Osmotic Properties of Amphibian Muscles

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ABSTRACT Changes in the volume of fiber water in hypotonic and hypertonic Ringer's solution were determined for the sartorius, stomach, and cardiac muscle of the frog using two methods. Loss of water in hypertonic solutions was nearly the same in all muscles, but swelling in hypotonic solutions was greatest in the sartorius, smallest in the heart. For the sartorius the deviation from the properties of an osmometer can be accounted for by a loss of electrolyte and by assuming that a small part of the fiber water is bound, but this appears insufficient to explain the behavior of stomach and cardiac muscle in hypotonic solutions. In very dilute solutions of $CaCl_2$ and $MgCl_2$ a large difference in concentration of electrolytes is maintained between the fibers and the medium. Under these conditions divalent cations, accumulating in the fibers, produce a change in physical properties which indicates increased internal cross-linking. It is suggested, therefore, that swelling is limited as in a gel and that a considerable hydrostatic pressure may develop within the fibers.

It has been demonstrated (9, 13, 16, 18) that muscle fibers gain or lose less water in solutions of different osmotic pressures than expected from an osmometer. An understanding of this deviation would give information on the physicochemical properties of the cells. To throw light on this problem the osmotic properties of skeletal, cardiac, and smooth muscles were compared. The last two of these types of muscle, which do not seem to have been used by others for such investigations, were studied because of some anomalous osmotic phenomena previously reported (3).

To determine osmotic movements of water not only the volume of the whole muscle, but also that of the extracellular space must be known. In the first such measurements by Fenn (9) chloride was used for this purpose. It is now known that this ion is not entirely extracellular, but the results obtained agree with those reported by others (9, 18) and in the present paper. The osmotic properties of skeletal muscle have recently also been determined by measuring the diameter of isolated fibers (14).

The results reported here confirm that the osmotic properties of skeletal muscle can be explained as a simple osmotic process if one takes into consideration small losses of solutes by the fibers and possibly binding of a small part of the fiber water. In other types of muscle, swelling in hypotonic solutions

is surprisingly small. Evidence that it is limited mechanically as in a gel is presented especially for muscles immersed in very dilute solutions of $MgCl_2$ and $CaCl_2$. Ernst (8) and Ling (11), who assume that most of the K in the fibers is bound, have explained the volume changes of skeletal muscles as being like those of a gel.

METHODS

Tissues Stomach muscle, the ventricle of the heart, and the sartorius of the frog (*Rana pipiens*) were used. Stomach muscle was prepared as described previously (3, 5). The isolated ventricle was opened by a longitudinal incision on one side. The region of the origin of the aorta and the valves was removed and several cuts were made to open the large sinuses. Inulin space was on the average smaller and less variable than in the other muscles used. Before weighing the muscles were blotted according to a standardized procedure, the sartorius for 10 seconds, the heart for 1 minute, stomach muscle for 2 minutes, except when noted otherwise. The dry weight of the sartorius and stomach muscle was assumed to be 20 per cent of net weight; that of the ventricle was on the average 16.8 per cent in fresh preparations, but rose to about 19 per cent after a few hours. Because some dilute solutions produced a contracture, weighing experiments with stomach and cardiac muscle were usually carried out with rings stretched over a small frame to prevent shortening (5).

Measurement of Fiber Volume In one of the methods used, not applicable to cardiac muscle, weight and inulin space were determined in two matched muscles, in one after bathing in an experimental solution, while the other was kept in Ringer's solution throughout. The change in fiber volume was determined by subtracting the change in extracellular water from the difference in wet weight between the muscles. In contrast to the other muscles used, inulin space of cardiac muscle was on the average not significantly different in the solutions used; it was 18.8 ± 1.8 per cent in normal Ringer's solution, 17.5 ± 1.8 per cent in solutions with twice the normal osmotic pressure, and 18.2 ± 2.3 per cent in solutions with half of normal osmotic pressure. Inulin space was determined by immersing the tissues in solutions containing 1 per cent inulin for an hour, extracting with Ringer's solution for 90 minutes, and determining inulin in the extract by the method of Bacon and Bell (1).

In another method, which will be called the dilution method, muscles were first equilibrated in Ringer's solution containing radioactive dextran, then in the experimental solution, to which also dextran was added. The gain or loss of water by the muscle fibers, w, was computed from the change in the concentration of dextran in the experimental solution due to the movement of water using the equation

$$w = (v + e) \cdot \left(\frac{c_1}{c_2} - 1\right)$$

where v is the volume of experimental solution, e the extracellular space of the muscle, c_1 and c_2 the counts of the solution before and after equilibration.

