ULTRASTRUCTURAL STUDIES ON THE CONTRACTILE MECHANISM OF SMOOTH MUSCLE

ROBERT E. KELLY and ROBERT V. RICE

From the Mellon Institute of Carnegie-Mellon University, Pittsburgh, Pennsylvania 15213

ABSTRACT

Fresh taenia coli and chicken gizzard smooth muscle were studied in the contracted and relaxed states. Thick and thin filaments were observed in certain (but not all) cells fixed in contraction. Relaxed smooth muscle contained only thin filaments. Several other morphological differences were observed between contracted and relaxed smooth muscle. The nuclear chromatin is clumped in contraction and evenly dispersed in the relaxed state. The sarcolemma is more highly vesiculated in contraction than in relaxation. In contraction, the sarcoplasm also appears more electron opaque. Over-all morphological differences between cells fixed in isometric and in unloaded contraction were also noticeable. The results suggest a sliding filament mechanism of smooth muscle contraction; however, in smooth muscle, unlike striated muscle, the thick filaments appear to be in a highly labile condition in the contractile process. The relation between contraction and a possible change in pH is also discussed.

INTRODUCTION

The similarity between striated and smooth muscle with respect to the force velocity and length tension curves suggests a sliding filament mechanism of smooth muscle contraction (2, 5). Panner and Honig (17) identified only thin filaments in turkey gizzard and have proposed a sliding mechanism of contraction involving a single array of actin filaments which "interdigitate and slide by the force of small myosin units." We have reported a double array of filaments in glycerinated chicken gizzard and have shown that the appearance of thick filaments in the glycerinated smooth muscle is pH dependent (18). We were unable to correlate the double array of filaments with contraction since the glycerinated tissue showed little contractility. Fresh smooth muscle contracts readily; it is difficult, however, to control the intracellular pH of this tissue. Results of early investigations are summarized in recent reviews by Needham and Shoenberg (15, 24).

The purpose of the present experiments is to show that a double array of filaments can be identified in fresh tissue and to correlate the appearance of thick and thin filaments with the contractile mechanism of smooth muscle.

Both fresh chicken gizzard and guinea pig taenia coli were used in these experiments. The fresh gizzard was used in an attempt to corroborate the previous findings on glycerinated gizzard. The taenia coli was used because, unlike gizzard, it has a longitudinal arrangement of the cells which allows the contraction (by shortening) to be measured more accurately.

MATERIALS AND METHODS

Strips of taenia coli of guinea pig were quickly dissected away from the cecum after sacrifice of the animal with ether. The taenia was moistened with Krebs-Ringer solution (10) during the dissection. In these studies, it was found that if the taenia was stretched during the removal of the strip from the animal, a coiled shortening of the muscle occurred and the muscle became rigid. When this rigid muscle was placed in Krebs-Ringer solution containing

0.25% atropine sulfate, the muscle extended and became flaccid. This coiled contraction could also be prevented if care was taken not to stretch the muscle in the excision process. Flaccid muscle obtained with or without atropine sulfate treatment was considered to be relaxed smooth muscle. All relaxed muscles were fixed for 3 hr in 6.25% glutaraldehyde in Krebs-Ringer's adjusted to pH 7.2.

For studies of contraction, individual strips of taenia were immediately stretched on corks and placed for 5 min in one of three oxygenated Krebs-Ringer solutions containing 5 mM ATP (ATP disodium, Sigma Chemical Co., St. Louis, Mo.), at pH 6.2, 7.2., or 8.2, respectively. Other stretched strips were placed in Krebs-Ringer solutions at the above pH values without ATP. For determining if these conditions of stretch caused contraction, other strips of taenia were immediately secured to a Statham strain gauge in a cylindrical glass chamber containing Krebs-Ringer solution. The Krebs-Ringer solution was removed and replaced by Krebs-Ringer's containing 5 mM ATP. That contraction occurred was evidenced by a rapid rise in the polygraph record. Each stretched specimen in Krebs-Ringer's, with or without ATP, was accompanied by a 1-cm free strip of taenia. The length change of the free segment was measured immediately after careful excision and then again after the 5-min period in the various solutions. The strips in the solutions containing ATP contracted at least 30% of the excised length.

All contracted tissues were fixed for 3 hr in 6.25% glutaraldehyde made up in a Krebs-Ringer's or Krebs-Ringer and ATP solution, the pH of which was adjusted to that of the solution in which the tissue was bathed for the 5-min period prior to fixation.

For studies of gizzard, thin strips were either fixed immediately unstretched in 6.25% glutaraldehyde or were washed for 30 min in a medium containing 0.1 M KCl, 1 mM $MgCl_2$, 5 mM ATP, 0.0166 M phosphate buffer pH 5.8. In this ATP-salt medium the gizzard contracted noticeably; however, measurements of contraction of gizzard are highly variable probably owing to the nonlinear arrangement of the muscle cells. The contracted gizzard was fixed for 3 hr in a solution of 6.25% glutaraldehyde, 5 mM ATP, and standard salt solution at pH 6.2.

All of the above tissues were postfixed in 1% osmium tetroxide (Palade's fixative [16]) and dehydrated in graded alcohols prior to embedding in Vestopal (Vestopal W; Martin Jaeger, Geneva, Switzerland) or were dehydrated in graded acetones and embedded in an Epon-Araldite mixture (25). Vestopal-embedded sections were stained for 3–5 min in a 1% solution of phosphotungstic acid in 75% ethanol. Epon-Araldite sections were stained with 2% uranyl acetate in methanol and poststained with lead citrate (21). Sections were viewed in a Philips 200 electron microscope at 80 kv with a 30-μ objective aperture.

