A non-ideal replacement for the Boyle van't Hoff equation

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Abstract

A non-ideal osmotic equilibrium equation is proposed as a replacement for the Boyle van't Hoff equation to describe the equilibrium volume of a living cell as a function of external osmolality. Contrary to common understanding, the Boyle van't Hoff equation is only valid for ideal, dilute solutions. However, the Boyle van't Hoff equation is commonly used to determine the osmotically-inactive fraction of the cell. This involves extrapolating to infinite osmolality, which violates the ideal, dilute solution constraint. It has been noted that the osmotically-inactive fractions obtained from the Boyle van't Hoff equation for human erythrocytes are markedly larger than measured values of the dry volume fraction of the cell. Using the new osmotic equilibrium equation to analyze experimental osmotic equilibrium data reduces the inferred osmotically-inactive fraction of human erythrocytes by approximately 20 %.

Key words

Osmotically-inactive fraction; erythrocytes; osmotic response; osmotic equilibrium;

Introduction

For many endeavors in which biological samples are manipulated ex-vivo, including biopreservation, a description of the osmotic equilibrium (or equilibrium cell volume as a function of osmolality) is required. Currently cellular osmotic equilibrium is described with the Boyle van't Hoff relation which states that the product of osmolality and equilibrium volume of the osmotically-active portion of the cell is constant. Written in its usual form, the Boyle van't Hoff equation is

$$\frac{V}{V_o} = \left(\frac{V_w^o}{V_o}\right)\frac{\pi_o}{\pi} + \frac{V_b}{V_o} = (1-b)\frac{\pi_o}{\pi} + b \qquad (1)$$

where V is the cell volume, V_o is the isotonic volume, V_w^o is the isotonic volume of water in the cell, π is the osmolality, π_o is the isotonic osmolality, V_b is the osmotically-inactive volume of the cell (i.e. the volume of the cell that does not participate in the osmotic response) and $b = V_b/V_o$ is the osmotically-inactive fraction of the cell volume.

To determine the osmotically-inactive fraction, equilibrium cell volumes are measured after the cells are exposed to solutions of known osmolality. These measurements have been performed using several techniques, including electronic particle counters [10,13,18,32,36], optical measurements under a microscope [34,35], assessing packing volume following centrifugation [2,24,26], and light scattering [28], among others. The Boyle van't Hoff equation is then used to determine the osmotically-inactive fraction of cells by extrapolating osmotic equilibrium measurements to infinite osmolality. This is commonly done using a Boyle van't Hoff plot, which is the equilibrium relative cell volume $\left(V/V_o\right)$ plotted as a function of inverse relative osmolality $\left(\pi_o/\pi\right)$. From equation 1, the value of the y-intercept of a linear fit of the data is the osmotically-inactive fraction, *b*. It can be noted from equation 1, that the osmotically-inactive fraction can also be determined from one minus the slope. Comparing the values obtained from the intercept and one minus the slope is a method to check the self-consistency of the osmotic equilibrium data.

It has been noted in the past that in some cases the Boyle van't Hoff relationship yields osmotically-inactive fractions higher than predicted from desiccation experiments [26] that measure the dry volume of the cell, which can be used as another estimate of the osmotically-inactive volume of the cell. The discrepancy between osmotically-inactive volume and dry volume has been discussed for human erythrocytes [26]. The osmotically-inactive fraction for human erythrocytes determined using the Boyle van't Hoff equation ranges from 0.41 to 0.48 [2,17,27]. This is at the higher end of the values found for other mammalian cell types (0.18 to 0.41 [6,10,12,18]). The dry volume fraction for erythrocytes measured by desiccation is 0.27 to 0.30 [2,26]. There have been many explanations for this difference, including bound water [2,26,31], a chloride ion shift [2], the osmotic properties of intracellular solutes [9,11,21,25], the large entropy of dilution characteristic of macromolecular solutes [4], and erythrocyte membrane characteristics [15,21,33].

