Polarization of Tryptophan Fluorescence in Muscle*

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ABSTRACT: The polarization of tryptophan fluorescence from glycerinated rabbit muscle was examined as a possible indicator of changes in orientation or conformation of muscle proteins. The fluorescence was excited at 300 mμ and was observed on axis after removing the exciting light with filters. When rigor muscle fibers were excited the polarization, p, was ~0.32 when the exciting light was polarized with the electric vector parallel to the muscle fiber axis, $p_{||}$, and ~0.08 when it was polarized with the electric vector perpendicular to the fiber axis, $p_{\perp}$. This anisotropy in p is associated with the structure of the muscle and is not due to dichroism in the usual sense. Relaxation of the muscle brought about by the addition of adenosine triphosphate (1 mm or higher) in a solution containing ethylene glycol bis(β-aminoethyl ether) *N,N*-tetraacetic acid plus magnesium ions caused a reversible increase in $p_{\perp}$ from ~0.08 to ~0.125 and no obvious change in $p_{||}$. Isometric contraction evoked by ATP and calcium ions produced intermediate values, *viz.*, ~0.10. High concentrations of glycerol (50–95%, v/v) also caused an increase in $p_{\perp}$ while having little effect on $p_{||}$ while pH, KCl concentration, D_2O, and several nucleotides and polyphosphates had little observable effect on either $p_{||}$ or $p_{\perp}$. We conclude that $p_{\perp}$ is a sensitive indicator when different functional states are imposed on muscle either by the binding of adenosine triphosphate or by the activation of adenosine triphosphate hydrolysis by calcium ions. We favor the view that the change in $p_{\perp}$ reflects changes in the average orientation of some tryptophan-containing element of muscle.

The polarization of tryptophan fluorescence from a protein, and particularly from an ordered array of the protein, can be a sensitive and easily measured indicator for changes in conformation or orientation (Konev, 1967). We have observed tryptophan fluorescence from glycerol-extracted striated muscle, a highly ordered array of the proteins actin and myosin. The major pattern of fluorescence polarization is related to the structural arrangement of tryptophanyl residues within the muscle rather than to restricted rotational freedom. This surmise is based on the lifetime of tryptophan fluorescence, the multiplicity of tryptophanyl residues, and the large size and general rigidity of myosin and F-actin.

Muscle structure cannot be predicted from the fluorescence polarization pattern directly and the usefulness of this property lies more in the ability to sense small changes in protein conformation and orientation. These changes can be correlated chemically with the binding and splitting of ATP and physiologically with the contractile state of the muscle.

The application of this approach to enzyme–substrate and protein–protein interactions in other crystalline systems seems potentially useful.

Materials and Methods

Optical Conditions. A 100-W mercury arc lamp (G. E. AH-4), quartz monochromator (Schoeffel QPM 30 S) used with a 1-mm slit which gave a 13-mμ band width, a $NiSO_4$ liquid filter, and a quartz achromatic condenser (Zeiss) were used for excitation. The angle between exciting and emitted light was 0°, *i.e.*, their respective rays moved parallel to the optic axis. The emitted light was collected with a numerical aperture 0.75 water immersion microscope objective (Zeiss), and imaged by focusing to maximum intensity onto a 2-mm aperture which was in turn imaged onto a photomultiplier tube (EMI 9524 B). A 2-mm Jena glass UG-5 filter was used in front of the phototube. This UG-5 filter and the glass of the optics limited the emitted light sampled to a fraction between about 340 and 480 mμ. Polarizing filters were Polaroid film (HNP'B, 0.006 in. thick) which had 30% transmission at 300 mμ, and for which $I_{\perp}/I_{||}$ equaled 0.017 at 300 mμ. The exciting light was polarized in a fixed direction, and the emitted light was analyzed by rotating a polaroid at 3.3 rpm in front of the phototube. The aperture above the ocular sampled light from about 10^{-7} g of muscle protein and a typical fluorescence signal from the photomultiplier (gain about 10^6) was 10^{-2} A. Background fluorescence measured adjacent to the muscle was 1–2% of this value. The present instrumentation can be improved for future work (Chance and Legallis, 1959). Excitation was normally at "300" mμ with a 1-mm monochromator slit and an 0.3-n.a. condenser aperture.