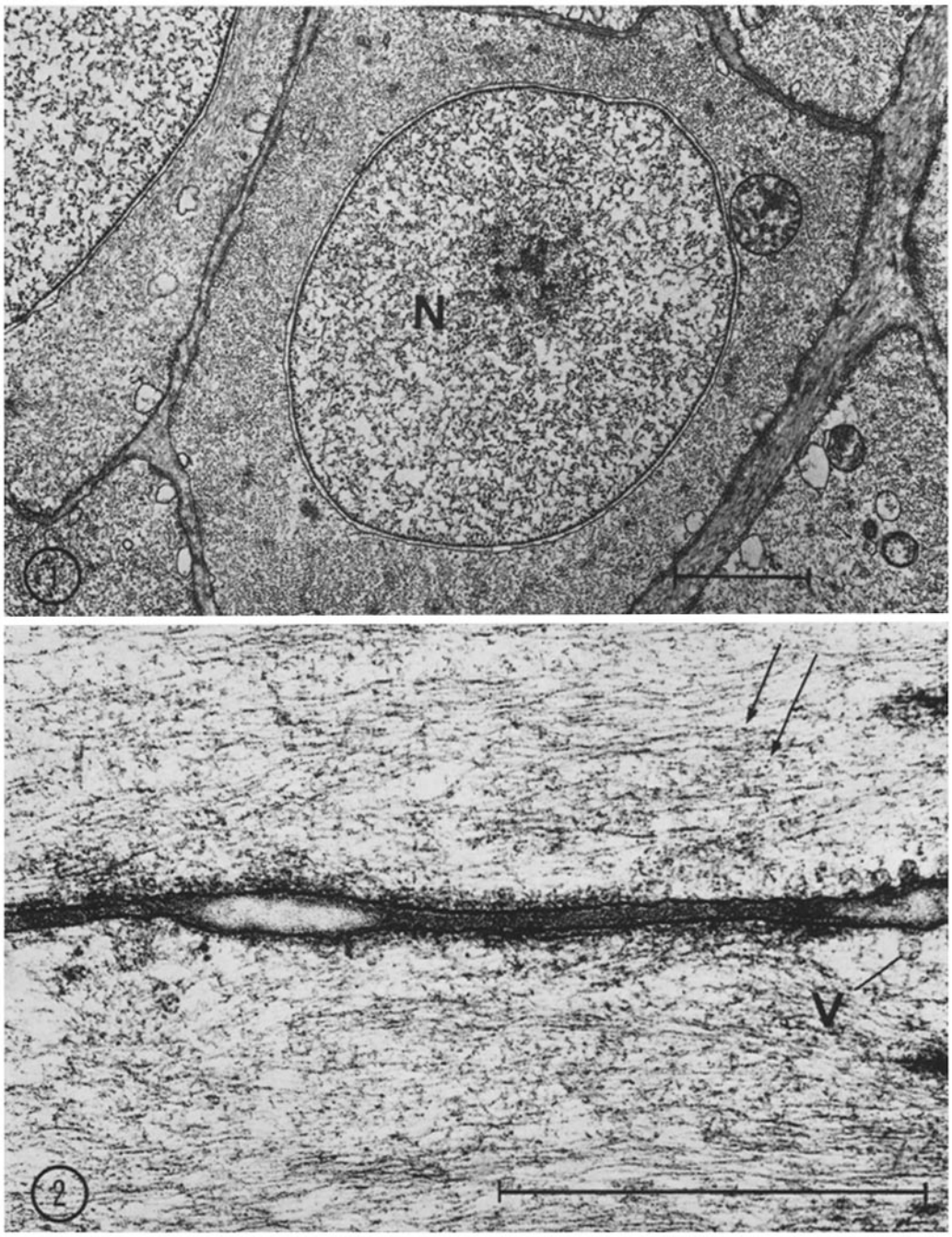
RESULTS

Relaxed Muscle

The fusiform cells of the relaxed taenia coli are highly attenuated and contain a nucleus elongated in the longitudinal axis of the cell. Only thin filaments approximately 60–80 A in diameter can be identified in any of the relaxed muscles studied (Figs. 1 and 2). The thin filaments have a somewhat wavy course generally in the longitudinal axis of the cell. At high power, the waviness of the filaments gives the relaxed smooth muscle a trabecular appearance (Fig. 2). Mitochondria are found dispersed throughout the cell but are most prominent in the perinuclear region, along with a small amount of smooth reticulum. The sarcolemma appears smooth except for an occasional vesicle. Often large, roughly circular vesicles 350–500 A in diameter are distributed throughout the cytoplasm (Fig. 1). One striking feature of relaxed smooth muscle is the moderate electron opacity of all the cells observed. Within the nucleus of moderate electron opacity, small chromatin granules are evenly dispersed throughout the karyoplasm (Fig. 1). The size of the chromatin granules varies; however, 170-A granules are prevalent. No clumping of the chromatin is ever observed in relaxed smooth muscle. The nucleus is enclosed in an even electron-opaque rim $\sim$ 315 A in width, and an outer nuclear membrane 90 A in width (Fig. 1).

Contracted Muscle (Stretched)

The smooth muscle cells fixed in isometric contraction are generally fusiform in shape and show a parallel alignment (Fig. 4). Both thick and thin filaments are often observed in isometrically contracted cells (Figs. 3, 5, and 6). The filaments have a parallel alignment with respect to the fiber axis (Fig. 4). The thick-to-thin filament relationship in cross-sections of stretched taenia fixed in the ATP-Krebs-Ringer solution cannot be accurately described as a rosette. There is often a halo of moderate electron opacity about 260 A in diameter surrounding an electron-opaque core of 175 A (Figs. 3 and 6). The distance between the thick filament core and the thin filament is variable, but approximates 260 A. Although many cells in isometric contraction contain both thick and thin filaments, it must be emphasized that some



The magnification line in all figures represents 1 μ except where noted.

FIGURE 1 Cross-section of a smooth muscle cell of the taenia coli relaxed with 0.25% atropine sulfate. The nucleus (*N*) contains evenly dispersed chromatin granules. Large irregular vesicles are dispersed throughout the sarcoplasm. Stained with 2% uranyl acetate and poststained with lead citrate. × 18,500.

FIGURE 2 Longitudinal section of a smooth muscle cell in natural relaxation. Only thin 50-A filaments (arrows) are present. Vesicles (*V*), 500 A in diameter, can be seen along the sarcolemma. Stained with 2% uranyl acetate and poststained with lead citrate. × 60,800.

