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X-ray diffraction of actively shortening muscle

(equatorial reflections/position-sensitive detector/myofilament lattice/cross-bridges/force–velocity relation)

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ABSTRACT **Low angle x-ray diffraction patterns were obtained from resting and activated frog sartorius muscles by means of a position-sensitive detector. Although the intensity ratio I_{10}/I_{11} decreased many-fold upon activation, it was nearly the same during isometric and isotonic contraction. Thus, motion has a much smaller effect on the low order equatorial pattern than the transition from rest to activity. Analysis of the *10* and *11* reflections separately showed that I_{10} and I_{11} change reciprocally upon activation, and that they both increase by a small amount in the transition from isometric to isotonic contraction. If the intensity ratio can be taken as a measure of cross-bridge number, the results provide evidence that the drop in force in an actively shortening muscle is due primarily to the influence of motion on the configuration, rather than the number, of cross-bridges.**

A distinctive characteristic of actively shortening muscle fibers is the dependence of the contractile force on the shortening velocity (1). At a given sarcomere length, the contractile force is a maximum when the velocity is zero and it decreases as the velocity increases. The detailed mechanism of this effect is not known. However, since contractile force is generated by cross-bridges that form between the myosin-containing and the actin-containing myofilaments, and the speed with which these filaments move past each other is directly related to the shortening velocity, the relation between force and velocity has been attributed to various effects of motion on the properties and the instantaneous number of cross-bridges (see, for example, refs. 2–4).

Information about the configuration of the myosin projections, which are involved in cross-bridge formation, can be obtained by low angle x-ray diffraction. In resting frog sartorius muscle, the intensity of the *10* equatorial reflection, I_{10}, is at least two times greater than the intensity of the *11* reflection, I_{11}. When the cells are activated and the length is held constant (isometric contraction), I_{11} increases relative to I_{10} (5, 6). This has been attributed to the formation of cross-bridges between the two types of myofilaments, by either radial (6) or azimuthal (7) movement of the myosin projections toward the actin filaments. There is a further increase of I_{11} relative to I_{10} in muscles put into rigor, suggesting that the number of cross-bridges in rigor is greater than that in isometric contraction (6, 8).

In the present study we examined the equatorial reflections in muscles shortening at different velocities. The main finding is that the intensity ratio I_{10}/I_{11} for actively shortening muscle is very close to that seen during isometric contraction. This indicates that the average configuration of the myosin projections generating a low force in rapidly shortening muscle is significantly different from that in muscle at rest. In addition, if the scattering of x-rays in the equatorial plane by myosin projections is sensitive mainly to factors that depend on whether a cross-bridge has been formed, it also implies that the number of cross-bridges during isotonic contraction is not closely proportional to the contractile force.

METHODS

A diagram of the experimental arrangement is given in Fig. 1. The experiments were carried out in the stroboscopic mode with a rotating anode x-ray generator (Elliott GX6, 6000 rpm) and a mirror-monochromator focusing camera. The specimen to focus distance was 48 cm.

Position-Sensitive Detector. The equatorial reflections were recorded with an electronic position-sensitive detector. The detector (spatial resolution, 100 μm) was a resistive wire design (9) pressurized to 8 atmospheres (800 kPa) with 90% argon, 10% methane and operated at 3190 V. The processed output of the position-sensitive detector was stored in a pulse height analyzer using a conversion gain of 12.5 channels per mm.

Physiological Aspects. Ten experiments were carried out in May and June with sartorius muscles dissected from *Rana temporaria* frogs. The muscles were activated at regular intervals for periods ranging between 10 and 25 hr. Except where otherwise noted the stimulus consisted of a 1 sec train of 1.25 msec, 10–20 Hz pulses. In the first seven runs (Exps. 4 through 10), the interval between stimuli was 4 min; in the last three, this was reduced to 2 min. The isotonic load was set at values ranging from 0.14 to 0.40 the steady isometric force. Since the steady isometric force fell during the course of a run, the load was decreased from time to time, which kept the relative load, and therefore the velocity, practically constant. The run was terminated when the steady isometric force fell below half the initial value.

