Water Transfer and Cell Structure in Isolated Crayfish Muscle Fibers

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ABSTRACT Changes in volume of crayfish single muscle fibers in response to changes in ionic or electrical conditions have been studied in conjunction with electrophysiological measurements and electron microscopic examinations. The occurrence of at least three mechanisms of water movements is revealed, two being processes which are superimposed on the normal osmotic water movement that results from a change in the concentration of solute in the medium. Differences between the time courses of the changes in volume and potential on changing K_i/K_o indicate that water may be distributed unequally for a time within compartments of the fiber. Electron micrographs reveal a selective accumulation of water at the periphery of the fiber under certain conditions. A correlation of H_2O transfer with a change in membrane potential is apparent in crayfish muscle fibers and is probably due to electroosmotic effects. Electrokinetic water movements are produced whenever the membrane potential is changed to a considerable degree by changing the level of K and/or Cl in the medium, or by applying currents with an intracellular microelectrode. Depolarizations cause shrinkage. Hyperpolarizations or repolarizations cause swelling. The volume changes are independent of the occurrence or absence of swelling in the anion-permselective transverse tubular system. They indicate that the fiber membrane along the surface is heterogeneous, not only with respect to the signs of its fixed charge sites, but also with respect to the sizes and relative permselectivities of these charged channels.

INTRODUCTION

Studies of the movement of ions and water across the cell membrane with correlated osmometric, electrophysiological, and morphological methods may be expected to yield more information on the properties of the membrane and about the structure of the cell than can be obtained with any one

method alone. The work to be described was on cells with a considerable degree of structural differentiation, single fibers isolated from crayfish leg muscles. It has uncovered the occurrence of volume changes under different experimental conditions which deviate from changes predicted on the basis of normal osmosis. Some of these anomalous effects appear to be related to changes in membrane potential, while others appear to be independent of the latter but correlated with morphological changes. Preliminary accounts of some aspects of this work have appeared (26, 27).

Across an uncharged homogeneous membrane water movements that result from changes in the relative concentrations of ionized solutes on the two sides of the membrane can be accounted for by the classical van't Hoff concepts. Water moves by "normal osmosis," from the dilute to the concentrated phase, in proportion to the change in osmotic pressure. On the other hand, water movement that is caused by changing the ionic composition across a fixed charge membrane is further dependent on factors such as the density and signs of the fixed charges, the structure and degree of permselectivity of the membrane, the membrane potential, the ionic species in the two solutions and their concentrations. Deviations thus arise from normal osmotic behavior. Using artificial membranes, these phenomena have been studied by a number of investigators (for references see 20). In particular, work begun by Sollner more than 30 years ago (33) and since continued with various colleagues (15, 16, 25, 35) has advanced considerably the experimental analysis of processes of "anomalous" water movements. Sollner and his colleagues uphold the view of the earlier workers that the effect is largely or exclusively a manifestation of electroosmosis, which is believed to be caused by circulation of local currents between areas of different permselectivity in heteroporous membranes. However, the theoretical analyses of a potential-coupled water flow by Schlögl (31, 32) and Kobatake (22) do not require that the membrane be heterogeneous, but that there should be merely an alteration of the potential across a homogeneous fixed charge boundary.

In systems which have a "mosaic" membrane (34) heterogeneous either as to pore size, or sign of fixed charges, or both, a change in potential across the membrane is also accompanied by circulation of a current (16, 25). Accordingly, the electrokinetic flow of water may also be regarded as an electroosmotic phenomenon under these circumstances. It has already been shown (12), and further evidence will be presented elsewhere (13), that the membrane of crayfish muscle fibers is a complex system, permeable to both K and Cl. To a considerable degree, permeability to Cl is localized at a special region, the transverse tubular system (TTS) which represents invaginations of the cell membrane (4, 12, and in preparation). The experiments to be described indicate that there is indeed a component of water movement in crayfish muscle fibers which is due to electrokinetic effects and which may thus also be termed electroosmosis.

METHODS

The work to be reported encompassed a period of nearly 3 years, and several (but not further identified) species of *Procambarus* were obtained at different seasons. No differences in results could be discerned ascribable either to the seasons or the species. The experiments were usually carried out at room temperatures, both in New York, and during the summer at the Marine Biological Laboratory, Woods Hole. Single fibers dissected from muscles of the walking legs, as already described (12), maintained their structural and functional integrity for long times, is some cases for more than 12 hrs. Their diameters ranged between 100 and 400 μ. All the fibers studied were of the group with 9 to 10 μ sarcomere lengths (12).

A fiber mounted in its chamber was photographed at chosen intervals (Fig. 1) while being subjected to various procedures. The peak changes in diameter obtained under the experimental conditions which were employed were usually a large percentage of the initial value. The diameters of the muscle fibers could be read from the enlarged records with an accuracy of ± 1.5 to 2 per cent. The error in the calculated volume therefore was less than about 5 per cent of the initial level. The fibers are essentially cylindrical over most of their length. However, errors introduced by nonuniformity of structure were minimized by measuring the diameter as it changed during the course of an experiment at a single topographically identified region of the fiber. In a few fibers the measurements were also made at several regions of somewhat different initial diameters. The differences in the calculated volumes were small compared with the error of the measurements. Some of the preparations were subsequently fixed for histological and electron microscopic examination (3, 4, 12, and in preparation).

Electrophysiological studies were carried out simultaneously with the volumetric measurements in some experiments. The stimulating and recording equipment used were standard for the laboratory. The microcapillary electrodes to be inserted into the muscle fibers were usually filled with 3 M KCl. However, for some experiments Cl-free microelectrodes were prepared with K propionate as the electrolyte.

Before an experiment was begun each muscle fiber was equilibrated for at least 2 hrs. in either of two control solutions (Table I), which were differentiated by the presence (A_1) or absence (B_1) of Cl. Magnesium was omitted from the media after experiments had shown that this could be done without affecting any of the parameters studied in the present work or in various other electrophysiological measurements (13). As is also the common practice in experiments on frog muscles (*cf.* discussion by Conway, 6, 7), the control solutions were high in K, containing 20 meq/liter as compared with the standard crayfish saline (5.3 meq/liter). The membrane of crayfish muscle fibers undergoes marked changes in its properties as the external K is increased above 5.3 meq/liter, but these changes are completed when the external K level is 20 meq/liter or higher (13, 26). Furthermore, contractions occur when solutions rich in K are applied to muscle fibers that are initially in the 5.3 meq/liter solution, but they are usually absent after the fibers have been equilibrated in the 20 meq/liter K medium. Absence of contraction was essential for the present work and it could be monitored by the change in sarcomere length which occurs in contracted

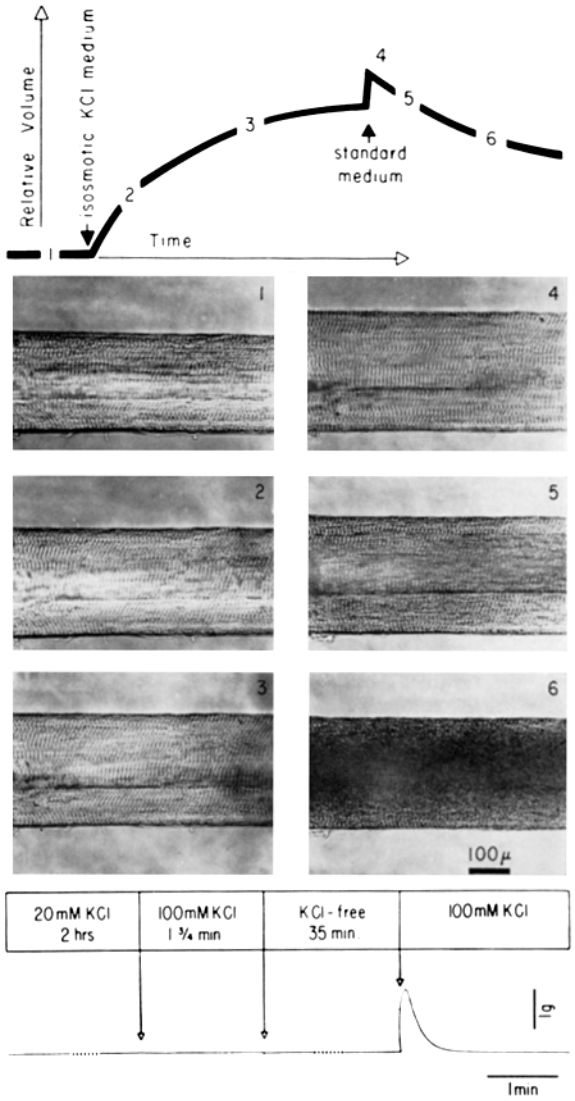

FIGURE 1. *Above*, The method of the volumetric measurements in a single crayfish muscle fiber. The diagram shows the time course of volume change when the fiber is exposed to an isosmotic KCl-enriched medium. The numbers on the diagram correspond approximately to the stages in the series of micrographs which were taken from one experiment. The standard medium, containing 20 mM KCl was replaced with one in which 80 mM KCl substituted for the same amount of NaCl. The swelling in stage 3 was 31 per cent, that in stage 4 was 55 per cent. Stage 5 shows the beginning of the optical change associated with vesiculation of the TTS, and stage 6 shows the change fully developed. *Below*, absence of contraction in a fiber which had been equilibrated in 20 mM KCl, and then exposed to a medium containing 100 mM KCl. Record of tension measurements made with strain gauge and ink recorder. After a brief exposure to the high KCl medium the fiber was soaked for 35 min. in a K-free medium. A second application of 100 mM KCl caused a contraction, the 300 μ fiber exerting a force of 2.6 kg/cm^2.

fibers. Experiments were terminated if a contraction ensued as a result of the experimental manipulations.