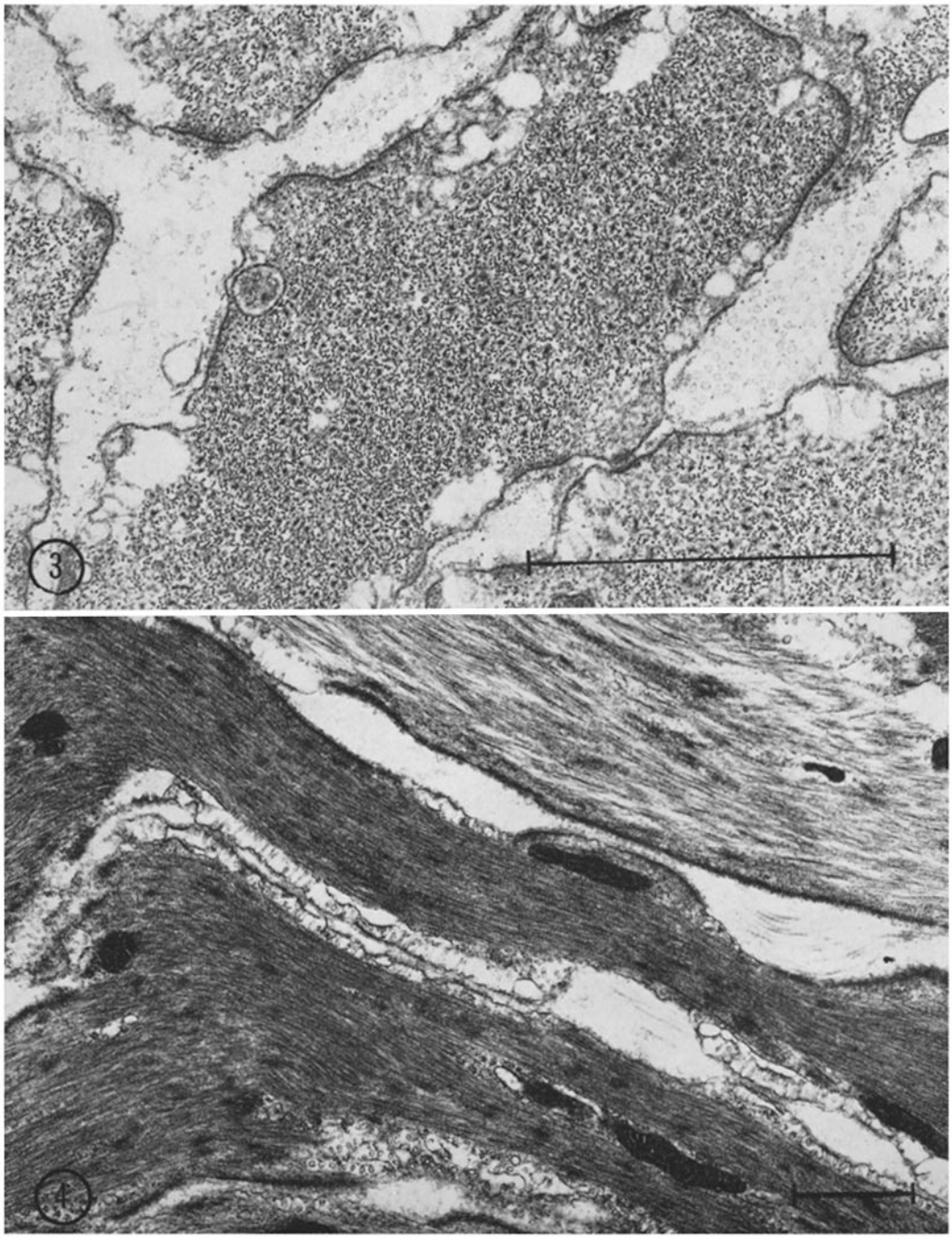


FIGURE 3 Cross-section of a smooth muscle cell of taenia coli isometrically contracted, in the ATP-Krebs-Ringer solution at pH 7.2. Thick and thin filaments are distributed throughout the cell. Thick filaments are 190 A and are surrounded by a halo of moderate electron opacity. Stained with 2% uranyl acetate and poststained with lead citrate. × 52,480.

FIGURE 4 Longitudinal section of taenia smooth muscle contracted isometrically in the ATP-Krebs-Ringer solution at pH 7.2. Filaments run generally in the longitudinal axis of the cell. The surface is highly vesiculated. The subsarcolemmal space is approximately 2,000 A. Sarcolemmal vesicles about 500 A in width can be observed traversing the subsarcolemmal space. Stained with 2% uranyl acetate and poststained with lead citrate. × 16,000.

