

Length-Tension Relation in *Limulus* Striated Muscle

BENJAMIN WALCOTT and MAYNARD M. DEWEY

Department of Anatomical Sciences, School of Basic Health Sciences, Health Sciences Center, State University of New York at Stony Brook, New York 11794

ABSTRACT Laser diffraction techniques coupled with simultaneous tension measurements were used to determine the length-tension relation in intact, small (0.5-mm thick, 1.0-mm wide, 20–25-mm long) bundles of a *Limulus* (horseshoe crab) striated muscle, the telson levator muscle. This muscle differs from the model vertebrate systems in that the thick filaments are not of a constant length, but shorten from 4.9 to ~ 2.0 μm as the sarcomeres shorten from 7 to 3 μm .

In the *Limulus* muscle, the length-tension relation plateaued to an average maximum tension of 0.34 N/mm² at a sarcomere length of 6.5 μm (L_0) to 8.0 μm . In the sarcomere length range from 3.8 to 12.5 μm , the muscle developed 50% or more of the maximum tension. When the sarcomere lengths are normalized (expressed as L/L_0) and the *Limulus* data are compared to those from frog muscle, it is apparent that *Limulus* muscle develops tension over a relatively greater range of sarcomere lengths.

Evidence is nearly irrefutable that, in vertebrate striated muscle, shortening of sarcomeres is accompanied structurally by a reduction in I-band width and by a constancy of the A-band width. This observation, in addition to the evidence for the constancy of lengths of the thick and thin filaments that make up the A- and I-bands, has led to the generally accepted sliding-filament theory of muscle contraction proposed by Huxley and co-workers (10, 11). Further support of this theory is the length-tension data of Gordon et al. (9), which suggest that the amount of isometric tension developed by a vertebrate striated muscle fiber is determined by the degree of overlap of the thick and thin filaments and thus, presumably, the number of myosin bridges that can interact with actin.

In one invertebrate muscle, however, it has been shown that a basic tenet of the sliding-filament model is not followed; in *Limulus* telson levator muscle the A-bands and constitutive thick filaments change length with sarcomere length. de Villafraña (4) was the first to report extensive changes in A-band length with sarcomere length in glycerinated *Limulus* muscle. This was confirmed by Dewey et al. (6), who additionally demonstrated by electron microscope observations that the shortening of the A-band below 5 μm resulted from shortening of the thick filaments. This observation was confirmed by a series of experiments in which thick filaments were isolated from muscle at different sarcomere lengths (8). Long, thick filaments (~ 4.2 μm) were isolated from muscles with long sarcomeres (8 μm), whereas short, thick filaments (~ 2.9 μm) were isolated from muscle with short sarcomeres (6 μm).

Given this significant variation from the structural aspect of the vertebrate system and model, the question arises as to its

effect on the function of the *Limulus* muscle. Therefore, we have determined the length-tension diagram for *Limulus* telson muscle and have correlated it with structural information previously determined.

MATERIALS AND METHODS

Adult *Limulus* (20–30 cm across the carapace) were obtained from the Marine Biological Laboratories (Woods Hole, Mass.) and were maintained at 14°C in artificial sea water (ASW) in large aerated tanks. Under these conditions they live for many months.

The Preparation

The dorsal carapace of the opisthosoma (abdomen) was cut away from the point of articulation of the telson to the first of a series of “pits” that run anteriorly in two rows. These pits represent a series of cuticular indentations that serve as places of muscle attachment. After removal of the underlying connective tissue, the dorsal bundles of the levator muscle to the telson were exposed. A small piece of cuticle ~ 1 cm² was cut to include the first pit on one side. The attached large muscle group was dissected to its tendinous attachment to the telson. This tendon was cut and the freed muscle was transferred to chilled ASW in a dissecting dish. Within the muscle group there were several small bundles of fibers that have long (2–3 mm) tendinous origins as well as tendinous insertions. These bundles were ~ 0.5 -mm thick, 1- to 2-mm wide and 20- to 25-mm long. The rest of the muscle group was cut away from the selected small bundle, and all loose connective tissue was removed. The remaining small bundle was then checked for damage under a dissecting microscope. The bundle had to show a clear translucence with no small “opacities” that indicated damage. None of the inherent fibers could be broken. After the bundle was checked, it was placed in the experimental chamber.

