

Volume Control by Muscle Fibers of the Blue Crab

Volume readjustment in hypotonic salines

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ABSTRACT Single isolated muscle fibers from the walking legs of the blue crab, *Callinectes sapidus* act as Boyle-van't Hoff osmometers with an osmotically inactive volume of 33 %. Fibers in hypotonic salines undergo a spontaneous volume readjustment toward the initial volumes of the cells found in isotonic salines. The volume readjustment is initiated by the increase in cell volume in hypotonic salines and appears to be dependent on the duration of exposure of the fiber to external sodium, the sodium concentration, and the pH of the external medium. The volume-readjusted cells continue to behave as osmometers, but with an increased relative osmotically inactive volume and a decreased internal resistivity. The decreases in cell volumes appear to be, in large part, due to losses of osmotically active nonelectrolytes from the cells.

INTRODUCTION

This study concentrates on a unique volume response of blue crab muscle fibers to hypotonic salines. When single fibers isolated from vertebrate muscle (Reuben et al., 1963; Blinks, 1965) or from invertebrate muscle (Reuben et al., 1964; Gainer and Grundfest, 1968) are placed into hypotonic salines, their volumes usually increase to new steady-state levels which remain constant until the fibers are returned to their control salines (after which they approach their initial volumes). However, when muscle fibers from *Callinectes sapidus* are placed in hypotonic salines, their volumes increase to new steady-state levels, but then with time spontaneously decrease towards their initial volumes in control salines. Upon return to control salines their volumes fall below their initial values. These volume responses of blue crab muscle fibers to hypotonic salines will be referred to as "volume readjustments."

A few other examples of volume readjustment have been reported in the literature. Kitching (1934) found that the protozoan, *Cothurnia curvula*, occasionally exhibited volume readjustment. Reuben et al. (1963) and Grundfest

(1967) demonstrated apparent volume readjustment in frog muscles and lobster axons, respectively. However, in contrast to this work on blue crab muscles, the latter findings were only obtained in the absence of external chloride in the salines. Fugelli (1967) observed volume readjustment in flounder erythrocytes placed in diluted blood plasma, and attributed the volume decreases to the loss of ninhydrin-positive substances from the cells. While volume readjustment has never before been described for crab muscles, there have been several reports which were suggestive of this phenomenon. For example, Shaw (1958) demonstrated that when the green crab, *Carcinus maenas*, was removed from 100% seawater and adapted to 40% seawater, the organic constituents of the muscles (mainly α -amino nitrogen compounds, trimethylamine oxide, and betaine) decreased more than could be explained by dilution alone. Furthermore, Duchateau et al. (1959) from similar experiments on *Carcinus maenas* muscles found that the major loss during adaptation was from the free α -amino acid pool, in particular the loss of glycine.

None of the above studies on crab muscles have related the volume decreases of cells in hypotonic solutions to the intracellular solutes lost under these conditions. In our view, both events represent essential aspects of the same process of volume readjustment. This paper characterizes the volume readjustment phenomenon in the crab muscle fibers by various osmotic and electrophysiological experiments. A future paper¹ will analyze some chemical changes in the fibers correlated with volume readjustment. A preliminary report of some of this work has been published (Lang and Gainer, 1968).

MATERIALS AND METHODS

Animals

Muscle fibers from the Chesapeake Bay blue crab, *Callinectes sapidus* Rathbun, were used. Crabs ranging in size from 12 to 17 cm across the lateral spines were obtained throughout the year from commercial dealers at the Maine Avenue Wharf, Washington, D.C. In preliminary experiments it was determined that the season, sex, and size of the animals had no effect on the osmotic behavior of their muscle fibers, and the muscle fibers were studied without regard to these factors. The crabs were kept in the laboratory in 50–75% artificial seawater (Rila Marine mix, Rila Products, Teaneck, N. J.). The seawater was well-aerated, and its temperature ranged between 19° and 21°C. The animals were not fed while in the laboratory. They were equilibrated to laboratory conditions for at least 2 days before use in the experiments. Preliminary tests showed that the experimental results were not influenced by maintaining the crabs in artificial vs. natural bay water, by feeding or starving them, nor by the length of time that they were equilibrated to the laboratory conditions.

¹Gainer, H., and Lang, M. A. Volume control by muscle fibers of the blue crab. Evidence against the sodium-gradient hypothesis of amino acid transport. Data to be published.

Solutions

The control saline contained 460 mM/liter NaCl, 10 mM/liter KCl, 20 mM/liter CaCl₂, 9.5 mM/liter MgCl₂, 5.8 mM/liter H₃BO₃ and was adjusted to a pH of 7.4 ± 0.1 with NaOH. All other salines were variations of this basic composition and will be discussed in the Results section with reference to specific experiments. Each saline will be described in the text according to its osmotic pressure (π_2) relative to that of the control saline (π_1) and its most abundant cation. For example, the saline made hyposmotic by removal of 174.4 mM/liter NaCl from the control saline had a relative osmotic pres-

TABLE I
COMPARISON OF MEASURED AND CALCULATED
RELATIVE OSMOTIC PRESSURES

	Relative osmotic pressure (π_1/π_2)	
	Calculated*	Measured†
Hyposmotic salines	1.26	1.26
	1.40	1.42
	1.52	1.52
	1.63	1.62
	1.79	1.79
	2.05	2.06
Hyperosmotic salines	0.74	0.75
	0.79	0.81
	0.65	0.68
	0.48	0.55
	0.32	0.33

*Osmotic pressures were calculated according to Gainer and Grundfest (1968). Hyposmotic salines were made by the reduction of NaCl in the control saline; and hyperosmotic salines were made by the addition of sucrose.

†Osmotic pressures were measured by an Advanced Cryomatic Osmometer, Model 31W (Advanced Instruments, Inc., Newton Highlands, Mass.). The calculated osmotic pressure of the control saline was 967 milliosmols/liter, whereas the measured value was 943 milliosmoles/liter.

sure $\pi_1/\pi_2 = 1.52$ and will be referred to as 1.5-Na-saline. Therefore, control saline will be referred to as 1-Na-saline.

The osmotic pressures of the salines were calculated according to methods described by Dydynska and Wilkie (1963) and Gainer and Grundfest (1968), and with the use of tables of molal osmotic coefficients (Robinson and Stokes, 1959). The validity of this approach was confirmed in the present study by comparing cryoscopic measurements of the relative osmotic pressures of the salines with the relative osmotic pressures which were calculated for the same salines. These data are presented in Table I. While the *absolute* values of the calculated osmotic pressures were slightly greater than the measured osmotic pressures of the same salines, their *relative* osmotic pressures (Table I) were very close.

