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Regulatory volume decrease in COS-7 cells at 22 °C and its influence on the Boyle van't Hoff relation and the determination of the osmotically inactive volume*

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Abstract

Cryobiological analyses assume that the direction and rate of water movements across cell membranes and equilibrium cell volumes are determined solely by differences in the chemical potentials of intra- and extracellular water. A consequence of this assumption is that cells obey the Boyle van't Hoff (BvH) law which states that cell volumes are a linear function of reciprocal osmolality. Extrapolation of the BvH plot to infinite osmolality yields a quantity b , the fractional volume of the cell occupied by solids. In many cells, however, a cell volume excursion above the isotonic volume initiates an energy-requiring response that causes the swollen cells to shrink back to or towards isotonic volume. It is referred to as Regulatory Volume Decrease (RVD). We have observed a strong RVD in COS-7 cells. If not eliminated by keeping exposure times short, this RVD produces a b that is 60% too high (0.48 vs. 0.30). These results indicate the importance of examining cells for volume regulatory mechanisms before performing measurements to determine their osmotic parameters.

The responses of cells to subzero temperatures depends to a major degree on their inherent osmotic properties and on the permeabilities of their plasma membranes to water and to solutes. In predicting and analyzing these responses, a fundamental underlying assumption is that the movement of water and solutes in and out of cells is totally passive and is solely in response to chemical potential differences between the cell and its exterior and at rates that are proportional to the magnitude of those differences. Essentially all animal cells swell or shrink in hypotonic or hypertonic solutions of nonpermeating solutes to a volume predicted by the Boyle van't Hoff law. At 0 °C and below, the enlarged or shrunken volume is maintained if the deviation from isotonicity is not damaging. For many cells that is also true between 20 °C and 37 °C; however, many other cells possess energy-driven volume regulatory responses which drive the cell volumes back close to the volume in isotonic conditions [1; 2]. In the case of a hypoosmotically induced swelling, this reaction is called a regulatory volume decrease (RVD). In the case of the re-swelling of a hyperosmotically shrunken cell, it is called a regulatory volume increase (RVI). If volume regulatory responses occur within the time frame of above-zero procedures used in cryobiology, it can

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confound or overwhelm the passive water and solute flows determined by differences in chemical potential. Such is the case in COS-7 fibroblast cells, reported here. When exposed to a hypotonic medium, they initially and rapidly swell to a volume predicted by the physical chemical law, but if they are held in that medium, they return to normal isotonic volume within 30 minutes.

The purpose of the current paper is several fold:

1. To document the extent and rapidity of the volume regulation in COS-7 cells in hypotonic media. It initiates about 2 minutes after the onset of the passive swelling and is nearly complete by 30 minutes at room temperature.
2. To determine whether the volume regulation requires energy. *It is likely to*, for the regulation is abolished at 0 °C.
3. To determine whether volume regulation also occurs in COS-7 cells that are shrunken in hypertonic media. It *does not*.
4. To determine whether ignorance of the existence of this volume regulation can lead to substantial error in cryobiological interpretation. It *can*.
5. To raise the question of how many other cell types exhibit cell volume regulation of sufficient magnitude and kinetics to influence cryobiological analyses and outcomes.

COS-7 cells (African Green Monkey kidney fibroblasts) were cultured as described [3] and suspended in isotonic Tyrode's buffered saline (TBS) or phosphate buffered saline (PBS), supplemented with 14.5 mM D-glucose, 0.3% bovine serum albumin and 1 μ M calcein AM (a live/dead fluorescent stain, Invitrogen, Carlsbad, CA) prior to exposure to anisotonic test solutions. The cell suspension was kept (in the dark) at 22 °C for at least 30 minutes before mixing the cells with the test solutions.

