CHANGES OF THICK FILAMENT STRUCTURE DURING CONTRACTION OF FROG STRIATED MUSCLE

N. YAGI, E. J. O'BRIEN, AND I. MATSUBARA, *Department of Pharmacology, University of Tohoku School of Medicine, Seiryo-machi, Sendai, Japan, and Medical Research Council Cell Biophysics Unit, King's College, 26–29 Drury Lane, London WC2 5RL, England*

ABSTRACT The strongest myosin-related features in the low-angle axial x-ray diffraction pattern of resting frog sartorius muscle are the meridional reflections corresponding to axial spacings of 21.4 and 14.3 nm, and the first layer line, at a spacing of 42.9 nm. During tetanus the intensities of the first layer line and the 21.4-nm meridional decrease by 62 and 80% respectively, but, when the muscle is fresh, the 14.3-nm meridional intensity rises by 13%, although it shows a decrease when the muscle is fatigued. The large change in the intensity of the 21.4-nm meridional reflection suggests that the projected myosin cross-bridge density onto the thick filament axis changes during contraction. The model proposed by Bennett (Ph.D. Thesis, University of London, 1977) in which successive cross-bridge levels are at 0, 3/8, and 5/8 of the 42.9-nm axial repeat in the resting muscle, passing to 0, 1/3, and 2/3 in the contracting state, can explain why the 21.4-nm reflection decreases in intensity while the 14.3-nm increases when the muscle is activated. The model predicts a rather larger increase of the 14.3-nm reflection intensity during contraction than that observed, but the discrepancy may be removed if a small change of shape or tilt of the cross-bridges relative to the thick filament axis is introduced. The decrease of the intensity of the first layer line indicates that the cross-bridges become disordered in the plane perpendicular to the filament axis.

INTRODUCTION

X-ray diffraction is one of the main techniques for investigating the molecular events taking place during the contraction of living muscle. It has been used to confirm the sliding filament mechanism (Elliott et al., 1965; Huxley et al., 1965) and to show that during contraction a movement of myosin cross-bridges takes place from the vicinity of the thick to that of the thin filaments (Haselgrove and Huxley, 1973). The use of improved x-ray sources and photon detection methods has made it possible to observe the changes taking place during contraction with increased time resolution. In particular, a study of the relation between tension development and the changes of the 1,0 and 1,1 equatorial reflections has shown that the myosin cross-bridges move towards the thin filaments before tension is produced (Huxley, 1975, 1976, and 1978; Matsubara and Yagi, 1978). This paper is concerned with a similar study in which the behavior of the meridional and layer-line reflections is investigated.

In their comprehensive analysis of the x-ray diffraction patterns of frog muscle in the resting, contracting and rigor states, Huxley and Brown (1967) showed that the well-developed system of layer lines attributed to the helical arrangement of myosin cross-bridges with a repeat of 42.9 nm largely disappeared when the muscle passed from the resting to the

BIOPHYS. J. © Biophysical Society · 0006–3495/81/01/121/17 $1.00
Volume 33 January 1981 121–138

contracting or rigor conditions. They showed that the strongest layer line in the pattern from resting muscle, the first, could arise from myosin cross-bridges at an average radius of 10–11 nm arranged on a 6/2 helix with 3 cross-bridge levels/42.9-nm repeat. The strong meridional reflection at 14.3 nm then corresponded to the axial separation of cross-bridge levels. During contraction the first layer line decreased by 69% of its resting value, indicating considerable movement and disordering of the cross-bridges, but the relatively small decrease of the meridional reflection (by 34%) implied this movement had only a small axial component. In rigor, the 42.9-nm layer line and its higher orders were absent, replaced by an actin-based system, but the 14.3-nm meridional reflection was still relatively strong. Electron microscopy and image analysis of thin filaments decorated with myosin subfragment-1 have indicated that in rigor the cross-bridges are attached at an angle of ~45° to the filament axis (Moore et al., 1970).

Huxley (1969) has incorporated the structural features deduced from x-ray diffraction and electron microscope data into a model for contraction involving rotation of the myosin heads about their point of attachment with actin. This model has been refined on the basis of kinetic observations on contracting muscle by Huxley and Simmons (1971), who propose that the myosin head may rotate stepwise through several binding sites with actin.

Since the meridional reflections in the x-ray diffraction pattern arise from the muscle structure projected onto its long axis, the behavior of these reflections during contraction should be sensitive to changes of the cross-bridge angle. We have followed not only the 14.3-nm reflection but also that at 21.4 nm, which is also strong and is well-resolved by our apparatus. The behavior of the 42.9-nm layer line has also been recorded. To relate the changes of reflection intensities more closely to the rise and fall of tension, diffraction patterns were recorded during twitch as well as tetanic contractions. It was possible to cause twitches in a muscle many times without inducing fatigue, so that by accumulating the data over a large number of successive twitches the reflection intensities could be obtained over shorter intervals of time (10 vs. 100 ms).

MATERIALS AND METHODS

Specimen Technique

A sartorius muscle from the bullfrog *Rana catesbeiana* was used. The muscle was dissected in Ringer's solution of composition: 115 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$, 2.15 mM Na_2HPO_4, 0.85 mM NaH_2PO_4, pH 7.2. Each muscle was held horizontally in a specimen chamber by clamping the pubic bone at one end and connecting the tendon to a force transducer at the other end. The specimen chamber was filled with oxygenated Ringer's solution which was renewed continuously at 4°C. The specimen chamber had two mylar windows which allowed x-rays to pass through the middle part of the muscle. The length of the muscle was adjusted by moving the transducer; the sarcomere length (measured by the light diffraction method) was set at 2.2 μm. The muscle was isometrically stimulated with supramaximal rectangular pulses with a duration of 5 ms given through a pair of platinum electrodes placed parallel to the muscle axis. In one type of experiment the muscle was stimulated by single pulses to give twitches, with a total of 200 twitches at 20-s intervals. Alternatively, trains of pulses (20 Hz) were used to give tetani of 1-s duration and the tetanus was repeated up to 80 times. Tension was averaged over a series of contractions of each muscle by a multichannel analyzer combined with a V-F converter (Yagi et al., 1977).

X-ray Techniques

X-RAY GENERATOR AND CAMERA The x-ray source was a rotating-anode generator (Rigaku/USA, Inc., Danvers, Mass.) with a fine focus (1 × 0.1 mm) on a copper target. This was operated at 50 kV with a tube current of 70 mA: such a high power was possible with an anode of a large diameter (30 cm) rotating at a high speed (9,000 rpm). A mirror-monochromator camera (Huxley and Brown, 1967) was used at a source-to-crystal distance of 25 cm with a viewing angle of 6°. Both point and line-focused beams were produced and superimposed. Part of the x-ray beam was not reflected by the mirror and was focused to a 4-mm vertical line 55 cm from the center of the crystal. The rest of the beam was focused by both mirror and crystal to produce a point focus at the same position. The specimen-to-counter distance was 46 cm and most of the beam path was evacuated.

