Influence of the Partitioning of Osmolytes by the Cytoplasm on the Passive Response of Cells to Osmotic Loading

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ABSTRACT Due to the dense organization of organelles, cytoskeletal elements, and protein complexes that make up the intracellular environment, it is likely that membrane-permeant solutes may be excluded from a fraction of the interstitial space of the cytoplasm via steric restrictions, electrostatic interactions, and other long-range intermolecular forces. This study investigates the hypothesis that the intracellular partitioning of membrane-permeant solutes manifests itself as a partial volume recovery in response to hyperosmotic loading, based on prior theoretical and biomimetic experimental studies. Osmotic loading experiments are performed on immature bovine chondrocytes using culture conditions where regulatory volume responses are shown to be insignificant. Osmotic loading with membrane-permeant glycerol (92 Da) and urea (60 Da) are observed to produce partial volume recoveries consistent with the proposed hypothesis, whereas loading with 1,2-propanediol (76 Da) produces complete volume recovery. Combining these experimental results with the previous theoretical framework produces a measure for the intracellular partition coefficient of each of these solutes. At 1000 mOsm, 1,2-propanediol is the only osmolyte to yield a partition coefficient not statistically different from unity, $\kappa^{p}_{i} = 1.00 \pm 0.02$. For glycerol, the partition coefficient increases with osmolarity from $\kappa^{p}_{i} = 0.48 \pm 0.19$ at 200 mOsm to $\kappa^{p}_{i} = 0.80 \pm 0.07$ at 1000 mOsm; urea exhibits no such dependence, with an average value of $\kappa^{p}_{i} = 0.87 \pm 0.07$ for all osmolarities from 200 to 1000 mOsm. The finding that intracellular partitioning of membrane-permeant solutes manifests itself as a partial volume recovery under osmotic loading offers a simple method for characterizing the partition coefficient. These measurements suggest that significant partitioning may occur even for small membrane-permeant osmolytes. Furthermore, a positive correlation is observed, suggesting that a solute's cytoplasmic partition coefficient increases with increasing hydrophobicity.

INTRODUCTION

It is well known that solutes may not occupy all of the interstices of porous permeable gels and tissues. The concept of molecular partitioning has traditionally been used to explain passive equilibrium concentration differences in various types of media. Originally, a partition coefficient was used to quantify the differential solubility of a solute distributed between two immiscible solvents (1). When the solvents are oil and water, this relation is used to characterize the hydrophobicity of a molecule. Subsequently, the concept of a partition coefficient has been used to quantify concentration limitations in hydrogels because of the steric exclusion from the gel pores (2,3). Since the cytoplasmic environment of cells consists of a dense network of proteins, organelles, and cytoskeletal elements, its role in influencing solute partitioning can be an important consideration. Indeed, this crowded organization is known to play a role in hindering the lateral mobility of even small tracer solutes (4).

However, the role of solute partitioning in the cytoplasm has not been investigated extensively. In a recent theoretical study (5), it has been suggested that partitioning of a membrane-permeant solute by the cytoplasm can manifest itself as a partial volume recovery of the cell in response to a hyperosmotic shock. This theoretical framework, which generalizes the well-known Kedem-Katchalsky equations (6) by incorporating a cytoplasmic partition coefficient κ^{p}_{i} for membrane-permeant solutes, predicts a gradual decrease in equilibrium volume recovery with decreasing κ^{p}_{i}, as shown in Fig. 1. It is interesting that the responses predicted from theory are consistent with prior experimental observations from hyperosmotic loading experiments, as reported by Xu et al. (7) for chondrocytes loaded with glycerol (a cryopreservation agent), and by Levitt and Mlekoday (8) for erythrocytes loaded with urea. However, these observed partial volume recoveries were either overlooked, dismissed as measurement artifacts, or not generally interpreted to result from solute partitioning in the cytoplasm, perhaps because the more common expectation is that the cell's regulatory volume response (9) may be responsible for these observations (10,11).

To explore, experimentally, the potential influence of cytoplasmic partitioning, a biomimetic study was recently conducted that modeled the osmotic loading of spherical alginate hydrogels with dextran solutions (12). This model system precluded any biological volume regulation, while exploring the fundamental influence of solute partitioning in a hydrogel under hyperosmotic loading. Experimental results demonstrated partial volume recoveries for various solute molecular weights and concentrations, in excellent agreement with theory, and with a behavior very similar to the predictions of Fig. 1. Thus, it was experimentally confirmed that the partition coefficient of osmolytes in these gels directly modulates the equilibrium volume response,

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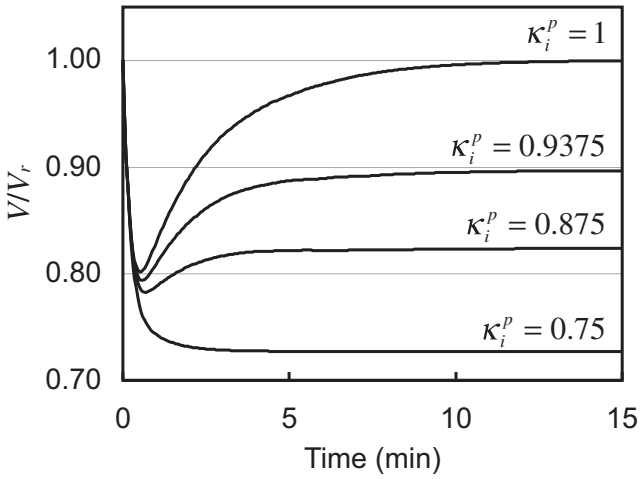

FIGURE 1 Normalized volumetric response of a cell modeled as a gel-filled semipermeable membrane, under osmotic loading with a membrane-permeant solute, using a theoretical framework that accounts for intracellular solute partitioning (5). The volume recovery at steady state decreases with decreasing partition coefficient κ^{P}_{i}.

explaining the occurrence of partial volume recovery through a passive volume exclusion mechanism. This finding suggests that molecular partitioning in a gel like cytoplasm may be similarly responsible for the partial volume recovery observed in cell osmotic loading.