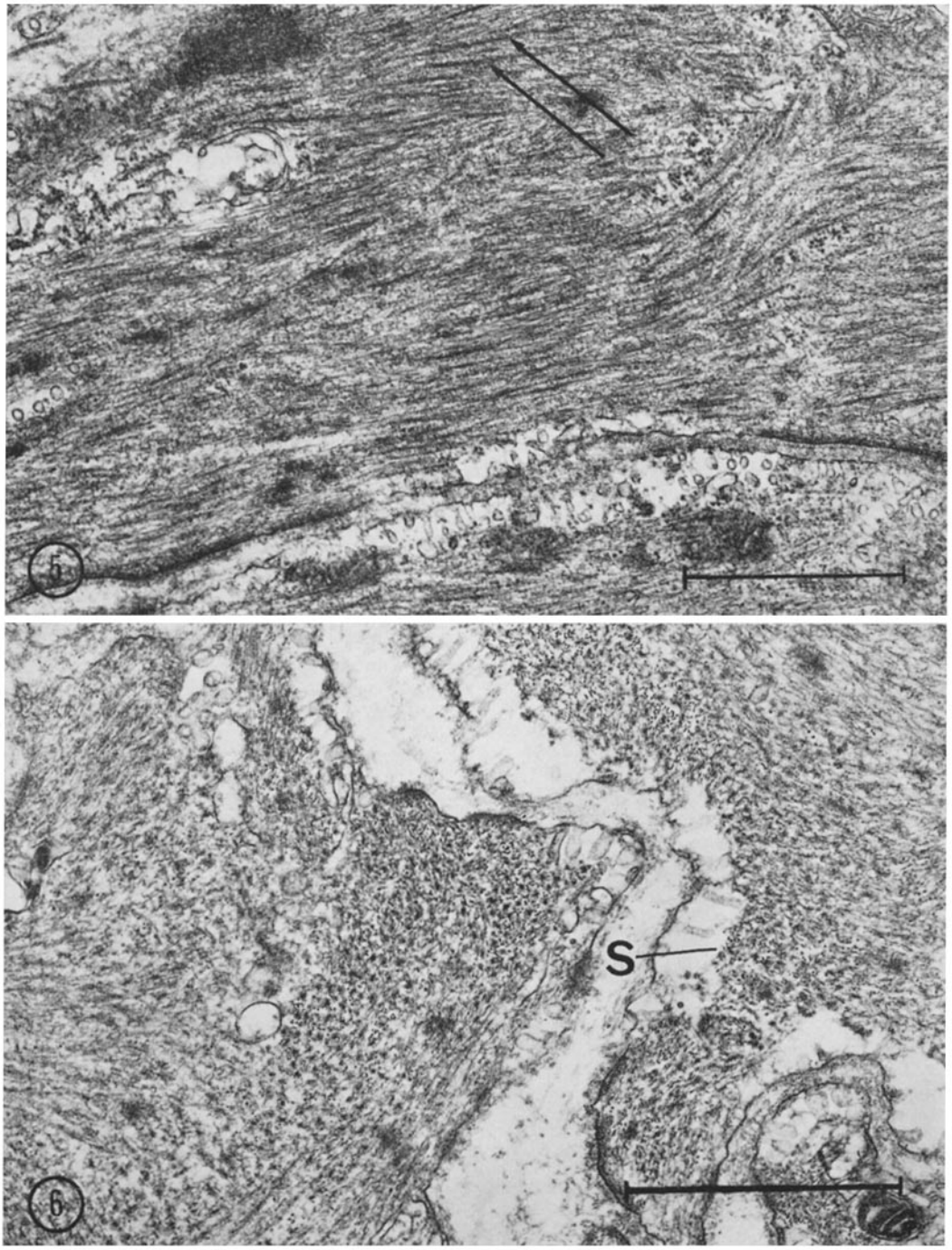


FIGURE 5 Longitudinal section of smooth muscle stretched in excision with subsequent contraction. Thick 150-A filaments (arrows) are observed. Vesicles 300 A in diameter and 1,000 A long extend in from the sarcolemma. Stained with 2% uranyl acetate and poststained with lead citrate. × 30,000.

FIGURE 6 Section of the smooth muscle of taenia coli contracted by stretch, showing both the transverse and logitudinal views of the thick and thin filaments. The sarcolemma is highly evaginated, forming a subsarcolemmal space (*S*), 2,000 A across. Vesicles 300 A in width traverse the space. Stained with 2% uranyl acetate and poststained with lead citrate. × 39,040.

cells contain only thin filaments. Even within the same block of one stretched segment of taenia, thick and thin filaments are observed at one level while cells of another region contain only thin filaments (Fig. 10). All cells, with or without thick filaments, in an isometrically contracted segment of taenia have a greater electron opacity than the cells fixed in relaxation. It must be emphasized also that in fresh smooth muscle, unlike glycerinated muscle, the appearance of thick and thin filaments cannot be correlated with the pH of the bathing solution. Thick filaments are observed in many cells fixed at pH 7.2, as well as in cells fixed at pH 6.2. Occasionally, 220-A circular tubules are observed in the interior of the cell (Fig. 3). These tubules are usually surrounded by a clear zone 100–200 A in diameter in which no filaments are evident. The sarcolemma of muscle fixed in isometric contraction has a greater number of invaginations or vesicles than the sarcolemma of relaxed muscle. The vesicles are arranged in rows along the length of the cell (Fig. 4). They are 500 A across and extend for variable distances into the cell. Often regions of vesiculation appear blister-like in that the sarcolemma separates sharply from the underlying myofilaments, accentuating a subsarcolemmal space as great as 2,000 A (Figs. 3–6). This region between the sarcolemma and myofilaments appears empty except for the vesicles which may traverse a subsarcolemmal space as large as 2,000 A. The vesicles are often contiguous with the underlying myofilaments (Figs. 4–6). If the space is too large (greater than 2,000 A), separation of the vesicles from the myofilaments occurs (Fig. 6). These bulges of the sarcolemma often occur between two dense attachment plaque areas (Figs. 3 and 4). Fig. 4 illustrates a typical arrangement of the vesicles and the subsarcolemmal space. At one point, a sarcolemmal evagination is observed between two dense attachment zones, while at another, the sarcolemma rejoins the main body of the cell at a point at which no density is evident beneath the membrane. Occasionally (Fig. 8), rows of circular vesicles are observed at the ends of the cell, extending for some distance between bundles of filaments. These vesicles are interpreted as the invagination of the sarcolemma sectioned in a transverse plane. The vesicles attached to the sarcolemma are often associated with highly elongated, dense mitochondria (Figs. 4 and 8), especially at the periphery of the cell.

The nuclei of the cells in isometric contraction are generally fusiform and have clumped chromatin especially at the periphery of the nucleus.

