CONTRACTILITY AND ULTRASTRUCTURE IN GLYCEROL-EXTRACTED MUSCLE FIBERS

II. Ultrastructure in Resting and Shortened Fibers

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ABSTRACT

Glycerol-extracted rabbit psoas muscle fibers were examined by electron microscopy both before and after ATP-induced isotonic shortening. Ultrastructural changes were correlated with the initial sarcomere length and the degree of shortening. The ultrastructural appearance of the resting fiber at rest length was identical with that described by H. E. Huxley and Hanson. At sarcomere lengths greater than 3.7 to 3.8 μ, the A and I filaments were detached and separated by a gap. The presence of "gap" filaments was confirmed, and evidence is presented which indicates that these filaments form connections between the ends of the A and I filaments. Shortening from initial sarcomere lengths at which the filaments overlapped took place through sliding of the filaments. If shortening was initiated from sarcomere lengths at which there was a gap, a narrowing of the I band was brought about by a curling of the I filaments at the boundary between the A and I bands. No evidence could be found that the I filaments moved into the A band.

INTRODUCTION

Glycerinated rabbit psoas muscle fibers stretched to sarcomere lengths greater than the combined lengths of the A and I filaments can still undergo appreciable shortening against small loads (1). This observation is contrary to the theoretical predictions (2, 3, 4) and raises the intriguing question of how shortening occurs in fibrils in which the primary and secondary filaments are presumably disengaged. It would be of interest to know, for instance, whether a sliding of filaments can still occur despite the absence of a preexisting zone of overlap. The finding that highly stretched fibers do not shorten to the same final length as fibers with overlap (1) suggests that some other mechanism of shortening may be involved under these circumstances. To investigate this question, we have examined electron micrographs of fibers with and without overlap which were fixed both at rest and during various stages of shortening.

METHODS

The procedures used for the electron microscopic examination of resting glycerinated fibers have been described in the preceding paper (1). To obtain electron micrographs of isolated shortened psoas muscle fibers, the arrangement used was the same as that employed for the measurement of shortening (1). Adenosine triphosphate (ATP) was added to the cuvette and the fiber was allowed to shorten a predetermined amount. Shortening could be rapidly

stopped by the addition of a few drops of osmium tetroxide. The solution in the cuvette was then replaced by 2 to 3 per cent osmium tetroxide and the degree of shortening measured. The fixation time was 1 hour. Fixation, dehydration, and embedding were carried out in the cuvette with load attached. As controls, resting fibers from the same bundle as the experimental fibers were fixed under isometric conditions, subjected to the same preparation procedure, and examined concurrently under the electron microscope.

RESULTS

FIBERS WITH OVERLAP, RESTING: A fiber fixed at rest length is shown in Fig. 1 *a*. The two-filament pattern observed agrees qualitatively with the ultrastructural pattern described by Huxley and Hanson (5).

FIBERS WITH OVERLAP, SHORTENED: Fibers fixed after ATP-induced shortening showed a sliding of filaments, also in agreement with the observations of Huxley (6). The fibril shown in Fig. 1 *b* is from a fiber which had an initial average sarcomere length of 2.7 μ and which shortened to a sarcomere length of 1.7 to 1.8 μ, or just short of the length at which the A filaments begin to impinge on the Z discs. The H zone has disappeared and parallel thick and thin filaments extend throughout the A band.

It has been shown by Huxley (6) in glycerol-extracted muscles that if shortening progresses to a sarcomere length less than the total I filament length, the I filaments do not collide and curl up in the center of the A band, as previously supposed (7), but slide past each other to form a zone of double overlap. Similar observations on longitudinal sections from shortened living frog muscle have also been made in this laboratory (Knappeis and Carlsen, unpublished experiments). This double overlap would presumably account for the formation of the contraction band at the M line. A cross-section through this zone should show thick filaments surrounded by twice the usual number of thin filaments. A cross-section from the same preparation illustrated in Fig. 1 *b* is shown in Fig. 1 *c*. The primary hexagonal lattice has been preserved and it is possible to see primary filaments surrounded by up to 12 secondary filaments. This 12-filament pattern in shortened glycerinated fibers has also been reported recently by Huxley (8). We could demonstrate a similar pattern in the shortened non-glycerinated frog muscle fiber (Fig. 1 *d*). Hence under these conditions the two sets of filaments slide past each other.

FIBERS WITHOUT OVERLAP, RESTING: If psoas fibers were stretched to sarcomere lengths greater than 3.7 to 3.8 μ, the two sets of filaments became disengaged and separated by a gap (Fig. 2 *a*). In cross-section (Fig. 2 *b*) one sees areas containing either thick or thin filaments exclusively; the double array characteristic of the overlap zone is absent.