Initially, many attempts were made to produce a single fiber preparation. In this muscle however, the fibers run the entire length of the bundles (up to 30 mm), and they are bound together by strands of connective tissue. In our hands, it proved impossible to free single fibers for their entire length without some

damage as shown by local membrane density changes and local contractions. Therefore we used intact small bundles that provided reproducible results.

The Apparatus

The experimental chamber was a rectangular optical glass dish ($12 \times 8 \times 3$ cm) with inlet and outlet tubes at each end. It was mounted on a large microscope stage that permitted x and y movements of the chamber. A stainless-steel bracket at one end of the chamber held the carapace portion of the preparation, and a transducer (Grass FT .03, Grass Instrument Co., Quincy, Mass.) attached to a manipulator was mounted on the stage at the other end of the chamber. The transducer was fitted with a stainless-steel clamp made from a pair of Dumont-type forceps that held the tendon insertion of the muscle bundle. The movement of the transducer parallel to the muscle long axis was calibrated with a micrometer accurate to 0.5 mm. A laser (model 132, 6328 nm wavelength, Spectra-Physics Inc., Laser Products Div., Mountain View, Calif.) was mounted vertically below the stage and was aligned so that the beam (~ 0.8 mm in diameter) passed through the center of the stage and chamber. A mechanical shutter was mounted between the laser and the stage, and a Polaroid camera back was mounted above the specimen chamber at an adjustable distance. The camera, stage, and laser were mechanically aligned and centered. Thus with this system, a muscle bundle could be mounted horizontally in the chamber and stretched to any desired length. The whole unit could then be moved relative to the laser and camera so that the entire bundle could be scanned from origin to insertion.

Muscle Stimulation

Silver electrode plates (4-cm long \times 0.8-cm high) were placed on either side and parallel to the muscle bundle. There was a 4- to 8-mm space between the edge of the bundle and the plates. The plates were attached to a stimulator (S-88, Grass Instrument Inc.), which delivered a train of 5-ms pulses at 80 Hz and usually at ~ 60 V. 2-s-long trains of pulses were manually initiated and timed. It was determined that this mode of stimulation produced as much tension as a contraction induced by high K^+ (8). However, unlike the K^+ -induced contractions, up to 10–15 repetitions with equivalent tension could be produced by electrical stimulation. Thus, electrical stimulation was exclusively used in these experiments.

Laser Diffraction Image Analysis

At each experimental length a photograph of the laser diffraction pattern was made by use of Polaroid PN-type 55 film. This provided a positive print and a negative. The sarcomere length could be roughly determined by measuring the distance between the middle of the first-order layer lines on the print, dividing by two, and placing that value in Bragg's equation (3, 5, 6). In addition, the distance between the muscle bundle and film was measured with calipers and was accurate to within ± 0.5 mm. This error, in a total distance of 70–80 mm, produces an error of $<0.2 \mu\text{m}$ in the calculation of sarcomere length.

For accurate measurements of sarcomere length, the density of the negative image was determined by use of a dual beam recording microdensitometer (Mk III; Joyce, Loebel & Co., Ltd., Gateshead-on-Tyne, England). The distance between the first-order peaks was measured, and when the first-order layer lines were close to the central spot, the second-order peaks were used. This value was used as previously described to determine the sarcomere length. The system was checked for accuracy by analyzing the diffraction image from a stage micrometer that has a $10\text{-}\mu\text{m}$ spacing.

Tension Measurements

The tension transducer was operated in its most sensitive range. However, the absolute tensions, both passive and active, were never large enough (usually 1–2 g) to permit any significant movement of the lever system. Thus, the muscle length was constant, even when developing tension. This was confirmed by comparison of laser images "at rest" and when the muscle was activated. Only when the muscle length was set below slack length was there a significant difference in the two sarcomere length measurements.

During the experiments as the muscle length was manually changed, passive tension was recorded and ultimately subtracted from the active tension produced by the electrical stimulus. In most experiments the tension was expressed as a percent of the maximum developed at L_0 . To estimate the absolute amount of active or passive tension the muscle can develop, we measured both the length of the bundle when it developed its maximum tension and the total fresh weight of the bundle. Assuming a density equivalent to water, the cross-sectional area is given by: $A = V/L$, where A is cross-sectional area, V is volume derived from the weight (assuming a density of 1.00), and L is the length. Force can then be related to cross-sectional area.