To create BvH plots, images of cells in five different tonicities were recorded after 5 –10 minutes or after 30 – 35 minutes of equilibration time. Details about the experimental conditions can be found in our previous publication [3]. Briefly, all test solutions were made from TBS or, for the 30 minutes BvH plot data, from phosphate buffered saline (PBS), by dilution with HPLC grade water or by addition of sucrose to the test solution (all compounds from Sigma Aldrich). For the kinetic experiments, the test solutions were prepared with a 20% higher or lower osmolality than the desired final concentration to compensate for the subsequent dilution with a) the cell suspension in isotonic TBS or PBS and b) with a 10 μ M solution of the live-dead dye calcein AM in isotonic TBS or PBS. The final osmolalities were measured (Vapro Osmometer 5520, Wescor Inc., Logan, UT) and amounted to (in case of the kinetic experiments after the addition of the respective volumes of isotonic TBS or PBS, adjusting for the dilution with small volumes of cell suspension and calcein AM) to: 152/157 $\pm$ 2, 227/242 $\pm$ 2, 308/308 $\pm$ 1, 602/602 $\pm$ 6, and 1010/986 $\pm$ 3 mOsm/Kg (values are mean $\pm$ S.E.M. The first value refers to the 30 minutes exposure experiments, the second to the 5 minutes experiments). The value of 308 is the milliosmolality of isotonic TBS.

Imaging of Cells and Estimations of Cell Volumes

To obtain data for the BvH plots, the cell suspension was added at a 1:99 ratio to the four anisotonic test solutions and sufficient volumes pipetted into the wells of a 24 well plate to fill them. After a waiting time of 30 minutes in the first set, and 5 minutes in the second set of experiments, 251 $\pm$ 15 cells (range: 170 – 314) were imaged with a Zeiss Axioscope microscope for each test solution over the ensuing 2 – 5 min, using both bright field and fluorescence microscopy. Cell diameters were measured with NIH Image J 1.42Q software,

averaged, and converted into volumes (Microsoft Excel software) assuming the cells to be spheres. However, the shapes of cells in 986 and 1011 mOsm/Kg deviated too much from spherical to justify that assumption. Consequently, for them, we calculated cell volumes using the formula for prolate spheroids.

The initial determinations of cell volume as a function of osmolality were made on cells that had been exposed to the anisotonic solutions for 30 – 35 minutes at room temperature. These times were used to ensure that these large cells had attained osmotic equilibrium. The results are shown in the BvH plot in Fig. 1 by the black circles and the dashed line. The Y-axis intercept at infinite osmolality is $1576 \mu\text{m}^3$, which translates to a volume of 0.47 relative to the mean measured volume of the isotonic cell ($3324 \mu\text{m}^3$). Not only is this rather high, but we were especially suspicious about the fact that the mean volumes of the cells remained constant at reciprocal osmolalities of 3.3, 4.5, 6.7 or osmolalities of 0.308, 0.227, and 0.152 Osm/Kg. Put differently, the volumes of cells in hypotonic solutions with concentrations that were 60% and 50% of isotonic were nearly the same as the volume of the cells in isotonic Tyrodes.

One possible explanation for the lack of volume change in the hypotonic solutions was that active volume regulation was occurring over that 30 minutes interval. To test that possibility, we repeated the BvH measurements after exposing the cells for only 5 minutes to the anisotonic solutions. The results are shown by the black diamonds and the solid line in Fig. 1, and are dramatically different from the 30 minutes exposure data. The slope of the regression line is nearly twice as high for the 5 minute line (614 vs. 354), and the Y-axis intercept (V_b) about half that of the 30 minute line ($993 \mu\text{m}^3$ vs. $1576 \mu\text{m}^3$). For the 5 minutes data, the resulting fractional volume b (relative to the mean measured volume of the isotonic cell) is 0.30, which is a factor of 1.6 lower than the value of b that results from the 30 minutes data plot.

