Comparison of energy output during ramp and staircase shortening in frog muscle fibres

Marco Linari* and Roger C. Woledge

Department of Physiology, University College London, Gower Street, London WC1E 6BT, UK

1. We compared the rates of work and heat production during ramp shortening with those during staircase shortening (sequence of step releases of the same amplitude, separated by regular time intervals). Ramp or staircase shortening was applied to isolated muscle fibres (sarcomere length, 2·2 μm; temperature, ~1 °C) at the plateau of an isometric tetanus. The total amount of shortening was no greater than 6% of the fibre length.

2. During ramp shortening the power output showed a maximum at about 0·8 fibre lengths per second (L_0 s^{-1}), which corresponds to 1/3 the maximum shortening velocity (V_0). For the same average shortening velocity during staircase shortening (step size, ~0·5% L_0) the power output was 40–60% lower. The rate of heat production for the same average shortening velocity was ~45% higher during staircase shortening than during ramp shortening.

3. The relation between rate of total energy output and shortening velocity was well described by a second order regression line in the range of velocities used (0·1–2·3 L_0 s^{-1}). For any shortening velocity the rate of total energy output (power plus heat rate) was not statistically different for staircase (step size, ~0·5% L_0) and ramp shortening.

4. The mechanical efficiency (the ratio of the power over the total energy rate) during ramp shortening had a maximum value of 0·36 at 1/5 V_0; during staircase shortening, for any given shortening velocity, the mechanical efficiency was reduced compared with ramp shortening: with a staircase step of about 0·5% L_0 at 1/5 V_0 the efficiency was ~0·2.

5. The results indicate that a cross-bridge is able to convert different quantities of energy into work depending on the different shortening protocol used. The fraction of energy dissipated as heat is larger during staircase shortening than during ramp shortening.

In the shortening muscle actin–myosin cross-bridges are cyclically and asynchronously formed in the region of overlap between thick and thin filaments (A. F. Huxley, 1957). In each interaction force is generated in the direction of shortening due to a structural change in the myosin head, the working stroke (H. E. Huxley, 1969). The work produced in the interaction is accounted for by the hydrolysis of one molecule of ATP (Lymn & Taylor, 1971; Huxley, 1974). According to the model of Huxley & Simmons (1971), the quick tension recovery, following a step length change, is due to the synchronous action of the cross-bridges formed. A step release imposed at the plateau of an isometric tetanus perturbs the equilibrium between different force generating states and promotes the subsequent synchronous transition to the final states of the working stroke. Multiple release experiments (staircase shortening) have shown that in frog muscle fibres at low temperature the ability of the cross-bridge to perform another working stroke following a step is regenerated with a rate constant of about 100 s^{-1} (Lombardi, Piazzesi & Linari, 1992). Thus, the repriming of the working stroke seems to be faster than the rate of cross-bridge cycling during steady shortening, as measured by the rate of ATP splitting (< 10 s^{-1}; for references see Woledge, Curtin & Homsher, 1985). This suggests that during staircase shortening the cross-bridge is able to perform more than one working stroke per molecule of ATP split. However, another possibility is that the stepwise shortening causes a more rapid cycling of the cross-bridges than the ramp shortening and extra ATP is split, so that a one-to-one relation could still be possible between working strokes and ATP splitting during ramp shortening. In this case, for the

* Present address: Dipartimento di Scienze Fisiologiche, Università degli Studi di Firenze, Viale G. B. Morgagni, 63-50134 Firenze, Italy.

Table 1. Heat production in two different regions of the fibre

Velocity (L_0 s^{-1})	Heat rate (μJ s^{-1})	
	A	B
0·12	7·4	7·14
0·33	11·58	11·4
0·82	19·94	20·19
1·59	35·73	34·91

The length of each region of the two points was 2 mm; the distance between the beginning of region and the extremity connected to the force transducer was 5 and 3 mm for regions A and B, respectively. The isometric heat rate (means ± S.E.M.), estimated in the isometric tetani delivered during the experiment, was 5·23 ± 0·15 μJ s^{-1} ($n = 11$, A) and 4·85 ± 0·14 μJ s^{-1} ($n = 13$, B).

same mean shortening velocity, during staircase shortening the muscle would split more ATP than during ramp shortening. Consequently the total rate of energy liberation (the sum of the power and rate of heat production) would increase. The experiments described in this paper were designed to investigate whether or not this is the case by comparing the total rate of energy liberation in staircase and ramp shortening.

METHODS

Preparation and mounting of the fibres

Frogs (*Rana temporaria*) were killed by decapitation followed by destruction of the spinal cord. Single fibres were dissected from the lateral head of the tibialis anterior muscle. The sarcomere length of each fibre was set to 2·2 μm by laser diffraction measurements in the dissecting chamber. The fibre length and the vertical (d) and horizontal (D) diameters were measured under a stereomicroscope with ×10 and ×100 magnification. Cross-sectional area ($A = \pi dD/4$) was determined as if the section of the fibre were elliptical. The fibres were horizontally mounted over a thermopile in a drop of Ringer solution between a length and a tension transducer; the connections between fibre tendons and the transducer hooks were made by means of T-shaped platinum foil clips similar to those described by Ford, Huxley & Simmons (1977); the total length of the tendon attachments outside the clips was less than 500 μm. After adjusting the fibre to the length corresponding to a sarcomere length of 2·2 μm, as determined in the dissecting chamber, most of the solution was removed; in this way there was only a very thin layer of Ringer solution around the fibre and between the fibre and the hot junctions of the thermopile.

Experimental protocol

During the experiment the temperature in the aluminium frame holding the cold junctions of the thermopile was about 1 °C and it was continuously monitored. After thermal equilibration, the sensitivity of the thermopile and the heat loss constant were determined by means of the Peltier heating method (Kretzschmar & Wilkie, 1975). Stimuli of 0·5 ms duration and 1·5 times threshold were applied to the fibre through the platinum clips. The optimal stimulation frequency for a fused tetanus was determined in each fibre and ranged from 16 to 25 Hz. Tetanic stimuli of 1 s duration separated by 5 min intervals were used. When the tetanus plateau was reached, ramp shortening or staircase shortening (a series of identical steps separated by regular time intervals) was applied at the plateau of isometric tetani; the total amount of shortening was not larger than 6% of the fibre length (L_0), in order to keep the sarcomere length in the plateau region of the tension–sarcomere length relation (Gordon, Huxley & Julian, 1966). An isometric contraction was interposed between each two shortening contractions. At the end of the experiment the fibre was dried in air, cut free from the tendons and weighed.

Solution

The Ringer solution had the following composition (mM): 115 NaCl, 2·5 KCl, 1·8 $CaCl_2$ and 3 sodium phosphate buffer at pH 7·1.

Calorimetric measurements

A metal-film thermopile similar to those described by Mulieri, Luhr, Trefry & Alpert (1977) and Curtin, Howarth & Woledge (1983) was used for calorimetric measurements. The thermopile was made of antimony and bismuth thermocouples evaporated onto a thin mica sheet (thickness, 6 μm). The length of the thermopile was 18·5 mm; there were four thermocouples per millimetre and usually a 2 mm long segment (resistance, 200 Ω) was used to detect the temperature change of the fibre. The Seebeck coefficient, determined as described by Kretzschmar & Wilkie (1972, 1975) was 89·3 μV K^{-1} per thermocouple. The output of the thermopile was amplified (gain 10^6) by an Ancom C-3A amplifier modified to chop at 1 kHz, as described by Dijkema, Elzinga & Holewijn (1985); the output was filtered at 50 Hz.