Preparation of Single Fibers

The experiments were done exclusively on muscle fibers from the musculus adductor carpopoditus (Cochran, 1933) which is found in the meropodite of the first, second, and third periopods (walking legs). The fibers of the musculus adductor carpopoditus have a diameter of $334 \pm 8.8 \mu$ (mean \pm SE) based on measurements from 61 fibers, and their sarcomere lengths ranged between 9 and 10 μ . Hays et al. (1968) have shown that these fibers are intermediate in properties between fast and slow fibers and resemble the so-called "intermediate" fibers described by Atwood (1963). The method of preparation of the single isolated muscle fibers was similar to that described by Reuben, Girardier, and Grundfest (1964).

Measurement of Fiber Volume

Volumetric data were obtained by photographing the single fibers and measuring their diameters (Reuben et al., 1964). Relative fiber volumes were calculated by the following relation:

$$\frac{V_2}{V_1} = \left(\frac{D_2}{D_1}\right)^2$$

where D is the diameter and V is the volume of the fiber. The subscripts 1 and 2 refer to the data obtained in the control and experimental salines, respectively. The above relation assumed that the fiber lengths were constant throughout. The sarcomere lengths of the muscle fibers were monitored during the course of each experiment and checked when the photomicrographs were examined. The only experiments which were accepted as valid were those in which the volume changes of the fibers produced no concurrent changes in the sarcomere lengths. The diameter is not necessarily uniform along the entire length of the fiber. Therefore, a topographical marker was produced in each fiber by leaving some loose tissue (connective or nervous tissue) on the fiber surface. In a given experiment, diameter measurements were always made at the identical known distance from the site indicated by the marker on the fiber. The error of the diameter measurement was about 2%, and the error of the relative volume measurement was less than 5%.

The solutions were changed by passing 50 ml of new solution through the 10 ml chamber by gravitational flow as the old solution was simultaneously removed by suction. A complete change required about 1 min. Isolated fibers were subjected to either short or long equilibration periods before the experiments were initiated. The fibers equilibrated for a short term were used within 2 hr after isolation. These would survive about 10–12 hr at room temperature. The fibers equilibrated for a long term were isolated and soaked in 1-Na-salines or 1-Li-salines for 10–12 hr at 4°C, and then were returned to room temperature for 1 hr to reach thermal equilibrium before the start of the experiments. These fibers survived a total of 24 hr. All the experiments were conducted at room temperature ($20^\circ\text{C} \pm 1^\circ\text{C}$).

Electrophysiological Techniques

Membrane potentials were measured and intracellular currents were passed using conventional glass microelectrodes filled with 3 M KCl. Intracellular potentials were

amplified by a high input impedance, negative capacity preamplifier and displayed on a conventional Tektronix oscilloscope. Only microelectrodes with tip potentials of less than 5 mv were used in these experiments. When potentials were measured from isolated muscle fibers, measurements were also made in regions of nonisolated fibers in the same preparation. These data showed that the isolated condition of the fibers had no effect on their membrane potentials.

RESULTS

The Osmotic Behavior of Isolated Muscle Fibers

Experiments were done which examined the relative volume changes of isolated muscle fibers that had been exposed to salines of varying relative osmotic pressures. The results of these experiments are depicted in Fig. 1 where the steady-state relative volumes (V_2/V_1) of the fibers are plotted against the reciprocals of the relative osmotic pressures (π_1/π_2) of the media.

In contrast to the experiments in isosmotic and hyperosmotic salines in which the cells approached definite steady-state volumes (Fig. 2 A and C) the cells placed into hyposmotic solutions showed more complex responses. In the latter case, the cells rapidly swelled to initial peak volumes which slowly declined with time (Figs. 2 B and 3). These initial peak volumes in the hyposmotic experiments were interpreted as most closely approximating the conditions of osmotic equilibrium comparable to the steady-state volumes found in the hyperosmotic experiments. Hence, the peak relative volumes (between 2–5 min after exposure to the hyposmotic solutions) were plotted in Fig. 1. The nature of the slow declines in fiber volumes in the hyposmotic experiments will be discussed in detail later on. If the cells obey the Boyle-van't Hoff relation, and if the above interpretation was correct, one would expect a straight line in Fig. 1. As can be seen in the figure, the single isolated muscle fiber of *Callinectes sapidus* behaves as an osmometer over a considerable range, with a semipermeable membrane which is effectively impermeable to sodium ions and sucrose. The data in this figure can be represented by $V_2/V_1 = K(\pi_1/\pi_2) + b$ where b represents the relative osmotically inactive space of the fiber (expressed as a fraction of the initial volume, V_1) and K equals a constant equal to the slope of the line. In Fig. 1 the least squares regression line for relative fiber volume (V_2/V_1) vs. relative saline osmotic pressure (π_1/π_2) has a slope equal to 0.65 ± 0.03 which corresponds to the constant K ; and the extrapolated ordinal intercept (α) is equal to 0.33 ± 0.01 which corresponds to b . The values are presented in Fig. 1 with their 95% confidence intervals based on a t -distribution. The 33% osmotically inactive space found in crab fibers was similar to values reported for frog and crayfish muscles (Reuben et al., 1963; Blinks, 1965; Reuben et al., 1964). The Boyle-van't Hoff relation predicts that the regression line should pass through the coordinate point (1,1) for $\pi_1/\pi_2 = 1$ and $V_2/V_1 = 1$. Constraints could be put on the line (Blinks, 1965; Reuben et al., 1964), but

the fact that it passed through the predicted point (within the experimental error) without constraints indicated that the fiber behaved as the same osmometer in both hyposmotic and hyperosmotic directions.

