Diffraction rings obtained from a suspension of skeletal myofibrils by laser light illumination

Study of internal structure of sarcomeres

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ABSTRACT Diffraction rings corresponding to the first, second, and third order were obtained by laser light illumination from a suspension of rabbit glycerinated psoas myofibrils (diameter, 1–2 μm; average length of the straight region, 44 μm; average sarcomere length, 2.2–2.6 μm) of which the optical thickness was appropriately chosen. Dispersed myofibrils were nearly randomly oriented in two dimensions, so that the effects of muscle volume were minimized; these effects usually interfere significantly with a quantitative analysis of laser optical diffraction in the fiber system. The diameters of diffraction rings represented the average sarcomere length. By using this system, we confirmed the ability of the unit cell (sarcomere) structure model to explain the intensity change of diffraction lines accompanying the dissociation from both ends of thick filaments in a high salt solution. The length of an A-band estimated from the relative intensity of diffraction rings and that directly measured on phase-contrast micrographs coincided well with each other. Also, we found that myofibrils with a long sarcomere length shorten to a slack length accompanying the decrease in overlap between thick and thin filaments produced by the dissociation of thick filaments.

INTRODUCTION

Laser light diffraction has been widely used in muscle research. The separation between diffraction lines is used for estimating the lattice constant (average sarcomere length) of skeletal and cardiac muscle fibers. This technique has been applied to detect, for example, uniform shortening of sarcomeres during tetanic contraction (Cleworth and Edman, 1972), the response of sarcomere shortening for quick release of a muscle fiber (Barden and Mason, 1978), and stepwise shortening of sarcomeres in a muscle fiber (Pollack et al., 1977). At the same time, it has been pointed out that optical artifacts appear in the fiber system due to the volume diffraction effects originating from the extremely high coherency of laser light and the thickness of the fibers (Rüdel and Zite-Ferenczy, 1979*a*; Altringham et al., 1984). In this respect, it has been shown recently that white light illumination can avoid the difficulty accompanying laser light illumination (Goldman, 1987).

On the other hand, only a limited number of structural studies based on diffraction intensities have been done (Kawai and Kuntz, 1973; Fujime, 1975; Baskin et al., 1979; Rüdel and Zite-Ferenczy, 1979*b*). The structural change in a sarcomere of skeletal muscle fiber has been studied by analyzing the intensities of optical diffraction lines based on a unit cell (sarcomere) structure model (Fujime, 1975; Fujime and Yoshino, 1978). The intensity of diffraction lines generally depends not only on the structure factor of a unit cell but also on several other factors such as the structure factor of the 3D arrangement of myofibrils (Fujime and Yoshino, 1978; Rüdel and Zite-Ferenczy, 1979*b*; Judy et al., 1982; Leung, 1982; Fujime, 1984; Brenner, 1985; Sundell et al., 1986; Zite-Ferenczy et al., 1986). Thus, the applicability of the unit cell structure model for quantitative analysis of the structure of sarcomeres in the fiber system has been limited to some specific problems such as the flexibility of myofilaments (Umazume and Fujime, 1975; Yoshino et al., 1978) and the dissociation from both ends of thick filaments (Ishiwata et al., 1985; Higuchi and Ishiwata, 1985; Higuchi et al., 1986).

Here, we try to extract information from the diffracted light intensities that arise from a structure factor of a myofibril by illuminating with a laser light a suspension of myofibrils, which are nearly randomly oriented in two dimensions. In this system, the volume effects are minimized. First, we show that diffraction rings can be obtained from a thin suspension of myofibrils; as a result, the internal structure of sarcomeres can be studied by measuring the intensities of diffraction rings. Moreover, the average length of sarcomeres in myofibrils can be estimated from the diameter of the diffraction rings. Recently, this diffraction method has been used for measuring the average length of sarcomeres in cardiac muscle cells (Krueger, 1988).

A preliminary report of this work has been presented (Ishiwata and Okamura, 1985).