To measure the heat production in a single muscle fibre it is necessary to minimize both the distance between the thermopile surface and the fibre and the volume of surrounding fluid. This could imply, during shortening, frictional effects of the fibre over the thermopile which, in turn, would affect the calorimetric and mechanical measurements. In a few fibres we tested whether heat measurements were significantly affected by friction by determining (i) whether in the fibre at rest there was a detectable heat production during shortening or lengthening and (ii) whether in the tetanized fibre there was a difference in the measured heat production during shortening at different positions along the fibre (the frictional effect should be greater near the motor lever and smaller near the force transducer lever). Table 1 shows heat rates, obtained during a period of ramp shortening with different velocities, in two different regions along the fibre, one close to the motor end (region A) and the other close to the force transducer end (region B). For each shortening velocity the heat rates in the two different regions were very similar; if the isometric heat rate is taken into account, the relative heat rate was actually higher in the region near to the force transducer end, a behaviour opposite to that expected by the frictional effect; this is in accordance with other work (Woledge, Wilson, Howarth, Elzinga & Kometani, 1988; Barclay, Constable & Gibbs, 1993). The results of these tests

lead us to conclude that, with the level of resolution of our heat measurements, friction during shortening does not produce any significant effect on heat production.

The conduction delay of the thermopile was estimated by electrically heating the muscle fibre (and surrounding Ringer solution) with a single rectangular pulse of 50 V amplitude and 1 ms duration; Fig. 1 compares the response of the thermopile–amplifier system for the heating pulse with the amplifier response to a square voltage input. The downward deflection at the start is probably an artifact due to the stimulus (Mulieri *et al.* 1977). The delay in the thermopile–amplifier system trace is about 2 ms and its rise time is ~8 ms.

Loudspeaker motor

Staircase (sequence of length steps of the same amplitude separated by regular time intervals) and ramp shortening were imposed on the muscle fibre by means of a servo-controlled loudspeaker length transducer similar to those described by Ambrogi Lorenzini, Colomo & Lombardi (1983). For different sizes of the step in the staircase, the same average shortening velocity was obtained by changing opportunely the interval between steps. The fibre was attached to the motor via a lever 70 mm long. Step displacements of the loudspeaker lever arm were complete in about 200 μs.

Measurements of force, work and power

Force was usually measured by means of a capacitance gauge transducer (Series 400, Cambridge Technology Inc., Cambridge, UK). The resonant frequency of the force transducer was 2·2 kHz, the sensitivity 4·5 mN V^{-1} and the noise 2 mV peak to peak. The force transducer lever was about 40 mm long. Due to the necessary misalignment between the fibre lying in contact with the thermopile and the force transducer lever, which must be clear of the thermopile surface, there is an oscillation of about 300 Hz in the tension response following a step length change. During staircase shortening a tension transient was evoked by each step. As described by Piazzesi, Linari & Lombardi (1993), both the force recorded at the end of the step length change (T_1) and the force attained before the next step (T_i) reached steady state characteristics after a few steps.

The power during the ramp shortening was calculated as the product of the velocity and the steady state force. During staircase shortening, ideally work is done by the fibre only during the step (Lombardi *et al.* 1992). Assuming that force during the step drops linearly with the length change, the work done by the fibre during each step can be calculated as $W = [(T_1 + T_i)/2]\Delta L$ (where $(T_1 + T_i)/2$ is the mean force during step length change and ΔL is the size of the length change). The power was calculated by the ratio of the work per step over time interval.

Due to the tendon compliance during the step, the actual length change delivered to the sarcomeres is smaller than that imposed on the fibre plus tendons. During the following time interval, the step tension recovers and the series compliance is restretched at the expenses of further shortening of the sarcomeres so that, before the next step, when tension attains the steady state prestep value (T_i), the length change delivered to the sarcomeres corresponds to that imposed on the fibre. To calculate the work per step by the product of the mean force per step and the step length change at the fibre end introduces an overestimate of the work at the time of the step because of the overestimate of both T_1 and ΔL. The overestimate will be larger the larger the tendon compliance; on the other hand the extra work delivered during the interval, and not detected by our method, will roughly compensate the overestimate of work during the step. In any case, in staircase shortening imposed in fixed-end mode, in contrast to the staircase in length-clamp mode (Lombardi *et al.* 1992), the total shortening per step is only partially stepwise at sarcomere level; therefore all the parameters that are affected by stepwise rather than ramp shortening are expected to show a smaller change.

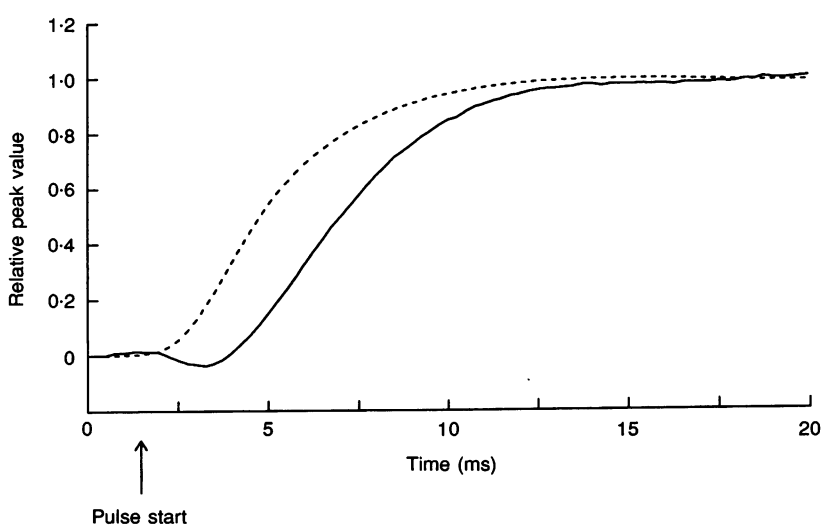

Figure 1. Estimate of the conduction delay in the calorimetric system

Continuous line, time response of the thermopile and amplifier to a heating pulse delivered to a dead muscle fibre and its attached Ringer solution. The fibre was killed with a very long period of tetanic stimulation, and then a voltage pulse (1 ms duration, 50 V amplitude) was applied to the fibre by means of the stimulating electrodes at the end of one experiment. The trace shows a downward deflection at the start; this is probably due to a stimulus artifact. Dashed line, time response of the amplifier to a current pulse. The arrow indicates the pulse start.

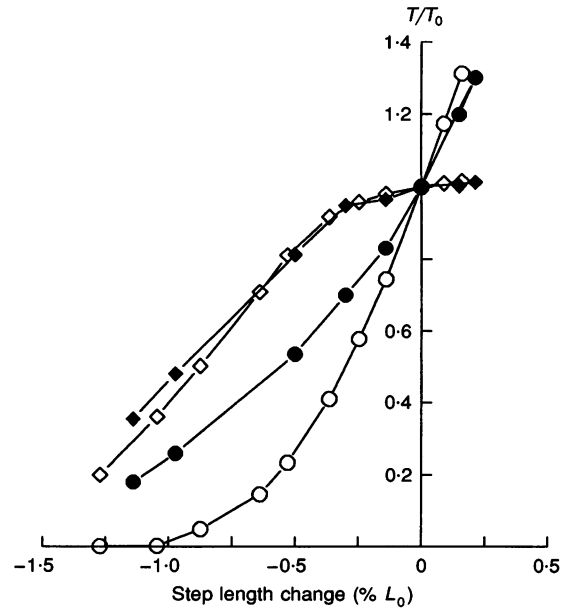

Figure 2. Comparison between T_1 and T_2 curves in the two different systems

T_1 (circles) and T_2 (diamonds) relations were determined either with a fibre in the thermopile system (filled symbols) or with a fibre in a system where a high frequency response force transducer was used (open symbols). T_1 and T_2 were made relative to the isometric tetanus plateau. Open symbols: fibre length, 7·57 mm; average sarcomere length, 2·24 μm; cross-sectional area, 17 100 μm^2; temperature, ~2 °C. Filled symbols: fibre length, 7·20 mm; average sarcomere length, 2·2 μm; cross-sectional area, 17 660 μm^2; temperature, ~1 °C.