In this method the count of the experimental solution is assumed to equal that of the Ringer's solution after equilibration because otherwise the former is altered by exchange with the extracellular space of the tissue. Since this condition cannot be

fulfilled accurately, a correction was made by adding the excess or deficit of radioactivity in the extracellular space to the radioactivity of the total volume of experimental solution ($v + e$), but this changed the final result generally less than 5 per cent. The value for e was also obtained by dilution technique, from the change in the concentration of dextran during the equilibration in Ringer's solution. The concentration of dextran, as well as that of inulin, was calculated on the basis of the weight before immersion in the experimental solution.

The procedure was as follows. Into small siliconized vials (about 2 ml) containing 0.4 ml Ringer's solution with added radioactive dextran were brought 200 to 300 mg tissue, usually 3 to 4 sartorii or stomach halves or 4 ventricles. The vials were tightly stoppered or placed into a closed glass bottle filled with water-saturated oxygen. The count of the solution before and after equilibration for 3 hours was determined. After blotting and weighing the muscles were brought into another small vial containing 0.4 ml of experimental solution for 2 hours. The amount of dextran added was chosen so that its concentration equalled the expected concentration of the first solution after equilibration. In practice the concentrations generally agreed within 1 per cent. The count of the experimental solution was determined before and after equilibration. The final tonicity of the experimental solution was calculated taking into consideration the amount of extracellular fluid and the gain or loss of water. During equilibration the vials were kept in a water bath slightly below room temperature, at about 22°C, and were agitated by an automatic shaker.

The time required for equilibration can be judged from the efflux of inulin and dextran. After equilibration in Ringer's solution containing inulin or dextran the sartorius retained on the average 1.2 per cent inulin and 4.4 per cent dextran, stomach muscle 0.6 per cent inulin and 2.8 per cent dextran after washing for 1 hour. In the ventricle equilibration was completed within 20 minutes. Therefore, it was considered satisfactory to equilibrate with inulin for 1 hour, with dextran for 3 hours. In the dilution method the second period of equilibration was made briefer. To test whether 2 hours were sufficient 9 groups of sartorii were equilibrated in the experimental solution for 150 minutes while the companion groups were in this solution for only 60, 90, or 120 minutes. No significant differences between paired groups were found if equilibration lasted for 90 or more minutes.

Techniques For counting 0.1 ml solution was brought into a counting vial with a Hamilton syringe. After adding 0.9 ml water and 8 ml scintillation fluid (6) radioactivity was determined by a liquid scintillation counter (Tricarb, Packard Instrument Co.), usually for 3 or 5 minutes. The same syringe was used in succession for all solutions; it was flushed out carefully with each solution before delivery. Counts of different samples of the same solution had a standard deviation (SD) of 0.38 per cent. Solutions before equilibration were made in quadruplicate, after equilibration in duplicate. The counts diminished on the average about 4 per cent in the first hour, more slowly later, because of deposition of dextran on the glass. Therefore, counting of each sample was started immediately after the scintillation fluid was added. Dextran-carboxyl-C^{14} with a molecular weight of 15,000 to 17,000, referred to here as dextran, was obtained from New England Nuclear Corp., Boston. To remove low molecular weight impurities which might penetrate into the fibers the material was extracted with methanol before it was dissolved in water.

For flame photometry the muscles were extracted in diluted acetic acid as recommended by Steinbach (17).

Solutions Ringer's solution contained in millimoles per liter: NaCl 111, KCl 3, $CaCl_2$ 1.8, $MgCl_2$ 0.6, and phosphate 2 (pH 7). Hypertonic Ringer's solution was made by adding NaCl, hypotonic solution by lowering the amount of NaCl, or by diluting normal Ringer's solution. Osmotic pressures of the solutions will be expressed as multiples of that of Ringer's solution. This factor will be called tonicity. All experiments were carried out near room temperature (22 to 25°C).