The appearance of a gap coincides with the appearance of a new structural entity, the so called "gap filaments", first noted by Huxley and Peachey (9) and Carlsen, Knappeis, and Buchthal (10), and recently described in more detail by Sjöstrand (11). A series of high-magnification electron micrographs of the gap region of stretched

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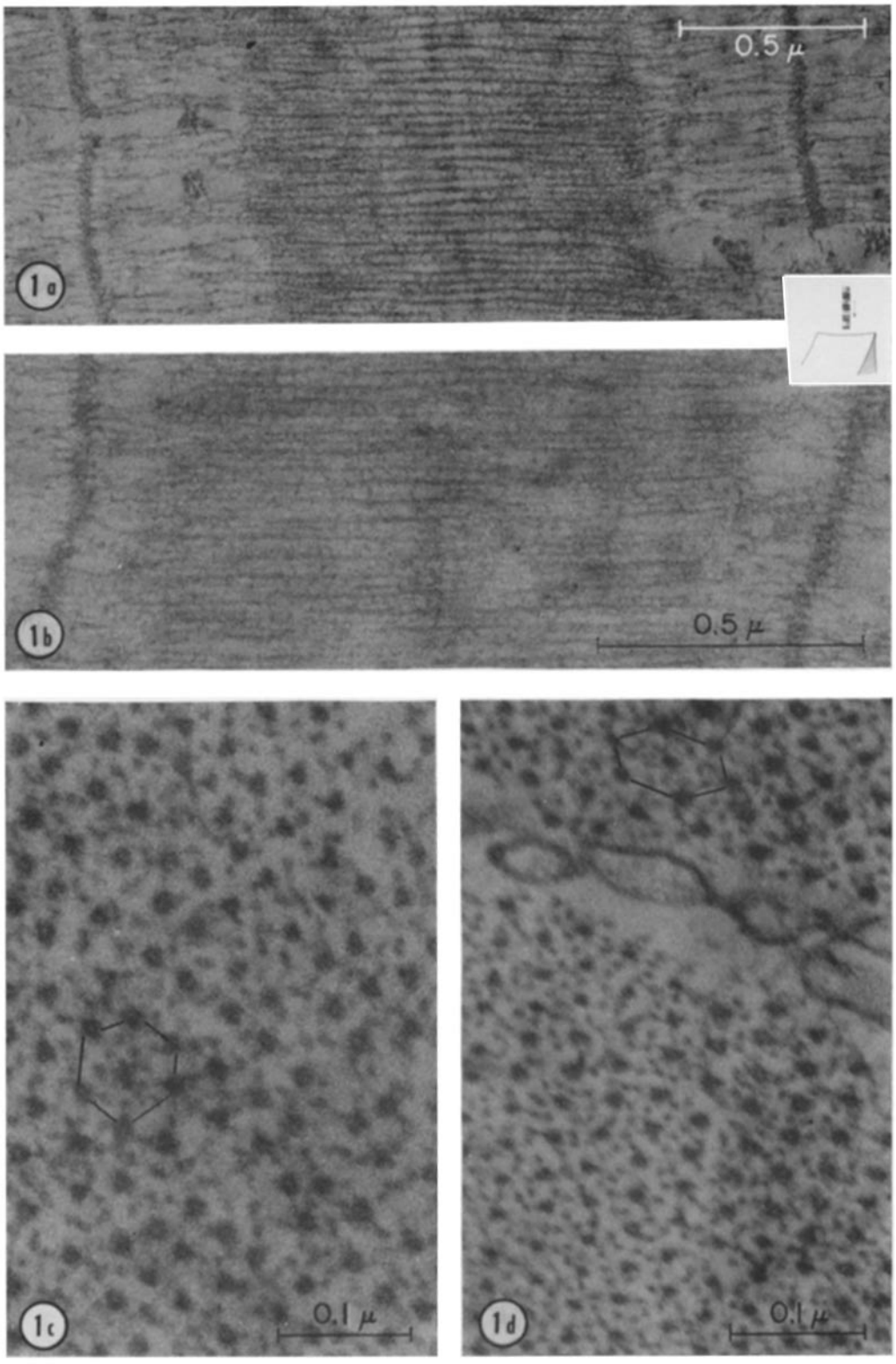
Unless otherwise stated, all preparations illustrated are from glycerol-extracted rabbit psoas muscle, held either at rest length or stretched before glycerol-extraction. Isotonic shortening was induced by the application of ATP to the preloaded fiber. The fibers were fixed with OsO_4, embedded in Vestopal W, and the sections stained with uranyl acetate. The cutting direction of all longitudinal sections is indicated by an inset diagram.

FIGURE 1 *a* Longitudinal section of a fiber at rest length illustrating overlap of primary and secondary filaments.

FIGURE 1 *b* Longitudinal section illustrating the sliding of filaments in an isotonically shortened fiber. The fiber shortened about 30 per cent from an initial sarcomere length of 2.7 μ.

FIGURE 1 *c* and *d* Cross-sections through the middle of the A bands of fibers which have shortened isotonically from rest length. Note thick filaments surrounded by 12 thin filaments. *c* Glycerol-extracted rabbit psoas fiber shortened by ATP. *d* Living semitendinosus fiber of frog shortened by electrical stimulation.


0.5 μ
1a
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1c
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fibers is shown in Fig. 3 *a* to *g*. In agreement with the interpretation of Sjöstrand (11), we see fine filamentary connections between the ends of the A and I filaments. The attachment of the gap filament to the very end of the A filament is evident in Fig. 3 *a* and *b*. A rather common feature is the convergence of two or more A filaments which seem to connect to the end of an I filament (Fig. 3 *d*). This convergence, which was absent in the rest length fiber, suggests that at high degrees of stretch some structure is exerting a force on the ends of the A filaments.

