Rectification of the Water Permeability in COS-7 Cells at 22, 10 and 0°C

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Abstract

The osmotic and permeability parameters of a cell membrane are essential physico-chemical properties of a cell and particularly important with respect to cell volume changes and the regulation thereof. Here, we report the hydraulic conductivity, Lp, the non-osmotic volume, Vb, and the Arrhenius activation energy, Ea, of mammalian COS-7 cells. The ratio of Vb to the isotonic cell volume, Viso, was 0.29. Lp, the activation energy required for the permeation of water through the cell membrane, was 10,700, and 12,000 cal/mol under hyper- and hypotonic conditions, respectively. Average values for Lp were calculated from swell/shrink curves by using an integrated equation for Lp. The curves represented the volume changes of 358 individually measured cells, placed into solutions of nonpermeating solutes of 157 or 602 mOsm/kg (at 0, 10 or 22°C) and imaged over time. Lp estimates for all six combinations of osmolality and temperature were calculated, resulting in values of 0.11, 0.21, and 0.10 μm/min/atm for exosmotic flow and 0.79, 1.73 and 1.87 μm/min/atm for endosmotic flow (at 0, 10 and 22°C, respectively). The unexpected finding of several fold higher Lp values for endosmotic flow indicates highly asymmetric membrane permeability for water in COS-7. This phenomenon is known as rectification and has mainly been reported for plant cell, but only rarely for animal cells. Although the mechanism underlying the strong rectification found in COS-7 cells is yet unknown, it is a phenomenon of biological interest and has important practical consequences, for instance, in the development of optimal cryopreservation.

Introduction

Water transport across cellular membranes is of crucial importance in animal and plant physiology. The permeability of a cell to water and the temperature coefficient of that permeability are two of its more important parameters. They, along with a cell’s permeability or lack thereof to solutes, determine the magnitude and kinetics of cell volume changes when the cell is subjected to conditions that depart from isotonic or isoosmotic. The permeability of a cell to water is usually referred to as the hydraulic conductivity, Lp. It has the units of volume divided by area×time, or commonly, μm/atm. min.

Water permeability is of particular importance in cryobiology, which is the main focus in our laboratory. The value of Lp is one of the chief factors determining the conditions under which ice forms or does not form in the interior of a cell [1,2]. Intracellular ice formation (IIF) is almost always lethal. If the cooling rate is low enough or if the Lp is high enough, the cell will dehydrate during cooling and will not undergo IIF. In contrast, if the cooling rate is too high or the Lp too low, the cell will not dehydrate rapidly enough to maintain osmotic or chemical equilibrium with the external ice and solution; the cell water will increasingly supercool and will eventually freeze in situ, usually with lethal consequences.

For the past seven years, our laboratory has been studying various conditions under which IIF occurs or does not occur in several cell types; namely, mouse oocytes and early embryos [3,4], oocytes of the frog Xenopus [5,6] at various stages of development, V79 Chinese hamster tissue culture cells, and the yeast Saccharomyces cerevisiae [7]. Based on physical chemical equations and knowledge of certain parameters such as Lp, and its temperature coefficient or activation energy, Ea, one can compute the likelihood of IIF as a function of temperature and cooling rate [2]. By comparing that likelihood with experimental observations on mouse oocytes, mouse embryos and on yeast cells, we have found the agreement to be excellent.

The current paper deals with the water permeability of COS-7 fibroblasts. This tissue cell line is widely used in cell biology as a convenient protein expression system when specific proteins are to be studied. The average diameter of COS-7 cells (18.51 μm) is about two to three times that of most other mammalian cells, making its volume 8 to 27 times higher. Individual cell diameters range from 9 to 33 μm; hence, individual (isotonic) cell volumes of COS-7 cell can differ by as much as 44-fold. These facts made it of interest to determine experimentally the relation between IIF in these cells and cooling rate, to determine the temperature at which IIF occurs, and to compare the observed relation between cooling rate and IIF with the computed relation. As mentioned, that computation requires knowledge of Lp and its activation energy, Ea. Determining these parameters was the purpose of the present study.
The standard way to determine $I_D$ is to transfer cells from an isonotic solution to a hypotonic or hypertonic solution of an impermeant solute and determine the rate at which the cell swells or shrinks, respectively. Usually, the $I_D$ that is calculated from the rate of swelling has about the same numerical value as the $I_P$ calculated from the rate of shrinkage. But occasionally, the two values for $I_P$ differ; i.e., the resistance of the membrane to inflow of water differs from its resistance to outflow. Such a difference is referred to as rectification. We have found that COS-7 cells exhibit extremely large apparent rectification; i.e., the value of $I_P$ for the influx of water is as much as 18-fold larger than the value for the outflow of water.

