X-Ray Evidence for a Conformational Change in the Actin-containing Filaments of Vertebrate Striated Muscle

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The control of vertebrate striated muscle from rest to activity is regulated by the combination of the regulatory proteins tropomyosin and troponin on the thin actin filament (see reviews by Ebashi and Endo, 1968; Ebashi et al., 1969). In the absence of calcium the regulatory proteins inhibit the actin-myosin interaction. Activation of muscle is caused by calcium binding to troponin which then removes the inhibition. Since there is only one troponin molecule for every seven actin molecules (Weber and Bremel, 1971) and troponin does not interact with actin, then the control of activation is presumably mediated by the tropomyosin molecules which run along the grooves in the actin filaments (Hanson and Lowy, 1963; Moore et al., 1971). It is, therefore, of importance to investigate any structural changes that occur in the thin filaments when actin-myosin interaction is allowed to take place, for such changes may give us information about the control mechanism. X-ray diffraction for the study of muscle has the great advantage that the structure can be studied at the molecular level without destruction of the specimen. The low-angle X-ray pattern (corresponding to spacings above about 20 Å) from vertebrate striated muscle consists of layer lines and meridional reflections that arise both from the myosin filaments and from the actin filaments (Huxley and Brown, 1967), so it is possible, by observing changes in the low-angle diffraction pattern, to follow changes occurring in the thin filament structure when the muscle contracts or passes into rigor. The great disadvantage of X-ray diffraction studies on muscle is that they do not give information directly about the muscle structure; the structure must first be assumed from other studies and the structure can be accepted if it gives a diffraction pattern like the observed pattern.

The earlier studies of the muscle low-angle X-ray diffraction patterns (Elliott et al., 1967; Huxley and Brown, 1967) showed the gross structure of the thin filaments was remarkably insensitive to the state of the muscle. The axial period of the helical structure was the same whether the muscle was resting, contracting, or in rigor, and this indicated that there is little difference in the structure of the basic helix of F-actin (composed of G-actin monomers). The first evidence for a significant change in the actin X-ray diffraction pattern of contracting muscle was obtained by Huxley (1970, 1971a,b), who found that a reflection corresponding to the 2nd actin layer line was present in the patterns from muscles contracting at rest length, although this reflection was absent from the patterns from relaxed muscles. We then noticed that similar changes occur when a striated muscle is put into rigor at rest length, the 2nd layer line being distinct while no sign was seen of the 3rd layer line (Fig. 1). Much stronger patterns from live muscles (Fig. 1) showed that the 3rd layer line is visible, although it is not as strong as the 2nd layer line from rigor muscles. We also found (Vibert et al., 1972a,b) that such changes also occur in the smooth muscles anterior byssus retractor muscle of *Mytilus* (ABRM) and taenia coli of the guinea pig. The increase in the intensity of the 2nd actin layer line is presumably related to the changes that occur in the thin filaments when actin and myosin are allowed to interact. O'Brien et al. (1971) showed with optical transforms of actin paracrystals, with and without tropomyosin, that the relative intensities of the layer lines are changed by the introduction of tropomyosin—specifically, the 2nd layer line becomes stronger. Although the changes in the X-ray diffraction pattern from whole muscle are not likely to be due to the complete removal and addition of tropomyosin to the thin filaments, they could well be caused by movement of the position of the tropomyosin molecules with respect to the actin, as suggested by Huxley (1970, 1971a,b) and Spudich et al. (1972). Their work shows that the tropomyosin is not held in the center of the groove between two strands and is thus in a position where it may interact with the myosin binding sites.

We therefore made calculations (using an IBM 360/44 computer at the Institute of Theoretical Astronomy in Cambridge) of the diffraction patterns that would be given by various simple

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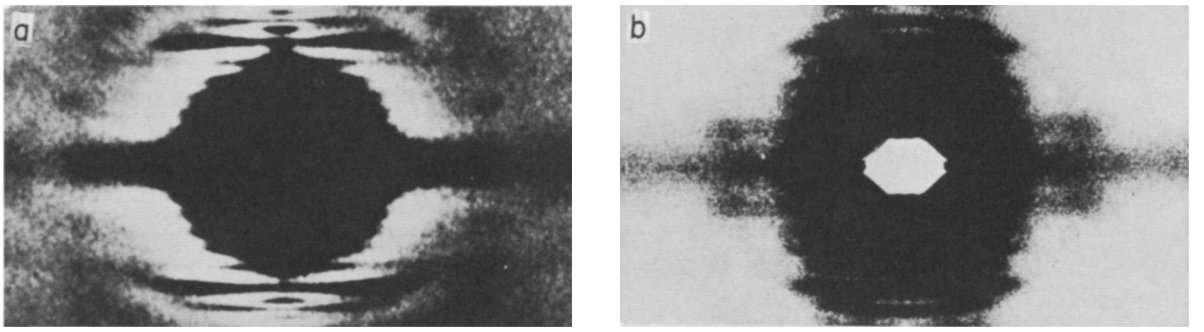

Figure 1. (*a*) The diffraction pattern from a live relaxed muscle at rest length. The 3rd layer line can be seen but not the 2nd. The layer lines are more easily seen if the picture is viewed at a glancing angle in the direction of the layer line. (*b*) The diffraction pattern of a muscle in a standard glycerol rigor at rest length. The 2nd layer line can be seen clearly but not the 3rd layer line. The same pattern is obtained from muscles in calcium-free rigor. (All the patterns were taken using a focusing X-ray camera (Huxley and Brown, 1967; Haselgrove, 1970) and have been reproduced by a background-leveling technique designed to enhance the weaker layer lines. The appearance of the pattern has thus been distorted to allow selected features to be demonstrated. We thank E. B. Neergaard for showing us this technique.)

models of the thin filament to see if we could account for the changes that occur in the observed diffraction patterns. The calculated patterns were compared with patterns obtained from live and rigor muscles. Rigor muscles were chosen for the comparison because in this state all possible myosin-actin attachments are made and we are investigating the changes associated with such interactions. However comparison of the patterns from vertebrate striated muscle contracting (patterns kindly lent by Dr. H. E. Huxley) and in rigor at rest length showed the second layer line to be of similar intensity compared with the rest of the pattern.

