ANOMALOUS CONTRACTION OF

INVERTEBRATE STRIATED MUSCLE

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ABSTRACT

The phenomenon of A band shortening or contraction has been investigated in glycerinated myofibrils of *Pecten irradians*, *Homarus americanus*, *Cambarus virilis*, and *Limulus polyphemus* through the techniques of ultraviolet microbeam inactivation and polarization microscopy. With the former method, it has been shown that these muscles, even though exhibiting the shortening effect, contract in a manner consistent with only the sliding filament model. Intrinsic birefringence studies have indicated no significant changes in mass distribution or orientation within the shortened A bands. Except in the case of *Limulus* muscle, the shortening effect was seen only in contraction under tension. The magnitude of this anomalous phenomenon was dependent upon glycerination time and has been duplicated in rabbit psoas muscle through brief trypsin treatment. A band shortening could not be observed in glutaraldehyde-fixed muscle or in myofibrils glycerinated for only short periods. It has been concluded that the phenomenon of A band contraction is an artifact induced by the glycerination procedure, possibly through weakening of the sarcomere structure. However, the fact that the A band shortens under tension rather than lengthens poses an interesting paradox.

INTRODUCTION

Implicit in the classical Hanson-Huxley sliding filament model for muscle contraction (13, 14) is the requirement that the A band remain constant regardless of sarcomere length. It has been shown recently, however, that the A band in glycerinated myofibrils decreases with decreasing sarcomere length in several phyla of invertebrates. De Villafranca (4) has observed this phenomenon in the horseshoe crab *Limulus polyphemus*, Baskin and Weise (1) in the giant barnacle *Balanus aquila*, Gilmour and Robinson (9) in the locust *Gastrimargus musicus*, and Sanger and Szent-Györgyi (19) in the swimming scallop *Pecten irradians*. This anomaly may also be seen in glycerinated myofibrils of the lobster *Homarus americanus* and the crayfish *Cambarus virilis* (22).

In cases in which detailed quantitative measure-

ments were carried out (i.e. *Limulus* (5), *Pecten* (19), *Homarus* and *Cambarus* (22)), plots of A and I band length *versus* sarcomere length showed sliding filament behavior above rest-length, while below that point most, if not all, of the changes in the length of the sarcomere were due to changes in the A band length.

De Villafranca and coworkers (6, 7) claim the A band of *Limulus* to be made up of actomyosin thick filaments rather than separate filaments of actin and myosin as commonly observed in vertebrate muscle. They suggest further that contraction of the sarcomere is dependent upon shortening of such actomyosin filaments (5). Their conclusions are based on the observation of only one type of filament in the A band of osmium tetroxide–fixed *Limulus* fibers (6) and on the fact that acto-

myosin, rather than myosin alone, can be extracted from the A band (7). However, employing the more recent glutaraldehyde fixation technique, Sanger (18) has shown both thick and thin filaments in the A band of *Limulus* and thus casts doubt on de Villafranca's interpretation.

The A band contraction report of Baskin and Weise (1) has been disputed by McAlear *et al.* (16) who, employing glycerinated *Balanus nubilus* fibers, have shown that the phenomenon was due to the thick filaments going out of register and thus giving the unusual band patterns reported by the former workers. Previous to the Baskin and Weise paper, Hoyle and McAlear (11) showed that supercontraction in *Balanus* was due to penetration of the Z disk by the thick filaments, with the thick filaments remaining of constant length; the discrepancy has been attributed to a preparation artifact (16).

Gilmour and Robinson (9) have attempted to explain their results in terms of stretching and coiling of thick filaments at the center and ends of the A band, such that their mechanism falls within the framework of the sliding filament model. They base this reasoning on an increase in density at the A band center and edges upon ATP contraction of *Gastrimargus* myofibrils; the resulting bizarre band patterns are surprisingly reminiscent of those reported by Baskin and Weise (1).

In the case of vertebrate muscle, an interesting example of A band contraction has been reported by Galey (8) for frog semitendinosus muscle fixed during stimulation; varying degrees of contraction were found within such a fiber. The length of the thick filaments decreased while the diameter of the filament increased with decreasing sarcomere length. This work essentially duplicates that of Sjöstrand and Andersson-Cedergren (21) carried out 7 years earlier. A. F. Huxley and Gordon (12) concluded that the effect reported by Sjöstrand was due to passive contraction or compression of sarcomeres in unstimulated fibrils adjacent to an actively contracting fibril. Recent work by Sjöstrand (20) shows constant A bands but marked increase in density at the A band ends, much the same as observed by Gilmour and Robinson (9) and Baskin and Weise (1) for locust and barnacle, respectively. Corsi and Perry (3) have observed anomalous band patterns and variation in A band length in both fresh and glycerinated rabbit psoas muscle, but the evidence here is difficult to interpret.

