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CELL VOLUME REGULATION STUDIES WITH THE ELECTRON MICROPROBE

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Abstract

Lumbricals from the hind feet of young rats are dissected free, stretched to approximately 125% of resting length, and mounted on individual, simple plastic forms. After recovery in physiological saline, the isolated muscles are incubated for periods of 40 to 60 min. in one of a series of hypertonic bathing solutions. The composition of each bathing solution is identical, except for osmolality which is increased with lactose. At least one muscle from each animal is incubated in a control solution to serve as a control muscle for that particular set of 5 to 8 muscles. Mounted muscles are removed from the bathing solutions and quickly plunged into chilled liquid propane. Tissue is freeze-dried at low temperature, fixed with osmium tetroxide vapor, and embedded in brominated EPON 826. Dilution of the embedding medium by tissue solids, assessed by reduction of the embedding plastic Br L_α signal, is used to establish intracellular hydration; or conversely, intracellular solids fractions. The Br L_α signal is monitored along with S K_α, K K_α, Cl K_α, Na K_α, and the continuum region from 4.2 to 7.2 keV using a simultaneous ED-WD spectrometer electron probe microanalyzer. This provides sufficient information to present intracellular concentrations in units of mmol/kg wet weight, mmol/kg water, or mmol/kg dry weight. Cell volume change relative to a suitable control is determined from the ratio of sulfur signals from the experimental and control cells. The response of cells to hypertonic challenge is assessed by comparing actual cell volume change with the change expected of a simple osmotic bag. Simultaneously, the intracellular electrolyte data provides information on the mechanism of cellular response to osmotic shifts.

Key words: Cell volume regulation, electron probe microanalysis, incubated tissue, regulatory volume increase, rat skeletal muscle, lumbricals, intracellular water, intracellular electrolytes, cell volume change

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Introduction

Many types of animal soft tissue cells have intrinsic properties that permit them to maintain cell volume approximately uniform when presented with an osmotic challenge (2,3,6). Cells exposed to a hypertonic bathing medium shrink rapidly and then undergo a regulatory volume increase returning cell volume toward normal. The precise mechanism for these regulatory maneuvers is unknown, but an energy dependent Na^+ - H^+ exchange mechanism is often implicated in cell volume recovery (4). Studies of the time course of the regulatory volume changes reveal that recovery can be rapid, with times of a few minutes being recorded in some experiments (15).

Cell volume regulation studies require measurement of parameters not directly available with conventional methods. It is necessary that accurate assessment be made of cell volume change, cellular water volume, cellular solids content and intracellular electrolyte concentrations. While these parameters can be computed only indirectly with conventional methods, they can be measured more directly in individual cells with electron probe microanalysis. The following presents methods that have proven useful for cell volume regulation studies using the freeze-dried, plastic embedded tissue preparation (12). Although these methods would be applicable for frozen hydrated tissue as well, they would be most inappropriate for dried, unembedded tissue.

Osmotic experiments are performed on isolated lumbricals from the hind feet of young rats. In the work described here, intact muscles are incubated in one of a series of hypertonic physiological solutions. The lumbricals are of a uniform fiber type, and because of their size, provide an almost ideal electron microprobe sample. They are less than one mm across and approximately 8 mm long, permitting excellent perfusion and cryofixation. In addition, it is possible to obtain up to 8 complete muscles from each animal, although often the number is closer to 5 or 6. The large number of identical muscles simplifies the assumptions concerning controls, in that one or two of the group make excellent controls for the other muscles. This paper will provide details of the methods that have proven useful for cell volume regulation studies with electron probe microanalysis, and will include typical data.

