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REPRIMING AND REVERSAL OF THE ISOMETRIC UNEXPLAINED ENTHALPY IN FROG SKELETAL MUSCLE

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SUMMARY

1. Heat production and high-energy phosphate hydrolysis by frog sartorius muscles in 6 s isometric tetani were measured to test the hypothesis that the isometric unexplained enthalpy (u.e.) and labile maintenance heat (l.m.h.) were of similar origin. Muscles were first given a conditioning tetanus to deplete the u.e. and l.m.h. A second (test) 6 s tetanus was given 6–300 s later to ascertain the extent to which l.m.h. and u.e. had recovered (reprimed).

2. The labile maintenance heat repriming was biphasic: 42% of the conditioning l.m.h. reprimed with a time constant of 10 s, the remainder with a time constant of 500 s.

3. The u.e. produced in the test tetanus 6 s after the conditioning tetanus was reduced to 18% of its conditioning value. By 30 s, u.e. had returned to conditioning values even though the amount of high-energy phosphate splitting was 17% less than that in the conditioning tetanus.

4. This observation is supported by measurements revealing that during the 30 s following a 6 s tetanus an amount of enthalpy was *absorbed* (less heat produced than expected from the measured metabolic changes) whose absolute value (after correction for oxygen consumption) was not different from the amount of unexplained enthalpy liberated during the tetanus.

5. The difference in repriming time course shows that l.m.h. and u.e. are not produced by the same reactions. The data are consistent with the hypothesis that calcium binding to troponin and parvalbumin produce u.e. while calcium binding to troponin and a non-linear time course of ATP hydrolysis produce l.m.h.

INTRODUCTION

Aubert (1956) showed that the heat production during an isometric tetanus, $h(t)$, was described by an equation of the form:

$$h(t) = A(1-e^{-\alpha t}) + Bt, \quad (1)$$

where t is time (s), α is a rate constant (s^{-1}), A is the labile maintenance heat (l.m.h.), and B is the stable maintenance heat (s.m.h.) rate. Subsequent studies of these

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empirical variables indicated that the s.m.h. is primarily (70%) associated with cross-bridge cycling because its rate is linearly dependent on the extent of the thick and thin filament overlap (Homsher, Mommaerts, Ricchiuti & Wallner, 1972; Curtin & Woledge, 1977). L.m.h. is thought to be independent of the number of interacting cross-bridges because it is only weakly dependent on sarcomere length (Aubert, 1956; Homsher *et al.* 1972) and is substantially reduced by prior activity (Aubert, 1956; Curtin & Woledge, 1977) even though tension is only slightly (*ca.* 10%) reduced.

High-energy phosphate utilization ($\Delta \sim P$) proceeds at an *approximately* linear rate during a tetanus (Marechal & Mommaerts, 1963; Homsher, Kean, Wallner & Sarian-Garibian, 1979; Curtin & Woledge, 1979). During the tetanus the energy liberated (observed enthalpy) significantly exceeds that produced by the high-energy phosphate usage (explained enthalpy) (Curtin & Woledge, 1978; Homsher & Kean, 1978). The difference between the observed and explained enthalpy, the unexplained enthalpy (u.e.), is large and significant (10–40 mJ/g), is not a consequence of protein phosphorylation, oxidative phosphorlyation, or glycolysis, and it is reversed by high-energy phosphate metabolism *during* recovery (see Kushmerick, 1983, for a review). Unexplained enthalpy is: (1) produced early in the tetanus (Homsher *et al.* 1979; Curtin & Woledge, 1979), (2) not reversed during relaxation (Curtin & Woledge, 1974), (3) only marginally affected by thick and thin filament overlap (Curtin & Woledge, 1981; Homsher & Kean, 1982), and (4) similar in magnitude to the l.m.h. (Curtin & Woledge, 1977, 1979, 1981). Curtin & Woledge (1977) have hypothesized that the production of l.m.h. and u.e. is of similar origin, a view supported by their observation that in the second of two isometric tetani separated by a 5 s time interval, *both* the l.m.h. and u.e. were reduced to about 25% of their value in the first tetanus.

To test this hypothesis and to more fully characterize the reversal (repriming) of the unexplained enthalpy, force, heat production, and $\sim P$ hydrolysis ($\Delta \sim P$) were measured in muscle pairs given two tetani, each of 6 s duration, separated by time intervals ranging from 6 to 300 s. If the l.m.h. and u.e. are produced by the same process, the amount of l.m.h. and u.e. in each tetanus should be identical. Measurements showed that l.m.h. and u.e. repriming are not identical so that l.m.h. and u.e. can be not of identical origin. An hypothesis is proposed which successfully accounts for the known behaviour of l.m.h. and u.e. A preliminary account of these experiments has appeared (Homsher, Lacktis, Wallner & Zohman, 1985).