Procedure

A small bundle was dissected as previously described and was mounted in the chamber. A continuous flow of ASW, at a rate of ~ 20 ml/min, entered the chamber at the transducer end and was aspirated out at the origin end. The bundle was set at just longer than slack length and was then stimulated with a 2-s train of pulses. This produced a tetanic tension response that plateaued within 500 ms (Fig. 1). At the end of the plateau, the laser diffraction pattern was photographed with an exposure of 1/60 s. We repeated this procedure every 2–3 min, changing the muscle length to determine the muscle length that produced maximum tension. Then the tetanic tension and the laser diffraction pattern were measured at longer and shorter sarcomere lengths.

For a preparation to be acceptable, a number of criteria were used: (a) Visual examination with a dissecting microscope showed no opacities or broken fibers. (b) Maximum tension had to be >0.25 N/mm². (c) When the muscle length was returned to L_0 , the tension had to be $>90\%$ of the original value. (d) The sarcomere length of L_0 had to remain within $0.5 \mu\text{m}$ of the initially determined value for that preparation. (e) The sensitivity of the preparation to the stimulus current had to remain constant. (f) A clear diffraction pattern with at least two orders at short lengths had to be obtained along the entire length of the bundle. At long lengths, more orders (up to seven) had to be obtained. (g) The variation in sarcomere length as evidenced in the diffraction pattern had to be $<10\%$ in the middle 90% of the muscle bundle. (h) The relation between sarcomere length and the amount of muscle stretch had to be linear and show no hysteresis or slippage.

In most cases, the determination of whether a preparation satisfied all these criteria could only be made after the data were analyzed. Hence, many complete runs were excluded from the sample after analysis.

Sarcomere Length Uniformity

A series of experiments was performed to determine whether there was variation in sarcomere length at the extreme ends of the bundles even though the middle 90% was uniform. In these cases, bundles that met all the criteria were set to different lengths, and at each length a diffraction image was obtained from the middle and each extreme end of the bundle. The patterns at the ends were obtained from the insertion or origin area where one could first obtain an image when scanning from tendon to muscle, which was about the first and last 0.5 mm of a 25-mm bundle.

RESULTS

Direct electrical stimulation of the muscle bundles produced a tetanic tension response that plateaued in ~ 500 ms after the onset of the 2-s train of pulses (Fig. 1). In most cases, the tension plateau was flat, showing neither significant creep nor decay. The amount of tension that a bundle produced was measured at the end of the plateau while the laser diffraction image was being recorded, and the maximum tension ranged from 0.24 to 0.50 N/mm², with an average value of 0.34 N/mm². The tension response of a given bundle was very consistent, with $<10\%$ variation in successive repetitions under the same conditions. Further, a preparation could be left for more than 2 h, with the same tension produced as before.

In these experiments, laser diffraction images of the bundles were recorded at the end of the tension plateau while tension was at a steady value (Fig. 1). In most cases, the number of layer lines obtained from the activated muscle was less than the ideal number ($n - 1$, where n is the sarcomere length).

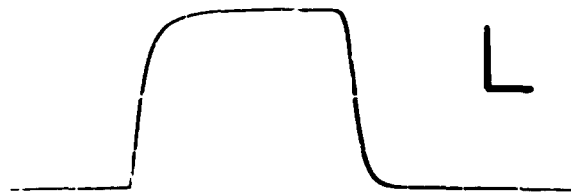


FIGURE 1 Record of tetanic force produced by a muscle bundle at $8\text{-}\mu\text{m}$ sarcomere length. Note that tension rises rapidly to a steady plateau that is relatively constant for the 2-s stimulus. Vertical calibration is 4 mN and horizontal calibration is 500 ms.