In the hyperosmotic direction the fibers could withstand osmotic stresses as

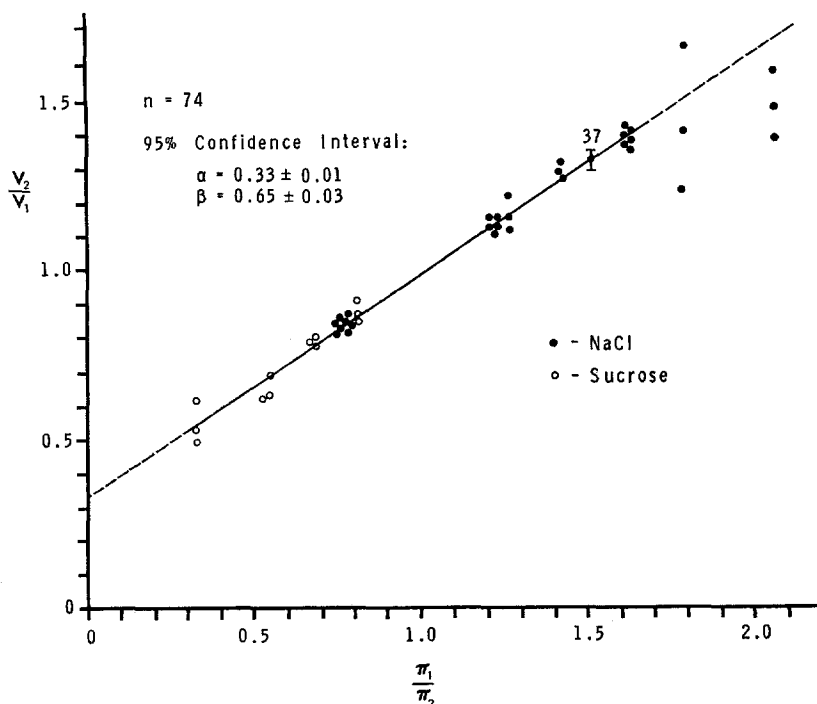


FIGURE 1. Relative volume changes of isolated muscle fibers exposed to solutions of different relative osmotic pressures. Ordinate, ratio of final volume (V_2) to initial volume (V_1). Abscissa, ratio of initial osmotic pressure (π_1) to final osmotic pressure (π_2). The variation of the osmotic pressure was by alteration of the NaCl concentration (closed circles) or by addition of sucrose (open circles). The point at π_1/π_2 equal to 1.52 represents the mean of 37 fibers ± 1 SEM. The broken lines are extrapolations of the solid regression line, based on relative osmotic pressures between $\pi_1/\pi_2 = 0.33$ and $\pi_1/\pi_2 = 1.6$. Beyond this range the fibers usually did not survive the return to the control solution. A total of 74 points was used to determine the regression line. Six additional points are shown on the graph from fibers which had been caused to swell in solutions in which π_1/π_2 was greater than 1.6. These fibers tolerated return to the control solutions, and the data from these fibers appeared to deviate from the regression line based on the other 74 fibers (broken line). This deviation was typical of the nonlinear osmotic behavior observed for other muscle fibers in extreme hypotonic conditions. The slope (β) of the regression line equalled 0.65 and the extrapolated intercept (α) equalled 0.33. Note that the line passes very close to the initial points (1, 1) without any constraints imposed on the line. The data in this figure are from fibers which either had been initially equilibrated in normal salines (π_1) for 1–2 hr at room temperature (20°C), or had been equilibrated in normal salines (π_1) for 10–12 hr at 4°C. This difference in pretreatment of the fibers had no effect on their Boyle-van't Hoff properties.

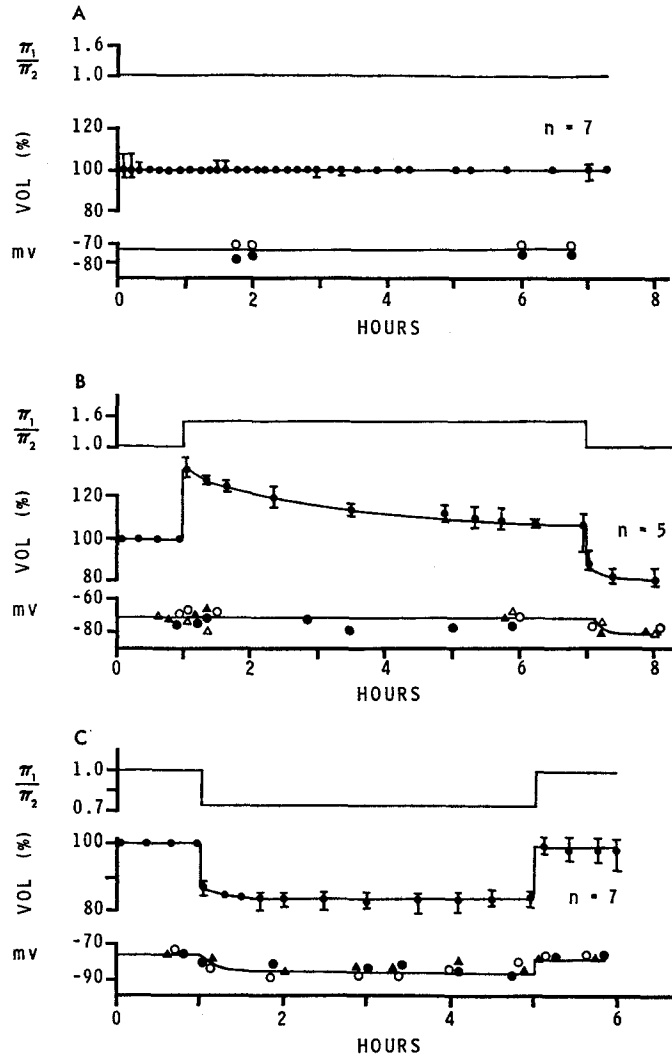


FIGURE 2. Comparison of the fiber volume and membrane resting potential responses to isosmotic, hyposmotic, and hyperosmotic media. A, seven isolated fibers were photographed in 1-Na-saline (control saline) over a period of 7 hr. The membrane resting potentials were measured over the same period for two of the fibers. Both fiber volume and membrane resting potential remained constant during the experimental time period. B, volume responses of isolated muscle fibers to immersion in salines from which sufficient NaCl had been removed to achieve a relative osmotic pressure of $\pi_1/\pi_2 = 1.5$ were measured for 6 hr. Membrane resting potentials were also measured in some of the fibers. Note that the membrane potential remained constant even when the fiber volume increased. When the fibers were returned to 1-Na-salines, their volumes decreased to a level below the initial volumes and the mean resting potential hyperpolarized 7 mv. C, fiber volume and membrane resting potential responses in hyperosmotic salines, in which NaCl was increased to achieve a relative osmotic pressure of $\pi_1/\pi_2 = 0.75$ were followed for 4 hr. The plotted points in A, B, and C represent the means and the vertical bars the ranges of the data.

great as $\pi_1/\pi_2 = 0.33$ and return to control salines without damage. From the data presented in Fig. 1, it was possible to clearly define the limits of safe osmotic and volume stresses of the muscle fibers. The osmometric experiments to be described in the following sections were all done under conditions which did not exceed these limits, and which maintained the cell volumes within the linear range of Fig. 1. The osmotic properties of the cells determined from the