The objective of our study is to test this hypothesis directly by performing hyperosmotic-loading experiments on cells, using membrane-permeant solutes. Here, isolated chondrocytes are used as a model cell since they have been previously shown to exhibit full and partial volume recovery when osmotically loaded with different cryopreservation agents (glycerol and 1,2-propanediol) (7). To extract a measure of the partition coefficient κ^{P}_{i} for these solutes, our prior theoretical framework (Fig. 1) is used to fit the transient volume response of the cell (5). As the governing equations of this framework require knowledge of additional material properties, such as the membrane hydraulic permeability, the membrane solute permeability and reflection coefficient, and the volume fraction of osmotically active water in the cell, a second osmotic loading test is performed on each cell, using a nonpermeating osmolyte, to provide a more complete assessment of these properties.

In response to osmotic perturbations, cells have been shown to attempt to modulate their volume through the activation of ionic cotransporters, exchangers, and nonselective cation channels (9). Through this mechanism, cells subjected to hyperosmotic loading can potentially exhibit a regulatory volume increase (RVI), which may appear qualitatively similar to the passive volumetric recovery exhibited under osmotic loading with a permeating osmolyte (11). It has been demonstrated that isolated chondrocytes may or may not exhibit this regulatory volume change in response to osmotic loading, as influenced by animal species, age, temperature, and other potential factors (13–16). A reduction from physiologic to room temperature has been suggested to inhibit the volume-regulatory capacity of chondrocytes (15) and has been demonstrated to completely abolish RVI in other cell types (17). Therefore, to avoid potential confounding effects of these regulatory processes in the interpretation of our findings, the experiments of this study are performed at room temperature and, additionally, the incidence of RVI is assessed through two independent experiments.

MATERIALS AND METHODS

Experimental design

Three experiments were performed in this study, each performed on a different batch of cells.

Experiment 1 consisted of hyperosmotically loading chondrocytes with sucrose and examining their volume response over 48 min. The objective of this experiment was to ascertain that the cell source and culture conditions employed in this study did not promote significant RVI over the typical duration of osmotic loading experiments when using a membrane-impermeant solute.

Experiment 2 consisted of osmotically loading chondrocytes with NaCl at various hyper- and hypo-osmolarities, and fitting their volume response to the standard Kedem-Katchalsky model to extract the membrane hydraulic permeability and intracellular fraction of osmotically active water. The objectives of this study were to ascertain that RV increase and RV decrease were not significant for this cell source and culture conditions; to confirm prior literature findings that the chondrocyte behaves as a perfect osmometer (7,15,18); and to examine whether the properties extracted from the experimental response remained valid over a broad range of volume changes.

Experiment 3 consisted of osmotically loading chondrocytes in two consecutive tests: Chondrocytes were hyperosmotically loaded with NaCl in the first test, and with a membrane permeant osmolyte in the second test. Three groups of cells were tested in this experiment, corresponding to each of the osmolytes (glycerol, 1,2-propanediol, and urea). The objectives of this experiment were to investigate whether partial or full volume recovery occurred with membrane-permeant osmolytes; to verify that RVI did not occur with NaCl in each tested cell; and to extract the properties of membrane hydraulic permeability and intracellular fraction of osmotically active water from the first test, and membrane solute permeability and reflection coefficient, and intracellular solute partition coefficient from the second test.

Cell isolation and culture

Articular cartilage was sterilely harvested from the wrist joint of freshly killed 4–6-month-old calves obtained from a slaughterhouse. Chondrocytes were isolated from cartilage tissue via enzymatic digestion (390 U/mL collagenase type V; Sigma, St. Louis, MO) for 11 h and plated at a high cell density (400,000 cells/cm^2) in a T-25 tissue culture flask. Cells were cultured in high-glucose Dulbecco's Modified Essential Medium supplemented with 10% fetal bovine serum (Sigma), amino acids (1 × minimum essential amino acids, 1 × nonessential amino acids), buffering agents (10 mM HEPES, 10 mM sodium bicarbonate, 10 mM TES, 10 mM BES), and antibiotics (100 U/mL penicillin, 100 μg/mL streptomycin). After 1–2 weeks after reaching confluence, cells were released from the tissue culture flask using a 0.025% trypsin/EDTA solution and suspended in media at 5,000,000 cells per mL.