FIBERS WITHOUT OVERLAP, SHORTENED: Fibers which had shortened from very high sarcomere lengths (about 4 μ) had an ultrastructural pattern which was strikingly different from that just described for fibers shortened from rest length. This is demonstrated in the electron micrograph in Fig. 4 *a*, chosen because it illustrates various stages of shortening in the same field. The most characteristic feature of the shortened fiber is the presence of dense bands at the boundaries of the A and I bands. With just a slight degree of shortening, this band appeared only faintly; as shortening progressed, it became heavier. This increasing density at the A-I border occurred coincidentally with a narrowing of the I band. It seems, then, that contraction is initiated at the A-I border and occurs through a rolling up of the filaments in this restricted zone. This pattern can be seen more clearly in an electron micrograph of some moderately shortened fibrils (Fig. 4 *b*).

A still higher magnification (Fig. 5 *a*) of a shortened fibril illustrates the gross disorganization of the I filaments and the absence of I filaments from the spaces between the A filaments. This can also be demonstrated in cross-sections from strongly shortened fibers (Fig. 5 *b*). They contain predominantly thick filaments rather than the two-filament array to be expected in a fiber of such a short sarcomere length. Thus shortening occurred through a curling rather than a sliding of filaments.

In connection with this particular shortening pattern, several relevant points should be mentioned. First, in all sections examined, the sarcomeres of the myofibrils were aligned in both the longitudinal and transverse directions. Secondly, for any given fiber, the ultrastructural pattern was uniform throughout the fiber cross-section; fibrils in the core of the fiber had the same appearance as those immediately adjacent to the sarcolemma. Thirdly, in examining many sections from different fibers, we have never found two different types of shortening patterns within the same fiber, that is, both sliding and curling. These observations have a bearing on some possible artifacts discussed in the next section.

DISCUSSION

The observations presented in this and the preceding paper (1) raise a number of questions with respect to both muscle structure and the mechanism of muscle contraction. Many of our observations and conclusions are in agreement with currently accepted concepts. Thus in resting fibers at "physiological" sarcomere lengths we found the same basic fine structure as described by Huxley and Hanson (5). We found the same overlapping hexagonal array of primary and secondary filaments, with changes in muscle length being accompanied by corresponding changes in the degree of overlap. Active shortening from initial sarcomere lengths at which there was a zone of overlap occurred through a sliding of filaments. Carlsen *et al.* (10) found, in addition, slight changes in filament length. The recent work of Page and Huxley (12) argues against this. However, the precision of current methods does not allow them to rule out the occurrence of small-scale length changes (1 to 2 per cent).

THE RESTING STRETCHED FIBER: With regard to the structure of the highly stretched fiber, the situation is still unclear. At sarcomere lengths in the region of 4 μ, the primary and secondary filaments no longer interdigitate but are separated by a sizeable gap. This gap is not empty but contains fine filaments which can be seen in electron micrographs published by Huxley and Peachey (9), Carlsen *et al.* (10), and Page and Huxley (12). None of these papers was specifically concerned with the ultrastructure of the stretched fiber, and the nature and pattern of connection of these filaments was not discussed in any detail. Sjöstrand's (11) study of the gap region in stretched fibers indicates that some structural continuity exists between the two sets of filaments, even when they no longer lie in apposition to each other. He believes that the gap filaments form connections between the ends of the A and I filaments. Our observations would seem to support this interpretation.

Other explanations have recently been put forth. Thus Huxley (13) suggests that the presence

