

THE DEPENDENCE ON EXTENT OF SHORTENING OF THE EXTRA ENERGY LIBERATED BY RAPIDLY SHORTENING FROG SKELETAL MUSCLE

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SUMMARY

1. Pairs of frog sartorius muscles were stimulated for 2 sec at 0 °C and, after 1 sec of isometric contraction, were released at a constant velocity. The total excess heat (shortening heat) and work associated with the release were determined by comparison with isometric control tetani.

2. Shortening heat and work production were non-linearly related to the distance shortened. There was proportionally more energy liberation for smaller releases.

3. The dependence of shortening heat on muscle length was investigated within the sarcomere length range 2.1–2.6 μm (as measured in resting muscle) and was found to be similar to that of isometric tension.

4. A simple model in which heat and work are produced in a two-state cycle can describe these and previous results concerning the energetics of rapidly shortening muscle.

INTRODUCTION

When an active muscle shortens it liberates more heat than in isometric contraction; the excess is known as shortening heat (Hill, 1938). If there are two periods of constant-velocity shortening in one tetanus, the shortening heat associated with the second is reduced due to the presence of the first, and the largest reduction is seen when there is no interval between the two shortenings (Irving & Woledge, 1981). The same effect should be apparent in another type of experiment: there should be a less than proportional increase in shortening heat with increasing extent of shortening. However Hill (1938) found that the shortening heat *was* linearly related to extent of shortening in isotonic releases. A linear relationship was also reported for releases against a small viscous load (Abbott, 1951). Several new facts about muscle heat production have come to light which make the interpretation of these early investigations difficult. There is a component of heat production (the thermoelastic heat) which is proportional to change in muscle tension (Hill, 1953; Woledge, 1961; Gilbert & Matsumoto, 1976). Both isometric heat production (Homsher, Mommaerts,

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Ricchiuti & Wallner, 1972; Smith, 1972; Aubert & Gilbert, 1980) and shortening heat production (Lebacqz, 1972; Irving, Homsher & Lebacqz, 1980) depend on muscle sarcomere length. Extra heat production associated with shortening is observed after the end of shortening itself (Irving, 1979; Irving & Woledge, 1981). The present experiments were intended to re-examine the relationship between shortening heat and extent of shortening, taking these factors into account.

A preliminary report has been made of some of these results (Irving & Woledge, 1979).

METHODS

Most methods and materials were as described by Irving & Woledge (1981). In some experiments, however, muscle length was controlled by the coaxial system described by Irving, Woledge & Yamada (1979). This was capable of only 4 mm axial movement but the system compliance (0.20 mm/N) was less than that with the lever system (Irving & Woledge, 1981). Similar results were obtained with both systems.

All tetani were of 2 sec duration and 3, 4 or 5 tetani were given at 3 min intervals in the period when the Ringer solution was drained from the muscles. No measurements were made on the first tetanus of the series. The muscles were immersed in Ringer solution for 15 min between series of tetani.

A small correction was made to the shortening heat values to allow for the thermoelastic properties of parallel elastic components in the muscle (Hill, 1952). These effects were assessed by measuring the heat absorbed by unstimulated muscles in releases taking place over the same range of muscle length as the active releases. For a release of 0.13 μm from initial sarcomere length 2.60 μm the heat absorption (per blotted weight of muscle) was 0.26 ± 0.14 mJ/g (mean \pm s.e. of mean, $n = 5$) and for a similar release starting at 2.47 μm the corresponding figure was 0.03 ± 0.02 mJ/g. No changes could be detected at shorter sarcomere lengths. These effects are small compared with the shortening heat (about 3.5 mJ/g), but in order to avoid this systematic error the observed shortening heat values have been corrected by subtraction of the thermal change in the corresponding unstimulated release.

Ten sartorius muscle pairs were used in the experiments. Their dimensions (mean and range) were: blotted weight, 137.2 (73.1–231.8) mg; muscle length (l_0) producing maximum isometric tetanic tension, 28.3 (23.0–32.8) mm; isometric tetanic tension (at l_0) per cross-sectional area, 211.3 (184.0–250.8) mN/mm².

