Polarization of Tryptophan Fluorescence from Single Striated Muscle Fibers

A molecular probe of contractile state

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ABSTRACT Instrumentation has been developed to detect rapidly the polarization of tryptophan fluorescence from single muscle fibers in rigor, relaxation, and contraction. The polarization parameter ($P_\perp$) obtained by exiciting the muscle tryptophans with light polarized perpendicular to the long axis of the muscle fiber had a magnitude $P_\perp$ (relaxation) > $P_\perp$ (contraction) > $P_\perp$ (rigor) for the three types of muscle fibers examined (glycerinated rabbit psoas, glycerinated dorsal longitudinal flight muscle of *Lethocerus americanus*, and live semitendinosus of *Rana pipiens*). $P_\perp$ from single psoas fibers in rigor was found to increase as the sarcomere length increased but in relaxed fibers $P_\perp$ was independent of sarcomere length. After rigor, pyrophosphate produced little or no change in $P_\perp$, but following an adenosine triphosphate (ATP)–containing solution, pyrophosphate produced a value of $P_\perp$ that fell between the contraction and relaxation values. Sinusoidal or square wave oscillations of the muscle of amplitude 0.5–2.0% of the sarcomere length and frequency 1, 2, or 5 Hz were applied in rigor when the myosin cross-bridges are considered to be firmly attached to the thin filaments. No significant changes in $P_\perp$ were observed in either rigor or relaxation. The preceding results together with our present knowledge of tryptophan distribution in the contractile proteins has led us to the conclusion that the parameter $P_\perp$ is a probe of the contractile state of myosin which is probably sensitive to the orientation of the myosin S1 subfragment.

INTRODUCTION

Present knowledge of the contraction of striated muscle suggests that the interdigitation of the myosin and actin filaments which occurs during shortening results from cyclic motion in the 190–260–A long myosin cross-bridges (1). The upper limit of the frequency of such a mechanical event is about 100 Hz since this is the frequency of the magnesium-activated actomyosin ATPase (2).

X-ray diffraction investigations (3–6) and also electron microscope examinations (7–8) of muscle have established that the myosin cross-bridge projections from the thick filaments do indeed assume different mean orientations in relaxation and rigor. The decreased intensity of the off-meridional myosin reflections when resting muscle is stimulated has been interpreted as a movement of the myosin cross-bridges, particularly in the azimuthal plane. But the X-ray methods, even with foreseeable improvements in technology, do not appear to be capable of obtaining an X-ray diagram within the time of a single cycle of cross-bridge activity.

Recently, Aronson and Morales (9) observed a structural parameter in glycerinated rabbit psoas which was found to change depending on whether the muscle fibers were relaxed, contracted, or in rigor. This parameter is the polarization ($P_\perp$) of fluorescent light from tryptophans which were selectively excited by ultraviolet light polarized perpendicular to the long axis of the fiber (the conjugate parameter, $P_\parallel$, i.e. the polarization of the fluorescence when the excitation plane was parallel to the fiber axis, showed no dependence on physiological state). These authors speculated that $P_\perp$ might result from the state-dependent orientations of the myosin cross-bridges; however, their experiments employed bundles of fibers, and their equipment could not distinguish between static and time-averaged values of $P_\perp$ (for instance, between a cyclic paddle motion or a static position), nor was fiber tension monitored. They had also observed that pyrophosphate, a known relaxant of glycerinated fiber tension, did not produce a value of $P_\perp$ like that produced by ATP. This paper reports a new apparatus enabling measurements at a rate well in excess of 100 Hz, and describes new results which seem to bear on the nature of the force-generating mechanism of muscle.

METHODS AND MATERIALS

Fluorescence Excitation and Detection Systems

A 75 w xenon lamp operated from a low ripple power supply (Pek, Inc., Sunnyvale, Calif., x75-1280) was used as a light source. The light was collimated by a quartz lens, passed through an $NiSO_4$ liquid filter (eliminating light in the range 340–500 nm), then through a monochromator (Schoeffel Instrument Corp., Westwood, N.J., QPM 30 S), through a polarizer (Polaroid film HNP'B, Polaroid Corporation, Cambridge, Mass., 0.006 in. thick), and then condensed onto the muscle fibers by a quartz condenser lens (Zeiss Achromat UV-kond., Carl Zeiss, Inc., New York, 0.8). In this way, the muscle fibers were illuminated with polarized ultraviolet light of wavelength 300 ± 7 nm (the bandwidth of the monochromator). The fluoresced light was collected with an objective lens (Zeiss Neofluar 16/0.40), passed through two filters (Corning 053 and 754, Corning Brand Glassware, Corning, N. Y.) restricting the transmitted light to 340–450 nm, and then split into its orthogonally polarized components ($I_\parallel$ and $I_\perp$) with a calcite Wollaston prism (Isomet Corporation, Palisades Park, N. J.) having an angular divergence of 15°, a 6×6 mm aperture, and 80% transmission at 300 mm. The two component beams transmitted by the prism then

fell on two photomultiplier tubes (Emitronics Inc., Plainview, N. Y., EMI 9524B) whose output voltage could be made equal with a potentiometer. A regulated power supply (Power Designs, Inc., Westbury, N. Y., 2K-10) applied 1450 v to each photomultiplier tube, while the outputs of the two tubes were amplified (Zeltex Inc., Concord, Calif., ZEL-1) and applied to the analogue channels of a PDP-12 computer (Digital Equipment Corp., Maynard, Mass.). The characteristics of the individual components of the light detection system were such that the time constant of all the steps in the system was approximately 5×10^{-5} sec. Occasionally, in order to check the performance of this two-channel system, the polarization was measured by the method of Aronson and Morales (9); a further check that was routinely carried out after each experiment was to make sure that the polarization was zero if the muscle fiber was replaced by a 3 mm thickness of 100 mM tryptophan solution.

