ANALYSIS OF MUSCLE CONTRACTION

BY ULTRAVIOLET MICROBEAM DISRUPTION

OF SARCOMERE STRUCTURE

R. E. STEPHENS

From the Department of Cytology, Dartmouth Medical School, Hanover, New Hampshire, and the Marine Biological Laboratory, Woods Hole, Massachusetts

ABSTRACT

In an effort to differentiate between the sliding filament theory for muscle contraction and alternative views which propose attachment between actin and myosin filaments at or across the H zone, rabbit psoas myofibrils were irradiated in various areas of the sarcomere with an ultraviolet microbeam. Irradiation of the I band appears to destroy the actin filaments; *in vitro* irradiation of F actin causes an irreversible depolymerization of the protein. Irradiation of the A band disorients the myosin but causes no apparent loss of dry mass. These effects are maximal at the wavelength of maximum absorption of the proteins involved. Actin filaments, released at the Z line of a sarcomere, are seen to slide into the A band on addition of ATP. Irradiation of a full A band prevents contraction, whereas irradiation of two-thirds of the A band, leaving a lateral edge intact, permits contraction at the non-irradiated edge. Thus contraction can occur in what is in essence only one-third of a sarcomere, eliminating any necessity for postulated H zone connections. These observations are in complete accord with the classical sliding filament theory but incompatible with either the contralateral filament hypothesis or the actin folding model for muscle contraction.

INTRODUCTION

By far the most widely accepted hypothesis for muscle contraction is the sliding filament theory of H. E. Huxley and Hanson (6) and A. F. Huxley and Niedergerke (3). In this model, actin filaments of the I bands interdigitate with myosin filaments of the A band, the movement occurring through some as yet undetermined cyclic actin-myosin interaction. This view is, in general, consistent with observations of ultrastructure, actin and myosin localization, and band pattern changes on contraction, but has not, until recently, offered adequate explanation of the unidirectional sliding of these filaments on activation. H. E. Huxley (4) has now shown a reversal of polarity in the myosin filaments at the center of the A band and a similar reversal in the actin filaments at the Z line, thus giving a molecular directionality to this model.

Alternative views, also consistent with protein localization and ultrastructure but having mechanical directionality, are the actin folding hypothesis proposed by Podolsky (12) and the contralateral filament theory of Szent-Györgyi and Johnson (16, 18). The Podolsky model postulates attachment of the ends of the actin filaments to myosin at the edge of the H zone; the contralateral filament theory proposes connections between the actin and myosin filaments across the
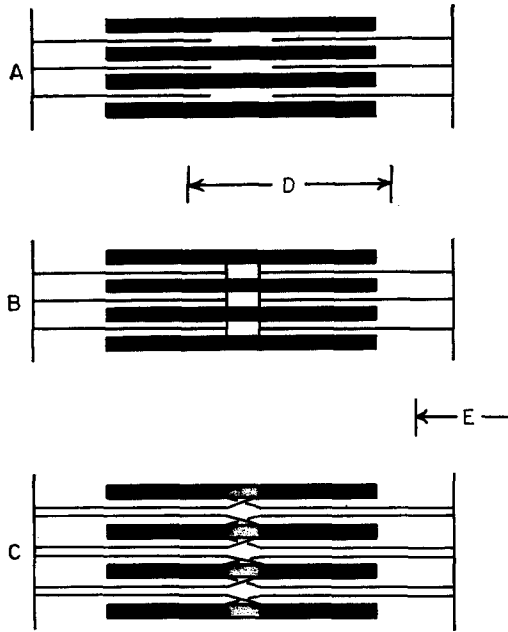
Figure 1 Comparison of the three current models of muscle contraction.

A. The classical Hanson-Huxley sliding filament model; by means of some unknown interaction, the I filaments move into the A band.

B. The Podolsky actin folding model; actin, attached to myosin at the H zone, folds within the A-I overlap during contraction.

C. The Szent-Györgyi–Johnson contralateral filament theory; migration of myosin toward the lateral edges of the A band draws in the contralaterally attached actin filaments.

D,E. Areas of ultraviolet irradiation employed in testing these models.

H zone. Contraction in the former model depends on folding of the actin filaments within the A-I overlap, whereas the latter theory involves a shift of myosin toward the lateral edges of the A band, drawing in the contralaterally attached actin filaments. These three models are compared in Fig. 1.

The Podolsky model implies constancy of the H zone. This has been described in the papillary muscle of the cat by Sonnenblick et al. (13), but H. E. Huxley has recently shown that the H zone of this muscle actually does increase with increasing sarcomere length and that the former authors were measuring the constant region of no side bridges on either side of the M line (5). At present there appear to be no bona fide examples of muscles wherein the H zone remains constant.