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TISSUE HYDRATION MEASUREMENT WITH BROMINATED PLASTIC

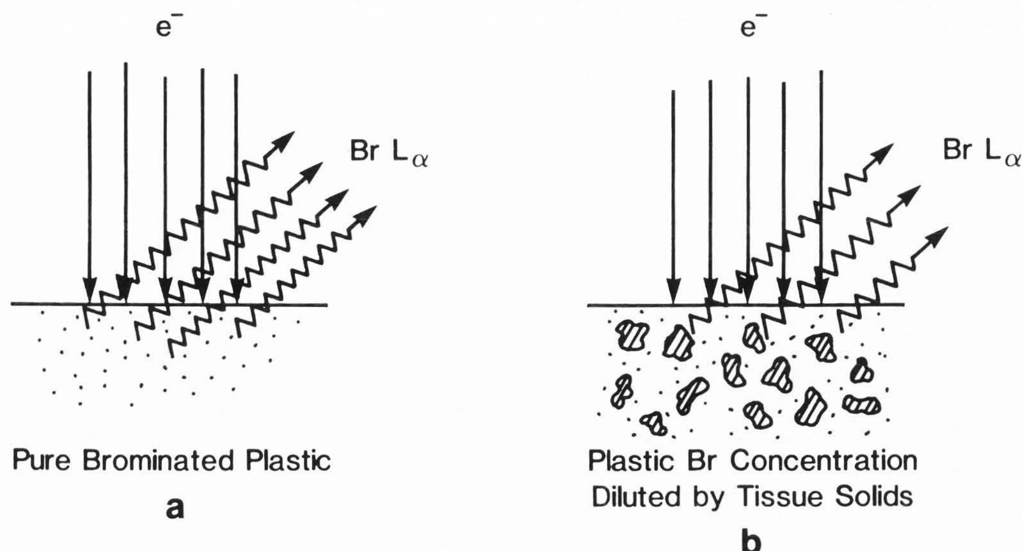

Figure 1. Conceptual diagram of the bromine dilution method for estimation of intracellular water with the freeze-dried, plastic embedded tissue preparation. The Br L_α signal from embedding plastic, EPON 826 complexed with dibromoacetophenone, is diminished when tissue solids are present. Electrons incident on embedding plastic generate characteristic x-rays from endogenous Br, (a). When tissue solids are present, (b), the characteristic Br x-ray signal is reduced because tissue solids dilute the embedding medium. The ratio of Br L_α signal with and without tissue solids has been calibrated to provide a measure of intracellular water concentration.

Materials and Methods

Lumbricals were dissected free from anesthetized, 250 g, Sprague-Dawley rats, stretched to approximately 125% of resting length, and mounted on individual plastic forms with color coded thread. Muscles recovered from the dissection for an hour at 37°C, in a normal bathing solution bubbled with 95% O_2, 5% CO_2 gas. At the end of the recovery period, all muscles were removed and distributed among the various experimental bathing solutions. These solutions were identical in composition, with the exception that osmolality was adjusted with lactose, Table 1. Scintillation vials placed in a temperature controlled water bath provided an efficient means of simultaneously incubating a variety of small muscles. After a 40 min. incubation in the oxygenated, 37°C experimental solutions, the muscles were removed from the bathing solutions and without blotting, quickly plunged into chilled propane.

The tissue was freeze-dried at a temperature near -80°C, fixed with osmium tetroxide vapor, and embedded in EPON 826 containing bromine (11). The embedded tissue was sectioned with a glass knife to expose the muscle in cross-section, and the exposed tissue block face was mounted along with pure-crystal secondary standards for thick sample analysis with the electron microprobe (12). An adjacent section was collected and stained for light microscopy.

The mounted tissue blocks were overcoated with approximately 20 nm carbon and analyzed with a focused 10 keV, 50 nA electron beam in a model SEMQ, ARL (Applied Research Laboratories, Sunland, CA) fully automated, four spectrometer electron probe microanalyzer fitted with a KEVEX model 7000 Si(Li) energy detector system (9). Simultaneously, S K_α and Cl K_α were measured with PET (pentaerythritol) diffraction crystals, Na K_α with RAP (rubidium acid phthalate), Br L_α with TAP (thallium acid phthalate), and K K_α and the continuum region from 4.2 to 7.2 keV were measured with the Si(Li) energy detector.