Data Acquisition and Processing

In this work control signals, as well as digitized data from four sensors, entered a PDP-12 computer through six input channels. Channel 10 received either simple "start" signals or received from a "slow" oscillator/function generator (Exact Electronics Inc., Hillsboro, Ore., Model 126) capable of generating trains of both sinusoidal and square waves. Channel 11 received from a "fast" oscillator (Hewlett-Packard Co., Palo Alto, Calif., 201C) whose sinusoidal waves were first shaped into square waves. Channels 14 and 15 received fluorescence intensity component data ($I_{\parallel}$ and $I_{\perp}$), and channels 16 and 17 received tension (T) and length (L) data.

The program used depended on whether the phenomenon being observed was aperiodic or periodic. Program 1 (aperiodic): following a start signal through channel 10, sensor data were acquired on each pulse of the fast oscillator, in successive and distinct batches of 500 "readings" per batch, for a usual total of 120 batches. Since the frequency of the fast oscillator was typically 500 Hz, this program caused readings to be taken every 2 msec, for an observational time of 2 min. Program 2 (fast periodic): the start signal was issued from the computer. The slow oscillator issued two wave trains of the same frequency (1, 2, or 5 Hz); its sinusoidal output was applied to a loudspeaker, which then imposed sinusoidal length changes on the muscle; its square wave train, transmitted to the computer, controlled data acquisition as follows. The beginning of each of its square waves initiated a period of collecting data (lasting 1, 0.5, or 0.2 sec). During such a period data were collected at each pulse of the fast oscillator, and sorted according to order of collection during the period; from all such periods, the computer grouped together the data first collected in each period, the data second collected, etc. The mean of each group then became a point in the curve depicting the "average" behavior of a parameter during an oscillation. Program 3 (slow periodic): control resided in the computer. Every 10 sec a square pulse from the slow oscillator, 0.5 sec in duration, stretched then returned the muscle to rest length a total of 100 times. At a present time just before each stretch, until a preset time after the stretch, the slow oscillator also caused data to be collected at the rate of the fast oscillator. The data collected during each period were sorted by the computer according to the order in which they had been collected in the period. The computer grouped together data first collected, second collected, etc. from all the periods. Each such group generated a point in the curve of average response to stretch.

Following data collection the computer immediately began printout, both by teletype and paper tape punch. Separate from the foregoing measurements there were determined experimentally after each experiment: (*a*) the polarization of the fluorescence from a solution of tryptophan; (*b*) the fluorescence intensity components from a location immediately adjacent to the fiber (neither component ever exceeded 5% of the corresponding fiber signal); (*c*) the fluorescence intensity equivalents of the "dark current" in each tube. Finally, a FOCAL program directed the computer to carry out the following operations on the averages. Intensity readings were corrected for dark current. One set of intensities ($I_{\parallel}$) was multiplied by a constant (very nearly unity) such that the polarization of the tryptophan fluorescence is made exactly zero. The polarization corresponding to all pairs of intensity readings were computed. In this way, polarization ($P_{\perp}$) length and tension were printed by the teletype for each batch of readings.

Mechanical Conditions

Although isometric conditions obtained in the Aronson-Morales work, tension could not be measured. In the present work, however, fiber tension was measured with an unbonded strain gauge (Shinkoh type UL, Shinkoh Communication Industry Co. Ltd., Kanagawa-Ken, Japan), the output of which was amplified (Keithley Instruments Inc., Cleveland, Ohio, 610B Electrometer—rise time 0.2 msec—when time resolution was required; or a Hewlett-Packard 410C Voltmeter for static measurements) and applied to the computer. The strain gauge was previously calibrated against a series of weights and was found to be linear within the range of force measured in these experiments.

In certain experiments to be described, it was necessary to make the length of the muscle fiber oscillate with a known amplitude and frequency. As explained above, these forced oscillations were achieved by attaching one end of the muscle fiber preparation to a stiff 0.05 in. tungsten wire linked to a loudspeaker which in turn was driven by a function generator capable of producing either sinusoidal or square wave displacements.

Length changes imposed by the speaker were measured by a length transducer. This transducer consisted of a small enclosed light source which emitted a beam of light that passed through a rectangular window and was detected by a photodiode (RCA Scientific Instruments, Camden, N. J., SQ2536) in a standard DC bridge circuit. A flag, firmly attached to the tungsten wire, interrupted the light falling on the photocell by an amount that was proportional to the position of the flag. The length transducer was calibrated by changing the position of the flag by a known amount with a micromanipulator (Narrishige model 2750, Labstron Scientific Corp., Farmingdale, N. Y.). Over the range of displacement used in these experiments (maximum 300 μ), this length-detecting apparatus was found to give a linear response. The complete experimental assembly is diagrammed in Fig. 1.

Muscle Fiber Preparation

Fresh strips of rabbit psoas 1–2 mm wide were tied to glass rods and immersed in 50% glycerol-water for 24 hr at 2°C, then transferred to fresh glycerol-water at

−15°C and stored for 2–24 wk. Using fine watchmaker's forceps, single psoas fibers were teased from these bundles and allowed to equilibrate to room temperature.