The contralateral filament theory is based on a quantitative shift in antimyosin labeling toward the lateral edges of the A band on contraction of chick myofibrils (17). This observation is difficult to reconcile under either the sliding filament theory or the actin folding model if this change in fluorescent antimyosin localization is actually measuring a shift in myosin concentration. However, employing the same antimyosin used in the above study, the present author (14) has shown that, in locally contracted sarcomeres, the shift in fluorescent antimyosin labeling occurs in the same half sarcomere in which contraction takes place. The contralateral filament theory would predict the opposite. It would be of considerable interest to determine independently which of these three models most clearly represents the actual case.

If, using stretched or rest length myofibrils, one could inactivate two-thirds of the A band of a sarcomere (area D in Fig. 1), leaving one lateral edge intact, this intact portion should, on addition of ATP, contract under the sliding filament theory, since only a small fraction of the A band would be needed for contraction. If the I filaments could not penetrate the inactivated area, one would expect contraction band formation at the boundary of the inactivated region and only partial contraction at the intact edge. No contraction in such a sarcomere should be observed, however, under the contralateral filament theory, since the mechanical connections necessary for contraction would no longer be operative and, furthermore, the myosin needed for contraction of the intact edge of the sarcomere would be in the inactivated side.

If such a method were used to test the actin folding model, one might argue that the I filaments reattach and fold within the remaining intact area, and thus one could not differentiate between this model and the sliding filament theory. Therefore, as a more definitive test, if the I filaments could somehow be released from the Z line (E in Fig. 1), on addition of ATP no movement of the A band toward the Z line in that half sarcomere should be observed under any of the theories. However, under the sliding filament and contralateral filament theories, the H zone should close symmetrically as the released I filaments either slide into, or are drawn into, the A band. The Podolsky model would suggest that the severed filaments should simply attach and fold, giving either no decrease in H zone width or else an asymmetrically positioned H zone.
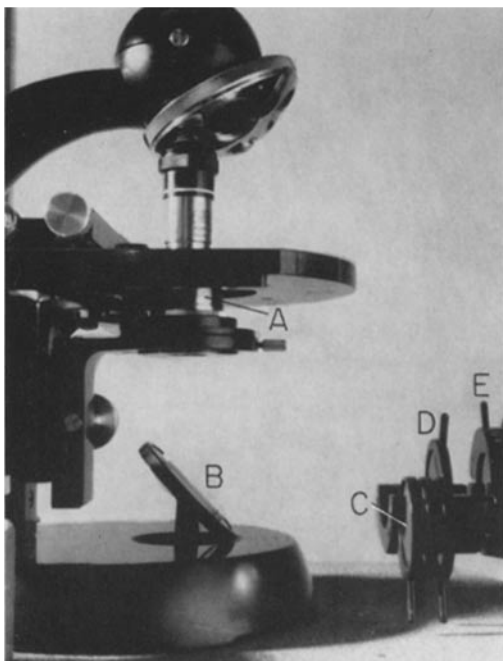
Figure 2 Ultraviolet microbeam apparatus. *A*, reflecting condenser with phase annulus; *B*, first-surface mirror; *C*, focusing cross-hairs; *D*, aluminum slit diaphragm; *E*, UV-blocking filter.

Through the use of the ultraviolet microbeam technique, the selective disruption and inactivation of fractions of a sarcomere can be accomplished.

METHODS

Microbeam Apparatus

Ultraviolet microbeam irradiation was carried out under either a phase contrast or a rectified polarizing microscope (7) employing the apparatus illustrated in Fig. 2.[1] An Osram HBO 200 mercury arc housed in a Wild illuminator fitted with quartz optics served as both a visible and an ultraviolet light source, eliminating the need for alignment of two sources and thereby greatly simplifying the method. An American Optical 50X reflecting objective (NA 0.56) served as a condenser. Used with a standard Zeiss 100X phase contrast objective, the aperture

[1] It has been brought to the author's attention, after this manuscript was submitted, that a description of a similar ultraviolet microbeam apparatus has been published previously by G. Czihak: Ein UV-Mikrostrahlenstichapparat, *Zeiss-Mitt.*, 1961, **2**, 165.

stop of this reflecting condenser acted as a phase annulus when a 3.0-mm opaque disk was suspended at its center. An aluminized first-surface mirror replaced the standard substage mirror. An aluminum foil diaphragm with a 0.05-mm slit in its center was placed in the filter rack of the lamp. By means of the reflecting condenser, an image of the slit was focused into the specimen plane and produced a beam roughly 1 μ in width. By this method, the specimen could be illuminated for visual observation by removing the diaphragm and using the microscope in the customary fashion, or could be irradiated by replacing the diaphragm and removing all filters from the optical path. Thus a specimen accurately positioned coincident with the slit image could be irradiated with ultraviolet light through the slit.

For narrow band width UV, Baird-Atomic interference filters were placed between the slit diaphragm and the lamp. Since the irradiations were carried out on 0.17-mm quartz coverslips, a correction for focal shift was necessary owing to the marked dispersion of quartz in the ultraviolet. To accomplish this, a cross-hair was placed at a calculated distance in front of the slit diaphragm such that when an image of the cross-hair was in focus in the specimen plane at 546 mμ, the slit would be in focus at the UV wavelength in question.