Under the contractile filament view of de Villa-

franca (5), contraction of the sarcomere and shortening of the A band must occur concomitantly since shortening of actomyosin filaments is thought to be responsible for contraction. The author (23) has presented previously a method for distinguishing among current contraction models through the use of an ultraviolet microbeam to locally inactivate regions within an individual sarcomere. Such a technique can be used to investigate this anomalous shortening phenomenon, first, in terms of plausible contraction mechanisms and, second, in regard to the question of dependence of sarcomere contraction upon A band shortening. The microbeam technique offers the added advantage of providing, within a single myofibril, both contracted and non-contracted sarcomeres for a comparative control.

If the thick filaments go out of register as postulated by McAlear *et al.* (16), then the apparent shortened A band would be that region in which the filaments are still coincident, with their ends extending into the I region. Gross changes in molecular orientation and mass distribution must occur under the mechanism put forth by Gilmour and Robinson (9) since the filaments are thought to coil or bend at the center and ends of the A band. Both out-of-register filaments and coiled filaments should be detectable through the use of polarization microscopy, particularly under matched refractive index conditions where only the intrinsic birefringence of the filaments is measured. Thus, coiling, stretching, and redistribution of mass should be evident through changes in intrinsic birefringence within the A band.

EXPERIMENTAL

Myofibrils

The adductor of *Pecten irradians*, the dorsal muscle between the cephalothorax and abdomen of *Limulus polyphemus*, the tail extensors of *Homarus americanus* and *Cambarus virilis*, and the rabbit psoas were glycerinated at various lengths in 50 per cent glycerol (Merck) for 12 hours at 0–4°C, placed in fresh 50 per cent glycerol, and then stored at −20°C. The muscles were homogenized in low-salt buffer with a high-speed blendor and used immediately. The low-salt buffer consisted of 0.1 M NaCl, 0.001 M $MgCl_2$, and 0.01 M phosphate buffer, pH 7.0.

Polarization Microscopy

The high-resolution rectified polarizing microscope of Inoué (15) was employed for these studies. In order to eliminate form and edge birefringence, the

refractive indices of *Pecten* and *Homarus* myofibrils were matched to that of the surrounding medium, using *o*-toluidine according to the method of Colby (2). The myofibrils were fixed in buffered 6 per cent glutaraldehyde, and then dehydrated with graded ethanols and finally with acetone before imbibition with the matching solvent. Photographs were taken on Kodak Tri-X and developed with Microdol-X. Slight positive compensation was introduced by means of a rotating mica compensator.

Ultraviolet Microbeam

The phase contrast microbeam apparatus, light source, and narrow band-pass filters used were identical to those previously described (23). Irradiations were carried out in low-salt buffer on 0.17-mm quartz coverslips with exposures of 45 to 60 seconds at a wavelength of 270 mμ and an energy of 1 × 10^{11} quanta/sec./μ^2. After irradiation, contraction of the myofibrils was obtained by diffusion of 2 mM ATP in low-salt buffer into the preparation.

Trypsin Digestion

Commercial (Worthington) 2X recrystallized trypsin and soybean trypsin inhibitor were employed. The trypsin was used at a concentration of 0.03 per cent and was prepared immediately before use by dissolving in low-salt buffer. The trypsin inhibitor was used at a concentration of ½ per cent in the same buffer. Myofibrils were exposed to trypsin for periods up to 1 minute; the action was stopped by perfusion of the trypsin inhibitor under the coverslip.

RESULTS

General Observations

When glycerinated muscle of *Pecten*, *Homarus*, and *Cambarus* at a variety of lengths between extreme stretch and complete contraction was homogenized, a variation in A band length, proportional to sarcomere length, was observed for all degrees of contraction below rest-length under conditions in which I bands were still present. Rest-length for *Pecten* adductor was considered to be that point at which the shell barely closed, and for *Homarus*, *Cambarus*, and *Limulus* it corresponded to that point at which the animal was fully extended. The *Pecten* A bands varied from 2.2 μ at and above rest-length to 0.9 μ for the most contracted sarcomeres; the A bands of *Homarus* and *Cambarus* myofibrils varied from 3.0 to 1.0 μ and from 3.2 to 1.5 μ, respectively. Beyond these lower limits, the I bands disappeared and C$_z$ bands formed. The results obtained for *Pecten* were in complete agreement with those reported by Sanger and Szent-Györgyi (19).