Kinetic Measurements in Hypotonic Media

To confirm that volume regulation was occurring, we made kinetic measurements of the volumes of the cells suspended in hypotonic Tyrodes (0.157 Osm/Kg) for 50 min. The results are shown in Fig. 2 and 3. Fig. 2 shows the bright field microscopy images taken from the cells in hypotonic Tyrodes at the peak of their initial volume increase at $t = 2 \text{ min } 46 \text{ s}$ (Fig. 2 A) and after completed RVD (Fig. 2 B) at $t = 56 \text{ min } 46 \text{ s}$, showing a volume close to the isotonic volume. The computed difference image $|\text{image A} - \text{image B}|$ of Fig. 2 C illustrates the diameter changes at the single cell level. Fig. 3 summarizes these results in the form of a plot of the averaged cell volume over time. The volumes were calculated from the averaged cell diameters. During the first 2 minutes, the cells displayed a passive volume increase that peaked at 180% of their isotonic volume (from $3,324 \pm 238 \mu\text{m}^3$ at isotonicity to $5,941 \pm 400 \mu\text{m}^3$). Subsequently, the cell volume started to decrease, reaching a value of 110% of the isotonic volume ($3,534 \pm 268 \mu\text{m}^3$) after 30 minutes and finally dropping to as low as 95% of isotonic ($3,123 \pm 234 \mu\text{m}^3$) after 50 min.

The imaging for the data points represented by black diamonds in Fig. 1 was initiated after the cells had been exposed to the anisosmotic media for five minutes. Fig. 3 shows that the volume regulatory decrease was already underway at that time in the 157 milliosmolal solution. This means that the maximum volume in that hypotonic medium ascribable to osmotic swelling was slightly higher than that depicted in Fig. 1. From the relative cell volumes at 2 and 5 min, we estimate about 9% higher. We assume it to be 3% higher for cells in 242 milliosmolal solution, although measurements analogous to those for 157 mosmolal were not made. These volume corrections have been applied in Fig. 4 to the two

hypotonic data points in the set labeled “corrected” (white squares). The effect of the correction on the resulting regression line (dashed line) is slight but discernable.

This return of the cell volume in hypotonic media to the isotonic value was abolished at 0 °C. At 0 °C, cells with an initial isotonic volume of $3,853 \pm 270 \mu\text{m}^3$ increased their volume by 184% ($7,106 \pm 509 \mu\text{m}^3$) after about 10 minutes, but failed to initiate a subsequent volume decrease; i.e., they remained in this enlarged state. (The higher isotonic value at 0 °C can be explained by inhibition of the $\text{Na}^+\text{-K}^+\text{-ATPase}$ under hypothermic conditions [4].) The lack of regulatory volume decrease (RVD) at 0 °C is consistent with the fact that this cellular response is achieved by specific membrane proteins, many of which provide energetically driven channel functionality [2; 5; 6; 7]. However, there could be a non-energetic contribution at 0 °C from phase transitions in plasma membrane lipids [8; 9].

Cell volumes were followed with video time-lapse microscopy. For room temperature measurements, suspensions of cells in isotonic Tyrodes were mixed 1:4 with the hypotonic test solutions and immediately loaded into the 50 μm deep chamber of a MicroCell slide (Conception Technologies, San Diego, CA). The slide was rapidly transferred to the stage of a fluorescence microscope where the imaging process was started after a brief searching and focusing period. Until its positioning onto the microscope stage, the MicroCell slides and the anisotonic test solutions were kept on ice to minimize the amount of cellular volume change that would occur before the first microscope image could be obtained. The first 6 images of an image series were recorded at 60 seconds intervals, starting 100 – 110 seconds after the mixing; the interval time was then increased to 5 minutes for the following 9 images. After the last image, a fluorescence image was taken to determine, and if necessary exclude, any dead cells from further analysis. To rule out any bias from the added calcein AM, we recorded control runs under exactly the same conditions, but without calcein AM. These time-lapse series were performed in 157 mOsm/Kg TBS ($n = 43$).

For the parallel series that were performed on cells in 157 mOsm/Kg at 0 °C ($n = 51$), we used a cryostage (BCS 196 cryostage, Linkam Scientific Instruments, Waterfield, UK). For these 0 °C experiments, the 1:4 cell suspension-test TBS mixture was loaded into a chamber made from two 12 mm round glass cover slips, separated by a 40 μm thick circular plastic spacer and placed in a Linkam quartz dish. The dish in turn was placed a metal holder that fitted into the cryostage. The test solutions, cover slips, and the metal holder were all kept at 2 – 4 °C prior to insertion into the cryostage that had been precooled to 0 °C. The microscope was not equipped for fluorescence.

