

# Differences in the Charge Distribution of Glycerol-Extracted Muscle Fibers in Rigor, Relaxation, and Contraction

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**ABSTRACT** Glycerol-extracted rabbit psoas muscle fibers were impaled with KCl-filled glass microelectrodes. For fibers at rest-length, the potentials were significantly more negative in solutions producing relaxation than in solutions producing either rigor or contraction; further the potentials in the latter two cases were not significantly different. For stretched fibers, with no overlap between thick and thin filaments, the potentials did not differ in the rigor, the relaxation, or the contraction solutions. The potentials measured from fibers in rigor did not vary significantly with the sarcomere length. For relaxed fibers, however, the potential magnitude decreased with increasing sarcomere length. The difference between the potentials measured for rigor and relaxed fibers exhibited a nonlinear relationship with sarcomere length. The potentials from calcium-insensitive fibers were less negative in both the rigor and the relaxation solutions than those from normal fibers. When calcium-insensitive fibers had been incubated in Hasselbach and Schneider's solution plus  $MgCl_2$  or Guba-Straub's solution plus MgATP the potentials recorded upon impalement were similar in the rigor and the relaxation solution to those obtained from normal fibers in the relaxed state. It is concluded that the increase in the negative potential as the glycerinated fiber goes from rigor to relaxation may be due to an alteration in the conformation of the contractile proteins in the relaxed state.

Recently considerable attention has been devoted to correlating the molecular interactions between the contractile proteins of muscle with changes in cross-bridge orientation. It is generally accepted that in the state of rigor the cross bridges of the myosin molecule (M), which are nearly free of bound nucleotide, bind to actin (A) in a chevron-like configuration (Reedy et al., 1965). In the resting state (relaxation) the cross bridges are at right angles to the thick filament and are distributed between two populations: M.ATP (Bag-

shaw and Trentham, 1973) and  $M.ADP.P_i$  ( $P_i$  = inorganic phosphate) (Taylor et al, 1970; Lymn and Taylor, 1970, 1971). Activation (contraction) leads to an increase in the rate of formation of  $M.ATP$ ,  $M.ADP.P_i-A$ ,  $M.ADP-A$ , and  $M-A$  (Lymn and Taylor, 1970, 1971). When actin and myosin are dissociated by agents which are either not hydrolyzed by myosin, such as pyrophosphate (Beinbrech et al., 1972) or are hydrolyzed at a 1,000-fold slower rate than  $MgATP$ , such as  $\alpha$ - $\beta$ -methylene- $ATP$  (Mannherz et al., 1973) there is some indication that the cross bridges may remain in the characteristic chevron configuration of rigor. If this observation is confirmed, the species  $M.ATP$  and  $M.ADP.P_i$  will have been assigned to particular cross-bridge configurations. Activation of the contractile system occurs when calcium binds to TN-C (calcium binding subunit of troponin) changing the precise interaction between the various subunits of troponin, tropomyosin, and actin (Ebashi et al, 1969; Hartshorne and Pyun, 1971; Margossian and Cohen, 1973) and thereby, activating the myofibrillar ATPase. X-ray diffraction studies suggest that during activation tropomyosin moves from a position overlapping the myosin binding site of actin to a position near the center of the grooves of the actin helix (Huxley, 1972; Spudich et al., 1972). This movement is thought to be independent of the presence of myosin (Vibert et al., 1972). Similarly, the cross bridges move independently of the presence of actin (Haselgrove, 1970).

In the present experiments electrophysiological techniques have been employed to investigate these interactions between the contractile proteins. Glycerinated muscle lacks a functional cell membrane (Szent-Gyorgyi, 1949). However, the thick and thin filaments provide effective electrolyte exclusion and a Donnan equilibrium operates between the immobile charged proteins and the medium (Collins and Edwards, 1971). The magnitudes of the potentials measured in glycerinated fibers during rigor, relaxation, and contraction will be compared in relation to the possible conformational states of these proteins. Brief accounts of some of these results have appeared (Pemrick and Edwards, 1972, 1973).

## METHODS

### *Principle of the Method*

The difference in potential upon microelectrode "impalement" of glycerinated fibers (Naylor and Merrillees, 1964; Weiss et al., 1967) has been shown to behave as if it were a Donnan effect caused by the contractile proteins (Collins and Edwards, 1971). The observations on which this conclusion was based included: the absence of potential difference between muscle and bath when measured by a potassium electrode, the invariance of the potential when sodium was replaced by potassium, the increase in the magnitude of the potential with a decrease in ionic strength, and the reversal of the sign of the potential beyond the approximate isoelectric point of the muscle proteins.