For work done under polarized light, the phase microscope was replaced with a rectified polarizing microscope, the reflecting condenser being used without the phase stop. To obtain polarized ultraviolet light for microbeam irradiation, a polished calcite rhomb was placed beneath the condenser (8, 19).

Accuracy of Focus

In order to judge the accuracy of the microbeam focus, a simple test was carried out. The microbeam was adjusted parallel to and touching (but not crossing) the Z line of a sarcomere. After an accurate irradiation, the half sarcomere adjacent to the irradiated Z line should contract on addition of ATP; if beam spreading or inaccurate positioning occurred, the half I band of this sarcomere should remain constant owing to disruption of its I filaments (a phenomenon discussed under Results). Normally, beam spreading was not seen; the exceptions occurred when a myofibril was several microns thick or when there was a layer of solution between the myofibrils and the quartz coverslip. Both exceptions were probably due to dispersion. With proper care, it appears possible to position and irradiate within the limits of resolution.

Myofibrils

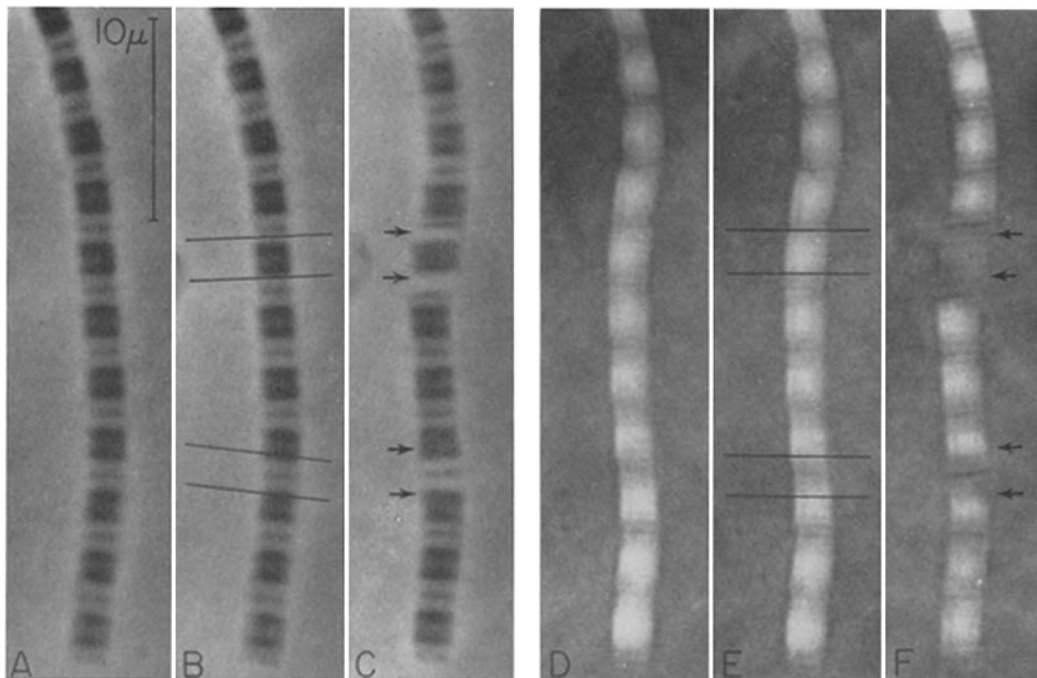Myofibrils were prepared by homogenizing glycerinated stretched or rest length rabbit psoas muscle

FIGURE 3 Sarcomere disruption by UV irradiation.

A. Phase image before irradiation. Scale marker, 10 μ.

B. Areas to be irradiated. The A band was irradiated for 8 minutes at 280 mμ, the I band for 6 minutes at 260 mμ. Microbeam width, 2 μ.

C. Phase image after irradiation. Note the clearing in the I band and retention of mass in the A band.

D. Polarization image before irradiation. Same myofibril as in A.

E. Areas to be irradiated.

F. Polarization image after irradiation. Note the loss of birefringence from the A band and the clearly visible Z line.

in low salt buffer for 20 seconds by means of a high speed blender. The homogenate was sedimented, the supernatant decanted, and the material resuspended in cold 50 per cent glycerol. The suspension was stored at −20°C until use, at which time it was transferred back to low salt buffer for all subsequent operations. The buffer consisted of 0.02 M NaCl, 0.001 M MgCl₂, and 0.01 M phosphate buffer, pH 7.0.

Long, single myofibrils were chosen as specimens, and, where possible, an entire experiment was carried out on one myofibril in order to standardize the results. After irradiation, the myofibrils were contracted either by allowing 2 mM ATP in low salt buffer to diffuse into the preparation, or by perfusion of dilute, graded ATP solutions (0.02 to 2 mM). The myofibrils used for irradiation were all roughly 1 μ thick and were lying directly on the quartz coverslip, both factors being chosen to minimize the possibility of dispersion. Contractions in which the myofibrils adhered to the coverslip were discarded owing to the possibility that tension on the contracting myofibril might invalidate the results.