DETECTION METHODS Two kinds of detectors were used, a scintillation counter combined with a mask and a position-sensitive counter. The second method enables several reflections along a line and the intervening background to be measured simultaneously. The scintillation counter, however, covers a larger area of the diffraction pattern, so that layer-line reflections in all four quadrants could be measured together. It is necessary with this detection method to make a separate assessment of the background associated with a reflection.

Scintillation Counter Combined with a Mask

The diffraction pattern was recorded on film placed at the focal position of the x-ray beam. A series of layer lines was observed at orders of 42.9 nm (Fig. 1). Using this recorded pattern as a guide, the film was replaced by a lead mask which passed only the required parts of the diffraction pattern. The positions and sizes of the apertures in the masks are shown in Fig. 1. For the measurements along a layer line, the mask had four apertures to cover the four quadrants of the pattern. For the meridional measurements, the mask had two apertures.

The x-ray photons which passed through the apertures were counted with a scintillation counter connected to a multichannel analyzer: the signals occurring at different times were registered in different channels. The multichannel analyzer and the stimulus given to the muscle were triggered by the same clock so that signals from the same period of contraction could be accumulated over many contractions. The intensities at the resting condition were measured before each contraction for 300 ms before twitch or 30 ms before tetanus. For each muscle the changes in intensity over 20–80 tetani or 200 twitches were recorded and the data from different muscles averaged.

Since the x-rays were focused into a line, the meridional reflection extended to the spacing of 72 nm in a direction parallel to the equator and the intensities measured on the meridian contained contribution from the off-meridional parts of the layer line. Comparison of Fig. 1 with a resting diffraction pattern recorded with a point focused x-ray beam showed that 5–10% of the resting intensities of the meridional reflections recorded in this experiment were contributed to by the off-meridional parts of the layer line. On the other hand, in measurements of layer-line intensities, there was no contribution from the meridional reflections.

Haselgrove (1975) showed that the meridional reflection at 14.3 nm is slightly (1%) displaced during contraction, suggesting a small change in the myosin periodicity. However, as can be seen in Fig. 1, such a small displacement (42 μm at the position of the mask) is negligible compared with the half-width of the aperture (0.6 mm) used in this experiment.

To determine the background contribution to the layer lines, the diffraction patterns on film from resting muscles were scanned axially at intervals of 1 mm across the positions of the mask apertures (Fig. 1), using a Joyce-Loebl microdensitometer (Joyce, Loebl and Co., Ltd., Gateshead-on-Tyne, England) with a spot size 40 μm (parallel to the meridian) × 1 mm (parallel to the equator). For each densitometer trace the background in the region of the reflection could be determined by extrapolating

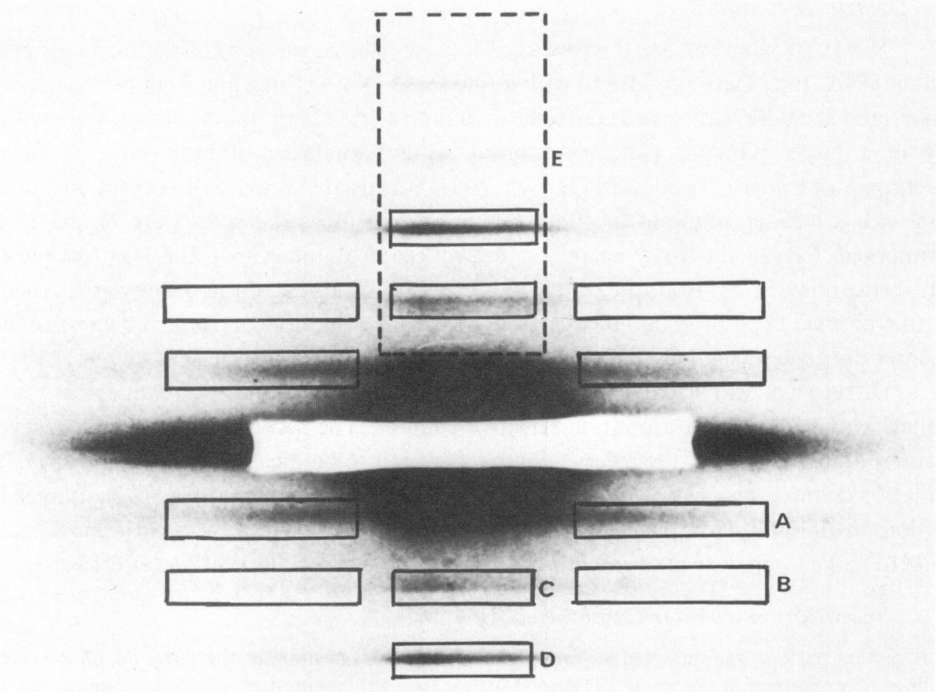

FIGURE 1 A typical diffraction pattern from a frog sartorius muscle in the resting condition. The axis of the muscle was vertical. The exposure time was 60 min. The positions and sizes of apertures of the mask used with the scintillation counter are shown. (*A*) the first order layer line at 42.9 nm, (*B*) the second order layer line at 21.4 nm, (*C*) the second order meridional reflection at 21.4 nm, (*D*) third order meridional reflection at 14.3 nm. When the position-sensitive counter was used, the intensity distribution was obtained from the area surrounded by the broken line (*E*).

the values either side. The density behind the backstop was taken as the zero intensity level. The areas above and below the background curve were measured, summed for each aperture position, and the background expressed as a percentage of the total intensity for that aperture. For the meridional reflections there was no variation in the ratio of background to reflection intensity at different transverse positions, so that measurements made in the middle of each reflection were sufficient. Changes in the background intensity during tetanus were measured with the scintillation counter midway between layer lines, both on and off the meridian. Along the meridian, the position-sensitive counter measurements indicated that the shape of the background curve did not change significantly during contraction. Hence the measurements with the scintillation counter on the meridian should provide a reliable estimate of the background changes at the reflection positions. No change was detected in the background between layer lines with the scintillation counter measurements made off the meridian, indicating that the background change on the layer lines was probably negligible.

Position-Sensitive Counter

The position-sensitive counter and the data collecting system were described in detail elsewhere (Yagi et al., 1977; Matsubara and Yagi, 1978). In the present experiment the conversion gain of the pulse height analyzer was 6.3 channels/mm and a xenon mixed gas (90% Xe + 10% CO_2) was used.