The source of the potential may be understood better if one considers the following three-phase system: the bathing fluid, the muscle protein gel, and the electrode tip filled with 3 M KCl. The muscle proteins actually behave as a layer of fixed charge separating the two solutions. The potential between the two solutions is therefore the sum of the Donnan potentials at the two solution-protein interfaces and the potential due to the differences between the ionic concentrations in the two solutions. The latter, for the case of a single uni-univalent ion in the two solutions is identical to the liquid junction potential and would, therefore, be negligible if KCl were the principal salt (Teorell, 1953). Thus the measured potential difference is essentially the sum of the Donnan potentials at the two boundaries. However, given the likely value for the density of the fixed charge in muscle protein, the magnitude of the Donnan potential between the muscle protein and 3 M KCl is also negligible, and so the potential measured is essentially the Donnan potential between the muscle protein and the bathing fluid. The magnitude of this will, of course, depend on the density of fixed charge in the muscle protein; a reduction in this charge density will reduce the potential.

The muscles used for these experiments were initially in a state of rigor, i.e., they were relatively inextensible and the number of "rigor complexes" between actin and nucleotide-free myosin was maximal (Bremel and Weber, 1972; Maruyama and Weber, 1972). If  $Mg^{2+}$  and appropriate MgATP ( $5 \times 10^{-5}$  M) are added, and the free  $Ca^{2+}$  level is kept below  $10^{-7}$  M, at an ionic strength (I) of 0.1, the muscles relax and become more extensible (Weber and Winicur, 1961; Portzehl et al., 1969). The number of rigor complexes for the relaxed fibril is negligible (Bremel and Weber, 1972). However, if the free  $Ca^{2+}$  level is raised ( $10^{-6}$  M) in the presence of  $Mg^{2+}$  and MgATP, the fibers contract, and are more extensible than in rigor due to an increase in the number of force-generating complexes between actin and myosin ( $A \sim M$ ) which probably contain ADP and/or  $P_i$  (Maruyama and Weber, 1972).

#### *Fiber Preparation*

Strips of rabbit psoas muscle (1 mm in diameter) were tied at rest-length, or stretched 10–40% over rest-length before being tied to wooden sticks, and placed in a solution of 50% glycerol, 2 mM  $K_2EGTA$ , 2.5 mM DTT (dithiothreitol), pH 7.2, for 2 days at 0°C. The solution was then exchanged and the muscles were stored at  $-15^\circ C$  for 2 wk before use. Muscles not used within 6 wk were discarded. Addition of 50 mM KCl to the glycerol solution prevented gradual extraction of the troponin-tropomyosin complex, but calcium sensitivity seemed to be reduced by storage much beyond 6 wk. To facilitate membrane destruction and extraction of soluble components, sometimes the tied fibers were shocked osmotically by bathing them alternately in the glycerol solution and a standard salt solution (100 mM KCl, 1 mM  $MgCl_2$ , 2 mM  $K_2EDTA$ , pH 7.2) for six 0.5-h intervals at 0°C as described by Rome (1972). After this treatment, the fibers remained in the glycerol solution at 0°C for 24 h before being transferred to fresh solution and stored at  $-15^\circ C$ .

Small bundles of glycerinated fibers (diameter approximately 200  $\mu m$ ) were rinsed in the rigor solution and fixed with Plasticene at both ends to a glass slide. Within the Plasticene boundaries a chamber was constructed by applying from a syringe a rectangular frame of petroleum jelly on the slide.

Sarcomere lengths were determined in some experiments from the optical diffraction patterns obtained from a helium-neon gas laser (Spectra-Physics, Inc., Mountain View, Calif., model 133). Parallel monochromatic light ( $\lambda = 632.8$  nm) was passed through the fiber bundle. The tangent of the angle  $\theta$  of the diffracted beam was calculated from the ratio of the mean distance between two diffraction lines and the length of the diffracted beam (Fig. 1). The average distance between Z-lines,  $d$ , or average sarcomere length was determined from the relationship,  $d \sin \theta = \lambda$ .

#### *Potential Measurements*

Glass microelectrodes (1- $\mu$ m tip diameter, 10–20 M $\Omega$ ) filled with 3 M KCl were used for the potential measurements. The microelectrode was inserted into the fiber

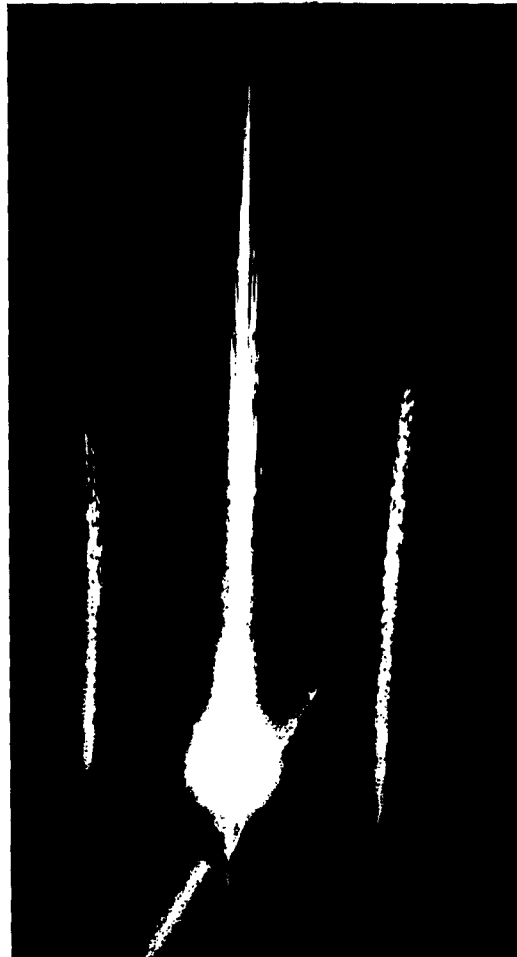


FIGURE 1. Optical diffraction pattern obtained by passing parallel monochromatic light from a helium-neon gas laser through a bundle of muscle fibers. The average sarcomere length can be calculated from the distance between diffractions.

