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Calculation of the polarized fluorescence from a labeled muscle fiber

(cross-bridges/dichroism/torsion)

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ABSTRACT **Equations are derived that explicitly relate fluorescence polarization observables on a labeled muscle fiber to attitude of the cross-bridges and to attitude of the labels within the cross-bridges.**

For some years the polarized emission from either intrinsic (1) or extrinsic (2) fluorophores imbedded in muscle cross-bridges has been used to deduce information about the spatial attitude of these cross-bridges. Until recently, however, the guiding analysis has been limited. For example, the measured quantities have been related to the fluorophore attitudes, not the cross-bridge attitudes, and the possibility of cross-bridge torsion [now suggested by electron microscope observations (3)] has been ignored. There has been a recent effort (4) to remove these limitations. In the present paper, as in ref. 5, the limitations are removed analytically; i.e., we obtain expressions for the measured intensities in terms of cross-bridge attitude and attitude of the fluorophore relative to the cross-bridge; furthermore, the possibility of torsion is included. These expressions also allow us to treat dichroism (6) with the same generality. Another paper (7) uses a model-independent approach to identify and define the information obtainable from fluorescence polarization and from dichroism experiments.

For visualization purposes we imagine (Fig. 1) that a cross-bridge is a cylinder that can rotate about its principal axis in "torsion," a motion to be described by angle ψ. When the cross-bridge "decorates" the Z axis of a "laboratory framework" of coordinates (when the center of one of the cylinder ends is placed on the Z axis in simulation of thick filament assembly), the principal axis of the cylinder acquires an attitude defined by the usual spherical angles, θ (for declination) and ϕ (for azimuth). Using some point on the lateral surface of the cylinder as center of coordinates, we construct a "moving framework." One of its axes (a) is parallel to the principal axis; another (r) is a radius of the cylinder; the third (t) is tangent to the cylinder and perpendicular to both a and r. In this t–r–a system we position a doublet of two unit vectors $\hat{v}_a$ and $\hat{v}_e$ (later to be associated with a fluorophore). The orientation of $\hat{v}_a$ is specified by two spherical angles, η (for declination from the a axis) and ξ (for azimuth). Thus,

$$\hat{v}_a = \hat{t}\sin\eta\cos\xi + \hat{r}\sin\eta\sin\xi + \hat{a}\cos\eta. \qquad [1]$$

Two additional angles position $\hat{v}_e$ relative to $\hat{v}_a$. One is the angle μ between $\hat{v}_a$ and $\hat{v}_e$. The other is the counterclockwise (viewed from the origin) angle, ζ, that the plane containing $\hat{v}_a$ and $\hat{v}_e$ makes with plane containing $\hat{v}_a$ and $\hat{a}$. Simple vectorial arguments (Fig. 1*A*) then show that

$$\hat{v}_e = \hat{v}_a\cos\mu + \left\{\frac{\hat{v}_a\times\hat{a}\times\hat{v}_a}{\sin\eta}\cos\zeta + \frac{\hat{a}\times\hat{v}_a}{\sin\eta}\sin\zeta\right\}\sin\mu. \qquad [2]$$

Substitution of Eq. **1** into Eq. **2** allows $\hat{v}_e$ to be written as

$$\begin{aligned}\hat{v}_e = {} & \hat{t}(\sin\eta\cos\xi\cos\mu - \cos\eta\cos\xi\sin\mu\cos\zeta \\ & - \sin\xi\sin\mu\sin\zeta) + \hat{r}(\sin\eta\sin\xi\cos\mu \\ & - \cos\eta\sin\xi\sin\mu\cos\zeta + \cos\xi\sin\mu\sin\zeta) \\ & + \hat{a}(\cos\eta\cos\mu + \sin\eta\sin\mu\cos\zeta). \qquad [3]\end{aligned}$$

The "moving framework" is thus embedded in the cross-bridge, and $\hat{v}_a$ and $\hat{v}_e$ are now taken as parallel to the absorption and emission dipoles of the fluorophore that "labels" the cross-bridge. As we said above, the unit vector, $\hat{a}$, is positioned in the "laboratory framework" by θ and ϕ, so (Fig. 1*B*)

$$\hat{a} = \hat{i}\sin\theta\cos\phi + \hat{j}\sin\theta\sin\phi + \hat{k}\cos\theta. \qquad [4]$$

Rotation of the cross-bridge around its own principal axis is measured by the counterclockwise (viewed from the origin) angle, ψ, that $\hat{r}$ makes with the plane containing $\hat{a}$ and $\hat{k}$. Vectorial arguments illustrated in Fig. 1*B* show how expressions for $\hat{r}$ and $\hat{t}$ in the laboratory framework can also be obtained:

$$\hat{r} = \frac{\hat{a}\times\hat{k}\times\hat{a}}{\sin\theta}\cos\psi + \frac{\hat{k}\times\hat{a}}{\sin\theta}\sin\psi \qquad [5]$$

$$\hat{t} = \hat{r}\times\hat{a}. \qquad [6]$$

By substituting Eq. **4** into Eqs. **5** and **6** we get

$$\begin{aligned}\hat{r} = {} & \hat{i}(-\cos\phi\cos\theta\cos\psi - \sin\phi\sin\psi) + \hat{j}(\sin\phi\cos\theta\cos\psi \\ & + \cos\phi\sin\psi) + \hat{k}(\sin\theta\cos\psi). \qquad [5']\end{aligned}$$

$$\begin{aligned}\hat{t} = {} & \hat{i}(-\sin\phi\cos\psi + \cos\phi\cos\theta\sin\psi) + \hat{j}(\cos\phi\cos\psi \\ & + \sin\psi\cos\theta\sin\psi) + \hat{k}(-\sin\theta\sin\psi). \qquad [6']\end{aligned}$$

