A COMPARISON OF THE TENSION RESPONSE TO

RAPID LENGTHENING OR SHORTENING STEPS IN

ISOMETRICALLY CONTRACTING FROG SKELETAL MUSCLE

By

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ABSTRACT

Rapid length steps of isometrically contracting single skeletal muscle fibres, or whole muscle, provide a measure of the elastic properties of a structure believed to be an integral part of the myosin cross-bridge. The experiments to be described were designed to compare the elastic properties of this structure when measured with small amplitude (2-6 nm/h-s), rapid (lms or 500 µs duration) lengthening steps versus shortening steps. All the experiments were carried out at $0°-4°C$, using small bundles (2-20 fibres) or single fibres from frog semitendinosus muscle. The preparation was given a rapid stretch or release at various times during the isometric twitch and tetanus. During the initial development of tension in an isometric twitch and tetanus, the stiffness was always seen to be rising faster than the force. During the relaxation phase of the isometric twitch, the stiffness was observed to lag the tension. During the relaxation phase of an isometric tetanus prior to the 'shoulder', the change in stiffness was seen to lag the tension change. Following the shoulder, there was a rapid fall in stiffness which corresponded to a similar decline in tension. In all instances, when stiffness values at a given force, and measured with a rapid release, were compared to those obtained with stretch, rapid lengthening produced a consistently higher stiffness than a shortening step. The difference was most pronounced during the late rising phase of tension, maximum tension, and the early relaxation phase of the twitch or tetanus. Several suggestions are discussed to explain this observed difference in stiffness between stretch and release. These include the possibilities that the instantaneous elasticity of the cross-bridge may be non-linear during stretch, and detachment of cross-bridges occurs during release but not stretch.

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I. INTRODUCTION

The tension produced by a contracting skeletal muscle is thought to be the sum of the tensions produced by a number of identical, independent force generators (Huxley and Simmons, 1971b). The tension generator is the head (subfragment 1 or S-1) of the myosin molecule (figure 1a,b). It is thought to attach to a binding site on the actin filament and undergo a series of rotations. This results in the production of tension and sliding of the actin filaments towards the center of the sarcomere. The tension is transmitted from S-1 to the myosin filament backbone through subfragment 2 (S-2).

It was proposed that cross-bridges behaved as independent force generators because tension was observed to decrease linearly with filament overlap as the muscle is stretched beyond its rest length (Gordon, Huxley and Julian, 1966) (figure 1c). If a sarcomere is stretched beyond its resting length, the amount of overlap between the actin and myosin filaments decreases. Thus the number of cross-bridges which can be formed decreases, and this accounts for the observed decrease in tension.

Similarly, stiffness (Huxley and Simmons, 1971a, Bressler and Clinch, 1974, Ford, Huxley and Simmons, 1981) was shown to decrease proportionately with decreased filament overlap and decreased tension, suggesting it is also a property of the cross-bridges. Stiffness, the tension required to cause a unit change in muscle length, is determined by measuring the tension response of isometrically contracting muscle to small rapid length steps (that is, more rapid than the maximum velocity of shortening of the muscle). A rapid shortening step results in a simultaneous decrease of tension which is believed to be due to unloading of an undamped elastic component in the cross-bridges (Huxley and Simmons,1970a) (see figure 7). This is followed by recovery of the

Figure 1. Summary of muscle mechanics terminology. (a)Illustration of the arrangement of myofilaments in the fundamental contractile unit of the muscle, the sarcomere. The diagram on the left shows maximum filament overlap. The A bands contain the thick (myosin) filaments, covered with projections (the cross-bridges), and overlapping, interdigitating thin (actin) filaments. The I band consists of the thin filaments only. The H zone is the central part of the A band between the ends of the two sets of thin filaments. The M line is due to thickenings in the centre of the thick filaments. The diagram on the right shows decreased filament overlap. (b)Illustration showing a force generator, or cross-bridge, which consists of the S-1 and S-2 portions of a myosin molecule. Curved arrow represents rotation of bound force generator. Arrow on left represents direction of sliding of actin filaments (modified from Huxley and Simmons, 1971b). (c)Relationship between tension generated by isometrically contracting skeletal muscle and sarcomere length. A sarcomere length of 2.0-2.25 μm corresponds to rest length (or maximum filament overlap) (modified from Gordon, Huxley and Julian, 1966). (d)Plots of the relationship between the instantaneous change in tension which occurs when an isometrically contracting muscle is given a quick release or stretch (T1) versus the amplitude of the applied length change (in nm per half sarcomere), at maximum filament overlap (solid line) and at reduced filament overlap (broken line). Tension is plotted relative to the maximum isometric tension at maximum overlap in both cases (modified from Huxley and Simmons, 1971a).

a

H-zone

Z line   Z line

—I-band—|←——A-band——→|←—I-band—

M

maximum filament overlap

H-zone

M

decreased filament overlap

b

Myosin filament backbone

S-2

S-1

binding site

Actin filament

c

Relative Tension

Sarcomere spacing (μm)

d

Relative Tension

Amplitude of length change (nm/h-s)
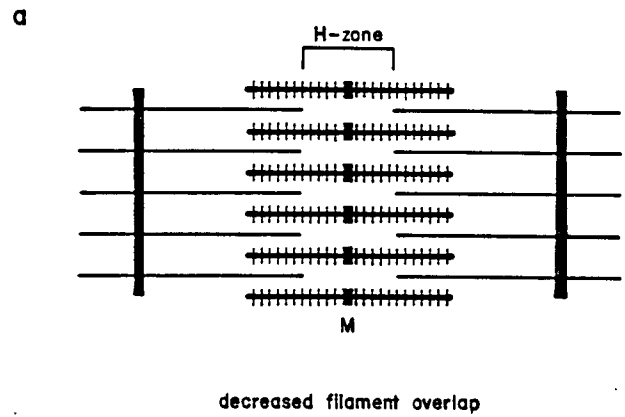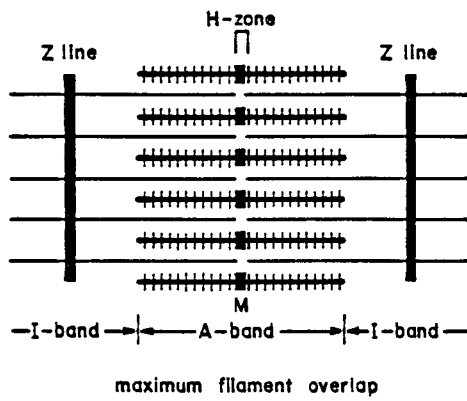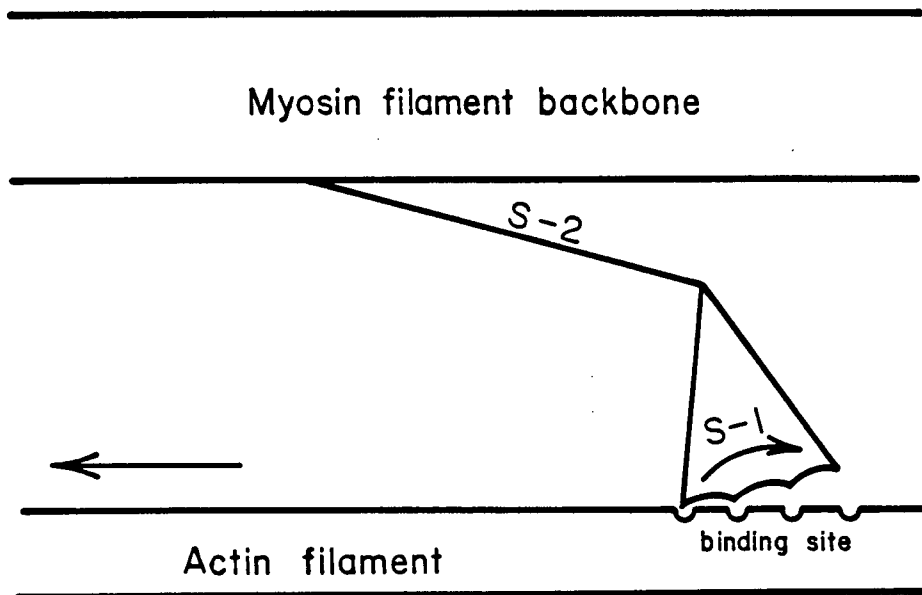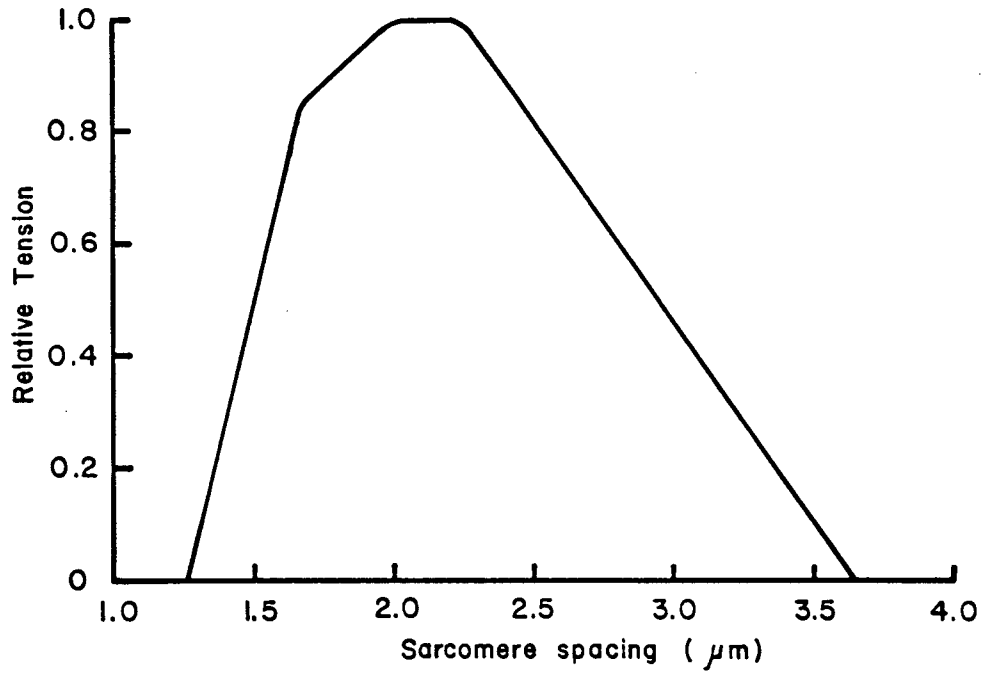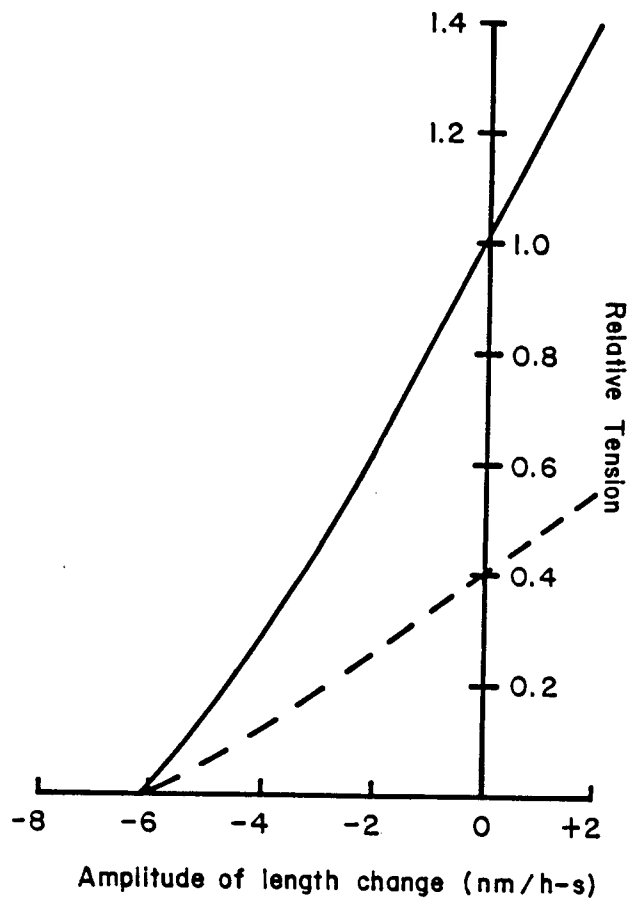
tension to a value approximating that preceding the length change, which would be expected since the small length change causes little or no change in the number of cross-bridges which can form (Huxley and Simmons, 1971b). The recovery of tension involves a reorientation and rotation of the cross-bridges as they proceed through their cycle and stretch out the elastic element to its pre-step length.

The instantaneous change in tension resulting from rapid releases and stretches, measured during the plateau of an isometric tetanus, can be plotted versus the amplitude of the step length change to give a 'T1 curve'. T1 curves can be constructed for various degrees of filament overlap. The T1 curve obtained when the overlap is small is a scaled down version of the T1 curve at maximal overlap (figure 1d) (Huxley and Simmons, 1971a, Ford et al, 1981). This indicates that the T1 curve represents the characteristics of the instantaneous elasticity of a formed, tension generating cross-bridge.

The object of this research project was to further characterize the instantaneous elasticity of isometrically contracting muscle by comparing the stiffness measured with a rapid release to that measured with a rapid stretch throughout the time course of the isometric twitch and tetanus in frog skeletal muscle at resting length (maximal filament overlap). It was found that stiffness correlated with tension throughout the time course of the twitch and tetanus. However, stretch gave a higher absolute value of tension change than release for the same amplitude of length change and thus a higher stiffness value. Several possible explanations for these results are discussed. The implications of these findings for the nature of the instantaneous elasticity are not yet clear.

II. REVIEW OF THE LITERATURE

Visco-elastic models

Before the introduction of the sliding filament theory (H.E. Huxley and Hanson, 1954, A.F. Huxley and Niedergerke, 1954) a considerable amount of mechanical data had been obtained for skeletal muscle. The earliest mechanical models of muscle contraction consisted of one or more elastic elements arranged in such a way that the model could simulate the mechanical properties of muscle but they were not correlated with any actual structure within the muscle. The earliest model was based on the work of Weber (1846) which suggested that when muscle was activated it behaved like a stretched spring. This basic idea was found to be insufficient to explain the apparent visco-elastic behavior of muscle. It was refined by introducing viscous forces, which caused damping of the spring (Blix, 1893, Hill, 1922, Gasser and Hill, 1924) and by adding an undamped spring in series (a series elastic component) (Levin and Wyman, 1927).

In 1935, Fenn and Marsh found that their force-velocity relationship could not be explained by the visco-elastic model. Using isotonically contracting muscle, they measured the shortening speed against different loads. They observed that force decreased exponentially with increased shortening velocity, whereas it should decrease linearly if the visco-elastic model were correct.

Introduction of a contractile element: Hill's model

Improved mechanical and thermal measurements of skeletal muscle by A.V. Hill (1938) led to the proposal of a new model. Hill measured heat production during isotonic contractions under various conditions and confirmed Fenn's (1924) results that extra heat (which Hill defined as the shortening heat) is produced if the muscle is allowed to shorten.

Hill showed that as the muscle shortened a distance, x, extra heat, ax, is released (where 'a' is a constant of proportionality between heat and shortening). The work done in lifting a load, P, is Px so that the extra energy released is Px + ax = (P + a)x. It follows that the rate of extra energy release is therefore $(P + a)dx/dt = (P + a)v$, where v is the velocity of shortening.

Hill found experimentally that the rate of extra energy release depended linearly on the load (P) on the muscle, increased as P decreased, was zero when P = Po, the isometric force, and had its maximum value when P = zero. Therefore $(P + a)v = b(Po - P)$, where b is a constant reflecting the absolute rate of energy liberation. This equation can be rearranged so that only constants are on the right side, in which form it is called Hill's characteristic equation : $(P + a)(v + b) = (Po - a)b$. This relationship also predicts the force-velocity relationship. Hill found that mechanical experiments used to test his equation fit the relationship, and the values of a and b agreed well with those obtained in thermal experiments.

Hill concluded that a new mechanical model was needed to explain his data. He proposed that active muscle could still be represented as a two-component system, but suggested that it consisted of an undamped elastic element in series with a contractile element whose properties were determined by his characteristic equation. He also stated that, although there must be visco-elastic and presumably purely viscous elements as well in resting muscle, their role in active muscle should be relatively small.

The requirement of an undamped elastic element, or series elastic component (SEC), in the new mechanical model arose from the work of Levin and Wyman (1927), which showed an instantaneous tension change in

response to a rapid length change in activated muscle. The interpretation of such an instantaneous response in terms of a passive spring-like mechanical element is compelling, and it has been retained in current models.

Hill's model differed from the previous visco-elastic models in that the contractile component (CC) was active, with mechanical changes determining the amount of energy it liberated. Stimulation of the muscle caused the rapid build-up of the active properties of the CC, resulting either in shortening, or in tension development if shortening is hindered in any manner. In relaxed muscle the CC contributed negligible force.

However, if resting muscle is greatly stretched it will produce tension. Hill (1950) accounted for this by adding the parallel elastic component (PEC) to his model. The length-tension relationship for the PEC was determined experimentally for relaxed muscle. The PEC does not come into play at normal body length (lo) and can therefore be ignored in experiments at lo. The PEC was thought to be made up mainly of the connective tissue sheaths (epimysium, perimysium and endomysium) of the muscle (Banus and Zetlin, 1938) and possibly the sarcolemma at sarcomere lengths greater than 3.0 μm (Casella, 1950, Natori, 1954, Podolsky, 1964, Rapoport, 1972).

The SEC is conceived to be the part of the muscle preparation which behaves like a purely passive, non-linear spring with properites that are unaffected by the state of activation of the muscle. It is characterized by a length-tension (tension-extension) curve. This relationship can be obtained by applying rapid length changes to muscle and measuring the resultant tension change, or by applying rapid tension changes and measuring the resultant length changes. The SEC of a muscle preparation is both external and internal. The external component is that found in

the connections between the muscle and the experimental apparatus. Hill (1938) recognized this and attempted to reduce the external component by using fine jewelry chains rather than thread to connect the muscle to the apparatus. This was important because length changes in the SEC are difficult to distinguish from length changes in the CC. One must use rigid connections to reduce the SEC, or length clamp a central region of the muscle preparation, by using a device such as a spot follower (Gordon, Huxley and Julian, 1966), to eliminate it. The internal SEC is found in the tendons and in the muscle itself (Jewell and Wilkie, 1958).

Hill's model can be used to explain a great deal of mechanical and thermal data from skeletal muscle (Aidley, 1971). The rise in tension during an isometric tetanus can be qualitatively predicted. At rest the muscle produces no tension, as the SEC is slack and the CC is inactive. When the muscle is stimulated, the CC will start to shorten at its maximum velocity since the tension is initially zero. Since the ends of the muscle are fixed, the shortening of the CC causes extension of the SEC. Tension will start to rise in accordance with the tension-extension curve of the SEC. As tension increases, the velocity of shortening will decrease in accordance with the force-velocity relationship of the CC, and therefore the SEC will be extended more slowly and tension will rise less quickly. This series of events will continue until the maximum isometric tension, Po, is reached at which point the velocity of shortening will be zero and the SEC will be fully extended.

The model can also explain the quick release results of Gasser and Hill (1924) using large amplitude releases. A quick release causes immediate shortening of the SEC, which results in a drop in tension. The new lower tension value will determine the new faster velocity at which the CC will shorten and re-extend the SEC, which will result in the

tension rising to its maximum isometric value at the new, shorter length.

The model also explains after-loaded isotonic contractions. In this situation the SEC is at a constant length and tension during shortening, and therefore the velocity of shortening of the muscle is that of the CC. The load on the muscle is then reduced to a new steady value, so that the tension in the SEC is reduced to a new steady value. This results in an immediate shortening of the SEC to a new constant length and the muscle shortens at a new velocity determined by the force-velocity relationship of the CC.

Critical test of Hill's model

Jewell and Wilkie (1958) tested Hill's two component model experimentally by measuring the force-velocity curve of the CC and the tension-extension curve of the SEC at lo, where the PEC would not contribute to tension. This data was then used to predict the rise of tension in an isometric contraction from the following relations : (1) $dP/dt = (dP/dx) (dx/dt) = (dP/dx) v$ where v is taken from the force-velocity curve and dP/dx is the slope of the tension-extension curve, and (2) $t = \int_{0}^{P_t} [1/(V) (dP/dx)] \times dP$ where Pt is the tension at time t after stimulation is initiated.

They measured the initial development of isometric tension, and the redevelopment of tension following a rapid release of amplitude sufficient to drop the tension to zero, given at the maximum isometric tetanic tension in the same muscle. They then compared the predicted development of tension to the measured development and redevelopment. It took the muscle longer to reach a given tension than predicted by the two-component model, about 80% longer than expected for initial development and about 50% longer than expected for redevelopment

following a release. Three possibilities for this disagreement were suggested: there might have been errors in their force-velocity curve; there might have been errors in their tension-extension curve; or muscle might not be correctly represented by Hill's two component model.

Sliding filaments and cross-bridges

Hill proposed his two-component model in 1938, before the structural changes which occur during contraction of the muscle were known. Actin and myosin were found to be the major proteins present in muscle (Straub, 1943) and it was suggested that contraction might be due to the shortening of continuous actomyosin filaments (see Huxley, 1974). However, the Hill model did not describe a structural basis for the contractile element.

In the 1950's light microscopic studies of the striation pattern of skeletal muscle provided such a structural basis. Using interference microscopy to observe isolated muscle fibres, A.F. Huxley and Niedergerke (1954) showed that during stretching or contraction of the fibres, the length of the A band remained constant. Using phase contrast to observe isolated myofibrils, H.E. Huxley and Hanson (1954) showed that during contraction the distance between the Z line and the edge of the H zone remained constant whereas the width of the I band and H zone shortened. The results of these two studies implied that the length of the myofilaments remained constant. In addition, A.F. Huxley and Niedergerke (1954) observed that contraction bands developed in the centre of the A band and at the Z line at short sarcomere lengths, implying localized folding of the ends of the I band filaments. The observations of these two groups, taken in conjunction with (1) low-angle X-ray diffraction studies (H.E. Huxley, 1953) showing an hexagonal double-array of

filaments, (2) electron micrographs of transversely sectioned skeletal muscle, showing each myofibril consisted of an overlapping, double array of thick and thin filaments (H.E. Huxley, 1952), (3) the discovery that myosin is localized in the A bands by selective protein extraction (Hasselbach, 1953, Hanson and H.E. Huxley, 1953), and (4) the observation that tension generated by isometrically contracting frog muscle was maximal at body length and decreased at shorter or longer muscle lengths, becoming zero at approximately one-half and twice body length, with the decrease in tension at longer muscle lengths being linearly related to the muscle length (Ramsey and Street, 1940), led to the following proposal. It was suggested that the striation pattern of skeletal muscle was due to two sets of overlapping, interdigitating filaments, and contraction resulted from the sliding of one set of filaments relative to the other without any appreciable change in length of either set of filaments.

Page and H.E. Huxley (1963) later confirmed that the filament lengths remain constant at different muscle lengths by electron microscopy of muscles fixed in resting condition and while contracting isometrically. In addition, Harman (1954) confirmed these observations using cine-photography with the phase contrast microscope to record contraction and relaxation of myofibrils. He observed that shortening occurred by obliteration of the I bands while the length of the A bands remained constant. Furthermore, the contraction bands in the centre of the A band were observed in electron micrographs showing the ends of the I filaments sliding past each other resulting in a region of double overlap (H.E. Huxley, 1964).