RESULTS

Determination of the shortening heat

The experimental design is shown in Fig. 1. The over-all range of sarcomere lengths employed was from 2.08 to 2.60 μm , as measured in unstimulated muscles. In order to investigate the dependence of shortening heat on sarcomere length in this range, the design included a set of releases (dashed horizontal arrows), all of extent 0.13 μm /sarcomere, but starting at different muscle lengths: 2.21, 2.34, 2.47, 2.60 μm /sarcomere. The other set of releases (full horizontal arrows) all ended at 2.08 μm /sarcomere, but varied in extent between 0.016 and 0.52 μm /sarcomere. This set was used to determine the dependence of shortening heat on extent of shortening. All releases were at a constant velocity of 1.3 μm /sec per sarcomere and started after 1 sec of isometric contraction. Control isometric tetani were also included in the design.

Total heat production was measured in the 1 sec period from the start of the release and control measurements were made on the corresponding period in isometric tetani. Measurement of net heat production at a time well after shortening is complete avoids

complications due to lag in thermopile response, thermoelastic effects and length changes in series elastic components. Any delayed component of shortening heat production is included (Irving & Woledge, 1981). The shortening heat was determined for each type of release by subtracting the isometric control heat from the heat production in the tetanus containing the release. The isometric control heat was calculated as a mean of heat production measurements in isometric tetani at the lengths involved, weighted according to the proportion of the measurement period the shortening muscle spends at each length. This procedure removes small effects due to the variation of isometric heat production with muscle length.

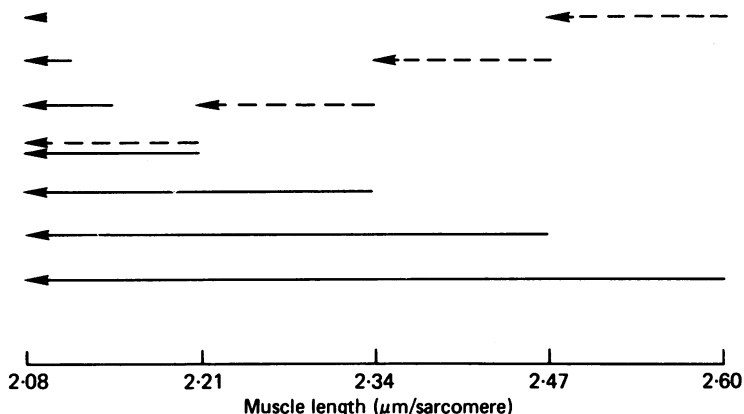


Fig. 1. Experimental design. Each arrow represents the extent of release. Two sets of releases were given. One set (dashed arrows) was of fixed extent, $0.13 \mu\text{m/sarcomere}$, and started from different muscle lengths: 2.21 , 2.34 , 2.47 and $2.60 \mu\text{m/sarcomere}$. The other set (continuous arrows) all ended at muscle length $2.08 \mu\text{m/sarcomere}$ but were of different extents: 0.016 , 0.032 , 0.065 , 0.13 , 0.26 , 0.39 , $0.52 \mu\text{m/sarcomere}$. All releases were at a velocity of $1.3 \mu\text{m/sec}$ per sarcomere and started after 1 sec of isometric contraction.

Isometric tetani were given at lengths 2.08 , 2.21 , 2.34 , 2.47 and $2.60 \mu\text{m/sarcomere}$.

Dependence of shortening heat on muscle length

Shortening heats for releases of extent $0.13 \mu\text{m/sarcomere}$ from different initial lengths are shown in Fig. 2 (circles). Each point is plotted above the mean length during the respective release. The releases from 2.34 to $2.21 \mu\text{m/sarcomere}$ and from 2.21 to $2.08 \mu\text{m/sarcomere}$ produced almost the same shortening heat, but the mean shortening heat is less at greater lengths. A paired comparison of the shortening heat for 2.60 – $2.47 \mu\text{m/sarcomere}$ with that for 2.34 – 2.21 shows that the difference is statistically significant ($P < 0.001$, $n = 8$, t test). Isometric tension, measured 1 sec after the start of stimulation in tetani at lengths between 2.08 and $2.60 \mu\text{m/sarcomere}$ is also shown in Fig. 2 (squares). Mean shortening heat and isometric tension were found to have a similar dependence on muscle length, in agreement with previous reports (Lebacqz, 1972; Irving *et al.* 1980).

