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X-ray diffraction of strained muscle fibers in rigor

(equatorial reflections/cross-bridges/filament order/contraction mechanism)

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ABSTRACT **The effect of strain on the equatorial x-ray diffraction pattern of glycerinated rabbit psoas fibers was studied in the rigor (ATP free) state. Strains between 30 and 100 Å per half sarcomere, measured directly by laser diffraction, did not change the intensity ratio, $_{(10)}/_{(11)}$. Because the intensity ratio depends on the distribution of mass within the myofilament lattice, the negative result indicates that strain does not change the angle of attachment of the subfragment 1 (S1) moiety of the myosin molecule to the actin filament. The effect of strain on the ordering of the actin filaments also was considered and judged to be negligible.**

Although there is considerable evidence that the formation of actomyosin cross-bridges is an essential feature of the contraction mechanism of vertebrate muscle cells, the detailed molecular changes that take place when force is generated are not known. A widely accepted mechanism is based on a hypothetical ability of the subfragment 1 (S1) moiety of the myosin molecule to "rock" from a 90° to a 45° orientation on the actin filament (1, 2). According to this idea, S1 initially interacts with the actin filament in a nearly 90° configuration; this step is followed by a transition to a more stable 45° configuration, which tends to move the distal end of S1 away from the Z line and generate force in the subfragment 2 (S2) moiety of the molecule.

There are several experimental implications of this force-generating mechanism. For example, if the distribution of cross-bridges between the two configurations depends on the mechanical force in the muscle fiber (3), it would be expected that the cross-bridge could be rotated back from the 45° configuration by application of an external force to the fiber. The same effect also should be seen if a rocking motion were the major source of compliance in the sarcomere (4). Because the transition from 45° to 90° would be expected to significantly change the equatorial x-ray diffraction pattern of the fiber (5), it is of interest that such changes were not detected when steady forces were applied to both frog and rabbit fibers in the rigor state (6).

As will be noted later in the present paper, there are a number of ways in which this unexpected result can be explained. However, several technical questions regarding the observation also can be raised. For example, because the length of S1 is 120 Å (7), the expected reach of the cross-bridge in a 90°–45° transition is ≈100 Å. Thus, sarcomere strains of 1% or less are of interest. These small changes were not measured during the experiment, and a possible explanation of the negative result is that such strains were not present in the observed region of the muscle.

Another question regarding the negative result is whether the applied force affects the ordering of the thin filaments in the A band. An increase in the ordering would decrease the intensity ratio $I_{(10)}/I_{(11)}$ of the first two measured reflections by increasing the actin contribution to the scattering (8). Because rocking the cross-bridge from 45° to 90° would be expected to increase this ratio, the observed result could come about if the two effects fortuitously balanced each other.

The present study was made with these questions in mind. We used a narrow x-ray beam to probe a small region of a glycerin-treated fiber bundle in rigor. A laser beam also was passed through the region of interest, and sarcomere length was measured electronically. Sarcomere strains of 0.3% and 1% were applied for 600 msec, and the equatorial x-ray diffraction pattern before, during, and after strain was built up by repeating the strain cycle many times. We found that strain had no effect on the intensity ratio, which is consistent with the results of the related experiments of Haselgrove (6). In addition, several methods were used to estimate the effect of strain on the ordering of the thin filaments, and we concluded that this also has a negligible effect on the diffraction pattern.

METHODS

Specimen Preparation. Glycerinated rabbit psoas muscle was used in all of the experiments. The glycerinating solution was 1:1 (vol/vol) in glycerin and a rigor solution containing 100 mM KCl, 2 mM $MgCl_2$, 4 mM EGTA, 7 mM KH_2PO_4, and 13 mM K_2HPO_4 (pH 7.0). Strips of muscle (≈4 mm diameter) were stored in this solution for 24 hr at 4°C, with the solution changed every 8 hr, and then were transferred to a freezer at −20°C for at least 3 wk before use (9).