Rabbit *psoas* muscle which had been soaked in 50% glycerol–water for 6 hr at 0° and which had been stored in 50% glycerol–water at −10° for less than 6 months was used for most measurements. Small bundles of these glycerol-extracted fibers were soaked in 1% Triton X-100 in 0.1 M KCl for 1 hr to reduce the nonmyofibrillar fluorescence, and were then stored on ice in 0.1 M KCl at pH 7. Fibers teased from these bundles were used over the next 3 days. Material with a sarcomere length of about 2 μ was usually

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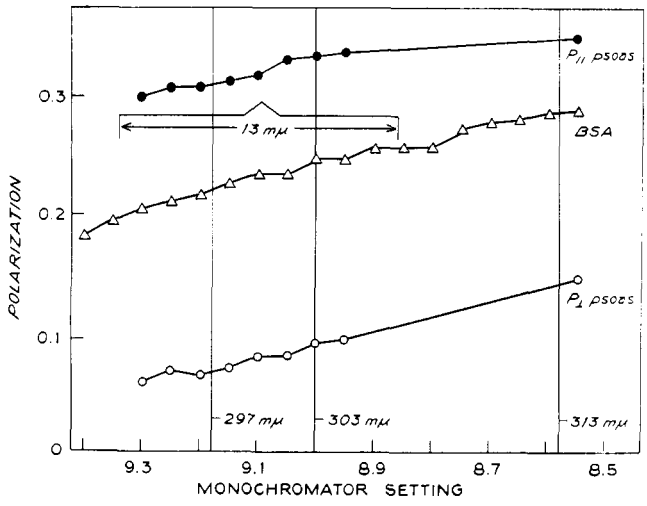

FIGURE 1: Sensitivity of polarization of tryptophan fluorescence to the wavelength of excitation. Most of the energy used for exciting tryptophan specifically in this study was in three complex mercury lines shown at 297, 303, and 313 mμ. With the usual monochromator setting of 9.1 the nominal band width was 13 mμ and both the 297 and the 303 mμ lines were included.

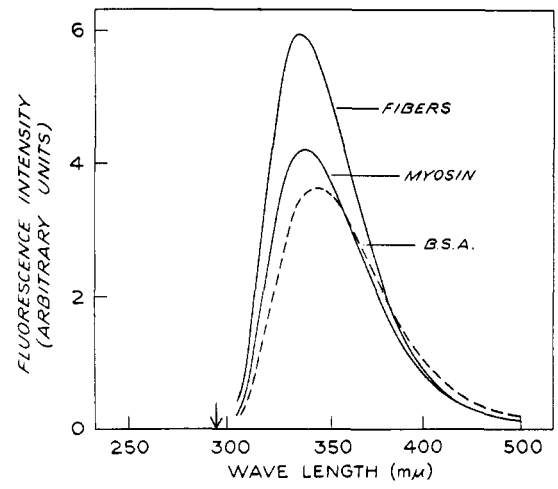

FIGURE 2: Uncorrected front surface emission spectra obtained using a macrospectrofluorometer. Excitation was at 295 mμ (↓) and a Corning 0-54 filter was used to remove scattered exciting light.

used in order to increase the proportion of myosin molecules in contact with actin. Several preparations glycerinated at 0° for 24 hr before storage relaxed poorly, which prompted the change to 6 hr at 0°.

The "relaxing" solution contained 0.1 M KCl, 10 mM $MgCl_2$, 1 mM EGTA,[1] 10 mM phosphate, and 3 mM ATP, and the pH was adjusted to 6.0. The "contracting" solution lacked EGTA and contained varying amounts of calcium, usually 0.1 mM.

Collodion dissolved in acetone was used to fasten a small bundle of fibers (diameter ~150 μ) to a quartz plate which in turn was fastened with silicone grease to the bottom of a small cell in which the objective could be immersed.

Surviving *psoas* fibers were obtained by cutting out a short length of muscle and teasing out a small bundle of fibers which was then covered by light mineral oil. Such bundles were easily stretched when mounted, and so were presumed to be relaxed.

The basic measurements are the intensities of fluorescence parallel, $I_{||}$, and perpendicular, $I_{\perp}$, to the plane of vibration of the exciting light, first when this plane is parallel to the fiber, and then when it is perpendicular to the fiber. To express our results we shall use the "polarization," p, where $p = (I_{||} - I_{\perp})/(I_{||} + I_{\perp})$, $p_{||}$ refers to the case in which the exciting light is polarized with its electric vector parallel to the fiber, and $p_{\perp}$ to the case in which the electric vector is perpendicular.