**Materials and Methods**

**Isotonic and anisotonic test solutions**

Test solutions were made from Tyrode’s Buffered Saline (TBS). It has a measured osmolality of 0.308, a value that we define as isotonic. Hypotonic solutions were made by diluting TBS with HPLC grade water; hypertonic solutions were made by adding sucrose to the TBS (all the above were from Sigma Aldrich). All test solutions were prepared with a 20% higher or lower osmolality than the desired final concentration to compensate for two subsequent dilutions with 10% volumes of isotonic TBS. One dilution was used for adding 10 μM of the live-dead dye calcein AM (Invitrogen, Carlsbad, CA); the second was for the subsequent addition of the COS-7 cell suspension. The osmolalities were measured with a Vapro Osmometer 5520 (Wescor Inc., Logan, UT). The final milliosmolalities of the TBS test solutions (3–5 samples each), after the 20% volume additions were: 157±2, 242±2, 308±2, 602±6, and 986±3 (Mean ± S.E.).

**Cell culture**

COS-7 cells (African Green Monkey kidney fibroblast) were obtained from ATCC (Manassas, VA). They were cultured in Dulbecco’s Modified Eagle Medium (DMEM), supplemented with 10% FBS (Invitrogen), in a 5% CO₂-air atmosphere, at 37°C. After reaching confluency in 3–4 days, the cells were harvested by rinsing in Dulbecco’s Phosphate Buffered Saline (Sigma Aldrich). We then dissociated the adherent layer with CellStripper (Mediatech, CA, USA), followed by a quench in DMEM. Harvested cells were pelleted in a 15 ml centrifuge tube (Falcon) at 60× g for 10 min. The pellet was resuspended in TBS. The centrifugation and resuspension was repeated once and the pellet was finally resuspended in a total volume of 180 μl of TBS supplemented with 14.5 mM D-glucose and 0.3% bovine serum albumin (BSA) (Sigma Aldrich). Then we added 20 μl of TBS with 10 μM calcein AM to the cell suspension to yield 200 μl of medium and 1 μM final concentration of calcein AM. The cells were left in this isotonic solution, in the dark at room temperature (RT, 22°C) for at least 30 minutes before starting the first experiment. The cell suspensions were continuously kept at 22°C until mixing with the test solutions.

**Osmotically induced volume change of individual cells after 5 min at 22°C (RT)**

Cell volumes were measured after equilibration in media that had osmolalities below, above, and at isotonicity, and plotted vs. the reciprocal of the osmolality of the medium. This result is referred to as a Boyle-vańt’s Hoff plot (BVH). When the result is linear, the cells are said to behave as ideal osmometers. To obtain a BVH for COS-7 cells, 40 ml of cell suspension (in isotonic TBS+calcein AM) was added to 160 μl of test TBS in the wells of a 96 well plate. The osmolalities of the test solution after the addition of the cells ranged from 157–986 mOsm/kg. After 5 min, the cells had sunk to the bottom of the wells and they were then photographed with a Zeiss Axioskope microscope at 400× over the ensuing 2 min, using both bright field and fluorescence microscopy. The fluorescent excitation maximum for calcein AM is 488 nm. Emission was collected through a 520–700 nm wide pass FITC filter. The diameters of the cells were measured with Image J 1.42Q software (NIH) and their volumes were calculated (Microsoft Excel software) assuming them to be spheres. The 5-min time period between mixing of cells and the measurement of diameters is important. We have shown that with additional time, swollen cells begin to return to their isotonic volume because of volume regulation [8].

The determination of $I_P$ of individual cells by following the dynamics of their volume change in anisotonic solutions at 22°C

When cells are placed in hypotonic or hypertonic solutions of solutes to which the cells are impermeable, they swell or shrink, respectively, and the rate of that swelling or shrinkage is a measure of the permeability of the cell to water; i.e., the hydraulic conductivity, $I_P$. To make these measurements for COS-7 cells, the cell suspension was mixed 1:4 with TBS solution that had been diluted with water, to yield a final osmolality of 157 mOsm/kg or mixed with TBS that had been supplemented with sucrose to yield a final osmolality of 602 mOsm/kg. These are approximately half and twice isotonic. These mixed solutions were immediately loaded into the 50 μm deep chambers of MicroCell slides (Concepcion Technologies, San Diego, CA) and then transferred to the stage of the microscope for photographing at frequent intervals, an example of the first image of such an imaging series is shown in Fig. 1a. To minimize the amount of cell swelling or shrinkage that occurred before obtaining the first microscope image, the MicroCell slides were kept near 0°C, on an aluminum block immersed in ice, all the test solutions were kept on ice too. First, 16 μl of anisotropic TBS test solution was pipetted into a 0.2 ml plastic tube. Next, 4 μl of cell suspension with calcein AM (held at 22°C) was added, and the resulting suspension was mixed for 2–4 s. The mixed solution warmed to about 6°C. Immediately after the mixing, 4 μl of this cell/test solution mixture was allowed to flow into the chamber of the MicroCell slide. The slide was on ice so the suspension again cooled towards 0°C. Finally, the slide was quickly transferred onto the microscope stage, which was at room temperature. We estimate that the slide converged on that temperature in 10–20 s. This time was used to adjust the focus and to locate a microscope field that displayed at least twenty cells. For each experiment, the time from the beginning of the mixing of the cell suspension with the test solution until the recording of the first image was determined to be 30–40 s. Images were recorded at 10 s intervals for 5 min. This procedure was repeated 2–4 times per test solution. After the last image of a sequence was obtained, the illumination mode of the microscope was set to fluorescence and a fluorescence image was taken, an example of such a fluorescence image is shown in Fig. 1b. The fluorescence images served to determine which cells were dead and therefore needed to be excluded from analysis. To exclude any bias from the added calcein AM, we recorded two control runs under exactly the same conditions, but without calcein AM.

