FILAMENT ULTRASTRUCTURE AND ORGANIZATION IN VERTEBRATE SMOOTH MUSCLE

Contraction Hypothesis Based on Localization of Actin and Myosin

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ABSTRACT

Using a variety of preparative techniques for electron microscopy, we have obtained evidence for the disposition of actin and myosin in vertebrate smooth muscle. All longitudinal myofilaments seen in sections appear to be actin. Previous reports of two types of longitudinal filaments in sections are accounted for by technical factors, and by differentiated areas of opacity along individual filaments. Dense bodies with actin emerging from both ends have been identified in homogenates, and resemble Z discs from skeletal muscle (Huxley, 1963). In sections, short, dark-staining lateral filaments 15–25 A in diameter link adjacent actin filaments within dense bodies and in membrane dense pataches. They appear homologous with Z-disc filaments. Similar lateral filaments connect actin to plasma membrane. Dense bodies and dense patches, therefore, are attachment points and denote units analogous to sarcomeres. In glycerinated, methacrylate-embedded sections, lateral processes different in length and staining characteristics from lateral filaments in dense bodies exist at intervals along actin filaments. These processes are about 30 A wide and resemble heavy meromyosin from skeletal muscle. They also resemble heads of whole molecules of myosin in negatively stained material from gizzard homogenates. Intact single myosin molecules and dimers have been found, both free and attached to actin, even in media of very low ionic strength. Myosin can, therefore, exist in relatively disaggregated form. Models of the contraction mechanism of smooth muscle are proposed. The unique features are: (1) Myosin exists as small functional units. (2) Movement occurs by interdigitation and sliding of actin filaments.

INTRODUCTION

No acceptable hypothesis to account for smooth muscle contraction has yet been proposed, largely because of lack of knowledge of the ultrastructure of myofilaments and of the disposition of myosin. Reflections characteristic of myosin filaments have not been identified in X-ray diffraction diagrams of vertebrate smooth muscle (Elliott, 1964, 1966), and structures comparable to the A band have not been observed in electron micrographs of sectioned material (Caesar, Edwards, and Ruska, 1957; Choi, 1962; Needham and Shoenberg, 1964). Although its location is unknown, myosin can be isolated from smooth muscle by chemical means (Needham and Williams, 1963), and the individual molecules of myosin so obtained have the same structure as those from striated muscles

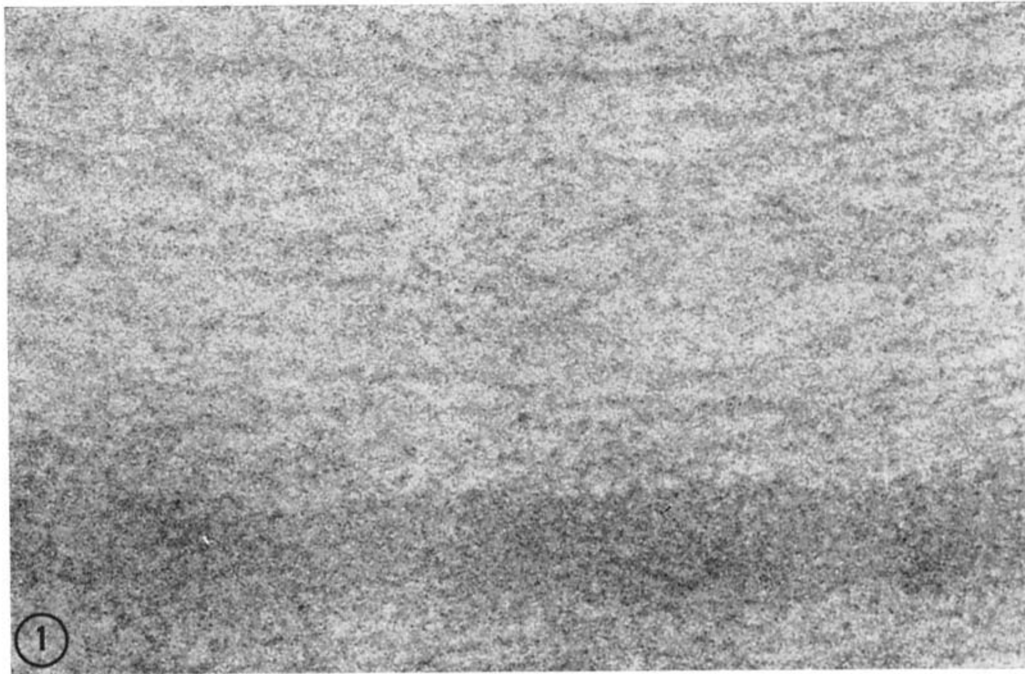


FIGURE 1 Conventional longitudinal section of nonglycerinated, OsO_4-fixed smooth muscle embedded in Epon and stained with lead citrate. Individual myofilaments show variability of diameter and electron opacity. Myofilaments within amorphous dense background are part of a dense body. × 250,000.

(Bárány et al., 1966). In the studies to be described, both actin and myosin molecules are identified in homogenates by use of negative staining. Some features of the organization of these proteins are described, and sliding models of contraction are suggested.

MATERIAL AND METHODS

Gizzards were removed from adult turkeys prior to defeathering, and within 2 min of slaughter. For sections, strips of muscle were obtained from the protoventricularis where the fibers are in parallel array. Some strips were immediately fixed in either cold, buffered 1% OsO_4 at pH 7.3 or 6.25% glutaraldehyde, pH 7.3. No attempt was made to maintain constant length. Other strips 1 cm long and 1 to 2 mm in width and thickness were tied to glass rods at approximately their rest length in situ. Some strips so prepared were placed in 50% glycerol-50% 20 mM Tris buffer, pH 7.4, for 24 hr at 2°C, and removed to fresh, buffered glycerol for 24–48 hr. Strips were maintained at constant length while being fixed by replacement of glycerol drop for drop by either OsO_4 or glutaraldehyde. Some muscles initially fixed in glutaraldehyde were postfixed in OsO_4. Tissue was dehydrated in graded ethyl alcohols; to some specimens 1% phosphotungstic acid (PTA) in absolute alcohol was added for 1 hr during dehydration. Specimens were embedded in either Epon 812 or butyl-methacrylate. Sections giving a gray-to-silver-gold light reflection were cut from specimen blocks on an LKB Ultrotome, mounted on carbon-coated grids, and stained with either lead citrate (Reynolds, 1963) or uranyl acetate (Watson, 1958), or both in succession.

Negative Staining

For examination of individual myofilaments, 10 g of fresh gizzard were homogenized at maximum speed in a Waring blendor for 5–15 sec at 0–2° C in 30 mM KCl, 20 mM imidazol, pH 7.0, or in 50 mM KCl, 20 mM Tris, pH 7.4. In some preparations the medium also contained 5 mM $MgCl_2$ or 0.1 mM $CaCl_2$, or both. Homogenate was either dropped or sprayed on grids and negatively stained with 1% PTA, pH 7.2, or with a 1% solution of uranyl acetate. Some grids were rinsed with 0.6 M KCl and again rinsed with 50 mM KCl prior to staining. First samples were stained within 2 min of homogenization.

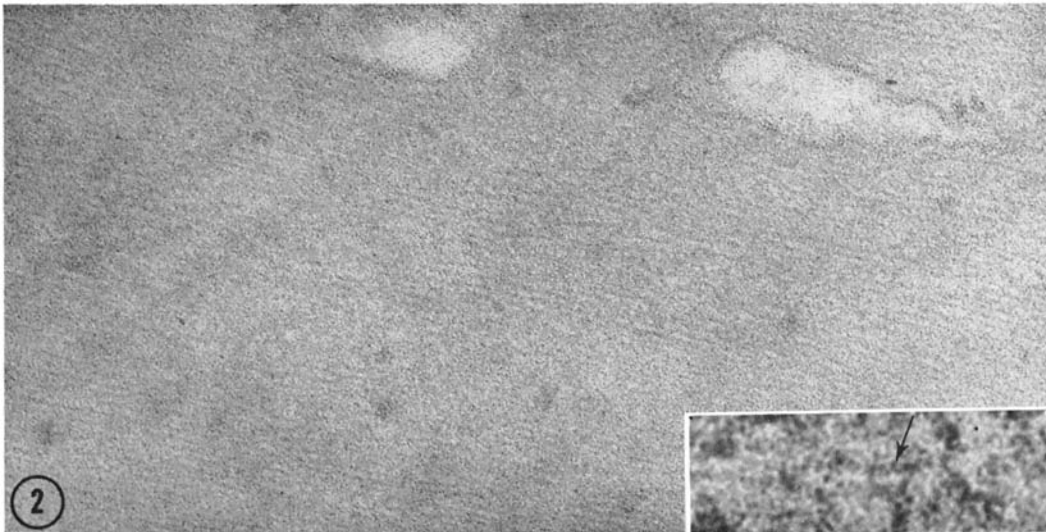


FIGURE 2 Nonglycerinated gizzard fixed in glutaraldehyde and postfixed in OsO_4 before Epon embedding. Section stained with uranyl acetate. Uniform 60 A filaments show no differentiated areas, and no lateral processes can be seen. Two vesicles are present. × 100,000. Inset: Gizzard extracted in glycerol, fixed in glutaraldehyde postfixed in OsO_4, stained with PTA and lead, embedded in methacrylate. Filaments are composed of globular subunits of about 55 A arranged in a manner consistent with a helix (arrow). × 250,000.

Heavy meromyosin was prepared[1] from rabbit skeletal muscle according to the procedures described by Lowey and Cohen (1962). This material was reacted with smooth muscle homogenates and negatively stained, both with and without previous fixation in cold, buffered 4% formaldehyde.