Electron micrographs of thin sections of myofibrils (H.E. Huxley and Hanson, 1954, Hanson and H.E. Huxley, 1955 and H.E. Huxley, 1957)

confirmed that the myofibrils contained two sets of interdigitating filaments of different diameter (figure 1a). The thin filaments were attached to the Z lines and extended through the I bands into the A bands. The thick filaments were localized in the A bands. The H zone was the central part of the A band between the ends of the two sets of thin filaments. The M line was observed to be due to thickenings in the centre of the thick filaments.

The suggestion of sliding filments raised the question of how such a system would work. A.F.Huxley (1957) suggested the existence within each overlap zone of some type of interaction sites between the myofilaments, or mechanical links, which could "make and break". These links would be responsible for the generation of force between the filaments necessary for sliding.

In Huxley's model, a 'side-piece', elastically connected to the myosin filament, was proposed to have at least two states; (1) attached to a site on an actin monomer of the actin filament and (2) detached from the actin site. During contraction, the side-piece would go through its states cyclically. Attachment and detachment were assumed to be biochemical reactions and the transition between states was controlled by probabilities per unit time.

The side-pieces were proposed to move randomly back and forth by thermal agitation (Brownian motion) which caused the side-pieces to enter a strained state. The range of motion of the side-pieces was less than the distance between the actin sites. Attachment occurred in this strained state with a moderate rate constant. This must be so to explain the decrease in the rate of energy release at high shortening speeds (Hill, 1938). In addition attachment was reversible and no ATP was hydrolyzed if the side-piece was unable to complete the working part of

its cycle . This explained the decrease in energy release during stretch (Fenn, 1924, Hill, 1938). As soon as attachment occurred the elastic connection produced a force in the direction of shortening.

Detachment occurred with the binding of ATP. The rate of detachment was slow when the side-piece was in a position such that it exerted positive tension. The detachment rate was rapid when it was in a position where it was unstrained or compressed, and resisted shortening. This would occur when shortening took place which allowed the side-piece to complete the working part of its cycle. In addition the difference in rate of detachment explained the increased energy release when shortening occurs (Fenn, 1924).

The side-pieces cyclically produced interfilament shear forces which caused the actin filament to slide past the myosin filament. This resulted in sarcomere shortening and contractile force. Sliding of the filaments caused the attachment site on the actin filament to move past the site of origin of the side-piece on the myosin filament, resulting in distortion of the side-piece. This distortion influenced the force produced by the side-piece and the probabilities per unit time of its subsequent changes in state. The total contribution from all other attached side-pieces and the external load on the muscle determined whether sliding occurred.

Soon after the proposal of A.F. Huxley's model, H.E. Huxley (1957) observed projections emerging from the thick filaments in high magnification electron micrographs of skeletal muscle. These projections, or cross-bridges, were suggested to be the side-pieces involved in cyclic attachment and force production.

Many lines of evidence supported and elaborated on Huxley's model: (a) X-ray diffraction showed the appropriate arrangement of molecules,

(b) biochemistry showed the energy transduction mechanism, the ATPase, and (c) the detailed tension-length curve supported the idea of independent force generators as did the speed of shortening at zero tension (d).

a) X-ray diffraction

The X-ray diffraction technique may be used to obtain accurate measurements of the spacing between repeating structures in an object which is made up of regularly-spaced molecules or macromolecular sub-assemblies, as is skeletal muscle. As in microscopy, X-rays are scattered by an object. However, unlike microscopy, X-rays cannot be re-focussed to produce an image of the object. Therefore in order to obtain an object's image the X-ray diffraction pattern (the pattern produced by the scattered or diffracted X-ray beam) must be mathematically reconstructed (Aidley, 1971, Bagshaw, 1982).

The spots and lines making up a diffraction pattern are referred to as reflections. Meridional reflections are produced by axially repeating structures in the muscle while equatorial reflections are produced by transversely repeating structures. Off-meridional reflections are produced by structures repeating in a manner other than axially or transversely, such as helically repeating structures. Layer lines are off-meridional reflections appearing as a series of lines parallel to the equator of the diffraction pattern (Aidley, 1971). The relative intensities of the reflections in a diffraction pattern provide information about the distribution of mass between the corresponding structures. Cnanges in reflection intensities indicate a movement of mass.

Low-angle X-ray diffraction produces a pattern in which the

diffracted X-rays diverge little from their original path. These patterns provide information about structures which repeat over relatively large distances, such as the periodicities of the myofilaments and the cross-bridges. Wide-angle X-ray diffraction patterns provide information about structures repeating over relatively small distances, mainly the alpha-helical myosin rod.

Low-angle X-ray diffraction accurately measures the distance between repeating structures within the range of resolution of the electron microscope. However X-ray diffraction cannot provide direct information as to the identity of the structures measured. Conversely, electron microscopy can identify structures, but the accuracy of size measurements is decreased due to such problems as tissue shrinkage and distortion caused by fixing, sectioning and staining procedures. When these two techniques are used in conjunction accurate information can be obtained about the ultrastructure of muscle which is consistent with the independent force generator (cross-bridge) theory.

Low-angle X-ray diffraction studies of relaxed skeletal muscle by H.E. Huxley and Brown (1967) showed a meridional spot at 14.3 nm and layer lines at 42.9 nm. The 14.3 nm axially repeating structure corresponded well with the periodicity of the cross-bridges observed in electron micrographs (H.E. Huxley, 1957). The diffraction pattern would indicate that cross-bridges are arranged on an X-stranded helical myosin molecule with an axial repeat of 14.3 nm and a pitch of 42.9 X nm (Bagshaw, 1982). Huxley and Brown (1967) originally suggested pairs of cross-bridges emerged opposite each other from the (1-stranded) myosin filaments, with each successive pair of cross-bridges rotated $120°$ to the preceding pair so that there was a true repeat of cross-bridge pairs every 42.9 nm. However, the majority of the recent evidence supports X =

3 for vertebrate skeletal muscle (Squire, 1981) so that the preferred cross-bridge position would be three cross-bridges emerging every 14.3 nm with a pitch of 128.7 (3 x 42.9) nm. In addition, 'forbidden' meridional reflections occur, which indicates that the spacing of the cross-bridges is not completely regular.

Negatively-stained electron micrographs of isolated actin filaments (Hanson and Lowy, 1963) showed these filaments to consist of two helical strands of globular repeating actin monomers. However, the pitch of the helix and the number of monomers per turn were uncertain, and were postulated to be 35 or 41 nm for the pitch and 13 or 15 for the number of monomers. Using low-angle X-ray diffraction, H.E. Huxley and Brown (1967) observed a meridional spot at 2.7 nm and layer lines at 5.1, 5.9, and 37 nm. The meridional spot indicated an axial distance of 2.7 nm between monomers. The two layer lines are believed to arise from the pitches of a right-handed and a left-handed primitive helix. The two primitive helices form the double non-primitive helix which has a pitch of 74 nm (2 x 37) and a cross-over at 37 nm, which gives the 37 nm layer line. The number of monomers per turn is thus 13.5.

Low-angle X-ray diffraction studies of relaxed muscle also have shown two main equatorial reflections, the 1.0 and the 1.1 reflections. These reflections arise from diffraction planes of the hexagonally arranged myofilaments. The 1.0 diffraction plane contains only myosin filaments while the 1.1 plane contains actin and myosin filaments. The relative intensities of these reflections indicate the position of the cross-bridges in a muscle state (H.E. Huxley, 1968, Haselgrove and Huxley, 1973). The 1.0/1.1 intensity ratio gives a high value in the relaxed state of about 2.5 (Haselgrove and Huxley, 1973).

X-ray diffraction studies have confirmed the finding from

microscopic studies (A.F. Huxley and Neidergerke, 1954, H.E. Huxley and Hanson, 1954, Harman, 1954) of the inextensibility of the myofilaments. If the filament length changed at different sarcomere lengths or during contraction, the axial distance between the repeating structures would change. The periodicity of the meridional reflections from low-angle diffraction patterns (H.E. Huxley, 1953) and the high-angle pattern (Astbury, 1947) of resting muscles were found to be independent of the length of the muscle. During isometric contractions, H.E. Huxley and Brown (1967) found a slight increase of about 1% in the axial spacing of the myosin filaments whereas there was no significant change in the axial spacing of the actin monomers. In addition Haselgrove and Huxley (1973) found that the 1.0/1.1 intensity ratio of muscles allowed to actively shorten to a pre-set sarcomere length just before X-ray exposure was the same as that for the final sarcomere length and was independent of the immediate history of the muscle.

Low-angle X-ray diffraction studies have shown that in relaxed muscle the position of the cross-bridges is determined by their attachment to the myosin filament backbone. Studies of frog muscle in the rigor state (absence of ATP) (H.E. Huxley and Brown, 1967, H.E. Huxley, 1968, Haselgrove and Huxley, 1973, Haselgrove, 1975) have shown that in rigor the myosin cross-bridges move towards, and presumable attach to, the actin filaments, as the cross-bridges take on the spacing of the actin filaments. The 42.9 nm myosin layer line decreases in intensity while the 5.1, 5.9 and 37 nm actin layer lines increase in intensity in rigor as compared to the relaxed state. The relative intensities of the equatorial reflections change during rigor, the 1.0 reflection decreases while the 1.1 reflection increases so that the 1.0/1.1 intensity ratio thus decreases in rigor to a value of about 0.2

to 0.3 (Haselgrove and Huxley, 1973). These studies indicate that cross-bridges have moved away from their myosin-centered, relaxed positions to become actin-centered, labeling the exteriors of the actin filaments (H.E. Huxley and Brown, 1967, Reedy, 1967, Miller and Tregear, 1972, Haselgrove Stewart and Huxley, 1976) and taking on the helical characteristics of the actin filaments (Bagshaw, 1982). This conclusion was supported by electron micrographs of transverse sections of relaxed and rigor muscles (H.E. Huxley, 1968). However, the 14.3 nm meridional myosin reflection decreases only slightly. This observation, taken in conjunction with the intensity changes in the equatorial reflections, indicates that cross-bridges may bind to actin "by a radial movement accompanied by a slewing about the azimuth, rather than extensive axial movement" (Bagshaw, 1982).

A.F. Huxley (1957) suggested that force generation is mediated via the cross-bridges. This would imply that cross-bridges may move during contraction. The difference in the X-ray diffraction data between the relaxed and rigor states in frog muscle would indicate that cross-bridge movement does occur. Further support has been obtained by Reedy, Holmes and Tregear (1965) studying glycerol-extracted insect flight muscle. They found similar differences in their X-ray diffraction patterns in the relaxed and rigor states as was observed in vertebrate muscle. In addition they observed that their electron micrographs differed in these two muscle states. In resting muscle, the cross-bridges were oriented $90°$ from the myosin filament backbone, while in rigor, the cross-bridges were attached to the actin filaments, and were oriented at an angle of $45°$ from the myosin backbone. Reedy et al interpreted the rigor position to correspond to the end of the working cycle of the cross-bridge. They proposed that the cross-bridge attached to actin and moved from the $90°$

to the 45° (rigor) position, during which the actin filament is pushed towards the centre of the sarcomere. Furthermore, electron micrographs of vertebrate myosin S-1 labeled actin filaments produced in rigor conditions have shown that cross-bridges attach to actin monomers in well defined positions (H.E. Huxley, 1963, Moore, Huxley and DeRosier, 1970). This idea has been widely accepted and incorporated into contraction models, although there is no direct evidence that the cross-bridges do exist in these angles at the start and end of their working stroke.

Based on the independent force generator theory, cross-bridges should attach to actin during contraction and it might be expected that similar X-ray diffraction patterns to those in rigor would be obtained. During isometric contraction the 42.9 nm myosin layer line decreases to about 30% of that in resting muscle and the 14.3 nm meridional reflection decreases to about 66% of that in resting muscle (H.E. Huxley and Brown, 1967). The 1.0/1.1 intensity ratio of the equatorial reflections increases during contraction and was very similar to the ratio in rigor (Matsubara, Yagi and Hashizume, 1975). Contracting vertebrate and molluscan muscles give a significant (although just marginally) increase in intensity of the 5.9 nm actin layer line (Haselgrove, 1975, Vibert, Haselgrove, Lowy and Poulsen, 1972, Lowy, 1972) supporting the idea that attachment to actin does occur. These results indicate that the cross-bridges move from their helically ordered state about the myosin filament backbone during contraction. The equatorial reflection changes would suggest that a significant number of cross-bridges are attached with a wide distribution in their angular distribution. The X-ray diffraction data show that there is some longitudinal movement (or rotating) of the cross-bridges and that a significant amount of radial movement occurs.

b) <u>Biochemistry</u>

By 1945, it was known that the energy released from ATP hydrolysis powered contraction and that myosin was an ATPase (see A.F. Huxley, 1980). In the 1960's it was found that the mysoin S-1, the cross-bridge, has one actin binding site and one ATP binding site (Young, 1967). Since the myosin cross-bridge is an ATPase and its activity is increased by the presence of actin (Eisenberg and Moos, 1968, 1970), it would appear that the cross-bridges are intimately involved in converting the chemical energy of ATP into mechanical work.

X-ray diffraction and electron microscopic studies have shown that the interaction of actin, myosin S-1 and ATP involved conformational changes in the proteins which results in sliding of the myofilaments (see section a). Rapid-reaction kinetic studies (see Eisenberg and Greene, 1980) have shown that the interaction of actin and myosin involved several intermediate steps, which indicated that the cross-bridges interact cyclically with actin. However, in muscle, the chemical reaction sequence would be influenced by mechanical constraints as the cross-bridge must move relative to actin for the reaction to proceed. Models of muscle contraction based on biochemical and mechanical data have been presented by Eisenberg and his co-workers (Eisenberg and Hill, 1978, Eisenberg, Hill and Chen, 1980, Eisenberg and Greene, 1980) (see discussion).

c) <u>Tension-length curve</u>

Gordon, Huxley and Julian (1966) re-investigated the relationship between tension and length (figure 1c) observed by Ramsey and Street (1940) in frog single fibres. The central region of the fibre was held at a constant length by a feedback circuit while its tension was

measured. This was done to remove sarcomere length non-uniformity which occurs at the ends of the fibres (A.F. Huxley and Peachey, 1961) and to remove external (series elastic) compliance in the experimental apparatus and tendons.

Their tension-length curve was found to correspond well with myofilament lengths obtained by Page and H.E. Huxley (1963); myosin filament, 1.6 μm, its bare zone, .15-.2 μm, actin filament, 1.0 μm, and the Z line, .05 μm. At a sarcomere length of 3.6 μm, where there would be no overlap of actin and myosin filaments, a small amount of tension is produced, probably as a result of a small amount of scatter in the sarcomere lengths of the central portion of the fibre. Between no filament overlap (3.6 μm) and maximal overlap (2.25 μm) there is a linear increase in the number of cross-bridges formed and in the tension generated. At sarcomere lengths of 2.05 to 2.25 μm, where the actin filaments would be moving into the central zone of the myosin filaments, which is devoid of cross-bridges, the tension generated remains constant, producing the plateau of the tension-length curve. These regions of the curve support the idea that tension generation is mediated via a set of independent force generators, the cross-bridges, evenly distributed throughout the A band. At shorter sarcomere lengths, less than 2.05 μm, the tension decreases. Two possible mechanisms have been suggested. One possibility is that mechanical interference occurs when the ends of the actin filaments slide into the opposite half of the sarcomere and interfere with tension generation, and when the ends of the myosin filaments come into contact with the Z line (at a sarcomere length of 1.65 μm). The other possibility is that the excitation-contraction coupling mechanism of the inner part of the fibre fails at short sarcomere lengths (Taylor and Rudel, 1970, Rudel and Taylor, 1971). It

may be that both of these mechanisms determine the shape of the tension-length curve at short sarcomere lengths.

This experiment supports the hypothesis that the cross-bridges are responsible for contraction, as the degree of tension generated was proportional to the amount of overlap between the filaments and thus the number of formed cross-bridges. Further support for the hypothesis was obtained in experiments of actively shortening muscle. It was shown that a fibre passively set to a long sarcomere length and then stimulated to contract against a light load shortened to the appropriate sarcomere length on the tension-length curve for that load (Edman, 1966, Gordon, Huxley and Julian, 1966). It was also shown that when a fibre actively shortened from a long to a short sarcomere length and was then held at the shorter length, it developed (within 10%) the same amount of tension as a fibre pre-set to the shorter length (see Simmons and Jewell, 1974). These experiments show that the myofilaments slide in both actively and passively shortening muscle.

d) Speed of shortening at zero tension

Resistance to shortening of a contracting muscle may be due to an externally applied load, a viscous resistance to relative sliding of the myofilaments, or to limitations of the cross-bridge cycling speed (A.F. Huxley, 1980). The effects of viscosity are negligible (Huxley, 1980). Therefore, when the external load on a muscle is zero, the speed of shortening is limited only by the cross-bridges. The resistance to shortening that must be overcome, and the tension generated by the muscle, will be proportional to the number of active cross-bridges, so the shortening velocity of the muscle should be independent of the number of active cross-bridges and the amount of overlap between the filaments.

The speed of shortening that decreases the net power output (and thus the tension generated) of any cross-bridge to zero will be identical and independent of the number of active cross-bridges in the same half sarcomere. It has been shown (A.F. Huxley and Julian, 1964, Gordon et al, 1966) that the speed of active shortening of frog muscle under a very light load from a number of different original sarcomere lengths was virtually constant.

Alternative models

The studies discussed in sections a to d support a model of muscle contraction in which the length of the actin and myosin filaments remain virtually constant as the sarcomeres shorten and in which shortening is produced, and tension generated, by myosin cross-bridges interacting independently with actin sites, that is, acting as independent force generators. Alternative models of muscle contraction exist, two main categories being the lateral expansion theories and the electrostatic attraction theories (see Huxley, 1980). The lateral expansion theories propose that a lateral repulsion between the myofilaments produces force, which is converted to shortening by some constraint which ensures that each sarcomere remains isovolumic. However, a skinned fibre (a fibre whose sarcomlemma has been removed) does not behave isovolumically (Matsubara and Elliott, 1972) yet it generates tension levels comparable to those generated by intact fibres.

The electrostatic attraction theories propose that the actin and myosin filaments are oppositely charged and the actin filaments are drawn towards the centre of the sarcomere by the resulting electrostatic force. However, if it is assumed that the charges on myosin are found on the cross-bridges, then filament overlap will be complete at sarcomere

lengths between 2.0 and 2.2 μm and would not proceed further. Therefore at these sarcomere lengths the tension produced would be zero, which disagrees with the tension-length curve (Gordon et al, 1966).

Although other theories exist, the independent force generator model is supported by the weight of the experimental evidence.

Cross-bridge mechanics

The properties of an average cross-bridge can be studied by examining the response of isometrically contracting muscle to rapid tension or length changes, which produce a velocity transient, or a tension transient respectively. Experiments with improved time resolution, as compared to experiments before the 1960's, have shown that the response of muscle to rapid changes of tension (load) or length are more complicated than those predicted by Hill's two-component model and Huxley's (1957) model.

a) Velocity transients

Experiments by Podolsky (1960, Civan and Podolsky, 1966) showed the more complicated response following a rapid change in load in frog single fibres and bundles. The muscle preparation was allowed to develop its maximal isometric tetanic tension (Po), and was then quickly released and allowed to shorten under a constant load which was less than Po. The time course of the change in length of the muscle preparation was recorded during the load change. It was found that an initial rapid shortening occurred, as would be predicted from the SEC. However, the initial shortening was followed by a complex sequence of changes in the shortening speed, the velocity transient. This was contrary to the prediction by Hill's two-component model of a constant shortening speed

following the initial shortening. Initially, the shortening speed was much faster than the steady-state speed for the same load. This showed that the force-velocity relation is not instantaneously obeyed when the load changes. The shortening speed then declined to a low value or reversed its direction, and then returned to its steady-state value, sometimes with a damped oscillation. This non-steady shortening was suggested to be due to the cyclic interaction of the cross-bridges with the actin sites (Civan and Podolsky, 1966). The time period necessary to attain steady shortening was found to have a large temperature coefficient which suggested a chemical process was responsible for the observed response rather than a physical process. They further suggested a time-dependent compliance within the sarcomere, probably within the cross-bridges.

b) Tension transients

Experiments by A.F. Huxley and his co-workers (Armstrong, Huxley and Julian, 1966, Huxley and Simmons, 1970a, 1971a,b) showed the complicated response following a rapid change in length in frog single fibres. Length changes were used by these investigators rather than load changes because they found the electronics of the feedback system were less complicated, and better time resolution could be attained in the former type of experiments.

Huxley and co-workers found that the initial tension change occurred simultaneously with the length change, as would be predicted by Hill's two-component model. However, the initial tension change was followed by a complex tension recovery, the tension transient. This was contrary to the nearly exponential recovery predicted by Hill's model. The tension transient contained an additional rapid component (phase 2) which had not

been previously observed because of technical limitations.

The tension transient was observed to be composed of four phases (Huxley and Simmons, 1970a). The first phase was the initial rapid change in tension occurring simultaneously with the length change. This was followed by a quick recovery of tension (phase 2) to a level approaching that prior to the length change. The recovery then dramatically slowed and sometimes reversed (phase3). Finally, tension reached a level approximating its original pre-length change level (phase 4). The rate of phase 2 was shown to be slow for stretches (lengthening steps) but increased rapidly with the amplitude of releases (shortening steps) (Huxley and Simmons, 1971b).

Huxley and Simmons (1971a) plotted the values of T1, the extreme tension value reached during the length change, and T2, the tension value immediately following the rapid recover, against the amplitude of length change to obtain T1 and T2 curves. T1 and T2 curves were measured at maximal filament overlap, 2.2μm, and at decreased filament overlap, 3.2μm. Based on the ratio of the isometric tension (Gordon et al, 1966), they then calculated T1 and T2 curves at a sarcomere length of 3.2 μm, which were found to fit the experimental points. Since the magnitude of the T1 and T2 values were found to scale in proportion to the overlap between the actin and myosin filaments and thus the number of formed cross-bridges, Huxley and Simmons proposed that the cross-bridges are the structures responsible for the instantaneous elasticity as well as the early recovery of the tension transient, assuming all other structures in the sarcomere are effectively rigid. If these curves were due to any other series elasticity residing elsewhere in the sarcomere (for example in the Z line or the non-overlap regions of the filaments) the T1 curve would have then had a slope that was independent of tension

(Huxley and Simmons, 1973). Recently, Ford, Huxley and Simmons (1981) have determined that the cross-bridges contain "at least 80% and probably well over 90%" of the measured instantaneous elasticity in frog single fibres. They indicated that the filamentary compliance would have "only small or negligible effects on the amplitude or time course of tension changes".

Bressler and Clinch (1974) confirmed the findings of Huxley and Simmons at sarcomere lengths longer than resting length in whole muscle. In addition, Bressler and Clinch (1975) showed that stiffness was correlated with tension at sarcomere lengths shorter than resting length. This was recently confirmed in single fibres by Julian and Morgan (1981b). These studies ruled out a significant contribution of the unbound portion of the actin filaments to the measured instantaneous elasticity.

Revised two-component model of contraction : Huxley and Simmons' model

The response of isometrically contracting skeletal muscle to rapid changes of length and load led to the proposal of a revised two-component model of muscle contraction in which the cross-bridge contains an instantaneous elasticity in series with a contractile element (Huxley and Simmons, 1971b). This model is an advance over that of Hill in that the structural and chemical basis of these elements is known in some detail.