Specimen Holder. This was a thermoelectrically cooled, anodized aluminum and acrylic plastic cell containing two double Mylar windows (Fig. 1). The pelvic end of the muscle was attached to a force transducer; the tibial end was attached to a spring-loaded lever which also served as the moving element of a displacement transducer. Sarcomere length was adjusted by passing a helium-neon laser beam through the Mylar windows and observing the optical diffraction pattern. The preparation was activated by passing current pulses between two platinum electrodes near the pelvic end of the muscle. An electromagnetic stop controlled the level release; the amount of shortening was limited to 3 mm by a second stop. The x-ray beam was generally limited to the central region of the muscle by placing a 2 mm wide slit in front of the Mylar window closest to the x-ray source.

The composition of the Ringer solution was (mM): NaCl, 115; KCL, 2.5; $CaCl_2$, 1.8; Na_2HPO_4 + NaH_2PO_4, 3.1 (pH 7.0). Water-saturated oxygen was bubbled through the solution, and the temperature was maintained at about 3°. The level of the solution in the cell was controlled by a motor-

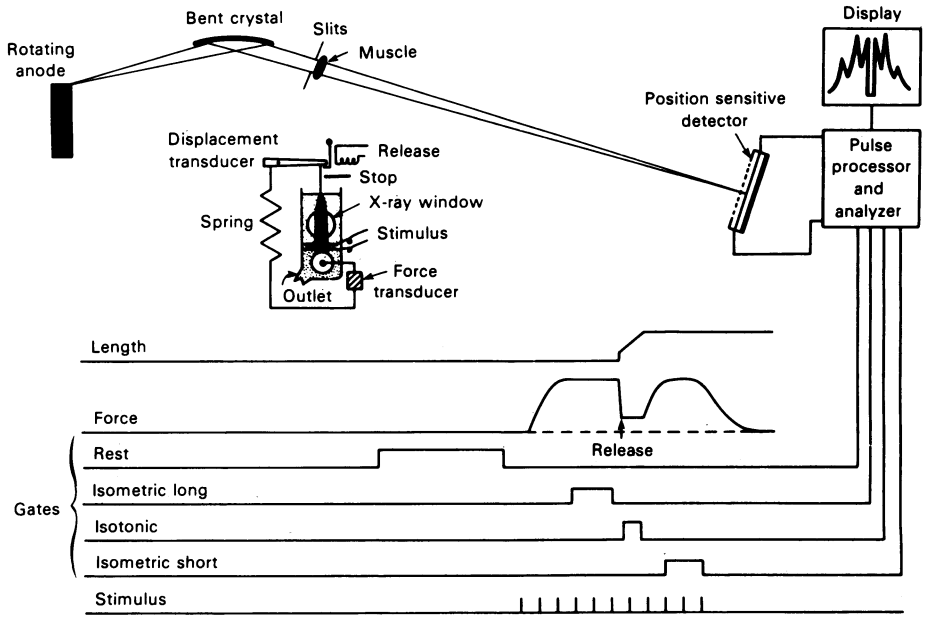

FIG. 1. Diagram of experimental setup. The upper part shows the x-ray source, camera, and the position sensitive detector. The data recorded by the detector are directed to different quarters of the analyzer memory by four gates timed to coincide with four different phases of the physiological activity cycle. The inset in the middle of the figure shows the main features of the specimen holder. Stimulus duration was generally 1 sec.

driven syringe. For most of the time the solution bathed the entire muscle, as indicated in Fig. 1. The level of the solution was brought below the Mylar windows 5–10 sec before the muscle was activated. The total count rate was then 300–700 counts/sec.

Control Gates. The primary timing signal was generated by the muscle stimulator. This signal triggered secondary pulse generators controlling the Ringer solution syringe drive motor, the lever release, and the four analyzer gates. The analyzer gates enabled each quarter of the pulse height analyzer to be addressed successively by the processed output of the position-sensitive detector. The lower part of Fig. 1 shows the gate configuration used in most of the experiments.

