Cat Heart Muscle in Vitro

IX. *Cell ion and water contents in anisosmolal solutions*

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ABSTRACT Cell contents of water, K, Na, and Cl have been determined in cat right ventricular papillary muscles immersed in solutions with and without NaCl when the external osmolality was varied with sucrose. The plot of cell water/kilogram dry weight (corrected for sucrose content) vs. (external osmolality)$^{-1}$ suggests that not less than 82% of water present in cells at physiological external osmolality is free to move across the cell membrane in response to an imposed osmotic gradient. Cells fail to increase their water content in very hypotonic solutions. For osmolalities greater than 5 times isosmolal, at which the mannitol space and the Cl^{36} space are both equal to 100% of muscle water, rather large amounts of univalent cation appear to remain "bound" to the tissue.

In interpreting previously published measurements of cellular ion concentrations (1), membrane potentials (2), potassium fluxes (3, 4), and active transport of Na (5) in cat papillary muscles, the cellular water and ion contents have been considered as an aqueous electrolyte solution. The observations to be reported in the present paper are an experimental test of the extent to which cell water participates in net water movements produced by imposition of an osmotic gradient. The results are consistent with the conclusion that all of cell water is osmotically active, and indicate that any nonsolvent water present does not exceed 18% of the cellular water content under isosmolal conditions.

METHODS

Experiments in NaCl-Containing Solutions

Papillary muscles from cat right ventricles were isolated and preequilibrated in vitro at 27.5°C in chambers suitable for incubation with a 1.0 ml volume of solution as previously described (6). The osmolality of the bathing solution was then varied

while the external ionic composition was kept constant. For this purpose half of the NaCl concentration of isosmolal cat Ringer's solution was replaced with variable concentrations of sucrose, to which cat heart muscle cells are relatively impermeable (6, 7). After a 1 hr preequilibration period in isosmolal solution, muscles were placed for 2 hr in a hypotonic or hypertonic medium labeled with H^3-mannitol, C^{14}-inulin, and Cl^{36}. In moderately hypotonic or hypertonic media incubation for 2 hr was usually sufficient for all net movements of ions and water to approach completion, thereby establishing a new steady state with respect to cellular ion concentrations. In very hypertonic solutions the cellular ion content was found to vary with time. Under these conditions, as well as in very hypotonic solution, analyses were also performed on muscles incubated for 15 or 30 min and for 4 hr. In order to render experiments utilizing incubation times of less than 2 hr comparable to those done with a 2 hr incubation period, it was necessary to find conditions which would lead to equilibration of the extracellular compartment with radioactive mannitol, inulin, and Cl, even for relatively short times in the anisotonic medium. For this purpose such muscles were preincubated for 105 or 90 min in an isosmolal solution containing the same concentrations (counts per minute per milliliter) of H^3-mannitol, C^{14}-inulin, and Cl^{36} as those in the anisosmolal medium to which these muscles were subsequently exposed during the final 15 or 30 min of the experiment. Unless otherwise stated, the total duration of equilibration with these radioactive substances (always at the same specific radioactivity) was thus 2 hr. The shorter exposure to very anisotonic solutions was necessary to minimize the resultant net ion movements. Such net ion movements were, in any case, rather slow, especially in comparison to the rapid net water movements in response to the imposed osmotic gradients. The osmolality of the bathing media was determined by measurement of the freezing point depressions with a Fiske Osmometer (Model G-62), calibrated using NaCl standards and the osmotic coefficients given by Robinson and Stokes for solutions at 25°C (8).

Experiments in NaCl-Free Media

Measurements of cell water and ion contents were also made at four external osmolalities in media in which all of external NaCl was replaced by sucrose. For these experiments muscles were first equilibrated for 1 hr in an isosmolal, phosphate-buffered, NaCl-containing medium, then immersed for 2 hr in the NaCl-free solution of the desired osmolality. Incubation in very hypertonic solutions without NaCl was carried out for 30 min, preceded by a 90 min exposure to approximately isosmolal NaCl-free solution containing the same concentrations of radioactive inulin and mannitol.

