INTRINSIC BIREFRINGENCE OF
GLYCERINATED MYOFIBRILS

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ABSTRACT

Patterns of intrinsic birefringence were revealed in formalin-fixed, glycerinated myofibrils from rabbit striated muscle, by perfusing them with solvents of refractive index near to that of protein, about 1.570. The patterns differ substantially from those obtained in physiological salt solutions, due to the elimination of edge- and form birefringence. Analysis of myofibrils at various stages of shortening has produced results fully consistent with the sliding filament theory of contraction. On a weight basis, the intrinsic birefringence of thick-filament protein is about 2.4 times that of thin-filament protein. Nonadditivity of thick- and thin-filament birefringence in the overlap regions of A bands may indicate an alteration of macromolecular structure due to interaction between the two types of filaments.

INTRODUCTION

Some of the earliest microscopical observations of muscle structure were made using polarized light (and were reviewed by A. F. Huxley, 1957). In the 1930s, the separate contributions of form birefringence (due to the alignment of submicroscopic myofilaments) and intrinsic birefringence (due to crystalline organization within molecules and within myofilaments) were distinguished by Noll and Weber (1934).

This paper reports detailed measurements of the intrinsic birefringence of myofibrillar bands, using the "rectified" microscope lenses of Inoue and Hyde (1937). Patterns of birefringence are shown as a function of sarcomere length and solvent refractive index, and ratios of retardation are calculated for various pairs of sarcomeric bands. The results are as expected according to the sliding filament hypothesis (H. E. Huxley, 1953), except for nonadditivity of thick- and thin-filament retardations in their region of overlap.

Observations of intrinsic birefringence are made after immersing the myofibrils in a solvent of refractive index near to that of myofibrillar protein. O-toluidine (n = 1.570) was the reagent chosen for this purpose; it minimized the contrast of myofibrils observed by dark-field and phase-contrast optics.

MATERIALS AND METHODS

Bundles of glycerinated muscle fibers were prepared from rabbit psoas muscle by the method of A. Szent-Györgyi (1951). To obtain a variety of sarcomere lengths, some bundles were stretched, and others were allowed to relax to equilibrium length before being tied onto wooden sticks for glycerination. Myofibrils were obtained by homogenizing short lengths of bundles in a Waring Blender; they were then washed and centrifuged several times in low salt solution (0.04 M KCl, 0.001 M MgCl₂, 0.01 M phosphate, pH 6.5). They were stored at −20°C as a concentrated suspension in low salt:glycerol (1:1).
All exchange of solvents was carried out under the microscope, in a chamber constructed between a large and a small cover slip separated along two edges by 70 µm-thick Teflon spacer strips (Arthur H. Thomas Co., Philadelphia, Pa.). Solvents could then be introduced dropwise at one of the remaining edges, and drawn through the chamber by filter paper wicks. I observed fibrils which were attached to one of the glass surfaces.

Optical components for birefringence measurements included rectified objective and condenser lenses (NA = 1.25) for oil immersion, and a rotating mica compensator (maximum retardation 27.3 nm) (Inoué, 1961). Using the green line of a mercury arc lamp, the extinction factor of the whole system ranged from day to day between 1 and 3 X 10^4 at NA 1.25.

In order to immerse myofibrils in o-toluidine, aqueous salt solution (containing 10% formalin) was first removed by perfusing the chamber with increasing concentrations of dimethyl sulfoxide (DMSO). In some experiments the myofibrils were eventually returned to their original aqueous environment by a reversal of this sequence.

Micrographs were exposed for 80 sec on Adox KB-17 film (no longer manufactured), and developed to a gamma of 0.9-1.0, linear over 2 units of optical density. They were analyzed directly with a microdensitometer (Joyce-Loebl, Mark III), using a fixed exit slit of length less than the projected width of the myofibril. The pen deflection was a measure of the optical density of the film, and hence of log (intensity) and log (retardation). The quantity actually determined in these experiments was retardation (r) rather than birefringence (BR), since the latter quantity depends upon the thickness (d) of the specimen, \[ \Gamma = BR \times d \], and I had no satisfactory way of measuring thickness. An assumption made throughout the investigation is that myofibrillar thickness remains constant through all the bands of a sarcomere. Densitometric measurements of myofibrillar widths in the various bands would seem to support this assumption.