(impalement) with a micromanipulator. Correct orientation of the microelectrode and impalement were determined with the aid of an inverted microscope. The microelectrode was connected to a bridge amplifier designed by John P. Hervey of The Rockefeller University. The circuit permits simultaneous measurement of potential and resistance changes following the technique of Purple (1964; also Purple and Dodge, 1966). The potential changes were displayed on one channel of a Tektronix Model 565 oscilloscope (Tektronix, Inc., Beaverton, Ore.) and also recorded from a digital voltmeter. The electrode resistance was first measured in the bathing solution and then compared with the resistance change during impalement of the fiber. Potential changes during impalement were accepted only if changes in electrode resistance were small compared to the values in the bathing solution (10–20%). This was to exclude the occasional large potential change which was invariably accompanied by a large increase in resistance and so was attributed to occlusion of the electrode tip.

For each experiment potentials were recorded first in the rigor solution, the solution removed, fresh solution added, and the measurements repeated to ensure that the solution change itself was without effect. The rigor solution was then exchanged for one maintaining relaxation or contraction. Before impalement the relaxed fibers were examined with an inverted microscope for evidence of contraction. Measurements were recorded in the relaxing solution only when the fibers appeared less taut within the Plasticene boundaries and there were no signs of contracted regions or sliding bundles of fibers. The calcium concentration of the contracting solution was kept slightly above  $10^{-6}$  M to minimize gross structural changes in fiber density which were observed at higher calcium concentrations. Under the present conditions the fibers were not prevented from shortening during contraction. However, at higher concentrations of calcium ( $10^{-4}$  M) the fibers not only shortened but also curled up, losing fiber alignment. This made it difficult to obtain suitable impalement potentials without a concomitant increase in resistance. Since it was possible to induce further shortening of these fibers in the contracting solution used (higher concentrations of calcium, long periods of incubation without changing the contracting solution) the shortening observed during the impalements was attributed to contraction and not to the onset of rigor.

For each fiber bundle the magnitudes of 10–15 impalements were recorded and the mean and standard deviation (SD) of these observations determined. The data were then grouped according to the nature of the solution and expressed as mean and standard error (SE). A paired difference test (Sokal and Rohlf, 1969) was used to analyze the statistical significance of differences in impalement potentials between various pairs of the three solutions.

Control experiments involved substitution of hard-boiled egg white for the glycerinated fiber. Potential measurements upon impalement of egg white in a rigor bathing solution were compared with measurements obtained in solutions producing relaxation or contraction of the muscles.

In a series of experiments strips of psoas muscle were extracted (48–72 h at 4°C) with Guba-Straub's solution plus MgATP or Hasselbach and Schneider's solution containing pyrophosphate and  $MgCl_2$  (Hanson and Huxley, 1955) to remove myosin and M-band proteins. At the end of the extraction period, the fibers were removed, rinsed, and the solutions were analyzed for protein by a modified microbiuret method

(Itzhaki and Gill, 1964). Potential measurements were then recorded for these fibers in the three solutions as described above.

The physiological solutions were designed for maintaining glycerinated fibers in either the rigor, the relaxed, or the contracted state (Table I). The rigor solution was modified from Marston and Tregear (1972). Two sets of solutions were used; one had an ionic strength of 0.069 and the other was 0.115.

Initially, to ensure the ionic similarity of the solutions, rigor solutions of slightly different compositions were used depending upon whether they were to be exchanged for the relaxation or the contraction solutions, respectively (Table I). The impalement potentials for fibers in rigor solutions A or B were identical. Therefore, to sim-

TABLE I  
PHYSIOLOGICAL SOLUTIONS

	Rigor			Relaxation	Contraction
	A	B	C		
	<i>mM</i>	<i>mM</i>	<i>mM</i>	<i>mM</i>	<i>mM</i>
Pipes (pH 6.8)	5	5	5	5	5
KCl*	5,50	12,62	5,50	5,50	12,62
Na <sub>2</sub> EGTA (pH 6.8)	5	1	5	5	1
MgCl <sub>2</sub>	5	1	5	5	1
MgSO <sub>4</sub>	2	1	1	0	0
Na <sub>2</sub> SO <sub>4</sub>	0	5	1	0	4
MgATP (pH 7.0)	0	0	0	2	1
Na <sub>2</sub> ATP (pH 7.0)	0	0	0	0	1
CaCl <sub>2</sub>	0	0	0	0	0.9
Creatine phosphate	10	10	10	10	10
Creatine phosphokinase (mg/ml)	0.5	0.5	0.5	0.5	0.5
Ionic strength*	73,123	67,129	72,122	75,125	67,129
Final pH 6.8 ± .05	—	—	—	—	—
Free Ca <sup>2+</sup> (mM)	<10 <sup>-5</sup>	<10 <sup>-5</sup>	<10 <sup>-5</sup>	<10 <sup>-5</sup>	1.86-2.75 ×10 <sup>-3</sup>