Solutions

The solutions used were buffered to pH 7.2 with phosphate and bubbled with 100% O_2 presaturated with water vapor. All solutions contained (in millimoles/liter) K 5.32, Ca 1.40, Mg 0.56, HPO_4 1.295, H_2PO_4 0.205, and dextrose 5.5. All media used for experiments in the presence of NaCl further contained Na 89.25 and Cl 95.8. The osmolalities (milliosmols/kilogram water) were made to 187, 290, 347, 388, 494, 932, and 1797, respectively, by adding the following amounts of sucrose (in g/100 ml of final solution): 0, 3.06, 4.88, 6.11, 9.17, 19.79, and 35.0. Relative to an

"isosmolal" value of 347 milliosmols/kg water, the osmolalities of the resulting solutions were, respectively, 0.539, 0.836, 1.00, 1.12, 1.42, 2.69, and 5.18. NaCl-free media had a Cl concentration (millimoles/liter) of 6.3. Their osmolalities (milliosmols/kilogram water) were made to 308, 368, 427, and 555, respectively, by adding the following amounts of sucrose (in g/100 ml of final solution): 8.80, 10.5, 12.2, and 15.6. The resulting relative osmolalities were 0.888, 1.061, 1.231, and 1.598.

RESULTS

Analysis of Data

As emphasized in a previous paper (6) and recent review (9), cell water and ion contents based on measurements of the extracellular compartment with mannitol differ significantly from those based on analogous measurements with the much larger inulin molecule. In the present series of experiments these differences become even more important, since the weight of the sucrose contained in the extracellular compartment had to be subtracted from the total dry weight. The dry weight thus corrected for sucrose content served as the reference for cellular ion and water contents. The relationship of the mannitol space and inulin space to the volume of the "true" extracellular compartment is at present obscure (9). In the analysis of the data it will accordingly be necessary to present duplicate results calculated on the basis of mannitol and inulin spaces measured simultaneously in all muscles.

Results in tables and text are presented as mean ± standard error. Unless otherwise stated, significant differences are those for which $P < 0.01$ using the Student t test.

The Time Course of Net Ion and Water Movements in Response to an Imposed Osmotic Gradient

In order to compare the relatively rapid response of muscle water content to an imposed osmotic gradient for solutions of different osmolality, it is convenient that cell solute content at the time of measurement be approximately the same for all external osmolalities tested. This condition may be satisfied in one of two ways. If no significant net ion movements result from the imposition of an osmotic gradient, cell water content may be measured at any time after the completion of the net water movement, when a new steady state will have become established. On the other hand, if the change in external osmolality is followed by a large net movement of intracellular solute across the cell membrane, the cell water content must be measured before a significant net solute movement has had a chance to occur. In making this measurement, advantage is taken of the fact that net water movements in response to the osmotic gradient take place rapidly relative to the secondary net movements of cellular solutes, to which the cell membrane is much less

permeable than to water. Although it is technically possible to preequilibrate the extracellular compartment with inulin and mannitol, the time resolution of the present method is limited by the necessity of equilibrating the extracellular compartment with sucrose after the osmolality has been changed to its final value (the extracellular sucrose content must be known for the expression of cell ion and water contents on a "sucrose-free" dry weight basis). At 27.5°C the minimum time needed for equilibration by diffusion in cylindrical structures having the diameter of papillary muscles (10) is from 15 to