Due to the non-optimal conditions for mechanical recording in the thermopile system (misalignment of the fibre with respect to the levers, limited frequency response of the force transducer), the T_1 value following each step length change during staircase shortening was overestimated. This produces an overestimate of the work done by the fibre during each step applied in staircase shortening. In order to quantify the error due to the limits of the mechanical recording in the thermopile system, the T_1 and T_2 (where T_2 is the tension reached at the end of quick early recovery after length step estimated as in Ford *et al.* 1977) relations determined with the thermopile system were compared with those determined in fibres mounted on a set-up for mechanical studies with high temporal resolution (Fig. 2). In this set-up the fibre was in a trough filled with Ringer solution, aligned between the levers of transducers. The force transducer was a capacitance gauge similar to that described by Huxley & Lombardi (1980) with resonant frequency 50 kHz. The T_1 values in the thermopile set-up were overestimated: for a step of 0·3% L_0 the ratio of T_1 in the thermopile system over T_1 in the fast set-up was 1·30 and for a step of 0·5% L_0 the ratio was 2·45. The amount of overestimate for each T_1 value can be estimated from the difference in the two T_1 curves and used to correct the T_1 values at the steady state of staircase shortening and consequently the work per step. The results for total energy liberation will be reported without and with this correction.

Recording and analysis of the responses

The signals from the thermopile, from the force transducer and from the motor position were recorded at 2 ms per point on a digital oscilloscope (type 4094, Nicolet, Madison, WI, USA) and stored on floppy disc. To record mechanical events (force and length) on a fast time scale during the shortening period, another digital oscilloscope was also used (type 310, Nicolet); in this case the sampling rate was 20–50 μs per point. The correction for the

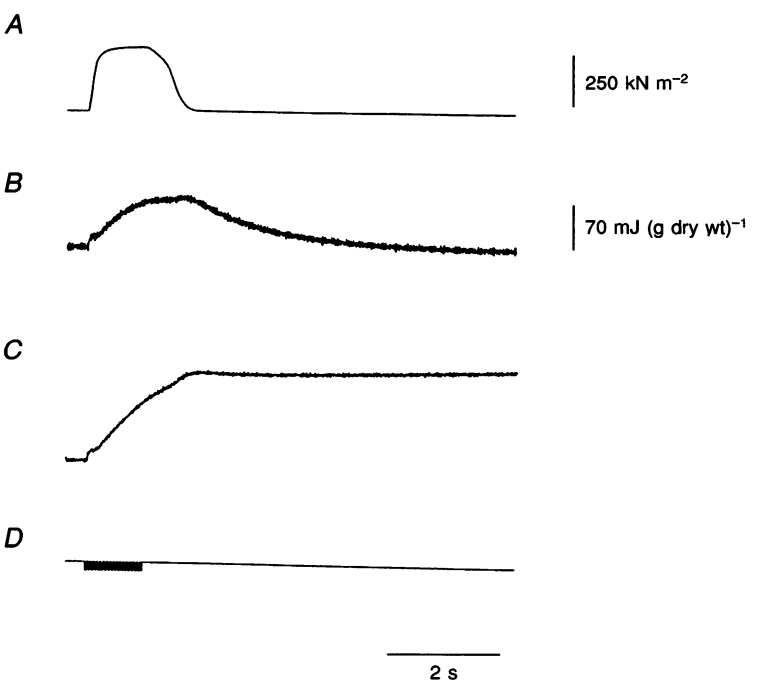

Figure 3. Tension and heat traces during the isometric tetanic contraction

Tension (*A*), heat (*B* and *C*) and stimulus (*D*) traces during an isometric tetanic contraction. The heat trace in *B* was taken directly from the thermopile amplifier without correction for heat loss; in *C* correction for heat loss was made and the 50 Hz noise was removed numerically. The exponential heat loss constant used in the correction, determined by using the Peltier heating method, was 1·48 s. Temperature, 1 °C; fibre length, 6·05 mm; average sarcomere length, 2·2 μm; cross-sectional area, 27 480 μm^2; dry weight, 79 μg.

thermopile heat loss was made as described by Curtin *et al.* (1983). Measurements of the responses were made with the Nicolet oscilloscopes by means of their internal reading system. In some cases a computer program was used to remove any residual 50 Hz noise present on the temperature traces, by subtracting a sine wave fitted to the baseline of the original trace. The rate of extra heat production during the shortening period was estimated from the ratio of the slope of a regression line fitted to the experimental records obtained during the whole shortening period over the slope of the regression line fitted to the record in the 300 ms isometric period preceding the shortening.

RESULTS

Isometric contractions

Records of the force and the heat production during an isometric tetanus are shown in Fig. 3. Figure 3*B* and *C* refers to the heat signal before and after corrections for the thermopile heat loss and the 50 Hz noise. There was a delay of about 16 ms between the start of the stimulation and the beginning of the temperature change and a delay of about 14 ms between the beginning of the temperature change