Live *Lethocerus americanus* were obtained from Steinhilber and Co., Inc., Oshkosh, Wis. The bugs were beheaded, their abdomens were removed, and their thoraces were divided along the dorsal midlines. Each half thorax was then immersed in 50% glycerol-water and evaculated with a rotary pump for 30 min to remove the gases trapped in the tracheal tubes. They were then maintained at 2°C for 24 hr and treated in the

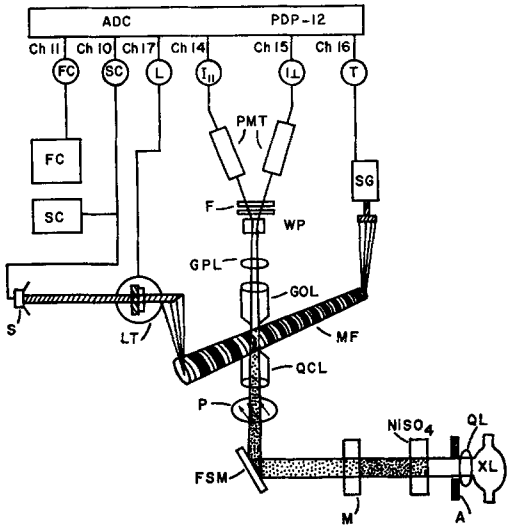

FIGURE 1. A diagrammatic explanation of the complete experimental assembly showing the origins of the analogue signals applied to the PDP-12 computer. *FC* is the signal from the fast oscillator; *L* is from the length transducer (*LT*); *T* is from the strain gauge (*SG*); $I_{\parallel}$ and $I_{\perp}$ are fluorescence intensity signals from the Wollaston prism; *S* is a loudspeaker which moves one end of the muscle fiber (*MF*). Light from the xenon lamp (*XL*) passes through a quartz lens (*QL*), through an aperture (*A*) and an $NiSO_4$ liquid filter, then through a monochromator (*M*); it is then directed up the axis of a microscope by a front silvered mirror (*FSM*), through a polarizer (*P*), through a quartz condenser (*QCL*), glass objective lens (*GOL*), and projector lens (*GPL*), then through the Wollaston prism (*WP*), through transmission filters (*F*), and finally impinges on the photomultiplier tubes (*PMT*).

same way as psoas muscle. The actual muscle fibers used in these experiments were the dorsal longitudinal flight muscles. Before use, small bundles of 10–15 fibers were removed from a half thorax, teased with fine watchmaker's forceps into single muscle fibers, and allowed to equilibrate to room temperature.

Living muscle fibers were obtained from the dorsal head of the semitendinosus of *Rana pipiens*. The muscle was first pared down to a small bundle of fibers which were still attached to their tendons. This preparation was transferred to the experimental cell and the tendons were clamped with fine jeweler's forceps, one end being attached to the strain gauge, the other to a micrometer. The bundle of fibers was then pared

down with electrolytically sharpened tungsten needles until a single fiber remained. This remaining fiber was essentially untouched by dissection instruments. The glycerinated fiber preparations (psoas and *Lethocerus* flight muscle) were glued to their mechanical supports with collodion dissolved in acetone. The experimental cell consisted of a Plexiglas block (Rohn and Haas Co., Philadelphia, Pa.) into which had been machined a shallow recess. A hole in the bottom of the chamber was covered by a recessed quartz cover slip to allow the condenser to make close contact with the cover slip. The volume of the chamber was approximately 3 ml. Changes in the bathing solutions were made by adding 12–16 ml of the new solution to the bath which was maintained at constant volume by a suction pump. In the case of the glycerinated muscle the 50% glycerol-water was removed from the fibers by allowing them to equilibrate at room temperature with a rigor solution for at least 15 min, and with at least three changes of solution.

In all of the experiments, rigor, relaxation, and isometric contraction were induced in the muscle fibers by exposing them to the following test solutions.

GLYCEROL-EXTRACTED MUSCLE *Rigor* was assumed to exist in 40 mM KCl, 10 mM histidine at pH 7.0; 2 mM EGTA[1] and 5 mM $MgCl_2$ could be added to the solution without changing its effect. *Relaxation* was induced in 40 mM KCl, 10 mM histidine at pH 7.0, 2 mM EGTA, 5 mM $MgCl_2$, and 5 mM ATP. A contraction was induced in 40 mM KCl, 10 mM histine at pH 7.0, 5 mM $MgCl_2$, 5 mM ATP, 2 mM EGTA, together with an added amount of $CaCl_2$ calculated to fix the pCa at some desired value between 8 and 5 (10).

FRESH MUSCLE Muscle fibers were dissected and maintained in a relaxed state in Ringer's solution (11): 115 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$, 2.2 mM Na_2HPO_4, and 0.9 mM NaH_2PO_4. Tension development was induced by changing the K^+ concentration to 400 mM, i.e., by inducing a potassium contracture. This contraction was readily reversed by returning the fiber to normal Ringer's solution. Rigor was irreversibly induced in the frog fibers by adding 0.5 mM NaF and 10 mM NaN_3 to the Ringer's solution, thereby blocking ATP generation through both glycolysis and oxidative phosphorylation.

RESULTS

Polarization of Glycerinated Psoas Fibers at Equilibrium Length during Relaxation, Contraction, and Rigor

Measurements of $P_\perp$ (when the exciting light is polarized perpendicular to the fiber axis) and $P_\parallel$ (when the exciting light is polarized parallel to the fiber axis) essentially confirm the original findings of Aronson and Morales (9), i.e. $P_\perp$ (relaxation) > $P_\perp$ (contraction) $P_\perp$ (rigor), whereas $P_\parallel$ is independent of physiological state. These measurements were made on fibers which were glycerinated at a normal, uncontracted (equilibrium) length and observed at, or very close to, that length. The results from 19 different single fibers are summarized in Table I. The sarcomere length of these muscle fiber prepara-

[1] EGTA = ethylene glycol bis(β-aminoethyl ether) N,N'-tetraacetic acid.

tions is between 1.9 and 2.0 μ where the overlap of myosin and actin filaments is nearly maximal.

