POTENTIAL AND K^+ ACTIVITY IN SKINNED MUSCLE FIBERS

Evidence Against a Simple Donnan Equilibrium

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ABSTRACT It has been suggested that potentials measured with conventional microelectrodes in chemically or mechanically skinned muscle fibers arise from a Donnan equilibrium due to myofilament fixed charges. This hypothesis was tested in mechanically skinned frog (*Rana pipiens*) semitendinosus fibers by measuring the distribution potential (E_d) between fiber and bath with 3 M KCl-filled microelectrodes and the K^+ activity gradient (a_K^i/a_K^o) with K^+ ion-selective microelectrodes (KISE). If skinned fibers are a Donnan system, E_d should become more positive as pH is decreased, altering the fixed charge on the myofilaments. Consistent with this expectation, E_d was -4.4, -0.6, and $+4.8$ mV in ATP-containing solutions and -6.5, -2.2, and $+8.4$ mV in ATP-free solutions at pH 7, 6, and 5, respectively. Donnan equilibrium also requires that all mobile ionic species be in electrochemical equilibrium. In ATP-containing solutions, this was true for K^+ at pH 7. At pH 5, however, KISE indicated that K^+ was not in equilibrium; average E_d was 5.9 mV positive to the K^+ equilibrium potential, and a_K^i/a_K^o was 1.04, while the Donnan prediction was 0.83. In contrast, KISE measurements in ATP-free solutions indicated that K^+ was in equilibrium at all pH studied. Skinned fibers in ATP-containing media are not equilibrium systems because ATPase reactions occur. Under our conditions, frog myofibrils hydrolyze 0.4 and 0.08 μmol ATP/min·mg myofibrillar protein at pH 7 and 5, respectively. It is suggested that in the presence of ATP, E_d is a superposition of Donnan and diffusion potentials, the latter arising from differences in the mobilities of anionic substrate and products that diffuse through the charged myofilament lattice. A coupling to diffusion of K^+, the predominant counter ion, is required for macroscopic electroneutrality. This coupling may be the origin of the nonequilibrium K^+ distribution.

INTRODUCTION

In a number of biological systems, electrical potentials are established without a functioning membrane. Using conventional microelectrodes, potentials can be measured in skeletal and cardiac muscle cells whose membranes have been made permeable by prolonged glycerination (1–10) or other chemical treatment (11–13) and in cells whose membranes have been mechanically removed ("skinned") (8, 9, 14–16). Potentials have also been measured with microelectrodes in gels made from various muscle proteins (17). In addition, potentials have been observed in myofibrils using a potential-sensitive dye (10), as well as across disks of myoplasm separating two compartments (18).

It has been proposed (4, 19) that these potentials arise from a Donnan equilbrium due to fixed charges on myofilament proteins, which are known to bear a net negative charge at near neutral pH. In accord with this notion, both the potential and the distribution of ions behave as predicted from Donnan theory, at least under some conditions. For example, the potential is unaffected by substitution of Na^+ for K^+ as the predominant cation in the bathing media, but can be decreased by increasing the ionic strength (4, 6, 10, 14). As expected, the sign of the potential is negative in solutions near pH 7 and reverses to positive at more acidic pH, below the presumptive isoelectric points of the major myofilament proteins (6, 14, 19). At near-neutral pH, the distribution of several charged species across the fiber boundary, determined both chemically (11, 14) and with ion-selective microelectrodes (4, 11), also appears to be consistent with a Donnan equilibrium.

Some investigators have advocated the use of potential measurements and Donnan theory to study molecular properties of the contractile apparatus (5, 6). For this reason, it is important to reexamine the origin of this putative Donnan potential in mechanically skinned skeletal muscle fibers of the frog, a widely studied tissue. Moreover, insofar as skinned muscle fibers in physiological media hydrolyze ATP, they are nonequilibrium systems.

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Consequently, one might expect deviations from Donnan equilbrium behavior under some conditions. In the present experiments, we investigated the potential difference and gradient of K^+ activity across the skinned fiber boundary at pH 5, 6, and 7 using both conventional and K^+ ion-selective microelectrodes. Brief descriptions of this work have appeared elsewhere (20, 21).

METHODS

Tissue Preparation and Solutions

Single fibers from the semitendinosus muscle of the frog *R. pipiens* were mechanically skinned in relaxing solution (pCa 8) at pH 7 (see Table I) using fine needles. The skinned fibers were stretched to a length slightly greater than slack length and pinned with fine insect pins to a small square (~1 × 1 cm) of Sylgard 184 (Dow Corning Corp., Midland, MI) elastomeric silicone polymer. Solutions were contained in 4-ml wells milled in a Plexiglas block. Except for records of continuous impalements, all data were taken from mounted fibers transferred manually from bath to bath with gentle blotting of the Sylgard mount in between.