Solution preparation

Sodium chloride solutions were prepared in deionized water at concentrations of 598, 400, 317, 254, 210, and 180 mOsm. A stock of culture media with all supplements except for fetal bovine serum was prepared for use as an isotonic reference solution (307 mOsm). A hypertonic solution

(600 mOsm) was prepared by supplementing the isotonic media with NaCl. Solutions of glycerol at 200, 400, 600, and 1000 mOsm, urea at 200, 600, and 1000 mOsm, and 1,2 propanediol at 1000 mOsm and sucrose at 1000 mOsm were also prepared through the addition of these solutes (Sigma) to isotonic media. These solutions were also supplemented with a small amount of additional NaCl to maintain their ionic concentrations. For culture media and NaCl solutions, osmolarities were measured with a Depression Point Osmometer (Model 3D; Advanced Instruments, Needham Heights, MA).

Testing apparatus

Osmotic loading of cells was achieved through the isolation of cells in a custom-made polydimethylsiloxane microfluidic device as previously described (19). A replica-molded polydimethylsiloxane template was sealed over a poly-L-lysine treated (2 μg/mL) sterile glass coverslip, creating a 1-cm-long Y-shaped flow channel of rectangular cross section (300-μm wide $\times$ 100-μm high). The channels were connected to two open reservoirs (ø4 mm) at the upstream end and one reservoir at the downstream end. For each test, chondrocytes were initially introduced to the flow channel through the addition of 120 μL of cell suspension. The channel was then incubated at 37°C for 30 min to allow cell adhesion to the poly-L-lysine-coated coverslip. Flow was induced through a hydrostatic pressure gradient after the addition of 80 μL of solution to each upstream reservoir and the continuous draining of the downstream reservoir through a syringe needle attached to a peristaltic pump (Varistaltic model; Vera, Barnant, OR). Upstream solution levels were maintained throughout each experiment. All experiments were conducted at room temperature (~21°C).

Image analysis

Throughout each test, cells were viewed through the glass coverslip with an inverted microscope (model No. CK40; Olympus, Melville, NY) with 100$\times$ oil objective, which focused on the cell's midplane as indicated by a sharply defined perimeter. Images were acquired at regular time intervals with an ExwaveHAD camera (Sony, Tokyo, Japan) and a computer-based data acquisition system (640 $\times$ 640 pixels, 0.1377 μm/pixel, METAVIEW, ver. 4.1; www.cochrane.org). Images were thresholded (Image J, National Institutes of Health, Bethesda, MD) and each captured cell's cross-sectional pixel area was subsequently determined with a customized routine (MATLAB, ver. 7; The MathWorks, Natick, MA) and used to calculate its volume under the assumption of a spherical geometry.

Analysis of volume response

The volume response of cells to osmotic loading was analyzed using equations formulated in the mixture theory framework (5). According to this model, the ordinary differential equations governing the time-dependent cell volume response $V(t)$ and the intracellular number of moles of membrane-permeant solute $n^{p}{}_{i}(t)$ are

$$\frac{dV}{dt} = AL_pR\theta\left[c_i^p - c_e^p + c_i^n - c_e^n - (1-\sigma^p)\left(\frac{c_i^p}{\kappa_i^p} - c_e^p\right)\right], \quad (1)$$

$$\frac{dn_i^p}{dt} = -AP^p\left(\frac{c_i^p}{\kappa_i^p} - c_e^p\right) + (1-\sigma^p)\frac{1}{2}\left(\frac{c_i^p}{\kappa_i^p} + c_e^p\right)\frac{dV}{dt}, \quad (2)$$

where A is the volume-dependent cell surface area ($A = 3\ V/a$, where a = cell radius, assuming that the cell is spherical), L_p is the membrane hydraulic permeability, R is the universal gas constant, θ is the absolute temperature, $c^{n}{}_{e}$ is the prescribed external solution osmolarity of a nonpermeating osmolyte, $c^{n}{}_{i}$ is the intracellular osmolarity of membrane-impermeant solutes, $c^{p}{}_{e}$ is the prescribed external osmolarity of a permeating osmolyte, $c^{p}{}_{i}$ is the intracellular osmolarity of that osmolyte, P^p is the corresponding solute membrane permeability, σ^p is its Staverman reflection coefficient, and $\kappa^{p}{}_{i}$ is its intracellular partition coefficient.

The intracellular number of moles $n^{\alpha}{}_{i}$ of the nonpermeating ($\alpha = n$) and permeating ($\alpha = p$) solutes is related to the corresponding intracellular osmolarity $c^{\alpha}{}_{i}$ via

$$c_i^\alpha = n_i^\alpha/\varphi_i^w V, \quad (3)$$

where $\varphi^{w}{}_{i}$ is the volume fraction of osmotically active water in the cytoplasm. As the cell shrinks or expands, this volume fraction changes as water leaves or enters the cell; according to the principle of conservation mass,

$$\varphi_i^w = 1 - \left(1 - \varphi_{ir}^w\right)\frac{V_r}{V}, \quad (4)$$

where the subscript r denotes the reference configuration (the resting condition, typically under isotonic conditions, before the first osmotic loading test). For intracellular membrane-impermeant solutes, $n^{n}{}_{i} = n^{n}{}_{ir}$ is constant in the absence of volume regulatory responses (which may otherwise bind or release such solutes from intracellular substrates).