However, acceptable bundles produced at least two layer lines from a sarcomere length of 3.5 μm and up to 7 lines from longer sarcomere lengths (Fig. 2). In addition, the width of the layer lines varied with wider lines originating from shorter sarcomere lengths, indicating more dispersion about the mean sarcomere length. However, the dispersion from bundles included in the sample never exceeded $\pm 0.5 \mu\text{m}$, and measurements of sarcomere length were accurate to within 0.5 μm at all sarcomere lengths (5). At sarcomere lengths $>7 \mu\text{m}$, there was no difference in the measured sarcomere length between active and resting muscle. At shorter muscle lengths, activation of the muscle produced sarcomere shortening to a new stable level, whereas relaxation was accompanied by sarcomere lengthening to 6–7 μm . Therefore, because the sarcomere length was constant during the majority of the tension plateau and was unaffected by activation at longer sarcomere lengths, our experiments were performed under isometric conditions.

In the course of these experiments, muscle bundles were set at various lengths relative to "rest length", which we defined as that length below which the muscle will go slack if not stimulated. Thus, one could relate stretch of the whole bundle to the sarcomere length determined by laser diffraction from a portion of the bundle. An example of this relation for a particular bundle is shown in Fig. 3. In this case most, but not all, of the laser images were obtained while the muscle was

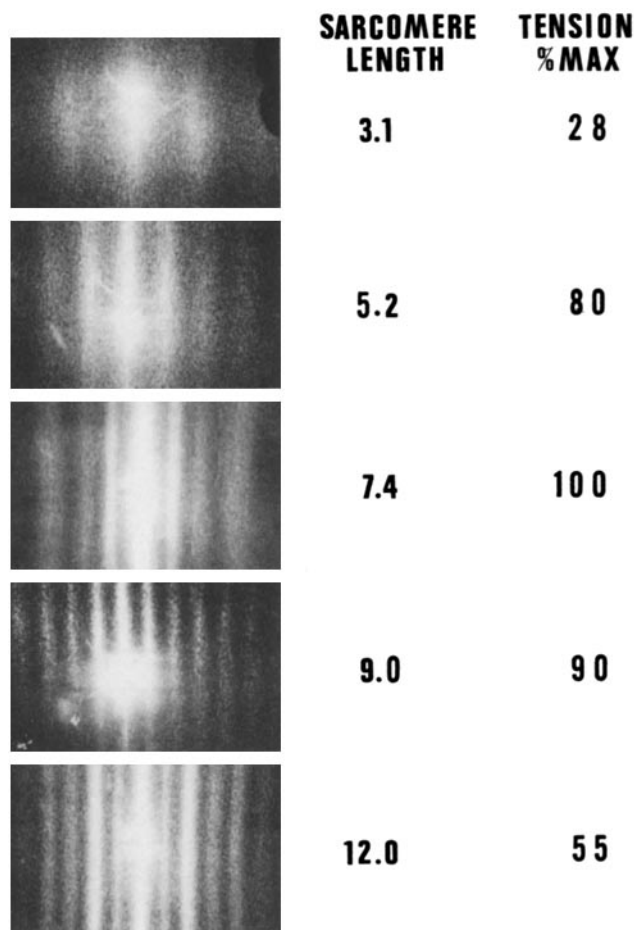


FIGURE 2 Laser diffraction patterns from tetanically contracted bundles with the sarcomere length calculated from the pattern and the percent of maximum tension developed at that time. The top three patterns are from one preparation, whereas the bottom two are from another.

stimulated at the set length. The result was a linear relation with little scatter of the data. Because the muscle length was not changed uniformly in one direction but rather was stretched, then shortened, then stretched again, the degree of dispersion of the points indicated the degree of hysteresis in the system as well as whether slippage of the muscle attachment had occurred. In this case, as with all the preparations included in the sample, the scatter was close to the $\pm 0.5\text{-}\mu\text{m}$ resolution of the system and thus there seems to be little hysteresis or irreversible stretching of some elastic component.