TABLE I*

CELL WATER CONTENTS AND EXTRACELLULAR SPACES IN SOLUTIONS CONTAINING NaCl

Ω		Cell water content		Weight of water in extracellular space	
		Based on mannitol space	Based on inulin space	Weight of water in whole muscle Mannitol	Inulin
		kg cell water/kg dry weight		*% total water*	
0.539‡	(8)	2.0±0.1	2.2±0.1	42±3	36±3
0.836	(7)	2.1±0.1	2.4±0.1	32±2	24±1
1.00	(7)	1.4±0.1	1.67±0.02	47±4	36±2
1.12	(7)	1.39±0.06	1.59±0.03	46±2	37±2
1.42	(8)	1.18±0.04	1.33±0.05	47±1	39±3
2.69‡	(7)	0.54±0.05	0.72±0.07	72±2	62±4

* Figures in parentheses give number of experiments. Throughout the tables and text dry weights of muscles have been rendered comparable by subtracting the weight of sucrose in the extracellular space, calculated from the measured volume of the mannitol and inulin spaces, respectively, and from tabulated values of the density of sucrose solutions (17). The corrected dry weights thus obtained therefore depend on whether the calculation of muscle sucrose content is based on inulin space or mannitol space data.

‡ Values for relative osmolalities of 0.539 and 2.69 were obtained after exposures of 15 and 30 min, respectively, to the anisotonic solution. All other values are for incubations lasting 2 hr.

30 min, depending on the viscosity (and therefore on the sucrose concentration) of the test solution.

The 15 or 30 min incubation periods chosen for experiments with short incubation times are a compromise between the longer times desirable for optimal extracellular equilibration with tracer and sucrose and the shorter times desirable to avoid significant loss of cellular solutes. The following considerations suggest that the error introduced by this compromise is small and does not seriously alter the experimental results: Diameters were measured in all muscles by a method previously described (3). In three experiments paired muscles were obtained from the same heart; the diameter of one muscle was greater by a factor of two or more than that of the other muscle from the same heart. The cell water contents and extracellular spaces of the larger of the pair were compared with those of its smaller partner after the short incubation in a solution of altered osmolality. For each of the three pairs,

there was no significant difference in the values obtained in the muscles with large and small diameters. The extracellular diffusion path is much longer in one of the two muscles; if a lag due to extracellular diffusion seriously limits the resolution of the method, one would expect this limitation to show up in a marked difference between paired muscles of grossly different diameter. Since such differences were not found, it was concluded that the error introduced by this effect is small.

TABLE II*
CELL ION CONTENT

Ω	K	Na	Cl
		mmole/kg dry weight	
	Calculated with mannitol space		
0.539	306±19	33±5	5±4
0.836	302±15	41±9	31±4
1.12‡	296±7	56±14	31±8
1.42	307±12	33±6	23±3
2.69	336±14	36±11	−4±2
	Calculated with inulin space		
0.539	305±20	50±5	20±5
0.836	297±20	65±8	57±4
1.12‡	280±13	75±17	50±5
1.42	302±12	51±6	40±4
2.69	326±16	58±10	19±4

* Dry weights corrected for sucrose contents. Number of experiments and duration of incubation are the same as for the corresponding value of Ω in Table I. Intracellular ion concentrations (mmole/kg cell water) may be derived by dividing the ion content in this table by the appropriate value of the cell water content in Table I.
‡ The ion and water contents of muscles at Ω = 1.12 in Tables I and II have been recalcuated to correct for extracellular sucrose content from experiments of Page, Goerke, and Storm (18).

Tables I and II summarize, respectively, the water and ion contents of muscles in NaCl-containing solutions under the conditions used for comparison of the cell water content as a function of external osmolality. The relative osmolality of the bath is Ω, defined as the ratio of the osmolality of the test solution to that of a medium (347 milliosmols/kg water) having the same freezing point depression as that of the physiological cat Ringer solutions previously described (1). It is apparent from Table II that the condition of constant intracellular ion content (assumed to be an index of constant cellular solute content) is sufficiently well approximated to justify comparisons of cellular water contents. Cell ion contents for Ω = 0.836 − 1.42, determined after 2 hr of incubation, did not differ significantly from one another or from cell ion contents measured after only 15 min of incubation.