When viewed under the rectified polarizing microscope at the matching refractive index of 1.569, no inhomogeneous distribution of birefringence was evident in the A bands of myofibrils of either *Pecten*, *Homarus*, or *Cambarus* at different degrees of contraction or stretch. This is illustrated for *Pecten* and *Cambarus* in Fig. 1. The retardation of these myofibrils, all roughly 1 μ in thickness, varied randomly from 1.8 to 2.2 mμ, regardless of A band size. No birefringent material was observed extending beyond the A region, and the A band size was the same (within the limits of resolution) whether observed under phase contrast, interference, or polarization microscopy.

When 2 mM ATP was added to myofibrils of *Pecten*, *Homarus*, or *Cambarus*, regardless of the original A band length, the A bands shortened further only when the myofibrils were under tension from the adhesion of both ends of the myofibril to the coverslip. Under similar conditions, *Limulus* myofibrils showed marked A band shortening in free contraction but the A bands shortened more so under tension. *Limulus* myofibrils, like the other myofibrils under matched refractive index conditions, showed homogeneous distribution of birefringence but, since the thickness and shape of the myofibrils varied considerably, no attempt was made to quantitate the observations.

Ultraviolet Microbeam Studies on Glycerinated Myofibrils

Under free ATP-induced contraction, *Pecten* muscle showed typical sliding filament band patterns. When a myofibril was irradiated across the A band, full A band irradiation prevented contraction of the sarcomere (Fig. 2 *a* to *c*) while contraction occurred at the non-irradiated lateral edges or ends of the A band (Fig. 2 *c*, arrows). Full I band irradiation prevented contraction of the adjacent half-sarcomeres but permitted symmetrical C$_m$ band formation (Fig. 2 *c*). Such results are consistent with only the sliding filament model since they demonstrate independence of the half-sarcomere and free movement of I filaments into the A band, thus eliminating from consideration those models postulating actin attachments at (17) or across (24) the H zone.

As mentioned previously, *Pecten* muscle under tension shows the A band shortening phenomenon. This can be most conveniently demonstrated in single myofibrils through contraction against a non-contracted section of the myofibril as obtained
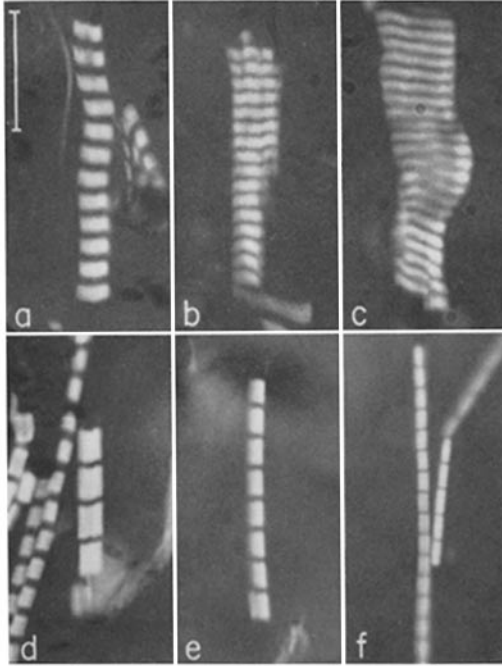
Figure 1 Myofibrilis of *Pecten irradians* (a to c) and *Cambarus virilis* (d to f) at increasing degrees of contraction and A band shortening. Rectified polarization microscopy; refractive index: 1.569. Positive compensation; A bands bright. Scale marker = 10 μ. × 1,500.

by means of a longitudinal irradiation (Figure 2 *d* to *f*). The A bands of sarcomeres adjacent to the tension-creating region have shortened from 2.0 to 1.2–1.5 μ (arrows); those farther to the right of this region have shortened to a lesser extent and show C_m and C_z bands. A full A band irradiation has been included to give a non-contracted sarcomere for comparison. In ribbon-like myofibrils such as those of *Pecten*, variation in focal level was evident; this factor was negligible in these experiments since "optical sectioning", *i.e.* focusing through the myofibril, revealed the same band patterns regardless of depth.

In free contraction, partial A and full I band irradiations of *Homarus* myofibrils have the same effects as above, namely, contraction at the non-irradiated lateral edge or end of the A band and prevention of contraction in the half-sarcomeres adjacent to the irradiated I region (Fig. *3 a* to *c*).

In ATP-induced contraction under tension through adhesion to the coverslip, the effect of I band irradiation in *Homarus* was the same as in free contraction (Fig. 3 *d* to *f*); C_m bands were

clearly seen. Partial A band irradiation prevented both contraction and A band shortening in the irradiated half-sarcomere, while central irradiation of a 3.0-μ A band with a 1.5-μ microbeam permitted contraction of the sarcomere but only partial A band shortening (3.0 to 2.5 μ). The non-irradiated sarcomeres, on the average, showed an A band shortening effect of 3.0 μ before contraction to 1.8 μ after contraction but before C_m band formation. The results of similar irradiations for *Cambarus virilis* were identical to those of *Homarus*.