In order to make further manipulations less cumbersome we implicitly define the vector components in the $\hat{t}$–$\hat{r}$–$\hat{a}$ system (Eqs. **1** and **3**) as $\hat{v}_a \equiv \hat{t}A_t + \hat{r}A_r + \hat{a}A_a$ and $\hat{v}_e \equiv \hat{t}E_t + \hat{r}E_r + \hat{a}E_a$; similarly, we define the components in the $\hat{i}$–$\hat{j}$–$\hat{k}$ system (Eqs. **4**, **5′**, and **6′**) as $\hat{a} \equiv \hat{i}a_i + \hat{j}a_j + \hat{k}a_k$, $\hat{r} \equiv \hat{i}R_i + \hat{j}R_j + \hat{k}R_k$, and $\hat{t} \equiv \hat{i}T_i + \hat{j}T_j + \hat{k}T_k$. Now, by substituting Eqs. **4**, **5′**, and **6′** into Eqs. **1** and **3**, we obtain expressions for $\hat{v}_a$ and $\hat{v}_e$ referred to the "laboratory framework" but explicitly recog-

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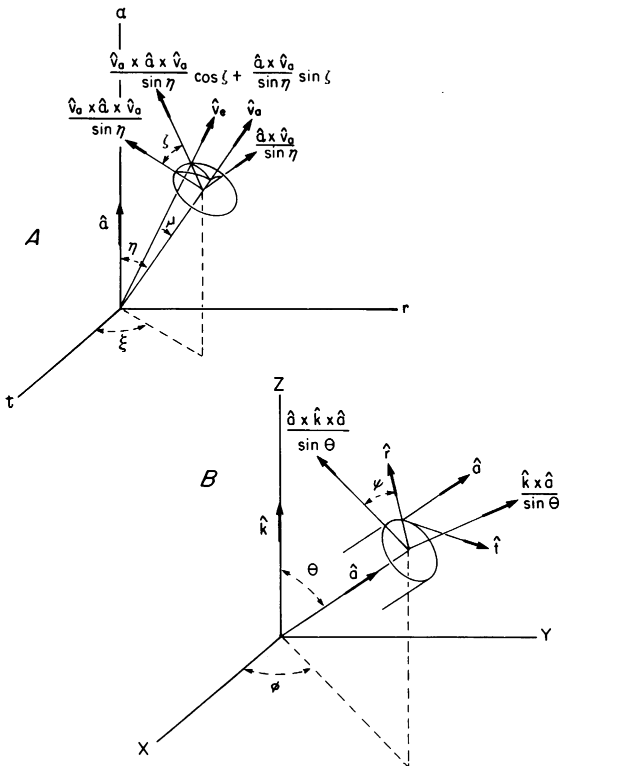

FIG. 1. (*A*) Derivation of Eq. **2**. Once $\hat{v}_a$ is positioned, $\hat{v}_e$ is written as the vector sum of components parallel (cos μ) and perpendicular (sin μ) to $\hat{v}_a$; to express the latter component it is necessary to introduce angle ζ. (*B*) Relation of the "moving" framework of coordinates, the $\hat{t}$–$\hat{r}$–$\hat{a}$ system, to the "laboratory" framework of coordinates, the *X–Y–Z* (or $\hat{i}$–$\hat{j}$–$\hat{k}$) system.

nizing how the fluorophore sits in the cross-bridge and how the cross-bridge is oriented in space. These expressions will be functions of six angles*: ξ, η, ζ, and θ, ϕ, ψ. It is convenient at this point, however, to take into account a special feature of the organization of cross-bridges in thick filaments, namely, helical symmetry about the *M* plane (a plane normal to the filament at its midpoint; see Fig. 2). Viewed from the *M* plane the helical arrangement of a cross-bridge "above" the plane is identical to that of one "below" the plane. A consequence of this circumstance is that certain terms in $\hat{v}_a$ and $\hat{v}_e$ will be negative, depending on whether the fluorophore in question is "above" or "below" the plane, thus,

$$\begin{aligned}\hat{v}_a = {} & \hat{i}(\pm A_tT_i \pm A_rR_i + A_aa_i) \\ & + \hat{j}(A_tT_j + A_rR_j \pm A_aa_j) \\ & + \hat{k}(A_tT_k + A_rR_k \pm A_aA_k)\end{aligned} \qquad [7]$$

and

$$\begin{aligned}\hat{v}_e = {} & \hat{i}(\pm E_tT_i \pm E_rR_i + E_aa_i) \\ & + \hat{j}(E_tT_j + E_rR_j \pm E_aa_j) \\ & + \hat{k}(E_tT_k + E_rR_k \pm E_aa_k).\end{aligned} \qquad [8]$$

In upcoming manipulations scalar products of these vectors will be raised to powers. In the resulting expressions many terms will be preceded by $(\pm)^m$ where *m* is a positive integer. Because it is equally likely for a cross-bridge to be "above" or "below" the *M* plane, and because it is anticipated that we will sum expressions from one cross-bridge with those from every other, it is obvious that in the summation all terms preceded by $(\pm)^m$, where *m* is odd, will cancel. For this reason we have found it expedient to omit altogether from the expressions those terms that we know will later cancel in the summation.

