

Solvent Water for Electrolytes in the Muscle Fiber of the Giant Barnacle

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ABSTRACT Seven experiments are described which permit estimation of the "solvent water" or the "osmotically active water" of the dissected fiber from the giant barnacle, *Balanus nubilus*. Each of the first four experiments includes the measurement of a free ion activity in the myoplasm by means of a Na⁺, K⁺, or Cl⁻ ion-specific microelectrode. The fifth experiment makes use of a membrane potential vs. [K]_o curve. The last two experiments measured fiber water and fiber volume as bath osmolarity was changed. The seven independent estimations of solvent water ranged from 0.64 to 0.72 of fiber water with a mean of 0.68. Since the extracellular space of single fibers was about 7% of fiber water, it was concluded that 25% of analyzable water was not acting as solvent for the osmotically active solutes in the myoplasm.

New and convincing evidence for water structuring in muscle has come from two lines of experimentation. First, studies with the K⁺-selective microelectrode (1-3) have consistently shown the free K⁺ concentration of myoplasm to be higher than the total concentration for the fiber (total fiber K⁺/total fiber water). These results suggest that only about 65-70% of the fiber water acts as solvent for the K⁺ ion in the myoplasm. Second, nuclear magnetic resonance (NMR) studies (4-6) have shown muscle water molecules to be highly structured and immobile compared to water in simple solution. According to Cope (5), about 27% of muscle water displays a transverse relaxation time only 1/200 that of normal water, which implies a very high degree of structuring.

When one combines this evidence for water structuring with other evidence for ion binding (1-3, 7-9)¹ it is no longer possible to imagine the fiber interior as a single homogeneous solution of ions and proteins. Yet, this is the model which has served the advancement of muscle physiology so well, particularly membrane physiology. No doubt, the old concept of an aqueous compartment which is in electrochemical and osmotic equilibrium with the out-

¹ Czeisler, J. L., O. G. Fritz, Jr., and T. J. Swift. Direct evidence from nuclear magnetic resonance studies for bound sodium in frog skeletal muscle. Personal communication.

side is still valid in spite of the existence of water, ion, and protein heterogeneity. What is in question now, however, is the size of such a compartment and the concentration of solutes in it. In order to define the myoplasmic concentration of Na^+ , for example, one can no longer make a quotient of the total fiber Na^+ and the total fiber water. Both the numerator and denominator of such a quotient are erroneous because of Na^+ -ion binding and water binding (1).

In this paper, seven experiments are described which permit calculation of "ideal water" in the myoplasm of the dissected fiber from the giant barnacle, *Balanus nubilus*. The first four calculations are based on the measurement of an ionic activity by means of an ion-specific microelectrode: a K^+ microelectrode for two experiments, a Cl^- microelectrode for one experiment, and a Na^+ microelectrode for another experiment. The last three calculations are independent of the microelectrodes and depend on information from the following experiments: (a) measurement of membrane potential as outside K^+ and Cl^- are changed (so that their product remains constant); (b) measurement of fiber water as bath osmolarity is changed; (c) measurement of fiber volume as bath osmolarity is changed.

All seven experiments have yielded surprisingly similar estimations for the ideal water in the myoplasm. It can now be said with considerable confidence that "solvent water" and "osmotic water" are similar and constitute 68% of the analyzable water in fibers from the giant barnacle.

METHODS

Dissection of single fibers from the depressor muscle of *Balanus nubilus* has already been described (1). The end product is a fiber, about 1–1.5 mm in diameter and 30 mm long, with a free tendinous end and a fleshy attachment to the stone. When such an undamaged fiber is stored in barnacle Ringer solution at 10°C, its membrane potential and electrolyte composition can remain constant for days (9). A fiber chosen for microelectrode impalement was cannulated at its tendon (but not through the muscle-tendon junction), and suspended vertically in a bath containing barnacle Ringer solution at room temperature. Fibers destined for chemical and radioisotope analysis were dissected apart and cleaned, but left attached as a group to a common baseplate.

Solutions The barnacle Ringer solution contained the following salts, in mm/liter: 450 NaCl, 8 KCl, 20 CaCl_2 , 10 MgCl_2 , 25 Tris(hydroxymethyl)aminomethane plus HCl to pH 7.6. When the K^+ content was elevated in some experiments, the Na^+ and Cl^- ions were replaced by K^+ and methanesulfonate ions so that $[\text{Na}^+] + [\text{K}^+]$ and $[\text{K}^+][\text{Cl}^-]$ remained constant. When the osmolarity of the Ringer solution was altered, only the NaCl was increased or decreased; the osmolarity was measured by means of a Fiske osmometer. Finally, a sucrose solution isosmotic with barnacle Ringer, was used routinely as a wash solution in analytic procedures.

Microelectrodes The construction and calibration of Na⁺, K⁺, and Cl⁻ microelectrodes used in this laboratory have been well-documented (10, 11, 3). The active tip was about 25 μ in diameter and 100 μ long for all ion electrodes. The Na⁺ tip was composed of NAS₁₁₋₁₈ glass (Corning, N. Y.) the K⁺ tip was NAS₂₇₋₄ glass, and the Cl⁻ tip was fused Ag-AgCl to Pt. The K/Na selectivity ratio was 10 for the K⁺ electrode, and about 0.01 for the Na⁺ electrode. All electrodes were routinely calibrated before and after each experiment using standard solutions. If the potential for a given solution varied by more than 2 mv, the electrode and the experiment were discarded.

Microelectrodes for membrane potential measurements were conventional micropipettes containing a Ag-AgCl wire and filled with 3 M KCl. Micropipettes with tip potentials in excess of 5 mv were discarded.

Chemical Analysis Grasped by its tendon with jeweller's forceps, each fiber was cut free from the baseplate, washed for 30 sec in isosmotic sucrose, blotted on filter paper, then dropped in a preweighed stoppered Pyrex bottle. After obtaining the wet weight, the fiber was dried overnight at 95°C and then reweighed for dry weight. For Na⁺ and K⁺ analysis, the dried fiber was digested with nitric acid, neutralized with NH₃, appropriately diluted, and analyzed with a Unicam S.P. 900 flame photometer. For Cl⁻ analysis, the dried fiber was digested in hot NaOH, the protein precipitated with 4% ZnSO₄ in 0.4 N HNO₃, the supernatant acidified and titrated on an automatic Buchler-Cotlove chloridometer (12).

Extracellular Space. In some experiments, fibers were equilibrated in sorbitol-¹⁴C, then analyzed for this isotope by means of a liquid scintillation β-counter. Handling, washing, and blotting procedures were identical to those described for chemical analysis. In other experiments, a Cl⁻ technique was used (12): half the fibers were soaked in 100% Cl⁻ Ringer, half in 50% Cl⁻ Ringer ([K][Cl] constant), then both groups were analyzed for Cl⁻ content. The first method makes use of a substance which presumably enters only the extracellular space of a fiber, and the second method measures a substance which presumably leaves only the extracellular space of a fiber. Both methods predict an extracellular space equal to 6% of fiber water for dissected fibers (9, 12).