Performing the same kind of irradiations on *Limulus*, one sees results paralleling those for *Homarus*. Fig. 4 illustrates these effects for a muscle glycerinated at $-20°C$ for 4 months (*a* to *c*) and another for only one week (*d* to *f*). In the first case, one can again see independent contraction of the half-sarcomere and shortening of the A band in the partial A band irradiation, while the I band irradiation prevents contraction of the adjacent sarcomere but does not show C_m band formation. It should be noted at this point that the irradiated area of the A band in Fig. 4 *c* shows higher density after irradiation than the non-irradiated region and that the shortened (non-irradiated) region has markedly decreased in volume compared to the non-contracted (irradiated) side. In the second case (Fig. 4 *d* to *f*), the 1-week glycerination, it is illustrated that one can observe contraction even after ⅛ of the A band has been irradiated. In this preparation, C_m bands are clearly visible. Very little shortening of the A band is observed and one cannot detect the irradiated area through high density.

Thus, these apparently anomalous muscles contract in accord with the sliding filament model in spite of the fact that the A bands shorten, since independence of the half-sarcomere, contraction of the half-sarcomere without appreciable A band shortening, and C_m band formation can all be demonstrated.

In light of the variation of band patterns observed in *Limulus*, it became apparent that the preparative precedure should come under close scrutiny.

Short-Term Glycerination, EDTA Treatment, and Glutaraldehyde Fixation

If fibers of *Pecten*, *Homarus*, *Cambarus*, or *Limulus* are glycerinated at 0–4°C for only 1 hour and then homogenized in low-salt buffer containing 0.01 M EDTA, *no shortening of the A band can be ob-*

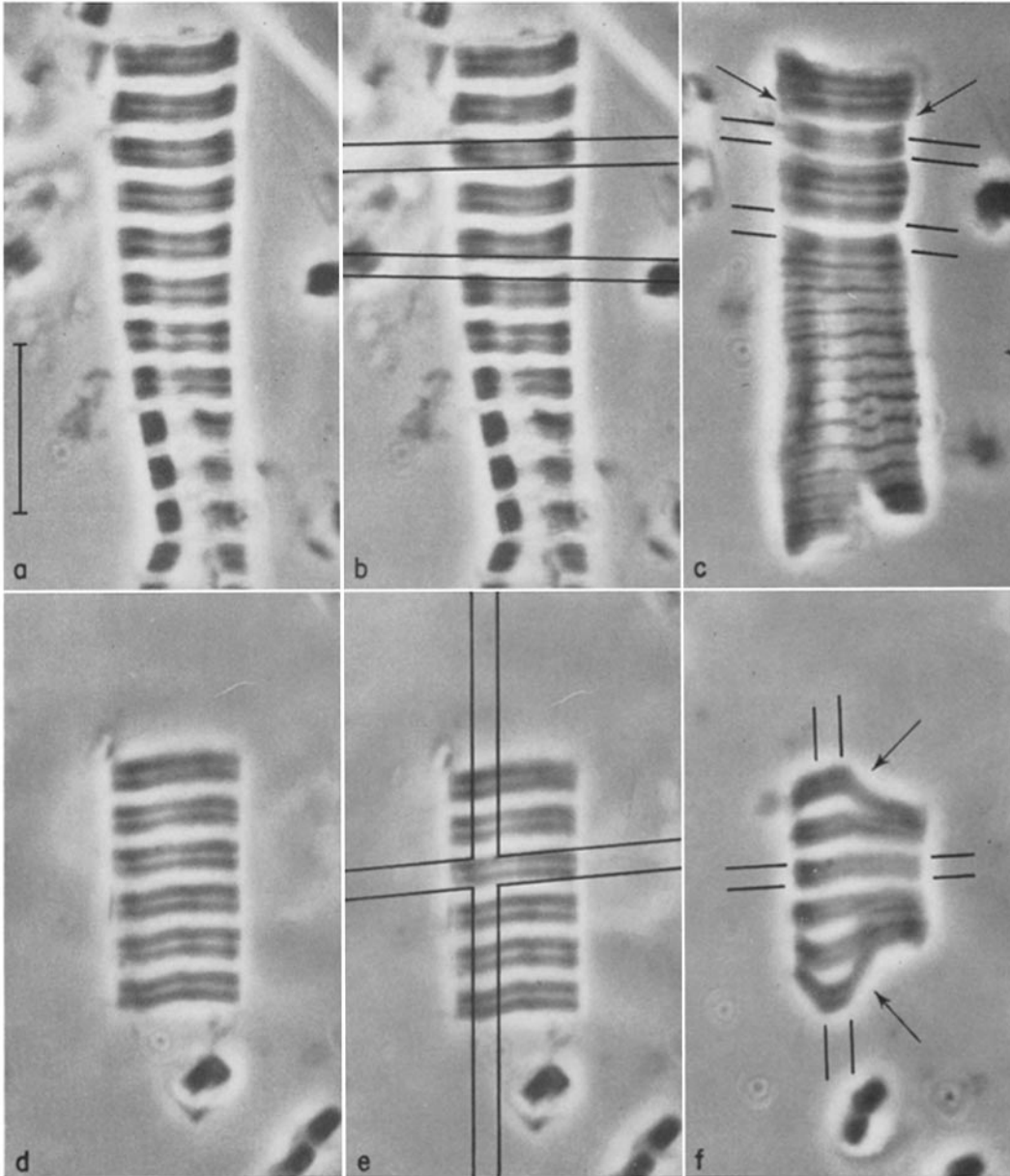THE JOURNAL OF CELL BIOLOGY · VOLUME 27, 1965