There are two types of experiments that capitalize on the directional feature of fluorescence. In the *dichroism mode* (Fig. 3*A*) excitation in the form of plane-polarized light inclined δ to the *X–Z* plane travels from $+X$ toward a fluorophore at the center of coordinates. The probability of exciting the fluorophore is proportional to $(\hat{v}_{EX}\cdot\hat{v}_a)^2$, where

$$\hat{v}_{EX} = \hat{j}\sin\delta + \hat{k}\cos\delta. \qquad [9]$$

If *all* the fluorescence is collected, the total emission intensity is proportional to the energy absorbed—i.e., to $(\hat{v}_{EX}\cdot\hat{v}_a)^2$. This geometric factor in the total emission intensity is thus obtained by substitutions from Eqs. **7** and **9**. Thus, the intensity contribution from one fluorophore is

$$\begin{aligned}\mathcal{I}_{tot} \propto {} & \{(A_tT_j)^2 + (A_rR_j)^2 + (A_aa_j)^2 + 2A_tA_rT_jR_j\}\sin^2\delta \\ & + \{A_t^2T_jT_k + A_r^2R_jR_k + A_a^2a_ja_k \\ & + A_rA_t(R_jT_k + R_kT_j)\}\sin 2\delta + \{(A_tT_k)^2 + (A_rR_k)^2 \\ & + (A_aa_k)^2 + 2A_tA_rT_kR_k\}\cos^2\delta.\end{aligned} \qquad [10]$$

In the next step we must sum the contributions from, say, *N* fluorophores [it is in this summation that terms preceded by $(\pm)^m$, with *m* odd, would disappear], each differing, possibly, with respect to one or more of the angles, ξ, η, ζ, θ, ϕ, and ψ. In the *fluorescence polarization mode* (Fig. 3*B*) the fiber laid along the *Z* axis and containing the fluorophore in question at the center of coordinates is excited by polarized light traveling along one axis (*X*, *Y*, or *Z*), and the polarized emission travels outward along one axis to be analyzed at the observation station. Along any polarized excitation or emission axis there are two possible orthogonal orientations of the electric vector. By writing out all the possibilities it is readily found that there are nine distinct arrangements, excitation-direction, observation-direction; therefore, nine distinct intensities are measurable. The "geometric factor" in each of these intensities is $(\hat{p}\cdot\hat{v}_a)^2(\hat{v}_e\cdot\hat{q})^2$, where $\hat{p}$ and $\hat{q}$ can

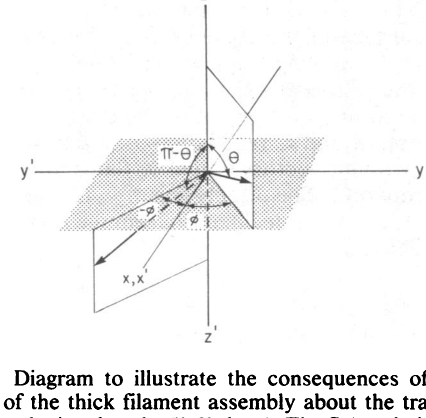

FIG. 2. Diagram to illustrate the consequences of the helical symmetry of the thick filament assembly about the transverse "*M* plane" (here depicted as the *X–Y* plane). The S-1 moieties of myosin molecules are in helical arrays about the *Z* and *Z'* axes. To an observer at 0,0 the sense of the "upper" helix must look the same as the sense of the "lower" helix, so vectors in the *X–Y–Z* and *X'–Y'–Z'* systems must have the same coordinates in their respective systems. But, if conjugate vectors are both viewed in the upper system, then angles θ and ϕ for an upper vector correspond to $\pi - \theta$ and $-\phi$ for a lower vector. Angle ψ (not shown) is the same for both vectors.

*The angle μ is wavelength dependent but will be otherwise regarded as a constant in this treatment.