**Dynamics of volume change of individual cells in response to anisotonic solutions at 10°C and 0°C**

The exposure of the cells to hypotonic and hypertonic TBS (157 and 602 mOsm/kg), at 10 or 0°C, and the image recording, were
AM was added because the Zeiss photomicroscope lacked out as described for the RT experiments, except that no calcein slip. Preparation, storage and mixing of the solutions were carried out as described for the RT experiments, except that no calcein AM fluorescence. Although the two damaged cells still display fluorescence, such cells were excluded from the analysis. Scale bar = 50 μm.

Figure 1. COS-7 cells suspended in the MicroCell chamber at 22°C. A) First image from a series of images initiated 21 s after cells had been exposed to hypotonic (157 mOsm/kg) TBS. The numbers indicate individually identified cells selected for analysis. The changes in their diameters were recorded until well after the cells had reached their maximal volume. Arrows point to two cells that show membrane damage in the form of a bleb of leaking cytosol. B) Fluorescence image of the same group of cells taken immediately after the last image of the bright light image series, about 5 min after A) was taken. Viable cells display green calcein AM fluorescence. Although the two damaged cells still display fluorescence, such cells were excluded from the analysis. Scale bar = 50 μm.

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performed in a BCS 196 cryostage (Linkam Scientific Instruments, Waterfield, UK) controlled with Pax-it control and capture software (v. 6.1, by Midwest Information Services, Franklin Park, IL). The cryostage was attached to a Zeiss bright-field photomicroscope equipped with a Paxcam digital CCD camera (800××600 resolution) and an Olympus 20× long working distance microscope objective. Prior to its being loaded with cells and inserted into the BCS 196 cryostage that had been precooled to 10 or 0°C, the metal sample holder was kept on an ice-cooled aluminum block. A 12 mm round glass cover slip (Ted Pella No. 26023) was inserted in the sample holder. To prevent squeezing of the cells, a 40 μm thick, round plastic spacer with 10 mm outer and 5 mm inner diameter was placed on this cover slip. Preparation, storage and mixing of the solutions were carried out as described for the RT experiments, except that no calcein AM was added because the Zeiss photomicroscope lacked fluorescence capabilities. After mixing the TBS test solution (cold) with the cell suspension (22°C), 4 μl of this solution was pipetted on the center of the cover slip. A second 12 mm round glass cover slip (precooled) was then placed with vacuum tweezers on this droplet, thereby enclosing the cells in a 40 μm deep cylinder of the test aqueous solution.

The sample holder was taken from the aluminum block and quickly placed into the cryostage, which also had been precooled and kept at 0 or 10°C. The focus was adjusted and an area that displayed at least twenty cells was located. The image sequences were recorded at 10 s intervals for 5 min. The intervals were then increased to 30 s and imaging continued for another 10 to 20 min. This procedure was repeated 2–4 times per test solution. For control purposes, we determined the cell volumes in isotonic TBS at 10 and 0°C. This was done by loading the imaging chamber with cells suspended in isotonic TBS, inserting the sample holder in the cryostage (held at 0 or 10°C), waiting for 5 min, and then imaging cells throughout the whole chamber over the ensuing 2 min. The time from the beginning of the mixing of the cell suspension with anisotonic TBS until the recording of the first image of the image sequence was documented for each experiment. That time was 25–40 s. Because this Zeiss microscope was not equipped for fluorescence imaging, we performed separate test runs with cells that had been preincubated with calcein AM and were subsequently treated exactly the same way as in the swell/shrink experiments at 10 and 0°C. After the imaging sequence in the Zeiss microscope, the sample holder and the imaging chamber were transferred to a fluorescence microscope (Zeiss WL Standard) equipped with FITC filters and the cells throughout the whole chamber were examined for their calcein AM fluorescence.

Statistics

Values are arithmetic means. Plus/minus values are standard deviations of the mean (standard errors). Tests of statistical significance were carried out by a two-tailed Students t test. All calculations were performed with Microsoft Excel software.

Determination of the non-osmotic volume (~volume of solids, Vb), the hydraulic conductivity, Lp, and its activation energy, Ea

A Boyle-van’t Hoff relationship was established by plotting the mean of the individual cell volumes (calculated from the recorded diameters, assuming the cells to be spherical), determined in each of the five different osmolality solutions, against the reciprocal of the osmolality of that respective solution; i.e.,

\[ V = V_c - V_b = a \left( \frac{1}{M} \right) \]

or

\[ V_c = a \left( \frac{1}{M} \right) + V_b, \]  

(1)

where V is the absolute volume of cell water, Vc is the cell volume, a is the slope, M is the osmolality of the test solution, and Vb is the absolute volume of the cell when 1/M is extrapolated to zero (i.e., M becomes infinite). It is the volume of solids and nonsmotic water in the cell.

