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Investigation of the Temperature Dependence of the Cross Bridge Parameters for Attachment, Force Generation and Detachment as Deduced from Mechano-Chemical Studies in Glycerinated Single Fibres from the Dorsal Longitudinal Muscle of *Lethocerus maximus*

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Abstract. 1) A study is presented on the effect of temperature on the mechano-chemical states involved in the cross bridge cycle of single glycerinated dorsal longitudinal fibres from Lethocerus. Contraction was induced by immersing the fibre in a MgATP-salt solution at $Ca^{2+} \sim 10$ μM (pH 6.7).

2) Rising the temperature increases the rates of isometric tension generation following an increase in the $[Ca^{2+}]$ from 0.01 to 10 μM as well as the steady state levels of isometric tension and the rates of ATP splitting.

3) Tension transients following stretches of rise times 250 μs and amplitudes up to 0.4% L_i comprise at least four phases: an elastic phase the amplitude of which decreases by raising the temperature; a biphasic quick phase of tension decay with a mean $Q_{10} = 2$; a delayed tension rise ($Q_{10} \sim 5$).

4) Tension transients following releases of fall time 250 μs and amplitudes up to 0.3% L_i also comprise four phases: an elastic phase comparable to that observed following stretches; a deactivation phase composed of a single exponential and a slow recovery phase.

5) The number of cross bridges attached to the actin at any moment is not changed during the elastic and quick recovery phase following a release as well as during the elastic and fast quick phase following a stretch. However, the number of attached cross bridges decreases during the deactivation phase.

6) The early phases of tension adjustment (T curves) which were recorded during the releases showed a marked dependence on temperature. *The T curves fitted with high accuracy the Huxley and Simmons (1971) predictions of cross bridge rotation.*

7) Analysis of the T curves in terms of the Huxley and Simmons (1971) model shows that a) the stiffness of a single cross bridge ($D = 1.2\ 10^{-4}$ N/m) obeys Hook's law; b) the number of myosin heads attached to actin (24% of the total number) is not altered during releases; c) rotation of myosin heads from a perpendicular to an acute angled position extends the elastic element of a cross bridge by 11 nm; d) at 25° C the rate constants for rotation from the perpendicular position to the acute angled position and vice versa are 8300 s^{-1} ($Q_{10} = 3.5$) and 3600 s^{-1} ($Q_{10} = 1.5$).

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8) Thermodynamics applied to cross bridge rotation predicts that during sudden releases the temperature within the fibre should fall and the driving force for tension generation is an increase in entropy of rotated bridges.

9) Rate constant of detachment of cross bridges from the actin is determined to $k_2 = 500\ s^{-1}$ (25° C; $Q_{10} = 2.3$).

10) The values of steady state rate of ATP splitting in conjunction with estimates of the number of attached cross bridges indicate that rate limiting steps of ATP cleavage occur while myosin heads are detached *and* while they are attached to the actin: Rate limiting for the attachment is the decay of the refractory myosin-product (Eisenberg and Kielley, 1973; $k_4 = 1.7\ s^{-1}$: 25° C; $Q_{10} = 2.5$). Rate limiting for detachment is the concentration ratio of actomyosin-ATP to actomyosin-product $K_1 = 0.018$ (25° C; $Q_{10} = 2.7$).

Key words: Myofibrillar ATPase – Mechano-chemistry of muscle – Insect fibrillar muscle – Muscular energetics – Kinetics of actin-myosin interactions.

Introduction

It is generally accepted that force in isometrically contracted muscle fibres is *maintained* by cross bridges attached to the actin filament. Based on this assumption, Huxley's (1957) two state model of cross bridge interaction with the actin correctly predicts the steady state relations of force and overlap between the myosin filaments and actin filaments (Gordon et al., 1966) as well as the mechanical and energetic aspects of steady state isotonic contraction (cf. Hill, 1938). Improvements of the stretching technique of muscle fibres (it is now possible to produce length changes of amplitudes up to 200 μm within less than 0.2 ms; Huxley, 1974) made evident the limits of the simple two state model. In 1971, Huxley and Simmons demonstrated that the time course of tension responses following sudden length changes (tension transients) cannot be predicted by Huxley's 1957 theory. Rather than to expand the two state model [as Podolsky and Nolan (1973) did] Huxley and Simmons proposed a contraction model in which at least three distinct cross bridge states are involved (cf. Huxley, 1974; Julian et al., 1974):

1) cross bridges detached from the actin (Fig. 1C);

2) cross bridges which attach their heads in a perpendicular position to the actin filament (Huxley, 1969; Beinbrech et al., 1976, Fig. 1A);

3) cross bridges the myosin heads of which are attached to the actin filaments in an acute angled position (Reedy et al., 1965, Fig. 1B). In the Huxley and Simmons (1971) model force is generated when the elastic elements of the cross bridges are extended by a rotation of their heads from the perpendicular position to the acute angled position while they remain attached to the actin filament.

On the basis of five adjustable molecular parameters the Huxley and Simmons (1971) model enables concise predictions on the very early phases of tension responses during and/or following quick length changes. These parameters are:

1) the number (N) of cross bridges attached at any moment of contraction to the actin filament.

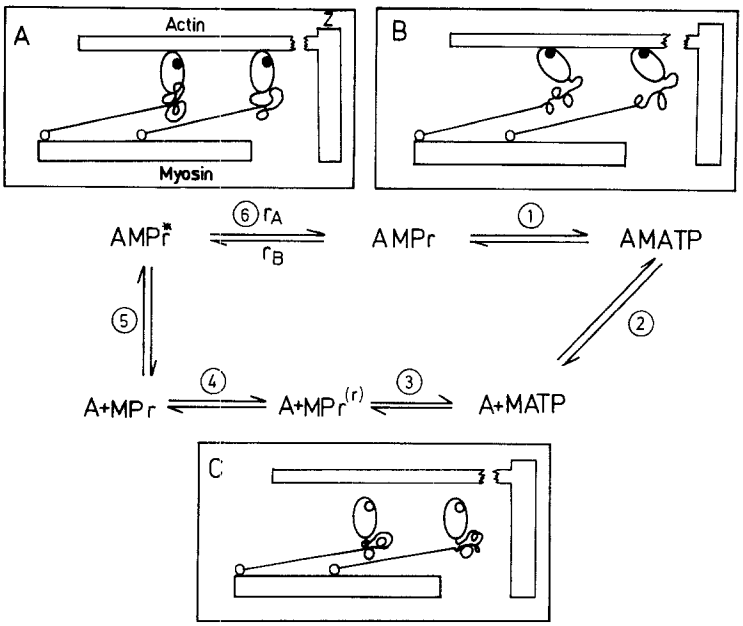

Fig. 1. Possible schematic representation of the link between biochemical events and mechanical events occuring during the cross bridge cycle. Mechanical cycle shows a three state contraction model similar to that proposed by Huxley and Simmons (1971; cf. also Huxley, 1969). Myosin heads of the cross bridges are attached to the actin in a perpendicular position in (**A**) and in an acute angled position in (**B**); myosin heads detached from actin in (**C**). Elastic element within the cross bridge is shown as randomly coiled protein portion in the neck part of the myosin molecule (cf. 1.6 of Discussion). Biochemical cycle shows six stages of the actin activated myosin ATPase. In step 1 product (Pr = ADP.P_i) dissociation from actomyosin (Lymn and Taylor, 1971) is involved. Step 3 represents the reversible hydrolysis of myosin-ATP (MATP) to myosin-product (MPr, Bagshaw and Trentham, 1973). $MPr^{(r)}$ is the refractory myosin-product complex (Eisenberg and Kielley, 1973). In step 6 a conformational change (White and Taylor, 1976) of attached cross bridges occurs

2) The stiffness (D) of the elastic element of a single cross bridge.

3) The extension by rotation (h) of the elastic element within the acute angled cross bridges. This parameter is defined as difference in the extension of the elastic elements of cross bridge in an acute angled conformation and in the perpendicular conformation.

4) The rate constants for the rotational movement of the attached myosin heads on the actin filament from the perpendicular position to the acute angled position (r_A) and vice versa (r_B).

The work of Huxley and Simmons (1970, 1971, 1973) and of Ford et al. (1974, 1976, 1977) has shown that in tetanized frog fibres the tension transients following sudden length changes can be interpreted by a set of these five parameters. The studies of these authors gave further evidence that the cross bridge elasticity is Hookean and that fibre stiffness varies in proportion to variation in the overlap between thin and thick filaments, i.e., fibre stiffness reflects the number of cross bridges attached to the actin filament at any moment. For skinned frog muscle fibres compare Yamamoto and Herzig (1978).

One of the aims of this paper was to study whether or not the effects of temperature on the very early phases of tension transients following sudden length changes are comparable in glycerinated fibres from *Lethocerus* to those observed in tetanized frog anterior tibialis fibres (Ford et al., 1977). The results given in the study of Abbott and Steiger (1977) seem to give evidence that such a similarity does not exist. But these authors analysed the effect of length changes on tension changes in glycerinated fibre bundles with an initial delay of 1–2 ms. Thus it seemed appropriate to use single fibres and to increase the time resolution in tension records even beyond the limits given by Huxley and Simmons (1971, 1973). This was achieved by recording tension responses *during* the length change itself (Güth and Kuhn, 1976; Ford et al., 1976). These improvements of the recording technique made possible to prove that glycerinated fibrillar muscle fibres and tetanized frog muscle exhibit very similar early phases of tension adjustment in response to sudden length changes.

A further aim of this paper was to study whether the number of cross bridges attached to the actin is constant during cross bridge rotation as predicted by the Huxley and Simmons (1971) model and whether there occurs cross bridge detachment from the actin within the very subsequent phase of tension transients as predicted by the contraction models of Julian (1969) and Thorson and White (1969). A suitable method to study the time course of stiffness following a length change is to apply length pulses of variable width and to record the length-tension diagrams obtained during the stretch and the release phases of the pulses (Güth and Kuhn, 1978). Fibre stiffness as calculated from the slopes of such diagrams reflects the number of cross bridges attached to the actin at any moment (Huxley and Simmons, 1971).