Dependence of shortening heat on extent of shortening

The shortening heat produced in releases of various extents but all ending at the same muscle length is shown in Fig. 3 (circles). Shortening heat is not linearly related to extent of shortening; proportionally more shortening heat is produced for smaller extent of release. The results can also be expressed as the shortening heat per unit extent of shortening, normalized by the maximum isometric tension in each muscle (α/P_0 ; Hill, 1938, 1964). In the present experiments α/P_0 was 0.50 ± 0.03 (mean \pm s.e. of mean, $n = 9$) for a release of $0.0325 \mu\text{m/sarcomere}$, but only 0.16 ± 0.01 ($n = 8$) for the largest release studied, which was of extent $0.52 \mu\text{m/sarcomere}$.

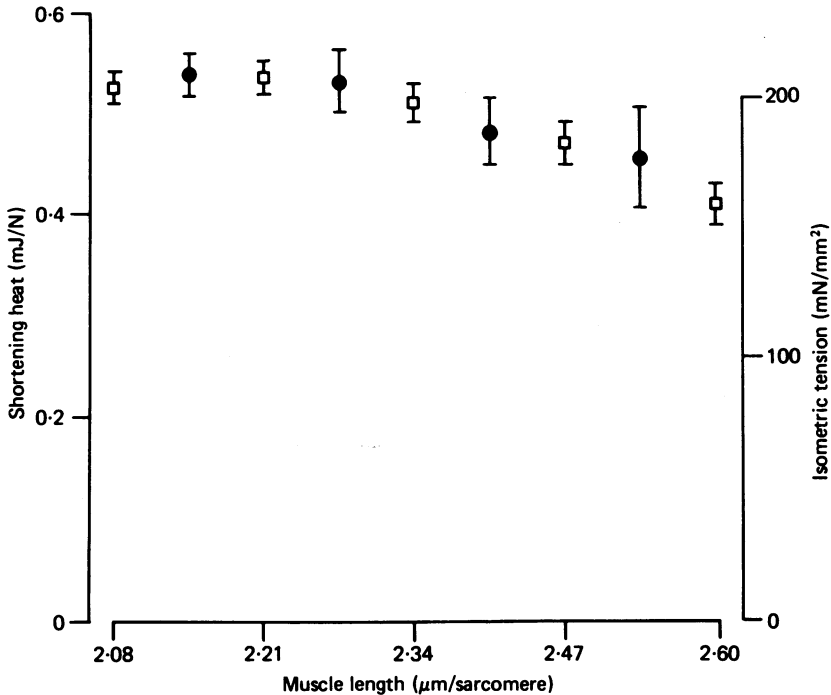


Fig. 2. Variation with muscle length of shortening heat (circles) and tension production at 1 sec in an isometric tetanus (squares). Points show mean \pm s.e. of mean for an average of nine muscle pairs. Shortening heat is the amount produced in a $0.13 \mu\text{m/sarcomere}$ shortening divided by the maximum isometric tension for the muscle pair. The values are plotted against the midpoint of the $0.13 \mu\text{m/sarcomere}$ range over which the shortening occurred.

The non-linearity in the relationship between shortening heat and extent of shortening is not due to the dependence of shortening heat on muscle length. The non-linearity is very clear for extents of release less than $0.26 \mu\text{m/sarcomere}$, in the length range within the plateau region of the relationship between shortening heat and muscle length (Fig. 2). For extents of release greater than $0.26 \mu\text{m/sarcomere}$, the initial muscle length was greater than $2.34 \mu\text{m/sarcomere}$ (Fig. 1), so the early part of these releases took place in a length range in which shortening heat is reduced (Fig. 2). The size of the consequent reduction in the shortening heat observed in

releases of 0.26, 0.39 and 0.52 $\mu\text{m}/\text{sarcomere}$ can be evaluated by calculating the shortening heat expected in these releases if shortening heat per unit shortening did not depend on previous shortening. In this case, the shortening heat would be the sum of the values observed in component 0.13 $\mu\text{m}/\text{sarcomere}$ releases in the corresponding length range (Fig. 2). These sums are shown in Fig. 3 (triangles). It is clear that the slight non-linearity between shortening heat and extent of shortening which would be produced by the length dependence of the shortening heat does not account for the effect observed.