Table III presents the time courses of the ion and water contents of muscles in a very hypotonic medium (Ω = 0.539) and in a very hypertonic medium

(Ω = 2.69). The results in the hypertonic medium are the more striking. The cells are observed to undergo a progressive loss of K between 30 min and 240 min after exposure to the solution of high osmolality. In the period between 15 min and 2 hr of incubation the total cellular ion content, and therefore the cell water content, are kept approximately constant by a net uptake of Na which compensates for the loss of K. The K content of the ex-

TABLE III

TIME COURSE OF CELL ION AND WATER CONTENTS*

Ω	Incubation time	Cell ion content			Cell water content	No. of experiments
		K	Na	Cl		
	min	*mmoles cell ion/kg dry weight*			*kg cell water/kg dry weight*	
		Calculated with mannitol space				
	15	306±19	33±5	5±4	2.0±0.1	8
0.539	120	251±12	35±5	19±3	2.0±0.1	7
	240	293±11	40±6	16±4	2.4±0.1	6
	30	336±14	36±11	−(4)±2	0.54±0.05	9
2.69	120	278±14	77±5	8±2	0.54±0.02	10
	240	195±19	48±3	−(9)±5	0.35±0.07	6
		Calculated with inulin space				
	15	305±20	50±5	20±5	2.2±0.1	8
0.539	120	262±12	70±3	56±8	2.6±0.1	7
	240	291±9	64±6	47±4	2.7±0.1	6
	30	326±16	58±10	19±4	0.72±0.07	7
2.69	120	268±15	91±6	26±3	0.69±0.02	10
	240	183±5	76±12	25±4	0.64±0.02	6

* Dry weights have been corrected for weight of extracellular sucrose. At Ω = 0.539, mannitol spaces at 15, 120, and 240 min were, respectively (% of total muscle water) 42 ± 3, 43 ± 2, and 35 ± 3, the corresponding inulin spaces being 36 ± 3, 30 ± 3, and 26 ± 2. At Ω = 2.69, mannitol spaces at 30, 120, and 240 min were, respectively, 72 ± 2, 68 ± 2, and 80 ± 2, the corresponding inulin spaces being 62 ± 4, 58 ± 1, and 61 ± 3. Total duration of exposure to mannitol and inulin was, in all cases, 120 min; the duration given as "incubation time" refers to the duration of exposure to the anisotonic medium.

tracellular space is a very small fraction of total muscle K. The observation of a progressive loss of cell K in the very hypertonic medium would therefore hold even if the cell membrane becomes permeable to mannitol or inulin in this solution. Moreover, the tissue K content per kilogram sucrose-free dry weight would change by less than 10%, even if all of the cell water equilibrates with extracellular sucrose. However, entrance of a significant amount of extracellular tracer or of sucrose into the cells would alter the values for cell contents of Na, Cl, and water obtained after 240 minutes at Ω = 2.69. These figures must accordingly be accepted with reservations. If, when Ω =

2.69, the cell membrane does effectively exclude mannitol, inulin, and sucrose, the results (Table III) show that after 4 hr both the total cellular univalent cation content (Na + K) and the cellular water content have fallen significantly.