Values for the water permeability coefficient, or hydraulic conductivity, \( I_w \) [μm²/μm² min/atm], were determined from the slopes of the plots of change in cell volume vs. time, in either the hypotonic solution (157 mOsm/kg) or in the hypertonic TBS (602
mOsm/kg). The volume of each individual cell at each point in time was calculated for the two tested osmolalities and for all three different temperatures. These individual values were then averaged for all the analyzed cells of an experiment (from 40 to 63 cells), generating six averaged swell/shrink curves. Average \( L_p \)’s for the six combinations of osmolality and temperature were then calculated using the following equation [9,10], which is the integrated form of the standard differential equation for cell shrinkage or swelling in anisotonic solutions:

\[
L_p = \frac{V_c (V_1 - V_2) + V_c \ln \frac{V_c - V_1}{V_c - V_2}}{(t_2 - t_1) A N R T}, \tag{2}
\]

where \( V_c \) is the volume of water in the cell after the cell has reached its equilibrium volume, \( V_1 \) is the cell water volume at time \( t_1 \) (all in \( \text{m}^3 \)), \( A \) is the surface area of the cell (\( \text{m}^2 \)), \( N \) is the number of osmoles of solute in the cell, \( R \) is the universal gas constant (\( \text{cal/mole.deg} \)), and \( T \) is the temperature in Kelvin. The several cell water volumes are obtained by subtracting the non-osmotic volume from the cell volume; i.e., \( V_c = V_c^0 - V_c^b \). \( A \) and \( N \) are assumed constant. The computations of \( L_p \) by Eq (2) were made using values of pairs of volumes \( V_1 \) and \( V_2 \) corresponding to 12 to 15 pairs of time intervals from the dynamic part of each averaged swell/shrink curve, and taking the average. Using this integrated equation has the advantage that mixing time artifacts do not affect the result. \( L_p \) only depends on the time difference \( (t_2 - t_1) \) and not the starting time. A list of the parameters used in our calculations, as well as their units and their numerical values, is given in Table 1.

The activation energy, \( E_a \), for \( L_p \) describes the dependence of the \( L_p \) of the plasma membrane on the environmental temperature. \( E_a \) was calculated from the slope of the Arrhenius plot (the natural logarithm of the \( L_p \) in \( \text{m}^3/\text{atm/mole.deg} \), against the reciprocal of the absolute temperature). In the present study, we intended to calculate \( E_a \) based on the mean \( L_p \) values obtained for the hypotonic and hypertonic media at the three tested temperatures: 0, 10 and 22°C, according to the equation \( E_a = -R^* \text{slope} \). Again, \( R^* \) is the gas constant, but here its units are cal/mole.K, and its value is 1.987. The slope is \( \ln(L_{p2}/L_{p1})/(1/T_1-1/T_2) \). However, for reasons we shall discuss later, we concluded that the \( L_p \)’s at 22°C are spurious.

### Results

Osmotic behavior of COS-7 cells after 5 min equilibration (the Boyle-van’t Hoff plot)

The volumes of COS-7 cells in isotonic media were measured at 0, 10 and 22°C. They were 3653 ± 120 \( (n=315) \), 3920 ± 158 \( (n=314) \), and 3324 ± 61 \( (n=829) \) \( \mu \text{m}^3 \), respectively. The mean isotonic cell volume \( (V_{c,iso}) \) and the volume of water in the isotonic cell \( (V_{v,iso}) \) at 22°C were about 15% lower than those measured at 0 and 10°C (Table 1). This difference is significant \((p<0.05)\). To examine whether a Boyle-van’t Hoff relationship exists, the cell volumes were determined at 22°C, after the cells had been exposed for 5 minutes to a series of hyper- or hypotonic TBS solutions (range: 157–986 mOsm/kg) and measured during the subsequent 2 min. The volumes of the COS-7 cells exhibited a classic linear Boyle-van’t Hoff osmotic response; namely, a monotonic linear relationship with the reciprocal of the osmolality of the medium; the higher the osmolality, the smaller the cells (see Fig. 2). Extrapolation of the linear Boyle-van’t Hoff plot to the \( V \)-axis generated an average non-osmotic cell volume \( V_b \) of 963 \( \mu \text{m}^3 \). Normalization, i.e., dividing \( V_b \) by \( V_{c,iso} \) (3324 \( \mu \text{m}^3 \)), yields a value of 0.29 for the ratio of the volume of cell solids to the volume of the isotonic cell, here referred to as \( n_b \).