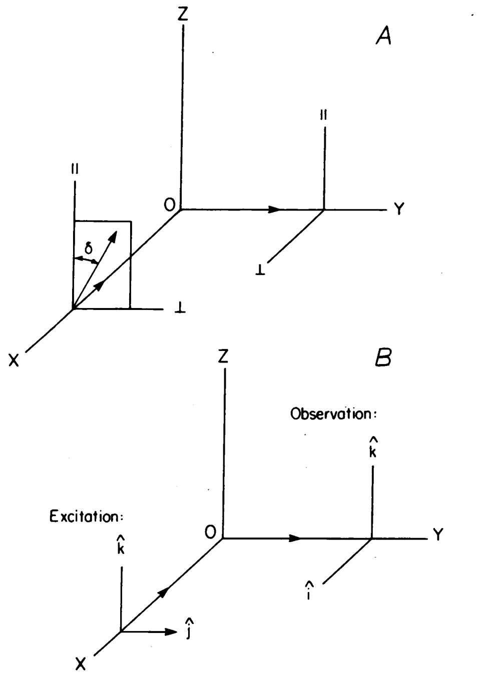

FIG. 3. Illustrations of the experimental layouts analyzed in this paper. (*A*) In the dichroism experiment the unit vector for excitation is (0, cos δ, sin δ). For each choice of δ, all three ($\hat{i}, \hat{j}, \hat{k}$) components of the emission are recorded and summed. Since (on energy balance grounds) the total emission is proportional to the total absorption, this experiment gives in effect absorption as a function of δ (hence the name "dichroism"). (*B*) The fluorescence polarization experiment consists in observing a directional emission intensity in four arrangements: (excitation direction, emission direction) necessary and sufficient to extract the desired information. The ones shown here, ($\hat{j}, \hat{i}$), ($\hat{j}, \hat{k}$), ($\hat{k}, \hat{i}$), and ($\hat{k}, \hat{k}$), commonly termed ($\perp, \perp$), ($\perp, \parallel$), ($\parallel, \perp$), and ($\parallel, \parallel$), are the most practical to use (see text).

each be $\hat{i}$, $\hat{j}$, or $\hat{k}$. For a general anisotropic sample, five of the nine intensities must be measured in order to deduce the orientation of the sample (to find the components of $\hat{v}_a$ and $\hat{v}_e$). A fiber, however, is commonly regarded as circularly symmetrical in the X–Y plane (see below). For such an object certain arrangements are equivalent ($\hat{i}, \hat{j}$ and $\hat{j}, \hat{i}$; $\hat{k}, \hat{j}$ and $\hat{k}, \hat{i}$; $\hat{i}, \hat{k}$ and $\hat{j}, \hat{k}$), and only four intensities have to be measured—let us say one from each equivalent pair ($\hat{j}, \hat{i}$; $\hat{k}, \hat{i}$; $\hat{j}, \hat{k}$) plus one more. For the fourth we choose $\hat{k}, \hat{k}$. The other two arrangements that could have been chosen ($\hat{i}, \hat{i}$ and $\hat{j}, \hat{j}$) are experimentally awkward in that they require either excitation and emission along the same axis (an arrangement that invites scattering errors) or using the long axis of the fiber for light transmission (which is very difficult). If we substitute from Eqs. **7** and **8** into $(\hat{p}\cdot\hat{v}_a)^2(\hat{v}_e\cdot\hat{q})^2$, for each of the four practical arrangements, we get individual fluorophore contributions of the form

$$\begin{aligned}\mathcal{I} \propto\ & [(A_tT_p)^2 + (A_rR_p)^2 + (A_aa_p)^2]\,[(E_tT_q)^2 + (E_rR_q)^2 \\ & + (E_aa_q)^2] + 2\,\{(A_tT_p)(A_rR_p)[(E_tT_q)^2 + (E_rR_q)^2 + (E_aa_q)^2] \\ & + (E_tT_q)(E_rR_q)[(A_tT_p)^2 + (A_rR_p)^2 + (A_aa_p)^2]\} \\ & + 4\,\{(A_tT_p)(A_rR_p)(E_tT_q)(E_rR_q) + [(A_tT_p)(A_aa_p) \\ & + (A_rR_p)(A_aa_p)][(E_tT_q)(E_aa_q) + (E_rR_q)(E_aa_q)]\}. \qquad \textbf{[11]}\end{aligned}$$

Upon substitution of the explicit expressions for the *A*s, *E*s, *T*s, *R*s, and *a*s, Eq. **12** generates $\mathcal{I}$ for each of the four arrangements, which we now write in the more customary notation ($\parallel\parallel$, $\parallel\perp$, $\perp\parallel$, $\perp\perp$ instead of $\hat{k}\hat{k}$, $\hat{k}\hat{i}$, $\hat{j}\hat{k}$, $\hat{j}\hat{i}$, respectively),

$$\mathcal{I}_{\parallel\parallel} \propto H^{(1)}_{\parallel\parallel} \sin^4\theta + H^{(2)}_{\parallel\parallel} \sin^2\theta \cos^2\theta + H^{(3)}_{\parallel\parallel} \cos^4\theta, \qquad \textbf{[12a]}$$

$$\begin{aligned}\mathcal{I}_{\parallel\perp} \propto\ & H^{(1)}_{\parallel\perp} \sin^4\theta \cos^2\theta + H^{(2)}_{\parallel\perp} \sin^2\theta \sin^2\phi \\ & + H^{(3)}_{\parallel\perp} \sin^2\theta \cos\theta \sin\phi \cos\phi \\ & + H^{(4)}_{\parallel\perp} \sin^2\theta \cos^2\theta \cos^2\phi + H^{(5)}_{\parallel\perp} \cos^2\theta \sin^2\phi \\ & + H^{(6)}_{\parallel\perp} \cos^3\theta \sin\phi \cos\phi + H^{(7)}_{\parallel\perp} \cos^4\theta \cos^2\phi, \qquad \textbf{[12b]}\end{aligned}$$