A third aim of this paper is to obtain information on the attachment rate of cross bridges to the actin. Provided that the number of cross bridges detached from the actin at any moment is known, this parameter is calculated from steady state ATP splitting rates which can be readily measured in chemically skinned fibres (cf. Rüegg and Tregear, 1966). Numbers of detached bridges may be estimated from the total number of cross bridges (Tregear and Squire, 1973) and the number of attached bridges as revealed from the analysis of cross bridge rotation (Huxley and Simmons, 1971).

Kinetic in vitro studies have revealed models for the mechanism of ATP cleavage by myosin in the absence (cf. Trentham et al., 1976; Mannherz et al., 1974) and in the presence of actin (Lymn and Taylor, 1971; Eisenberg and Kielley, 1973). Lymn and Taylor (1971) have shown that actin dissociates from the ternary actomyosin-ATP complex when the myosin-ATP complex is formed (step 2, Fig. 1). This step corresponds to cross bridge detachment in fibres. ATP is hydrolysed to its products (Pr = ADP.P_i) in a reversible step (3, Fig. 1; Bagshaw and Trentham, 1973) while myosin is detached from the actin. In the step which in fibres corresponds to cross bridge attachment, there is a controversy as to the interpretation of kinetic results obtained in vitro. According to Chock et al. (1976) the first order decay of the refractory state ($M.Pr^{(r)}$, Eisenberg and Kielley, 1973) to the myosin product complex within step 4 is slow, so that the fast bimolecular recombination step 5 does not affect steady state ATP splitting rates. According to White and Taylor (1976) the myosin-product (M.Pr) and the actomyosin-product are in a fast

equilibrium, so that the product dissociation from the actomyosin which also involves the conformational step 6 becomes rate limiting for steady state ATP splitting.

The interpretation of our mechanical results in terms of recent cross bridge models (Huxley and Simmons, 1971; Julian, 1969; Thorson and White, 1969; Julian et al., 1974) gives in conjunction with estimates of the rates of ATP splitting evidence that in muscle fibres recombination of M.Pr occurs according to the Eisenberg and Kielley (1973) kinetic scheme. There is also evidence suggesting a second reversible rate limiting step takes place, after the myosin heads attached to the actin filament have generated force by cross bridges rotation.

Methods

Solutions and Temperature Control: Contraction solution contained: imidazole, 20 mM; NaN_3, 10 mM; $MgCl_2$, 15 mM; H_2Na_2ATP[1], 15 mM; HDTA, 7.5 mM; KCl, 92 mM; CaEGTA, 4 mM. In relaxing solution CaEGTA was replaced by EGTA (4 mM) and it contained 8 mM KCl in addition to contraction solution. Rigor solution contained: imidazole, 20 mM; EGTA, 4 mM; EDTA, 4 mM; KCl, 50 mM; NaN_3, 1 mM; HDTA, 37 mM. Values of pH were adjusted with KOH or HCl to 6.7 at the temperatures (5–35° C) used. [The high ionic strength (I = 200 mM) is comparable to that used in "Ca-jump" experiments (cf. Ashley and Moisescu, 1975), so that it will be possible to compare the rates observed in future "Ca-jump" experiments with those reported here.] Extraction solution contained a mixture of 50 : 50 v/v double distilled glycerol to water, 20 mM histidine, 10 mM NaN_3 and 4 mM EGTA at pH 7 (5° C).

Fibre Preparation: Dorsal longitudinal flight muscle from *Lethocerus maximus* were glycerinated while fixed at the thorax by immersion in 150 ml extraction solution for 10 h at 5° C and at low atmospheric pressure. After transferring them to a fresh extraction solution the muscle was shaken for 24 h at 5° C and then transferred anew to a fresh extraction solution where it was kept at −16° C between 8 days and 6 months before use. In order to prepare single fibres, bundles (100–200 fibres) were dissected from the muscle and immersed in relaxing solution.

Mechanical Set Up: For a detailed description of the measurement device see Güth and Kuhn (1978) and Güth et al. (1979). Single fibres were glued by cellulose dissolved in acetone at their one end to a glass rod connected to the length step generator. The other end of the fibre was wound around the force transducer and glued to a platinum wire of fixed position. Fibre length (L_i, measured between the inner surfaces of the pin of the force transducer and the rod from the length step

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1 Abbreviations used: ATP: Adenosine-5′-triphosphate; ADP: Adenosine-5′-diphosphate; EGTA: 0,0′-bis-(β-aminoethyl)-ethylenglycol-N,N′-tetracetat; PEP: Phosphoenol-pyruvate; NADH and NAD: Nicotinamide-adenine-dinucleotide in reduced form and oxidized form respectively; HDTA: 1,6-Diamino-Hexan-N,N,N′,N′-tetraaceticacid

generator) was kept below 5 mm. From the diffraction produced by illuminating part of the fibre with a laser beam (Spectra Physics Model 135) sarcomere lengths of 2.4–2.5 μm were calculated (cf. also Reedy, 1968).

The force transducer was a Aksjeselskapet (AE 801) type strain gauge with low temperature drift and modified to obtain a resonance frequency of 15 kHz. The measured signal of the force transducer was corrected for a damped harmonic oscillation by means of an analogue computer (for details see: Güth et al., 1979). The corrected force signal was checked for linearity by means of a soft rubber strip which produced linear curves on a length-force diagram when the length was changed by 1% within 250 μs.

The relay type length step generator produced length changes (ΔL) which followed a potential function in time (t):

$$\Delta L = a\, t^b$$

Best fits of the exponent were $b = 2.5$. Typical time to produce a 50 μm length change was 250 μs. This set up allows tension signals to be measured with 10% accuracy already 25 μs after initiation of the length change (as checked with rubber strips).

Measurement of Tension Transients During and Following Rapid Length Changes

We measured tension *during* the length change by means of a storage oscilloscope (Nicolet Explorer 1090A). There result T curves when tension and length are plotted in a length-tension diagram (Güth and Kuhn, 1976; Ford et al., 1976). These T curves depend on the shape and size of the length signal. Typical T_1 curves (Huxley and Simmons, 1971) are expected only if stress-strain relaxation is slow compared to the duration of the length change. Otherwise T curves are obtained which entail the information on the fibre stiffness *and* of the early phases of stress-strain relaxation. It seems to be useful to record T curves rather than to try to obtain T_1 curves by increasing the release velocity. For, a further increase in the shortening velocity would increase the effects of the fibre ringing due to the finite transmission of elastic waves through the fibre (Schoenberg et al., 1974; Güth and Kuhn, 1978). For measurement of tension transients the last 2–4 μm of the movement of the relay anchor were damped by a film of butanol.

ATPase Assays: The steady state rate of ATP cleavage occurring in small bundles (8–10 fibres) was calculated by using the linked enzymatic system which quantitatively converts ADP, NADH and PEP into ATP, NAD and lactate by pyruvate kinase and lactodehydrogenase (cf. Pybus and Tregear, 1973). NADH extinction was monitored at 340 nm. All solution used in ATPase assays contained the adenylate kinase inhibitor diadenosine pentaphosphate (Feldhaus et al., 1975) purchased from Boehringer (Mannheim, FRG) in a final concentration of 0.2 mM. Prior to each ATPase estimate the bundles were incubated in rigor solution (5 min) and immersed in the test solution (5–15 min) until steady state was well established. Then the bundle was incubated for 5–15 min in 400 μl of a fresh test solution. The

molar amounts of ADP produced (reproducibility better than 2 nmoles) divided by the number of the fibres in the bundle, the fibre length (cm) and the duration of the incubation gave the ATP splitting rate.

Results

Isometric Contraction (Fig. 2)

The single glycerinated fibres from the dorsal longitudinal muscle of *Lethocerus maximus* were first incubated in relaxing solution containing 15 mM ATP and 4 mM EGTA. In the relaxed state the fibre was extended slightly above slack length, so that the passive elasticity characteristic to this muscle (cf. Pringle, 1977) caused a steady state tension of $\sim$ 20 μN at initial fibre length (L_i = 4.5–5.0 mm).

When contraction is induced by transferring the fibre from relaxing solution containing 4 mM EGTA ($[Ca^{2+}] \leq 0.01$ μM) to contraction solution, i.e., to a saline containing 15 mM MgATP and 4 mM CaEGTA ($[Ca^{2+}] > 10$ μM), isometric contraction (170 μN) was to 90% complete within less than 10 s at 35° C, whereas it took longer than 2 min to reach the same relative tension level at 5° C.

Steady state isometric tension increased by raising the temperature. Figure 2A shows the variations of the temperature effect on isometric tension when fibre preparations from three different animals were used. This demonstrates that the effect of temperature of the increases in isometric tension is reproducible.