Intracellular Water Measurement

Dibromoacetophenone is complexed with EPON 826 to provide an embedding plastic with uniform distribution of approximately 80 mmol kg^{-1} Br (10). Tissue solids dilute the embedding plastic in freeze-dried, plastic embedded tissue, resulting in a reduction of the measured Br L_α x-ray signal. This reduction in signal is proportional to the cell solids concentration, Fig. 1, provided that the embedding plastic faithfully replaces water of the hydrated cell. In practice, the Br L_α signal is measured simultaneously with the other characteristic x-rays during electron probe microanalysis, and the dilution of embedding plastic by tissue solids is used to estimate water

Table 1. Incubation solutions for osmotic experiments using isolated rat skeletal muscle. All units are in mmol/l.

	mOsM/L	K^+	Cl^-	Na^+	Ca^{++}	Mg^{++}	$HCO_3^=$	$HPO_4^=$	ACET	GLU	ALA	LEUC	LACT
1R	309	4.6	114	147	1.8	1	25	2.3	8	8	5	5	5
1.13R	350	4.5	114	144	1.8	1	25	2.3	8	8	5	5	45
1.22R	376	4.5	114	145	1.8	1	25	2.3	8	8	5	5	76
1.31R	405	4.5	114	145	1.8	1	25	2.3	8	8	5	5	100

content at each point of analysis within the cell. Complete drying of tissue is necessary for this method of estimating tissue water, and reproducible, accurate assessment of tissue water appears to be possible when a final drying step at $+50^oC$ is included in the drying schedule.

The ratio of net Br L_α signal from plastic containing cellular solids, compared to that of pure plastic, has been calibrated to provide a measure of intracellular hydration (10). The calibration was accomplished using a set of standards prepared from samples containing varying dry solids fractions of albumin. The calibration curve over a range of hydration states from 60% to 95% water was linear with a correlation coefficient of 0.991. The uncertainty in absolute concentration for any given measurement in the region of normal tissue hydration using this calibration was estimated to be 5%. The use of imperfect calibration factors results in systematic error of measurement, the consequences of which can be diminished by use of appropriate controls.

The necessity for calibration of the Br ratio with albumin comes, in part, because of the well-documented shrinkage that accompanies tissue dehydration (1,14). Implicit in this work is the assumption that shrinkage of prepared tissue is comparable to that of albumin standards. This assumption has been found to be reasonable for rat skeletal muscle, where determination of intracellular water was measured with electron probe microanalysis to be within 3% of that measured with conventional methods (8).

Intracellular Electrolyte Measurement

The electron microprobe characteristic x-ray signals are converted to numbers representing intracellular electrolyte concentrations by referencing electron microprobe signals through secondary standards to counting rates on fabricated albumin standards (12). Although the concentrations are determined in units of mmol $(kg\ ww)^{-1}$, the concentrations also can be expressed in units of mmol $(kg\ H_2O)^{-1}$ or mmol $(kg\ dw)^{-1}$ with the information on tissue water. In addition, data associated with fabrication of standards also permits expressing composition of dry solids per unit volume.

Measurement of Cell Volume Change

Measurement of cell volume change is straightforward when tissue is either frozen-hydrated or embedded with an appropriate embedding medium. For studies of cell volume regulation, in which the change in volume is needed rather than absolute cell volume, change in cell volume can be assessed from the characteristic x-ray data of an element endogenous to tissue solids. Sulfur has been found useful for this purpose with freeze-dried, embedded tissue.

Sulfur-containing amino acids, which occur as part of the protein in the cellular structure, are the source of the electron microprobe sulfur signal. Intracellular free amino acids total approximately 28 mmol $(kg\ ww)^{-1}$ (5). Of these, taurine is the only S-containing free amino acid that is expected to be present, and then at a level of approximately 12 mmol $(kg\ ww)^{-1}$. With normal plasma taurine levels of only approximately 0.07 mmol l^{-1}, an aggressive mechanism must exist for maintaining intracellular taurine levels. Because of the large concentration gradients, it is anticipated that most of the intracellular free amino acids are lost from the cell during dissection, resulting in an osmotic shift and contributing to swelling of cells in dissected tissue. To simplify interpretation of the electron microprobe S data, incubation solutions are used that do not include S-containing amino acids, Table 1. If movement of taurine from tissue occurs during dissection and recovery, the loss will not compromise the electron microprobe S K_α signal because control tissue and experimental tissue are treated identically. Incubation of the cells after the recovery period should not result in further loss of taurine, and it should be quite safe to assume that the difference in S K_α signal between experimental tissue and control tissue results only from changes in the cell volume.