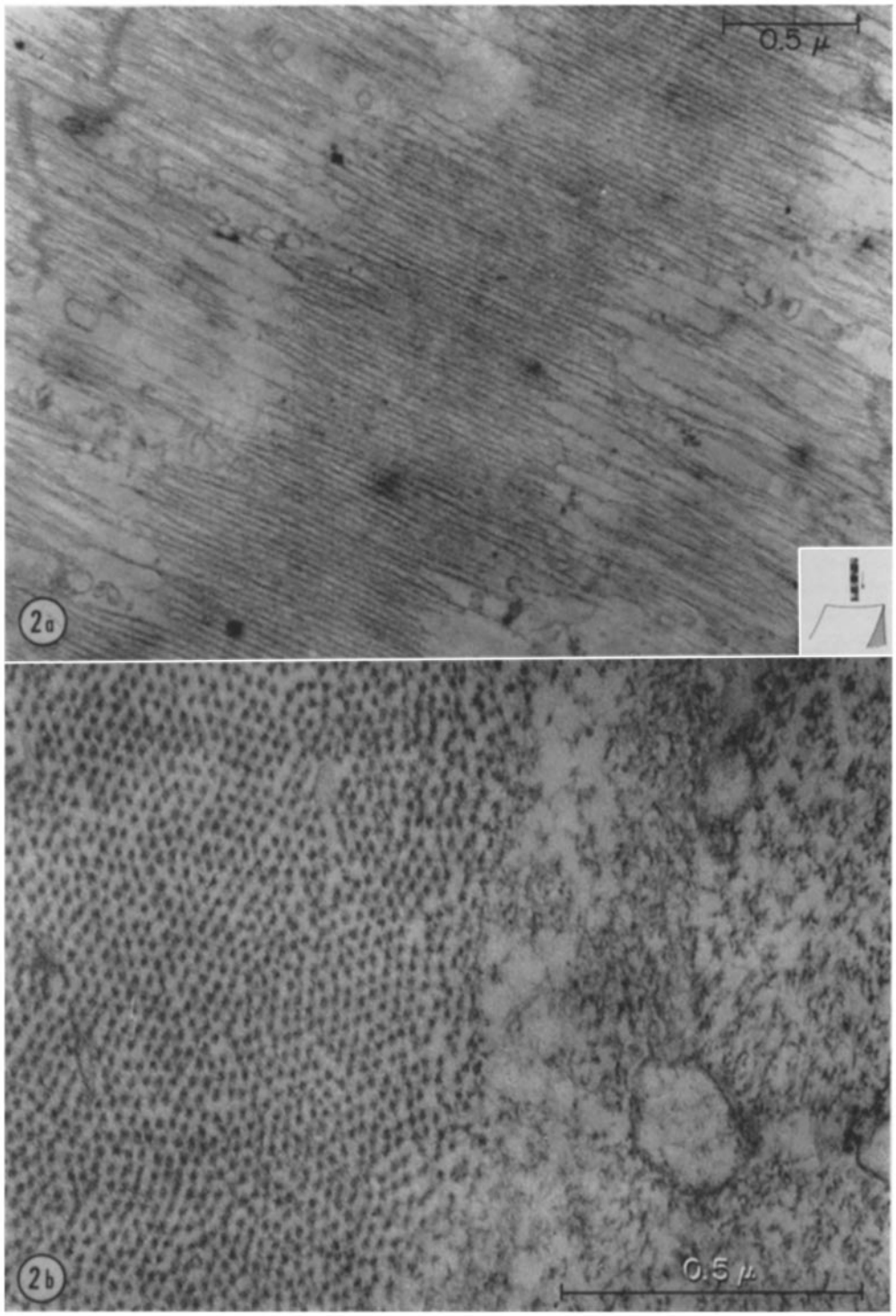


FIGURE 2 *a* Longitudinal section of a glycerinated psoas fiber stretched to a sarcomere length of about 4.2 μ. The thin filaments have been withdrawn from the A band.

FIGURE 2 *b* Cross-section of a highly stretched fiber (sarcomere length 4.1 μ) through a region of A (left) and I (right), illustrating absence of a double hexagonal array.

of gap filaments may simply be due to a superposition of filaments lying in two different planes. If this were the case, we would expect to see the gap filaments more or less randomly located with respect to the ends of the A and I filaments. However, we found that these filaments had a specific localization, each one joining an I filament to the ends of one or more A filaments. We have already mentioned the convergence of groups of A filaments when the I filaments were withdrawn from the A bands. This suggests that some structure, most likely the gap filaments, is exerting a tension on them.