Figure 2B shows the effect of temperature on the ATP splitting rate of fully Ca-activated fibres (pCa $\sim$ 5) reached under steady state isometric conditions. At 5° C

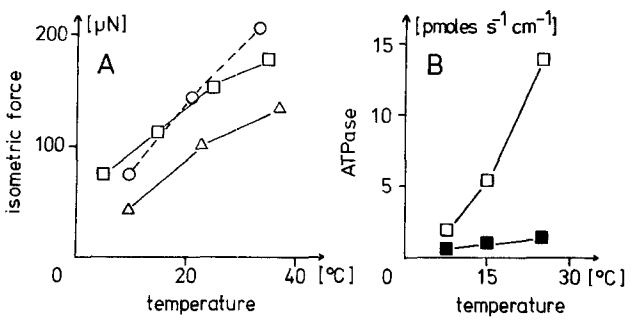

Fig. 2. Effect of temperature on isometric tension (**A**) and steady state rate of ATP splitting (**B**) in glycerinated fibres (dorsal longitudinal muscle) from *Lethocerus maximus*. (**A**) shows the isometric contractile force in single fibres in dependence of temperature for three different fibre preparations: (○), (△), and (□) were glycerinated on the 4. May 1977, 27. Nov. 1977, and the 23. Dec. 1977; experiments of 9. Dec. 1977, 12. Dec. 1977, and 8. Jan. 1978 respectively. At each temperature the relaxed fibre was extended to a force level of 20 μN prior to initiate isometric contraction by increasing the Ca^{2+} concentration from 0.01 μM to 10 μM. (**B**) shows ADP production from small bundles (8–10 fibres) of length 5 mm prepared the 23. Dec. 1977 in the relaxed state (■) and contracted state (□) when recalculated to one fibre of 1 cm length (mean from 10 experiments of march 1978). *Conditions:* pH 6.7; contraction solution: imidazole, 20 mM; NaN_3, 10 mM; $MgCl_2$, 15 mM; ATP, 15 mM; KCl, 92 mM; CaEGTA, 4 mM; HDTA, 7.5 mM. Relaxing solution: imidazole, 20 mM; NaN_3, 10 mM; $MgCl_2$, 15 mM; KCl, 100 mM; CaEGTA, 4 mM; HDTA, 7.5 mM

the rate of ATP cleavage was 1.8 pmoles s^{-1} in 1 cm of a fibre. It increased by a constant factor of $Q_{10} = 2.5$ when temperature was increased by 10° C. The rate of ATP cleavage was more strongly affected by temperature than the isometric tension (Fig. 2A, 2B). Consequently, the tension costs (the ratio of ATPase to tension; cf. Pybus and Tregear, 1973) increased by raising the temperature (cf. also Schädler, 1967; Paul and Peterson, 1977; Breull et al., 1973).

The rate of ATP splitting at the high ionic strength ($I \sim 200$ mM) used in this study is comparable to the rates of ATP splitting found before at lower ionic strength ($I \sim 100$ mM; Rüegg and Tregear, 1966; Breull et al., 1973; Pybus and Tregear, 1975). This indicates that ionic strength in the range between 100 and 200 mM does not appreciably affect steady state ATP splitting rates. Furthermore, at 25° C the presence of a powerful ATP regenerating enzymatic system (10 mM phosphoenol-pyruvate and 40 effective units/ml pyruvate kinase) did only slightly (by a factor of 1.2) increase ADP production the temperature dependence of which was not altered at all by this "back up" system. Hence, it can be concluded that at the high (15 mM) concentrations of ATP used the fibre did not enter the high tension state (Jewell and Rüegg, 1966) which was observed at 0.2 mM MgATP in the presence of 10 μM Ca^{2+} and of the ATP regenerating enzymatic system.

Tension Transients Following Stretch (Figs. 3 and 4)

A sudden stretch of amplitude 0.4% L_i resulted in tension transients which are at least triphasic (Fig. 3):

1) an elastic tension response during lengthening;
2) biphasic quick phase of tension decay following the end of the stretch;
3) an increase in force at constant degree of extension (delayed tension rise, Jewell and Rüegg, 1966).

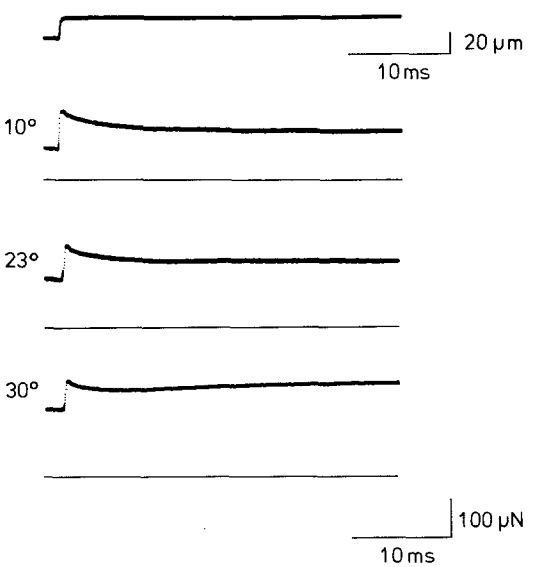

Fig. 3. Effect of temperature on early phases of force responses following stretch (top) in contracted glycerinated single fibres from the dorsal longitudinal muscle from *Lethocerus maximus*. Amplitude of stretch was 0.45% L_i (ΔL 19 μm; L_i 4.3 mm); *S*-shaped length change of duration 250 μs achieved by butanol damped relay stop. Temperatures given opposite to each force record. Signal of force transducer (15 kHz resonance frequency) corrected for its harmonic oscillations by means of an analogue computer. Base line of force given by horizontal line for each force transient. For conditions cf. legend to Figure 2

In the temperature range 10–30° C the amplitudes of the elastic tension responses were decreased by rising the temperature, although the levels of isometric tension before stretch were much higher at 30° C than at 10° C. This indicates that the ratio of apparent stiffness to isometric tension decreases by increasing temperature in these fibrillar muscle preparations. Ford et al. (1977) reported similar results from their studies on tetanized frog anterior tibialis fibres. These fibres also showed a positive effect of temperature on isometric tension and they exhibit only a slight increase of stiffness when temperature rises.

The tension decay during the quick phase following the 0.4% L_i stretches (Fig. 3) is much slower at low temperature (10° C) than at the higher ones (23° C, 30° C). Time to reach 50% of the tension decay was 2.3, 1.1, and 0.6 ms at 10, 23, and 30° C respectively; corresponding to a Q_{10} value of about 2. However, the time course do not follow a single exponential in time: Times to reach 80% of the tension decay (7, 4, and 2 ms at 10, 22, and 30° C) were by a factor of $\sim$ 3 larger than half decay times whereas a single exponential would predict a factor 2.3. These rough estimates agree with those of Abbott and Steiger (1977) who carefully computed two distinct phases of tension decay within the quick phases following stretch in glycerinated rabbit psoas muscle and insect flight muscle.

The quick phase of tension decay is followed by a phase of delayed tension rise; in the tension transient at 30° C (Fig. 3). This delayed rise of tension begins at about 8 ms after completion of the stretch whereas tension didn't appreciably rise within 40 ms in the transients at 23° C and 10° C.

Figure 4 shows the effect of temperature on the delayed tension rise following a stretch of amplitude 1.8% L_i. At the slow sweep time used in recording the transients of Figure 4, the elastic phase (and at higher temperatures even parts of the quick phases) are not resolved. Stretching of the fibre at 35° C induced a biphasic tension response within 200 ms. At this temperature the peak tension was already reached

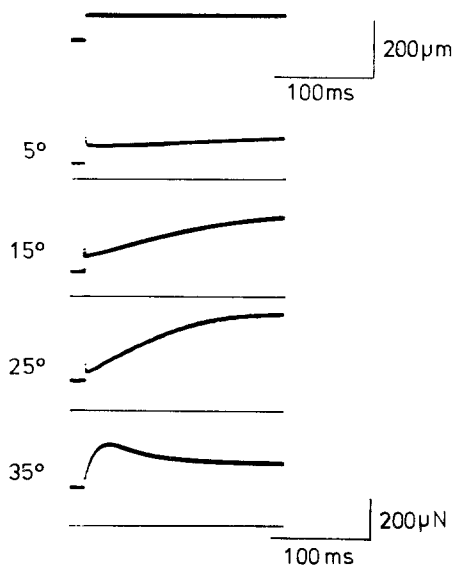

Fig. 4. Effect of temperature on late tension responses following sudden stretches of a single glycerinated fibre from the dorsal longitudinal muscle form *Lethocerus maximus*. Top: stretch of 1.8% L_i (90 µm at L_i 5 mm). Duration of length change 350 µs; relay stop damped by butanol. Temperature indicated at the left of digital oscilloscope record of force. Elastic phase and quick phase not clearly resolved by the digital oscilloscope. Base line of force represented by horizontal full lines. Note: late phase of tension decay seen at 35° C also is present (but not shown) in all other force transients. For conditions cf. legend to Figure 2

about 40 ms after stretch. Lowering the temperature by 10° C increased the time to reach peak tension to about 200 ms corresponding to a Q_{10} value of about 5. The generation of delayed tension is further slowed down when the temperature is decreased to 15° C or 5° C. At 5° C the tension still increases at the end of the record (i.e., 200 ms after the length has ceased to change). Similar results for the speeding up of the delayed tension rise by increasing the temperature were reported by Steiger and Rüegg (1969) and by Abbott and Steiger (1977).

Tension Transients Following Release (Fig. 5)

Figure 5 shows the effect of temperature on the tension transient following a release of amplitude 0.2% L_i. Such a small amplitude of the length change was chosen in order to ensure that the single fibre doesn't become slackish in any phase following the release. At temperatures from 10–23° C three transient phases of tension adjustment can be detected:

1) the elastic phase induced by the release;

2) a quick recovery of tension towards its isometric value;

3) a deactivation phase (Jewell and Rüegg, 1966) during which the tension is falling although the length remains constant.

In addition to these phases there appears (10 ms after release) at 34° C slow recovery of tension toward the isometric level prior to release. This phase was also present in all other transients, but it was then too slow to become visible within 40 ms following the release.