The composition of the experimental solutions are given in Table I and were calculated using a computer program similar in principle to that described by Fabiato and Fabiato (22), run in BASIC on a PET 2001 microcomputer using binding constants given in Godt and Lindley (23). Solutions were chosen to have K^+, Mg^{2+}, MgATP concentrations, and a calculated ionic strength approximating their normal intracellular values at room temperature (22–23°C). Tris maleate buffer was used in all solutions to obviate any possible buffer effects on the measurement.

Electrical Arrangements

The potential difference across the skinned fiber boundary was recorded with conventional 3 M KCl-filled microelectrodes (12–20 MΩ) coupled to a high-input resistance, unity-gain amplifier (W P Instruments, Inc., model 750, New Haven, CT), and the potential detected by the ion-selective microelectrodes was recorded through a very high input resistance, unity-gain amplifier (W P Instruments, Inc., model FD-223). A 3 M KCl–3% agar bridge was used for ground. The signals were monitored on an oscilloscope, chart recorder, and digital voltmeters with 0.1 mV resolution from which measurements were taken.

Ion-Selective Microelectrodes

Potassium ion-selective microelectrodes (KISE) based on Corning 477317 liquid ion-exchanger (Corning Medical and Scientific, Corning Glass Works, Medfield, MA) (24) were fabricated from Pyrex 7740 glass blanks that were first silanized by exposure to vapors of trimethylsilyldimethylamine (25). The potential, E_o, observed in KCl or the experimental solutions is given by Eq. 1:

$$E_o = V_o + S \log a^o_K. \tag{1}$$

V_o is an empirically determined constant; S is the slope of the KISE response in KCl solutions; and a^o_K is the activity of potassium. These constants were determined both before and after an experiment by measuring the response of the KISE to 100, 150, and 300 mM KCl solutions. S ranged from 57 to 60 mV per 10-fold increase in a^o_K. The selectivity of KISE are sufficient (24) that in the high-K^+, low-Na^+ experimental solutions, only a^o_K significantly contributes to the KISE response. A typical calibration curve is shown in Fig. 1.

Upon impaling a fiber, the change in KISE potential, ΔE, is a function of both the electrical potential difference, E_d, and the K^+ activity gradient:

$$\Delta E = E_d + S \log (a^i_K/a^o_K) \cong E_d - E_K. \tag{2}$$

Because S is very close to the theoretical value of 59 mV, ΔE directly gives the difference between E_d and E_K, the change in the electrochemical potential of K^+ across the fiber boundary. In a Donnan equilibrium, E_d must equal E_K, and, therefore, ΔE is expected to equal 0 mV. With independent measurements of ΔE and E_d, the potassium activity inside the fiber, a^i_K, can be calculated from a rearrangement of Eq. 2 using the calibration data and the known composition of the bathing media.

Calculation of a^i_K assumes that E_d detected by the KISE and the conventional microelectrode are the same. A possible problem is that the conventional microelectrode and KISE are at different points within the fiber, and E_d may vary somewhat with the location of the impalement (see Results). To minimize this problem, the values of a^i_K/a^o_K presented in Table III were calculated from the average values for E_d and ΔE. Such averaging is commonly necessary in experiments with ion-selective microelectrodes (for examples and discussion, see reference 24). Another possible problem is that the diffusion potential at the tip of the conventional microelectrode may change on impaling a fiber. Use of 3 M KCl should minimize systematic errors of this type. The above reservations do not apply to conclusions based on the ΔE upon impaling the KISE, however. ΔE directly gives the difference between E_d and E_K at the tip of the KISE. A separate measurement of E_d, and a comparison of recordings made at different sites is not necessary.

TABLE I

SOLUTION COMPOSITION

	With ATP			Without ATP		
	pH 7	pH 6	pH 5	pH 7	pH 6	pH 5
Total Mg	2	2	2.2	1	1	1
Total ATP	1.1	1.2	2.8	0	0	0
Mg^{2+}	1	1	1	1	1	1
$MgATP^{-2}$	1	1	1	0	0	0
EGTA	1	1	1	1	1	1
KCl	118	118	110	124	124	124
Total K^+	130	128	118	134	132	129
a^o_K	96	95	87	99	97	95
Total Na^+	2.1	2.4	5.5	0	0	0
Tris maleate	6.9	8.5	14.9	6.9	8.5	14.9
Ionic strength	150	150	150	150	150	150

Nominal concentrations are given in mM. a^o_K was calculated using an activity coefficient of 0.738 (49).