Tension measurements on single muscle fibers confirmed the visual observations regarding the absence of contractions under the various experimental conditions employed in the present work. When a fiber is exposed to 20 meq/liter K it contracts and then relaxes, while still in the same medium. Further increase of K does not cause another contraction (Fig. 1) although the fiber is still capable of responding to this challenge if it is first returned for a time to a low K medium. The fibers did not contract when they were challenged by media made hyperosmotic or hyposmotic by increasing or decreasing the Na salt of the medium.

TABLE I

STOCK SOLUTIONS

	Na^+	K^+	Ca^{++}	Cl^-	Propionate	HCO_3^-
	meq/liter	*meq/liter*	*meq/liter*	*meq/liter*	*meq/liter*	*meq/liter*
A. Cl salines						
1. Control	197.6	20	27	242	—	2.6
2. High Na	1,197.6	20	27	1,242	—	2.6
3. High K	197.6	1,020	27	1,242	—	2.6
4. "Zero" Na	2.6	20	27	47	—	2.6
B. Propionate salines						
1. Control	197.6	20	27	—	242	2.6
2. High Na	1,197.6	20	27	—	1,242	2.6
3. High K	197.6	1,020	27	—	1,242	2.6
4. Zero Na	2.6	20	27	—	47	2.6

A comparative study was made (unpublished data) of impermeant anions as substitutes for Cl. Propionate, which was found to be impermeant in a variety of electrophysiological experiments (13), was chosen in preference to SO_4. Measurements showed that Ca is present in crayfish blood in as high a concentration as in the van Harreveld-Ringer's solution. The membrane properties of the muscle fibers are markedly changed on reducing the Ca (13). The high solubility and activity of Ca propionate permitted the required high level of Ca to be maintained in the various solutions used (Table I), whereas the use of SO_4-Ringer's solution restricts the Ca activity to about 1 meq/liter. After an initial transient effect which reflects the redistribution of Cl (*cf.* references 19, 13, 27) when the fibers are immersed in the propionate medium they could be maintained indefinitely at the same resting potential and volume as in the Cl-saline (Fig. 15). After the fibers had remained in the solution for the equilibration time a further period in the control solution was allowed during which it was ascertained that the fiber volume remained at a steady value. Changes in osmolarity were made by mixing measured volumes of the various experimental solutions (Table I) with the known amount of the control solution already in the chamber. When the fibers were to be returned to the control conditions the bathing medium was aspirated off and the preparation was washed several times in the appropriate control medium. The changes could be made within 1 min., so that

most of the time course of their effects on fiber volume and membrane potential could be observed.

It would have been desirable to include a series of experiments in which the osmotic pressure was changed with a non-ionic medium. Unfortunately, however, we have been unable to find such a medium among the commonly available sugars. Additions of sucrose or glucose affect the properties of the crayfish muscle fiber membrane in the same way as does diminution of Ca (12, 13). A similar action of various sugars has also been observed in the case of muscle fibers of the mealworm, *Tenebrio molitor* (23). It has been reported (36) that sucrose binds Ca as well as heavy metal ions. We were unable to demonstrate chelation of Ca by sucrose with either conductance measurements or with Ca-sensitive glass electrodes (11), while the effects of a chelating agent (EDTA) could be readily observed.[1] Presumably, therefore, sucrose and some other sugars act, at least to a large extent, directly on the cell membrane. Accordingly, only a few experiments were carried out using sucrose as an additive to the saline medium in order to study some specific points and thus they cannot be directly compared with the bulk of the data in which propionate solutions were used.

The following considerations were employed in presenting data in which the volume or potential is plotted as a function of osmotic pressure. The relative osmotic pressure of a solution obtained by variation of the sodium salt concentration is given by the equation

$$\frac{2k(\text{Na salt concentration})_{\text{initial}} + R}{2k(\text{Na salt concentration})_{\text{final}} + R} = \text{effective osmotic pressure.}$$

The coefficient k is the product of an osmotic coefficient and a reflection coefficient. The reflection coefficient for the Na salts was assumed to be unity since a steady displacement of volume occurred with variation of the concentratoin of either NaCl or Na propionate (Figs. 2 and 4). The osmotic coefficient for NaCl is essentially constant over the range of concentrations used and a value of 0.93 was assigned to it (30). The same value was used for the Na propionate although some difference might be expected. R represents the contribution of the invariant osmotically active constituents in the medium, which in the present experiments are the K and Ca salts (Table I). Again, both their respective osmotic and reflection coefficients determine the effective osmotic pressures of these constituents. In the chloride medium the reflection coefficient for KCl is less than unity since small additions of this salt do not produce a steady displacement of the cell volume. In the propionate medium the reflection coefficient becomes unity, since propionate is impermeant. A value of 0.92 was used for the osmotic coefficient. Both calcium salts were assumed to have an osmotic coefficient of 0.86 (30) and a reflection coefficient of unity.

[1] These experiments and the measurements of Ca activity in crayfish blood mentioned above were done at the Marine Biological Laboratory during the summer of 1962. We wish to thank Professor Garrels for providing us with 2 Ca-sensitive glass electrodes each of which responded with a change of 28 mv/decade change in Ca concentration. While we were unable to demonstrate chelation of Ca by sucrose with the electrometric techniques, a recent paper (5) reports that lactose does chelate Ca in appreciable amounts, the chelated complex being detected by chromatographic analysis.

RESULTS

The Muscle Fiber as an Osmometer for Na

There is a considerable body of data demonstrating that frog muscles are osmometers for Na (6, 7). On altering the concentration of NaCl in the medium a weight (or volume) change results which is proportional to the relative osmotic pressure. Studies of volume changes in single frog muscle fibers, analogous to those reported here, place important restrictions on the osmometric properties of frog muscle (28, 29). However, crayfish muscle fibers respond in a relatively simple manner to a change in Na concentration under different conditions (Figs. 2 and 4). The data which have been obtained in the present work on single crayfish muscle fibers have the added features that the

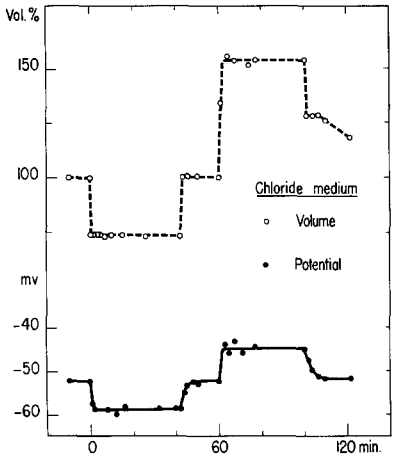

FIGURE 2. Changes in volume and in membrane potential when a fiber was exposed first to a medium made hyperosmotic by addition of 165 mM NaCl, returned to the standard medium, and then exposed for a time to a hyposmotic medium with the removal of 97.5 mM NaCl. Note hyperpolarization associated with shrinkage and depolarization related to swelling, except that the fiber remained swollen while the membrane potential regained its control level on replacing the hyposmotic medium with the control solution.

findings also relate to the kinetics and locations of the water distribution as disclosed by volume changes, by changes in membrane potential, and by electron microscopic observations. In this section two types of experiments will be reported; those which were carried out in Cl-containing media, and others in Cl-free media. The use of Cl-free media avoids changing the K × Cl product. The results therefore may be expected to be somewhat simpler than in the case of changes in the concentration of NaCl.

The fiber of the experiment shown in Fig. 2 was first subjected to an increase in osmotic pressure by adding 165 mM NaCl to the control solution. This represents an increase of osmotic pressure ($P_{\text{final}}/P_{\text{initial}}$; P_f/P_i) to 1.78 of the control saline. The relative volume change predicted from a simple van't Hoff relation with no dead space ($P_iV_i = P_fV_f$) is the reciprocal of the relative increase in osmotic pressure, or a shrinkage of 56 per cent. In the experiment, the fiber shrank only by 27 per cent, to 73 per cent of its initial