The steady-state response to osmotic loading may be deduced from these equations in the limit when $dV/dt \to 0$ and $dn^{p}{}_{i}/dt \to 0$, in which case the intracellular and extracellular osmolarities must match, $c^{p}{}_{i} + c^{n}{}_{i} = c^{p}{}_{e} + c^{n}{}_{e}$, and the intracellular osmolarity of the membrane-permeant solute is partitioned relative to the external environment according to $c^{p}{}_{i} = \kappa^{p}{}_{i}\ c^{p}{}_{e}$. In the special case when the steady-state response corresponds to the initial reference configuration, where the cell has not yet been loaded with a membrane-permeant solute, we may set $c^{p}{}_{er} = 0$, which produces $c^{p}{}_{ir} = 0$ and $c^{n}{}_{ir} = c^{n}{}_{er}$, and thus $n^{p}{}_{ir} = 0$ and $n^{n}{}_{ir} = \varphi^{w}{}_{ir}V_r c^{n}{}_{ir}$ according to Eq. 3. These relations, along with $V = V_r$, establish the initial conditions for solving the system of differential equations in Eqs. 1 and 2, complemented by the constraints of Eqs. 3 and 4.

Using these relations, it is also possible to deduce that the general steady-state response for the cell volume is given by

$$\frac{V_\infty}{V_r} = 1 - \varphi_{ir}^w + \frac{\varphi_{ir}^w c_{er}^n}{c_e^n + (1-\kappa_i^p)c_e^p}. \quad (5)$$

The osmolarities appearing in this equation represent extracellular quantities only, in the reference configuration ($c^{n}{}_{er}$, which is the osmolarity of the reference bath) and the osmotically loaded configuration ($c^{e}{}_{n}$ and $c^{e}{}_{p}$). This relation is very informative because it encompasses a number of important concepts relevant to this study: First, it reproduces the Boyle-van 't Hoff relation for a perfect osmometer in the case when osmotic loading is performed only with a nonpermeating solute ($c^{p}{}_{e} = 0$); second, it predicts full volume recovery when loading with a permeating solute ($c^{p}{}_{e} \neq 0$, $c^{n}{}_{e} = c^{n}{}_{er}$), which is not partitioned by the cytoplasm ($\kappa^{p}{}_{i} = 1$); and finally, it predicts partial volume recovery when loading with a permeating solute that is partitioned by the cytoplasm ($0 < \kappa^{p}{}_{i} < 1$). The latter concept is the main subject of this study.

In addition, note that Eqs. 1 and 2 reduce to the classical formulation of Kedem and Katchalsky (6) in the limit when $\kappa^{p}{}_{i} = 1$. When osmotically loading with a nonpermeating solute only ($c^{p}{}_{e} = 0$), Eq. 2 is not needed and the only material parameters remaining in the governing equations are L_p and $\varphi^{w}{}_{ir}$. Therefore, osmotic loading of a cell with a nonpermeating solute can be used to characterize these two material parameters. If, subsequently, the cell is osmotically loaded with a permeating solute, the more general system of equations may be used to obtain the remaining material parameters, P^p, σ^p, and $\kappa^{p}{}_{i}$. In principle, if steady-state is achieved experimentally, $\kappa^{p}{}_{i}$ may also be extracted from the measured partial volume recovery V_∞/V_r using Eq. 5, with the prior knowledge of $\varphi^{w}{}_{ir}$; nevertheless, the values of $\kappa^{p}{}_{i}$ reported below were obtained from fitting the experimental transient response of $V(t)$ to the solution of the differential equations, since steady state was not achieved in some cases.

Testing protocols

Experiment 1

Chondrocytes ($n = 6$) were initially exposed and equilibrated in the isotonic reference solution for 5 min, and then subjected to hyperosmotic loading with the 1000 mOsm sucrose solution for 48 min. The volume response was monitored over this entire duration.

Experiment 2

Chondrocytes ($n = 8$) were initially exposed and equilibrated in $c^{n}_{er} = 317$ mOsm NaCl for 5 min and subsequently exposed to solutions of $c^{n}_{e} = 598$, 400, 317, 254, 210, and 180 mOsm for ~5 min each. The transient volume response was fitted to Eq. 1 with $c^{p}_{i} = c^{p}_{e} = 0$ to extract the material parameters L_p and φ^{w}_{ir}.

Experiment 3

Cells were initially exposed and equilibrated in the isotonic reference solution for 5 min. The cells were then subjected to a hypertonic solution of NaCl-supplemented media for 4 min ($c^{n}_{e} = 600$ mOsm). After this period, cells were reexposed to the isotonic reference solution for an additional 4 min, allowing their volume to return to its initial state. The second osmotic test consisted of loading with a solution of glycerol at $c^{p}_{e} = 200$, 400, 600, or 1000 mOsm, urea at $c^{p}_{e} = 200$, 600, 1000 mOsm, or 1,2 propanediol at $c^{p}_{e} = 1000$ mOsm. The number of cells tested for each combination of osmolyte and osmolarity is presented in Table 1. The transient volume response from the first test was fitted to Eq. 1 with $c^{p}_{i} = c^{p}_{e} = 0$ to extract the material parameters L_p and φ^{w}_{ir}. The response from the second test was fitted to Eqs. 1 and 2, with $c^{p}_{er} = 0$ and $c^{n}_{er} = c^{n}_{ir} = 307$ mOsm, to extract the material parameters P^p, σ^p, and κ^{p}_{i}.

Statistical analysis

Two-way analysis of variance was used to determine differences in the values of κ^{p}_{i}, σ^p, and P^p in the eight groups of cells tested with a permeating osmolyte (glycerol at 200, 400, 600, 1000 mOsm, urea at 200, 600, 1000 mOsm, and 1,2-propanediol at 1000 mOsm), with $\alpha = 0.05$ and statistical significance set at $p < 0.05$. Tukey's post-hoc test was used to detect differences in the means. A one-tailed test using a T-statistic was performed to determine whether the equilibrium volumes (V/V_r) and partition coefficients (κ^{p}_{i}) for all permeating osmolyte groups were less than unity.