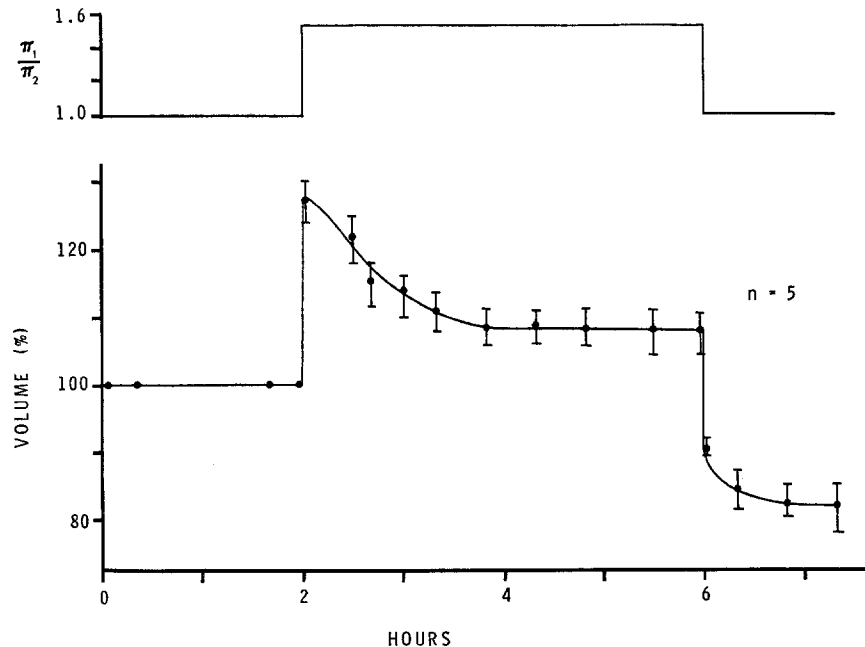


FIGURE 3. Pattern of standard fiber volume readjustment in hyposmotic (hypotonic) saline. Five isolated fibers, which had maintained their volumes constant in 1-Na-salines for 2 hr, were placed in 1.5-Na-salines made hyposmotic by partial removal of NaCl. The initial volume increases corresponded to the expected values determined from Fig. 1. Within 5 min the fiber volumes began to decrease, at first rapidly and then slowly. The decrease in volume (volume readjustment) rarely reached the initial level (100%). When the fibers were returned to 1-Na-salines their volumes fell below the initial level. The plotted points and vertical bars represent means and ranges of the data, respectively.

data depicted in this figure were independent of the specific impermeant solutes (i.e., sucrose and NaCl) which were used in this study. Similar results were obtained in independent studies in which $MgCl_2$, $CaCl_2$, or $LiCl$ were used as the impermeant solutes.

*Effects of Saline Tonicities upon the Volumes and
Electrophysiological Properties of Muscle Fibers*

The data presented in Figs. 2 to 8 are measurements of the rates of changes in relative volumes of single, isolated, muscle fibers which had been placed in

external salines of varying relative osmotic pressures (π_1/π_2). The per cent volume changes were plotted against time, and in some cases the resting membrane potentials of the cells were simultaneously recorded and presented in the figures.

The fibers were challenged by either hyperosmotic, isosmotic, or hyposmotic salines; and typical fiber volume responses to these salines are illustrated in Figs. 2 and 3. As routine, the volumes of the cells were measured in the control salines for 1 or 2 hr in order to guarantee that they remained constant. This provided evidence that the cells were healthy and in osmotic equilibrium with the external salines. The fibers were then placed into the experimental salines and their volumes were measured usually from 3 to 6 hr before they were returned to the isosmotic (control) salines.

When a fiber was placed into hyposmotic Na-saline, the volume invariably rose rapidly to a peak at which it remained for 1–5 min, and then gradually decreased over several hours to a level close to the control volume. When this fiber was returned to the control saline, the fiber volume shrank below the initial level. This phenomenon which is illustrated in Figs. 2 B and 3 was never observed in either control 1-Na-salines (Fig. 2 A), or in hyperosmotic salines (Fig. 2 C).

Hays et al. (1968) showed that *C. sapidus* muscle fibers behaved as if their osmotic and electrophysiological spaces were identical. In that study the resting membrane potentials of the crab fibers could be satisfactorily predicted by the internal and external potassium activities according to the Nernst relation ($E_M = -RT/nF \ln (a_{K_i}/a_{K_o})$) and the fibers appeared to be characterized by a Donnan-like distribution for potassium and chloride. The data presented in Fig. 2 show that the fibers also behaved in accordance with the Nernst equation for potassium in hyperosmotic Na-saline but not in hyposmotic Na-saline. The membrane potentials of the fibers remained constant in isosmotic 1-Na-saline for 7 hr (Fig. 2 A). When placed in 0.75-Na-saline (Fig. 2 C) the total fiber volume decreased by 16%, which indicated that the osmotically active space was decreased by 24%. The measured resting potentials under these conditions hyperpolarized from -76 mv to -84 mv. This hyperpolarization was consistent with the prediction of -83 mv from the Nernst equation which took into account the expected increase in the internal potassium concentration due to the decreased fiber volume. When the fibers were returned to 1-Na-salines, they returned to a resting potential of -76 mv as expected. However, when the fibers were placed into 1.5-Na-salines and swelled (Fig. 2 B), the resting potentials held constant for the entire 6 hr period of volume readjustment. The absence of depolarization of the fibers swelled in hypotonic salines indicates that they deviate from their usual potassium electrode properties. Only when these fibers were returned to the 1-Na-salines, thereby terminating the process of volume readjustment, did the resting potentials