F Actin Irradiation

Actin was prepared by the method of Mommaerts (11). Excess nucleotide was removed from G actin by the use of Dowex 1 resin (15) and the G-ATP actin thus obtained was polymerized in 0.1 M KCl. The resulting F actin was diluted to 1.0 mg/ml with 0.1 M KCl and irradiated in a quartz cuvette using the HBO 200 illuminator and filters previously described. The light output of the lamp-filter system was standardized with a uranyl oxalate actinometer at each wavelength employed (10). Viscosity was determined directly in the cuvette by means of a 1-ml bulb and capillary used as an Ubbelohde viscometer. The flow time was compared with that of water. Controls were run to show that repeated viscosity measurements caused no significant change in viscosity. The decrease in viscosity with time of irradiation was determined at various wavelengths.

For comparison, a sample of F actin resulting from the polymerization of G-ADP actin (2), obtained through the courtesy of Dr. Teru Hayashi and R. J.

Grant, was irradiated at 260 mμ and its viscosity change determined in the manner outlined above.

Ultracentrifuge observations of irradiated actin solutions were carried out at room temperature by standard sedimentation velocity methods in the Spinco Model E ultracentrifuge.

RESULTS

Ultraviolet Microbeam Effects on Sarcomere Structure

When narrow band width ultraviolet light of varying wavelength was used to irradiate the I band between two sarcomeres, an apparent intensification of the Z line resulted as observed under polarized light. This effect was due to the loss of the weakly birefringent material of the I band. Under phase contrast, a clearing effect was seen in the irradiated I region; often a myofibril would be severed at the point of irradiation. Irradiation of the A band resulted in the loss of birefringence, but little apparent loss of dry mass was evident under phase contrast. The effects of UV on the A and I bands are illustrated in Fig. 3. One can clearly see the Z line intensification and I band clearing in the I band irradiation. In the A band irradiation, the A band has increased slightly in width; increases in half I band length of the irradiated sarcomere and shortening of the
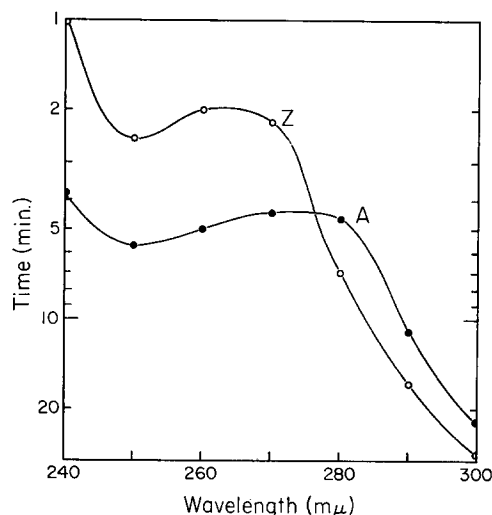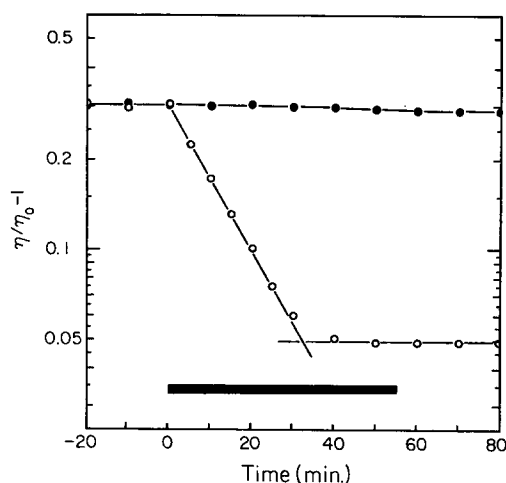

FIGURE 5 Plot of the log of specific viscosity $(\eta/\eta_0 - 1)$ *versus* irradiation time in minutes for F actin. The bar indicates the length of irradiation at 260 mμ. Non-irradiated control, solid circles; irradiated sample, open circles. The sample in this case was polymerized from G-ADP actin.

FIGURE 4 Plot of minimum irradiation time in minutes needed to see Z line intensification (open circles, *Z*) or birefringence loss (solid circles, *A*) *versus* wavelength of irradiating beam. The times are corrected for lamp output relative to that at 260 mμ.

adjacent half I bands may also be observed. These latter two effects may be due to tension on the myofibril drawing the irradiated sarcomere apart and releasing the adjacent half I bands, since the I filaments are disrupted on irradiation. It might be noted that the polarized light images obtained for muscle with rectified optics differ significantly from those commonly observed with unrectified systems. Birefringence in the I bands may be seen, and bands within the A band, due to edge birefringence, are also visible. The energies of irradiation employed were 0.8×10^{11} and 1.9×10^{11} quanta/second/μ^2 at 260 and 280 mμ, respectively. For irradiation done under phase contrast (as used in the ATP contraction experiments), these values were reduced by a factor of 0.44 owing to the phase annulus. The values represent the radiation *in* the specimen plane and have been corrected for quartz absorption and reflectivity of the condenser.