Na⁺ Influx (9) Groups of fibers attached to a common baseplate were immersed in a Ringer solution containing 2 μci/ml ²²Na. At periods of 1, 3, 6, 12, 24, and 48 hr, three to five fibers were removed, analyzed for Na⁺ and K⁺, then counted for ²²Na content. A γ-radiation analyzer was used to select a 0.3 Mev energy range centered at 1.28 Mev, the principal energy peak of ²²Na. Each sample was counted for 15 min and corrected for background. The lowest counting rate was at least 10 times higher than background (8 cpm).

RESULTS

I. *Solvent Water from Myoplasmic (K⁺)_m in the Resting Fiber*

The distribution of K⁺ in a single fiber may be described as follows:

$$V[K^+] = \alpha_o V[K^+]_o + \alpha_m V(K^+)_m + B_K \quad (1)$$

where V is total fiber water; α_o and α_m are the fractions of fiber water in the extracellular and myoplasmic compartments, respectively; $[K^+]$, $[K^+]_o$, and $(K^+)_m$ are the analyzed concentration, the bath concentration, and the myoplasmic concentration of K^+ , respectively; and B_K represents any K^+ which may not be located in the two fluid compartments named.

When fibers are equilibrated in normal Ringer, α_o is about 0.06, $[K^+]_o$ is 0.008, and $[K^+]$ is about 0.160. These figures show that the term, $\alpha_o V [K^+]_o$, is very small compared to $V [K^+]$. Hence, equation 1 can be simplified to $V [K^+] = \alpha_m V (K^+)_m + B_K$. Furthermore, if one now assumes $B_K = 0$, i.e. all the analyzable K^+ is measurable as soluble K^+ in the myoplasm, then $[K^+] = \alpha_m (K^+)_m$. It follows that the fiber water fraction, α_m , can be calculated if the myoplasmic $(K^+)_m$ and analytic $[K^+]$ concentrations of a fiber are known.

Table I gives the average results of an experiment in which fibers from two barnacles were handled in one of three ways: (a) impaled with a K^+ microelectrode, (b) impaled with a Na^+ microelectrode, (c) analyzed for water, Na^+ , and K^+ content. In order to combine the information from the three procedures, one must assume that each group is representative of the three groups combined.

The calibration potentials (in standard solutions) for the Na^+ and K^+ microelectrodes were remarkably constant throughout the duration of the experiment (2 days). Hence, their electrical performance can be described by

TABLE I
 Na^+ AND K^+ CONCENTRATION IN SINGLE FIBERS*
 BATHED IN RINGER SOLUTION AT 24°C

Microelectrode measurements†			Analytic measurements‡		
$(a_K)_m$	$(a_{Na})_m$	M_p	$[K^+]$	$[Na^+]$	% H_2O
0.150	0.0064	-74.5	0.156	0.069	75.8
± 0.004	± 0.001	± 0.4	± 0.0035	± 0.003	± 0.2
(7)	(10)	(17)	(10)	(10)	(10)

The numbers in parentheses are number of determinations.

* All fibers were from two barnacles.

† Calibration equations for the Na^+ and K^+ microelectrodes were:

$$E_{Na} = 109 + 58 \log_{10} (a_{Na} + 0.01 a_K)$$

$$E_K = 70.5 + 57.5 \log_{10} (a_K + 0.15 a_{Na})$$

Where E_{Na} and E_K are the electrode potentials in myoplasm after correcting for membrane potential (M_p).

‡ Flame photometry on companion fibers, not impaled with electrodes.

two equations:

$$E_{\text{Na}} = 109 + 58 \log_{10} (a_{\text{Na}} + 0.01 a_{\text{K}}) \quad (2)$$

$$E_{\text{K}} = 70.5 + 57.5 \log_{10} (a_{\text{K}} + 0.15 a_{\text{Na}}) \quad (3)$$

where E_{Na} and E_{K} are the measured potentials (millivolts), corrected for membrane potential if necessary, of the Na^+ and K^+ electrodes. In order to calculate $(a_{\text{Na}})_m$ from equation 2 for each fiber in *b*, a value of 0.15 was assigned to a_{K} . The average value of $(a_{\text{Na}})_m$ was then used in equation 3 to calculate $(a_{\text{K}})_m$ for each fiber in group *a*.

The average value for $(a_{\text{K}})_m$ was exactly 0.150, which indicates the insignificance of the term, $0.15 a_{\text{Na}}$, in the calibration equation 3. If one assumes that the myoplasm has a mean activity coefficient equal to the coefficient of Ringer solution (0.65), then an $(a_{\text{K}})_m$ of 0.150 represents a $(\text{K}^+)_m$ of 0.231. Since the $[\text{K}^+]$ of these fibers was 0.156 mole/kg water, it follows from $[\text{K}^+] = \alpha_m (\text{K}^+)_m$ that $\alpha_m = 0.675$.

The values of % water, Mp, and $[\text{Na}]$, though not required for the calculation of α_m , are included in Table I to indicate the good health of the fibers. Based on past experience, damaged fibers in Ringer solution invariably have water contents in excess of 78%, membrane potentials less than 65 mv, and Na^+ concentrations higher than 90 mm/kg water.

II. Solvent Water from Change in Myoplasmic $(\text{K}^+)_m$ during Water Entry

When the bathing solution of a fiber impaled with a K^+ microelectrode is changed from isotonic to hypotonic Ringer, the $(a_{\text{K}})_m$ decreases continuously as water enters the fiber (3). If one assumes that the total free K^+ in the myoplasm remains constant, at least during the first few minutes of osmotic stress, then one may write $V_m (\text{K}^+)_m = \text{constant}$ where $V_m = \alpha_m V$. The derivative is $V_m \delta (\text{K}^+)_m + (\text{K}^+)_m \delta V_m = 0$. Now assume $\delta V_m = \delta V$ in the first few minutes of water entry and rearrange the derivative to give:

$$\frac{\delta V}{\delta (\text{K}^+)_m} = - \frac{\alpha_m V}{(\text{K}^+)_m} \quad (4)$$

It follows from this expression, that solvent water α_m can be obtained from the slope $\delta V / \delta (\text{K}^+)_m$ of the curve obtained by plotting the decrease in $(\text{K}^+)_m$ with increase in fiber water content. Based on the assumptions used, equation 4 is only valid when no free K^+ leaves the myoplasm and when all the water which enters the fiber acts as diluent for the K^+ ion. Probably such conditions exist only in the first few minutes of water movement.

Fig. 1 shows the average curve of experiments in which fibers were treated in one of two ways: (a) impaled with K^+ microelectrode and $(a_{\text{K}})_m$ measured continuously as the bath changed from normal Ringer to hypotonic Ringer

(50%); (b) from a group of 24 fibers, 4 removed at 0, 2, 5, 10, 20, and 30 min after transfer to hypotonic Ringer (50%) and analyzed for % H₂O. The ordinate of each point is the mean of 16 determinations and the abscissa of each point is the mean of 8 determinations. An indication of the degree of variation is shown by the standard error bars about the 2 min point.