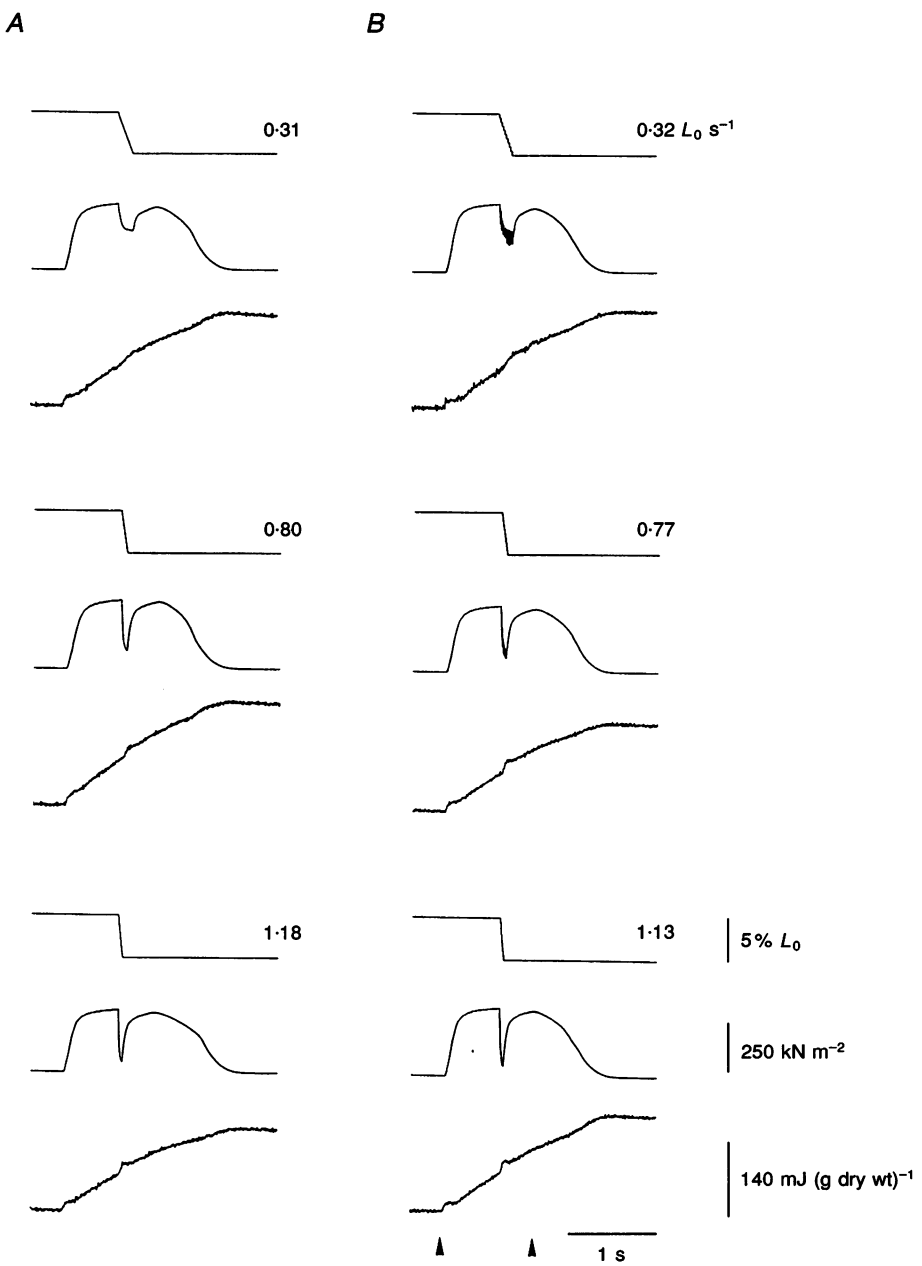

Figure 4. Tension and heat traces during ramp and staircase shortening

Tension response and heat production during ramp shortening (*A*) and during staircase shortening (*B*) imposed at the isometric tetanus plateau. In each frame, from top to bottom, traces show motor position, tension response and heat production (corrected for heat loss and removal of 50 Hz noise). Figures close to the records indicate the shortening velocity, expressed in L_0 s^{-1}. Staircase step was 0·51 % L_0. Arrowheads indicate start and end of stimulation. Temperature, 1 °C; same fibre as in Fig. 3.

and the force rise. In different experiments the rate of heat production was 25–35 μJ s^{-1} during the tension rise and then fell to 4–9 μJ s^{-1} by the end of stimulation; the presence of these two different rates was first described by Aubert (1956) in whole muscle as labile and stable heats. During relaxation a clear acceleration of heat production can be seen after the shoulder which separates isometric from chaotic relaxation (Huxley & Simmons, 1970). These features of the record depend critically on the degree of alignment of the fibre with the thermopile (Curtin, Howarth, Rall, Wilson & Woledge, 1984).

Heat rate during ramp and staircase shortening

When a muscle fibre contracting isometrically was made to start shortening at a constant velocity, there was an increase in the rate of heat production above the isometric rate (Fig. 4*A*). A similar increase in heat rate was seen when staircase shortening was superimposed on the isometric tetanus (Fig. 4*B*). With both types of shortening the increase in heat rate was larger the higher the shortening velocity. It can also be seen from these records that, when the shortening is over, the heat rate fell below the isometric value during the period of rapid force redevelopment, as in records made with whole muscles

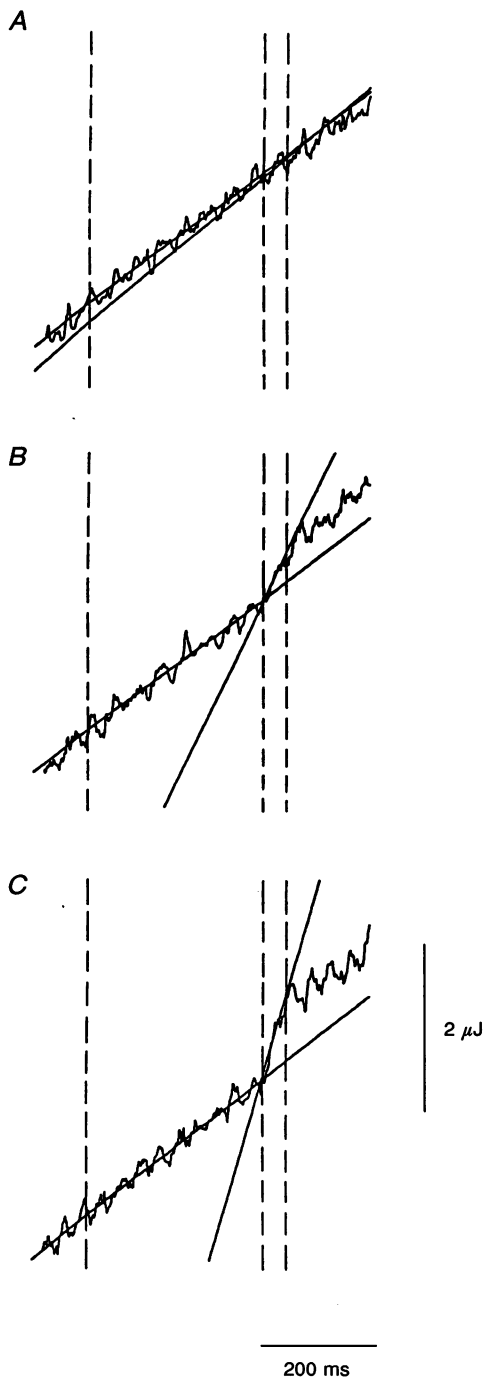

Figure 5. Heat traces during ramp and staircase shortening

Heat traces after corrections for heat loss and 50 Hz noise, during contractions in the isometric condition (*A*), with superposed ramp shortening (*B*) and with superposed staircase shortening (*C*). Ramp and staircase shortening had the same mean velocity (about 1·0 L_0 s^{-1}); staircase step was 0·49 % L_0. The three vertical dashed lines mark two different time periods, as indicated in the text (the first one corresponds to the isometric period lasting for the 300 ms before beginning of shortening; the second period corresponds to the shortening period). In each period the heat rate was calculated by the slope of first order regression lines fitted to the heat trace (Table 2). Temperature, 1 °C; fibre length, 6·4 mm; average sarcomere length, 2·2 μm; cross-sectional area, 20 610 μm^2; dry weight, 78·8 μg.

Table 2. Slopes of the regression lines fitted on heat traces

	S_0 (μJ s^{-1})	S (μJ s^{-1})	S/S_0
Isometric	5·37	5·95	1·11
Shortening			
Ramp	5·27	14·72	2·79
Staircase	5·53	24·43	4·42

The slopes of the regression lines, expressed in μJ s^{-1}, during isometric (S_0, 300 ms before shortening starts) and shortening periods (S, for both ramp and staircase shortening conditions). Data from experiment in Fig. 5.

(Woledge, 1961). This reduction in the heat rate is presumably due to the thermoelastic behaviour of the muscle, which will be discussed below.

Figure 5 shows heat traces on a faster time base. The noise present in the trace during staircase shortening was of the same order as that in ramp shortening and showed no relation to the phase and the period of staircase steps and pauses. The rate of heat production was almost constant during the whole shortening period not only for ramp shortening, but also for staircase shortening. In any condition the rate of heat production was estimated by the slope of the linear regression fitted to the heat trace. The three vertical dashed lines mark two time periods; the first period lasted for the 300 ms before the shortening started, and the second period corresponds to the duration of ramp or staircase shortening. The two straight lines in each trace were obtained by linear regression analysis on the part of the traces referring to the isometric (S_0) and shortening (S) periods, respectively. As shown in Table 2, in this experiment the ratio of the heat rate during the shortening period to the rate during the preceding isometric period (S/S_0) was 58% higher for staircase (Fig. 5*C*) than for ramp shortening (Fig. 5*B*). Since the slope during the shortening period was calculated on a shorter trace, it is more affected by the noise. To test the reliability of the method, the fit has been repeated for the 'shortening period' in the tetanus with no shortening (first row of the table). It can be seen that the error is much smaller than the changes introduced by the different shortening protocols.