Only the meridional part of the axial pattern was recorded (Fig. 1). A typical resting pattern is shown in Fig. 2. The second (at 21.4 nm) and the third (at 14.3 nm) order meridional reflections were studied because these were stronger than higher order reflections and had lower background intensities than the

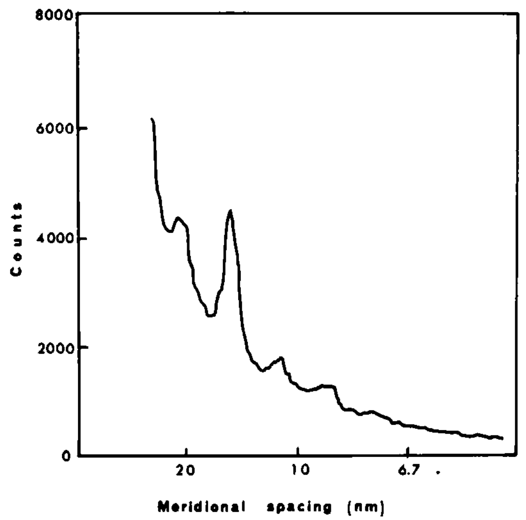

FIGURE 2 A typical intensity distribution along the meridian from a resting frog sartorius muscle recorded with the position-sensitive counter. Five peaks are seen, corresponding to the second through sixth orders (from left to right) of the 42.9-nm repeat. The third order reflection is the strongest. The exposure time was 180 s.

inner meridional reflections. With a specimen-to-counter distance of 46 cm, the third order meridional reflections in each half of the diffraction pattern were separated by ~2 cm. Since the position-sensitive counter had an active length of 4 cm and high resolution was achieved only in its middle part, it was impossible to record both sides of the diffraction pattern with reasonable resolution and only one side of the pattern was recorded.

The memory was divided into 15 segments each of 256 channels and set to record over one period in the contraction cycle. The length of each period was shorter at the times when rapid intensity changes were occurring. In experiments using the scintillation counter, data over a large number of tetani and twitches (40 or 200) were accumulated. In the resultant intensity profile the background was drawn by eye and the area under each peak measured. The results from different muscles were averaged and the standard error obtained.

RESULTS

First Order Layer Line at 42.9 nm

TETANUS The intensity of the off-meridional part of the first order layer line was measured with the scintillation counter. The intensity decreased on contraction and stayed at a level 11 ± 1% ($N = 7$) lower than the resting level throughout the duration of tetanus (Fig. 3 *b*). The background level did not change during contraction and in the resting case represented 82.4 ± 0.9% (SEM, $N = 12$) of the measured intensity. The corrected results indicated that the layer-line intensity decreased by ~62% of its resting value. The intensity started to return to the resting level on cessation of stimuli (Fig. 3 *b*). However, the return was slower than the tension decline: the intensity reached the resting level 4 s after the tension had fallen to zero. A similar delay in the return of the layer line intensity after tetanus has been reported by Huxley (1972).

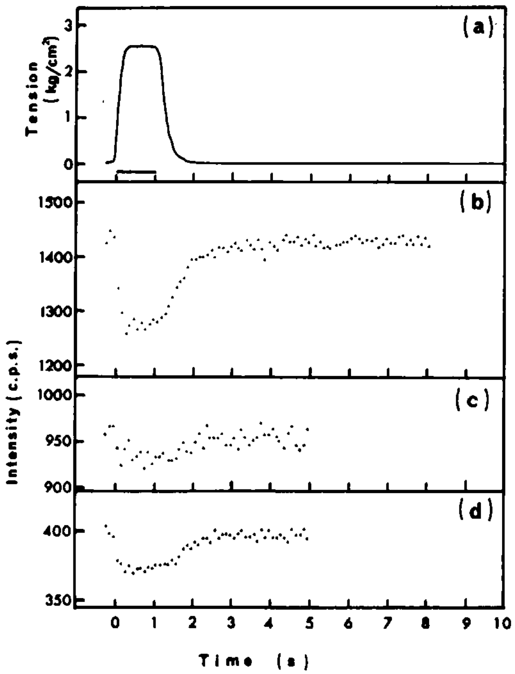

FIGURE 3 Intensity changes of the myosin-related reflections during tetanus measured with the scintillation counter. (*a*) A typical tetanic tension averaged for 40 tetani. The horizontal bar represents the period of stimulation. (*b*) First order layer line at 42.9 nm. The intensity was average for seven muscles. The initial tetanic tension of the series of contractions was 2.92 ± 0.32 kg/cm^2 (mean ± SD). The tetanic tension averaged for the 40 contractions was 76.4 ± 6.1% of the initial tetanus. (*c*) Second order layer line at 21.4 nm. The intensity was averaged for six muscles. The initial tension: 3.11 ± 0.25 kg/cm^2. The averaged tension for 40 tetani: 80.0 ± 3.5% of the initial tension. (*d*) Second order meridional reflection at 21.4 nm. The intensity was averaged for six muscles. The initial tension: 3.23 ± 0.37 kg/cm^2. The averaged tension for 40 tetani: 80.5 ± 4.1% of the initial tension.

TWITCH The intensity of the first order layer line decreased as the twitch tension developed (Fig. 4 *b*) by a maximum of 9 ± 1% ($N = 12$) of the resting intensity. Allowing for the background, the corrected decrease was 49%.

The intensity curve in Fig. 4 *b* formed an approximate mirror image of the tension curve (Fig. 4 *a*). However, a careful comparison of the two curves revealed that the intensity reached the minimum 20–30 ms before the tension reached its peak.

Second Order Layer Line at 21.4 nm

The off-meridional part of the second order layer line was much weaker than the first, and its intensity change could be followed only during tetanus (Fig. 3 *c*). The intensity decreased with a time course similar to that of the first order layer line during tetanus.

Second Order Meridional Reflection at 21.4 nm

TETANUS Fig. 3 *d* shows that the intensity of the second order meridional reflection measured with the scintillation counter decreased by 8 ± 1% ($N = 6$) during tetanus. The background during contraction increased by 2 ± 1% and in the resting case represented