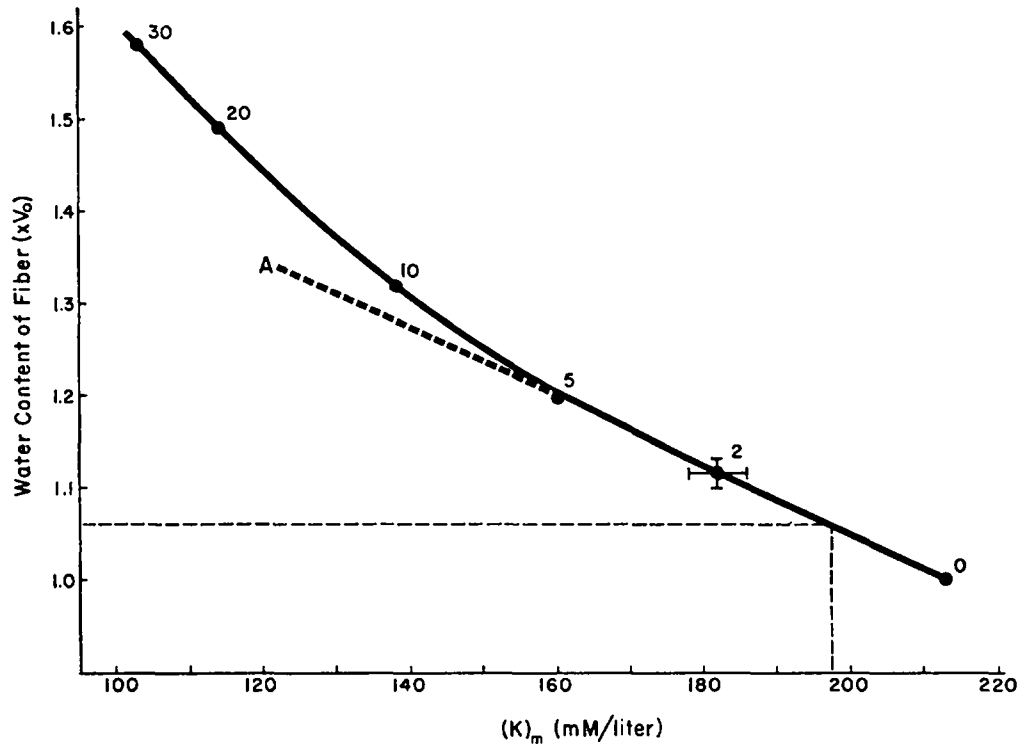


FIGURE 1. Changes in water content of fiber and in the free K⁺ concentration in the myoplasm (K⁺)_m after fiber had been placed in hypotonic (50% NaCl) Ringer. Numbers beside each experimental point indicate time in minutes. Line A is drawn between points 0 and 2. Lengths of bars at point 2 are twice the standard error.

In Fig. 1, line A is drawn through the 0 and 2 min points. If $\delta V/\delta(K^+)_m$ of the curve equals the slope of line A at the 1 min mark (midway between 0 and 2) and since $V = 1.06 V_0$ and $(K^+)_m = 197.5$ at the 1 min mark, α_m calculates as 0.69 using equation 4.

III. Solvent Water from Change in Myoplasmic (Cl⁻)_m during Water Entry

An experiment similar to the previous one was carried out using a Cl⁻ microelectrode rather than a K⁺ microelectrode in the myoplasm. The resulting plot of myoplasmic (Cl⁻)_m and fiber water V is shown in Fig. 2. If we make assumptions similar to the previous ones, the slope of this curve can be de-

rived:

$$\frac{\delta V}{\delta(\text{Cl}^-)_m} = - \frac{\alpha_m V}{(\text{Cl}^-)_m} \quad (5)$$

If one makes $\delta V/\delta(\text{Cl}^-)_m$ equal to the slope of line A, $V = 1.06 V_0$, and $(\text{Cl}^-)_m = 47$ at the 1 min mark, and then α_m becomes 0.64 from equation 5.

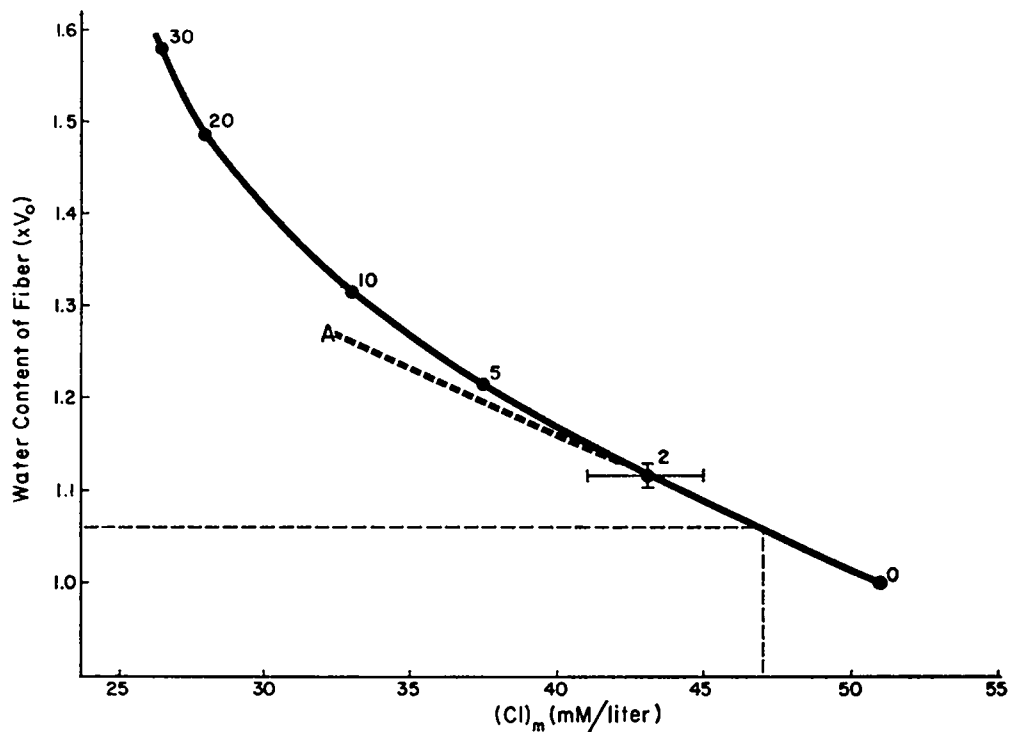


FIGURE 2. Changes in water content of fiber and in the free Cl^- concentration in the myoplasm $(\text{Cl}^-)_m$ after fiber had been placed in hypotonic (50% NaCl) Ringer. See legend to Fig. 1 for more details.