The technique used to determine the relative retardations of sarcomeric bands has been described by Inoué and Sato (1966). Exposures were made of each myofibril, oriented at 45° to the polarizer-analyzer axes, at three to eight settings of the compensator on each side of the background extinction position. Then, after densitometric scanning of the photographic negatives, the averaged optical densities corresponding to each sarcomeric band were plotted against compensator angle, and a graph such as Fig. 6 was constructed. The minimum point on each curve of Fig. 6 corresponds to the extinction compensation for a particular sarcomeric band. The horizontal displacements (θ) of these minima, measured from the background extinction angle, are a function of the retardation, according to the equation

\[ \Gamma = \Gamma_{comp} \sin \theta, \quad (1) \]

where \( \Gamma_{comp} \) is the retardation of the compensator (27.3 nm).

This procedure for determining retardation avoids many of the stringent requirements of Allen and Nakajima (1965) for the “two-exposure” method of analysis: the gamma-value of the film need not be constant from batch to batch, the extinction factor of the microscope is not critical, and photographic exposures need not fall in the linear region of the gamma-curve.

**RESULTS**

Fig. 1 b–i shows the appearance, in polarized light, of a myofibril perfused with a series of solvents. The phase-contrast image (Fig. 1 a) includes A bands, H bands, I bands, and Z lines typical of slightly stretched sarcomeres, in this case 2.40 µm long. Using polarized light, a complex pattern is seen in an aqueous solvent (Fig. 1 b–c, showing opposite compensations), due presumably to sharp discontinuities in refractive index at the edges of A bands, H bands, and Z lines. Edge birefringence (Inoué, 1959) is so prominent that it precludes the quantitative measurement of form birefringence. The pattern in Fig. 1 b–c is simplified by the introduction of solvents with higher refractive indices (Fig. 1 d–e, f–g) until, in Fig. 1 h–i, with o-toluidine, the pattern reduces to that expected from a simple overlap of strongly birefringent thick filaments and weakly birefringent thin filaments. In solvents of still higher refractive index the more complex pattern begins to reappear, but with reversed contrast.

To illustrate the effects of organic solvents, Fig. 2 shows a similar myofibril under phase-contrast optics. The successive immersing liquids are (a) low salt solution, (b) DMSO, (c) benzyl alcohol (n = 1.53, in which most of the contrast has been lost), (d) DMSO again, and (e) low salt solution again. O-toluidine was also perfused, but it rendered the myofibril invisible. Note that there
has been considerable lateral shrinkage upon introduction of organic solvents, and also longitudinal shrinkage to the extent of about 7%. Although the reintroduction of low salt solution produces the highly refractile appearance of Fig. 2 e, the birefringence pattern of such a myofibril is identical to that obtained before the introduction of nonaqueous solvents. This criterion for structural integrity has proven quite sensitive in other filamentous systems, such as the mitotic spindle (Kane and Forer, 1965). It may also be relevant to recall that α-helical protein structure is not disrupted by organic solvents such as ethanol.

Fig. 3 shows the patterns of intrinsic bire-
fringence in sarcomeres of successively decreasing length. The changes are in qualitative agreement with expectation according to the sliding filament theory. Birefringence is seen in the I band as well as the A band (with subtractive compensation: Fig. 3 a, c, e; also Fig. 1 f), but neither the Z line nor the M line is visible. An H band is visible at sarcomere lengths between 2.09 and 2.50 µm (Figs. 3 a-h). The increased birefringence toward the lateral edges (non-H band region) of each A band is interpreted as the result of overlap between thick and thin filaments. In shorter sarcomeres, between 1.44 and 1.74 µm, a more highly birefringent zone appears in the center of each A band (Figs. 3 i-l), corresponding to the Cm band seen with phase-contrast optics. From the magnitude of the retardation in this band, as well as the way it spreads in shorter sarcomeres, I would propose that it results from the overlap of thin filaments from opposite ends of a sarcomere, crossing in the center of the A band. A few fibrils were observed with sarcomere lengths of about 1.25 µm (Figs. 3 m–q), much less than the length of an A band. They show generally reduced birefringence, and a loss of sharpness at edges, especially at the ends of sarcomeres. (It is possible to distinguish between the middle and the end of a sarcomere by referring to the end of the myofibril, which always breaks at a Z line.) There is no evidence that thick filaments can interdigitate with their neighbors in adjacent sarcomeres, in contrast to the findings in some invertebrate muscles (Aronson, 1963; Hoyle et al., 1965).