FIGURE 2 *Pecten irradians* myofibrils under free contraction (*a* to *c*) and contraction against a tension-creating region (*d* to *f*).

a, Myofibril before irradiation.

b, Irradiation areas. Full and partial A band; full I band.

c, After ATP contraction. Note contraction at the non-irradiated lateral edges (arrows) of the A band in the upper irradiation and prevention of sarcomere contraction under full irradiation; heavy C_m bands are evident in the lower full I band irradiation.

d, Myofibril before irradiation.

e, Irradiation areas. Longitudinal sarcomere irradiation and full A band.

f, After ATP contraction. Note shortening of A bands (arrows) adjacent to the longitudinal irradiation but the C_z and C_m bands further to the right of this region. Phase contrast; scale marker $= 10\ \mu$. $\times$ 2,300.
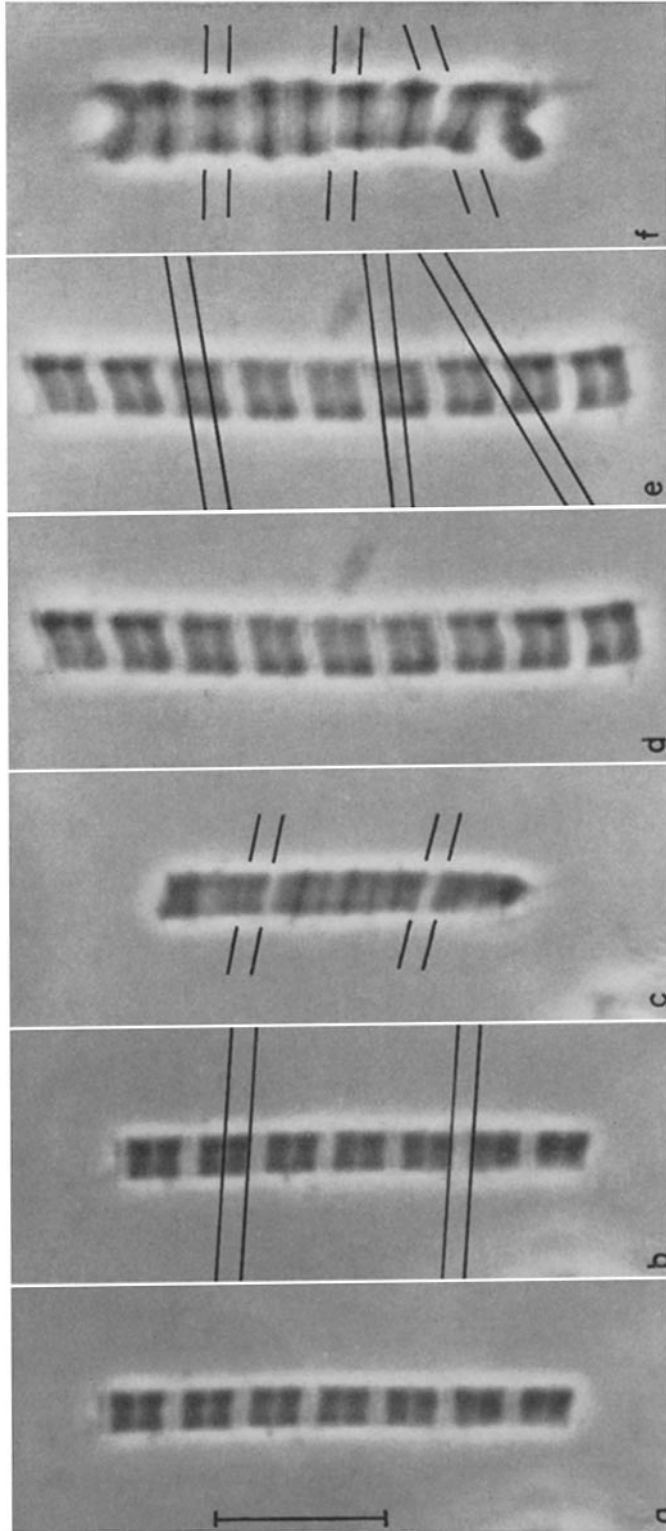
FIGURE 3 *Homarus americanus* myofibrils under free contraction (*a* to *c*) and contraction under tension (*d* to *f*).