RESULTS

a. Volume Change Swelling was much less in stomach and cardiac muscle than in the sartorius, while hypertonic solutions produced about the same effect in all types of muscle (Fig. 1). In agreement with Fenn (9) and Tasker *et al.* (18) extracellular space of the sartorius was increased in hypertonic, decreased in hypotonic solutions, but in stomach muscle extracellular space was diminished in hypotonic as well as in hypertonic solutions, probably due to a weak contracture. As mentioned above, extracellular space of the heart remained on the average nearly constant in all solutions. Recently it has been reported that in hypertonic solutions extracellular space of the sartorius remains unchanged and that shrinking is slightly in excess of that of an osmometer (7). This result may be due to the use of sucrose, a substance which has been found to penetrate into the fibers to some extent (2).

In very dilute solutions of $CaCl_2$ or $MgCl_2$ (1 to 4 mM) stomach muscle swells much less than in diluted Ringer's solution of the same osmotic pressure (3). Swelling was even smaller in cardiac muscle (Fig. 2); it was rather variable and often followed by shrinking below the original weight. These volume changes were not reversible because after return into Ringer's solution permeability to electrolytes was severely altered (3). The results were the same whether the muscles were allowed to shorten or not.

b. Electrolytes Changes in concentration of Na and K in the sartorius and stomach muscle were determined by comparing controls with matched groups of muscles equilibrated in experimental solutions. In the heart the difference between concentration of muscles immersed in experimental solutions and a control group kept in Ringer's solution was determined. Intracellular concentrations were calculated on the basis of the weight before transfer to the experimental solutions assuming that fiber water is 60 per cent of net weight in the sartorius (extracellular water 20 per cent), 53 per cent in the stomach (extracellular water 27 per cent), and 63 per cent in the heart (extracellular water 18 per cent).

As observed in the sartorius by Steinbach (16) strongly hypotonic solutions produced a loss of K; it was smallest in the sartorius. A small amount of K may also escape in strongly hypertonic solutions (Table I).