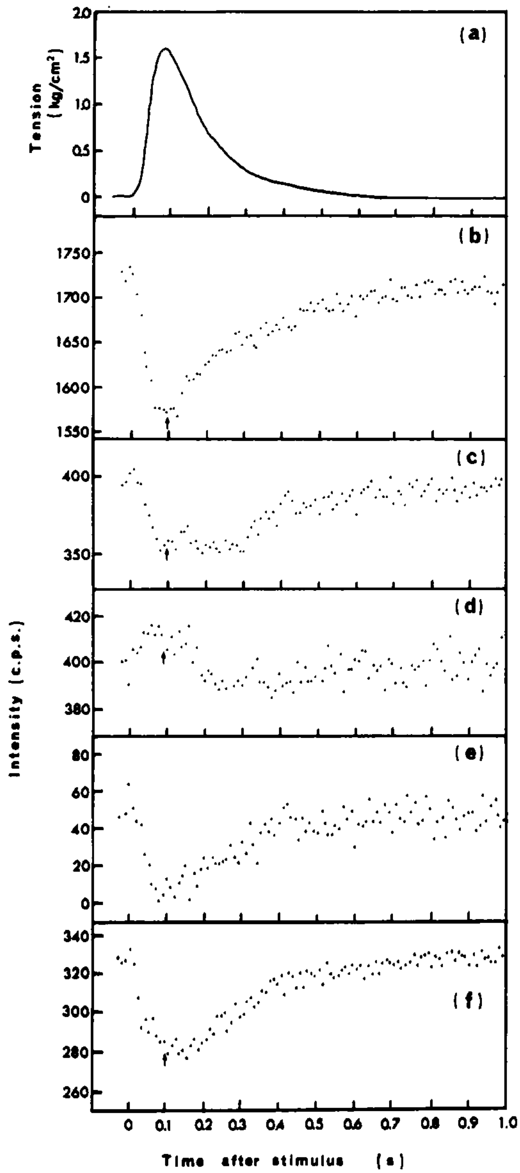

FIGURE 4 Intensity changes of the myosin-related reflections during twitch measured with the scintillation counter. The arrows indicate the time the peak tension occurred. (*a*) A typical twitch tension averaged for 200 twitches. (*b*) The first order layer line at 42.9 nm. The intensity was averaged for 12 muscles. The initial twitch tension of the series of contractions was 1.78 ± 0.24 kg/cm^2. The twitch tension averaged for the 200 contractions was 72.5 ± 5.1% of the initial twitch. The peak tension occurred at 97.5 ± 4.9 ms (mean ± SD). (*c*) The second order meridional reflection at 21.4 nm. The intensity was averaged for 13 muscles. Initial tension: 2.21 ± 0.38 kg/cm^2. The averaged tension for 200 twitches: 74.1 ± 4.8% of the initial tension. The peak tension occurred at 92.8 ± 6.9 ms. (*d*) The background intensity in the vicinity of the second order meridional reflection. The intensity was averaged for six muscles. Initial tension: 2.11 ± 0.24 kg/cm^2. The averaged tension for 200 twitches: 74.3 ± 4.2% of the initial tension. The peak tension occurred at 92.8 ± 6.9 ms. (*e*) The intensity of the second order meridional reflection corrected for the background. (*f*) The third order meridional reflection at 14.3 nm. The intensity was averaged for 13 muscles. Initial tension: 2.04 ± 0.22 kg/cm^2. The averaged tension for 200 twitches: 76.2 ± 5.9% of the initial tension. The peak tension occurred at 98.2 ± 8.3 ms.

87.8 ± 1.2% ($N = 12$) of the measured intensity. The corrected result indicated that the intensity of the reflection decreased by ~80% of its resting value when the muscle was producing a steady tetanic tension. The intensity returned to the resting value after the tension had fallen to zero.

Fig. 6 *a* shows that the reflection intensity decreased by 70 ± 6% of its resting value when measured with the position-sensitive counter. This decrease may not be significantly smaller than that recorded by the scintillation counter. However, the difference in background determination (see Materials and Methods) might be expected to produce a smaller decrease. This is because there are several minor reflections in the region of the second order meridional reflection (Haselgrove, 1975) which may not vary in intensity during contraction. With the scintillation counter method these reflections were measured and subtracted from the main reflection but with the position-sensitive counter they were unresolved and included with the main peak.

TWITCH The intensity of the second order meridional reflection, measured with the scintillation counter, decreased also during twitch (Fig. 4 *c*). The apparent time course of the decrease was complicated by the contribution of the background intensity which itself underwent a complex change during twitch (Fig. 4 *d*). The time course of changes in the corrected intensity (Fig. 4 *e*) was similar to that of the first order layer line (Fig. 4 *b*), namely, the intensity reached the minimum before the tension reached its peak and returned to the resting value after the tension had fallen to zero. The maximum decrease of the reflection intensity during twitch was 80% of its resting value.

Third Order Meridional Reflection at 14.3 nm

TETANUS The behavior of the third order meridional reflection during tetanus was found to change significantly as the muscle went into fatigue on repetition of tetani. Therefore, the time course of the intensity was studied at various stages of fatigue.

Initially the time course was obtained from the first 20 tetani of fresh muscles using the scintillation counter. The muscles showed little sign of fatigue: the steady tension of the 20th tetanus was ~78% of the initial tetanus, and there was no tension decline during each tetanic stimulation. Fig. 5 *a* represents the averaged time course of the intensity for the 20 tetani. The intensity decreased slightly in the first 100 ms of tetanus when the tension was rising, and then increased to a level 7 ± 1% ($N = 9$) greater than the resting intensity. This high level was maintained during the period when the muscle was producing a steady tetanic tension. On cessation of stimuli the intensity decreased markedly to below the resting level. The minimum intensity was reached at ~500 ms after the end of stimulation when the tension was almost zero. The intensity returned to the resting value ~1 s after the tension had fallen to zero. After correction for the background the intensity was 13% greater than the resting value when the muscle was producing a steady tetanic tension. The correction indicates the background level as 62.1 ± 2.4% ($N = 12$) of the measured intensity for the resting muscle and as increasing by 2% during contraction.

In the second set of measurements the time course of the intensity was averaged for the first 40 tetani of fresh muscles. The tension of the 40th tetanus was ~60% of the initial tetanus. Fig. 5 *b* shows the time course of the intensity measured with the scintillation counter. The increase in the intensity during tetanus was smaller than that obtained from the first 20 tetani.

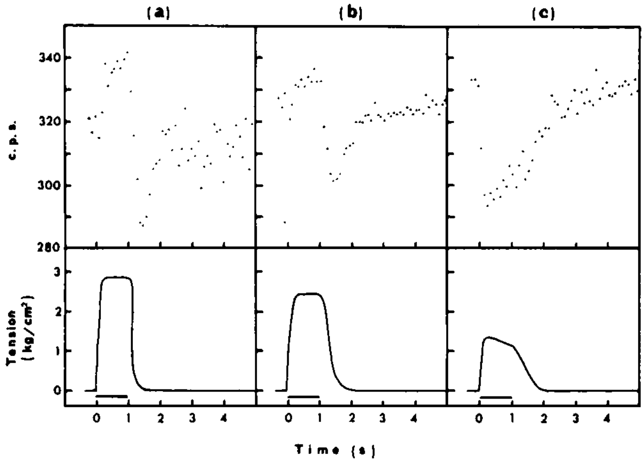

FIGURE 5 Averaged tension recordings and intensity changes of the third order meridional reflection at 14.3 nm during tetanus. The intensity was measured at various degrees of fatigue using the scintillation counter. (*a*) Tension and intensity averaged for the first 20 tetani of fresh muscles and for the intensity the average for nine muscles is shown. The maximum tension during the initial tetanus: 3.21 ± 0.23 kg/cm^2. The averaged tension for 20 tetani: 86.3 ± 3.2% of the initial tension. (*b*) The results averaged for the first 40 tetani of fresh muscles. The intensity was averaged for 29 muscles. Initial tension: 3.09 ± 0.18 kg/cm^2. The averaged tension for 40 tetani: 80.7 ± 5.9% of the initial tension. (*c*) The results averaged for the latter 40 tetani of 80 tetani. The intensity was averaged for 13 muscles. The initial tension (the tension developed by a muscle during the first tetanus of the 80 tetani) was 3.17 ± 0.17 kg/cm^2. The averaged maximum tension for the 40 tetani: 42.1 ± 9.4% of the initial tension.