Data Analysis. To obtain the intensities of the reflections, the data were smoothed by calculating the 1,2,1 running average (10) and the background was stripped away. Stripping was done by assuming that (*i*) the background under a peak is linear, and (*ii*) the peaks have no "tails." The intensity of a given reflection was taken as the mean value of the peaks on the two sides of the pattern.‡

RESULTS

Fig. 2 shows diffraction data from a typical experiment in which the rest, isometric long, isotonic, and isometric short states were sampled. The isotonic force in this case was 0.15 times the steady isometric value. The total exposures for the four patterns were (clockwise from the upper left) 690, 72, 19, and 86 sec. The spectra were normalized according to the amplitude of the central peak in each pattern, which is the main beam attenuated 10^6 times.

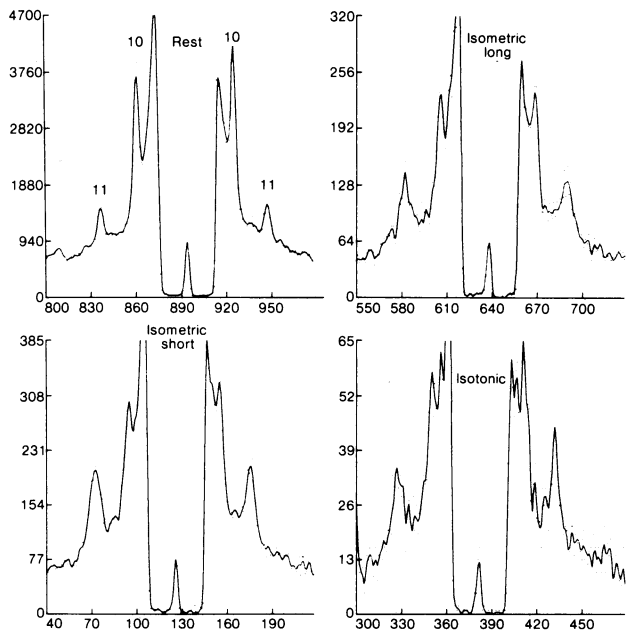

FIG. 2. Diffraction patterns from different phases of the activity cycle. The numbers on the abscissa give the analyzer channel; the ordinates are the total counts in each channel. The small dots are the data points; the curves are the 1,2,1 running averages. Gate duration (msec): rest, 2000; isometric long, 210; isotonic, 53; isometric short, 240. Exp. 10, June 9, 1975.

‡ The accuracy with which the intensity of a reflection could be measured was limited at the outset by the total number of counts in the stripped peak. A "worst case" example of this is the isotonic pattern in Fig. 2, which contains the smallest number of counts of all the patterns in the present study. In this pattern the *10* and *11* reflections on each side of the central peak contain about 125 and 250 counts, respectively, so that the uncertainty in the mean intensity ratio is about 8%. When the data were normalized according to the intensity of the central peak, the counting error in this peak also figured in the uncertainty. Taking the data in Fig. 2 as an example again, the isotonic central peak contained 44 counts above background, while the isometric long and short central peaks contained 246 and 215 counts, respectively, so that the expected uncertainty in the normalization factor is 15% for the isotonic pattern and about 7% for the two isometric patterns. The corresponding counts in the other experiments were greater than this by as much as a factor of 10, which reduced these uncertainties 3-fold.