When the muscle was set at various lengths and tetanically stimulated, the active tension produced varied with the sarcomere length (Fig. 4). These results combined from 11 different preparations show that significant tension was produced over a wide range of sarcomere lengths. Over 50% of the maximum tension was produced by a bundle in a sarcomere length range of 3.8–12.5 μm . The shape of the curve consisted of a relatively steep portion rising to maximum tension at an average sarcomere length of 6.5 μm . There was a plateau of maximum tension that extended from L_0 (6.5- μm sarcomere length) to 8.0 μm . Then the tension fell as the muscle was stretched further. Although bundles were never stretched so far as to produce zero tension, the data suggest that that point is at a sarcomere length of 14.0 μm . As the muscle bundle was stretched, it exhibited some passive tension. This force was first observed at a sarcomere length of 9–10 μm and increased gradually as the bundle was stretched further. At a sarcomere length of 12 μm ,

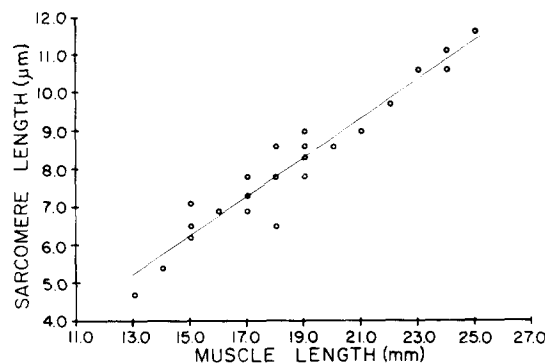


FIGURE 3 The relation between muscle length and the sarcomere length of the fibers measured by laser diffraction. The line is the calculated regression line for the data and the correlation coefficient is 0.961. The small scatter indicates little hysteresis or slippage in the muscle.

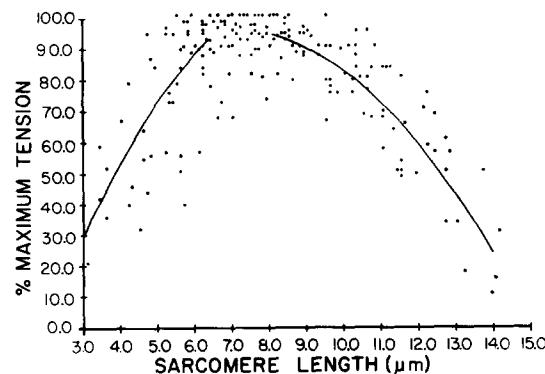


FIGURE 4 The length-tension relation determined for *Limulus* telson levator muscle bundles. The two lines are third-order regression lines calculated between sarcomere lengths 3–6.5 μm and 8–14 μm . The correlation coefficients are 0.673 and 0.857, respectively.

the passive tension was still small, 5–10% of the maximum active tension of the bundle, and by 13–14 μm it reached at most 30%.

To compare them with other preparations, such as frog muscle, in which the sarcomeric dimensions are significantly different, these length-tension data have to be normalized. This had been done by expressing the sarcomere lengths as a fraction of the L_0 length (6.5 μm) and comparing them to those of frog (Fig. 5) (9). From this comparison, it was clear that *Limulus* muscle has a significantly wider length-tension curve than frog muscle. The plateau region was similar to that of frog but both the short and long sides of the curve differ from that seen in the frog muscle.

It could be argued that this difference is attributable to sarcomere length nonuniformity within the muscle. In these preparations, however, the laser diffraction images from over 90% of the length of the bundle showed that the sarcomere lengths in these different regions varied by $<0.5 \mu\text{m}$. In another series of experiments we examined the extreme ends of the bundles. The results of some of these experiments are given in Table I. It was clear that there was greater variation in the extreme ends than in the majority of the bundle. However, the

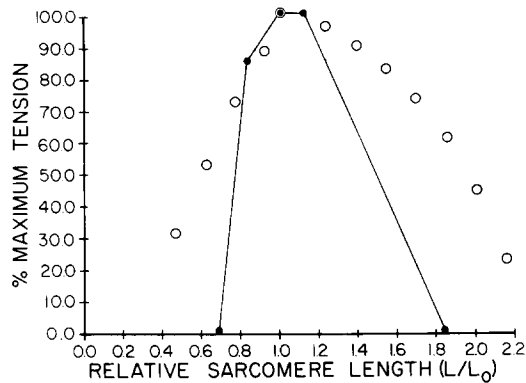


FIGURE 5 A comparison of the length-tension curve for frog (solid circles) and *Limulus* (open circles). In both cases the sarcomere lengths have been normalized by expressing them as L/L_0 where L_0 in frog is 2.0 and in *Limulus* is 6.5 μm . Note that *Limulus* muscle can develop significant tension over a relatively greater range of sarcomere lengths.