Myofibrillar ATPase Activity

The actomyosin ATPase activity of myofibrils prepared from frog sartorius muscle was measured at room temperature in a 1 ml reaction solution at pH 7 and 5 under ionic conditions identical to those used for

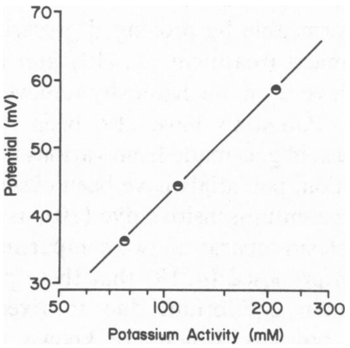

FIGURE 1 Calibration of a potassium ion-selective microelectrode (KISE). Preexperiment (•) and postexperiment (o) data are nearly identical.

KISE measurements. Myofibrils were prepared essentially as described by Solaro and Shiner (26). Myofibrillar ATPase activity in solutions at pH 7 and 5 (see Table I) was monitored by the determination of liberated phosphate using a colorimetric assay (27). Protein concentrations were determined by the method of Lowry et al. (28). Control experiments demonstrated no hydrolysis of ATP in the absence of protein at either pH over the time course of the reaction (15 min). These experiments were performed by R.J. Solaro and M. Rocklin (Department of Physiology, University of Cincinnati).

RESULTS

Conventional microelectrodes were used to determine if potentials could be observed in mechanically skinned frog muscle fibers, and how those potentials respond to pH and ATP. Upon impaling a fiber, potentials, which we shall term "distribution potentials" (E_d), could be observed.[1] Typical impalements are shown in Fig. 2. Values for E_d were accepted from impalements exhibiting the traditional criteria for successful impalements of intact cells, i.e., a rapid change of potential upon impalement, maintenance of a stable recording, and a rapid return to baseline upon withdrawal. An additional requirement for acceptance was visual confirmation of the impalement, as documented by dimpling of the fiber surface, movement of the fiber when the electrode was gently rocked from side to side, or adherence of the fiber to the electrode upon withdrawal. Visual confirmation was especially important under conditions where low potentials were observed. Data were not accepted if the recording became markedly noisy, as was infrequently observed, implying blockage of the electrode. Other workers have implied that the potential observed could be influenced by changes in electrode resistance upon impalement (4, 14). This possibility was examined in several experiments. Electrode resistance was measured before and during impalement. Neither changes in electrode resistance during impalement nor the initial resistance were related to the E_d observed.

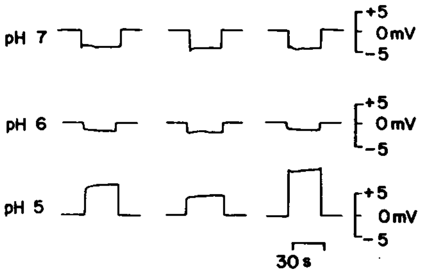

FIGURE 2 Distribution potentials (E_d) in ATP-containing solutions. Typical 3-M KCl-filled microelectrode impalements are shown. E_d was always negative in pH 7 solution, positive in pH 5 solution, and near 0 in pH 6 solution.

Values for distribution potentials in the presence and absence of ATP in solutions of different pH are shown in Table II (also see Fig. 2). In almost all cases, data were obtained from an individual fiber at more than one pH and with or without ATP. The responses of individual fibers to solution changes were not qualitatively different from the pooled data (Table II). In any one of the solutions, E_d was quite variable from fiber to fiber, at different locations along a fiber and at different depths within a fiber at the same location. Nevertheless, in ATP-containing solutions, E_d was always negative at pH 7, positive at pH 5, and near zero at pH 6. In the absence of ATP, E_d at pH 7 and 6 became more negative, whereas at pH 5, E_d became more positive.

Measurements of E_d in the presence of ATP reported here are in qualitative agreement with those of others (4, 6, 7, 11), in that E_d becomes more positive as pH declines. The exact values of E_d at any pH differ in these various studies, however. This is not unexpected given the differences in solution composition (e.g., ionic strength), procedures used to disrupt the sarcolemma, and fiber type and species. In addition, variability in E_d in our hands and from group to group in the literature may be due to variable compression of the myofilament lattice, which would lead to variation in local fixed charge density. Moreover, one cannot rule out the possibility that physical contact between a microelectrode and the myofilaments might damage them and alter their surface charges.