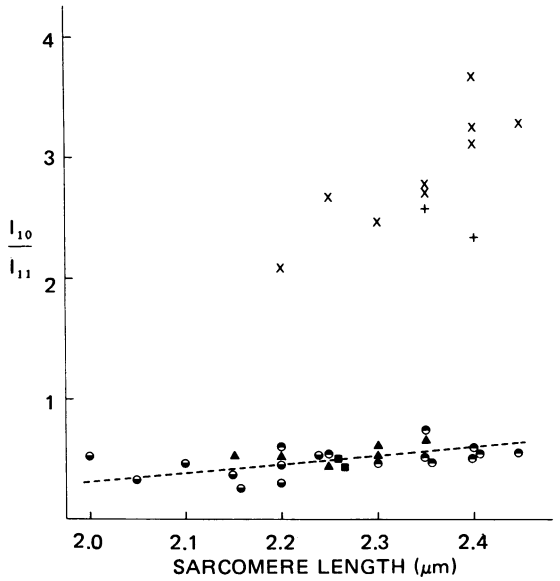

FIG. 3. Intensity ratio I_{10}/I_{11} as a function of sarcomere length. Symbols: ×, rest before activation; +, rest after activation; ⊖, isometric long; ⊖, isometric short; ▲, isotonic, relative load 0.25–0.40; ■, isotonic, relative load 0.14–0.19. The line was fitted to the isometric long and isometric short points by the method of least squares.

Isometric Contraction. The resting pattern shows strong *10* and weaker *11* reflections. Upon activation, the normalized intensity of the *10* reflection decreased and that of the *11* reflection increased.[§] The isometric pattern at the short length is similar to that at the long length, except that I_{10} is somewhat weaker, and I_{11} is a little stronger, relative to the central peak. This is consistent with previous reports that the ratio I_{10}/I_{11} decreases when the sarcomere length decreases (8, 11). Some of the patterns also contain a very weak peak between the *10* and the *11* reflections, which may be a reflection from the Z disc (5, 12).

The spacing between reflections was smaller by 3–5% in the isometric short pattern than in the isometric long. This would be expected if the distance between the lattice filaments increased when the muscle shortened (5, 11, 13).

Isotonic Contraction. The isotonic pattern was noisier than that for the two isometric states because the exposure time was about four times shorter. However, the number of counts was sufficient to define the diffraction pattern, since the left and right sides of the intensity distribution are essentially the same.

The spacings between the reflections and the intensity ratio, I_{10}/I_{11}, are both bracketed by the values found for the two isometric patterns. The normalized intensities of both reflections appear to be stronger than in two isometric states; this was not a consistent finding, however, and a possible explanation for the variability will be discussed below.

Fig. 3 shows the intensity ratios for the four states shown in Fig. 2 and similar data from nine other experiments, plotted as a function of sarcomere length. The crosses are for resting muscle, the circles for isometric contractions, and the triangles and squares for isotonic contractions.[¶] Within the limits of error, the data for various activated states appear to fall within the same group.

Table 1. Absolute intensity of the central peak in the diffraction patterns for different physiological states

Exps.	Isometric long	Isotonic	Isometric short
4	1.00	1.02 (0.13)	0.94 (0.08)
5	1.00	1.27 (0.07)	0.95 (0.04)
6	1.00	1.16 (0.14)	0.79 (0.06)
7	1.00	0.97 (0.07)	0.77 (0.03)
8	1.00	0.97 (0.09)	1.01 (0.05)
9	1.00	0.73 (0.12)	0.92 (0.06)
10	1.00	0.79 (0.12)	0.96 (0.06)
13	1.00	0.98 (0.06)	1.05 (0.03)

The absolute intensity of the central peak is taken as N, the counts above background in this peak, divided by the sampling time. The quotient is normalized to the value for the isometric long state. The statistical uncertainty, given in parentheses, is equal to the normalized absolute intensity divided by $\sqrt{N}$.

Changes in Muscle Shape During the Contraction Cycle. Normalizing a given reflection relative to the central peak in the diffraction pattern makes it possible, in principle, to obtain information about the filament lattice in different physiological states. However, the normalized intensity also depends on the amount of the muscle in the beam path, which may change when the muscle is activated, and necessarily changes when shortening occurs.

In the present study I^*, the absolute intensity of the central peak in the diffraction pattern, was taken as a measure of the amount of muscle in the beam path. I^* is the total counts above background in the central peak divided by the sampling time for that pattern.

Table 1 gives the values of I^* for the experiments in which isometric long, isotonic, and isometric short patterns were recorded. Since the muscle shortened 10% of its length, if shortening were uniform the amount of muscle in the beam path would have increased 5% and I^* for the isometric short state should have been 5% less than I^* for the isometric long. This turned out to be the case in most of the runs (Table 1, final column), although muscle shape seemed to change in three experiments (Exps. 6, 7, and 13).