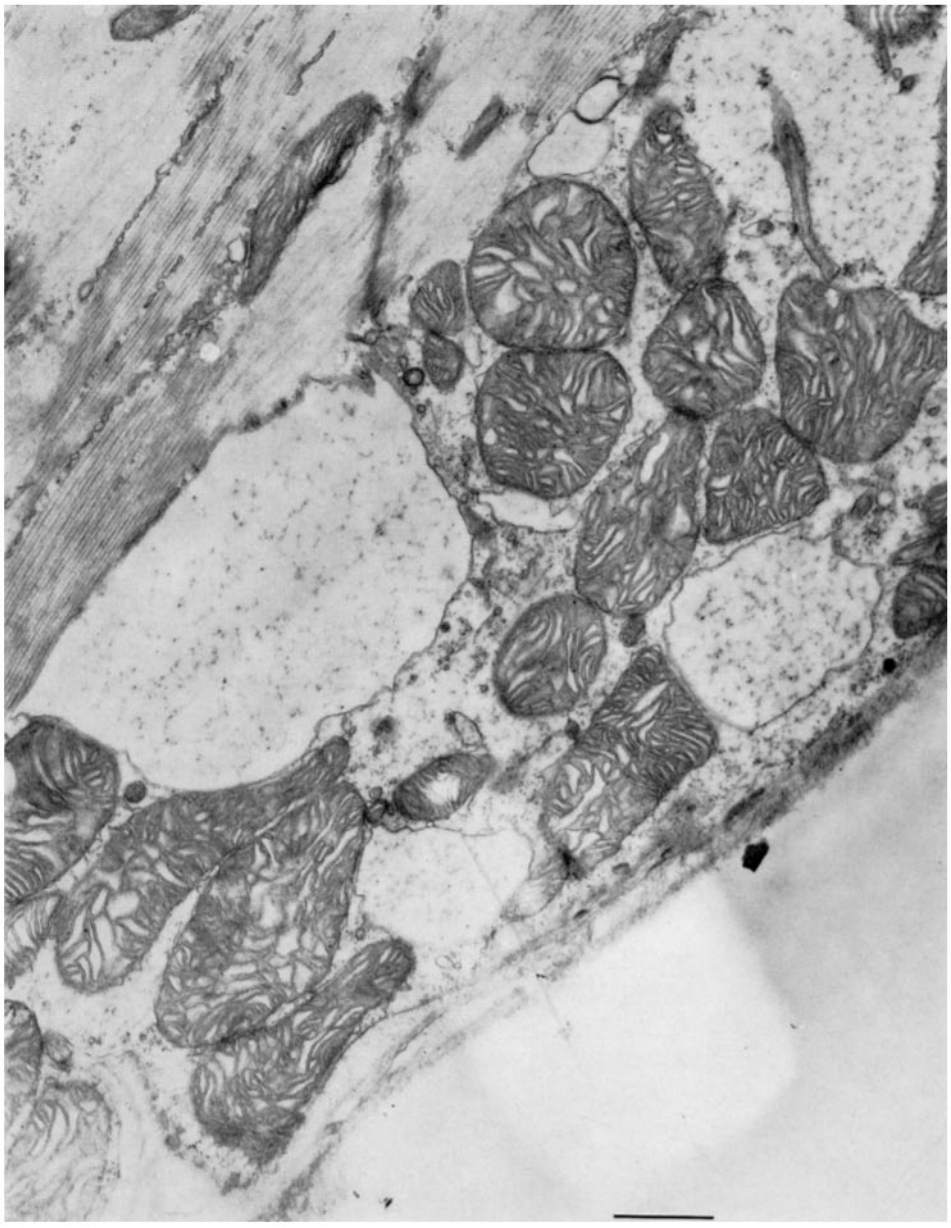

FIGURE 3. Electron micrograph of the periphery of a crayfish muscle fiber which had been returned to the control saline after exposure to a hyposmotic medium, as in Fig. 2. The sarcolemma and basement substance are seen diagonally in the lower right. The myofibrils are in the upper left. The layer between contains numerous mitochondria and under the conditions of this experiment there are also large vesicles which correlate with the persistent swollen state of the fiber × 13,000.

volume. The shrinkage was rapid and remained steady as long as the fiber remained in the hyperosmotic medium. When the control medium was restored in the bath the fiber promptly returned to its initial volume.

On exposing the fiber to a medium of decreased osmotic pressure by diminishing the Na salt by 97.5 mM (P_i/P_f = 1.84) the volume increased by 55 per cent. The swelling was also rapid in onset and the volume remained steady thereafter. Thus, per unit change of osmotic pressure the volume change in the hyposmotic medium was larger relatively than the change in the hyperosmotic medium.

TABLE II

CORRELATION OF CHANGES IN VOLUME AND POTENTIAL IN THE EXPERIMENTS OF FIGS. 2 AND 4

Medium	Relative volume* per cent of initial		Membrane potential	
	Calculated P_i/P_f	Observed	Measured	Calculated‡
			mv	*mv*
Cl saline	56	73	59	60.3
	184	155	44.5	41.3
Propionate saline	59	81	56	57.7
	172	165	41	39.7

* Osmotic changes made by addition of 165 meq/liter of the Na salt or by removal of 97.5 meq/liter.

† Calculations of membrane potential are based on a value of K_i (160 meq/liter) estimated from the initial resting potential, which was 52 mv in both fibers, and on the observed volume change.

When the control medium was reintroduced, instead of returning to the initial volume, the fiber now lost only part of the water. It remained swollen at about 28 per cent above the initial volume for nearly 10 min., when it began to shrink again, but rather slowly. A change in optical appearance of the muscle fiber also occurred which was somewhat similar to that shown in Fig. 1 (frame 6). However, electron micrographs[2] revealed (Fig. 3) that the residual swelling was accompanied by an accumulation of water at the periphery of the fiber. This was denoted by large vesicles formed in this region, which is demarcated in crayfish muscle fibers by a dense layer of mitochondria and is free of myofibrils. Thus, the electron microscopic picture was quite different from that which was obtained when the efflux of Cl from the cell caused swelling of the transverse tubular system (12).

[2] We wish to thank Dr. P. W. Brandt for permission to use this figure. A preliminary account of the morphological studies has appeared (4) and a full report is in preparation.

Correlated with the various changes in the volume there were also changes in membrane potential (Fig. 2). Since the fiber was maintained in 20 meq/liter K (Table I) the initial resting potential was only 52 mv. The membrane hyperpolarized by 7 mv in the hyperosmotic medium, the change in potential occurring somewhat more slowly than the change in volume. The return from the hyperpolarization was also slower than the increase in volume when the fiber was returned from the hyperosmotic to the control medium. When the fiber swelled in the hyposmotic medium there was a depolarization of 7.5 mv, which occurred about as rapidly as did the swelling. However, when the swollen fiber was returned to the control medium the return of the potential toward its control level was at about the same rate as for the changes in the

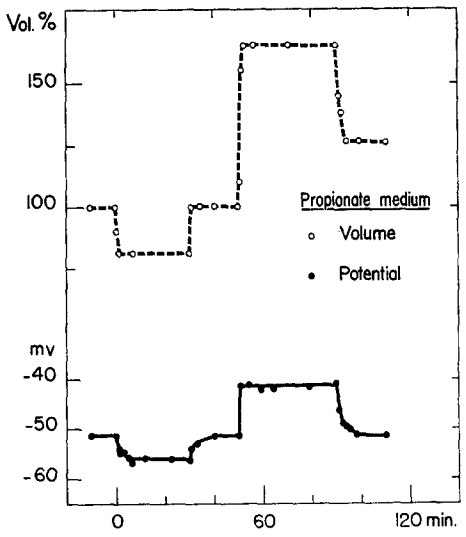

FIGURE 4. Changes in volume and membrane potential of a fiber treated as in Fig. 2, but in Cl-free media.

hyperosmotic medium. Thus, the initial resting potential was attained while the fiber was still in the somewhat swollen state at its secondary plateau level.

It seems likely that only water could have moved in significant amounts under the experimental conditions of Fig. 2. Accordingly, the relative intracellular concentration of K must have increased when water left the cell and it must have decreased as water entered. Thus, the amount of hyperpolarization and depolarization respectively, might be correlated with the change in volume under steady-state conditions (1). The changes in potential during the observed steady-state changes in volume could be calculated from the measurements of the latter on the basis of the Nernst relation:

$$E_M = -58 \text{ mv} \log \frac{K_i}{K_o}$$

The relevant data are given in Table II and indicate fair agreement with

the above assumption. A similar experiment, but performed in Cl-free media, is shown in Fig. 4. The changes in the concentration of the Na propionate were the same as those made in the NaCl of the experiment of Fig. 2. However, the relative changes in osmotic pressure were somewhat different (Table II) since, as described in the section on Methods, the reflection coefficient for the K propionate now was taken as unity. The changes in volume which resulted on exposing the fiber to the hyperosmotic and hyposmotic media had essentially the same time courses as those observed with the changes induced in the fiber exposed to Cl-containing media. Again, there was a relatively larger swelling than shrinkage per unit change of osmotic pressure.

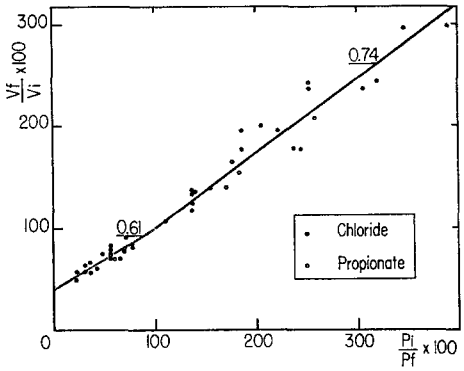

FIGURE 5. Relative volumes of single muscle fibers in media of different osmotic pressures. *Ordinate*, percentage ratio of final volume, (V_f) to initial volume (V_i). *Abscissa*, percentage of ratio of initial osmotic pressure (P_i) to final pressure (P_f). Changes in osmotic pressure were made by increasing or decreasing the Na salt concentration. Calculated regression lines for hyperosmotic (broken line) and hyposmotic (continuous line) conditions, have different slopes, but have a common origin at 100 on both axes. The regression line for the hyperosmotic conditions extrapolates to an intercept of 40 per cent on the ordinate.

In this experiment also, after the fiber became swollen in the hyposmotic medium it did not return rapidly to its initial volume when the control medium was reintroduced. The plateau (at about 25 per cent above the initial volume) was long lasting, the fiber remaining at the plateau volume during the subsequent 20 min. The same changes in optical appearance, which were also accompanied by accumulation of water at the periphery of the fiber, were observed as in the experiment of Fig. 2. This finding serves to differentiate further the changes caused during reversal from hyposmotic conditions from those which occur when the TTS is swollen. Swelling of the TTS was never observed in fibers which had been equilibrated in Cl-free media (12).

The changes in membrane potential which occurred simultaneously with the volume changes are also shown in Fig. 4. As in the experiment of Fig. 2, hyperpolarization ensued when the fiber shrank and depolarization occurred

when the fiber swelled. The expected changes in potential, calculated as described above, are shown in Table II. The time courses of the changes in potential were also similar, with the depolarization associated with the swelling being particularly rapid as was also the case in the experiment of Fig. 2. Again, the potential returned to its initial level while the fiber still remained swollen after being replaced in the control medium.