On the other hand, there is considerable discrepancy in the literature as to the effect of deletion of ATP on E_d. Our results at pH 7 agree with some reports (10, 16, and for A band, 8 and 9), but not others (5 and, for I band, 8 and 9). The present data are best compared with those of Pemrick and Edwards (5), in that they also estimated E_d with random microelectrode penetrations. In contrast to our observations at pH 7, however, they found that E_d of glycerinated rabbit psoas muscle became more positive in the absence of ATP at pH 6.8. We are uncertain of the basis for this discrepancy.

TABLE II
EFFECT OF pH AND ATP ON DISTRIBUTION POTENTIALS IN SKINNED FIBERS

	With ATP			Without ATP		
pH	E_d ± SD	n	No. of fibers	E_d ± SD	n	No. of fibers
	mV			*mV*		
7	−4.4 ± 0.7	26	6	−6.5 ± 0.8	32	6
6	−0.6 ± 0.9	24	4	−2.2 ± 0.4	24	4
5	+4.8 ± 1.0	37	6	+8.4 ± 1.1	33	6

Mean (E_d) and standard deviation (SD) are based on pooled data from all fibers; n is the number of impalements. Differences in E_d with ATP and with pH are highly significant ($p < 0.001$) in all cases.

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[1] In previous experiments by Godt et al. (29), only very small potentials were observed in mechanically skinned fibers. We have no ready explanation for this except to point out that, in the previous experiments, values were only accepted if electrode resistance did not change more than 5% upon insertion. This criterion was stricter than that used by us or others (Pemrick and Edwards [5] accepted changes of 10–20%) and could have resulted in the rejection of most insertions with good contact between the electrode tip and the myoplasm.

As reported in the literature, the effect of deletion of ATP on E_d appears to vary with species (5, 8, 9, 16), electrode position within the sarcomere (8, 9, however cf. 16), and technique for measuring E_d (5, 10). The present findings indicate, in addition, that the effect of ATP deletion on E_d is also dependent upon pH, a factor not previously explored.

Potassium Activity Gradient

One expectation in a Donnan system is that all ions that are not physically excluded from the fiber must be in electrochemical equilibrium regardless of the sign of E_d. This was tested for K^+ in fibers exposed to solutions at pH 5, 6, and 7 using K^+ ion-selective microelectrodes. Because in a Donnan system $E_K = E_d$, the potential recorded by KISE should not change upon impaling the fiber (see Eq. 2). The criteria for acceptance of KISE data were the same as previously described for conventional microelectrodes.

Under some conditions the expectations of Donnan equilibrium were not fulfilled. As is clearly shown in Fig. 3 and Table III, K^+ is not in electrochemical equilibrium at pH 5 in the presence of ATP. Fig. 3 shows continuous impalements of a fiber with both KISE and conventional microelectrodes. At pH 5, E_d was positive, and the KISE potential became more positive upon impalement. This implies that the K^+ activity inside the fiber is higher than expected for a Donnan system. The observed a^i_K/a^o_K ratio calculated from these impalements was 0.96, while Donnan equilibrium requires an a^i_K/a^o_K ratio of 0.63. When the pH 5 solution was exchanged for one of pH 7, E_d changed sign and KISE potential approached its bath value. This indicates that the K^+ distribution approached equilibrium. On readmitting pH 5 solution, the original potentials were restored, and once again, K^+ was out of equilibrium. When the order of exposure to solutions was inverted (i.e., pH 7 to 5 to 7 again), the K^+ distribution showed an identical pH dependence.

To obtain continuous records of the sort illustrated in Fig. 3, gentle flushing of the experimental chamber was required. This resulted in an incomplete solution exchange and in small differences (~0.1 pH unit) between the pH of the introduced solution and the actual final pH attained with exchange. The data on K^+ activity shown in Table III, however, were obtained from experiments in which the fiber was transferred between solutions as described in Methods. As can be seen in Table III, in the presence of ATP, nonequilibrium behavior was clearly demonstrated in solutions at pH 5 and was suggested at pH 6. On the other hand, at pH 7 and under all conditions in the absence of ATP, KISE potentials are consistent with electrochemical equilibrium for K^+.