Although the characteristic x-ray signal generated in the electron microprobe is sensitive to the mass under the beam, knowledge of sample density permits expressing the electron microprobe data in units of sample volume. When appropriate controls are used so that data can be expressed as ratios, knowledge of exact sample density is not necessary.

With bulk sample analysis, for any given accelerating voltage the excitation volume will be essentially the same in experimental tissue samples as in control tissue samples, dictated primarily by the density of plastic. In this case, calibration of the S K_α signal is not necessary, and the ratio of net S K_α counts from control and experimental samples will vary inversely as the ratio of cell volumes. Cell volume change, then,

is computed from the electron microprobe sulfur data:

$$\frac{V_e}{V_c} = \frac{(S\ K\alpha)_c}{(S\ K\alpha)_e} \qquad (1)$$

A more universal relationship between cell volume change and tissue S concentration, [S], can be derived which is also applicable to thin sections using the definition of tissue sulfur concentration:

$$[S] = \frac{M_s}{V \rho} \qquad (2)$$

or

$$M_s = V \rho [S] \qquad (3)$$

where M_s is mass of sulfur in a given cell with cell volume, V. Note that since [S] is measured with the electron microprobe in units of mmol/unit mass, it is necessary to include sample density, ρ. With the assumption that no detectable change in cell sulfur content occurs in response to an osmotic stress; i.e.,

$$(M_s)_e = (M_s)_c \qquad (4)$$

We have from Eqs. 3 and 4

$$V_e \rho_e [S]_e = V_c \rho_c [S]_c \qquad (5)$$

Or, if sample density is approximately the same in experimental tissue samples as in controls; i.e.,

$$\rho_e \simeq \rho_c \qquad (6)$$

Then,

$$\frac{V_e}{V_c} = \frac{[S]_c}{[S]_e} \qquad (7)$$

The accuracy of this estimation of cell volume change is dependent upon the validity of the assumption that the density of embedded experimental cells is approximately the same as the density of embedded control cells. With the freeze-dried, plastic embedded preparation, the embedding plastic has a density of approximately 1.152 kg l^{-1}. This is little different from the density of tissue solids which is expected to be between approximately 1.15 and 1.30 kg l^{-1}. Also, since the embedded muscle samples are typically 60% to 80% plastic, even if there were a substantial difference in density between tissue and embedding plastic, subtle changes in tissue solids content would not change significantly the density of embedded tissue. As an example of an extreme case, consider the comparison of a control cell that is 25% solids with a cell that has been exposed to an osmotic stress with resultant loss of 25% of cell volume. If no tissue solids left the dehydrated cell, solids would comprise 33% of the dehydrated cell. Upon freeze-drying and embedding, the cell volume of normal tissue is reduced by approximately 20% by the well-known drying artifact (1,14). Thus, the embedded control cell would be approximately 69% Epon and the experimental cell would be approximately 59% Epon. Estimating cell solids to be approximately 1.3 kg l^{-1} and Epon to be 1.15 kg l^{-1} provides estimations of 1.18 and 1.19 kg l^{-1} respectively, for control and experimental cell densities. It has not been established that dehydrated tissue will experience the same shrinkage during freeze-drying as fully hydrated tissue. If there is a difference in shrinkage artifact between the two types of tissue, the dehydrated cell would be expected to experience the least shrinkage, diminishing the difference in densities between embedded control and dehydrated cells. Thus, an error in determination of cell volume change of about 1% is estimated to result from the density difference between embedded dehydrated and embedded control cells in this hypothetical case.

Alternatively, cell volume change can be determined from information on tissue solids derived from the intracellular water data. Similar to the sulfur ratio method, the ratio of experimental to control cell solids concentration will indicate a change in cell volume. The derivation is the same, except tissue solids concentration is used in place of [S] in each equation above. The determination of cell volume change using data on tissue solids will be accurate only if there is no net movement of tissue solids across the cell membrane. When material enters the cell, volume change will be underestimated with the tissue solids method. Because there are occasions when material is accumulated across the cell membrane, this method of estimating cell volume change is not as reliable as that using the ratio of sulfur concentrations.