When the result was corrected for the background, the intensity of the reflection during tetanic stimulation (except at its beginning) was found not to be significantly different from its resting value. The behavior of the reflection intensity at the end of the stimulation was similar to that observed in the first 20 tetani.

Measurements over 40 tetani were carried out also with the position-sensitive counter and the averaged time course is shown in Fig. 6 *b*. The intensity decreased slightly during the rising phase of the tension, but then increased promptly to a level which was maintained during tetanus. This level was not significantly different from the resting intensity, in agreement with the results obtained with the scintillation counter.

In the third set of measurements the muscles were tetanized 80 times and the intensity of the reflection was recorded for the last 40 tetani. Fatigue was prominent in these tetani: the maximum tension of the 80th tetanus was ~36% of the initial tetanus, and the tension declined markedly during each tetanus. The falling phase of the tetanic tension was prolonged: it took ~1 s for the tension to fall to zero after cessation of stimuli (whereas in nonfatigued muscles it took only ~0.5 s). There was a significant decrease in the intensity of the reflection throughout the duration of tetanus (Fig. 5 *c*). The recovery of the intensity to the resting level after the end of stimulation was very slow, probably reflecting the slow fall of the tension.

TWITCH The 200 twitches required for recording the time course of the intensity caused a slight fatigue to the muscles: the peak tension of the last twitch was ~60% of the initial twitch. This degree of fatigue roughly corresponded to that of the second set of

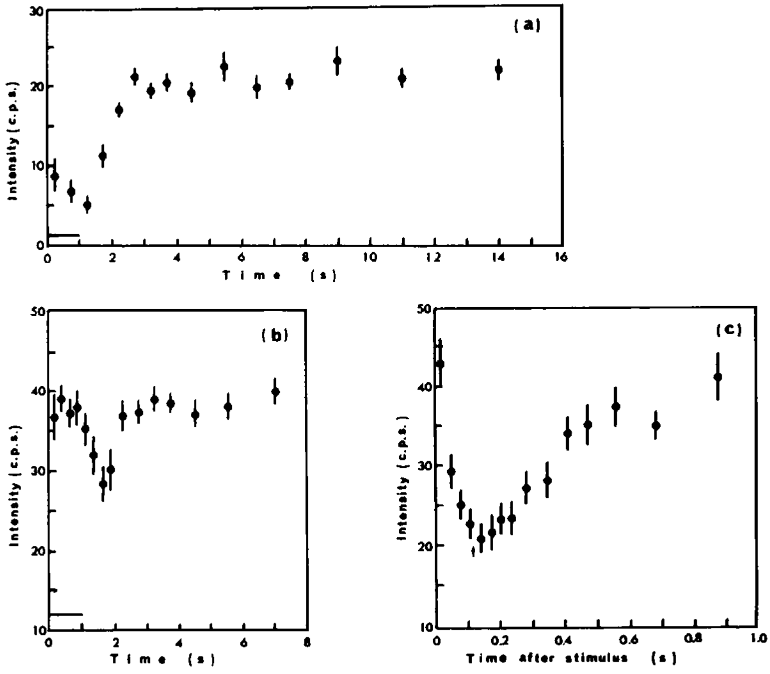

FIGURE 6 Intensities of myosin-related reflections measured with the position-sensitive counter. (*a*) The intensity of the second order meridional reflection at 21.4 nm during tetanus. Each point represents a mean value (±SEM) for 17 muscles. The tension during the initial tetanus: 2.94 ± 0.11 kg/cm^2. The tension averaged for 40 tetani: 78.5 ± 4.5% of the initial tension. The 16-s period after the onset of each tetanus was divided into 15 phases of 0.5–4 s duration: the first 4 s after the onset of tetanus was divided into eight 0.5-s phases, the following 4 s into four 1-s phases, the next 4 s into two 2-s phases, and the last 4 s into one phase. (*b*) The intensity of the third order meridional reflection at 14.3 nm during tetanus. Each point represents a mean value (±SEM) for eight muscles. The tension during the initial tetanus: 2.97 ± 0.15 kg/cm^2. The tension averaged for 40 tetani: 75.4 ± 3.3% of the initial tension. The 8-s period after the onset of each tetanus was divided into 15 phases of 0.25–2 s duration: the first 2 s after the onset of tetanus was divided into eight 0.25-s phases, the following 2 s into four 0.5-s phases, the next 2 s into two 1-s phases, and the last 2 s into one phase. (*c*) The intensity of the third order meridional reflection at 14.3 nm during twitch. Each point represents a mean value (±SEM) for 21 muscles. The initial twitch tension: 2.22 ± 0.10 kg/cm^2. The averaged tension for 200 twitches: 72.3 ± 9.7% of the initial tension. The peak tension occurred at 117.1 ± 5.4 ms (mean ± SD) as indicated by the arrow. The 1-s period after the onset of each stimulus was divided into fifteen phases of 30–240 ms duration: the first 240 ms after stimulation was divided into eight 30-ms phases, the following 280 ms into four 70-ms phases, the next 240 ms into two 120-ms phases and the last 240 ms into one phase.

measurements carried out on tetanized muscles in which the last (i.e., 40th) tetanic tension was also ~60% of its initial value.

Fig. 4*f* shows the intensity change during twitch measured with the scintillation counter. The intensity decreased promptly on stimulation and stayed below the resting value throughout the duration of contraction. The maximum decrease, 14% of the resting intensity, was observed immediately after the time of peak tension. When the results were corrected for the background, it was found that the intensity of the reflection decreased by 42% of its resting intensity 100–160 ms after the stimulus.

The same type of measurements were carried out using the position-sensitive counter (Fig. 6 *c*). The intensity of the reflection decreased by 48% of its resting value during twitch, in approximate agreement with the result obtained with the scintillation counter. The minimum intensity occurred 120–170 ms after the stimulus, immediately after the time of peak tension.

Comparison of the intensity curves during twitch (Figs. 4 *f* and 6 *c*) with those during tetanus (Figs. 5 *b* and 6 *b*) indicates that the average intensity in the first 50 ms of twitch was lower than that during the first 100 ms of tetanus (during which time two pulses were given). This suggests that the intensity during the second 50 ms of tetanus was higher than during the first, giving the higher average value over the initial 100 ms.

DISCUSSION

Intensity Changes during Contraction

During the plateau phase of tetanus we observe that the intensities of the 42.9-nm layer line and the 21.4-nm meridional reflection decrease by 62 and 80%, respectively, relative to their values in the diffraction pattern from resting muscle. Huxley and Brown (1967) observed a similar decrease, 69%, in the intensity of the layer line, but did not monitor the behavior of the 21.4-nm meridional reflection during tetanus, although they observed that it disappeared when the muscle went into rigor. For the 14.3-nm meridional reflection, these authors observed a 34% decline of intensity when the muscle was tetanized, whereas our results indicate that, when the muscle is fresh, there is a rise of 13% in intensity on activation. Huxley (1978) has also observed a rise in the intensity of this reflection during a relatively short series of twitches or tetani of freshly dissected muscle, and has shown that this result is not due to a narrowing of the reflection about the meridian, since the effect occurs when the muscle is tilted relative to the x-ray beam.

