Some comments on recent discussion of the Boyle van't Hoff relationship

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Abstract

The estimation of several cellular biophysical parameters must be made in order to mathematically predict optimal cryopreservation protocols. These parameters include total cell volume, osmotically inactive volume, cell surface area, and relative water and solute permeabilities. Recent attention has been paid to the determination of the osmotically inactive volume and, specifically, an argument was made suggesting that this volume was incorrectly determined in the literature (Katkov, 2011). Here we show that this assertion is false.

Keywords: cryobiology, Boyle van't Hoff, osmotically inactive volume, osmotic pressure, linear regression

After the work of Meryman (see e.g. [6]), one of the principle focuses of cryobiology has been to develop and understand the relationship between the water volume of cells and their biophysical history. The first and most commonly acknowledged application of this idea in mathematical terms was presented by Mazur [5], and many more followed. The relationship between intracellular water content and the likelihood of ice formation is fundamental to cryobiology, and as such much deserved attention has been paid to this quantity.

In order to understand this relationship, models are constructed that describe the transport induced by concentration gradients across cell membrane

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encountered during cryopreservation, typically of a form similar to

$$\begin{aligned} \frac{dw}{dt} &= P_w A(\mu_w^e(t) - \mu_w^i(w,s,n)), \\ \frac{ds}{dt} &= P_s A(\mu_s^e(t) - \mu_s^i(w,s,n)), \\ \frac{dn}{dt} &\equiv 0, \end{aligned} \tag{1}$$

where w, s and n indicate moles of intracellular water, permeating and nonpermeating solute, respectively, A is the fixed cell surface area, and μ_w and μ_s and P_w and P_s are chemical potentials and permeability coefficients for water and solute, respectively [9] [1].

Although many methods for estimating P_w and P_s exist, one commonly employed in the cryobiological literature is to use the relationship

$$v = \bar{v}_w w + \bar{v}_s s + \bar{v}_n n + v_b^* \tag{2}$$

where v is the total cell volume, $\bar{v}_w$, $\bar{v}_s$, and $\bar{v}_n$ are the partial molar volume of the water, and permeating and nonpermeating solutes, respectively, and v_b^* is the so called "osmotically inactive volume" of the cell, thought to consist of (among others) solids such as cell and organelle membrane, organelles, large protein complexes such as actin, and water which is "bound" to these structures in such a way as to not be available to contribute to the intracellular chemical potential in a significant way, and certainly not available to be transported across the semipermeable cell membrane [8]. It can be shown that the volume occupied by isotonic potassium chloride in a cell makes up less than 1% of the isosmotic volume and this volume remains relatively constant (up to concentration dependence of the partial molar volume). If one assumes $d\bar{v}_n/dt \equiv 0$, the volume of the nonpermeating solutes $\bar{v}_n n$ may be accounted in the v_b term (e.g. $v_b := v_b^* + \bar{v}_n n$).

Assuming $d\bar{v}_w/dt \equiv d\bar{v}_s/dt \equiv d\bar{v}_n/dt \equiv 0$, equation (2) may be differentiated

[1] Except in the paragraph on page 5 describing relative volume, all variables indicate non-normalized values.

with respect to time to yield

$$\frac{dv}{dt} = \bar{v}_w \frac{dw}{dt} + \bar{v}_s \frac{ds}{dt}. \tag{3}$$

With equation (3) and system (1) in hand, one may then use measurement techniques that estimate total cell volume to estimate best-fit parameters P_w and P_s. Although we may assume that $v(0)$ is known through current or prior measurement, this coupling requires knowledge of the initial conditions for system (1), namely $w(0)$ and $s(0)$. However, in the absence of some physical way to estimate initial w and s, one must make an assumption about the osmotic behavior of the cell.