Huxley and Simmons (1971b) explained their results using the illustration of a 'rocking' cross-bridge in which the head of the myosin molecule contained the active contractile element and the S-2 portion between the head and myosin filament backbone contained the elastic element (figure 1b). They pointed out that this was only one possibility as to the location of these two components.

Huxley and Simmons proposed that there are two attached states, with the second state having less potential energy than the first, and in each state a different angle is formed between the myosin head and the actin site. In the isometric state, each cross-bridge spends an equal amount of time in each attached state as a result of the potential energy differences between the two states causing forward movement (from state 1 to state 2), and the tension in the elastic element causing backward movement (from state 2 to state 1). Thus the 'average' position of a cross-bridge is half-way between these two attached states. In the isometric state, it is not necessary for the filaments to slide relative to each other to develop force, as the elasticity of the cross-bridge allows it to rotate about the actin site and develop tension, which will then be transmitted to the myosin filament through the elastic element.

In order to move forward the cross-bridge needs activation energy to move from the state it is in, and to perform the work required to stretch the elastic component sufficiently to allow it to move to the next state. The higher the tension, the greater the activation energy required and the greater the rate of movement to the next state.

This model can account for the observed difference in the rate of the rapid early recovery phase (phase 2) of the tension transient. When isometrically contracting muscle is rapidly shortened the elastic element will shorten before the contractile element can change its angle with respect to the actin site. Thus the tension in the elastic element will decrease instantaneously. This allows the contractile element of the cross-bridge to move into the higher tension state (2) which restretches the elastic element and returns tension to its pre-length change level. A large amplitude of release, results in a large drop in the tension in the elastic element. This decreases the amount of activation energy

required for the forward movement of the contractile element which results in a more rapid early recovery phase.

As in Huxley's 1957 model, the transition between the attached states depends on thermal agitation and is thus relatively slow. As a result, the two attached state model predicts low values for the force and work per cross-bridge (Huxley and Simons, 1971b). Therefore, Huxley and Simmons suggested that at least three attached states would be required to explain physiological data.

Stiffness

X-ray diffraction studies (H.E. Huxley and Brown, 1967) as well as mechanical studies (Ford, Huxley and Simmons, 1981) have shown that the compliance of the myofilaments is very small. Ford et al (1981) proposed that filament compliance would have only small or negligible effects on the amplitude or time course of tension transients.

If it is assumed that the instantaneous elasticity of the cross-bridge, reflected by phase 1 of the tension transient, is independent of the force-generating mechanism (Huxley and Simmons, 1971b), then the stiffness associated with it can be studied. Stiffness is defined as the change in tension resulting from a given change in length during a quick length step, that is, it is the slope of the T1 curve. If the dependence of the amplitude of phase 1 on the amount of overlap between the myofilaments indicates the cross-bridge contains an undamped elasticity (Huxley and Simmons, 1971a), then stiffness provides a measure of the number of formed cross-bridges.

The recovery of tension is assumed to occur independently of the changes in the instantaneous elasticity and begins before the length change is complete (Huxley and Simmons, 1971b). This results in

truncation of Tl, that is, an underestimation of the tension change. Therefore to obtain the true Tl, truncation must be taken into account (see discussion).

Because of the arrangement of the experimental apparatus, the length change involves the apparatus, the tendons and connections to the apparatus, and the muscle itself. The length change does not occur simultaneously in all sarcomeres, as it must be propagated from the end where it originates to the other end where it is recorded. This is taken into account by multiple segment models (Ford et al, 1977, 1981, Gott, 1979). Stiffness behavior can be tested against these models of the cross-bridge.

III. METHODS

All experiments were carried out using single fibres or small fibre bundles of the semitendinosus muscle from the frog, Rana pipiens or Rana temporaria. The experiments are conceptually simple, but are subject to certain complex and subtle technical interferences. Therefore the methods will be described in detail, and the relevant technical considerations will be discussed.

Muscle preparation

Frogs were killed by a blow to the head. The head was quickly removed and the spinal cord was pithed. The legs were removed from the body and pinned onto a parafin dissecting board so that the cut surface was facing down. The legs were kept moist throughout the dissection by rinsing with cooled, unbuffered Ringer's solution. The leg to be dissected was skinned from the region of the pelvis to just past the knee. The location of the semitendinosus muscle could be approximated by observing the two proximal tendons and the distal tendon through the overlying muscle which was initially removed. Subsequently one head of semitendinosus was isolated and removed from the thigh as follows. After isolating the tendons, surgical silk (5-0) was tied onto one proximal tendon and onto the distal tendon. The proximal tendon was cut and the muscle was gently lifted and dissected away from the surrounding connective tissue and its neurovascular supply. Near the distal end the two heads of the muscle insert into a common tendon so the head not being used was carefully dissected away. The distal tendon was freed and the muscle was quickly transferred to a perspex dissecting dish, filled with cooled, unbuffered Ringer's solution. The threads on each tendon were looped around stainless steel hooks at each end of the floor of the dissecting dish and the length of the muscle was adjusted to just past

slack length. The threads were secured to the rim of the dissecting dish with plasticine. The remainder of the dissection was done with the aid of a Zeiss dissecting microscope and dark field illumination. The remaining nerve stump was located and about one-half to three-quarters of the muscle was dissected away from this region by either starting at one tendon and cutting along the long axis of the muscle, or by making an incision in the middle of the muscle, peeling and cutting away the fibres towards the tendons. Thus, the fibres to be used were on the side of the muscle opposite to the point of entry of the nerve. Damaged fibres were removed until the preparation was clean. Damaged fibres could be distinguished from living fibres by opaque swellings at the site of injury or by opacity of the whole fibre. Living fibres looked clear under the dissecting microscope. Fibres were removed one at a time or in small bundles by carefully taking hold of them with fine (#5) stainless steel forceps, making a small incision, peeling them towards the tendons and cutting them away with fine scissors. This was continued until the muscle preparation was the desired size. To untwist the muscle or to remove damaged fibres more readily, the preparation could be rotated in the dissecting dish by twisting the threads at one end. When the dissection was complete the muscle preparation was stored overnight at 5° C. The following day any fibres that appeared damaged were removed. Usually single fibre preparations were used the same day as the dissection.

Experimental apparatus

Two slightly different experimental arrangements were used during the course of this work. In both systems the muscle preparation was attached to a motor at one end and a force transducer at the other end

(figure 2). The motor was used to maintain the muscle preparation in the isometric state or to give rapid length changes during a contraction. In the initial series of experiments, a Ling shaker (model 201) was used as the servo-motor and this was subsequently replaced for all the single fibre experiments by a Cambridge model 300S servo-system. In addition, an RCA 5734 force transducer (1.2 KHz resonant frequency) was used with the shaker system and a model 404 capacitive type force transducer (2 KHz resonant frequency) was used with the Cambridge system. The analog outputs of both the motor and the force transducer could be displayed on two oscilloscopes (Tektronics D13 Dual Beam Storage Scope and the Digital Nicolet 3091) and photographed for later analysis. A digital timer (Digitimer D4030) triggered the stimulator (Medical Systems Corp. 2533), the function generator (Exact, model 330), the oscilloscopes, and the analog to digital converter at pre-set times during a contraction.

a) The Ling Shaker system

The Shaker system was used for the initial experiments on small muscle bundles. After dissection, the tendons of small muscle bundles (2-20 fibres) were retied with 5-0 silk thread very close to the myotendinous junction to reduce stray series compliance. The preparation was subsequently transferred to the experimental chamber which consisted of two pieces of perspex each containing a transverse array of platinum wire electrodes at 1mm intervals. The arrangement of the electrodes permitted all-over transverse stimulation of the fibres. The thread on one end of the preparation was firmly tied to a wire stirrup, which was glued to the anode pin of an RCA 5734 force transducer (1.2 KHz resonant frequency). The other end was tied to a straight annealed stainless steel wire which was firmly connected to an aluminum rod (the moving arm)
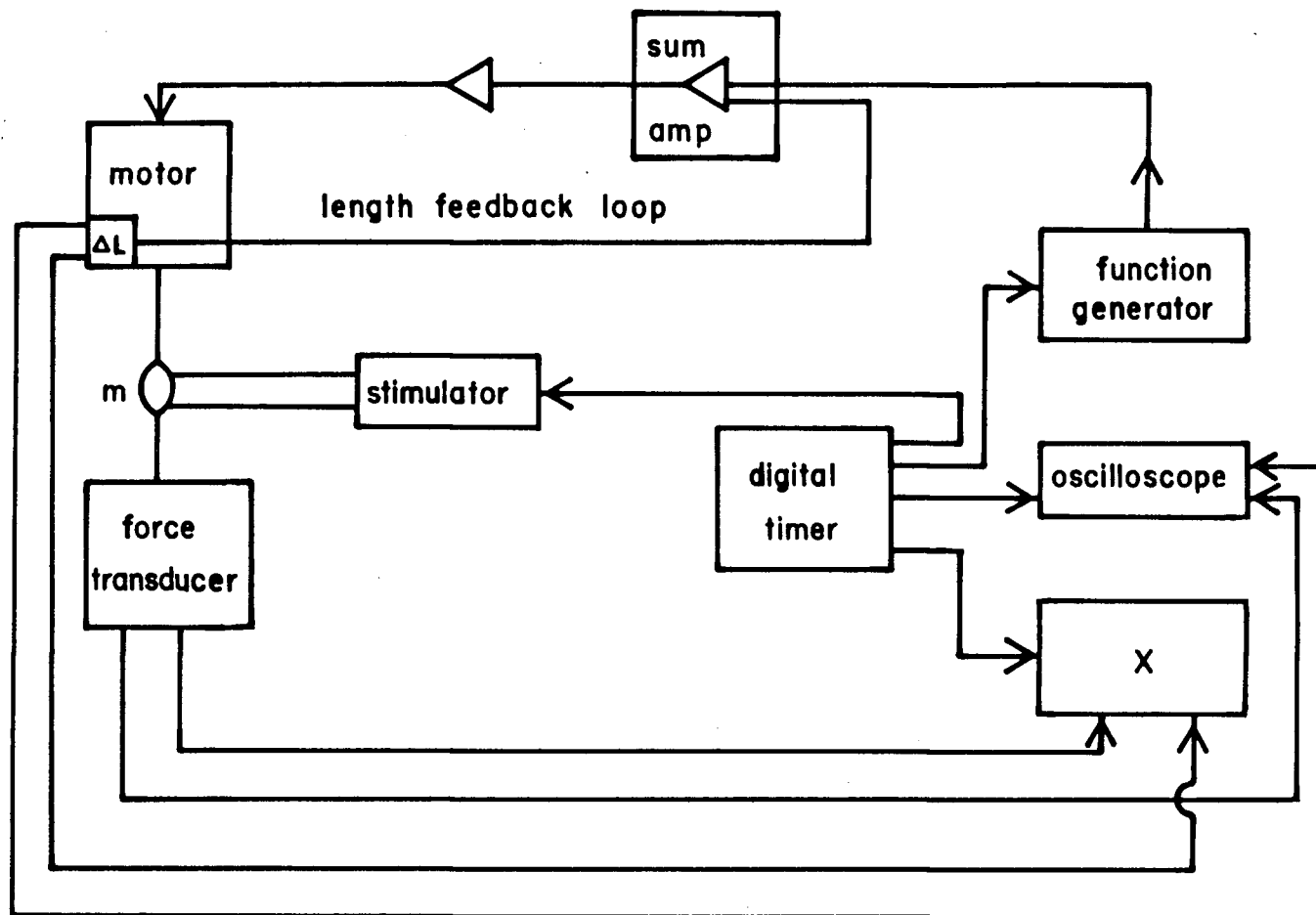
Figure 2. Block diagram of the experimental apparatus. The m stands for muscle preparation. The length feedback loop is shown by the length transducer (ΔL) and the two amplifiers. X stands for the analog to digital converter and Dec Writer in the Shaker system, or the Nicolet digital oscilloscope in the Cambridge system. See text for details.

extending from the moving element of the Ling Shaker (model 201). A
lucite block attached to the mounting plate of the Shaker contained a
short brass tube through which the moving arm passed. The brass tube
limited any non-axial movements of the Shaker's moving arm. A glass
chamber was placed over the perspex block and the whole apparatus was
then set vertically in a 40L Dewar flask containing constantly aerated
ice and water mixture. This maintained the preparation at less than 1°
C. The glass chamber was filled with buffered Ringers solution and
bubbled with 100% oxygen throughout the experiment. The composition of
the Ringer's solution was (mM): NaCl, 115.0, KCl, 2.5, CaCl, 1.8,
Na_2HPO_4, 2.1, NaH_2PO_4, 0.9 at pH = 7.1. Temperature was
monitored throughout the experiment using a thermistor probe inserted
into the chamber and lying in close proximity to the preparation.

Figure 3 shows the response of the Shaker to a step input. The
response is critically damped and is complete within 1.0 ms. This was
the duration of the step length change used in all experiments done with
the Shaker system. The stiffness of the system was 10.2 g/μm.

b) The Cambridge Dual Mode Servo-System

The Cambridge system was primarily used for single fibre
experiments. Two methods of attachment to the motor and force transducer
were used in this experimental set-up. In the bundle experiments and
some single fibre experiments, a needle was used to make a small hole in
the tendons close to the myotendinous junction. The muscle preparation
was then transferred into the experimental chamber using a small piece of
longitudinally sectioned plastic tubing. The tendons were positioned so
that the wires attached to the force transducer and motor went through
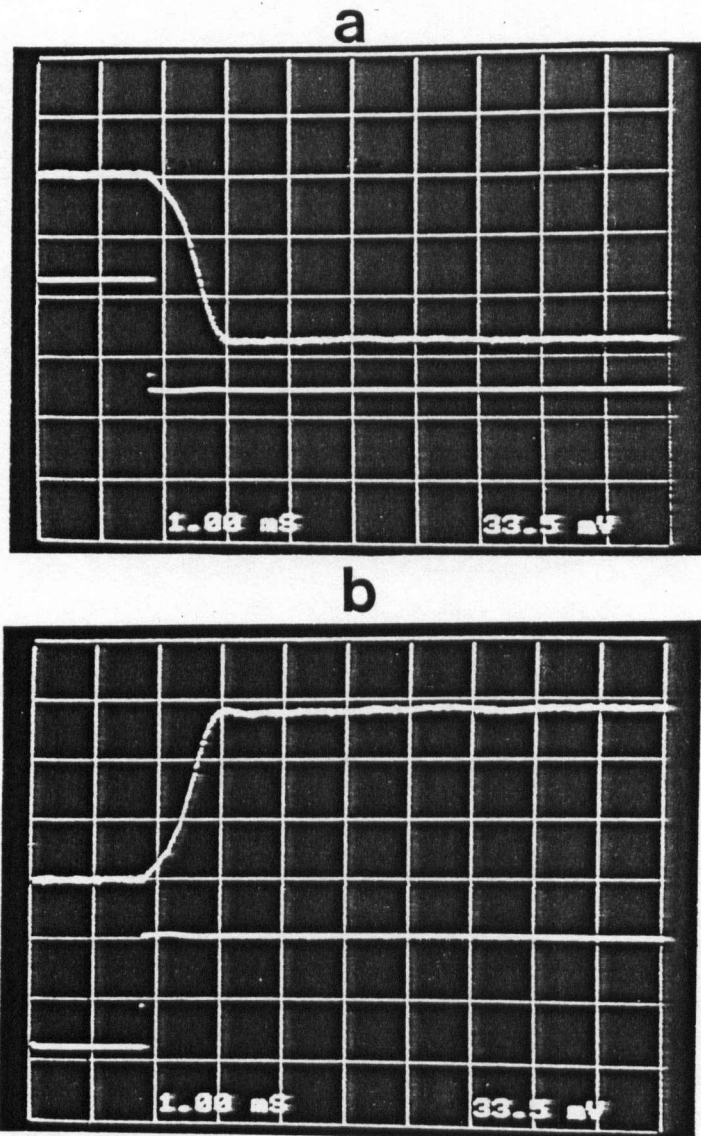the pre-made holes. The tendons distal to the holes were then tied

Figure 3. Response of the Shaker system (upper trace) to step inputs from the function generator (lower trace): (a) release (b) stretch. Responses are critically damped and complete within 1 ms. Time scale 1.0 ms per division. Output of Shaker 33.5 mv per division.

securely with 10-0 monofilament nylon thread onto the wires. In the remainder of the single fibre experiments aluminum foil clamps were used (Ford, Huxley and Simmons, 1977). The clamps were T-shaped with a hole in the tail of the T. Under 40x magnification, the two arms of the T were folded over and firmly clamped onto the tendon of the fibre close to the myotendinous junction. The preparation was then transferred to the experimental set-up and the hole in the tail of the T-shaped clamp was placed over the wires connected to the force transducer and the motor. The sides of the tail of the clamp were then firmly squeezed around the hook in the wires to ensure that the clamps would not move during the course of the experiment.

The experimental chamber in this set-up was horizontally oriented. The chamber was made of hardened aluminum, coated with teflon. It consisted of two plates between which were sandwiched two thermoelectric modules used to maintained the chamber at a constant pre-set temperature. The temperature for most experiments was less than 1° C however in some of the later experiments the temperature was $3-4^{\circ}$C. The chamber contained a double glazed floor which allowed light to pass through the muscle fibre so that sarcomere length could be measured by laser diffraction or photomicroscopy (described below). The sides of the chamber contained two platinum plate electrodes which allowed maximal transverse stimulation along the whole length of the muscle preparation.

Figure 4 shows the response of the Cambridge servo motor to a step input. The response was critically damped and complete within 500 µs. The shortest step duration possible with no overshoot was 350 µs however, 500 µs was used as the resonant frequency of the force transducer was only 2 KHz. The stiffness of the servo system was 50.0 g/µm.

The force transducer and motor were mounted on micromanipulators
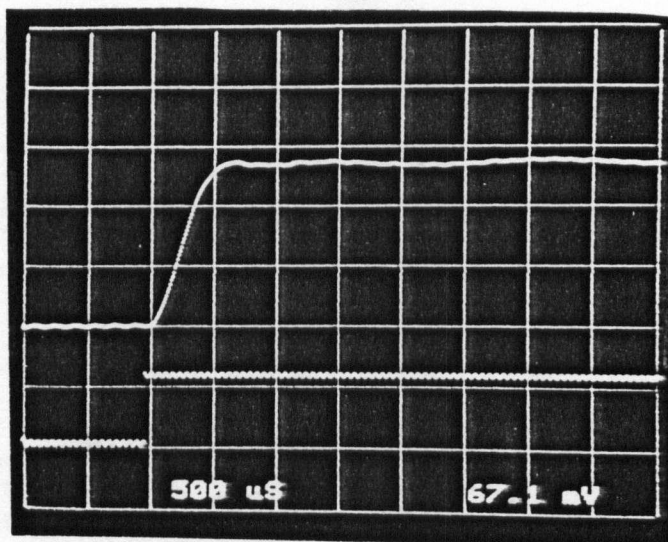
a
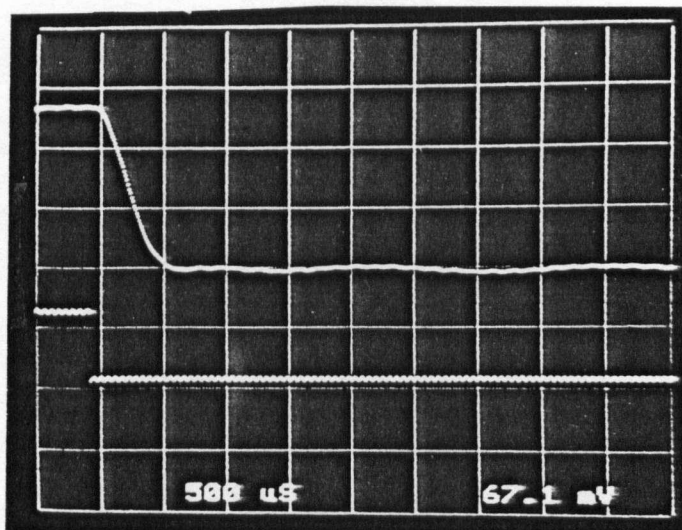
b

Figure 4. Response of the Cambridge system (upper trace) to step inputs from the function generator (lower trace) (a) release (b) stretch. Responses are critically damped and complete within 500 μs. Time scale 500 μs per division. Output of motor 67.1 mv per division.

which were used for fine adjustment of fibre length and position. The whole system (micromanipulators and experimental chamber) was mounted on the stage of a Leitz microscope (figure 5). The microscope had been modified so that sarcomere length can be measured by laser diffraction or photomicroscopy.

Sarcomere length measurements

As the functional unit of striated muscle, the sarcomere consists of alternating A-bands and I-bands of dissimilar refractive indices, and are in constant register, they act as a phase diffraction grating, so that when laser light was shone through muscle a diffraction pattern is observed. The distance from the zero to the first order intensity maxima of the diffraction pattern represented the sarcomere length. A 5mW Helium-Neon laser was rigidly mounted to the light port of the microscope base (figure 5). The beam was then deflected via a mirror, through the glass floor of the experimental chamber, and onto the muscle fibre. The resultant first and zero order of the diffraction pattern were collected in a 4x convex lens, passed through the nosepiece of the microscope and was then condensed via a cylindrical lens. The zero order was focussed onto a phototransistor and the first order onto a photodiode array (Fairchild CCD 143 High Speed Linear Image Sensor) consisting of 1024 elements. The output of this sensor was then analyzed by a computer which compared the distance from the zero order to the mean position of the first order and gave a digital readout of the static sarcomere length. This system is a modification of that of Iwazumi and Pollack (1979).

For photomicroscopy, the laser sensor attachment was removed from the microscope and replaced by the photography attachment (figure 6a).