After equilibration first in a 25% (vol/vol) glycerin solution and then in the rigor salt solution, a small specimen (200–350 μm in diameter and 9 mm in length) was dissected under the microscope. The fiber diameter was measured to within 25 μm, and the fiber was assumed to be circular in calculating its cross section. The specimen was mounted in a Plexiglas chamber between two Mylar windows positioned ≈400 μm apart (Fig. 1 *Inset*). At one end the bundle was tied with 6-0 surgical silk to a force transducer with a compliance of 0.2 μm/mg. The response time of the force transducer was 30 msec because the output was filtered to reduce high frequency noise. The other end of the bundle was tied to a relay mechanism that applied small length changes to the specimen. The amplitude of these changes could be varied by manual adjustment of a screw stop.

X-Ray Diffraction. An Elliott GX13 generator operating at 2.5 kW was used as the x-ray source, and a camera with a single Franks mirror produced a line focus approximately 1.5 mm high at the specimen (Fig. 1). A position-sensitive gas flow detector was used with 90% argon/10% methane at a pressure of 7.0 kg/cm^2 and with an anode voltage of 3 kV (10). The specimen-to-detector distance was 75 cm, and a semitransparent (transmit-

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Abbreviations: S1, myosin subfragment 1; S2, myosin subfragment 2.

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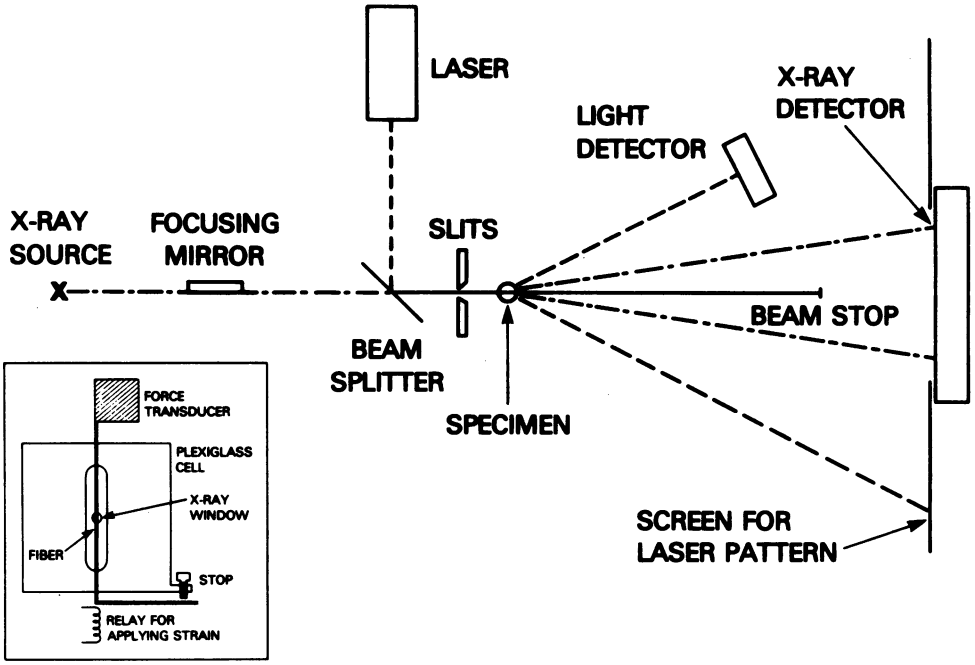

FIG. 1. Diagram of the experimental setup. It shows the x-ray source, camera, x-ray position-sensitive detector, and the laser system for measuring small changes in sarcomere length. The output of the x-ray detector was computer controlled and directed to two different memories according to the strain state of the preparation. (*Inset*) Main features of the specimen holder.

tance $\simeq 10^{-6}$) backstop was positioned at ≈25 cm from the detector. The x-ray path was filled with helium to reduce absorption and scatter. The output of the detector was fed into a computer-controlled multichannel analyzer. The total exposure time was typically 200 sec; the long exposure time was taken to reduce the statistical noise. A typical pattern is shown in Fig. 2.

Each half of the diffraction pattern was analyzed separately with a least squares technique. The pattern was first smoothed with a five-point Gaussian routine, and then the (1,0), (1,1), and (2,0) peaks and usually the Z line were fitted to a Gaussian equation, and the background was fitted to a fifth-order polynomial. This gives the intensities and the lattice spacing. In some experiments an experimental background was obtained with only solution in the beam path. Subtracting this background from the patterns made the patterns visually much clearer, but the computer fit remained unchanged.