As stated when these equations first appeared [19,23,29,30], the Boyle van't Hoff and the van't Hoff equations are only valid for ideal, dilute solutions (even though osmolality appears in the equations). Importantly, in this work we stress that that the product of osmolality and osmotically active cell volume is not constant in solutions that are not ideal and dilute. It has been recognized that intracellular solutions are not always ideal and dilute [16,20] and thus do not always adhere to the Boyle van't Hoff relation. This was pointed out by Lucke and McCutcheon [19], who stated "To be an ideal osmometer both cell and medium would have to behave as ideal solutions and the membrane must be perfectly semipermeable". The osmolality of erythrocyte cytoplasm is not equal to the

intracellular solute molality, thus it is not an ideal, dilute solution, which can be seen in Figure 1. Since the osmotically-inactive fraction is found by extrapolating osmotic equilibrium measurements to infinite osmolality, the ideal, dilute solution constraint is obviously violated.

Recognizing that the cytoplasm of the erythrocyte is not an ideal, dilute solution, others have made adjustments to the equations for the *kinetics* of water movements [8,16,20]. We note that, the kinetic water transport equations are derived from a chemical potential driving force, thus the non-ideality of the cytoplasm can be taken into account by using osmolality where concentration (or molality) had been used. While extracellular and intracellular osmolalities are equal at osmotic equilibrium, the equations to describe osmotic equilibrium (i.e. the Boyle van't Hoff equation) result from applying conservation of mass to the intracellular solutes. Therefore, simply using a nonideal osmolality in the Boyle van't Hoff equation is not the correction that should be made to account the non-ideality of the cytoplasm. In the osmotic equilibrium equation, contrary to common understanding, molality, not osmolality, should be used, even for non-ideal, non-dilute solutions. This will be shown in the derivation below.

Our hypothesis is that the anomalous osmotic behaviour of erythrocytes can be corrected by using a non-ideal osmotic equilibrium equation in place of the Boyle van't Hoff equation. Experimentally, the observed anomalies in the osmotic equilibrium of erythrocytes disappear when the hemoglobin is removed from the cytoplasm and the cells become red cell ghosts [1,17]. This supports our hypothesis that the non-ideality of

the cytoplasm (due in large part to the hemoglobin and its interactions with the other intracellular solutes) contributes to the observed difference between inferred osmotically-inactive volume from the Boyle van't Hoff equation and the measured dry volume.

Theory

Osmotic equilibrium equations result from assuming that the number of intracellular solute molecules remains constant and only cell water crosses the cell membrane in response to the changes in extracellular osmolality. This is the same as assuming ideal semipermeablity of the membrane and applying conservation of mass to the intracellular solutes.

$$N_s = N_{s_o} \quad (2a)$$

or,

$$m\rho_{water}V_{water} = m_o\rho_{water}V_{water_o} \quad (2b)$$

where N_s is the number of intracellular solute molecules, m is the molality of intracellular solute molecules, ρ_{water} is the density of water, V_{water} is the volume of osmotically active intracellular water and the subscript *o* refers to the isotonic condition. The water density, ρ_{water}, cancels out of equation 2b.

The osmotically-*active* water volume of the cell is, by definition, the total cell volume minus the osmotically-inactive volume (V_b).