RESULTS

Experiment 1

Chondrocytes subjected to hyperosmotic loading with 1000 mOsm sucrose exhibited a monotonic decrease in volume, equilibrating in <1 min (Fig. 2). Subsequently, the cell volume remained nearly constant, demonstrating <5% decrease over the next 48 min.

Experiment 2

When subjected to the multiple-step NaCl solution changes, chondrocytes exhibited a monotonic volume change, achieving a near-steady-state value at the end of each step (Fig. 3 *A*). Curve-fitting of the transient response to the theoretical equations produced $L_p = (6.84 \pm 2.23) \times 10^{-14}$ m^3/N·s and $\varphi^{w}_{ir} = 0.73 \pm 0.06$, with the quality of the fit provided by the coefficient of determination $R^2 = 0.993 \pm 0.004$. The chondrocytes exhibited the behavior of a perfect osmometer (Eq. 5 with $c^{p}_{e} = 0$), as illustrated in Fig. 3 *B*.

Experiment 3

Representative experimental results and theoretical curve-fits are provided for NaCl in Fig. 4, 1,2-propanediol in Fig. 5, glycerol in Fig. 6, and urea in Fig. 7. Material coefficients extracted from the theoretical curve-fits are summarized in Table 1, along with the coefficients of determination for the quality of the fits.

Cells loaded with all concentrations of glycerol (Fig. 6) and urea (Fig. 7) exhibited only partial volume recovery, returning to a normalized volume V/V_r significantly less than unity ($p < 0.005$), and producing partition coefficients also less than unity ($p < 0.005$, Table 1). At 1000 mOsm, 1,2-propanediol was the only osmolyte to yield a partition coefficient not statistically different from unity, $\kappa^{p}_{i} = 1.00 \pm 0.02$. For glycerol, the partition coefficient linearly increased with concentration ($R^2 = 0.63$, Fig. 8) from $\kappa^{p}_{i} = 0.48 \pm 0.19$ to $\kappa^{p}_{i} = 0.80 \pm 0.07$ while urea exhibited no such concentration dependence, with an average value of $\kappa^{p}_{i} = 0.87 \pm 0.07$ for all concentrations. Staverman's reflection coefficient σ^p yielded values very close to unity for glycerol at 200 and 400 mOsm and significantly decreased at higher concentrations ($p < 0.05$, Table 1). The reflection coefficient for urea showed no such concentration dependence. The solute membrane permeability P^p was significantly higher for 1,2-propanediol than glycerol and urea ($p < 0.005$) and

TABLE 1 Curve-fitted material parameters from Experiment 3

		Nonpermeating osmolyte curve-fit			Permeating osmolyte curve-fit			
Osmolyte	Osmolarity (mOsm)	Hydraulic permeability L_p (10^{-14} m^3/N•s)	Water content φ^{w}_{ir}	R^2	Solute permeability P^p (10^{-10} m/s)	Reflection coefficient σ	Partition coefficient κ^{p}_{i}	R^2
1,2 Propanediol	1000 ($n = 8$)	3.06 ± 1.35	0.55 ± 0.08	0.98 ± 0.01	843 ± 334	0.78 ± 0.15	1.00 ± 0.02	0.94 ± 0.04
	200 ($n = 7$)	2.47 ± 0.57	0.67 ± 0.06	0.99 ± 0.01	28.6 ± 14.4	0.82 ± 0.11	0.86 ± 0.09	0.93 ± 0.04
Urea	600 ($n = 8$)	3.14 ± 0.81	0.65 ± 0.08	0.98 ± 0.01	26.7 ± 15.7	0.77 ± 0.18	0.90 ± 0.02	0.95 ± 0.02
	1000 ($n = 8$)	3.16 ± 0.79	0.63 ± 0.06	0.98 ± 0.02	21.1 ± 12.9	0.75 ± 0.25	0.84 ± 0.07	0.93 ± 0.04
	200 ($n = 5$)	4.76 ± 1.94	0.58 ± 0.07	0.97 ± 0.03	5.38 ± 4.82	1.00 ± 0.00	0.48 ± 0.19	0.89 ± 0.05
	400 ($n = 5$)	4.40 ± 1.61	0.59 ± 0.06	0.99 ± 0.01	5.01 ± 3.11	1.00 ± 0.00	0.59 ± 0.11	0.93 ± 0.05
Glycerol	600 ($n = 5$)	4.36 ± 1.08	0.66 ± 0.08	0.99 ± 0.01	8.50 ± 3.58	0.85 ± 0.19	0.67 ± 0.04	0.92 ± 0.07
	1000 ($n = 7$)	5.50 ± 1.39	0.66 ± 0.06	0.99 ± 0.01	10.6 ± 2.83	0.59 ± 0.25	0.83 ± 0.06	0.94 ± 0.03