TABLE I
Sarcomere Length Uniformity along Bundles

Date	Origin	Middle	Insertion
	μm	μm	μm
8/5/77, II	10.1	9.9	9.9
	12.1	12.1	12.1
	15.9	14.5	13.7
8/5/77	10.0		10.9
	11.1	11.6	11.1
	12.5	12.5	12.8
	13.3	12.5	12.5
8/24/77	10.7	9.2	9.0
	10.4	10.0	11.0
	11.8	12.1	12.4
	12.1	12.1	13.0
	7.4	5.9	5.2
	4.5	4.6	4.4
	4.7	3.8–3.9	3.5

variation was not consistent in magnitude and often did not appear at all.

DISCUSSION

One of the major purposes of length-tension determinations is to examine the relationship between sarcomeric structure and the tension that it can produce. In all these experiments, however, the tension produced by a large population of sarcomeres is measured while the sarcomeric structure of only a fraction of the population is determined either visually (9) or by laser diffraction. Therefore, it is important to determine the degree of uniformity of sarcomere structure in the whole bundle. In these experiments this was possible because we used living, intact bundles with tendon on both sides. Because we included bundles in the sample only if their sarcomere lengths were uniform (i.e., within 0.5 μm) over 90% of their length, we are confident of that degree of uniformity. Our experiments show that often, but not always, there was some sarcomere length nonuniformity at the extreme ends of the bundles. The variation seldom exceeded 10% of the length measured in the middle of the bundle and was variable in its appearance from bundle to bundle and from run to run on the same bundle. However, the number of sarcomeres involved is small when compared to the total number present in the entire bundle. Because the total amount of tension produced by this muscle is large even at long or short lengths, it seems unlikely that this small population of sarcomeres could greatly affect the tension response of the entire bundle. It could also be argued that the laser image provides an average sarcomere length measurement and that there could be small populations of very short sarcomeres that were not observed. We believe that the large number of layer lines and their sharpness argues against this, particularly in stretched bundles. Therefore, we believe that we can relate the tension produced by the whole bundle to the sarcomere structure determined by our sampling method.

With the resolution of our technique we can differentiate three parts of the length-tension curve: the plateau, the descending limb as sarcomeres get shorter, and the descending limb as the sarcomeres get longer. In *Limulus* the plateau is that region, from 6.5- to 8.0- μm sarcomere length, where maximum tension is developed. In frog muscle, Gordon et al (9) interpreted this region to extend from where there was maximum overlap between thick and thin filaments to the shorter sarcomere length where thin filaments from opposite Z-disks begin to interfere. In *Limulus* this does not seem to be the case; L_0 seems to occur when the thick filaments are at their maximum length and yet are still relatively aligned with each other (6). At this point, the overlap of the thin filament over the thick is only ~68% of the maximum possible. Thus, there is an extensive region of thick filament that cannot interact with the thin filaments. The right end of the plateau occurs in *Limulus* when there is less overlap and when relative skewing of the thick filaments begins to occur. The exact point at which tension begins to decline is difficult to determine, and this does not seem to occur at a particularly precise point.

As the sarcomeres shorten below 6.5 μm in length, the thick filaments of the A-band also shorten (6, 7, 8). Thus, an I-band always appears to be present even though extensive sarcomere shortening has occurred. However, presumably as the thick filaments shorten, fewer myosin sites are able to interact with the thin filaments that surround them. This could explain the reduction in tension that one sees as the sarcomeres shorten from 6.5 to 5.0 μm . As the sarcomeres shorten further, the

length of the thick filaments is reduced and the thin filaments from opposite Z-disks begin to overlap. Thus, one might predict and one indeed observes that the tension declines. However, in comparison to frog muscle, *Limulus* muscle develops relatively more tension at these shorter lengths (see Fig. 5) and, in fact, develops significant tension at relative sarcomere lengths at which frog muscle is unable to develop tension. This would be the expected result if active tension development by the frog muscle were prevented by interaction between the thick filaments and the Z-disk. In *Limulus* muscle, because the thick filaments shorten, there is always an I-band and the thick filaments do not limit sarcomere shortening.

