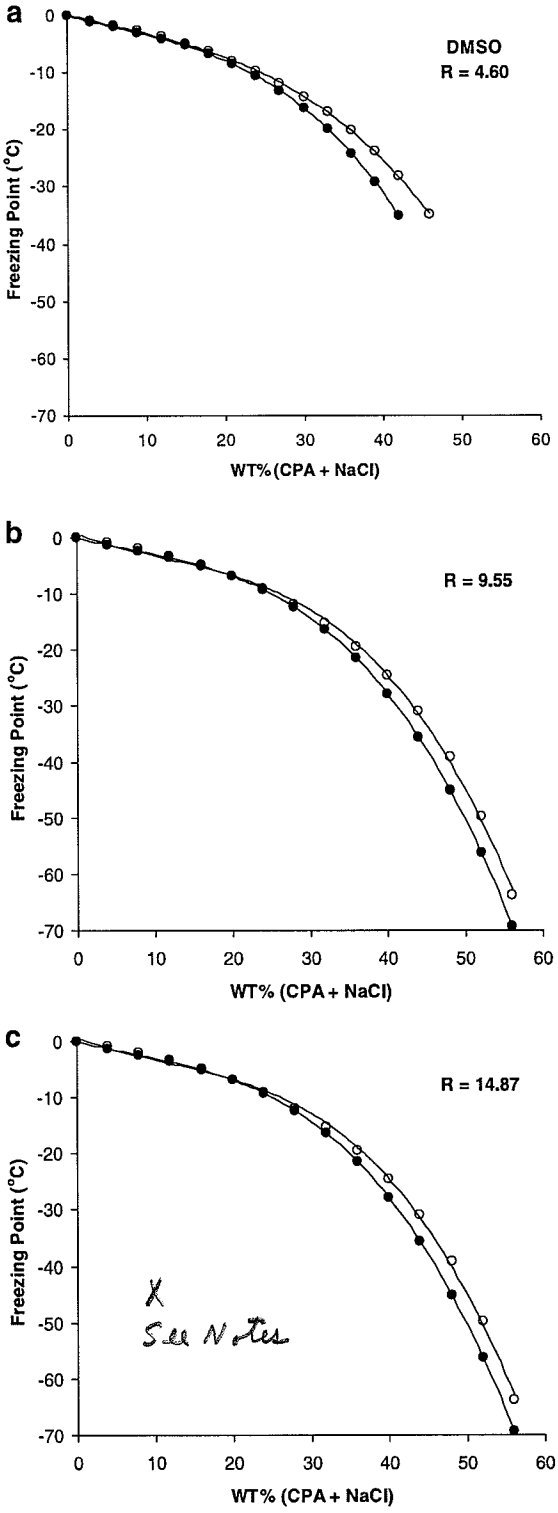

Fig. 4. A comparison of published experimental partial phase diagrams for the ternary system DMSO/NaCl/water (closed circles) with synthesized ternary phase diagrams (open circles) for R values (isopleths) of 4.60, 9.55, and 14.87. These R values apply to 0.5, 1.0, and 1.5 molar DMSO, respectively, in isotonic saline. The experimental curve is from the fitting equations of Pegg [7] based on the ternary data of Hildebrand et al. [3]. Specifically: $FP_T = AW_T + BW_T^2 + CW_T^3$ where $A = -0.6 + 0.17\tan^{-1}(R)$, $B = (1/132)\tan^{-1}(R/2) - 0.001$, and $C = -4.5 \times 10^{-4}$. The points indicate concentrations for which freezing points were calculated.