Smooth muscle myosin B was prepared by extraction in Weber-Edsall solution at 2°C for 12 hr. Myosin B was precipitated by rapid dilution, redissolved in 0.6 M KCl, and reprecipitated. Samples of the dilution precipitate and of myosin B to which ATP had been added were negatively stained as described above.

Electron Microscopy

Grids were examined with an Hitachi HU-11A electron microscope operated at 75 kv with beam currents of 10–25 μamp and an objective aperture of 30–40 μ. Primary magnifications of 15,000–50,000 were confirmed by photographing a diffraction-grating replica. We then used plates of the grating replica to calibrate the photographic enlarger.

[1] H-meromyosin was prepared by Drs. Zobel, Moos, and Eisenberg, Department of Biophysics, State University of New York at Buffalo. Homogeneity of the fraction obtained after 4 min of tryptic digestion was tested by ultracentrifugation.

RESULTS

Localization of Actin

TISSUE SECTIONS

To determine the influence of preparative techniques upon the appearance of myofilaments, we compared the effects of OsO_4 and glutaraldehyde fixation, alone or in succession, on Epon-embedded tissue sections stained with lead citrate, uranyl acetate, or PTA, either alone or in combination. None of these techniques permits visualization of myofilaments with the degree of contrast and resolution possible with striated muscle. In OsO_4-fixed gizzard in longitudinal section, individual myofilaments may be followed for up to 2 μ. Diameters range from 20 to 60 A, with 40 A the most common measurement (Fig. 1). No difference in diameters was observed between filaments from muscles fixed in contracture and those from muscle fixed at rest length. Glutaraldehyde used alone or followed by postfixation in OsO_4 yields uniform-appearing filaments about 60 A in diameter (Fig. 2). The difference in appearance of filaments in Figs. 1 and 2 may reflect the greater distorting effect of OsO_4 on contractile proteins

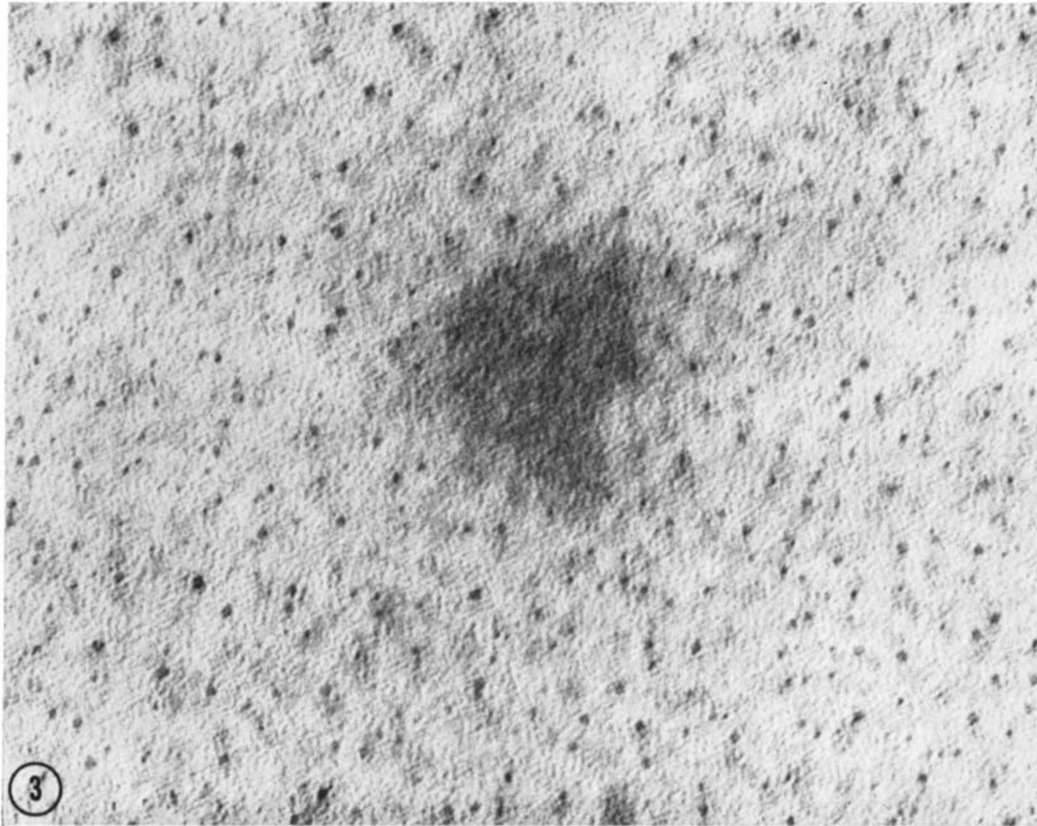


FIGURE 3 Cross-section of OsO_4-fixed smooth muscle embedded in Epon. Myofilaments are easily distinguished from lighter uniform granularity due to phase contrast effect. A dense body is seen in cross-section. Fig. 4 was obtained by measurement of filaments in this print. × 250,000.

(Page, 1964). When strips are extracted in glycerin, then fixed in glutaraldehyde, and postfixed in OsO_4, similar uniform 60 A filaments are seen. Use of double cationic staining with PTA in dehydration fluid and lead citrate on sections results in filament diameters up to 80 A, the diameter of actin filaments from gizzard as seen in negatively stained material.

In glycerinated muscle fixed in glutaraldehyde, postfixed in OsO_4, and doubly stained, filaments exhibit substructure. Many filaments appear to be composed of globular subunits about 50 A in diameter. In some areas, these subunits are arranged in a fashion consistent with a two-dimensional projection of a helix (Fig. 2, inset). We, therefore, can identify some of the longitudinal filaments as actin. Because of technical factors it is impossible for one to identify actin substructure in every filament, even in the case of striated muscle (Huxley, 1965). The uniform appearance of glutaraldehyde-fixed filaments suggests, however, that all longitudinal filaments are the same. This interpretation is consistent with results obtained with cross-sections of OsO_4-fixed, Epon-embedded muscle stained with lead citrate. When photographed over a hole in the supporting film (Fig. 3), filaments appear as round-to-ovoid densities. When magnified by projection to × 1,100,000 these densities can be resolved into two units consistent with the two-stranded substructure of actin. Each unit of density is about 30 A, or the diameter of an actin monomer. Triads and tetrads were also observed, as would be expected were these poorly aligned filaments cut with varying degrees of obliquity and resulting superimposition of subunits. Fig. 4 was obtained by plotting maximum and minimum diameters of 106 filaments from Fig. 3, magnified by projection. The

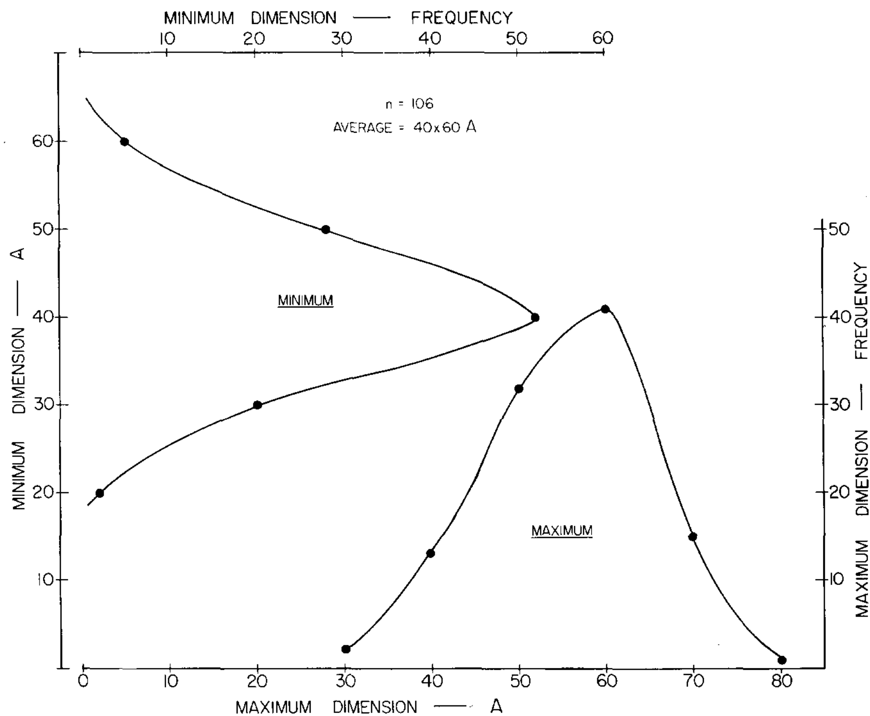


FIGURE 4 Frequency distribution of maximum and minimum diameters of myofilaments seen in cross-section (Fig. 3), and measured at final magnification of 1,100,000, obtained by projection. Absence of bimodal distribution indicates that all filaments are from a single population.

frequency curves fail to reveal the bimodal distribution which would be expected were two populations of filaments present. The average dimensions, 40 × 60 A, are characteristic of I-band filaments as seen in tissue sections (Huxley, 1957).