It should be mentioned that experiments similar to those in sections II and III have been done using hypertonic Ringer (200%). Compared to hyposmotic stress, fiber water leaves at a much faster rate and both $(\text{K}^+)_m$ and $(\text{Cl}^-)_m$ increase very rapidly in the first few minutes of hyperosmotic stress (3). Unfortunately, when equations 4 and 5 are applied to the resulting $(\text{K}^+)_m$ vs. V and $(\text{Cl}^-)_m$ vs. V plots, irrational values for α_m are obtained ($\alpha_m > 1$). It is possible that the changes are too rapid to be detected with any degree of precision, or it may be that the assumptions are not applicable when water leaves the myoplasm.

IV. Solvent Water from Myoplasmic (Na^+)_m and ^{22}Na Influx

The exchange of ^{22}Na with fiber Na^+ over a 50 hr period is shown in Fig. 3. Notice the clear delineation of two exchange rates. The fast one (dashed line)

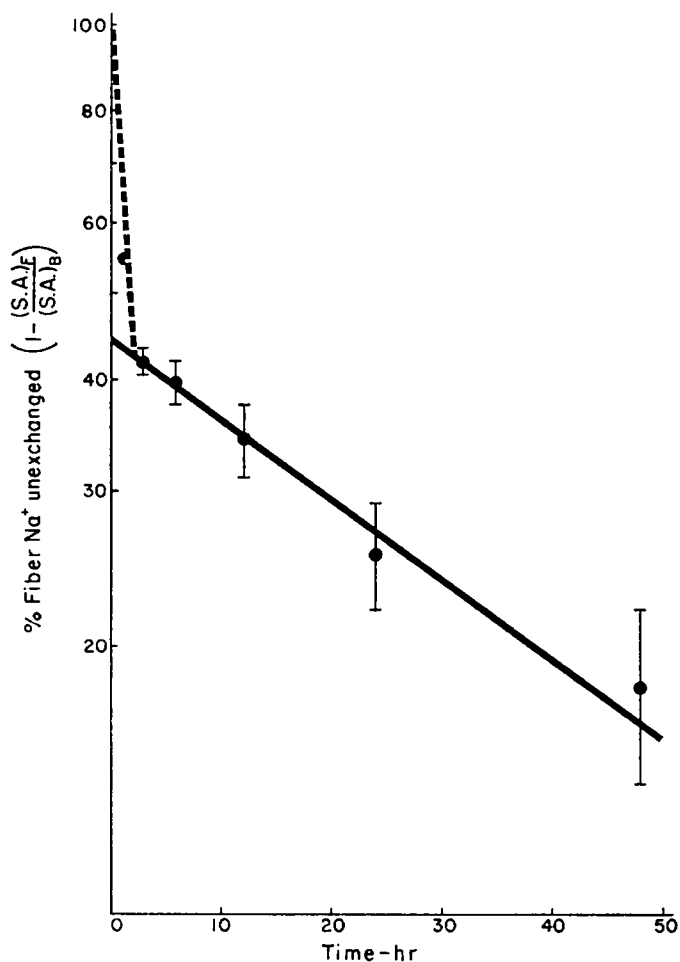


FIGURE 3. Average exchange of ^{22}Na in bath ($2 \mu\text{Ci/ml}$) with Na^+ of single fibers. Ordinate is calculated as shown from the measured specific activity of the fiber, $(S.A.)_F$, and the specific activity of the bath $(S.A.)_B$. See Table II for the fiber concentration which was relatively constant for 50 hr.

has been satisfactorily identified as ^{22}Na exchange with myoplasmic free Na^+ (9). The slow rate reveals a Na^+ pool which is either tightly bound or well compartmentalized. Extrapolation of the slow exchange line to zero time indicates that the pool of slowly exchanging Na^+ contains about 44% of the intra-fiber Na^+ (excluding Na^+ of extracellular space). This means that about 56%

of the intrafiber Na^+ is located in the myoplasm as free Na^+ . Such a statement can be written as follows:

$$\alpha_m V(\text{Na})_m = 0.56 \{V[\text{Na}^+] - \alpha_o V[\text{Na}^+]_o\} \quad (6)$$

where $[\text{Na}^+]$, $[\text{Na}^+]_o$, and $(\text{Na}^+)_m$ are the analyzed concentration, the bath concentration, and the myoplasmic concentration of Na^+ , respectively. Dividing by V and solving for α_m , equation 6 becomes:

$$\alpha_m = \frac{0.56 \{[\text{Na}^+] - \alpha_o [\text{Na}^+]_o\}}{(\text{Na}^+)_m} \quad (6a)$$

Companion fibers to those for the ^{22}Na influx study (Fig. 3) were treated in one of three ways: (a) impaled with the Na^+ microelectrode to obtain $(a_{\text{Na}})_m$; (b) equilibrated in 50% Cl^- Ringer ($[\text{K}^+]_o[\text{Cl}^-]_o$ constant) to obtain extracellular space (see Methods); (c) analyzed for % H_2O , $[\text{K}^+]$, and $[\text{Na}^+]$. The pertinent data for solving equation (6a) for α_m are given in Table II. Assuming as before that $\gamma = 0.65$ in the myoplasm, $(\text{Na}^+)_m$ becomes 0.0206 from an average $(a_{\text{Na}})_m$ of 0.0134. The free water can now be solved from equation 6a,

$$\alpha_m = \frac{0.56 [0.051 - 0.058(0.450)]}{0.0206} = 0.68$$

Notice that the estimation of α_m is similar whether it is dependent on the data in Table I or in Table II. Yet, the average $[\text{Na}^+]$ and $(a_{\text{Na}})_m$ were quite different in the two experiments. This illustrates the danger in combining measurements from different families of barnacles when calculating a fraction.

V. *Solvent Water from Membrane Potential vs. $[\text{K}^+]_o$*

Fig. 4 displays the results of an experiment in which a single fiber, impaled with a K^+ microelectrode, was bathed in a series of solutions containing increasing amounts of K^+ ($[\text{K}^+][\text{Cl}^-]$ and $[\text{K}^+] + [\text{Na}^+]$ were constant in all

TABLE II
ANALYSIS OF COMPANION FIBERS
TO THOSE IN FIG. 3

Na^+ activity ($a_{\text{Na}})_m$	Membrane potential	Na^+ content $[\text{Na}^+]$	Extracellular space*
	<i>mV</i>	<i>moles/kg H₂O</i>	α_o
0.0134	-76.3	0.051	0.058
± 0.0018	± 0.6	± 0.0028	± 0.004
(27)	(27)	(24)	(24)

* Determined by the 50% Cl^- Ringer wash method.

solutions). The fiber was allowed to equilibrate in each solution for 30 min before potentials were recorded. Throughout the duration of the experiment, the myoplasmic activity, $(a_K)_m$, remained relatively constant at 0.149 ± 0.0019 (SE). This indicates that electrochemical equilibrium was maintained even though large changes in membrane potential occurred.