Both the above effects were found to be wavelength dependent as judged from the irradiation time needed to cause equivalent effects after correction for lamp output. The Z line intensification was found to be optimum at 260 to 270 mμ; the loss of A band birefringence was peaked at 270 to 280 mμ. Fig. 4 gives the relative action spectra for these effects based on the times needed to see the Z line effect and the beginning of A band birefringence loss.
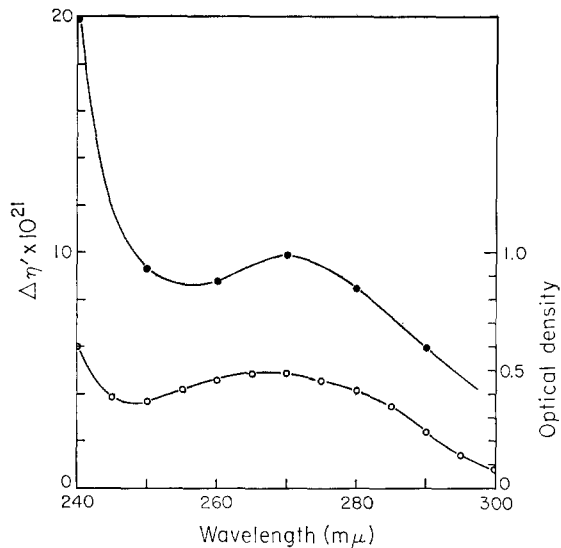
FIGURE 6 Action spectrum for the irreversible radiation depolymerization of F actin. The change in the log of the specific viscosity per second per quantum is plotted *versus* wavelength. Action spectrum, solid circles, left ordinate; absorption spectrum, open circles, right ordinate.

When the A and I bands were irradiated with polarized UV at 280 and 260 mμ, respectively, no dichroic effect could be seen in the times of birefringence loss in the A band or of Z line intensification.

Irradiation of F Actin

Irradiation of F actin in 0.1 M KCl resulted in a marked loss of viscosity. The log of the specific viscosity was found to decrease linearly (within an experimental error of ±10 per cent) with time of irradiation. Radiation depolymerization continued until the specific viscosity reached that of the solvent, and showed no signs of repolymerization after the irradiation was stopped (Fig. 5). Non-irradiated controls showed no significant viscosity change, indicating that repeated viscosity measurements had no detrimental effects. The decrease in specific viscosity was found to be wavelength dependent when corrected for lamp output. Representative dosages were 11×10^{15} and 25×10^{15} quanta/second at 260 and 280 mμ, respectively. An action spectrum for this effect of UV irradiation on viscosity, in terms of change in specific viscosity per second per quantum *versus* wavelength of irradiation, is given in Fig. 6. The effect was found to be optimum at 265 to 275 mμ. The absorption spectrum of the F actin employed in the irradiations is also given in the figure. The samples contained a small amount of free nucleotide.

The rate of depolymerization for F actin prepared from G-ADP actin was found to be approxi-

mately five times that for the comparable F actin polymerized from G-ATP actin when measured at 260 mμ.

Ultracentrifuge patterns of the irradiated materials indicated a F actin peak which decreased with irradiation time, followed by a slow-moving inhomogeneous material whose presence increased with time, implying that the F actin was indeed broken down into smaller units.

Effects of Irradiation on Contraction

When ATP was added to a myofibril in which a Z line and the adjoining half I bands had been irradiated with 270 mμ UV, no contraction was seen in the irradiated I band area, whereas the other halves of the two sarcomeres involved contracted normally (Fig. 7 F). Though this was difficult to discern, it appeared as if the H zones of the sarcomeres decreased. The minimum irradiation time needed to stop contraction at this wavelength was 10 seconds; no dichroic effect on the minimum irradiation time was seen when polarized UV was used. No contraction could be observed in the case of a sarcomere in which the A band had been irradiated with a 1.2 μ wide beam of 270-mμ UV centered on the H zone (Fig. 7 C and F). For wavelengths of 260, 270, and 280 mμ, the dosage needed to stop contraction in A band irradiation was roughly 75 per cent of that needed for I band irradiation.

