POLARIZATION FROM A HELIX OF FLUOROPHORES AND ITS RELATION TO THAT OBTAINED FROM MUSCLE

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ABSTRACT Expressions for the polarization of fluorescence from fluorophores in a helical array have been obtained. These predictions have been compared with experimental data obtained by reacting fluorescent S-1 subfragments of myosin (extrinsically labeled with *N*-(iodoacetylaminoethyl)-5-napthylamine-1-sulfonic acid [1,5-IAEDANS]) with glycerated rabbit psoas fibers in rigor. A comparison with data obtained by directly labeling fibers with this fluorophore is also presented.

INTRODUCTION

The tryptophan content of the contractile proteins of muscle is sufficient to produce measurable fluorescence when excited by an ultraviolet light. When the exciting light is polarized, so is the resultant fluorescence, and the polarization observed is dependent both on the physiological state of the muscle and on the relative orientation of the muscle fibers and the polarization axis of the exciting light (Aronson and Morales, 1969; dos Remedios et al., 1972; Steiger et al., 1972). Recently an extrinsic fluorophore 1,5-IAEDANS has been attached to the cysteine on the head of myosin within the intact contractile matrix. The resultant fluorescence showed qualitatively similar polarization characteristics to those of the intrinsic tryptophan (Nihei et al., 1974 *b*). In particular, a reproducible and reversible change in the fluorescence polarization from 1,5-IAEDANS occurred when a muscle in rigor was relaxed by the addition of MgATP in the absence of Ca^{2+}. Since muscle relaxation is known to be accompanied by change in angle of the myosin heads relative to the fiber axis (Reedy et al., 1965) it has been assumed that the polarization change is caused by this particular angular movement of the heads (Nihei et al., 1974 *a*). This assumption was based on the physical fact that an ensemble of dipole emitters with a net orientation in space generates polarized radiation dependent on that orientation.

If appropriate fluorophore geometry could be utilized to predict the observed polarizations, then the issue of whether the myosin head angle actually changes during contraction (Huxley and Simmons, 1971) might be resolved. X-ray diffraction has not, so far, succeeded in directly detecting the active crossbridges, although their presence may be inferred from the diffraction pattern (Huxley and Brown, 1967; Armitage et al., 1972; Hazelgrove and Huxley, 1973).

The contractile proteins of vertebrate skeletal muscle are helically arrayed within the filaments (Huxley and Brown, 1967). Recent fluorescence depolarization studies have indicated that 1,5-IAEDANS binds specifically to myosin (Mendelson et al., 1973). A helical array of fluorophores is therefore expected from the reaction of this fluorophore with muscle. We have calculated the properties of a helical array of fluorophores and tested certain experimental results against the helical case.

POLARIZATION OF FLUORESCENCE FROM A HELICAL SET

Assumptions

(*1*) Each fluorophore may be represented by two dipoles, one for absorption ($\vec{A}$) and the other for emission ($\vec{E}$) separated by a fixed angle (λ). (*2*) The fluorophores are immobile. (*3*) Fluorophores do not interact with one another. (*4*) The fluorophores lie on a single helix. (*5*) The helix is circularly randomized, i.e. all azimuthal positions are taken up with equal probability. (*6*) The direction of observation is in line with the incident light beam.

Calculation of Intensities

The methods used here were originally developed by Weber (1952).

The Individual Fluorophore. If light is polarized with its electric vector parallel to a particular axis then the probability of absorption (p) is proportional to the square of the cosine of the angle between that axis and the absorption dipole direction. For excitation by light passing along the x-axis, perpendicular to the fiber or z-axis, we have

$$
\begin{aligned}
{}_{\parallel}p &= k_1 \cos^2 \theta_A \\
{}_{\perp}p &= k_1 \sin^2 \theta_A \cos^2 \phi_A ,
\end{aligned}
$$

where θ_A is the angle between the absorption dipole and the z-axis and ϕ_A is the angle between the y-axis and the projection of the absorption dipole on the xy plane (Fig. 1). The prescripts refer to the angle of the polarizer relative to the z-axis.

The fluorescent intensity is proportional to the square of the cosine of the angle between emission dipole and the analyzer. Thus the analyzed intensities are

$$
\begin{aligned}
i_{\parallel} &= k_2 p \cos^2 \theta_E \\
i_{\perp} &= k_2 p \sin^2 \theta_E \cos^2 \phi_E ,
\end{aligned}
$$

where θ_E is the angle between emission dipole and z-axis and ϕ_E is the angle between the y-axis and the projection of the emission dipole onto the xy plane (Fig. 1). The subscripts refer to the angle of the analyzer relative to the z-axis.