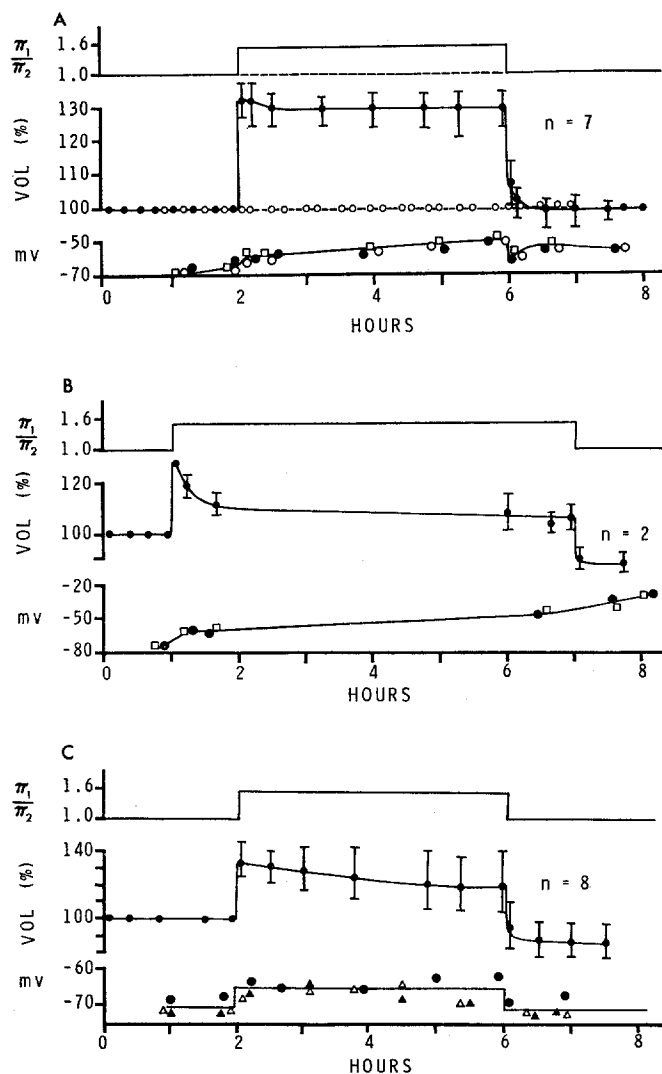


FIGURE 4. The effect of substitution of lithium or magnesium for sodium on volume readjustment. Isolated fibers were treated with salines in which the NaCl was isototically replaced with LiCl (A, B) or $MgCl_2$ (C). A, fibers were placed in 1-Li-salines for 2 hr or more, and then challenged with hypotonic 1.5-Li-salines. The membrane resting potentials were recorded from three of the experimental fibers. The open circles on the vol (%) axis represent data from a single control fiber which remained in 1-Li-saline throughout the experiment. The lithium saline treatment blocked fiber volume readjustment. B, two fibers were placed in 1-Li-salines for 1 hr, and then were placed into 1.5-Li-salines as described in A. In this case, fiber volume readjustment was not blocked. C, fibers were placed in 1.5-Mg-salines for 2 hr or more and then challenged with hypotonic 1.5-Mg-salines. The membrane resting potentials were recorded from three of the fibers. The magnesium treatment tended to inhibit fiber volume readjustment. The average membrane potentials at the peak of swelling for A, B, and C were

change. The resultant hyperpolarization reflected the 18% net decrease in fiber volume.

Due to the fact that the magnitude of the net decrease in fiber volume during volume readjustment was a function of both the size of the osmotic perturbation and time, it was necessary for comparative purposes to establish standard experimental conditions for the volume readjustment experiments. The data in Fig. 3, taken from fibers placed in 1.5-Na-saline for 4 hr, represent the standard volume readjustment response which was used. The average net decrease in fiber volume, based on the relative volumes in 1-Na-salines before and after the standard volume readjustment, was equal to 18%.

The effects of volume readjustment on the cable properties of single isolated muscle fibers were also examined. Six fibers were caused to swell in 1.5-Na-salines (these fibers underwent volume readjustment for 10 hr at 4°C, followed by 1–2 hr at room temperature) and were then returned to 1-Na-salines. Another set of six control fibers was always maintained in 1-Na-salines but otherwise was treated identically to the experimental fibers. The mean decrease in net volume of the experimental fibers equalled 18 ± 3.6 (SD) %, while the control fibers underwent no volume changes. Furthermore, in the experimental fibers only there was a significant hyperpolarization of the resting potential comparable to the data shown in Fig. 2 B. The mean (\pm SD) internal resistance (R_i) of the six experimental fibers ($333 \pm 91 \Omega \cdot \text{cm}$) was significantly less ($P < 0.05$) than that of the six control fibers ($525 \pm 41 \Omega \cdot \text{cm}$). There were no other significant differences in cable constants between the experimental and control groups. In another related experiment, nine fibers underwent volume readjustment in hypotonic Na-salines for 1 hr, were returned to 1-Na-salines for 1 hr, and then were again challenged with the same hypotonic salines. In every case, the first relative volume change was greater than the second, which suggested that volume readjustment produced an increase in the relatively inactive (dead) space. This implied that solutes were being lost from the osmotically active space during volume readjustment. The concurrent decrease in internal resistivity described above suggests that the solutes lost from this space were, in large part, nonelectrolytes.

Factors Affecting the Volume Readjustment

The process of volume readjustment was found to be dependent upon the duration of the fiber's exposure to sodium ion, the sodium concentration of the external saline, and the pH of the external saline. The data illustrated in Fig. 4 A and B were obtained when sodium was isosmotically replaced by lithium

equal to -60 mv, -60 mv, and -65 mv, respectively. For A, B, and C the Nernst equation for potassium predicted the values of -55 mv, -65 mv, and -61 mv. The plotted points and vertical bars represent the means and ranges of the data, respectively.

in the external salines.² In Fig. 4 A the fibers were equilibrated in 1-Li-salines for a minimum of 2 hr before they were challenged with hypotonic lithium salines. When placed in the 1.5-Li-salines, the fibers rapidly swelled to stable volumes which, in contrast to the experiments in sodium salines (Figs. 2 B and 3), remained constant for 4 hr. The lack of volume readjustment under these conditions was confirmed by the return of the volumes to their initial levels when the fibers were returned to 1-Li-salines (Fig. 4 A). Also in contrast to the experiments with sodium (Fig. 2 B), in which the resting membrane potential of the fibers remained constant during swelling, the fibers caused to swell in lithium (Fig. 4 A) underwent marked depolarization. The data illustrated in Fig. 4 B indicated that the volume and potential changes during volume readjustment were not necessarily related. When the fibers (Fig. 4 B) were equilibrated in 1-Li-salines for only 1 hr and then placed in hypotonic lithium salines, they clearly exhibited volume readjustment similar to that found in hypotonic sodium salines (Fig. 2 B). However, the membrane potentials did not remain constant as in Fig. 2 B, but underwent considerable depolarization. It was not possible to test the effects on the fibers of immediate replacement of the 1-Na-salines with 1.5-Li-salines, since the fibers invariably contracted violently and died under these conditions. It is not clear at this time why this effect takes place, nor is it clear why the 1 hr exposure to 1-Li-salines, before the transfer of the fibers to 1.5-Li-salines, eliminates this effect.

When the NaCl of the external saline was isosmotically replaced by MgCl₂, the net decrease in fiber volume in the hypotonic 1.5-Mg-saline was only 10% (Fig. 4 C). This was significantly less than the decrease observed under comparable conditions for the control fibers (Fig. 3). In the case of the magnesium experiments (Fig. 4 C), the resting potentials reflected the predicted changes in the internal potassium concentration due to the increase in fiber volume.

