

OVERLAP OF THE BIREFRINGENT
COMPONENT OF ADJACENT A REGIONS
DURING THE INDUCED SHORTENING OF
FIBRILS TEASED FROM *DROSOPHILA* MUSCLE

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ABSTRACT

Fibrils from the indirect flight muscle of *Drosophila melanogaster* which have been teased into a solution containing 0.1 M KCl, 2 mM EDTA, 4 mM MgCl₂, and 2.5 mM ATP at pH 7.0 can be made to shorten to 10 per cent of their initial length by reducing the level of ATP at a pH of about 8 or by briefly treating the fibrils with trypsin before lowering the level of ATP. Fibrils shortened in either of these ways, when dehydrated and immersed in nitrobenzene, display a strong positively birefringent band at the level of the Z band. In the trypsin-treated fibrils the width of this Z band increases as the fibril shortens. The data obtained are in agreement with the view that the positively birefringent Z band results from the interdigitation of A filaments in adjacent sarcomeres. With shortening to about 35 per cent of the initial length, the cytological pattern suggests that the A filaments of alternate as well as of adjacent A regions interdigitate.

INTRODUCTION

During investigations on the *in vitro* responses of fibrils from the indirect flight muscle of *Drosophila*, it was noted that a fibril, under certain experimental treatments, could shorten well below its usual physiological range. Shortening below about 85 per cent of the initial length was accompanied by the formation of a birefringent band at the level of the Z band, and an isotropic I region was no longer present. The strength of birefringence of this "Z" band was considerably greater than that of the A region. Such a birefringent Z band has not been described previously.

METHODS

Fibrils for these experiments were teased from the indirect flight muscle of *Drosophila melanogaster* into a solution containing 0.1 M KCl, 5 mM KH₂PO₄, 4

mM MgCl₂, 2 mM EDTA, and 2.5 mM ATP¹ (disodium salt). The pH of this solution was adjusted to 7.0 with KOH. These fibrils were briefly washed by passing under the coverslip a similar solution which lacked EDTA.²

Fibrils prepared as described above were shortened by treatment with a solution of trypsin which contained ATP at a concentration below that causing elongation (1). The trypsin concentration was 0.01 mg/cc in 0.1 M KCl + 0.005 M KH₂PO₄ at pH 7. Mixing of the incoming trypsin solution with the ATP-containing solution in which the fibrils were mounted was frequently enough to cause strong shortening, but in other cases it was necessary to add a small amount of ATP to the trypsin. The usual method for causing fibrils to shorten at about

¹ ATP, adenosine triphosphate.

² EDTA, ethylenediaminetetraacetate.

pH 8 was to wash the initial preparation, which is at pH 7.0 and contains magnesium ions and ATP, with 0.1 M KCl containing 0.005 M K_2HPO_4 at a pH adjusted to 8.5. When the shortening was insufficient, it was often possible to wash with an ATP-containing solution and repeat the cycle to get further shortening. The extent of shortening with trypsin and with alkaline pH was variable but in some fibrils reached 10 per cent of the initial length.

The thickness of the fibrils in aqueous media (about 3 μ) and the optical properties of the Z region, which often mask changes in the birefringent components of the I region, could both be minimized by dehydrating the fibrils with acetone and immersing them in a media of similar refractive index. The decrease in diameter on dehydration permits more precise measurements of length, minimizes the contribution of the out-of-focus image, and increases the contrast. Dehydration causes little change in sarcomere length. The A band appears to increase by about 10 per cent, but this is probably due to an inability to determine accurately the length of the A region in aqueous media rather than to an actual increase in length. Nitrobenzene ($n = 1.552$) was used as the immersion fluid. Its refractive index differs slightly from that of the dehydrated fibril which is between 1.538 and 1.547, as determined with the phase contrast microscope. This mismatch is apparent in some instances at the edge of refractive index gradients.

Observations were made with a model P-42 American Optical Company polarizing microscope³ using na 1.25, 97 \times selected strain-free objectives, which had been "rectified" (1) for the objective and for the condenser. The condenser aperture was

³ I am indebted to the American Optical Company, New York, for the loan of this microscope.