$$\begin{aligned}\mathcal{I}_{\perp\parallel} \propto\ & H^{(1)}_{\perp\parallel} \sin^4\theta \sin^2\phi + H^{(2)}_{\perp\parallel} \sin^2\theta \cos\phi \\ & + H^{(3)}_{\perp\parallel} \sin^2\theta \cos\theta \sin\phi \cos\phi \\ & + H^{(4)}_{\perp\parallel} \sin^2\theta \cos^2\theta \sin^2\phi + H^{(5)}_{\perp\parallel} \cos^2\theta \cos^2\phi \\ & + H^{(6)}_{\perp\parallel} \cos^3\theta \sin\phi \cos\phi + H^{(7)}_{\perp\parallel} \cos^4\theta \cos^2\phi, \qquad \textbf{[12c]}\end{aligned}$$

$$\begin{aligned}\mathcal{I}_{\perp\perp} \propto\ & H^{(1)}_{\perp\perp} \sin^2\phi \cos^2\phi + H^{(2)}_{\perp\perp} \sin^4\theta \sin^2\phi \cos^2\phi \\ & + H^{(3)}_{\perp\perp} \sin^2\theta \sin^4\phi + H^{(4)}_{\perp\perp} \sin^2\theta \sin^2\phi \cos^2\phi \\ & + H^{(5)}_{\perp\perp} \sin^2\theta \cos^4\phi + H^{(6)}_{\perp\perp} \sin^2\theta \cos\theta \sin^3\phi \cos\phi \\ & + H^{(7)}_{\perp\perp} \sin^2\theta \cos\theta \sin\phi \cos^3\phi \\ & + H^{(8)}_{\perp\perp} \sin^2\theta \cos^2\theta \sin^2\phi \cos^2\phi \\ & + H^{(9)}_{\perp\perp} \cos\theta \sin^3\phi \cos\phi + H^{(10)}_{\perp\perp} \cos\theta \sin\phi \cos\phi \\ & + H^{(11)}_{\perp\perp} \cos^2\theta \sin^4\phi + H^{(12)}_{\perp\perp} \cos^2\theta \sin^2\phi \cos^2\phi \\ & + H^{(13)}_{\perp\perp} \cos^2\theta \cos^4\phi + H^{(14)}_{\perp\perp} \cos^3\theta \sin^3\phi \cos\phi \\ & + H^{(15)}_{\perp\perp} \cos^3\theta \sin\phi \cos^3\phi \\ & + H^{(16)}_{\perp\perp} \cos^4\theta \sin^2\phi \cos^2\phi. \qquad \textbf{[12d]}\end{aligned}$$

The *H*s (see *Appendix*) are functions of dipole position in the cross-bridge (*A*s and *E*s) and of the angle, ψ. Again, the next step is to sum the contributions from *N* fluorophores. These *N* fluorophores will all make contributions of the same form (one of the four forms above, depending on the experimental arrangement) but with possibly different values of the angles ξ, η, ζ, θ, ϕ, and ψ.

What angles are going to characterize the cross-bridge orientations and the orientations of the fluorophores embedded in the cross-bridges is decided by physical information (or conjecture), but how the calculated observable intensity is obtained once the decision is made can be illustrated here. Suppose, for simplicity, that in the processes to be studied the fluorophores are going to retain their orientations in the cross-bridges, then $\mathcal{I}_{tot}$ in Eq. **10**, or one of the $\mathcal{I}$s in Eqs. **12**, can be written as $\mathcal{I}$ (constants, θ, ϕ, ψ)—i.e., dependent only on cross-bridge orientation. We can invent a function that tells for each choice of θ, ϕ, and ψ what *fraction* of the cross-bridges have their angles between θ and $\theta + d\theta$, ϕ and $d\phi$, and ψ and $d\psi$. Integration of such a function over the allowable ranges of the three angles must give unity. Because the three angles are independent of one another, the function must be separable—i.e., expressible as $\Theta(\theta)\Phi(\phi)\Psi(\psi)$. Such a function has the characteristics of a probability distribution. Therefore (if we neglect interference effects), the summated—i.e., the observed—intensity is *N* times the "mathematical expectation" of $\mathcal{I}$:

$$I \propto N \iiint \Theta\ \Phi\ \Psi\ \mathcal{I}\ (\text{constants}, \theta, \phi, \psi) \sin\theta\, d\phi\, d\psi\, d\theta. \qquad \textbf{[13]}$$