The relation of heat rate to shortening velocity is shown in Fig. 6*A* and *B* for two experiments with various amplitudes of staircase step. Figure 6*C* shows the mean relation obtained from five fibres with a staircase step of 0·49 ± 0·02% L_0 (mean ± S.E.M.). In the range of velocities used, the rate of heat production was linearly related to shortening velocity and the slope of the relation was greater for staircase than for ramp shortening. Consequently, while for velocities lower than 0·4 L_0 s^{-1} there was no significant difference in heat rate between ramp and staircase shortening, at 0·8 L_0 s^{-1} this value was ~45% greater. Heat rate changes also with the size of the step in the staircase, slightly increasing with the size of the step (see for instance Fig. 6*A*, where circles refer to a step two times larger than that of triangles).

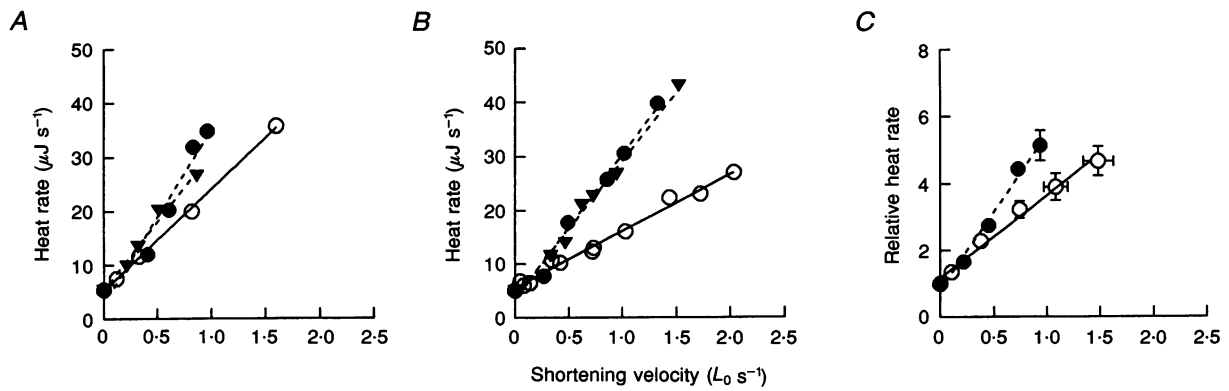

Figure 6. Heat rate–velocity relations during ramp and staircase shortening

A and *B*, relations of heat rate to shortening velocity obtained at the steady state of force response during ramp (open symbols and continuous lines) and staircase shortening with two different step sizes (filled symbols and dashed lines) in two different experiments. ○, ramp. Amplitude of staircase step (expressed as % L_0): *A*: ▼, 0·26; ●, 0·49; *B*: ▼, 0·38; ●, 0·53. *C*, mean relations for five fibres: ○, ramp; ●, staircase with a step size of 0·49 ± 0·02% L_0 (mean value ± S.E.M.). Lines were obtained by linear regression analysis. The intercept and the slope of linear regression in *C* are 1·17 and 2·49 s L_0^{-1} for ramp shortening and 0·83 and 4·66 s L_0^{-1} for staircase shortening. *A*: fibre length, 6·95 mm; average sarcomere length, 2·2 μm; cross-sectional area, 19144 μm^2; dry weight, 115·4 μg. *B*, same fibre as Fig. 5. Temperature, 1 °C.

Power output during ramp and staircase shortening

Force responses to ramp and staircase shortening are shown with a high time resolution in Fig. 7. During ramp shortening there was a drop of force towards a steady state value characteristic of a Hill (1938) $T-V$ relation. During staircase shortening for each step the force dropped to a minimum value (T_1) and then recovered to a maximum value (T_i) which remained constant independent of the number of steps (Piazzesi *et al.* 1993).

Figure 8*A* shows power–velocity relations for the same fibre as in Fig. 6*B* at the steady state of the response to ramp shortening (open symbols and continuous lines) and staircase shortening (filled symbols and dashed lines) with a staircase step of 0·38% L_0 (triangles) and of 0·53% L_0 (circles). The continuous line was obtained by fitting $T-V$ data with Hill's equation. It can be seen that, in accordance with Piazzesi *et al.* (1993), the power during staircase shortening was lower with respect to ramp shortening and that during staircase shortening, for the same shortening speed, the larger the size of the step the lower the power. The mean relations obtained for the same fibres as in Fig. 6*C* are shown in Fig. 8*B*, where open circles refer to mean $T-V$ data points obtained with ramp shortening, filled circles are staircase data points referring to a step size of 0·49 ± 0·02% L_0 and diamonds are obtained from the circles data, after correction for the overestimate of T_1 values, according to the procedure described in Methods. The continuous line was obtained by fitting $T-V$ data with Hill's equation. For a velocity of about 1/3 V_0, the reduction in power with staircase was ~40% before correction (circle) and ~60% after correction (diamonds).

Total energy rate and mechanical efficiency

Rate of total energy liberation for each velocity was calculated by the sum of power (data from Fig. 8*B*) and heat rate (data from Fig. 6*C*). Figure 9*A* shows the relations between rate of total energy liberation and shortening velocity either for ramp shortening (open symbols) or for staircase shortening (filled symbols). Filled circles were obtained by using power values not corrected for the overestimated T_1, filled diamonds by using corrected power values. At low shortening velocity the energy rate was similar for the two shortening protocols; at high velocity the energy rate data for staircase shortening lay above those for ramp shortening. Without the correction, for a velocity of 0·8 L_0 s^{-1}, the rate of energy production during staircase shortening was higher by about 10% than the rate with ramp shortening, but the difference is not significant ($P > 0·1$; Student's t test for independent data). After correction of power values, the difference was even smaller ($P > 0·2$), and again not significant.

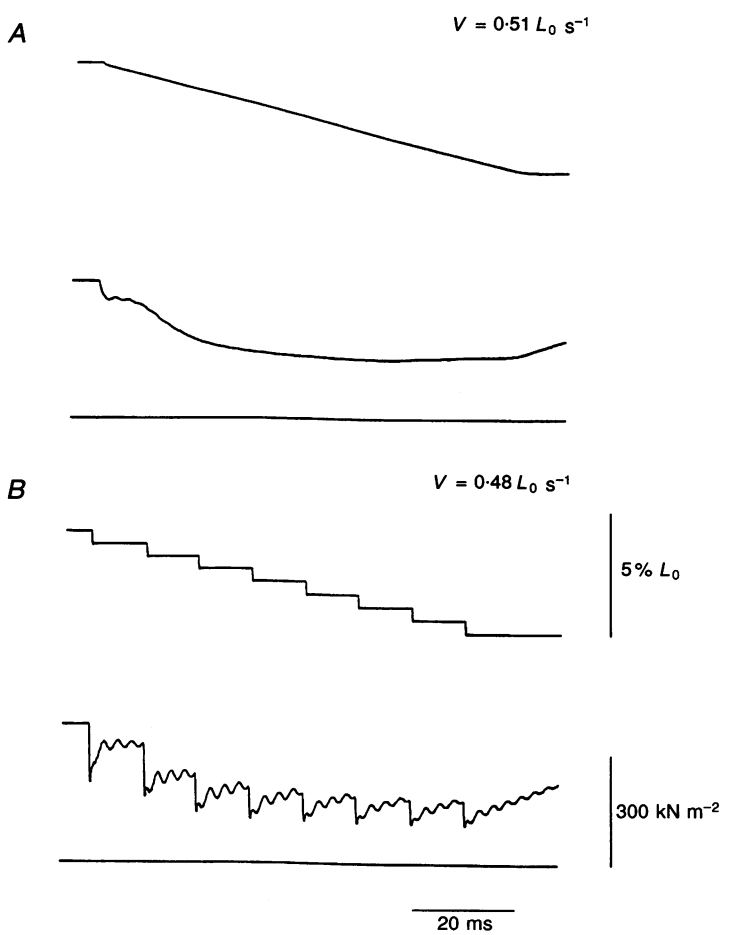

Figure 7. Tension traces during ramp and staircase shortening on a fast time base
Tension responses to ramp shortening (*A*) and to staircase shortening (*B*) at the same mean shortening velocity (about 0·5 L_0 s^{-1}). Size of step in staircase, 0·53% L_0. In each frame upper trace is length change, middle trace is tension response, lower trace is resting tension. On the tension trace, during staircase shortening there are two different types of oscillations, both evoked by the step release: an oscillation at about 2·2 kHz, hardly visible in the traces shown, which corresponds to the frequency response of the force transducer and an oscillation at about 300 Hz, which was due to a transversal oscillation arising as a consequence of the misalignment between the fibre, lying on the thermopile, and the force transducer lever. Temperature, 1 °C; fibre length, 6·8 mm; average sarcomere length, 2·2 μm; cross-sectional area, 7860 μm^2; dry weight, 43·8 μg.