A concern in all applications of ion-selective electrodes is that the presence of interfering ions may confound interpretation of the measurement. Therefore, several control experiments were done to test whether the apparent nonequilibrium behavior of K^+ under acidic conditions could be attributed to interference. One possibility was an effect of pH on the KISE or conventional microelectrode. Several control experiments, including one conducted with the electrodes used to obtain Fig. 3, demonstrated that exchange of pH 7 and 5 solutions had no effect on electrode behavior, however, and H^+ was not detected (30). Moreover, control experiments indicated, as expected, that electrode behavior was not influenced by ATP. Another possible interfering ion is NH_4^+. NH_4^+ is produced during the conversion of ATP to inosine monophosphate via the sequential action of ATPase, myokinase, and adenylate deaminase. The selectivity coefficient for the KISE for NH_4^+ is typically 0.27 (30). This means that the activity of NH_4^+ must be 66.7 mM to explain the 18 mM excess a^i_K found at pH 5 in ATP-containing solution (see Table III). Such an enormous accumulation of reaction product is highly improbable. Further, the rate of the adenylate deaminase reaction is markedly slowed by lowering pH from 6 to 5 (31). This would tend to result in greater interference from NH_4^+ at pH 6 than at pH 5, and if the accumulation of NH_4^+ was significant, K^+ would appear to be further out of equilibrium at pH 6 than at pH 5. Just the opposite was found. It might be argued that an unidentified interfering ionic species was present within the fiber at pH

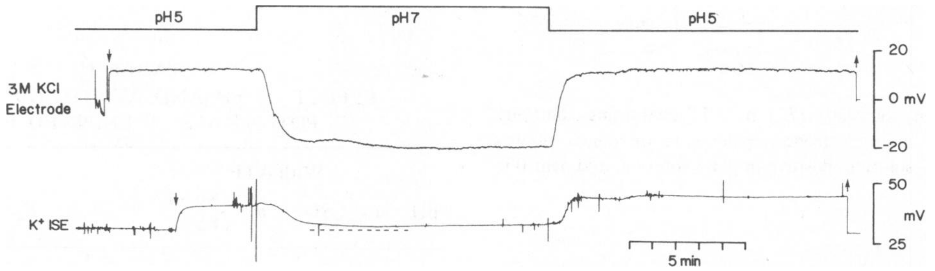

FIGURE 3 The effect of pH on the KISE potential and the distribution potential (E_d). Impalements were made at downward arrows, and the electrodes were withdrawn at upward arrows. E_d was +12 mV in pH 5 media, became −19 mV on switching to pH 7 solution, and +12 mV when pH 5 media was readmitted. The change in the KISE potential (ΔE) was +11 mV on impaling in pH 5. This implies that E_d was 11 mV positive to E_K and that a^i_K is greater than expected for a Donnan equilibrium. Upon switching to pH 7 solution, the KISE potential decreased by 9.5 mV and closely approximated the Donnan prediction (dashed line, ---). The ΔE of +13 mV on withdrawing the KISE in pH 5 media indicates that the nonequilibrium distribution of K^+ was reestablished under acidic conditions.

TABLE III
ION-SELECTIVE MICROELECTRODE POTENTIALS AND POTASSIUM ACTIVITY GRADIENTS IN SKINNED MUSCLE FIBERS

Solution	$\Delta E \pm$ SD	n	No. of fibers	a^i_K/a^o_K expected	a^i_K/a^o_K observed
	mV				
pH 7 +ATP	0	17	7	1.19	1.19
pH 7 −ATP	0	25	4	1.29	1.29
pH 6 +ATP	+2.1 ± 1.1	18	4	1.02	1.11
pH 6 −ATP	0	16	4	1.09	1.09
pH 5 +ATP	+5.9 ± 3.0	12	7	0.83	1.04
pH 5 −ATP	0	32	4	0.72	0.72

Mean change in KISE potential upon impalement (ΔE) and standard deviation (SD) are based on pooled data from all fibers; n is the number of impalements. ΔE of <1.5 mV could not be reliably detected and were tabulated as 0 mV. Expected a^i_K/a^o_K was calculated from E_d values in Table II assuming a Donnan equilibrium. Observed a^i_K/a^o_K was calculated from ΔE and E_d using Eq. 2. ΔE is significantly greater than 0 mV in both pH 5 +ATP and pH 6 +ATP solutions ($p < 0.001$) and equals E_d minus E_K.

5 but not at pH 7, with the result that an artifactually high K^+ activity in the fiber was recorded at pH 5. If this were the case, however, it is difficult to see how deletion of ATP would abolish the interference. Thus we must conclude that the observed activity differences were real.

Myofibrillar ATPase

The initial rates of ATP hydrolysis by frog myofibrils in our experimental solutions was 0.40 μmol inorganic phosphate (Pi)/min·mg myofibrillar protein at pH 7 and was 0.08 μmol Pi/min·mg protein at pH 5. These data show that at pH 5 significant amounts of ATP were hydrolyzed by the myofibrils under our conditions.