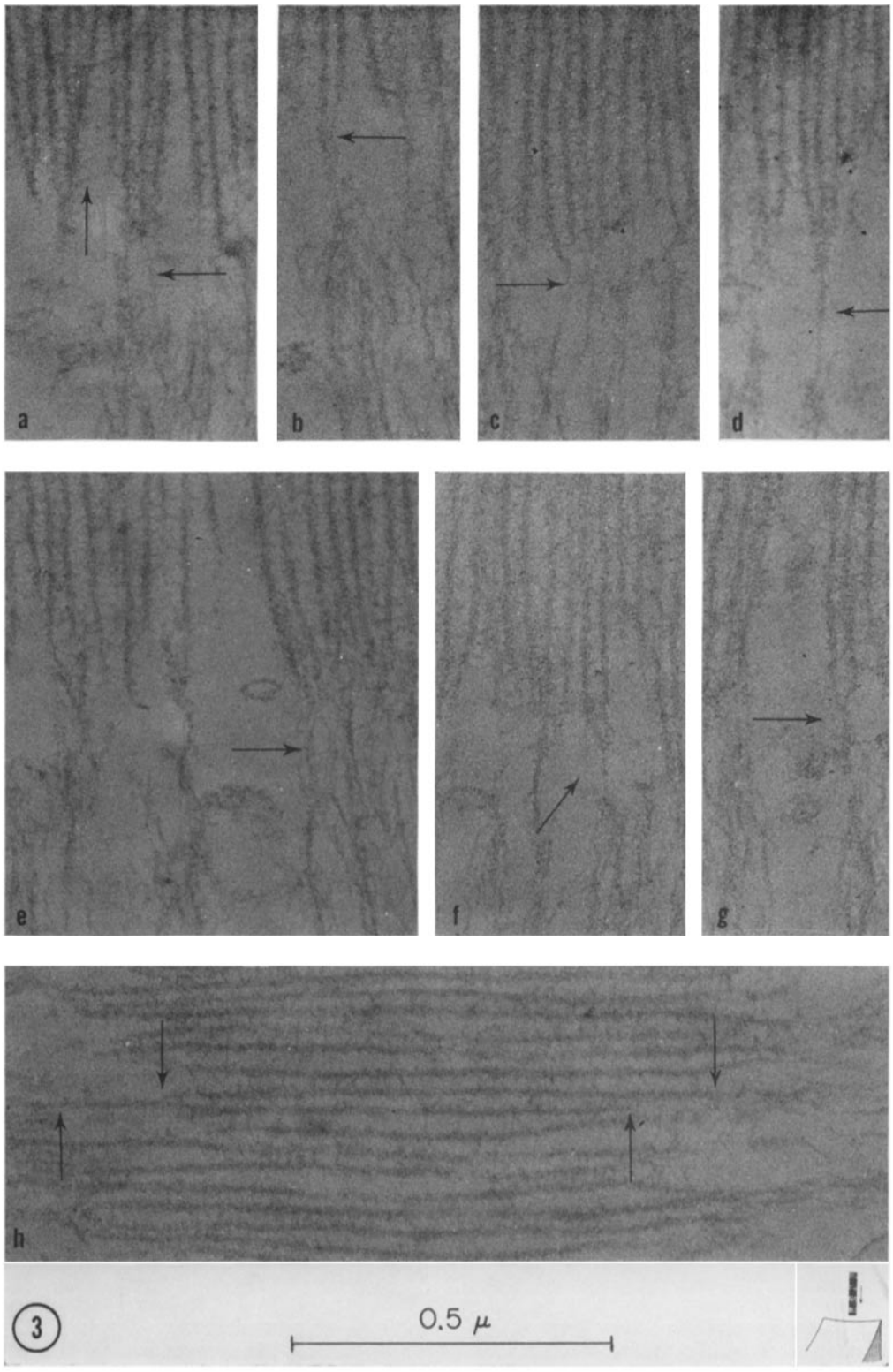
It has also been suggested by Page and Huxley (12) and by Huxley (13) that the gap filaments may be "S" filaments which have been withdrawn from the A band. The existence of S filaments, which connect the ends of opposite I filaments across the H zone, was postulated by Hanson and Huxley (7) to account for the elastic properties of the resting muscle fiber. Some reservations were later expressed by Huxley (14) in view of his observations on the double overlap of I filaments with strong shortening. More recently, Podolsky (15) argued that such filaments must exist to account for the reversibility of the loss of contractility in his experiments. The reversible nature of the active length-tension diagram in living intact fibers is also well known (16). Although direct experimental evidence is lacking, it seems reasonable to assume that when a muscle fiber is stretched and released, the I filaments slide back into their original positions. Nevertheless, the evidence available, both morphological and physiological, provides little support for the contention that the gap filaments are exposed S filaments or that the S filaments exist at all. The gap filaments, when visible, always appear to join the ends of the A filaments. We have never seen them continue into the spaces between the A filaments. Similarly, in none of the published electron micrographs of fibers with overlap is there any indication of some structure connecting the ends of the I filaments across the H zone. The drawing together of the ends of the A filaments during stretch is also incompatible with the view that structural continuity exists only between the I filaments. On the other hand, the occurrence of a double overlap of I filaments with strong shortening seems well established, particularly in view of our consistent finding of A filaments surrounded by twelve I filaments. This would argue against the existence of elastic connections between the ends of the I filaments.

It might be supposed that, although the average distance from the Z line to the ends of the I filaments is about 1.1 μ, there are some filaments which are significantly longer and would appear as "gap" filaments when the fibril is stretched to a length at which the great majority of I filaments no longer lie in the overlap zone. However, such abnormally long I filaments should also be present in equilibrium or rest length preparations where distortion of the filament alignment is minimal. Furthermore, one should see these filaments at least as often as one sees gap filaments. In all such preparations made in this laboratory, as well as in those illustrated in the numerous publications of Huxley and colleagues, where the ends of the individual I filaments can possibly be seen, they always appear to lie in fairly good register.

We must also consider the possibility that the gap filaments are simply misaligned I filaments which appear to connect to the ends of the A filaments. However, the diameter of the gap filaments was estimated by us to be about 40 A and by Sjöstrand (11) to be at least 30 A. On the other hand, the diameter of the I filaments, as measured on Vestopal-embedded frog muscle in this laboratory (17), is about 70 A. Hanson and

FIGURE 3 *a* to *g* Longitudinal sections of highly stretched fibers showing "gap" filaments connecting the ends of the A and I filaments (arrows). In some cases gap filaments are split into two filaments which attach to two different A filaments (horizontal arrow, Fig 3 *a*). Also, two gap filaments can be seen diverging from the end of a single A filament (vertical arrow, Fig. 3 *a*). Several A filaments often converge and attach to a single filament protruding from the I band (Fig. 3 *d*).