The value of I^* for the isotonic state was expected to be equal to the average of the two isometric states. This was the case, within experimental error, in half the runs (Exps. 4, 7, 8, and 13). In the remaining runs the difference between I^*_{isotonic} and $\frac{1}{2}(I^*_{\text{isometric long}} + I^*_{\text{isometric short}})$ indicated that the muscle probably twisted while it shortened, bringing more (Exps. 9 and 10) or less (Exps. 5 and 6) mass into the beam path during isotonic contraction.

Changes in the Equatorial Reflections During Isotonic Contraction. Although the data in Fig. 3 indicate that the intensity ratios, I_{10}/I_{11}, for the isometric and isotonic states are close to each other, analysis of the normalized intensities of the individual reflections in the two states revealed

[§] This was also the case in three other experiments where the absolute intensity of the central peak in the resting and the isometric long pattern was nearly the same. The average value of $I_{\text{rest}}/I_{\text{isometric long}}$ for the four runs was 2.0 for the *10* reflection and 0.4 for the *11* reflection.

[¶] Each point is the mean of the intensity ratio for the left and right sides of a diffraction pattern. The average deviation of the ratios from the mean was 11% for the rest pattern, 18% for the isometric long, 15% for the isometric short, and 20% for the isotonic. In two experiments the isotonic pattern was very asymmetric and the intensity ratios from the two sides differed by a factor of 4; these points were omitted from Fig. 3.

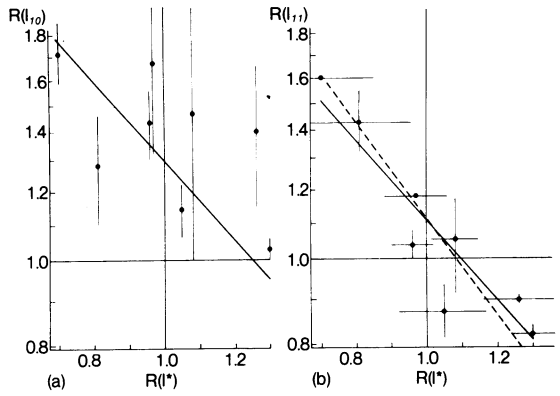

FIG. 4. Intensity of the equatorial reflections during isotonic contraction relative to average isometric state. $R(I^*) = I^*_{\text{isotonic}}/(1/2)(I^*_{\text{isometric long}} + I^*_{\text{isometric short}})$, where I^* is the counts above background in the central peak divided by the sampling time. $R(I_{10})$ and $R(I_{11})$ are corresponding ratios for the normalized values of I_{10} and I_{11}. Ordinates were found by averaging the ratios obtained from the two sides of the diffraction patterns; the ordinate error bars show the range of the two ratios divided by $\sqrt{2}$. The abscissae were calculated from Table 1; the abscissa error bars, which were omitted from (a) for clarity, give the counting error in the central peak of the isotonic diffraction pattern. The solid line in (b) is the least squares fit assuming the error is in $R(I_{11})$; the broken line is the fit assuming the error is in $R(I^*)$; the $R(I_{11})$ intercept is the same in both cases. The solid line in (a) has the same slope as that in (b), but it was displaced vertically to fit the data.

measurable differences. This is shown in Fig. 4. In both panels the abscissa is $R(I^*) = I^*_{\text{isotonic}}/(\frac{1}{2})(I^*_{\text{isometric long}} + I^*_{\text{isometric short}})$; according to the argument in the previous paragraph, this ratio is a measure of the muscle mass in the beam path during isotonic contraction, relative to that for isometric contraction. The ordinates are the corresponding ratios (plotted logarithmically) for the normalized values of I_{10} and I_{11}; these ratios, $R(I_{10})$ and $R(I_{11})$, give the intensity of each reflection in the isotonic state, relative to that for the average isometric state.