Dynamics of cell volume change in hypertonic TBS (602 mOsm/kg) at 22, 10 and 0°C and the computed \( L_p \)

We performed kinetic shrink experiments at 0, 10, and 22°C by placing cells in TBS solution made hypertonic (602 mOsm/kg) with sucrose. In the two or three runs per temperature, we analyzed a total of 153 individual cells \((n=51 \text{ at 22°C}, n=54 \text{ at 10°C} \text{ and } n=48 \text{ at 0°C})\) by measuring their time-dependent volume changes. Fig. 3 shows the averaged cell volume change at the respective temperatures. The graphs were constructed by first averaging the measured diameters of each individual cell at each recorded point in time. Subsequently the average diameters were converted into cell volumes. As expected, the higher the temperature, the shorter the time until the cells reached equilibrium. At 22°C, they reached their equilibrium volume after 185 s, at 10°C after 230 s and at 0°C after 790 s. The cell volumes at equilibrium were 1762 ± 100 at 22°C, 2626 ± 235 at 10°C and 2199 ± 200 \( \mu \text{m}^3 \) at 0°C. The integrated equation, Eq (2), requires the use of absolute cell volume values (as opposed to

### Table 1. Initial values and constants used to calculate \( L_p \) with Equation (2).

| Symbol | Parameter | Temperature | units |
|--------|-----------|-------------|-------|
| \( V_c \) | Cell volume | 0°C | 10°C | 22°C | (\( \mu \text{m}^3 \)) |
| \( V_{c,iso} \) | Mean volume of isotonic cell | 3853 | 3920 | 3324 | (\( \mu \text{m}^3 \)) |
| \( V_b \) | Volume of non-osmotic materials in cells | 1116 | 1135 | 963 | (\( \mu \text{m}^3 \)) |
| \( V_{iso} \) | Volume of water in the isotonic cell | 2737 | 2785 | 2361 | (\( \mu \text{m}^3 \)) |
| \( A \) | Surface area of the cell | 1188 | 1202 | 1021 | (\( \mu \text{m}^3 \)) |
| \( R \) | Gas constant | \( 8.21 \times 10^{-13} \) | \( 8.21 \times 10^{-13} \) | \( 8.21 \times 10^{-13} \) | (\( \text{cal/mole.K} \)) |
| \( R^* \) | Gas constant | 1.987 | 1.987 | 1.987 | (\( \text{cal/mole.K} \)) |
| \( M_{iso} \) | Intracellular osmolality of isotonic cell = osmolality of Tyrode’s | 0.308 | 0.308 | 0.308 | (Osm) |
| \( N \) | Osmoles of solute in cell = \( M_{iso} \times V_{iso} \) | \( 8.43 \times 10^{-13} \) | \( 8.58 \times 10^{-13} \) | \( 7.27 \times 10^{-13} \) | (Osm) |

Note: The volumes of cells and cell solids relative to their volumes in an isotonic cell are shown by a lower case \( V \).
Comparison of water permeabilities ($L_p$) in hypo- and hypertonic TBS at 22, 10 and 0°C

All $L_p$ values are displayed in Fig. 5 and in Table 2. Comparing the $L_p$ values obtained under hypotonic conditions with their hypertonic counterparts at the same temperature reveals that the values for water influx (hypotonic TBS) are 7 to 18 times higher than the values for water efflux (hypertonic TBS). The differences are highly significant for all three temperature pairs ($p<0.001$) and increased with rising temperatures, from an $L_p$ hypertonic/$L_p$ hypotonic ratio of 7.5 at 0°C, to 8.7 at 10°C and up to 18.0 at 22°C (Table 1). There was also an effect of temperature causing the $L_p$ values in hypotonic conditions at 22°C and at 10°C to be significantly higher than the one at 0°C. In hypertonic conditions, only the $L_p$ value at 10°C, but not the one at 22°C was significantly higher than the one at 0°C. The $L_p$ value at 22°C was half of that at 10°C and equal to that at 0°C.

Activation energy ($E_a$) of $L_p$ in hypo- and hypertonic TBS

Arrhenius plots were generated for both the hypo- and the hypertonic conditions, by plotting the natural logarithms of the $L_p$ values gained at 0 and 10°C, against the reciprocal of the absolute temperature. The activation energy, $E_a$, as calculated from the slope of the Arrhenius plot, was 10,700 cal/mol at 602 mOsm/kg and 12,000 cal/mol at 157 mOsm/kg. (see Fig. 6). We did not include the $L_p$ values gained at 22°C in the Arrhenius plot for reasons given in the Discussion.

Discussion

The osmotic and permeability characteristics of cells play an important role in their physiology and they play a central role in determining their responses to the strongly anisotonic conditions occurring during cryobiological preservation. The studies reported here were concerned with determining osmotic parameters and the permeability of COS-7 cells to the osmotically driven inflow and outflow of water. The osmotic questions of concern were do the cells behave as ideal osmometers and what is the fractional volume occupied by cell solids and bound water ($v_s$)? Answers to both questions can be obtained by measuring the volumes of cells equilibrated in solutions of a range of concentrations of non-permeating solutes, and generating a Boyle van’t Hoff (BVH) plot; i.e., a plot of cell volume vs. the reciprocal of the osmolality of the solutions. These are static equilibrium measurements. In contrast, the permeability to water ($L_p$) is determined by kinetic measurements in which cells are placed in an anisotonic solution made of non-permeating solutes of known osmolality, and recordings are made of their change in volume with time. In hypertonic solutions, the cells shrink with time as cell water flows out. In hypotonic solutions, the cells swell with time as water flows into the cell. $L_p$ is a function of the slope of these fluxes. One can determine $E_a$, the activation energy of $L_p$, by conducting the shrink/swell experiments at several temperatures. We chose 0, 10 and 22°C.