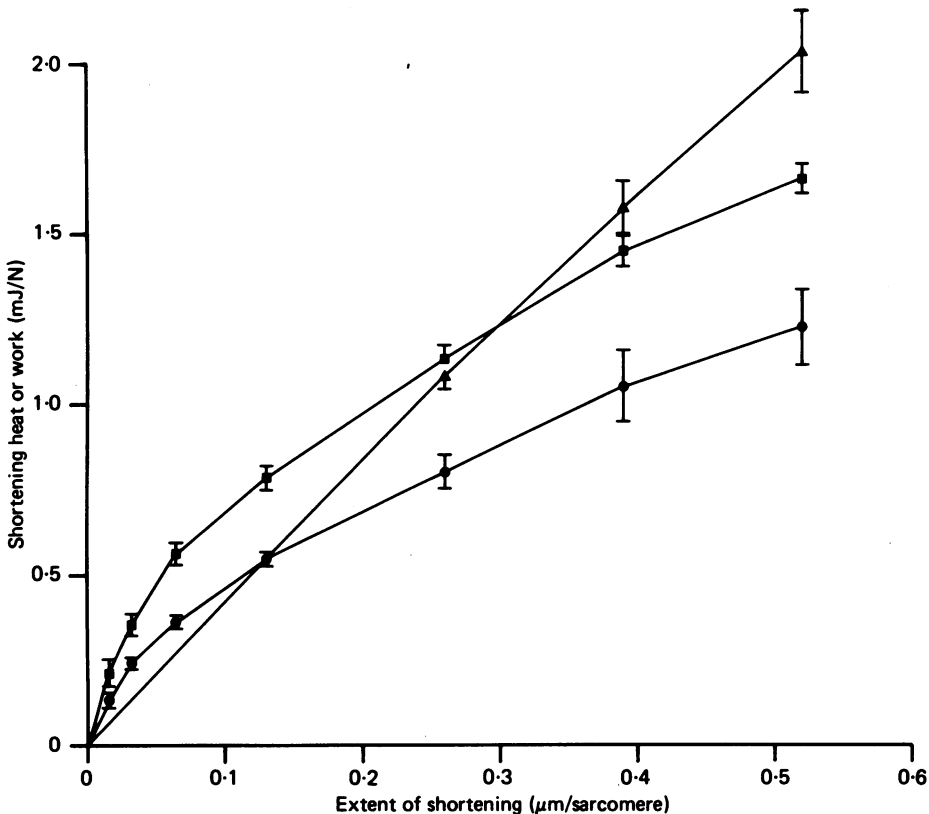


Fig. 3. Shortening heat or work production in releases of various extents; means \pm s.e. of mean for between four and ten muscle pairs. Circles: observed shortening heats in releases of varying extent all ending at muscle length 2.08 $\mu\text{m}/\text{sarcomere}$. Squares: work production (excluding series elastic work) in these releases. Triangles: predicted shortening heat for releases of extent 0.26, 0.39 and 0.52 $\mu\text{m}/\text{sarcomere}$ ending at the same length, calculated from the results in Fig. 2 on the assumption that shortening heat is independent of previous shortening.

Tension and work production

Muscle tension at length 2.08 $\mu\text{m}/\text{sarcomere}$ at the end of isovelocity releases of various extents is shown in Fig. 4. For extents of release greater than 0.2 $\mu\text{m}/\text{sarcomere}$, tension at the end of the release was almost independent of extent of shortening; the tension at the end of the 0.26 $\mu\text{m}/\text{sarcomere}$ release was $35.4 \pm 1.9 \text{ mN}/\text{mm}^2$

(mean \pm s.e. of mean, $n = 8$) and the corresponding figure for the $0.52 \mu\text{m}/\text{sarcomere}$ release was $32.4 \pm 1.8 \text{ mN}/\text{mm}^2$. The early phase of tension fall is reasonably well fitted by an exponential dependence on extent of release, with an e-fold decrease in tension for $0.049 \mu\text{m}/\text{sarcomere}$ shortening (line in Fig. 4).

The work produced in releases of various extents was calculated by numerical integration of the tension during releases, and a small correction was made for work exchange with series elastic elements as described previously (Irving & Woledge, 1981). Work done on such elements during tension redevelopment was included in