When a sarcomere was irradiated in an area extending from the A-I junction to a point two-thirds of the way across the A band, leaving a
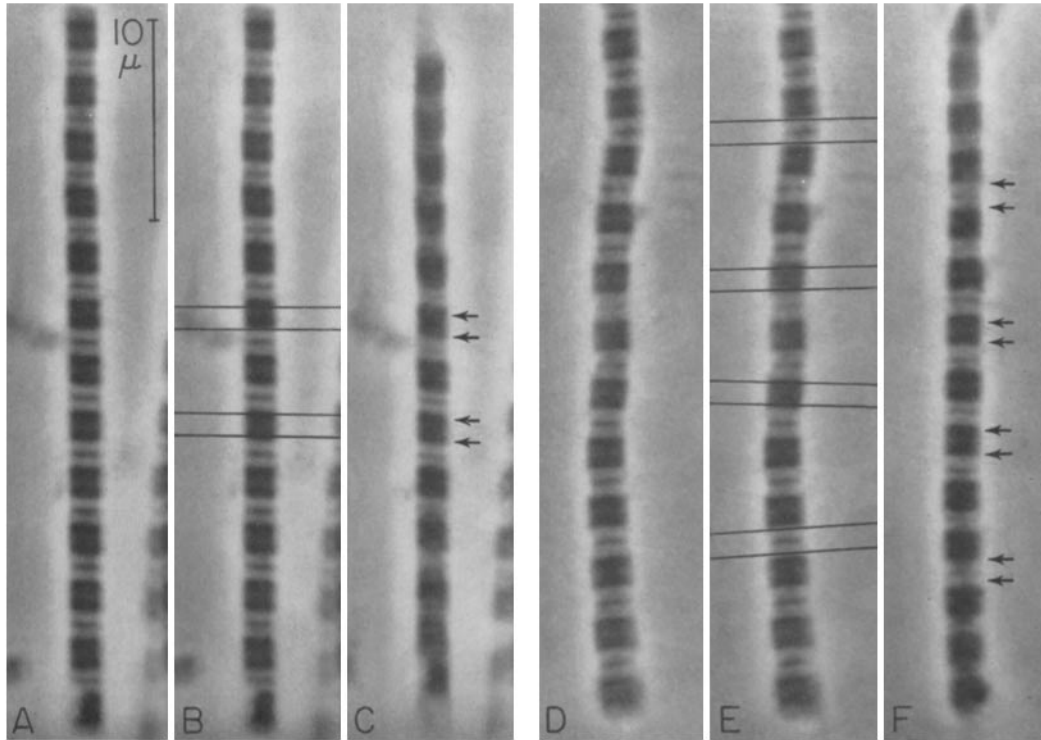
FIGURE 7 Irradiation effects on contraction.

A. Myofibril before irradiation. Phase contrast. Scale marker, 10 μ.

B. Areas to be irradiated. Two-thirds A band and full A band, from top to bottom. Thirty seconds at 270 mμ. Microbeam width, 1.2 μ.

C. After contraction with ATP. Note that there is no contraction in the full A band irradiated sarcomere, whereas the sarcomere in which two-thirds of the A band had been irradiated contracted at the non-irradiated lateral edge.

D. Myofibril before irradiation.

E. Areas to be irradiated. I band, three-fourths A band, full A band, and I band irradiations, from top to bottom. Forty seconds at 270 mμ.

F. After contraction with ATP. Note that the three-fourths and full A band irradiated sarcomeres have not contracted, whereas those irradiated in the I bands have contracted on the side of the non-irradiated half I bands.

TABLE I

Comparison of Band Pattern Changes of Sarcomeres in Fig. 8

Relative length measurements were made on enlarged photographs and rounded off to the nearest 0.05 μ. The left- and right-hand numbers in the last two columns correspond to the respective sides of the sarcomeres in the figure.

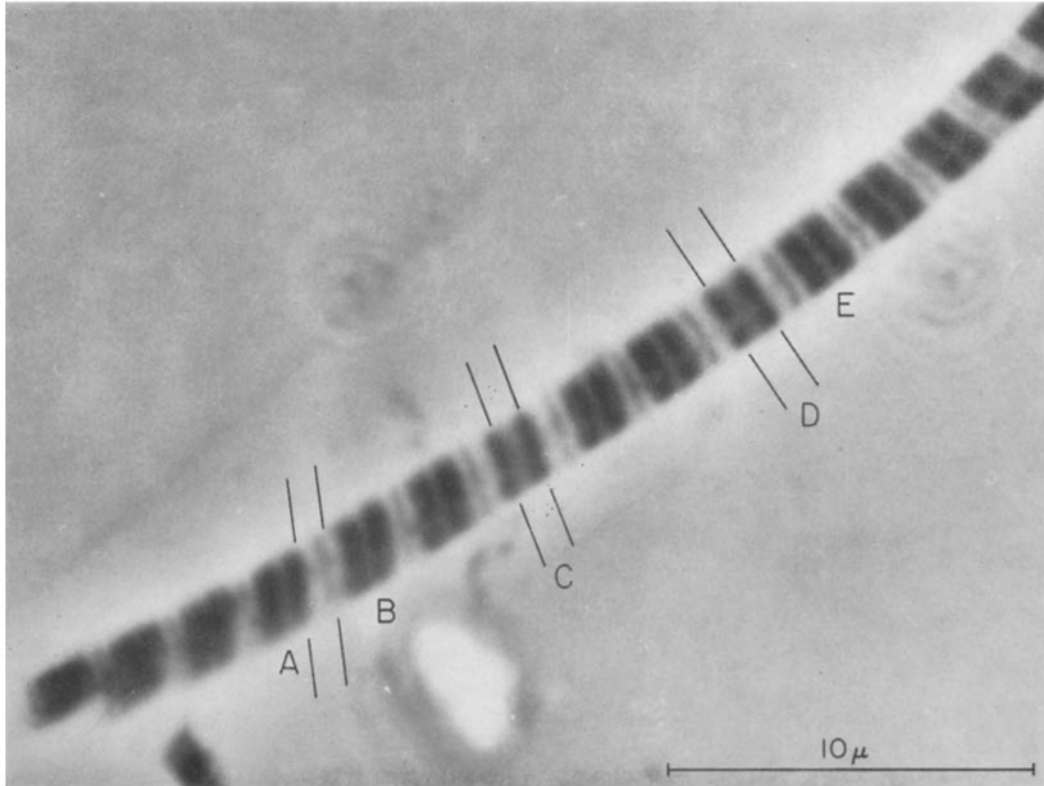| Sarcomere	Sarcomere length	A band length	H zone length	A-I overlap	Half I band
	μ	μ	μ	μ	μ
A	2.45	1.50	0.25	0.70, 0.55	0.35, 0.55
B	2.50	1.50	0.20	0.65, 0.65	0.55, 0.45
C, D	2.70	1.50	0.50	0.50, 0.50	0.60, 0.60
E	2.30	1.50	0.20	0.65, 0.65	0.40, 0.40