$$V_{water} = V - V_b = V - bV_o \quad (3)$$

Combining equations 2b and 3 gives

$$m_o(V_o - bV_o) = m(V - bV_o). \quad (4)$$

Equation 4 can be rearranged to give

$$\frac{V}{V_o} = (1-b)\frac{m_o}{m} + b. \quad (5)$$

Equation 5 is the general osmotic equilibrium equation arising directly from conservation of mass and does not contain dilute solution assumptions. It should be noted that this equation is correct for ideal and non-ideal solutions. Equation 5 should be used to determine the osmotically-inactive fraction, but a problem arises in applying the equation since the molality of intracellular solutes is not known. Thus, the *van't Hoff* relation has been used to relate intracellular solute concentration to osmolality. The van't Hoff relation [29,30] states

$$\pi = m \quad (6)$$

where π is the osmolality. Under equilibrium conditions, the intracellular osmolality (π^i) is equal to the extracellular solution osmolality (π^e). Combining equation 6 with 5 yields the well-known Boyle van't Hoff equation (equation 1). The fact that the van't Hoff relation (equation 6) is applicable only to ideal, dilute solutions is well established [14]. By using equation 6 to relate osmolality to molality, the Boyle van't Hoff equation

includes an ideal, dilute solution assumption. Although this assumption may be valid at isotonic conditions, intracellular osmolality diverges increasingly from ideal at higher equilibrium osmolalities. Use of the Boyle van't Hoff relation may lead to osmotically-inactive fractions which are incorrect.

In deriving a replacement osmotic equilibrium equation for the Boyle van't Hoff relation, it is important to note that (i) if the cytoplasm is not an ideal, dilute solution, solute molality in equation 5 cannot be replaced with osmolality and (ii) an accurate description of the intracellular solute molality as a function of equilibrium osmolality is required. Herein, we combine a non-ideal equation of state for the relationship between osmolality and intracellular solution molality [7] with the assumption that the number of intracellular solute molecules remains constant, to derive a non-ideal replacement for the Boyle van't Hoff equation that is applicable for many non-ideal, non-dilute solutions.

When the osmolality begins to diverge from ideal, an expression for the intracellular osmolality as a function of molality, $\pi(m)$, is required. The osmolality is related to the water activity and is a property of the solution. For multisolute solutions, such as a cytoplasm, the osmolality can be written as a function of the individual solute molalities (shown in equation 9) or the individual solutes can be grouped together to create an effective concentration of one 'grouped solute' and the osmolality written as a function of that 'grouped solute' molality (shown in equation 12). This function can be inverted to give:

$$m = m(\pi) \qquad (7$$

a)

and its value at isotonic conditions

$$m_o = m(\pi_o) \quad \text{(7b)}$$

where m is either the molality of one of the intracellular solutes or is the molality of the grouped solute.

Equation 5 can then be written:

$$\frac{V}{V^o} = (1 - b^*)\frac{m(\pi_o)}{m(\pi)} + b^* \quad \text{(8)}$$

where b^* is a new osmotically-inactive fraction of the cell obtained without any ideal, dilute solution assumptions.

The intracellular molality of solutes is generally not known, and for non-ideal solutions, the molality is related to the osmolality in a non-linear manner. The relationship, $m(\pi)$, can be determined using a non-ideal equation of state. We have chosen to use the osmotic virial equation, which we have previously shown can accurately predict the osmotic behaviour of the erythrocyte cytoplasm [7]. The parameters in the equation are predicted by a model or found by fitting experimental data.

Osmotic virial equation: Model for $\pi(m)$

In a previous study, the cytoplasm of a human erythrocyte was modelled as a solution of hemoglobin (Hb) and other solutes, where all other solutes were assumed to behave osmotically as ideal, dilute solutes [7]. The hemoglobin, which is highly non-ideal, and

1 the interactions between the hemoglobin and ideal solute contribute a significant portion
2 to the cytoplasm osmolality and thus the cytoplasm is a very non-ideal solution. The
3 form of the osmotic virial equation used to predict the osmolality of the erythrocyte
4 cytoplasm is [7]:

$$\pi = m_{Hb} + m_I + B_{Hb} m_{Hb}^{\ 2} + B_{Hb} m_{Hb} m_I + C_{Hb} m_{Hb}^{\ 3} \qquad (9)$$