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MATERIALS AND METHODS

Myofibrils were prepared in a rigor solution containing 60 mM KCl, 5 mM $MgCl_2$, 10 mM Tris-maleate buffer (pH 6.8), and 1 mM EGTA (solution A) by homogenizing rabbit glycerinated psoas muscle fibers with a homogenizer (Ultra Turrax, IKA-WERK, Staufen, FRG; Ishiwata, 1981). Most of the myofibrils were 1–2 μm in diameter and were composed of a single myofibril or a bundle of a few myofibrils. A small number of larger bundles of myofibrils was also present. The membrane was dissolved by treating the myofibrils with solution A containing 1% (vol/vol) Triton X-100. To remove glycerol and Triton X-100, myofibrils were washed with solution A by repeating low-speed centrifugation (1,000 *g*, 10 min). Phase-contrast micrographs of myofibrils were taken with a Biophoto VBS-UWT (objective lens, CF Plan DM 40× (NA = 0.65) for the observation of a suspension of myofibrils and CF Plan DM 100× (NA = 1.25) for the measurement of sarcomere lengths and the length of thick filaments; Nikon Co., Ltd., Tokyo, Japan) by using Kodak Plus-X 35-mm films.

A drop of suspension of myofibrils (1 mg/ml) was put on a glass slide and covered with a cover slip (the gap between the two glasses was ~15 μm). Myofibrils were easily oriented by the flow of solution. In the present work, we selected a part in which myofibrils appeared to be randomly oriented; a typical part is shown on a phase-contrast micrograph (Fig. 1). Solvent was exchanged by pouring a new solvent at one side of a cover slip and absorbing it with a piece of filter paper from the other. The length of the straight region of myofibrils was estimated to be 44.0 ± 25.2 μm (average ±SD; number of myofibrils measured, 100). This means that the average number of sarcomeres in a straight region is 17 for myofibrils of which the average sarcomere length is 2.5 μm. If we assume that the protein concentration inside a myofibril is 0.1 g/ml and the average volume of a straight region of myofibril is $1 \times 1 \times 44\ \mu m^3$, the weight of proteins in a myofibril is estimated to be 4.4×10^{-12} g on the average.

Diffraction rings were obtained by means of a simple set-up as schematically illustrated in Fig. 2. The light beam from a He-Ne laser (632.8 nm, 15 mW nominal; type GLG-5601, NEC Inc., Tokyo, Japan) was passed perpendicularly through a thin layer of the myofibril suspension (the diameter of the laser beam was ~1.5 mm). To obtain sharp and strong diffraction rings, we examined several combinations of the protein concentration (0.1–20 mg/ml myofibrils) and the sample thickness (a few micrometers to a few millimeters). We found that the

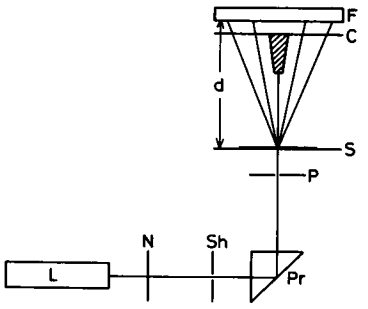

FIGURE 2 Schematic illustration of the experimental apparatus for optical diffraction (side view). *L*, laser; *N*, neutral density filter (percentage transmission, variable); *Sh*, shutter; *Pr*, prism; *P*, pin hole; *S*, sample; *C*, cone-shaped beam stopper; *F*, film; *d*, sample-to-film distance.

combination of ~1 mg/ml myofibrils and about 15 μm thickness of myofibril suspension gives good diffraction rings. The total weight of proteins illuminated by laser light is estimated to be 2.7×10^{-8} g ($=\pi \times (1.5\ mm/2)^2 \times 15\ \mu m \times 1$ mg/ml). Thus, the number of myofibrils (44 μm long and 1 μm thick) in the myofibril suspension of 1 mg/ml illuminated by laser light is ~6,000 ($=2.7 \times 10^{-8}/4.4 \times 10^{-12}$). The diffraction pattern was directly recorded on a 60-mm roll film (Neopan F, 120 type, Fuji Photo Film Co., Ltd., Tokyo, Japan; exposure time, 1/8 s with an ND filter giving 20% transmission was used), which was mounted on a film holder (Mamiya Camera Co., Ltd., Saitama, Japan). The sample and the film holder were set on the stage of the optical microscope. A cone-shaped beam stopper was placed between the sample and film. The sample-to-film distance (d) was 3.8 cm. The average sarcomere length (L) was estimated by the equation, $L = n\lambda (d^2 + r(n)^2)^{1/2}/r(n)$, where λ is the wavelength of laser light. $r(n)$ is the average radius of the nth order diffraction ring, which was obtained by taking an average of the longest and the shortest diameters of each diffraction ring; the difference between the longest and the shortest was a few percent of the average. The intensity profile of the diffraction pattern was obtained by densitometry of the negative roll film with a