Now $\phi_E = \phi_A - \beta$, where β = angle between projections of emission and absorp-

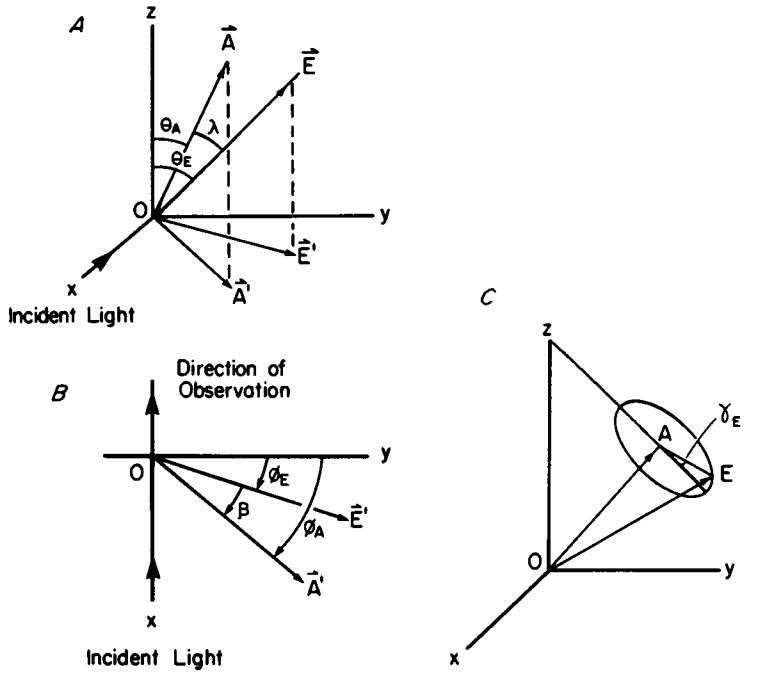

FIGURE 1 Diagrams to illustrate angles defined in text. (*A*) The absorption ($\vec{A}$) and emission dipole ($\vec{E}$). Both polarizer and analyzer directions lie in the *xy* plane; the *z*-axis coincides with that of the helix. (*B*) Projection of the dipoles onto the *xy* plane. (*C*) Definition of γ_E in the random case (after Pesce et al., 1971).

tion dipoles onto the *xy* plane (Fig. 1). Hence for an individual flurorphore

$$
\begin{aligned}
{}_{\parallel}i_{\parallel} &= k_h \cos^2\theta_A \cos^2\theta_E, \\
{}_{\parallel}i_{\perp} &= k_h \cos^2\theta_A \sin^2\theta_E \cos^2(\phi_A - \beta), \\
{}_{\perp}i_{\parallel} &= k_h \sin^2\theta_A \cos^2\theta_E \cos^2\phi_A, \\
{}_{\perp}i_{\perp} &= k_h \sin^2\theta_A \sin^2\theta_E \cos^2\phi_A \cos^2(\phi_A - \beta),
\end{aligned}
$$

where $k_h = k_1 k_2$.

The Helical Set. For any circularly symmetrical set of n_h fluorophores, of which a circularly randomized helical set is a special case, θ_A,θ_E are constant and ϕ_A takes up all values between 0 and 2π with equal probability. The total intensity of any one component from the set is given by

$$
I = \frac{n_h}{2\pi} \int_0^{2\pi} i \, d\phi_A,
$$

$$
{}_{\parallel}I_{\parallel} = n_h k_h \cos^2\theta_A \cos^2\theta_E, \tag{1}
$$

$$
{}_{\parallel}I_{\perp} = \tfrac{1}{2} n_h k_h \cos^2\theta_A \sin^2\theta_E, \tag{2}
$$

$$_{\perp}I_{\parallel} = \tfrac{1}{2}n_h k_h \sin^2\theta_A \cos^2\theta_E, \tag{3}$$

$$_{\perp}I_{\perp} = (\tfrac{1}{8}n_h k_h \sin^2\theta_A \sin^2\theta_E)(1 + 2\cos^2\beta), \tag{4}$$

where β is related to θ_A, θ_E, and λ by the expression

$$\cos\beta = (\cos\lambda - \cos\theta_A \cos\theta_E)/(\sin\theta_A \sin\theta_E). \tag{5}$$