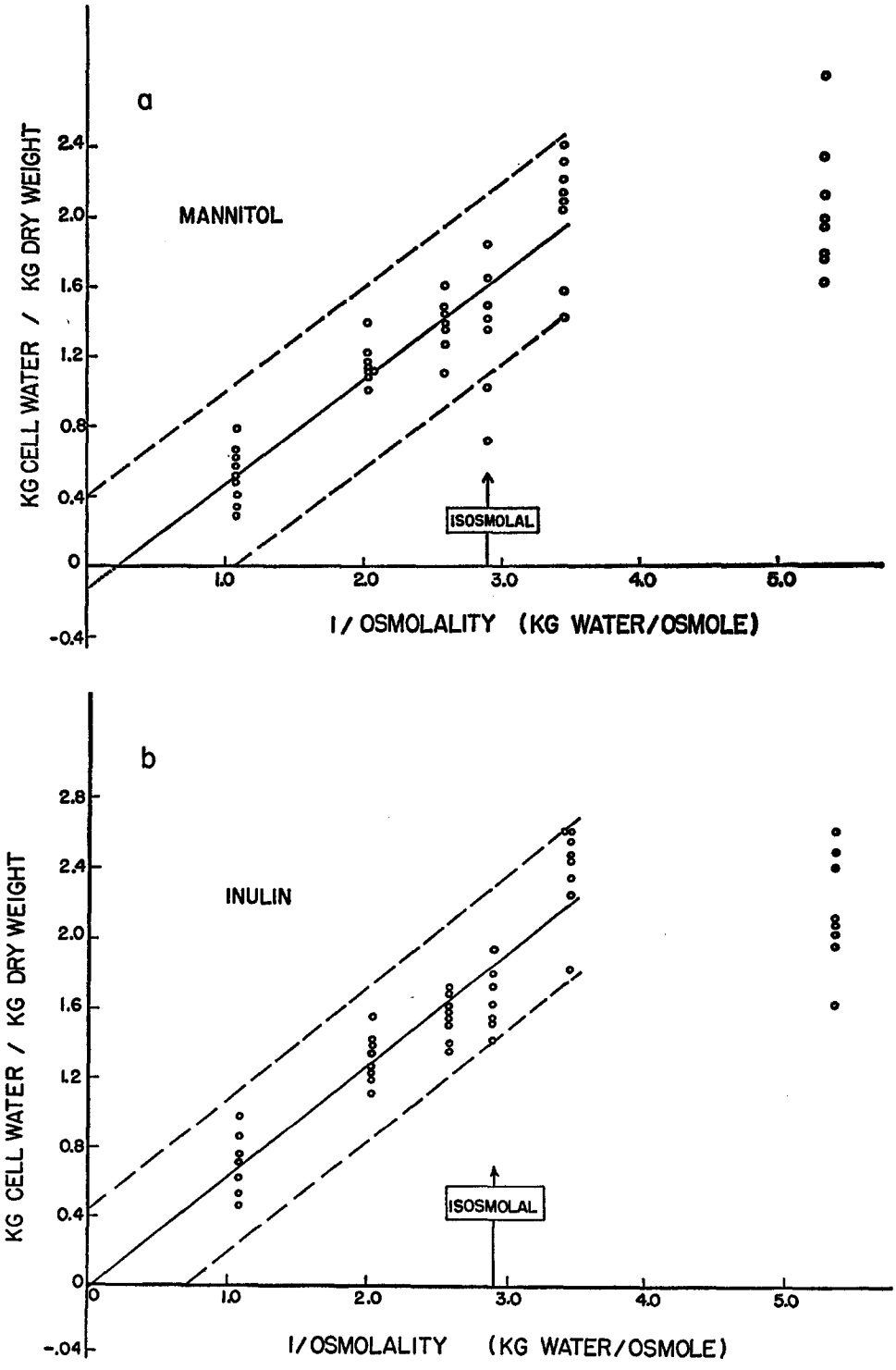
By contrast, the cellular K and Na contents after 15 and 240 min of incubation in the very hypotonic medium ($\Omega = 0.539$) do not significantly differ from each other or from steady-state values. The table shows an unexplained transient decrease in cell K content at 2 hr, the cell K content differing from that at 15 and 240 min at the $P < 0.05$ level of significance. In addition, the cell Cl content increases between 15 and 120 min of incubation.