DISCUSSION

Exactly what is measured by a conventional microelectrode in these preparations may not be intuitively obvious. Because the tip of a microelectrode is much greater in diameter (~200–500 nm) than either the distance between myofilaments (~40 nm) or the Debye length (~1 nm), the potential detected must represent a complex average. Recent theoretical analyses of a Donnan equilibrium system consisting of parallel polyelectrolyte rods with fixed charge density and interparticle spacing appropriate to the myofilament lattice indicate that the potential detected will closely approximate the expected Donnan potential, at least in this simplified model of muscle (32, 33).

To make the situation clearer, consider a dialysis bag containing impermeable polyionic molecules in a solution of permeable ions. If one measures the potential inside this bag (with respect to the outside) with an electrode, a Donnan potential due to separation of the permeable ions will be measured. We believe the skinned fiber is analogous. The impermeable polyionic molecules are primarily the myofilaments retained within the skinned fiber because of their interactions. One measures a potential due to separation of mobile ions in response to these fixed charges.

In another context, some observations have been interpreted as suggesting that potentials measured with conventional 3 M KCl-filled microelectrodes in biological cells in the absence of membranes may be artifactual. Rehm and co-workers (34), using isolated frog cornea, measured the potential of the stroma (with respect to the aqueous side) and Nelson et al. (35) studied the potential in isolated frog epithelial cells in which the plasma membrane was first ruptured by freezing and thawing. Both groups obtained negative potentials upon impaling with microelectrodes filled with 3 M KCl but zero potential when the electrodes were filled with bathing solution. They suggested that the true potential was zero and that the negative potential was an artifact due to the fixed negative charges in stromal connective tissue or cytoplasm, respectively.[2] In their view, this artifact arises from a significantly higher mobility for K^+ than for Cl^- in a regime of fixed negative charges (e.g., skinned muscle fibers at pH 7) that produces a significant negative liquid-junction potential at the electrode. In fact, any such artifact would likely be of the opposite polarity, since in a fixed-charge matrix diffusion of the counter ion (K^+ for negative fixed charges) will be slower than the co-ion (here Cl^-) (37). In our case, the liquid-junction potentials will, of course, be summed with the true E_d at open-tipped microelectrodes. The error in E_d and in the calculated a^i_K should be small, because according to the Henderson equation (38, pp.48–49), liquid-junction potentials will be minimized by filling with a high concentration of KCl as is customary. These considerations do not apply to ΔE measurements made with ion-selective electrodes where diffusion is nil. Consequently, uncertainty regarding liquid-junction potentials fails to affect the conclusion that E_d does not equal E_K, a conclusion that can be drawn solely from the observation of a nonzero ΔE (see Eq. 2).

While the effects of pH on E_d appear to be consistent with a Donnan origin for the distribution potential in skinned muscle fibers, a more critical test of this hypothesis is provided by measurements of ion activity. In the presence of ATP, our KISE measurements document that K^+ is not in electrochemical equilibrium across the boundary

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[2] Overbeek (36) discusses why it is appropriate to use 3 M KCl-filled electrodes to measure a Donnan potential. In contrast, attempts to measure a Donnan potential with a bathing solution–filled electrode (34, 35) will always fail to detect a Donnan potential when one is present. The failure results because a Donnan potential is established between the tissue and the bathing solution-filled electrode. This potential is exactly equal and opposite in sign to the Donnan potential of interest between the bulk phase of the bathing solution and the tissue. Consequently, no change in potential will be observed on impaling a tissue exhibiting a Donnan potential because two equal but opposite potentials are being summed.

of frog skinnned fibers at pH 5 or, probably, at pH 6. Donnan theory cannot explain the deviation of K^+ from electrochemical equilibrium.

To the contrary, the distribution of K^+ activity at pH 7 in ATP-containing solutions is consistent with Donnan theory. To conclude from this that E_d is a purely Donnan potential under these circumstances would be premature, however. This conclusion requires equivalent measurements of more than a single ionic species. The only other study of ionic distribution in skinned fibers in the presence of ATP is that of Stephenson et al. (14) on barnacle muscle. They made chemical determinations of the concentration of a number of ions in the myofibrillar space in pH 7.1 solutions and concluded that the observed cation accumulation and anion depletion were quantitatively consistent with a Donnan mechanism. However, the E_d predicted from the concentration ratios between myofibrillar and extramyofibrillar space for both K and EGTA (their Table I, V^{K}_{pred} and V^{EGTA}_{pred} their notation for predicted E_d) is not in good quantitative agreement with their observed E_d at three of six ionic strengths examined (their Fig. 2).