Figure 5. The Cambridge servo-system. The force transducer and motor are mounted on brass plates attached to micromanipulators (3-way positioners). This arrangement allows fine adjustment of fibre length and position. The micromanipulators and experimental chamber are mounted on a large brass plate. This plate sits on the microscope stage and can be moved in the X and Y planes so as to scan the length and width of the fibre when measuring sarcomere length. The plate is removable so that fibres may be attached to the motor and force transducer with the aid of the dissecting microscope. Ice water is circulated through ports on either side of the experimental chamber, acting as a heat sink. The temperature is monitored by a calibrated thermistor probe mounted just outside the chamber (represented as large black dot). The laser diffraction attachment is shown in this figure (see sarcomere length measurement section for details). Illustration by Bruce Stewart (Biomedical Communications, UBC).
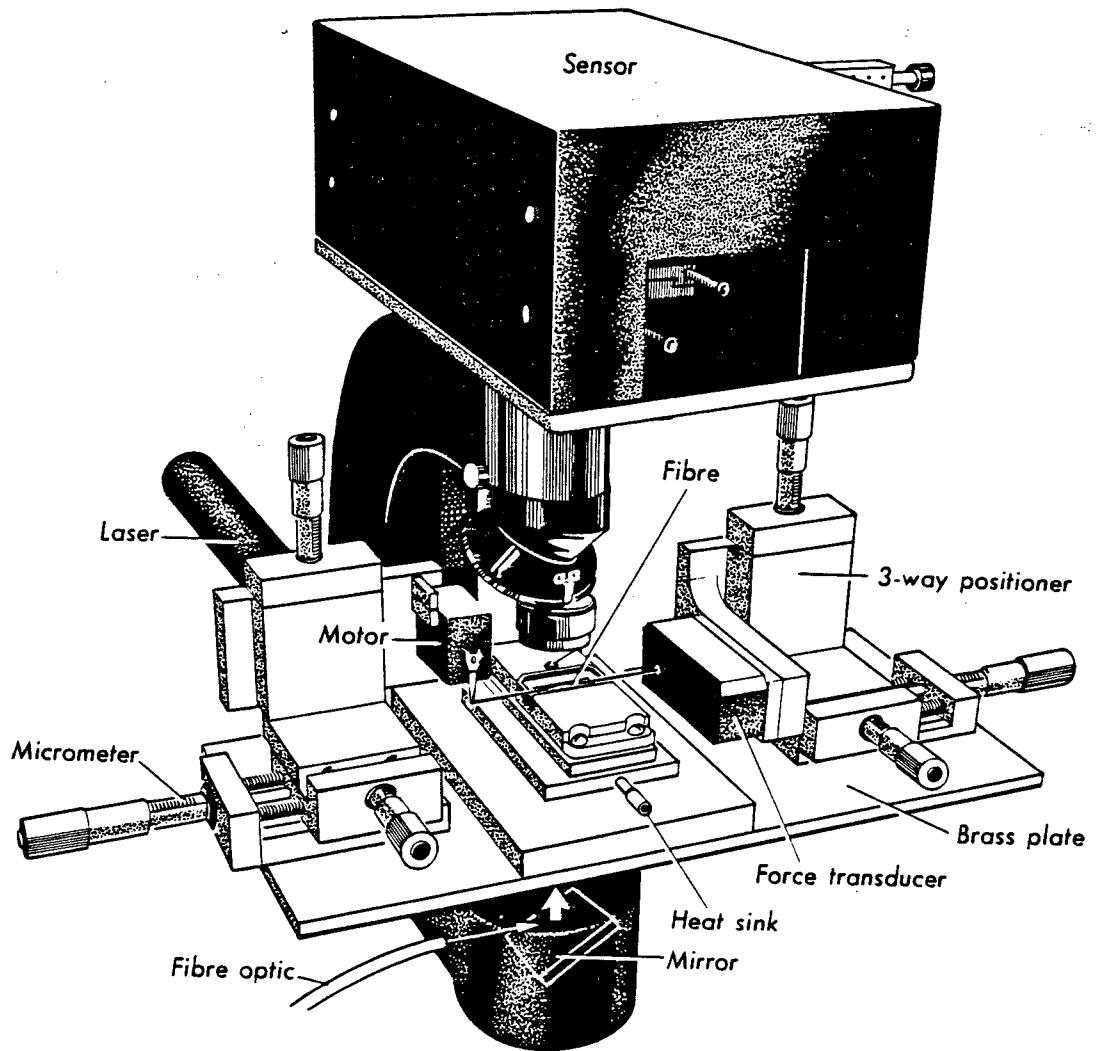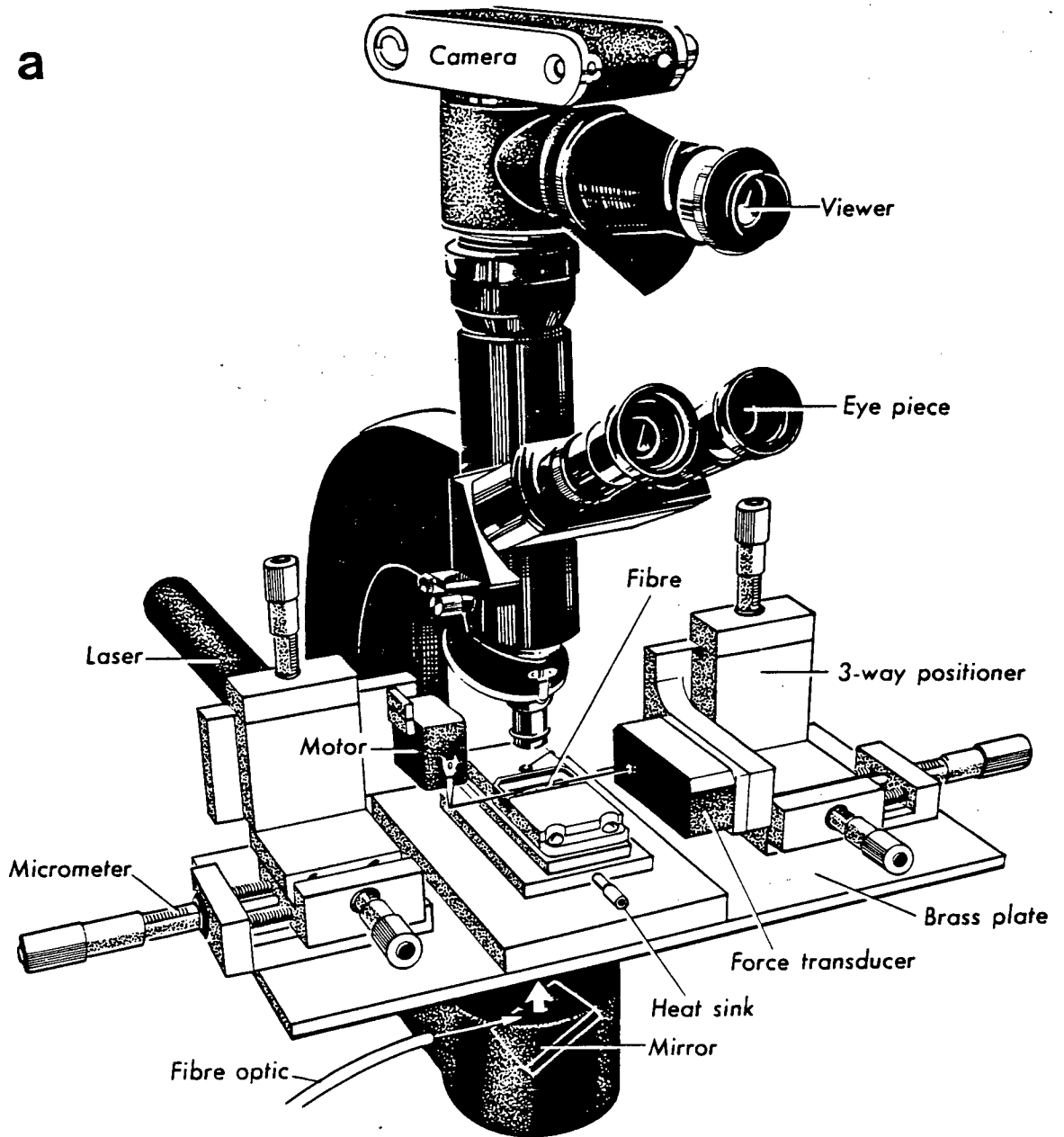
Light from a fibre optic source (Volpi, 150 H) was reflected by a mirror mounted below the stage. The muscle was observed and the image focussed through the viewer and a 10x objective lens. Five areas of the fibre were photographed: the motor end, between the motor and centre, the centre, between the centre and the force transducer, and the force transducer end. In addition a calibrated diffraction grating was photographed. Sarcomere spacing was obtained from prints of the various areas of the fibre (figure 6b) by direct comparison with a print of the diffraction grating. The sarcomere spacing of the fibre was taken as the average of the sarcomere spacings of the five areas photographed.

Experimental procedure

Small fibre bundles or single fibres were prepared and mounted for stimulation and isometric tension recording as described above. Throughout an experiment the preparation was stimulated at regular 90 second intervals with a regime consisting of 3 twitches followed by a tetanus. The stimulus was a supramaximal square wave of 1 ms duration. The optimum stimulus voltage was determined by noting the voltage which produced the maximum twitch tension and adding 1 volt to this value. The frequency and duration of stimulation for tetani were determined in each experiment so as to obtain a fused isometric tetanus. All experiments were carried out at lo, the length at which the maximum isometric twitch was recorded. At the end of every experiment, the total length of the muscle preparation between the knots securing the tendons was measured with fine calipers.

Throughout the experiment representative twitches and tetani were recorded in order to ensure that there was no significant decline in isometric tension.

a

Camera

Viewer

Eye piece

Fibre

3-way positioner

Laser

Motor

Micrometer

Brass plate

Force transducer

Heat sink

Mirror

Fibre optic

- 48 -

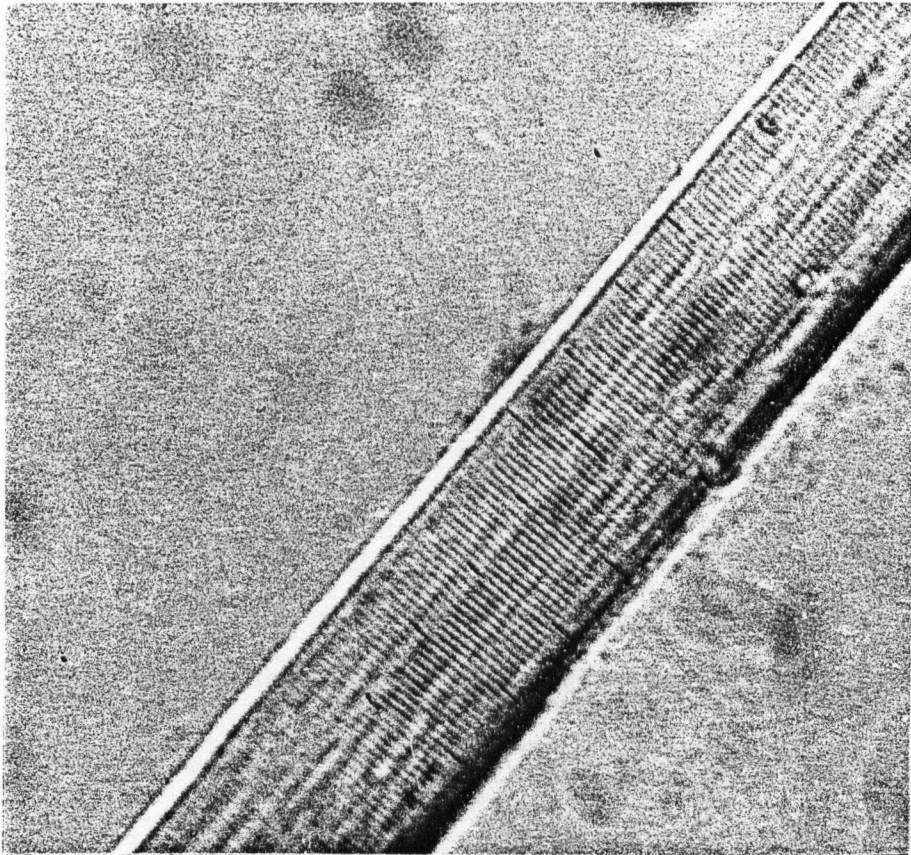Figure 6. Modification of the Cambridge system for photomicroscopy. (a)Same as figure 5 except the laser diffraction attachment has been replaced by the photomicroscopy attachment. (b)Representative photograph of central segment of a fibre. Experiment of August 4, 1983, sarcomere length, 2.15 μm. Illustration in (a) by Bruce Stewart (Biomedical Communications, UBC).
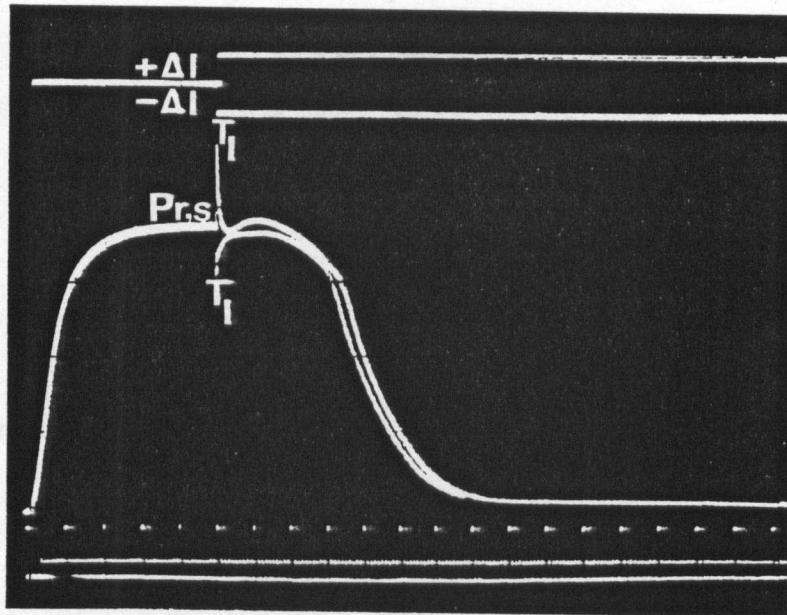
The instantaneous stiffness measurements were obtained with two different methods. In experiments using the Shaker system tension-extension curves were obtained by imposing a length step (1 ms duration) on an isometrically contracting muscle at 25, 50, or 100 ms time intervals throughout a twitch or a tetanus. The corresponding analog outputs of the length and tension transducers were digitized in an analog-to-digital converter at the rate of 50 µs/address. Force values were then normalized with respect to the maximum tetanic force (Po) and the changes in length were expressed as a fractional change of the body length (lo). Plotting force against the change in length produced a tension-extension curve. The linearity of a portion of this curve (Bressler and Clinch, 1974) permitted the use of linear regression of the points from 0.4Po to Po for releases and from Po to T1 (the extreme tension value reached) for stretches, and use the slope of the line as a measure of the instantaneous stiffness of the muscle.

In experiments using the Cambridge servo-system the length change was complete in 500 µs and therefore the A-D converter did not provide sufficient resolution to record the points directly. For these experiments stiffness values were obtained by comparing the ratio of the tension change (ΔP) to the length change (Δl) as read from the oscilloscope records (figure 7). In addition it was possible to read values directly from the Nicolet digital oscilloscope when it became available. All stiffness values were expressed in terms of the maximum stiffness measured at the plateau of an isometric tetanus.

The amplitude of the length changes throughout the experiments were less than 6 nm/half-sarcomere, or less than 0.5% of the length of the muscle preparation. Small amplitudes were chosen so as not to move the fibre along its tension-length curve during the shortening or lengthening

Figure 7. Representative film records of tension and length changes at the plateau of an isometric tetanus (a) on a slow time scale, 100 ms between upper row of time dots and (b) on an expanded time scale, 1 ms per division. In (a) length is the upper trace and tension is the lower trace, while in (b) tension is the upper trace and length is the lower trace. The various tension and length values read from film records are : Pr,s, the tension value immediately preceding the tension change, T1, the extreme tension value, ΔP, the change in tension, T2, the rapid early recovery tension level and Δl, the amplitude of length change. The tension values were normalized with respect to Po and the length values were normalized with respect to lo. Stiffness (S) was calculated as the change in tension for a given change in length, $\Delta P/\Delta l$ or $\Delta P/(\Delta l/lo)$. S values were normalized with respect to So, stiffness values obtained at the last shock of a tetanus with releases of the same amplitude. The length change was converted to nanometers/half-sarcomere (nm/h-s) by multiplying $\Delta l/lo$ by the sarcomere length /half-sarcomere (nm).

a

b
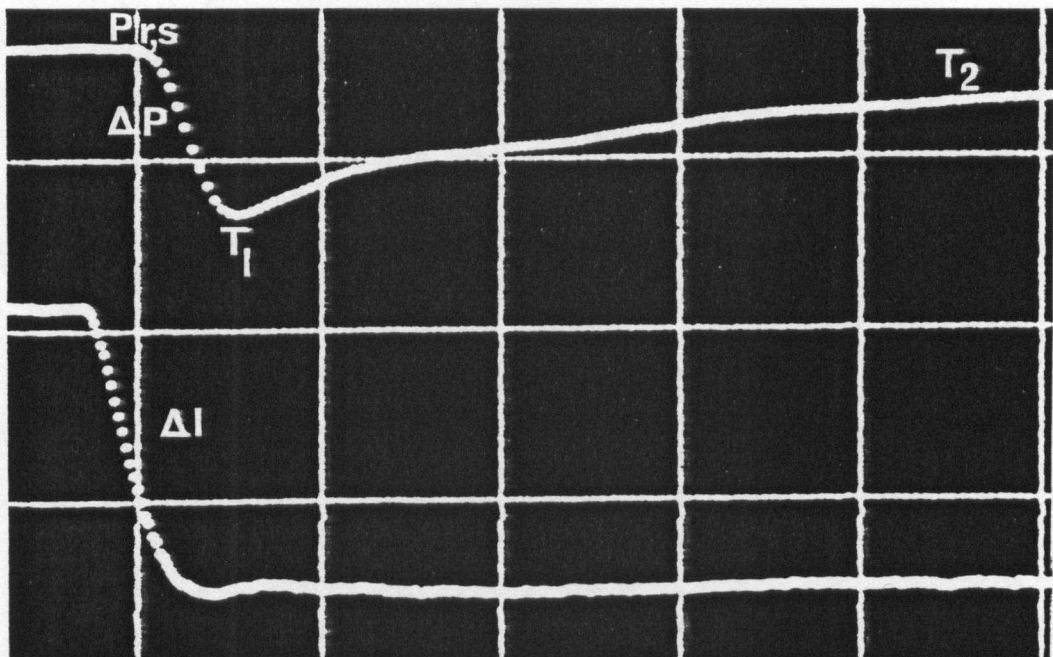
step. Small amplitudes of length change are desirable when comparing the difference in instantaneous stiffness measured with release versus stretch. The rate constant of the rapid early recovery phase (phase 2) of the tension transient increases as the amplitude of the release increases. With stretch, this rate constant decreases as the amplitude of the length change increases (Huxley and Simmons, 1971b). Theoretically, this would result in an underestimation of the instantaneous tension response (phase 1) to a release as the rapid early recovery phase would be taking place at a faster rate than in a stretch and would truncate part of phase 1 (see Discussion).

Technical considerations and corrections

The information that was desired from the mechanical experiments described in this thesis was the elastic properties for an average, yet ideal cross-bridge, that is, the relationship between tension and length. However, several complicating factors existed due to dynamic effects of the experimental system. These factors were (a) the inherent response characteristics of the force transducer, (b) series elastic compliance in the tendons and attachments to the motor and force transducer, and (c) fibre inertia and friction forces retarding the movement of the fibre.

a) Force transducer response

It was necessary to determine to what extent the tension signal reflected the actual behavior of the muscle and how much was due to the inherent characteristics of the transducer itself. This may be determined by measuring the response time and damping of the force transducer during rapid length changes by a technique originally

described by Ford, Huxley and Simmons (1977).

A small piece of 10-0 monofilament nylon thread with a loop at each end was attached to the wire hooks on the motor arm and force transducer. A large release was then given to the thread so that it went slack and the loop lost contact with the transducer hook. From a time just before the first minimum in the output signal, the force transducer was completely unloaded and should have performed a damped oscillation at its natural frequency about the level corresponding to zero tension. The oscillations should have decayed exponentially with a specific time constant. However, complications in determining the resonant frequency and damping time constant arose due to a superimposed resonance of a different frequency believed to be due to the glass capillary tube which extended from the moving part of the force transducer and contained the wire to which muscle preparations were attached. Since the force transducer response during free oscillation appeared to be the sum of a damped sinusoidal oscillation due to the capillary tube and another damped sinusoidal oscillation due to the moving part of the force transducer itself, the damped oscillation due to the capillary tube was subtracted from the transducer signal. An exponentially decaying sinusoidal oscillation with the same frequency as that of the capillary tube was calculated and subtracted. The subtracted record still appeared to be composed of more than one oscillation so a second oscillation was calculated based on parameters from the difference record. This oscillation was then subtracted from the difference record. This correction routine resulted in a final record with a resonant frequency of 2 KHz. The damping time constant could not be determined and was assumed to be 3 periods (Ford et al, 1977), or 1500 μs.

Representative tension records have been corrected for the force

transducer response using the equation of Ford et al (1977) :

$$Tc = [X - \frac{2(mt/kt)X}{(h)^2}] + [\frac{(bt/kt)Y}{2h} + \frac{(mt/kt)Y}{(h)^2}] + [\frac{-(bt/kt)Z}{2h} + \frac{(mt/kt)Z}{(h)^2}]$$

where $mt/kt = 1/[(2\ pi\ v)^2 + (1/t)^2]$ and $bt/kt = (2/t) \times (mt/kt)$.

The values in these equations are : Tc is corrected tension, X is the tension point to be corrected, Y is the tension point following X, Z is the tension point preceding X, h is the time interval between tension points, v is the resonant frequency of the force transducer and t is the transducer's damping time constant.

Figure 8 shows a corrected record of a stretch and a release. Note that the correction changed both records in the same direction and did not eliminate the difference in tension change observed with stretch versus release.

b)Series elasticity

It was necessary to determine to what extent the tension response reflected the actual response of the muscle and how much was due to the tendons and the attachment of the muscle to the motor and the force transducer. Since the attachments were not ideally rigid this led to the problem that the applied length change was equal to the length change of the tendons and attachments plus the length change of the muscle. The amount of length change due to the tendons and attachments was minimized in the experiments reported on in this thesis by using stiff attachments and reducing the amount of tendon present by placing the attachments very close to the myotendinous junction. This reduced stray series compliance. Other investigators (eg. Gordon, Huxley and Julian, 1966) eliminated series compliance in their preparations using segment length clamps. However, it is possible to measure the compliance and correct
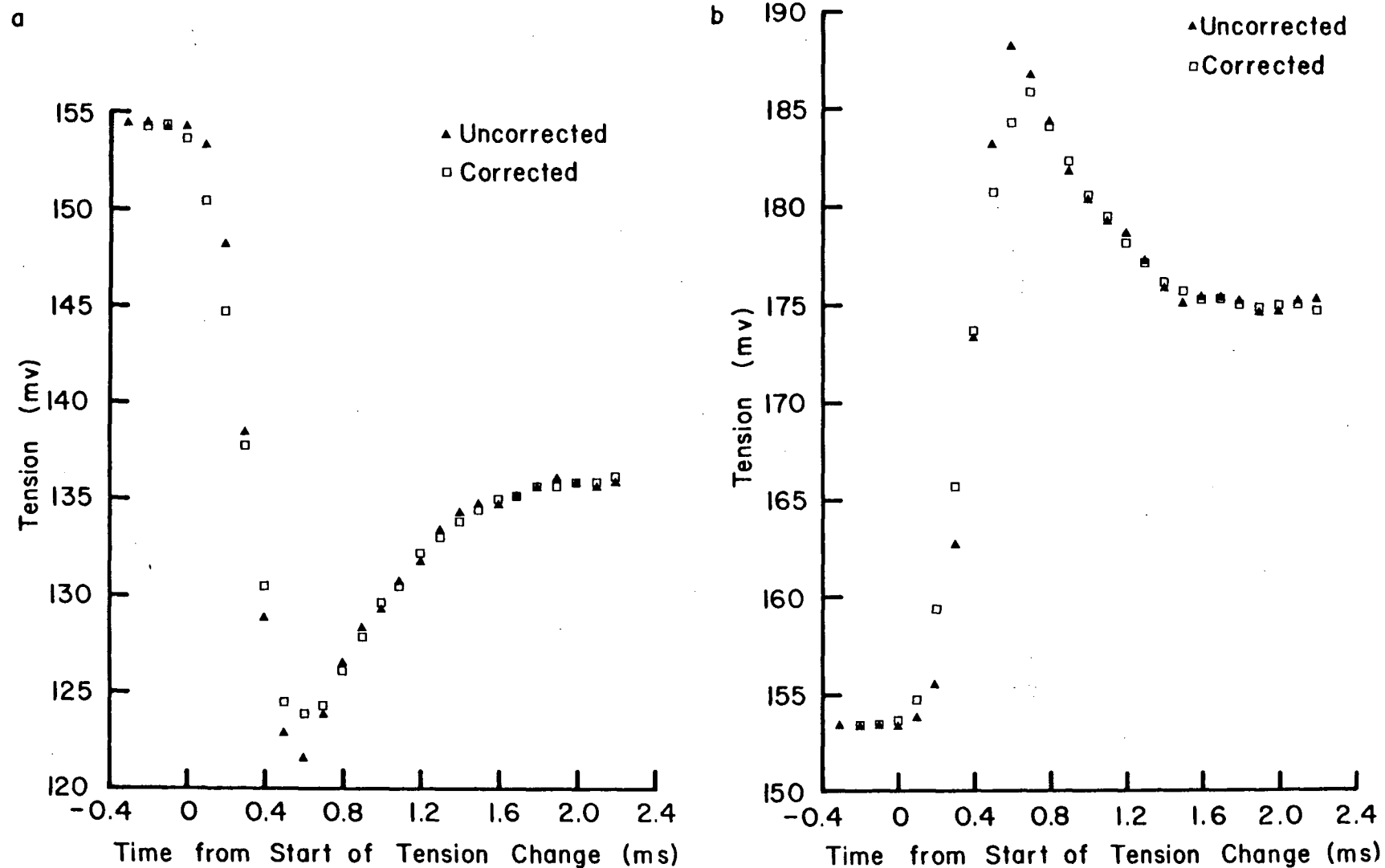
Figure 8.   Representative tension transients corrected for response of force
transducer (a) release (b) stretch.   Records from experiment of July 26, 1983.

for it. When this was done by Bressler and Clinch (1974), compliance was found to be negligible in their preparations. Although a contribution from the attachments and tendons can not be ruled out in the preparations used in this study, their contribution to the results obtained was believed to be minimal.

c) Fibre inertia and friction

It was necessary to determine to what extent the tension changes measured by the force transducer were due to changes in the muscle and how much was due to the effects of inertia and frictional forces. The effect of inertia was that it took a certain amount of time for the applied length change to be propagated down the fibre. Consequently, the motor end of the preparation received the length change and produced a tension change before a similar segment near the force transducer responded, so that the tension signal from the force transducer was the sum of the tension changes along the length of the fibre. The effect of friction was to oppose the force produced by the cross-bridges by drag due to the surrounding fluid and internal viscosity of the fibre. When a length change was applied to the fibre, fluid drag opposed the initial movement of the fibre. The continuing motion of the fibre during the length change caused the fluid adjacent to the fibre to move with it. When the length change ceased and the fibre stopped moving, it was dragged towards the force transducer by the continuing movement of the fluid. When the muscle was stimulated, the filaments had to displace water as they interdigitated and the internal viscosity of the fibre resulted in the production of frictional forces.