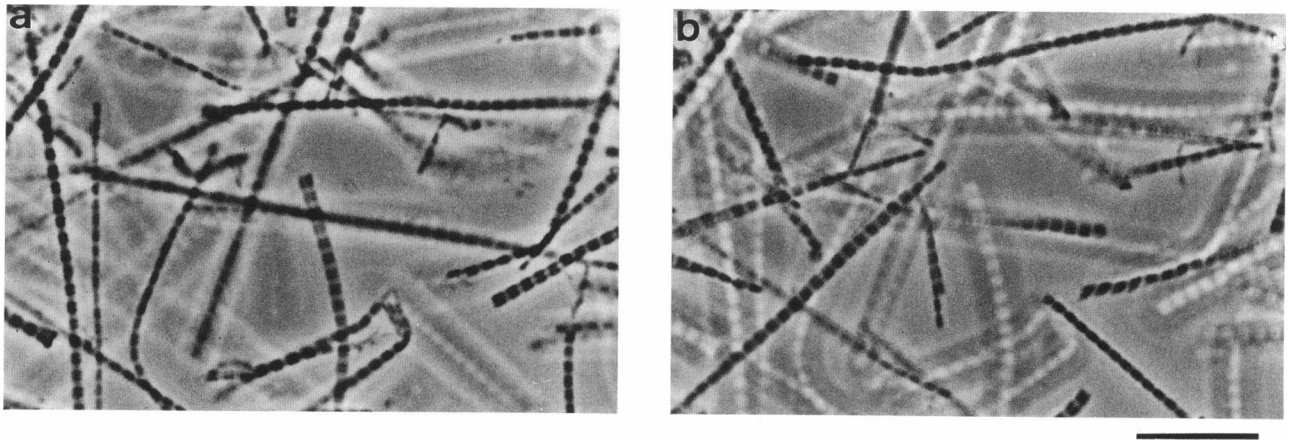

FIGURE 1 Phase-contrast micrographs of a suspension of myofibrils in the rigor solution (solution A) taken with an objective lens of 40×. (*a*) Taken at a place close to a cover slip; (*b*) taken close to a glass slide. Myofibrils in which contrast is reversed were out of focus. Room temperature. Bar, 20 μm.

chromatoscanner (CS-910, Shimadzu Co., Kyoto, Japan; transmission mode with a logarithmic amplifier at a wavelength of 550 nm). The intensity change of the first- and second-order diffraction rings was directly measured by focusing about one-twentieth part of the circumference of the diffraction rings with condenser lenses onto photodiodes. Photographs of diffraction rings were taken under a condition of no flow of solution, whereas the photoelectric measurements of diffraction intensities were performed under continuous flow of solution. All observations and experiments were done at room temperature.

The change of diffraction intensity accompanying the dissociation of thick filaments was calculated at each sarcomere length according to model *a* of Ishiwata et al. (1985). This model assumes that thick filaments dissociate from both ends and the change of diffraction intensity occurs through the change of a scattering form factor of a single sarcomere as schematically shown in the inset of Fig. 3. Fig. 3 shows an example of this model calculation at a sarcomere length of 2.6 μm.

RESULTS AND DISCUSSION

Figs. 4 and 5, respectively, show photographs of diffraction patterns and their radial intensity profiles obtained from a suspension of myofibrils. First-, second- and third-order diffuse diffraction rings, which originate from dispersed thin myofibrils, are observed in a rigor solution (Figs. 4 *a* and 5 *a*); sharp short lines observed at several places along the diffraction rings originate from a small number of contaminating thick bundles of myofibrils; the diffraction rings are overlapped with the strong zeroth-order diffraction (the central part of the diffraction pattern in Fig. 4, of which the radial intensity profile is shown by the dashed line in Fig. 5). The radial intensity profile of the diffraction rings shows a steep increase inside and gentle decrease outside (noticeable in the second-order diffraction ring). Such a feature of the diffraction pattern is consistent with that expected for straight myofibrils oriented at random on a plane perpendicular to the incident laser beam. In other words, the diffraction pattern coincides with that obtained by rotating a single myofibril on the plane. The intensity of the sharp short lines is asymmetrical, probably because of the tilting of the diffraction plane of the fibers, i.e., one of the volume effects observed in the fiber system.