Volume Regulation of Cells Shrunken in Hypertonic Media

Like most cells, COS-7 cells undergo abrupt passive osmotic shrinkage when placed in hypertonic media. Thus, when placed in double- or even triple-strength hypertonic TBS (602 or 986 mOsm/Kg), they shrink to 53% and 44% of their isotonic volume, respectively. At this point, some cell types exhibit a regulatory volume increase (RVI) that drives the cell volume back towards the isotonic value. However, that is not the case with COS-7. As opposed to the response in hypoosmotic media, the cells at 22 °C remained shrunken and did not show any signs of a regulatory volume increase over 60 minutes of observation. This difference in the regulatory response to swelling and shrinkage is not especially surprising since the postulated mechanisms differ in the two cases. A similar unidirectional volume regulation has also been reported for other cell types such as pancreatic beta-cells [10], renal cells [11] and an undifferentiated type of tumor cell [12]. One difference is thought to be a consequence of the increased concentration of intracellular Cl^- resulting from cell shrinkage; it hampers several ion transport mechanisms needed for RVI [13]. Also, in RVI, a central mechanism appears to be an energy-requiring increased uptake of NaCl coupled to

the subsequent intracellular replacement of Na^+ by K^+ . In invertebrate cells, amino acids and other nonelectrolytes can also be involved [1; 13]. In contrast, the mechanisms responsible for RVD appear to require a **mainly** energy-driven extrusion of K^+ and Cl^- , as well as organic molecules such as amino acids, polyacohols, and amines.

Implications of Volume Regulation for Boyle Van't Hoff plots and Estimates of Non-Osmotic Water

Returning to Fig. 1, we see that unawareness of the operation of a volume regulatory response can introduce large errors in a Boyle van't Hoff plot where measured cell volumes are assumed to be equilibrium cell volumes solely determined by passive osmotic flows. This unawareness introduces nearly 2-fold errors for COS-7 cells in the slope of the line derived from a best linear fit and its Y-axis intercept. A close inspection of Fig. 1 shows that all the discrepancies between the cell volumes after 5 and 30 minutes exposures to the test solution lie in the hypotonic region – not in the hypertonic region. This, of course, is in accord with our observation that volume regulation in COS-7 cells occurs in the former but not in the latter.

V_b , the Y-axis intercept, is considered to be a measure of the solids and bound water content of the cell. Usually, it is expressed as b , the ratio of V_b to $V_{\text{iso cell}}$. But we have expressed it and all other volumes as absolute volumes rather than volumes normalized to the isotonic cell volume. One reason for our doing so is that there is ambiguity as to the proper value for $V_{\text{iso cell}}$. We have taken it, as do most investigators, as the mean measured volume of the cell in isotonic media; i.e. $3324 \mu\text{m}^3$. On that basis, b for the uncorrected 5 min line in Fig. 4 is 0.301 and 0.254 for in the corrected line. One could however take $V_{\text{iso cell}}$ to be the value calculated from the equation of the best fit line at an osmolality of 0.308 or reciprocal osmolality of 3.25. For the uncorrected line, that would be $2989 \mu\text{m}^3$, and b would be $993/2989$ or 0.332 instead of 0.301. The difference is small because here the measured volume of the isotonic cell is close to the value calculated from the best-fit line. Katkov [14] has argued that the standard method of calculating b is wrong on mathematical grounds. He states that the correct regression line must pass through the measured cell volume in isotonic media. As a consequence, this regression line will be displaced from the regression line we have drawn but parallel to it. In the case of uncorrected 5 minutes data, we would have that $V_b = V_{\text{iso cell}} - (614)(1/M_{\text{iso}}) = (614)(3.25) = 1304 \mu\text{m}^3$, and $b = 1304/3299 = 0.40$, a rather large difference from the values above. However, Benson [15] has recently reported that Katkov's arguments can be rejected on mathematical grounds. Consequently, we do not accept his premise that the regression line must be forced to go through the point $1/M_{\text{iso}}$, $V_{\text{iso cell}}$ (measured).