In Fig. 1 the cell water contents at approximately constant cellular univalent cation content, calculated on the basis of the mannitol space (Fig. 1 *a*) and inulin space (Fig. 1 *b*), are plotted against the reciprocal of the external osmolality. It is apparent that cell water content decreases in hypertonic solutions and increases in hypotonic solutions, no matter which of the two measures of the extracellular water content is used. In very hypotonic solutions ($\Omega = 0.539$) cell water content measured after 15 min in the hypotonic medium apparently fails to increase in response to the lowered external osmolality. After 4 hr, however, the cell water content has significantly increased as measured with both mannitol and inulin space determinations (Table III). Since the univalent ion content of the cell has risen only slightly, this observation suggests that unidentified osmotically active substances are being produced within the cells.

The results for $\Omega = 0.836 - 2.69$, plotted in Fig. 1, have been fitted by the method of least squares to a line $y = \alpha + \beta x$. The resulting Equation (Fig. 1 *a*) is $y = -0.12 + 0.60x$ (SD of $\alpha = 0.12$, SD of $\beta = 0.050$, SE of estimate = 0.26, correlation coefficient = 0.89 ± 0.05); the corresponding equation for Fig. 1 *b* is $y = -0.014 + 0.65x$ (SD of $\alpha = 0.11$, SD of $\beta = 0.04$, SE of estimate = 0.21, correlation coefficient = 0.93 ± 0.04). If the lines are constrained to pass through the origin, the two equations become $y = 0.55x$ and $y = 0.64x$ for Figs. 1 *a* and 1 *b*, respectively. Because of the rather large scatter of the data, the least squares lines in Fig. 1 are clearly not statistically different from lines passing through the origin. The vertical axis intercepts of lines 3 SD of α above the least squares lines are, respectively, 0.25 (Fig. 1 *a*) and 0.31 (Fig. 1 *b*). These values correspond to 18% and 19% of the cell water in isosmolal solution.

Experiments in NaCl-Free Solutions

Table IV presents the cell contents of K and water in muscles incubated in NaCl-free sucrose Ringer's solutions of varying osmolality, having the physiological (5.32 mM) external K concentration. Comparisons of cell water con-


a
MANNITOL
KG CELL WATER / KG DRY WEIGHT
2.4
2.0
1.6
1.2
0.8
0.4
0
-0.4
ISOSMOLAL
1.0
2.0
3.0
4.0
5.0
1/ OSMOLALITY (KG WATER/OSMOLE)
b
INULIN
KG CELL WATER / KG DRY WEIGHT
2.8
2.4
2.0
1.6
1.2
0.8
0.4
0
-.04
ISOSMOLAL
0
1.0
2.0
3.0
4.0
5.0
1/OSMOLALITY (KG WATER/OSMOLE)

tents were again carried out under the conditions of approximate constancy of cellular K. As previously noted (5, 18), the residual Na content of muscles equilibrated in NaCl-free solution is below the resolution of the flame photometric method used. Since heart muscle cells in solutions of such low ionic strength do not tolerate large changes in cell volume well, the range of osmolalities tested was necessarily somewhat smaller than that feasible in the presence of NaCl. In Fig. 2, the cell water contents for individual muscles measured using mannitol space data are plotted against (osmolality)$^{-1}$ as in Fig. 1. Table IV and Fig. 2 indicate that cells in NaCl-free medium apparently fail to respond to hypotonic solutions by an increase in their water con-

TABLE IV

EFFECT OF EXTERNAL OSMOLALITY ON CELL WATER AND K CONTENTS IN NaCl-FREE SOLUTIONS*

Ω		Cell water content	Cell K content	$[K]_i$	Weight of water in extracellular space / weight of water in whole muscle
		kg water/kg dry weight	*mmole K/kg dry weight*	*mmole/kg cell water*	*% total water*
Calculated with mannitol space					
0.888	(7)	1.63±0.07	309±17	190±9	39±2
1.061	(7)	1.54±0.05	312±4	204±6	43±1
1.231	(6)	1.30±0.04	321±5	249±9	54±2
1.598	(8)	0.97±0.05	311±5	326±19	53±2
Calculated with inulin space					
0.888	(7)	1.91±0.05	301±17	158±9	24±1
1.061	(7)	1.72±0.05	304±4	177±4	34±2
1.231	(6)	1.72±0.10	296±5	176±11	31±2
1.598	(7)	1.16±0.05	302±3	264±10	44±3