a, Myofibril before irradiation.

b, Irradiation areas. Half A band and full I band.

c, After ATP contraction. Note contraction of the non-irradiated half-sarcomere and the formation of C_m bands in the sarcomeres adjacent to the irradiated I region.

d, Myofibril before irradiation.

e, Irradiation areas. Central A band, half A band, and diagonal I band.

f, After ATP contraction. Note contraction of the centrally irradiated sarcomere with formation of C_m bands but almost no decrease in A band length. Half A band irradiation permitted contraction at the non-irradiated lateral edge, while I band irradiation allowed C_m band formation. Phase contrast; scale marker = 10 μ. $\times$ 2,200.

FIGURE 4 *Limulus polyphemus* myofibrils glycerinated at −20°C for 4 months (*a* to *c*) and for 1 week (*d* to *f*).

a, Myofibril before irradiation.
b, Irradiation areas. Half A band and full I band.
c, After ATP contraction. Note contraction and A band shortening in the partial A band irradiation and the absence of C_m bands adjacent to the full I band irradiation. z lines have been labeled for clarity.

d, Myofibril before irradiation.
e, Irradiation areas. ⅞ A band and full A band.
f, After ATP contraction. Note contraction at non-irradiated lateral edge of the partially irradiated sarcomere and prevention of contraction in the full A band irradiation. C_m bands and nearly constant A bands are evident. Phase contrast; scale marker = 10 μ. × 1,400.

served, regardless of the sarcomere length, with or without tension. Fig. 5 illustrates all stages of contraction in Homarus myofibrils, showing constant A bands and band patterns consistent with the sliding filament model. These patterns serve as a striking comparison to those of Fig. 3 f. If the short-term glycerinated fibers are returned to the glycerol at 0–4°C and homogenized 12 hours later, the A band contraction phenomenon is easily demonstrable. If previously glycerinated rabbit psoas muscle is incubated for 12 hours at 0–4°C in the used glycerol from the invertebrate glycerinations, short A bands may be observed when compared to non-incubated glycerinated rabbit psoas myofibrils. Thus, the A band contraction effect is dependent upon glycerination time, and the anomaly can be induced in rabbit psoas with "used" glycerol.

Even at −20°C, a time-dependent effect is observed. Fig. 4 d to f illustrates Limulus muscle glycerinated for 1 week; its band-pattern changes and degree of A band shortening are significantly different from these same effects in the 4-month glycerination (Fig. 4 a to c).

No shortening of the A band can be observed in any of the aforementioned muscles when fixed under tension with 6 per cent buffered glutaraldehyde. As mentioned above, glutaraldehyde-fixed, glycerinated myofibrils of Pecten and Cambarus under matched refractive index conditions showed a retardation of 2 mμ. Fixed, non-glycerinated myofibrils of these same species (of approximately equal thickness) have a retardation 50 to 75 per cent higher than their glycerinated counterparts.

Trypsin Digestion of Glycerinated Rabbit Psoas Myofibrils

Because of the action of "used" glycerol in inducing A band contraction in rabbit psoas, enzymatic action was investigated as a source of the anomaly.

Glycerinated rabbit psoas myofibrils were treated with 0.03 per cent trypsin for 1 minute and the action stopped with ⅓ per cent trypsin inhibitor. The myofibrils were irradiated in the full A band and ¾ A band and then subsequently contracted with 2 mM ATP. The non-irradiated A bands shortened considerably (Fig. 6) and the irradiated areas remained of relative higher density when compared to the shortened A bands (cf. Fig. 4 c), mainly due to the increase in width of the non-irradiated A bands. Full A band irradiation prevented both contraction of the sarcomere

and A band shortening, while partial A band irradiation permitted contraction at the non-irradiated lateral edge. The results were the same whether trypsin treatment followed or preceded the irradiation.