Polarization of Glycerinated Psoas Fibers at Different Degrees of Stretch in Relaxing and Rigor Conditions

In these experiments, stretch was always applied to a single muscle fiber while it was in the ATP relaxing solution. The sarcomere length was observed from 10 different areas in the field of view and an average was obtained. At sarcomere lengths greater than 3 μ, longitudinal dislocations of the fibrils within the fiber made these measurements difficult to determine with certainty. Fig. 2 demonstrates that the polarization value, $P_\perp$, in the relaxed state remains remarkably constant as the fiber is stretched but that $P_\perp$ in rigor shows a progressive increase. However, it is uncertain whether there is a real difference in $P_\perp$ in rigor and relaxation at sarcomere lengths where the overlap of thick and thin filaments is reduced to zero, because of: (*a*) the difficulty in measuring precisely the sarcomere length, and, (*b*) the distribution of sarcomere lengths

TABLE I

FLUORESCENCE POLARIZATION ($P_\perp$* AND $P_\parallel$‡) IN GLYCERINATED RABBIT PSOAS FIBERS

State	$P_\perp \pm$ SE mean§	$P_\parallel \pm$ SE mean§
Rigor	0.090 ± 0.001	0.304 ± 0.004
Relaxation	0.126 ± 0.001	0.305 ± 0.004
Contraction	0.110 ± 0.001	—

* Exciting light polarized perpendicular to the muscle fiber axis.
‡ Exciting light polarized parallel to the muscle fiber axis.
§ Standard error of the mean.

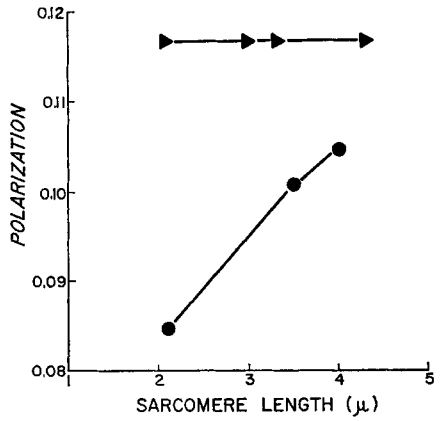

FIGURE 2. A plot of the polarization ($P_\perp$) vs. sarcomere length (μ) for a single glycerinated rabbit psoas fiber in rigor (●) and relaxation (▲). Note the increase in $P_\perp$ with sarcomere length in rigor and the constant relationship of the two in relaxing solution.

which exists in a given area of fiber at this high degree of stretch. Nevertheless, this increase in $P_\perp$ for rigor fibers and a constancy of $P_\perp$ during relaxation was observed in three other fibers, and suggests that the difference in $P_\perp$ for rigor and relaxation is due, at least in part, to the area of overlap, i.e. to the ability of the myosin and actin filaments to interact.

Polarization ($P_\perp$), Tension, and Stiffness in Glycerinated Psoas Fibers

It is not certain that $P_\perp$ and tension are the results of the same process and are therefore absolutely related to one another. Force recorded in a muscle fiber is the net result of viscous and elastic forces and is therefore a function of time. Fig. 3 illustrates the time-course of changes in both $P_\perp$ and tension, and suggests that $P_\perp$ reaches its steady-state value before the tension has stabilized, particularly in the case of the ATP-relaxation of tension. Fig. 4 demonstrates that, in rigor, the elastic properties are markedly different from those of the relaxed fiber. From this plot it can be seen that the fibers are approximately three times as stiff in rigor as in relaxation.

The Effects of Pyrophosphate Relaxation on Glycerinated Psoas Fibers

Experiments with actomyosin dissociation, as well as with the relaxation of glycerol-extracted psoas fibers, have always suggested that pyrophosphate and ATP do qualitatively the same thing, namely dissociate myosin and actin. The results obtained in the present study do not dispute this conclusion—at least

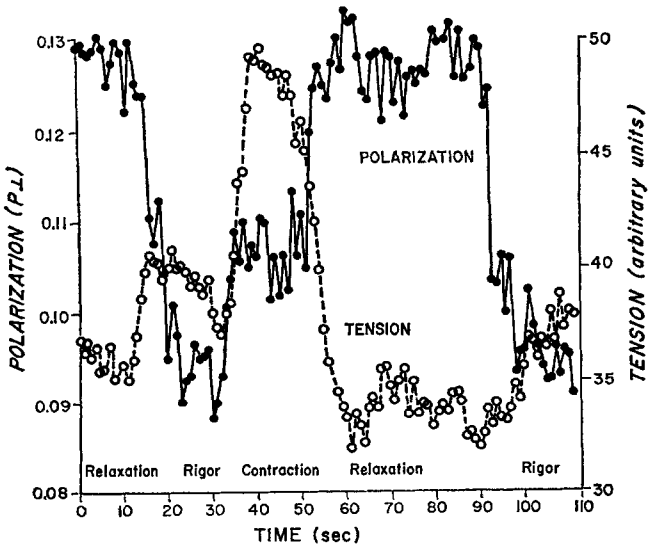

FIGURE 3. A plot of polarization, $P_\perp$ (●), and tension (○) vs. time (seconds) for a single psoas myofiber exposed to the three test solutions. These data were obtained by the aperiodic computer program.

in so far as reduction of the tension can be taken to reflect reduction in cross-bridging. However, the present work does show that, for the same reduction in tension, $P_\perp$ changes differently depending on whether the reduction is achieved by pyrophosphate (5 mM) or ATP (5 mM) (Fig. 5).

Aronson and Morales (9) showed, and it has been confirmed here, that when ATP is used to effect relaxation, the order of $P_\perp$ is relaxation > contrac-

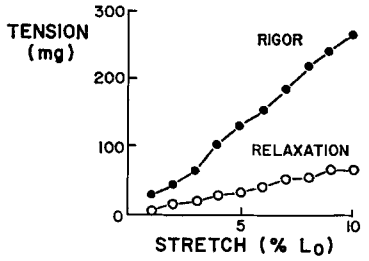

FIGURE 4. A plot of tension vs. stretch for a small bundle of glycerinated rabbit psoas fibers in rigor (●) and relaxation (○). Zero per cent stretch is taken to be the in vivo, uncontracted (L_o) length of the muscle which was maintained during the process of glycerination. Note that the fibers in rigor are approximately three times as stiff as relaxed fibers.