In dilute solutions of $CaCl_2$ and $MgCl_2$ stomach and cardiac muscle lost

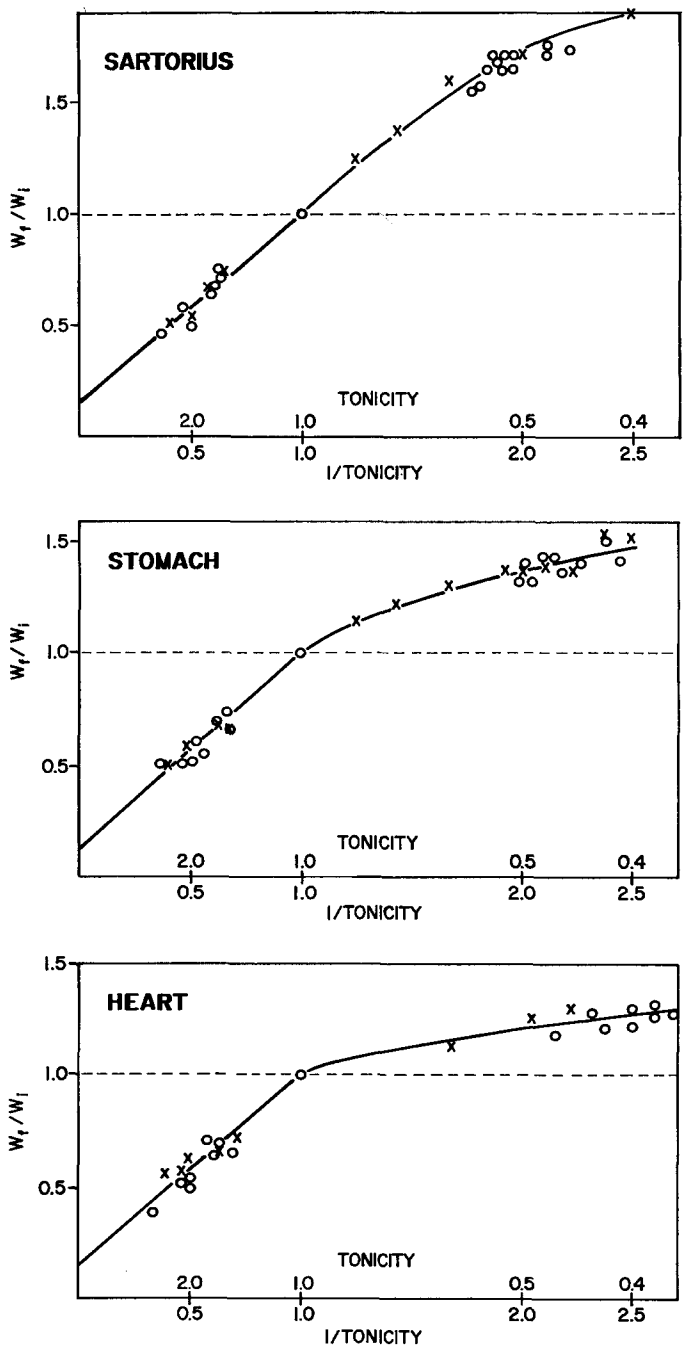

FIGURE 1. Change in volume of fiber water with change in concentration of Ringer's solution expressed as tonicity. Ordinate, ratio of final volume of fiber water (W_f) to initial volume (W_i). Circles, values obtained with the dilution method. Crosses, averages of 6 to 8 determinations obtained by comparing fiber volume in normal with that in modified Ringer's solution. SD was on the average 0.08.

about half of their K in 1 hour. The loss was diminished if the muscles were first immersed for a few minutes in more concentrated solutions of these salts. The concentration of the cations remaining under such conditions, computed on the basis of the final weight of the muscles, was many

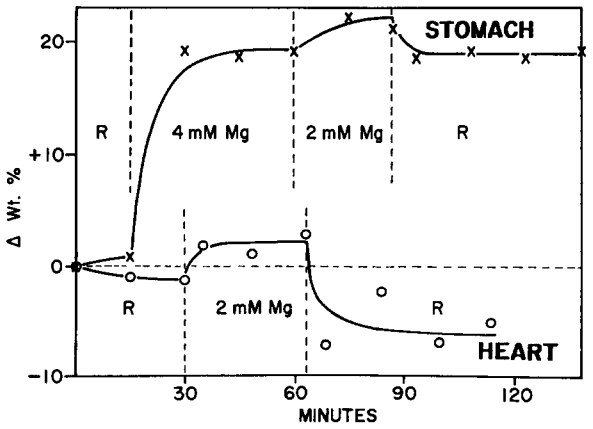

FIGURE 2. Effect of dilute solutions of $MgCl_2$ on weight of stomach and cardiac muscle. Ordinate, difference between final and initial weight given as per cent of initial weight. *R*, Ringer's solution. Before each weighing muscles were blotted for 30 seconds.

TABLE I
LOSS OF INTRACELLULAR K IN HYPERTONIC AND HYPOTONIC RINGER'S SOLUTION

Tonicity	Sartorius	Stomach muscle	Heart
2.0 (8)	3±2	2±3	4±3
0.8 (6)	1±3	2±3	3±4
0.7 (8)	5±4	5±3	5±3
0.5 (12)	8±3	16±6	13±6
0.4 (12)	9±5	20±6	17±4

Values are differences in concentration of K between controls and muscles kept in modified Ringer's solution for 1 hour, expressed as milliequivalents per liter fiber water (averages ± SD). In parentheses, number of determinations.

times higher than that of the electrolytes in the medium (Table II). The muscles were not in a state of equilibrium in these experiments because of a slow loss of electrolytes. However, because equilibration with inulin is practically completed within an hour it can be assumed that the concentration in the extracellular space approaches that in the medium within a short time.

DISCUSSION

That volume changes in hypotonic and hypertonic solutions are smaller than those of an osmometer can be due to several causes. Movement of solutes is

important, as demonstrated by the loss of K in strongly hypotonic solutions. How large the net transfer of solutes would have to be to explain the deviation from the behavior of an osmometer can be calculated as follows: The amount of solute in the fibers can be expressed as $s = k \cdot v.p$, where s is the original amount of solute, k a constant, v the volume of fiber water, and p the osmotic pressure. If osmotic pressure changes to $a.\ p$, volume to b, v, the amount of solute after equilibration is $s' = k.a.v.b.p = a.b.s.$ To give an example, the volume of fiber water of the sartorius increased by a factor of 1.75 in 50 per cent Ringer's solution. Therefore, the total amount of solute should be $0.5 \times 1.75 \times 240 = 210$ meq, a diminution by 30 meq while the loss of K alone was 8 meq per liter fiber water. On the other hand, the loss of solutes