There is evidence, on the other hand, for the buckling of filaments near the ends of these short sarcomeres: I found a distinctive dependence of the pattern upon the orientation of the myofibril with respect to the polarizer-analyzer axes. By turning the microscope stage so as to put the myofibril at a narrow, 15°, angle to the polarizer axis, a skew pattern appeared in each sarcomere (Figs. 3 m, e–p). To explain such a pattern, one could suppose that portions of the myofilaments, near the ends of sarcomeres, become buckled and oriented at a skew angle to the long axis of the myofibril. At a narrow stage angle, skew myofilaments in opposite quadrants of each sarcomere would make an angle closer to 45° with respect to the polarizer-analyzer axes of the microscope, and would thus show up more brightly in polarized light. Myofibrils with longer sarcomeres do not show this skew effect at intermediate stage angles.

Quantitative information on the structure of myofibrils was obtained by densitometric scanning of the photographic negatives. To reduce the effects of photographic and electronic "grain," the densitometric tracings, such as Fig. 4, were manually averaged over a length of three or more sarcomeres, at intervals corresponding to about 0.125 µm along the length of a myofibril. To correct for the effects of absorption and light scatter (Allen and Nakajima, 1965), averages obtained from photographs with opposite compensator settings were then themselves averaged to yield corrected distributions of intrinsic birefringence in myofibrils. Five such averages, each showing two adjacent sarcomeres, are presented in Fig. 5, along with indications of the lengths expected for thick filaments (thick bars: 1.60 µm) and thin filaments (thin bars: 1.125 µm) according to Page and H. E. Huxley (1963).

It will be seen at once that the filament lengths revealed by polarization microscopy are shorter than those found by Page and Huxley. I obtained average A- and I filament lengths of 1.44 and 1.07 µm, respectively, with some variability, from myofibrils both with H- and with Cm bands. It has already been shown that sarcomere shortening can occur upon immersion of fibrils in DMSO (Fig. 2 a–b); furthermore, Page and Huxley suggest that fixatives can cause shortening of in-
individual filaments in the range of 1–7%, and that thin filaments shorten more than thick filaments. It is also possible that birefringence may be reduced at the ends of filaments.

Allowing then for filament shrinkage and/or end effects, Fig. 5 shows how the birefringence of each sarcomeric band can be accounted for. The shortest sarcomeres shown (Fig. 5 e; 1.40 µm) appear to have extraordinarily short A bands. This fibril showed no evidence of filament buckling, so I can only presume that macromolecular structure had been disrupted where thick filaments from adjacent sarcomeres had come into contact.

Many densitometric scans of myofibrils (such as Fig. 4), at a series of compensator settings, were used to calculate the relative retardations of the I-, H-, A_lu-, and C_m bands. (A_lu refers to the lateral portion of an A band, in which thick and thin filaments overlap.) The treatment of data is shown in Fig. 6 (for the fibril illustrated in Fig. 3 g–h). The minimum point of each parabolic curve represents the compensation required to produce extinction, and is related to retardation (relative to background) according to equation 1.

An important factor in interpreting these calculations is the decision as to whether a band has been fully resolved by the microscope. The theoretical limitation of 0.2 µm applies to the resolution of two point-sources of light, and is not applicable to the resolution of a thin sharp edge. H. E. Huxley and Hanson (1957) showed empirically that resolution of myofibrillar bands by interference microscopy required a distance of 0.3 µm on each side of the edge. Similarly, I measured the spreading of clearly resolved edges in densitometric scans such as Fig. 4, and found that the distance required on each side of an edge depended upon the difference in density at the edge. In the case of the A-I boundary, 0.25 µm was required on either side for resolution. Less contrasty boundaries, including the edges of the H- and C_m bands, required only 0.125 µm on either side.