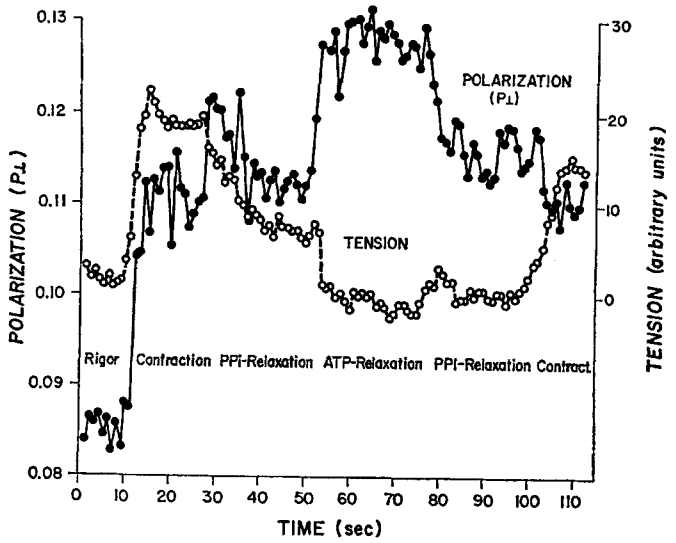

FIGURE 5. The effect of adding pyrophosphate-relaxing solution to a single psoas fiber following contracting and ATP-relaxing solutions. The pyrophosphate solution was identical to the ATP-relaxing solution (5 mM ATP) except that the ATP was replaced with 5 mM sodium pyrophosphate. Note that the fall in tension (○) induced by pyrophosphate is further lowered by the ATP-relaxing solution. A small (about 5%) stretch was applied to the fiber before the test solutions were applied. This experiment employed the aperiodic type of program.

tion > rigor. This relationship remains true with pyrophosphate if the pyrophosphate relaxation is induced after contraction; however, it is not true if the pyrophosphate relaxation is induced after rigor (Fig. 6).

Length Oscillations of Glycerinated Psoas Fibers in Rigor Solution

On the basis of X-ray diffraction (3–6) and electron microscopy (7–8) it is thought that, in rigor, the myosin cross-bridges project from the long axis of the thick filaments toward the center of the sarcomere and form a stable bond with the actin of the thin filaments at an angle considerably different from $\pi/2$. This being so, one might expect small oscillations in fiber length to pro-

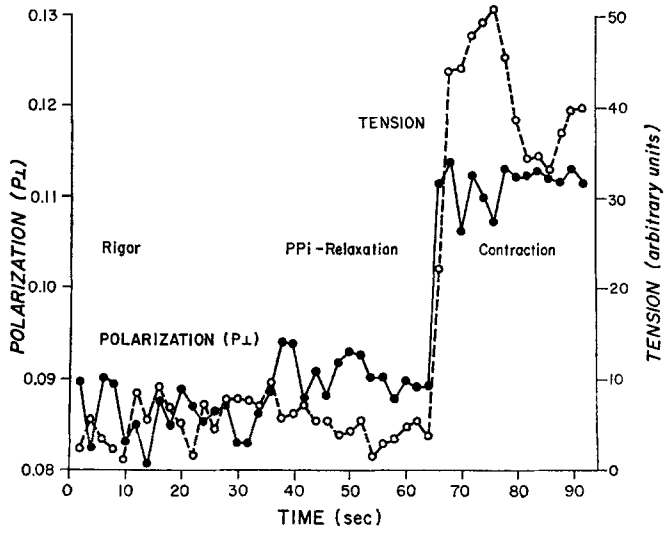

FIGURE 6. When pyrophosphate (5 mM) is added to a single psoas fiber after the rigor solution, there is little or no change in either the polarization, $P_\perp$ (●), or tension (○). Compare this response with that shown in Fig. 5.

duce oscillations in this angle. If the fluorescence effect ($P_\perp$) is mediated by the cross-bridges, then oscillations in length might produce oscillations in $P_\perp$. Accordingly, single fibers of glycerinated rabbit psoas were oscillated by 0.5, 1, and 2% of their sarcomere lengths, employing either an approximately sinusoidal cycle of length change or an abrupt (approximately square pulse) change in length at frequencies of 1, 2, or 5 Hz. At 5 Hz, the computer sampled the input analogue channels at the analogue-to-digital (A/D) converter as described in a previous section, resulting in readings which represent a sum of a total of 500 oscillations. From the data plotted in Fig. 7, it is evident that at this frequency (as well as others) there are no large scale oscillations in the polarization ($P_\perp$) corresponding to the oscillations in the length and tension under conditions that are known to produce rigor. A similar result was ob-

served for muscle fibers oscillated in the ATP-relaxing solution. The viscoelastic properties of the muscle fibers makes the interpretation of slow sinusoidal oscillations subject to the criticism that the small length changes could be obscured by the viscous elements in the muscle fibers. For this reason, we employed a second type of oscillation in which the length changes were applied very rapidly (stretching was completed in less than 10 msec) in an approximately square wave fashion. Fig. 8 shows the effects of the rising portion of an applied square wave displacement on tension and polarization. Once again, there were no large-scale effects of the length oscillation on the polarization. Note the viscoelastic behavior of the fiber tension, showing the characteristic