Calculations

The actin filament without any tropomyosin present may be considered for the purpose of calculation as a series of spherical subunits, each representing one actin monomer, joined in two long strands which wind around each other forming a slowly turning double helix (Hanson and Lowy, 1963). Mathematically the helix can be thought of in terms of the genetic helix with a repeat length of 51 Å and two subunits in slightly more than one turn of the helix. The structure repeats again after 15 turns of the genetic helix and in this distance there are 28 subunits. This distance (764.4 Å) is the pitch length of one of the two long strands of monomers. It makes very little difference to the diffraction patterns if the whole filament is twisted slightly so that the structure does not repeat exactly after one period of the long helix; so for convenience this 28/15 genetic helix was taken as our model for the F-actin helix which gives rise to reflections only on defined layer lines with indices (l), given by the selection rule $l = 28m + 15n$ (Cochran et al., 1952). Values for l and n for the actin layer lines are shown in Table 1. The intensity on a given layer line is the square of the amplitude

Table 1. Comparison of Observed and Calculated Layer Lines

Layer Line[a]	Index (l)	Bessel Order (n)	Intensity obs.[b]	Intensity calc. ($\times 10^{-4}$)	Radial Position Å^{-1} obs.	Radial Position Å^{-1} calc.
1 Relaxed	2	2	S	760	0.011	0.016
Activated			W	460	—	0.016
2 Relaxed	4	4	—	17	—	0.030
Activated			W	93	0.024	0.024
3 Relaxed	6	6	VW	30	0.029	0.028
Activated			—	10	—	0.032
5	11	−3	W	31	0.018	0.017
6	13	−1	S	401	0.008	0.009
7	15	+1	S	209	0.007	0.009

[a] The first three layer lines were calculated using the following parameters: radius of actin monomer, 20 Å; actin helix 24, Å; tropomyosin cylinder, 10 Å; $\phi = 50°$ for relaxed pattern, 70° for activated pattern. The 5th, 6th, and 7th layer lines were calculated using a 24 Å sphere at a radius of 24 Å. Diffraction from a 28/13 helix gives two exceedingly weak layer lines in the region between the 3rd and 5th layer lines. These layer lines have not been discussed because their intensity is so low. Data for the first three layer lines for a model with 8 Å radius tropomyosin can be read from Fig. 4.

[b] Intensity classification: S, strong; W, weak; VW, very weak.

F_A calculated from the equations

$$F_A = \Phi(x) Jn(X)$$
$$\Phi(x) = \frac{3}{x^3}(\sin x - x \cos x) \quad (1)$$
$$X = 2\pi r R$$
$$x = 2\pi r_0 s$$

where $Jn(X)$ is an nth order Bessel function, n is given by the selection rule. $\Phi(x)$ is the form factor for a sphere. R = the radius in reciprocal space of the point where the diffraction intensity is considered, s = distance from the origin in reciprocal space of this point, r = radius of the helix, and r_0 = radius of the sphere. No correction was made for the Lorentz factor because no evidence was ever obtained that the actin layer lines are sampled by a lattice repeat.

The actin filament structure is often thought of in terms of the 28/13 genetic helix which has a pitch of 59 Å and a repeat (or near repeat) of the structure after 28 subunits in 13 turns of the helix. It is useful when calculating the diffraction intensities from helices to follow the conventions of Cochran et al. (1952) for describing the helices, since their equations can then be used for calculations without adjustment of signs. Following their convention the 51 Å genetic helix describes an actin filament with a right-handed, long double chain, and the 59 Å helix describes an actin filament with a left-handed long chain. Actin is now thought to be a right-handed structure (Depue and Rice, 1965; Reedy, 1967, 1968). For the calculations described here, the results are not dependent on which model is chosen since X-ray diffraction cannot distinguish between two structures which are mirror images. However later calculations in which the angle or position of attachment of subunits is dependent on the direction of twist of the helix may well be confused if the wrong basic model is taken at this stage.

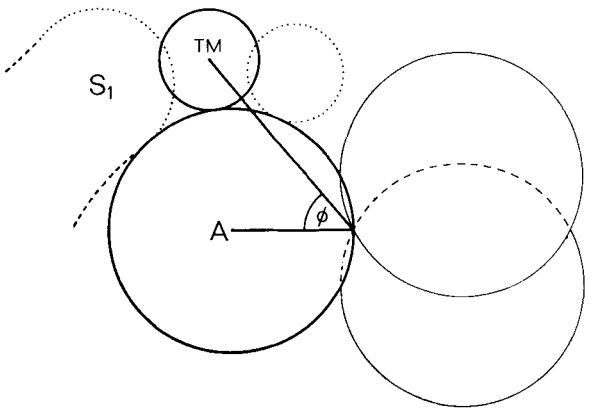

Figure 2. Cross section through a thin filament showing the relative positions of actin (*A*), tropomyosin (*TM*) and the S_1 subfragment of myosin when attached as in a decorated thin filament. The tropomyosin is shown both in the relaxed position (full circle) where $\phi = 50°$ and in the activated position (dotted circle) where $\phi = 70°$. Only one of the two tropomyosin molecules associated with the filament is shown; the other sits in a corresponding position in the other groove. For consistency with the equations of Cochran et al. the angle ϕ as shown should be negative.

For calculations of the diffraction expected from the thin filaments the tropomyosin molecules were included in the model. The tropomyosin was represented by two continuous cylinders, of radius r_{0T} and wound into a double helix of radius r_T so that one cylinder followed each groove in the actin structure. Since the cylinder representing tropomyosin runs so nearly parallel to the helix axis, it was assumed that the form factor f_{TM} of tropomyosin could be represented by the form factor of a thin disc of radius r_{0T}. The intensity of diffraction from the thin filament on a given layer line is then the square of the amplitude F given by

$$F = F_A + W_{TM} f_{TM} Jn(2\pi r_T R) \exp\{-in\phi\}$$
$$f_{TM} = \frac{2}{2\pi r_{0T} R} J_1(2\pi r_{0T} R) \quad (2)$$