Tension records may be corrected for the effects of inertia and friction using the equations of Ford et al (1977). These investigators
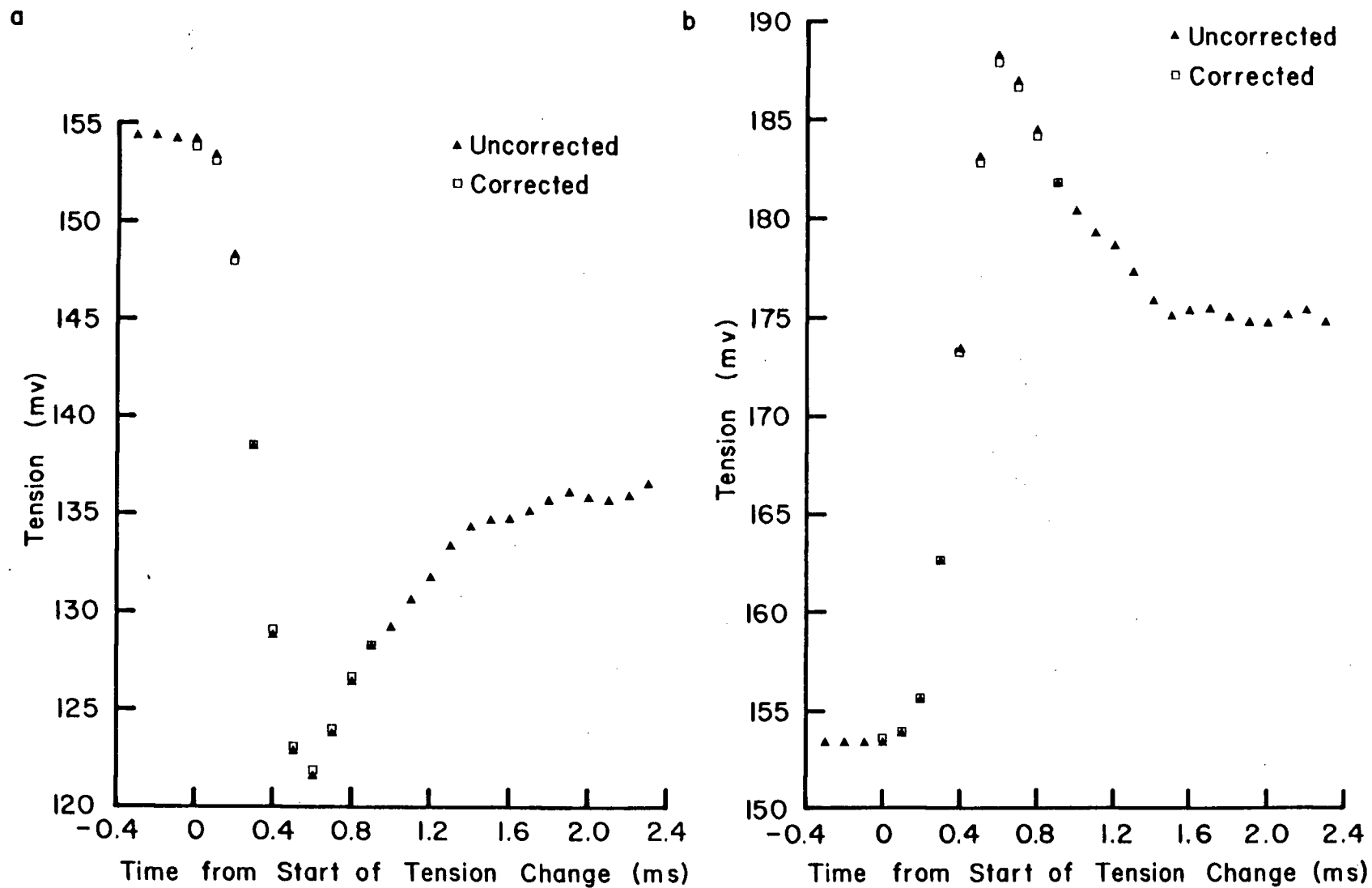
Figure 9. Representative tension transients corrected for inertia (a)release
(b)stretch. Records from experiment of July 26, 1983.

found that when they corrected tension records for the effects of friction, the largest correction value obtained was about 0.5% of the total tension change. Since most of their frictional forces were due to fluid drag from markers on the surface of their fibres and these were not used in this study, friction would have had a smaller effect in our preparations and therefore this correction was not performed.

Representative tension records were corrected for the effect of inertia using the equation:

$$\Delta T = (pi/d)^2 \ Wf \ Nt \ (Ys/6) \ (1 + 2c) \ sin \ (pi/d \ x \ t)$$

where ΔT is the change in tension due to inertia, d is the duration of the length change, Wf is the weight of the fibre, Nt is the total number of sarcomeres in the fibre, Ys is the change of length per half-sarcomere at the end of the length step, c is the tendon compliance as a fraction of the fibre compliance, and t is time. The value used for c was taken from Ford et al (1977) as it was not measured in any experiments. It was found that the inertial correction had little effect on tension records (figure 9). Furthermore, the correction had the same effect on records of releases and stretches and did not eliminate the difference in tension change between stretches and releases.

Inertia would have less of an effect in shorter muscle preparations, as the propagation time for an applied length change would be shorter. In order to determine whether inertia would affect the results of this study, the experimental protocol was repeated on a tibialis anterior fibre, as these fibres are approximately one-half the length of semitendinosus fibres. It was found that the difference in stiffness measured with a stretch versus a release was still present in the shorter tibialis anterior fibres.

In summary, the potential complicating technical factors in this

experimental apparatus and preparation have been identified and characterized. In particular, no differential effect of these factors on the measured stiffness in stretches versus releases has been found.

IV.   RESULTS

In figure 10 are original records from experiments both in small muscle bundles and single semitendinosus fibres which were given length steps complete in 1 ms (figure 10a) or 500 μs (figure 10b). In (a) the lower trace is tension and the upper trace is length while in (b) the lower trace is length and the upper trace is tension. The characteristic tension transients first observed by Armstrong, Huxley and Julian (1966) may be seen. The tension response consists of four phases (Huxley and Simmons, 1970a) : a) phase 1, a rapid change in tension to an extreme level, T1, that occurs simultaneously with the length change, b) phase 2, a rapid initial recovery in tension to an apparent plateau, T2, c) phase 3, a slowing or even reversal of the tension recovery and d) phase 4, a final slow recovery of tension.

In figure 11 are typical T1 and T2 curves obtained from a semitendinosus fibre measured from records similar to those in figure 10. The T1 curve is obtained by plotting the extreme tension reached during the initial length step, expressed as a fraction of the maximum tetanic tension. Extrapolation of the linear portion of this curve to the abscissa provides a theoretical measure of the size of length step that would be required to reduce the tension to zero from the plateau of an isometric tetanus. The value of 6.5 nm/half-sarcomere (nm/h-s) compares favourably with the published values of 8nm/h-s for semitendinosus fibres using 1 ms length steps (Huxley and Simmons, 1971a,b) or 4 nm/h-s for tibialis anterior fibres with steps complete in 200 μs (Ford et al, 1977). Previous work by Huxley and Simmons (1971a) and Bressler and Clinch (1974, 1975) has shown that the slope of the T1 curve provides an estimate of the stiffness of an elastic structure within the sarcomeres, most likely, the cross-bridges.

The T2 curve is obtained by plotting the tension level reached

a

-4.0   **+4.0**

b

-3.2   **+3.2**
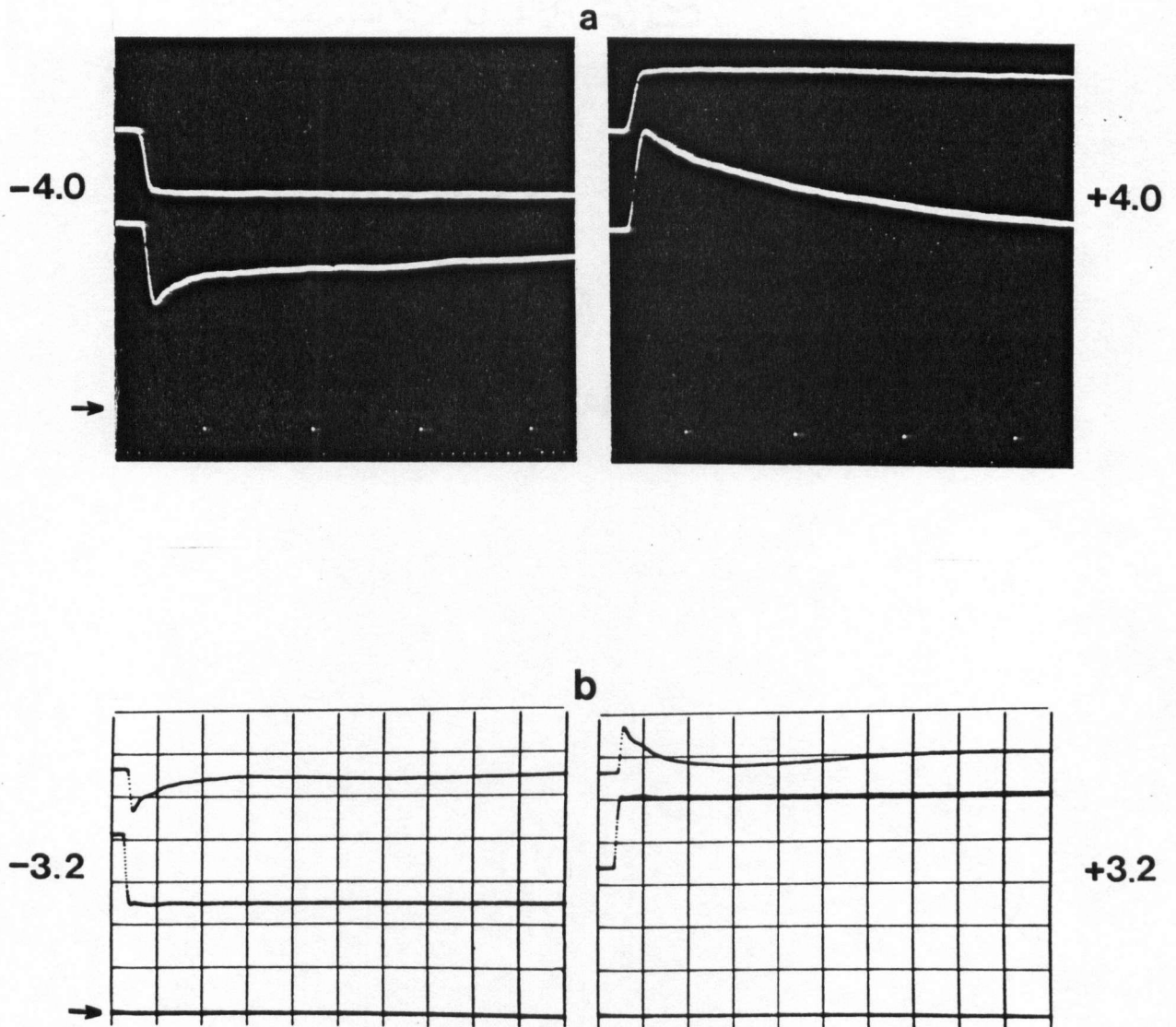
Figure 10. Original records of tension responses to step length changes
complete in (a) 1 ms and (b) 500 μs. Length changes given at last shock
of tetanus.   In (a) length is upper trace and tension is lower trace
while in (b) length is lower trace and tension is upper trace.   Tension
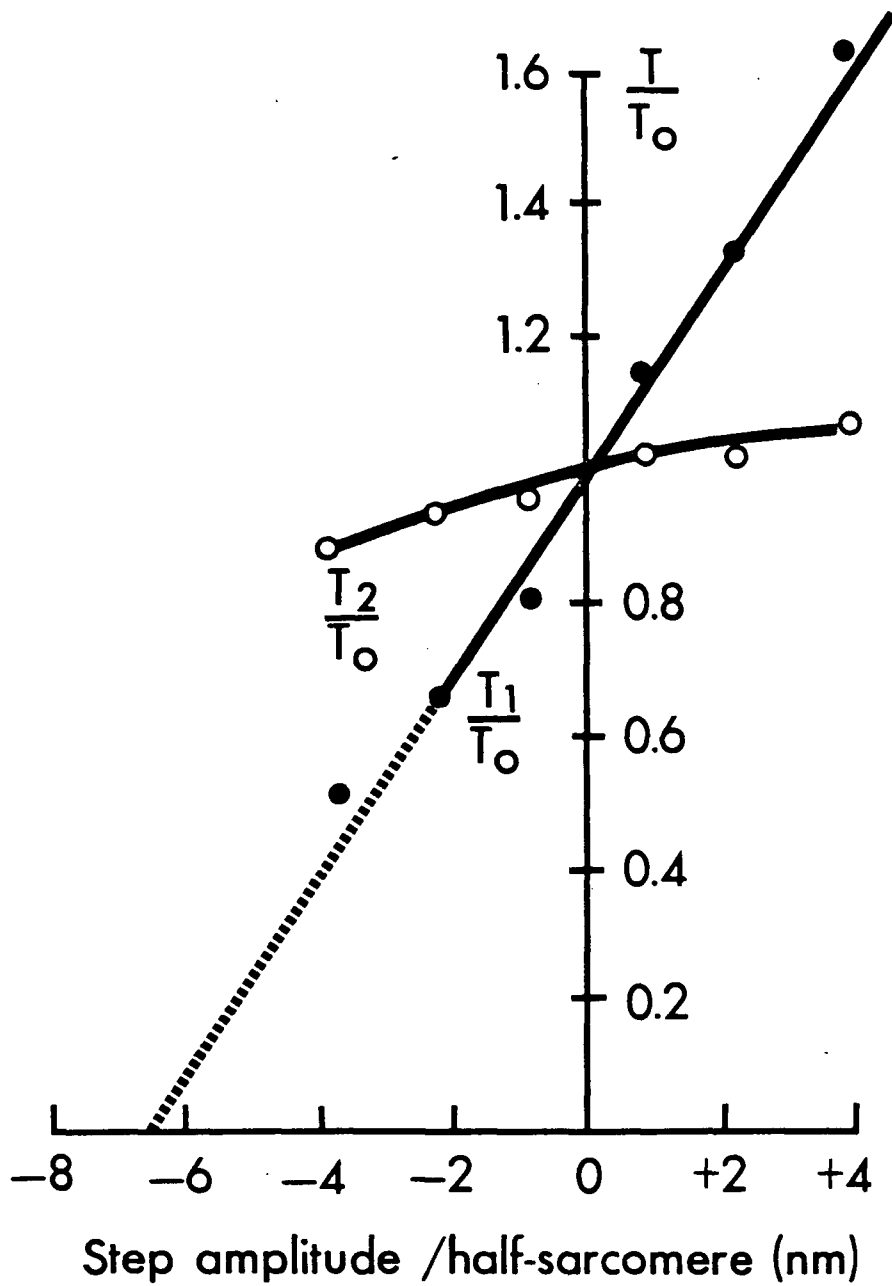baseline is indicated by arrow. Experiments of (a)December 10, 1981 and
(b)January 24, 1984.

Figure 11. Curves of T1, extreme tension, and T2, tension approached during early recovery phase, obtained with length steps of various amplitudes. Both T1 and T2 are expressed as fractions of To, the maximum isometric tetanic tension. Experiment of June 24, 1983, sarcomere length, 2.2 μm.

immediately following the initial rapid recovery phase, once again, expressed as a fraction of the maximum tetanic tension. This curve may be seen to be highly non-linear. This is due to the fact that the rate constant of the initial recovery phase varies with the amplitude of the length step, being slow for stretches and becoming faster as the size of the release increases (see discussion).

Original records of a typical experiment designed to measure the difference is stiffness between stretch and release are shown in figure 12. Tension records with a rapid release have been superimposed on (a) a twitch and (b) a tetanus with a rapid stretch. In both records, the upper trace is length and the lower trace is tension. The upper row of time dots at the bottom of the records are 100 ms apart. The length change is expressed in nanometers/half-sarcomere (nm/h-s). Near the peak of the twitch, the stretch produced a tension change that was 7.5% greater than the release. At the plateau of an isometric tetanus there was a 10.1% difference in the tension change between stretch and release.

Twitch

Figure 13 is a comparison of the pooled stiffness to tension relationship throughout the isometric twitch obtained with 4 different amplitudes of length change in 15 single fibres. Irrespective of the amplitude of the length change, a rapid stretch produced a consistently higher stiffness value than a release.

In order to determine whether the difference in the measured stiffness between stretch and release varied in different phases of the twitch, the tension immediately preceding the length step was compared to the stiffness values during the rising phase (figure 14), peak (0.95 -

a

$^+_-$ 3.7

0.49
mN
100
msec

b

$^+_-$3.7

0.49
mN
100
msec
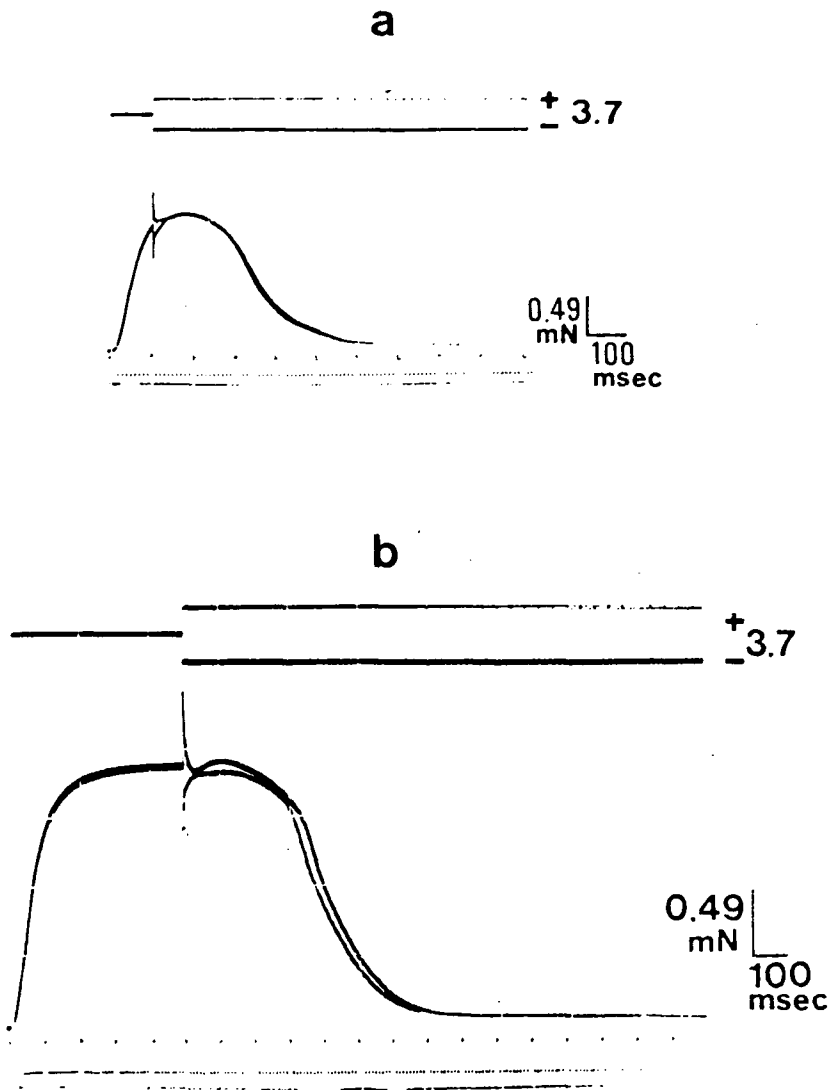
Figure 12. Original records of (a) an isometric twitch with a rapid stretch superimposed on a twitch with a rapid release and (b) an isometric tetanus with a rapid stretch superimposed on a tetanus with a rapid release. The time of the length change was (a) 150 ms after stimulation and (b) at the last shock of the tetanus. The amplitude of the length change is expressed in nm/h-s. Experiment of January 18, 1984, sarcomere length 2.27 μm, temperature 3.1°C.