The intensity ratio $I_{(10)}/I_{(11)}$ was measured to better than 10%, based on the variability of repeated exposure of the same specimen. Variability between specimens was somewhat larger than this.

Sarcomere Length Determination. A light-diffraction arrangement was added to the x-ray camera to measure the length change applied by the relay that was transmitted to the sarcomeres. A 4-mW He/Ne laser was used, and the beam was reflected into the x-ray path with an x-ray-transparent pellicle beam splitter (Melles Griot, Irvine, CA). Coincidence with the x-ray path was accomplished by aiming the laser to pass between the collimating slits and to hit the narrow backstop. The slits reduced the light source to approximately the same size as that of the x-ray source, so they both sampled the same region of the muscle.

The first order line was focused upon a position-sensitive light detector (UDT-SC-10, United Detector Technology, Culver City, CA) by means of a cylindrical lens. A signal proportional to $(A - B)/(A + B)$, in which A and B are the outputs from the two ends of the detector, was recorded on a chart recorder. The device measures the centroid of the incident beam, and the output depends on the background light, including that scattered from the preparation. The detector was calibrated for each experiment by a known movement of the detector.

The detector output was slightly sensitive to intensity and position. Stepping down the intensity of the laser by 50% produced a signal of <10 Å per half sarcomere. Because in the experimental maneuvers the intensity did not vary by more than 25%, this effect was ignored.

For strains larger than 100 Å, two difficulties were encountered. One was that the laser pattern did not return to its original position after the strain. Close examination under the microscope showed that the slack length of the sample increased with very large stretches because of slippage at the knots tying the ends of the fiber. There also could have been internal disordering or yielding of the fiber, or both (e.g., see refs. 11 and 12). A second difficulty was that the apparent sarcomere length change often did not scale with the end displacement or the measured force. However, these problems very rarely arose for small strains (30 Å per half sarcomere); larger strains of 75–100 Å were more difficult but were possible occasionally. The data from experiments having these problems were discarded.

Experimental Procedure. All experiments were done at room temperature (25°C) unless otherwise noted. After the fiber was mounted, it was placed in the x-ray camera, and the transducer was adjusted to take up the slack in the muscle. This was monitored by allowing a small tension (<20 mg for a 200-μm bundle) and a sarcomere length increase of less than 10 Å per half sarcomere. The stop on the relay mechanism was then adjusted to give the required stretch.

At the beginning of the experiment, an x-ray exposure of the unstretched state was taken as a control. The experiment was then carried out under computer control applying the stretch for 0.6 sec and resting for 10 sec before repeating the procedure. The x-ray pattern was collected for the last 0.5 sec of the stretched state (allowing 0.1 sec for the muscle to produce a steady force). The force and sarcomere-length change remained fairly constant during this time interval (Fig. 3). A no-strain control x-ray pattern for 0.5 sec was collected just prior to applying the strain. The whole cycle was repeated several hundred times to build up the x-ray exposures, and then, at the end of the experiment, another x-ray pattern in the zero strain state was taken as a control.

Experiments in which the intensity ratio $I_{(10)}/I_{(11)}$ in the three control x-ray patterns (before, during, and after the cycling) did not agree to within 15% were discarded. The strained force level sometimes dropped slightly during the course of an experiment.