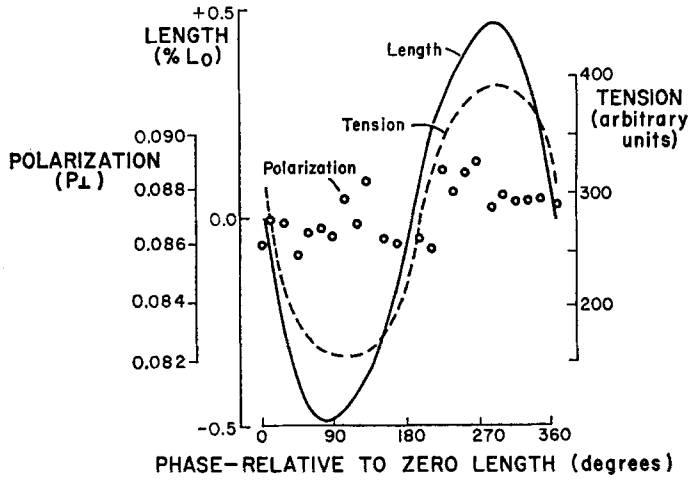

FIGURE 7. A plot of length, tension, and polarization ($P_\perp$) vs. the phase of a sinusoidal oscillation. These data were obtained from the fast periodic type of program described in the text. The tissue is a single glycerinated rabbit psoas fiber oscillated at 5 Hz with an amplitude of $\pm 0.5\%$ of the length of the fiber. Each data point represents the average of 500 oscillations.

stress-relaxation phenomenon. This plot is the averaged result of 100 sweeps, with a time resolution of 2 msec.

Glycerinated Lethocerus Longitudinal Flight Muscle

Pringle (12) and his associates have shown that it is possible to set into oscillation at its resonant frequency the glycerol-extracted dorsal longitudinal flight muscle of *Lethocerus cordofanus*. This capability of insect muscle, together with its highly ordered structure (8), suggested that it might be ideal material for the present study in that these cross-bridges might conceivably oscillate synchronously with one another. Static observations in Table II show that the dependence of $P_\perp$ on physiological state is qualitatively the same in insect muscle and in mammalian muscle, but the differences between the states are

much smaller in the former, and, therefore, much harder to quantify for the present purposes. Experiments with forced oscillations were attempted with this material, but no correlated oscillations in $P_\perp$ could be established with certainty in any given physiological state.

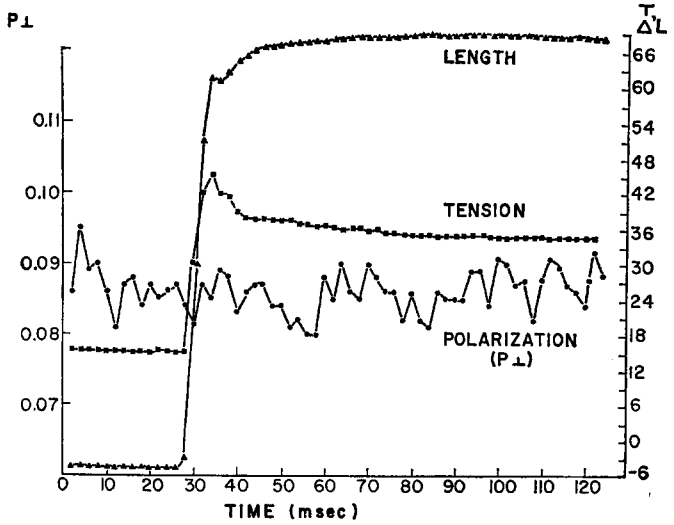

FIGURE 8. Periodic (about 1.35% of the rest length), approximately square wave stretches of 500 msec duration were applied to a glycerinated rabbit psoas fiber every 10 sec for 1000 sec; data were collected using the slow periodic program. This figure shows, as the averages over 100 cycles, $P_\perp$ (●), length, ΔL (▲), and tension, T (■), as a function of time. The values are shown for a period of approximately 28 msec before the stretch, for approximately 10 msec during which the stretch takes place, and for a portion of the time that the stretch is held. ΔL and T before 28 msec thus correspond to the unstretch state; the units of the parameters L and T are arbitrary, and are indicated with the same set of numerals.

TABLE II

FLUORESCENCE POLARIZATION ($P_\perp$* AND $P_\parallel$‡) IN INSECT DORSAL LONGITUDINAL FLIGHT MUSCLE

State	Mean SE mean§
Rigor	$P_\perp = 0.104 \pm 0.025$ $P_\parallel = 0.32 \pm 0.01$
Relaxed	$P_\perp = 0.112 \pm 0.005$ $P_\parallel = 0.32 \pm 0.01$
Contraction	Tension rising $P_\perp = 0.106$ (N = 1) Tension falling $P_\perp = 0.109$ (N = 1)

* Exciting light polarized perpendicular to the muscle fiber axis.
‡ Exciting light polarized parallel to the muscle fiber axis.
§ Standard error of the mean.

Frog Semitendinosus Muscle Fibers

Dissected single fibers were assumed to be in the relaxed state. After experimenting with both electrical stimulation and potassium contractures, we chose the latter method because in our hands it produced longer and smoother contractions. The tension developed in the high potassium solution (400 mM KCl in Ringer's solution) could readily be reversed at least the first, second, and sometimes a third time by replacing the solution with ordinary Ringer's. As already explained in the Methods and Materials section, rigor could be induced by poisoning ATP generation by the fibers with fluoride and azide, but of course this poisoning could not be reversed. As might be expected in the presence of permeability barriers to free diffusion, it takes time to change the fiber from one state to another. In Fig. 9 is illustrated the transition from relaxation to the azide-fluoride–induced rigor, and the concurrent transition in $P_\perp$. Table III summarizes the $P_\perp$ values obtained from the frog muscle relaxed in ordinary Ringer's solution, contracted in high potassium solution, and in the azide-fluoride–induced rigor. The actual polarization values obtained are rather more variable than those obtained from the glycerinated psoas fibers and are also somewhat higher. This is in agreement with the findings of Aronson and Morales (9) who measured $P_\perp$ and $P_\parallel$ in living rabbit psoas fibers. Finally, Fig. 10 demonstrates the relationship between stress and strain in living frog fibers in Ringer's solution (relaxed) and after rigorization. As in the glycerinated psoas material, the stiffness of the fibers in rigor is about three times that of the relaxed fibers.