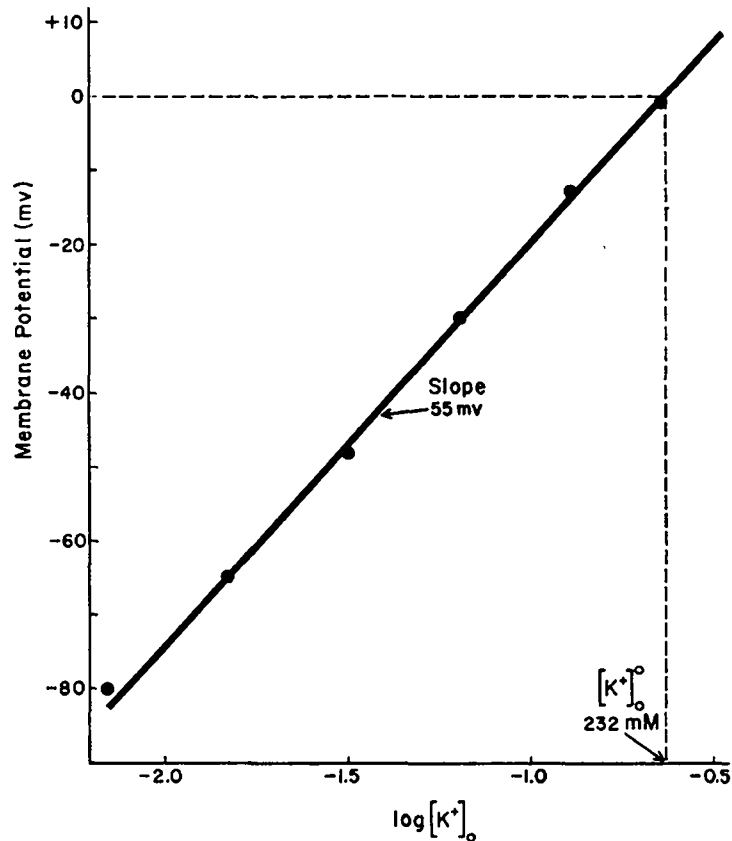


FIGURE 4. Change in membrane potential of a typical fiber bathed in high $[K^+]_o$ solutions. $[K^+]_o[Cl^-]_o$ and $[K^+]_o + [Na^+]_o$ were constant for all bathing solutions. Fiber was allowed to equilibrate for 30 min in each solution. This fiber was impaled with a K^+ microelectrode which measured $(a_K)_m$ as 0.149 ± 0.0019 throughout the experiment.

The line drawn through the experimental points in Fig. 4 has a slope of 55 mv per unit log change in $[K^+]_o$ and predicts that a $[K^+]_o$ of 232 mM will produce a zero potential. Thus, the equation for the line is:

$$(Mp) = 55 \log \frac{[K^+]_o}{[K^+]_o} = 55 \log \frac{[K]_o}{232}$$

Now, if the membrane of this fiber were acting as a perfect K^+ electrode, the

equation would be:

$$(Mp) = 59 \log \frac{[K^+]_o}{(K)_m}$$

Thus, the fiber membrane can be treated as a good K^+ electrode under the conditions described.

Table III lists the average $[K^+]_o$ at zero Mp obtained from seven experiments as illustrated in Fig. 4, and the average analytic K^+ concentration obtained from companion fibers. Also included are data from a similar study re-

TABLE III

SUMMARY OF $[K^+]_o$ WHICH PRODUCES ZERO Mp IN SINGLE FIBER *

	$[K^+]_o$ † at zero mp	$[K^+]$	$\frac{[K^+]_o}{[K^+]_o} = \alpha_m$
	<i>mM/liter</i>	<i>m/kg H₂O</i>	
1969 study §	0.225 ±0.010 (7)	0.160 ±0.004 (14)	0.71
1966 study (1)	0.242 ±0.012 (5)	0.174 ±0.006 (15)	0.72

* $[K^+]_o$, $[Cl^-]_o$ was constant for all external solutions. Methanesulfonate was used as the substitute anion.

† Obtained graphically, as in Fig. 1.

§ Barnacles for the 1969 and 1966 studies were obtained from Vancouver B. C., and Campbell River, B. C., respectively.

ported in 1966. Assuming now that $[K^+]_o$ at zero Mp equals $(K^+)_m$ and using the reasoning of method I, one can write $[K^+] = \alpha_m [K^+]_o$. Notice in Table III that the average α_m was 0.71 for the present study and 0.72 for the 1966 study.

It is worth emphasizing that the predicted $(K^+)_m$ for the fiber in Fig. 4 is 0.232 m but the measured K^+ activity for the fiber $(a_K)_m$ was 0.149 ± 0.0019 . These two values predict an activity coefficient of about 0.64 in the myoplasm. In all experiments, so far, a value of 0.65 has been assigned to the activity coefficient of myoplasm.

VI. Solvent Water and Osmotically Active Water

Many investigators have demonstrated (13–21) by a variety of techniques that the muscle fiber does not behave as a perfect osmometer. Instead, the fiber behaves as if part of its analyzable water is osmotically inactive (20, 21). Ob-

viously it is of importance to know how the osmotically active water of the fiber relates to the myoplasmic solvent water which has been determined here in four ways. With this in mind, two methods were used to study the changes in fiber water of a single fiber subjected to varying osmotic stress.

In one experiment, the water content and sorbitol- ^{14}C space were determined on fibers which had reached an equilibrium in a solution of known osmotic strength. In a typical experiment, about 20 undamaged fibers were equilibrated in normal osmotic Ringer solution and another 20 fibers from the same barnacle were equilibrated in a hypo- or hypertonic Ringer solution. Each solution contained a constant amount of sorbitol- ^{14}C . After 30 min, three-fourths of the fibers in each solution were cut from the stone, gently blotted (in a constant manner), then placed in preweighed bottles for determination of water content. The remaining one-fourth of the fibers were washed for 30 sec in a solution which was identical to the equilibrating solution minus sorbitol- ^{14}C , then analyzed for their sorbitol space.

The relation between the water content of the fiber (V) and the water fraction by weight (a) is:

$$\frac{V}{V_0} = \frac{(1 - a_0)a}{(1 - a)a_0} \quad (7)$$

where V_0 and a_0 refer to the fiber in normal osmotic Ringer solution; V and a refer to the fiber in another osmotic solution. Since V contains extracellular water, the factor V/V_0 must be multiplied by $(1 - e)/(1 - e_0)$, where e_0 and e are extracellular spaces at the normal and experimental states, respectively, before intrafiber water content is obtained.

The average results of four experiments, each from a different barnacle and each for a different osmotic state, are shown as four solid point (its diameter equals 2 (SE) of the mean) in Fig. 5. In effect, a solid point contains all the data (V , V_0 , a , and a_0) from a given experiment. Rather than draw a line for each point (all through the open circle) we have simplified Fig. 5 by drawing an average line for the two hypotonic experiments (line A) and an average line for the two hypertonic experiments (line B). The y -intercept for line A is 0.32 and for line B is 0.26. The y -intercept for the line through all five points by least squares fit is 0.287. Thus, one may say that the osmotically inactive water inside the fiber is equal to 0.29 $(1 - e_0) V_0$. Furthermore, since the average value for e_0 in these experiments was 0.069, the inactive water content becomes 0.27 V_0 . Put another way, this method predicts that about 66% of the analyzable fiber water ($100 - 27 = 73\%$) is osmotically active which is about equal to the myoplasmic solvent water obtained by other methods.