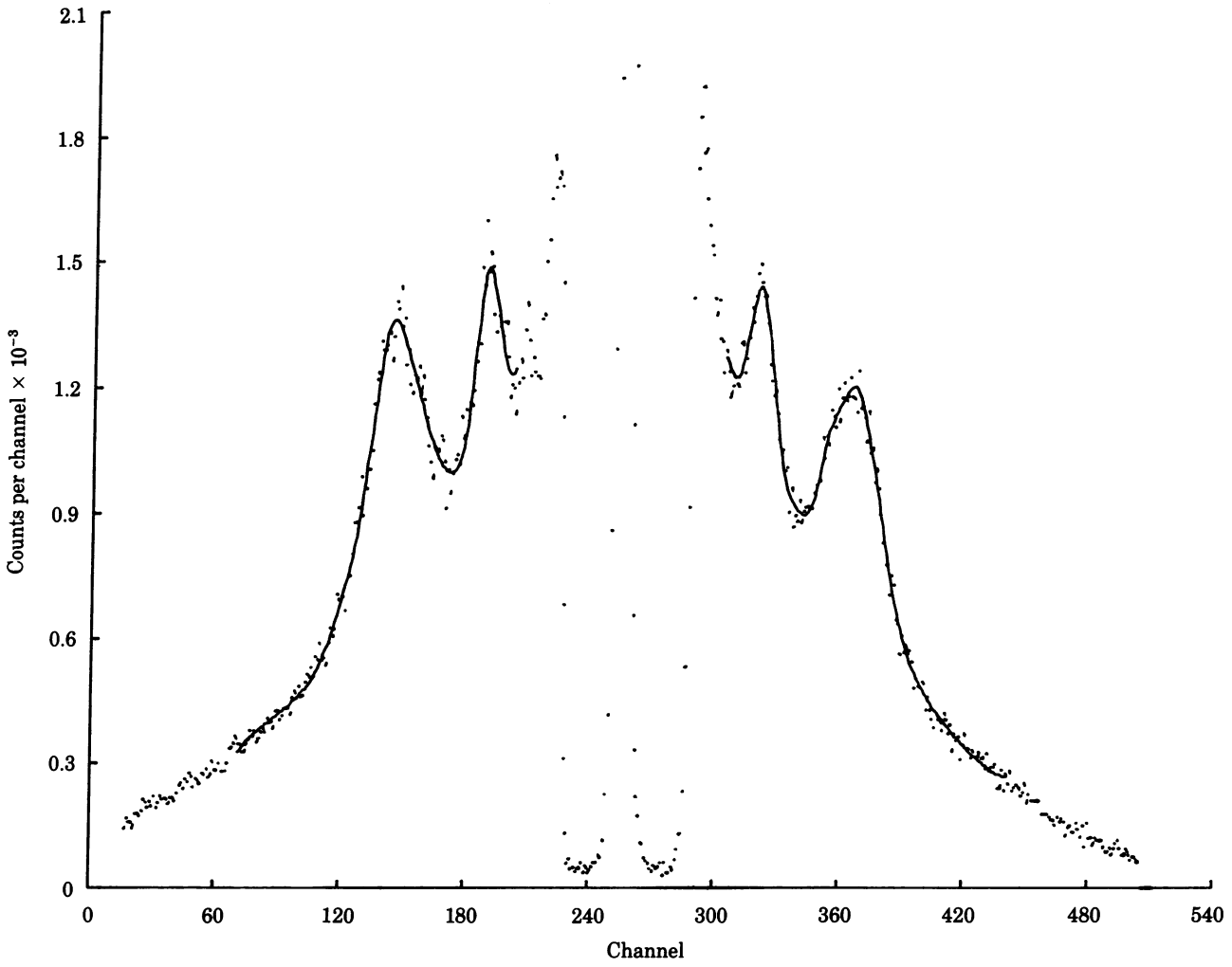

FIG. 2. A typical x-ray equatorial diffraction pattern for fiber preparation in rigor. ·····, Experimental data; ——, fitted curve with the assumption of Gaussian peaks at the (10), Z line, (11), and (20) positions and a fifth order polynomial as background. The specimen–detector distance was 75 cm, and the conversion gain was 25 channels per mm. The sarcomere length was 2.6 μm, and the accumulated exposure time was 200 sec.

This effect was small in the stretches of <50 Å per half sarcomere, but in the larger stretches the force level sometimes dropped to ≈60% of its initial value by the end of the experiment.

In earlier experiments the windows on the x-ray cell were further apart (5 mm) to avoid possible rubbing of the fiber on the windows. The solution was removed from the cell during the x-ray exposure in this instance and returned to the cell for the rest period of the cycle. No difference in the results was detected with this procedure.

RESULTS

Results of a typical experiment in which strain was applied to the rigor muscle are seen in Figs. 2 and 3. Fig. 2 shows a typical x-ray exposure for 200 sec and also gives the computer fit to the peaks. Fig. 3 shows the output from the force transducer and the light detector on applying a length change at the end of the fiber. The strain of 35 Å per half sarcomere seen by the light detector is uniform for the duration of the stretch, and the force level drops by less than 10%.