METHODS

Sartorious muscles of *Rana temporaria*, obtained from Charles Sullivan of Nashville, TN, U.S.A., were used in these experiments. Two shipments of frogs were used. The first (1984) was used for both heat measurements and energy balance studies in repriming experiments, and the second (1985) for measurements of energy balance in the 30 s following an isometric tetanus. Maintenance of the frogs, muscle dissection, sarcomere length measurement, Ringer solution composition, pre-experimental treatment, mounting of the muscles on thermopiles, and force measurement were as previously described (Homsher, Irving & Wallner, 1981).

Heat measurement

Two electroplated thermopiles (Ricchiuti & Mommaerts, 1965), E-9 and E-10 (Homsher *et al.* 1979), were used in the experiments and sampled temperature from 19 to 24 mm of the muscle length respectively. Calibration, amplification, recording and correction of recordings for heat loss,

conduction lag and stimulus heat were all as previously described (Homsher *et al.* 1981). The stimulus heat averaged 8·4 ± 0·3 % of the total initial heat in these experiments. The myothermal records were read at 0·1 s intervals and were fitted to Aubert's eqn. (1) using a modified Levenberg–Marquardt least-squares routine (Brown & Dennis, 1972) for fitting non-linear equations (available from International Mathematical and Statistical Libraries, Inc. Houston, TX, U.S.A.). The fit of the equation to the data was excellent in that the average deviation of the fitted equation from the data points was less than 1 %. Plots of the residuals against time showed random scatter suggesting that there were no systematic errors inherent in the analysis.

Chemical analyses

Mounting of the muscles on the rapid-freezing apparatus, temperature equilibration, freezing and extraction of the muscles were as previously described (Homsher *et al.* 1979). Analyses for phosphorylcreatine (PCr), free creatine (Cr), ATP, ADP and AMP were performed by high-pressure liquid chromatography (h.p.l.c.) using a C_{18} Bondapak 5 μm pellicular cartridge (Waters Associates, Inc., Milford, MA, U.S.A.) according to a protocol similar to that described by Dubyak & Scarpa (1983). Duplicate 20 μl samples of the neutralized muscle extract were injected onto the column and were eluted isocratically by a buffer containing 0·3 M-$NH_4H_2PO_4$ (pH 4·2) at 2·0 ml/min. Under these conditions separate peaks of PCr, Cr, ATP, ADP and AMP (read at 214 nm) elute at 1·45, 2·0, 5·2, 6·0 and 12·5 min respectively after injection onto the column. The peak heights are linearly proportional to the amount injected between 0–6 nmol (for PCr and Cr) and 0–2 nmol for the adenine nucleotides. Standard curves for each metabolite were made at the beginning of each day of analysis. The h.p.l.c. analyses of muscle extracts for total creatine were within 1 ± 1 % (n = 54) of those obtained using a standard colorimetric technique (Ennor & Rosenberg, 1952). The free creatine (Cr_f), ATP, ADP and inorganic phosphate (P_i) content of each muscle was divided by the total creatine (Cr_t) content of the muscle (to normalize the values) and the differences between the experimental and control muscles ($\Delta Cr_f/Cr_t$, $\Delta ATP/Cr_t$ etc.) were calculated from paired muscles. These differences were converted to micromoles per gram blotted muscle weight (ΔCr_f, ΔATP etc.) by multiplication of the ratio by the total creatine content per gram of blotted muscle weight determined on the muscle pairs used for myothermal experiments (Homsher *et al.* 1981). For the 1984 and 1985 batches of frogs these values were 32·1 ± 0·3 and 33·9 ± 0·9 μmol/g respectively. In twenty-one pairs of muscles a pair-wise comparison of ΔCr_f revealed that the value determined using the h.p.l.c. technique was not significantly different (−6·4 ± 9·7 %) from that using the method of Ennor & Rosenberg (1952). Analysis of muscle extracts using h.p.l.c. requires < 1 % of the muscle extract and half as much time as the techniques used previously. The assay for inorganic phosphate (P_i) was also simplified. After the P_i in an aliquot of the muscle extract had been quantitatively isolated by $Ca(OH)_2$ precipitation (Seraydarian, Mommaerts, Wallner & Guillory, 1961), the P_i content of the precipitate was assayed using a Phosphorus Rapid Stat Diagnostic Kit (Sherwood Medical Inc., St Louis, MO, U.S.A.); this method, based on the semi-microcolorimetric technique of Grindler & Ishizaki (1969), requires 30 % less time than the technique we have used in the past (Berenbloom & Chain, 1938). Comparison of the Rapid Stat method to the Berenbloom & Chain (1938) method on muscle pairs yielded results that were within 5·4 ± 4·9 % of each other (n = 8).