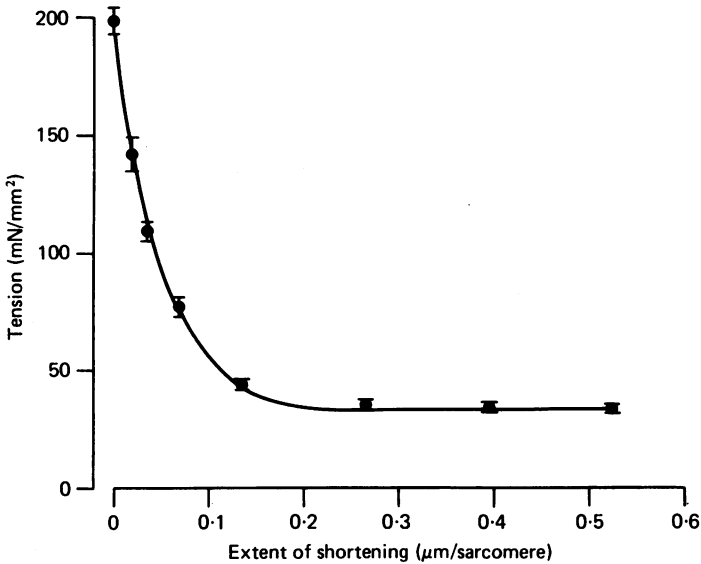


Fig. 4. Muscle tension at length $2.08 \mu\text{m}/\text{sarcomere}$ at the end of isovelocity releases of various extents; means \pm s.e. of mean for an average of eight muscle pairs. The line is an exponential representing an e-fold fall of tension per $0.049 \mu\text{m}/\text{sarcomere}$ extent of release.

the total in order that the results would be comparable with the shortening heat measurements (in which the tension redevelopment period is also included). Work production for each extent of release is shown in Fig. 3 (squares). Like the shortening heat (Fig. 3, circles) work production is non-linearly related to extent of shortening, with proportionally greater work production for smaller releases. The fraction of the total extra energy liberation due to shortening (shortening heat plus work) which is liberated as work showed no significant variation with extent of shortening over the wide range of extents studied.

DISCUSSION

Dependence of shortening heat on extent of shortening

Our main conclusion, that shortening heat per unit shortening is greater for small releases, appears to conflict with earlier work, particularly that of Hill (1938), who found a linear relationship between shortening heat and extent of shortening. There

are several differences between the designs of the two experiments which may be responsible for the different results.

(1) In Hill's experiments, releases of different extents all started at the same muscle length, one considerably greater than that (about $2.1 \mu\text{m}/\text{sarcomere}$) at which the maximum isometric heat was produced (Hill, 1938, Figs. 9, 10). Thus the smaller releases occurred in a length range in which the shortening heat is reduced (Fig. 2), perhaps by 10–20%. This reduction would have been sufficient to obscure a large part of the non-linearity we have found in the relation between shortening heat and distance shortened.

(2) Hill's small releases would also have been affected by the thermoelastic behaviour of the parallel elastic component (Hill, 1952; Lebacqz, 1972) again because they were at long muscle length. This thermoelastic effect would have led to a proportionally larger underestimate of the shortening heat for small releases.

(3) In the present experiments shortening heat was measured 1 sec after the start of shortening to avoid complications due to the thermoelastic heat effect, shortening against series elasticity and the need to correct for thermopile lag. The effect of these factors in Hill's experiments cannot be calculated but they may have contributed to the difference between his results and ours.

(4) In Hill's experiments the releases occurred at constant tension rather than at constant velocity. In the former case work per unit distance shortened is almost constant whereas in the latter it is greater for small releases (Fig. 3). It might be that shortening heat behaves similarly. Points (1) and (2) on this list are probably sufficient to explain why Hill did not observe the non-linearity in the shortening heat, but the influence of (4) needs further investigation.

The heat produced in large rapid releases against a small viscous load was measured by Abbott (1951). By comparing the time courses of extra heat production and total shortening in a single release, an approximately linear relationship between shortening heat and extent of shortening was derived. This method measures only the shortening heat produced *during* shortening; it was assumed that there was no lag between shortening heat production and its recording. More recently, the time course of extra heat production has been measured in isovelocity releases (Hill, 1964, Fig. 8; Homsher & Rall, 1973, Fig. 2), with a reasonable correction applied for the lag in heat conduction between muscle and thermopile. If one applies Abbott's method to this data the resulting relationship between shortening heat and extent of shortening is non-linear; the shortening heat is proportionally greater for small releases.

Gilbert & Matsumoto (1976) found no net shortening heat production for releases less than 1% of the standard muscle length (l_0). We find that for a release of $0.016 \mu\text{m}/\text{sarcomere}$ (about 0.7% l_0), shortening heat production is $0.134 \pm 0.025 \text{ mJ/N}$ (mean \pm s.e. of mean, $n = 5$), which is significantly different from zero ($P < 0.01$). The cause of this discrepancy, which represents a very small quantity of heat, is unknown.