Two different strain levels of approximately 30 Å and 100 Å were studied. For the larger strain, the force level and applied strain seemed to scale (Fig. 4). The maximum strain (100 Å per half sarcomere) produced a force of about 700 gm/cm^2. The lattice spacing measured to 1% accuracy was independent of the applied strain.

Table 1 summarizes the measured intensity ratio and its changes. For the smaller strains, the effect of sarcomere length also was investigated. In all cases, no measurable change in $I_{(10)}/I_{(11)}$ was detected in the strained state. There was no apparent change in the strength of the diffraction pattern in the strained and unstrained states under our conditions. As a control, similar experiments were carried out with three preparations in relaxing solution. These also showed no effect of strain on $I_{(10)}/I_{(11)}$.

$I_{(10)}/I_{(11)}$ decreased by 50% between pH 7.0 and 8.5, making $I_{(10)}/I_{(11)}$ less accurate as the $I_{(10)}$ became very small. In this experiment the 15% change in $I_{(10)}/I_{(11)}$ observed during the strain was not statistically significant. Lattice spacing changed from 368 to 410 Å on going from pH 7.0 to 8.5. Similar changes were found by Rome (13).

Contribution of Thin-Filament Flexibility to $I_{(10)}/I_{(11)}$. First, we undertook some modeling studies based on the intrinsic flexibility of the thin filament measured in the *I* band (14, 15). In these studies we approximated the filaments as cylinders with uniform electron densities and calculated the expected intensity ratio $I_{(10)}/I_{(11)}$. A diameter of 170 Å for the thick filament and 70 Å for the thin filament gave good agreement with the observed intensity ratio. Flexibility or disorder of the thin filament was modeled by smearing out the thin filament mass into a cylinder of larger radius and correspondingly lower density. A mean radius of curvature of ≈5 μm [based on a flexibility λ

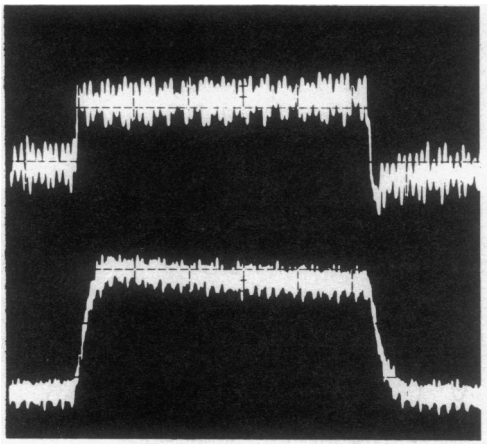

FIG. 3. Strain and force records for a fiber bundle. The upper trace is the output of the laser detector and shows a strain of 35 Å per half sarcomere applied for 500 msec. The lower trace is the output of the force transducer. The force increment was 60 mg.

$= 0.2\ \mu m^{-1}$ (14) and an assumption that the filament forms a simple arc] gives a mean deviation of 20 Å from the trigonal position—i.e., the effective diameter increases from 70 Å to 110 Å. This results in only a 20% effect on $I_{(10)}/I_{(11)}$, which is almost certainly an overestimate because a more complicated bending than a simple arc would decrease the deviation from the original position.

Second, it was noted that the flexibility decreases markedly with temperature (16, 17). We looked at $I_{(10)}/I_{(11)}$ in rigor bundles at 10°C and 20°C. The results are summarized in Table 2. $I_{(10)}/I_{(11)}$ appears to be independent of temperature over this range. This agrees with the data of Lymn (18), who studied more fully the effect of temperature on the x-ray equatorial pattern