5 where π is the osmolality of the cytoplasm, m_{Hb} is the molality of hemoglobin, m_I is the
6 molality of the ideal solutes, B_{Hb} is the second osmotic virial coefficient for hemoglobin
7 ((mole Hb / kg water)$^{-1}$), and C_{Hb} is the third osmotic virial coefficient for hemoglobin
8 ((mole Hb / kg water)$^{-2}$). Note B_I and C_I are equal to zero and thus do not appear in
9 equation 9. Since m_I will be a parameter that is fit for at the isotonic condition, the
10 dissociation of ions will be included in m_I. The osmotic virial coefficients for hemoglobin
11 were determined by fitting osmolality as a function of molality data from aqueous
12 solutions of hemoglobin [7]. The virial coefficients take into account the interactions
13 between the solutes in the solution. The values for the osmotic virial coefficients in
14 equation 9 were determined in a previous study [7] and are listed in Table 1a.
15
16 As the cell dehydrates in hypertonic solutions, the contents of the cell concentrate at the
17 same rate, assuming that none of the solute molecules precipitate out of the solution.
18 The osmolalities of aqueous hemoglobin solutions were measured up to 12.3 millimolal
19 with no solute precipitation [3]. Applying our osmotic virial model of the cytoplasm to the
20 most hypertonic data point, we extrapolate to a hemoglobin molality of approximately 30
21 millimolal. Incorporating the no precipitation assumption, the relative molality of

hemoglobin is the same as the relative molality of the ideal solute as the cell dehydrates:

$$m_{Hb} / m_{Hb_o} = m_I / m_{I_o} = m / m_o \quad (10)$$

where m_{Hb_o} is the isotonic molality of hemoglobin, m_{I_o} is the isotonic molality of the ideal solute, m is the total intracellular solute molality and m_o is the total isotonic intracellular solute molality.

Equation 10 can be used in equation 9 to express the ideal solute concentration as a function of the hemoglobin concentration and the isotonic concentrations of the ideal solutes and hemoglobin [7].

$$\pi = m_{Hb} + \left(\frac{m_{I_o}}{m_{Hb_o}}\right) m_{Hb} + B_{Hb} m_{Hb}^{\ 2} + B_H \left(\frac{m_{I_o}}{m_{Hb_o}}\right) m_{Hb} m_{Hb} + C_{Hb} m_{Hb}^{\ 3} \quad (11)$$

At the isotonic condition, the only unknown in equation 11 is the molality of the ideal solutes. The isotonic osmolality of the cytoplasm, which includes contributions from the hemoglobin, the ideal solute, and their interactions, is assumed to be 305 milliosmoles. The isotonic concentration of hemoglobin taken from the literature is 35.1 gram / 100 mL cells [9]. This value was determined spectrophotometrically and when converted to molality, gives a value of 7.3 millimolal [27]. Using the above values and the values of the osmotic virial coefficients for hemoglobin listed in Table 1 in equation 11

$$305 = 7.3 + \left(\frac{m_{I_o}}{7.3}\right) 7.3 + 0.0493(7.3)^2 + 0.0493\left(\frac{m_{I_o}}{7.3}\right)(7.3)(7.3) + 0.0307(7.3)^3$$

and solving this equation for m_{I_o} yields an isotonic concentration of the ideal solute of 208 millimolal.

This is not the actual composition of the cytoplasm of the erythrocyte, but rather this is the effective concentration of an ideal solute needed to model the osmotic behaviour of the cytoplasm once the effects of the known amount of hemoglobin are taken into account (i.e. the ideal solute is used to model the contribution from the rest of the molecules and the dissociated ions in the cytoplasm). This model works extremely well for predicting the osmotic behaviour of the cytoplasm (see Figure 1).

For each osmolality of interest, Equation 11 can be inverted to give the intracellular molality of hemoglobin at that osmolality, $m_{Hb}(\pi)$. The hemoglobin molalities can then be used in equations 10 and 8 to determine the osmotically-inactive fraction.