Eqs. **10** and **13**, and the *Appendix*, show that $\mathcal{I}$ itself is the sum of terms each of which is separable, and the factors are thus individually subject to the expectation operator. It is for this reason that each I/N is expressible in terms of simple averages. There is experimental justification for assuming that thick filaments are circularly symmetric in the transverse plane (for example, see ref. 8). Tregear and Mendelson (9) advanced the further argument that when whole fibers with unavoidable imperfections are viewed, Φ is not only symmetric but constant. This assumption greatly simplifies the integrand in Eq. **13**, for the expectation operator converts the ϕ-containing factors in Eq. **10** or in Eqs. **12** either into zero or into simple fractional coefficients; the price, however, is loss of information about this important coordinate. There is much less rationale for choosing either Θ or Ψ. Choosing them both to be delta functions, $\Theta(\theta - \theta_0)$ and $\Psi(\psi - \psi_0)$, is simplest in appearance. In this "model" the distribution function would then be $(\text{const}/2\pi)\ \Theta(\theta - \theta_0)\ \Psi(\psi - \psi_0)$. If we believe that the cross-bridge can assume a declination anywhere in the range θ_a to θ_b (midpoint, θ_m) with equal likelihood, then the distribution function would be $(\text{const}/2\pi)\ ((\theta_b - \theta_a)\sin\theta_m)\Psi$, provided the band is narrow.

In analysis of the dichroism experiment the assumption that the distribution function is $(\text{const}/2\pi)\ \Theta(\theta - \theta_0)\ \Psi(\psi - \psi_0)$ leads to the elimination of the coefficient of $\sin 2\delta$ in Eq. **10**, and we find

$$I_{tot} \propto N[F \sin^2\delta + G \cos^2\delta], \qquad [14]$$

where

$$F = (1/2)[A_t^2(\cos^2\psi_0 + \cos^2\theta_0 \sin^2\psi_0) + A_r^2(\cos^2\theta_0 \cos^2\psi_0 + \sin^2\psi_0) + A_a^2\sin^2\theta_0 + 2A_tA_r\,(\cos\theta_0 \sin\psi_0 - \cos^2\theta_0 \sin\psi_0 \cos\psi_0)]$$

and

$$G = [A_t^2\sin^2\theta_0 \sin^2\psi_0 + A_r^2\sin^2\theta_0 \cos^2\psi_0 + A_a^2\cos^2\theta_0 - 2A_tA_r\sin^2\theta_0 \sin\Psi_0 \cos\Psi_0].$$

Eq. **14** is of the form used by Borejdo *et al.* (6). Exactly their equation is obtained if the absorption dipole of the fluorophore is parallel to the principal axis of the cross-bridge [as it should be, of course, because Borejdo *et al.* (6) derived their equation in terms of fluorophores only]. In that case ψ becomes irrelevant, $A_a = 1$ and $A_t = A_r = 0$, so

$$I_{tot} \propto N[(1/2) \sin^2\theta_0 \sin^2\delta + \cos^2\theta_0 \cos^2\delta]. \qquad [14']$$

If Eq. **14′** is assumed to hold, then the particular circumstance in which I_{tot} is insensitive to δ occurs only for a "magic" value of θ_0 such that $(\sin^2\theta_0)/2 = \cos^2\theta_0$. In the more general case of Eq. **14** the circumstance $F = G$ can be obtained in a variety of ways—for example, when ψ is randomized and $A_a^2 = (A_t^2 + A_r^2)/2$.

As already mentioned, the analysis of fluorescence polarization is also greatly simplified by the assumption Φ = constant. This can be seen by inspection of Eqs. **12b–12d**. Terms containing the functions of ϕ raised to odd powers vanish as a result of calculating the expectation (Eq. **13**), $\sin^2\phi$ or $\cos^2\phi$ becomes 1/2, $\sin^4\phi$ or $\cos^4\phi$ become 3/8, and $\sin^2\phi \cos^2\phi$ becomes 1/8. It does not seem advisable in this paper to work out the Is for particular "models" (choices of Θ and Ψ) because of the inherent arbitrariness in the choices.

In actual practice the multiplicative constant ("physical factor") that would convert the proportionality of Eq. **13** into a true equation is very hard to measure, and recourse is sought in using ratios. Among 4 Is, 6 nontrivial ratios can be formed and experimentally measured. If we view these relations as equations, we have 6 equations in 4 variables; however, since only ratios are involved, there are $4 - 1 = 3$ variables. If we have 6 equations in 3 variables, the variables are overdetermined by 3; i.e., only 3 experimental ratios have to be measured. These are the 3 "polarization functions" set forth by Tregear and Mendelson (9)—e.g., $P_{\parallel} \equiv (I_{\parallel\parallel} - I_{\parallel\perp})/(I_{\parallel\parallel} + I_{\parallel\perp})$, $P_{\perp} \equiv (I_{\perp\parallel} - I_{\perp\perp})/(I_{\perp\parallel} + I_{\perp\perp})$, and, as Q, either $(I_{\parallel\parallel} - I_{\perp\perp})/(I_{\parallel\parallel} + I_{\perp\perp})$ or $(I_{\parallel\perp} - I_{\perp\parallel})/(I_{\parallel\perp} + I_{\perp\parallel})$. Previous analysts have been well aware, of course, that changes in these Ps can result as well from reorientation of the fluorophores in the cross-bridges (cross-bridges constant) as from reorientation of the cross-bridges (fluorophores constant), but the calculation of the first effect is made easy by the present results, and we close with an illustration. Suppose that in a physiological process $P_{\parallel}$ is observed to change; how large a change in $P_{\parallel}$ could be expected from *fluorophore* reorientation (say from coincident dipoles parallel to the S-1 axis to coincident dipoles perpendicular to the axis) even when cross-bridge orientation remains constant? Assuming for simplicity that $\mu = 0$, this change in $P_{\parallel}$ can be estimated by supposing that in the initial state of the process, $A_a = E_a = 1$ (all other components are zero), while in the final state $A_a = E_a = 0$ (and all other components are $\sqrt{2}/2$). Even though cross-bridge orientation (angles θ, ϕ, and ψ) remains constant, Eqs. **12** show that $P_{\parallel}$ changes significantly, from $(2 - 3\sin^2\theta)/(2 - \sin^2\theta)$ to $[2 - 3(1 - 2\sin\psi\cos\psi)\sin^2\theta]/[2 - (1 - 2\sin\psi\cos\psi)\sin^2\theta - 4]$, as the fluorophore dipoles rotate through 90° within the S-1.