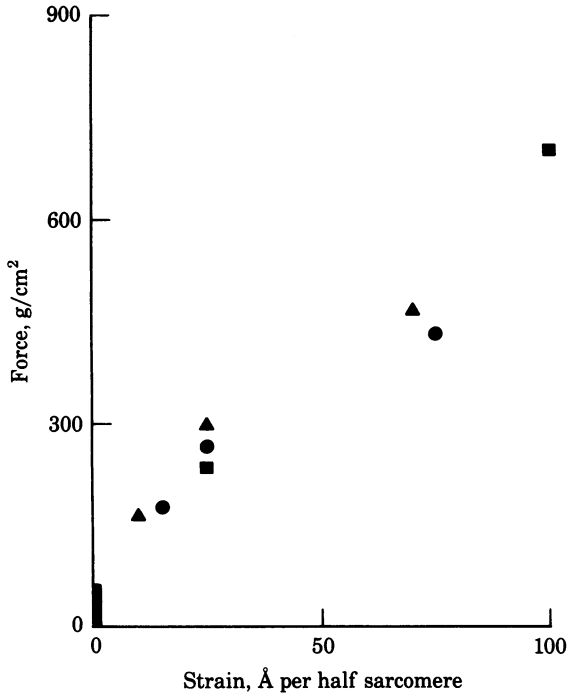

FIG. 4. The relationship between axial force and sarcomere strain. The strain was measured by laser diffraction, and the force was measured at one end of the fiber bundle as shown in Fig. 3. The symbols refer to three experiments with bundle diameters ranging from 200 to 300 μm. The initial force at zero strain, indicated by the vertical bar at the origin, was ≤60 g/cm².

Table 1. The effect of applied strain on the equatorial x-ray diffraction pattern of small bundles of glycerinated rabbit psoas fibers in rigor

Sarcomere length, μm	pH	No. of exp.	Strain*	$I_{(10)}/I_{(11)}$ range	$\Delta I_{(10)}/I_{(11)}$†
2.0–2.2	7.0	4	30	0.21–0.48	2 ± 6%
2.4–2.6	7.0	16	30	0.38–1.2	1 ± 4%
2.8–3.0	7.0	6	30	1.3 –2.4	1 ± 3%
2.2–2.6	7.0	3	70–100	0.18–0.44	6 ± 4%
2.3	8.5	1	30	0.25	15%

* Å per half sarcomere.

† This is the average value of the difference between the intensity ratio in the strained and unstrained states for each experiment; results are expressed as mean ± SEM.

(at pH 8), and indicates that reducing the flexibility does not change the intensity ratio.

Thus, it seems likely that the x-ray equatorials are insensitive to disorder of the thin filament arising from their known flexibility.

DISCUSSION

The main result of the present study is that the first two equatorial reflections are insensitive to the applied strain—i.e., both the lattice spacing and the intensity ratio $I_{(10)}/I_{(11)}$ are practically the same before and during applied strains of up to 100 Å per half sarcomere (Table 1). This contrasts with the 3-fold change in $I_{(10)}/I_{(11)}$ expected from a 45° to 90° rotation of S1 attached to the actin filament (5).

To aid in understanding this result, a secondary question of the contribution of thin-filament flexibility to $I_{(10)}/I_{(11)}$ was examined. This is important because an applied strain could increase the ordering of the thin filament and, thereby, change the ratio $I_{(10)}/I_{(11)}$. Two different approaches to this question both lead to the conclusion that $I_{(10)}/I_{(11)}$ is relatively insensitive to the flexibility of the thin filament. One computational approach, which probably overestimates the effect, gave a maximum of 20% change in $I_{(10)}/I_{(11)}$, and the other more direct experimental approach, examining the effect of temperature on the pattern, could detect no change in $I_{(10)}/I_{(11)}$. Thus, this complication in interpretation was ruled out.

The experiment was designed to apply strains at the sarcomere level of 30–100 Å, which is thought to correspond to a substantial fraction of the cross-bridge reach (3, 19). Over our time scale of measurement, the corresponding force level maintained by the muscle was less than the calcium-activated physiological force, giving an apparent stiffness of about one-half that

Table 2. The effect of temperature on the equatorial x-ray diffraction pattern of small bundles of glycerinated rabbit psoas fibers in rigor.

Experiment	Sarcomere length, μm	$I_{(10)}/I_{(11)}$ 20°C	$I_{(10)}/I_{(11)}$ 10°C
18 viii 80	2.8	0.76	0.78
		0.72	—
4 ix 80	2.6	0.85	0.85
		0.78	—
8 ix 80	2.6	0.48	0.43
		0.46	0.50
		0.48	0.50
		0.48	—

The temperature was cycled between 20°C and 10°C. The intensity ratio $I_{(10)}/I_{(11)}$ was unaffected by this temperature change.

of an active fiber. This is reasonable if the instantaneous stiffness of rigor and Ca^{2+}-activated fibers are nearly the same (20, 21) and if there is a fast force transient in the rigor preparation (21) that is not seen by the relatively slow force transducer used in the present experiments. It would have been interesting to extend the present study to larger strains and forces. However, this was not possible with the present setup because in such cases (*i*) the laser pattern did not return to its original unstrained position (*ii*) force and displacement no longer scaled. Apparently our experimental protocol placed requirements on the stability of the attachment system and the homogeneity of the specimen that were not satisfied during such large displacements.