W_{TM} is the weight/unit length of tropomyosin relative to the weight/unit length of actin in the structure. ϕ is the angle subtended at the center of the filament by the tropomyosin cylinder and the actin chain with which it is associated (Fig. 2). ϕ goes from 0 to $\pi/2$ as the tropomyosin moves from the very edge of the filament towards the center of the groove. Because tropomyosin is represented by a continuous helix it makes little or no contribution to the intensities of the 5th, 6th, 7th, and 8th actin layer lines which occur near the meridian because the actin-monomer helix is discontinuous. Therefore the intensity of the first four layer lines was calculated using Eqs. 2 taking both the tropomyosin and actin components into account, and the other layer lines were calculated using Eqs. 1 assuming that tropomyosin does not contribute to the intensity of these layer lines. (For details of the helical diffraction see Cochran et al., 1952 and Klug et al., 1958.) Little is known about the position or shape of the troponin in the thin filament except that it occurs regularly every 385 Å (Ohtsuki et al., 1967). We therefore did not take the troponin into account when performing the calculations, although we recognize that it may well affect the diffraction pattern detectably because the total weight of troponin in the thin filament ($2 \times 80{,}000$ daltons every 385 Å (Ebashi et al.,

1971) is about the same as the tropomyosin ($2 \times 70{,}000$ daltons every 385 Å (Holtzer et al., 1965; Woods, 1967). The occurrence of similar intensity changes in the frog muscle, which contains troponin, and the ABRM (Vibert et al., 1972a,b), which is troponin-free, encouraged us to make this simplification at this stage.

The object of the calculations was to simulate the diffraction patterns expected from different models of the thin filament structure and by comparison with the observed diffraction patterns to define some of the parameters of the structures that give the best agreements. Thus we could determine the structural changes that might be expected to occur when a muscle contracts or passes into rigor and that give rise to the changes in the diffraction pattern. Intensity measurements of the actin layer lines are very difficult because the reflections are very weak and diffuse (the 2nd layer line in a contracting muscle is probably about 100 times weaker than the 1st myosin layer line), making quantitative measurements of the intensity of the weaker lines impossible. Therefore we had to make the comparison of calculated and experimentally obtained patterns using a qualitative comparison by eye of the shape, intensity, and radial position of the layer lines (Table 1). The computer was programmed to give both the calculated intensity and a "visual" output of characters of different visual density to simulate a diffraction pattern; all the intensities below a selected level appeared blank and the others appeared more or less dark according to the intensity (Fig. 3). Comparison of the calculated and observed patterns was thus made straightforward.

In order to reduce the number of calculations that need be made (each model contains six parameters that can be varied) and to simplify the interpretation of the patterns, we imposed the following restrictions: (a) The helix of actin monomers has the same parameters in the relaxed muscle and the contracted muscle. (b) The radius of the tropomyosin cylinder is constant. (c) The tropomyosin cylinder always touches one long chain of actin monomers but the angle ϕ (Fig. 2) can vary, and with it the radius of the tropomyosin helix. (d) The values for the parameters should be physically meaningful in that the actin subunit should be about 20 Å in radius (Moore et al., 1971), and the tropomyosin cylinder should be about 10 Å radius (Holtzer et al., 1965). (e) Each 385 Å of filament length contains two tropomyosin molecules of weight 70,000 daltons (Woods, 1967)

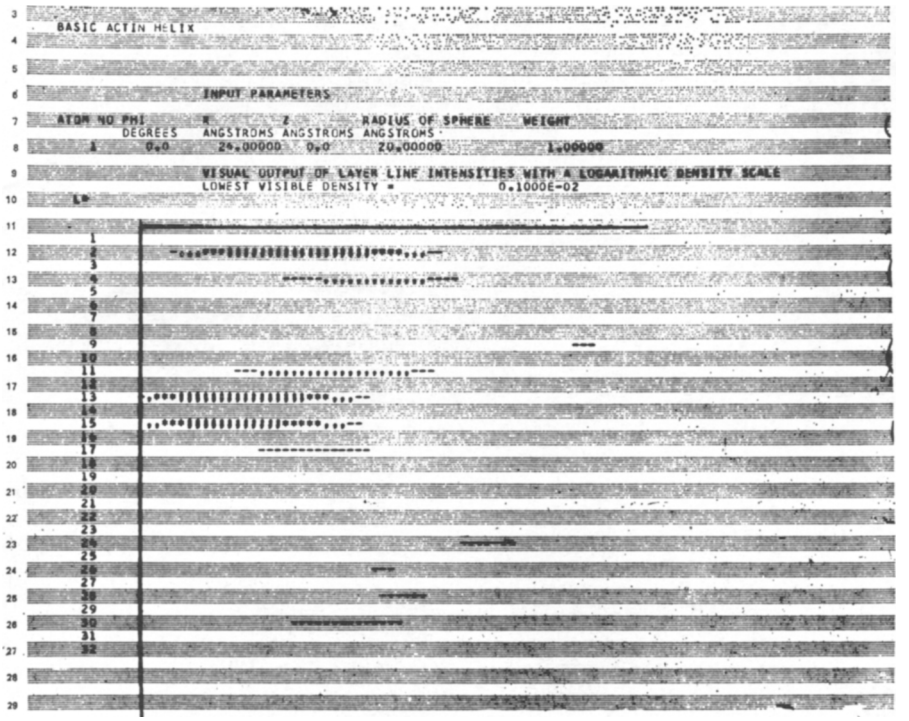

Figure 3. Computed visual output of the lower right-hand quadrant of the diffraction pattern from an actin helix consisting only of spheres 20 Å radius arranged into a helix 24 Å in radius. Points with an intensity of less that 0.001 are not shown. Each character printed represents a density level of $\sqrt{10}$ greater than the next visually less dense character. The intensity distribution along the first three layer lines is shown in Fig. 5b.

and 14 actin monomers of weight 46,000 daltons (Rees and Young, 1967). The value of W_{TM} was therefore held constant at 0.22 for all calculations.