\* The concentration of KCl was varied as indicated to give two sets of solutions of different ionic strengths.

ply the procedure, a composite solution (C) was designed with no observable difference in the measured potentials for fibers in rigor. The contraction solution contains 4 mM Na<sub>2</sub>SO<sub>4</sub> to replace the Na<sup>+</sup> lost in lowering Na<sub>2</sub>EGTA from 5 (relaxation and rigor solutions) to 1 mM; the Mg<sup>2+</sup> concentration has been lowered from 5 to 1 mM; and ATP was added at neutral pH as the Mg<sup>2+</sup> or the Na<sup>+</sup> salt. The free Mg<sup>2+</sup> concentration is lower than in either of the other solutions and its possible effect on the ATP-regenerating system was not ignored. The composition of the present solutions evolved experimentally. To obtain impalement potentials of sufficient magnitude to confirm the statistical significance of the data, it was necessary to work at an ionic strength (0.069) at which the relaxed fiber was very sensitive to activation. The conditions found to be optimal for relaxation were not optimal for contraction even at 10<sup>-6</sup> M calculated free Ca<sup>2+</sup>. Therefore, activation was achieved by varying the levels

of several components in the contraction solution which are known to alter  $\text{Ca}^{2+}$  sensitivity: MgATP, free  $\text{Mg}^{2+}$  (Weber and Bremel, 1971).

## RESULTS

*Egg White*

As a control for the ionic similarity of the solutions, potentials were measured after impalement of hard-boiled egg white. It was found that the potential changes upon impalement do not differ significantly among the three solutions (Table II).

TABLE II  
CHANGE IN POTENTIAL UPON IMPALEMENT OF HARD-BOILED EGG WHITE

Physiological solutions (ionic strength $I = 0.069$ )		
Rigor	Relaxation	Contraction
<i>mV</i>	<i>mV</i>	<i>mV</i>
$-13.9 \pm 0.7^*$	$-13.5 \pm .04$	
$-14.3 \pm 0.4$		$-14.8 \pm 0.5$

\* Each potential in this table is the mean and standard error for four series of 10 impalements.

*Normal Glycerol-Extracted Muscle Fibers*

Potentials on impalement were measured in two sets of solutions which differed only in KCl concentration and ionic strength. As reported by Collins and Edwards (1971), the potential difference was greater at the lower ionic strength (Table III).

For solutions of either of the two ionic strengths used significant differences are found in the potentials measured from rest-length glycerinated fibers in the three solutions (Table III). The potential change upon impalement of the relaxed fibers is significantly more negative than the potential change upon impalement of fibers in either the rigor or the contracted states. However, the difference between the potentials recorded from impalements of fibers in the rigor and the contracted states is not significant. In all three solutions, decreasing the ionic strength increases the magnitude of the potential change upon fiber impalement but does not alter the 45–65% increase in the potential measured in impalements of relaxed fibers as compared to contracted fibers or fibers in rigor. The potential measurements from fibers which were glycerinated rapidly are more negative than those from fibers which were subjected to longer glycerination procedures (Table III). In addition, this treatment increases the magnitude of the difference between the potentials found for impalements of relaxed fibers and those in rigor.