We studied COS-7 cells for four reasons. One is because this cell line is widely used as a cellular expression system for recombinant proteins and for experiments in molecular biology, biochemistry and cell biology. The second reason is the future possibility of using transfected COS-7 cells that express specific membrane proteins involved in the facilitation of osmotic transport, like aquaporins. Such modified cells would open new ways for detailed studies of cellular mechanisms occurring during freezing and thawing. The third reason is that we wish to compare our so far unpublished observations on the relation between intracellular ice formation in COS-7 and cooling rate with the calculated relationship. To calculate the relationship, we need to...
know $I_p$ and its activation energy. The fourth reason is that the average COS-7 cell has nearly twice the diameter of most mammalian cells, and is about a quarter the diameter of a mouse oocyte. This will allow us to further test the growing impression that the larger a cell, the higher is the temperature at which intracellular ice formation occurs during cooling.

Cells behave as osmometers because of the semipermeable nature of their plasma membrane [11]. The osmotic or chemical potential of water across this membrane depends on both the extracellular osmotic pressure and the intracellular water potential. The former is constant; the latter varies as water leaves or enters the cell. The cellular water volume at a given time can be determined by subtracting the non-osmotic volume, $V_b$, from the total cell volume. $V_b$ refers to the absolute volume of the intracellular space occupied by lipids, proteins, carbohydrates, and bound water that is tightly hydrogen-bonded to the hydrophilic surfaces of these molecules. It is commonly obtained from the ordinate intercept of a Boyle-van’t Hoff plot of the relative cell volume, $V_c$ (the absolute cell volume divided by the volume of the isotonic cell) as a function of the reciprocal of the osmolality of the external medium [13]. Then, the osmotically active cell volume $(V_{c_{iso}}-V_b)$ indicates the amount of water in isotonic cells that can be lost (unbound water) during the osmotic stress the cell encounters through cryopreservation.

| Temperature [°C] | $I_p$ hyperosm | $I_p$ hypoosm | $I_p$ hyperosm/$I_p$ hypoosm |
|------------------|----------------|---------------|----------------------------|
| 22               | 0.10±0.01      | 1.87±0.10     | 18.0                       |
| 10               | 0.21±0.01      | 1.73±0.06     | 8.2                        |
| 0                | 0.11±0.01      | 0.79±0.05     | 7.5                        |

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Figure 3. Normalized shrink curves of COS-7 cells in hypertonic (602 mOsm/kg) TBS. The cell volumes were normalized to the mean isotonic volume at each temperature; namely, 3324, 3920, and 3853 μm³. The data represent averages and S.E. values of 153 individually measured cells. Cells reached their equilibrium volume faster the higher the temperatures.
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Table 2. Lp values and their ratios, determined under hypertonic and hypotonic conditions at 0, 10 and 22°C.
and permitted us to exclude dead or damaged cells from the analysis. This high survival rate confirmed that our protocol was well adjusted to keep the vast majority of the cells in a viable state during the experiments.

The first parameter we determined was the degree to which the volume of COS-7 cells is related to the reciprocal of the osmolality of non-permeating solutes in which they are suspended. A linear relation in such a BVH plot is indicative of ideal osmotic behavior, and from such plots, as indicated, one can also obtain the non-osmotic volume, V_b. We found this to be the case for cells suspended for 5–7 minutes in anisotonic TBS solutions (made by the addition of defined amounts of sucrose or of water). The mean diameter of the cells in isotonic media was 18.5 μm and the computed mean cell volume V_c,iso was 3324 μm³ at 22°C. The value of V_b at 22°C was 963 μm³, which translates to a fractional volume θ_b of 963/3324 or 0.29. This value of θ_b or b is close to but slightly lower than the values of 0.34 to 0.36 reported for other mammalian fibroblast and epithelial cell lines [14–16].

Next, we determined the hydraulic conductivity L_p, or water permeability of the cells. In this procedure, the cells are mixed with the desired anisotonic solution, loaded into a counting chamber, and permitted to exclude dead or damaged cells from the analysis. This high survival rate confirmed that our protocol was well adjusted to keep the vast majority of the cells in a viable state during the experiments.