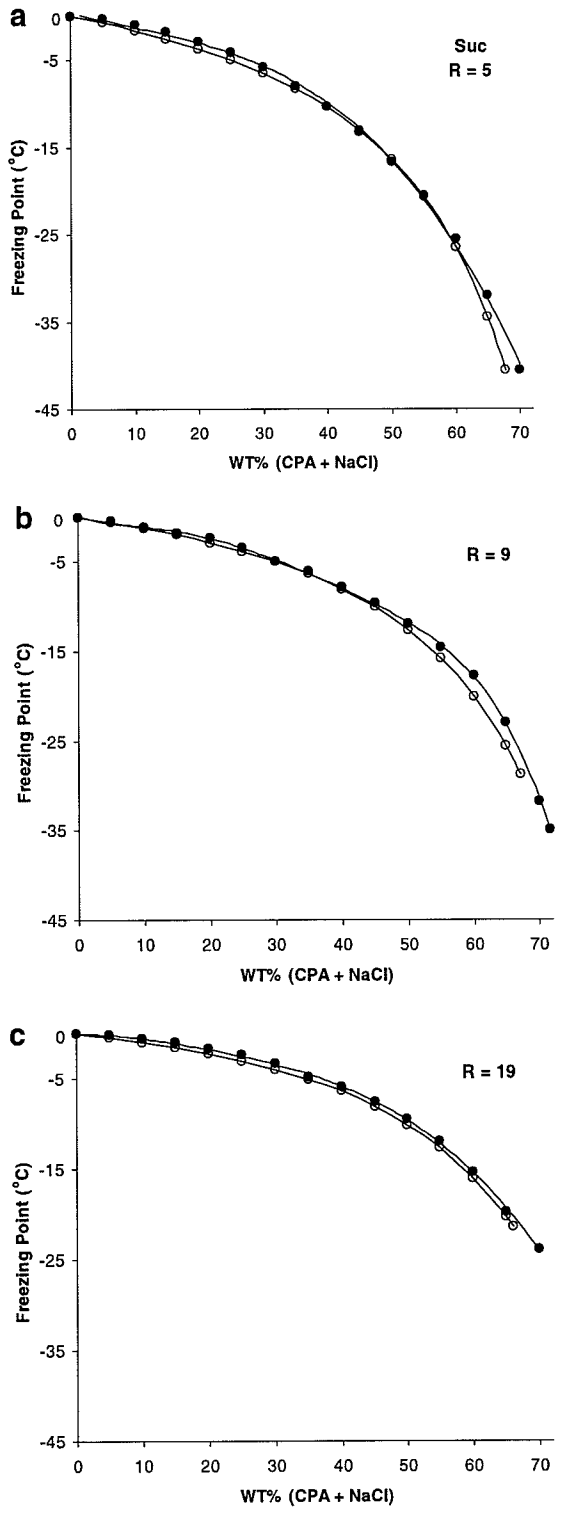

Fig. 5. A comparison of published experimental partial phase diagrams for the ternary system sucrose/NaCl/water (closed circles) with synthesized ternary phase diagrams (open circles) for R values (isopleths) of 5, 9, and 19. These R values apply to 0.125, 0.220, and 0.442 molar sucrose, respectively, in isotonic saline. The experimental data are from Gayle et al. [1]. Their fitted experimental curves were digitized to read off freezing points corresponding to a range of weight percents. On the synthetic curve, the points indicate concentrations for which freezing points were calculated.