The difference in the magnitudes of the potentials measured in fibers in

TABLE III  
CHANGE IN POTENTIAL UPON IMPALEMENT OF REST-LENGTH GLYCERINATED FIBERS

Ionic strength	Fiber preparation	Physiological state of myofibril			$d = X_2 - X_1$ where $X_2 > X_1$
		Rigor	Relaxed	Contracted	
		<i>mV</i>	<i>mV</i>	<i>mV</i>	<i>mV</i>
0.115	A	$-5.7 \pm 0.3^*$	$-10.5 \pm 0.6^*$		4.8
		$-7.0 \pm 0.1$	$-13.0 \pm 1.1$		6.0
		$-6.3 \pm 0.3$	$-13.9 \pm 0.4$		7.6
		$-7.8 \pm 0.4$	$-11.2 \pm 0.5$		3.4
		$-6.7 \pm 0.4$ (4) ‡	$-12.2 \pm 0.7$ (4) ‡		$5.5 \pm 1.5$ §
0.115	A	$-7.3 \pm 0.3$		$-6.7 \pm 0.4$	0.6
		$-6.6 \pm 0.3$		$-6.6 \pm 0.6$	0.0
		$-7.7 \pm 0.3$		$-7.9 \pm 0.6$	-0.2
		$-8.3 \pm 0.4$		$-8.3 \pm 0.4$	0.0
		$-7.8 \pm 0.1$		$-7.3 \pm 0.1$	0.5
		$-7.4 \pm 0.7$		$-7.3 \pm 0.5$	$0.2 \pm 0.3$
0.115	B	$-10.8 \pm 0.1$	$-16.3 \pm 0.6$		5.5
		$-10.4 \pm 0.1$	$-18.2 \pm 0.7$		7.8
		$-8.3 \pm 0.5$	$-12.5 \pm 0.5$		3.9
		$-9.5 \pm 0.5$	$-13.8 \pm 0.6$		4.3
		$-8.8 \pm 0.7$	$-11.7 \pm 0.5$		2.9
		$-9.6 \pm 0.4$	$-14.5 \pm 1.1$		$4.9 \pm 1.7$
0.069	C	$-7.9 \pm 0.7$	$-17.5 \pm 0.5$		9.6
		$-10.1 \pm 0.4$	$-16.8 \pm 0.6$		6.7
		$-10.9 \pm 0.7$	$-15.8 \pm 0.6$		4.9
		$-11.0 \pm 0.8$	$-18.1 \pm 0.4$		7.1
		$-9.7 \pm 0.4$	$-16.6 \pm 0.6$		6.9
		$-10.2 \pm 0.6$	$-17.3 \pm 1.3$		7.1
		$-12.3 \pm 0.5$	$-19.0 \pm 0.8$		6.7
		$-12.7 \pm 0.6$	$-18.7 \pm 0.9$		5.4
		$-9.8 \pm 0.4$	$-17.7 \pm 0.5$		7.8
		$-8.0 \pm 0.5$	$-15.1 \pm 0.4$		7.1
		$-10.3 \pm 0.5$	$-17.2 \pm 0.3$		$6.9 \pm 1.2$
0.069	D	$-13.4 \pm 0.8$	$-31.6 \pm 2.0$		18.2
		$-13.3 \pm 0.5$	$-26.0 \pm 0.8$		12.7
		$-12.3 \pm 0.7$	$-22.6 \pm 1.0$		10.3
		$-14.5 \pm 0.8$	$-27.1 \pm 0.7$		12.6
		$-14.0 \pm 0.7$	$-25.3 \pm 0.4$		11.3
		$-13.5 \pm 0.3$	$-26.5 \pm 1.3$		$13.0 \pm 2.7$
0.069	C	$-10.2 \pm 0.6$		$-8.7 \pm 0.8$	+1.5
		$-9.4 \pm 0.7$		$-8.8 \pm 0.5$	+0.6
		$-7.2 \pm 0.5$		$-13.3 \pm 0.8$	-6.1
		$-12.3 \pm 1.5$		$-9.2 \pm 0.5$	+3.1
		$-9.7 \pm 0.9$		$-10.0 \pm 1.0$	$0.2 \pm 3.5$

\* Mean and standard error for 10-15 impalements on one fiber bundle.

‡ Mean and standard error for ( $X$ ) number of fiber bundles.

§  $d \pm SD$  = the average difference  $\pm$  the standard deviation of the difference measurements from which the  $t$  statistic may be calculated.

|| Fibers subjected to osmotic shock for a rapid glycerination procedure as described by Rome (1972).



solutions causing rigor or relaxation is a function of the extent of overlap between thick and thin filaments and the interfilament distance. Fibers stretched before glycerination, so there is no overlap between thick and thin filaments (sarcomere lengths  $>3.6 \mu\text{m}$ ) showed no significant differences in the potentials measured in impalements in the rigor, the relaxation or the contraction solutions (Table IV). Furthermore, at the higher ionic strength the measured potentials from stretched fibers in the rigor solution are similar in magnitude to those obtained from rest-length fibers in rigor (Table III). In the rigor solution of lower ionic strength, the magnitude of the potentials

TABLE IV  
CHANGE IN POTENTIAL UPON IMPALEMENT OF STRETCHED GLYCERINATED FIBERS ( $s = 3.7 \mu\text{m}$ )

Ionic strength	Fiber	Physiological solutions		
		Rigor	Relaxation	Contraction
		<i>mV</i>	<i>mV</i>	<i>mV</i>
0.115	1	$-7.6 \pm 3.6^*$	$-6.8 \pm 1.3$	
	2	$-8.7 \pm 1.7$	$-8.4 \pm 1.6$	
	3	$-6.2 \pm 1.1$	$-6.5 \pm 1.2$	
	4	$-7.4 \pm 0.7$		$-6.6 \pm 1.6$
	5	$-6.5 \pm 1.3$		$-7.1 \pm 1.8$
0.069	6	$-28.5 \pm 4.5$	$-30.1 \pm 4.5$	
	7	$-13.8 \pm 3.4$	$-12.1 \pm 5.0$	
	8	$-11.9 \pm 2.1$	$-14.5 \pm 4.2$	
	9	$-20.9 \pm 5.6$		$-18.4 \pm 3.3$
	10	$-42.7 \pm 13.3$		$-34.4 \pm 6.0$
	11	$-12.6 \pm 1.5$		$-14.5 \pm 2.7$

\* Mean and SD of at least 10 impalements.

from stretched fibers varies considerably. This is due probably to volume differences between fibers at these sarcomere lengths ( $s = 3.6 \mu\text{m}$ ). However, the potential measurements do not indicate a change in charge density upon changing solutions (Table IV).