The volume changes observed in all the experiments in which the osmotic pressure was changed by varying the level of Na in the medium are shown

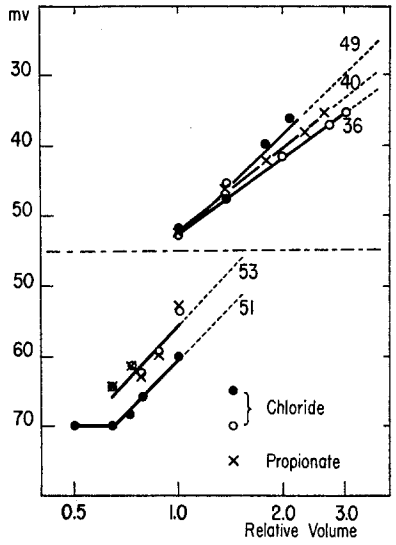

FIGURE 6. Changes in membrane potential correlated with volume changes of fibers placed in media of different osmotic strength. Volume scale is logarithmic. Each symbol represents a different fiber. *Below*, measurements on 3 fibers which were exposed to different hyperosmotic media. Intervals between change of solution and measurements were 40 to 60 min. in all cases. *Above*, measurements on 3 other fibers which were exposed to hyposmotic media. The numbers near each line represent the change in membrane potential calculated for a tenfold change in volume.

in Fig. 5. For the hyperosmotic conditions the linear regression equation used was determined by the fact that the line had to intersect the 100 per cent locus. The variance is: $S = \left[\left(100 - \frac{V_f}{V_i}\right) - b\left(100 - \frac{P_i}{P_f}\right)\right]^2$ with the slope b obtained for $\sum \frac{dS}{db} = 0$.

The regression line has an intercept at 40 per cent on the ordinate (relative volume). In hyposmotic media the muscle fibers could swell to at least 3 times their intial volume. The slope of the linear regression through the 100 per cent locus was approximately 20 per cent greater than for the relation between pressure and volume obtaining in hyperosmotic media.

The magnitudes of changes in membrane potential with volume were studied further in six experiments that are detailed in Fig. 6. Four of the experiments were on muscle fibers in Cl media, the others on preparations in propionate media. In each experiment 4 or 5 steps of change in osmotic pressure were made. Each change was maintained for 40 to 60 min., when the membrane potential of the fiber as well as the volume was recorded. Each

value of the potential represents the mean from 4 to 6 penetrations at different locations along the fiber. The deviations among the individual readings were usually less than 1 mv.

The slopes of the potential-log relative volume relation approached the theoretical expectation discussed above more closely when the fibers were exposed to hyperosmotic (lower part of Fig. 6) than to hyposmotic media (upper graphs), the difference being evidenced in the experiments with Cl-containing as well as Cl-free media. The lower values of the slopes obtained in the hyposmotic media, in which the greater volume displacements occurred relative to the changes in osmotic pressure (Figs. 2, 3, and Table II), suggest that the distribution of water within the swollen cell might have been non-uniform, at least during relatively short (40 to 60 min.) periods of equilibration in the different media.

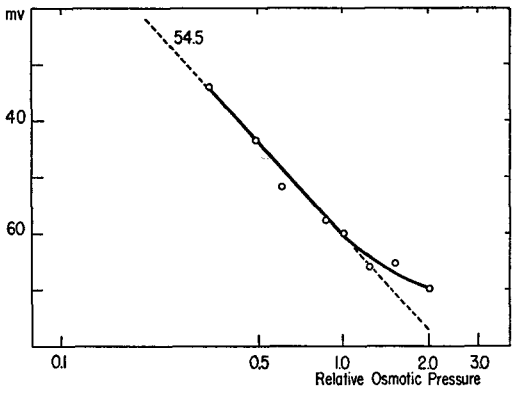

FIGURE 7. Resting potentials of fibers in muscles which had been exposed for 24 hrs. to solutions of different osmotic pressures. Further description in text.

In order to obtain comparable data on equilibrated single muscle fiber preparations it would have been necessary to ensure viability of all the fibers for many hours. Accordingly, to test the possibility of a slow equilibration of water among intracellular components electrophysiological measurements on whole muscles were used. For the measurements of Fig. 7, 47 whole muscle preparations were each equilibrated for 24 hrs. at 2–4°C in a Cl-free solution of given osmotic strength, the latter being varied by changing the concentration of Na propionate. The resting potentials of representative fibers in each muscle were measured and in Fig. 7 are shown plotted against the logarithm of the relative osmotic pressure. Each point is an average measurement at room temperature from 5 to 10 superficial fibers in each of 4 or 5 muscles, the whole experiment altogether representing measurements on more than 200 fibers.

The depolarizations which were produced in the hyposmotic media yielded a slope of 54.5 mv/decade decrease in osmotic pressure, or approximately the same as was observed for hyperosmotic conditions in Fig. 6. As the osmotic

pressure of the medium increased the slope of the membrane potential–log osmotic pressure relation decreased. However, the smaller degree of volume change in fibers exposed to hyperosmotic media (Figs. 2, 4, 5, and Table II) can account for this decrease and it therefore is likely that with equilibration the water in the muscle fibers tends to become more uniformly distributed.

One experiment of the same type was made on a muscle which was exposed to 5 different osmotic changes and in each of which the mean potential of 10 surface cells was determined only 20 min. after each change of the medium. The time-dependence of the relation between volume and membrane potential was then greatly exaggerated. The measurements gave a slope of 12 mv for the change in membrane potential per decade change in osmotic pressure. This low slope is in marked contrast with the change in membrane potential when the superficial fibers of a whole muscle are exposed to changes in the K in a Cl-free medium. The membrane potentials of the fibers then change within 1 min. and the slope of the relation E_M *vs.* log K_o is 58 mv/decade change of K_o (13).

Movement of Water in Media Made Hyperosmotic with Addition of K Salts

When the concentration of permeant ions is increased in the medium by adding KCl to the standard solution all ion species may be regarded as initially contributing to the osmotic pressure difference across the cell membrane, since the movement of water is much faster than the redistribution of ions. Thus, a muscle fiber should shrink initially and only as the permeant ions redistribute should there be a return towards the control volume.

An experiment on a single fiber, comparing the effects of additions of identical amounts of NaCl and KCl to the control medium is shown in Fig. 8 (right). The fiber was first exposed to a medium which was made hyperosmotic with addition of NaCl. After it had been returned to the control medium it was exposed again, this time to a medium made hyperosmotic with an equivalent amount of KCl. The shrinkage in the latter case was considerably larger. The subsequent reentry of water is also shown and it should be noted that the fiber eventually swelled almost 20 per cent above the control level.

The maximum initial shrinkages observed in all such experiments on single fibers are shown as a function of the osmotic pressure in Fig. 8 (left). Two opposing water movements are obviously operating in experiments of this type. The loss of water due to the initial differential of osmotic pressure causes a shrinkage while the entry of KCl into the fibers leads to reentry of water. Thus, the peak shrinkage may be expected to be somewhat smaller than that which ought to be obtained with an impermeant salt. In point of fact, however, the shrinkages in the experiments of Fig. 8 (left) were in general considerably larger than those that were produced when the medium was made hyperosmotic with a Na salt. Indeed, the rapidity of the initial efflux of water

as compared with the subsequent influx that accompanied the entry of KCl is shown by the fact that the amount of the initial shrinkage was independent of the presence or absence of Cl. In all but a few of the 26 experiments plotted in Fig. 8 (left) the shrinkage was considerably greater than predicted from the regression line for the hyperosmotic Na salts (Fig. 5) which is also included on the graph. A line drawn by inspection would project to an intercept at about 12 per cent on the volume ordinate. Thus, these experiments show clearly that in addition to the osmotic efflux of water which occurs in media made hyperosmotic with increase of Na salt, some additional process must be

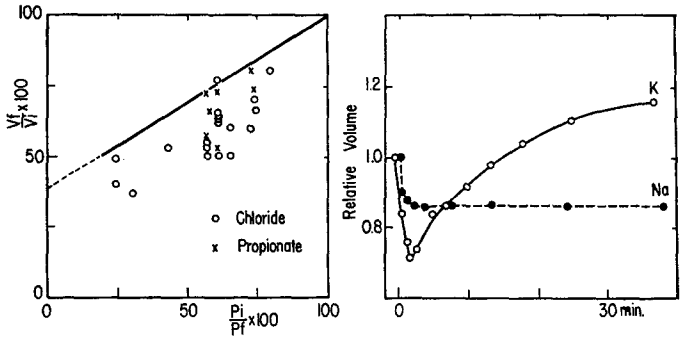

FIGURE 8. *Right*, volume changes in a single muscle fiber which was first exposed to a Cl-saline made hyperosmotic by addition of 165 mM NaCl (filled circles and broken line). After return to the control medium, it was subsequently exposed to a saline to which 165 mM KCl had been added (open circles and continuous line). Note the greater peak shrinkage in the latter case. The starts of the 2 curves are displaced slightly for clarity. *Left*, the peak shrinkages of muscle fibers exposed to media to which various amounts of K salt had been added. Note that the scatter of values obtained in 7 preparations exposed to propionate salines was the same as for 19 preparations in Cl media. The coordinates are the same as those for the portion to the left of the control conditions in Fig. 5 and the line for the hyperosmotic data of the graph is drawn in.

involved in removing water when K is added either in the presence or absence of Cl.

Effects of Different K Salts on Fiber Volume and Potential

The volume changes that resulted on changing the osmotic pressure of the medium by varying external K depended markedly on the anions in the medium. This is illustrated in Fig. 9 in experiments on 3 different fibers. In all 3 cases the osmotic pressure was increased by raising the external K to 153 meq/liter, but with different anions. The latter was Cl in the experiment of Fig. 9*A*, and the excess of KCl was added to the standard Cl saline. The initial medium was the Cl-free propionate saline for the experiment of Fig. 9*B*, and it was made hyperosmotic by adding K propionate. For the experiment

of Fig. 9*C* the initial medium was the Cl saline, but the hyperosmotic solution was a mixture in which KCl and K propionate were proportioned so that the product $K_o \times Cl_o$ would be equal to $K_i \times Cl_i$ calculated for the condition that the fiber volume decreased to 59 per cent of the initial volume, the percentile reciprocal of the relative osmotic pressure for the experiments on fibers B and C. For fiber A this value was 56 per cent.