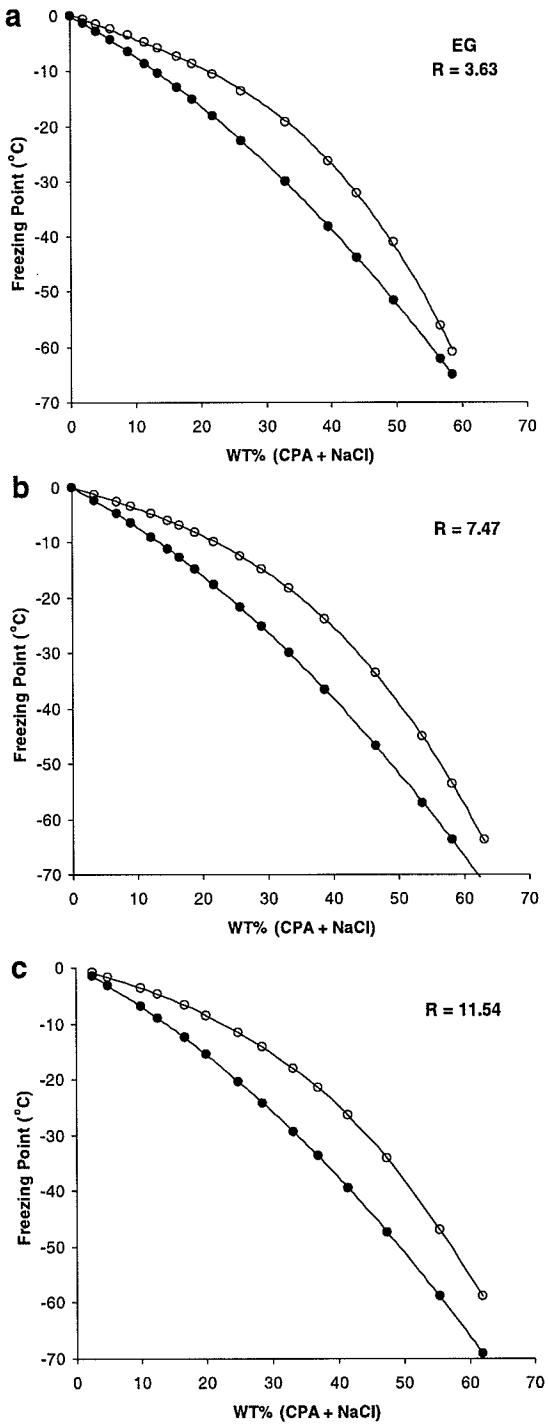

Fig. 6. A comparison of published experimental partial phase diagrams for the ternary system ethylene glycol/NaCl/water (closed circles) with synthesized ternary phase diagrams (open circles) for R values (isopleths) of 3.63, 7.47, and 11.54. These R values apply to 0.5, 1.0, and 1.5 molar EG, respectively, in isotonic saline. The experimental data are from Woods et al. [13], The freezing points for the solutions with the indicated values of R were calculated by their fitting equation; namely, $FP_T = [-0.676 + (4.77 \times 10^{-3})R]W_T + [(-7.64 \times 10^{-3}) + (-2.75 \times 10^{-5})R]W_T^2$. The points on both curves indicate concentrations for which freezing points were calculated.

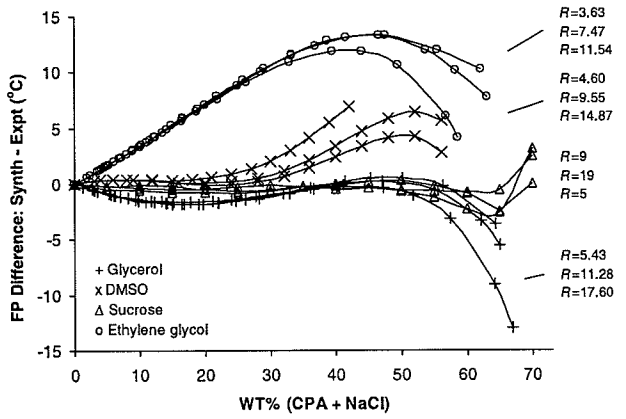

Fig. 7. The difference between the synthetic and experimental ternary solution freezing points as a function of the wt% solute for glycerol (pluses), DMSO, (x's), sucrose (open triangles), and ethylene glycol (open circles). The CPA/NaCl mass ratios (R) are listed to the side of the graph for each curve. Further details of the solutions are shown in Tables 3 and 4. The glycerol and sucrose synthetic freezing points match the experimental to 55 wt% and beyond. DMSO begins to show deviations above 30–35 wt% but these never exceed 7 °C up to the wt% limits of the graph. For ethylene glycol, the synthetic and experimental freezing points diverge rapidly with increasing wt% and reach as high as 13 °C at 40 wt%.

In all three cases, as the total weight percent increases above 0%, the two curves diverge with the curve for the synthesized ternary diagram lying above the experimentally, derived data of Woods et al. The difference increases to a maximum at around 40 wt% (EG + NaCl). The two curves then begin to slowly converge at still higher solute concentrations, up to the curve limits of ≈60 wt%. In the region of greatest divergence at around 40 wt%, the freezing points differ by 12–13 °C.

Results summary

The difference (error) between the experimental and synthetic ternary freezing points are collected and displayed in Fig. 7 as a function of wt%. It is readily apparent in this figure that for glycerol and sucrose, the synthetic FP error is small up to 55 wt%. The DMSO synthetic errors appear earlier, at 30–40 wt%. In the case of EG, the errors are clearly larger than for the other CPA's and appear at less than 10 wt%.