We close with the important fifth question posed in the introduction; namely, how many other cell types exhibit cell volume regulation of sufficient magnitude and kinetics to influence cryobiological analyses and outcomes? We do not know. But we do know that volume regulation is common among cell types across a wide range of phyla [16]. This long-time conservation of a cellular process means that it is of high evolutionary importance to maintain cells at close to their isotonic volume, which in turn means maintaining their cytosolic components at the optimum concentrations. Chamberlin and Strange [16] point out that volume regulatory mechanisms are crucial for cell survival. Thus, the maintenance or reestablishment of a constant intracellular milieu is required for the proper hydration state of cytosolic proteins and for the control of macromolecular crowding. Excessive alterations of cell volume endanger structural integrity and basic cellular physiology. Animal cells are therefore well equipped with a range of counteracting options they can activate [2]. We have cited a few examples.

The existence of volume regulatory processes does not necessarily mean, however, that they have cryobiological significance. They almost certainly do not act during the actual cooling and freezing of cells. Since they are enzymatically driven, the rates of these processes will likely slow by factors of two or three for every 10 °C drop in temperature. Thus by –20 °C, the rate will have dropped perhaps 100-fold. In COS-7 cells, we did not detect them at all at 0 °C.

But many important processes in cryobiology and cryopreservation occur at room temperature, and as we have shown for COS-7 cells, volume regulation could substantially and significantly perturb them. For example, prior to freezing, cells are usually equilibrated in hyperosmotic solutions of cryoprotective agents (CPA) such as glycerol or ethylene glycol. In that exposure, they undergo an abrupt decrease in volume followed by a much slower return to near normal volume. This is assumed to be totally a consequence of passive osmotic flows, but volume regulation could also be operating, and affecting subsequent analyses. Two cryobiologically important parameters derived from such shrink/swell curves are the permeability of a cell to water and to cryoprotective solutes. The estimation of these two parameters also assumes that cell volumes are being determined solely by passive flows of solvent and solute. If such is not the case, the calculated permeability coefficients could be significantly in error.

The findings in this paper on COS-7 cells thus constitute a warning flag to be sensitive to potential contributions from volume regulation. Based on our data, any such contribution should be simple to detect: A cell volume anomaly will be present at room temperature but not at 0 °C and the extent of the anomaly will increase with time at room temperature or above.

References

1. Hoffmann EK, Simonsen LO. Membrane mechanisms in volume and pH regulation in vertebrate cells. *Physiol Rev.* 1989; 69:315–82. [PubMed: 2538851]
2. Lang F, Busch GL, Ritter M, Volkl H, Waldegger S, Gulbins E, Haussinger D. Functional significance of cell volume regulatory mechanisms. *Physiol Rev.* 1998; 78:247–306. [PubMed: 9457175]
3. Peckys DB, Kleinhans FW, Mazur P. Rectification of the Water Permeability in COS-7 Cells at 22, 10 and 0 degrees C. *PLoS One.* 2011; 6:e23643. [PubMed: 21887290]
4. Hochachka PW. Defense strategies against hypoxia and hypothermia. *Science.* 1986; 231:234–41. [PubMed: 2417316]
5. Furst J, Gschwentner M, Ritter M, Botta G, Jakab M, Mayer M, Garavaglia L, Bazzini C, Rodighiero S, Meyer G, Eichmuller S, Woll E, Paulmichl M. Molecular and functional aspects of anionic channels activated during regulatory volume decrease in mammalian cells. *Pflugers Arch.* 2002; 444:1–25. [PubMed: 11976912]
6. Okada Y, Hazama A. Volume-Regulatory Ion Channels in Epithelial Cells. *Physiology.* 1989; 4:238–242.
7. Hoffmann EK, Lambert IH, Pedersen SF. Physiology of Cell Volume Regulation in Vertebrates. *Physiological Reviews.* 2009; 89:193–277. [PubMed: 19126758]
8. Carruthers A, Melchior DL. Study of the relationship between bilayer water permeability and bilayer physical state. *Biochemistry.* 1983; 22:5797–5807.
9. Grisham CM, Barnett RE. The role of lipid-phase transitions in the regulation of the (sodium + potassium) adenosine triphosphatase. *Biochemistry.* 1973; 12:2635–7. [PubMed: 4268111]
10. Engstrom KG, Sandstrom PE, Sehlin J. Volume regulation in mouse pancreatic beta-cells is mediated by a furosemide-sensitive mechanism. *Biochim Biophys Acta.* 1991; 1091:145–50. [PubMed: 1995074]