In contrast to the above results, several studies of vertebrate smooth muscle have been interpreted as evidence for existence of both thick and thin filaments in vertebrate smooth muscle (Choi, 1962; Needham and Shoenberg, 1964; Lane, 1965). The possibility that the apparently thick filaments are due to superimposition is eliminated by cross-sections and by the use of very thin longitudinal sections (Fig. 5). We believe that technical factors account for what previous investigators regarded as a class of thick filaments. Gizzard embedded in methacrylate, regardless of fixative, and examined either with or without heavy metal staining of the sections, regularly exhibits dense regions along individual myofilaments rather than two separate classes of myofilaments. This appearance is not a consistent finding in Epon-embedded material. The dense regions of individual myofilaments are up to 1 μ long (Figs. 5, 6). In filaments which can be followed for distances greater than 1 μ, dense regions of an individual filament appear continuous with less dense regions of the same filament (Fig. 6, inset). In the dense regions, myofilaments have diameters of about 60 A in sections stained with lead citrate or uranyl acetate. After double cationic staining with PTA and lead, filament diameter in the dense regions is about 80 A. These diameters are not measurably different from those of the less dense adjacent regions of the same filament. In both light and dense regions, globular subunits have been identified in some of the filaments.

Homogenates

CONDITIONS FOR ISOLATION OF FILAMENTS: To obtain individual filaments from skeletal muscle, one must carry out homogenization in the presence of high concentrations of ATP, Mg, and EDTA (Huxley, 1963). In this "relaxing medium" cross-links between actin and myosin are dissociated, and shearing forces are rendered effective. In

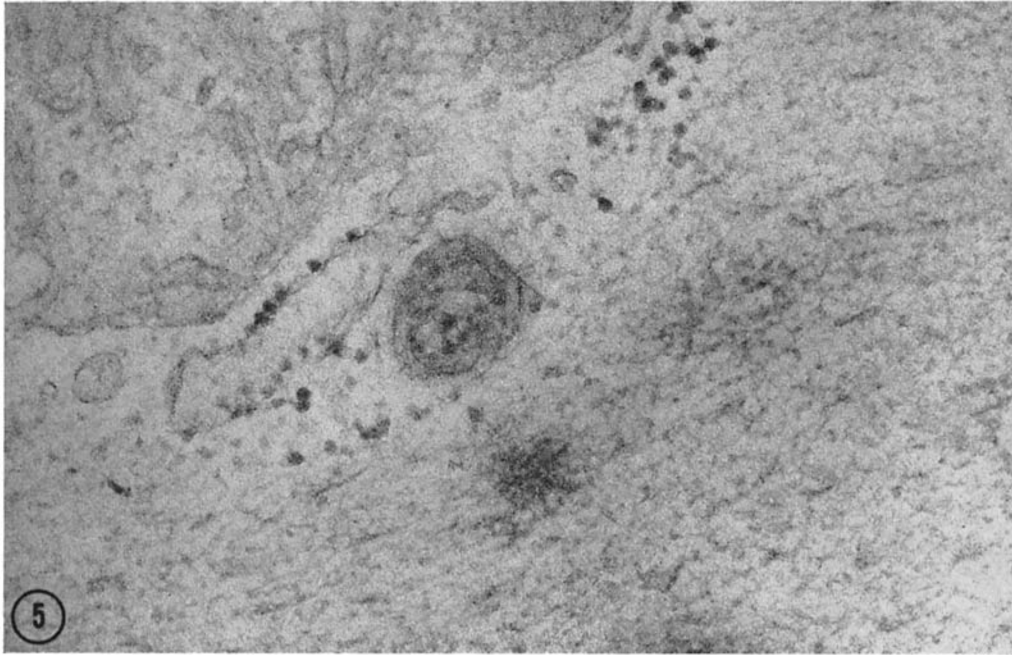


FIGURE 5 Nonglycerinated smooth muscle fixed in OsO_4, embedded in methacrylate, and stained with lead citrate. Dense areas of individual filaments appear continuous with less dense portions of the same filaments. This thin section (gray interference color) shows that dense areas along a particular myofilament are not due to superimposition. × 100,000.

contrast, individual actin filaments can be readily obtained from gizzard in the absence of added ATP, without Mg or EDTA in the medium, and at ionic strengths less than 1/10 those necessary for disruption of skeletal muscle. Individual filaments can be obtained even in 20 mM Tris buffer, in the presence of 0.1 mM Ca, and from gizzard allowed to consume endogenous ATP for up to 8 hr. These findings indicate that linkages between actin and myosin are either weaker than those of striated muscle, or that each unit of myosin is smaller, and hence attached to actin through fewer links. The second possibility is consistent with the fact that neither we nor others (Hanson and Lowy, 1964; Rüegg, Strassner, and Schirmer, 1965; Shoenberg et al., 1966) have found thick myosin filaments in fresh homogenates or in smooth muscle extracts. Thick filaments are also absent from homogenates of low ionic strength allowed to stand for up to 8 hr. At these ionic strengths, myosin from skeletal muscle is known to aggregate (Huxley, 1963; Zobel and Carlson, 1963; Josephs and Harrington, 1966).

ACTIN NEGATIVELY STAINED: The structure of negatively stained actin filaments from gizzard is similar to the structure of actin from other muscles, as described by Hanson and Lowy (1963). Filaments are about 80 A wide and composed of 50 A globular subunits arranged in a manner consistent with a two-dimensional projection of a helix. In some filaments, one can identify 13 subunits per turn, with cross-over points at about 350 A intervals (Fig. 7). Substructure varies considerably among preparations, however, and even along a particular filament in the same preparation, in agreement with recent observations of Hanson (1967). No differentiated regions comparable to those seen in tissue sections could be identified.

Localization and State of Aggregation of Myosin

SECTIONED MUSCLE

In glycerinated gizzard embedded in methacrylate, it is possible to observe lateral processes between longitudinal filaments (Fig. 6). With appropriate preparative technique, some of the longitudinal filaments to which the processes are at-

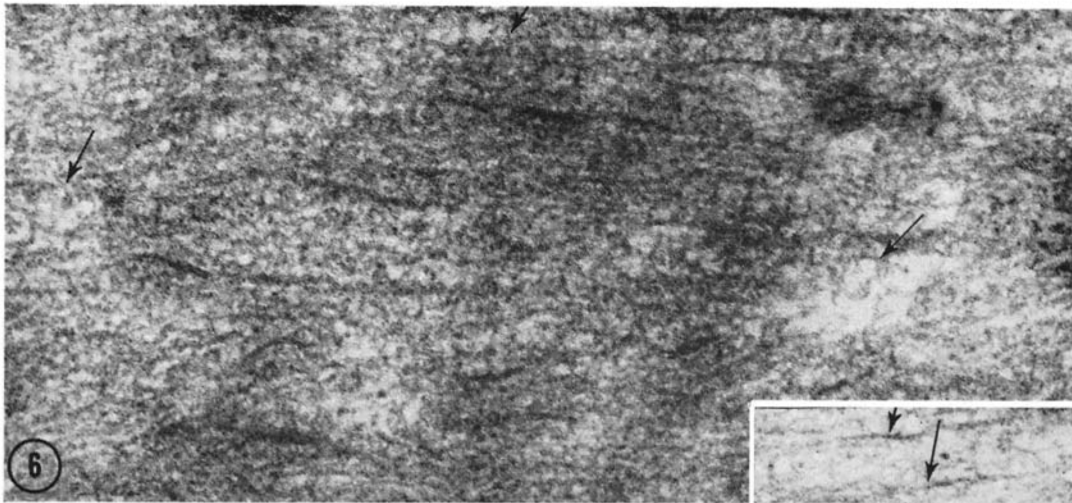


FIGURE 6 Glycerinated smooth muscle fixed in glutaraldehyde, postfixed in OsO_4, stained in the block with PTA before methacrylate embedding. Section stained with uranyl acetate. Numerous processes project from longitudinal filaments at approximately right angles (arrows denote representative samples). × 100,000. Inset: Dense areas of individual filaments appear continuous with less dense regions of the same filament (arrows). × 100,000.

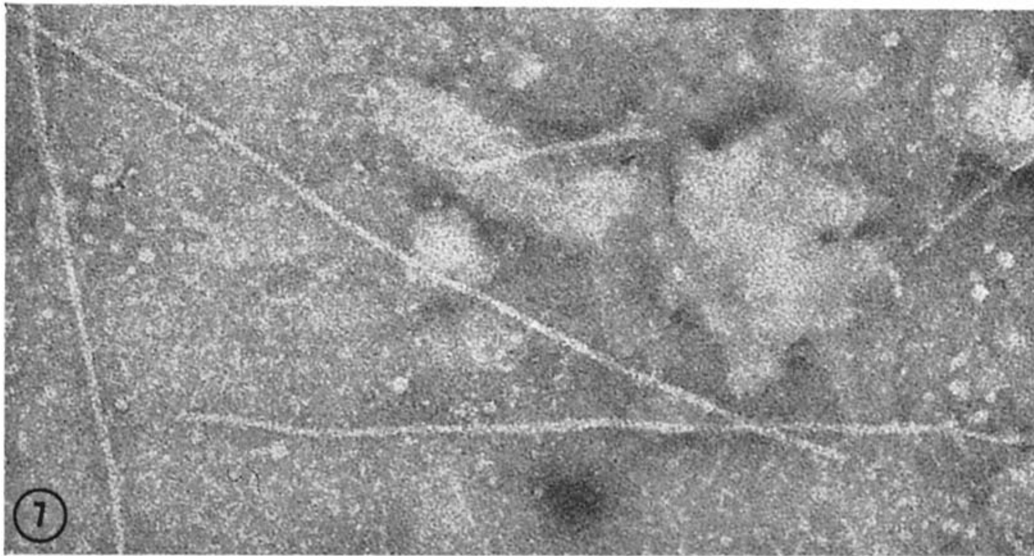


FIGURE 7 Filaments obtained from gizzard homogenized in 30 mM KCl exhibit helically arranged globular subunits characteristic of actin. Differentiated areas of filaments seen in methacrylate-embedded sections are not visible in negatively stained material. × 150,000.