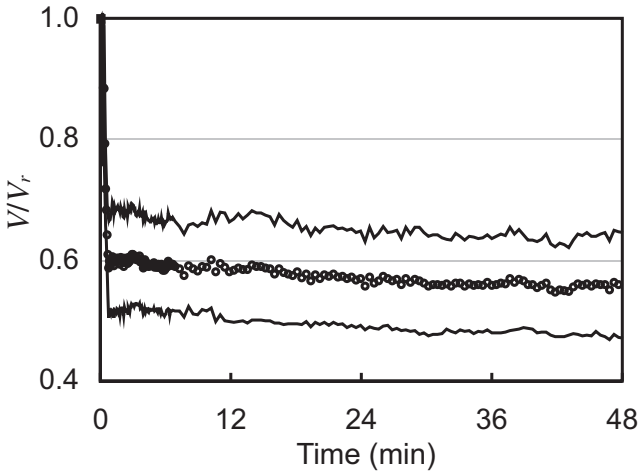

FIGURE 2 Mean and standard deviation of the normalized volumetric response of chondrocytes to osmotic loading with sucrose (Experiment 1).

exhibited an increasing trend when plotted against the partition coefficient on a semi-log scale (Fig. 9).

DISCUSSION

The objective of this study was to test the hypothesis that the intracellular partitioning of membrane-permeant solutes manifests itself as a partial volume recovery in response to hyperosmotic loading. A strong foundation for this hypothesis had been set forth in our previous theoretical study (5), as summarized in Fig. 1 and Eq. 5, and confirmed experimentally in a biomimetic study using osmotic loading of alginate beads with dextran (12). The experimental aim of this study was to investigate whether partial volume recovery could be observed in cells loaded with various permeating osmolytes, under conditions where the alternative mechanism of cell volume regulation would not produce a confounding interpretation. A measure of the intracellular solute partition coefficient κ^{P}_{i} was also obtained, by combining experimental volume measurements with the theoretical framework. Chondrocytes and cryopreservation agents (glycerol and 1,2-propanediol) or urea were used as a model system to test this hypothesis, since prior studies (7,8) had suggested these combinations might exhibit partial volume recoveries. No particular insight specific to chondrocytes and their in situ function is sought from our study, whose primary focus is on the concept of intracellular solute partitioning in general. The fact that partial volume recovery has been reported in erythrocytes loaded with urea (8) suggests that our results are not specific to chondrocytes.

Several tests were used to ascertain that regulatory volume changes did not contribute significantly to the measured cell volume responses of this study. Experiment 1 demonstrated that sustained hyperosmotic loading with sucrose, a membrane-impermeant solute, did not produce noticeable RVI for at least 50 min (Fig. 2). Experiment 2 demonstrated that hyperosmotic and hypo-osmotic loading with NaCl produced monotonic changes in volume, reaching a near-steady-state value at each step, over a time span of nearly 30 min (Fig. 3 *A*); the steady-state volume response at various osmolarities demonstrates a straight-line behavior consistent with the Boyle-van 't Hoff relation for a perfect osmometer (Fig. 3 *B*), which is expected only if regulatory volume changes are negligible. These results are also consistent with the treatment of NaCl as a membrane-impermeant osmolyte, under the understanding that ion leakage through channels is compensated by pumping mechanisms to produce no net transport of NaCl into or out of the cell (9).

Since Experiment 3 included osmotic loading with NaCl for each tested cell, the absence of RVI and the steady volume response observed consistently in all cells (Fig. 4) provided further confirmation that regulatory volume changes were not significant for this cell source and culture conditions used here. Based on these findings, it was assumed that regulatory volume changes would also be negligible when loading with the various permeating osmolytes used in this study. To the best of our knowledge, there is no evidence in the literature that osmotic loading with glycerol, 1,2-propanediol, or urea might specifically trigger regulatory volume responses when such responses are not significant with NaCl or sucrose. Furthermore, regulatory volume changes, had they been triggered with permeating osmolytes, would presumably have returned the cell closer to its original volume rather than keeping the steady-state volume depressed as observed with glycerol (Fig. 6) and urea (Fig. 7); therefore the observation of partial volume recovery after osmotic loading with these solutes is entirely

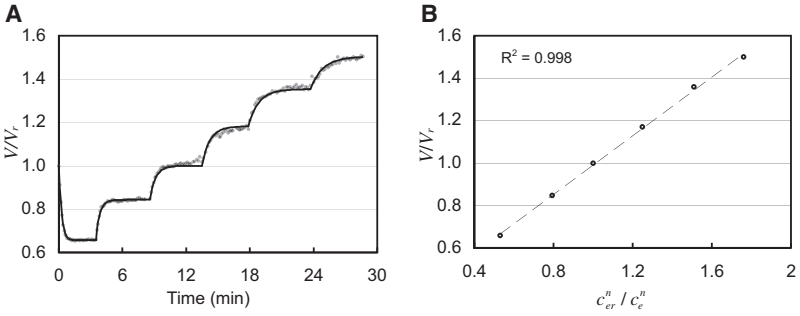

FIGURE 3 (*A*) Representative normalized volumetric response of an isolated chondrocyte to NaCl step osmotic solution changes with theoretical curve-fit of Kedem-Katchalsky equations for a nonpermeating osmolyte. (*B*) The steady-state response at the end of each step obeys the Boyle-van 't Hoff relation for a perfect osmometer (Experiment 2).