Deviations from Donnan equilibrium are not unexpected in ATP-containing solutions. Hydrolysis of ATP by ATPase reactions within the fiber necessarily results in net fluxes of both charged substrate and products across the fiber boundary, while a Donnan equilibrium mandates no net flux for all charged species. Thus, whenever ATP hydrolysis occurs, at least some species must violate Donnan conditions.

In contrast to the anomalous behavior of ionic distributions in ATP-containing solutions, in the absence of ATP the situation appears to be more straightforward. Deviations from electrochemical equilibrium have never been detected, although the distribution of a number of ions have been examined under a variety of conditions. The present results show that K^+ activity was in electrochemical equilibrium in mechanically skinned frog fibers at pH 5, 6, and 7. This is in agreement with the observations of Collins and Edwards (4) for K^+ activity in glycerinated rabbit psoas muscle at pH 7.5 and with those of Hinke (11) for K^+, Na^+, and Cl^- activity in detergent-skinned barnacle muscle at pH 7.4.

Numerous investigators have attributed the distribution potential observed upon impalement of skinned or glycerinated muscle fibers with conventional microelectrodes to the establishment of a Donnan equilibrium due to the presence of fixed charges on the cellular proteins (4–11, 13–16). In the case of muscle cells, these are predominantly the contractile proteins. Some have gone further to utilize the observed potentials and Donnan theory to calculate the net charge density on the proteins (4–9, 11, 17) and have used these calculations to make inferences concerning the physiological and biochemical mechanisms of contraction (4, 6, 7, 9, 17).

The assumption that the distribution potential, under all conditions, arises solely from a Donnan mechanism, i.e., the basis for the calculation of fixed charge density, must be considered suspect. In the first place, Overbeek (36) has stressed that Donnan systems are (or should be) in equilibrium. If the system is not in equilibrium, Donnan theory does not apply. One would expect a priori that skinned or glycerinated muscle fibers in ATP-containing solution are not equilibrium systems, since ATPase reactions occur within the fiber. Moreover, the ATPase experiments indicate that frog myofibrils hydrolyze significant amounts of ATP under our experimental conditions. Secondly, the present experiments have shown that, under certain conditions, K^+ is not in electrochemical equilibrium, a result impossible under simple Donnan theory. Thirdly, if E_d was a Donnan potential, in the simplest case, removal of negative charge associated with MgATP should make E_d more positive at all pH studied. Such was not the case at pH 6 and 7. Lastly, even if the system were a simple Donnan one, the considerable variability of E_d, the possibility of mechanical distortion of the myofilament fixed charge density by the microelectrode, and the possibility of systematic diffusion potential errors in the measurement of E_d lead to large uncertainties in the calculated charge densities because of the logarithmic relationship between E_d and fixed charge density in Donnan theory (36, 38, pp. 53–66).

This is not to say that fixed charges play no role in the potential and the distribution of ions across the skinned fiber boundary. Rather, factors in addition to Donnan theory must be considered. One might imagine, for example, that a diffusion potential will result from the hydrolysis of ATP within the fiber, if there is an unequal transfer of charge due to diffusion of charged substrate, products, and their associated counter ions (in our case almost exclusively K^+). This might result from differences in charge and/or mobility of the myriad forms of substrate and products (see Table IV). Such diffusion potentials seem probable. In this vein, DeSimone and co-workers (41, 42) have shown theoretically in an analogous system that an enzymatic reaction at a charged surface leads to a large perturbation of the equilibrium double-layer potential when mobilities of charged substrate and product are different.

If, in the presence of ATP, E_d is a superposition of Donnan and diffusion potentials, the occurrence of nonequilibrium distribution of K^+ under acidic conditions could be explained. With diffusion of charged substrate and products, there is obligatory diffusion of counter ions, in the present case primarily K^+, as required for macroscopic electroneutrality. This coupling of K^+, substrate, and product diffusion may be the origin of the deviation of the K^+ distribution from equilibrium. Similar couplings between chemical reactions and transport have been shown to occur in a number of systems (43–47).

In this view, one would expect that removal of ATP would abolish the diffusion potential and hence alter E_d. Such alterations have been observed by us and by others (5, 8–10, 16). However, removal of ATP might also mod-