The initial shrinkage was nearly the same in all 3 experiments. It was large compared with that which occurred in the case of fibers exposed to hyperosmotic Na-containing media (Figs. 2, 4, 5, and 8). The fiber which was in the KCl medium (Fig. 9*A*) then began to swell, returning to a volume 8 per

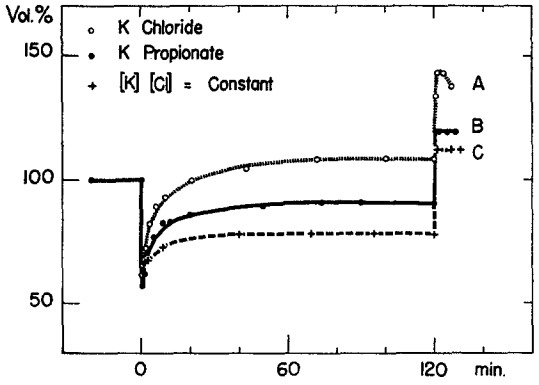

FIGURE 9. Time courses of volume changes in 3 muscle fibers exposed to solutions in which the K salt was increased to 153 meq/liter. Further description in text.

cent greater than that of the control. The other two fibers (B, C) also began to swell, but the time course of the changes and the final volumes differed markedly from those observed in fiber A. When the fibers were returned to their respective control media after 2 hrs., they all swelled by approximately equal amounts, using the steady-state volumes in the hyperosmotic media as the base line.

Four experiments were performed under the same osmotic and ionic conditions as in Fig. 9*A*. In these experiments, when the fibers were equilibrated in the hyperosmotic medium containing excess KCl the increase in volume ranged between 8 per cent (Fig. 9) and 20 per cent (Fig. 8).

Although the volumes of the fibers in Figs. 9*B* and *C* remained below the control level as long as they were maintained in their respective hyperosmotic media, the fibers nevertheless had gained water after the initial shrinkage instead of responding with a stepwise shrinkage, as did the fibers of Figs. 2 and 4. Their increase in volume cannot be ascribed to the simplest explanation, that propionate could replace Cl. If the membrane were permeable to propionate the final volume of the fibers should have been the same under all 3 experimental conditions, assuming a homogeneous intracellular distribution. However, in more than 20 experiments with cells exposed to hyperosmotic

KCl solutions the equilibrium condition was with fibers swollen above the control volume. K propionate was used in 7 experiments. The final volume never attained the control level. The largest amount of return in all these experiments is that shown in Fig. 9*B* and a smaller amount is shown in the experiment of Fig. 10*B*. In another experiment the shrinkage had a "square pulse" form as in the fiber of Fig. 4, when Na propionate was used to make the medium hyperosmotic. Furthermore, when the change to the hyperosmotic medium was made by stepwise increase of K propionate (5 successive additions of 30 meq/liter each, at 30 min. intervals) the maximum shrinkage

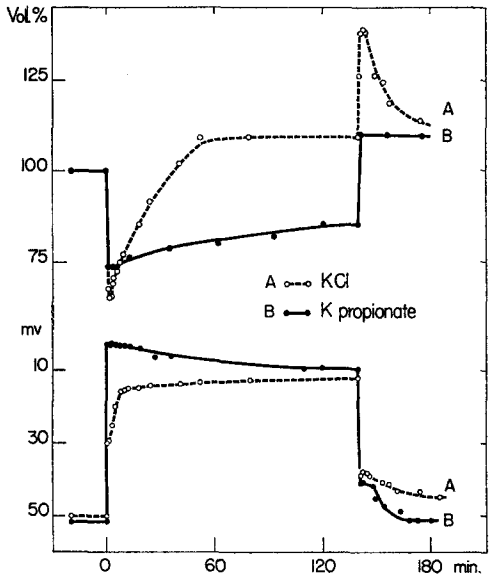

FIGURE 10. Correlation of changes in volume and membrane potential of 2 fibers each of which was exposed for a time to a medium made hyperosmotic by increasing the concentration of the K salt. Further description in text.

was no greater than that produced by addition of 150 meq/liter Na propionate, and the time course of the shrinkage also had a square pulse form. Only 2 experiments were carried out under the constant product condition of Fig. 9*C*. Similar values were obtained in both.

While all 3 fibers of Fig. 9 swelled immediately when they were returned to their control media the subsequent volume changes had different time courses. Fibers B and C remained at essentially the same volume during the 10 min. period which is shown in Fig. 9. Fiber A shrank rather rapidly during this time. This fiber, however, then underwent the marked optical changes that are shown in Fig. 1. These optical changes which are due (12) to disarray of the striations because of the appearance of large vesicles (some up to 4 μ in diameter), which have been shown to occur in the TTS, were never observed under the conditions that were specified for fibers B and C.

Simultaneous measurements of membrane potentials and the volume changes (Fig. 10) provided some further insight into the effects that were

produced in the hyperosmotic KCl and K propionate media. When a fiber was exposed to the hyperosmotic KCl medium (A) there was an initial step of depolarization which was far short of the theoretically expected value, as predicted by the Nernst relation. This could not have been due to the marked shrinkage of the fiber, since the partial depolarization was absent in the case of fiber B, which also shrank nearly to the same degree. Fiber B (in the Cl-free medium) depolarized to a greater extent and rapidly, whereas in the case of fiber A time was required for redistribution of Cl (13). However, as the 2 fibers underwent further changes in volume in their respective media their

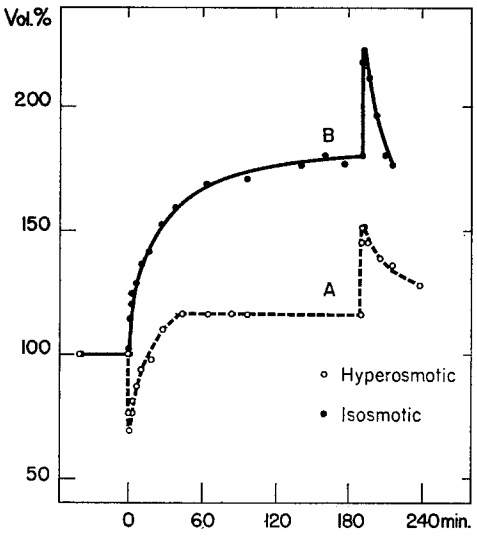

FIGURE 11. Time courses of volume changes in 2 fibers which were exposed to KCl-enriched saline media. In B an equivalent amount of NaCl was deleted when 100 mM KCl were added.

membrane potentials tended to approach each other, that of fiber A rising slowly and that of fiber B falling.

A simple relation between volume and potential could not be predicted for the fiber immersed in the KCl medium, since the electromotive forces of both the K and Cl batteries contribute to the measured membrane potential (10, 13). However, in fibers which are exposed to the Cl-free propionate medium the membrane potential should depend only on the ratio of $\frac{K_i}{K_o}$. Thus, a direct correlation between volume and potential would be expected in this case if the distribution of K_i were homogeneous. The membrane potential expected at the peak of the shrinkage of the muscle fiber was calculated from the Nernst equation, taking into consideration the increased levels of both K_o (arising from the addition of K propionate) and K_i (arising from the shrinkage). The calculated membrane potential was -9 mv, whereas the observed value of the depolarization was significantly greater, the potential becoming

−3 mv. The calculated value was attained only after a period of 2 hrs., during which time the volume also increased by approximately 12 per cent.

The changes in both parameters when the 2 fibers were returned to their control media were also significantly different. Both fibers swelled and in both there was repolarization. However, the fiber in the Cl-free medium (*B*) swelled only about 10 per cent above the control level, or to about the equilibrium level for fiber A. The latter at first exhibited a very marked degree of further swelling. Subsequently, this swelling began to subside and the volume approached the value which was found in fiber B. The course of the changes in membrane potential differed considerably. After the initial step of repolari-

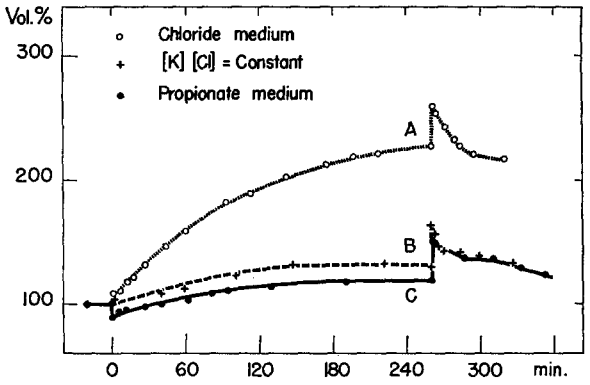

FIGURE 12. Comparison of volume changes in 3 muscle fibers each of which was exposed for a period to an isosmotic medium in which the K salt was increased to 153 mM with concomitant deletion of the Na salt.

zation there was a plateau in both experiments, but the potential then returned rapidly to the resting value in fiber B. Fiber A, however, remained depolarized by some 6 to 7 mv. The discrepancy may have been caused by the presence of Cl in this experiment, since the electrochemical potential for Cl would remain low until a new equilibrium was established by redistribution of KCl.

The volume changes that were produced when KCl was increased, but with the medium maintained isosmotic, differed considerably from those which occurred in hyperosmotic KCl (Fig. 11). The preparation in the isosmotic solution did not shrink initially, and its volume increased markedly. However, when the two fibers were returned to the standard medium both showed approximately similar secondary swellings. In both cases the return toward the initial volume was slowed and the fibers developed the grainy appearance (Fig. 1) associated with vesiculation of the TTS (12).