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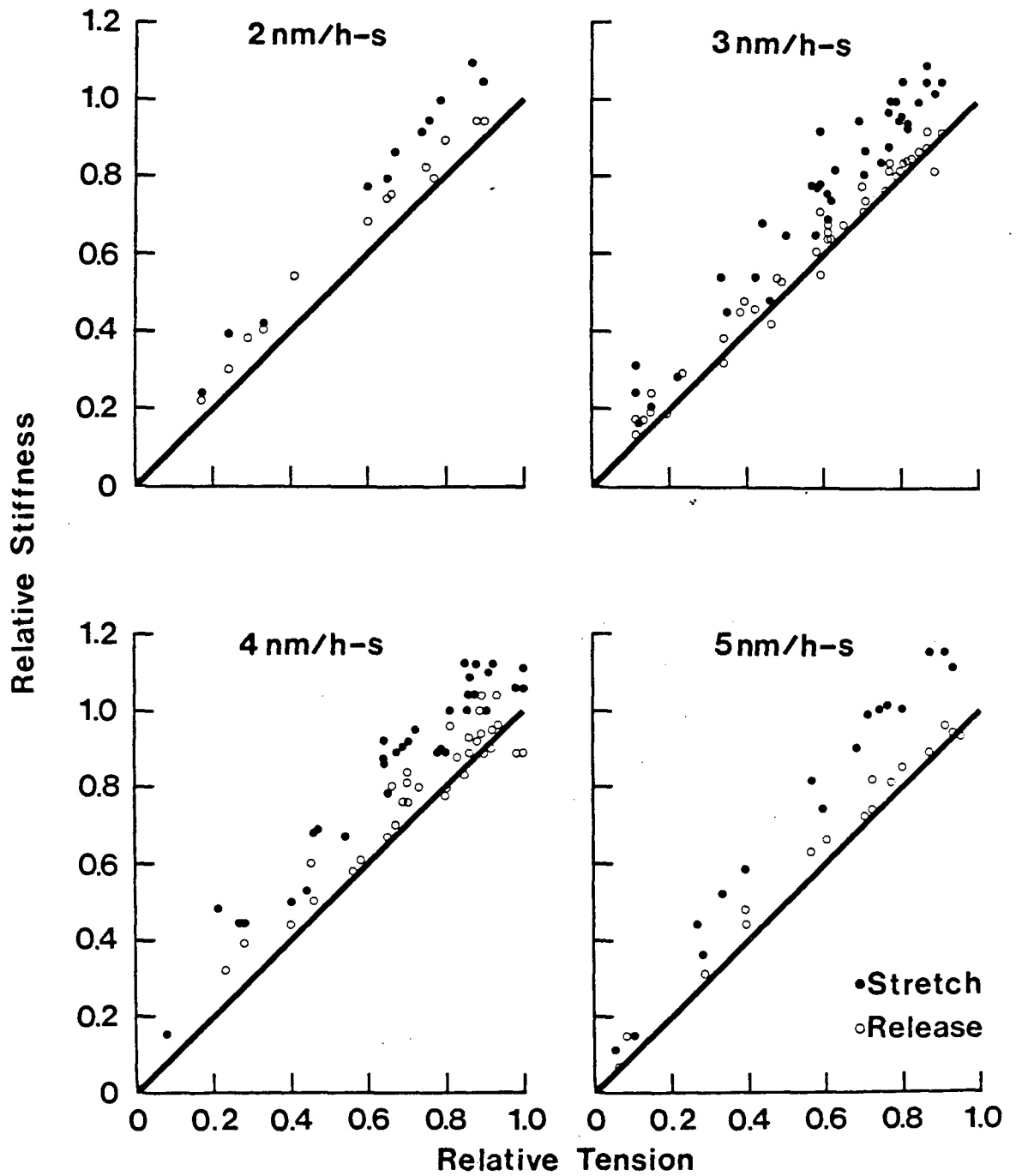
Figure 13. Comparison of tension during the isometric twitch to stiffness values obtained with 4 amplitudes of length step for 15 single fibres. All values are expressed as a fraction of the corresponding maximum values obtained at the plateau of an isometric tetanus. The solid line represents a 1:1 correlation between stiffness and tension.

Figure 14. A comparison of tension values during the rising phase of the isometric twitch to stiffness values obtained with 4 amplitudes of length change. All values expressed as fraction of the maximum values at the plateau of an isometric tetanus.

1.0 Pr,s/Pt) (figure 15) and relaxation phase (figure 16) for the 4 amplitudes seen in figure 13. It may be seen that the largest difference in stiffness measured with stretch versus release occurred during the late rising phase of tension, the peak and early in the relaxation phase.

The stiffness-tension relationship during the twitch was also investigated using muscle bundle preparations ranging in size from 2 to 20 fibres. Similar results to those in figure 13 are seen in figure 17.

Tetanus

a) Rising phase

Figure 18 is a comparison of the stiffness to tension relationship during the initial development of tension in an isometric tetanus obtained with 2 amplitudes of length change in 4 muscle bundles (a) and 2 single fibres (b). As in the twitch, a rapid stretch resulted in a higher stiffness value than a release.

Figure 19 is a comparison of the stiffness-tension relationship to time during the initial development of isometric tetanic tension from a representative experiment. In may be seen that stiffness follows the time course of tension however, it increases faster than tension during this phase of the tetanus. This supports previous studies by Bressler and Clinch (1974) and Cecchi, Griffiths, and Taylor (1982, 1984) using releases or sinusoidal oscillations respectively to measure stiffness.

b) Plateau

Figure 20 is a comparison of the stiffness to tension relationship during the plateau of an isometric tetanus obtained with 1 amplitude of length change in 1 single fibre experiment. In may be seen that the
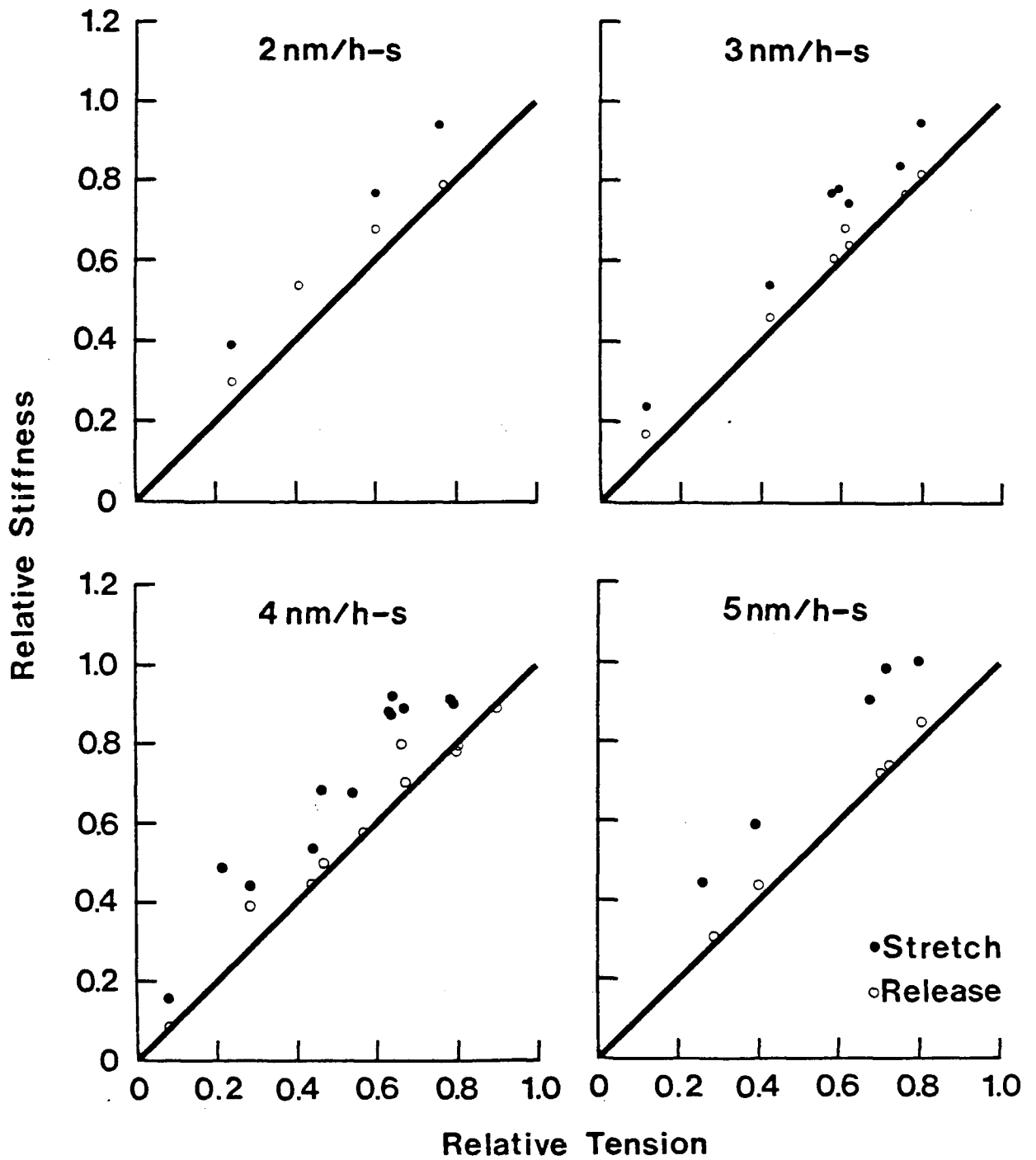
Figure 15. A comparison of tension values during the peak of the isometric twitch to stiffness values obtained with 4 amplitudes of length change. All values are expressed as a fraction of the maximum values at the plateau of an isometric tetanus.

Figure 16. A comparison of tension values during the relaxation phase of the isometric twitch to stiffness values obtained with 4 amplitudes of length change. All values are expressed as a fraction of the maximum values at the plateau of an isometric tetanus.
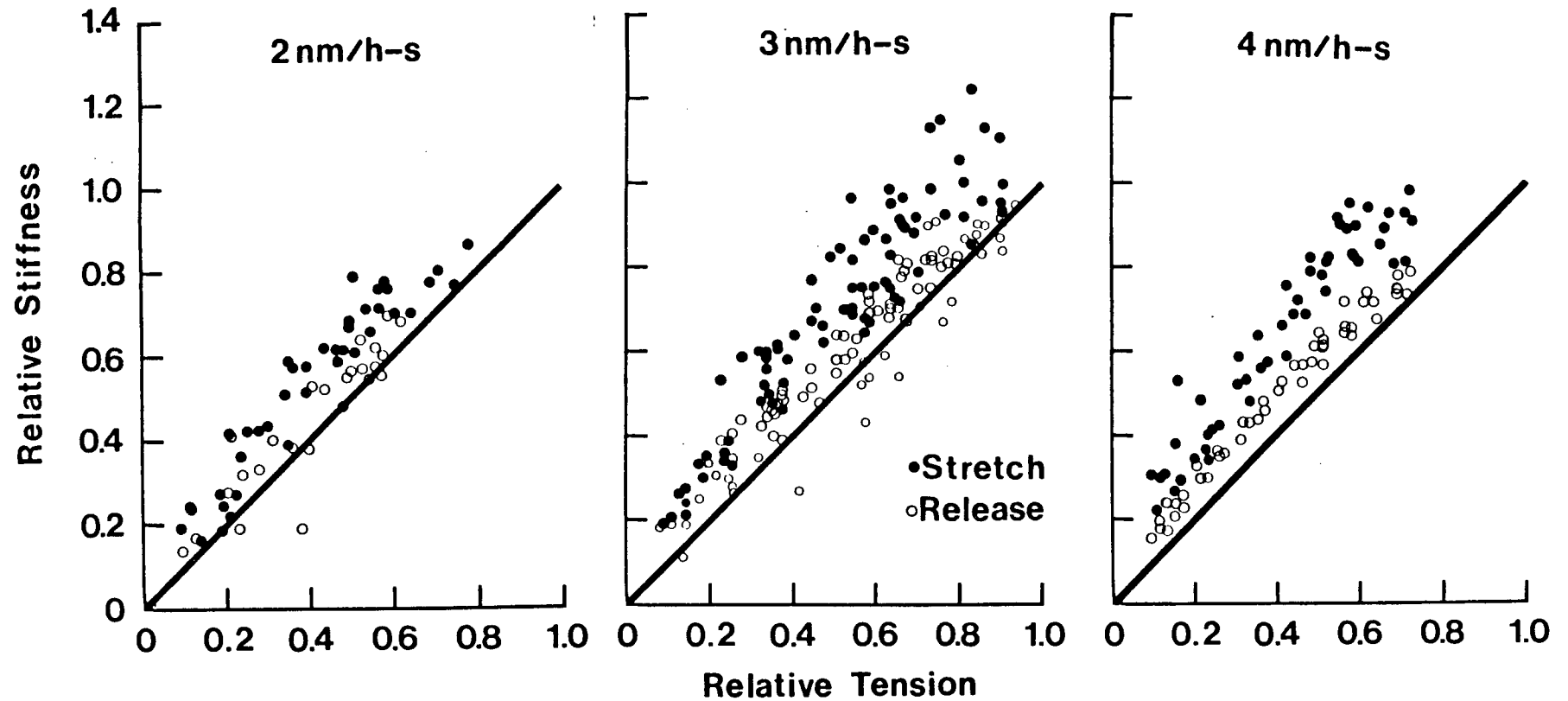
Figure 17. A comparison of tension immediately preceding the length change to stiffness values obtained with 3 amplitudes of length step for 17 fibre bundles during the isometric twitch. All values are expressed as a fraction of the corresponding maximum values obtained at the plateau of an isometric tetanus.
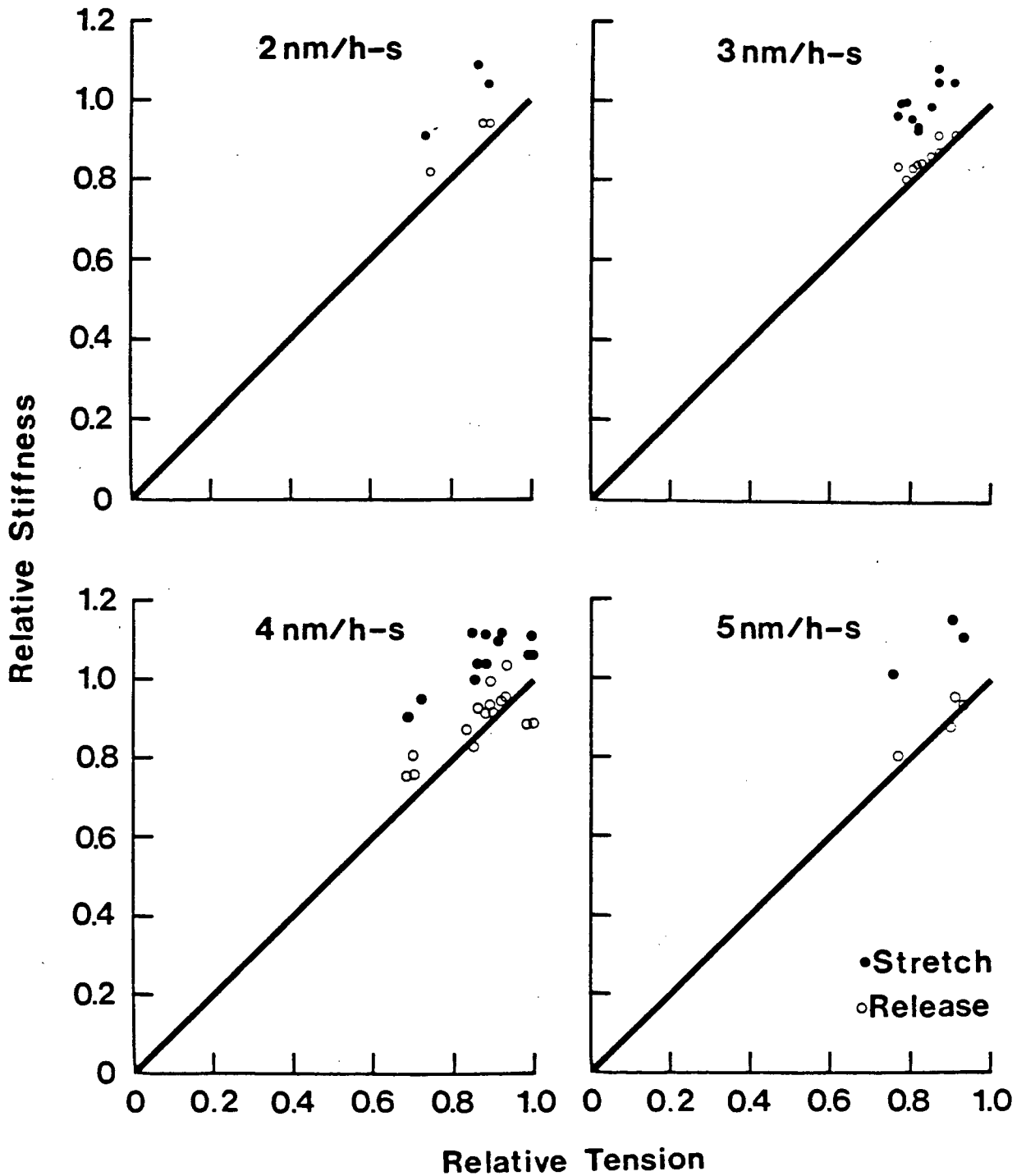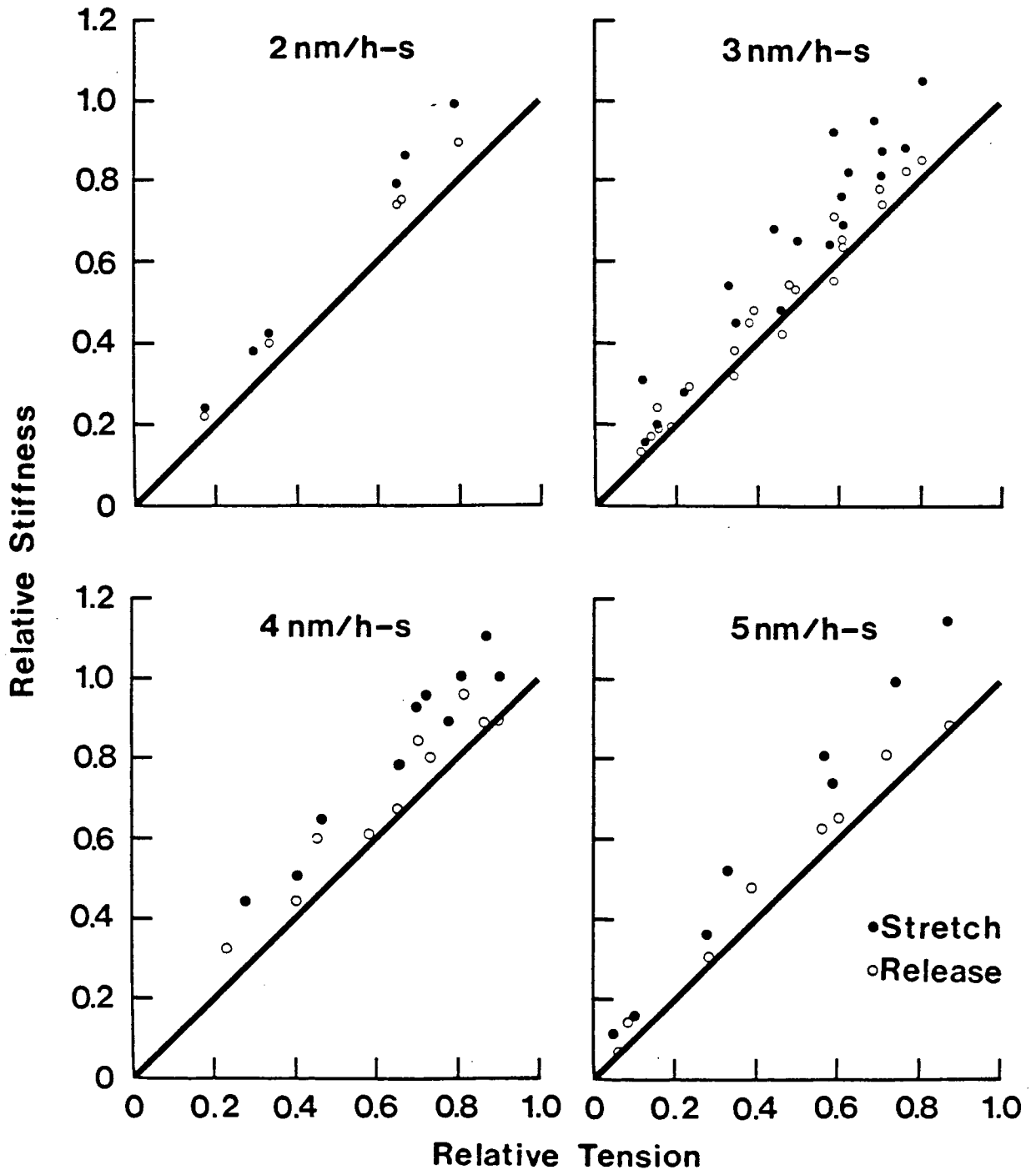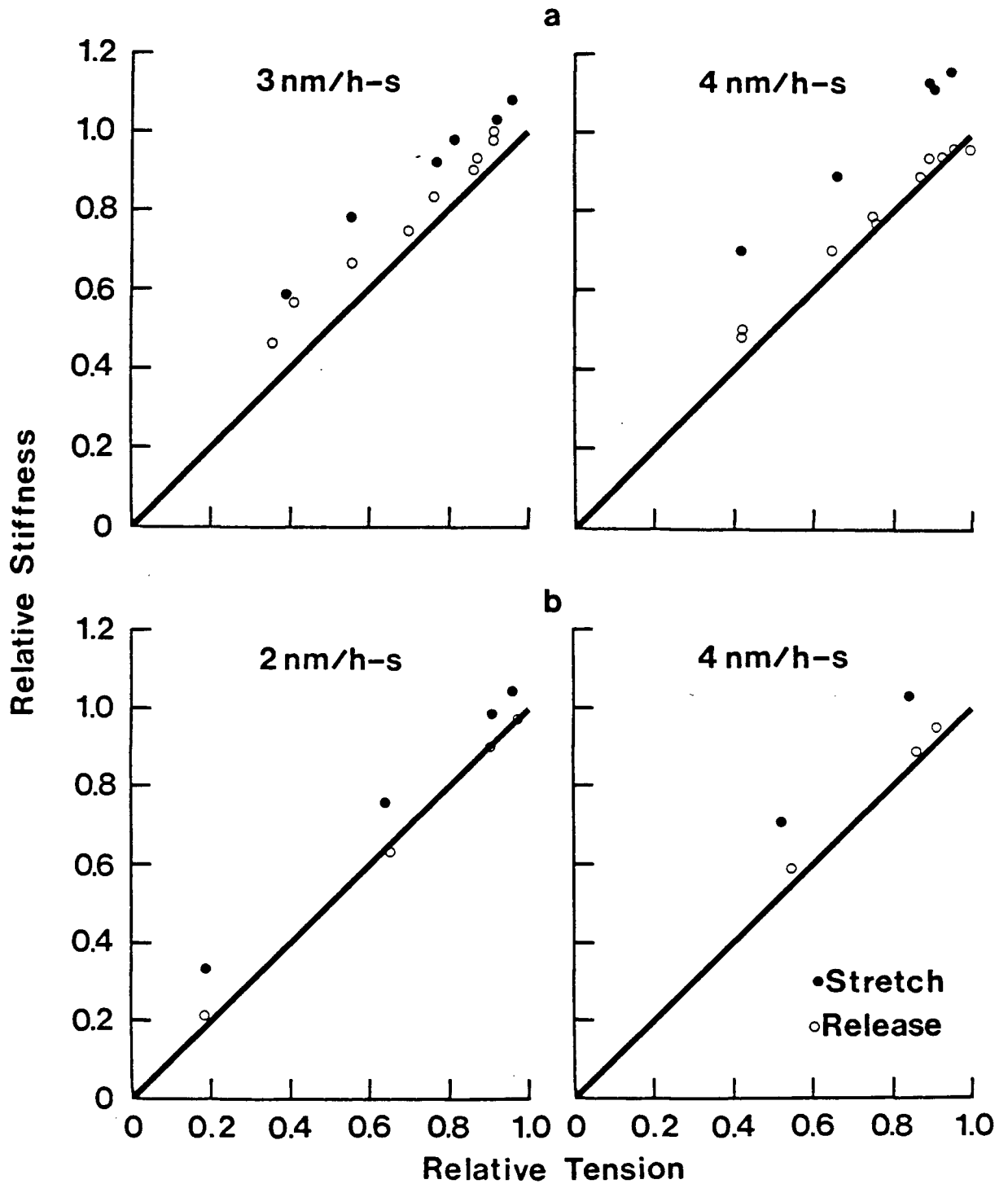
Figure 18. A comparison of tension during the rising phase of the isometric tetanus to stiffness values obtained with (a) 2 amplitudes of length change for 4 muscle bundles and (b) 2 amplitudes of length change for 2 single fibres. All values are expressed as a fraction of the maximum values obtained at the plateau of an isometric tetanus.