TABLE IV
PREDOMINANT SUBSTRATE AND PRODUCT SPECIES AS A FUNCTION OF pH

Species	Concentration		
	pH 7	pH 6	pH 5
	M	*M*	*M*
$MgATP^{2-}$	1×10^{-3}	1×10^{-3}	1×10^{-3}
$MgHATP^{1-}$	2.1×10^{-6}	2.1×10^{-5}	2.1×10^{-4}
ATP^{4-}	2.9×10^{-5}	2.9×10^{-5}	3.0×10^{-5}
$HATP^{3-}$	1.3×10^{-5}	1.3×10^{-4}	1.3×10^{-3}
H_2ATP^{2-}	1.9×10^{-8}	2.0×10^{-6}	2.0×10^{-4}
$KATP^{3-}$	3.0×10^{-5}	3.0×10^{-5}	2.8×10^{-5}
$MgADP^{1-}$	4.3×10^{-6}	2.2×10^{-6}	3.6×10^{-7}
$MgHADP$	4.5×10^{-8}	2.4×10^{-7}	3.8×10^{-7}
ADP^{3-}	2.8×10^{-6}	1.5×10^{-6}	2.4×10^{-7}
$HADP^{2-}$	9.1×10^{-7}	4.9×10^{-6}	7.9×10^{-6}
H_2ADP^{1-}	1.1×10^{-9}	5.9×10^{-8}	9.4×10^{-7}
$KADP^{2-}$	2.0×10^{-6}	1.1×10^{-6}	1.6×10^{-7}
PO_4^{3-}	2.0×10^{-11}	3.4×10^{-13}	3.7×10^{-15}
HPO_4^{2-}	2.6×10^{-6}	4.5×10^{-7}	4.8×10^{-8}
$H_2PO_4^{1-}$	5.4×10^{-6}	9.2×10^{-6}	9.9×10^{-6}
$KHPO_4^{1-}$	1.1×10^{-6}	1.8×10^{-7}	1.8×10^{-8}
$MgPO_4^{1-}$	negligible since PO_4^{3-} is so low		
$MgHPO_4$	8.6×10^{-7}	1.4×10^{-7}	1.5×10^{-8}

Species calculated for a solution identical to the experimental solutions, which contain 1 mM Mg^{2+} and 1 mM MgATP (the species hydrolyzed), except that it also contains 0.01 mM total ADP and inorganic phosphate due to ATPase reactions (i.e., 1% of MgATP hydrolyzed). Binding constants were taken from references 23, 39 and 40.

ify fixed-charge densities, and, therefore, changes in E_d alone cannot provide a decisive test of the proposed mechanism. The experiments with KISE are more compelling since this model requires that K^+ be in electrochemical equilibrium in ATP-free solutions. Under all conditions examined, such was the case.

One question that might be raised is why is K^+ apparently in electrochemical equilibrium in the presence of ATP at pH 7 but not at pH 5? The most likely explanation is that at pH 7 charge transfer associated with the ATPase reactions is fortuitously near balance whereas at pH 5 it is not. This difference may arise from pH-induced alterations in the relative amounts and/or mobilities of the various diffusing species. Certainly the relative concentrations of diffusing species varies markedly with pH (see Table IV). Furthermore, the fixed-charge milieu provided by the myofilament lattice also varies with pH. At pH 7, the major myofilament proteins of the lattice bear a net negative charge, while at pH 5, below their isoelectric points, the lattice bears a net positive charge (4, 19). Because product and substrates are anionic at either pH, one would expect a decreased mobility of these species when the lattice is cationic (pH 5), but little effect when the lattice is anionic (pH 7) (37). At present, the absence of data on the relative mobilities of the various diffusing species and the actual field geometry surrounding the charged proteins precludes modeling the changes in ΔE and E_d that might be expected in skinned muscle fibers. An additional effect of pH under our conditions will be on the free Ca^{2+} levels in the solutions. The apparent Ca^{2+} binding constant of EGTA decreases markedly as pH declines (pK′ is 6.42, 4.42, and 2.54 at pH 7, 6, and 5, respectively, calculated from data in reference 48); thus, free Ca^{2+} will be higher in the more acidic solutions. This might well modify fixed charges. Nevertheless, changes in pCa do not alter the expectation that in a Donnan system all ions must be in electrochemical equilibrium.

Linkages between enzyme activity and ion distribution have important implications for biological function. As DeSimone and co-workers (41, 42) have pointed out, enzymatic reactions at a charged surface can, under some circumstances, act as an amplifier where comparatively small variations in bulk concentration of product or substrate can provide large changes in surface concentration of these substances. Our experiments demonstrate that in ATP-containing solutions at acidic pH there is more K^+ inside the skinned fiber than expected from electrochemical equilibrium. Similar increases in other cations, e.g., Ca^{2+}, might also occur. One might speculate that with activation, as Ca^{2+} in the myofibrillar space increases, ATPases are activated that, in turn, further increase the Ca^{2+} concentration in this space. A similar process might occur at the sarcoplasmic reticulum. Thus, the control of contraction by Ca^{2+} would tend to be regenerative, an intriguing possibility that bears further study.

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