Most often the assumption made is that the osmotic pressure and solvent volume are governed by the Boyle van't Hoff relationship. In the most used case in cryobiology, this states that

$$\pi \bar{v}_w w = c \tag{4}$$

where c is a constant that may depend on temperature [7] and π is the osmotic pressure in the system. Using relationship (4) one then may conduct experiments to determine c, and when used in conjunction with equation (2) and putting $s \equiv 0$, one may use total volume measurements to determine v_b. In particular, one may set $\pi_0 \bar{v}_w w_0 = \pi \bar{v}_w w$ where subscript 0 indicates a particular value (e.g. isosmotic). Dividing through by $\pi \neq 0$ yields

$$w = \frac{\pi_0}{\pi} w_0, \tag{5}$$

and to recover total volume, we add v_b to both sides:

$$v = \bar{v}_w w + v_b = \frac{\pi_0}{\pi} \bar{v}_w w_0 + v_b. \tag{6}$$

Prickett et al. [7] argue that the application of equation (4) in the context of non-ideal solutions is incorrect. Because inferring v_b from extrapolation to infinite concentrations by definition involves non-ideal solutions, using the classical Boyle van't Hoff relationship in eq. (4) to find v_b is also incorrect. Instead they

use a conservation of mass argument. By system (1), $n_0 = n$, which is equiv-
54 alent to $m_0 \rho_w w_0 = m \rho_w w$, where m is the molality of nonpermeating solutes and ρ_w the density of water.

56 Rearranging as in equation (5), we arrive at

$$v = \bar{v}_w w + v_b = \frac{m_0}{m} \bar{v}_w w_0 + v_b. \tag{7}$$

The challenge here is that it requires the estimation of the intracellular non-
58 permeating molality, not osmolality which is assumed in equilibrium with the external media.

60 Prickett et al. use the inverse of the osmotic virial equation described by Elliott et al. [1] to solve for the concentration of nonpermeating solutes as a func-
62 tion of osmolality, and show that this relationship is nonlinear when the presence of certain highly active intracellular solutes are present (e.g. hemoglobin). On
64 the other hand, Elmoazzen et al. [2] show that in the absence of these large intracellular proteins, and in particular, in the presence of salts alone, the ap-
66 proximation $\pi \propto m$ is valid for a fairly large range of osmolalities, a case not discussed in [7].

68 We adopt this argument noting that in the ideal dilute case $\pi = RT\delta m$, where δ is a dissociation constant. Letting $x := 1/m$ and $a := m_0 \bar{v}_w w_0$ we have

$$v = ax + v_b. \tag{8}$$

70 In order to determine v_b the generally accepted method (with some exceptions) has been to fit equation (8) for the independent parameters a and v_b.

72 Recently Katkov argued that this method for the determination of v_b was incorrect [3] on several grounds. In particular Katkov follows the derivation
74 of equation (6), but then continues (up to normalization of the volume and concentration terms, addressed below):

$$\begin{aligned} v = \bar{v}_w w + v_b &= \frac{m_0}{m} \bar{v}_w w_0 + v_b \\ &= \frac{m_0}{m}(v_0 - v_b) + v_b \qquad (9) \\ &= ax + v_b. \qquad (10) \end{aligned}$$

Note Katkov divides (9) by V_{iso} to arrive at eq. 2 in [4] (we address this specifically below). Katkov's first complaint is that now $a = a(v_b)$ and thus a and v_b are not independent. Katkov then goes on to provide several fitting techniques that account for this dependence.

We argue here that Katkov is incorrect on this point. First, Katkov's argument that because $a = a(v_b) = m_0(v_0 - v_b)$ it is dependent on v_b is incorrect: by definition $a = m_0\bar{v}_w w_0$. Although it is possible to add and subtract other variables to imply dependence (here we have added and subtracted v_b from w_0 to arrive at $\bar{v}_w w_0 = v_0 - v_b$), this does not mean that w_0 is dependent on v_b. If this were true, we could infer considerably more complicated dependencies! In short, it is not wrong to use this substitution, but it is incorrect and unnecessary to fit equation (10) with $a = a(v_b)$.