Agreement between the two methods of estimating cell volume change provides strong evidence that mass of cell solids has not changed significantly as a result of the experimental procedure. When there is disagreement between the two estimations of cell volume change, it can be determined by the sense of the disagreement whether solids have entered or have left the cell.

A third technique for estimating cell volume change with the electron microprobe might be developed around the technique proposed by Tosteson and Hoffman (17) for use with conventional methods. This technique involves comparing the sum of intracellular electrolyte concentrations, [K] + [Na] + [Cl], in control tissue with the sum of electrolyte concentrations in experimental tissue. There are inherent weaknesses in this method, however, that limit its usefulness. The measured electrolytes account for only approximately one-half of the free particles required to establish the osmolality of intracellular fluid. As an additional complication, the electron microprobe measures total concentration and not activity, which is needed for accurate assessment of contribution to the osmotic pressure. The most serious disadvantage of the method of using intracellular electrolyte concentrations to estimate cell volume change, however, lies in the implicit assumption that the only response of cells to an osmotic challenge is the movement of electrolytes and water across the cell membrane, and it does not accommodate the involvement of other entities.

Because accurate measurement of cell volume change is possible only with methods sensitive also to changes in concentration of the particles not normally detected with the electron microprobe, a method of estimating cell volume based solely on electron microprobe electrolyte concentration measurements is not appropriate for general application.

Cell Membrane Potential

Information on the magnitude of cell membrane potential is necessary for proper understanding of the events involved in transmembrane electrolyte movement. While direct measurements of membrane potential often are not available, the electron microprobe measurement of intracellular chloride provides a means of estimating membrane potential, and of identifying occasions when profound changes of membrane potential have occurred. If Cl^- is in electrochemical equilibrium and extracellular Cl^- concentration is known, then changes in membrane potential will be reflected in electron microprobe chloride data. The Nernst potential for Cl^- should then provide a good estimate of the membrane potential (7):

$$E = -61 \log \frac{[Cl^-]_o}{[Cl^-]_i} \quad (8)$$

Where the Cl^- concentrations outside, $[Cl^-]_o$, and inside the cell, $[Cl^-]_i$ are expressed in units of mmol per liter cell water. The estimation of cell membrane potential is not exact, because, while the assumptions concerning electrochemical neutrality of the Cl^- ions are probably correct in most cases with skeletal muscle, the electron microprobe does not measure activity; it measures total concentration. Changes in cell membrane potential may be determined reasonably accurately, however, if the activity coefficients of extracellular and intracellular Cl^- are approximately the same in experimental as in control tissue. With these cautions in mind, significant disturbances to cell membrane potential are recognizable.

Assessment of Osmotic behavior

If a cell were a bag of fluid contained within an ideal semipermeable membrane, the response to osmotic stress would be described accurately by the Van't Hoff relationship which relates osmotic pressure to cell water volume:

$$\pi = \frac{\Sigma n_i \phi_i RT}{W_v} \quad (9)$$

where n_i is the number of particles with osmotic coefficient ϕ_i, R is the universal gas constant, T is the absolute temperature, and W_v is the volume of cell water. For the simplified case in which the cell membrane is an ideal semipermeable membrane through which nothing passes except water, changing the osmolality of the bathing solution will result in a change only in cell water volume. In such a case, the numerator of Eq. (9) is a constant, and cell water volume will vary inversely as osmotic pressure. Thus, a change of bath osmolality from π_c to π_e results in a change of cell water volume from W_c to W_e as

$$\frac{W_e}{W_c} = \frac{\pi_c}{\pi_e} \quad (10)$$

Cell volume can be expressed as the sum of cell water volume and tissue solids volume; i.e,

$$V_c = W_c + V_{dry\ solids} \quad (11)$$

Using the assumption that cellular tissue solids content does not change, the expected volume of a cell in an experimental solution will be

$$V_e = W_e + (V_c - W_c) \quad (12)$$

Eq. 12 can be combined with Eq. 10 to produce:

$$V_e = \frac{\pi_c W_c}{\pi_e} + (V_c - W_c) \quad (13)$$

where the volume of cell water in control tissue, W_c, is determined from the electron microprobe data. This provides the volume expected of a passive bag of fluid contained within a perfect semipermeable membrane.