TABLE II

EFFECT OF IMMERSING STOMACH AND CARDIAC MUSCLE IN 1 mM $MgCl_2$ ON CONCENTRATION OF Na AND K AND ON WEIGHT

	Stomach muscle					Heart				
	Na	K	Na + K	Δ Weight per cent	*n*	Na	K	Na + K	Δ Weight per cent	*n*
Ringer's	43±3	106±6	149±6		16	57±3	90±4	147±3		10
$MgCl_2$, 30 min.	8±2	67±4	75±4	20±4	10	15±4	61±4	76±4	14±3	6
$MgCl_2$, 60 min.	7±2	47±5	54±4	24±3	10	8±2	48±3	56±3	22±4	6

First line, Na and K concentrations (averages ± SD) of muscles immersed in Ringer's solution for 2 to 3 hours. Second and third lines refer to muscles which were in 10 mM $MgCl_2$ for 5 minutes, then immersed in 1 mM $MgCl_2$ for 30 or 60 minutes. Concentrations given as milliequivalents per liter fiber water at the end of experiment. Δ weight is the average percentage increase in weight in $MgCl_2$ solution; *n*, number of determinations.

would have to be 75 meq in smooth muscle and 95 meq in cardiac muscle, while only respectively 10 and 13 meq K escaped.

Another factor which may reduce volume changes is binding of water. From experiments on single striated muscle fibers of the frog (14) the non-solvent space has been estimated to be about 25 per cent of the volume of fiber water, but in our experiments on the sartorius the intercept of the volume-tonicity relation curve with the volume axis was only 0.15. The volume of red cells in solutions of different tonicity can be explained quantitatively by assuming a non-solvent space of 20 per cent (15). This value corresponds to a binding of about 30 ml water per 100 gm hemoglobin and agrees with other estimates of water bound by hemoglobin and proteins generally. If the lower protein content of muscle is taken into consideration bound water would be expected to be about 9 per cent of fiber water. Bound water and loss of solutes together may explain the movement of water in the sartorius but they seem insufficient for cardiac and stomach muscle. The assumption that swelling of these muscles is much smaller because a

larger part of the water is bound is contradicted by the fact that shrinking in hypertonic solutions is not very different in the three types of muscles.

In cardiac and smooth muscle swelling may be limited also by internal structure. Whether this applies to normal tissues is not established. The fact that smooth and cardiac muscle swell only slightly or not at all in isosmotic solutions with high concentrations of KCl (3, 15) could be explained on this basis. However, in very dilute solutions of $CaCl_2$ and $MgCl_2$ a large difference in osmotic pressure between the fibers and the medium is maintained for a long time as shown by the fact that the concentration in the fibers of Na and K alone is many times greater than the concentration of electrolytes in the medium. The muscles swell only moderately in such solutions probably because Ca, presumably also Mg, penetrate rapidly into the fibers (4) and form strong cross-links. That these metals can transform the fibers into a stiff gel is shown directly for stomach muscle by a marked change in physical properties, by an increase in opacity, and by the observation that stress relaxation becomes many times slower (5) . That accumulation of Ca causes increased cross-linking is also confirmed by the shrinking of the fibers in isosmotic sucrose solution containing small amounts of Ca (3). Cross-linking probably explains also that excitability and contractility can be restored after muscles have been bathed for an hour or longer in the dilute solutions mentioned (5), even in pure water (unpublished). In contrast to this the sartorius swells rapidly in such solutions and becomes irreversibly inexcitable within 15 minutes. The difference probably is in part due to the fact that Ca penetrates much less readily into this muscle than into smooth and cardiac muscle (10).

The assumption that swelling is limited by a gel structure implies that inside the fibers a considerable hydrostatic pressure can be produced which balances the osmotic pressure of the solutes. Because in 1 mM $CaCl_2$ or $MgCl_2$ the concentration of Na and K combined is about 75 meq per liter fiber water after 30 minutes, 56 meq after 60 minutes, the pressure due to these ions alone would be respectively about 1.6 and 1.1 atmospheres if the activity coefficient of the ions is the same as in the medium. Several factors may contribute to this pressure. Contracture induced by dilute solutions does not seem to be a significant factor because the effects were the same whether the muscles were allowed to shorten or not. Passive stretching of bonds probably is also not the principal factor because in dilute solutions of $CaCl_2$ or $MgCl_2$ swelling of cardiac muscle often is transient and is followed by shrinking. Accumulation of Ca in isosmotic sucrose solution containing traces of Ca also is associated with shrinking of the fibers (3). It seems, therefore, necessary to assume that the divalent cations while establishing new cross-links cause a syneresis of the cytoplasm.

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