The amplitude of the elastic phase induced by the release (0.2% L_i) is smaller at 23 and 34° C than at 10° C. Thus apparent fibre stiffness decreases with increasing

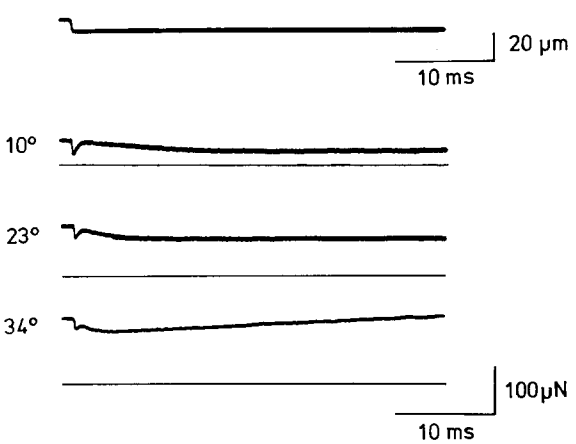

Fig 5. Effect of temperature on early phases of tension changes in responses to a sudden release (top) during contraction. Single glycerinated fibre from the dorsal longitudinal muscle from *Lethocerus maximus*. Release of amplitude 0.25% L_i (ΔL 11 µm; L_i 4.3 mm) ceased to change within 200 µs; relay stop damped by butanol. Temperature is given by numbers opposite to digital oscilloscope traces of force (upper traces) and base line of force (lower traces). Forces recalculated by analogue computation from damped oscillations of the strain components in the force transducer. For conditions cf. legend to Figure 2

temperature irrespective of whether the length change used to measure fibre stiffness was a release or a stretch (cf. Fig. 3).

Figure 5 shows that the quick recovery following release is altered in amplitude and shape by temperature. At high temperature (34° C) the time to reach the relative peak of recovery is shorter than at 10° C (0.5 ms and 2.5 ms, respectively). This increase in recovery peak time with decreasing temperature might be caused partly by a decrease in the rate constant of the quick phase and partly by the much slower deactivation of tension at the lower temperature (10° C) compared to that of 23° C and 34° C.

At 34° C the deactivation phase seems to be over 4–5 ms after the end of the release whereas at 10° C it is not completed within 40 ms. The deactivation phases could be fitted by one exponential function in time. The half times of these phases were 0.7, 1.5, and 4.7 ms at 34, 23, and 10° C ($Q_{10} = 2.3$).

The apparent decrease of the amplitudes of the quick tension recovery following release with increasing temperature might suggest that at high temperature part of the tension transient may have already taken place during the quick release (truncation effects, cf. Huxley and Simmons, 1973). In order to test this suggestion we plotted tension versus length during the release and obtained T curves in a length-tension diagram.

Adjustment of Tension During Release: T Curves (Figs. 6 and 7)

In Figure 6 are shown four T curves recorded during the release of 1.7% L_i amplitude while the single fibre was incubated in the solution producing contraction and four T curves while it was incubated in relaxing solution. Temperatures were 5, 15, 25, and 35° C. The T curves obtained while the fibre was in the relaxed state were hardly affected by temperature. There was a great variability in size and shape of the

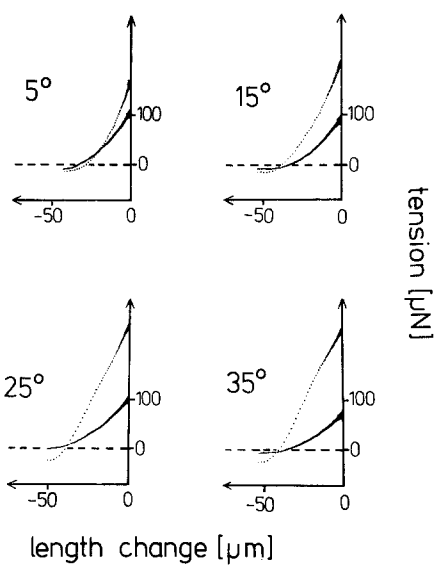

Fig. 6. *X-y* diagrams obtained during quick release from digital oscilloscope records of force (ordinate) and release (abszissa) in contraction (upper traces) and relaxation (lower traces). Single glycerinated fibre from the dorsal longitudinal muscle of *Lethocerus maximus*. Number on the top of the diagrams give temperature. Release of 87 µm (L_i = 5 mm) completed within 350 µs; relay used undamped; force not corrected for damped oscillations of the force transducer. For conditions cf. legend to Figure 2

T curves obtained from contracted fibres. At low temperature (5° C) the slope of the T curve decreases with increasing release, so that the plot becomes upwards curved and appears similar in shape to the T_1 curves observed in tetanized frog muscles (Huxley and Simmons, 1971, 1973; Ford et al., 1977) and in skinned frog muscle (Yamamoto and Herzig, 1978). The curvature of the T curves disappears gradually when the temperature is raised from 5° C to 25° C. At 25° C the T curve is quite linear over the whole range of positive forces. At even higher temperature (35° C) the T curve becomes S-shaped: the slope of the T curve is relatively small for small release amplitudes (up to 0.15% L_i) it increases in the middle part of the curve and decreases again at high releases (0.8—1.0% L_i). Note that for a short time the fibre could even exert negative forces before it becomes slack.

White et al. (1977) have presented evidence that the elasticity of relaxed fibrillar insect flight muscle is passive and parallel to immediate elasticity in contracted fibres. Hence, active tension is the difference between tension in contraction and relaxation (cf. Fig. 6). The effects of temperature on the curves of active tension versus length as shown in Figure 7 are characteristic to glycerinated dorsal longitudinal muscle fibres from *Lethocerus maximus* and could be reproduced in fibre preparations form three different animals. At 5° C the slope of the active T curve decreases gradually with increasing the amplitude of the release; zero tension is attained at a release amplitude of 4.5 nm at a finite positive slope. The active T curve at 15° C shows a subtle curvature. The slope to this curve firstly falls, reaches a minimal value at 6 nm release amplitude and begins then to increase again. The minimal slope of the T curve is shifted to lower release amplitudes when the temperature is raised to 25° C. At even higher temperatures (35° C) the slope to the active T curve appears to be minimal at zero release amplitude; it then increases gradually. At 35° C the active T curve intercepts with positive slope zero tension at 11 nm.

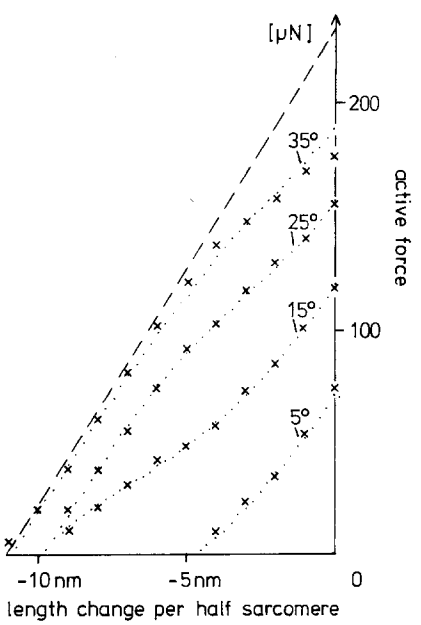

Fig. 7. Tension-length diagrams of active force (ordinate) versus length change per half sarcomere (abszissa). Active force (×) on the ordinate is the difference between the force values reached in contraction and in relaxation given in Figure 6. Dots are best fit estimates to experimental values as revealed from computations predicted by the Huxley and Simmons (1971) model using the parameters of Tables 1 and 2 (for details see Discussion). Slope of broken straight line gives fibre stiffness as calculated under the assumption of absence of cross bridge rotation. For conditions cf. legend to Figure 2

Stiffness Transients Following Length Changes (Fig. 8)

In order to get information on the time course of fibre stiffness within the quick phases of tension transients following sudden length changes we applied short length pulses to the single fibre preparations and recorded the T curves during both length changes of the pulse on the x-y mode of a digital storage oscilloscope.

The time course of tension after a quick release is given in the upper part of Figure 8. Release amplitude was 22 μm ≙ 0.44% L_i and temperature was 25° C. At

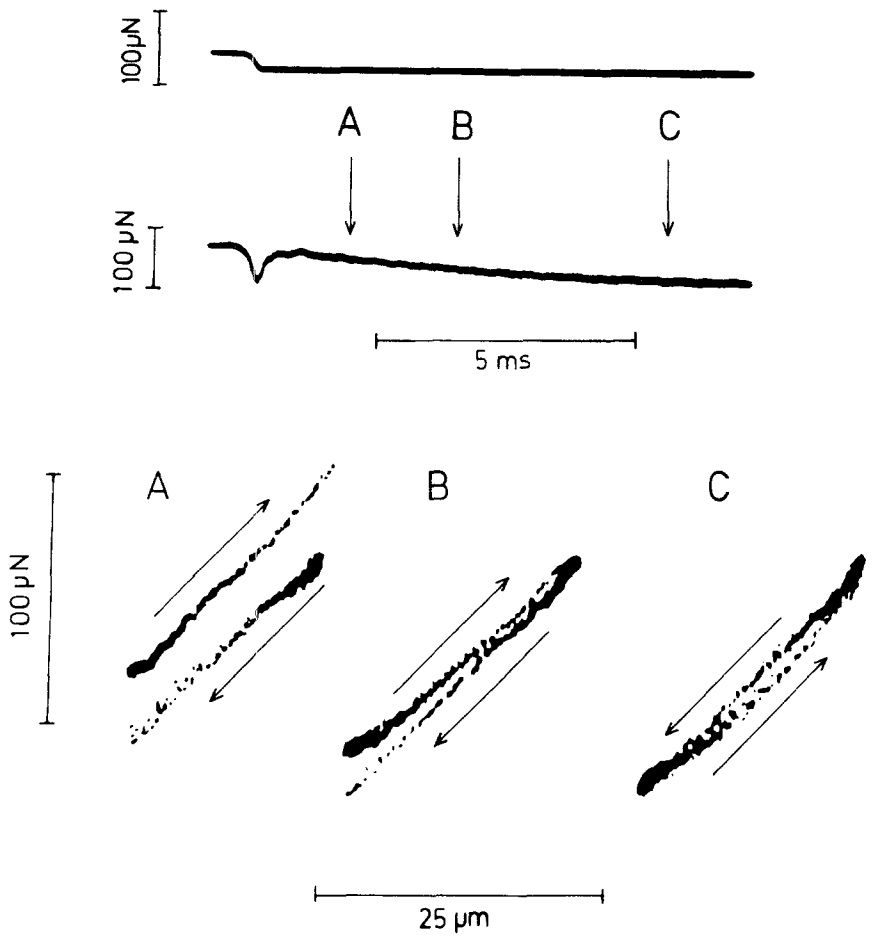

Fig. 8. Change in fibre stiffness following a release. Single fibre glycerinated the 23. Dec. 1977 from the dorsal longitudinal muscle of *Lethocerus maximus*. Isometric contraction (180 μN) was induced by increasing the Ca^{2+} from 0.01 μM to 10 μM in the MgATP-Salt solution of the incubation bath (1 ml). *Upper part:* records of time courses of length and contractile force. Length change of amplitude 0.5% L_i (22 μm; L_i 4.5 mm) was performed within 200 μs; relay stop damped by butanol; force signal corrected for damped oscillations (15 kHz) of the force transducer. *Lower part: X-y* diagrams of force (ordinate) and length (abszissa) recorded during the length change of releases (←) and restretches (→) performed at **(A)**, **(B)**, and **(C)**, i.e., 2, 4, and 8 ms after initiation of the releases. For records of the lower part the relay was used undamped and the analogue computer for force recalculation was not activated. For conditions cf. legend to Figure 2 (25° C)