During twitch, we observe that the 21.4-nm meridional reflection decreases in intensity by the same amount as during tetanus, that is, by 80%. This change is comparable with that observed by Huxley et al. (1980), who reported a nearly complete disappearance of the reflection during twitch contraction. The decrease we observe for the 42.9-nm layer line during twitch is 49%, considerably less than that observed during twitch by Huxley et al. (>90%). Their results were obtained with high intensity synchrotron radiation, so that data could be collected from relatively thin muscles compared with the bullfrog muscles we used. Sufficient oxygen may not be available to the fibers at the center of thick muscles, and this may have hindered complete activation of our muscle preparations, leading to a smaller intensity change. Another factor which may have contributed to the smaller intensity decrease is the lower temperature at which our experiments were carried out (4° vs. 10°C).

We observe that the 14.3-nm meridional reflection shows a decrease of ~50% during twitch, and declines also at the onset of tetanus, subsequently recovering during the plateau phase. For this reflection, Huxley et al. (1980) showed a biphasic behavior during twitch in which the intensity decrease, plotted as a percentage of the maximum, did not build up monotonically: the intensity recovered during the peak of the twitch and then fell to its minimum value. We also observe the minimum intensity to occur after the peak tension, and the fall to the minimum does not take place at a uniform rate, being faster at first (Fig. 4 *f*), though it is not clear whether there is any recovery of intensity during this period.

The decrease of the 14.3-nm meridional intensity during twitch and at the beginning and relaxing phases of tetanus may be related to internal length changes in the muscle. It is known that shortening of individual sarcomeres takes place during the rising, and lengthening during the relaxing, phases of twitch and tetanus (Kawai and Kuntz, 1973; Paolini et al., 1977). When the muscle is fatigued, there is a decrease of the 14.3-nm meridional intensity during tetanus. In this case, some fibers may not respond to every pulse of the tetanic stimulation (Luttgau, 1965), and even in fibers responding to all pulses, contraction may not be uniform along each fiber due to deterioration of the excitation-contraction coupling (Aljure and Borrero, 1968). These factors would cause internal changes of sarcomere length throughout tetanus. There are two possible mechanisms for the intensity decrease of the 14.3-nm meridional reflection in a muscle which undergoes an internal length change. The first is the disorder in the relative axial positions of the thick filaments, which makes the projected density of the cross-bridges onto the fiber axis less well defined at the 14.3-nm intervals. The type of disorder and its effect on the meridional reflections has been discussed by Haselgrove (1975) and Huxley et al. (1980). The meridional reflections become broader, extending along the layer lines, so that, if their intensities are measured only at the meridian, smaller values are obtained. In our experiments, in which the x-ray beam was linearly focused parallel to the equator (see Materials and Methods), values approximating the integrated intensities of the meridional reflections were obtained and the measurements would have been insensitive to reflection broadening, unless it were extreme. The second possible mechanism is tilting or axial displacement of the myosin heads attached to actin. Since a relative sliding of thick and thin filaments takes place in a muscle which undergoes an internal length change, the myosin heads attached to actin may be tilted to various angles, or axially displaced from the position where they initially attached to actin.

Thick Filament Structure and the Meridional Reflections

The main features of the low-angle x-ray diffraction pattern from resting muscle have been attributed by Huxley and Brown (1967) to a helical arrangement of myosin cross-bridges (see Introduction). Meridional reflections arising from this helix occur on every third layer line at orders of 14.3 nm so that, as mentioned by these authors, the presence of the 21.4-nm and other meridional reflections on the intermediate layer lines implies some departure from the helical symmetry. This is also true for the helix given by Squire (1972) in which there are three rather than two bridges every 14.3 nm along the thick filament.

C-protein has been identified as a component of the thick filaments of vertebrate striated muscle, with an axial repeat of 42.9 nm (Craig and Offer, 1976) or slightly more (Sjöström and Squire, 1977), and might be expected to contribute to the 21.4-nm reflection. This protein forms stripes across the A-band each of ~7-nm axial extent (Craig and Offer, 1976) and only a very large change of the stripe width could seriously affect the intensity of the 21.4-nm meridional reflection. It is unlikely therefore that the large change of this reflection observed during contraction involves C-protein directly.

It should be possible to explain the behavior of the 21.4-nm meridional reflection from consideration of the cross-bridge density projected onto the filament axis. Although the packing of the myosin tails in the filament backbone might also contribute, this packing involves a variation of scattering density along the filament that is small compared with that introduced by the globular heads spaced by sarcoplasm. Since we are considering only the axial arrangement of the cross-bridge density, azimuthal or radial parameters are irrelevant. The presence of the 21.4-nm and other meridional reflections forbidden by the selection rule for a helix with a pitch of 42.9 nm and a subunit repeat of 14.3 nm may be due to a variation in the number of myosin molecules at each 14.3-nm interval and/or to axial departures of the bridges from this interval. The first possibility seems the more radical, since the addition or omission of even one cross-bridge per 42.9-nm axial interval would severely disrupt the helical symmetry. Moreover, in electron micrographs of sections of myofibrils treated with antibody to myosin subfragment-1 (Craig and Offer, 1976), there is no evident variation in the amount of antibody attached, apart from a gap of ~15 nm near the end of the A-band. An axial perturbation of the cross-bridge arrangement therefore seems more likely, and has been suggested by Marvin and Wachtel (1975) by analogy with their studies of filamentous viruses, one class of which gives rise to extra meridional reflections in the x-ray diffraction pattern.

Axial Perturbation of Cross-bridge Arrangement

There is evidence from electron microscopy that the cross-bridges in relaxed muscle do not have an exact 14.3-nm repeat. Craig (1977) has shown in negatively stained A-segments from frog muscle that along the main central portion of the thick filament, the cross-bridges are grouped in threes, with a strong line of stain separating each group. It is difficult to measure accurately the cross-bridge interval but from the drawing given by Craig (in his Fig. 4 *a*) it seems possible that the separation between bridges within each group is greater than that between groups. Sjöström and Squire (1977) have also observed a variation from a strict 14.3-nm repeat in cryosections of human muscle, but attributed it to the superimposition onto the cross-bridge pattern of C-protein with a slightly larger spacing than 3 × 14.3 nm.

From their electron micrographs of sections of glycerinated muscle stained with antibody to C-protein, Craig and Offer (1976) considered that this protein had an axial periodicity of exactly rather than slightly $>3 \times 14.3$ nm. The observation that C-protein was located at 42.9-nm intervals rather than 14.3 nm was taken to imply that not all myosin molecules in the thick filament were equivalent, though this did not necessarily involve any axial perturbation.