* For Ω = 0.888 to 1.231 measurements were made after 120 min in a solution of the indicated osmolality. For Ω = 1.598, muscles were incubated for 100 min in a solution with Ω = 1.061, followed by 20 min in a solution with Ω = 1.598. The numbers of experiments are given by the figures in parentheses.

tent. The experimental results for isosmolal and hypertonic solutions plotted in Fig. 2 have also been fitted to a line by the method of least squares. The line so obtained, $y = -0.15 + 0.62x$, (SD of $\alpha = 0.17$, SD of $\beta = 0.073$, SE of estimate = 0.13, correlation coefficient = 0.88 ± 0.07), is again not statistically different from a line, $y = 0.56x$, constrained to pass through the origin.

FIGURE 1. Plot of cell water content against (osmolality)$^{-1}$ in NaCl-containing solutions (*a*) calculated using mannitol spaces and (*b*) calculated using inulin spaces. Broken lines in this and the following figure are two standard errors of the estimate from the least squares (solid) line. Each point is the experimental result for a single papillary muscle.

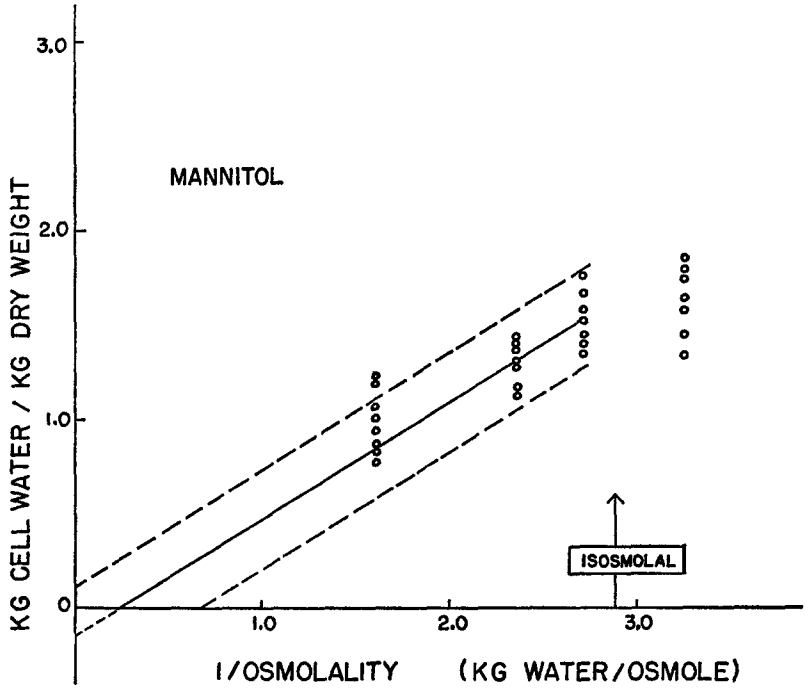


FIGURE 2. Plot of cell water content against (osmolality)$^{-1}$ in NaCl-free solutions, calculated using mannitol spaces.

Experiments in Solutions of Very High Osmolality

In solutions of very high osmolality (Ω = 5.18) the water contents of the volumes equilibrating for 2 hr with mannitol and Cl^{36} become equal to the total muscle water content determined gravimetrically from the difference between the wet and dry weights (Table V). It would seem that the extreme hypertonicity has made the cell membrane permeable to mannitol and caused

TABLE V

EFFECTS OF EXPOSURE TO A VERY HYPERTONIC SOLUTION (Ω = 5.18)*

Duration of incubation	(Weight of water in space) / (Weight of water in whole muscle)			Residual Na	Residual K
	Mannitol	Cl^{36}	Inulin		
hr	*% total water*			*mmols ion/kg dry weight*	
2	98±3	99±3	73±3	112±4	43±6
4	98±2	106±3	—	117±8	18±3