APPENDIX

The functions (of the fluorophore dipole components relative to the cross-bridge, and of the cross-bridge "torsional" angle, ψ) are to be appropriately substituted into Eqs. **12** in order to get the complete expressions for the four observable polarized intensities produced by a single fluorophore. The symbols s and c have been used as shorthand for $\sin\psi$ and $\cos\psi$, respectively.

$$H_{\parallel\parallel}^{(1)} = (A_tE_ts^2 + A_rE_rc^2)^2 + (A_tE_r + A_rE_t)^2s^2c^2 - [A_tA_r(E_t^2s^2 + E_r^2c^2) + E_tE_r(A_t^2s^2 + A_r^2c^2)]sc$$

$$H_{\parallel\parallel}^{(2)} = A_a^2(E_ts - E_rc)^2 + E_a^2(A_ts - A_rc)^2 + 4A_aE_a[(A_tE_ts^2 + A_rE_rc^2) - (A_tE_r + A_rE_t)sc]$$

$$H_{\parallel\parallel}^{(3)} = A_a^2E_a^2$$

$$H_{\parallel\perp}^{(1)} = E_a^2(A_ts - A_rc)^2$$

$$H_{\parallel\perp}^{(2)} = (A_tE_rs^2 - A_rE_tc^2)^2 + (A_tE_t - A_tE_r)^2s^2c^2 - [A_tA_r(E_r^2s^2 + E_t^2c^2) - E_tE_r(A_t^2s^2 + A_r^2c^2)]sc$$

$$H_{\parallel\perp}^{(3)} = 2[A_tA_r(E_t^2 - E_r^2) + E_tE_r(A_t^2 - A_t^2)]s^2c^2 + 2[2A_tE_tA_rE_r(s^2 - c^2) + (A_t^2s^2 + A_r^2c^2)(E_r^2 - E_t^2)]sc - 2E_tE_r(A_t^2s^4 - A_r^2c^4) + 4A_aE_a[A_tE_rs^2 + (A_tE_t - A_rE_r)sc - A_rE_tc^2]$$

$$H^{(4)}_{\parallel\perp} = [(A_tE_ts^2 + A_rE_rc^2) - (A_tE_r + A_rE_t)sc]^2 + A_aE_a[A_aE_a - 4(\langle A_tE_ts^2 + A_rE_rc^2\rangle - \langle A_tE_r + A_rE_t\rangle sc)]$$

$$H^{(5)}_{\parallel\perp} = A_a^2(E_rs + E_tc)^2$$

$$H^{(6)}_{\parallel\perp} = 2A_a^2[(E_r^2 - E_t^2)sc - E_tE_r(s^2 - c^2)]$$

$$H^{(7)}_{\parallel\perp} = A_a^2(E_ts - E_rc)^2$$

$$H^{(1)}_{\perp\parallel} = A_a^2(E_ts - E_rc)^2$$

$$H^{(2)}_{\perp\parallel} = (A_rE_ts^2 - A_tE_rc^2)^2 + (A_tE_t - A_rE_r)^2s^2c^2 + 2[A_tA_r(E_t^2s^2 + E_r^2c^2) - E_tE_r(A_r^2s^2 + A_t^2c^2)]sc$$

$$H^{(3)}_{\perp\parallel} = 2[2E_tE_r(A_t^2 - A_r^2) + A_tA_r(E_r^2 - E_t^2)]s^2c^2 + 2[-2A_tE_tA_rE_r(s^2 - c^2) + (E_t^2s^2 + E_r^2c^2)(A_t^2 - A_r^2)]sc + 2A_tA_r(E_t^2s^4 - E_r^2c^4) + 4A_aE_a[(A_tE_rc^2 - A_rE_ts^2) + (A_rE_r - A_tE_t)sc]$$

$$H^{(4)}_{\perp\parallel} = [(A_tE_ts^2 + A_rE_rc^2) - (A_tE_r + A_rE_t)sc]^2 + A_aE_a[A_aE_a - 4(\langle A_tE_ts^2 + A_rE_rc^2\rangle - \langle A_tE_r + A_rE_t\rangle sc)]$$