Fig. 7 *a* shows the helix proposed by Huxley and Brown (1967), with pairs of cross-bridges occurring at 14.3-nm intervals. When only the meridional reflections in the diffraction pattern are considered, the structure may be regarded as projected onto the filament axis, as illustrated in Fig. 7 *b*. This projection would give rise to reflections at axial intervals of $1/14.3$ nm^{-1}. To give rise to a strong reflection at $1/21.4$ nm^{-1}, that is, on the second order of $1/42.9$ nm^{-1}, it is necessary that the bridges are displaced to bring greater density on planes spaced at one-half rather than one-third of 42.9 nm. Fig. 7 *c* and *d* show the most effective way in which this may be achieved, where the bridges at two levels are displaced to positions which would increase the intensity of the second order meridional reflection without decreasing too greatly the strength of the third. Since the second order reflection is very weak in the diffraction pattern from contracting muscle, the cross-bridge perturbation is almost removed, approximating the structure in Fig. 7 *a* and *b*. This would lead to an increase in the intensity of the third order meridional reflection, as we have observed experimentally.

The perturbation in relaxed muscle may be due to the myosin heads protruding at different angles along the thick filament, so that at the pairs of levels shown displaced in Fig. 7 *c* the

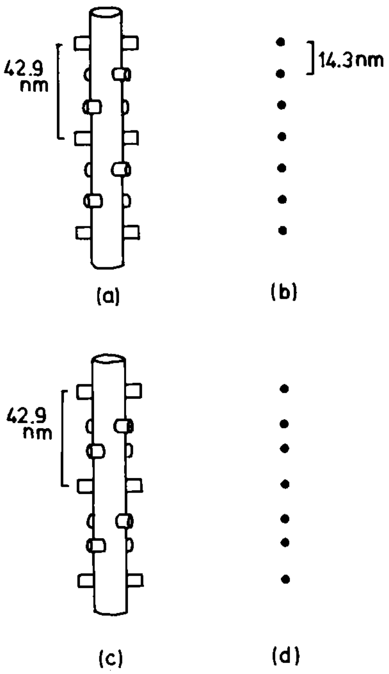

FIGURE 7 (*a*) Arrangement of cross-bridges on 6/2 helix, proposed by Huxley and Brown (1967). (*b*) The arrangement of *a* projected onto the filament axis. (*c*) Perturbed arrangement in which the bridges at two levels per 42.9-nm repeat are displaced axially. (*d*) The projection of *c*. In the model proposed by Bennett (1977) the axial intervals are successively 3/8, 2/8, and 3/8 of 42.9 nm. The projections in *b* and *d* are independent of the number of cross-bridges at each axial level and would therefore be valid for the model with three bridges given by Squire (1972).

heads are more inclined towards each other. Alternatively, or additionally, there may be an underlying displacement in the packing of the myosin tails in the thick filament backbone. The perturbation is not induced by the proximity of the thin filaments, since the 21.4-nm and other meridional reflections forbidden by the helical selection rule are still present in diffraction patterns from very stretched muscles (Huxley, 1972; Haselgrove, 1975).

When muscle is in rigor the 21.4-nm and other forbidden meridional reflections are not observed (Huxley and Brown, 1967), indicating that a removal of the perturbation has taken place similar to that occurring during activation. The 14.3-nm meridional reflection becomes considerably weaker than when the muscle is at rest (Huxley and Brown, 1967), suggesting that the myosin heads are inclined to the filament axis (Reedy, 1968) to a greater extent than during rest or contraction.

Perturbation Proposed by Bennett

Bennett's (1977) proposal was based on her observations of the packing in light meromyosin paracrystals. All paracrystals showed a repeat of 42.9 nm but in some the sub-banding showed an interval of 14.3 nm whereas in others the packing was based on an interval of 3/8 rather than 1/3 of 42.9 nm. Bennett proposed that the packing of myosin molecules in the thick filament in relaxed muscle might be displaced from the positions 0, 1/3, 2/3 to 0, 3/8, 5/8, that is, to positions similar to those shown in Fig. 7 *c* and *d*.

If f_m is taken as the scattering factor of the myosin cross-bridge assembly at each axial level, the structure factor for the meridional reflections has the form: $F(l) = f_m(\exp 2\pi i l z_1 + \exp 2\pi i l z_2 + \exp 2\pi i l z_3)$, where z_1, z_2, z_3 are the fractional coordinates at the three levels in each 42.9-nm period. If $z_1 = 0$, $z_2 = 3/8$, and $z_3 = 5/8$, then $F(1) = -0.4f_m$, $F(2) = f_m$, and $F(3) = 2.4f_m$. The intensities of the second and third meridional reflections are therefore in the ratio 1:5.8 if the variation of scattering factor is ignored. The observed ratio (Figs. 2 and 6) is ~1:2.5, though there is considerable variation from muscle to muscle. The scattering factor depends on the projected axial width of the cross-bridge, but would be expected to decline between the second and third order meridional positions. Allowance for this factor would bring the intensity ratio calculated from the model nearer to the observed value. A correction for the effects of specimen disorientation, curvature of the sphere of reflection, and divergence of the incident beam (Wang and Worthington, 1975) would also decrease the discrepancy between calculated and observed ratios. It is likely, therefore, that the model represents a reasonable approximation to the experimental situation. At higher angles of diffraction, the fifth, eighth, and eleventh order meridional reflections are predicted to be relatively strong by the model, and as pointed out by Bennett (1977), this is in agreement with the x-ray data of Huxley and Brown (1967).

If when the muscle contracts the perturbation is removed so that the myosin cross-bridges are located at 14.3-nm intervals (Fig. 7 *a* and *b*), then $z_1 = 0$, $z_2 = 1/3$, and $z_3 = 2/3$, and $F(1) = 0$, $F(2) = 0$, and $F(3) = 3f_m$. On this model, therefore, the second order meridional reflection would disappear, which is in approximate agreement with the 80% decrease we have observed. The third order meridional reflection would increase in intensity by 50%, whereas we have observed an increase of 13%. This discrepancy may be due to a change of shape or angle of attachment of the cross-bridges during contraction. If the cross-bridges become more tilted or elongated in the axial direction, the intensity of the 14.3-nm reflection would be reduced, and this would counteract the effect of the axial rearrangement in the thick filament discussed here. This type of change in cross-bridge shape or angle would be consistent with the model for isometric contraction developed by Huxley and Simmons (1971) from that of Huxley (1969), in which the myosin head rotates between three stable positions.

When a muscle contracts or passes into rigor, an increase of ~1% in the spacing of the 14.3-nm meridional reflection has been observed (Huxley and Brown, 1967; Haselgrove, 1975; Huxley, 1978). This may be taken as evidence for a structural change in the thick filament during contraction (Haselgrove, 1975), and may be incorporated within the model for the axial rearrangement without significantly affecting the calculations or conclusions made.