Figure 19. Stiffness-tension relationship during the rising phase of an isometric tetanus. Tension values are those immediately preceding the length change. Amplitude of length changes was 2nm/h-s. Experiment of October 24, 1983, sarcomere length 2.2 μm.

Figure 20. Stiffness-tension relationship during the plateau of an isometric tetanus. Tension values are from a representative tetanus. The amplitude of the length changes was 2nm/h-s. Experiment of October 24, 1983, sarcomere length 2.2 µm.

stiffness and tension values remain constant during the plateau. In addition, stiffness measured with stretch is higher than that measured with release.

c) Relaxation phase

Figure 21 is a comparison of the stiffness-tension relationship to time during the relaxation phase of the isometric tetanus from a representative experiment. It may be seen that stiffness follows the time course of the decline in tension, however it does not decrease as rapidly as tension (Dusik and Bressler, 1984). This agrees with recent results of Cecchi, Griffiths and Taylor (1984) and Shoenberg and Wells, (1984). The former group used single tibialis anterior fibres and high frequency sinusoidal oscillations to measure stiffness, while the latter group used semitendinosus muscle bundles and a transmission time technique to measure stiffness. For a short time following the last shock, tension and stiffness remain at the plateau values. During the initial stage of relaxation prior to the tension shoulder, the decline in stiffness lags the tension change. Following the shoulder, both stiffness and tension fall precipitously. The difference between stiffness values obtained with a stretch and stiffness values obtained with a release is most pronounced during the plateau and the early phase of relaxation. The magnitude of this difference in stiffness begins to decrease in the region of the shoulder and continues to decrease as relaxation proceeds.

Figure 22 is a comparison of the pooled stiffness to tension relationship during the relaxation phase of the isometric tetanus obtained with 3 amplitudes of length change in 10 single fibres. Again, it may be seen that a rapid stretch produced a consistently higher
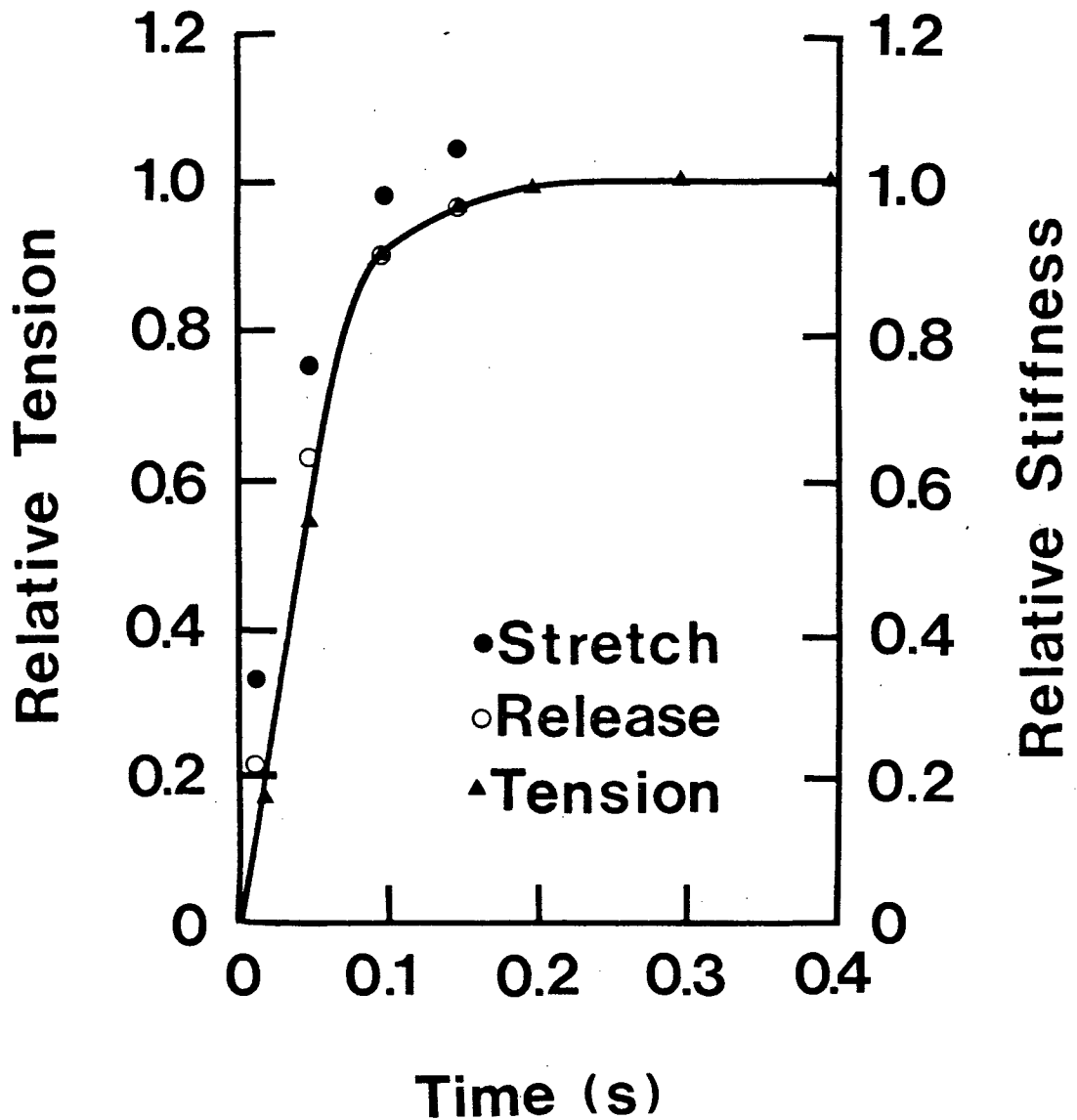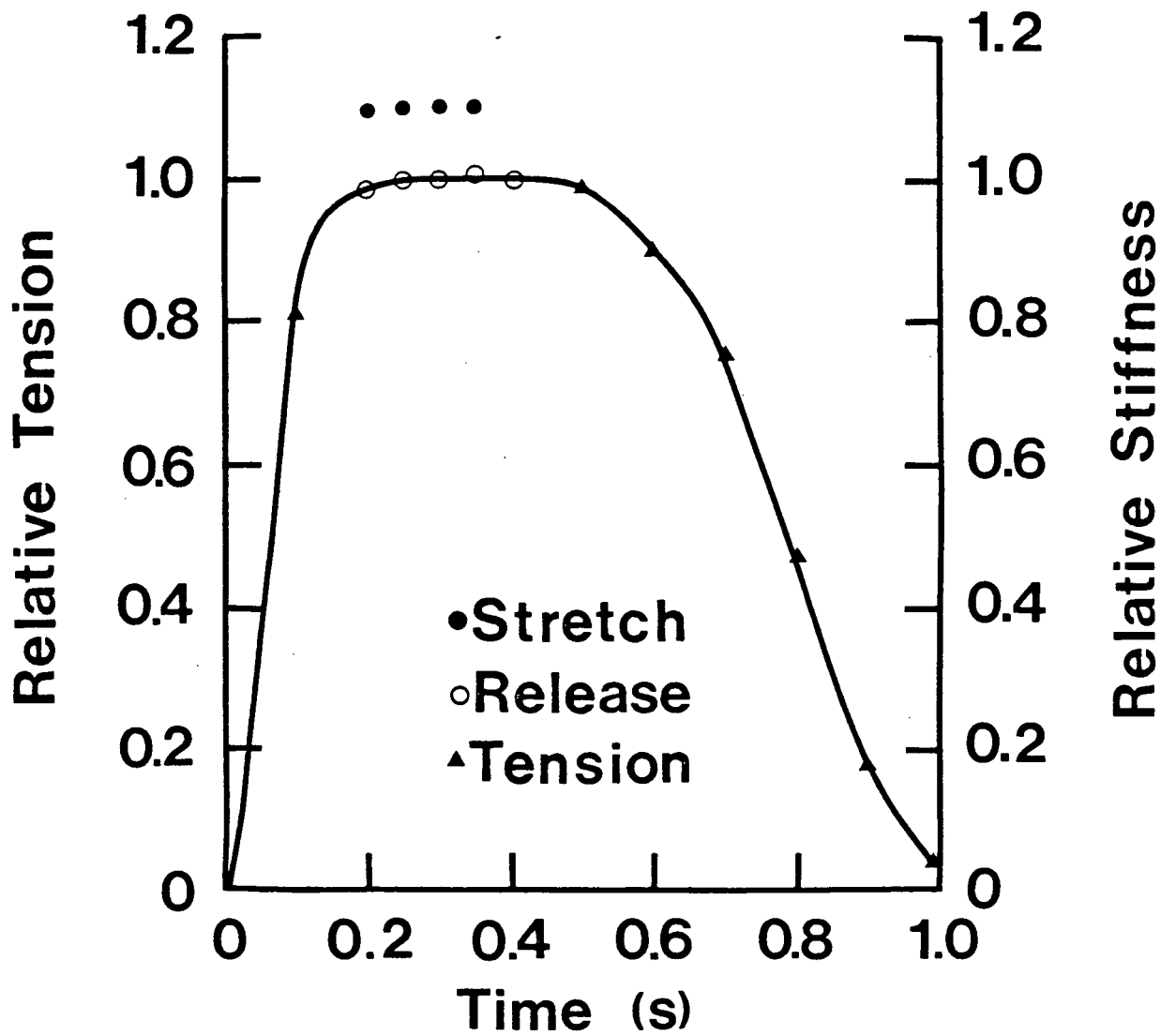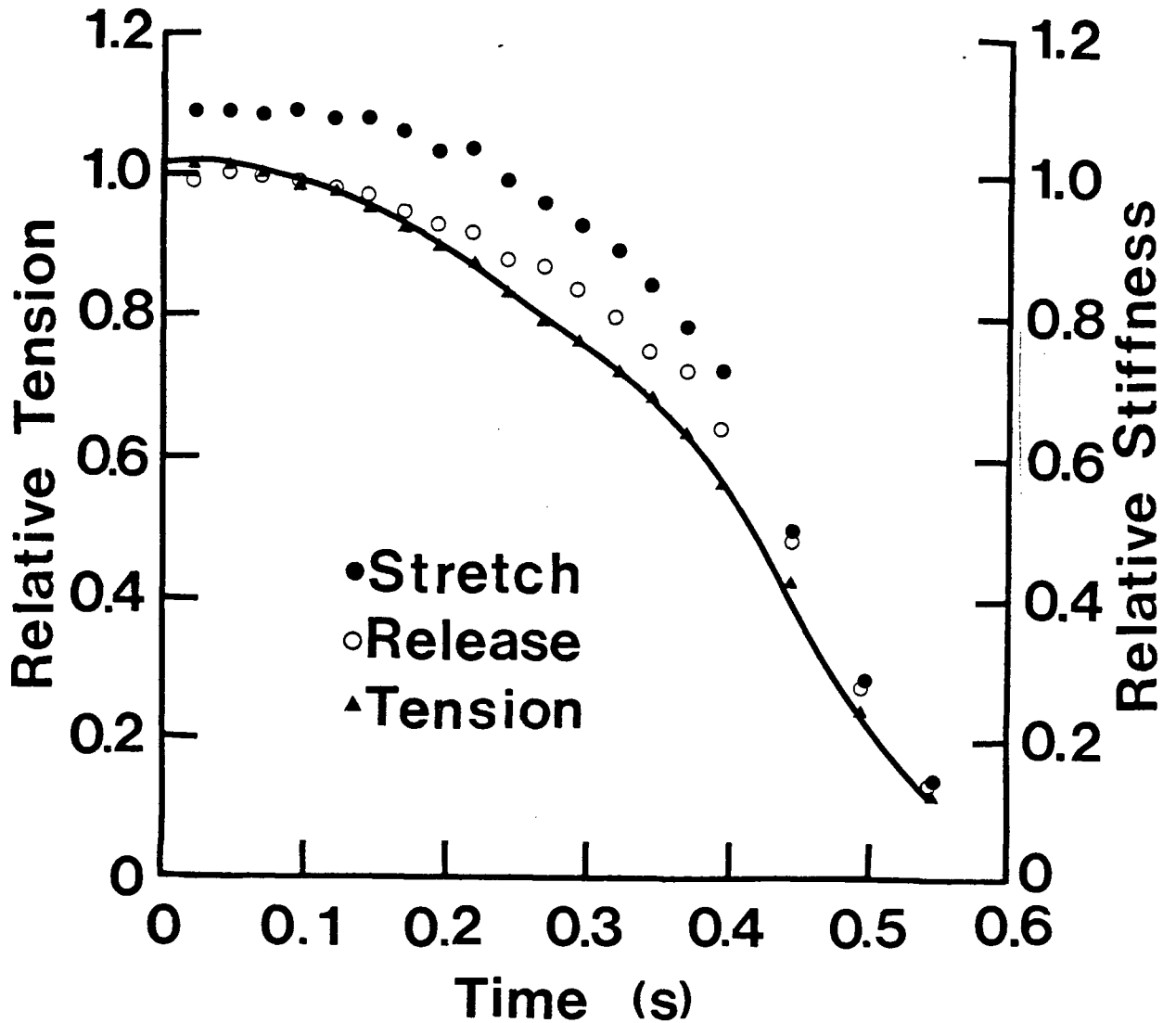
Figure 21. Stiffness-tension relationship during the relaxation phase of an isometric tetanus. Tension values are those immediately preceding the length change. Time is measured from the last shock of the stimulus train. The amplitude of the length changes was 2nm/h-s. Experiment of October 24, 1983, sarcomere length 2.2 μm.
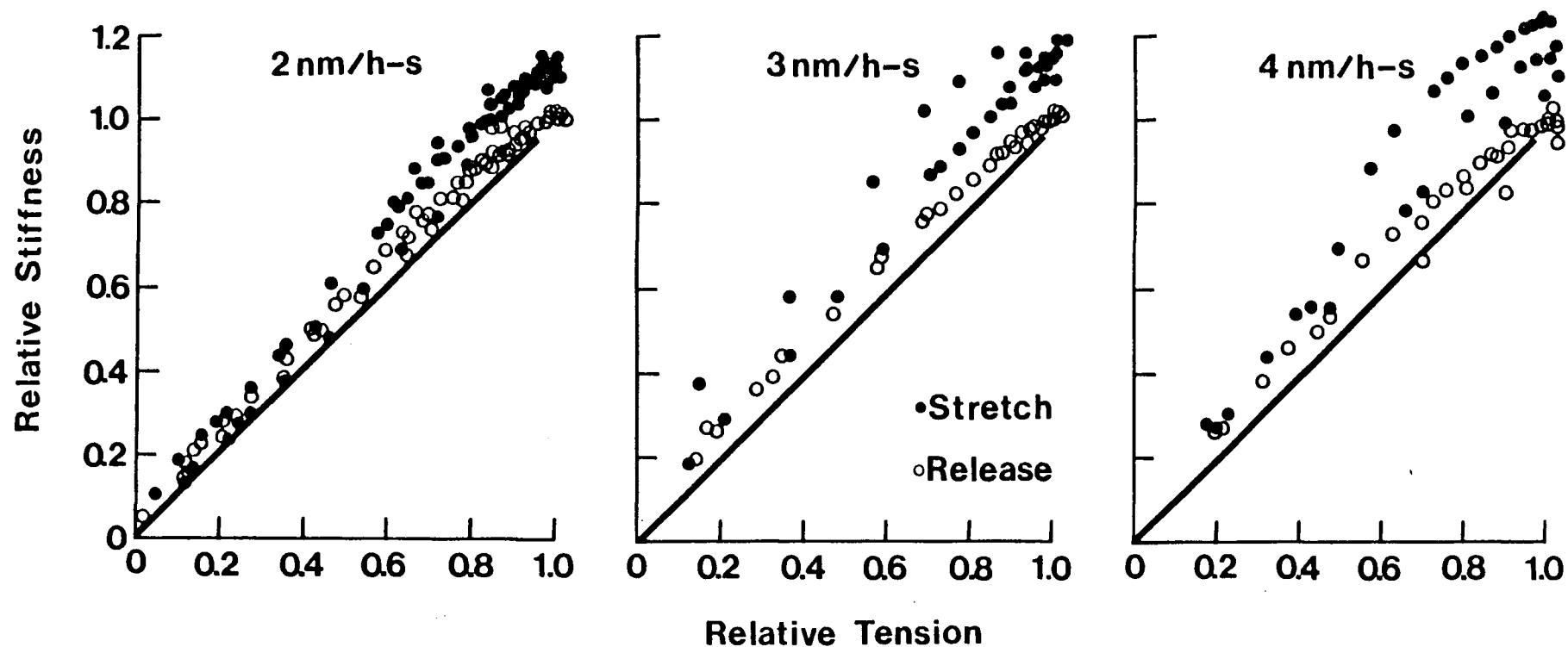
Figure 22. A comparison of tension during the relaxation phase of the isometric tetanus to stiffness values obtained with 3 amplitudes of length step for 10 single fibres. All values are expressed as a fraction of the maximum values obtained at the plateau of an isometric tetanus.

stiffness value than a release. As in the twitch, it may be seen that the largest difference in stiffness measured with stretch versus release occurred during the late rising phase of tension, the plateau, and early in the relaxation phase of the tetanus (figures 19 to 22).

The stiffness-tension relationship during the relaxation phase of the tetanus was also investigated using muscle bundle preparations ranging in size from 2 to 20 fibres. Similar results were seen in these preparations (figure 23) as in single fibres (figure 22).

Figure 23. A comparison of tension during the relaxation phase of the isometric

tetanus to stiffness values obtained with 2 amplitudes of length change for 4

muscle bundles. All values are expressed as a fraction of the maximum values

obtained at the plateau of an isometric tetanus.

V. DISCUSSION

Stiffness-tension relationship during the isometric twitch and tetanus

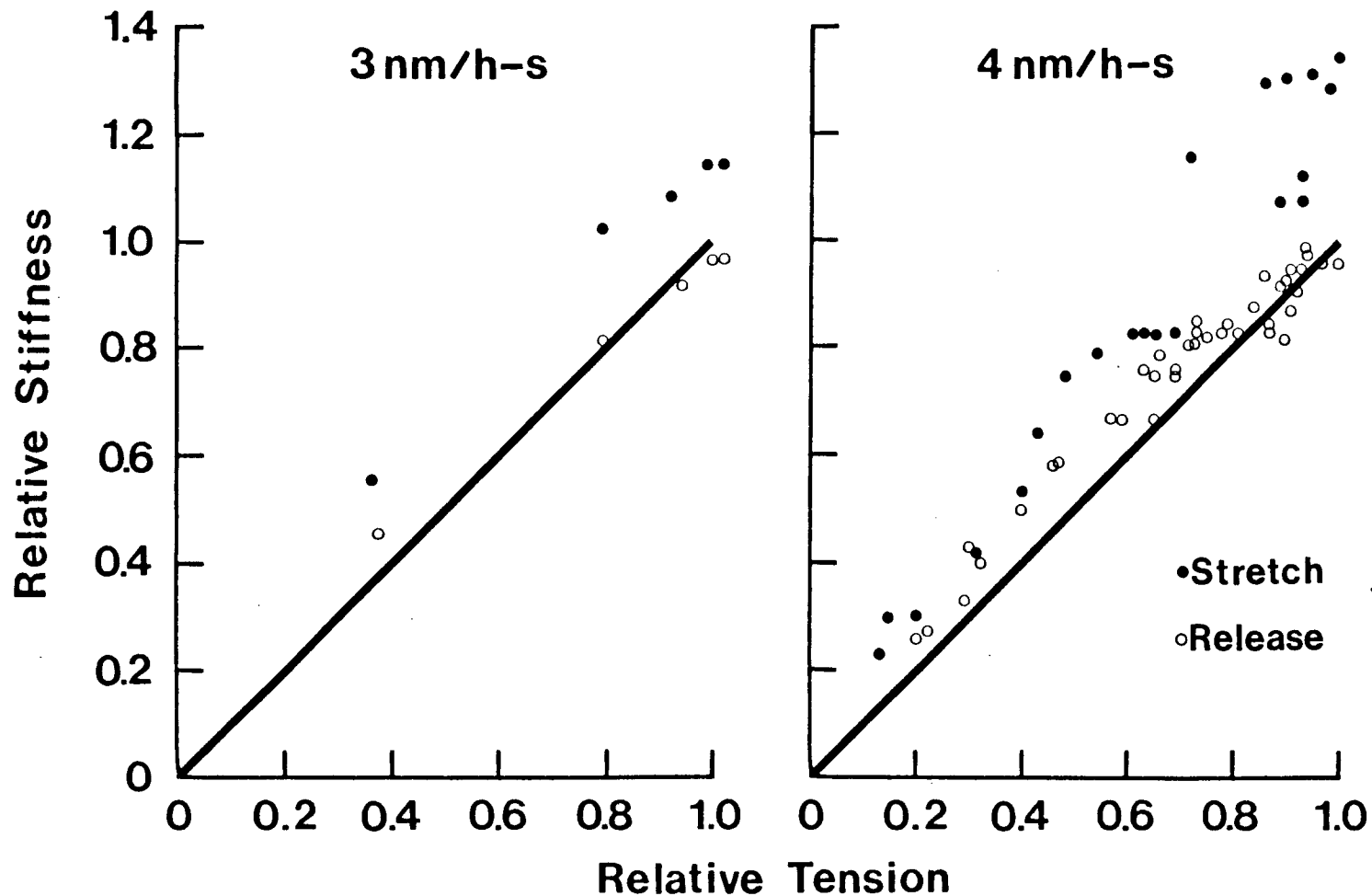This study has shown that relative stiffness values are greater than relative tension values throughout the isometric twitch and during the rising and relaxation phases of the isometric tetanus. Both the stiffness and the tension are proportional to the number of attached cross-bridges. An independent measurement of the number of attached cross-bridges is provided by X-ray diffraction.

Low-angle equatorial X-ray reflections are derived from the hexagonal array of the myofilaments, and the intensity ratio changes of these reflections have been interpreted to indicate the re-distribution or movement of mass between the myosin and actin filaments, that is, cross-bridge formation (see introduction). Changes in the intensity ratio of these equatorial reflections during the isometric twitch and tetanus have been shown to precede tension during the rising phase (H.E. Huxley, 1979, Matsubara and Yagi, 1978), peak at approximately the same time as tension, and then lag tension during the relaxation phase (Yagi, Ito, Nakajima, Izumi, and Matsubara, 1977, Matsubara and Yagi, 1978). That is, the time course of the intensity ratio changes of the equatorial reflections is quite similar to the time course of stiffness changes during the isometric twitch and tetanus. Stiffness measurements indicate the number of attached cross-bridges. Thus the results of two independent experimental approaches suggest that cross-bridges move towards and attach to actin before tension is generated.