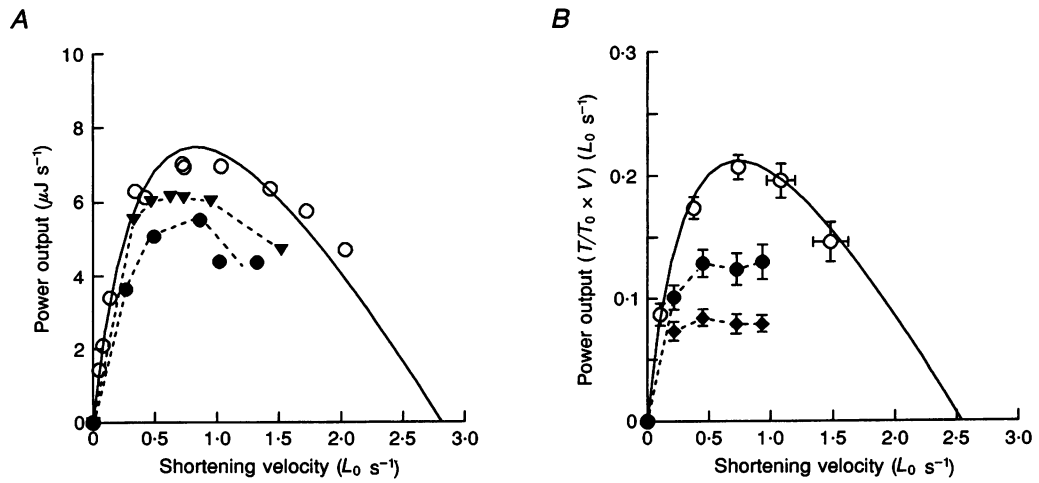

Figure 8. Power–velocity relations during ramp and staircase shortening

Relations of the power output and shortening velocity obtained at the steady state of force response to ramp shortening (open symbols and continuous lines) and staircase shortening (filled symbols and dashed lines). In *A* the relations are reported for the same experiment as in Fig. 6*B*. Amplitude of staircase step (expressed as % L_0): ▼, 0·38; ●, 0·53. In *B* the mean relations obtained after grouping the data for five fibres in classes of velocity are reported. ○, data for ramp shortening. Filled symbols, staircase shortening: ●, uncorrected; ◆, corrected, as described in Methods. In both graphs the continuous line was calculated from values of Hill's hyperbolic equation fitted to the force–velocity data. Parameters of Hill's equation in *B* are $a/P_0 = 0{\cdot}20$; $V_0 \approx 2{\cdot}5\ L_0\ s^{-1}$. Dashed lines are drawn to join experimental points.

The mechanical efficiency (ratio between power and total energy rate) is shown in Fig. 9*B* (symbols refer to the same cases as in Fig. 9*A*). Mechanical efficiency for ramp shortening had a maximum value of 0·36 at about 1/5 V_0; the peak of the efficiency preceded the peak of the power (about 1/3 V_0), as already described for whole frog sartorius muscle by Hill (1938, 1939). For staircase shortening the efficiency was lower than during ramp shortening. At 1/5 V_0 the efficiency was 0·25 for uncorrected power and 0·18 for corrected power, which indicates a reduction of 30 and 50%, respectively, with respect to ramp shortening.

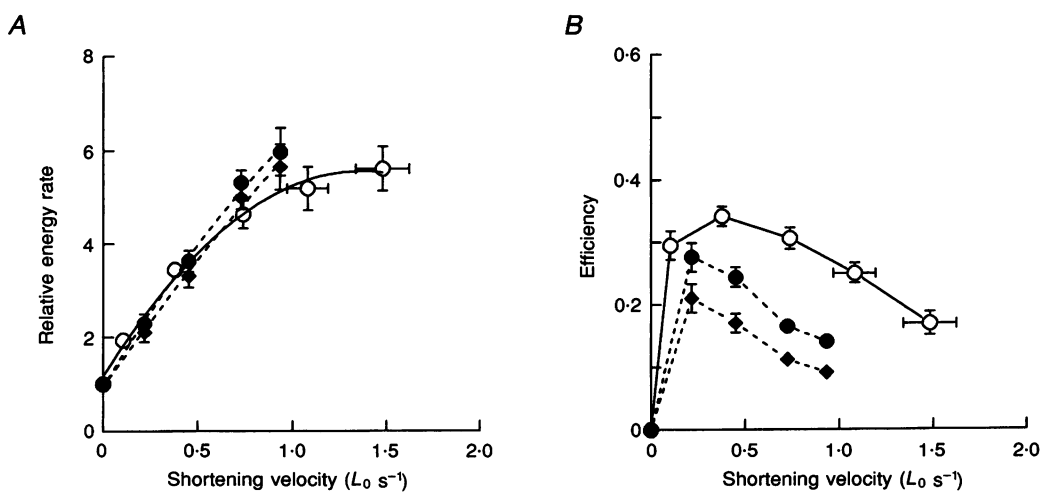

Figure 9. Energy rate and mechanical efficiency *versus* shortening velocity during ramp and staircase shortening

A, total energy rate *versus* shortening velocity for ramp (open symbols and continuous lines) and staircase shortening (filled symbols and dashed lines) obtained from five fibres; in staircase shortening the step size was 0·49 ± 0·02% L_0 (mean ± S.E.M.). For staircase shortening filled circles refer to uncorrected values and filled diamonds to corrected values, as described in Methods. Continuous and dashed lines were obtained by second order regression analysis. *B*, mechanical efficiency *versus* shortening velocity. Symbols as in *A*. Lines are drawn to join experimental points.

DISCUSSION

The main conclusion of this work is that the rate of heat production during staircase shortening is, in general, higher than during ramp shortening (see Fig. 6*C*) but, because of the reduction in the power output, the rate of total energy liberation is not significantly increased (see Fig. 9*A*). Since in the whole range of velocities used the power output is lower in staircase than in ramp shortening, the mechanical efficiency reduced with staircase shortening anyway (see Fig. 9*B*). This suggests that during the staircase shortening there is internal dissipation as heat of the energy that could have become work in a ramp shortening. During the shortening period a cross-bridge is able to utilize a portion of the energy available from ATP hydrolysis in doing mechanical work. This portion of free energy depends on the mechanical conditions under which the cross-bridge works: for the same shortened distance the work done is lower if the shortening occurs in steps.