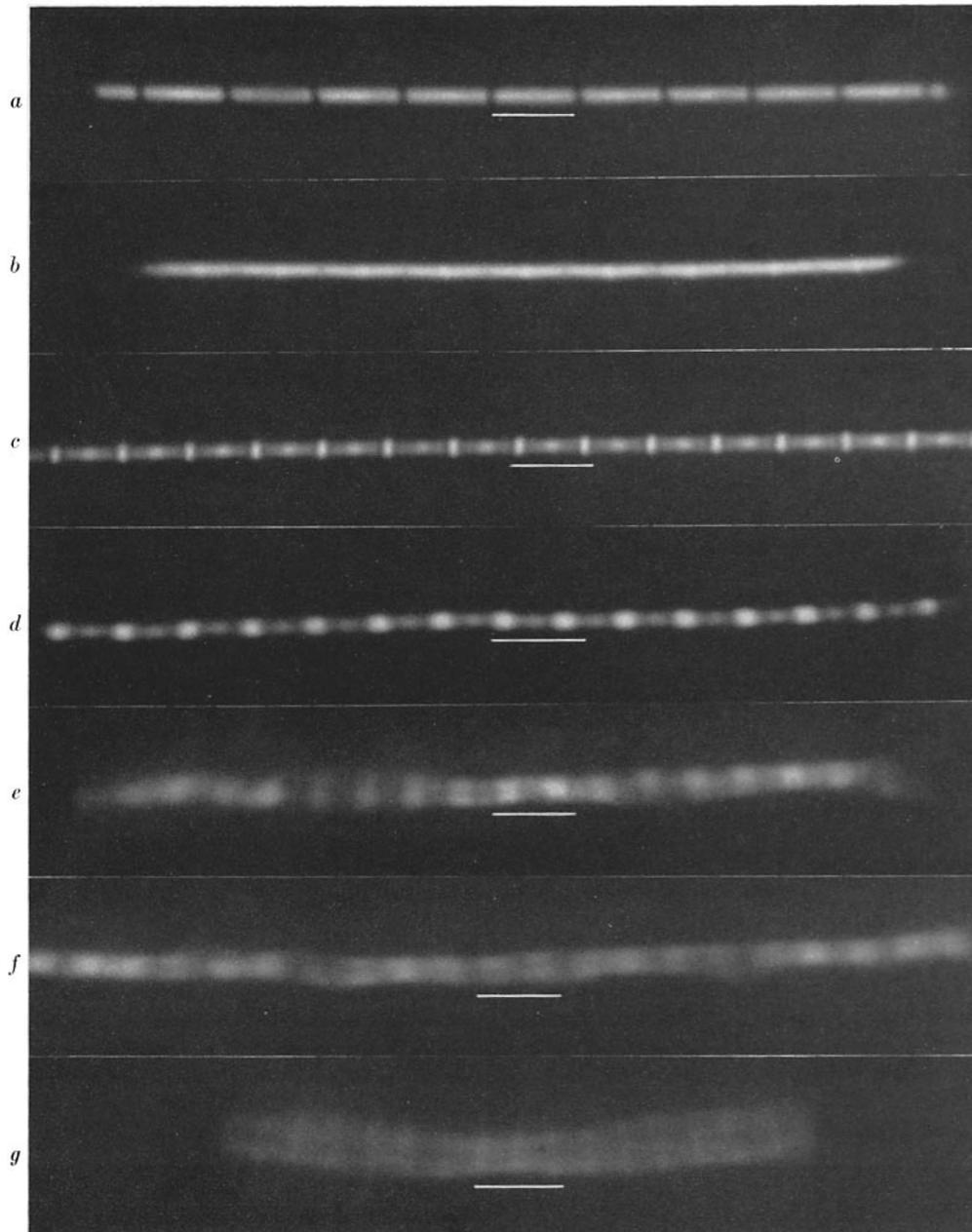
usually of the order of 0.9, and a variable amount of compensation was used to increase the visual contrast. This use of the compensator to adjust the contrast may be confusing when the pictures are examined since the light intensity at a given point will not necessarily reflect the strength of the birefringence at that point. In discussing the observations I have used the term "length" to refer to the dimension that lies in the axis of the fibril.