Osmotic virial equation: Best fit to measurements of $\pi(m)$

Since creating a model for the cytoplasm involves making an assumption about the composition of the intracellular solutes, an alternative is to fit experimental measurements of cell water volume using the osmotic virial equation. Electron spin resonance (ESR) has been used to measure the relative cell water volume (cell water volume / cell water volume at isotonic) of human erythrocytes as a function of inverse relative equilibrium osmolality (isotonic osmolality / osmolality) in two studies [5,22]. To fit the ESR data, we assumed all of the intracellular solutes (including hemoglobin) can be treated as one "grouped solute", to determine $\pi(m)$. This "grouped solute" represents the effects of all the solutes in the cytoplasm and their interactions are taken into account with the virial coefficients of the "grouped solute". The ESR data is presented

as the relative cell water volume (which can be converted to relative intracellular solute molality) as a function of inverse relative osmolality, so the data was fit as π/π_o as a function of m_{GS}/m_{GS_o}. The single "grouped solute" osmotic virial equation used is

$$\frac{\pi}{\pi_o} = A_{GS}\left(\frac{m_{GS}}{m_{GS_o}}\right) + B_{GS}\left(\frac{m_{GS}}{m_{GS_o}}\right)^2 + C_{GS}\left(\frac{m_{GS}}{m_{GS_o}}\right)^3 \quad (12)$$

where m_{GS} is the molality of all of the intracellular solute molecules grouped together, A_{GS} is the grouped solute linear coefficient, B_{GS} is the second virial coefficient for the grouped solute, C_{GS} is the third virial coefficient for the grouped solute (see Table 1b) and the subscript *o* refers to the isotonic condition. This fit, along with the osmotic virial model (equation 11) expressed as π/π_o versus m/m_o (or m_{GS}/m_{GS_o}) is shown in Figure 1.

For each osmolality of interest, Equation 12 can be inverted to give the relative grouped solute molality $\left(m_{GS}/m_{GS_o}\right)$ as a function of the relative osmolality $\left(\pi/\pi_o\right)$. The relative grouped solute molalities can then be used in equation 8 to determine the osmotically-inactive fraction.

This makes it possible to re-analyze osmotic equilibrium data with equation 8 and either the hemoglobin and ideal solute model (equation 11) or the best fit of the grouped solute data (equation 12).

Materials & Methods

Equilibrium volume measurements were performed by suspending human erythrocytes in hypertonic solutions of Dulbecco's phosphate buffered saline (PBS) (Invitrogen, Burlington, Ontario). A 10X isotonic solution of PBS was diluted with an appropriate amount of distilled, deionized water (CORNING Mega-Pure™ system ACS) to obtain 1X, 2X, 3X, 4X and 5X isotonic PBS solutions. The osmolalities of the PBS solutions were measured using a calibrated freezing point depression osmometer (μOSMETTE™, Model 5004 Automatic Osmometer, Precision System Inc.™, Natick, MA) before the addition of the cells.

The erythrocytes used in this study were obtained from a normal human blood donor. Ten (10) mL of blood was aliquoted into each of four 50 mL sterile plastic centrifuge tubes (Fisherbrand, Fisher Scientific Ltd., Nepean, Ontario), washed three times by centrifugation at 1500 g for 10 minutes and re-suspended in 1X isotonic PBS to a final volume of 45 mL. Our isotonic PBS solution had an osmolality of 305 mOsm. After the third wash, the cells were diluted to a final concentration of 1.0 - 1.7 $x10^6$ cells/mL. One hundred (100) μL of this cell suspension was added to 10 mL of the hypertonic solution of interest. The cells were added to the hypertonic solution at room temperature and were exposed to the solution for 30 seconds before being analyzed on the particle counter.