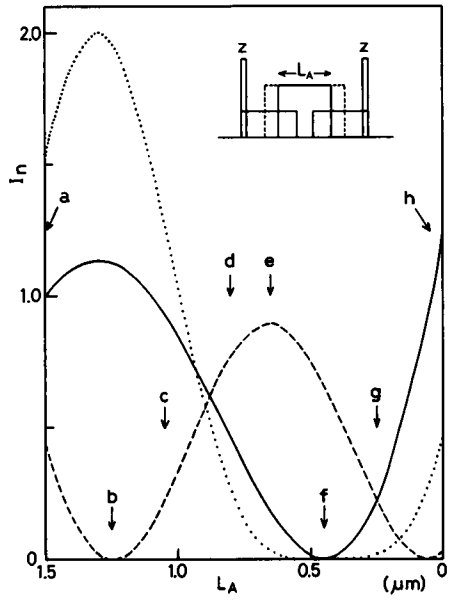

FIGURE 3 Model calculation of the intensity change of the first- (*solid curve*), second- (*dashed curve*), and third-order (*dotted curve*) diffraction lines (rings) accompanying the dissociation of thick filaments in myofibrils at a sarcomere length of 2.6 μm. These curves were obtained according to model *a* in Ishiwata et al. (1985), in which the ratio among refractive indices of an A-band, an I-band, and a Z-line is assumed to be 1:0.5:1.5, and thick filaments dissociate from both ends as schematically shown by the profile of optical density of the sarcomere (see inset). Ordinate, relative values of the diffraction intensity (I_n); abscissa, length of thick filaments (L_A), where the original length is assumed to be 1.5 μm. Arrows from *a* to *h* roughly correspond to the diffraction patterns *a* to *h* in Fig. 4.

Accompanying the dissociation from both ends of thick filaments, the diffraction intensities changed as predicted by the unit cell structure model. As the model calculation suggests (Fig. 3), the intensity of the first-order diffraction ring gradually decreased (Figs. 4, *a–e*, and 5, *a–e*), then was extinguished (Figs. 4 *f* and 5 *f*) and finally increased (Figs. 4, *g* and *h*, and 5, *g* and *h*); the intensity of the second-order diffraction ring rapidly decreased and was extinguished (Figs. 4 *b* and 5 *b*), then increased to the maximum (Figs. 4 *e* and 5 *e*) and finally decreased again (Figs. 4, *f–h* and 5, *f–h*); the intensity of the third-order diffraction ring gradually decreased (Figs. 4, *a–c*, and 5, *a–c*) and was extinguished at nearly the same length of thick filaments as that at which the second-order diffraction ring reached the maximum (Figs. 4 *e* and 5 *e*). The intensity itself was relatively weak compared with those of the first- and second-order diffraction rings, and this observation is not consistent with the model calculation. This can be ascribed to the fact that the higher the order of diffraction is, the more affected the diffraction intensity is by the disorder of structure. Also, it should be noted that the intensity of the zeroth-order diffraction decreased with the decrease of the protein concentration in myofibrils. The intensity decreased at nearly the same rate irrespective of the radial direction of diffraction (see dashed lines in Fig. 5).

The diameter of the diffraction rings started to increase between Fig. 4 *c* and *d*, and finally increased by 7–8% of the initial value (see Table 1), indicating that sarcomeres shortened by 7–8%. It appears that sarcomeres started to shorten when the overlap between thick and thin filaments disappeared owing to the dissociation of thick filaments, that is, when the filament length became ~0.6 μm. The sarcomeres did not shorten to less than some