The relation between shortening heat, work and tension production

Shortening heat and work production have a similar dependence on extent of shortening (Fig. 3). In small releases the mean tension during the release is large (Fig. 4) as are the work output and shortening heat per unit shortening. Shortening heat,

work and tension also have a similar dependence on the interval since a previous period of shortening (Irving & Woledge, 1981). In that case it was argued that the effect could not be explained by the dependence of shortening heat on tension observed in isotonic releases (Hill, 1964). The same conclusion holds for the results of the present experiments. The tension-dependence described by Hill (1964) would cause the shortening heat per unit shortening (α/P_0) to be 31% less in a 0.52 μm /sarcomere release than in a 0.0325 μm /sarcomere release, but the observed reduction was 68%.

Another possible explanation for the present results and those of the previous paper (Irving & Woledge, 1981) is that the muscle is 'inactivated' by rapid shortening (Edman, 1975). The inactivation would have to be very large in order to cause the observed reduction in α/P_0 with increasing extent of shortening. There is no evidence of a large differential effect in tension values measured at the end of shortenings of various extents, however (Fig. 4); the tension at the end of a 0.52 μm /sarcomere release is only 8% less than that at the end of a 0.26 μm /sarcomere release. Any substantial inactivation would have to have a time course similar to that of the fall of tension during the isovelocity release.

It is likely that shortening heat and work are produced by a common mechanism: the interaction between thick and thin filaments in contracting muscle. This would explain the similarities in shortening heat and work production described above, and receives additional support from the observation that shortening heat decreases in the same way as isometric tension as muscles are stretched beyond rest length (Lebacqz, 1972; Irving *et al.* 1980).

A two-state model for shortening-dependent energy liberation

The present results and those of the previous paper (Irving & Woledge, 1981) can be quantitatively described by a model in which heat and work are produced in a two-state cycle. Such a model has clear parallels with cyclical cross-bridge models following that of Huxley (1957) and with the mechanism of actomyosin ATPase activity (Lymn & Taylor, 1971).

The idea that shortening heat is produced in one step of a cyclical process has been suggested before (Woledge, 1971) and schemes involving specific intermediates in the actomyosin ATPase mechanism have been proposed (Curtin & Woledge, 1978; Kodama & Yamada, 1978; Rall *et al.* 1976). The emphasis here will be on the kinetics of the mechanism; only the total energy liberation (heat plus work) will be considered. The extra energy associated with shortening (shortening heat plus work) will be referred to as the 'shortening enthalpy'.

The scheme is shown in Fig. 5. *A* and *B* are two relatively long-lived intermediates in the interaction of cross-bridges with thin filaments in contracting muscle. The transition *B* to *A* is coupled to the dephosphorylation of ATP; since the concentration of ATP is effectively constant, this reaction is assigned the first order rate constant k_R . The molar enthalpy of the reaction is ΔH_R . The transition *A* to *B* is assumed to have rate constant k_I during isometric contraction, increasing to k_S during shortening at the velocity used in the present experiments; the molar enthalpy of this reaction is ΔH_F . If k_R is greater than k_I , *A* will be the predominant intermediate during isometric contraction, as the reaction cycle hydrolyses ATP at a steady rate. When

shortening is allowed the rate of reaction A to B increases, B becomes the predominant state and, if the shortening is sufficiently extensive, a new steady state is reached in which ATP is hydrolyzed at a faster rate. However the burst of enthalpy resulting from the transition A to B (and its subsequent reversal) occurs even for very small releases; this is responsible for the non-linear relationship between shortening enthalpy and extent of shortening. If two closely spaced periods of shortening occur, so that there is insufficient time for intermediate A to be resynthesized after the first shortening, the transitional burst, and hence the shortening enthalpy, will be reduced in the second shortening. During shortening, the reaction A to B is responsible for enthalpy production without simultaneous ATP hydrolysis, as observed by Rall *et al.* (1976). In the period immediately following shortening extra ATP hydrolysis should be seen corresponding to the transition B to A . Such delayed ATP hydrolysis was suggested by further results of Rall *et al.* (1976), and has now been demonstrated directly (Homsher, Irving & Wallner, 1981).