The computation of the extent of ATP hydrolysis (ξ_{ATP}), PCr splitting (ξ_{PCr}), and the amounts of explained and unexplained enthalpy were as previously described (Homsher *et al*, 1981).

The experimental design for the repriming studies

Experiments were performed at an initial sarcomere length of 2·3 μm and a temperature of 0 °C. The design for the repriming experiments was as follows. Muscles were first given a 6 s conditioning isometric tetanus during which force and heat production were measured. They were then rested for 6, 30, 60 or 300 s from the last stimulus of the conditioning tetanus (the repriming interval) whereupon a second 6 s tetanus (test tetanus) was given and the force and heat production measured. Separate experiments were used to study the chemical change accompanying contraction. For the conditioning tetanus, a pair of muscles was treated so that one of the pair was an unstimulated control while the second, or experimental muscle, was frozen at 6 s into an isometric tetanus. For the test tetanus, muscle pairs were stimulated and frozen so that the control muscle was frozen 6, 30 or 300 s after the last stimulus of the conditioning tetanus, while its contralateral mate, the experimental muscle, was frozen at a point 6 s into its second (test) tetanus which had

begun 6, 30 or 300 s (respectively) after a 6 s conditioning tetanus. The difference in high-energy phosphate content between the experimental and control muscles was the amount of high-energy phosphate consumed during the test tetanus.

Data rejection

A total of sixty-two pairs of muscles were used to measure the heat production. Of these three pairs were rejected because their heat production or peak isometric force was more than 2 S.D. away from the mean of their group with the suspected value included. A total of eighty-one muscle pairs were frozen for chemical analysis. Of these eight pairs were rejected prior to analysis because the peak isometric force developed by each muscle of a pair differed by more than 10%. Of the remainder, six pairs were rejected because the change in P_i or Cr in an experimental group was more than 2 S.D. away from the mean of that group with the suspected value included.

RESULTS

Heat and force measurements

The upper panels of Fig. 1 contain typical force recordings of conditioning tetani superimposed upon test tetani from 6, 30 and 300 s test intervals. The lower panels show the corresponding time course of the heat production (filled circles) along with the regression equation for the data points (continuous line). A summary of results is given in Table 1. Several features should be noted. First, although the magnitude of isometric peak tetanus tension is similar to that usually reported (Curtin & Woledge, 1978; Homsher & Kean, 1978), the average rate of tetanus tension decline in the conditioning tetanus is greater than in the test tetanus (Fig. 1). The average rate of tension decline was computed by dividing the difference between the peak tension and that at the time of the last stimulus by the time interval between the two. The average rate of tension decline in the conditioning tetanus was about twice that in the test tetanus. This behaviour was observed in both the myothermal and chemical experiments and appears to be a consequence of a rapid phase of force decline in the first 3 s or so of the conditioning tetanus (upper panels, Fig. 1). Table 1 shows that for intervals up to 300 s little repriming of the peak tension or rate of loss of tension occurs. When several longer test intervals (40–60 min) are allowed, both the peak tetanus tension and rate of loss of tension returned to values similar to those seen in the conditioning tetanus. Thus the rapid loss of tetanus tension is seen only when the muscle is rested for a long interval, i.e. an interval similar to that needed for the resynthesis of high-energy phosphate.

Table 1 and Fig. 1 show there is no significant difference in the s.m.h. rate between the conditioning and test tetani. However, the l.m.h. is significantly reduced by the conditioning tetanus, and the extent of l.m.h. repriming is dependent on the duration of the repriming interval. The relationship is plotted in Fig. 2 and is biphasic. The fast phase amounts to 13 mJ/g and recovers with a time constant of *ca.* 10 s, while the slow phase, about 18 mJ/g, reprimes with a time constant of about 500 s (8·3 min). The time constant of the slow phase is similar to that for the resynthesis of creatine phosphate (Carlson, Hardy & Wilkie, 1967; Kushmerick & Paul, 1976). Table 1 and Fig. 3 also show that the l.m.h. is evolved over a shorter period of time in the test tetani than in the conditioning tetanus. A plot of the time course of the return of α to control values as a function of repriming interval (Fig. 3) is similar (i.e.