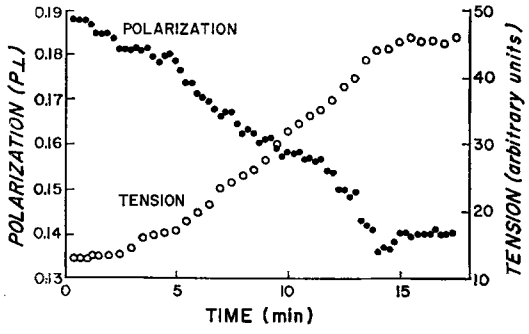

FIGURE 9

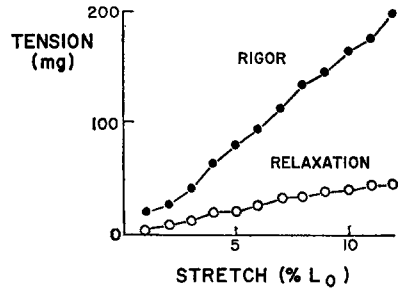

FIGURE 10

FIGURE 9. A plot of polarization, $P_\perp$ (●), and tension (○) vs. time for a single live frog semitendinosus fiber after the addition of 10 mM NaN_3 + 0.5 mM NaF to the relaxed fiber in Ringer's solution. The rise in tension is due to the rigor "contraction."

FIGURE 10. A plot of tension vs. stretch for a single live frog semitendinosus fiber relaxed in Ringer's solution (○) and 60 min after the onset of azide-fluoride–induced rigor (●). Zero per cent stretch is the relaxed length of the fiber. Note the threefold increase in stiffness after rigorization.

TABLE III

FLUORESCENCE POLARIZATION ($P_\perp$) FROM LIVE FROG SEMITENDINOSUS

Experiment	Relaxed ($P_\perp$)	K^+ contracture ($P_\perp$)	Rigor ($P_\perp$)
7. 7.70C	0.207	—	0.144 (15 min) 0.149 (24 hrs)
8.24.70D	0.204	0.186	—
8.25.70C	0.195	—	0.153
8.25.70E	0.191	—	0.140
10.23.70A	0.207	0.174	0.141
10.23.70B	0.192	0.163	—
10.27.70B	0.200	0.175	—
10.30.70B	0.189	0.161	0.149
10.30.70C	0.183	0.167	—
11. 5.70A	0.203	0.176	—
Mean	0.197	0.172	0.142
SE mean*	±0.003	±0.003	±0.002

* Standard error of the mean.

DISCUSSION

Aronson and Morales (9) suggested that there are two different populations of trytophan residues that are responsible for the polarization obtained when the exciting light is polarized perpendicular ($P_\perp$), and parallel ($P_\parallel$), to the muscle fiber axis. The lack of change in $P_\parallel$, observed by them and confirmed here using slightly different techniques, might arise either because the tryptophan residues responsible for that fluorescence do not change their orientation in the three physiological states (e.g., they may be located in the light meromyosin [LMM] subfragment of myosin) or because the changes in each half sarcomere are equal but opposite. On the other hand, the values of the $P_\perp$ are more interesting because they do change when the muscle fibers are transferred from rigor to contraction to relaxation and back again.

Distribution and Abundance of Tryptophans in the Contractile Proteins

There is now quite strong evidence from both electron microscopy and X-ray diffraction studies that the myosin cross-bridge projections have different average orientations in rigor, relaxation, and contraction. These cross-bridges now play an important role in the various contraction schemes and so it is attractive to consider whether the parameter ($P_\perp$) somehow reflects the orientation of these cross-bridges. The first pertinent question to ask is what is the distribution of the trytophan residues in the contractile proteins. The data in Table IV indicate that more than 50% of the trytophan residues in the contractile proteins are located in the myosin molecules. As Aronson and Morales (9) have already pointed out, the fluorescence intensity of the actin tryptophans is about the same as the myosin fluorescence intensity on a weight basis even though

actin contains nearly twice as much tryptohan. This evident quenching of the actin fluorescence together with the greater quantity of myosin suggests that more than 70% of the fluorescence from the contractile proteins comes from the myosin. Until very recently, little data were available on the distribution of tryptophan residues in the various subfragments of myosin. Lowey et al. (13) concluded that the S2 subfragment contained no tryptophans. On the basis of published extinction ceofficients for LMM and HMM-S1 (heavy meromyosin–subfragment 1), assuming that the two S1 subfragments are very nearly identical, one may conclude that approximately 70% of the tryptophan residues in myosin are located in S1 (Weeds—personal communication). Very recently, Shimizu et al. (14) determined the tryptophan con-

TABLE IV

ESTIMATES OF THE TRYPTOPHAN DISTRIBUTION IN CONTRACTILE PROTEINS

	% Fibrillar protein	Tryptophan per 10^5 g	% Fibrillar tryptophan	Recalculated % fibrillar tryptophan considering F-actin quenching
Myosin*	55	3.9	56	73
HMM S1‡	(24)	(7.0)	(39)	(51)
HMM S2‡	(14)	(0.0)	(0)	(0)
LMM‡	(16)	(5.0)	(17)	(22)
Actin*	15	10.0	39	20
Tropomyosin*	15	0.0	0	0
Troponin*	7	2.4	5	7
Light myosin chain‡	5	1.3	1	1

* Aronson and Morales (9).

‡ Estimate made on the basis of the extinction coefficient ($E^{1\%}_{280}$) where the contribution from the tyrosine residues was known. We are indebted to Dr. A. Weeds for this information.

tents of HMM and S1 by several different methods and concluded that 95% of the tryptophan residues in HMM were contained in the S1 subfragment. Thus, on the basis of tryptophan distribution and abundance, it is not unreasonable to think that the fluorescence effect (changes in $P_\perp$ with contractile state) arises from the cross-bridges, particularly from their S1 subfragments.