The data in Fig. 4b show a good correlation between $R(I_{11})$ and $R(I^*)$, indicating that changes in muscle shape during shortening significantly affect I_{11} in the isotonic state. In the absence of a shape change [$R(I^*) = 1.0$] the value of $R(I_{11})$ is close to 1; the intrinsic change in I_{11} in the transition from isometric to isotonic contraction appears to be about 10%.

Fig. 4a is a similar plot for the normalized *10* reflections. Although the uncertainties in the measurements are large, the ordinates of the data points are all greater than 1, indicating that I_{10} is clearly greater in isotonic than in isometric contraction. The line in Fig. 4a has the same slope as the solid line in Fig. 4b, but it was shifted vertically to fit to the data. The $R(I_{10})$ intercept is about 1.3, which indicates that, in the absence of a shape change, I_{10} for isotonic contraction increases relative to the isometric contraction by about 30%.

The observation that the intrinsic increase in I_{10} is greater than that for I_{11} implies that the intensity ratio I_{10}/I_{11} for the isotonic state is greater than that for the isometric state. The increase in the intensity ratio appears to be 10–20%.

Recovery of the Resting Pattern Following Activation. Fig. 5 shows an experiment in which the diffraction pattern was sampled both before and after an activity cycle. The duration of the stimulus train in this case was 0.6 sec. The analyzer gates for resting before activation (not shown), isometric long (top trace), and isotonic (second from top trace) were triggered in the usual way. After the stimulus ended and the lever extended the muscle to its initial length, a "rest after" gate was triggered (third from top trace). The intensity ratio I_{10}/I_{11} in the pattern sampled by this gate was 2.3 ± 0.2, which is almost back to the value of the intensity ratio for the rest before activation (Fig. 3). Therefore, in this type of experiment the rest pattern returns soon after the contractile force falls to zero.

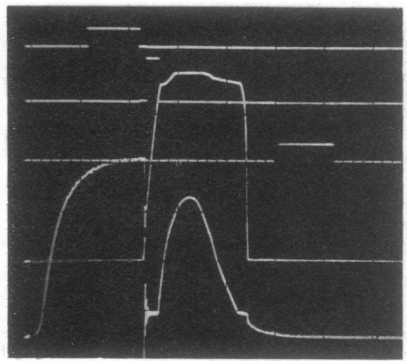

FIG. 5. Muscle contraction and analyzer gates in experiment showing quick recovery of the resting diffraction pattern. Traces, from top to bottom: isometric long gate, isotonic gate, rest after activation gate, muscle length, force. The rest before activation pattern was sampled just before the sweep was triggered. Duration of sweep, 1.5 sec; steady isometric force, 50 g weight (0.49 N); total displacement, 3.0 mm; muscle weight, 70 mg. Exp. 12, June 13, 1975.

DISCUSSION

Diffraction patterns for the different physiological states were analyzed in several ways. In one, the conventional method, the intensity ratio I_{10}/I_{11} was measured. This parameter has the advantage of being independent of beam intensity and relatively insensitive to small changes in muscle shape and orientation; the main disadvantage is that information about the individual reflections is lost. The analysis showed that (*i*) I_{10}/I_{11} increased 400–500% in the transition from isometric contraction to the resting state, and (*ii*) I_{10}/I_{11} changed very little on this scale in the transition from isometric contraction to isotonic contraction at relatively low loads (Fig. 3).

The second analysis, a new procedure, dealt with the reflections separately, which is a more sensitive procedure for detecting small changes in intensity. It showed that in the isometric to isotonic transition I_{10} definitely increased (Fig. 4a) while I_{11} probably increased slightly (Fig. 4b). We estimate from these data that the ratio I_{10}/I_{11} increased by about 10–20%. This is consistent with the direct intensity ratio analysis, since in Fig. 3 the isotonic points tend to lie above, rather than below, the line fitted to the isometric points.