OBSERVATIONS

All the fibrils in a preparation initially have a similar appearance by phase contrast microscopy and by polarized light microscopy, although, as previously noted (2), there may be small variations in length for a given pattern. A variety of conditions will cause these fibrils to shorten slightly (2-4), but extreme shortening, such as is found when glycerinated rabbit fibrils are treated with ATP, could be obtained only by (a) using trypsin with dilute solutions of ATP or (b) by decreasing the ATP concentration below levels which cause elongation in solutions having a pH of about 8. Fibrils shortened at pH 8 show a loss of birefringence, which centers on the H gap, and show little increase in width of the birefringent Z band once it forms. Fibrils shortened with trypsin retain a more even distribution of birefringence throughout the A region, and the width of the birefringent Z band increases with continued shortening. Preliminary studies indicate that it is the enzymatic activity of the trypsin, rather than its being a basic protein, which is necessary for shortening. Acetyltrypsin was active in causing strong shortening, and a variety of basic proteins were inactive. The follow-

FIGURE 1 This figure shows a representative series of fibrils which were obtained by treating with trypsin and ATP. These fibrils have been dehydrated in acetone and mounted in nitrobenzene. The white line indicates the expected position of an A filament. Polarized light. About $\times 3200$.

- a. An unshortened fibril showing I gaps.
- b. The birefringence of this fibril is almost uniform, and identification of the bright band can not be determined visually. In this instance it is probably an M band, but there may be some confusion between the M band and the A overlap, at this stage.
- c. The strongly birefringent A overlap is easily seen as well as a broader birefringent region about the M band.
- d. The A overlap is considerably wider.
- e. The same general pattern as in d, but the fibril is more swollen.
- f. The A overlap here is about sixty percent of the repeating period.
- g. This pattern is interpreted as showing double overlap. By comparing this with Fig. 2, it can be seen that there is usually some variation in the lateral dimensions for a given pattern.



ing observations and discussion relate to fibrils shortened with trypsin.

Fig. 1 shows a series of photographs of fibrils having different sarcomere lengths which presumably have shortened to different degrees. That these changes are sequential is demonstrated by fibrils in which a gradient of changes in a single fibril can be observed as is shown in Fig. 2.

The fibrils when initially isolated have an A region of fairly uniform birefringence which makes up about 90 per cent of the sarcomere length. This

the length of the birefringent Z band. When the sarcomere length is about 1.6μ , the image becomes unclear, as is shown in the central part of Fig. 2 *c*, but with further shortening of the sarcomere a well defined striation appears which is considerably narrower than that observed previously. The shortening of a sarcomere is associated with varying amounts of lateral swelling which appears to depend on the degree of trypsin treatment as well as on the degree of shortening (compare Figs. 1 *g* and 2 *c*).

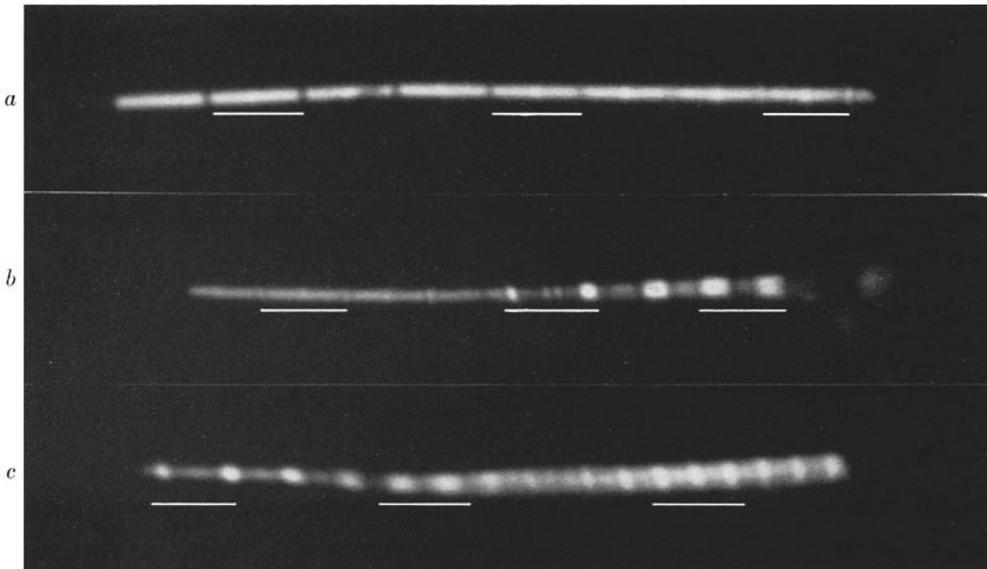


FIGURE 2 The fibrils in this figure have a gradient of sarcomere lengths in a single fibril. All micrographs were taken in polarized light. The white line indicates the expected position of an A filament. About $\times 3500$.