arrows A, B, or C, the fibre was restretched (transient not shown). The T curves presented in Figure 8A were recorded during release (lower trace) and during restretch performed 2 ms later (cf. arrow A). Both T curves are largely linear. The slope to these T curves indicate a fibre stiffness of 4.5 N/m. The T curve for restretch is parallel shifted to higher forces which were generated during the quick recovery phase of tension following the release (cf. also Güth et al., 1978). Comparable results for tetanized frog fibres were reported by Ford et al. (1974).

When the restretch was performed 4 ms after the release (arrow B) the T curve remains largely linear, however the slope of the T curve recorded during restretch is lower than the slope to the T curve recorded during release; fibre stiffness at the moment of restretch was 3.5 N/m. This gives evidence that during the deactivation phase there occurs a decrease of fibre stiffness. It would be interesting to study the time courses of stiffness and tension during the deactivation phase in response to a quick release in more detail.

When the restretch was performed 8 ms after the release (arrow C, Fig. 8), we observed a change in the quality of the T curve. At the beginning of the restretch the slope to the T curve (3.0 N/m) becomes appreciable smaller than the slope to the T curve of the release (Fig. 8C). During the restretch the T curves become steeper; at the end of the restretch it has even become higher (6.7 N/m) than the slope of the release curve. At present we cannot interpret this curvature.

Discussion

1. The Parameters of Cross Bridge Rotation

It will be shown in this section that a three state cross bridge model similar to the contraction theory proposed by Huxley and Simmons (1971) describes the early phases of tension changes in response to releases in single glycerinated fibrillar fibres from the flight muscle of *Lethocerus maximus*.

This model may be tested by recording the very early phases of the tension responses following an applied length signal. Huxley and Simmons (1971, 1973) checked their model by analysing the tension transients in tetanized frog muscle fibres observed after completion of the length change (cf. also Ford et al., 1977). Güth and Kuhn (1976) and Ford et al. (1976) extended this method by analysing the tension responses obtained during the length change, i.e., by analysing T curves as shown in Figure 6.

As described in Appendix 1 the observed values of tension (crosses) in the tension-length diagram of Figure 7 were fitted by a family of computed T curves as predicted from the Huxley and Simmons (1971) model. The result of this calculation is shown in Figure 7 as dotted lines. The absence of significant deviations between calculated and observed values of forces at any length (maximal deviation less than 8 μN) convincingly demonstrates that the force values predicted by appropriate parameters of the Huxley and Simmons (1971) model fit the observed values of force. This is taken to prove that the three state model of Huxley and Simmons (1971) is with high accuracy able to predict the active tension responses observed during quick releases (T curves, cf. Fig. 7).

Clearly, by means of mechanical studies alone it is not possible to check the structural evidence in favour of the rotational hypothesis of H. E. Huxley (1969) which is adopted in the Huxley and Simmons (1971) model. More recent structural evidence for cross bridge rotation derives from studies in connection with the ATP analogue AMP-PNP (Yount et al., 1971) in fibrillar insect flight muscle. Beinbrech et al. (1976) have observed an increase in the number of cross bridges attached to the actin in a perpendicular position on electron micrographs (Fig. 1A) when AMP-PNP binds to the myosin heads whereas the number of cross bridges attached to the actin in an acute angled position decreased (Fig. 1B). The results of X-ray diffraction studies in presence and absence of AMP-PNP (Goody et al., 1975; Marston et al., 1976) may also be taken to support the rotational hypothesis, although model calculations suggest that other processes than rotation must also play a role when AMP-PNP binds to attached myosin heads (Barrington Leigh et al., 1977) in a stretch dependent manner (Kuhn, 1977).

The Huxley and Simmons (1971) model could fit quantitatively the observed experimental data, if the five parameters of the theory were assumed to have the values discussed below (cf. also Appendix I).

1.1 Stiffness of a Single Cross Bridge. By analysing the early tension changes in response to step changes in length in tetanized frog muscle fibres, Huxley and Simmons (1971) estimated a value $D = 2.5 \times 10^{-4}$ N/m for the stiffness per cross bridge (cf. also Julian et al., 1974). Kuhn (1978) calculated the stiffness of a single cross bridge ($D = 1.2 \times 10^{-4}$ N/m) in fibrillar insect flight muscle by analysing the stretch dependent binding of the non cleavable ATP-analogue β-γ-imido-ATP (Yount et al., 1971) to the active centre of the myosin heads.

The T curves of Figure 7 could be fitted to the Huxley and Simmons (1971) model by assuming length independent stiffness values of a single cross bridge between $D = 1 \times 10^{-4}$ N/m and 1.4×10^{-4} N/m which is in the range of Kuhn's (1978) prediction. The independence of the stiffness of a single cross bridge on length implies that the elastic elements within the cross bridges are Hookean.

1.2 The number of cross bridges attached to actin at any moment was determined to be 1.7×10^8 in a half sarcomere (length 1.2 μm) or 2.4 pmoles in 1 cm of the fibre in order to account for the T curves. This parameter was not affected by temperature; its best fit estimates varied by only 3% in the range of the temperatures (5–35° C) tested.

The total density of myosin heads in fibrillar insect flight muscle was estimated by Tregear and Squire (1973) to 8–10 pmoles in 1 cm fibre (corresponding to a concentration of 200 μM; cf. Marston and Tregear, 1972). Hence, about 25% of the myosin heads present in these fibre preparation seems to be attached to the actin filament at any moment of calcium induced contraction. This number compares well with the estimates of Armitage et al. (1973) who calculated from equatorial X-ray diffraction intensity ratios that in contracted insect flight muscle 10–20% of the myosin heads are attached to the actin. In contrast to the calculation of Huxley and Simmons (1971) for living frog muscles, there is in case of insect fibrillar fibre preparation no discrepancy between the number of attached myosin heads and the total number of heads.

Since neither stiffness of the single cross bridge nor the number of attached myosin heads is appreciably altered by temperature, it follows that fibre stiffness (which resides to a large extent within the cross bridges themselves; Huxley and Simmons, 1971) is also not appreciably affected by temperature. This conclusion implies that dynamic fibre stiffness measured at high frequencies should not depend on temperature. Such calculations imply furthermore that (at 15° C) dynamic fibre stiffness should rise with increasing the frequency of the length signal.

It seems furthermore that the net number of attached cross bridges is not affected by release (otherwise the fits in Figure 7 would not attain their high accuracy), i.e., the curvature seen on the T curves of Figure 7 are *not* due to a net attachment and/or detachment of bridges. Further evidence for this conclusion is apparent from Figure 8A where the "linear" T curves of release and of the restretch performed 1 ms or 2 ms later are of similar slope and parallely shifted. Analysis of these T curves by the Huxley and Simmons (1971) model indeed indicates that the number of attached bridges was not affected during the phases within which cross bridge rotation is assumed to occur. This shows that force is generated within the quick recovery phase without further attachment of cross bridges. Rather, a release induces changes in the free energies within the Hookean elastic elements of the attached bridges, so that by the release the acute angled myosin head position becomes more frequent than the perpendicular position. Thus, during the force generation which follows a release the myosin heads rotate from the perpendicular position (Fig. 1A) to the acute angled position (Fig. 1B) of attachment thereby stretching elastic elements located within the cross bridges.

During the delayed tension rise following 1% L_i stretches of fibrillar insect muscle fibres there is an increase in fibre stiffness to up to 200% of its value before stretch (Schädler et al., 1971; White et al., 1977; Herzig, 1977). Following the interpretation of immediate stiffness given by Huxley and Simmons (1971), this indicates that the number of attached cross bridges is about doubled when such a fibre is activated by stretch (Jewell and Rüegg, 1966). Assuming that one myosin head attaches per force generating cross bridge, this indicates that in a stretch activated fibre nearly all cross bridges are in a force generating position at the top of the delayed tension rise (50% of the total myosin heads compared to 25% before stretch).

1.3 The extension of the elastic element within the force generating cross bridges was determined as $h = 11$ nm. This parameter did not vary with temperature (mean variation less than 5% in the range of temperature tested). Similar values ($h =$ 12 nm) were reported by Kuhn (1978) from analysing immediate and static stiffness of fibrillar insect flight muscle fibres in rigor. From the extrapolation of the T_2 curves to zero tension reported by Huxley and Simmons (1971, 1973) for tetanized frog semitendinousus fibres and by Ford et al. (1977) for living frog tibialis anterior fibres a value of $h = 12$ nm is expected.

These estimates of h are based on the assumption that attached cross bridges can push following releares to negative extensions of their elastic elements. Pushing cross bridges are likely to exist, since the T curves of Figure 7 intercept zero tension with non vanishing slopes. Consequently, at 5° C the zero tension reached at a release amplitude of about 5 nm per halfsarcomere is the sum of roughly 50% pull-

ing cross bridges with their heads in an acute angled position and 50% pushing cross bridges the heads of which are attached in a perpendicular position.