The volume changes in the isosmotic condition with high K also depended upon the character of the anion (Fig. 12). There was a small swelling in fiber

B, which was in a constant product medium while fiber C, in the Cl-free medium shrank at first, then swelled somewhat. The initial small shrinkage was also observed, but less frequently, in the isosmotic KCl-enriched salines. All 3 fibers shown in Fig. 12 swelled by about the same amount on return to the control solution and the subsequent loss of water was slow. The secondary swelling above the control volume after they were returned to the control medium occurred in all fibers that had been exposed to isosmotic K-rich media, no matter what the anion was. In the Cl-saline media a prompt shrink-

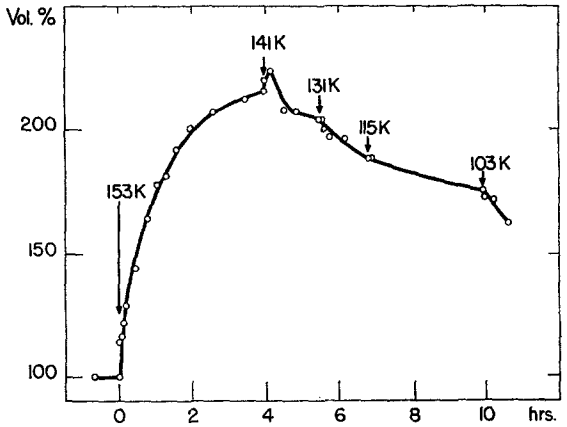

FIGURE 13. Modification of volume changes by stepwise decrease in KCl from an isosmotic medium which had initially contained 153 mM KCl. The arrows indicate the changes in the medium, and the numbers show the concentration of KCl at each stage. Corresponding additions of NaCl were made to maintain isosmotic conditions. Compare with Figs. 11*B* and 12*A*.

age of the swollen fibers was to be expected. The anomalous increase in volume therefore represents a "negative" movement of water. No change in volume was to be expected in the Cl-free media.

The swelling induced by removal of the high external K could be diminished or abolished by stepwise decrease of the K (Fig. 13). In the experiment shown, the muscle fiber had been first exposed to an isosmotic solution containing 153 mM KCl. As has already been noted in the experiments of Figs. 11*B* and 12*A* there was a large swelling. The level of K was then reduced while maintaining isosmotic conditions by substituting Na for K. Each step was accompanied by a repolarization calculated to be about 2 to 3 mv. Only on the initial removal of 12 meq/liter of K was there a further increase in volume, and this was small in comparison with the very large increase that was shown in Figs. 11*B* or 12*A*. In the course of the 3 subsequent removals of K the fiber volume only diminished, although there may have been changes in the rate of the fall as a consequence of the changes in K. Four experiments of this type were performed. The swelling produced by the first reduction in

external K was no larger than 8 per cent and in 2 experiments no swelling was produced. Optical and structural changes (Fig. 1) associated with efflux of Cl (12) were observed in all 4 preparations after the external K was reduced to about half the initial level.

In the experiment illustrated in Fig. 14, K was increased by addition of KCl to the standard medium to a final value of 152 mM, but in 3 equal steps of 44 mM each. The successive changes in membrane potential were calculated to be depolarizations of approximately 22.5, 18, and 8.5 mv, respectively. The excessive initial shrinkage which was described above (Fig. 8)

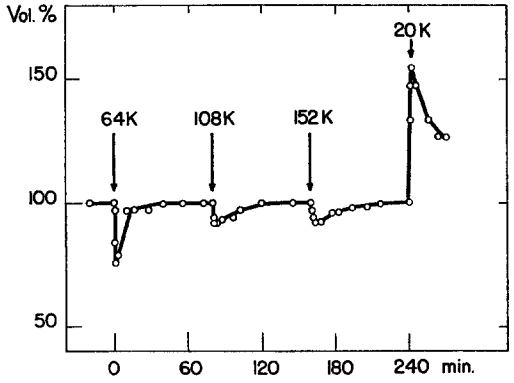

FIGURE 14. Modification in volume of a muscle fiber by stepwise additions of KCl to the standard medium, and the subsequent return to the standard medium. First 3 arrows show the successive additions of KCl to concentrations given on the graph. The fiber did not swell in the high K medium (compare with Figs. 8, 9*A*, 10*A*, and 11*A*). However, there was a large swelling when the fiber was returned to the medium containing only 20 mM KCl.

occurred only at the first increase of KCl. In the 2 subsequent steps the shrinkage was of the same order as that which was produced by additions of NaCl (Figs. 2, 5, and 8). Very conspicuous also was the absence throughout of the secondary swelling above the control volume which always occurred when 100 to 160 mM KCl were added at one time. Nevertheless, when the muscle fiber was replaced in the control medium it swelled rapidly. In the experiment of Fig. 14 the peak value was 54 per cent above the control level, or within the range of swelling observed in experiments like those of Figs. 9*A* and 10*A*. The structural changes shown in Fig. 1 (frame 6) and which were associated with swelling of the TTS also occurred in all the experiments of this type.

Volume Changes Accompanying Cl-Induced Changes In Membrane Potential

When the external Cl is altered under isosmotic conditions crayfish muscle fibers exhibit a transient change in membrane potential (13, 27), similar to but smaller in magnitude than that observed in frog muscle fibers (19).

Reduction of the Cl from 247 to 1 meq/liter caused a peak depolarization of the crayfish fibers of 15 to 25 mv which was over in 10 to 20 min. in media containing 20 meq/liter K. On returning to the initial level of Cl the membrane was transiently hyperpolarized. While the amplitude of the hyperpolarization was somewhat smaller than the depolarization the general course of the response was the same as that of the transient depolarization.

These changes in membrane potential were accompanied by transient changes in volume (Fig. 15). During the depolarization caused by removing Cl the fibers shrank briefly and they then swelled transiently on restoring the normal level of Cl. The magnitudes of the volume changes differed somewhat

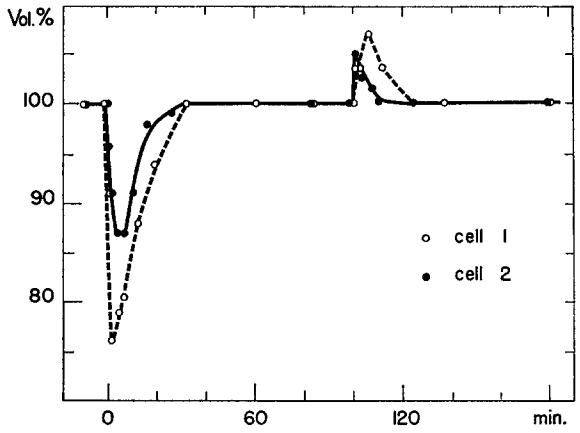

FIGURE 15. Transient volume changes which were associated with transient depolarization on substitution of propionate for Cl in the medium, and with subsequent transient hyperpolarization on restoration of the Cl. Two experiments are shown.

in the two fibers of Fig. 15, but a large scatter in peak values of the changes in membrane potential was also observed (13). The time course of the volume changes approximated very closely the time course of the changes in potential observed in experiments of this type. Since the volume changes were transient, water must have moved out and in during the depolarization and again diphasically in the opposite direction during hyperpolarization. Ionic redistribution could have been only unidirectional in each change of external Cl. Thus, another process is clearly implicated in the volume changes. During the temporary efflux of water and Cl there was also a temporary change in appearance like that of the fiber in Fig. 1. Preparations fixed at this stage showed vesiculation of the TTS (12). During the exposure of the muscle fiber to the low Cl medium the volume returned to its control value, rather than remaining below that level. A steady-state shrinkage which might have been expected due to the loss of all Cl would have amounted to only about 5 per cent. However, analysis of single fibers showed that after 5 to 6 hrs. of soaking in the

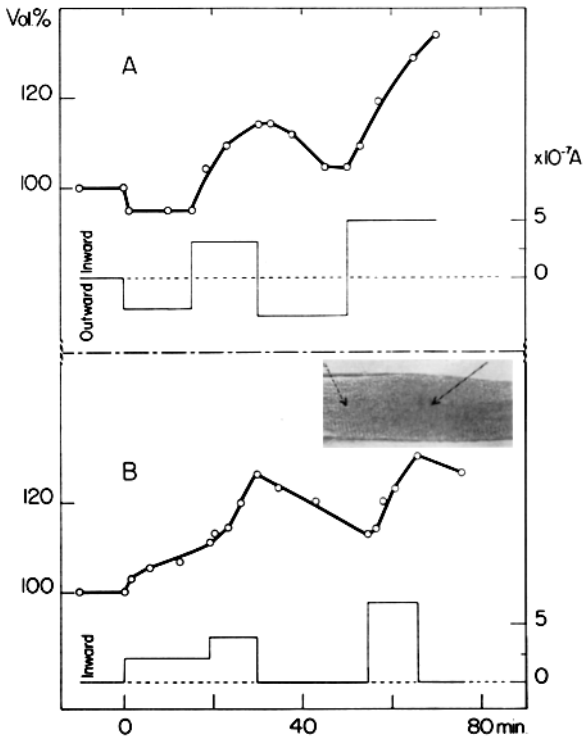

FIGURE 16. Volume changes during application of currents with an intracellular microelectrode. *A*, volume changes (circles and heavy line) while current was applied through an intracellular electrode filled with 3 M K propionate. Duration, magnitude, and direction of the current are shown on the lower, thin line. Current scale on right ordinate. *B*, a similar experiment, but with the current applied through a microelectrode filled with 3 M KCl. Only inward current, associated with swelling, was used in this experiment. The inset shows the darkening which occurred in the region of the intracellular cathode (represented by continuous arrow) during application of the current. This darkening is similar to the change which was shown in stage 6 of Fig. 1. It always occurred, only when inward currents were applied and only with a KCl-filled electrode and it was due to swelling of the TTS. A recording electrode which monitored the current is indicated by the broken arrow.

propionate saline they still contained about 40 meq/liter Cl^-. Retention of Cl was also observed in lobster muscle (9*a*).