Contracted Smooth Muscle (Unstretched)

In smooth muscle allowed to shorten freely, the cells, even neighboring ones, show great variation in shape and electron opacity (Figs. 7, 9, 11, and 12). The most prevalent cell shapes observed in the unloaded contraction of gizzard and taenia are the crenulate and the serpentine. Crenulate appearance is due to numerous cytoplasmic projections of the cell which interdigitate with projections from neighboring cells (Fig. 11). These projections are tightly abutted at both crest and trough so there is little intercellular space. Often the processes lack filaments, but contain many vesicles which are usually attached to the sarcolemma. The cells of serpentine shape seldom have cytoplasmic projections (Figs. 7 and 12). Often, a large intercellular space separates neighboring cells; however, the gap at the end-to-end abutment of the cells is very narrow (~240–350 A). As in the stretched contracted muscle, both thick and thin filaments are observed in certain cells of both taenia coli and chicken gizzard allowed to contract freely (Figs. 7 and 9).

DISCUSSION

The results of these experiments show that a double array of filaments can be identified in fresh smooth muscle of both chicken gizzard and guinea pig taenia coli in contraction. The presence of thick filaments in a number of smooth muscle cells in contraction and the complete absence of thick filaments in the relaxed state suggest that the myosin filament may aggregate in contraction and disperse in relaxation. It is interesting that thick and thin filaments have now been observed in glycerinated smooth muscle at low pH (8), and in fresh smooth muscle in contraction. In fresh muscle, unlike glycerinated muscle, the appearance of thick filaments cannot be correlated with change in pH, since thicl and thin filaments are observed in fresh contracted smooth muscle in standard salt solution at neutral pH. The intracellular pH changes which accompany fresh smooth muscle contraction are, however, difficult to measure and hard to interpret. Dubuisson (3) measured the intracellular pH changes which accompanied contraction and relaxation in the stomach of the frog and tortoise, by use of a glass electrode method, and found four pH changes which were superim-

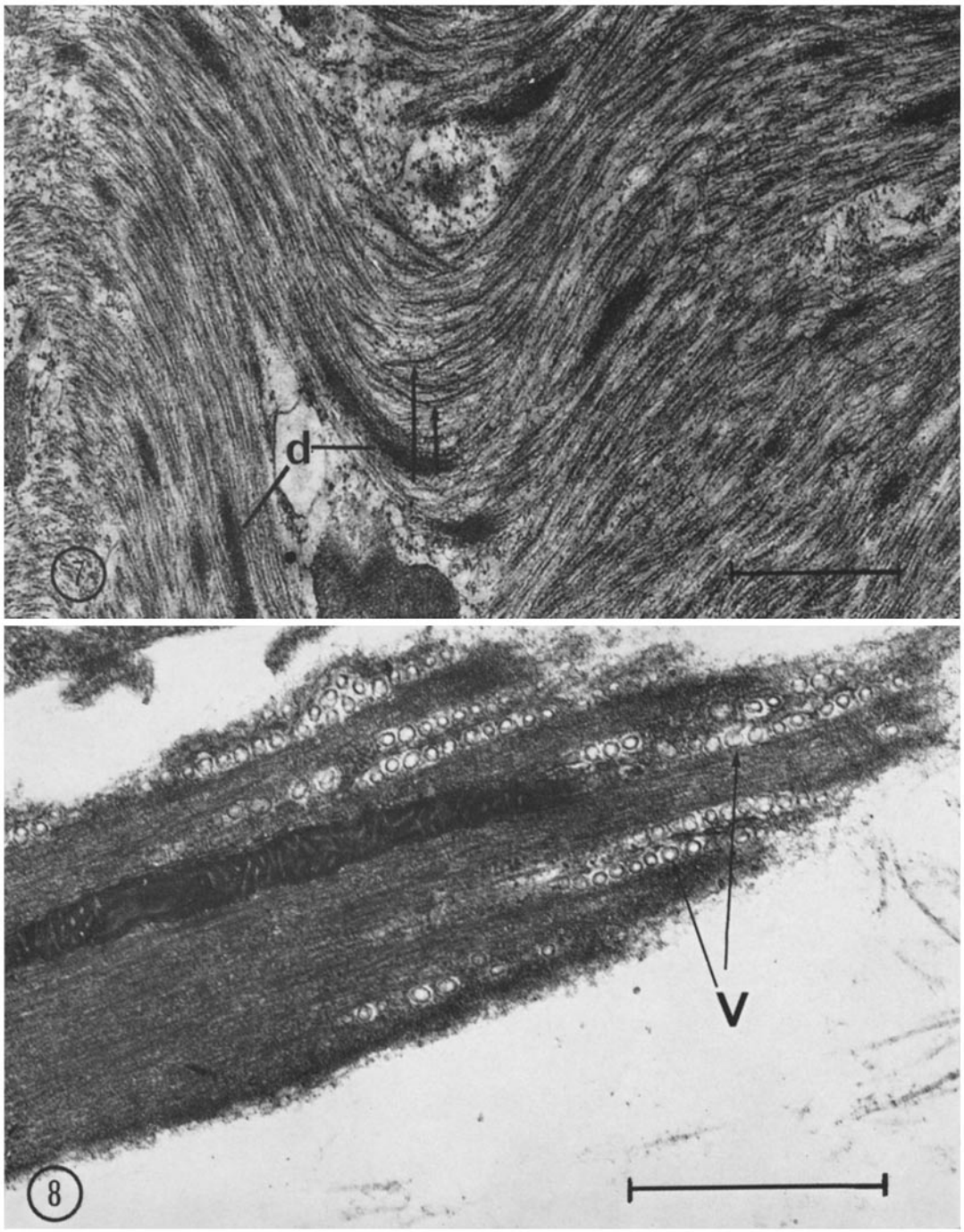


FIGURE 7 Longitudinal section of a gizzard smooth muscle cell isotonically contracted. Thick 160-A filaments (arrows) and thin 60–80-A filaments can be seen. Dense bodies (*d*) of various shapes are numerous. Stained with 1% PTA in 75% ethanol. × 20,400.

FIGURE 8 Longitudinal section of taenia contracted under stretch in the ATP-Krebs-Ringer's, pH 7.2. The tapered fiber contains circular vesicles (*V*) ranging from 500 to 750 A in diameter. The vesicles are arranged in rows between the filaments. The elongated mitochondrion is 0.2 μ in width and 2 μ in length. Stained with 2% uranyl acetate and poststained with lead citrate. × 36,800.