Discussion

When the CPA is glycerol, the synthesized ternary phase diagrams yield excellent agreement (±2 °C) with the experimentally derived phase diagrams up to solute concentrations of 55 wt% which corresponds to freezing points of ≈−30 to −35 °C. With DMSO the correspondence is not quite as good. The phase curves agree within 3 °C up to 33, 39, and 42 wt% (FP = −20, −26, and −31 °C) for $R = 4.6$, 9.55, and 14.87, respectively. However, the error never exceeds 7 °C up to the highest wt% determined. With sucrose, the agreement between synthesized and experimental ternary phase diagrams is excellent (±2 °C) up to 60–65 wt% solute for all three R values depicted. In the case of EG, the agreement is much poorer. The synthesized and experimentally, derived curves rapidly diverge with the freezing point difference exceeding 3 °C for all wt%'s above 9%. The error exceeds 10 °C for significant portions of all three ($R = 3.63$, 7.47, and 11.54) curves. The results for all four CPA's are graphically displayed in Fig. 7.

There would seem to be two possible explanations for the EG discrepancy. The first is that the interactions among EG, NaCl, and water are much different than are those for the three other CPA's tested and thus our synthetic method breaks down. However, EG is a chemical homolog of glycerol, differing only in having two carbon atoms instead of three. It would be surprising if the interactions of two close homologs with NaCl and water would differ so markedly when the phase behavior of glycerol is so similar to that of two non-homologous solutes (DMSO and sucrose).

The second possible explanation for the large discrepancy between the synthesized ternary diagrams for EG and the ones derived experimentally, by Woods et al. [13] is that there are sizeable systematic errors in the experimental data. In favor of this argument are the apparent internal problems in the EG/NaCl experimental results. When experimentally, derived phase diagram plots are made, e.g. for glycerol/NaCl/water solutions, the isopleths curve downwards more and more sharply as the R value decreases; i.e. as the relative mass of NaCl in the medium increases (see Fig. 1). This downward movement is primarily a consequence of the fact that a molal unit of NaCl, which dissociates into two ions, exerts nearly twice the freezing point depression as a molal unit of non-electrolytes like glycerol or EG. However, when one plots Woods et al's isopleths for $R = 5$, 15, and 45 on the same graph

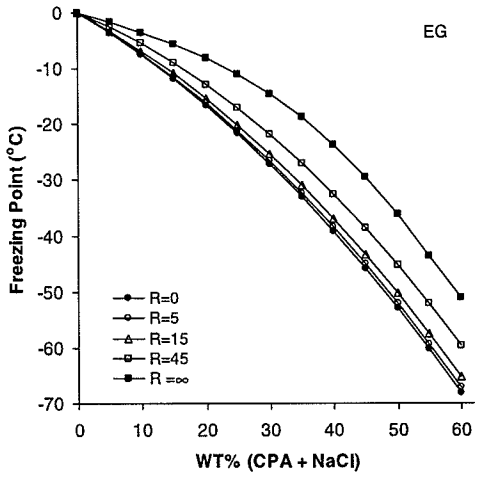

Fig. 8. A comparison of the experimentally derived isopleths published by Woods et al. [13] for EG/NaCl/water for R values of 5, 15, and 45 with those for the binary systems EG/water ($R = \infty$) and NaCl/water ($R = 0$) taken from Fig. 2e and a, except that solute concentrations are expressed as weight percents. The Woods et al. isopleths are inconsistent with the $R = 0$ and infinity cases; see text.

(Fig. 8), along with the EG/water binary isopleth ($R = \infty$) and the NaCl/water binary curve ($R = 0$) taken from Fig. 2 (and used by Woods et al. [13]), one notes two anomalies. First, the $R = 0$, 5, and 15 curves nearly superimpose, rather than diverging as expected and seen for glycerol (Fig. 1), DMSO, and sucrose (not shown). Second, as R increases, the isopleths should approach the limiting case of a pure binary EG/water solution ($R = \infty$). However, the Woods et al. curve for $R = 45$, which is nearly pure EG/water, diverges significantly from the $R = \infty$ pure EG/water isopleth. Ultimately, further experiments will be required to determine if the behavior of EG is anomalous or if there are errors in the Woods et al. EG/NaCl/water data. Our analysis leads us to conclude the latter is more likely.