Results

Fig. 2 describes the accumulation of K in tissue incubated in hypertonic solutions. [Na] and [Cl] are not presented because their concentrations in fibers incubated in hypertonic solutions were not changed from their respective concentrations in control fibers as dramatically as was [K]. The major ion movement occurred with K^+, and a major accumulation of undetected particles was observed in tissue incubated in the most hypertonic solutions. Rather than absolute concentrations, the difference in K concentrations from [K] of tissue incubated in the isotonic solution have been plotted. Electron microprobe tissue water measurements were combined with the electrolyte measurements to express concentrations in units of mmol $(1\ water)^{-1}$. This permits direct comparison of the amount of K^+ accumulated with the increased tonicity of the experimental bathing solutions.

The corresponding cell volume change in response to hypertonic challenge is shown in Fig. 3. Sulfur concentration increased in fibers incubated in 1.13R and 1.22R solutions, indicating that cell shrinkage had occurred and that subsequent cell volume recovery had not been complete by the time of cryofixation. The observed shrinkage of fibers incubated in the 1.13R solution was little different from that expected of a bag of fluid contained within a perfect semipermeable membrane. Fibers incubated in the 1.31R solution apparently had regulated their volumes back nearly to normal during the period of incubation. The exquisite regulatory volume increase occurred even though intracellular electrolyte accumulation was insufficient to provide the needed additional particles. Consequently, accumulation of unmeasured particles must have occurred.

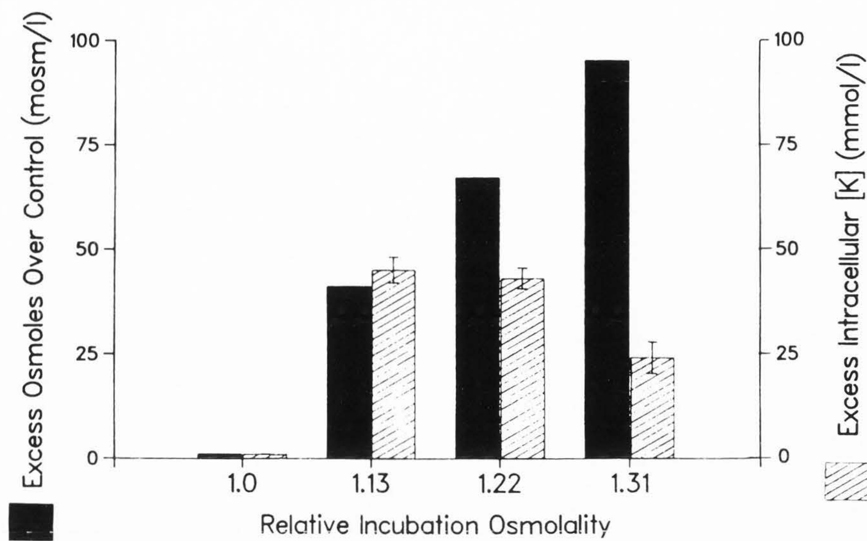

Figure 2. Change of intracellular K concentration is plotted for rat muscle fibers incubated in each of the 3 hypertonic solutions. In the first hypertonic solution, K was accumulated in sufficient quantities to compensate for the additional osmolality. Although cell volume regulation was most effective with fibers incubated with the third, or most hypertonic solution, K accumulation was not adequate to explain the cell volume response. The data suggest the involvement of additional unmeasured entities.