There are several points in the interpretation of p which should be considered. (1) Muscle fibers are birefringent and relatively thick so that the results are most easily interpretable when the exciting and emitted light are polarized parallel or perpendicular to the fiber axis. The geometry of the sarcomere, with cross bridges oriented in opposition in each half-sarcomere, is a further cause for measuring along these directions.

(2) Fibers as well as their component fibrils and filaments should be parallel in order to distinguish between gross orientation changes and molecular changes. Alignment was set by switching to birefringence optics in the same arrangement. Angle could thus be set well within 2°. The effect of rotating an entire fiber bundle 5° off the extinction position was to produce a decrease in $p_{||}$ and in $p_{\perp}$ of about 0.01.

(3) Absorption of the emitted light and reemission can decrease p but is likely to have little effect in the measurements to be described, since the overlap of the absorption and emission bands is small in tryptophan and the absorbance of a 125-μ thick fiber bundle is about 0.2 at 300 mμ.

(4) p is sensitive to the angle of observation which in these experiments remained fixed as long as the condenser and objective apertures were unchanged.

(5) Tryptophan fluorescence is excited near 300 mμ in order to avoid exciting tyrosine fluorescence too. There are, however, at least two overlapping electronic transitions for tryptophan in this region according to Weber (1960) and only one of these gives high positive values of p. This makes p in the region near 300 mμ extremely sensitive to the exciting wavelength and to small shifts in absorption and emission bands. Figure 1 shows this sensitivity relative to small changes in the exciting wavelength and it could be more marked relative to shifts in absorption maxima.

(6) Depolarization due to scatter and differential effects on fluorescence intensity resulting from scattering are also factors to be considered. Depolarization on passing through a 125-μ thick fiber bundle is 1–2% at 366 mμ and it is only slightly greater when the light is vibrating perpendicular to the fiber axis than when it is vibrating parallel. Because of the glass optical system we could not determine the depolarization at 300 mμ; presumably it is somewhat greater than at 366 mμ, but still small. Differential scattering of the exciting light or of the emitted light will affect p, but we were unable to study this experimentally. It would be expected to decrease both $p_{\perp}$ and $p_{||}$.

[1] Abbreviation used is: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid.

TABLE I: The Major Protein Components of the Myofibril with Estimates of Their Relative Proportion in Muscle, the Number of Tryptophan Molecules per 10^5 g of Protein, and Their Contribution to the Total Fluorescence.[a]

Protein	% by Wt	Trp/10^5 g[d]	% "Total" Trp	Recalcd Considering F-Actin Quenching
Myosin	55[c]	3.9	56	73
Light meromyosin	(16)	6.0	(25)	(33)
Heavy meromyosin	(39)	3.1	(31)	(40)
Actin	15	10.0	39	20
Tropomyosin	15	0.0	0	0
Troponin[b]	7	2.4	5	7

[a] The quenching correction was made assuming that actin and myosin have the same fluorescence intensity on a weight basis (see text). Reliable chemical estimates for the actin, tropomyosin, and troponin content of muscle are not available. [b] We are indebted to Dr. S. Watanabe for the data concerning troponin. [c] Hanson and Huxley (1957). [d] Kominz *et al.* (1954).

TABLE II[a]

Protein	Medium	p	Trp/10^5 g
Myosin A	0.1 M KCl	0.21	3.9
Myosin B (Weber Edsall)	0.1 M KCl	0.21	
F-actin (KCl polymerized)	0.05 M KCl	0.17	10.0
"I filaments" (fibril ghosts)	0.1 M KCl	0.15	
Bovine serum albumin	H_2O	0.23	2.9

[a] Polarization of tryptophan fluorescence, p, for muscle proteins. The same optical arrangement used for determining the polarization of fiber fluorescence was used. "I filaments" refers to the randomized insoluble material obtained after extracting the myosin from glycerinated myofibrils using the procedure of Hanson and Huxley (1957). Excitation was at 300 mμ.

Results

Equilibrium length muscle in rigor in 0.1 M KCl when excited at 300 mμ with light vibrating parallel to the muscle fiber axis gave p values of 0.32 ($p_{\parallel}$) and when excited perpendicular to the muscle axis gave p values of 0.08 ($p_{\perp}$). As shown in Figure 1, these values are higher when excitation is at 313 mμ. Repeated measurements of p on the same specimen fell within ±0.01 of the mean; measurements on different specimens fell within ±0.02 of the mean. p was consistently 0.02 larger at a sarcomere length of 2.8 μ than it was at 2.0 μ in the two preparations compared. The difference seems real although further work on more preparations is needed to establish that it is related to sarcomere length rather than to improved orientation.