The first parameters to fix were those of the actin-monomer helix. Calculations of models with different helix radii were compared with diffraction patterns from the frog sartorius, from the smooth muscles ABRM and taenia coli of the guinea pig. We have already found that the actin diffraction patterns from all these muscles is very similar (Vibert et al., 1972a,b). In making the comparison of calculated and observed patterns more weight was placed on the agreement of the 5th, 6th, and 7th lines, which are not affected by tropomyosin, than on the first three lines, which are affected. The best fit for all the patterns (Figs. 1, 3) came from a model with spherical subunits each 24 Å in radius at a helix radius of 24 Å. The same values were also obtained by Miller and Tregear (1971) from a study of the diffraction pattern from insect muscle. The 1st layer line of the calculated pattern is the strongest and extends as far from the meridian as any of the other lines. The 6th and 7th layer lines (the well-known layer lines at 59 and 51 Å) are also strong and are flanked nearer the equator by the 5th layer line. The 2nd layer line, although weak, is significantly stronger than the peak of the 3rd layer line which lies in the region in which the subunit transform is zero.

A helix of 24 Å radius spheres at a helix radius of 24 Å has a total diameter of 96 Å, which is slightly greater than the value of 80 Å usually accepted for the thickness of the thin filaments. In view of our restrictions to spherical subunits, a 24 Å radius sphere is an acceptable approximation to a subunit which is expected to be about 55 Å long (the repeat length of subunits along one chain) and about 40 Å thick in a cross section as found by Moore et al. (1970). Indeed if we were to reduce the cross-sectional size of the subunits to turn them into prolate ellipsoids, then the 6th layer line would extend slightly further from the meridian than it does relative to the 7th layer line, and the 5th layer line would extend slightly further still and so improve the agreement between the calculated and observed patterns. Nearer the equator of the pattern, the first few layer lines are more dependent on the cross-sectioned shape of the subunits than they are on the length of the subunit. Therefore when we calculated the intensity of these layer lines from models containing both actin and tropomyosin, we tried different models for the actin structure: (a) a 24 Å radius sphere in a 24 Å helix, (b) a 20 Å radius sphere in a 24 Å helix, and (c) a 20 Å radius sphere in a 20 Å helix.

The tropomyosin cylinder is sufficiently thin to give an appreciable contribution to the diffracted intensity in the region of the 2nd and 3rd layer lines, where the diffraction from actin is very weak, although it only has one-fifth of the weight of actin. As has already been mentioned, the changes in the 2nd and 3rd actin layer lines could be brought about by a movement of the tropomyosin in the groove of the actin helix. If we consider the structure of the thin filament at low resolution, then we can see the type of change that we would expect. With tropomyosin situated near the edge of the groove it would appear to divide the actin helix at one-third of its repeat length and correspondingly the 3rd layer line would be strong. With the tropomyosin near the center of the groove the actin repeat would appear to be half that of the actin monomer chain and so the 2nd layer line will be strong. Using the constraints described above we calculated the diffraction patterns expected from models with different diameter tropomyosin cylinders (in the region of 20 Å) at different positions in the actin groove. To determine the best models, a comparison was made of only the 2nd and 3rd layer lines of the calculated and the observed patterns. The first layer line in patterns from vertebrate striated muscle at rest length is obscured by the myosin diffraction pattern, the 4th line is too weak to see, and the 5th, 6th, and 7th layer lines are not dependent upon the position of the tropomyosin.

As expected, we found that it was possible to simulate qualitatively the changes we see in the X-ray pattern by changing the position of the tropomyosin in the actin groove. What we did not expect was that the changes in diffracted intensity could be accomplished by a relatively very small change in position, viz., a movement of the tropomyosin to alter the angle ϕ by 20° and corresponding to a movement of about 15 Å (Figs. 4, 5). The exact parameters chosen for the size of the actin and tropomyosin molecules were not very critical. All the parameters we used for the actin monomer helix gave acceptable patterns—24 Å spheres in a helix of radius 24 Å and 20 Å spheres in helices with radii of 20 or 24 Å. It was not possible to make the 3rd layer line much stronger than the 2nd if the tropomyosin was represented by a cylinder as large as 12 Å radius, but 8 and 10 Å cylinders gave patterns in which a change in the angle ϕ between the tropomyosin and actin helices of only 20° from 50° to 70° produced considerable changes in the pattern. It was not possible to choose between these six models with confidence because of the lack of quantitative data on the layer line intensities, but the models all showed the same characteristics as follows:

The 3rd layer line was strongest when the angle ϕ was about 50°, near which point the 2nd layer