These expressions may be generalized for any angle of observation. For observation along the y-axis only the $_{\perp}I_{\perp}$ component is changed, becoming:

$$_{\perp}I_{\perp} = (\tfrac{1}{8}n_h k_h \sin^2\theta_A \sin^2\theta_E)(1 + \sin^2\beta).$$

The Random Set. The intensity from a random set of n_r fluorophores may be obtained in this notation by the treatment of Soleillet (1929):

$$_{\parallel}I_{\parallel}(r) = \frac{n_r k_r \int_0^{\pi/2}\int_0^{2\pi}\int_0^{2\pi} \cos^2\theta_A \cos^2\theta_E \sin\theta_A \,d\gamma_E\, d\phi_A\, d\theta_A}{\int_0^{\pi/2}\int_0^{2\pi}\int_0^{2\pi} \sin\theta_A \,d\gamma_E\, d\phi_A\, d\theta_A}$$

$$_{\perp}I_{\parallel}(r) = \frac{n_r k_r \int_0^{\pi/2}\int_0^{2\pi}\int_0^{2\pi} \sin^3\theta_A \cos^2\theta_E \cos^2\phi_a \,d\gamma_E\, d\phi_A\, d\theta_A}{\int_0^{\pi/2}\int_0^{2\pi}\int_0^{2\pi} \sin\theta_A \,d\gamma_E\, d\phi_A\, d\theta_A},$$

where γ_E is the angle between the planes zOA and AOE (Fig. 1 C), and

$$\cos\theta_E = \cos\theta_A \cos\lambda - \sin\theta_A \sin\lambda \cos\gamma_E.$$

Hence

$$_{\parallel}I_{\parallel}(r) = \frac{1}{15} n_r k_r (1 + 2\cos^2\lambda) \tag{6}$$

$$= {}_{\perp}I_{\perp}(r).$$

Similarly,

$$_{\perp}I_{\parallel}(r) = \frac{1}{15} n_r k_r (2 - \cos^2\lambda) \tag{7}$$

$$= {}_{\parallel}I_{\perp}(r).$$

Random and helical components may be combined by addition of the appropriate (incoherent) intensities.

EXPERIMENTS

The polarization of fluorescence was measured from 1,5-IAEDANS, bound to subfragment-1 (SF_1) itself diffused into and bound to glycerol-extracted rabbit muscle fibers.

Methods

Muscle Fibers. Thin strips of rabbit psoas muscle were extracted for 2 days to 5 wk at −18°C in a 50% glycerol solution at pH 7 (50% glycerol, 5 mM NaH_2PO_4: Na_2HPO_4, 40 mM KCl, 5 mM $MgCl_2$, 2 mM EGTA). Bundles of 10–40 fibers were washed for 1 h in a low ionic strength buffer solution (5 mM NaH_2PO_4:Na_2HPO_4 at pH 7, 40 mM KCl, 5 mM MgCl, 2 mM EGTA); this solution was used throughout the experiments, unless otherwise stated. In one series, myosin was eluted overnight at 5°C in a high ionic strength pyrophosphate solution (600 mM KCl, 10 mM $Na_4P_2O_7$, 20 mM K_2HPO_4: KH_2PO_4 at pH 7). The fibers were then immersed for 3–20 h at 2°C in a 1–5 μM subfragment-1 solution which had been previously reacted with 1,5-IAEDANS, washed briefly, returned to the 50% glycerol solution, dissected down to bundles of from one to four fibers, mounted by attachment with cellulose nitrate/acetate glue to Lucite blocks over a glass slide, and reimmersed beneath a supported coverslip.

In one series the fiber bundles were not immersed in SF_1, but directly reacted for 30–60 min with 30 μM 1,5-IAEDANS at 25°C, washed, dissected down, and mounted as above.

A solution of SF_1 in 20% sucrose was also used for observation.

Subfragment-1. Myosin was prepared by a modification of the method of Tonomura et al. (1966), reacted for 18 h at 4°C with 1,5-IAEDANS in a 1:1 molar ratio (SF_1:dye, 1:0.5) and proteolyzed by insoluble papain on *p*-aminobenzoate cellulose for 15 min at 25°C (Lowey et al., 1969). The resultant SF_1 was purified on G200 Sephadex and dialyzed against the low ionic strength buffer used in these experiments. All reactions were performed at pH 7.