In another experiment, fiber volume was determined at a given equilibrium state by measuring its buoyant weight in two media of known densities. The experimental procedure was as follows. A single fiber was isolated with an

intact attachment to a fragment of the stony baseplate. After fiber and stone had been carefully cleaned of extraneous tissue, a platinum loop was tied to the tendon of the fiber without damaging the fiber. Both fiber and stone were then suspended from a weighing arm of the Cahn electrobalance (via a hangdown wire) in a special density chamber containing Ringer solution.

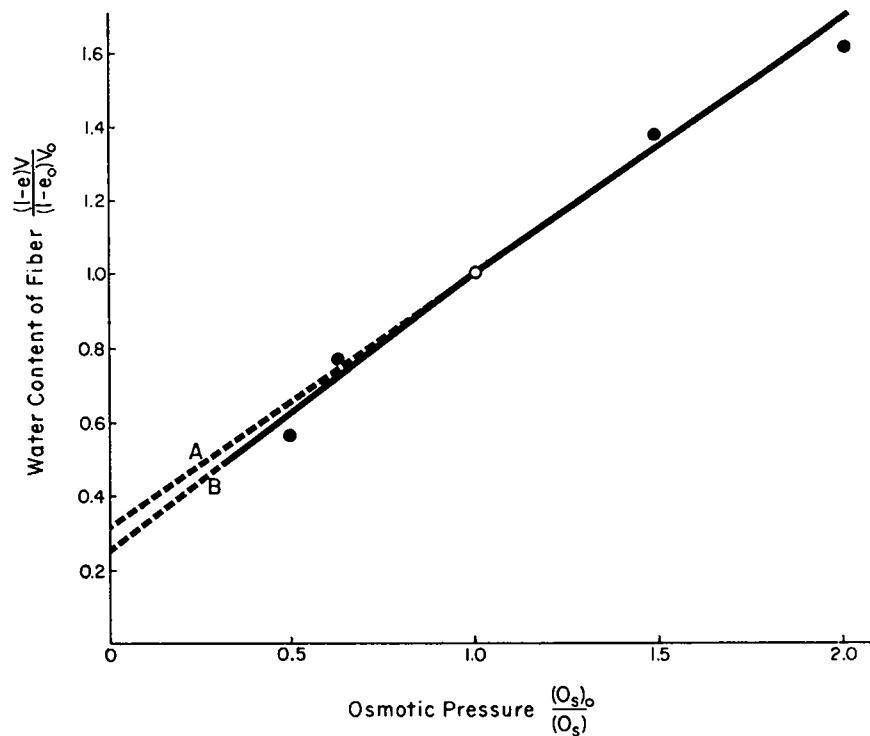


FIGURE 5. Changes in intrafiber water content of single fibers with changes in osmotic pressure. Intrafiber water $(1 - e)V$ is determined from total water of fiber (V) and extracellular space of fiber (e), both of which vary with osmotic stress. Line *A* is the best line for the hypotonic points and line *B* is the best fit for the hypertonic points. Diameter of the points equals $2 \times \text{SE}$ of the mean.

The buoyant weight of the fiber and stone in Ringer was monitored until it was constant (about 20–30 min). Then the chamber was lowered and the fiber blotted gently for 30 sec. Both fiber and stone were quickly weighed in air, then submerged in a hypertonic or hypotonic solution and the buoyant weight measured. When constant weight was reached, the chamber was lowered and the fiber reweighed in air after blotting as before. Finally, the fiber was cut away and the whole procedure repeated with stony fragment, platinum loop, and hangdown wire.

The fiber volume (V_f) for a given osmotic equilibrium state is related to

density (d) of the suspending media and buoyancy weight (W) as follows:

$$\frac{V_f}{(V_f)_o} = \frac{(d_o - d_{ao})(W_a - W_b)}{(d - d_a)(W_{ao} - W_{bo})} \quad (8)$$

where subscripts a and b refer to the suspending media, air, and solution, respectively; and the o subscript refers to the initial equilibrium state.

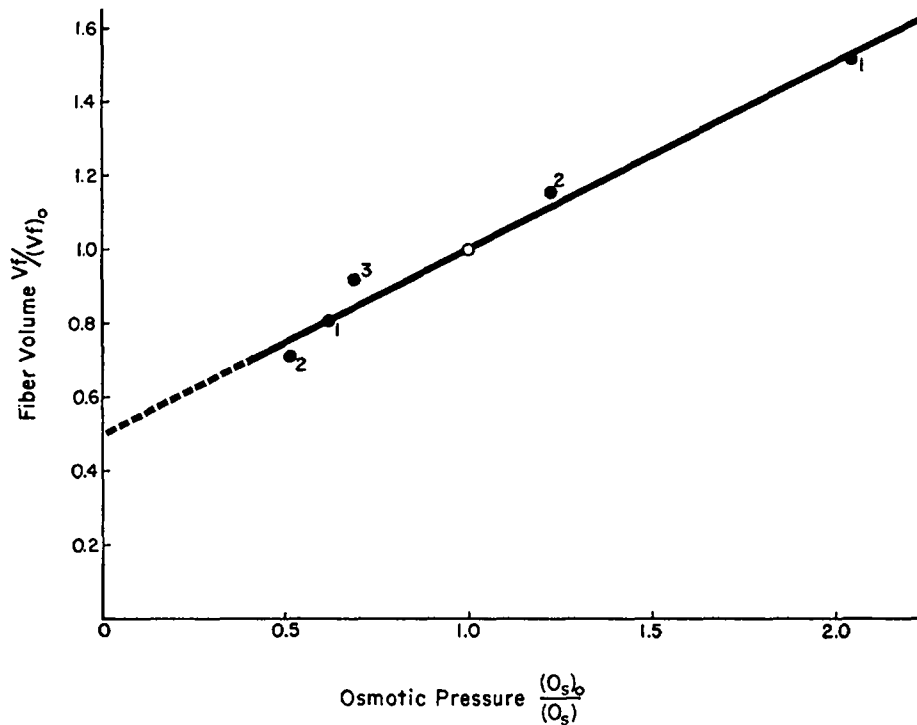


FIGURE 6. Changes in intrafiber volume, V_f , of single fibers with changes in osmotic pressure. Each point represents data from one fiber. The number beside each point refers to the solution listed in Table IV. The line through the points was determined by the method of least squares fit; it passes through the reference point (0) and crosses the y-axis at $V_f/(V_f)_o = 0.49$.

The density of each test solution was determined by measuring the buoyant weight of a glass plummet in each solution, using the equation:

$$d = \frac{(W_{ap} - W_{bp})}{V_p} + d_a$$

where W_{ap} and W_{bp} are buoyant weights of the plummet in air and solution; and V_p is volume of the plummet.