Anisotropy of fluorescence polarization was visible in preparations of surviving muscle mounted in mineral oil. The difference between $p_{\parallel}$ and $p_{\perp}$ was not as great as in rigor glycerinated muscle, presumably because of contributions from randomly oriented fluorescent material and because of the relaxed state of the surviving muscle. In muscle from three individuals the average values were $p_{\parallel}$ = 0.286, 0.305, and 0.314 and $p_{\perp}$ = 0.200, 0.197, and 0.198.

Evidence that the measured fluorescence comes mainly from tryptophan is provided by the emission spectra of glycerinated muscle fibers. Uncorrected emission measurements were made from the front surface of the fibers using a spectrofluorometer constructed by Dr. H. C. Cheung. As shown in Figure 2, the fiber curve seems comparable to those obtained for bovine serum albumin and for myosin in the same instrument. There is a small blue shift of fiber fluorescence relative to bovine serum albumin but the peak (~337 mμ) falls well within the range of values for tryptophan in other proteins (Teale, 1960) and near Burshtein's values for actomyosin (cited in Konev, 1967). The glass elements in the microscope exclude radiation below about 335 mμ, which means that about half the tryptophan fluorescence shown in Figure 2 is not measured. A direct means of demonstrating that excitation was mainly at 297 and 302 mμ was to use a sharp cut off Corning 0-53 filter which has about 50% transmission at 300 mμ as determined with a Zeiss spectrophotometer. Placing this filter in the exciting beam reduced the emission from muscle to the extent expected by excitation at a wavelength of 298 mμ.

The fluorescence intensities of F-actin, myosin, and bovine serum albumin were measured with the microscope arrangement used for muscle fibers. F-actin had nearly the same fluorescence intensity as that measured for myosin on a weight basis even though actin contains more tryptophan (Table I). Because of this stronger quenching of tryptophan fluorescence in actin and because muscle contains three or four times more myosin than actin it is likely that more than 50% of the muscle fluorescence measured comes from myosin (Table I). Neither F-actin nor myosin solutions had unusually low values of p when examined with the same optical conditions used for muscle (Table II) although, as expected from its greater tryptophan content, F-actin had a lower value than myosin.

Exciting tryptophan fluorescence with polarized light and measuring the total emission intensity can be used to study dichroism although one can be misled because of differential quenching. Muscle excited with light vibrating parallel to the fiber axis had an emission intensity (without the analyzer) which was only 15% greater than when excitation was vibrating perpendicular to the fiber axis. Similarly, Perutz *et al.* (1950), who looked specifically for tryptophan dichroism directly in alcohol-fixed frog muscle, were unable to detect it. It is unlikely that internal compensation of actin by myosin reduces the dichroism greatly since the absorbance of flow-oriented F-actin at 300 mμ is only 5% greater perpendicular

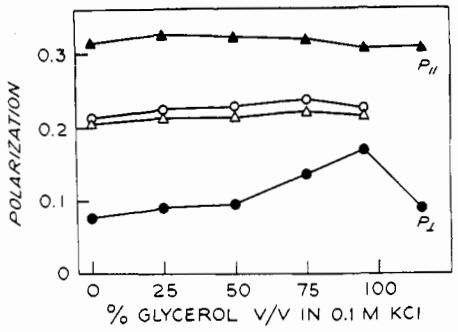

FIGURE 3: The effect of increasing glycerol concentrations on $p_\perp$ (●) is shown along with measurements of $p_{||}$ (▲) on the same fiber and p for gelled actomyosin (△) and gelled myosin (○). Excitation was at 300 mμ.

to the long axis of F-actin than it is parallel (Higashi *et al.*, 1963).

These observations showing that there is not a high degree of tryptophan dichroism in the fiber and that there is not an unusual amount of energy transfer in the major muscle proteins indicate that most of the anisotropy in p is related to the structure of the sarcomere and particularly to the structure of the A and I filaments. This is also shown when the sarcomere structure is disrupted. A salt solution (Hanson and Huxley, 1957) used for extracting myosin from glycerinated rabbit *psoas* caused a decrease in $p_{||}$ from 0.34 to 0.24 and an increase in $p_\perp$ from 0.10 to 0.14. Almost complete randomization as measured by p was achieved by suspending fibers in 0.6 M KI or in 3 M urea containing 0.1 M KCl, although there was some recovery of anisotropy when the fibers were returned to 0.1 M KCl. Both the above solutions partially disrupt the I filaments as well as the A filaments so it can not be concluded that the I filaments are isotropic.