$$H^{(5)}_{\perp\parallel} = E_a^2(A_rs + A_tc)^2$$

$$H^{(6)}_{\perp\parallel} = 2E_a^2[(A_t^2 - A_r^2)sc + A_tA_r(s^2 - c^2)]$$

$$H^{(7)}_{\perp\parallel} = E_a^2(A_ts - A_rc)^2$$

$$H^{(1)}_{\perp\perp} = (A_rE_rs^2 + A_tE_tc^2)^2 + (A_tE_r + A_rE_t)^2s^2c^2 + 2[A_tA_r(E_r^2s^2 + E_t^2c^2) + E_tE_r(A_r^2s^2 + A_t^2c^2)]sc$$

$$H^{(2)}_{\perp\perp} = A_a^2E_a^2$$

$$H^{(3)}_{\perp\perp} = A_a^2(E_rs + E_tc)^2$$

$$H^{(4)}_{\perp\perp} = -4A_aE_a[A_rE_rs^2 + (A_tE_r + A_rE_t)sc + A_tE_tc^2]$$

$$H^{(5)}_{\perp\perp} = E_a^2(A_rs + A_tc)^2$$

$$H^{(6)}_{\perp\perp} \quad + -2A_a^2[E_tE_r(s^2 - c^2) + (E_t^2 - E_r^2)sc] - 4A_aE_a[(A_tE_rs^2 - A_rE_tc^2) + (A_tE_t - A_rE_r)sc]$$

$$H^{(7)}_{\perp\perp} = 2E_a^2[A_tA_r(s^2 - c^2) + (A_t^2 - A_r^2)sc] + 4A_aE_a[(A_rE_ts^2 - A_tE_rc^2) + (A_tE_t - A_rE_r)sc]$$

$$H^{(8)}_{\perp\perp} = A_a^2(E_ts - E_rc)^2 + E_a^2(A_ts - A_rc)^2 + 4A_aE_a[(A_tE_ts^2 - A_rE_rc^2) - (A_tE_r + A_rE_t)sc]$$

$$H^{(9)}_{\perp\perp} = 2A_tA_r(E_r^2s^4 - E_t^2c^4) + 2[A_tA_r(E_t^2 - E_r^2) + 2E_tE_r(A_t^2 - A_r^2)]s^2c^2 + 2[(A_t^2 - A_r^2)(E_r^2s^2 + E_t^2c^2) + 2A_tE_tA_rE_r(s^2 - c^2)]sc$$

$$H^{(10)}_{\perp\perp} = -2E_tE_r(A_r^2s^4 - A_t^2c^4) - 2[E_tE_r(A_t^2 - A_r^2) + 2A_tA_r(E_t^2 - E_r^2)]s^2c^2 - 2[(E_t^2 - E_r^2)(A_r^2s^2 + A_t^2c^2) + 2A_tE_tA_rE_r(s^2 - c^2)]sc$$

$$H^{(11)}_{\perp\perp} = (A_tE_rs^2 - A_rE_tc^2)^2 + (A_tE_t - A_rE_r)^2s^2c^2 + 2[-A_tA_r(E_r^2s^2 + E_t^2c^2) + E_tE_r(A_t^2s^2 + A_r^2c^2)]sc$$

$$H^{(12)}_{\perp\perp} = 4\{(A_r^2 - A_t^2)(E_t^2 - E_r^2)s^2c^2 - [A_tA_r(E_t^2 - E_r^2) + E_tE_r(A_t^2 - A_r^2)](s^2 - c^2)sc - A_tA_rE_tE_r(s^2 - c^2)^2\}$$

$$H^{(13)}_{\perp\perp} = (A_tE_rc^2 + A_rE_ts^2) + (A_tE_t - A_rE_r)^2s^2c^2 + 2[A_tA_r(E_t^2s^2 + E_r^2c^2) - E_tE_r(A_r^2s^2 + A_t^2c^2)]sc$$

$$H^{(14)}_{\perp\perp} = -2E_tE_r(A_t^2s^4 - A_r^2c^4) + 2[2A_tA_r(E_t^2 - E_r^2) + E_tE_r(A_t^2 - A_r^2)]s^2c^2 - 2[(E_t^2 - E_r^2)(A_t^2s^2 + A_r^2c^2) - 2A_tE_tA_rE_r(s^2 - c^2)]sc$$

$$H^{(15)}_{\perp\perp} = 2A_tA_r(E_t^2s^4 - E_r^2c^4) - 2[A_tA_r(E_t^2 - E_r^2) + 2E_tE_r(A_t^2 - A_r^2)]s^2c^2 + 2[(A_t^2 - A_r^2)(E_t^2s^2 + E_r^2c^2) - 2A_tE_tA_rE_r(s^2 - c^2)]sc$$

$$H^{(16)}_{\perp\perp} = (A_tE_ts^2 + A_rE_rc^2) + (A_tE_r + A_rE_t)^2s^2c^2 - 2[A_tA_r(E_t^2s^2 + E_r^2c^2) + E_tE_r(A_t^2s^2 + A_r^2c^2)]sc.$$

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