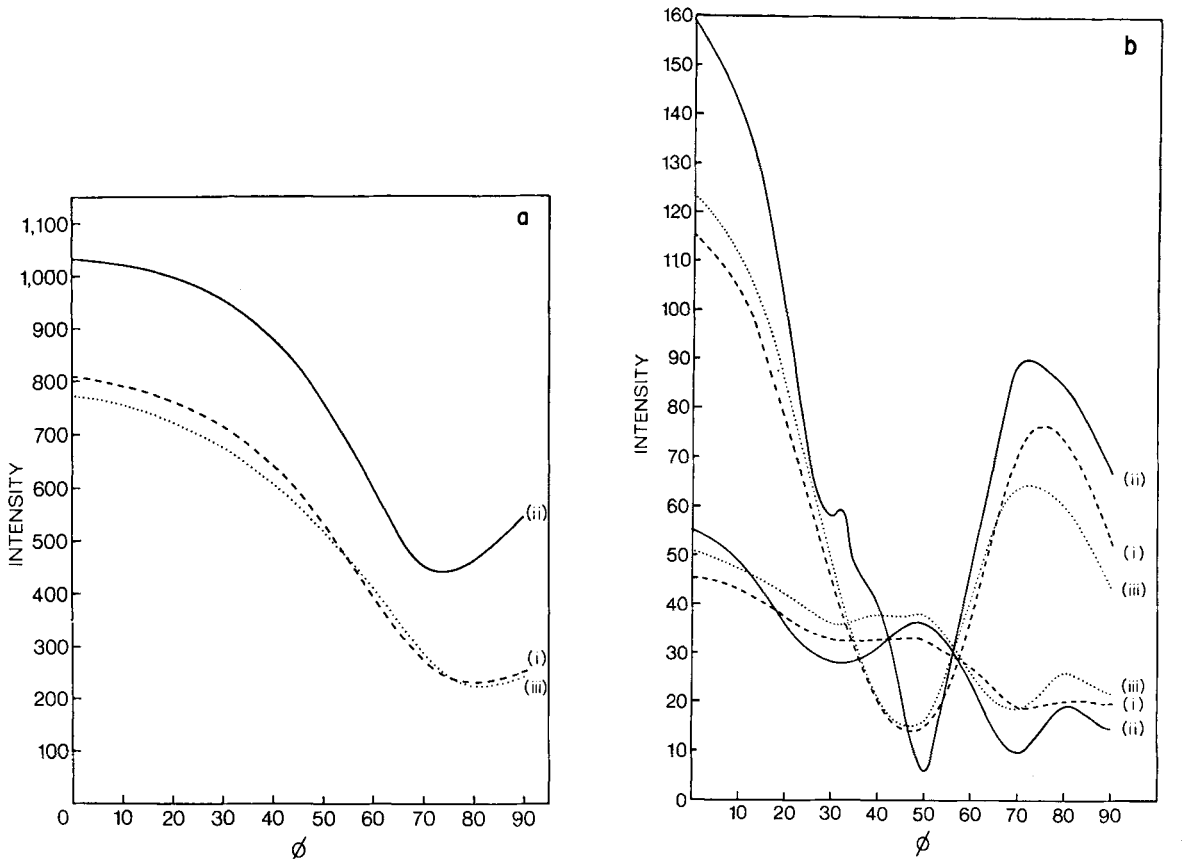

Figure 4. Graphs showing how the calculated peak intensities of the first three layer lines depend on the position of tropomyosin in the thin filament. (*a*) First layer line. (*b*) Top curves: 2nd layer line; lower curves: 3rd layer line. The position of tropomyosin is defined by the angle ϕ in Fig. 1. Tropomyosin radius = 8 Å. Curves are plotted for different models of actin: (i) 20 Å sphere in 20 Å radius helix; (ii) 20 Å sphere in 24 Å radius helix; and (iii) 24 Å sphere in 24 Å radius helix. Calculations with a tropomyosin cylinder of 10 Å radius give very similar curves showing that the exact parameters chosen for the model are not important.

line was at its weakest (Fig. 4). The ratio of the intensities of the 2nd and 3rd layer lines was then about 0.5:1. An increase of only 20° in ϕ decreased the intensity of the 3rd layer line to its weakest where it was only about one-half to one-third as strong. The same structural change strengthened the intensity of the 2nd layer line between four and ten times (depending on the model chosen) so that the relative intensities of the 2nd and 3rd layer lines was about 5:1 or more (Figs. 4, 5). The intensity of the 2nd layer line at its strongest is considerably stronger than the 3rd layer line at its strongest, in agreement with the patterns from vertebrate striated muscle. Further increase in the angle ϕ from 70° to 90° caused a very slight further increase in the intensity of the 2nd layer line from some models, but then the intensity began to drop again whereas the 3rd layer line intensity rose again slightly. The 1st layer line is worthy of note. Although the 1st layer line arising from actin alone is so strong and the weight of the tropomyosin component so small, the strength of the 1st layer line decreases by over one-third when the angle ϕ increases from 50–70°. This change in the position of the tropomyosin molecule in the thin filament corresponds to a linear movement of only about 15 Å (Fig. 1), which is sufficiently small to be explained in terms of conformational changes of molecules within the thin filament.

Thus we have shown using calculations from a very elementary model that the large change in intensity of the 2nd layer line that occurs when vertebrate striated muscles contract or pass into rigor (Table 2) can satisfactorily be explained by a movement of the tropomyosin molecule in the groove of the filament, as suggested by Spudich et al. (1972). Moreover only a very small structural change is necessary to produce quite pronounced changes in the diffraction pattern. A most pleasing aspect of the model that we deduce is that it is essentially the same as the structure of the actin-tropomyosin-troponin filaments in the presence of