The procedure for weighing an intact undamaged fiber, as outlined above,

was successfully completed only five times. Many experiments were abandoned along the way because of visible damage or contraction of the fiber. The results of the five volume determinations (using equation 8) are shown as a plot against osmolarity in Fig. 6. The osmolarities and densities of the solutions in these experiments are listed in Table IV. In Fig. 6, the line through the five points was determined by the technique of least squares fit. Notice that the line passes through the open dot, which defines the common initial state of all fibers, and crosses the y -axis so that $(V_f/(V_f)_o)_o = 0.49$ when $(Os)_o/(Os) \rightarrow 0$.

TABLE IV
OSMOLARITY AND DENSITY OF
SOLUTIONS FOR FIG. 6

Experiment and solution	Osmolarity*	Density†
	<i>osmoles/liter</i>	<i>g/cm³</i>
1. Ringer Hypertonic 1	0.951	1.02201
	1.539	1.03585
2. Ringer Hypertonic 2	0.953	1.02247
	1.861	1.04139
3. Ringer Hypertonic 3	0.956	1.02287
	1.392	1.03074
4. Ringer Hypotonic 1	0.955	1.02289
	0.467	1.01132
5. Ringer Hypotonic 2	0.955	1.02289
	0.780	1.01824

* Measured on Fiske osmometer calibrated with NaCl standards.

† From buoyancy weight (using Cahn electrobalance) of a plummet with a known weight and volume.

In a companion experiment, the density of a fresh intact fiber was determined by measuring its buoyant weight in a Ringer solution and in a sucrose solution which was precisely isosmotic to the Ringer solution. The matching of the two solutions was verified by a water analysis of companion fibers. The average density of the dissected barnacle fiber was found to be 1.0626 ± 0.0015 ($n = 3$).

When the osmotically inactive fraction of fiber volume, $\{V_f/(V_f)_o\}_o = 0.49$, and the density of the fiber in normal Ringer solution, $d_f = 1.06$, are known, we can calculate the osmotically active fraction of intrafiber water $(\alpha_m)'$ from the equation:

$$(\alpha_m)' = \left[1 - \left\{ \frac{V_f}{(V_f)_o} \right\}_o \right] \cdot \frac{(1 - e')}{d_f(a - e')} \quad (9)$$

where a is the weight fraction of fiber water and e' is the weight fraction of extracellular space ($e' = ae$) in the single fiber. The derivation of this expression becomes clear when one defines the intrafiber volume as $(1 - e')W/d_f$ and the weight of intrafiber water as $(a - e')W$, where W is the measured weight of the fiber. Since a was 0.75 and e' was 0.045 ($e = 0.06$) for the fibers in this experiment, then $(\alpha_m)'$ calculates to be 0.65. This value for $(\alpha_m)'$ is similar to the value of α_m obtained from Fig. 5 based on fiber water content. It should be realized, however, that $(\alpha_m)' = \alpha_m/dw$, where dw is the density of the osmotically active water of the fiber. Thus, $(\alpha_m)'$ and α_m are numerically identical if dw is 1.0.

DISCUSSION

The seven estimations of intrafiber solvent water for the barnacle muscle fiber are listed in Table V with a summary of the main measurements and assumptions required for each calculation. Notice that the first four experiments make use of an intrafiber measurement by one of three ion-selective microelectrodes (K^+ , Na^+ , or Cl^- microelectrode), and the last three experiments make use of such well-established relationships as membrane potential vs. $[K^+]_o$ and V vs. osmotic pressure. Experiment IV is particularly interesting because no assumption was required to calculate α_m . The influx curve for $^{22}Na^+$ indicated the size of the free Na^+ pool in the myoplasm and the Na^+ microelectrode indicated the concentration of Na^+ in this pool. Combined, these measurements lead to the water content of the pool and α_m .

The α_m values range from 0.64 to 0.72 with an average of 0.68. This number means that only 68% of the analytic water in a single muscle fiber is acting as "ideal solvent" (experiments I-V) or osmotically active water (experiment VI). Since 6-7% of fiber water is extracellular in the sarcotubular and cleft system, it appears that about 25-26% of the fiber water is either not behaving as an ideal solvent in the myoplasm or not physically available to the myoplasm as solvent. Dick (22) considered both these explanations for osmotically inactive volumes of cells and concluded that they are in reality restatements of the one fact that the cell interior is not like an ideal solution.

Perhaps the most important and newest conclusion arising out of these experiments is the fact that the water fraction required as solvent for free Na^+ , K^+ , and Cl^- in the myoplasm is identical to that fraction of the fiber water which is osmotically active and makes the fiber behave as a perfect osmometer. With this information one can now speak realistically of a myoplasmic compartment of a given size and composition which behaves as an ideal solution, which makes contact with the limiting membrane of the fiber, and which is in electrochemical and osmotic equilibrium with the exterior.

According to Robinson and Stoke (23) the general equation relating osmotic pressure (Π), molality (m), and the molal osmotic coefficient (ϕ) of an

aqueous solution is:

$$\Pi = \frac{RTW_a}{1000\bar{V}_a} \cdot \phi vm$$

where W_a is the molecular weight of water, \bar{V}_a is a partial molal volume of water, v is the number of ions produced by the solute, and R and T have their usual significance. This equation can be adapted to describe the osmotic be-

TABLE V
SUMMARY OF EXPERIMENTAL ESTIMATIONS OF
SOLVENT WATER IN MUSCLE FIBERS

Fraction of total water α_m	Primary measurement	Secondary measurement	Assumption
I. 0.675	$(a_K)_m$ by microelectrode	$[K^+]$ flame analysis	Zero K^+ binding
II. 0.690	$(a_K)_m$ by microelectrode	Fiber water content	$\alpha_m V(K^+)_m$ constant initially
III. 0.640	$(a_{Cl})_m$ by microelectrode	Fiber water content	$\alpha_m V(Cl^-)_m$ constant initially
IV. 0.680	$(a_{Na})_m$ by microelectrode	^{22}Na influx, $[Na^+]$ and ECS*	-----
V. 0.715	Mp by micropipette	$[K^+]$	$Mp = \frac{RT}{F} \ln \frac{(K^+)_o}{(K^+)_i}$ Zero K^+ binding
VI. 0.660	Fiber water content	ECS	$\frac{V}{V_o} = \frac{K'(Os)_o}{Os} + b'$
VII. 0.650	Fiber volume	Fiber density	$\frac{V_f}{(V_f)_o} = \frac{K''(Os)_o}{Os} + b''$

* ECS = extracellular space.

havior of the fiber interior in the following way: (a) assume \bar{V}_a is constant for most hypertonic and hypotonic states and define $RTW_a/1000\bar{V}_a$ as a constant k ; (b) define $(vm)_m$ for muscle interior as $(M)_m/V$, where $(M)_m$ is the total effective molar equivalent of osmotic particles in the myoplasm and V is the total water (by weight) of the system. Therefore, the above equation transforms to:

$$\frac{\Pi V}{\phi_m} = k(M)_m$$

Now, if $(M)_m$ remains constant for a given fiber in a given experimental situation then $\Pi V/\phi_m$ is a constant and the term, V/ϕ_m , becomes that fraction of

intrafiber water which permits the fiber to behave as an ideal osmometer. But this water fraction has been shown to be identical to $\alpha_m V$, defined in equation 1 as the solvent water for free ions (Na^+ , K^+ , and Cl^-) in the myoplasm. Therefore, $\alpha_m = 1/\phi_m$ and $\Pi\alpha_m V = \text{constant}$.