Pyrophosphate Effect

An observation which kept Aronson and Morales (9) from the foregoing hypothesis was that they observed no changes in $P_\perp$ when a bundle of fibers in rigor was exposed to a relaxing solution containing pyrophosphate. The present studies confirm the observation; moreover, they also show that pyrophosphate causes little change in $P_\perp$ when it is added to a fiber in *contraction.* Our new tension-measuring capability makes it possible to say that in both cases—when it is added after rigor and when it is added after contraction—pyrophos-

phate does cause mechanical relaxation, presumably by dissociating actin-myosin bonds. Thus the dilemma of Aronson and Morales is explained; we can now say that $P_\perp$ originates in a structure whose state is dependent on ATP concentration, i.e. on functional state, but not on actin-myosin bonding per se. These considerations seem closely related to the work of White (15), who reported that in passing from rigor to pyrophosphate relaxation the "dynamic stiffness" changed very little, whereas in passing from rigor to ATP relaxation it changed a great deal. An inference of both investigations is that, beyond affecting actin-myosin bonding, ATP affects the state of an important structural element (presumably the bridge).

Dependence of $P_\perp$ on Sarcomere Length in Rigor

Gordon et al. (11) observed that the force developed by a muscle fiber is proportional to the degree of overlap of the thick and thin filaments and consequently to the number of myosin-to-actin contacts. If this observation is extended to muscle fibers in rigor, one might predict that the number of cross-bridges that can make an actin contact with an orientation that is specific to the rigor condition will also decrease as the degree of overlap is progressively reduced. We have found that $P_\perp$ (rigor) does increase with decreasing overlap. If we ignore the uncertainties associated with heterogeneity of sarcomere length (see above), however, we note that the asymptotic value of $P_\perp$ (rigor) is well below the value of $P_\perp$ (relaxation). From this difference the simplest inference is that the structural feature generating $P_\perp$ is only *indirectly* affected by actin-myosin bonding; for instance, if we think that $P_\perp$ reflects the arrangement of cross-bridges we would have to say that some structural feature of cross-bridges is different when ATP is absent (rigor) and when it is present (relaxation). On the other hand, if subsequent measurements show that with better sarcomere length control $P_\perp$ (relaxation) = $P_\perp$ (rigor at no overlap), we would have to say that when bonding is impossible (for whatever reason) the cross-bridges assume the same time-average position.

Oscillation Experiments in Rigor

Huxley (6) has pointed out on the basis of his X-ray diffraction data that when rigor is induced in a previously relaxed fiber, there is a shift in the mass of the myosin of the thick filaments (e.g., a movement of the myosin S1 subfragment) towards the thin actin filaments. Later Huxley (1) proposed that the junction between the globular head of the myosin (S1) and the linear part of the HMM (S2) might be a flexible coupling point. Were this so, rigor might allow the S1 subfragment to maintain a constant orientation with the actin monomer of the thin filament while the S2 (with few or no tryptophans) subfragment changes *its* orientation towards both the LMM and the S1 fragments. This idea visualized in Fig. 11, would thus accommodate an oscillation of 0.5–2.0% without

disturbing the orientation of tryptophan-containing S1 subfragment relative to its actin. Pertinent to these observations is the finding of Dr. A. Miller (personal communication), who applied a constant stretch to insect flight muscle and observed the intensity of the 145 A cross-bridge reflection. He observed no change in its intensity and concluded that the angled connection of the cross-bridge in rigor is very rigid. Thus, the data reported here are consistent not only with Miller's unpublished observations but also with Huxley's concept of the force-generating mechanism.

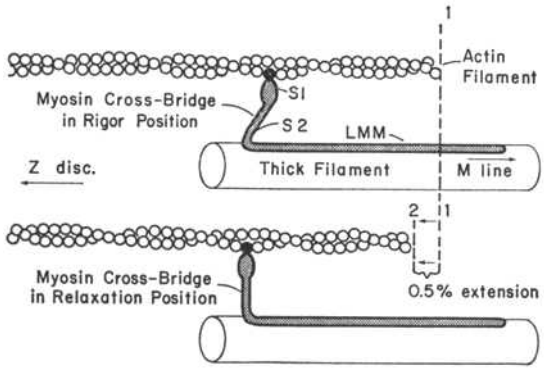

FIGURE 11. Diagram showing a single "head" of a myosin cross-bridge with its S1 and S2 subfragments projecting from a thick filament and making contact with an actin monomer in a thin filament. In the upper drawing, the myosin cross-bridges project towards the M line at a characteristic angle. When a small stretch is applied (either statically or in an oscillating fashion) the thin filaments are moved from position 1 to position 2. The lower diagram proposes that the attitude of the S1 subfragment to the actin monomer (●) remains unchanged and that the new position of the cross-bridge must be accommodated by the S2 subfragment. As pointed out in the text, the S2 portion of the myosin contains little or no tryptophan and consequently would contribute little or nothing to the observed fluorescence signals.

Is $P_\perp$ a Method of Detecting Myosin Cross-Bridge Orientation?

Because X-ray diffraction methods have already shown that the disposition of the myosin cross-bridges is different in rigor and in relaxation, and that they may undergo movement during contraction, it is natural to wonder whether $P_\perp$ also reflects cross-bridge orientation. It has been shown here that the difference in $P_\perp$ in rigor, relaxation, and contraction occurs not only in glycerinated rabbit psoas fibers but also in insect flight muscle and in live frog myofibers. Furthermore, the dependence of $P_\perp$ in rigor on the sarcomere length (and hence the degree of overlap of myosin and actin filaments), the effect of pyrophosphate, and the oscillation experiments in the rigor state, all fit with the hypothesis that the observed parameter indeed reflects the disposition of the myosin cross-bridges.

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