As *Limulus* bundles are stretched beyond 8.0- μm sarcomere length, the thick filaments remain at a constant length of 4.9 μm and are skewed relative to each other (6). Thus the I-band remains remarkably constant in width, whereas the A-band becomes wider because of the skewing of the thick filaments. Because the Z-lines are not similarly skewed, thick filaments (4.9- μm long) cannot interact with the thin filaments (2.4- μm long) in one half of the sarcomere at long (10- μm or more) sarcomere lengths. However, the length-tension data clearly show that the muscle can develop significant tension (50% of maximum) at long (12- μm) sarcomere lengths. Therefore some connection must exist across the sarcomere. The evidence for the thin filament length comes from the determination that double overlap occurs at a sarcomere length of 4.8 μm , that extraction of thick filaments leaves I-segments with central Z-disks that are 5.0- μm wide (8), and that Fourier reconstructions of laser-diffraction images also show double overlap to occur at a sarcomere length of 4.8 μm (6). This evidence, however, does not preclude there being a population of thin filaments that are longer than 2.4 μm . This nonuniformity of length is known to occur in some vertebrate muscles (14). However, if there is a population of long, thin filaments, what happens at short sarcomere lengths? One would expect to see some disorder in the I-band, particularly if the thin filaments were up to 4.0- μm long, which is the length necessary to ensure thick-thin filament interactions at both ends of each thick filament in a 12.0- μm -long sarcomere. This disorder is not observed (6), and thus a small population of long, thin filaments seems to be an unlikely explanation for the tension at long lengths.

A more plausible hypothesis is that there is some interaction between adjacent thick filaments in the center of the A-band. Such an interaction would provide a "bridge" effectively creating a very long thick filament out of two. The interaction could involve electrostatic forces between the filaments or possibly some thick-thin filament interaction. In many tonic muscles, including *Limulus*, there is no M-band and a clear H-zone is not visible in longitudinal sections (6). Close examination of these regions in sections often reveals short lengths of material similar to the thin filaments. It could be that these are short segments of thin filament, unattached to the Z-disk, which "couple" the thick filaments at long sarcomere lengths. Such a coupling could account for the large tension development at long sarcomere lengths in *Limulus* muscle. It is also possible that some other constituent, such as connectin (13), acts to link the sarcomeric structure together at long lengths. However, if such structures do exist, they must make a small contribution to passive tension (resistance to stretch), because in *Limulus* muscle passive tension becomes measurable only above 9- to 10- μm sarcomere length and never exceeds 30% of active tension even at long (13- μm) sarcomere lengths.

It is assumed from the sliding-filament model (9, 10, 11) that tension is developed by an energy-requiring interaction be-

tween thick and thin filaments. In *Limulus* we have no evidence to suggest that this is not so here as well. However, another tension-generating mechanism must be present. We know that the *Limulus* thick filament length can vary and that it can exist at lengths between the extremes, depending on the sarcomere length (8). During muscle activation, the interaction between thick and thin filaments would effectively pull the end regions of each thick filament with respect to the central region. Because during an isometric contraction the A-band and thick filaments remain at a constant length, the thick filaments themselves, to maintain the constant length, must be able to generate a force at least equal and opposite to that produced by the thick and thin filament interaction. Whether this intra-filament force is energy requiring or is some mechanical molecular "catch" remains to be seen. Because in vitro shortening of thick filaments requires ATP and because the nonhydrolyzable analogue, adenylyl imidodiphosphate, is ineffective (2), it is likely that the length change requires the hydrolysis of ATP. Thus, in these muscles there may be an additional tension-developing system beyond the thick and thin filament interaction.

A length-tension diagram similar to what we have obtained for *Limulus* was obtained earlier for crayfish single fibers (1). In this system, caffeine contractures were obtained at different sarcomere lengths, but otherwise the curve is almost identical to that for *Limulus*. L_0 is at ~ 6.5 μm , and the plateau extends to ~ 8 μm . Significant tension is generated over a sarcomere length range of 5-13 μm , with 50% maximum tension or more being developed from ~ 5 or less to 11.5 μm . These tonic crayfish fibers are structurally similar to those of the *Limulus* telson muscle (12) and have a similar extensive range of sarcomere lengths. This gives us confidence that our data, even though they were obtained from small bundles of fibers, are valid and that the phenomena we have observed are of general interest.

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