1.4 Temperature Dependence of the Rotational Rate Constants. Ramp shortenings of tetanized frog muscle fibres have shown that the shape of T curves changes when the velocity of the release is decreased under isothermic conditions (Ford et al., 1977; cf. their Figs. 29, 30). We observed comparable alterations in shape of T curves from glycerinated fibrillar fibres when temperature is raised without alteration in shape of the length signal (Figs. 6, 7). This suggests that rising the temperature increases the rate of tension adjustment occurring during the releases. In order to get fits for the active T curves (Fig. 7) increasing values of the rate constant of the myosin head rotation on the actin with increasing temperature and size of the release had to be assumed.

The effect of temperature on the rotational rate constants for the conformational changes of attached cross bridges from the perpendicular position to the acute angled position (r_A) and vice versa (r_B) extrapolated to zero release amplitude are shown in Figure 9. Both rate constants increase when the temperature is raised. The temperature dependence of the rate constants follows the Arhenius law fairly well: in the log rate $-1/T$ (absolute temperature) plots of Figure 9 the values of the fitted rate constants r_A and r_B lay on straight lines. The activation energy of the rate constant r_A ($E_A{}^a = 74.20$ kJ/mole) is markedly higher than the activation energy ($E_B{}^a = 28.1$ kJ/mole) of the rate constant r_B, indicating that the rate constant of rotation from the perpendicular attached cross bridges state (A) to the acute angled position (Fig. 1B) is more affected by temperature than the reverse rotation.

Cross bridge rotation is a very fast process in the dorsal longitudinal muscle fibres studied: from the rate constants r_A and r_B (Table 1, Fig. 9) a mean life time of the acute angled conformation of about 100 μs at 25° C and 300 μs at 10° C is calculated. Thus, Abbott and Steiger (1977) who analysed their tension transients 1.7 ms after initiation of the length change were unable to discuss tension transients due to rotation.

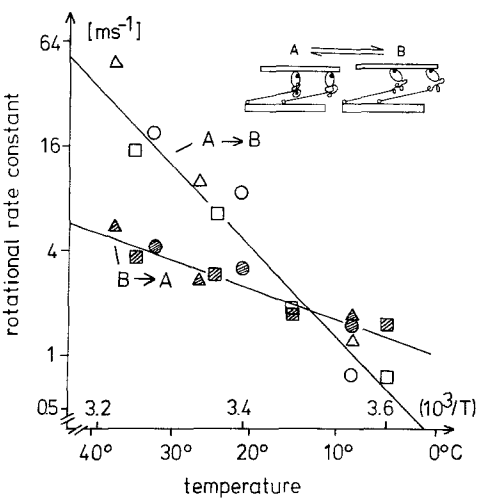

Fig. 9. Arhenius plot showing the temperature dependence of the rotational rate constants of cross bridge movements as revealed from the analysis of the early tension responses tn sudden length changes (T curves; Fig. 7) by means of the Huxley and Simmons (1971) model. On the *ordinate* are given the rate constants for rotation from the perpendicular position of attached myosin heads to their acute angled position (open symbols, cf. Fig. 1) and vice versa (full symbols). *Abszissa* gives temperature on a reciprocal Kelvin scale. For symbols and conditions see legends to Figures 2 and 6

Table 1. Kinetic Parameters of cross bridge cycle (cf. Fig. 1) and Q_{10} values in single glycerinated fibres from *Lethocerus maximus*. K_1 = ratio of AM-ATP : Am.Pr; k_2 = detachment rate constant; K_3 = ratio of $MPr^{(r)}$: MATP at equilibrium deduced from stop flow measurement of Mg^{2+} activated myosin ATPase (Bagshaw and Trentham, 1973); k_4 = apparent attachment rate constant; r_A and r_B rotational rate constants between state A and B (step 6 of Fig. 1) extrapolated to zero release amplitude; ATPase = ATP splitting rate per s in one fibre of 1 cm length

	25° C	Q_{10}
K_1	0.018	2.7
k_2	500 s^{-1}	2.3
K_3	10	–
k_4	1.7 s^{-1}	2.5
r_A	8300 s^{-1}	3.5
r_B	3600 s^{-1}	1.5
ATPase	13.9 pmoles s^{-1} cm^{-1}	2.5

Table 2. Parameters of cross bridge rotation (25° C, zero release amplitude). D = stiffness of a single cross bridge; h = difference between the extensions of the elastic element of the cross bridges in the acute angled conformation; N = number of myosin heads attached to the actin; N_A = number of myosin heads attached in the perpendicular position; N_B = number of myosin heads attached in an acute angled position

D	1.2 10^{-4} N/m
h	11 nm
N	2.4 pmoles cm^{-1}
N_A	0.8 pmoles cm^{-1}
N_B	1.6 pmoles cm^{-1}

Similar high rate constants were reported in a whole tetanized frog sartorius muscle by Truong (1974) who analysed the velocity and retardation of elastic waves in these preparations and estimated from the frequency dependence of these parameters a maximum in the distribution of rate constants of about 10 ms^{-1} (25° C). However, the value of the apparent rate constant as revealed by our study ($r = r_A + r_B = 1.0$ ms^{-1}) is at comparable temperatures higher than that reported by Huxley and Simmons (1971) in tetanized frog semitendinousus fibres ($r = 0.4$ ms^{-1}).

The changes in the shapes of the active T curves (Fig. 7) induced by rising the temperature are linked to a speeding up of the rotational rates at constant value of the number of the attached cross bridges. Consequently, the shapes of the T curves change from a slightly truncated T_1 curve (5° C; cf. Huxley and Simmons, 1973) to a curve approximating a T_2 curve at 35° C, i.e., to the T curve which describes the rotational equilibrium. At 25° C the T curves were mainly "linear" (Fig. 6). However, the slope of this T curve does not measure instantaneous stiffness of the fibre: when interpreted by the Huxley and Simmons (1971) model, instantaneous stiffness corresponds to the slope of the broken straight line in Figure 7 which is by more than a factor of two higher than the mean slope of the T curve at 25° C.

1.5 Entropy and Energy Changes During Cross Bridge Rotation. Szent-Györgyi (1951) concluded from mechanical experiments with different muscle preparations

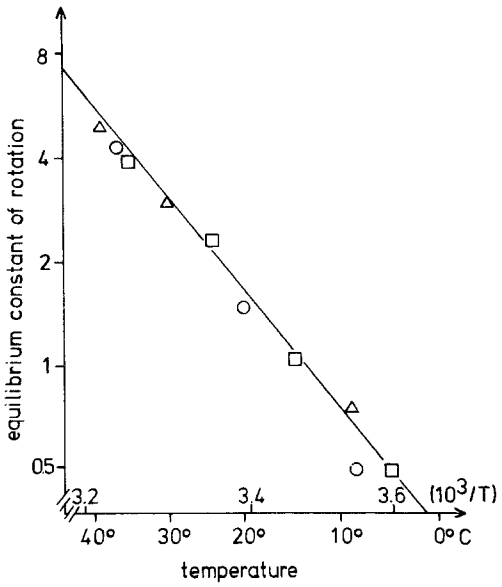

Fig. 10. Van't Hoff plot of equilibrium constant for cross bridge rotation as revealed from analysis of the early tension responses to sudden length changes (*T* curves; Fig. 7) by means of the Huxley and Simmons (1971) model. *Ordinate* gives on a logarithmic scale the ratio of the number of myosin heads attached to the actin in a perpendicular position to those in an acute angled (force generating) position. *Abszissa* gives temperature on a reciprocal Kelvin scale. The linear regression line indicates that phase transitions do not occur during force generation in muscle. For symbols and conditions see legends to Figures 2 and 6

that large changes in entropy take place during muscular contraction (cf. also Wöhlisch, 1926; Pryor, 1950; Kuhn et al., 1960). It will be shown in this section that our results confirm this conclusion: force generation in muscle is a process during which entropy of attached cross bridges increases.

As shown in the discussion of paragraph 1.2 the increase in isometric tension with increasing temperature (Fig. 2A) occurs at a constant number of cross bridges attached to the actin at any moment. When interpreted in terms of the Huxley and Simmons (1971) model, this result reflects that by rising the temperature there is a transition of cross bridges from the perpendicular position into the acute angled position: the ratio of the rate constants for rotation to and from the acute angled position of attached myosin heads (Fig. 9) is increased by rising the temperature.

Hence, low temperatures stabilize the perpendicular conformation (Fig. 1A). This indicates that the internal energy of the perpendicular conformation is lower than the internal energy of the acute angled attached cross bridges (Fig. 1B; $\Delta E = E_B - E_A > 0$). At 15° C the rate constants from and to conformation A are equal (Fig. 9); van't Hoff's law then implies that the standard free energy ($\Delta F^\circ = \Delta E - T\Delta S^\circ$) of the rotational reaction vanishes at 15° C. Thus the standard entropy change (ΔS°) of the cross bridge rotation from A to B (Fig. 1) has a positive value: the standard entropy of conformation B is greater than the standard entropy of cross bridge conformation A. It is thus a positive change in standard entropy which allows (*against* a positive energy change) the spontaneous rotation from the perpendicular cross bridge position (A) into the acute angled position (B) during the force generating step of the cross bridge cycle.

Figure 10 shows a plot of the rotational equilibrium constant, i.e., the ratio of myosin heads in the perpendicular position to heads in the acute angled position on a logarithmic scale versus the reciprocal of absolute temperature. The values of the rotational equilibrium constant ($= r_A/r_B$) lay on a straight line. According to van't Hoff's law, this indicates that the energy changes $\Delta E = 46.1$ kJ/mole and the

changes in standard entropy $\Delta S^\circ = 0.16$ kJ/mole/K do not depend on temperature. A temperature dependence of energy changes and/or entropy changes would, however, be expected, if a phase transition (e.g., a helix-coil transition, cf. Flory, 1956; Doty et al., 1957) is involved in the force generating step of the cross bridges cycle (cf. Davies, 1963; Harrington, 1971; Holmes, 1977; Mason, 1978). The lack of a curvature in the van't Hoff plot (Fig. 10) excludes this possibility. However, the values of ΔE and ΔS° could be accounted for by a conformational change of attached cross bridges.