The Ca^{2+}-activated ATPase of 1,5-IAEDANS-reacted myosin was approximately 2.5 times that of untreated myosin at this reaction mixture. On addition of 2 mol dye/mol myosin activation was approximately five- to six-fold.

Observation. Fluorescence was observed along the line of excitation, as in previous studies from this laboratory (dos Remedios et al., 1972; Nihei et al., 1974 *a,b*). The fluorescent emission from the fiber was measured by the difference between the signal from the fibers and that from an adjacent area of solution. The background intensity from unstained fibers was found to be less than 2% of that from the fibers containing the fluorophore.

Excitation was restricted by means of a 370 nm three-element interference filter (full width at half maximum [FWHM], 11 nm; Ditric Optical Co., Marlboro, Mass.). The excitation cone half-angle was approximately 3° and the illuminated field 100–300 μm in diameter. Emission was collected by a 10× Tijoda objective with the aperture reduced by a stop (cone half-angle approximately 10°). An ultraviolet absorbing filter

(3/72; Corning Glass Works, Corning, N.Y.) was placed immediately behind this lens and a 2.5 mm aperture placed at the first image plane (equivalent to 250 μm at fibers) to reduce the effect of fluorescence from this filter. Observation was restricted by means of a two-element 480 nm interference filter (FWHM 10 nm, Oriel Corp., Stamford, Conn.) placed immediately after the Wollaston prism. The electronics and signal averaging system were similar to those used previously (Nihei et al., 1974 *b*). One observation was made on each selected field of view; not more than two observations were made on any one set of fibers.

Each set of observations consisted of four intensities (I) from which three independent ratios could be derived. We have cited the conventional polarizations:

$$P_{\parallel} = ({}_{\parallel}I_{\parallel} - {}_{\parallel}I_{\perp})/({}_{\parallel}I_{\parallel} + {}_{\parallel}I_{\perp});$$
$$P_{\perp} = ({}_{\perp}I_{\perp} - {}_{\perp}I_{\parallel})/({}_{\perp}I_{\perp} + {}_{\perp}I_{\parallel}),$$

and in addition a similar parameter, $Q_{\parallel}$, derived from alteration of the excitation orientation:

$$Q_{\parallel} = ({}_{\parallel}I_{\parallel} - {}_{\perp}I_{\parallel})/({}_{\parallel}I_{\parallel} + {}_{\perp}I_{\parallel}).$$

The intensity ratios (${}_{\parallel}I_{\parallel}/{}_{\parallel}I_{\perp}$, etc.) may be derived from these functions.

Modeling

The functions $P_{\parallel}$, $Q_{\parallel}$, and $P_{\perp}$ were computed in the following cases:

(*1*) A single helix, from Eqs. 1–4. (*2*) A set of helices equally distributed about a median cone angle, θ_A, with the restriction that $\theta_E - \bar{\theta}_A = \delta$ is held constant, i.e.,

$$\theta_A = \bar{\theta}_A \pm \Delta\theta$$
$$\theta_E = \bar{\theta}_A + \delta \pm \Delta\theta.$$

Here

$${}_{\parallel}I_{\parallel} = \int_{\bar{\theta}_A - \Delta\theta}^{\bar{\theta}_A + \Delta\theta} \cos^2\theta_A \cos^2(\theta_A + \delta) \sin\theta_A \, d\theta_A$$

$${}_{\perp}I_{\perp} = \tfrac{1}{8} \int_{\bar{\theta}_A - \Delta\theta}^{\bar{\theta}_A + \Delta\theta} \sin^3\theta_A \sin^2(\theta_A + \delta)(1 + 2\cos^2\beta) d\theta_A,$$

etc., where $\beta = \beta(\theta,\lambda)$, defined by Eq. 5. The term $\sin\theta_A$ is introduced to fulfill the condition of equal space-filling. The integrals were computed numerically. (*3*) A single helix plus an immobilized random component, according to Eqs. 1–4, 6, and

7 with the assumption that $k_r = k_h = k$. Here

$$_{\parallel}I_{\parallel} = nk\left[(1 - \alpha)\cos^2\theta_A\cos^2\theta_E + \frac{\alpha}{15}(1 + 2\cos^2\lambda)\right]$$

$$_{\perp}I_{\perp} = nk\left[\frac{1}{8}(1 - \alpha)\sin^2\theta_A\sin^2\theta_E(1 + 2\cos^2\beta) + \frac{\alpha}{15}(1 + 2\cos^2\lambda)\right],$$

etc., where α = fraction of the total number of fluorophores which are randomized and n is the total number of fluorophores. β is defined by Eq. 5.