Changes in Cell Volume Induced by Intracellularly Applied Currents

The data of the experiments illustrated in Figs. 13 to 15 suggest that there is a correlation between the changes in membrane potential and the changes in volume of the muscle fibers. If the deviations from the volume changes expected on the basis of normal osmosis are due to electrokinetic effects,

volume changes should also be produced by the passage of a current across the cell membrane, as a result of electroosmosis.

The electrical properties of the muscle fibers create certain difficulties for the demonstration of current-induced volume changes. The length constant of the isolated fibers is about 1.5 mm (12, 13). The change in membrane potential that is produced by a current applied through an intracellular microelectrode accordingly falls off sharply with the distance along the fiber. The volume changes, if any did occur, thus may be expected to develop in only a small region of the fiber close to the site of insertion of the current electrode. Lack of longitudinal equilibration complicates the experiments further, since water movements that would occur at the site of the electrode would tend to spread longitudinally along the axis of the fiber. During prolonged periods of applying currents, rectification, both in the fiber membrane and in the electrodes, adds to the difficulties.

Nevertheless, qualitative data demonstrating changes in the fiber volume during applications of current were obtained with surprising ease (Fig. 16). Experiments on two fibers are shown involving both depolarizing (outward) and hyperpolarizing (inward) currents in one (*A*) and only hyperpolarizing currents of different durations and magnitudes in the other (*B*). The measurements of the diameter of the fiber were made at the level of impalement with the current microelectrode, and the inset of Fig. 16*B* shows the swelling that was obtained during the course of the experiment on fiber B. In fiber A, outward current of 0.28 μa applied for 15 min. caused a maximum shrinkage of 5 per cent. Hyperpolarization with a slightly higher current (0.32 μa) caused a reversal with a continuing swelling of 15 per cent. The swelling was diminished when the current was reversed. An inward current of 0.5 μa caused a swelling of nearly 35 per cent above the initial level. These changes and those in the fiber of Fig. 16*B* obviously do not represent equilibrium conditions, but nevertheless they are clear-cut.

In the experiment of Fig. 16*A* the microelectrode delivering the current was filled with 3 M K propionate. However, when electrodes were filled with KCl (Fig. 16*B*), in addition to causing a swelling, hyperpolarization always also initiated a local change in the appearance of the fiber (inset) like that seen in Fig. 1 (frame 6). This was associated with the vesicle formation characterizing a swelling of the TTS (12). In a few experiments the fibers were in K-free media. When an inward current was applied through a KCl-filled microelectrode the fiber became intensely dark very rapidly, as well as developing a local swelling. The rapid, intense darkening resulted from the fact that the current was largely carried by efflux of Cl. Longitudinal currents applied with 2 intracellular electrodes caused no discernible changes in the volume of the fiber, nor in its appearance (12).

DISCUSSION

The Factors Which Affect Volume of the Muscle Fibers The results described in the foregoing indicate that at least 3 different types of processes can occur during osmotically or ionically induced changes in volume in isolated crayfish muscle fibers. One of these is due to a normal osmotic flow of water. A second arises from the presence of intracellular compartments with different kinetics of water equilibration. A third is dependent upon and occurs during a variation of the membrane potential.

Normal osmotic distribution of water is characterized by rapid and reversible equilibration of the cell volume after alteration of the external medium. However, as is shown in Figs. 2 to 4, a complication can occur which is due to the second type of effect, the unequal distribution of water.

Osmotic water movement is proportional to the osmolarity of the external medium and is independent of the electrical forces across the cell membrane. Water flow which is correlated with a change in membrane potential may occur (31, 32) no matter whether there can be a sizable redistribution of salts (as in the Cl media) or not (as in propionate salines).

Normal Osmotic Phenomena The volumetric data reported here agree with electrophysiological and other findings (12, 13) and indicate that the membrane of crayfish muscle fibers, like that of frog muscle fibers (2, 6, 7, 19), is permeable to K and Cl, but is effectively impermeable to Na. On altering the osmotic strength of the medium by increasing or decreasing the concentration of the Na salt there was a rapid change in volume (Figs. 2 and 4). This change was stable as long as the osmotic pressure change was maintained, in the present experiments for as long as 4 hrs. The changes in membrane potential which were observed in fibers of whole muscle preparations that had been exposed to media of different osmotic strength for about 24 hrs. (Fig. 7) also indicate that osmotic movements of water, once they have occurred, remain stable in a given medium.

Adrian (2) has reported changes in the membrane potential of frog fibers resulting from changes in internal K when the muscles were placed in hyperosmotic and hyposmotic media. However, the conditions of those experiments differed considerably from those of the present work. Sucrose was used in the media and the external K was kept at the normal value (2.5 meq/liter). Accordingly the slopes obtained in that work, on the order of a 35 mv change for a tenfold change in K_i, are not strictly comparable with the findings shown in Fig. 6.

The rate of water movement in the hyperosmotic NaCl was about 3 μ^3 μ^{-2} min.$^{-1}$ At^{-1}. This is considerably higher than the movement in most eggs (24) or other cells that have been studied (17; Table 2.8), except erythro-

cytes. It is of the same order as that of frog ovarian eggs. The normal osmosis appears to be independent of the anion in crayfish muscle (Figs. 2, 4, and 5).

From the data of Fig. 5 a question arises with regard to the behavior of the muscle fibers as osmometers for Na. The intercept on the volume ordinate is as large as it is in measurements on whole frog muscle (6) or on frog single fibers (28). The commonly accepted explanation of this value (0.35 to 0.4), that the intercept represents a component of extracellular space as well as osmotically inactive intracellular material, was plausible for the case of whole muscle. However, such an explanation cannot be valid for either type of single fiber preparation. The data of Fig. 8 support the conclusion that the intercept of Fig. 5 cannot be interpreted in terms of one or several fixed dead spaces. A linear relation between the initial shrinkage and the osmotic pressure when the latter is raised by adding K salt to the control medium would clearly yield an intercept which is much smaller than that obtained in media made hyperosmotic by adding Na salts.

There was relatively little scatter of the measured volume changes at a given osmotic pressure (Fig. 5). This finding is in agreement with our observations that the fibers undergo little change in shape during shrinkage or swelling. Direct measurements on single frog sartorius muscle fibers (30*a*) also showed that they tended to maintain their shape under different osmotic conditions.

The data of Fig. 5 provide further support for the conclusion that dynamic factors, among which may be alterations in the state of the intracellular constituents of the sarcoplasm, affect the volume-pressure relation. Crayfish muscle fibers can swell to about 4 times their initial volume and the presence or absence of Cl in the hyposmotic medium does not alter the relation. Whole frog muscle preparations, however, are limited in their water uptake to about 60 per cent above the initial level (14). This limitation on swelling is also seen in single frog muscle fibers that are exposed to hyposmotic media containing Cl and enriched in KCl to 10 or 12.5 mM (28).

Unequal Distribution of Water Even when only Na salt concentration is changed, the kinetics of volume changes indicate that processes other than normal osmosis are also involved in the water redistribution. This is revealed by measurements of the relation between volume and membrane potential (Figs. 2, 4, 6, 7, and 10), and by morphological changes observed with electron microscopy (Fig. 3 and references 4 and 12).

The discrepancies between membrane potential and volume were seen only in transitional conditions and were essentially absent when the muscle fibers had attained osmotic equilibrium (Figs. 5 and 6). They indicate, accordingly, that the muscle fiber possesses at least 2 compartments with different kinetics of water movement. Since the membrane potential can reach the control level while the fiber still contains an excess of water, as in the terminal portions

of the experiments in Figs. 2, 4, and 10*B*, one of these compartments may be designated as an "electrophysiological space." The absolute amounts of intracellular K in this space must have remained nearly constant, since the two branches of the potential-volume relation (Fig. 6) were both straight lines. Furthermore, the membrane potential returned promptly to the control value in all experiments in which osmotic pressure was changed by increasing or decreasing the Na salt (Figs. 2 and 4).

Per unit change in osmotic pressure the swelling in hyposmotic media was greater than the shrinkage in hyperosmotic solutions, while the change in membrane potential per unit volume change was greater in the hyperosmotic media. These data suggest that another compartment, which has a relatively low K concentration, can swell more rapidly than it can shrink. A space which retains water after the electrophysiological space must have reached its equilibrium condition lies at the periphery of the muscle fiber (Fig. 3). The electron microscopic data (4, and in preparation) reveal still more complex morphological conditions.

Unlike water movements due to normal osmosis, those associated with equilibration of water and ions among intracellular compartments would not be expected to occur rapidly. It should be noted that if the volume and potential measurements had been made solely in preparations which had been equilibrated for long periods of time, the existence of compartmentalization within the cell would not have been observed.

Electrokinetic Movement of Water Whether it can be detected or not, electrokinetic movement of water (or electroosmosis) must necessarily occur if a boundary of sufficient fixed charge density is interposed between two electrodes which are delivering a current (18, 20). While the theory of electroosmosis is still incomplete (*cf.* references 8, 18, 21, 22, 31, 32), the magnitude of water translocation appears to be proportional to both the membrane and the electrokinetic (zeta) potentials. Since the latter decrease with increased ionic strength of the medium, clear-cut electrokinetic effects are best observed, even in model systems, when the solutions have relatively low concentrations of salts (16). The effects should be small, therefore, when the ionic strength of the solutions approaches that of physiological media. Electroosmosis as a possible mechanism for water movement in cells has been discussed by a number of physiologists (20), but evidence for its occurrence in living cells has been meager. Indeed, the occurrence in living cells of electroosmotic water transfer in significant amounts has been doubted by most investigators, although Sollner and his colleagues consider it a possibility (15, 16, 25, 33–35) which would arise from heterogeneity in membrane properties.