Most physical–chemical textbooks state that it is not permissible or is dangerous to obtain the colligative properties of a multi-solute solution by summing the properties of each solute in water independently. Our data refute that view in three of the four ternary systems we have examined; namely glycerol, DMSO, and sucrose. The synthesized ternary diagram agrees with experimentally derived ones within 2 °C for wt%'s up to 55%, 30%, and 60%, respectively. This conclusion may be of substantial practical value since there are far more binary data on potential CPA/water solutions than there are ternary data. We also expect this synthetic methodology to be useful for estimating the properties of more complex solutions, say of two CPA's in addition to water and salt. Finally, a synthesized ternary phase diagram provides an easy way to check on the validity of an experimentally derived phase diagram. Sizeable discrepancies between the two would be a cause for concern.

Appendix A

In the body of this paper, solute concentrations have been expressed as weight percents or molalities, both of which are based on solute and solvent mass. The use of molality rather than molarity in this paper is not an accident. In a multi-component solution it is the concentration of each solute in water that, to first approximation, determines the solution properties of relevance to biology. Also, conveniently, molality is temperature independent whereas molarity is not. However, in the laboratory, one usually prepares solutions to a given molarity. So the question arises, how can we convert molarity information into the molalities needed to determine the solution properties? Below we show a method based on the partial molar volumes of the solutes. Molalities can also be computed from molarities if the densities of the ternary solutions are known, but that is not discussed here. A second, related factor, concerns the way cryobiologists typically make solutions. It is often the case that we start with a stock solution of some buffer and add additional solutes, such as CPA to the buffer. Alternatively, solutions can be made by adding everything directly to water, rather than starting with a buffer. We will return to this point later.

Here, we consider the addition of CPA to buffer. For simplicity, let the buffer contain a single solute (salt). Using our previous notation, let the subscripts W, A, and B refer to water, CPA, and salt, respectively. To distinguish between the properties of the buffer versus those of the final solution, we use primes (') to denote the buffer properties. Additionally, V, M, and m stand for volume, molarity, and molality, respectively. Then:

$$V'_{\text{buffer}} = V' = V'_{\text{W}} + V'_{\text{B}} \tag{A1}$$

The solute volume is, by definition:

$$V'_{\text{B}} = M'_{\text{B}} * V' * \overline{V}_{\text{B}} \tag{A2}$$

where $\overline{V}_{\text{B}}$ is the average molar volume of the solute, salt, in water and M'_{B} is the molarity of the salt. Then:

$$V'_{\text{W}} = V' - V'_{\text{B}} = V'(1 - M'_{\text{B}} * \overline{V}_{\text{B}}) \tag{A3}$$

Finally, solving for the ratio of solute to solvent volume, we obtain:

$$V'_{\text{B}}/V'_{\text{W}} = M'_{\text{B}} * \overline{V}_{\text{B}}/(1 - M'_{\text{B}} * \overline{V}_{\text{B}}) \tag{A4}$$

Now we add CPA to the buffer with a final concentration M_{A}. The volume of this final solution is

$$V_{\text{sol}} = V = V_{\text{W}} + V_{\text{A}} + V_{\text{B}} \tag{A5}$$

and substituting for V_{A}, we have:

$$V = V_{\text{W}} + M_{\text{A}} * V * \overline{V}_{\text{A}} + V_{\text{B}} \tag{A6}$$

Now, a key step: adding CPA to the buffer does not change the mass ratio of salt to water. It is the same in the buffer and in the final solution. That is why the molality of NaCl is constant in Table 3 at 0.151 molal, independent of the CPA concentration. The volume ratio of salt to water will also remain constant if the average molar volume of the salt does not change appreciably on addition of the CPA. We believe this is a reasonable approximation for the solution concentrations shown in Table 3. i.e, estimated variations in $\overline{V}_{\text{B}}$, say 10%, yield changes of <0.1% in the molar–molal conversions shown in Table 3. However, ultimately, this is one of the approximations we make to synthesize the properties of ternary solutions. With this approximation, we have:

$$V_{\text{B}}/V_{\text{W}} = V'_{\text{B}}/V'_{\text{W}} = M'_{\text{B}} * \overline{V}_{\text{B}}/(1 - M'_{\text{B}} * \overline{V}_{\text{B}}) \tag{A7}$$