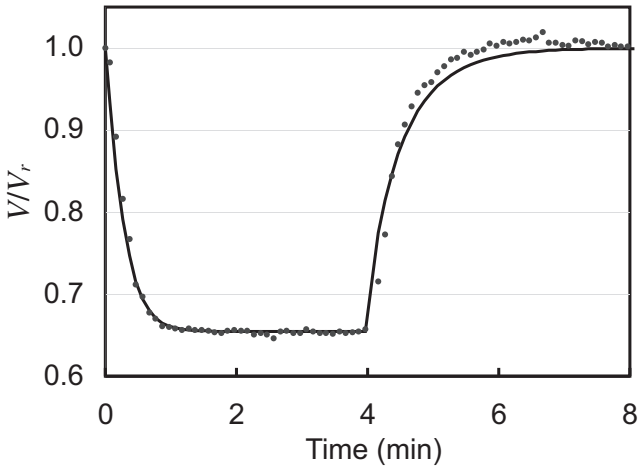

FIGURE 4 Representative normalized volumetric responses of chondrocytes osmotically loaded with 600 mOsm hypertonic culture media, followed by isotonic recovery. The solid curve represents the curve-fit of this response using the Kedem-Katchalsky equations for nonpermeating solutes (Experiment 3).

consistent with the mechanism of intracellular solute partitioning.

Consequently, the results of this study are in strong support of the proposed hypothesis, and the measures of κ^{P}_{i} reported in Table 1 and Fig. 8 demonstrate that meaningful values of the intracellular partition coefficient of membrane-permeant solutes may be obtained easily from osmotic loading experiments. In general, due to the dense organization of organelles, cytoskeletal elements, and protein complexes that make up the intracellular environment, the mechanism behind this partitioning effect is likely to be the exclusion of solutes from some of the interstitial space of the cytoplasm via steric restrictions, electrostatic interactions, and other long-range intermolecular forces, as

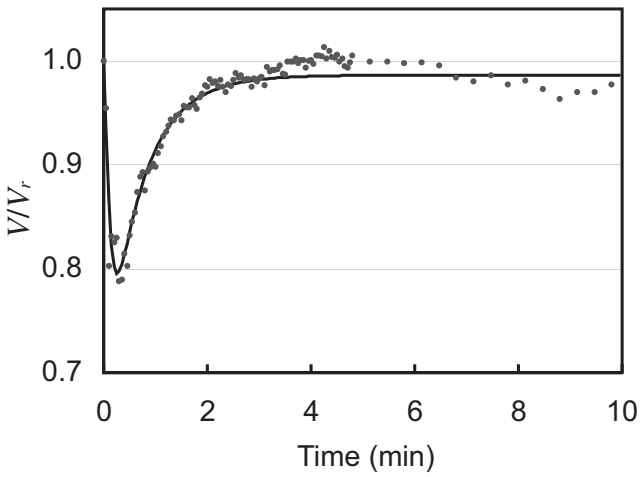

FIGURE 5 Representative normalized volumetric response of chondrocyte osmotically loaded with 1,2-propanediol, with theoretical curve-fit using equations described in the text (Experiment 3).

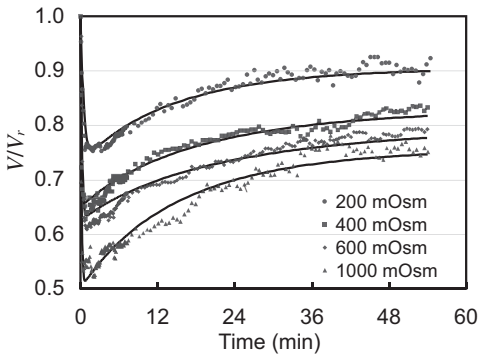

FIGURE 6 Representative normalized volumetric responses of chondrocyte osmotically loaded with glycerol, with theoretical curve-fits (Experiment 3).

commonly reported in porous gels and tissues (20). The concept of solute partitioning in a porous structure implies that some interstices are too small, or induce too much electrostatic repulsion, to accommodate the solute, whereas others provide sufficient space and negligible repulsive forces; the fraction of the interstitial space accessible to the membrane-permeant solute is given by κ^{P}_{i} (21).

In general, solute partitioning does not imply that the interstitial space has been saturated with the solute in a manner preventing further increases in intracellular concentration (although this does represent a limiting condition under sufficiently high concentrations); doubling of the extracellular concentration of solute may indeed lead to doubling of the interstitial concentration, with the understanding that the additional solutes will similarly occupy those interstices that can accommodate them. The physicochemical explanation behind this concept is that, at equilibrium, the solute electrochemical potential is in balance between the extracellular solution and the intracellular interstitial space accessible to the solute; there cannot be solute electrochemical balance with the interstitial space that is inaccessible to the solute.

Although this is the first study to place partial cytoplasmic exclusion of solutes in the context of osmotic loading, the

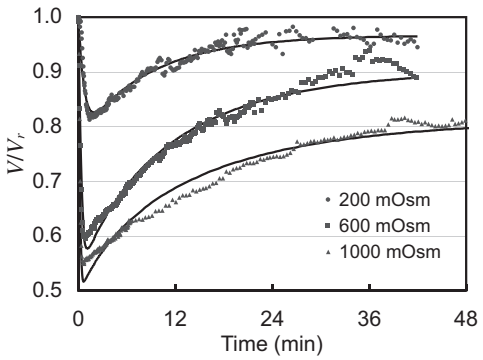

FIGURE 7 Representative normalized volumetric responses of chondrocyte osmotically loaded with urea, with theoretical curve-fits (Experiment 3).