The treatment with 1-Li-saline also had an effect on the volume readjustment in 1.5-Na-saline. This effect is presented in Figs. 5 and 6. The fibers in Fig. 5 A were first equilibrated in 1-Li-saline for 10 hr at 4°C, and then for 1 hr at 20°C before they were challenged with hypotonic sodium salines. After 4 hr in the 1.5-Na-saline, the average net volume readjustment of the long-term lithium-treated fibers was only 5%, which was significantly less than the 18% decrease in volume found for the control fibers (Fig. 3). The resting potentials of the fibers depicted in Fig. 6 A were also affected by their immersion in 1.5-Na-salines. In the 1-Li-salines, the resting potentials of the fibers, appeared to be decreasing. However, upon introduction of the 1.5-Na-salines there was a small immediate depolarization (presumably due to the dilution

²The immediate increase in volume of the fibers when they were transferred from 1-Na-saline to 1-Li-saline (Fig. 5 B) reflected the fact that the Li salines which were calculated to be isosmotic with the control Na-salines were actually slightly hyposmotic. Freezing point osmometry showed that the π_1/π_2 of these two salines actually equalled 1.05.

of the internal K^+), and then a slow rise in potential which continued throughout the 4 hr that the fibers were in the hypotonic sodium salines. When the fibers were returned to isosmotic sodium salines (at 5 hr), there was an immediate rise in potential, followed again by a slow hyperpolarization. Although

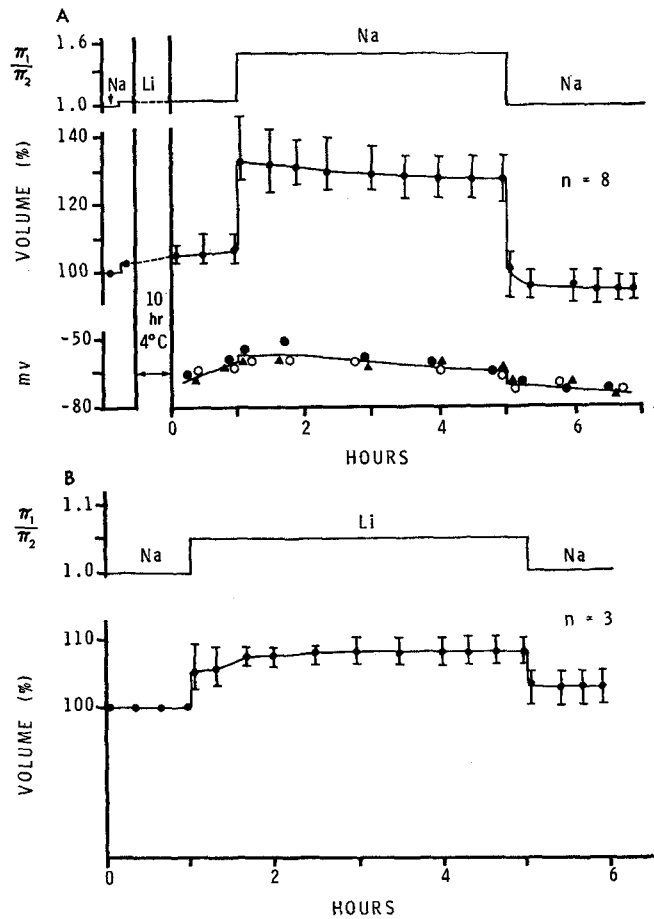


FIGURE 5. Effect of lithium saline on volume readjustment in sodium saline. A, isolated fibers were placed in 1-Li-saline for 10 hr or more, then challenged with hypotonic 1.5-Na-saline. The membrane resting potential was recorded in some of the fibers. Fiber volume readjustment in hypotonic sodium saline was inhibited by the lithium saline treatment. B, fibers in 1-Na-saline were placed in 1-Li-saline for 4 hr and returned to 1-Na-saline. The relative osmotic pressure of the salines was $\pi_1/\pi_2 = 1.05$. This was reflected in the immediate fiber volume changes when the fibers were exposed to a change of solution. The slow rise in volume of the fibers in the lithium salines suggested that LiCl was entering the fiber. This was consistent with the 2% net change in volume when the fibers were returned to 1-Na-saline. No volumetric evidence of a LiCl exit from the fibers was observed. The plotted points and vertical bars represent the means and ranges of the data, respectively.

the evidence is poor at present, these data are suggestive of an active transport mechanism (presumably for Na^+) which is dependent on the external Na^+ , and which can rapidly influence the membrane potential either by a direct electrogenic action or by the rapid exchange of external K^+ for internal Na^+ .

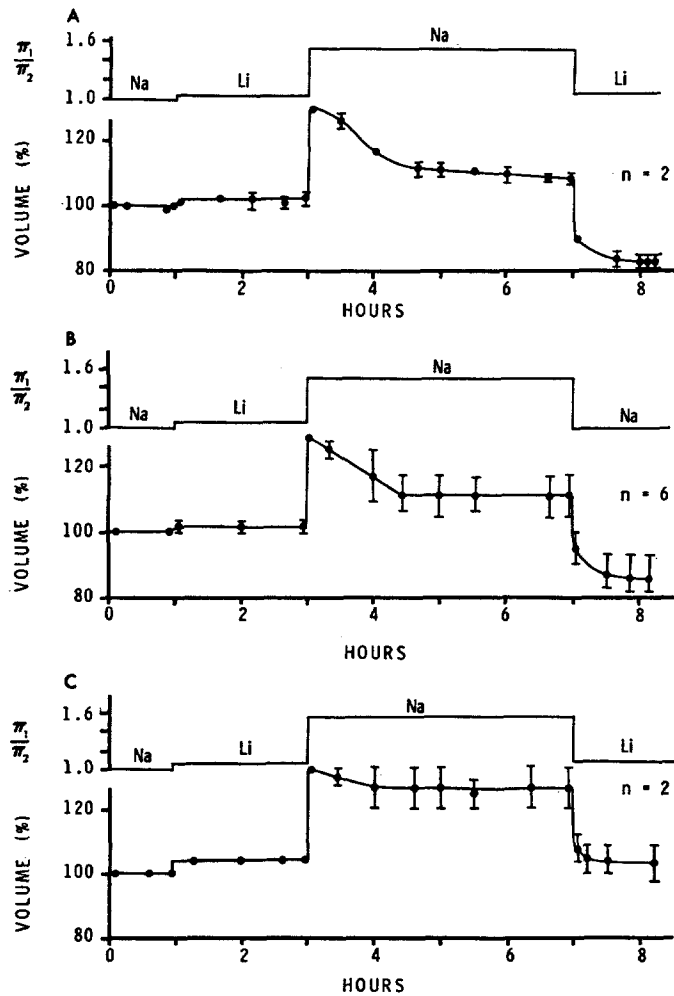


FIGURE 6. Effects of long- and short-term exposures of the fibers to external sodium. Isolated fibers were placed in 1-Li-saline for 2 hr, challenged with hypotonic 1.5-Na-saline for 4 hr, and returned to 1-Na-saline or 1-Li-saline. A and B, the data were taken from fibers equilibrated in 1-Na-saline at 4°C for 10 hr (long-term soaks) before any measurements were made. The fiber volume readjustment process was not influenced by the lithium treatment. C, these fibers were not soaked for an extended period of time as in A and B, but were used soon after isolation (short-term soaks). In this case, the 2-hr exposure to lithium saline significantly inhibited the fiber volume readjustment. The plotted points and vertical bars represent the means and ranges of the data, respectively.

