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Challenge from the simple: Some caveats in linearization of the Boyle–van't Hoff and Arrhenius plots ☆

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ABSTRACT

Some aspects of proper linearization of the Boyle–van't Hoff (BVH) relationship for calculation of the osmotically inactive volume v_b, and Arrhenius plot (AP) for the activation energy E_a are discussed. It is shown that the commonly used determination of the slope and the intercept (v_b), which are presumed to be independent from each other, is invalid if the initial intracellular molality m_0 is known. Instead, the linear regression with only one independent parameter (v_b) or the Least Square Method (LSM) with v_b as the only fitting LSM parameter must be applied. The slope can then be calculated from the BVH relationship as the function of v_b. In case of unknown m_0 (for example, if cells are preloaded with trehalose, or electroporation caused ion leakage, etc.), it is considered as the second independent statistical parameter to be found. In this (and only) scenario, all three methods give the same results for v_b and m_0. AP can be linearized only for water hydraulic conductivity (L_p) and solute mobility (ω_s) while water and solute permeabilities $P_w \equiv L_pRT$ and $P_s \equiv \omega_sRT$ cannot be linearized because they have pre-exponential factor (RT) that depends on the temperature T.

Determination of the cell osmotic characteristics, namely the osmotically inactive cell volume (V_b), the cell membrane permeability to water (L_p and P_w) and to a permeable cryoprotective agent (ω_s and P_s), activation energy (E_a) for the permeabilities and the limits of osmotic tolerance (often expressed as the minimal V_{min} and maximal V_{max} tolerable volume excursions) are the key parameters for optimization of cryopreservation protocols, avoiding intracellular ice during equilibrium freezing, and preventing osmotic damage during addition and dilution of permeable CPAs [1,5,6,15,16,19,21].

The two formulas commonly used in such experiments are the Boyle–vant'Hoff (BVH) relationship for estimation of V_b and the Arrhenius plot (AP) for calculations of E_as. They both can be easily linearized, which has made them a powerful and popular tool among cryobiologists and for other osmotically related research: key words "*Boyle–van't Hoff*" produced 52 hits in PubMed search, "*activation energy* AND *permeability*" refers as much as 649 hits, and at least half of the publications is related to biological membranes.

However, despite seemingly straightforward approach and simple calculations, there are several caveats on a "*streamline road*" of the BVH and AP linearizations, particularly in selecting the method of linearization (BVH), and application of the Arrhenius relationship to the proper permeability parameters.

Below, we will analyze those two aspects in a hope that it may help researchers to clarify experimental design, methods of calculation, and interpretation of the results.

Boyle–van't Hoff plots: what linearization?

The osmotically inactive volume (formerly called osmotic ballast, thus V_b) can be determined in series of exposures of the cell to media containing an impermeable solute of different external (equilibrium, final) osmolality (M_f) for time long enough for equilibration. The equilibrium cell volume V_f then plotted against the reverse equilibrium osmolality, and the osmotically inactive volume is determined by extrapolating the cell volume to infinite osmolality. The key question is what would be the proper linearization method(s), and which is (are) not.

Cell model used in calculations

The BVH relationship [18], which states that the amount of the impermeable solute(s) defined as a product of the intracellular molality (M) and cell water volume (W) remains constant and in commonly used term can be written as following:

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$$MW = M_0W_0 = M_fW_f = \text{const} \quad (1)$$

where subscripts "0" and "f" refer to the initial and final (equilibrium) values, respectively. We assume that osmotic equilibration is reached so M_f is the molality of the external medium. We consider ideal diluted solutions with the osmotic coefficient equals a unit, so osmolality of the solutes will be equal to its molality. We also assume that no permeable solutes are present neither inside nor outside the cell.