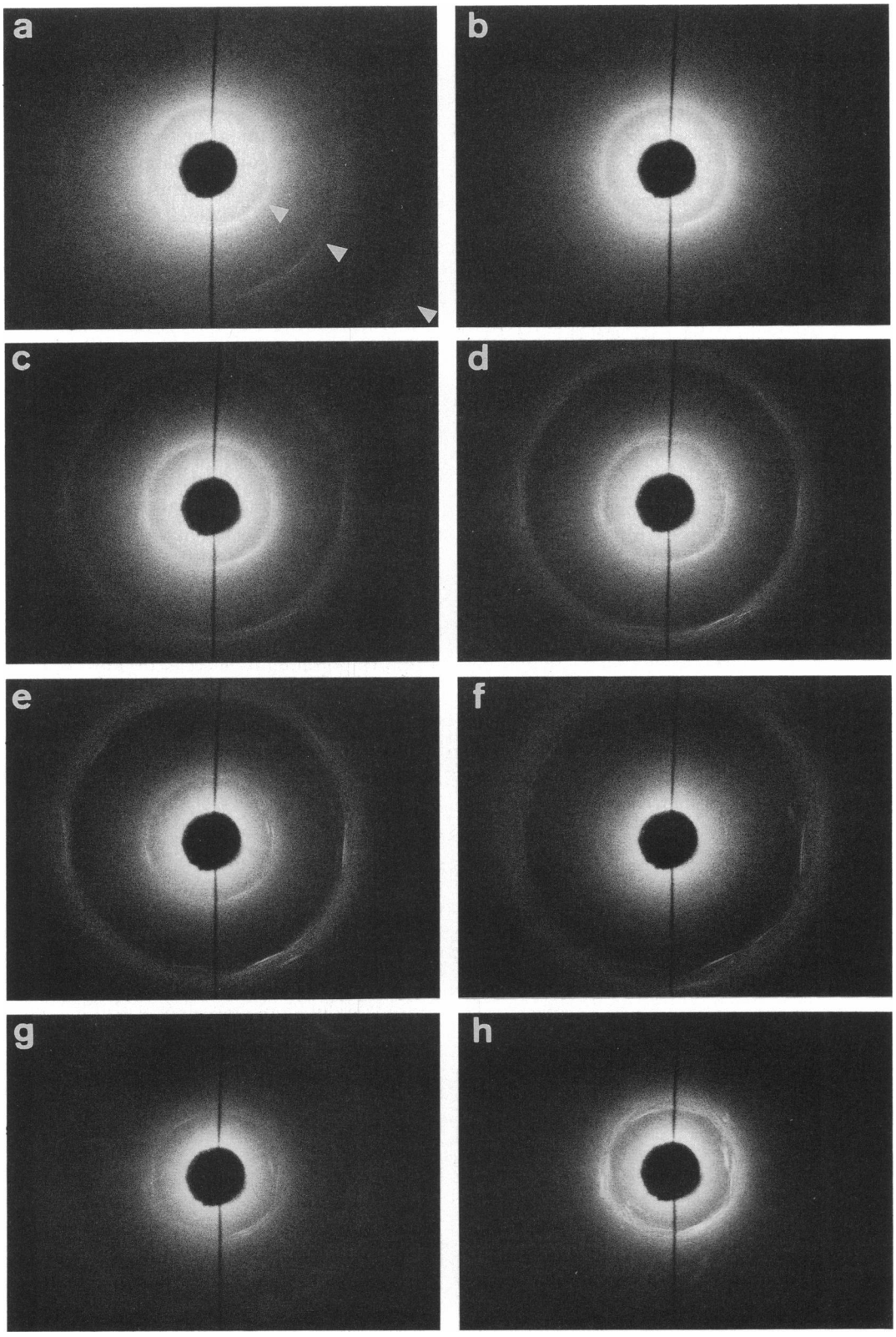

FIGURE 4 Diffraction patterns of a suspension of myofibrils. The change of diffraction pattern accompanying dissociation from both ends of thick filaments in a high salt solution was examined. (*a*) In rigor solution (solution A). (*b* and *c*) After exchanging solution A for that containing 0.275 M KCl and 10 mM PPi. (*d–g*) Gradual change with time after exchanging the former solution for solution A containing 0.3 M KCl and 10 mM PPi. (*h*) After exchanging the former solution for solution A containing 0.5 M KCl and 10 mM PPi. Arrowheads in *a* indicate the first-, second-, and third-order diffraction rings, respectively, from the inside to the outside of the concentric diffraction rings. The average sarcomere length estimated from the diameter of diffraction rings was ~2.6 μm before the dissociation of thick filaments (*a*), and ~2.4 μm after the dissociation (*h*) (cf. Table 1). Room temperature.