Additionally, the mass balance derivation of the nonideal van't Hoff relationship depends only on the molality of intracellular solutes and the volume of water. Again we note that by the above definition v_b is the volume unaccounted for by $\bar{v}_w w$ and $\bar{v}_s s$ and thus, since n is constant, m_0 and w do not depend on v_b. Therefore, the slope of equation (8) is *a priori* independent of v_b. One may claim that if $d\bar{v}_n/dt \neq 0$, then $dv_b/dt \neq 0$. In this case, we may repeat the above arguments with v_b^* while accounting for this condition.

Next we address Katkov's proposition that the only valid regression is one in which the volume in isosmotic is fixed to be an *a priori* determined value. In particular, Katkov's derivation of the BVH relationship continues by dividing eq. (9) by v_0, where we define the relative volume $v^r := v/v_0$, relative osmotically inactive volume v_b^r (sometimes, and in Katkov's case, known as the osmotically inactive fraction), and relative concentration variable $x = m_0/m$, yielding

$$\begin{aligned} v^r &= x(1 - v_b^r) + v_b^r, \\ &= a(v_b)x + v_b^r. \end{aligned} \tag{11}$$

Therefore evaluate at the particular value m_0, we have

$$1 = a(v_b) + v_b^r. \tag{12}$$

Katkov concludes that valid regressions, therefore, must have this relationship.

There are several flaws with this argument. The first and most mathematical argument is that by definition, the derivation of eq. (11) is incorrect: a is independent from v_b. Therefore, eq. (12) should be

$$\begin{aligned} 1 &= v_0^{-1}\bar{v}_w w_0 + v_b^r, \\ &= v_0^{-1} a + v_b^r, \end{aligned}$$

as including v_0 into the a term causes a to be v_b dependent (since $v_0 = \bar{v}_w w_0 + v_b$).

The second argument is that it is statistically inappropriate to determine, independently, v_0 and then regress all remaining data. There is certainly a confidence interval around v_0, and as such one should expect that the regression, with arguably considerably more information should determine v_0 more accurately than at m_0 alone. In other words, volumes determined at multiple concentrations refine the measurement at a particular concentration. This leads to the last and possibly most intuitive argument: we have been completely arbitrary in our choice of v_0. Why do we choose to fix the "known" volume at isosmotic? From our derivations above, there is nothing that precludes us "choosing" m_0 to be anisosmotic. If we choose v_0 to be determined at a high osmolality (say, $m_0 = 2$ mol/kg) and "fix" the volume as Katkov recommends, then, because of its proximity to the intercept, v_b will be skewed to values corresponding to hyperosmotic volume measurements.

For a concrete example of the last argument we used the mean volume data presented in [4] in figure 2A. Without the original data, some slight differences will be produced, but for the purposes of this demonstration, they will suffice. We now perform a sequence of linear regressions. First we perform the correct, non-fixed regression, with a independent of b, arriving at $a = .58$ and $v_b^r = .26$, matching the values presented in figure 2A. Next we fix the regression to pass through each of the 10 volume-concentration points. Values are presented in Table 1. The mean b value is 0.266 ± 0.084 (mean$\pm$ SD), with the highest b value corresponding to the isosmotic point. This is due to the concavity of the

data, a commonly encountered phenomena in the literature [10]. This example serves to illustrate the huge dependence of v_b^r on the arbitrary fixing of a point for the regression to pass through.

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Table 1: Data presented in Figure 2A [4] were regressed with equation (8) and the indicated point fixed, yielding coefficients a and b dependent on the fixed point.

Fixed Point	a	b
(0.22, 0.3)	0.647	0.158
(0.29, 0.35)	0.640	0.164
(0.35, 0.41)	0.621	0.193
(0.43, 0.51)	0.579	0.261
(0.67, 0.73)	0.532	0.372
(1, 1)	0.593	0.405
(1.31, 1.07)	0.611	0.269
(1.51, 1.26)	0.674	0.242
(1.7, 1.23)	0.566	0.266
(2.15,1.33)	0.464	0.332
Mean $\pm$ S.D.	0.593 ± 0.062	0.266 ± 0.083