Interpretation of the Results. As regards the question of how motion affects the number of cross-bridges in a sarcomere, the answer from the present experiments depends on the relation between the intensity of the *10* and the *11* reflections and the cross-bridge number, which is not yet known. However, it seems reasonable to assume that the intensity ratio I_{10}/I_{11} is sensitive mainly to cross-bridge number, as the intensity ratio for the isometric state is very different from, and intermediate between, that for muscle at rest and in rigor, states which presumably represent ex-

tremes in the range of possible cross-bridge number (6). If this assumption is correct,[‖] the data in Fig. 3 indicate that during rapid steady motion, where the contractile force is considerably less than the steady isometric value, the cross-bridge number remains close to that present during isometric contraction. In this case, the decrease in force associated with motion must be largely an effect arising from a decrease in the average force developed by a cross-bridge during its activity cycle. This result is consistent with the idea that cross-bridges can generate negative as well as positive force, and that the number of negative-force cross-bridges (or possibly the amount of force per negative-force cross-bridge) increases during shortening. It should be pointed out in this connection that quick release experiments also provide evidence that cross-bridges can exert negative forces (16).

Another reason for thinking that the intensity ratio I_{10}/I_{11} is a measure of cross-bridge number is that, when muscle is activated and isometric force is developed, the intensity ratio changes through a reciprocal change in I_{10} and I_{11}; that is, the change in intensity of the *10* reflection is balanced by a nearly equal but opposite change in the *11* (6). [This behavior is clear in the present study, when I_{10} and I_{11} for the same muscle in the two states are normalized with respect to the central peak in the diffraction pattern (Fig. 2)]. The reciprocal nature of the individual intensity changes indicates that the formation of cross-bridges, at least in the transition from rest to isometric contraction, is associated with gross movement of mass from one reflection plane to another, and it is reasonable to suppose that similar movements for other changes in physiological state will cause similar intensity changes.

With this argument in mind, it is significant that the small intensity changes that accompany the transition from isometric to isotonic contraction do not appear to follow a reciprocal relation (Fig. 4), as this suggests that these intensity changes are not caused by simple mass movements between reflection planes. Rather, these intensity changes probably reflect differences in the distribution of cross-bridge configurations in the two states. This possibility is attractive because an influence of motion on cross-bridge configuration is an essential feature of many current models of the contraction mechanism (2–4). The extent to which the configurational changes are accompanied by a change in cross-bridge number is impossible to say on the basis of the present data, but, as already noted, the fact that the change in intensity ratio is small relative to the change that occurs when the muscle is activated suggests that the change in cross-bridge number due to motion is likely to be small.

This conclusion runs counter to explanations of the force–velocity relation in which the decrease in force with motion is attributed mainly to a nearly proportional decrease in the total number of force generators, as is the situation, for example, in the phenomenological model described by A. V. Hill (1), and the early cross-bridge model put forward by A. F. Huxley (2). It appears to be more consistent with a model in which force decreases with velocity primarily because an increasing number of negative-force generators balances out the effect of a slowly decreasing number of positive-force generators, since in this case the total number of force generators is relatively insensitive to velocity (3).

We are grateful to Charles Crist of the Laboratory of Physical Biology for constructing the specimen holder used in this study.

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[‖] By scaling an estimate of the change in cross-bridge number according to the difference in the intensity ratio for the resting and the isometric state, we imply that essentially all the detached myosin projections in activated muscle are in the resting configuration. It is possible, however, that the many-fold change in the intensity ratio in the transition from rest to the isometric state is a direct effect of calcium on the myosin projections (14) rather than an effect of cross-bridge formation *per se*. The recent report (15) that calcium does not affect the polarization of the fluorescence emitted by a dye linked to the S1 subfragment of myosin in glycerinated psoas muscle fibers unless the myosin-containing and actin-containing myofilaments overlap can be taken as evidence against a direct calcium effect. Another possible complication is that, after a cross-bridge is broken, the time taken for the myosin projection to relax (that is, to return to the configuration that produces a large I_{10}/I_{11} ratio) is long compared to the interval between successive reactions of the projection with the actin-containing filament. Although this possibility cannot be completely excluded, it too appears unlikely, because the intensity ratio was found to return to the resting value soon (within 100 msec) after the force in an activated muscle fell to zero (Figs. 3 and 5).