tached are identified as actin. Longitudinal spacing of the lateral processes is difficult to determine because of possible superimposition, but processes may be found as close together as 160 A and separated by as much as 300 A along an individual filament. The lateral processes in stained sections are thinner than actin (about 20–30 A in diameter), and in their lateral extent may span several

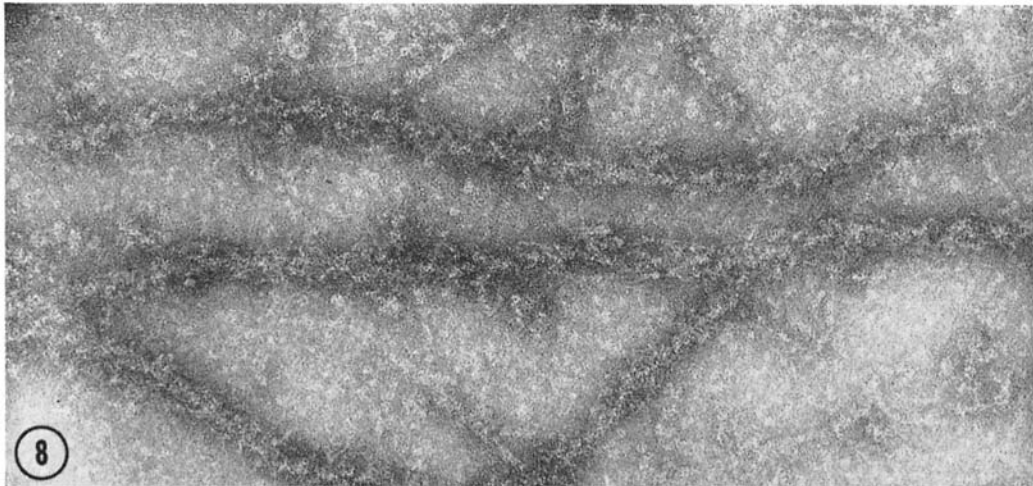


FIGURE 8 Synthetic acto-H-meromyosin prepared by reacting H-meromyosin from rabbit skeletal muscle with actin from gizzard homogenate; negatively stained with uranyl acetate. Arrowheads of paired H-meromyosin molecules maintain the same direction for at least 1.2 μ. $\times$ 125,000.

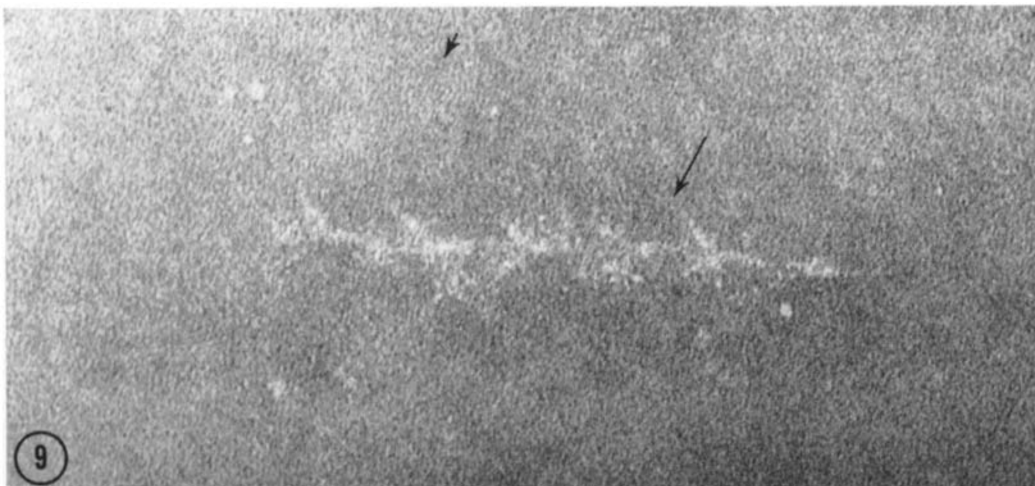
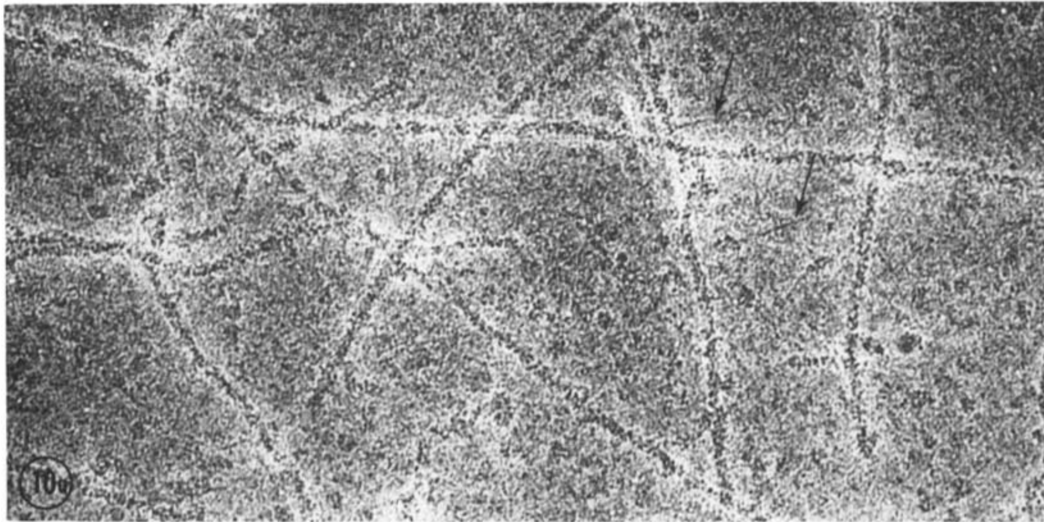


FIGURE 9 Naturally occurring compound filament of actomyosin isolated from fresh gizzard homogenized in 30 mM KCl and negatively stained with PTA within 2 min of disruption. Myosin heads attach in pairs to form arrowheads at about 350 A intervals along the actin filament. Long arrow indicates junction of head of myosin molecule with its tail. The heads are about 350 A long, and a tail extends an additional 1200 A (short arrow). $\times$ 300,000.

longitudinal filaments. The angle between actin and lateral processes varies, but many of the processes are nearly perpendicular. Similar processes are visible along the dense areas of filaments, but it is impossible for us to determine whether the processes are attached at these points or superimposed. Substructure of lateral processes cannot be resolved in sections; in particular, we cannot determine whether lateral processes are thicker near their attachment points.

HOMOGENATES

HETEROLOGOUS ACTO-H-MEROMYOSIN: H-meromyosin from skeletal muscle was reacted with



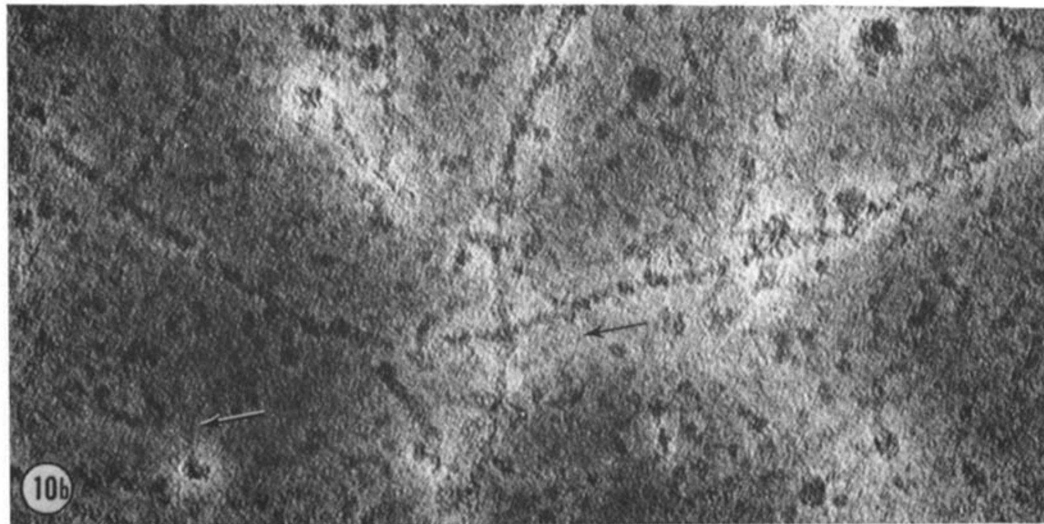


FIGURE 10 *a* Reverse contrast print of fresh gizzard homogenate, negatively stained with uranyl acetate. Medium contained 2 x 10^{-4} M Ca^{++}. Numerous linear structures 20 A wide and up to 1200 A long are present, both free and attached to actin (arrows). × 200,000.