FIGURE 3 *h* Longitudinal section of a stretched fiber showing "slipping" of the A filaments. Relative movement of two adjacent filaments is indicated by arrows pointing to the ends of the filaments.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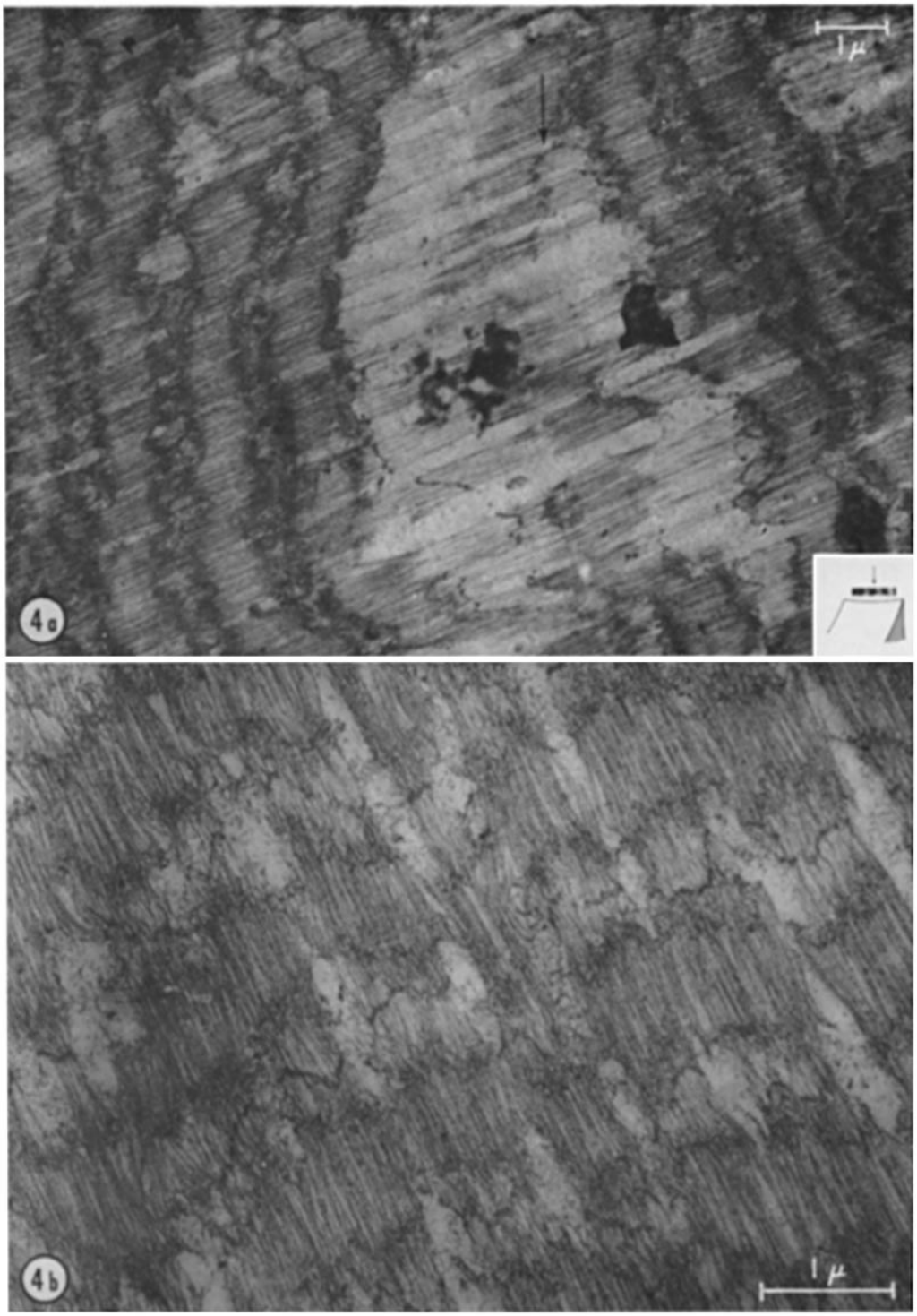

FIGURE 4 Low-power electron micrographs of longitudinal sections of fibers which have shortened isotonically from an initial sarcomere length of 4.2 μ. *a* Section showing strongly shortened (left and right) and stretched and slightly shortened fibrils (center) in the same field. The strongly shortened fibril is characterized by shortened I bands and dense condensations at the borders of the A and I bands. Note condensation which has begun to form at one A to I border of a slightly shortened fibril (arrow). *b* Section of a moderately shortened fiber showing condensations at the lateral borders of the A bands.