In each case a fit was sought between the model predictions and the values observed.

Results

Subfragment-1 (SF_1) bound slowly to muscle fibers. After 3 h immersion at 2°C in 1 μM SF_1 the fibers contained sufficient fluorescence to observe the polarization from two to four fibers, but after 20 h there was much more, and single fibers gave sufficient fluorescence for accurate measurement.

When the fibers were excited by light whose electric vector was parallel to the fiber axis the observed polarization was much greater than that from a random set of immobilized fluorophores and varied only slightly between different SF_1-binding conditions ($P_{\parallel}$, Table I). The "excitation change polarization," $Q_{\parallel}$, was also much greater than that from a random immobile set. It was slightly but significantly less than $P_{\parallel}$ and remained relatively constant throughout the experiments (Table I). On

TABLE I

POLARIZATION OF FLUORESCENCE FROM 1,5-IAEDANS REACTED WITH SUBFRAGMENT-1

	SF_1 binding conditions		
	$P_{\parallel}$	$Q_{\parallel}$	$P_{\perp}$
3 h to eluted fibers (20)	0.475 ±0.004	0.453 ±0.005	0.156 ±0.011
3 h to normal fibers (9)	0.486 ±0.012	0.465 ±0.007	−0.039 ±0.029
20 h to normal fibers			
1 μM SF_1 (19)	0.484 ±0.003	0.459 ±0.003	−0.043 ±0.005
5 μM SF_1 (12)	0.504 ±0.002	0.461 ±0.003	−0.089 ±0.002
Free in 20% sucrose (4)	0.326 ±0.009	0.339 ±0.006	0.338 ±0.003

$P_{\parallel}$, $Q_{\parallel}$ and $P_{\perp}$ are defined in the Methods section. All error values are standard errors of the mean; numbers of experiments in brackets. 1 μM SF_1 used except where otherwise stated. All experiments at pH 7, 25°C.

the other hand, $P_\perp$ was much less than that from a random immobile set and varied greatly. Thus the fiber acted like a plane polarizer: much more light was emitted when the fiber was excited parallel to its axis than when it was excited perpendicular (maximum ratio approximately 2:1).

In one series of experiments 1,5-IAEDANS was directly reacted with muscle fibers. This gave very different results to those obtained from SF_1 attached to fibers: both $P_\parallel$ and $P_\perp$ were positive and slightly below that from a set of randomized immobile fluorophores and $Q_\parallel$ was closer to $P_\perp$ than to $P_\parallel$ ($P_\parallel = 0.226 \pm 0.007$, $Q_\parallel = 0.267 \pm 0.004$, $P_\perp = 0.285 \pm 0.004$ [15]).

The polarization observed from 1,5-IAEDANS bound to SF_1, freely diffusing within a 20% sucrose solution (P_{random}) was approximately 0.33 (Table I); calculation from the observed rotational correlation time of SF_1 (Mendelson et al., 1973) showed that this was probably 5% lower than the value from totally immobilized SF_1. P_{random} fell to approximately 0.28 on changing the excitation wavelength to 350 nm. ($\Delta P_{random} = -0.05 \pm 0.01$ [5]).

DISCUSSION

Relation of Experiment to Theory

The assumptions on which the helical theory was based can be justified in the present case. 1,5-IAEDANS in solution behaves as a two-dipole fluorophore whose properties vary greatly with wavelength in the excitation region 340–380 nm (Hudson and Weber, 1973). Under the present experimental conditions the apparent angle between the dipoles (λ) was determined to be approximately 28° when corrections for residual mobility were included.[1] The fluorophores on SF_1 are effectively immobile when SF_1 is bound to actin (Mendelson et al., 1973) and are too distant fron one another to interact. Reaction of 1,5-IAEDANS with myosin produced an activation of Ca^{2+}-activated ATPase equivalent to that which has been seen using a "spin-label" with a similar iodoacetamide reactivity (Quinlivan et al., 1969; Seidel et al., 1970). Electron paramagnetic resonance spectra indicated that the "spin-label" was highly specific to one fast reacting thiol on each myosin head. It is therefore probable that 1,5-IAEDANS also reacts predominantly with this "fast" SH of myosin and hence should form a single helix. The large numbers of myofibrils observed ensures that all azimuthal positions will be taken up equally.