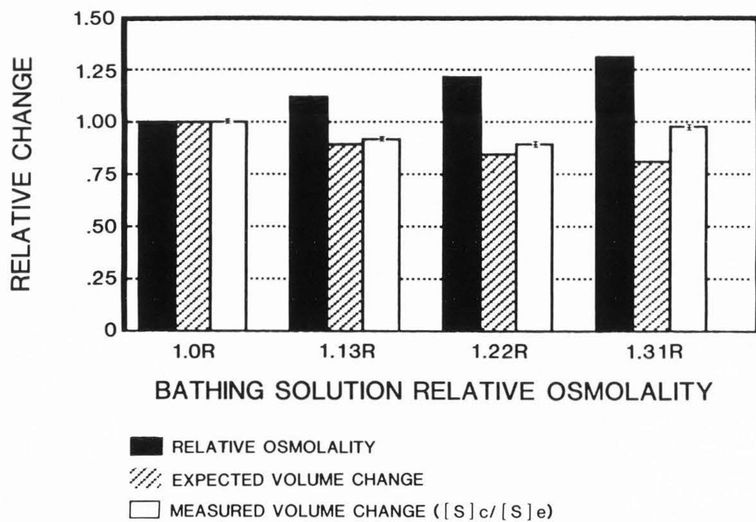

Figure 3. Volume change of isolated lumbricals in response to hypertonic challenge is compared with the volume change expected of a perfect osmometer. Averages and SEM of muscles from 5 animals, twenty fibers per muscle, are included. Note that evidence for cell volume regulation is present for tissue incubated in each of the hypertonic solutions, with the most effective volume regulation experienced by the fibers incubated in the most hypertonic solution.

Discussion

Cell volume regulation has been demonstrated in this series of experiments. It is just recognizable in muscle fibers incubated in the 1.22R solution and is obvious in muscles incubated in the 1.31R solution. Muscle fibers in the 1.13R solution and to a major extent in the 1.22R solution accommodated the increased osmolality by accumulation of K^+ ions. However, volume regulation was not effective in those fibers in the forty minute incubation period, despite the large accumulation of potassium. In muscle from the 1.13R solution, the extra intracellular potassium per liter water was roughly equivalent to the extra osmoles per L water. This was sufficient to establish osmotic equilibrium across the cell membrane, but not to result in the volume expansion necessary to return cell volume to control value. Although significant regulatory volume increase had not occurred, muscles incubated in the 1.22R solution clearly were shrunken less than predicted

by the Van't Hoff relationship, suggesting some cell volume regulation. Fig. 2 indicates that in fibers incubated in the 1.22R solution, and especially those incubated in the 1.31R solution, the ratio of extra potassium to extra osmoles was insufficient to account for the recovery of cell volume. Apparently, particles other than potassium were used for volume regulation.

The additional potassium in muscles incubated in the 1.13R and 1.22R solutions had been accumulated against an electrochemical gradient, and hence required an active transport process. Unpublished data indicates that the accumulation of both K^+ and undetected particles is effectively inhibited when tissue is incubated in hypertonic solutions at 0^oC. Also, data has been presented showing that when Na^+-K^+ ATPase was inhibited with ouabain, there was no change in cellular response except that Na^+ and K^+ apparently had changed roles (13). Accumulation of undetected particles apparently was unaffected.

Accounts in the literature indicate that both amino acids and urea have been found to effect cell volume regulation, although nearly all of those studies have been performed on invertebrate cells. One study, however, describes a mild osmotic stress produced by hypernatremia in rats (16), where, over a period of more than 24 hours, maintenance of hypernatremia caused brain tissue to accumulate amino acids to regain normal hydration. Also, the high plasma-to-cell gradient observed for some amino acids suggests the presence of a highly effective transport mechanism in the cell membrane. It is therefore reasonable to expect that the phenomena of cell volume regulation observed in skeletal muscle fibers also might entail accumulation of amino acids. Work is presently underway to determine if the undetected particles are, in fact, amino acids.

It is clear that further study is necessary to elucidate the mechanisms responsible for cell volume regulation in skeletal muscle, the molecules involved, and the receptors responsible for maintaining normal hydration. The electron microprobe methodology is difficult and tedious, but in studies of cell volume regulation, the electron microprobe can be an invaluable tool when used in such a manner as to provide accurate, simultaneous information on cell volume change, intracellular water content, and concentrations of important intracellular electrolytes.

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