DISCUSSION

Under free, ATP-induced contraction, glycerinated myofibrils of Pecten, Homarus, and Cambarus show typical Hanson-Huxley band pattern changes. However, these same myofibrils when under tension, as in glycerination at various fixed lengths or through attachment to the coverslip in ATP contraction, show the anomalous A band shortening phenomenon. In myofibrils glycerinated at different lengths, the intrinsic birefringence of the A band remains essentially constant and homogeneous regardless of the sarcomere length, implying no significant changes in orientation or localization of mass within the A band. This result could be explained in terms of a uniform shortening and thickening of the myosin filaments with concomitant increase in A band thickness or decrease in orientation to offset any increase in mass which would occur in shortening; such an effect would not then be detectable with polarization optics but would explain the constancy of intrinsic birefringence. No significant amount of positively birefringent material extending beyond the edges of the A band is detectable and, considering the fact that phase-contrast, interference, and polarization microscopy all indicate identical A band lengths, it is reasonable to conclude that no appreciable number of thick filaments are out of register in the shortened A bands of the muscles studied. This does not discount, of course, the explanation of McAlear et al. (16) in the case of Balanus.

All of the muscles studied behave in a manner consistent with only the Hanson-Huxley model when subjected to ultraviolet microbeam analysis since contraction can be observed in a fractional sarcomere (23). Full I band irradiation prevents contraction of the irradiated half-sarcomeres but allows C_m band formation, presumably through release of I filaments at the Z line. Myofibrils irradiated in this manner frequently show a widening of the I region along with C_m band formation in the adjacent sarcomeres, resembling an effect reported by Hanson (10) for Calliphora myofibrils broken at the Z line. The A band shortening phenomenon is consistently seen in myofibrils con-
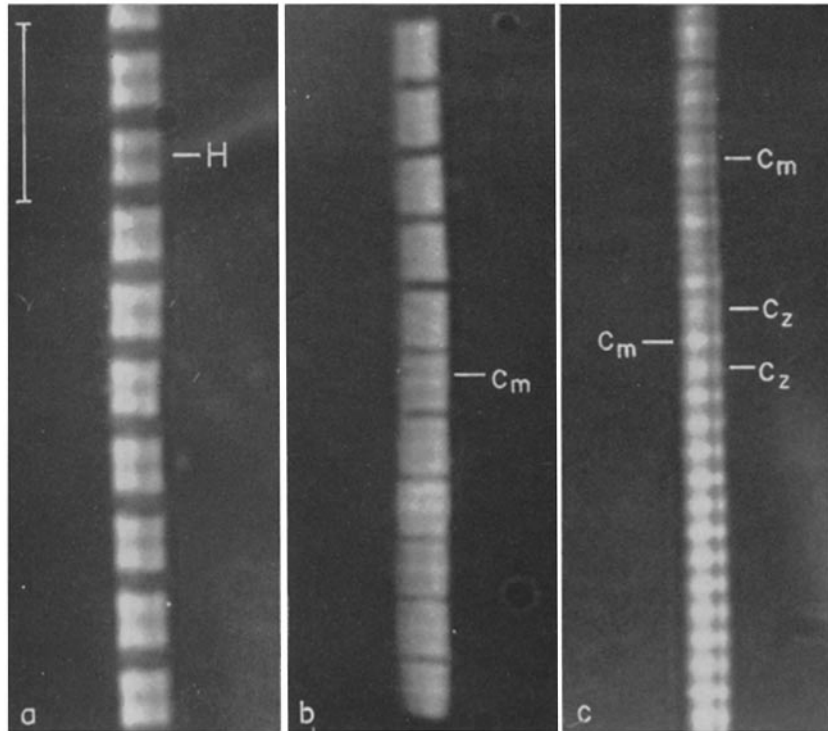
FIGURE 5   *Homarus americanus* myofibrils glycerinated for 1 hour and homogenized in low-salt buffer containing 0.01 M EDTA. The A bands are constant, regardless of the degree of contraction. H zones are evident in *a*, C_m bands in *b*, and both C_m and C_z bands can be seen in *c*. Interference microscopy; scale marker = 10 μ. X 2,300.

tracting under tension. Full A band irradiation prevents both contraction of the sarcomere and A band shortening, while $\frac{3}{4}$ or $\frac{7}{8}$ A band irradiation permits complete contraction at the non-irradiated lateral edge or end of the A band but prevents nearly all shortening in the A band. Thus, it is clearly possible to have full contraction of the half-sarcomere without appreciable A band contraction.