The data, however, do *not* fit our first model, the pure single helix. The relatively invariant parameters, $P_\parallel$ and $Q_\parallel$, fit to helices in the range $\theta_E = 39$–$40.5°$, $\theta_A = 40$–$41.5°$ (Fig. 2). For such helices $P_\perp = -0.39$–-0.41 ($\beta \approx 46°$) whereas the observed values of $P_\perp$ ranged between $+0.16$ and -0.09 (Fig. 2). This misfit is unlikely to be due to the optical properties of single rabbit muscle fibers, which produce less than 5% depolarization due to light scatter measured at 370 nm (Nihei and Mendelson, unpublished observations). Birefringence does not interfere with results obtained with elec-

[1] The adjusted $P_{random} = 0.35 = (3\cos^2\lambda - 1)/(\cos^2\lambda + 3)$.

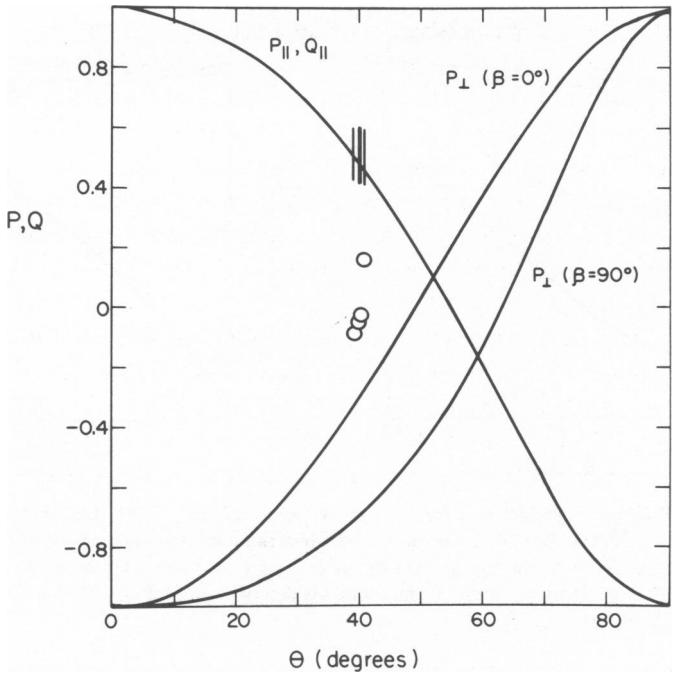

FIGURE 2 Theoretical predictions from single helical model. $P_\parallel$ and P are plotted as a function of θ_E. $Q_\parallel$ is plotted as a function of θ_A. The two lines of P correspond to $\beta = 0°$ and $\beta = 90°$. Experimental values of $P_\perp(\theta_E)$ fitted by fixing θ_E via $P_\parallel$ (vertical lines). Computation as described in Methods section.

tric vectors analyzed perpendicular or parallel to the fiber axis (Aronson and Morales, 1969; Rozanov et al., 1971) when the excitation and emission directions are normal to this axis. On the other hand, there are many possible causes of disorder at the molecular level: disorientation of the dye's attachment to the "fast" SH on SF_1, of SF_1 on actin, or of actin relative to the fiber could cause a distribution of helical angle, while *either* dye bound to other SH groups of SF_1, *or* SF_1 bound to proteins other than actin in the fibers could constitute an immobile random component in parallel with the helix.

Both these types of disorder have been theoretically simulated. In our second model, θ_A was allowed to take up all values within a specified range ($\pm\Delta\theta$) with probability weighted by the volume of space occupied (i.e., $x \sin \theta$); θ_E was maintained with constant azimuth relative to θ_A. This situation is a first approximation to the condition where the actin longitudinal axis is somewhat randomized by internal flexibility but no torsional movement is possible. The motivation for this comes from the observation that fibers from which myosin had been eluted showed less orientation than normal fibers and thus the randomization observed in the normal case could also arise from this source. This particular type of disorder produced a large increase in $P_\perp$ when $\Delta\theta > 10°$ with only a slight rise in $P_\parallel$ and $Q_\parallel$ (Fig. 3 A). Thus an approximate fit to the data could be obtained at high values of $\Delta\theta$ (Fig. 3 A); the median values of θ_A