At those assumptions, the BVH relations in terms of total cell volume ($V \equiv W + V_b$) can be written as follows:

$$M_f(V_f - V_b) = M_0(V_0 - V_b) \quad (2)$$

For the sake of simplicity, we will use normalized variables. We normalize the total cell volume to its initial value at time zero ($v \equiv V/V_0$; $v_b \equiv V_b/V_0$;); the osmolality of the solutes (both extra and intracellular) is normalized to a reference osmolality ($m = M/M_{ref}$). We specifically emphasize that M_{ref} is not necessary equal to the isotonic value M_{iso} (as it is commonly suggested) or to the initial (prehistoric) value M_0 (also commonly suggested to be equal M_{iso}) for the reason that will be discussed in a special sub-Chapter I.2 of BVH considerations (m_0 is unknown) after the major case (sub-Chapter I.1). We use any arbitrary M_{ref}, which makes our consideration more general. The special case $m_0 = 1$ will be considered in Appendix. All formulas for absolute values of V_b and M_0 (when it is unknown) can be easily derived from the normalized values multiplied by M_{ref} for the osmolalities and by V_0 for the volumes. Thus, a unit in formulas below represents V_0, the initial cell volume. Very often, the reference osmolality is either the initial intracellular osmolality M_0 or the isotonic osmolality M_{iso}. In general, however, there can be an important exclusions, so we will keep m_0 in general form in th major text In normalized form, the BVH Eq. (2) can be re-written as follows:

$$m_f(v_f - v_b) = m_0(1 - v_b) \quad (3)$$

Now, we will introduce reverse osmolality ($\chi_f \equiv 1/m_f$). The BVH formula in linear form is:

$$v_f = m_0(1 - v_b)\chi_f + v_b \quad (4)$$

Eq. (4) will be used as the basic (theoretical or "*true*") expression for the BVH plot.

Major common case (m_0 is known)

Preset experimental model. Let us now assume that the cell was exposed in three different solution containing 2*X* ($i = 1$, the first point on the plot), 1*X* and ½*X* of the values of M_{ref} ($i = 1$, 2, and 3, respectively). We also presume that the initial intracellular osmolality $m_0 = 0.5$. And, finally, we assume that the "*true*" (theoretical) value of osmotically inactive volume comprises 20% of the initial total value. Eq. (4) is then can be written as:

$$v_{f_{THEOR}} = 0.4\chi_f + 0.2 \quad (5)$$

The plot of this line v_f vs. χ_f is shown on Fig. 1 (circles represent three different reversed osmolalities 0.5, 1, and 2). The theoretical ("true") values of the cell volume are 0.4, 0.6, and 1.0, respectively (empty circles on the line "*Theory*"). The slope of this line *a* equals 0.4, the intercept $b = 0.2$.

Let us then assume that during experiments due to inaccuracy of the method or any other reasons the experimental value of the cell volume $v_{f_{EXP_i}}$ are 0.3, 0.8, and 0.9 for $i = 1$, 2, and 3, respectively (Fig. 1, filled rhombs).

The deviation between the theoretical (fitted) and the experimental values at point *i* are defined as:

$$d_i \equiv v_{f_{THEOR\,i}} - v_{f_{EXP_i}} \quad (6)$$

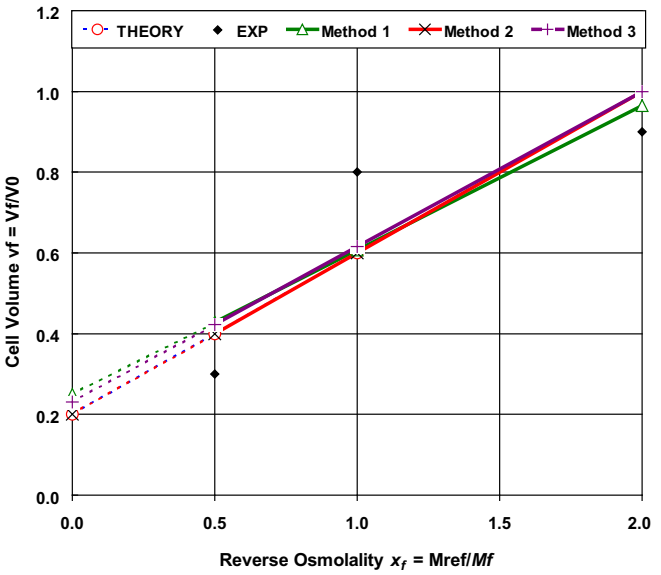

Fig. 1. Linearization methods for Boyle–van't Hoff plots of experimental data described in the text. *X*-axis: inverse normalized osmolality $\chi_F \equiv 1/m_F$; *Y*-axis; total cell volume normalized to the initial value $v_F \equiv V_F/V_0$; v_b-partial osmotically inactive volume $v_b \equiv V_b/V_0$. "*Theory*": theoretical ("*true*") BVH equation at known normalized initial intracellular osmolality $m_0 = 0.5$ and $v_b = 0.2$ (formula (5)). "*Method 1*": regression line (15) in assumption of independent slope (16) *a* and intercept $b \equiv v_b$, which is calculated from (17) and equals 0.25. "*Method 2*": regression line (21) in assumption of independent *b* only ($v_b = 0.2$ from (22)) while the slope is the function of m_0 and v_b (formula (23)) and equal to 0.4. This line is completely merged with the theoretical line (for these experimental conditions, not always the case). "*Method 3*": independent intercept is fit by using the Least Square Method (LSM, formulas (24)–(26); $v_b = 0.231$ (27), the slope $a = 0.385$ (28). If m_0 is unknown, all three methods give the identical results: $a = 0.357$ (formulas (29) and (34)), $v_b = 0.250$ (formulas (30), (32), and (39)), and $m_0 = 0.476$ (formulas (31), (33), (35), (40), and (41). Note that the osmolality *M* is normalized to an arbitrary osmolality M_{ref} that is not equal its "*physiological*" value. For the depicted plots, $M_{ref} = 2 \times M_{iso}$. As the result, the theoretical line crosses the points (2; 1), and (1; 0.6). Such normalization allows to calculate the initial intracellular osmolality in case it is not known at the beginning of the BVH experiments, for example if the cell was preloaded with trehalose, ion leakage caused by electroporation, etc. In this case, all three methods would give the same result a Method 1 for known m_0, namely: $v_b = 0.25$; $m_0 = 0.476$. That means, for example, that the cell with $M_{iso} = 300$ mOsm as "physiological" tonicity, and assuming $M_{ref} = 600$ mOsm, would have the prehistoric intracellular osmolality lower than 300 mOsm ($M_0 = 286$ mOsm), due to, for example ion leakage after E-poration. Alternatively, if M_0 turns to be higher than 300 mOsm, the cells would be loaded with osmotically active molecules such as trehalose by inducing transient permeability to that xeroprotective agent, and later before BVH experiments start, closing the pore so the agent is trapped inside the cell. Thus, this method allows to estimate the amount of the compound that penetrated the cell and later became impermeable.

where $i = 1$, 2 and 3. It is also called an offset or a residual from the theoretical curve. The squared deviation is then defined as follows:

$$D_i \equiv d_i^2 \equiv \left(v_{f_{THEOR\,i}} - v_{f_{EXP_i}}\right)^2 \quad (7)$$

We now introduce a set of average values for the experimental data as follows:

$$\overline{\chi_f} \equiv \frac{\sum_{i=1}^{n}\chi_{f_i}}{n} = \frac{0.5 + 1 + 2}{3} = 1.167 \quad (8)$$

$$\overline{\chi_f^2} \equiv \frac{\sum_{i=1}^{n}\chi_{f_i}^2}{n} = \frac{0.5^2 + 1^2 + 2^2}{3} = 1.750 \quad (9)$$

$$\overline{v_f} \equiv \frac{\sum_{i=1}^{n}v_{f_i}}{n} = \frac{0.3 + 0.8 + 0.9}{3} = 0.667 \quad (10)$$