- a. Sarcomeres with both an I gap and a short positively birefringent Z line (the A overlap) are visible.
- b. This fibril has sarcomeres with both a short A overlap and a wide overlap region.
- c. This fibril shows the single overlap changing to the double overlap pattern.

refers to the image seen after the refractive index has been matched and not to the image seen in aqueous media. The sarcomere length at this time is about 3.5μ . With shortening, the I region becomes less obvious until a positively birefringent band appears at the level of the Z line. Associated with this there is usually a small increase in the birefringence at the center of the A band. The less birefringent bands flanking the birefringent Z band, at this time, are probably optical effects associated with the increased density of the Cz band seen by phase contrast microscopy. As is shown in Figs. 1 and 2, a further decrease in sarcomere length is associated with an increase in

It is characteristic of these fibrils that they remain attached to the glass when they shorten, even though the fibril is exposed to the force of the flowing solution. This might be pictured as the continual gluing of the fibrils to the glass by protein gels.

DISCUSSION

These observations suggested that the A filaments, which are considered to be the major birefringent component of the sarcomere (2, 5 page 201), might interdigitate to give a positively birefringent band which centers on the Z line. Another possibility is that material migrates from the A region to the

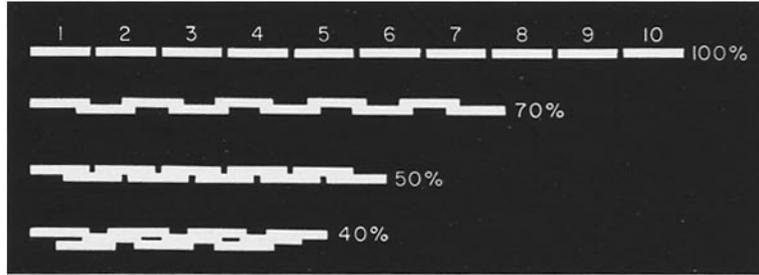


FIGURE 3 This figure demonstrates diagrammatically the cytochemical changes that might be seen as a striated muscle shortens, if the length of the birefringent component of the A region remains constant. The light regions are intended to represent the birefringence of the A region, and the gap, which is assumed to be $\frac{1}{2}$ of the sarcomere length, is the I region. When a sarcomere shortens to 70 per cent of its initial length, there is a region where the A bands are superimposed which has been called the "A overlap". With further shortening, this overlap increases and at 50 per cent the A bands of alternate sarcomeres approach each other. Further shortening causes alternate A bands to overlap, and the region where they are superimposed is called the "double overlap".

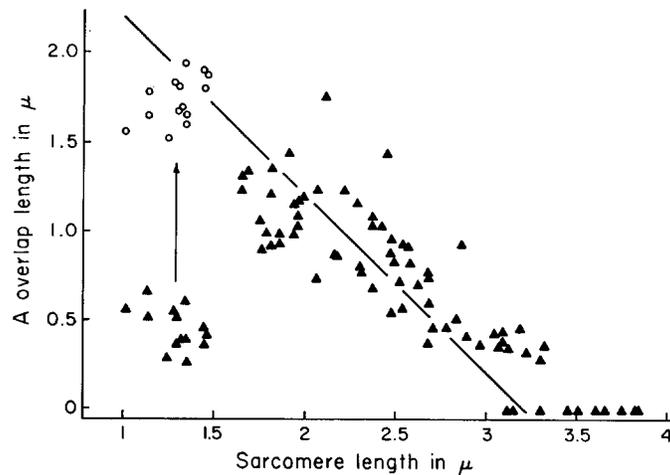


FIGURE 4 Measurements of A overlap were made on fibrils that had been shortened with trypsin and ATP, dehydrated in acetone, and immersed in nitrobenzene. The open dots are calculated points obtained by adding the value of the sarcomere length to all points below 1.5μ . The rationale for this is apparent in Fig. 3 where the length of the A overlap in a 40 per cent shortened fibril corresponds to the repeating unit plus the double overlap width. Sarcomeres at zero overlap all had appreciable I bands.

Z line (3, 6, 7) and localizes in an oriented manner to give an increase in birefringence. In this case, the length of the dense Cz band would be expected to correspond with that of the birefringent Z line.