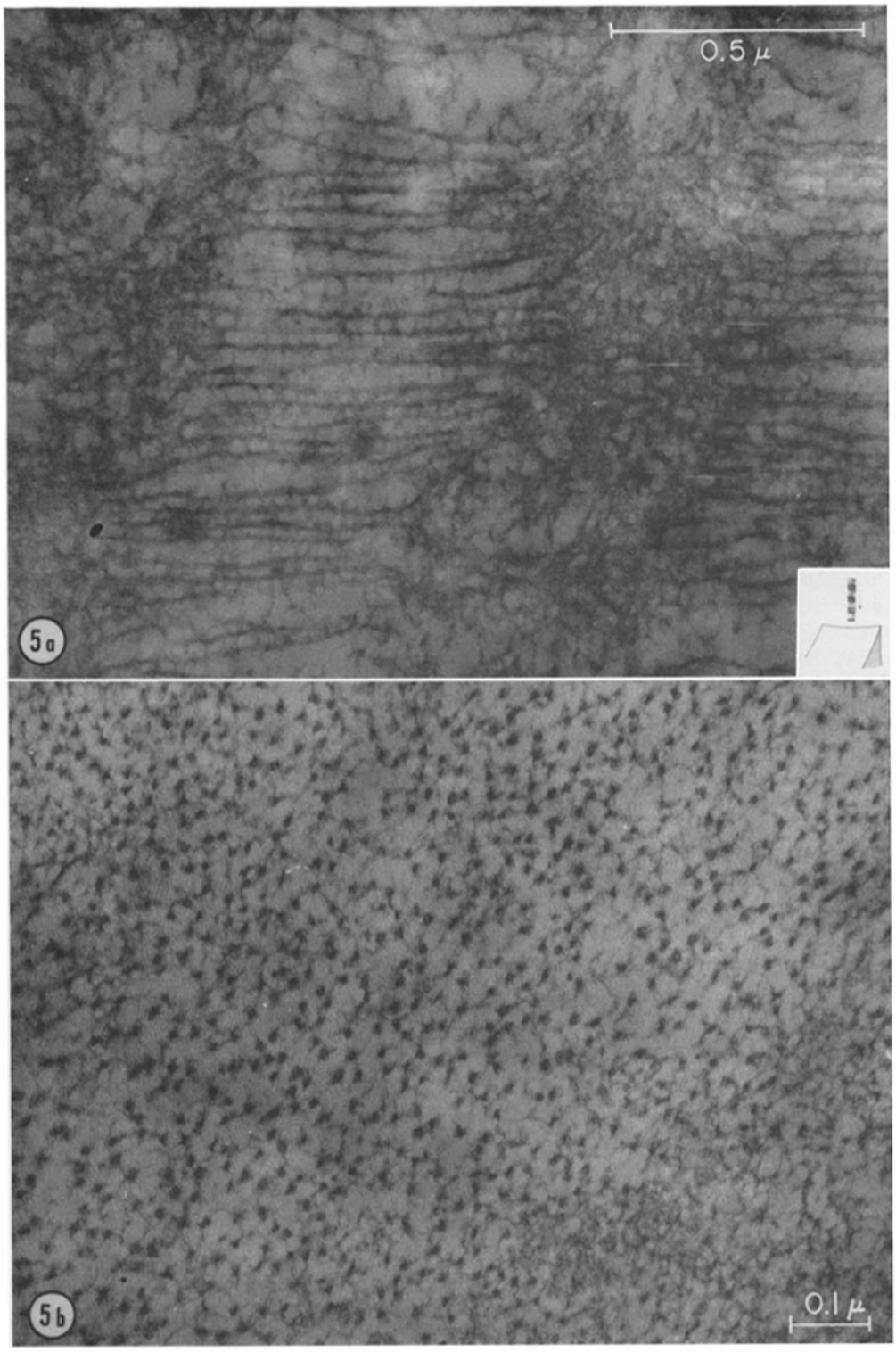

FIGURE 5 *a* Longitudinal section of a fibril from a fiber which has shortened about 60 per cent from an initial sarcomere length of 4.1 μ. Note the disorganization of the thin filaments and the absence of thin filaments from the A band.

FIGURE 5 *b* Cross-section through the A band of a preparation similar to that shown in Fig. 5 *a*. The double hexagonal array is absent.

Lowy (18, 19) have shown that, on molecular structural grounds, the diameter of the I filaments must be considerably more than 60 A. Although measurements of filament diameters in longitudinal sections are subject to some uncertainty, the observed difference in diameter between the I filaments and gap filaments does suggest that these are not identical structures.

How, then, does one account for the gap filaments? There can hardly be permanent connections between the ends of the A and I filaments or the range of sliding would be severely restricted. It is possible that temporary connections form as the ends of the I filaments slip out from between the A filaments. Since each I filament is surrounded by three A filaments, the simplest arrangement would be a three-point attachment of I filaments to A filaments. Evidence on this point is still lacking. It is also tempting to speculate on the possible identity of the gap filaments and tropomyosin, in view of recent evidence (18–20) that tropomyosin is present in the Z line and I filaments. It is conceivable that the tropomyosin forms, or is part of, some continuous structure which becomes visible when the actin and myosin are separated by stretch. Clearly an area for future research is the elucidation of the cellular structures which guide the rather precise movements of the I filaments both in release from stretch and during active shortening to low sarcomere lengths.

SHORTENING OF HIGHLY STRETCHED FIBERS: With regard to the shortening of fibers lacking filament overlap and the peculiar ultrastructural pattern associated with this shortening, we encounter difficulties if an explanation is sought within the framework of the sliding filament model. We may consider first some of the more obvious possibilities.