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Next, we determined the hydraulic conductivity L_p, or water permeability of the cells. In this procedure, the cells are mixed with the desired anisotonic solution, loaded into a counting chamber,
which in turn is rapidly placed under a microscope, where the cells are photographed at frequent intervals as they shrink or swell. \( L_p \) is calculated from the rate of shrinkage or swelling [9,17–19]. One potential shortcoming with this procedure is that about 35 s elapsed between mixing the cells with the test solution and the acquisition of the first photographic image. Two steps were taken to minimize the loss of data in that time gap and the consequences of that loss. First, to slow the initial volume changes in that time gap, the solutions and the imaging chamber were kept on ice until the latter was placed on or in a microscope stage. We describe in Materials and Methods the temperature excursions the cells experienced in that interval. With this procedure, we see in Fig. 3 that the relative cell volumes in the first image collected during shrinkage were 1.0 at 10° C and 0° C and 0.9 at 22° C, indicating that little or no shrinkage had occurred at this point. In the swelling experiments, the cell volumes in the first image were 1.2 to 1.4 indicating that some, but not an excessive amount of swelling had already occurred. Second, to calculate \( L_p \), we used the integrated version of the water permeability equation. It does not require data from the very early portions of the shrink or swell curves but instead uses cell volume/time pairs from randomly chosen intervals of the dynamic part of the curves. This formula has been used previously to calculate the \( L_p \) of mouse ova [10].

In Fig. 5, we see a striking and highly significant (p <0.001) difference between the \( L_p \) values calculated for water inflow (hypotonic solutions) and water outflow (hypertonic solutions). At 10° C the \( L_p \) values differ by a factor of 8 (see Table 2). By analogy with electrical properties, such differences in water permeability between influx and efflux are referred to as rectification. It has been much more commonly observed in plant cells and algae [20–22] then in mammalian cells. However, rectification was recently reported for transepithelial water flow through confluent mammalian epithelial cells [23], and it has been previously reported in two studies on other mammalian cells. Toupin et al. [24] used a Coulter cell sizer to determine the shrinkage and swelling kinetics of human granulocytes in isotonic solutions. They derived \( L_p \) values of 2.04 \( \mu m/min/atm \) for exo- and 6.05 for endoosmotic flow at 20° C. Another study, by Muldrew et al. [25], also found, by the same Coulter method, that the \( L_p \) values of V79 Chinese hamster fibroblast cells were higher for endo- than for exoosmotic flow, i.e., 2.9–1.1 \( \mu m/min/atm \) versus 1.3–0.4 \( \mu m/min/atm \). Although the ratio of endo to exo is 2 or higher, the authors specifically reject calling it rectification, for reasons we find obscure. Rather, they argue that the difference in \( L_p \) is a consequence of the initial volume of the cells; i.e., the larger the cell, the higher is \( L_p \). But if so, then the value of \( L_p \) should continually change as the cell changes volume in both hypertonic and hypotonic solutions. Available evidence does not support that supposition, e.g., [26]. Furthermore in our study, we found that differences of up to 5-fold in the initial isotonic volumes of individual COS-7 cells had no influence on \( L_p \) (data not shown).

One final example of rectification has been reported in zebrafish embryos by Hagedorn et al. [26]. Interestingly, the direction of the rectification there is opposite what we and the other authors cited above have found in mammalian cells; i.e., in zebrafish, the \( L_p \) for endosmosis is much lower than that for exosmosis. It is attractive to assign an evolutionary explanation for this. Zebrafish are fresh water animals and their oocytes are shed into freshwater and fertilized there. Consequently, there is a strong osmotic force to drive water into the cells. Due to the fact that the \( L_p \) for endosmotic flow is near 0, the rate of that endosmosis is greatly slowed down.

It should be noted that in our experiments the osmolarities of the external media used to determine \( L_p \) differed by a factor of 3.8 (602 mOsm/157 mOsm) in the water efflux and water influx experiments, respectively. The \( L_p \) values differed by a factor of 7–10, depending on the temperature. The question which is responsible for this difference – the direction of water flow or the difference in the external osmolality? House [27] has discussed this question in detail in his monograph on water transport in cells and tissues. In some cases, the apparent rectification is due to the fact that \( L_p \) shows a reciprocal relation to the osmolality of the external medium. That is to say, the lower osmolarities used to assess the \( L_p \) during swelling yield higher values of \( L_p \), than the high osmolarities used to assess \( L_p \) during cell shrinkage. Armitage [28] has published an elegant demonstration that the apparent rectification in human platelets is due entirely to this effect of the external osmolality on \( L_p \). A second class of findings is that the \( L_p \) is essentially independent of the external osmolality and that differences in its value between swelling and shrinkage represent true rectification. The third case is where the two explanations are experimentally confounded. The fourth and perhaps most common case is where \( L_p \) is independent of the direction of water flow and independent, or nearly so, of the osmolarity of the external solution; i.e., there is no evidence of rectification of any sort.

The third case is the situation in our experiments. Armitage’s approach was to subject the platelets to small changes in external osmolality (mostly ranging between 20–40 mOsm/kg). He found that the direction of water flow had no effect on the estimated \( L_p \), but the final osmolality of the external medium had a major effect. However, his method is not applicable to our procedure. He used a photometric method that allowed instantaneous detection of volume changes in thousands of cells at the time of mixing and with a resolution of fractions of a second. We measured the volumes of individual cells with the first image being taken ~35 s after mixing cells and medium. In addition, platelets differ significantly from COS-7 cells in many aspects, such as a 40 fold higher surface area to volume ratio, a 100 to 2000-fold lower initial volume, and a three-fold smaller value of \( \eta_0 \).