Calorimetric measurements on a single muscle fibre impose some restrictions on the fibre mounting on the experimental set-up: (i) the fibre lies on the thermopile under the surface tension exerted by the thin layer of Ringer solution and (ii) the fibre is not aligned with the transducer levers and its response is in some degree affected by friction over the thermopile. As discussed in Methods, this does not affect heat measurements but does influence mechanical measurements. Together with the reduced frequency response of the force transducer, friction and misalignment can explain why, in the experiment on the thermopile, T_1 is overestimated with respect to T_1 determined in a classical set-up for high resolution mechanics (Fig. 2). As already discussed in Methods, this overestimate affects all mechanical parameters referring to staircase shortening and therefore the mechanical measurements for staircase shortening are presented either as original or corrected for the degree of overestimate of T_1. The difference between original and corrected values is large in terms of power (~30%) and in terms of efficiency (25–30%), in the whole range of velocities used. At the same time, since power is a relatively small fraction of total energy rate, the difference is much less evident in terms of total energy rate (~5%). In any case the conclusion that total energy rate is not significantly increased in staircase shortening with respect to ramp shortening is not contradicted but indeed is reinforced by the correction.

Another limit of the mechanics of these experiments is that staircase shortening is imposed in fixed end mode: the step release taken by sarcomeres is somewhat smaller than that imposed on the fibre (tendons included). The difference, which varies from fibre to fibre according to the compliance in the tendon attachments, corresponds to the further shortening by the sarcomeres during the quick tension recovery, so that there is a compensation during the pause of the overestimate of the work during the step. However, since in staircase shortening the heat rate is larger than in ramp shortening, if the shortening in staircase is only partially stepwise (see Methods), the increase in the rate of heat production will be smaller than that with the real stepwise shortening by a factor that is larger the larger the compliance in series with the sarcomeres. The effects of staircase to those on heat rate on power output are in the opposite direction; thus, in this case, the reduction of power output with a staircase in fixed-end mode is smaller than with the staircase in length-clamp mode. If we assume that at the sarcomere level the size of the step is ~25% smaller (Cecchi, Colomo, Lombardi & Piazzesi, 1987) than that at the motor end, the step in Fig. 8*B* is actually 0·37% and the observed effects on power and heat rate should be referred to this step size.

The error introduced by the series compliance is expected to be larger with larger step releases, because of the nonlinearity in the series compliance at low tension levels (Cecchi *et al.* 1987, Fig. 5*B*). For instance in Fig. 6*A* the large step (0·49% L_0) will be more reduced at the sarcomere level than the smaller step (0·26% L_0). This could explain why we found a small effect of the size of the step on the increase of heat rate. These considerations do not affect the main conclusion of this work that the total energy rate is similar in staircase and in ramp shortening.

Thermoelastic and shortening heat

It has long been known that a period of shortening interrupting an isometric tetanus causes two changes in the heat rate. Firstly the drop in force causes a heat liberation which is reversible, proportional to the change in force and thought to arise from the thermoelastic properties of the myofibrillar structures (Woledge, 1961). Secondly the shortening itself is accompanied by the shortening heat (Hill, 1938) which is proportional to the distance shortened and thought to arise from the complete or incomplete cross-bridge ATPase cycles (Woledge, Curtin & Homsher, 1985). From the size of these effects, observed previously in whole muscle experiments, it would have been expected that, in the observations made in this paper, the thermoelastic heat would contribute, depending on the speed of shortening, 20–40% of the total heat produced during shortening. These experiments have provided a clear indication of the existence of the thermoelastic heat in our preparations: the reduced rate of heat production during the rapid tension recovery at the end of shortening (Fig. 4, bottom panels), for which there is no other explanation. It follows that the extra heat produced during shortening in these experiments was not wholly due to the shortening heat, and were we able to correct the records for thermoelastic heat production the values might be somewhat less. However, we have also neglected a systematic underestimate of the heat produced during shortening, that due to thermopile lag. We felt that the records we had available did not have a sufficient signal-to-noise ratio to justify any attempt at correction for these two effects. The appearance of the records has been that during the shortening period

there is a steady heat rate (Fig. 5). The contribution of the thermoelastic heat should have been to make the heat rate higher in the first part of the shortening; the effect of the thermopile lag should have been to then make the heat rate lower. Presumably the actual appearance of the records is due to the approximate cancelling of these effects.

Rate of working-stroke regeneration per myosin head

The observation by Lombardi *et al.* (1992) that, during a sequence of step releases of ~4 nm per half sarcomere separated by 8 ms intervals ($V \approx 0{\cdot}5\ \mu m\ s^{-1}$), the working stroke can be regenerated at a rate ($70\ s^{-1}$) one order of magnitude higher than the known ATP hydrolysis rate during steady shortening (Kushmerick & Davies, 1969), leads to the conclusion that multiple working strokes can occur per molecule of ATP split. On the other hand the ATP hydrolysis rate could be accelerated by the staircase-shortening protocol and thus correspond to the rate of working stroke regeneration. The evidence given here that during staircase shortening the rate of energy liberation is not greater than that observed during steady shortening strengthens the idea of multiple cross-bridge working strokes per molecule of ATP split. This conclusion relies on the assumption that, during an isometric contraction, a large fraction of myosin heads are attached and force generating. In this case the regeneration of working stroke in the staircase experiments is in a great part due to detachment and reattachment of cross-bridges already exerting force before each step.

According to Lombardi *et al.* (1992), during staircase shortening the rate of working stroke per myosin head is given by $r_{ws} = f_0 f s$, where f_0 is the fraction of myosin heads attached in an isometric contraction, f is the fraction of isometric heads attached at the steady state of the staircase and s is the step frequency. At the steady state of a staircase similar to that used here (step of 0·5% L_0 separated by 10 ms intervals), f is ~0·7 (Lombardi *et al.* 1992); so $r_{ws} = f_0 \times 0{\cdot}7 \times 100\ s^{-1}$. Unfortunately there is considerable uncertainty about the value of f_0. Goldman & Simmons (1977) suggested a value of 0·8, based on comparison, in skinned fibres, of the stiffness in an isometric contraction to that in rigor. If $f_0 = 0{\cdot}8$, $r_{ws} = 0{\cdot}8 \times 0{\cdot}7 \times 100\ s^{-1} = 56\ s^{-1}$. On the other hand, according to others f_0 can be as small as 0·2 (Cooke, Crowder, Wendt, Barnett & Thomas, 1984; Huxley & Kress, 1985). In this case, $r_{ws} = 0{\cdot}2 \times 0{\cdot}7 \times 100\ s^{-1} = 14\ s^{-1}$.

Number of working strokes per ATP split

Our calorimetric and mechanical measurements can be used to estimate the number of working strokes that can be performed by a myosin head for each ATP molecule split. The total energy rate can be used to find the rate of ATP hydrolysis by the molar enthalphy change for PCr (34 kJ mol^{-1}; Woledge & Reilly, 1988) and by the myosin content (1·2 μmol of myosin head per gram dry weight; Bagshaw, 1993). For a staircase shortening at the velocity of 0·5 $L_0\ s^{-1}$ (100 steps s^{-1} and step size of 0·5% L_0), the relative total energy is ~3·3 times the isometric value (68 ± 9 mW (g dry wt)$^{-1}$; mean ± S.E.M., $n = 5$ fibres). The rate of ATP hydrolysis per myosin head can be calculated as $r_{ATP} = (3{\cdot}3 \times 68\ \text{mW (g dry wt)}^{-1}/34\ \text{mJ}\ (\mu\text{mol of ATP split})^{-1})/1{\cdot}2\ \mu\text{mol of myosin head (g dry wt)}^{-1} = 5{\cdot}5\ s^{-1}$.