FIGURE 8 Myofibril which has been irradiated with 270 mμ UV for 30 seconds in the indicated areas and subsequently contracted with ATP. The two full A band irradiations (C and D) which prevented contraction permit direct comparison of contracted (E) and non-contracted sarcomeres. Note change in H zone width with contraction. The sarcomere in which the I filaments were released at the Z line shows a normal A band with shortened H zone (B), but the half I band, where the filaments were irradiated, has not shortened. The adjacent sarcomere, receiving full half I band irradiation and partial A band irradiation owing to the microbeam width (A), shows an asymmetrically positioned, shortened H zone. Scale marker, 10 μ.

lateral edge of 0.4 to 0.5 μ, the sarcomere contracted at this edge not irradiated with UV. The irradiated side usually showed a widening of the half I band as the A band was drawn to the opposite side of the sarcomere (Fig. 7 C). When three-fourths or more of the A band was irradiated, contraction was inhibited completely (Fig. 7 F). In all cases of partial A band irradiation, a full A band irradiation control was run in order to demonstrate that the radiation received by the A band alone was sufficient to stop contraction and that the effect was not simply a disruption of the I filaments at the point where the microbeam overlapped the A-I junction (cf. area D in Fig. 1).

Irradiation of the edge of the half I band, next to the Z line, appeared to release the I filaments

from the Z line, for, on addition of ATP, the length of the involved half I band either remained constant or increased while the H zone closed symmetrically—the implication being that the released I filaments slide into the A band in the same manner as those still attached at the Z line on the opposite side of the sarcomere. The adjacent sarcomere, receiving full half I band irradiation and partial A band irradiation because of the microbeam width, showed an asymmetric A band due to the fact that the H zone closed from one side only. These effects are seen in Fig. 8; full A band irradiations are included for comparison of H zone width in contracted and non-contracted sarcomeres within the same myofibril. The relative band pattern changes are compared in Table I.
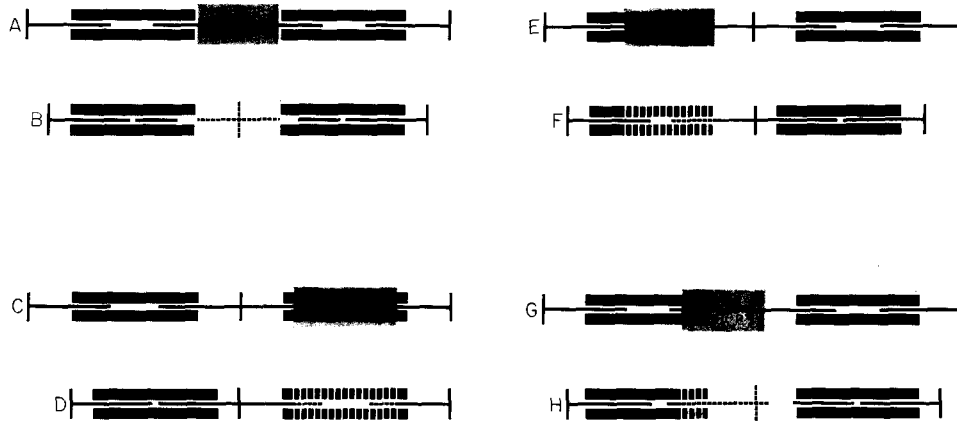
Figure 9 Summary of the effects of UV microbeam irradiation on contraction shown schematically in terms of the sliding filament theory. Shaded areas are areas of irradiation.