If the change in potential upon impalement of fibers in rigor is examined as a function of sarcomere length (Fig. 2 *a*) no obvious relationship is apparent. A least squares analysis of the data indicates a slope of  $+0.202 \text{ mV}/0.1 \mu\text{m}$  change in sarcomere length which does not differ significantly from zero (*t* test). Therefore, it is concluded that the potential measurements are constant in the  $2.7\text{--}3.6\text{-}\mu\text{m}$  range of sarcomere length. However, a similar plot of potential measurements from relaxed fibers for the same range of sarcomere lengths (Fig. 2 *b*) suggests a decrease in the potential as the sarcomere length ( $s$ ) increases, especially between  $s = 2.9\text{--}3.2 \mu\text{m}$ . A least squares fit of the data indicates an average change of  $-0.96 \text{ mV}/0.1\text{-}\mu\text{m}$  change in sarcomere length with 97% confidence limits that the slope differs from zero.

The difference between potentials measured in impalements of fibers in solutions causing relaxation and rigor is plotted as a function of sarcomere length in Fig. 3. The graphical relationship is similar to plots of the 1,0 lattice spacing (distance between myosin filaments) versus the sarcomere length for

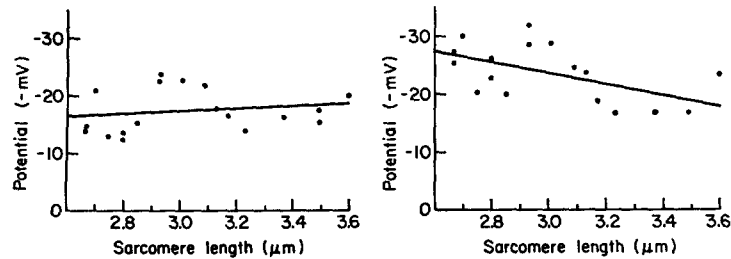


FIGURE 2. (a) Potential changes upon impalement of glycerinated fibers in the rigor solution as a function of sarcomere length. Each point is the mean of at least 10 impalements. Assuming a linear relationship the best straight line has been drawn. (b) Potential changes upon impalement of glycerinated fibers in the relaxation solution as a function of sarcomere length. Each point is the mean of at least 10 impalements. A linear regression line has been drawn.

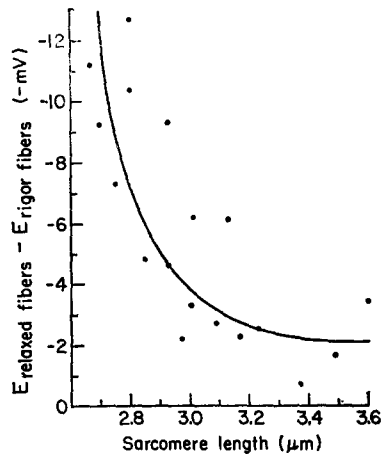


FIGURE 3. A comparison of the difference in potential upon impalement of relaxed and rigor fibers as a function of sarcomere length.

fibers which maintain a constant lattice volume: relaxed and contracted fibers, living or glycerinated from toad or rabbit striated muscle (Rome, 1972; Elliott, 1967). In these cases a plot of the inverse square of the lattice spacing versus the sarcomere length appears to be linear.

#### *Modified Glycerol-Extracted Fibers*

During these experiments various conditions were observed to have an effect on the magnitude of the potential difference measured upon impalement of

relaxed fibers and fibers in rigor (Table V). If the fibers contract in the relaxation solution or if contracted regions of the fibers are noted (i.e., the fibers have lost calcium sensitivity) the impalement potentials are lower in both the rigor and relaxation solutions than those obtained from calcium-sensitive fibers. It should be noted that the potential measurements from fibers in rigor differ from preparation (Table III preparations A-B and C-D) to preparation (Table V) but vary little within a given preparation (e.g. Table III prepara-

TABLE V  
EFFECT OF VARIOUS CONDITIONS ON THE MAGNITUDE OF THE POTENTIAL DIFFERENCE IN RELAXED AND RIGOR FIBERS AT REST-LENGTH

Fiber condition	Physiological solutions (ionic strength $I = 0.069$ )	
	Rigor	Relaxation
	<i>mV</i>	<i>mV</i>
Ca <sup>2+</sup> -sensitive fibers	-7.1±0.3*	-11.3±0.4*
Ca <sup>2+</sup> -insensitive fibers‡	-6.4±0.9 -5.5±1.8	-8.8±1.1 -8.2±1.3
After extraction in H and S§ solution (48 h at 4°)	-13.5±3.7 -10.8±1.9	-9.6±0.8
After extraction in H and S solution (72 h at 4°)	-8.5±1.7 -8.4±1.2	-4.9±1.2
Extraction in Guba-Straub + MgATP solution (48 h at 4°)	-12.3±3.3 -12.1±1.5	-11.7±1.3

Mean and SD of a minimum of 10 impalements unless otherwise indicated.