FIGURE 10 *b* Reverse contrast print of fresh gizzard homogenate, negatively stained with uranyl acetate. Medium contained 2 x 10^{-4} M Ca^{++}. An apparent three-dimensional effect, possibly due to nonuniform charge distribution with secondary lens effect, emphasizes linear structures like those shown in Fig. 10 *a*. Black arrow indicates representative linear structure with globular region at site of attachment to actin. White arrow indicates an unattached structure typical of others seen throughout the field. × 200,000.

freshly prepared gizzard homogenates in which abundant free actin was present. In the synthetic compound filaments thus formed (Fig. 8) H-meromyosin appears as lateral projections from actin. These projections are 30–50 A wide and about 450 A long. Two H-meromyosin units on either side of the actin filament form an arrowhead. If sufficient H-meromyosin is present the actin filament is completely coated, indicating that smooth muscle actin can combine with myosin

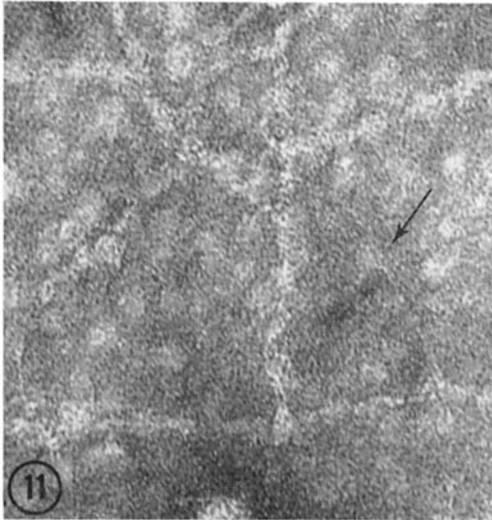


FIGURE 11 Fresh gizzard homogenized in 50 mM KCl shows actin filaments and three myosin molecules, two of which (arrow) appear to be joined tail to tail. The dimer connects two widely separated actin filaments. × 250,000.

along its entire length. Since the arrowheads of H-meromyosin point in the same direction along the entire visible length of the filament, the directionality of actin is constant over at least 1.2 μ.

HOMOLOGOUS NATURAL ACTOMYOSIN: Perhaps the most important result we have obtained with smooth muscle myosin is shown in Fig. 9. When fresh gizzard is homogenized and immediately sampled, longitudinal filaments are found with lateral processes attached at regular intervals of about 350 A. The number of these compound filaments can be increased if 0.1 mM Ca is present in the homogenate. The longitudinal filaments exhibit substructure characteristic of actin. The size and substructure of the lateral processes enable us to identify them as individual whole molecules of endogenous myosin The molecules attach as pairs opposite one another, and give an arrowhead appearance to the compound filaments of natural actomyosin. The markers in Fig. 9 indicate one myosin molecule. It is composed of a head 30 A wide and about 350 A long, which resembles the heads of myosin molecules from skeletal muscle (H-meromyosin) shown in Fig. 8. The myosin head becomes continuous with a tail about 15 A wide which can be followed for 1200 A. Tails of two other myosin molecules in Fig. 9 can be followed for shorter distances. The maximum total length of the smooth muscle myosin molecules which we have observed in this and other negatively stained preparations is 1500–1600 A, a value in good agreement with results of Bárány and coworkers (1966) on gizzard myosin based on hydrodynamic measurements and on shadow casting. At the junction of myosin head and myosin tail there is a slight angular displacement of the tail. Similar bending at junction of head and tail has been

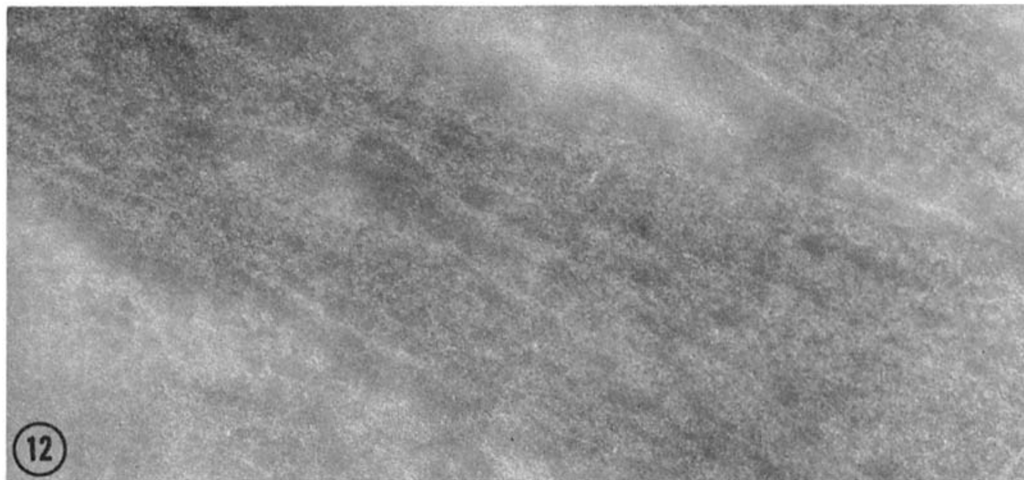


FIGURE 12 Array of filaments from myosin-B syneresed with ATP. Longitudinal actin filaments are maintained in a raftlike array by lateral processes. Negatively stained with PTA. × 150,000.

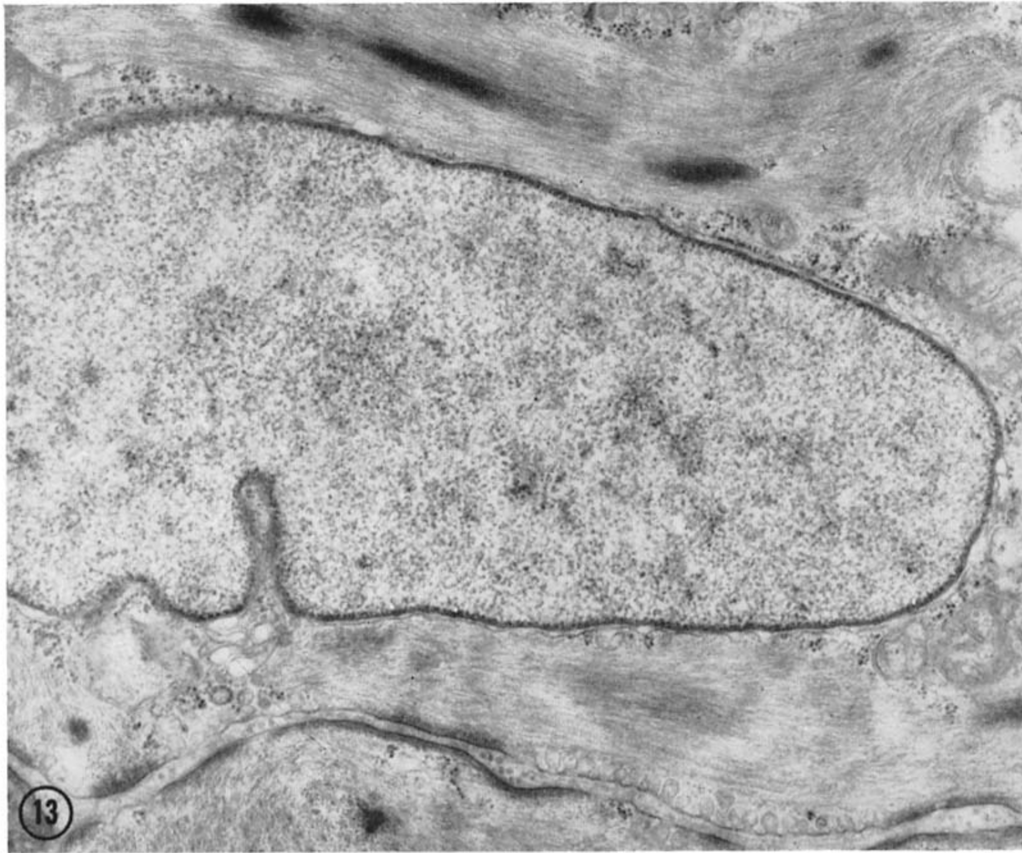


FIGURE 13 Longitudinal section of smooth muscle allowed to shorten during fixation in OsO_4, and embedded in Epon. Folding of the cell is denoted by irregularity of nuclear and plasma membranes, and by transverse and longitudinal orientations of dense bodies. Dark patches along the plasma membrane alternate with light areas which contain vesicles. × 19,500.

described by Zobel and Carlson (1963) in shadow-cast preparations of myosin from skeletal muscle.

MYOSIN FRAGMENTS: The number of intact myosin molecules which can be found in homogenates is much too small to account for either the amount of myosin known to be present, or for the actin-myosin ratio of smooth muscle (Needham and Williams, 1963). It is, therefore, significant that in all negatively stained preparations abundant unattached material 20–30 A in diameter and 200–1200 A long may be seen (Figs. 10 *a* and *b*). Some of these structures exhibit a globular region at one end. Similar structures too short to be intact myosin molecules are also found attached as lateral processes to actin, suggesting that both the attached material and some of the unattached material are myosin which was fragmented during homogenization and specimen preparation.