An axial perturbation of thick filament structure has been proposed by Millman and Bennett (1976) for relaxed scallop striated muscle. Alternate cross-bridge levels are displaced by 1.5 nm from the repeat of 14.5 nm, to give rise to the extra meridional reflections observed at orders of 29 nm. This displacement is removed when the muscle passes into rigor. In support of their proposal, these authors note that light meromyosin from this muscle forms paracrystals (Szent-Györgyi et al., 1973) with a 58-nm repeat (2 × 29 nm) rather than 43 or 14.3 nm. In this proposal, therefore, as in the model of Bennett (1977) outlined here, the perturbation involves displacements in the filament backbone, rather than affecting the heads only.

Movement of Cross-bridges in Three Dimensions

The above discussion is limited to the interpretation of the changes in intensity of the meridional reflections during contraction. In the pattern from resting muscle the 42.9-nm layer line is strong but in contraction it is weak, showing that in addition to the rearrangement along the filament axis there must be considerable movement of cross-bridges in the plane perpendicular to this axis. The axial change is from a perturbed to a regular 14.3-nm repeat, but the movement in the perpendicular plane is from more to less ordered cross-bridge positions. In the contracting muscle the cross-bridges have lost most of their helical ordering, though retaining a precise axial repeat.

Conclusions

The strength of the 2.14-nm meridional reflection as well as the 14.3-nm reflection in the diffraction pattern from resting muscle is adequately explained by the model proposed by Bennett (1977) involving an axial perturbation of the cross-bridge repeat. This perturbation is removed when the muscle contracts or passes into rigor. In the pattern from contracting muscle the 42.9-nm layer line is weak, indicating that the removal of the perturbation in the axial direction is accompanied by loss of order in the azimuthal and radial directions.

We thank Dr. P. M. Bennett for helpful discussion, Doctors A. Elliott and G. Offer for helpful comments on the manuscript, and Professor M. Endo for encouragement.

Received for publication 7 May 1980 and in revised form 8 September 1980.

REFERENCES

ALJURE, E. F., and L. M. BORRERO. 1968. The evolution of fatigue associated with isometric contraction in toad sartorius. *J. Physiol.* **194**:289–303.

BENNETT, P. M. 1977. Structural studies of muscle thick filaments and their constituents. Ph.D. Thesis. University of London.

CRAIG, R. 1977. Structure of A-segments from frog and rabbit skeletal muscle. *J. Mol. Biol.* **109**:69–81.

CRAIG, R., and G. OFFER. 1976. The location of C-protein in rabbit skeletal muscle. *Proc. R. Soc. Lond. Biol. Sci.* **192**:451–461.

ELLIOTT, G. F., J. LOWY, and B. M. MILLMAN. 1965. X-ray diffraction from living striated muscle during contraction. *Nature (Lond.).* **206**:1357–1358.

HASELGROVE, J. C. 1975. X-ray evidence for conformational changes in the myosin filaments of vertebrate striated muscle. *J. Mol. Biol.* **92**:113–143.

HASELGROVE, J. C., and H. E. HUXLEY. 1973. X-ray evidence for radial cross-bridge movement and for the sliding filament model in actively contracting skeletal muscle. *J. Mol. Biol.* **77**:549–568.

HUXLEY, A. F., and R. M. SIMMONS. 1971. Proposed mechanism of force generation in striated muscle. *Nature (Lond.).* **233**:533–538.

HUXLEY, H. E. 1969. The mechanism of muscular contraction. *Science (Wash. D.C.).* **164**:1356–1366.

HUXLEY, H. E. 1972. Structural changes in the actin- and myosin-containing filaments during contraction. *Cold Spring Harbor Symp. Quant. Biol.* **37**:361–376.

HUXLEY, H. E. 1975. The structural basis of contraction and regulation in skeletal muscle. *Acta Anat. Nippon.* **50**:310–325.

HUXLEY, H. E. 1976. The relevance of studies on muscle to problems of cell motility. *In* Cell Motility. Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y. 115–126.

HUXLEY, H. E. 1978. Time resolved x-ray diffraction studies on muscle. *In* Cross-bridge Mechanism in Muscle Contraction. University of Tokyo Press, Tokyo. 391–405.

HUXLEY, H. E., W. BROWN, and K. C. HOLMES. 1965. Constancy of axial spacing in frog sartorius muscle during contraction. *Nature (Lond.)*. **206**:1358.

HUXLEY, H. E., and W. BROWN. 1967. The low-angle x-ray diagram of vertebrate striated muscle and its behaviour during contraction and rigor. *J. Mol. Biol.* **30**:383–434.

HUXLEY, H. E., A. R. FARUQI, J. BORDAS, M. H. J. KOCH, and J. R. MILCH. 1980. The use of synchrotron radiation in time-resolved x-ray diffraction studies of myosin layer-line reflections during muscle contraction. *Nature (Lond.)*. **284**:140–143.

KAWAI, M., and I. D. KUNTZ. 1973. Optical diffraction studies of muscle fibers. *Biophys. J.* **13**:857–876.

LÜTTGAU, H. C. 1965. The effect of metabolic inhibitors on the fatigue of the action potential in single muscle fibres. *J. Physiol.* **178**:45–67.

MARVIN, D. A., and E. J. WACHTEL. 1975. Structure and assembly of filamentous bacterial viruses. *Nature (Lond.)*. **253**:19–23.

MATSUBARA, I., and N. YAGI. 1978. A time-resolved X-ray diffraction study of muscle during twitch. *J. Physiol.* **278**:297–307.

MILLMAN, B. M., and P. M. BENNETT. 1976. Structure of the cross-striated adductor muscle of the scallop. *J. Mol. Biol.* **103**:439–467.

MOORE, P. B., H. E. HUXLEY, and D. J. DEROSIER. 1970. Three-dimensional reconstruction of F-actin, thin filaments and decorated thin filaments. *J. Mol. Biol.* **50**:279–295.

PAOLINI, P. J., K. P. ROOS, and R. J. BASKIN. 1977. Light diffraction studies of sarcomere dynamics in single skeletal muscle fibers. *Biophys. J.* **20**:221–232.

REEDY, M. K. 1968. Ultrastructure of insect flight muscle. *J. Mol. Biol.* **31**:155–176.

SJÖSTRÖM, M., and J. M. SQUIRE. 1977. Fine structure of the A-band in cryosections. *J. Mol. Biol.* **109**:49–68.

SQUIRE, J. M. 1972. General model of myosin filament structure. *J. Mol. Biol.* **72**:125–138.

SZENT-GYÖRGYI, A. G., E. M. SZENTKIRALYI, and J. KENDRICK-JONES. 1973. The light chains of scallop myosin as regulatory subunits. *J. Mol. Biol.* **74**:179–203.

WANG, S. K., and C. R. WORTHINGTON. 1975. Low-angle X-ray data processing for collagen. *Biophys. J.* **15**:322 *a*. (Abstr.).

YAGI, N., M. H. ITO, H. NAKAJIMA, T. IZUMI, and I. MATSUBARA. 1977. Return of myosin heads to thick filaments after muscle contraction. *Science (Wash. D.C.)*. **197**:685–687.