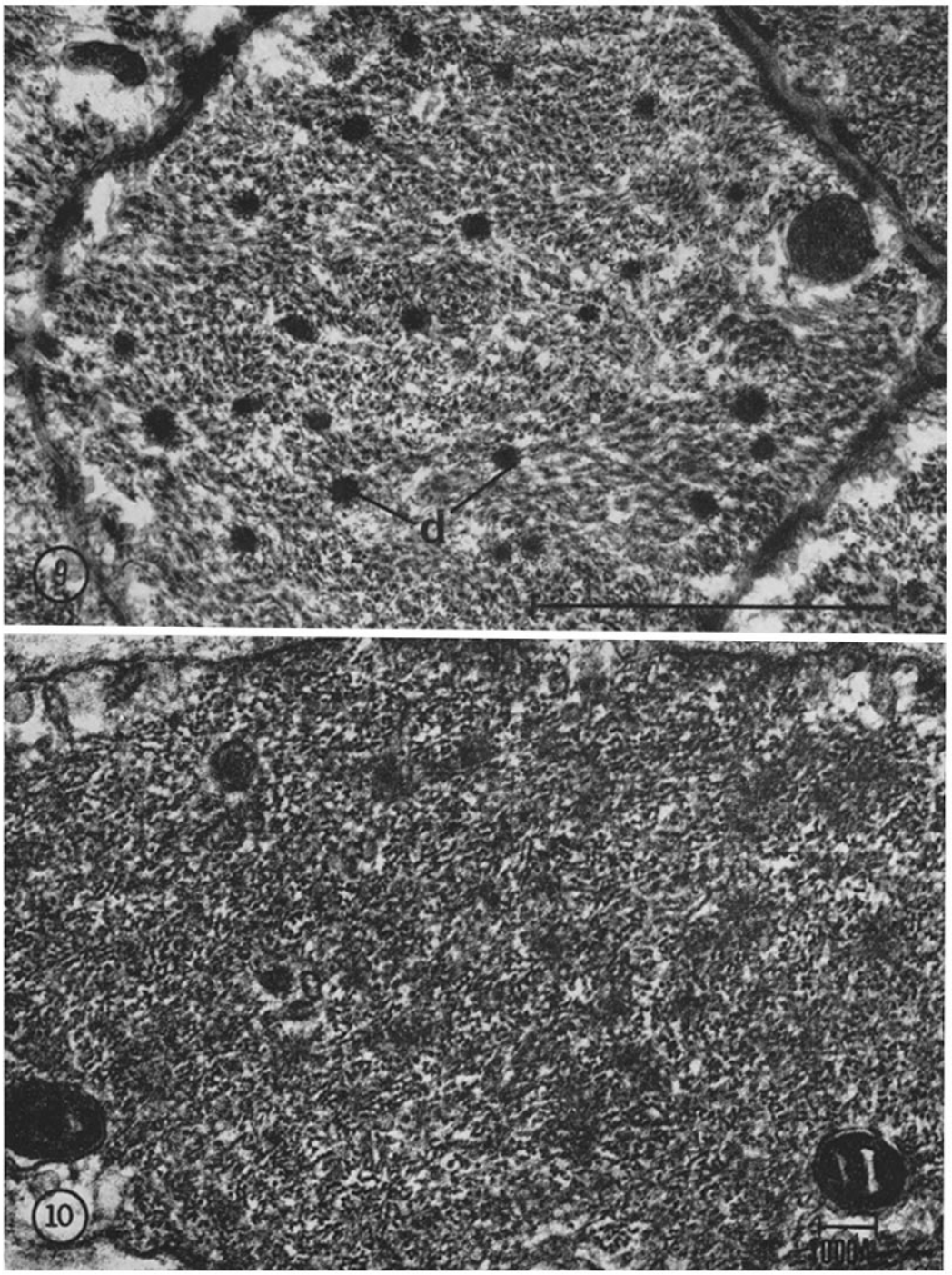


FIGURE 9 Cross-section of a fresh gizzard smooth muscle cell contracted in the ATP-standard salt solution under isotonic condition. The arrangement of thick to thin filaments is irregular and cannot be defined as a rosette. The 160-A thick filament is surrounded by a halo of moderate electron opacity. Dense bodies (*d*) and attachment plaques are prominent. Stained with 1% PTA in 75% ethanol. × 52,480.

FIGURE 10 Cross-section of taenia coli from the same segment of muscle as Fig. 6. Only thin filaments can be identified. × 85, 400.