FILAMENT ARRAYS: In a few favorable fields, whole myosin molecules have been found which approximate one another tail-to-tail to form what appear to be dimers connecting two well separated actin filaments (Fig. 11). Dimers are oriented nearly perpendicular to the long axes of the actin filaments. More commonly, groups of actin filaments are found, closely aligned side by side. Identical arrays occur in smooth muscle myosin B superprecipitated with ATP (Fig. 12). Numerous laterally oriented structures, some of which seem to be attached to actin, give the arrays a bristly appearance. Though it is not possible to identify with certainty individual lateral processes as myosin in such incompletely separated arrays, in both homogenates and myosin B the processes resemble the individual myosin molecules and dimers of

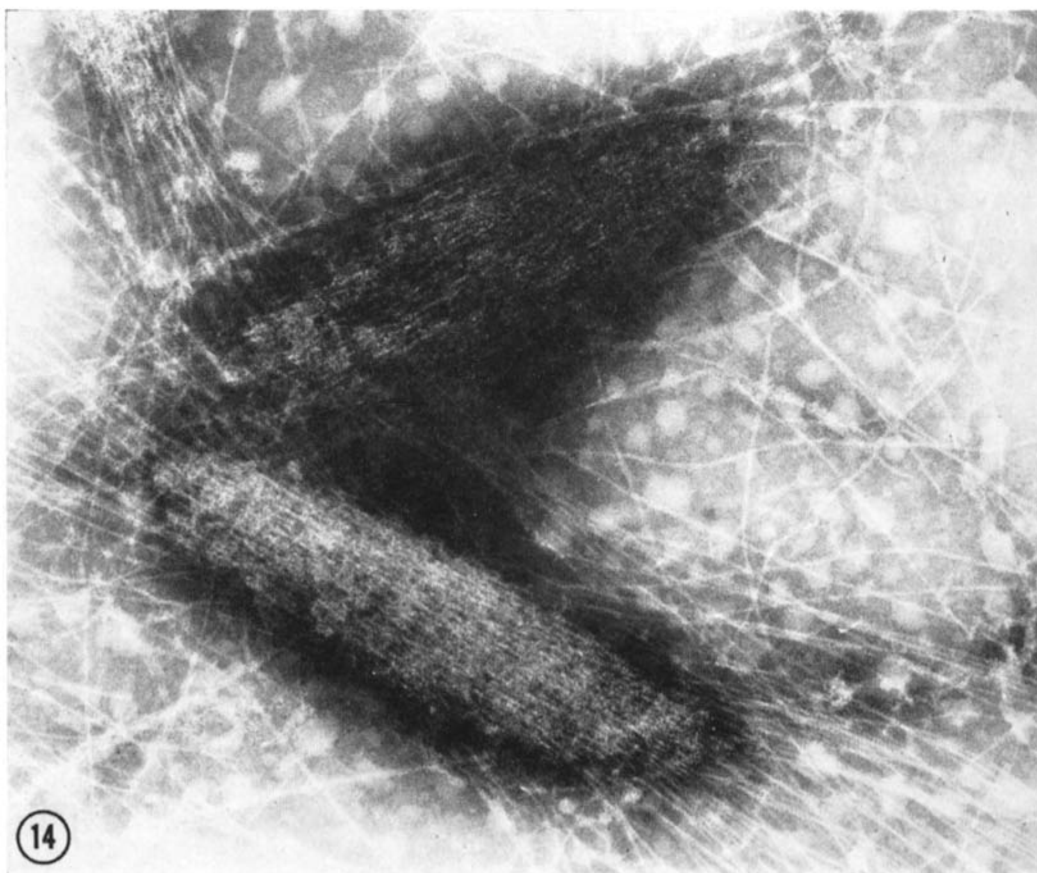


FIGURE 14 Two negatively stained dense bodies obtained from fresh gizzard homogenate are largely composed of actin filaments some of which emerge from both ends. Substructure of filaments within dense bodies is the same as that of free actin filaments. × 100,000.

myosin found attached to isolated, or widely separated actin filaments (Figs. 9, 11).

Organization of Myofilaments

Elliptical dark regions referred to as dense bodies are found frequently throughout the cytoplasm of smooth muscle cells in intimate relation to longitudinal filaments (Figs. 1, 3, 13). Dense bodies are up to 3 μ long and 0.2–0.5 μ in diameter. In muscle fixed at approximately rest length, dense bodies are arranged with their long axes parallel to the long axis of the cell. Lateral distances between dense bodies vary from 0.5 to 2.0 μ, and longitudinal distances from 1 to 3 μ. No order in their spacing has been discerned. An amorphous background material contributes to the dark appearance. The substructure of myofilaments within dense bodies cannot be resolved in tissue sections. Dense bodies can be isolated from muscle homogenates either by differential centrifugation and positive staining of sectioned pellets or by negative staining of unfractioned homogenates. Dimensions of dense bodies are the same in homogenates and intact muscle. In negatively stained material, longitudinal filaments within and attached to dense bodies exhibit typical actin substructure (Fig. 14). Consequently, filaments seen in tissue sections in relation to dense bodies can be identified as actin.

The fact that dense bodies survive the shearing stresses of homogenization suggests that structures exist which hold them together. In cross-sections of muscle embedded in either Epon or methacrylate, thin lateral filaments are visible within dense bodies. They are 15–20 A wide in Epon-

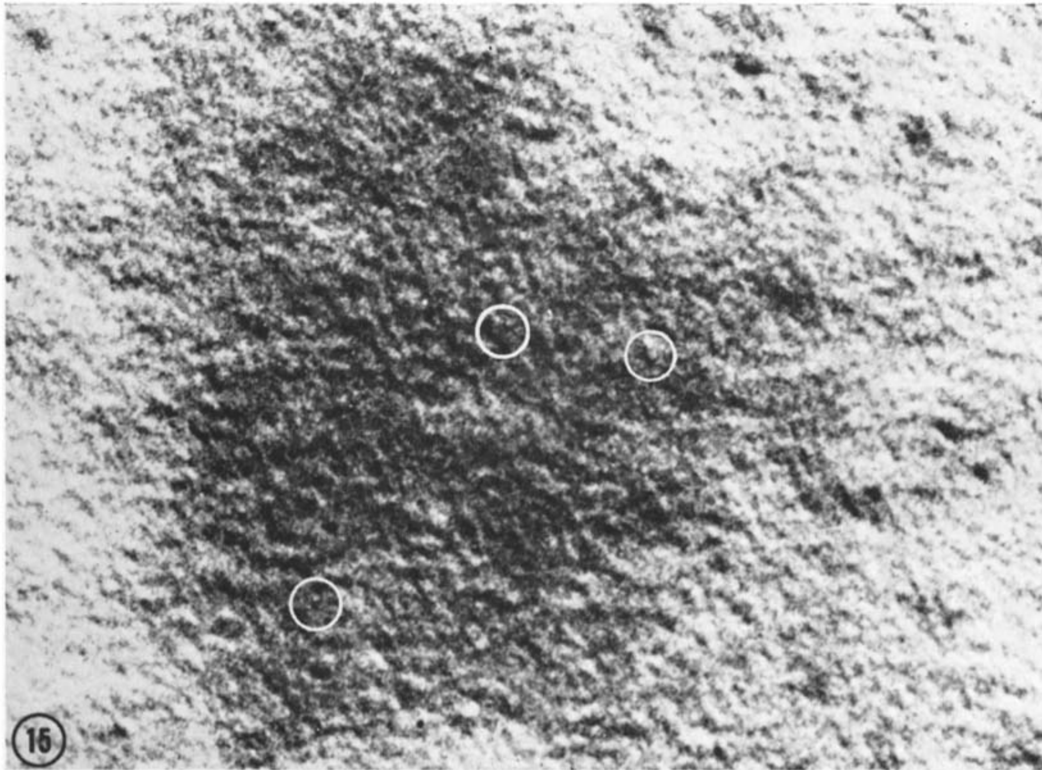


FIGURE 15 Cross-section of dense body shown in Fig. 3. Circled areas indicate stellate structures formed by short lateral processes 15 A in diameter which link actin filaments within the dense body. × 375,000.

embedded muscle, and up to 25 A wide in methacrylate-embedded muscle. In both embedding media, they are somewhat wavy filaments about 40 A long which link adjacent actin filaments. Up to five thin lateral filaments extend from a particular actin cross-section to form a stellate structure (Fig. 15). We are unable to determine whether adjacent sets of lateral filaments form a lattice comparable to that in the Z line described by Knappeis and Carlsen (1962).

Dense regions of cytoplasm can be seen at intervals beneath the plasma membrane in sections prepared by any conventional technique (Fig. 13). These alternate with lighter regions which contain vesicles. Actin filaments enter the dense patch and terminate there. In so doing, they maintain an almost parallel orientation to the cell surface. At high magnification, dense patches exhibit substructure like that of dense bodies. Thin lateral filaments identical in appearance to those described within dense bodies form linkages not only between adjacent actin filaments but also between actin and plasma membrane per se (Fig. 16).

DISCUSSION

The principal observations which we report support the following conclusions: (1) Longitudinal filaments seen in tissue sections are composed of actin. (2) Myosin exists in small functional units. (3) Dense bodies and membrane-dense patches organize filaments into sets analogous to sarcomeres. In the following discussion, these findings are considered in relation to the results of others and sliding models of contraction are suggested.