If the A filaments interdigitate with little change in length, then the length of the positively birefringent Z band should have an inverse relationship to sarcomere length. Fig. 3 is a diagram which may help to visualize the expected pattern of birefringence in this case. At 100 per cent length there is a sharply demarcated I gap defined as $\frac{1}{2}$ of the sarcomere length. With shortening to

92 per cent the I gap will disappear and the fibril will be evenly birefringent. Below 92 per cent a more strongly birefringent region, which centers on the Z line, will appear which increases in length on further shortening of the sarcomere. At about 45 per cent of the initial length, the birefringent regions of alternate sarcomeres approach each other and the fibrils will again become evenly birefringent. Further shortening will show a new narrow birefringent band with decreased contrast.

The regions where the birefringent areas overlap will be referred to as "A overlap" regions and

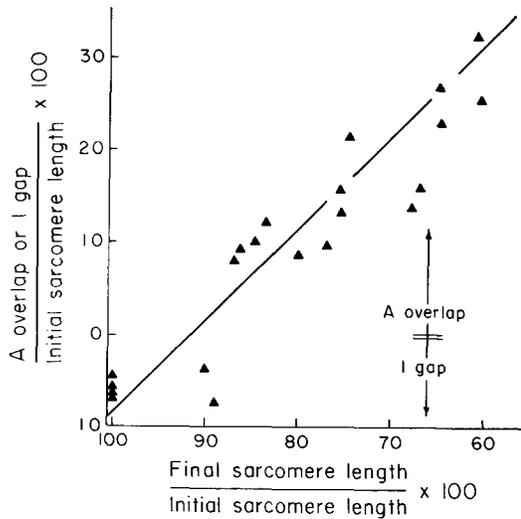


FIGURE 5 Measurements in which an initial sarcomere length, a contracted sarcomere length, and an A overlap length could be obtained from the same fibril. The A overlap and I gap were measured after the fibrils had been dehydrated and mounted in nitrobenzene.

than 1.6μ . The discontinuity probably corresponds to sarcomere lengths where the A overlap regions abut and the fibrils appear too homogeneous to measure easily. From Fig. 3, this effect would be expected when a fibril shortens to 45 per cent of its initial length, which is equivalent to about 1.6μ in Fig. 4. For this reason sarcomeres with lengths below 1.6μ are considered to have regions of double overlap. For purposes of comparison, single overlap values have been calculated and are shown as open dots but these points have not been included in any statistical calculations.

The relation between the A overlap region and the sarcomere length can be shown by a straight line with a slope of -1.0 , if it is assumed that the length of the A region remains constant. This is indicated by the arbitrarily positioned solid line in Fig. 4. The plotted points fall fairly close to this expected line, but a least mean squares line has a considerably lower slope of -0.65 . However, there is no statistical basis for assuming that this is a different slope. When one includes the data calculated from the "double overlap" region, the

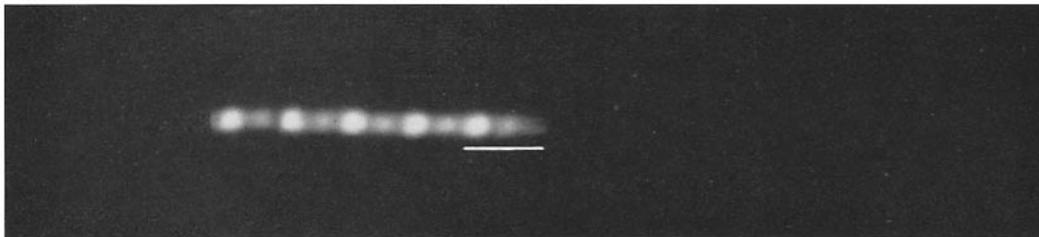


FIGURE 6 The broken end of a shortened fibril after mounting in nitro-benzene. The strongly birefringent band is the A overlap, and the position of an A filament in the terminal sarcomere is indicated by the white line. Note that the M band is positioned asymmetrically and that the fibril does not terminate in a strongly birefringent band. Polarized light. About $\times 3500$.

those where alternate birefringent regions overlap will be termed "double overlap" regions. The A overlap of a sarcomere might be expected to correspond to the birefringent Z band. The following data are a test of this view.