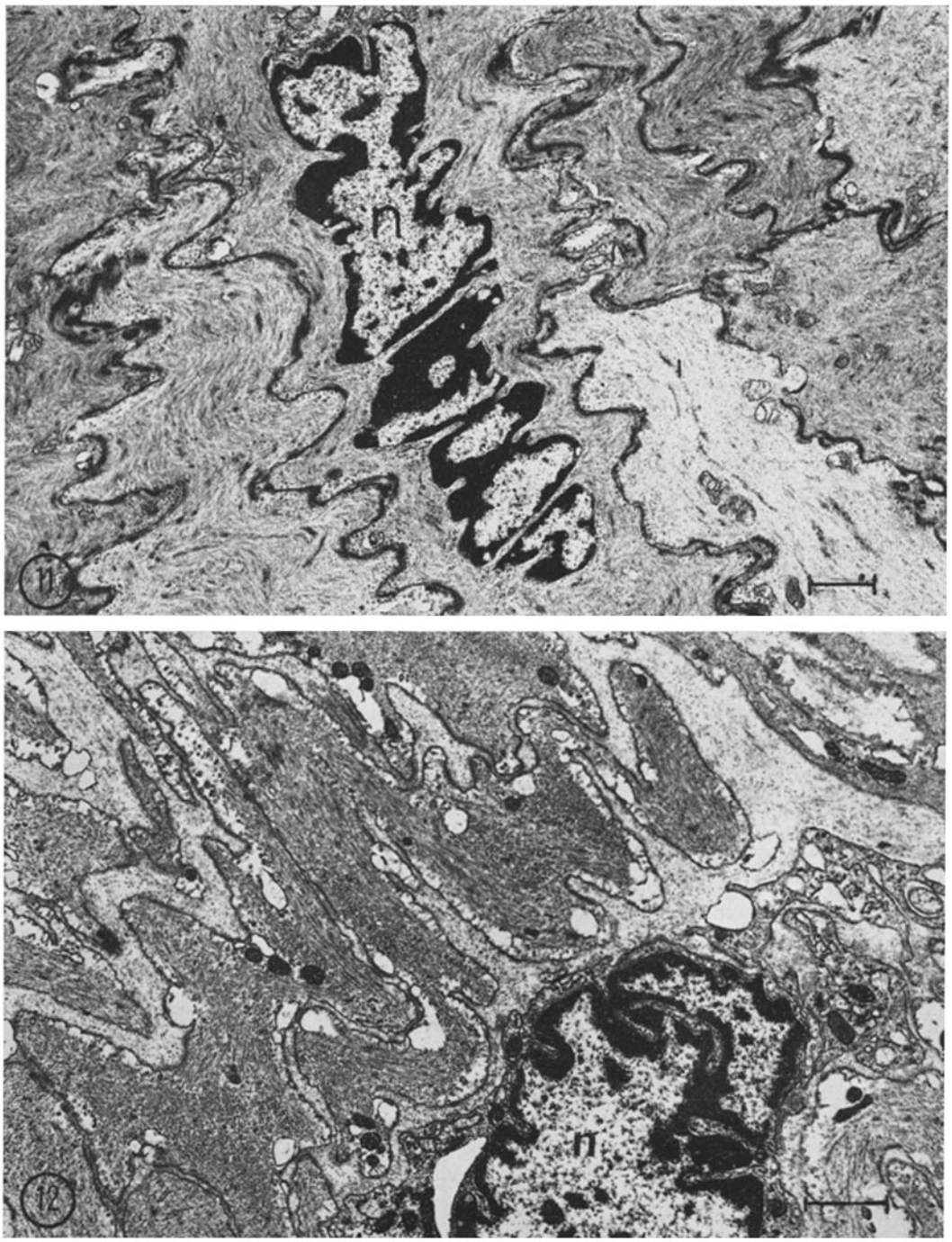


FIGURE 11 Longitudinal section of taenia cells in isotonic contraction. The cells have a crenulate appearance. Adjacent cells show large variation in electron opacity. The clumped chromatin appears along the periphery of the nucleus (*n*). Note the tight abutment of the cells. Stained with 2% uranyl acetate. × 9,900.

FIGURE 12 Longitudinal section of taenia cells with serpentine-shaped cells. The cells were fixed in isotonic contraction. Clumped chromatin appears along the periphery of the nucleus (*n*). The surface of the cell is highly vesiculated. Large regions of the cell contain a subsarcolemmal space. Large extracellular spaces are evident. Stained with 2% uranyl acetate and poststained with lead citrate. × 10,200.

posed on one another during isometric contraction and relaxation. In our previous experiments with glycerinated smooth muscle, it was assumed that the intracellular pH was in equilibrium with the surrounding medium. The work of Dubuisson demonstrates that this assumption is questionable with fresh tissue. Therefore, until more is known of the intracellular pH of fresh smooth muscle, the correlation between pH change, the appearance of thick filaments, and contraction will remain conjectural. X-ray diffraction has failed to detect reflections which could be assigned to myosin thick filaments, even when taenia coli was kept in simulated peristaltic contraction (4). This negative result may be a reflection of the labile nature of the thick filament structure. Thick filaments in fresh smooth muscle have been previously observed with the electron microscope by Lane (11), and a thick and thin filament preparation has been recently isolated from intact muscle by Schoenberg (24). In addition, Pease (18) has reported the presence of thick filaments in unfixed muscle prepared by either "inert dehydration" or by "freeze substitution." As we point out in this report, not all cells of contracted smooth muscle show thick filaments. If thick filaments are transitory, present X-ray diffraction methods may not be able to detect the characteristic myosin aggregate. The 115-A X-ray reflection assigned to regular actin filament packing (4), in living unstimulated taenia coli, would suggest a regular lattice arrangement of thin filaments which has never been observed in the electron microscope. Whereas the negative result of X-ray diffraction might be ignored by electron microscopists, this positive result must be considered. Better preparative methods may yet confirm the suggested lattice.

The thick and thin filaments, which we observe, run generally parallel to the longitudinal axis of the cell; otherwise, no apparent order in the arrangement of the filaments during contraction is evident. Certain evidence suggests, however, that thick filaments may be localized in regions along the segment of taenia fixed in isometric contraction. It is often found that thick and thin filaments are observed in cross-sections of one region of a segment of taenia contracted by stretch (Fig. 6), whereas in sections cutting deep into the block of the same segment the smooth muscle cells contain only thin filaments (Fig. 10). The cells of that portion of the segment with only thin filaments, however, have none of the morphological characteristics of relaxed smooth muscle, but are similar to the other cells of the same segment which have both thick and thin filaments. McGill (14) studied actively contracting smooth muscle with the light microscope and found that when the tissue was stimulated it contracted, and that contraction waves were observed passing over the fibers, causing distinct enlargement of the fibers as thy passed. In fixed tissue, she found that in most cases the wave appeared to be localized as densely staining, transverse regions along the cell. In total contraction, these transverse bands were wider, and in some cells the densely staining regions took up the entire contracting cell. Lewis and Lewis (12) observed similar wavelike nodes in actively contracting individual cells in tissue culture. The contraction wave is unique to smooth muscle and, upon initial consideration, appears to be incongruous with a sliding filament mechanism of contraction. However, unlike striated muscle, in which contraction and relaxation are reflected only in changes in the spatial arrangement of existing filaments, vertebrate smooth muscle has a contraction which appears also to involve aggregation and dispersal of the myosin filaments, the thin actin filaments remaining intact and supposedly being anchored to the sarcolemma. The localized regions of thick and thin filaments we observe in cross-section appear to cover a number of cells, and therefore these regions may be difficult to observe in longitudinal section owing to the limited field of view of the electron microscope. It seems possible, however, that the densely staining bands seen in the light microscope are a reflection of the localized regions of thick and thin filaments which we observe. If this is the case, the contraction wave may then be only an apparent phenomenon due to the periodic contraction of a series of fixed localized regions along the muscle fiber. The results emphasize that smooth muscle contraction must be considered as a whole-cell process.