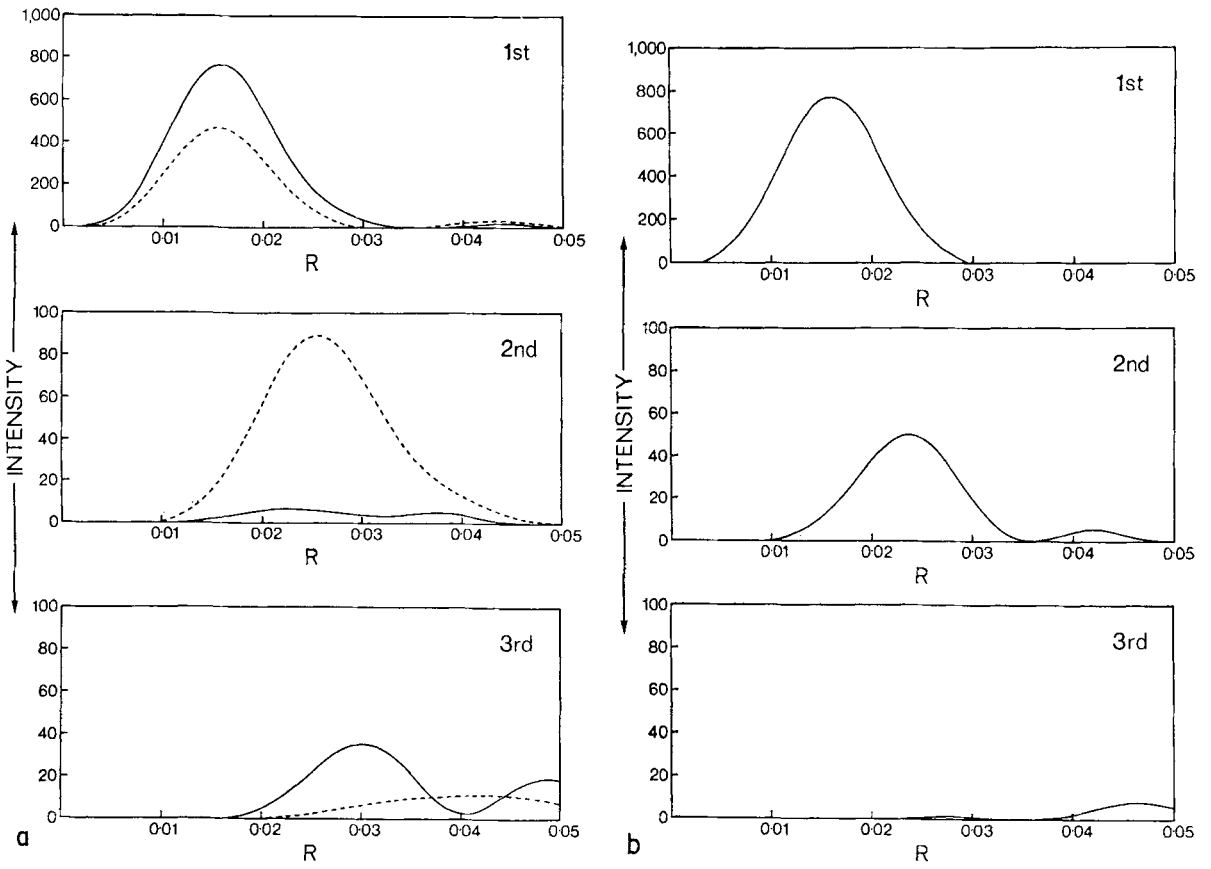

Figure 5. Intensity distribution along the first three layer lines calculated for a model with an actin monomer 20 Å radius at a helix radius of 24 Å. Note that the scale of the plot for the 1st layer line is 10× smaller than that for the 2nd and 3rd layer lines. (*a*) Layer lines calculated for actin filament with a 10 Å radius cylinder of tropomyosin. (——) Tropomyosin in relaxed position with $\phi = 50°$; (– – –) tropomyosin in activated position with $\phi = 70°$. (*b*) Layer lines calculated for actin alone.

calcium found by Spudich et al. (1972). They found that the tropomyosin was associated with one actin chain and the tropomyosin and actin chains subtended an angle of 60° with the center of the filament, which is in the narrow region where we think the tropomyosin sits. We should stress here that the agreement between the observed diffraction patterns and those calculated from a model of spheres and cylinders does not prove that the thin filament structure is the same as our model. It only shows that at low resolution the filament structure could be represented by such a model, and the agreement of our model structure with the structure deduced by other means supports the correctness of our model. We have no proof, for example, that the change in the X-ray pattern when a muscle contracts or goes into rigor is caused by a movement of the tropomyosin from a position where $\phi = 50°$ to one where $\phi = 70°$. Figure 4 shows that qualitatively a similar change would be produced if ϕ increased only to 60°, or up to 90° and the tropomyosin moved right to the center of the groove, or even moved in the opposite direction towards the edge of the filament. We choose to discuss the tropomyosin movement from $\phi = 50°$ to $\phi = 70°$ because this very small movement gives the largest change and therefore the most easily visible change in the diffraction pattern. Also the changes in the first three layer lines can all be interpreted by this one movement.

Table 2. Strength of the 2nd Layer Line from Vertebrate Striated Muscle

	Sartorius $s = 2.2\,\mu$	Semitendinosus nonoverlap
Live resting	–	–
Contracting (Huxley, 1970)	+	
Rigor in presence of Ca^{++}	+	+
Rigor in absence of Ca^{++}	+	–

\+ 2nd layer line distinct, 3rd layer line not visible.
– 2nd layer line weaker than 3rd layer line, which is visible but very weak.

In the relaxed muscle where actin-myosin interaction is inhibited, the 2nd actin layer line is weaker than the 3rd layer line and tropomyosin is situated towards the edge of the groove in the filament. When actin-myosin inhibition is removed and the cross-bridges interact with actin (Table 2), the 3rd layer line becomes weaker and the 2nd layer line becomes much stronger because the tropomyosin has moved, or has been moved, to a position nearer the center of the groove in the actin helix. We call these two positions of the tropomyosin the relaxed and the activated positions, corresponding to the relaxed and activated (or rigor) X-ray patterns. There seems little doubt that this movement of tropomyosin is associated with the mechanism that controls actin-myosin interaction, but the immediate cause of the movement still has to be identified. Two possible mechanisms seem likely:

1. Calcium ions released into the sarcoplasm when muscles are activated bind to troponin which then undergoes a conformational change (Wakabayashi and Ebashi, 1968), causing the tropomyosin to move relative to the actin. This mechanism would be calcium sensitive and independent of the number of bound cross-bridges and would occur even if no cross-bridges were bound to actin.

2. Cross-bridges bind to actin tangentially on one side (Moore et al., 1970) so that they are projecting into the filament. The present X-ray data provide no way of distinguishing which side of the groove the tropomyosin is associated with (i.e., whether ϕ is negative as in Fig. 3 or whether it is positive); but if the tropomyosin were on the side on which the cross-bridges attach, then upon activation the cross-bridges would bind to actin and in so doing would move tropomyosin away from the point of attachment of myosin towards the center of the groove.

Experiments show that both of these mechanisms may operate.

Experimental Results

The dependence of the tropomyosin movement on the attachment of cross-bridges was investigated by putting muscles into rigor in conditions where the cross-bridges cannot interact with actin (Haselgrove, 1970). By stretching a semitendinosus muscle beyond overlap length (the length at which the actin and myosin filaments just overlap), muscles can be put into rigor either by soaking them in a frog Ringer solution containing 1 mM iodoacetate, or by soaking them in a glycerol solution for 24 hr followed by a frog Ringer solution or a standard salt solution (Rome, 1972). Pictures from such muscles (Fig. 6a) show a pronounced 2nd actin layer line although the cross-bridges have been physically prevented from attaching to the thin filament. Although densitometric measurements were not possible, the 2nd layer line looks as strong relative to the 6th and 7th lines as it is in the rest length rigor muscle.

One advantage of studying the actin pattern from such stretched muscles is that there are no layer lines from the myosin filaments to obscure the actin pattern; the myosin layer lines seen in relaxed muscle have completely disappeared, and the rigor series arising from the attachment of cross-bridges to the actin are not generated. On the other hand, the disadvantage of working with nonoverlap muscles is that the filaments when separated do not all remain parallel and result in a disordered pattern. However even in the best-ordered patterns no trace of the third layer line could be seen. The 1st layer line was much weaker than would have been expected from the calculations of the thin filament in the rigor state and in many patterns was very difficult to see at all. The discrepancy may be accounted for by the simplicity of the model chosen for calculations and the omission of the effect of troponin which may well help weaken the first layer-line further than does the tropomyosin alone. The patterns did show the 5th actin layer line predicted from the calculations.