\* Mean and SE for four sets of 10 impalements.

‡ Calcium-insensitive fibers contract in the relaxing solution.

§ Hasselbach and Schneider's solution plus MgCl<sub>2</sub>.

|| Two sets of rigor measurements on the same fiber to show the effect of rinsing on the impalement potential.

tion C). Table V shows the effect on the impalement potential of the loss of calcium sensitivity of a particular group of fibers.

Fibers which had lost calcium sensitivity (Table V) were extracted with Hasselbach and Schneider's solution (sodium pyrophosphate + MgCl<sub>2</sub>) or Guba-Straub's solution plus MgATP (48-72 h at 4°C). These solutions are expected to dissociate the actin and myosin filaments and then to extract the myosin and M-band proteins. Protein analysis indicates that even after 72 h only one-half of the available myosin has been extracted. However, after 48 h the treated fibers do not have a striated appearance and do not contract in the contraction solution. The magnitude of the potential change upon impalement of these fibers in the rigor solution is similar to that obtained upon impalement of the calcium-sensitive fibers in the relaxation solution (Table V, line 1). The

magnitude of the potential change upon impalement of the treated fibers in the rigor solution does not decrease significantly with repeated rinses or a 24-h equilibration in the rigor solution (no pyrophosphate). Furthermore, upon exchanging the rigor solution for the relaxation solution the measured potentials do not differ from those obtained in the rigor solutions. An exception is observed after 72 h in Hasselbach and Schneider's solution, in which case the potential measurements from fibers in the rigor solution are lower than those obtained after a 48-h extraction period (in either Hasselbach and Schneider's or Guba-Straub's solution) and decrease further upon exchanging the rigor for the relaxation solution (Table V).

#### DISCUSSION

The negative charge on the contractile proteins may be estimated from the Donnan equation (Collins and Edwards, 1971). For rest-length fibers at an ionic strength of 0.115 where KCl is the principal electrolyte, the negative charge ( $zC$ ) on the contractile protein is about 40 meq/liter (Collins and Edwards, 1971; Elliott, 1973).

The total negative charge at pH 7 for the proteins within rat muscle has been estimated at 100 meq/liter (Conway, 1957). This value includes both the soluble sarcoplasmic proteins and the contractile proteins. In calculating  $zC$  for the contractile proteins it is assumed that glycerinated muscle is 12% contractile protein with a density of 1. Myosin at 45% of the contractile protein contributes 105 charges/mol (Kominz, 1966) and 12.1 meq/liter to the  $zC$  of glycerinated muscle. Values for the myosin fragments (LMM, HMM, and S-1) are more difficult to determine. However, by comparing the data of Kominz (1966) for myosin and the 3-S subunit (LMM) with the data for LMM, HMM, and S-1 (Lowey et al., 1969), it is possible to estimate values: LMM, 6.5; HMM, 8.1; and S-1, 2.3 meq/liter. Actin comprises 28% of the contractile protein and from the amino acid sequence (Elzinga et al., 1973) a value of 24 charges/mol and 19.2 meq/liter is obtained. The values of tropomyosin and troponin are estimated from the amino acid compositions of Cohen et al. (1972) and only tropomyosin is corrected for amide  $\text{NH}_2$  (Kominz, 1966). Tropomyosin comprising approximately 4.2% of the myofibrillar protein (Hartshorne and Pyun, 1971) has a charge density of 60/mol and a  $zC$  of 4.3. The charge density of troponin at 5.6% of the myofibrillar protein (Hartshorne and Pyun, 1971) is between 33/mol (assuming 22% of the Glu and Asp are Gln and Asn) and 79/mol (assuming no Gln and Asn) from which  $zC$  is estimated to be between 3 and 7 meq/liter. Therefore, the total  $zC$  for the contractile proteins is approximately 39–43 meq/liter.

The estimated values for the total  $zC$  (vide supra) are similar to the value of 46 meq/liter which can be calculated from the change in potential observed upon impalement of relaxed fibers at a sarcomere length of 2.7  $\mu\text{m}$  (Table