Another interesting process occurring during smooth muscle contraction is the increase in the vesiculation of the sarcolemma and the formation of a subsarcolemmal space. Prosser et al. (20) have observed vesiculation in all cells from a wide variety of stretched smooth muscle types. Gansler (6) has reported an increase in size and number of vesicles in guinea pig colon during contraction. We also observe this increase in size of the vesicle; however, only the vesicle's depth (extension into the cell) increases, while the width of the vesicle remains relatively constant. The vesicles appear to contain an amorphous substance of moderate

electron opacity. Rostgaard and Barrnett (23) have localized ATPase activity within the vesicles of smooth muscle. The arrangement of the vesicles in rows between bundles of myofilaments, together with the fact that the base of the vesicles often remains contiguous with the myofilaments, suggests that the vesicles may be involved in activation-coupling during smooth muscle contraction. This, as Rhodin (22) has suggested, may explain the small development of the sarcoplasmic reticulum in smooth muscle. Vesiculation never occurs in the regions of the electron-opaque plaques beneath the sarcolemma. These plaquelike densities are believed to be the points where the actin filaments anchor to the sarcolemma (19, 7, 9). It is interesting that the subsarcolemmal space often develops between two such dense areas. It is possible that the subsarcolemmal space is a reflection of the tension developed during smooth muscle contraction. Thus, if fixed regions along the sarcolemma (attachment plaque area) begin to approximate each other by the force exerted by the interaction of the thick and thin filaments, the unfixed flexible membrane between (vesiculated region between attachment plaques) would evaginate and the sarcolemma would pull away from the filaments, forming a subsarcolemmal space.

We have observed increased electron opacity in the cells in contraction as have Gansler (6) and Rhodin (22). This increase in electron opacity is difficult to interpret, since density depends on a number of parameters (staining, thickness of sections, etc.). We have attempted to keep these parameters constant by comparing only contracted and relaxed muscle treated under identical conditions. In isometrically contracted muscle, almost all of the cells observed have a greater electron opacity than those fixed in relaxation. The great variation in density of the isotonically contracted muscle may indicate that all of the cells are not contracted under these conditions. The increased density of contracted smooth muscle appears to be due to an increased density in the sarcoplasmic matrix rather than an increased filament packing since the space between filaments appears to be more electron opaque in the contracted than in the relaxed state.

It is difficult to assess the reason for chromatin clumping in contraction and dispersal in relaxation. Changes in nuclear morphology have been described in light microscopic studies of smooth muscle contraction by McGill (13, 14). She measured the volume of the nucleus and found that it remained constant in the contracted and relaxed state. Isolated rat liver nuclei have been shown to swell and shrink according to changes in pH, ionic strength, and ionic composition (1). Therefore, a more careful control and measurement of these intracellular parameters is necessary before the problem of nuclear chromatin clumping can be approached in smooth muscle.

In summary, the results of these experiments shows that there are a number of morphological differences between smooth muscle cells fixed in contraction and those fixed in relaxation. Thick and thin filaments are observed in a number (but not all) of the cells fixed in contraction, whereas relaxed smooth muscle cells contain only thin filaments. The sarcolemma of the contracted cell is highly vesiculated, while only an occasional vesicle is observed in the relaxed state. The nuclear chromatin of contracted cells is always clumped, especially along the periphery of the nucleus, but in relaxation the chromatin is granular and evenly distributed throughout. In addition, over-all electron opacity appears to be much greater in contracted, as opposed to relaxed, smooth muscle. Differences between unloaded cells and cells fixed in isometric contraction are also noticeable. Smooth muscle cells allowed to contract freely with no restriction on the change of length assume a number of shapes, while the cells fixed in isometric contraction show little variation in over-all morphology.

The results of these experiments indicate the complex nature of smooth muscle contraction. Fresh smooth muscle is a difficult tissue to control, and more work must be done on development of more quantitative measurements of the intracellular milieu before the mechanism of smooth muscle contraction can be fully understood. We have shown that thick filaments occur in contracted smooth muscle. It is not known, however, if thick filaments are a prerequisite for all smooth muscle contractions.

After submission of this manuscript, two reports appeared which generally support our results. Nonomura (26) published a brief note in which he reported the presence of thick and thin filaments in freshly dissected guinea pig taenia coli. The thin filaments varied between 40 and 70 A, and the thick ones were 120–170 A. He did not observe a regular lattice arrangement of the filaments. Fixation was carried out at pH 7.2, but he did not attempt to show the state of contraction. Otherwise, his preparative methods were similar

to those reported here, except that he used the technique of "block staining" with 2% uranyl acetate which he suggested might be a prerequisite for demonstrating both sizes of filaments. In view of our results, this additional technique does not appear to be necessary although it may prove to be helpful.

Shoenberg (27) has recently published a more complete account of her already mentioned studies (15, 24). From her studies on negatively stained homogenized chicken gizzard, she suggests that Ca^{++}, Mg^{++}, and ATP must be present for thick filament formation. Although we have not systemically studied the effect of each of these substances, it should be noted that all these substances were present in at least physiological concentrations in the fresh tissue. She further suggests that the release of Ca^{++} after excitation triggers the aggregation of myosin. Schirmer (28) has made a similar suggestion from studies of smooth muscle actomyosin, but he suggested the lowering of pH as the trigger.

These results on homogenates and actomyosin should serve to stimulate work on the whole tissue. We cannot yet pick either Ca^{++} influx or pH lowering as the trigger in fresh tissue, but the results showing both thick and thin filaments may serve to encourage the workers who use in vitro preparation.

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