11. Grunewald JM, Grunewald RW, Kinne RK. Regulation of ion content and cell volume in isolated rat renal IMCD cells under hypertonic conditions. *Am J Physiol.* 1994; 267:F13–9. [PubMed: 8048552]
12. Hoffmann EK, Lambert IH, Simonsen LO. Mechanisms in volume regulation in Ehrlich ascites tumor cells. *Ren Physiol Biochem.* 1988; 11:221–47. [PubMed: 3074400]
13. Hoffmann EK, Dunham PB. Membrane mechanisms and intracellular signalling in cell volume regulation. *Int Rev Cytol.* 1995; 161:173–262. [PubMed: 7558691]
14. Katkov. On proper linearization, construction and analysis of the Boyle-van't Hoff plots and correct calculation of the osmotically inactive volume. *Cryobiology.* 2011; 62:232–41. [PubMed: 21376029]
15. Benson JD. Some comments on recent discussion of the Boyle van't Hoff relationship. *Cryobiology.* 2012; 64:118–20. [PubMed: 22202670]
16. Chamberlin ME, Strange K. Anisotonic cell volume regulation: a comparative view. *Am J Physiol.* 1989; 257:C159–73. [PubMed: 2669504]

Figure 1.

Two different Boyle-van't Hoff plots displaying the absolute volume of COS-7 cells as a function of the reciprocal of the osmolality of the anisotonic medium in which the cells were suspended for 5 – 10 minutes (black diamonds) or 30 – 35 minutes (black circles) at 22 °C. Each point is the mean value of 170 – 314 individually analyzed cells. Due to the effects of RVD, which occurred under hypotonic conditions and lowered the corresponding volumes to levels similar to the isotonic volume, the regression line of the 30 minutes data (dotted line) was much flatter than the 5 minute data (solid line), had a lower correlation coefficient (r^2), and resulted in a 60% higher value for V_b . The volumes were calculated from the measured diameters as described in the text. Data were fitted by the method of least squares.

Figure 2.

Bright field microscopy images showing the regulatory volume decrease (RVD) that COS-7 cells exhibit after having reached their maximum volume in 157 mosmolal hypotonic solution. Figure 2 A shows the cells swollen to their maximum volume at $t = 2 \text{ min } 46 \text{ s}$. Figure 2 B was taken 54 min later and demonstrates that the same cells had reduced their cell volume values. Figure 2 C is a computed difference image of A and B that illustrates, at the single cell level, the differences of the cell diameters at the peak of the initial swelling reaction (A) and at the end of the RVD (B). All scale bars: $50 \mu\text{m}$.

Figure 3.

Dynamic volume response curve of COS-7 cells after exposure to hypotonic (157 mOsm/Kg) TBS. The first data point (open circle) at $t=0$ represents the average diameter (and S.E.M value) of 828 individually measured cells at isotonicity. All other data points (solid circles) represent averages and S.E.M. values of 43 individually measured cells. Cells reached their maximum volume within 2 min, then reversed the swelling due to the onset of regulatory volume decrease mechanisms, and shrunk slowly back to a final volume that was 95% of their initial isotonic volume.

Figure 4.

Corrected and uncorrected BVH plots for COS-7 cells after exposure to anisotonic test solutions for 5 minutes. Figure 2 showed that in the 157 mosmolal hypotonic solution, the process of volume regulatory decrease (RVD) begins to reverse the passive osmotic swelling of the cells at about 2 min. Consequently in this, Figure 3, the measured cell volumes for the two points in the hypotonic range (242 and 157 mosm/kg water) have been multiplied by 1.03 and 1.09, respectively to obtain the plotted corrected points (white squares and dotted regression line). The uncorrected points (black diamonds) and (solid) regression line are the same as in Figure 1.