One problem we have with the external osmolality hypothesis is why should an effect of osmolality on \( L_p \) be restricted to the external osmolality? Why not an effect from the intracellular osmolality? In our swelling experiments, the external osmolality is fixed at 157 mOsm/kg, but the internal osmolality decreases from 308 mOsm/kg at 0 time to 157 mOsm/kg at equilibrium. In our shrinkage experiments, the external osmolality is fixed at 602 mOsm/kg, but the internal osmolality increases from 308 mOsm/kg at 0 time to 602 mOsm/kg at equilibrium. If such changes in internal osmolality had substantial effects on \( L_p \), then the later the pairs of times chosen for the calculation of \( L_p \) by Eq (2), the greater should have been the difference from the \( L_p \)’s calculated from early pairs of times; however, we could not detect any such effect in our data, in fact it is tantamount to saying that a single value of \( L_p \) appears to hold sway during the entire shrinkage or swelling curve.

There is another way to examine the question of whether a single value of \( L_p \) operates over the whole range of shrinkage and swelling of the COS-7 cells. First, take the mean \( L_p \) values obtained from applying the integrated equation (Eq. 2) to pairs of V and t experimental values and incorporate those \( L_p \)’s into the differential equation that describes the kinetics of water loss or water gain. Second, use that equation to compute the entire kinetic curve. And third, compare those simulated curves with the experimental points. These calculations and comparisons are shown in Fig. 7 for each of the six conditions used. In each graph, the points are the experimental values and the thin solid line is the simulated shrinkage or swelling curve. These simulated curves are
generated by solutions to the differential equation

\[ \frac{dV}{dt} = L_p A R T N \left( \frac{1}{V} - \frac{1}{V_{ref}} \right) \]  

where \( V \) refers to the absolute volumes of water, and the other symbols have the same units and meaning as in Eq. (2). The values of the \( L_p \) used in each condition are given in Table 2. We see that the shapes of the simulated curves appear similar to the experimental but
are shifted both with respect to time and cell volume. The heavy solid curves are the result of manually shifting the simulated curves with respect to X and Y so as to provide the best fit by eye to the experimental points. In almost every case, the shifted simulated curves coincide almost exactly with the experimental points.

In the case of the time axis, the shift is always to the right and amounts to a mean 30.8 s increase in time. In Fig. 7, as well as in Figs. 3 and 4, the value of 0 on the time axis is the time the cells are first mixed with the test solution. In the simulated curve, that mixing is assumed to be instantaneous and complete. Consequently, the cells are assumed to begin shrinking or swelling instantaneously. In the experiments, a measured 25 to 40 seconds elapsed between the initial mixing and the acquisition of the first photographic image.

In the volume axis there are two differences between the experimental and the simulations. The first is that the volume of the cells at 0 time in the simulation is assumed to be the mean measured value of the isotonic cells at each temperature. In the experimental runs, we had no measurement of the volume of a given cell at 0 time. A second difference is that the simulation curves assume that the final, equilibrium, water volume obeys the Boyle-van’t Hoff law, which means that the ratio of the equilibrium water volume to the isotonic water volume (mean = 2626 μm{	extsuperscript{3}}) is equal to the ratio of the isotonic osmolality (0.308) to the osmolality of the test solution (0.157 or 0.602); i.e., 1.96 or 0.512. Or, in absolute terms, the equilibrium volumes of cell water V, after swelling or shrinking are 5147 and 1345 μm{	extsuperscript{3}}. To calculate the equilibrium cell volumes, one must add the V{	extsubscript{C}} of 963 μm{	extsuperscript{3}}. This leads to calculated equilibrium cell volumes of 6110 and 2308 μm{	extsuperscript{3}} for the hypertonic and hypotonic cells, respectively.

In conclusion, we describe here for the first time the osmotic activation energy for the process; namely, the movement of water through aquaporin pores, but the Ea is also very high (12.0 kcal/mol), which opposes the idea of water flow through aquaporin pores. However, in the case of endosmosis, the low Lp values of <0.10 μm/min/atm in combination with an Ea value of >10 kcal/mole clearly suggests bilayer diffusion.

Conclusions about the mechanisms of water transport in COS-7 based on the Ea values must be viewed cautiously. First of all, although we made measurements of Lp at three temperatures, we have concluded that the only valid measurements are those made at 10 and 0°C. The values of Lp at these two temperatures are based on measurements of the volumes of 142 cells, and, therefore, we believe they are accurate. Adding measurements at one or two temperatures between 10 and 0°C might have increased our comfort level in the value of Lp, but it would not have told us whether this value can be extrapolated to subzero temperatures. It is the value below 0°C that is especially important to an analysis of the probability of a cell undergoing intracellular ice formation.

To solve the remaining open questions related to osmotic and permeability matters described here, future work with COS-7 cells might use transfected cells expressing different aquaporins and might examine the influence of cytoskeleton associated proteins, like integrins and cadherins, that may be candidates for transferring osmolality related signals to the membrane [30].
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