A. Full I band irradiation.

B. After ATP contraction. The irradiated area shows no contraction, whereas the other halves of the two sarcomeres involved contract normally.

C. Full A band irradiation.

D. After ATP. No contraction is observed in the irradiated sarcomere.

E. Fractional A band irradiation.

F. After ATP. Contraction is observed at the non-irradiated lateral edge.

G. Release of I filaments at the Z line.

H. After ATP. The H zone of the right sarcomere closes while its half I band remains constant. The H zone appears off center in the left sarcomere.

DISCUSSION

Irradiation of the I band of a sarcomere appears to disrupt the I filaments, an optimum effect being seen at 260 to 270 mμ as judged from relative disappearance of birefringence. That the actin filaments are destroyed or dissolved is supported by (a) the loss of dry mass under phase contrast, (b) the sliding of released filaments into an A band with retention of the I band length, (c) widening of the half I band on contraction as the A band is drawn to the opposite side of the sarcomere in cases of two-thirds A band irradiation, and (d) breakage of irradiated myofibrils at the point of irradiation. In vitro irradiation of F actin indicates that the protein is irreversibly depolymerized, with a wavelength optimum of 265 to 275 mμ. The action spectrum for this effect and that for myofibril I band irradiation closely parallel the absorption spectrum of F actin. The unique F actin prepared from G-ADP actin shows a fivefold higher rate of depolymerization than F actin from G-ADP actin, an effect one might expect in view of its relatively labile nature (1). Specifically which form of F actin is present in the sarcomere is open to question, but

in either case the radiation effect is the same. In all probability, then, the F actin of the sarcomere depolymerizes in the same manner as F actin in solution, eliminating—either through breakage or through dissolution—any actin connections which may occur.

Irradiation of the A band of a sarcomere results in the disordering of myosin as indicated by the loss of birefringence in polarized light and the retention of dry mass in phase contrast. The effect, optimum at 270 to 280 mμ, approximates an actomyosin absorption spectrum. The lack of dichroic effects with polarized UV irradiation of the A and I bands indicates that there is no preferential arrangement of wavelength-specific chromophores in these bands (cf. 9).

The results of UV irradiation on contraction are summarized schematically in Fig. 9. As expected under any of the three contraction theories, full I band irradiation stops contraction in the half sarcomeres involved (Fig. 9 A and B), since no connections would exist at the Z line. Full A band irradiation prevents contraction in both halves of an irradiated sarcomere (Fig. 9 C and D), this finding again being consistent with all of

the theories. Irradiation of two-thirds of an A band permits contraction at the non-irradiated lateral edge, an observation consistent with the sliding filament theory but in direct contradiction to the contralateral filament theory (Fig. 9 E and F). Such an irradiation could either (a) disrupt or cross-link contralateral or H zone connections or (b) inactivate the myosin needed to draw in the contralaterally attached I filaments. In either case, no contraction should be observed in the non-irradiated third of the sarcomere under the Szent-Györgyi–Johnson hypothesis. It has been argued that myosin in the irradiated side may still be active and could be attached through cross-linkage to the contralateral I filaments, and thus contraction still might occur. However, contraction can be observed on the intact side of a sarcomere in which one-half of the A band has been irradiated to the point of complete birefringence loss, a condition under which the myosin could not be functional.

Admittedly, if UV cross-linkage is taking place within the A band, the partial A band irradiation method presented here would not distinguish between the Hanson-Huxley and Podolsky models if the folding I filaments attach to myosin through UV cross-linkage in the A-I overlap region. Such could be the case, since the ends of the I filaments interdigitating with the non-irradiated lateral edge are also irradiated (area D in Fig. 1). As pointed out previously, if these I filaments simply reattach on activation, one would likewise not have a true test of the folding model by this method.

Sliding of released I filaments into an A band on contraction (as seen in sarcomere B of Fig. 8; schematically in Fig. 9 G and H) is clearly in agreement with both the sliding filament and the contralateral filament theories, but not with the actin folding hypothesis. Of course, decrease in

H zone width in normally contracting sarcomeres (6) likewise supports this same conclusion.

It can be concluded therefore that no attachments appear to be necessary between actin and myosin filaments either at or across the H zone, since contraction can be observed in what is in essence only one-third of a sarcomere. These results are in complete accord with the classical sliding filament theory; they are clearly not in agreement with the contralateral filament theory, since the necessity for contralaterally attached filaments has been eliminated. The fate of the actin folding model is questionable, since it can be argued that the actin in the partially irradiated sarcomere is reattached to the myosin filaments even though its original connections at the H zone may have been disrupted. However, the fact that the H zone width decreases on contraction of a sarcomere in which the I filaments have been severed at the Z line is difficult to reconcile with the folding model if one considers the latter to be valid. Thus the data presented here are consistent only with the classical Hanson-Huxley sliding filament theory.

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