We have discussed (1) the evidence for believing that shortening at high sarcomere lengths cannot be explained in terms of internal variation in sarcomere length, and the electron microscopic observations would seem to support this contention. Let us consider the hypothetical case of a highly stretched fiber in which a small number of fibrils containing sarcomeres with overlap are distributed at random. It is already established that such fibrils shorten through a sliding of filaments (see also reference 8). In the context of the sliding filament model, one can envision two possible mechanisms to account for the shortening of the fiber (1). In one case, the fibril possessing overlap at the beginning could initiate a sideward spread of overlap by pulling on adjacent fibrils. In the second case, the few fibrils possessing overlap would account for the total tension development and shortening, the remainder undergoing passive shortening. A precondition for both mechanisms would be that at the very start of contraction most of the load is being carried by extrafibrillar structures and would gradually be transferred to the fibrils as shortening progressed and more overlapping sites were formed (1). In both cases we can make definite predictions about the ultrastructural appearance of the shortened fiber: If there were a sideward spread of overlap, most if not all of the shortened fibrils would show evidence of shortening through sliding. In principle the appearance of most of the fibrils should be indistinguishable from the appearance of those which shortened from rest length. This is obviously not the case, and hence we can discard this mechanism. For the alternative mechanism, whereby a few fibrils possessing overlap at the beginning account for the total tension development and shortening, we would expect to see the characteristic crumpling up of the passively compressed fibrils, with both sets of filaments being involved. Furthermore we should find, interspersed among the passively compressed fibrils, those fibrils which exerted the tension and which have shortened through a sliding of filaments. As we have already pointed out at the end of the Results section, just the opposite has been observed. Both the longitudinal and transverse alignments of the sarcomeres of the fibrils were typical of a fiber in which all of the shortened fibrils are exerting tension. Contrary to what we would expect to see with passive folding, the shortening occurred in localized regions of the fibril, namely the A-I borders. The pattern shown in Fig. 4 *a*, in which both highly stretched and highly shortened fibrils are found next to each other in a very small area, is difficult to explain on the basis of any assumption other than that the shortened fibrils are exerting tension. Similarly, in examining many such sections, we have failed to find any fibrils which have shortened through a sliding of filaments, although in fibers which have shortened more than 50 per cent we would expect to find a considerable number of them if this were the true explanation of the observed shortening. This failure can, in fact, be taken as supporting evidence in favor of our previous conclusion that the internal variation in sarcomere length is not great

enough to explain the shortening observed in fibers with sarcomere lengths greater than 4 μ. It also argues against the possibility that a transfer of load from parallel elastic structures to the contractile structure is of any quantitative significance in the experiments.[1]

It might be argued that under our experimental conditions there was a diffusion gradient for ATP which would give rise to differences in morphological appearance between the periphery and the core of the fiber. In fibers which have shortened from both long and short sarcomere lengths, we have examined fibrils at the core and at the sarcolemmic border of the same fiber and could not find significant ultrastructural differences. This indicates that, with single fibers and an ATP concentration of 5 mM, there was complete penetration of ATP into the fiber.

ends of the filaments are no longer in register. This "staggering" of the A filaments presents the possibility that, as the fibril is stretched, the ends of the I filaments are pulled into positions where they approach and actually overlap the ends of the A filaments, thus creating new zones of overlap at each A-I border. This is shown diagrammatically in Fig. 6. Assuming that there would be no steric hindrances, there would be the possibility for an interaction between the two sets of filaments, with a movement of the I filaments relative to the ends of the A filaments and a subsequent shortening of the I band. In agreement with observation, contraction would be localized at the A-I border, and sliding would not occur because of the distortion of the filament lattice. This hypothesis would also explain Podolsky's (15) observation that weak contractions sometimes occurred in fibers which

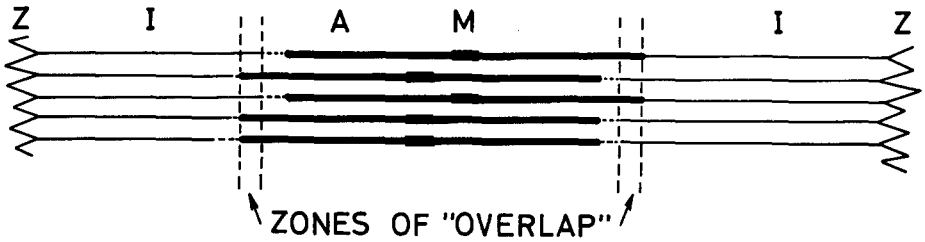

FIGURE 6 Diagram illustrating how "staggering" of the A filaments could create new reaction sites between A and I filaments in fibrils stretched to a degree at which normal overlap is absent.

One other possible explanation might reconcile our observations with the current formulation of the sliding filament model. We have previously alluded to the question of variation of overlap within the fibril (1). It was observed in some electron micrographs of stretched fibers that the A filaments were pulled out of their regular alignment as a result of the unequal distribution of force on the myofilaments. An example is given (Fig. 3 *h*) of a stretched fibril in which neighboring filaments have slipped past each other so that the had been stretched so far that the striation pattern disappeared. Furthermore, the non-alignment of the A filaments was not present in all electron micrographs of stretched fibers. A more detailed quantitative analysis is required to determine whether this slipping of the filaments would furnish enough overlapping sites to account for the observed tension development.

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[1] Shortening occurred through sliding in sarcomeres of frog muscle made to shorten maximally (>4 μ to ~1.4 μ) by electrical stimulation (Carlsen and Knappeis, unpublished). In highly stretched non-glycerinated muscle fibers the parallel elastic component carries an essential portion of the load. This might account for the structural difference between glycerinated and non-glycerinated fibers during contraction.

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