Analysis of myofibrils with appropriate sarcomere lengths and appropriately resolved band edges yielded the following retardation ratios

| Sarcomere Lengths | Retardation Ratios |
|-------------------|--------------------|
| 2.50 µm           | C_m: I, H, A_lu, C_m |
| 2.40 µm           |                     |
| 2.23 µm           |                     |
| 2.09 µm           |                     |
| 1.74 µm           |                     |
| 1.44 µm           |                     |
| 1.25 µm           |                     |
| 1.18 µm           |                     |

Figure 3 Rabbit myofibrils, observed with the polarization microscope and in the solvent o-toluidine.

All are formalin-fixed except a–b. All fibrils were oriented at a stage angle of 45° to the polarizer axis, except m and o–p, which were oriented at 15°. Bright myofibrils have been additively compensated; dark myofibrils have been subtractively compensated. The sarcomere lengths are: (a–b) 2.50 µm, (c–d) 2.40 µm, (e–f) 2.23 µm, (g–h) 2.09 µm, (i–j) 1.74 µm, (k–l) 1.44 µm, (m–n) 1.25 µm, (o–p) 1.18 µm. × 8450.
FIGURE 4 Densitometric scans of the fibril shown in Fig. 3 i (lower tracing, upper baseline) and Fig. 3 j. The subtractively compensated tracing has been inverted. The baselines are scans of the background regions alongside the myofibrils.

(Their significance will be discussed in the next section. The number of fibrils examined is given in parentheses; the error is one standard deviation.)

(a) \( \frac{\Gamma_H}{\Gamma_T} = 4.5 \pm 0.8 \) (5 fibrils)
(b) \( \frac{(\Gamma_{A\text{lat}} - \Gamma_H)}{\Gamma_T} = 0.21 \pm 0.03 \) (5 fibrils)
(c) \( \frac{(\Gamma_{A\text{lat}} - \Gamma_I)}{\Gamma_{A\text{lat}}} = 0.05 \pm 0.01 \) (5 fibrils)
(d) \( \frac{(\Gamma_{A\text{lat}} - \Gamma_{A\text{lat}})}{\Gamma_{A\text{lat}}} = 0.10 \pm 0.06 \) (2 fibrils)

It is of interest to compare the data from myofibrils with that from dry threads of the proteins myosin and actin. The threads were prepared with circular cross-sections so that their diameters could be measured and their retardations converted to units of birefringence. Five myosin threads and five actin threads had the following birefringences (in units of refractive index):

Myosin: \( 10.1 \times 10^{-3} \pm 1.4 \)
Actin: \( 3.3 \times 10^{-3} \pm 0.3 \)

\( \frac{\text{BR}_{\text{myosin}}}{\text{BR}_{\text{actin}}} = 3.1 \pm 0.2 \)

DISCUSSION

What is the significance of the above ratios? Ratio a gives the relative birefringence of the thick and thin filaments. It indicates that the thick filaments have four to five times more retardation than the thin filaments. The ratio is much lower than that obtained by W. J. Schmidt (1934) from alcohol-fixed insect muscle fibers embedded in Canada balsam: Schmidt found the A bands to be 10 times more birefringent than the I bands. The fibers he used were quite thick (about 2.5 µm), and the refractive index of Canada balsam (1.51–1.52) may have allowed some form birefringence to influence his observations.

FIGURE 5 Representative manual averages of densitometric scans such as in Fig. 4. Two full sarcomeres are shown for each fibril. Both additively and subtractively compensated images were averaged to produce each pattern. Thick- and thin-filament length calibrations (1.60 and 1.125 µm, respectively) are drawn to scale under each pattern. The sarcomere lengths are: (a) 2.40 µm, (b) 2.15 µm, (c) 2.00 µm, (d) 1.74 µm, (e) 1.40 µm.
FIGURE 6 Illustrates method for obtaining the retardations of sarcomeric bands. The myofibril analyzed is that shown in Fig. 3 g–h, with sarcomere length 2.09 µm. Relative optical densities (in centimeters, measured relative to an unexposed piece of film) are obtained for the background (X), I band (●), H band (○), and A1alt band (△), from a series of densitometric scans such as in Fig. 4. The compensator angle corresponding to the minimum of each parabolic curve is estimated by folding the graph paper so as to superimpose the two sides of each parabola. The horizontal displacements (θ) of the minima, relative to background, are entered into equation 1 to calculate the retardations of each sarcomeric band.