The effect on p of a number of environmental factors was examined under conditions in which filament structure is stable.

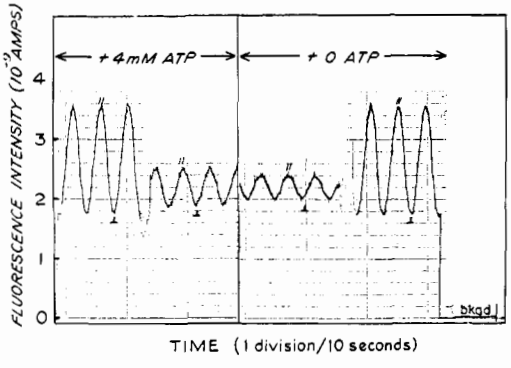

FIGURE 4: This tracing shows the kind of data recorded and the magnitude of the change seen when a fiber changes from rigor (0 mM ATP) to the relaxed state (4 mM ATP in the presence of EGTA and Mg^{2+}). The oscillations are caused by the rotation of a polaroid film between the photomultiplier and the fluorescence source. The symbols || and ⊥ refer to the relation of this "polaroid" to the plane of vibration of the exciting light. The large oscillations are seen when the fiber is oriented parallel to the plane of vibration of the exciting light and the smaller oscillations when oriented perpendicularly. Excitation was at 300 mμ.

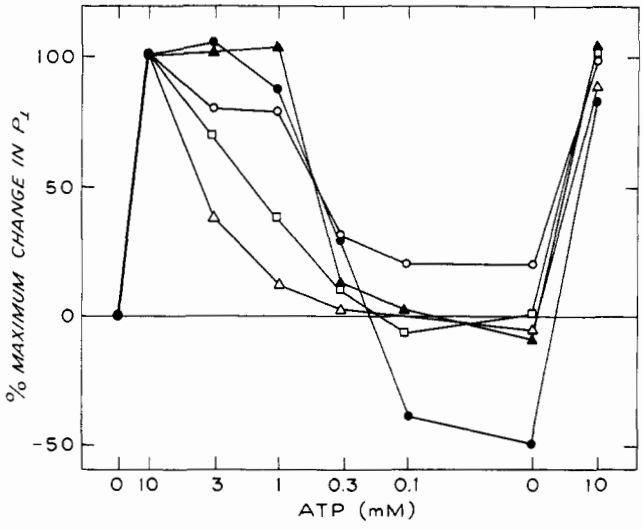

FIGURE 5: ▲, ○, ●, □, and △ are values of $\Delta p_\perp$ for individual fiber bundles as the ATP level in the relaxing solution was varied; 100% was defined as the change in $p_\perp$ in going from 0 to 10 mM ATP at the beginning of the experiment. Excitation was at 300 mμ.

pH. p seemed insensitive to pH between 6 and 9 in 0.1 M KCl. At pH 5 there is a small decrease in both $p_{||}$ and $p_\perp$ which may be related to the irreversible functional changes which occur at this pH.

KCl. Varying the KCl concentration between 0.01 and 0.4 M in a solution buffered with 5 mM phosphate at pH 7 did not have an appreciable effect on $p_{||}$ or $p_\perp$. Changes in p, presumably associated with a loss of structure, were apparent with 0.6 M KCl or with 0.3 M KCl containing 4 mM ATP, 1 mM EGTA, and 10 mM Mg^{2+}.

D_2O. D_2O (95%) containing 0.1 M KCl and at a pH near 7 did not cause an observable change in $p_{||}$ or in $p_\perp$.

Urea. Urea (2 M) containing 0.1 M KCl caused a small decrease, −0.01, in $p_{||}$ and a small increase, +0.01, in $p_\perp$. Irreversible changes, presumably associated with loss of structure, were seen in 3 M urea.

Glycerol. Fibers mounted in 25–95% glycerol–water solutions containing 0.1 M KCl showed an increase in $p_\perp$ (Figure 3) which was reversible and showed little change in $p_{||}$. Similar changes in $p_\perp$ were apparent with glycerol–water solutions without salt and also when excitation was at 313 mμ. The fluorescence intensity increased by about 75% when the glycerol concentration was raised from 0 to 75 vol %. Gelled actomyosin and gelled myosin both showed at best a small increase in p when mounted in glycerol (Figure 3).