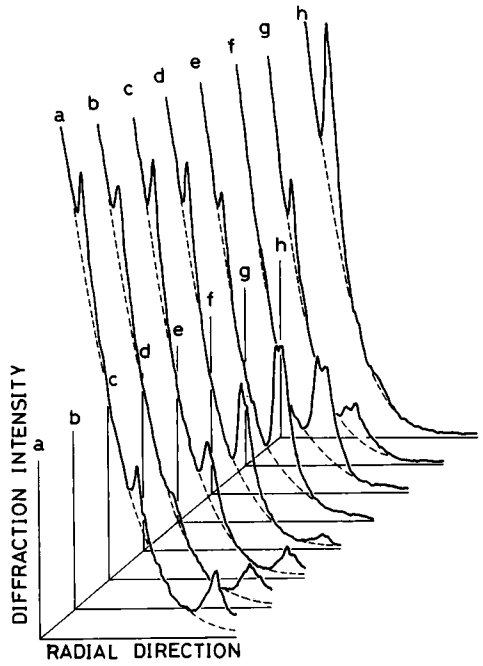

FIGURE 5 Intensity profiles of diffraction patterns of a suspension of myofibrils. The intensity profile was obtained by densitometry (measured along a diagonal line, i.e., a radial direction, with a 0.1 × 3 mm rectangular illumination window) of a negative film of Fig. 4. The diffraction intensity is shown in a logarithmic scale with an arbitrary unit. Vertical lines with *a–h* show the centers (the position of the center of incident laser beam) for diffraction patterns corresponding to Fig. 4, *a–h*, respectively.

particular length (see Table 1, *g* and *h*). The final length of shortened sarcomeres corresponded to the so-called slack length, below which the resting tension of the myofibril disappears. No sarcomere shortening was observed for myofibrils of which the initial sarcomere length was less than ~2.3 μm. Thus, the reason sarcomeres are shortened is thought to be that at a long sarcomere length, passive tension is developed due to the stretching of parallel elastic components. In the present case, such internal stress in myofibrils will be sustained by a small number of cross-bridges present under partial relaxation obtained by the presence of pyrophosphate (PPi) at room temperature (cf. Ishiwata et al., 1986). Then, myofibrils will start to shorten when the force balance is broken by the decrease of the number of cross-bridges with the decrease of the overlap between thick and thin filaments. Such sarcomere shortening was observed under a phase-contrast microscope mainly in thin myofibrils but rarely in the large bundle of myofibrils, probably because the latter is apt to adhere to a glass surface. This is consistent with the fact that the position of the strong diffraction line, which originates from a large bundle of myofibrils, hardly changed, whereas the diameters of the diffraction rings changed (this is especially evident on the second-order diffraction ring [Figs. 4, *e–g*]; also, see the split of the second-order diffraction peak [Figs. 5, *e–g*]).

The sarcomere length and its shortening with the dissociation of thick filaments were examined by phase-contrast microscopy. When the sarcomere length (the separation between Z-lines) was 2.51 ± 0.10 μm before the dissociation of thick filaments, it became 2.34 ± 0.17 μm after the dissociation (average ± SD; number of sarcomeres measured, 100 = 10 myofibrils arbitrarily

TABLE 1 Change of sarcomere length in myofibrils accompanying the dissociation of thick filaments

Stage*	*a*	*b*	*c*	*d*	*e*	*f*	*g*	*h*
SL1‡ (μm)	2.61	2.61	2.61	2.55	2.49	—	—	2.38
SL2‡ (μm)	2.55	—	2.53	2.50	2.46	2.41	2.37	—

*Stages *a–h* correspond to Figs. 4, *a–h*, respectively.
‡Sarcomere lengths were obtained by taking the average diameters of diffraction rings, SL1 and SL2 from the first- and the second-order diffraction rings, respectively. A blank sarcomere length could not be estimated because diffraction rings were absent or faint.

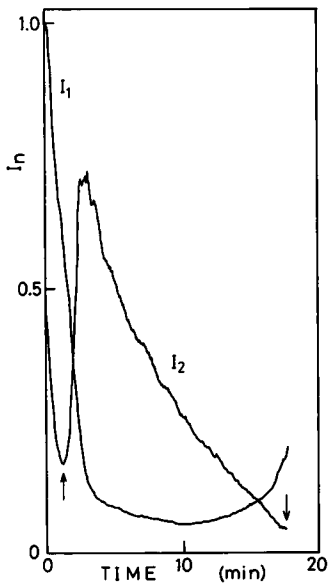

FIGURE 6 A typical example showing the time course of intensity change of the first- (I_1) and second-order (I_2) diffraction rings accompanying the dissociation of thick filaments in myofibrils. At time zero, the rigor solution (solution A) was exchanged for that containing 0.3 M KCl and 10 mM PPi. The average sarcomere length before the dissociation was ~2.5 μm in this preparation. Ordinate, diffraction intensity (in a linear scale with an arbitrary unit for each order of diffraction); abscissa, dissociation time. Room temperature. Arrows pointing up and down show the first and the second minima of I_2, respectively.