Eqs. (A6) and (A7) give us two equations with two unknowns: V_{B} and V_{W}. Solving them for V_{W} yields:

$$V_{\text{W}} = V(1 - M_{\text{A}} * \overline{V}_{\text{A}})(1 - M'_{\text{B}} * \overline{V}_{\text{B}}) \tag{A8}$$

To find molality requires the mass of the water. Taking the density of water as 998.2 g/cm^3 at 20 °C, we let 1 l of water equal 1 kg of water with an error of less than 0.2%. Then the molality of the CPA, m_{A}, is:

$$\begin{aligned} m_{\text{A}} &= (\text{moles}A)/(\text{mass water}) = M_{\text{A}}V/V_{\text{W}} \\ &= M_{\text{A}}/[(1 - M_{\text{A}} * \overline{V}_{\text{A}})(1 - M'_{\text{B}} * \overline{V}_{\text{B}})] \end{aligned} \tag{A9}$$

The inverse calculation is:

$$M_A = m_A(1 - M'_B * \overline{V}_B)/(1 + m_A * \overline{V}_A - m_A * \overline{V}_A * M'_B * \overline{V}_B) \quad (A10)$$

Note that these two equations apply accurately only at the temperature at which $\overline{V}$ is calculated (usually 20 °C).

Before working a few numeric examples, we need to discuss exactly what we mean by $\overline{V}_A$ and $\overline{V}_B$. Physical chemists use these symbols to denote the partial molar volume of solutes A and B, respectively. e.g.

$$\overline{V}_A = (\partial V/\partial n_A)_{T,P} \quad (A11)$$

where n_A refers to moles of A and everything else is held constant. i.e. The partial molar volume of A is the infinitesimal change in solution volume when an infinitesimal amount of solute A is added to a solution (which may already contain some solute A). In cryobiology, we use the same notation, but we generally mean the 'average molar volume' of A in solution. i.e. the macroscopic change in solution volume when a macroscopic amount of solute A, say 1.5 moles, is added to the solution (initially containing no solute A). If the partial molar volume were concentration independent, these two definitions would be identical. But it is not. Imagine adding 1 ml of glycerol to a liter of water versus adding a liter of glycerol to 1 ml of water. In the former case, the glycerol volume diminishes by 3%. In the latter case its volume is virtually unchanged. That is because the partial molar volume of the first drop of glycerol added to 1 ml of water will be very different from that of the last drop of glycerol added. As a consequence, the 'average molar volume' of glycerol will be different in the low and high concentration glycerol cases. ~~We note, parenthetically, that by definition, it is the solute which is presumed to change volume on going into solution, and not the solvent.~~

For NaCl, glycerol, sucrose, and ethylene glycol, the 'average molar volume' can be computed from the solution tables in the CRC Handbook of Chemistry and Physics [12]. These differ by a few tenths of a percent over the concentration ranges in Table 3. We list average values in Table A1. For DMSO we use the value of Kiyohara et al. [4].

Now, we consider a numeric example relevant to Table 3. Consider 1.0 molar DMSO in NaCl buffer. The NaCl buffer contains 0.151 molal salt which is found to equal 0.1504 molar salt by interpolation of the CRC binary solution tables [12]. This same starting buffer is used to prepare all the solutions in Table 3. Then from Table A1, above, the 'average molar volume' of NaCl and DMSO is 0.01745 and 0.0689 l/mole, respectively. Using these values in Eq. (A9) yields a DMSO concentration of 1.08 molal in the ternary solution. In some cases, the above, Table A, 'average molar volumes' will not yield the exact CPA molality results shown in Table 3. This is because we have attempted to be more precise in that table by using the, concentration dependent molar volumes and by taking into account the slight difference between 1 kg and 1 l of water. However, the differences are at the level of a few tenths of a percent and of no practical consequence.

Finally we note that if 0.1504 molar salt and 1.0 molar DMSO are mixed in one step (rather than buffer first and CPA second) we obtain final, ternary solution concentrations of 0.162 m salt and 1.08 m DMSO. The salt value differs from that shown in Table 3 (0.151 m). It matters how solutions are mixed and this is left as an exercise for the reader!