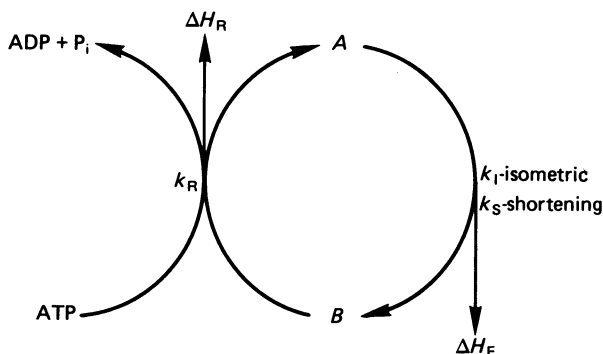


Fig. 5. Two-state model for shortening heat and work production by contracting muscle. A and B are intermediates in the cross-bridge interaction with thin filaments. The transition $B \rightarrow A$ is linked to the hydrolysis of ATP and is assumed to have pseudo first-order rate constant k_R and molar enthalpy ΔH_R . The transition $A \rightarrow B$ has first-order rate constant k_I during isometric contraction and k_S during shortening at $1.3 \mu\text{m} \cdot \text{sec}^{-1}$ per sarcomere; its molar enthalpy is ΔH_F .

We have made a quantitative comparison of the predictions of the scheme in Fig. 5 with the results of this and the previous paper (Irving & Woledge, 1981). The energy liberation measurements were made 1 sec after the start of shortening, by which time the isometric steady-state has been re-established. Consequently the recorded enthalpy production corresponds to complete cycles of the reaction scheme, and it is not necessary to know the values of ΔH_F and ΔH_R . In addition, ATP is continuously replenished by the creatine phosphokinase reaction, so the net chemical change per cycle is the hydrolysis of one molecule of phosphocreatine, yielding 34 kJ/mole (Curtin & Woledge, 1978). The best fit to the experimental data was obtained with the following values for the rate constants: $k_I = 1.5 \text{ sec}^{-1}$, $k_R = 5.0 \text{ sec}^{-1}$, $k_S = 30 \text{ sec}^{-1}$. The total concentration (M) of intermediates A and B was assigned the value $0.41 \mu\text{mole/g}$.

With these values the results of three types of experiment can be predicted.

(1) The rate constant with which the extra energy liberation in the second of two periods of shortening is related to the interval between shortenings is $(k_I + k_R) \text{ sec}^{-1}$ in the model in Fig. 5; this is the rate constant with which the system returns to the steady-state after a period of shortening. The experimental value of the rate constant was 6.5 sec^{-1} (Irving & Woledge, 1981), in agreement with the values $k_I = 1.5 \text{ sec}^{-1}$, $k_R = 5.0 \text{ sec}^{-1}$.

(2) The relationship between the shortening enthalpy (ΔE) and the extent of shortening (x , $\mu\text{m}/\text{sarcomere}$) was calculated by kinetic analysis of the two-state model, as

$$\Delta E = 34 M k_R^2 \Phi \{x/v + \Phi [1 - \exp(-(k_S + k_R) x/v)]\} \quad \text{mJ/g}$$

$$\text{where } \Phi = (k_S - k_I)/(k_S + k_R) \cdot (k_I + k_R) \quad (\text{sec})$$

$$v = \text{velocity of shortening} \quad \mu\text{m} \cdot \text{sec}^{-1} \cdot \text{sarcomere}^{-1}$$

The relationship between shortening enthalpy and extent of shortening predicted from the values of the constants listed above is compared with the experimental data in Fig. 6. The agreement between the experimental points and the predicted curve is excellent, over the entire range of extents of release studied.