Localization of Contractile Proteins

ACTIN

Though substructure typical of actin has not been observed previously in sectioned smooth muscle, X-ray diffraction data indicate that at least some longitudinal filaments are composed of

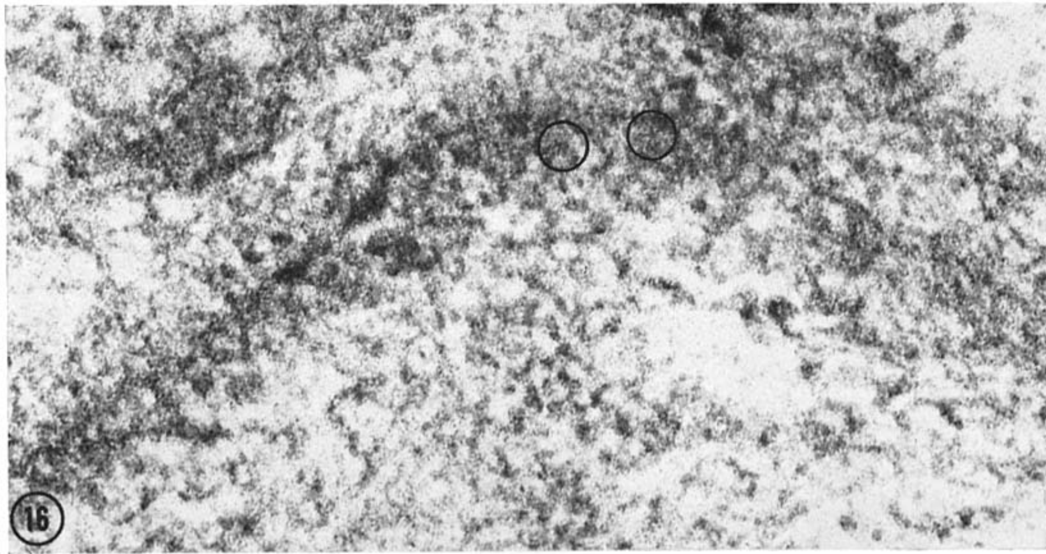


FIGURE 16 Portions of adjacent smooth muscle cells embedded in methacrylate following glycerol extraction, showing dense patches beneath plasma membranes. Lateral filaments 15–25 A in diameter form stellate structures about actin filaments seen in cross-section. Circled area shows lateral filaments which link actin filaments to each other and to the plasma membrane per se. × 200,000.

actin (Elliott, 1964 and 1966). We are able to confirm this view. In glycerol-extracted gizzard fixed in glutaraldehyde, postfixed in OsO_4, and stained with both phosphotungstate and lead, longitudinal filaments are 80 A in diameter. Some filaments are composed of 50 A globular subunits which in a few areas are arranged in a structure consistent with a two-dimensional projection of a helix (Fig. 2, inset). Moreover, in negatively stained material longitudinal filaments in and attached to dense bodies can be identified as actin. The striking uniformity of filament appearance after glutaraldehyde fixation (Fig. 2) strongly suggests that even those longitudinal filaments which cannot be traced to dense bodies are also composed of actin. Lack of a bimodal distribution of filament diameters in OsO_4-fixed muscle adds supporting evidence that uniformity in Fig. 2 is not simply the result of glutaraldehyde. Abundant actin filaments long enough to account for the longest filaments seen in sectioned material can be found in gizzard homogenates by use of negative staining, and only actin and collagen reflections have been observed in X-ray diffraction diagrams (Elliott, 1964). On the basis of these, and the supporting observations summarized below, it seems highly probable that all thin, longitudinally oriented filaments are composed of actin, and that thick, longitudinally oriented myosin filaments are absent.

MYOSIN

EVIDENCE FROM TISSUE SECTIONS: The principal obstacle to an understanding of contraction mechanism of smooth muscle has been lack of knowledge of the locus and state of aggregation of myosin. Choi (1962) and Needham and Shoenberg (1964) described what appeared to be short, thick, dark-staining filaments in addition to more abundant thin ones. Recognizing that the number of apparently thick filaments was inadequate to account for the amount of myosin actually present in the tissue, Shoenberg suggested that the appearance of thick filaments might be due to superimposition. This possibility can be eliminated by use of very thin longitudinal sections (Fig. 5), and by the separation of filaments in cross-sections (Fig. 3).

Needham and Shoenberg (1964) also suggested that ostensibly thick filaments might represent myosin which had been either aggregated into thick filaments or deposited upon actin during specimen preparation. We can certainly coat actin from gizzard homogenates with H-meromyosin to produce thick filaments of acto-H-meromyosin, but we have never observed parts of a particular

actin filament free of coating if sufficient H-meromyosin is present to give the appearance of a thick filament. In other words, myosin coats actin uniformly. Since the dark, apparently thick filaments seen in tissue sections are of relatively uniform length and since dense regions alternate with thin, light-staining regions of the same filament (Figs. 5, 6), it is unlikely that the dense regions are deposits of myosin on actin. Moreover, the diameter of filaments in the dense regions is not measurably greater than in the light regions; it only appears larger because of its greater contrast with respect to surrounding areas. In view of (*a*) the continuity of light and dense regions, (*b*) uniform filament diameters, and (*c*) the mode of attachment of myosin to actin as revealed by negative staining, we can eliminate the possibility that the dark-staining regions along actin filaments represent a separate class of thick actomyosin filaments.

The identity of the material responsible for the dark-staining regions, and of the similar material found in dense bodies and membrane-dense patches, is unknown. Since tropomyosin B is associated with actin in striated muscle (Pepe, 1966), the dark material may be tropomyosin. The amount of tropomyosin in smooth muscle is compatible with this idea (Needham and Williams, 1963). The solubility of tropomyosin could account for the observation that prolonged washing in solutions of low ionic strength, especially after procedures which injure cell membranes, decreases the number and electron-opacity of ostensibly thick filaments (Needham and Shoenberg, 1964).

MYOSIN IN HOMOGENATES AND MYOSIN B: In agreement with Hanson and Lowy (1964), Rüegg, Strassner, and Schirmer, (1965), and Shoenberg et al. (1966), we have never observed thick filaments of myosin in homogenates or extracts of smooth muscle. Since filaments from the A bands of striated muscle remain intact during homogenizations four to six times longer than those which we employed (Huxley, 1963), it seems unlikely that all thick filaments could have been completely disrupted had they existed in vivo. Even if this were the case, reaggregation would be anticipated were a homogenate of low ionic strength allowed to stand at room temperature (Huxley, 1963; Carney and Brown, 1966; Josephs and Harrington, 1966). Even under these conditions, we observe no thick filaments. Failure to find a structure cannot prove that it does not exist, and the capacity of smooth muscle myosin to form thick filaments has been demonstrated by Hanson and Lowy (1964). On the other hand, presence in vitro does not establish relevance in vivo. Hanson and Lowy obtained thick filaments by dialyzing solutions of uterine myosin. Whether myosin is capable of aggregating in the presence of all cell constituents, however, is unknown, for in vitro conditions strongly influence the aggregation (Josephs and Harrington, 1966; Kaminer and Bell, 1966). Moreover, chemical changes in myosin produced by aging (Zobel, personal communication) and proteolysis (Shoenberg, 1965) favor aggregation. Absence of X-ray reflections characteristic of myosin filaments at both low and high angles in formalin-fixed, stained, or even living smooth muscle constitutes further evidence that large myosin aggregates do not exist (Elliott, 1964, 1966). While results with X-ray diffraction are not in themselves conclusive, they are consistent with the solubility of smooth muscle actomyosin at low ionic strengths (Needham and Williams, 1963; Rüegg, Strassner, and Schirmer, 1965), and with the electron microscopic observations described in this paper.

Requirements for filament isolation bear on the organization of myosin and its interaction with actin. To isolate filaments from skeletal muscle, it is necessary for one to dissociate actin and myosin by chelating Ca, and applying high concentrations of ATP and Mg (Huxley, 1963). In contrast, we can readily obtain filaments from smooth muscle homogenized in 20 mM Tris buffer alone, even in the presence of added Ca 1×10^{-4} M. In terms of structure, solubility of actomyosin and extractability of filaments mean that linkages between myosin and actin are easily broken, as would be expected were each structural unit of myosin quite small, and hence attached to actin at relatively few points. If thick filaments of myosin are absent in living smooth muscle, myosin must exist in relatively disaggregated form. Our results provide direct morphological evidence that this is the case. We find individual whole molecules of myosin in homogenates of fresh gizzard sampled within 2 min of disruption (Fig. 9). At the low ionic strengths which we employed, myosin from striated muscle would be expected to have been aggregated (Huxley, 1963; Josephs and Harrington, 1966; Carney and Brown, 1966). Pairs of smooth muscle myosin molecules attach on either side of actin filaments to form arrowheads spaced at regular

intervals about equal to one-half the actin period. This spacing is consistent with the molar ratio of actin to myosin (1:4) obtained from chemical data (Needham and Williams, 1963). Were all actin filaments so coated, the spacing could account for the amount of myosin in the muscle. Most actin filaments are not coated, however, and the number of intact myosin molecules which can be found is much too small to account for the amount of myosin present. This discrepancy is due, in part, to the fact that myosin "tails" are about at the theoretical limit of resolution by negative staining (Valentine and Horne, 1962). We believe, however, that a more important cause for the discrepancy is fragmentation of the long, thin myosin molecule as a result of shearing forces during homogenization, and surface forces during specimen preparation. The distribution of particle lengths observed in shadow-cast preparations by Zobel and Carlson (1963) indicates that the myosin molecule can be so fragmented. In every homogenate we find numerous linear structures of irregular length, but with diameters characteristic of myosin molecules (Figs. 10 *a* and *b*). Structures of generally shorter length have recently been described by Shoenberg et al. (1966). Some of the thin structures in Fig. 10 *b* terminate in a wider region suggestive of a myosin "head." The presence of both whole molecules of myosin and short fragments of similar width attached to actin in the same preparation strongly suggests that the linear structures are myosin molecules showing varying degrees of fragmentation. It is possible that some of the shortest unattached fragments which do not possess globular ends may be tropomyosin.

According to Schirmer (1965), the concentration of actomyosin within smooth muscle cells greatly exceeds that at which it is soluble at the Mg-ATP concentration and pH thought to exist in vivo. Consequently, even unaggregated myosin is not in solution, but is rather part of a contractile structure. In homogenates and in myosin B syneresed with ATP, we find raftlike side-by-side arrays of actin apparently held together by myosin (Fig. 12). Since only the heads of myosin molecules have actin-binding capacity (Huxley, 1963), the smallest myosin unit which could hold actin in such arrays is a dimer. It is of interest, therefore, that in a few instances we have found pairs of actin filaments connected by structures similar to myosin dimers observed by Zobel and Carlson (1963) in shadow-cast material from skeletal muscle.