The absence of this hyperpolarizing effect in lithium salines (Fig. 4 A and B) indicated that the Li^+ was replacing Na^+ at some critical site necessary for the active transport process. Furthermore, the situation is complicated by the replacement of internal K^+ by Li^+ after long-term soaks of the fibers in 1-Li-salines (Hays et al., 1968), and a slight (2%) slow increase in the volume of the cells (Fig. 5 A and B) due presumably to the net entry of LiCl under these conditions.

The volume readjustment also appeared to be influenced by the duration of the fiber's exposure to external sodium. The data presented in Fig. 6 were taken from fibers equilibrated in 1-Li-salines for 2 hr before they were placed into hypotonic sodium salines. The experiments depicted in Fig. 6 A and B were done on fibers that had been soaked in 1-Na-salines for 10 hr at 4°C before the 2 hr lithium treatment. The volumes of these fibers were then measured for 1 hr in 1-Na-salines at 20°C. The fibers depicted in Fig. 6 C were not given an extended exposure to the 1-Na-salines, and were used immediately after isolation. The 2 hr 1-Li-saline treatments which followed the 1-Na-salines were able to significantly inhibit volume readjustment in the fibers exposed to sodium for a short term (Fig. 6 C), but had little effect on the fibers exposed to sodium for a long term (Fig. 6 A and B).

The volume readjustment process was also influenced by the pH of the external media. The data illustrated in Fig. 7 A were from fibers which were placed immediately after isolation into 1-Na-salines (pH = 7.4) for 1 hr, then placed into 1-Na-salines (pH = 4.8–5.6) for 2 hr, and then caused to swell in 1.5-Na-salines (pH = 4.8–5.6) for 4 hr before being returned to the 1-Na-salines (pH = 4.8–5.6). The data presented in Fig. 7 B were taken from fibers which had been soaked in 1-Na-salines (pH = 7.4) for 10 hr at 4°C and then then subjected to the same treatment as described above (at 20°C). The acid salines contained 1 mM sodium phosphate buffer. These experiments required a reduction of the calcium chloride concentration of the salines to 1 mM due to the low solubility of calcium phosphate. The osmotic deficit due to the removal of CaCl_2 was made up by the addition of sodium chloride. Preliminary experiments showed that fibers soaked in salines either with 1 mM calcium chloride and borate buffer or 1 mM calcium chloride and phosphate buffer, both at pH = 7.4, did not significantly differ in their kinetics and magnitudes of volume readjustment from controls at normal calcium levels (Fig. 3). Low pH completely blocked volume readjustment in the fibers exposed to sodium for a short term (Fig. 7 A), but only partially blocked it in the fibers exposed to sodium for a long period (Fig. 7 B). The average net volume decrease of the fibers exposed to sodium for a long period in acidic saline was about 10%, which was still significantly less than the net volume readjustment observed in control fibers (Fig. 3) and in fibers exposed to sodium for a long term which were treated for 2 hr in 1-Li-salines (Fig. 6 A and B).

The fiber volumes can also be increased without involving an osmotic stress, by using a permeant solute such as potassium chloride. Fig. 8 illustrates the effect of isosmotic replacement of sodium in the saline by potassium. With the use of the equations developed by Hays et al. (1968), which assumed that the muscle fiber's ionic distribution was based on a Donnan-like distribution for

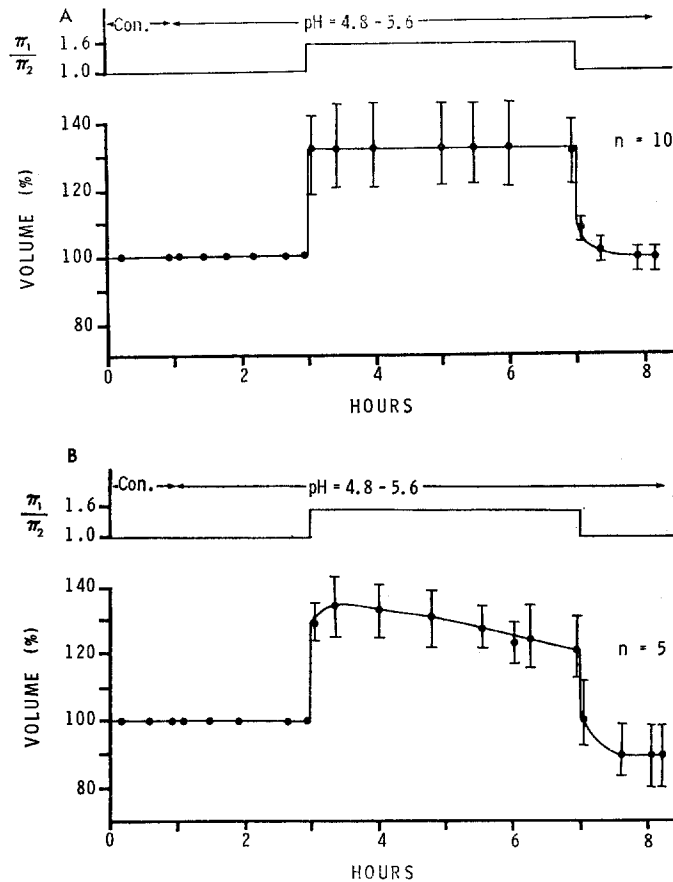


FIGURE 7. Effects of low pH and duration of exposure of the fibers to sodium on the volume readjustment. The sodium saline was made acidic by lowering Ca^{++} to 1 mM and substituting a 1 mM phosphate buffer at $\text{pH} = 4.8$. The fiber's natural buffering capacity tended to raise the pH of the external saline towards a pH of 7.4. Frequent change of external saline maintained the pH of the acidic salines between 4.8 and 5.6. Isolated fibers were transferred from control 1-Na-salines (Con.) at a pH of 7.4, to acidic 1-Na-salines for 2 hr; after which they were challenged with acidic 1.5-Na-saline for 4 hr and finally returned to acidic 1-Na-salines. A, fibers were used within 2 hr after isolation. The acidic salines effectively blocked fiber volume readjustment. B, the fibers were equilibrated in 1-Na-saline at a pH of 7.4 for 10 hr at 4°C before measurements were made at 20°C . The acidic salines significantly inhibited, but did not completely block, the fiber volume readjustment. The points and vertical bars represent the means and ranges of the data, respectively.