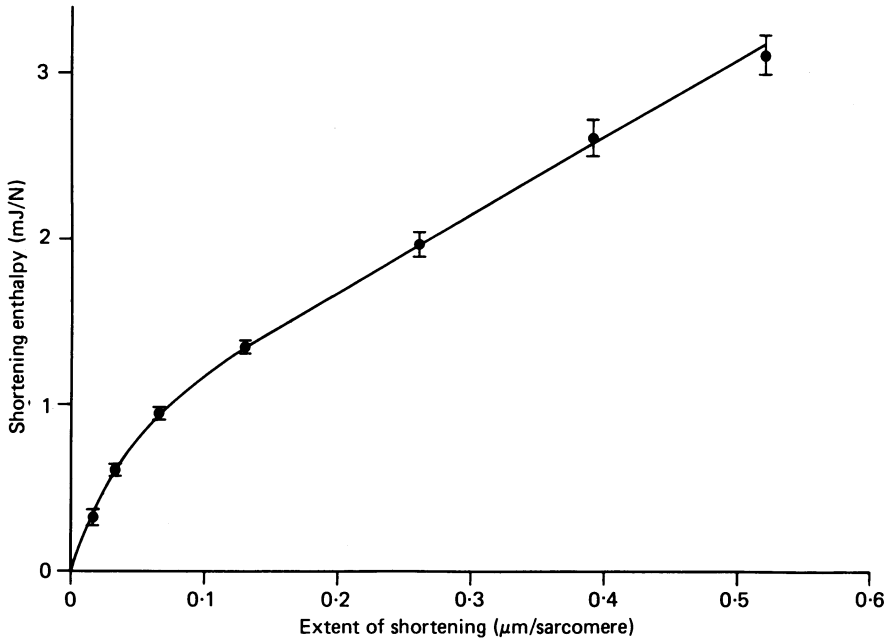


Fig. 6. Shortening enthalpy (shortening heat plus work) as a function of extent of shortening. In order to eliminate small effects due to the variation of energy liberation with sarcomere length (Fig. 2) the experimental values (means \pm s.e. of means for an average of eight muscle pairs) have been normalized by the mean isometric tension in the length range over which shortening occurs, instead of by isometric tension at $2.21 \mu\text{m}/\text{sarcomere}$ as in previous Figures, but only the points at 0.39 and $0.52 \mu\text{m}/\text{sarcomere}$ are affected. The curve was calculated from the model in Fig. 5 with parameters given in the text.

(3) The contribution of the reaction scheme to the isometric energy liberation rate (dE_I/dt) can be shown to be

$$dE_I/dt = 34 M k_I k_R / (k_I + k_R) \quad \text{mW/g.}$$

Substituting the values for k_I , k_R and M given above, the predicted isometric heat rate is 16.3 mW/g . The observed heat rate between 1.0 and 2.0 sec in an isometric tetanus at muscle length $2.21 \mu\text{m}/\text{sarcomere}$ was $24.1 \pm 0.8 \text{ mW/g}$ so, according to the model, some 68% of the total isometric heat is produced by the cross-bridge system. This is in reasonable agreement with the conclusions drawn from the experiments of Homsher *et al.* (1972) and of Smith (1972).

The simple two-state model is therefore able to provide an accurate quantitative description of the results of three types of experiment. The best fit was obtained with the total concentration of intermediates (M) set at $0.41 \mu\text{mole/g}$, which can be

compared with the measured concentration of myosin subfragment-1 in the sarcoplasm, $0.28 \mu\text{mole/g}$ (Ebashi *et al.* 1969). As far as the rate constants are concerned, one would like to be able to draw comparisons with *in vitro* measurements on frog actomyosin ATPase. The *in vitro* state, with its lack of mechanical and geometric constraints on protein interactions, probably corresponds most closely to the rapidly shortening state in intact muscle. The rate-limiting step in this case is 5.0 sec^{-1} (k_R) in the two-state model and this is similar to the *in vitro* actin-activated myosin subfragment-1 ATPase rate, estimated as 4.4 sec^{-1} for frog proteins at $0\text{--}2^\circ\text{C}$ (Ferenczi, Homsher, Trentham & Weeds, 1978). The rate constant of reaction $A \rightarrow B$ is 30 sec^{-1} during shortening (k_S) and this is similar to the rate constant for tension fall during the isovelocity release, which can be calculated from the fit in Fig. 4 as 27 sec^{-1} (tension fell to $1/e$ in $0.049 \mu\text{m/sarcomere}$ and the shortening velocity was $1.3 \mu\text{m/sec}$ per sarcomere). In terms of cross-bridge kinetics, the transition $A \rightarrow B$ might therefore correspond to detachment of the cross-bridge from the thin filament. The transition $B \rightarrow A$ may be interpreted as a transition within the detached cross-bridge. At the velocity of shortening used in these experiments the filaments have a relative velocity of 650 nm/sec and, assuming that an attached cross-bridge can undergo a distortion of about 15 nm (Ford, Huxley & Simmons, 1977), the mean attachment time must be less than 25 msec . The transition $B \rightarrow A$ is therefore too slow (5 sec^{-1}) to be associated with an attached state.

M.I. was a M.R.C. Research Scholar.

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