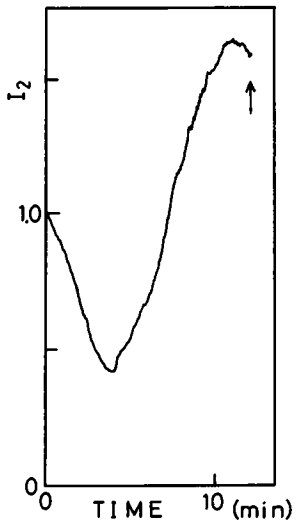

FIGURE 7 An example showing the time course of intensity change of the second-order diffraction ring accompanying the dissociation to some intermediate length of thick filaments in myofibrils. In this example, the rigor solution (solution A) was exchanged at time zero for that containing 0.25 M KCl and 10 mM PPi, and just after the diffraction intensity had passed the maximum (at the time shown by the arrow), phase-contrast micrographs were taken to measure the length of thick filaments (A-band width). Note that the dissociation rate is slower than that of 0.3 M KCl shown in Fig. 6 (cf. Higuchi and Ishiwata, 1985). The average sarcomere length before the dissociation was ~2.3 μm in this preparation. Ordinate, diffraction intensity (in a linear scale with an arbitrary unit); abscissa, dissociation time. Room temperature.

chosen × 10 sarcomeres for each myofibril). These values are consistent with those estimated above (cf. Table 1).

Next, we tried to examine photoelectrically the intensity change of the diffraction rings. Fig. 6 shows the time course of the intensity change of the first (I_1)- and second (I_2)-order diffraction rings accompanying the dissociation of thick filaments in a high salt solution. It should be noted that both I_1 and I_2 include the background intensity, which is large in size and steep in radial decay, and moreover decreases greatly with dissociation of thick filaments (Figs. 4 and 5). This is the major reason for the apparent discrepancies discussed below between the observed changes of diffraction intensities (Fig. 6) and calculated ones according to the unit cell structure model (cf. Fig. 3 and model *a* in Ishiwata et al., 1985): First, I_1 and I_2 at the minimum points have finite values, which should be zero according to the model calculation. This is due to the background intensities at these points; indeed the diffraction rings were extinguished (Figs. 4 *b* and 5 *b* for the second-order and Figs. 4 *f* and 5 *f* for the first-order diffraction rings). Second, the initial increase of I_1 is difficult to observe, despite its being expected (although it is not large in size) from the model calculation for myofibrils with a sarcomere length shorter than ~ 2.8 μm. This is due to the decrease in background intensity. In fact, I_1 stays almost constant in the very early stage of dissociation of thick filaments, and then decreases very rapidly. Third, I_2 at the second minimum is smaller than that at the first minimum. This is also due to the decrease

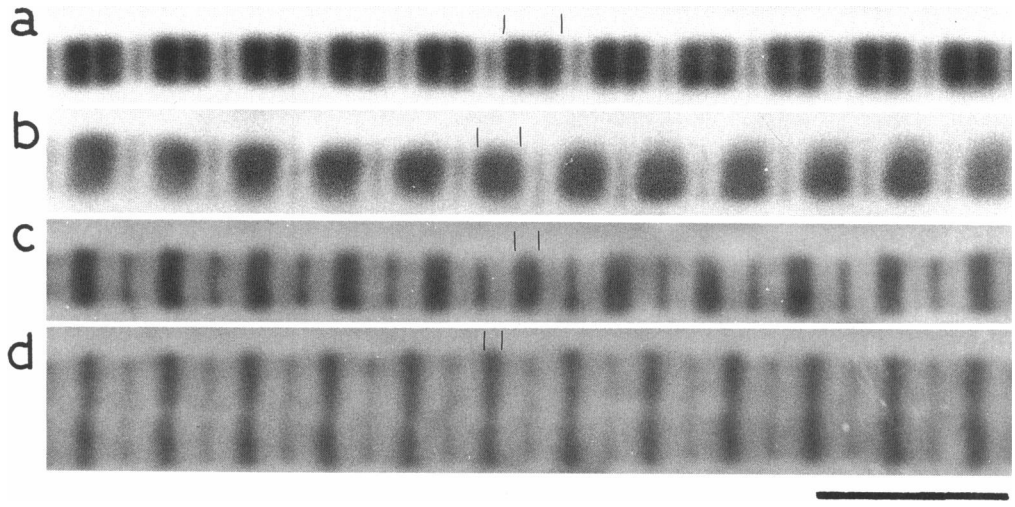

FIGURE 8 Phase-contrast micrographs of myofibrils at different stages of dissociation of A-bands taken with an objective lens of 100×. (*a*) A myofibril before the dissociation of thick filaments in the rigor solution (solution A). (*b–d*) Myofibrils of which A-bands were partially dissociated from both ends; typical myofibrils were selected from a suspension of myofibrils which showed the first minimum (*b*) and the maximum (*c*) of the intensity of the second-order diffraction ring, and the minimum (*d*) of the intensity of the first-order diffraction ring. The dissociation of thick filaments was induced at room temperature by exchanging solution A for that containing 0.25 M KCl and 10 mM PPi. The intensity change of diffraction rings was monitored as shown in Fig. 7. The length of thick filaments was estimated by measuring the width of the A-band, i.e., the distance between two thin lines shown in the figure.