1.6 The Nature of the Elasticity Within the Elastic Elements of the Cross Bridges. The analysis of the temperature effects on tension also enables to discuss the thermoelastic phenomena (Wiegand and Snyder, 1934; Hill, 1953; Woledge, 1961; Huxley and Simmons, 1973) within the elastic elements. It is to be shown in this section that the elasticity of the elastic elements is of entropic origin, i.e., it may be accounted for by a randomly coiled protein.

The Wiegand and Snyder equation relates the temperature coefficient of isometric tension ($\partial F/\partial T$) and the entropy changes induced by unit length ($\partial S/\partial L$) under isothermic conditions:

$$\frac{\partial F}{\partial T} = -\frac{\partial S}{\partial L}. \tag{1}$$

Equation 1 predicts that an increase of isometric tension with increasing temperature (cf. Fig. 2A) is correlated with a stretch induced flux of entropy from the contractile elements to the sarcoplasm which would in turn warm up by stretching the fibre.

The entropy changes induced by stretching the fibre under isothermic conditions are composed of at least two components:

1) entropy changes within the elastic element;

2) entropy changes due to a length dependent rotation of the attached cross bridges.

A release induces the cross bridges to rotate from the perpendicular position (A) to the acute angled position (B). Conversely, some cross bridges rotate from B to A (Fig. 1) when the fibre is stretched. Since the standard entropy of the latter process was found in section 1.5 to be negative, the stretch induced cross bridge rotation from B to A gives rise [according to the Wiegand and Snyder equation (1)] to a positive temperature coefficient of isometric tension (cf. Fig. 2A).

The number of cross bridges in a half sarcomere which are expected to rotate per unit amplitude of stretch from position B to position A can be calculated from the Huxley and Simmons (1971) model (cf. Appendix 2) to 22.8 nmoles cm^{-1}. This number multiplied with the standard entropy change of rotation $\Delta S^\circ = 0.16$ kJ/mole/K (cf. section 1.5) gives at 15° C the stretch dependent entropy increase

$$\left(\frac{\partial S}{\partial L_{rot}}\right) = -3.8\ \mu N/K. \tag{2}$$

The slope of the isometric tension-temperature curve (□, Fig. 2A) gives at 15° C.

$$\left(\frac{\partial F}{\partial T}\right) = 4.1 \text{ μN/K} . \tag{3}$$

There is thus a small difference between the temperature coefficient of isometric tension expected (cf. eqs. 1 and 3) from the stretch induced rotation and the observed values. This difference is small; but for all experiments performed it was positive and of the same order of magnitude.

This suggests that the elastic elements of the cross bridges undergo negative entropy changes when the fibre is stretched: $(\partial S/\partial L)_{\text{elast. el.}} = -\partial F/\partial T - (\partial S/\partial L)_{\text{rot.}} = -0.3$ μN/K (cf. eqs. 1–3). This implies that the mechanical work ($F\Delta L \cong 100\ \Delta L$ μN; cf. Fig. 2A) performed by extending the elastic elements within the cross bridges is nearly completely transformed into heat [$T\,\partial S/\partial T)_{\text{elast. el.}}\ \Delta L \cong -90\ \Delta L$ μN], i.e., the elastic elements behave isoenergetically upon stretching. This is characteristic of a randomly coiled macromolecule (Kuhn, 1934; Guth and Mark, 1935) the stiffness of which was given by

$$D = 3\ k\ T/r^2 , \tag{4}$$

where r is the radius of the coil and $k\ T = 4.2 \times 10^{-21}$ J at 15° C. The stiffness of 1.2×10^{-4} N/m (cf. section 1.1) could thus be described by a random coil of 8 nm radius. The randomly coiled protein portion within the myosin molecules would be comparable in size to that of the myosin heads (length about 15 nm). Studies of intrinsic viscosity and sedimentation of polypeptides in solution suggest that such a coil would comprise about 60 amino acid residues (cf. Benoit, 1948). Such a random coil could be stretched by about 20 nm without appreciable increase in stiffness (Kuhn and Grün, 1942).

The Wiegand-Snyder equation (1) implies that a stretch would induce a heat flux ($Q_{\text{rev.}} = T\Delta S$) from the cross bridges rotated to the perpendicular position to the sarcoplasm of the fibre. Following a stretch of 1% L_i an increase of temperature of about 5×10^{-4} °C is expected from equation (2) assuming a fibre diameter of 80 μm (Jewell and Rüegg, 1966) and a specific heat capacity of 0.25 $JK^{-1}\ cm^{-3}$. Conversely, a 1% L_i release would give rise to a temperature fall of 5×10^{-4} °C within the sarcoplasm due to cross bridges which rotate (release dependently) from position A to B (Fig. 1). Apparently, there is a discrepancy between the signs of these calculated temperature changes and those measured following a release in tetanized toad sartorius muscles (Woledge, 1961; cf. also Hill, 1953). However, the temperature measurements in the estimates of Woledge (1961) and of Hill (1953) had a time lag of more than 20 ms, so that the processes involved in the deactivation phases are also comprised in these estimates. This suggests that in the fibres there is more heat produced during the deactivation than can be absorbed during the very fast length dependent rotational movements of the attached cross bridges.

2. Attachment Kinetics of Cross Bridges

The glycerinated fibres used made possible to determine not only the number of myosin heads attached to the actin at any moment but also the rates of ATP split-

ting under comparable conditions. This makes possible to discuss the attachment and detachment kinetics of cross bridges to actin.

In fibres the attachment process of cross bridges to the actin seems to take place according to the kinetic scheme of Eisenberg and Kielley (1973) for the following reasons: in section 1 we have presented evidence that the early phases of tension adjustment following releases can be accounted for by cross bridge rotation during which the cross bridges constantly remain attached to the actin. Thus, a rapid attachment-detachment equilibration as proposed by Lymn and Taylor (1971) is unlikely to occur. Furthermore, such a rapid detachment-attachment process could allow slippage of cross bridges along the actin filament (Flitney and Hirst, 1978; Güth and Kuhn, 1978): it is possible to decide whether or not slippage occurs from analysing the tension transients induced by short length pulses. Flitney and Hirst (1978) and Güth and Kuhn (1978) have shown that slippage is induced by large stretches ($\Delta L_i > 0.5\%\ L_i$) but, that slippage is absent if this limit of stretch is not exceeded. The results of the study of Güth et al. (1979) showed that the time to reach a new steady state following slippage is several tens of milliseconds. This indicates that the processes following large distortions in which attachment and detachment are involved are of a different kind than those involved in the fast processes of cross bridge rotation.

In fibres attachment should thus be discussed by means of the Eisenberg and Kielley (1973) scheme which postulates that the fast recombination of the myosin product complex with actin

$$\mathrm{M.Pr} + A \underset{k_{5-}}{\overset{k_5}{\rightleftharpoons}} \mathrm{AM.Pr}^* \tag{5}$$

is preceeded by a slow reversible conformational change between the myosin product complex and the refractory state [$\mathrm{MPr}^{(r)}$]

$$\mathrm{M.Pr}^{(r)} \underset{k_{4-}}{\overset{k_4}{\rightleftharpoons}} \mathrm{M.Pr}\,. \tag{6}$$

Provided that the conditions $k_5\,[A] \gg k_{4-}$, k_{5-} are fulfilled the *apparent* attachment rate becomes (cf. Chock et al., 1976):

$$k_4\,[\mathrm{M.Pr}^{(r)}] \tag{7}$$

which (under steady state conditions) is equal to the ATP splitting rate.

The analysis of the T curves (Fig. 7; section 1.2) by means of the Huxley and Simmons (1971) model has revealed that 7.6 pmoles cm^{-1} myosin heads are in a detached position at any moment of a steady state. With the values of the equilibrium constant for the hydrolytic step 3 ($K_3 = 10$; Bagshaw and Trentham, 1973) this gives a density of [$\mathrm{MPr}^{(r)}$] = 6.9 pmoles cm^{-1}. Under steady state conditions the ADP production in 1 cm of a fibre was 13.9 pmoles $cm^{-1}\ s^{-1}$ (25° C; Fig. 2B; cf. Rüegg and Tregear, 1966; Breull et al., 1973; Pybus and Tregear, 1975). Hence the specific attachment rate constant becomes $k_4 = 1.7\ s^{-1}$. This value compares well with values of the actin activated myosin ATPase in biochemical kinetic studies at saturating actin concentrations ($k_4 = 0.9\ s^{-1}$: Chock et al., 1976).

At the high (15 mM) MgATP concentrations used in fibrillar muscle fibres the attachment of cross bridges to the actin seems to be rate limited by a conformational change within the detached cross bridges. This confirms the theory of Thorson and White (1969) who proposed that in these preparations showing stretch activation (Jewell and Rüegg, 1966) the attachment rate constant for cross bridges to the actin depends on the strain within the thick filaments. For further experimental evidences in favour for the Thorson and White (1969) model see White (1973) and Abbott (1973).

3. Detachment Kinetics of Cross Bridges

Detachment of cross bridges may occur during the deactivation phase following a release (Fig. 5; Jewell and Rüegg, 1966; for theories cf. Julian, 1969; White and Thorson, 1972): during this phase occured a decrease in tension concomitantly with a decrease in fibre stiffness (Fig. 8).

Based on the results of their overlap experiments, Huxley and Simmons (1971, 1973) showed that immediate fibre stiffness reflects the number of cross bridges attached to the actin at any moment. An analysis of the T curves shown in Figure 8A and B for deactivation by means of the Huxley and Simmons model (1971) has revealed that the decrease in fibre stiffness is due to a net detachment of cross bridges, although cross bridge rotation also takes place during these "linear" T curves. Consequently, the decay of tension observed during deactivation (Figs. 5 and 8) is due to a net detachment of cross bridges from the actin.