A particle counter (Coulter® Z_2^{TM} series, Beckman Coulter, Mississauga, Ontario, Canada) and size analyzer (The Great Canadian Computer Company, Spruce Grove, Alberta, Canada) were used to determine the volume of the cells in the various hypertonic solutions. Cells suspended in each hypertonic solution were run three times on the Coulter counter, except for the 5X PBS which was done twice. Between each hypertonic solution run, a calibration, consisting of three drops of the 10 μm beads (Beckman Coulter, Mississauga, Ontario) in 10 mL of the hypertonic solution, was run yielding a calibration factor that was used to calculate the equilibrium volume of the cells in that hypertonic solution.

The standard error in the five equilibrium cell volume measurements was determined for each hypertonic solution. The standard errors are shown on the figures as the error bars.

Osmotic equilibrium data were also obtained from two literature sources [26,36]. Savitz *et al.* measured the equilibrium volume of erythrocytes using both the hematocrit and isotope dilution method in anistonic solutions ranging from 192 mOsm to 480 mOsm [26]. Since both methods gave the same equilibrium volume results, we have used the hematocrit data. The 1964 study did not indicate the isotonic osmolality, but in a later paper [27], the authors used 290 mOsm as the isotonic osmolality. The hematocrit at 290 mOsm was not measured. For this study, we calculated the isotonic hematocrit from a second order polynomial of the reported data. This resulted in an isotonic hematocrit of 0.4413. Zhao *et al.* utilized an electronic particle counter to measure the

equilibrium volume of human erythrocytes in anisotonic solutions ranging from 118 mOsm to 3186 mOsm [36]. We used an isotonic osmolality of 302 mOsm, with a corresponding isotonic volume of 74.42 ± 3.640 fL for these data.

Since the data from each source had a different isotonic osmolality, and therefore isotonic volume, each data set was divided by its own isotonic values before analyzing, ensuring that all data had a relative volume (V/V_o) of one when the inverse relative osmolality (π/π_o) was one. For lack of more specific information, It was assumed that the isotonic concentration of hemoglobin was 7.3 millimolal [9,27], and this resulted in a slightly different value for the isotonic concentration of ideal solute for each data set (see Table 2 for the values).

Combining the osmotic equilibrium data from our study and the literature, the osmotically-inactive fraction was determined by linear regression. The results are plotted on graphs where the ordinate values are the relative equilibrium cell volume measurements (V/V_o) and the abscissa values are the inverse relative osmolality (π_o/π) for equation 1 or the inverse relative solute molality (m_o/m) for equation 8 . The slope is equal to [1-b] for equation 1 (or [1-b*] for equation 8) and the intercept is equal to b for equation 1 (or b* for equation 8). To evaluate the error in the osmotically-inactive fraction determined from the linear regression, the standard errors in the slope and the intercept were calculated using the LINEST function in EXCEL (Microsoft, Redmond, WA, USA).

Results

The osmotic equilibrium data were analyzed using (i) the Boyle van't Hoff equation (equation 1), (ii) our non-ideal replacement equation for osmotic equilibrium (equation 8) using the osmotic virial model of the cytoplasm to determine $m(\pi)$ (equation 11) or (iii) our non-ideal replacement equation for osmotic equilibrium (equation 8) using the ESR data for human erythrocytes best fit with the osmotic virial equation to determine $m(\pi)$ (equation 12).

(i) Boyle van't Hoff equation

The osmotic equilibrium measurements from our study and the literature [26,36] for human erythrocytes are shown in Figure 2 as relative cell volume $\left(V/V_o\right)$ versus inverse relative equilibrium osmolality $\left(\pi_o/\pi\right)$. These data were fit to equation 1, the Boyle van't Hoff equation, and the osmotically-inactive fraction (b) determined from the y-intercept was 0.51 ± 0.020. This value of the osmotically-inactive fraction is similar to other reported values for human erythrocytes which range from 0.41 to 0.48 [2,17,27].

(ii) Non-ideal osmotic equilibrium equation, with osmotic virial model prediction.