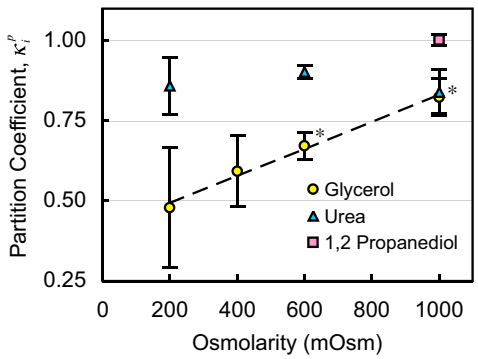

FIGURE 8 Measured cytoplasmic partition coefficient of permeating osmolytes in chondrocytes. For glycerol, $*p < 0.05$ indicates a significant increase above κ^{P}_{i} at 200 mOsm (Experiment 3).

occurrence of cellular partitioning has been alluded to in the prior literature. In particular, actin-rich cellular domains have been suggested to act as a molecular sieve, partitioning solutes from subdomains of the cytoplasm (22). At high concentrations, certain osmolytes have been found to be excluded, preferentially, from the immediate domain of a protein due to unfavorable interactions with its surface residues (23). Osmoprotectants have been shown to stabilize a protein's configuration and maintain their enzymatic activity, thus providing a basis for their use as osmoregulators in yeast cells (24). Under a variety of conditions, glycerol has been shown to act in this fashion, being preferentially excluded from the domain of tubulin (25) and several globular proteins (23). It is likely that these composite interactions with many types of cellular proteins can contribute to the cellular partitioning effect observed in this study. However, the value of κ^{P}_{i} reported in this study represents an average of the partition coefficient over the entire intracellular space, with no particular attribution to specific subcellular domains.

For glycerol, the partition coefficient in the cytoplasm increased nearly linearly with concentration, as shown in Fig. 8. This finding implies that at higher concentrations, glycerol molecules are able to occupy a greater fraction of the available cytoplasmic interstitial space. In a more general context, the dependence of the partition coefficient on concentration has been previously reported for polystyrene in finely porous glass and was originally attributed to conformational changes of linear macromolecules at high concentrations (26). However, this behavior has also been demonstrated with globular protein molecules such as serum albumin (27), which are more representative of the relatively fixed morphology of glycerol. One potential explanation for the increase in κ^{P}_{i} is that glycerol may possess a decreased hydration sphere at higher concentrations, as alluded to in previous studies (28).

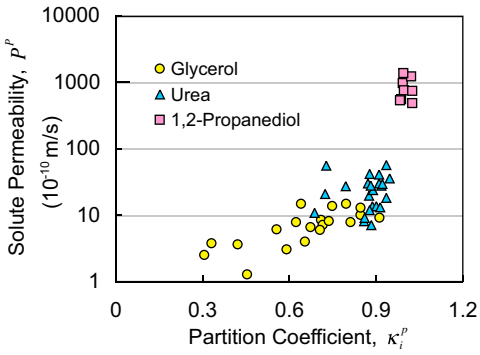

FIGURE 9 Solute permeability versus cytoplasmic partition coefficient for all tested cells (Experiment 3).

The permeability P^{P} is a measure of the diffusivity of the solute across the membrane (5,29). Solute permeabilities P^{P} measured in this study vary over two orders of magnitude (Table 1 and Fig. 9), despite the three osmolytes possessing similar molecular weights (92 Da for glycerol, 60 Da for urea, and 76 Da for 1,2-propanediol). However, independent diffusion measurements on planar lipid bilayers have also reported values of this magnitude ($P^{P} = 5.4 \times 10^{-10}$ m/s for glycerol and $P^{P} = 2.8 \times 10^{-8}$ m/s for 1,2-propanediol (30)). Traditionally, it has been believed that the membrane permeability of small nonelectrolytes is governed by their lipid solubility (31,32), as stated by the Overton Rule (33). This solubility is typically measured by a molecule's octanol-water partition coefficient as characterized by its hydrophobicity. The octanol-water partition coefficient values for 1,2-propanediol, urea, and glycerol, are, respectively, 0.13, 0.026, and 0.011 (34), which, as expected, also correlate with the membrane permeabilities for these osmolytes.

An interesting observation regarding the partitioning mechanism may relate to interactions with hydrophobic molecular domains in the cytoplasm, as suggested by the observed positive correlation between the solute membrane permeability, P^{P} and the intracellular partition coefficient, κ^{P}_{i} (Fig. 9). In addition to 1,2-propanediol permeating the membrane much faster than the other two osmolytes, it is also the only osmolyte not to be partitioned among the three tested. Similarly, for the other osmolytes, a lower membrane permeability correlated with a lower partition coefficient. This observation suggests that the two behaviors may be mechanistically related.

In summary, this experimental study proposes that the partial volume recovery of cells to osmotic loading with permeating osmolytes may be explained substantially by the passive mechanism of solute partitioning in the cytoplasm. This mechanism was previously suggested from theory and verified in a biomimetic experiment, and our study provides direct experimental evidence in support of it. There are at least two practical implications of this result. First, the observation of cell partial volume recovery in response to hyperosmotic loading with a membrane-permeant solute need not be automatically attributed to active cell volume regulation mechanisms, since the familiar mechanism of solute partitioning may explain this response; and

second, measurements of the partial volume recovery may be used to extract the intracellular solute partition coefficient, yielding a potentially useful property from a relatively simple experiment. These findings bring new insights into our understanding of the cell's response to its osmotic environment.

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