Provided that by a length change the cross bridges are distorted far enough from their steady state distribution and provided that the attachment rate constant of undistorted bridges is far slower than the detachment rate constant, Abbott and Steiger (1977; cf. their Appendix) have pointed out that the time constants of the tension decay within the slower part of the quick phase following stretch reflects the apparent rate constant of the detachment process itself. This is taken to mean that the stiffness decreases observed during the phase of tension decay shown in Figure 8 reflect detachment without concomitant attachment.

A reliable value of the detachment rate constant at 25° C is calculated from the time constant of the single exponential time course of deactivation in Figure 5 ($k_2 = 500\ s^{-1}$, $Q_{10} = 2.3$; Table 1).

It will now be shown that a second rate limiting step (in addition to that in which the refractory state is involved) takes place when the myosin heads are attached to the actin. From biochemical studies of the actin activated myosin Mg ATPase it is well established that dissociation of actin takes place from the ternary actomyosin-ATP complex (step 2, Fig. 1; Lymn and Taylor, 1971). Furthermore, it is assumed in the biochemical kinetic schemes of ATP splitting cycles by actomyosin that the rate limiting step of ATP break down is the slow and mainly irreversible product dissociation from actomyosin (White and Taylor, 1976). In fibres there seems also to occur a rate limiting step of ATP cleavage on the actomyosin pathway: the high number of force generating cross bridges (1.6 pmole cm^{-1} at 25° C, section 1.4) and the high value of the detachment rate constant ($500\ s^{-1}$) cannot account for the observed low ADP production (13.9 pmoles $cm^{-1}\ s^{-1}$, 25° C; Fig. 2B). In conse-

quence, a step between the state of force generating cross bridges (AM.Pr) and the ternary actomyosin-ATP complex must limit the rate of ATP break down. Since the decay of force generating cross bridges occurs with a high rate constant of detachment, the rate limiting step cannot be slow: there seems to be a fast equilibrium between the force generating bridge (AM.Pr) and the ternary actomyosin-ATP complex (AM.ATP, step 1 of Fig. 1):

$$\text{AM.Pr} + \text{ATP} \rightleftharpoons \text{AM.ATP} + \text{ADP} + P_i\,. \tag{8}$$

If shifted to 98.3% to the actomyosin product complex (= 1.6 pmoles cm^{-1} of force generating cross bridges) this equilibrium would give rise to a low density of the actomyosin-ATP complex (0.028 pmoles cm^{-1}). Under these assumptions the product from the high detachment rate constant (500 s^{-1}) and the low AM.ATP density could account for the low rate of ATP cleavage (0.028 pmole $cm^{-1} \times 500\ s^{-1} = 13.9$ pmole cm−1 s^{-1}).

Possible values of the ratio between the number of actomyosin complexes with ATP and product are thus; AM.ATP : AM.Pr = 0.017 with a $Q_{10} = 2.7$ (Table 1).

4. Further Conclusions

The major aim of this study was to show that the cross bridge rotation model of Huxley and Simmons (1971; for extensions cf. Julian et al., 1974 and for alterations cf. Eisenberg and Hill, 1978) predicts the early tension responses to quick releases (cf. Figs. 6 and 7) observed in glycerinated fibrillar single fibres of insect flight muscle. The study has provided evidence that force generation in these preparation is at least a two stage process between attached cross bridge states. Muscular force is generated when attached myosin heads rotate with a highly temperature dependent rate from a perpendicular position to an acute angled position. The equilibrium between these states is quickly (within less than 100 μs) attained. Consequently, alterations in contractile tension may reflect changes of the rotational equilibrium, i.e., force generation per se does not dissipate free energy, it rather transforms chemical free energies set free by conformational changes occurring in attached myosin heads into elastic free energy of randomly coiled protein portions located presumably within a hinge region of the myosin molecule. Furthermore, since cross bridge rotation quickly leads to an equilibration between two attached states which are roughly equally populated, rotation cannot rate limit the cross bridge cycle.

After cross bridge rotation is completed, there may occur a detachment of myosin heads from the actin as suggested by the theories of Julian (1969) and White and Thorson (1972) as well as by the experiments of Abbott and Steiger (1977). This cross bridge detachment is, for the first time, experimentally verified by this study: Figure 8 shows a decrease of fibre stiffness within the deactivation phase following release. On the basis of the Huxley and Simmons (1971) model the enhanced detachment during these phases is interpretable in terms of the release dependent increase in the number of cross bridges attached to the actin in the acute angled position (Fig. 1B). Note that a first order reaction is equal to the product of the rate constant times the concentration of a species (or its number per half sarcomere). So, even at a

length independent value of the detachment rate constant (k_2; Table 1) an increase in the rate of detachment is expected from the increased number of acute angled cross bridges per se. Conversely, following a stretch a decrease of the detachment rate is expected from the increase in the number of cross bridges which rotated stretch dependently to the perpendicular myosin head position. This prediction of the Huxley and Simmons (1971) model was verified by Abbott and Steiger (1977) who found in glycerinated psoas muscle and in fibrillar flight muscle that the slower part of the quick phase (i.e., cross bridge detachment) had a rate constant which decreases with increasing the amplitudes of the stretches to up to 0.2% L_i.

Table 1 shows values of the kinetic parameters of different stages of ATP splitting by the myosin heads in fibrillar fibres. The Q_{10} values of these parameters are with one exception (cross bridge rotation) near to 2.5. This indicates in terms of the temperature dependence of rate constants that the number of cross bridges attached to the actin is not affected by temperature. But, such a phenomenological description cannot explain whether or not the number of actin-myosin interactions is independent of temperature by a superimposed mechanism within the stages of actin activated myosin ATPase.

The delayed tension rises following large stretches (1.8% L_i; Fig. 4) exhibit a Q_{10} of about 5. This indicates that these phases cannot be described by a single stretch induced attachment process of cross bridges (of $Q_{10} = 2.5$; Table 1). Evidences that activation by large stretches is not a single process also derives from the studies of Schädler et al. (1971) and Herzig (1977) who showed that the ratio of stiffness to tension decreases during the later phases of the delayed tension rises.

Appendix I

Computations of T Curves Using the Huxley and Simmons (1971) Model

The force (F) developed in a half sarcomere of a striated muscle fibre is described by the Huxley and Simmons (1971) model by the following three equations:

$$F = F_0(y + n)\,; \quad F_0 = DN \cdot h\,; \quad y = L/h\,, \tag{A1}$$

where D is the stiffness per cross bridge, N is the total number of attached bridges, h is the extension of the elastic element, n is the relative number of cross bridges in the acute angled position (B) and L is the displacement between the thin and thick filament. The relative number of cross bridges in the acute angled position is calculated using the first order rate equation of rotation:

$$\dot{n} = -(r_{Ay} + r_B)n + r_{Ay}\,, \tag{A2}$$

where r_B is the rate constant for a transition from the acute angled position (Fig. 1B) to the perpendicular position (Fig. 1A) and

$$r_{Ay} = r_A \exp(-\alpha y)\,; \quad \alpha = Dh^2/kT \tag{A3}$$

is the length dependent rate constant for the transition from A to B [cf. Hill's (1974) Fig. 11; for another model of the length dependence of the rotational rate constants see White and Thorson (1973)].

In order to solve the differential equation (A2) the whole length change of amplitude (y_0) was divided into 500 intervals of equal length and the length signal approximated by 500 step functions in length. The applied release signal had the shape of a potential function in time (cf. Methods):

$$y = -y_0(t/t_0)^{2.5}, \tag{A4}$$

where the duration of the release was $t_0 = 350$ μs. Equation (A4) allows to calculate the duration $t_i = t_i - t_{i-1}$ of the i-th length step. During the i-th length step the relative number of cross bridges in conformation B tends to attain the equilibrium value

$$G_i = r_{Ay_i}/(r_{Ay_i} + r_B). \tag{A5}$$

The solution of the rate equation for rotation (A2) gives:

$$n_i(t) = G_i + (n_{i-1} - G_i) \exp[-(r_{Ay_i} + r_B) \cdot \Delta t_i]. \tag{A6}$$

The values of n_i can thus be calculated from the values at zero time (n_0) by iteration.

(These simple calculations could be performed with the help of a Texas instruments pocket calculator TI-59.)

The parameters F_0, $r_A + r_B$, r_A/r_B and h were varied at each temperature (whereas the parameter α was only varied for the whole set of data; cf. eqs. 4 and A3) until a best least square fit was obtained (minimal variance). Minima of variance were sharpest for variations of F_0 and decreased in sharpness from h, r_A/r_B, $r_A + r_B$ in this order.

Appendix II

The Effect of Stretch on the Number of Acute Angled Cross Bridge Conformations

Under equilibrium conditions the relative number of cross bridges attached to the actin in an acute angled position is given by equation (A3) and (A5). These equations imply that cross bridges rotate from the acute angled position to the perpendicular position by stretching the fibre. The derivative of the number of acute angled cross bridges ($= N \cdot n$) over fibre length ($= h \cdot y$) is easily calculated from equations (A3, A5):

$$\frac{\partial N_B}{\partial L} = \frac{N}{h} \cdot \frac{\partial G}{\partial y} = -N \cdot \frac{Dh}{kT} \cdot \frac{r_A/r_B}{(1 + r_A/r_B)^2}. \tag{A7}$$

With the numbers given in the discussion (section 1): $N = 1.73 \times 10^8$, $D = 1.2 \times 10^{-4}$ N/m, $h = 11$ nm (Table 2) and $r_A/r_B = 1$ (15° C, Fig. 10) equation (A7) yields:

$$\frac{\partial N_B}{\partial L} = -2.28 \times 10^{-8} \text{ moles m}^{-1}. \tag{A8}$$

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