Relation to Other Results. The absence of any observable change in $I_{(10)}/I_{(11)}$ is consistent with other similar experiments. Haselgrove (6) saw no change in the intensity ratio when large static loads were added to rigor frog muscle and glycerinated rabbit psoas bundles.‡ However, as mentioned above, the extent to which the loaded sarcomeres in his preparations were strained was not measured in his study, and the reversibility of the strain was also uncertain. Haselgrove (6) also observed that the pattern was considerably weaker in the muscle under tension, which was not the case in our experiments. The difference in results might be due to the increased disorder caused by the larger forces in the former study.

Yagi and Matsubara (22) stretched frog muscle during isometric tetanus and found no change in the x-ray intensity ratio in two 500-ms time cuts after the end of the stretch. Amemiya *et al.* (23), using 10-msec time cuts, found no change in the intensity ratio after a quick release. These experiments are different from ours in that the cross-bridges are cycling and presumably can detach and reattach in the sampling interval after the length change. More recently, however, Huxley *et al.* (24) made quick-release and quick-stretch experiments with 1-msec time resolution. They also found little or no change in the equatorial pattern after the change in length (although the 143-Å meridional reflection showed large changes in intensity). These experiments are comparable to ours because it is unlikely that cross-bridges in activated muscle attach or detach to a significant extent in 1 msec. However, the biochemical states are different because the cross-bridges in activated muscle contain ATP or its hydrolysis products, or both, whereas in our case only one biochemical state (actomyosin) is present.

The observed change in lattice spacing of $<1\%$ is consistent with the calculations of Schoenberg (25) relating radial and axial stiffness. These predict a lattice-spacing change of approximately 0.5% because of the applied axial force.

The experiment also was performed at pH 8.5, and the result was the same as at pH 7.0. This is not unexpected because at pH 8.5 the S2 moiety may be released from the surface of the myosin filament and introduce additional compliance to the rigor linkage (26).

'It is interesting to note that the present results are in agreement with experiments in which tryptophane fluorescence (27) nd electron paramagnetic resonance spectra of spin labels (28) were used as probes of cross-bridge orientation. In the fluorescence study, cyclic end displacements of 0.5–2% were applied to single rabbit psoas fibers in rigor; in the electron paramagnetic resonance study, a tension of 1 kg/cm^2 was applied. In both cases the resulting strain did not affect the signal of interest.

CONCLUSION

Although the x-ray intensity ratio would be expected to be very sensitive to S1 orientation (5), no such effect was observed on applying strains of up to 100 Å per half sarcomere to the rigor fiber. This indicates that, in the actomyosin state in rigor, the S1 orientation is not very changeable and that the major compliance in the sarcomere in this state originates in a process other than rocking of S1. The compliance could be in S2, a shape change in S1, or in the filament structure elsewhere (ref. 29).

With regard to the contraction mechanism, the inability to reorient S1 with an applied force appears to be at variance with the proposal of Huxley and Simmons (3) that force is generated by the transition of cross-bridges through a series of attached states in thermal equilibrium, which rocks the S1 moiety relative to the actin filament, and that the distribution among these states depends only on the force in the system. However, these authors (29) also pointed out that similar transitions would produce structural changes other than rocking, and it is certainly conceivable that S1 does not rock at all during the cross-bridge cycle. Another possibility that preserves the idea that S1 rocks during the cross-bridge cycle is that this process is strongly coupled to changes in biochemical state (30). Our results then would imply that the orientation of S1 in at least one of these states is very stable and that the compliance originates in an independent structure which, when strained, does not affect the x-ray intensity ratio.

‡ The same result has also been found with stressed insect flight muscle in rigor (R. T. Tregear, personal communication).

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