III). This is important since for the relaxed fiber the value of  $zC$  calculated from the Donnan relation is approximately equal to that estimated from the net charge on the proteins. This suggests the negative and the positive charges are more or less entirely available in the relaxed state. For at least one of the myofibrillar proteins, troponin A (TN-C), the net negative charge is important in regulating interactions between troponin subunits. This can be inferred from the observation of Hartshorne (1970) that certain polyanions can substitute for troponin A in a nonspecific interaction with troponin B (inhibitory protein). In addition, McCubbin and Kay (1973) have implicated carboxylate groups in the binding of calcium to troponin A. However, except for troponin A, there is no evidence for the other contractile proteins that the conformational changes involved in going from rigor to relaxation are associated with a change in charge. Although considerable care was taken to design, within experimental limits, solutions of similar ionic composition, it should be remembered that this was accomplished by adjusting the concentration of physiologically neutral ions. The binding affinities of  $MgSO_4$  and  $MgATP$  for the catalytic site of myosin may be quite dissimilar and the structural changes associated with binding may not be equivalent for  $MgSO_4$  and  $MgATP$ . The increase in the negative potential upon going from rigor to relaxation may be due in part to the potential difference between bath and protein due to a change in the amount of bound ions ( $MgADP$ ,  $P_i$ ,  $Mg^{2+}$ ,  $MgATP$ , etc.). However, this can be shown not to be the only explanation for the more negative charge on the relaxed filaments. Assuming, in the relaxed condition there is the addition of 2 mol each of  $MgADP$  and  $P_i$  per mole of myosin, this could contribute 12 charges/mol of myosin (the maximum number possible) and the change in  $zC$  should be about 2.7 meq/liter. This is equivalent to a change in potential of approximately 0.78 mV, which would not be detected. It is concluded, therefore, that the more negative potential observed upon impalement of relaxed fibers over those in rigor must include an alteration in the conformation of the contractile proteins.

Conformational changes associated with going from rigor to relaxation are: a maximal decrease in the number of rigor links (Bremel and Weber, 1972; Rome, 1972), a change from the chevron to right-angle cross-bridge configuration (Reedy et al., 1965), and a reorientation of tropomyosin from the center of the grooves of the actin helix to a position which sterically blocks myosin attachment to actin (Huxley, 1972; Spudich et al., 1972). In the contracted fiber, there would be an intermediate number of "rigor-like" links (Bremel and Weber, 1972; Bremel et al., 1972), a cycling between the chevron and right-angle cross-bridge configuration (Huxley and Brown, 1967), and tropomyosin would be in the same position as in the rigor state. Therefore, the major difference between rigor and contracted fibers would be the steady-state range of orientations of the cross bridges. Although the "relaxed"

X-ray pattern is missing from contracted muscle (Huxley and Brown, 1967), the lifetime of the relaxed conformation of the cross bridges is not known. It is unlikely that the present technique is sensitive enough to register the small steady-state level of cross bridges which are at right angles to the thick filament (relaxed configuration) during contraction.

It is suggested that the more negative potential found in relaxed fibers over those in rigor is due to a conformational change *unique* to the relaxed state. This would include an increase in the number of cross bridges at right angles to the thick filament, and the steric blocking by tropomyosin of myosin binding to actin. For the simplest case, it can be assumed that tropomyosin, troponin, and HMM are changing from a conformation where all the charged residues are buried (in rigor) to one in which all the positive and the negative charges are distributed equally along the surface of the molecules (in relaxation). In terms of the estimated  $zC$  tropomyosin contributes 4.3 meq/liter, troponin between 3 and 7 meq/liter; and HMM approximately 8.1 meq/liter. The total  $zC$  contribution of these three components is between 15–19 meq/liter, in excellent agreement with the observed difference of 20 meq/liter between the two states. For this calculation, conformational changes in myosin (associated with the cross bridge assuming the relaxed configuration) are restricted to the HMM portion of the molecule. In support of this assumption are studies of fluorescence polarization by Mendelson et al. (1973) which indicate considerable flexibility within HMM near the (S-1)–(S-2) connection and the small contribution in charge of the S-1 moieties (2.3 meq/liter) to the total charge of HMM (8.1 meq/liter).

Several aspects of the present results appear to be at variance with the results of recent volume determinations for relaxed glycerinated fibers (Rome, 1972), and for those in rigor (Rome, 1967) as a function of the sarcomere length. For example, it is expected that in a constant-volume system (i.e., relaxed glycerinated fibers) the charge density of the contractile proteins would not change as a function of sarcomere length (Matsubara and Elliott, 1972). The present data suggest the opposite (Fig. 2 *b*). Similarly, glycerinated fibers in rigor do not maintain a constant-volume relationship with changes in sarcomere length in salt solutions of physiological concentrations (Rome 1967); however, the impalement potentials for these fibers do not indicate a significant decrease in the charge density in the 2.7–3.6- $\mu\text{m}$  range of sarcomere lengths (Fig. 2 *a*). One obvious explanation is that the fibers in the present experiment behave as a constant-volume system in the rigor and not in the relaxed state. However, we have no other evidence to support this explanation. An alternative conclusion is that the charge density of the thick and thin filaments in the relaxed condition is decreasing in the 2.7–3.6- $\mu\text{m}$  range of sarcomere lengths without a change in volume. Although recent evidence suggests that the conformations of the thick and thin filaments are independent

of the degree of overlap (Haselgrove, 1970; Vibert et al., 1972), the present results can be explained if the degree of ionization of the myofilaments is altered as the sarcomere length increases from 2.7–3.6  $\mu\text{m}$  (negating assumption 4 in the Appendix of Matsubara and Elliott, 1972). The X-ray data of Elliott et al. (1963) indicate that in the absence of a sarcolemma, the I band has less structural coherence than the A band. Therefore, as Elliott (1973) has suggested, the effective phase boundary of fibers that are not constant-volume systems may be around each A band.

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