The experiment shows then that in standard conditions the change in the state of the thin filament from the relaxed to the activated state can occur even if no cross-bridges can attach to the thin filaments. The mechanism causing this change of tropomyosin position must therefore be contained within the thin filaments, as is the case with the first of the possible mechanisms described above. This experiment does not disprove the second mechanism since it shows only that cross-bridge attachment is not a necessary requirement in the given conditions; it does not show that the cross-bridges cannot move the tropomyosin when they do attach.

If the movement of the tropomyosin is controlled by the troponin component of the thin filaments, then we would expect the movement to be calcium sensitive. We were able to investigate the calcium dependence of the change in intensity of the first three layer lines by putting muscles into rigor at different sarcomere lengths in the complete absence of calcium. Soaking a live muscle for 24 hr in a calcium-free frog Ringer solution containing 2 mM EGTA completely destroyed the ability of the muscle to be excited electrically, presumably by binding all the available calcium. The X-ray diffraction pattern was not altered by such treatment, indicating that the structure of the filaments was identical to that of a fresh live muscle. Subsequent

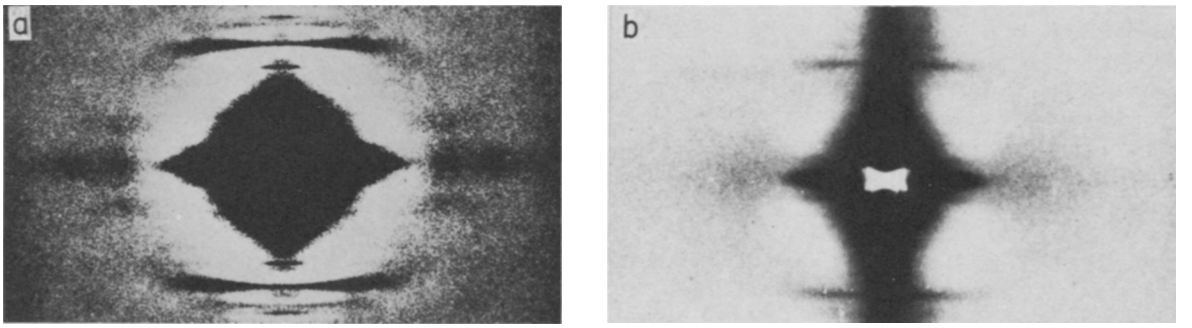

Figure 6. Diffraction patterns from semitendinosus muscles put into rigor at nonoverlap lengths. (*a*) Standard iodoacetate rigor. The 2nd layer line is visible. (*b*) Calcium-free iodoacetate rigor. The 2nd layer line is absent, but the first layer line is clearly visible. It was not possible to reproduce this pattern to show the 3rd layer line.

treatment of the relaxed muscle, either with the same Ringer solution containing 1 mM iodoacetate or by glycerination using solutions without calcium but containing 2 mM EGTA, depleted the muscle of ATP and put it into rigor. The glycerinated muscles were X-rayed either in standard salt solution containing 2 mM EGTA or in calcium-free frog Ringer containing 2 mM EGTA. Tension records were made of some muscles being put into rigor by glycerination at rest length. Muscles in calcium-containing solutions generate tensions up to about 20 g when returned from the glycerol solution to either frog Ringer solution or standard salt. Only a very small (less than 0.5 g) effect was seen when muscles were glycerinated in the absence of calcium. We thus feel confident that our treatment of muscles with calcium-free solutions has affected the calcium-dependent, tension-generating mechanism of the muscle. As with muscles put into rigor in solutions containing calcium there was no difference in the patterns from muscles put into rigor using iodoacetate or using glycerol.

Semitendinosus muscles stretched to nonoverlap lengths and then put into a calcium-free rigor give a pattern that is different from that given by muscles put into a standard rigor (Fig. 6). The first layer line is now distinct whereas the 2nd layer line is much weaker than the weak 3rd layer line. The pattern is very similar to the actin component of a relaxed muscle, indicating that little if any change had occurred in the thin filament structure although the loss of the myosin layer line pattern showed that changes had occurred in the myosin structure. The observed pattern was pleasingly similar to that calculated for the thin filament in the relaxed state, with the 1st and 6th layer lines strong, the 3rd and 5th layer lines weak, the 2nd very weak, and the 4th absent (Table 1). We have thus been able to demonstrate that the whole of the low-angle pattern predicted from the actin thin filament structure is present in vertebrate striated muscle. It therefore seems likely that the 400 Å reflection seen in live striated muscle by Huxley and Brown (1967) does have components from both the 1st myosin layer line and the 1st actin layer line as they suggested. Furthermore it is now possible to see that the diffraction pattern from striated muscle actin is remarkably similar to the diffraction pattern from smooth muscles (Vibert et al., 1972a,b).

We conclude therefore that the tropomyosin had not moved when the muscle went into calcium-free rigor at nonoverlap length and that the thin filament was still in the relaxed state. We were worried that the lack of change in the thin filaments might be due to the muscle's being incompletely in rigor, but the complete loss of the myosin layer line pattern showed that the whole of the muscle had been affected by the rigor process. Confirmation that the thin filament structure is affected by the presence of calcium came from the following experiment. Glycerinated muscle in rigor at nonoverlap lengths were X-rayed both in calcium-containing and calcium-free solutions. No 2nd layer line was visible in the calcium-free solution but it was visible in the calcium-containing solution. Therefore when cross-bridges cannot attach to the thin filament, the position of the tropomyosin molecule is sensitive to the concentration of calcium. It is known that the combination of tropomyosin and actin is not calcium sensitive with respect to the interaction of actin and myosin, so there seems no reason to assume that these two molecules alone should be sensitive to calcium with respect to their structural relationship. We have assumed, however, that the changes we are investigating are related directly or indirectly to the mechanism controlling actin-myosin interaction. Calcium sensitivity is conferred on the relaxing system by troponin, which is situated regularly along the thin filaments (see Ebashi and Endo, 1968). It seems, therefore, that the movement of tropomyosin in the thin filament is either caused or controlled by the troponin.