Table A1
Average solute molar volume

Solute	Average molar volume in water[a](l/mole)
NaCl	0.01745
Glycerol	0.0711
Dimethyl sulfoxide	0.0689
Sucrose	0.212
Ethylene glycol	0.0545

[a] These are mean values for the concentration ranges shown in Table 3 (~0.5 to 1.5 molar CPA). In the notation of the CRC Solution Tables [12], the 'average molar volume' is given as $(C_0 - C_W) * (\text{gram mol wt})/(1000 * C_s)$ where $(C_0 - C_W)$ is the water displaced by solute in g/l and C_s is the solute concentration in g/l.

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Notes:

- Fig4C is a duplicate of Fig 4B in the published manuscript. This was a final manuscript assembly error and does not affect any of the numbers or results reported in the paper. The correct figure is below:

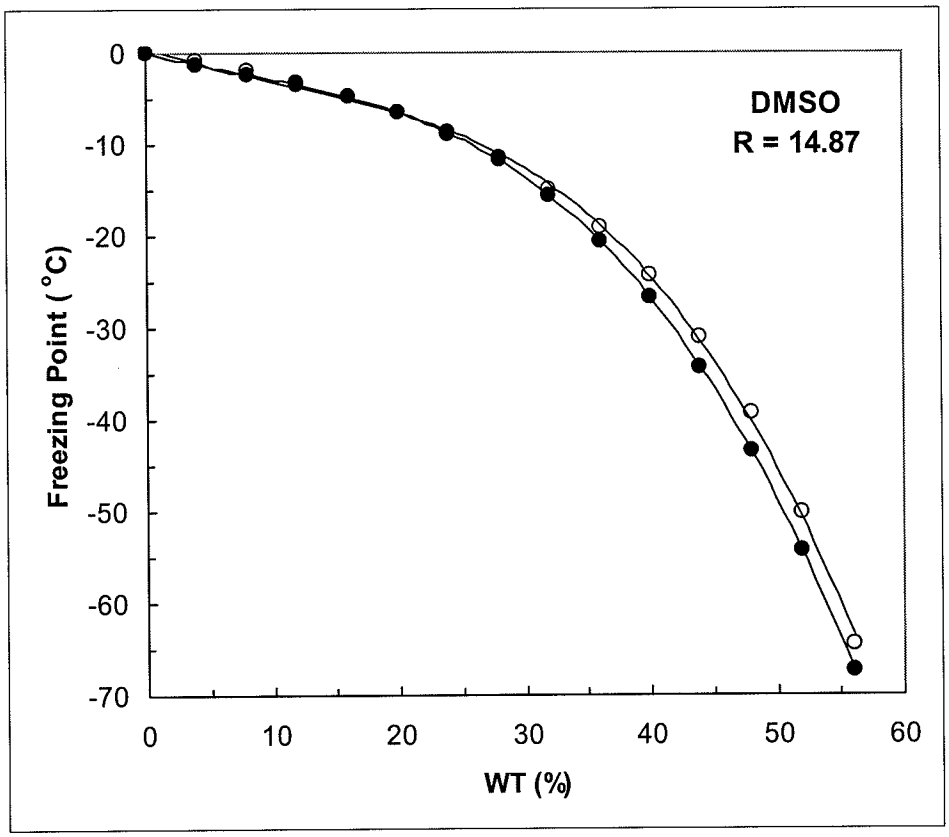

Fig 4C (corrected):
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- **A good paper published just after ours:**

Elliott, Prickett, Elmoazzen, Porter, and McGann
A multisolute osmotic virial equation for solutions of interest in biology.
J Phys Chem B **111**, 1775-1785 (2007)

The osmotic virial equation was used to predict osmolalities of solutions of interest in biology. The second osmotic virial coefficients, Bi, account for the interactions between identical solute molecules. For multisolute solutions, the second osmotic virial cross coefficient, Bij, describes the interaction between two different solutes. We propose to use as a mixing rule for the cross coefficient the arithmetic average of the second osmotic virial coefficients of the pure species, so that only binary solution measurements are required for multisolute solution predictions. Single-solute data were fit to obtain the osmotic virial coefficients of the pure species. Using those coefficients with the proposed mixing rule, predictions were made of ternary solution osmolality, without any fitting parameters. This method is shown to make reasonably accurate predictions for three very different ternary aqueous solutions: (i) glycerol + dimethyl sulfoxide + water, (ii) hemoglobin + an ideal, dilute solute + water, and (iii) bovine serum albumin + ovalbumin + water.