The intracellular solute molality of human erythrocytes was calculated as a function of equilibrium osmolality using the osmotic virial model in equation 11 and the same osmotic equilibrium data shown in Figure 2 were re-plotted in Figure 3 as relative cell volume $\left(V/V_o\right)$ versus inverse relative *intracellular solute molality* $\left(m_o/m\right)$. The total

solute molality ratio is equal to the hemoglobin solute molality ratio (equation 10). Using the new non-ideal osmotic equilibrium equation (equation 8), the osmotically-inactive fraction (b^*) determined from the y-intercept is 0.38 ± 0.027.

(iii) Non-ideal osmotic equilibrium equation, with ESR data osmotic virial equation best fit.

Figure 4 is the plot of the same osmotic equilibrium data as in Figures 2 and 3, but using the ESR data fit [5,22] to equation 12 to determine $m(\pi)$ of the cytoplasm. The relative cell volume is plotted versus relative inverse intracellular solute molality in Figure 4. The osmotically-inactive fraction (b^*) determined from the y-intercept is 0.41 ± 0.020. The osmotically-inactive fraction, b^*, that results from this "grouped solute" fit agrees, within error, with the b^* resulting from the hemoglobin and ideal solute model.

Table 3 displays all the values of the inferred osmotically-inactive fraction. Comparing the values from the two methods of obtaining the osmotically-inactive fraction from the same equation (i.e. intercept, and one minus the slope) is a test of the self-consistency of the data. The maximum difference between the values from the intercept and slope is 0.03.

Discussion

Compared to an analysis of the same data with the Boyle van't Hoff equation (equation 1) (yielding osmotically inactive fractions of 0.49 - 0.51), the new non-ideal osmotic equilibrium equation (equation 8) resulted in osmotically-inactive fraction of human

erythrocytes 20 % lower (0.35 - 0.41) and closer to the results of dry volume obtained from desiccation experiments (0.27 - 0.30) [2,26].

ESR measures the water volume of the cell, independent of the total cell volume. Isotopically determining cell water mass is another approach to measure cell water independent of total cell volume (or mass). Both of these methods should result in an osmotically-inactive fraction of zero, since they are not measuring the cell solids. However, using the Boyle van't Hoff equation (equation 1), the authors [5,22,31] determined osmotically-inactive fractions in the range of 0.07 - 0.24, which we interpret as a measure of the non-ideality of the cytoplasm (i.e. the non-ideality in $\pi(m)$). These values are in the same range as the difference between the ideal b (0.49 - 0.51) and the non-ideal b^* (0.35 - 0.41) that was obtained here (see Figure 5).

The cytoplasm of human erythrocytes does not behave as an ideal, dilute solution, particularly when cells are shrunken in hypertonic solutions. The Boyle van't Hoff equation commonly used to describe cellular osmotic equilibrium is not valid for non-ideal, non-dilute solutions (even though osmolality appears in the equation). The non-ideal osmotic equilibrium equation presented in this paper (equation 8) describes the cytoplasm without making ideal, dilute solution assumptions and should be used to replace the Boyle van't Hoff equation.

To use the replacement equation, the typical osmotic equilibrium measurements are still used (equilibrium cell volume as function of osmolality). A non-ideal equation of state

may be used to obtain $\pi(m)$ for cytoplasm, which is inverted to obtain $m(\pi)$. The non-ideal equation of state may be predicted from a model (for example, our osmotic virial equation model for hemoglobin and an ideal solute) or found from a fit of experimental measurements (for example, ESR data). The non-ideal osmotic equilibrium equation (equation 8) can then be used to determine the osmotically-inactive fraction, b^* from the appropriate plot of osmotic equilibrium measurements. The non-ideal osmotic equilibrium equation is a simple, thermodynamically-correct equation that can be applied to the entire range of solutions, both ideal and non-ideal, that are encountered during the biopreservation process.