In Fig. 4, measurements of A overlap and sarcomere length have been plotted for a population of fibrils. These measurements were made on fibrils similar to those shown in Fig. 1. There is a gap which centers around 1.6μ in which no measurements were recorded. Such a discontinuity is difficult to explain if the observed A overlap of these points were to arise by shortening of the overlap observed at sarcomere lengths greater

slope is much closer to one. The lower slope is suggestive of some shortening of the A region, but the significance has not been evaluated as the dimensions measured are small and systematic errors may have been introduced. Figure 5 is similar to Fig. 4 except that the A overlap values were measured on individual fibrils in which an initial length was determined, the fibril was permitted to shorten to a final length and then the A overlap length was measured. Again, the data are in reasonable agreement with expected values, although the range covered is not so extensive.

A teased fibril which has not shortened usually breaks at the I region. If these fibrils are then

made to shorten, no sign of a terminal positively birefringent Z band is seen nor is there shortening of the terminal half sarcomere (Fig. 6). The position of the M band is also off center. These observations are in agreement with the view that the length of the A region remains fairly constant, but their significance is uncertain since a break might be expected to interfere with the functioning of the terminal half sarcomere.

There is a large decrease in the total amount of birefringence of a fibril which has shortened strongly (down to about 25 per cent or more), but from measurements which have been made on less strongly shortened fibrils⁴ there is no reason to think that the positive birefringence of the Z region could not arise by the interdigitation of A filaments.

For these reasons I favor the view that the positive birefringence seen at the level of the Z line arises by the interdigitation of A filaments. Since the birefringent structure of the sarcomere seems to behave in a predictable way, it should be possible to see if there is a migratory component whose behavior is independent of the A overlap. If so, this should both establish the reality of the migratory component, about which there is some question (2), and permit an estimate of the proportion of muscle protein involved. Observations by phase contrast microscopy on dehydrated fibrils in which the mismatch between refractive indices could be adjusted indicate that there is a component of appreciable density whose distribution about the Z band does not coincide with the A overlap. However, an unequivocal demonstration of this does not appear possible by phase contrast microscopy because the density of the Cm band may interfere with an accurate determination of the width of the Cz line. This is particularly so as the Cz and Cm bands approach each other at short sarcomere lengths. This point would appear to be settled most conclusively by the use of the electron microscope.

The action of trypsin in permitting the sarcomeres to shorten so extensively is uncertain. One possibility is that trypsin removes the Z band from *Drosophila* fibrils just as it does from rabbit muscle (8, see reference 4 also), permitting the filaments to intrude on each other.

⁴ Estimates of the birefringence of the A region, of an overlap region, and of a "double overlap" are in the ratio of 1.0:1.6:2.8 for fibrils immersed in nitrobenzene.

Many striated muscles do not appear to behave like trypsin-treated *Drosophila* fibrils. The birefringent regions of glycerinated frog and rabbit muscles were not observed to overlap during ATP-induced shortening. Electron micrographs of shortened amphibian muscle (9-11) have given no indication of overlap of the A filaments, and the published measurements indicate that the A region shortens (9, 11). Observations by Engelmann (12) have shown that the Cz band of *Telophorus* muscle is isotropic and that the length of the birefringent A region decreases with shortening. Polarized light images figured by Van Gehuchten (13) for several contracted insect muscles and the photographs of contraction nodes in *Vespa* and *Dytiscus* muscle by Schmidt (14) also can be considered to show shortening of the A region.

The moderate decrease in birefringence of *Drosophila* fibrils on shortening contrasts with the large decrease in birefringence that glycerinated rabbit muscle shows with strong shortening (15) and the almost complete loss of birefringence which Meigs noted (16) when fibrils teased from the thorax of blowflies shortened.⁵

If A filaments do interdigitate and in as extreme a manner as the data suggest, one may wonder what bearing this has on models for muscle contraction in which the A and I filaments interact (5). When A filaments in adjacent sarcomeres interdigitate, a situation arises in which A filaments will be moving in different directions in the same region and relative to the same segment of I filament. There is, of course, no way to tell with the light microscope how orderly the interdigitation is. That shortening, as far as the double overlap occurs, indicates that the directionality of movement of a half sarcomere is retained despite the intruding filaments. If both sets of filaments develop tension equally, one would expect the sarcomere to become stalled before a "double overlap" region is seen.

The restriction suggested by these observations is that shortening cannot involve a tension-bearing element which extends between the adjacent ends of A filaments, at least at short sarcomere lengths.

⁵ Meigs induced shortening by teasing fibrils in a 1:1 mixture of egg white and 2 per cent sodium chloride. This observation has been repeated, and the strong shortening appears to be caused by the alkaline pH of egg white.

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