The A band length of myofibrils glycerinated at fixed length is dependent upon glycerination time (Fig. 1 *d* to *f* versus Fig. 5). Brief glycerol treatment produces myofibrils which maintain constant A bands even under tension; fixation of fresh tissue with glutaraldehyde likewise produces A bands of constant length. In *Limulus* myofibrils, in which A band shortening is seen even under no tension, the type of band pattern observed depends also upon the length of glycerination. In Fig. 4 *a* to *c*, the edges of the A band show higher density after contraction, an effect comparable to that reported

by Gilmour and Robinson (9) and Baskin and Weise (1). In addition, C_m bands are not observed in such *Limulus* myofibrils. In the shorter glycerination (Fig. 4 *d* to *f*), however, the A bands remain uniform in density, even in the irradiated areas, heavy C_m bands are evident, and little shortening of the A band is seen.

The anomalous A band shortening phenomenon can be duplicated through brief trypsin treatment of glycerinated rabbit psoas myofibrils, followed by ATP contraction, implying that partial enzymatic digestion of the sarcomere might be the cause of the shortening A band. Indeed, all of the invertebrate muscles studied showed heavy bacterial contamination, even after washing with cold glycerol prior to the actual glycerination. This view is further supported by the fact that rabbit psoas will show the shortening effect after 12 hours' incubation in glycerol used previously for the invertebrate muscle. It is certainly conceivable that bacterial enzymatic action during the 0–4°C glyc-
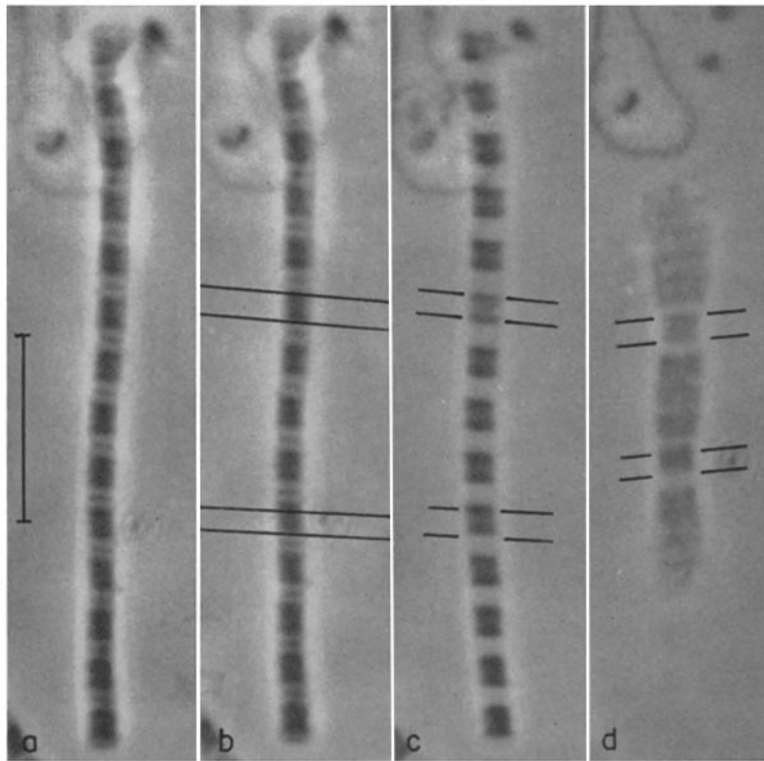
FIGURE 6 Contraction in trypsin-treated rabbit psoas myofibrils.

a, Myofibril before irradiation and trypsin treatment.

b, Irradiation areas. Full A band and ⅔ A band.

c, After 1-minute treatment with 0.03 per cent trypsin.

d, After ATP contraction. Note absence of contraction in the full A band irradiation and contraction at the non-irradiated lateral edge in the partial A band irradiation. The A bands show considerable shortening and the irradiated areas remain relatively phase dark. Phase contrast; scale marker = 10 μ. × 2,400.

erination period may weaken the integrity of the sarcomere in a manner similar to the trypsin treatment. The possibility remains, of course, that the glycerol itself also acts on the sarcomere structure in the course of the procedure.

In any event, the results presented here show that 1) the A band shortening phenomenon is a consequence of contraction and not its cause, 2) muscles showing this anomalous effect contract in a manner consistent with only the sliding filament model, and 3) the A band shortening phenomenon occurs only after glycerination with its magnitude depending upon the length of glycerol treatment.

While this effect appears to be an artifact, it poses the rather interesting question of why the presumably weakened A band shortens under tension instead of increasing in length as one might

surmise from physical reasoning. One possibility is that disruption of the integrity of the Z line through the combined action of glycerination and tension would permit segments of the Z disk to be pulled into the ends of the A band by the I filaments, thus causing a self-compression of the A band and also the formation of contraction bands at the A-I junctions. Under free (isotonic) contraction, however, there would be very little stress on the Z disk and the contraction might then proceed normally.

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