Weber and Bremel (1971) have shown that at very low levels of ATP concentration the tropomyosin-troponin relaxing system is unable to suppress actin-myosin interaction which then occurs regardless of the calcium concentration. This is confirmed by Spudich et al. (1972) who have shown that actin filaments can be decorated with S, subfragments of myosin in the absence of both ATP and calcium. It is therefore expected that when a muscle at rest length is depleted of ATP it will go into rigor and the cross-bridges will attach to actin even in the absence of calcium. Indeed sartorious muscles at rest length put into a calcium-free rigor gave diffraction patterns which were indistinguishable from the usual patterns of rigor muscles (Fig. 1); viz., the 6th and 7th layer lines appear closer to the meridian than in a relaxed muscle, and also close to the meridian are the two series of layer lines associated with the attachment of cross-bridges to the actin filament. Further from the meridian the 2nd actin layer line is prominent but the 3rd layer line is not visible. On well-ordered patterns the end of the 1st layer line can be seen extending out from the meridian almost as far as the 2nd layer line, and, as predicted by the calculations (Fig. 5), are about the same intensity in this region. Although the muscle contains no calcium, the presence of a distinct 2nd layer line shows that the position of tropomyosin in the thin filament is very similar, if not identical to, the activated state that can be induced by the presence of calcium. However we would not expect the mechanism operating here to be the same as the mechanism operating in nonoverlap muscles, since the latter mechanism is sensitive to calcium. As far as we know the only difference between muscles put into calcium-free rigor at different lengths is that at nonoverlap no cross-bridges attach to actin, and the diffraction pattern indicates that the actin filament is in a relaxed state; whereas at rest length cross-bridges attach to actin and the diffraction pattern indicates that the actin filament is in an activated state. The experiments with nonoverlap muscles showed that the attachment of cross-bridges to actin is not a necessary requirement for the change if actin is present, but it did not show that the cross-bridges cannot cause the change. We must now conclude that in the absence of calcium, the attachment of cross-bridges to the thin filament can cause movement of the tropomyosin towards the center of the groove in the filament, thus supporting the second possible mechanism proposed earlier.

This demonstration of actin-myosin attachment and the enhancement of the 2nd actin layer line even in the absence of calcium allows us to demonstrate that local changes the cross-bridges make on the thin filaments are not transmitted for long distances along the thin filaments. Muscles were stretched so that the length of overlap of the thick and thin filaments was considerably reduced, but not eliminated, so that cross-bridges could interact with actin at the end of the filaments but not along the rest of their length. These muscles were then put into a calcium-free rigor; X-ray patterns from the muscles did not show a strong 2nd layer line, indicating that along most of the length of the thin filament the tropomyosin was in the relaxed state. Since we have just shown that in the region where interaction can take place the tropomyosin is moved to the activated position, we presume that this is happening at the end of the filament but that the movement is not propagated along the whole of the filaments.

Discussion

These results show then that the changes in intensity of the first three actin layer lines in X-ray diffraction patterns may be accounted for by movement of the tropomyosin molecule within the filament. The total movement necessary to affect the X-ray pattern in the way we observe is relatively small and is about 15 Å towards the center of the groove in the filament. In relaxed muscles the tropomyosin is sitting at the edge of the groove in the thin filament and is very close to the point on the actin at which S_1 subunits of myosin attach (Fig. 3; Moore et al., 1970). In this position tropomyosin may well inhibit actin-myosin interaction by physically blocking the active site for myosin attachment. On addition of calcium the tropomyosin is moved to a position nearer the center of the groove where it no longer interferes with the actin-myosin interaction. Since this interaction is not inhibited if no troponin is present, it seems likely that the normal position for tropomyosin in the thin filament is in the activated position near the center of the filament and inhibition of actin-myosin interaction by troponin in the absence of calcium is caused by troponin moving the tropomyosin to the relaxed position. In this way it would be possible for one troponin molecule to exert a controlling influence over all seven actin monomers in contact with the tropomyosin molecule controlled by that troponin. Although such a mechanism involving simple steric blocking of an active site would explain the control of activation by calcium in a live contracting muscle, the whole system is not likely to be quite so straightforward, as the following discussion shows.

Relaxation of actin-myosin interaction is dependent on the presence of ATP as well as upon the level of calcium present (Weber and Bremel, 1971).

Our X-ray patterns have shown that in the absence of ATP and calcium the thin filament is in the relaxed state if the cross-bridges are physically prevented from attaching by stretching the muscles to beyond overlap length. So at rest length the cross-bridges are able to attach firmly to the thin filament although the tropomyosin has not been moved before the attachment. Once attached, the cross-bridge is then able to move in a way that will cause local movement of the tropomyosin. It seems, therefore, unlikely that the tropomyosin covers the active site completely, but must inhibit the interactions in a way that prevents attachment of myosin loaded with ATP (or its split products; Lymn and Taylor, 1971) but allows attachment of unloaded myosin. Moreover Bremel et al. (this volume) have shown recently that the presence of tropomyosin activates the actomyosin ATPase. Sequence work on tropomyosin by Smillie, (personal communication) shows that tropomyosin does not have a repeating sequence that would enable tropomyosin to interact equivalently with each actin monomer near the active site and in this way control the actin-myosin interaction.

Smooth muscles also give X-ray diffraction patterns showing that upon activation the 2nd layer line increases in intensity with respect to the third layer line (Vibert et al., 1972a,b), although the change is less than in vertebrate striated muscle. Studies on the muscle ABRM have shown (Kendrick-Jones et al., 1970) that the thin filaments of molluscan smooth muscle contain no troponin and that it is the myosin which is calcium sensitive and controls activity. Thus the changes in the thin filament in the ABRM cannot be brought about by the action of troponin but must be caused by the attachment of cross-bridges. The cycling of cross-bridges during contraction means that not all cross-bridges are attached to actin at once, so only some regions of the thin filament are affected by cross-bridges attaching. This in turn produces the change in the X-ray pattern which is small compared with the complete change produced by the action of troponin in vertebrate striated muscle. Therefore there remains much about the causes and effect of the movement of tropomyosin in the thin filament that must still be investigated.

At the same time as the calculations described here were being made, Drs. Parry and Squire independently performed similar calculations and arrived at a similar conclusion about the movement of tropomyosin. Their work is now in press. I would like to thank Drs. Spudich, Huxley, and Finch for allowing me to study and quote from their paper, and especially Dr. H. E. Huxley for many helpful and stimulating discussions.

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