ATP. The preceding measurements were made on muscle in which no ATP was present and at sarcomere lengths at which much of the myosin can be considered to be bound to actin, *i.e.*, the fibers are in rigor. Addition of 4 mM ATP in the presence of 10 mM Mg^{2+} and 1 mM EGTA, *i.e.*, a relaxing medium, caused an increase in $p_\perp$ and little or no change in $p_{||}$. The change in the measured signal is shown in Figure 4. A small increase in $p_{||}$ was sometimes observed and may be related to reduced depolarization from scattering, improved fibril and filament orientation or possibly to a structural change. This ATP-induced change in $p_\perp$ was also seen with excitation at 313 mμ.

The concentration dependence for the ATP-induced

change in $p_\perp$ is shown in Figure 5. In the range of ATP where the change in $p_\perp$ is not maximal, signs of continued contraction were usually observable making measurements less reliable than in either the relaxed or rigor states. Variability in reaching a saturating level of ATP for different fibers could be real. However, lumped measurements from fibers which were not included in Figure 5 suggest that the two lower curves are less typical than the more abrupt changes shown by the other fibers.

The increase in $p_\perp$ is easily reversed by washing out the ATP and a fiber bundle can usually be cycled several times, especially at pH 6. The procedure in most experiments was to measure p in a non-ATP-containing medium, then in a high ATP-relaxing medium at least once, and finally again in a non-ATP-containing medium.

Mg^{2+}. There is a magnesium ion requirement for the increase in $p_\perp$ which is satisfied by 1 mM Mg^{2+} in the presence of 4 mM ATP. Mg^{2+} (0.1 mM) in the presence of 4 mM ATP gave about 20% of the change seen with 10 mM Mg^{2+}, and on visual inspection there were signs of prolonged contraction.

Ca^{2+}-(Tension Development). The addition of 0.1 mM Ca^{2+} to fibers in a solution containing 10 mM Mg^{2+} and 4 mM ATP caused the fibers to develop tension isometrically, and associated with this increase in tension there was a decrease in $p_\perp$ (Table III). Lower concentrations of calcium ions gave a similar effect and the higher level was used only to avoid complications from residual EGTA and to activate quickly. If the increase in $p_\perp$, in going from rigor to relaxed, is considered to be 100%, then 0.1 mM Ca^{2+} caused a decrease to about 30% of this value. The variation was large because local contraction, broken fibers, and the tendency to hurry the measurements before the fiber breaks interfere, but in all cases there was a decrease in $p_\perp$ of much more than a few per cent and in no case did $p_\perp$ return to the rigor value. The Ca^{2+}-induced decrease in $p_\perp$ was reversed by an EGTA-containing relaxing medium.

TABLE III[a]

Solvent (mM)	$p \pm$ sem	$p \pm$ sem
1 ATP (0)–Mg^{2+} (10)	0.086 ± 0.003	0.316 ± 0.004
2 ATP (4)–Mg^{2+} (10)–EGTA (1)	0.130 ± 0.002	0.328 ±0.005
3 ATP (4)–Mg^{2+} (10)–Ca^{2+} (0.1)	0.100 ± 0.002	0.317 ± 0.003
4 ATP (4)–Mg^{2+} (10)–EGTA (1)	0.129 ± 0.003	0.320 ± 0.003
5 ATP (0)–Mg^{2+} (10)–EGTA (1)	0.091 ± 0.002	0.318 ± 0.004

[a] Data from 14 fibers in which the addition of calcium ions did not cause breakage or extensive distortion has been lumped and averaged. All solutions contained 0.1 M KCl and 10 mM phosphate, had a pH of 6, and were exchanged in the order 1–5.

Nucleotide and Polyphosphate Effects. ADP (7 mM), AMP (10 mM), pyrophosphate (5 mM), methyl triphosphate (5 mM), or adenosine (10 mM) + tripolyphosphate (10 mM) in the presence of 8–10 mM Mg^{2+} and 1 mM EGTA did not change $p_\perp$ in rigor fibers. There was a significant increase in $p_\perp$ with 8 mM ITP but not as much as when fibers were relaxed with ATP. A low concentration of ATP (0.1 mM) which should almost saturate the hydrolytic site in the presence of EGTA did not appear to complement either 7 mM ADP or 5 mM pyrophosphate so as to produce a detectable change in p.