For the barnacle muscle fiber under isotonic conditions, the average value of ϕ_m is 1.47 because $\alpha_m = 0.68$. Such a value for ϕ_m compares well with ϕ values for pure protein solutions at a 10% concentration (Table VI) but it is not what one expects for a protein-salt solution. The osmotic coefficient for a protein-salt solution should be close to the osmotic coefficient of the salt. This should be close to 1.0 for a protein dissolved in a KCl solution.

When the fiber was equilibrated in a series of solutions in which $[\text{K}^+]_o[\text{Cl}^-]_o$ remained constant, the fiber membrane behaved as a respectable K^+ electrode mainly because $(\text{K}^+)_m$ remained constant as $[\text{K}^+]_o$ was changed (Fig. 5 and

TABLE VI
OSMOTIC COEFFICIENTS OF PROTEINS
AT 10 G/100 ML SOLUTION*

Protein	ϕ
Horse hemoglobin	1.36
Horse myogen	1.25
Horse serum albumin	1.54
Horse serum globulin	1.39
Bovine serum albumin	2.45

* Dick, D. A. T. 1959. *Int. Rev. Cytol.* 8:387.

Table III). A detailed report of more comprehensive experiments confirming these findings is now in preparation.²

This procedure provides a way of estimating α_m when cation-selective microelectrodes are not available or not applicable to a given cell system. It can be used to verify that $\phi_m = 1/\alpha_m$ when the osmotic behavior of the cell system is known.

Several investigators have reported M_p vs. $[\text{K}^+]_o$ plots for a variety of muscle fibers but few combined their studies with chemical analysis. Hagiwara et al. (24) reported two M_p vs. $[\text{K}_m]_o$ curves for barnacle muscle but neither one represented conditions in which $[\text{K}^+]_o$ $[\text{Cl}^-]_o$ was kept constant. The two $[\text{K}_m]_o$ values for these curves were 200 and 330 mM/liter. When Hagiwara's value for $[\text{K}^+] = 157$ mM/liter is combined with the two $[\text{K}^+]_o$ values, one calculates α_m to be between 0.48 and 0.78. Dunham and Gainer (25) reported on a M_p vs. $[\text{K}^+]_o$ curve for lobster muscle which yields a $[\text{K}^+]_o$ value of 240 mM/liter. They also reported a $[\text{K}^+]$ of 155 mM/liter for the single fibers under normal conditions. If one combines these numbers, α_m becomes 0.65 which is

² Gayton, D. C., and J. A. M. Hinke. In preparation.

in excellent agreement with estimates of α_m for the barnacle muscle (Table V). Other curves for lobster muscle (26) yielded $[K^+]_o$ values between 240 and 270 but no $[K^+]_i$ values were included to permit an α_m calculation.

The osmotically inactive volume $[V_f/(V_f)_o]_o$ has been determined for several single fiber preparations (Table VII). These fractions have been used to calculate an α_m (using equation 9) which leads in turn to ϕ_m and Ponder's R values for each of the fibers. Since reliable data were not available on e' , a , and d_f for each preparation, the value of 1.3 was assigned to the factor, $(1 - e')/d_f(a - e')$, in equation 9 and d_f was made equal to 1.06 for all fibers. Thus, all the (α_m) values in Table VII are relative to $\alpha_m = 0.66$ for the barnacle muscle. Only the α_m value of 1.17 for lobster muscle, based on a

TABLE VII
OSMOTIC VOLUME OF SINGLE FIBERS AND $(\alpha_m)'$

Muscle type	$(V_f/(V_f)_o)_o$	Reference	$(\alpha_m)'$	$\frac{1}{(\alpha_m)'} = \phi_m$
Frog	0.35	16, 20, 21	0.85	1.18
Crayfish	0.40	17	0.78	1.28
Barnacle	0.49	—	0.66	1.51
Blue crab	0.33	27	0.87	1.15
Lobster	0.10	26	1.17	0.85

$\alpha_m = (\alpha_m)'dw$, where dw is the density of the osmotic volume $(V-b)$ of the fiber. Also $(\alpha_m)'$ equals Ponder's R .

* Equation (9), assuming $\frac{(1 - e')}{(d_f(a - e'))} = 1.3$ for all fibers.

$[V_f/(V_f)_o]_o$ of 0.1 (27), is unreasonable because it exceeds unity. The α_m value for frog muscle predicts that about 15% of intrafiber water is not osmotically active. This estimate conforms with most interpretations of the osmotic behavior of the frog muscle (16, 20, 21).

Lev (28) measured the $(K^+)_m$ of frog muscles by means of K^+ glass micro-electrodes and found it to be about 130 mM/liter when $[K^+]_o$ was about 125 mM/kg fiber water. For these numbers to give the same α_m as in Table VII (0.85), one has to postulate that about 10% of the fiber K^+ is bound, i.e., $B_K = 0.1 V[K^+]_o$. Thus, using equation 1:

$$\alpha_m = \frac{V[K^+]_o - B_K}{(K^+)_m} = \frac{0.9 V[K^+]_o}{(K^+)_m} = 0.86$$

Another estimation of α_m for frog muscle is possible from an M_p vs. $[K^+]_o$ curve by Hodgkin and Horowicz (29) in which $[K^+]_o[Cl^-]_o$ was constant in the solutions. If one concentrates only on the equilibrium crosses of their Fig. 4 a line can be drawn with a slope of 55 mv and $[K^+]_o$ of about 150 (their line had a slope of 58 mv and $[K^+]_o = 140$). If Adrian's value of 139 mM/kg fiber

water is accepted for $[K^+]$ (30) and if 10% of fiber K^+ is assumed to be bound as with Lev's data, then $\alpha_m = 0.84$ using equation 1.

Neither of these two calculations should be taken seriously enough to be invoked as evidence in favor of 10% K^+ binding inside frog muscle fibers. This small correction may be based equally well on the supposition that extracellular space was badly estimated in both cases. Neither Adrian (30) nor Lev (28) gave evidence that extracellular space was measured in their studies. Since Tasker et al. (15) have demonstrated how widely extracellular space can vary in the toad sartorius muscle, one cannot really select an average value in order to convert a tissue concentration to a fiber concentration without introducing a significant error. Hopefully, these speculations on the state of fiber water and K^+ will stimulate a careful study in which $[K^+]$ and e' are carefully measured on single fibers subjected to methods V and VI. Methods I to IV may not be necessary to verify that $\alpha_m = 1/\phi_m$ in frog muscle as well as in barnacle muscle.

In summary, strong evidence has been presented which defines the solvent water of myoplasm of the barnacle muscle as 68% of analyzable water. Of the remaining 32%, 25% of the fiber water does not appear to operate as solvent water for solutes and may be comparable to that fraction of muscle water which appears "bound" by NMR measurements (5, 6).

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