The number of working strokes per ATP split (R) can be calculated by dividing the rate of working stroke per myosin head by the rate of ATP hydrolysis per myosin head. If f_0 is 0·8, $R = 56\ s^{-1}/5{\cdot}5\ s^{-1} \approx 10$, in accordance with the early works by Yanagida and coworkers (Yanagida, Arata & Oosawa, 1985; Ishijima, Takashi, Katsuhiko & Yanagida, 1991), while if f_0 is 0·2, $R = 14\ s^{-1}/5{\cdot}5\ s^{-1} \approx 2{\cdot}6$, a value similar to that estimated by Finer, Simmons & Spudich (1994) in a recent *in vitro* motility assay. It seems from our results very unlikely that R can be equal to 1.

Ambrogi Lorenzini, C., Colomo, F. & Lombardi, V. (1983). Development of force–velocity relation, stiffness and isometric tension in frog single muscle fibres. *Journal of Muscle Research and Cell Motility* **4**, 177–189.

Aubert, X. (1956). *Le Couplage Energetique de la Contraction Musculaire*. Arscia, Brussels.

Bagshaw, C. R. (1993). *Muscle Contraction*. Chapman & Hall, London.

Barclay, C. T., Constable, J. K. & Gibbs, C. L. (1993). Energetics of fast- and slow-twitch muscles of the mouse. *Journal of Physiology* **472**, 61–80.

Cecchi, G., Colomo, F., Lombardi, V. & Piazzesi, G. (1987). Stiffness of frog muscle fibres during rise of tension and relaxation in fixed-end or length-clamped tetani. *Pflügers Archiv* **409**, 39–46.

Cooke, R., Crowder, M. S., Wendt, C. H., Barnett, V. A. & Thomas, D. D. (1984). Muscle cross-bridges: do they rotate? In *Contractile Mechanisms in Muscle*, ed. Pollack, G. H. & Sugi, H., pp. 413–423. Plenum Press, New York.

Curtin, N. A., Howarth, J. V., Rall, J. A., Wilson, M. G. A. & Woledge, R. C. (1984). Simultaneous heat and tension measurements from single muscle cells. In *Contractile Mechanisms in Muscle*, ed. Pollack, G. H. & Sugi, H., pp. 887–899. Plenum Press, New York.

Curtin, N. A., Howarth, J. V. & Woledge, R. C. (1983). Heat production by single fibres of frog muscle. *Journal of Muscle Research and Cell Motility* **4**, 207–222.

Dijkema, F. K., Elzinga, G. & Holewijn, A. J. (1985). Measurement of temperature changes in muscle contraction with a thermopile and amplifier designed to improve signal-to-noise ratio and resolution in time and place. *Journal of Physiology* **366**, 4*P*.

Finer, J. T., Simmons, R. M. & Spudich, J. A. (1994). Single myosin molecule mechanics: piconewton forces and nanometre steps. *Nature* **368**, 113–119.

Ford, L. E., Huxley, A. F. & Simmons, R. M. (1977). Tension responses to sudden length change in stimulated frog muscle fibres near slack length. *Journal of Physiology* **269**, 441–515.

Goldman, Y. E. & Simmons, R. M (1977). Active and rigor muscle stiffness. *Journal of Physiology* **269**, 55–57*P*.

Gordon, A. F., Huxley, A. F. & Julian, F. J. (1966). The variation in isometric tension with sarcomere length in vertebrate muscle fibres. *Journal of Physiology* **184**, 170–192.

HILL, A. V. (1938). The heat of shortening and the dynamic constants of muscle. *Proceedings of the Royal Society* B **126**, 136–195.

HILL, A. V. (1939). The mechanical efficiency of frogs' muscle. *Proceedings of the Royal Society* B **127**, 434–451.

HUXLEY, A. F. (1957). Muscle structure and theories of contraction. *Progress in Biophysics and Biophysical Chemistry* **7**, 255–318.

HUXLEY, A. F. (1974). Muscular contraction. *Journal of Physiology* **243**, 1–43.

HUXLEY, A. F. & LOMBARDI, V. (1980). A sensitive force transducer with resonant frequency 50 kHz. *Journal of Physiology* **305**, 15–16*P*.

HUXLEY, A. F. & SIMMONS, R. M. (1970). Rapid 'give' and the tension 'shoulder' in the relaxation of frog muscle fibres. *Journal of Physiology* **210**, 32–33*P*.

HUXLEY, A. F. & SIMMONS, R. M. (1971). Proposed mechanism of force generation in striated muscle. *Nature* **233**, 533–538.

HUXLEY, H. E. (1969). The mechanism of muscular contraction. *Science* **164**, 1356–1366.

HUXLEY, H. E. & KRESS, M. (1985). Crossbridge behavior during muscle contraction. *Journal of Muscle Research and Cell Motility* **6**, 153–161.

ISHIJIMA, A., TAKASHI, D., KATSUHIKO, S. & YANAGIDA, T. (1991). Sub-piconewton force fluctuations of actomyosin in vitro. *Nature* **352**, 301–306.

KRETZSCHMAR, K. M. & WILKIE, D. R. (1972). A new method for absolute heat measurements, utilizing the Peltier effect. *Journal of Physiology* **224**, 18–21*P*.

KRETZSCHMAR, K. M. & WILKIE, D. R. (1975). The use of the Peltier effect for simple and accurate calibration of thermoelectric devices. *Proceedings of the Royal Society* B **190**, 315–321.

KUSHMERICK, M. J. & DAVIES, R. E. (1969). The chemical energetics of muscle contraction. 2. The chemistry, efficiency and power of maximally working sartorius muscles. *Proceedings of the Royal Society* B **174**, 315–353.

LOMBARDI, V., PIAZZESI, G. & LINARI, M. (1992). Rapid regeneration of the actin–myosin power stroke in contracting muscle. *Nature* **355**, 638–641.

LYMN, R. W. & TAYLOR, E. W. (1971). Mechanism of adenosine triphosphate hydrolysis by actomyosin. *Biochemistry* **10**, 4617–4624.

MULIERI, L. A., LUHR, G., TREFRY, J. & ALPERT, N. R. (1977). Metal-film thermopiles for use with rabbit right ventricular papillar muscles. *American Journal of Physiology* **233**, C146–156.

PIAZZESI, G., LINARI, M. & LOMBARDI, V. (1993). Work and power during staircase shortening of tetanized single frog muscle fibres. *Journal of Physiology* **459**, 451*P*.

WOLEDGE, R. C. (1961). The thermoelastic effect of change of tension in active muscle. *Journal of Physiology* **155**, 187–208.

WOLEDGE, R. C., CURTIN, N. A. & HOMSHER, E. (1985). *Energetics Aspects of Muscle Contraction*. Academic Press, London.

WOLEDGE, R. C. & REILLY, P. J. (1988). Molar enthalpy change for hydrolysis of phosphorylcreatine under conditions in muscle cells. *Biophysical Journal* **54**, 97–104.

WOLEDGE, R. C., WILSON, M. G. A., HOWARTH, J. V., ELZINGA, G. & KOMETANI, K. (1988). The energetics of work and heat production by single muscle fibres from the frog. In *Molecular Mechanisms of Muscle Contraction*, ed. SUGI, H. & POLLACK, G., pp. 677–688.

YANAGIDA, T., ARATA, T. & OOSAWA, F. (1985). Sliding distance of actin filament induced by a myosin crossbridge during one ATP hydrolysis cycle. *Nature* **316**, 366–369.

Acknowledgements

The authors wish to thank Professor G. Elzinga for provision of the thermopile used, Dr V. Lombardi for stimulating criticism, suggestion and encouragement during the preparation of this manuscript, and Dr G. Piazzesi for critical reading of the manuscript. We also wish to thank Mr Mario Dolfi, Mr Duncan Farquason, Mr Bill Potter and Mr Paul Stukas for skilled technical assistance and Mr Adrio Vannucchi for the preparation of illustrations. This work was supported by Commission of the European Communities (grant ERB4001GT922141).

Received 24 October 1994; accepted 15 March 1995.