8-Anilino-1-naphthalenesulfonate was tried as a possible indicator for conformational changes and for changes in rotational freedom in glycerinated muscle since it binds to muscle with a large (75 ×) increase in quantum yield and has little effect on function (Aronson *et al.*, 1968). Unfortunately there was no sign of a single class of well-defined binding sites which could be studied. The polarization of fluorescence proved quite sensitive to the amount of dye bound, and some time dependence was detectable. Although these are not insurmountable difficulties gross changes were not apparent on contraction and it was felt that very detailed measurements would be necessary to define the system.

Discussion

Isometric contraction and relaxation of glycerinated muscle do not involve gross disorientation of the filament array (Sjöstrand and Jagendorf-Elfvin, 1967) so that it is reasonable to infer that the fluorescence polarization and particularly $p_\perp$ varies because of a change in some smaller element of structure. This change may be a conformational change affecting quenching, energy transfer,[2] or a shift in absorption axis, maximum or strength of tryptophan, or it may be a structural change involving the simultaneous movement of a number of tryptophanyl residues.

A quantitative change in fluorescence affecting one or a few tryptophan residues superimposed on an optically inert background of 10–20 other tryptophan residues seems less likely to have been observed than an orientation change involving the simultaneous movement of a number of tryptophan residues. Since there is evidence from both electron microscopy (Reedy *et al.*, 1965) and X-ray diffraction (Huxley *et al.*, 1965) for this latter possibility, the increase in $p_\perp$ on relaxation appears interpretable as a tendency for projecting segments of myosin to become oriented more nearly perpendicular to the fiber axis[3] when the actin–myosin contact is

[2] As already noted, this is not an efficient process due to limited spectral overlap, but we cannot rule out that the effect is responsible for low polarization or even for change in polarization, since it certainly occurs in model compounds (Edelhoch *et al.*, 1968).

[3] It is perhaps surprising that the anisotropy in p is as great as it is considering the low degree of dichroism, the structural complexity, and the multiplicity of tryptophanyl residues which exist in muscle. However, if we assume that the absorption axis at 300 mμ and the emission axis of tryptophan are parallel and in the plane of the indole ring [the orientation of the two transition moments is known (Weber, 1960; Konev, 1967; Yeargers, 1968) but we are not yet in a position to make use of this information], and that muscle contains two populations of tryptophanyl residues, one of which is oriented parallel to the fiber axis and the other is oriented more nearly perpendicular (but not at 90°) to this axis on helically arranged projections which are opposed in each half-sarcomere, it is possible to envisage at least one way in which $p_\parallel$ and $p_\perp$ might differ considerably, and how $p_\perp$ might be differentially increased. In this case exciting along the axis would excite mainly the parallel tryptophanyl residues giving a large p while exciting perpendicu-

dissociated by ATP. However, other conditions (ADP, pyrophosphate, and low levels of ATP) thought to dissociate an appreciable proportion of actin–myosin contacts had little or no effect on $p_{\perp}$. The change in $p_{\perp}$ on activation of contraction with calcium ions also suggests that actin–myosin dissociation is not a complete explanation. One might feel on the basis of the decrease in $p_{\perp}$ that more than 50% of the actin–myosin contacts existing in rigor muscle re-form when glycerinated muscle is activated with calcium ions; yet the X-ray data discussed by Huxley and Brown (1967) indicates that there are probably few actomyosin contacts (less than 20% of those in rigor) during maximal activation of surviving frog muscle.

The effect of high concentrations of glycerol may be interesting in this respect. Glycerol does not plasticize muscle, and yet it selectively increased $p_{\perp}$ while having little or no effect on $p_{||}$. Rome (1967) has previously pointed to this similarity in relation to changes in filament spacing of surviving relaxed muscle as compared with rigor muscle mounted in a 50% glycerol–water solution. It is therefore conceivable that high concentrations of glycerol induce a change in rigor muscle protein comparable with that occurring when ATP is bound without being split and without dissociating actin from myosin.

lar to the fiber axis would excite a population which in over-all projection has a more "random" orientation and therefore a lower p. A change in which the helically arrayed tryptophanyl in each half-sarcomere becomes more nearly perpendicular would tend to make the population on the helical projection more nearly parallel and therefore increase the value of $p_{\perp}$.

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