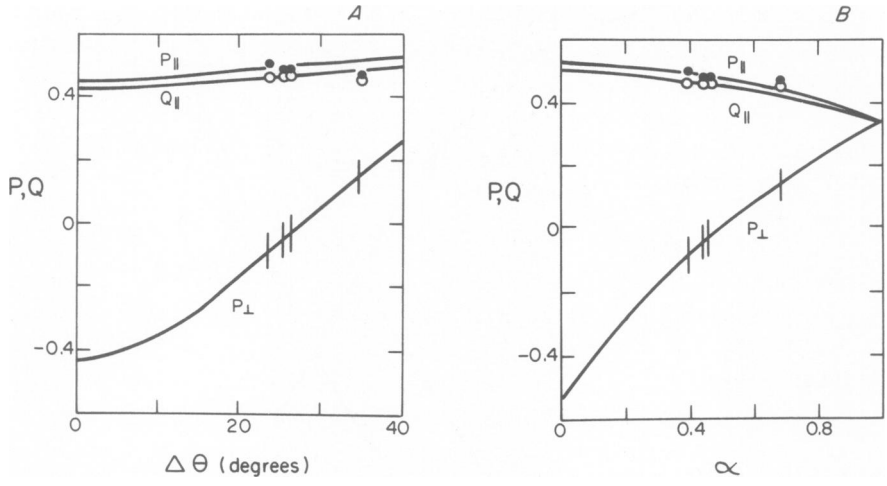

FIGURE 3 Theoretical predictions from more complex models. (*A*) Distributed helices; median $\theta_A = 42°$; median $\theta_E = 41°$. (*B*) Single helix plus random component; $\theta_A = 39°$; $\theta_E = 38°$. In these separate cases experimental points ($P_\parallel$, ●; $Q_\parallel$, ○) were fitted to theoretical curves by fixing $\Delta\theta$ or α via $P_\perp$ (vertical lines). Computation is described in Methods section; $\lambda = 28°$ assumed throughout.

and θ_E which gave the best fit to the data were slightly greater than those from fitting $P_\parallel$ and $Q_\parallel$ to the single helix; θ_A, median = 41–42°, θ_E, median = 40–41°. It should be noted that this is only one case of this type of disorder, in which rotation of one vector around the other has been disallowed.

The third model consisted simply of adding an immobile random component (α). Increase of α caused a large regular increase of P and a slight but definite decrease in $P_\parallel$, $Q_\parallel$ (Fig. 3 B). This model also produced a fit to the data (Fig. 2 B), with slightly decreased value of θ(θ_A = 38–39°, θ_E = 37–38°).

Both types of disorder could therefore produce the observed results, and we cannot determine the type present. Furthermore, the models used *assume* the presence of a single helix and generate a fit to the results by adding disorder. They therefore do not prove its existence, which remains an assumption based on the evidence of Seidel et al. (1970), Moore et al. (1970), Miller and Tregear (1972), and Mendelson et al. (1973). If the helix does exist, the models show that the data give quite accurate estimates of its parameters: in both models of disorder the estimate of the helical angle remains close to that derived from the simple model, so that the data obtained from the dominant excitation, $P_\parallel$ and $Q_\parallel$, give good estimates of the helix cone angles. In other words the emission favored by helix, ${}_\parallel I_\parallel$ in this case, is relatively undisturbed by these forms of disorder, whereas the disfavored emission, ${}_\perp I_\perp$, is grossly altered.

If this is correct, the cone angles of the absorption and emission dipoles of 1,5-IAEDANS attached to the "fast" SH of SF_1 are well-defined by the data; they are both approximately 40° relative to the filament axis when the SF_1 is attached to actin. The major axis of the rod-shaped SF_1 is known to be 50–60° to the actin filament in this

case (Moore et al., 1970; Miller and Tregear, 1972). It follows that the minimum angle between the fluorophore dipoles and the major axis of SF_1 is 10–20°. Mendelson et al. (1973) have shown that the dipoles are probably not more than 30° off from the major axis. On this basis the position of the dipoles relative to the acto-SF_1 complex may be closely defined; they lie between the SF_1 major axis and the filament axis and near to the plane defined by these two axes.