The difference in the time course between stiffness and tension (rising phase : twitch - Bressler and Dusik, 1983, Shoenberg and Wells, 1984 , tetanus - Bressler and Clinch, 1974, Mason and Hasan, 1980 Cecchi, Griffiths and Taylor, 1982, 1984, Ambrogi-Lorenzini, Colomo and Lombardi, 1983 , relaxation phase : twitch - Bressler and Dusik, 1983, Shoenberg

and Wells, 1984, Stein and Parmiggiani, 1979, tetanus - Dusik and Bressler, 1984, Shoenberg and Wells, 1984, Cecchi et al, 1984) may be explained in a number of ways. The rising phase will be discussed first. It may be that attachment of the cross-bridges to the actin sites is a two step process in which the first step is rapidly reversible and tension is generated only after both steps have occurred (A.F. Huxley, 1973). Another possibility is that following attachment, the next chemical step in the cross-bridge cycle must occur before tension is generated, with this step having a relatively slow rate constant (H.E. Huxley, 1979, Cecchi et al, 1982, 1984). Alternatively, the difference between stiffness and tension may be due to sarcomere shortening during the rising phase (Mason and Hasan, 1980, Ambrogi-Lorenzini et al, 1983, Shoenberg and Wells, 1984). Huxley and Simmons (1971a, 1973) found that stiffness increased proportionally with tension during the rising phase of the isometric tetanus, while the other investigators cited above found stiffness increased faster than tension. The results of Huxley and Simmons may differ from those of other investigators as their experiments were done using a length clamp. Ambrogi-Lorenzini et al (1983) have pointed out that with fixed-end experimental conditions, a small amount of series compliance and inertial artefacts may contribute to the observed difference between stiffness and tension, particularly at low tension levels.

The difference between stiffness and tension during the relaxation phase may be explained by various possibilities. The relaxation process may result in a hindrance of the transition to the force producing state by cross-bridges which have just attached (see below). Other possibilities include increased detachment of force producing cross-bridges relative to non force producing cross-bridges, detachment

followed by rapid re-attachment of some cross-bridges, transition of force producing cross-bridges to a non force producing state, or transition of force producing cross-bridges to a state generating relatively less tension during relaxation than at the plateau (see Cecchi et al, 1984). As was suggested for the rising phase, a relatively slow transition between attachment and force generation in the cross-bridge cycle is assumed to occur during relaxation in these possibilities. Finally, factors independent of the cross-bridge reaction mechanism such as inhomogeneities in the sarcomere length and/or an effect of calcium ions (Ca++) may account for the observed difference between stiffness and tension.

It has been shown that the shoulder of the tension record marks the beginning of changes in sarcomere length (Huxley and Simmons, 1970b, 1973, Julian and Morgan, 1979, Edman and Flitney, 1977, 1982). Sarcomere shortening and lengthening occur in different regions of the relaxing muscle fibre following the shoulder, and become maximal when tension nears zero (Edman and Flitney, 1977, 1982). The resting sarcomere length is not re-established until after tension has disappeared (Edman and Flitney, 1982). This may explain why the intensity ratio changes of the equatorial X-ray reflections do not return to their resting pattern until well after tension had reached zero (Yagi et al 1977, Matsubara and Yagi, 1978, H.E. Huxley, 1979). The pattern of sarcomere length changes appears to be similar during relaxation of twitches and tetani (Edman and Flitney, 1977).

Blinks, Rüdel and Taylor (1978) found that the sarcoplasmic Ca++ concentration, measured with aequorin, decreased rapidly during relaxation of the twitch and tetanus and preceded the decline in tension. However, two later studies, using a much higher gain on the

aequorin signal, obtained different results. Ashley and Lignon (1981) found that during the twitch there was a prolonged phase to the decay of the aequorin response which continued until tension reached zero. Furthermore, Cannell (1982) found that the decrease in the aequorin signal during the tetanus is reduced or reversed at the tension shoulder and a small increase in the signal remained after tension had reached zero. He suggested this might be due to shortening sarcomeres. Allen and Kurihara (1982) and Ridgway and Gordon(1984) measured Ca++ transients with aequorin following slow (5-6 ms), large (up to 6% lo) length changes. Length changes of this amplitude would move the sarcomeres along their tension-length curve. These investigators found that following a stretch, there was no change (Allen and Kurhara, 1982) or a small decrease (Ridgway and Gordon, 1984) in the aequorin signal whereas following a release the aequorin signal increased. Ridgway and Gordon (1984) suggested that following sarcomere lengthening the sarcoplasmic Ca++ concentration decreases due to Ca++ uptake by the filaments and following sarcomere shortening the sarcoplasmic Ca++ concentration increases due to Ca++ release from the filaments. This would agree with Cannell's suggestion and indicates that following the tension shoulder the increased rate of tension and stiffness decline will be due, in part at least, to sarcomere shortening and lengthening and the accompanying Ca++ changes that these changes in sarcomere length bring about.

The difference between stiffness and tension during relaxation may be the result of hindrance of the transition to the force producing state of cross-bridges which have just attached. Biochemical in vitro studies by Chalovich and Eisenberg (1982) have suggested that the troponin-tropomyosin complex does not block the binding of myosin to actin, but acts by blocking the rotation of myosin on actin (or the

transition to the force generating state), increasing the activation energy for this transition and decreasing the cross-bridge cycling rate. If the rotation of the cross-bridges is blocked or slowed, less tension will be generated per cross-bridge than if it were allowed to complete its cycle. However, stiffness measurements indicate the number of attached cross-bridges, whether or not they are generating tension. This would result in a more rapid decline of tension values than stiffness values during the relaxation phase.

Stretch versus release

This study has shown that a rapid stretch produced a consistently higher stiffness value than a rapid release throughout the time course of the isometric twitch and tetanus. One possible explanation for these results may be attained from the Eisenberg, Hill and Chen (1980) model of muscle contraction. This model of contraction relates in vitro biochemical studies to in vivo physiological studies. In order to develop a complete cross-bridge model of muscle contraction, the physiological property of cross-bridge elasticity had to be combined with the biochemical states. One of the main differences between this model and the model of Huxley and Simmons (1971b) is that in the latter the cross-bridge contains a tension generator and an independent elastic element. This elastic element is "completely independent of any chemical changes in state occurring elsewhere in the attached cross-bridge". In the Eisenberg et al model the elastic properties of the cross-bridges and chemical state changes are intimately related so that changes in state are directly related to changes in elasticity. Furthermore, each of the attached states has individual elastic and chemical properties.

The biochemical basis for the model was the actomyosin cycle of

Chock, Chock and Eisenberg (1976). In this cycle, actomyosin is rapidly dissociated by ATP which is then hydrolyzed on the myosin head to ADP and inorganic phosphate (Pi). Next, a conformational change must occur in the myosin head, from a refractory to a non-refractory state, before it can re-bind to actin. This is the rate-limiting step in the cycle. Subsequently myosin, with bound ADP and Pi, binds to actin. This is followed by the relatively quick release of Pi, and then ADP, and the return to the beginning of the cycle. It was assumed that two unattached states, the refractory and non-refractory states, and two attached states A-M-ADP-Pi (A is actin, M is myosin) and A-M-ADP occurred in significant concentrations in vivo and that the other states in the cycle were transient intermediates. A-M-ADP-Pi (or state 1) was assumed to be the 90° state and A-M-ADP (or state 2) was assumed to be the 45° state. The 90° and 45° states were based on structural studies (Reedy, 1967, Reedy, Holmes and Tregear, 1965) of glycerol-extracted insect flight muscle which showed that in relaxed muscle the detached cross-bridges were oriented at a 90° angle from the myosin filament while in rigor the attached cross-bridges were oriented at a 45° angle. In the Eisenberg et al model the cross-bridges do not exist solely at 90° or 45° angles. These are the preferred angles for the attached states. The cross-bridges can rotate to a larger angle where they will exert positive force or to a smaller angle where they will exert negative force. "The key point in this model is that the elastic properties of the cross-bridge states do not determine the rate constants between these states. Therefore the rate constants between the 90° and 45° states do not depend on thermal (Brownian) motion to stretch an elastic element as in the Huxley-Simmons model". The binding of ATP was assumed to have no effect on the angle of attachment so that work done by a cross-bridge was a result of myosin

binding to actin.

In biochemical studies, each biochemical state has specific thermodynamic properties and is defined as a thermodynamic state. It will always exist at its minimum free energy level (Eisenberg and Hill, 1978). In physiological studies, however, the thermodynamic properties and free energy levels of an attached cross-bridge state will vary because of the elasticity of the cross-bridge. Therefore "a biochemical thermodynamic state is a physiological cross-bridge state at a specific value of x", the axial position of the actin binding site relative to the attached cross-bridge. The free energy profile in figure 24 shows the basic free energy of the four cross-bridge states in the Eisenberg et al model as a function of x. The free energy profiles relate the chemical parameter of free energy to the physiological parameters of the position of the attached cross-bridge and the mechanical force. The free energy profiles of the unattached states are independent of the position of the actin binding sites and therefore independent of x. The free energy profiles of attached cross-bridge states will be dependent on x because of the cross-bridge elasticity. The parabolic shape of state 1 and 2 free energy curves is based on the isometric tension transient data of Ford, Huxley and Simmons (1977) which showed that the corrected experimental curve for the elasticity of the cross-bridge (T1 curve) is linear. Force development depends on the presence of a gradient of free energy as a function of the axial position of a cross-bridge relative to its rest position in that state (Eisenberg and Hill, 1978). Thus, the slope of the free energy curve for an attached state, at any value of x, is equal to the force exerted by that cross-bridge state at that value of x. Zero force is generated at the bottom of the free energy curve. "Because cross-bridge states are at their minimum free energy levels in solution,
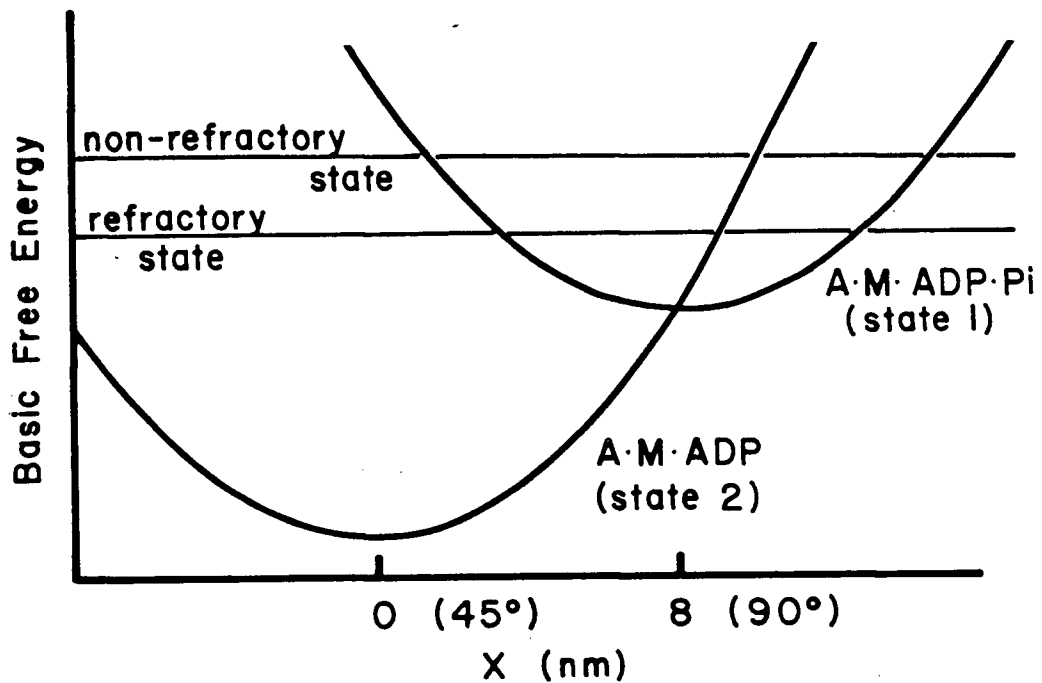
Figure 24. Basic free energy profile for the Eisenberg-Hill-Chen cross-bridge model. The ordinate shows the relative basic free energy for the cross-bridge states. "The profile repeats indefinitely above and below, with one ATP being hydrolyzed during each cycle." The abscissa shows the axial position of the actin binding site relative to the attached cross-bridge, x, with reference to its position when state 2 is at its minimum free energy. The angle of attachment at X=0 is 45° and at x=8 is 90°. (Modified from Eisenberg et al, 1980).

the relative vertical positions of the minimum of the free energy curves are based on the equilibrium constants between cross-bridge states in solution". Finally,"the basic free energy drop for 1 complete cycle is equal to the free energy of the hydrolysis of 1 ATP molecule which is about 12 kcal/mole in vivo (Kushmerick and Davies, 1969, and Curtin et al, 1974 in Eisenberg and Hill, 1978)".

As there are four states in this model there are four pairs of rate constants. "The ratio of the forward and reverse rate constants between any t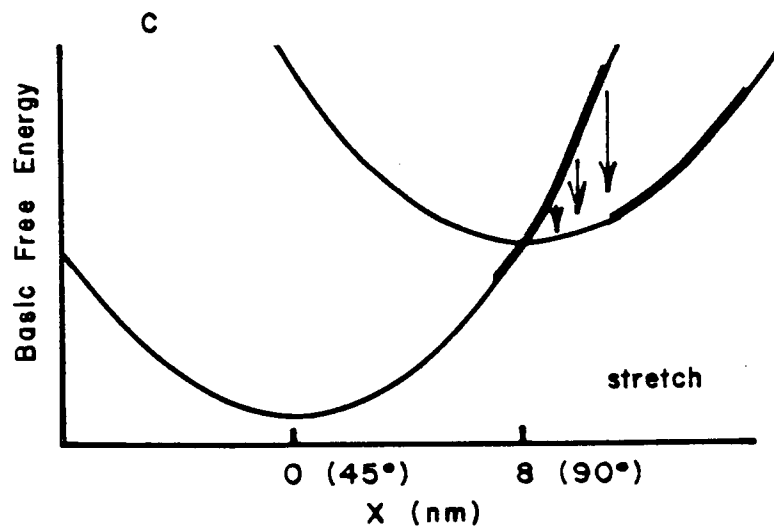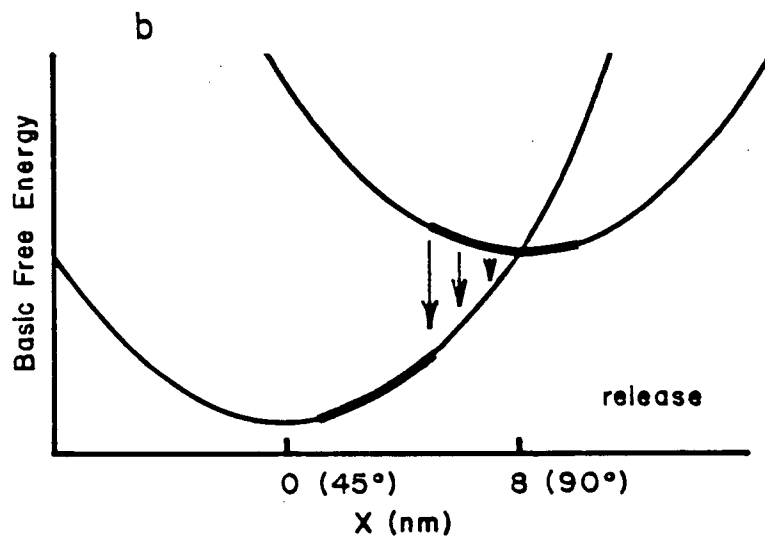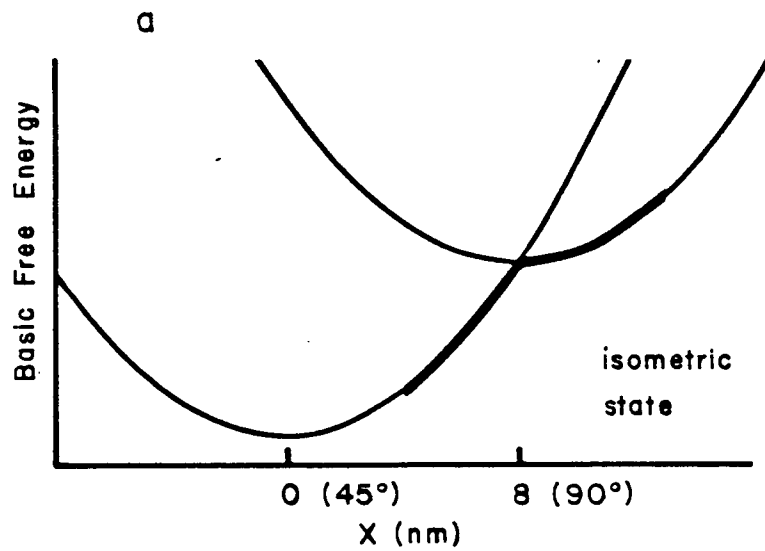wo states is determined by the free energy difference between the two states (Eisenberg and Hill, 1978). Like the free energy curves themselves, the values of these rate constants were chosen to be consistent with both biochemical and physiological data".

In the isometric state there will be a distribution of cross-bridges among the various cross-bridge states as a function of x (figure 25a). This distribution is due to the difference in the axial repeat distance of the two sets of myofilaments (H.E. Huxley, 1969), and the assumption that during steady isometric contraction no axial displacement of the filaments occurs. Therefore, cross-bridges will be attached to actin binding sites over a range of angles but each individual cross-bridge will be attached only at the angle where it is able to attach to an actin binding site and will go through its cycle at that angle. If a cross-bridge is attached at an angle greater than $90°$, the free energy of state 2 will be much higher than that of state 1 so that few cross-bridges will be capable of changing states. Thus these cross-bridges will neither complete their cycle nor hydrolyze ATP. Instead, an equilibrium will occur between the unattached states and state 1. If a cross-bridge is attached at an angle less than $90°$, the free energy of state 2 will be lower than that of state 1 so these

cross-bridges will cycle and hydrolyze ATP.

Figure 25 shows the cross-bridge distribution in the isometric state (a) and immediately following a small amplitude release (b) and stretch (c). Eisenberg and Hill (1978) assumed that the tension transients were completely due to cross-bridges, and primarily to a rapid change of attached states. It can be seen that the cross-bridge distribution is shifted simultaneously with the length change and that the cross-bridges have been forced to rotate to angles at which they exert a different amount of tension than in the isometric state. As stated above the steeper the slope of the free energy curve the higher the tension generated. During a release the cross-bridges in state 1 have been forced to rotate to angles where they produce little tension, no tension (bottom of curve) or negative tension (negative slope). The cross-bridges in state 2 have been forced to rotate to angles at which they exert less force than in the isometric state. During a stretch the cross-bridges in both states have been forced to rotate to angles where they produce more tension than during the isometric state. Re-distribution during the length change results in cross-bridges at each angle being forced into an inappropriate state for that angle. The recovery phase of the tension transient is due to "readjustment to the appropriate distribution for each angle". The difference in the rate of recovery between stretch and release is due to the transition between states following the length change. Following a release the transition from the 90° state to the 45° state occurs rapidly as the rate of this transition increases as the cross-bridge angle decreases below 90°. Following a stretch the transition from the 45° state to the 90° state occurs relatively slowly as the rate of this reverse transition remains nearly constant as the cross-bridge angle increases above 90°. Thus

Figure 25. Distribution of cross-bridges among the attached cross-bridge states as a function of x. The slopes of the free energy curves equal the tension generated by a cross-bridge state at a specific value of x. The thick line indicates the states a cross-bridge will occupy at each value of x under specific conditions. (a)Distribution of cross-bridges during isometric contraction. (b)Distribution of cross-bridges immediately following a small amplitude, rapid release. The arrows indicate the re-distribution of cross-bridges to the appropriate state for each value of x responsible for tension recovery. (c)Distribution of cross-bridges immediately following a small amplitude rapid stretch. Arrows as in (b). (Modified from Eisenberg and Hill, 1978).

a

Basic Free Energy

isometric
state

0 (45°)   8 (90°)

X (nm)

b

Basic Free Energy

release

0 (45°)   8 (90°)

X (nm)

c

Basic Free Energy

stretch

0 (45°)   8 (90°)

X (nm)

Eisenberg and Hill (1978) and Eisenberg et al (1980) explained the isometric tension transient as a transition between two attached states, as did Huxley and Simmons (1971b). However, in the Eisenberg et al model, the transition between states was accounted for "without postulating an independent elastic element in the cross-bridge".

Thus the Eisenberg-Hill-Chen model can account for the decrease in tension following a rapid release and the increase in tension following a rapid stretch. It may also account for the higher stiffness values obtained with a stretch as follows. Recent studies in which stiffness has been measured during the rapid tension recovery using quick length steps (Ford et al, 1974) or sinusoidal oscillations throughout the tension transient (Julian and Morgan, 1981a, Cecchi et al, 1981, 1982, 1984) have shown that stiffness decreases following a small (less than 1% lo) rapid release but does not change significantly during a small, rapid stretch. This would suggest that some of the bound cross-bridges detach during a release but stay on during a stretch. If is it assumed that cross-bridges may be cycling at a faster rate than the time course of the length changes used to measure stiffness (Guth, Kuhn, Tsuchiya and Ruegg, 1981, Ford et al, 1977), it may be that during release the cross-bridges in state 2 being forced down their energy well are responsible for the decrease in stiffness. The cross-bridges in state 1 being forced into a negative force generating mode will resist further change which may cause some truncation of the tension response. In addition cross-bridges will be detaching during the release, most likely cross-bridges near the bottom of the state 2 free energy curve as these bridges will be forced to an angle where detachment is favored. During stretch cross-bridges will stay on and are forced into higher tension generating angles, resulting in higher stiffness values.

It is also possible that truncation of the tension transient with releases contributes to the results obtained. Huxley and Simmons (1971b) have shown that the rate of the rapid recovery phase in frog semitendinosus fibres decreases as the amplitude of stretches increases, and increases as the amplitude of the release increases. However, Abbott and Steiger (1977) have found in glycerinated rabbit psoas fibres that the rate of the rapid recovery increased as the amplitude of length change increased up to 2 nm/h-s and then remained constant for both stretches and releases. The difference in results between these studies has been suggested to be due to the different muscle preparations used, the difference in speed of length step, and differences in experimental apparatus and methodology (Abbott and Steiger, 1977, A.F. Huxley, 1980). The results of Huxley and Simmons would indicate that the effect of truncation of the instantaneous tension change due to rapid recovery would be smallest when comparing stretch and release at small length changes while the results of Abbott and Steiger would indicate the truncation would have the same effect on stretches and releases at any amplitude of length change.

In any event, the difference in stiffness between stretch and release is still evident at small amplitudes of length change. This suggests that truncation due to rapid recovery cannot fully explain the results of this study. Furthermore it has been found that the rapid early recovery of tension following a release is significantly slowed by hypertonic solution (Vaughan, Bressler, Dusik and Trotter, 1983) which would reduce the effect of truncation. It has been observed that the stiffness measured with stretch is higher than that measured during release in hypertonic solution (Bressler and Dusik, unpublished results). This would again suggest that truncation cannot fully explain

the results obtained in this study.

Another possible explanation for the results of this study is that the instantaneous elasticity is non-linear. Ford et al(1977, 1981) found that using computer simulation they could reproduce the tension response to releases but not to stretches. From this they concluded that the instantaneous elasticity is non-linear in stretches, its tension-extension curve being concave upwards.

At present it is not possible to tell which of these explanations best fits the data. Many complex and subtle technical considerations were identified and characterised during this study, and improved technical features, such as correction for truncation and faster length steps made possible by computer-assisted data collection with a Nicolet digital oscilloscope linked to an Apple IIe, have been developed. Experiments using these improved techniques are being conducted to determine which of these possibilities is responsible for the results obtained.

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