* Dry weights corrected for weight of sucrose in mannitol space. Residual Na and K are amounts of these ions remaining after subtraction of K and Na contents of mannitol space. Number of experiments at 2 and 4 hr are 9 and 6, respectively.

any membrane-limited compartments which remain impermeable to mannitol and sucrose to lose their water because of the osmotic gradient. On the assumption that the chemical composition of the mannitol space is identical with that of the bathing medium, the K and Na contents of this space may be subtracted from the total tissue contents of these ions. The large residual Na content of 112 mmoles/kg dry weight so obtained remains approximately constant between 2 and 4 hr of incubation. By contrast, the smaller residual K content decreases significantly during this period.

DICUSSION

Response of Cell Water to an Osmotic Gradient

A recent review by one of us (9) points out that the question of "free" vs. "bound" water has not previously been experimentally examined in heart muscle. In the human erythrocyte as much as 20% of cell water may be present as nonsolvent water bound to hemoglobin (11–13). By contrast, so large a fraction of protein-bound water which does not act as a solvent for cytoplasmic solutes appears unlikely in heart muscle, assuming that the weight of water associated with each gram of extractable cellular protein is of the same order of magnitude as the weight of water bound to hemoglobin or myoglobin (9).

The observation that the vertical axis intercepts in Fig. 1 are not statistically different from the origin is consistent with the interpretation that all of cell water is free to participate in net water movement in response to a change in activity of extracellular water. The standard deviation of the intercept supports the conclusion (at the 99% probability level) that not less than 82% of water present in the cells of papillary muscles in isosmolal solution is free to respond by a net water movement to an imposed osmotic gradient. However, no rapid increase in cell water content occurred after a 15 min exposure to a very hypotonic medium in NaCl-containing medium ($\Omega = 0.539$, Tables I and III and Fig. 1). This apparent departure from the behavior expected for a "perfect osmometer" raises the possibility that mechanical restrictions to expansion of cell volume may become important at very low external osmolality. Such restrictions might be imposed by the elastic properties of cellular structures and of the extracellular connective tissue. In this connection, failure of the volume of single frog skeletal muscle cells to expand in very hypotonic media has been observed with a photographic method by Reuben, Lopez, Brandt, and Grundfest (14). Analogous restrictions have been described in the swelling of highly cross-linked ion exchange resins (15).

Experiments in Solutions of Very High Osmolality

At very high external osmolality (Table V, $\Omega = 5.18$) all of muscle water becomes accessible to mannitol, and presumably to sucrose, either because

normally membrane-limited structures are rendered permeable to these molecules or because all water measured by our gravimetric method is osmotically withdrawn from those membrane-limited structures remaining impermeable to mannitol and sucrose. The experimental observation is that the weight of water equilibrating with mannitol becomes equal to that in the Cl^{36}-space, the water contents of both spaces being equal to the total muscle water content determined gravimetrically. Nevertheless, there is a substantial residual tissue content of Na and K after subtraction of the K and Na contents of the mannitol space. The observations are open to two alternative interpretations. On the one hand, the assumption that the ion content of the mannitol space is the same as that of an equivalent volume of bathing solution may be incorrect. This explanation seems improbable, in view of the fact that the assumption leads to an exact prediction of the tissue content of Cl ion. Alternatively, the muscle (K + Na) content remaining after subtraction of the K and Na contents of the mannitol space may reflect the univalent cation-binding capacity of the residual muscle anions at pH 7.2. The residual univalent cation content is very much larger than the known K and Na binding capacities of the isolated and purified contractile proteins of muscle (16). A possibility meriting further exploration is that a fraction of the residual univalent cation represents the K and Na content of mitochondria. The progressive loss of tissue K between 2 and 4 hr of incubation (Table V) is consistent with this possibility.

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