Application of Theory to Other Results from Muscle

It is possible to study physiological states only by direct reaction of a fluorophore within the muscle, or by use of the indigenous tryptophan fluorescence. Most of the 1,5-IAEDANS reacted with muscle fibers is believed to attach to the fast SH of myosin (Nihei et al., 1974 *b*). However, it produces rigor polarizations very different from that pre-reacted attached SF_1 (Table II). Again, the data do not fit to a single helix. They fit to a helix plus a random component, with high values of θ_A, θ_E and very large values of the random fraction (Table II). Relaxation of muscle increaes both $P_\parallel$ and $P_\perp$ from 1,5-IAEDANS (Table II). In terms of the third model this is characteristic of a slight diminution of θ_E and a further increase of α (Table II).

It is clear that none of these data approximate to the single helix case. Either the reaction of 1,5-IAEDANS with muscle fibers is not as specific as has been supposed (Nihei et al., 1974 *b*) or the heads of myosin in rabbit muscle attach to actin in a different manner in rigor than does infused SF_1, possibly because of the duplex nature of myosin. The low intensities of the outer layer lines of the rigor X-ray diffraction pattern of vertebrate skeletal muscle (Huxley and Brown, 1967; Rome, 1972) relative to those from insect flight muscle (Miller and Tregear, 1972) is consistent with this idea.

In view of the poor approximation to the single helix, the meaning of the supposed helical angles derived from the model (Table II) is uncertain. If they do represent an average helical angle, it is definitely higher in rigor than that obtained from infused SF_1, and falls slightly when the muscle is relaxed. The X-ray diffraction patterns of

TABLE II

FLUORESCENCE POLARIZATION FROM MUSCLE FIBERS DIRECTLY REACTED WITHIN 1,5-IAEDANS, AND BEST-FIT CONDITION OF THE RANDOM AND HELIX MODEL

	Condition					
	$P_\parallel$	$Q_\parallel$	$P_\perp$	θ_E	θ_A	α
Rigor						
Present results	0.23	0.27	0.29	53	56	0.68
Nihei et al. (1974 *b*)*	0.34	—	0.22	46	(46)	0.74
Relaxed						
Nihei et al. (1974 *b*)*	0.37	—	0.26	42	(42)	0.83

*These data have been adjusted from λ_{ex} = 350 nm to λ_{ex} = 370 nm by adding 0.05 to both polarization values (cf. Results).

relaxed muscle have been interpreted as showing that the myosin heads lie with their major axes perpendicular to the filament axes (Huxley and Brown, 1968; Miller and Tregear, 1972). If most of the 1,5-IAEDANS does react with the fast SH of the myosin head (Nihei et al., 1974 *b*) and the fluorophore axes lie within 30° of the major axis of the head (Mendelson et al., 1973), then such a position of the head would produce greatly different results: $P_\perp$ would be much greater, and $P_\parallel$ much less, than that observed (cf. Fig. 2). Neither of these assumptions is proven, so the diffraction model is not excluded; it should also be noted that all the fluorescence measurements using extrinsic labeling have been made on glycerol-extracted muscle, on which relatively few X-ray diffraction measurements are available (Rome, 1972). However, there are two indications that the head angle is variable, at least in glycerol-extracted muscle. Firstly, the model interpretation of the 1,5-IAEDANS data indicates that on relaxation the randomicity of the fluorophores increases, and the rise in both $P_\parallel$ and $P_\perp$ from tryptophan fluorescence on relaxation (Nihei et al., 1974 *b*) is consistent with this. Secondly, direct measurement of the mobility of 1,5-IAEDANS reacted with muscle fibers shows that a large proportion of the fluorophores become slightly mobile on relaxation (rotational correlation time 500–1,000 ns; Mendelson and Tregear, unpublished observations). If the average position of the myosin heads were near to 90° but were free to rotate over a considerable axial angle, the apparent conflict between the X-ray and fluorescence results might be resolved.

General Use of Theory

Three independent functions, $P_\parallel$, $Q_\parallel$, $P_\perp$, can be meaningfully measured for any fibrous system of fluorophores. If the same fluorophores are placed in a randomized, immobilized configuration an additional parameter, P_{random}, can be determined. The first three parameters can be used to test for the presence of a pure single helix geometry. If they fit to such a helix the fourth parameter provides an independent test of the fit, and hence can affirm the existence of the helix. If they do not fit a single helix, the fourth parameter allows theoretical simulation of forms of disorder about a single helix in order to generate a fit. In this case the existence of a helix has to be justified by external information. If this can be done the polarizations measured when using the excitation favored by the helix are good measures of its angular characteristics even when considerable disorder is present. More complex systems can only be handled if additional external information is available. These principles should be of use in biological systems other than muscle.

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