To calculate the relative birefringence of A- to I filament protein, retardation ratio α must be converted to a weight basis. We can use data based on dry mass in the myofibril (obtained by interference microscopy), or on extractable protein (obtained by quantitative biochemical isolation procedures), or on filament cross-sectional areas (obtained by electron microscopy). The first method was used by H. E. Huxley and Hanson (1957), and gave a value of 1.90 ± 0.06 for the ratio of A- to I filament material per unit length of myofibril, depending upon the method of calculation. The same paper contains tabulated data from five biochemical investigations on glycercinated myofibrils, both washed and unwashed. Assuming that the protein residue after myosin extraction is localized uniformly in the I bands, these data yield a ratio of 1.88 ± 0.29. By the third method, if one assumes that cross-sectional diameters are 100 and 55 Å for the thick and thin filaments, respectively (Young, 1969), and that there are twice as many thin filaments as thick, the ratio of cross-sectional areas becomes 1.64. I would judge the latter estimate to be the least accurate, due to uncertainty in the interpretation of data from fixed and embedded material. Combining the first mass estimate with ratio α, then, one concludes that on a weight basis, A filaments have 2.4 ± 0.5 times more intrinsic birefringence than I filaments. (To obtain this number, divide [Γ_A = 4.5Γ_I] by [mass_A = 1.9 mass_I].)

The fact that A-filament protein has more birefringence than I-filament protein may reflect myosin's higher content of α-helical structure (56% compared with actin's 30%—A. G. Szent-Györgyi, 1960), or may reflect a difference in macromolecular packing within filaments. From the thread data (ratio b), myosin might be expected to have 3.1 times more birefringence than actin. Some possible explanations for the discrepancy include the question of molecular orientation in the glassy threads, and the effects of tropomyosin and other I-band proteins on the birefringence of thin filaments.

Ratio b compares the birefringence of thin filaments overlapping the A band with those in the I band. It suggests that some birefringence is lost in the region of overlap. Indeed, if the deficiency of birefringence below what would be expected on the basis of thick- and thin-filament additivity were due entirely to the thin filaments, then the deficiency would amount to almost 80%. Whichever type of filament is responsible, one can suggest that the deficiency is due either to normal interactions between thick and thin filaments in glycercinated muscle (such muscle is devoid of adenosine triphosphate [ATP] and hence may be considered to be in rigor—it is not stretchable), or to interactions mediated by the formaldehyde fixative so as to interfere with the normal structure of the filaments.

In this connection it is noteworthy that Eberstein and Rosenfalck (1963) detected a 9% decrease in total birefringence (including form- and edge components) in fresh contracting fibers isolated from frog muscles. Here, too, it is possible that interaction between filaments may have interfered with the structures responsible for intrinsic birefringence. I made no attempt to examine "relaxed" myofibrils, or to compare them with myofibrils in rigor; it may be possible to make

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such an investigation in the future, using the fixation methods of Reedy et al. (1965).

Ratios $c$ and $d$ compare the retardation of thin filaments in the $C_m$ region (of contracted sarcomeres) with that of thin filaments in the $A_{th}$-bands (of rest-length sarcomeres). It is interesting to find that $C_m$ thin filaments (ratio $d$) have twice the relative birefringence of $A_{th}$ thin filaments (ratio $c$). Perhaps once past the center of the $A$ band, thin filaments are released from interactions which would have reduced their birefringence. Of course the possible error associated with ratio $d$ is quite large. And one should remember that, in a living vertebrate, sarcomeres are not known to shorten to the point where $C_m$ bands are formed.

The results presented here are all qualitatively consistent with the sliding filament theory of muscle contraction. With respect to the currently more interesting question of what is happening in contracting myofibrils at the molecular level, these results suggest only that interactions between thick and thin filaments entail some loss of intrinsic birefringence to one or both structures. I have no way of evaluating, for example, Elliott's hypothesis (1967) that long-range interactions between thick and thin filaments may be fundamental in effecting contraction.

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