potassium and chloride, it was possible to calculate a level of ion substitution (i.e., K^+ for Na^+) which would produce a volume change in the fibers comparable to that produced by the 1.5-Na-salines. The amount of volume readjustment in the experiment depicted in Fig. 8 was significantly less than that found in the control experiment (Fig. 3). Furthermore, the isosmotic potassium saline completely blocked volume readjustment in one fiber, an event which was never observed with the control fibers. Attempts to cause the mus-

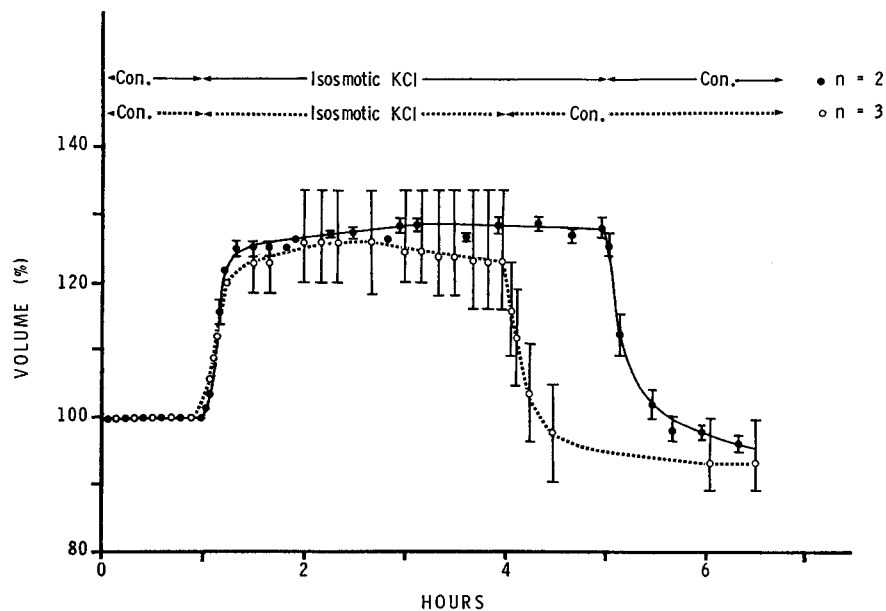


FIGURE 8. Effects of isosmotic potassium salines on the fiber volume readjustment. Single isolated fibers in 1-Na-saline (*Con.*) were challenged with isosmotic, but hypotonic salines in which 101 mM sodium was replaced with potassium. The broken line refers to three fibers observed in the hypotonic saline for 3 hr, and the solid line refers to two fibers followed for 4 hr. The isosmotic potassium salines effectively inhibited fiber volume readjustment. The high potassium saline also had a depolarizing effect which caused a brief (less than 200 msec) contracture in each fiber. The points and vertical bars represent the means and ranges of the data, respectively.

cle fibers to swell by 30% (as in Fig. 8) in salines made isotonic with a nonelectrolyte (glycerol) were unsuccessful. These failures were due to the unexpectedly destructive effects on the fibers of the large amounts of glycerol that had to be added to the salines.

Factors Unrelated to Volume Readjustment

Although calcium ion is known to decrease membrane permeability, it appeared to have no effect on the volume readjustment process. The average volume readjustments found for fibers tested in low calcium (1 mM) and high

calcium (100 mM) salines were not significantly different from the control volume readjustment (Fig. 3), which took place in salines that contained 24 mM of calcium.

DISCUSSION

Muscle fiber volume readjustment in *C. sapidus* appears to be an example of what Schoffeniels (1967) refers to as "isosmotic intracellular regulation" whereby the cells adjust to changes in body fluid osmotic pressure while cellular hydration is kept constant. Maintenance of the osmotic pressures of the body fluids above those of the external medium in freshwater forms and below in marine forms is referred to as "anisomotic regulation." The former has been found in all cases of euryhaline marine invertebrates while the latter has not. Consequently, anisomotic regulation has been characterized by Schoffeniels (1967) as an evolutionary advance over isosmotic intracellular regulation. This would relieve the intracellular mechanism of a difficult task, and afford the euryhaline organism a new mode of adaptation.

While the model of isosmotic intracellular regulation described above implies a decrease in the osmotically active volumes of the cells exposed to hypotonic solutions, this report on volume readjustment is the first to rigorously describe this phenomenon. Thus far, attention has been largely focused on the changes in concentrations of the intracellular osmotically active substances. In crustacean muscles, the only substances which have been shown to be significantly involved in intracellular osmoregulation were the intracellular free amino acids (Potts and Parry, 1964). For example, the analysis of the intracellular osmotically active contents of *Eriocheir sinensis* (Bricteux-Gregoire et al., and *Carcinus maenas* (Shaw, 1958) muscles showed that when animals in full seawater were adapted to diluted seawater, the free amino acids were the only substances which were greatly reduced.

Since volume readjustment in *C. sapidus* muscle fibers produced an increased relative osmotically inactive space, the free amino acids could have either moved from the intracellular to the extracellular space, or have been incorporated into protein. The former is suggested by the work of Fugelli (1967), who found an increase in extracellular ninhydrin-positive substances with the decrease in intracellular ninhydrin-positive substances during volume readjustment in flounder erythrocytes. Schoffeniels (1967) found no change in the electrophoretic pattern of cellular protein during osmotic adaptation of *E. sinensis*. Furthermore in *C. sapidus*, volume readjustment began immediately after the fibers had reached osmotic equilibrium with the hypotonic saline. Hence, it is postulated that free amino acids were transported out of the *C. sapidus* muscle fibers during volume readjustment. Evidence in favor of this hypothesis will be presented in a future report.¹

We wish to thank Dr. Elizabeth A. Hays, Mrs. Jenny R. Zollman, and Dr. Robert Williams for their valuable assistance and discussions.

This work was supported by a United States Public Health Service grant (NB-05043) to Dr. Gainer from the National Institute of Neurological Diseases and Blindness, and by support from Natural Resources Institute, State of Maryland, to Dr. Lang.

Received for publication 13 August 1968.

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