The importance of electrokinetic phenomena in biological systems has recently been emphasized in the work of Diamond (9). Streaming potentials of considerable magnitude were recorded when an osmotic gradient was im-

posed across the gall bladder of fresh water fish. This effect is related to, though the opposite of, electroosmosis. The membrane of crayfish muscle fibers is a mosaic of anion- and cation-permselective regions (12). Thus, a change in the EMF of one of the membrane batteries relative to another will give rise to a flow of current and to electroosmosis. The occurrence of an electroosmotic transfer of water therefore may be expected and it is indicated by several types of experimental data presented in the foregoing.

Changes in membrane potentials, which are the motive force of electroosmotic phenomena, are small when muscle fibers are exposed to media in which the Na salt concentration alone is varied (Figs. 2 and 4). The contribution of electrokinetic phenomena to water movements therefore is probably small under these conditions. Nevertheless there must be some current flow between the cationic sarcolemma and the anionic TTS, since transient swelling of the TTS occurs in crayfish muscle fibers when they are exposed to a hyperosmotic NaCl solution (12).

Electrokinetic water movements were prominent in the present work when the change in membrane potential was large and rapid. The flow was in the same direction as the movement of water in normal osmosis when the medium was made hyperosmotic with addition of K (Figs. 8 to 11A and Fig. 14). It was clearly opposite to that of osmotic flow when the fiber was restored to the control medium after exposure to a K-rich isosmotic medium (Figs. 11 to 13).

Potential-dependent water movements may reach a maximum rapidly when the membrane potential is altered. However, the water movement lasts only as long as does the disequilibrium of the electrochemical potentials which contribute to the membrane potential of the cell. In the present work this transient character of the electrokinetic movement of water was best illustrated by the volume changes which occurred when Cl was first removed from, and then reintroduced into, the bathing medium (Fig. 15). These changes in ionic composition lead to a transient depolarization of the cell and to a transient hyperpolarization, respectively. Thus, the depolarization was associated with a transient shrinkage and the hyperpolarization with a transient swelling. It should be noted that the volume changes in these experiments occurred under isosmotic conditions when water movement by normal osmosis could not have taken place.

The relations between the transient changes in volume and the potential were opposite to those which were observed during osmotic movement of water (Figs. 2, 4, 6, and 7). There was also a swelling of the TTS during the efflux of Cl which could be reversed by the reintroduction of Cl into the medium or which disappeared if the fibers were maintained in the Cl-free medium. Since the initial shrinkage of the fiber when Cl was removed was only transient it may be concluded that the swelling of the TTS was probably associated largely with a redistribution of intrafiber water while the transient

swelling of the whole fiber must have involved entry of water from the external solution. However, the water movements and the forces which act on them must be quite complex in these transient responses. This is denoted by the diphasic courses of the volume changes. The ion fluxes must be undirectional, KCl leaving the cell when external Cl is removed and reentering when the latter is restored.

Volume changes were also observed, as predicted, with currents which shifted water into or out of the cell (Fig. 16). The direction of current flow determined the direction of the flow of water. Furthermore, the volume

TABLE III

CONDITIONS FOR PRODUCING, AND THE DIRECTIONS OF THE POTENTIAL-INDUCED VOLUME CHANGES IN CRAYFISH MUSCLE FIBERS

Procedure	Change in potential	Change in volume
Internal anode	Depolarization	Shrinkage
Internal cathode	Hyperpolarization	Swelling
Decreased Cl_{out}	Depolarization	Shrinkage
Increased Cl_{out}	Hyperpolarization	Swelling
Hyperosmotic KCl	Depolarization	Initial shrinkage
Hyperosmotic K propionate	Depolarization	Initial shrinkage
Isosmotic KCl	Depolarization	Initial shrinkage*
Isosmotic K propionate	Depolarization	Initial shrinkage
Reversal from KCl	Repolarization	Initial swelling
Reversal from K propionate	Repolarization	Initial swelling

* Seen only occasionally.

changes were in the same direction as those which occurred on changing the membrane potential by modifications of the ionic milieu (Table III).

The electroosmotic volume changes were independent of the nature of the salts in the intracellular electrode (Fig. 16). This would not have been the case if the volume changes were due entirely to changes in the intracellular ionic composition. The net flow of water always moved in the same direction as did the cation. This fact suggests a preponderance of the effects of negative fixed charges in determining the electroosmotic flow of water.

The electrical resistance of the channels formed by the TTS and RT (radial tubules) is about 10 times lower than the resistance of the surface membrane of the resting crayfish muscle fiber exposed to its normal level of K_o (12, 13). However, when the fibers are exposed to high K the transport number of K rises toward unity (13). Thus, most of the current is then carried in the predominantly negatively charged surface membrane. Furthermore, the radially oriented channels form a relatively poor pathway for the electroosmotic exchange of water between the cell and the external medium. Electrokinetic

effects will be minimized because of the large diameter of the RT (*ca.* 200A), and of the sarcolemmal invaginations (*ca.* 0.1 μ) since the zeta potential then becomes an insignificant factor (8).

An electroosmotically induced shrinkage may also be expected to occur when the membrane is depolarized under isosmotic conditions by increasing the concentration of K in the medium. This was infrequently observed in experiments with Cl saline media (Figs. 11*B*, 12*A*, and 13), but it was seen in all the experiments in propionate media (Fig. 12*C*). In the experiments with isosmotic KCl media the fibers swelled rapidly as a result of the influx of water with the salt. The electroosmotic efflux of water induced by the depolarization would be opposed by the osmotic flow arising from the rapid entry of KCl. At best, the electroosmotic effect could be only transient, during the time when the ions are undergoing redistribution. When the K of the medium is above 20 meq/liter the equilibration is rapid (13).

In the media in which propionate replaced Cl, the reflection coefficient for K propionate must have been similar to that for the Na salt, since the available evidence (12, 13) indicates that propionate is an impermeant anion. Little or no redistribution of water during the movement of ions is to be expected under this condition. However, electroosmotic efflux of water might be expected if the depolarization of the muscle fiber in high K causes an initial transient flow of current. This appears to be likely in the case of hyperosmotic K propionate media (Figs. 8–10) since the shrinkage was much larger than expected on the basis of osmotic effects alone. This interpretation is supported by experiments in which the change to a strongly hyperosmotic medium was made by stepwise additions of K propionate. The maximum swelling in this case was not greater than that obtained in comparably hyperosmotic media, made so by increasing NaCl. When a given shrinkage was attained, the volume remained constant until the next addition of the propionate.

Further evidence for the occurrence of electroosmosis is provided by the swelling which was shown in all experiments in which the muscle fibers had been initially exposed to a K-rich isosmotic medium and then returned to the control medium (Figs. 11*B*, 12, and 13). This swelling occurred independently of the volume at that time. The fibers in the Cl media were already swollen markedly while the fibers of Fig. 12*B* and 12*C* showed small swelling. Even if the new steady-state volume in an isosmotic KCl medium could be explained by Donnan considerations (6, 7), the swelling that also occurred in a constant product medium (Fig 12*B*) is contrary to the expectation from a Donnan equilibrium. The amount of the secondary swelling when the fibers were returned to their respective media was about the same in the 3 muscle fibers of Fig. 12, whereas from osmotic considerations alone it would have been expected that returning fiber A from a low Na to the relatively high Na in the control medium would have led to a rapid shrinkage.

Experiments like that of Fig. 13, which indicated a dependence of the swelling upon the degree of change in membrane potential, provide additional evidence that the swelling is a potential-dependent effect. When a fiber which had been swollen in an isosmotic high KCl medium was exposed successively to a series of small reductions in the external KCl, only the first small decrease in KCl resulted in a swelling and this was relatively small. As already noted, no swelling at all occurred in 2 of the 4 experiments of this type.

A model which may serve to explain an electroosmotic flow of water in a heterogeneous fixed charge membrane has been fully discussed by Höber (20, see Figs. 65–67). This model requires the flow of local currents to induce a water flux and thus the term electroosmosis becomes applicable.

Factors Influencing the Steady-State Volume While the level of Na salt concentration in the medium correlates well with cell volume (Fig. 5), it is clear from Fig. 14 that the new steady-state volume also depends upon the amount of change in the KCl concentration and on the osmotic pressure that is imposed simultaneously. In the experiment of Fig. 14, the KCl concentration was eventually raised to the same level as that shown in Figs. 9 and 10, but the membrane was never exposed to as large an electrical and osmotic gradient. Under this procedure the new steady-state volume was essentially equal to the control volume. This type of experiment suggests several features which are conceivably characteristic of muscle fibers and perhaps of cells in general. The steady volume may at least in part be regulated by a dynamic state of ionic and water fluxes. Thus, the new volume level can be dependent upon the salt that is used to increase the osmotic pressure and the amount of change of the ionic and osmotic gradients across the membrane. A coupling between water flow and ionic flux may also be operative in the system. This type of coupling is characteristic of heterogeneous membranes with water-filled pores (37, 38). In such a system a sizable change in the influx/efflux ratio may occur when an osmotic (or hydrostatic) gradient exists across a barrier. If the phenomenon occurs in the muscle fiber membrane, the transient alteration of the fluxes during the period of water flow may result in a permanant displacement of the fluxes and volume beyond that predicted for a simple Na salt osmometer. Such changes appear to play a particularly important role in frog muscle fibers (reference 28, and unpublished data). Conjectures regarding mechanisms of this type in such a complex system as a muscle fiber are probably not too useful at this time. However, the time-dependent non-homogeneous distribution of water within the crayfish muscle fiber is substantiated by the swelling of the TTS (12) and the transient vesiculation at the periphery of the fiber (Fig. 3). A further and detailed study of the distribution of water among the intracellular organelles and myofibrils under many of the conditions described will be detailed elsewhere (4, and in preparation).

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