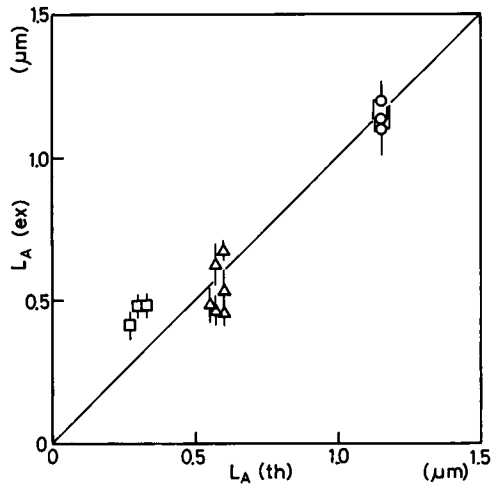

FIGURE 9 Comparison between the length of thick filaments in myofibrils estimated from the intensity of diffraction rings and that measured from phase-contrast micrographs of the same sample. Abscissa, the length of thick filaments, $L_A(th)$, estimated from the first minimum (○) and the maximum (▲) of the intensity of the second-order diffraction ring, and the minimum of the intensity of the first-order diffraction ring (□). The average sarcomere length used for these experiments was 2.2–2.3 μm, where the shortening of sarcomeres was not observed. Model calculation was performed at each sarcomere length as shown in Fig. 3 (note that the length of thick filaments showing the maximum and minimum of diffraction intensity depends on sarcomere length [Ishiwata et al., 1985]). Ordinate, the average length of thick filaments, $L_A(ex)$, measured from phase-contrast micrographs (number of measurements, 100 = 10 myofibrils arbitrarily chosen × 10 A-bands for each myofibril). Vertical bars, standard deviation. The conditions for the dissociation of thick filaments were the same as that in Fig. 8.

with time in the background intensity. I_1 at the final stage is much smaller than that at the initial stage; this is, however, mainly ascribed to the fact that the dissociation of thick filaments was not yet complete. If these facts are taken into consideration, the unit cell structure model is concluded to describe quantitatively the change in diffraction intensities.

It is noticeable in Fig. 6 that the intensity change slowed down with time (compare with Fig. 3). This indicates that the dissociation velocity of thick filaments, i.e., the dissociation rate of myosin molecules from the ends of thick filaments, decreases with the shortening of filament length; that is, the shorter the filament length is, the more stable the filament structure is. This supports the previous work on muscle fibers that suggested the accumulated strain mechanism for the length regulation of thick filaments (Higuchi and Ishiwata, 1985; Higuchi et al., 1986; Davis, 1988).

The length of thick filaments during the dissociation was estimated according to the model calculation of diffraction intensities at the three points marked *b*, *e*, and *f* in Fig. 3. The estimated length was compared with that measured on phase-contrast micrographs (cf. Ishiwata, 1981; Higuchi et al., 1986). An example showing the time course of the intensity change of the second-order diffraction ring used for such experiments is shown in Fig. 7. Phase-contrast micrographs used for measuring the length of thick filaments are shown in Fig. 8. Fig. 9 summarizes these results. The filament lengths obtained in two such different ways coincided with each other within the limits of their systematic errors except at the shortest filament length, where the measurement from phase-contrast micrographs tends to show a longer length because of the limited resolution of microscopy (Fig. 8 *d*). Thus we conclude that the applicability of the unit cell structure model for analyzing the dissociation from both ends of thick filaments in myofibrils is confirmed.

Finally, we should mention that myofibrils are easily oriented under the shear of flow, so that it is highly probable that diffraction lines will be obtained from a suspension of myofibrils. The application of the optical diffraction method not only to such a randomly oriented system of myofibrils as presented here but also to an oriented system will open up new possibilities of analyzing the structure and function of skeletal myofibrils.

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