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Figure Captions

Figure 1. Relative osmolality as a function of relative intracellular molality for human erythrocytes. The closed diamonds are the ESR data from Du [5] and the open circles are the ESR data from Moronne *et al.* [22]. The solid line is predicted using our osmotic virial equation [7] with hemoglobin and an ideal solute (equation 11). The long-dashed line is the osmotic virial equation best fit of the cytoplasm data, assuming all of the intracellular solutes are one grouped solute (equation 12). The short-dashed line is the prediction of the relative osmolality of the cytoplasm assuming an ideal, dilute solution.

Figure 2. Boyle-van't Hoff plot for human erythrocytes in phosphate buffered saline solutions. The open circles are from Savitz *et al.* [26], the grey squares are from Zhao *et al.* [36] and the closed diamonds are our measured data points. The solid line is the best linear fit to equation 1.

Figure 3. Non-ideal osmotic equilibrium plot for human erythrocytes in phosphate buffered saline solutions, using our osmotic virial equation model for hemoglobin and an ideal solute (equation 11) to determine $m(\pi)$. The open circles are from Savitz *et al.* [26], the grey squares are from Zhao *et al.* [36] and the closed diamonds are our measured data points. The solid line is the best linear fit to equation 8.

Figure 4. Non-ideal osmotic equilibrium plot for human erythrocytes in phosphate buffered saline solutions, fitting the ESR data to the osmotic virial equation for one "grouped solute" (equation 12) to determine $m(\pi)$. The open circles are from Savitz *et*

al. [26], the grey squares are from Zhao *et al.* [36] and the closed diamonds are our measured data points. The solid line is the best linear fit to equation 8.

Figure 5. Osmotic equilibrium plot for human erythrocytes in phosphate buffered saline solutions. The solid line is the Boyle van't Hoff equation (equation 1). The dashed line is the non-ideal osmotic equilibrium equation (equation 8) using the fit to the ESR data to determine $m(\pi)$. The difference between the osmotically-inactive fraction from the Boyle van't Hoff (b = 0.51) and the osmotically-inactive fraction from the non-ideal osmotic equilibrium equation (b^* = 0.41) is a measure of the non-ideality of the cytoplasm. Figure 5(a) is the osmotic equilibrium plot over the entire osmolality range and 5(b) is the osmotic equilibrium plot over the initial osmolality range.

Table 1a. Solute osmotic virial coefficients in the osmotic virial model (equation 9).

	Osmotic Virial Coefficients	
Solute	**B** (millimole/kg water)$^{-1}$	**C** (millimole/kg water)$^{-2}$
Ideal, dilute solute [7]	0	0
Hemoglobin [7]	0.0493	0.0307

Table 1b. Osmotic virial coefficients for the cytoplasm of human erythrocytes as a 'grouped solute' (equation 12).

	Osmotic Virial Coefficients		
Solute	**A**	**B**	**C**
Cytoplasm of Human Erythrocyte as a Grouped Solute [5,22]	1.03	-0.230	0.185

Table 2. Isotonic values for each data set.

	Isotonic vales		
Data source	**Osmolality** (mOsm)	**Hemoglobin concentration** (millimolal)	**Effective ideal solute concentration** (millimolal)
Savitz et al. [26]	290	7.3	197
Zhao et al. [36]	302	7.3	206
Our data	305	7.3	208

Table 3. Values of osmotically-inactive fraction for erythrocytes obtained from the traditional Boyle van't Hoff equation and from the non-ideal osmotic equilibrium equation.

	Traditional BvH (eq. 1)	**Non-ideal replacement (Hb + ideal)** (eq. 8)	**Non-ideal replacement (Grouped solute fit)** (eq. 8)
b from intercept	0.51 ± 0.020	0.38 ± 0.027	0.41 ± 0.020
b from (1-slope)	0.49 ± 0.020	0.35 ± 0.028	0.40 ± 0.020