The above observations on homogenates appear relevant to intact smooth muscle. In glycerin-extracted, methacrylate-embedded tissue, actin exhibits lateral projections 20–30 A wide, consistent with individual myosin molecules attached to actin in the same manner as in homogenates (Fig. 6). Review of Plate 88 of Needham and Shoenberg (1964), which shows Araldite-embedded chicken gizzard, reveals similar structures.

Filament Organization

Isolated dense bodies with actin emerging from both ends (Fig. 14) bear a striking resemblance to the isolated Z discs with attached I-band filaments found by Huxley (1963) in homogenates of skeletal muscle. The homology is strengthened by finding thin lateral filaments which connect actin filaments within dense bodies (Fig. 15). These lateral filaments are to be clearly distinguished from the lateral processes thought to be composed of myosin found outside dense bodies. The former differ from the latter in location, shape, staining qualities, and the embedding medium necessary for their visualization. It is probable that individual actin filaments are not continuous within the dense body but rather, as in the Z disc (Knappeis and Carlsen, 1962), are linked end-to-end to form a compound filament each half of which has opposite sense. We have not succeeded in finding dense bodies with sufficient H-meromyosin or endogenous myosin attached at both ends to be certain of the direction of the actomyosin or acto-H-meromyosin arrowheads. The crucial matter of the directionality of smooth muscle actin therefore remains unresolved.

Lateral filaments identical to those in dense bodies are also found in membrane dense patches (Fig. 16), where they attach actin filaments to each other, and to the plasma membrane as well. We conclude that dense bodies and dense patches serve as attachment points, and also denote sets of actin filaments analogous to sarcomeres.

Sliding Models of Smooth Muscle Contraction

Within limits of measurement, diameters of longitudinal filaments from muscles fixed in contraction and fixed at rest length are the same, suggesting that contraction takes place through sliding rather than folding of filaments. This view is consistent with filament counts in resting and contracted smooth muscle (Shoenberg, 1962). The qualitative similarity between the force-

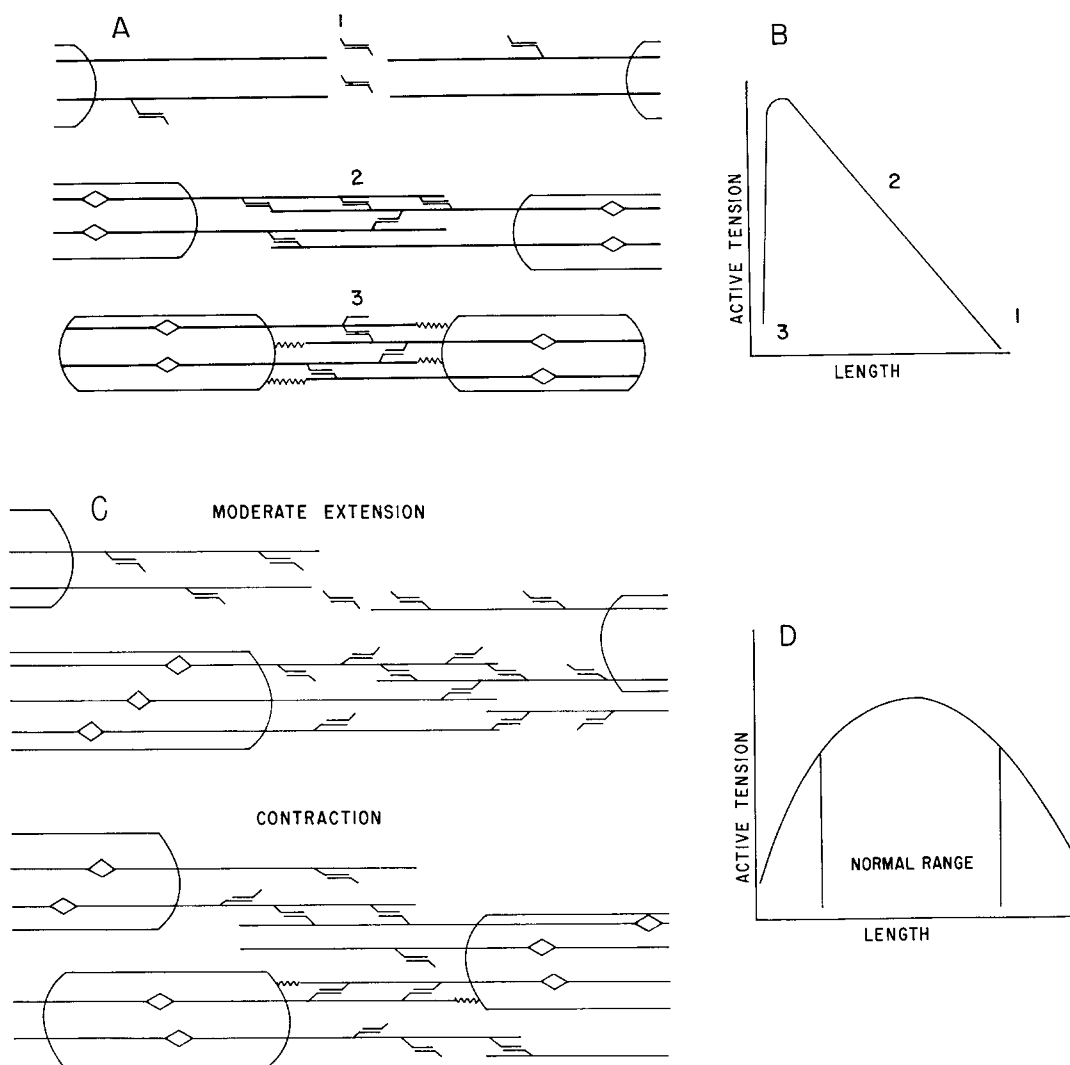


FIGURE 17 Two models of contraction mechanism of vertebrate smooth muscle; long lines denote actin; ellipses represent dense bodies; club shapes denote myosin. In both models, shortening is due to interdigitation and sliding of actin filaments driven by interaction with small myosin units. *B* and *D* are length-tension diagrams derived from models *A* and *C*, respectively.

velocity and length-tension relations of smooth and striated muscles (Csapo, 1960) also suggests a sliding mechanism. Since we believe all longitudinal filaments in sectioned smooth muscle to be composed of actin, the unique feature of the model we propose is that actin filaments interdigitate and slide with respect to one another, driven by small units of myosin.

The smallest myosin unit capable of interacting with two actin filaments is the one which we have observed in homogenates, namely a dimer. Though we have not seen larger aggregates, and the width of lateral processes in tissue sections is consistent with single myosin molecules, we cannot exclude the possibility that the functional unit is a tetramer. Aggregates larger than tetramers should, however, be identifiable in tissue sections and by negative staining. If dimers or

tetramers were arranged parallel to actin, they could link two sets of actin filaments which interdigitate only with each other. If the two sets were of opposite sense, interaction produced by many myosin units would increase the extent to which actin filaments overlap and so shorten the muscle. Developed tension would fall to zero if the muscle were stretched sufficiently to eliminate overlap of actin filaments (Fig. 17 *A1* and *B1*). Tension should also fall with extreme contraction because of contact between actin filaments and the dense body of the opposite "half-sarcomere" (Fig. 17 *A3* and *B3*). Over the range of lengths at which the muscle might normally function the number of possible links, and hence isometric tension, should decrease with length (Fig. 17 *A2* and *B2*). According to Csapo (1960), tension normally increases with length, and a broad length optimum is a salient physiological characteristic of smooth muscle. It might be possible for us to reconcile the observed length-tension relation with the arrangement shown in Fig. 17 *A* were a range of "sarcomere" lengths present in the muscle, or through some effect of length on excitation-contraction coupling, but these possibilities seem unlikely. Moreover, in most smooth muscles the number and size of dense bodies seem inadequate to impose the high degree of order the model requires.

An arrangement which does predict the correct length-tension relation, and requires fewer dense bodies, is shown in Fig. 17 *C*. In this model, each set of filaments collated by a particular dense body interdigitates with more than one set of filaments of opposite sense. Since dense bodies are not in register, linkages and hence tension are possible at lengths sufficient to eliminate overlap of some sets of actin filaments. Note that tension can also be developed at very short lengths, even though some dense bodies may be separated by a distance less than that of the free actin which emerges from a dense body. Because smooth muscle appears less well ordered than striated muscle, fewer links may be formed for comparable amounts of actomyosin. This may be one of the factors responsible for the relatively low tension per unit fiber cross-section characteristic of smooth muscle.

A third possible arrangement is one in which myosin is oriented almost perpendicular, rather than parallel, to actin. This model is similar in principal to that shown in Fig. 17 *C*, in that the long myosin units would link well separated actin filaments which would not necessarily interdigitate. The actin filaments would, nevertheless, slide relative to one another. Although perpendicular arrangement of myosin yields the correct length-tension relation, longitudinally directed forces are more likely to be developed by longitudinally oriented tension-bearing units.

How might a small myosin unit, regardless of orientation, apply force to an actin filament from which it might, on occasion, be completely detached? As shown schematically in Fig. 17, actin filaments are thought to be connected by many myosin units. Load-dependent interaction and shortening could, therefore, occur, provided linkages form and break asynchronously along each actin filament. Slow contraction and absence of a system of sarcotubules in relation to filaments are consistent with asynchronous activation. Thus, we visualize contraction of smooth muscle as interdigitation and sliding of actin filaments driven by relatively disaggregated myosin. In this view, failure of myosin to form thick filaments in vivo is an adaptation which facilitates shortening at the expense of speed and force.

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