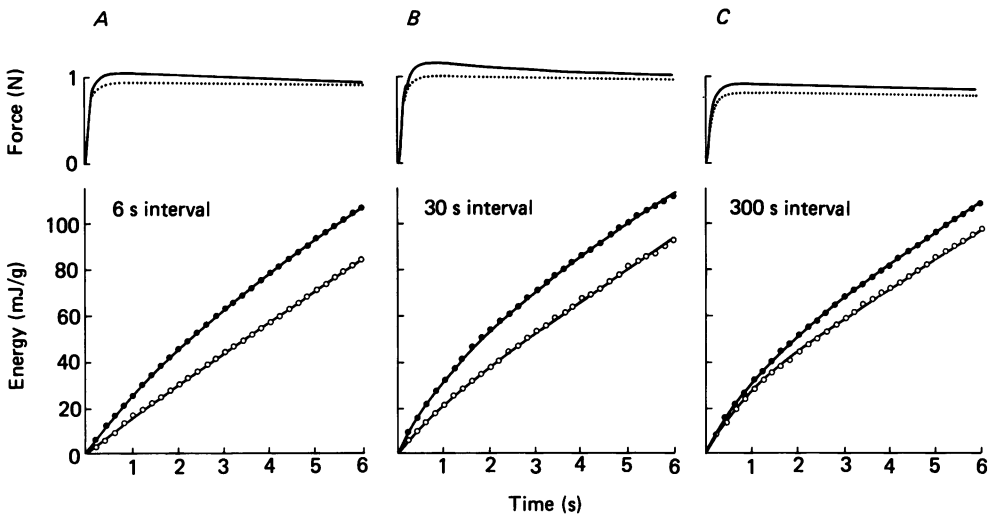

Fig. 1. Force and heat production in conditioning and test tetani. *A*, *B* and *C* correspond to repriming intervals of 6, 30 and 300 s respectively. The upper panels are force recordings of conditioning (continuous lines) and test tetani (dotted lines), while the lower panels are the corresponding heat recordings (● for conditioning and ○ for test tetani). The circles in the lower panels represent readings of the heat records (corrected for heat loss and stimulus heat) taken at 0·1 s intervals. The continuous lines are the results of the non-linear curve-fitting routine to the data points. The equations describing these lines are of the form given in eqn. 1.

TABLE 1. Mechanical and myothermal parameters in tetani*

Parameter	Conditioning tetanus	Test tetanus (s)			
		6	30	60	300
Number	48	10	9	11	11
Peak tension (N/cm^2)	21·1	18·5	19·4	19·5	17·7
	±0·5	±0·8	±0·9	±1·0	±1·2
Rate of tension	2·20	0·88	0·76	0·72	0·84
decline (%/s)	±0·02	±0·04	±0·06	±0·08	±0·15
Total energy (mJ/g)	113·6	80·8	102·5	98·8	103·4
	±2·7	±4·3	±4·6	±5·2	±6·0
s.m.h. ($mJ\ g^{-1}\ s^{-1}$)	13·3	12·1	14·4	13·3	13·4
	±0·4	±0·7	±0·6	±0·6	±0·8
l.m.h. (mJ/g)	31·6	5·8	13·3	16·4	21·1
	±1·3	±0·4	±1·1	±1·9	±2·5
α (s^{-1})	0·8	1·7	1·2	1·0	1·0
	±0·1	±0·2	±0·1	±0·1	±0·1

* Values given as mean ± S.E. of mean.

biphasic) to that for l.m.h. repriming. These data are qualitatively like those of other less complete characterizations of tension, s.m.h., and l.m.h. repriming (Aubert, 1956, 1968; Curtin & Woledge, 1981) and suggest (because of the latter's biphasic time course) that more than one reaction is involved in the evolution of the l.m.h.

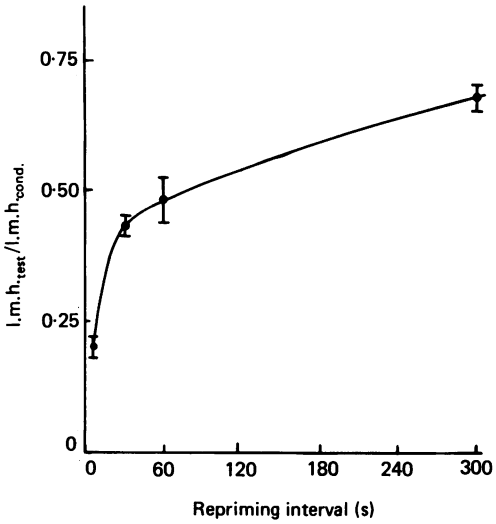

Fig. 2. Plot of the repriming of the l.m.h. as a function of the repriming interval. Values are given as the mean ± S.E. of mean. The continuous line is the equation: $\text{l.m.h.}_{test}/\text{l.m.h.}_{cond.} = 0{\cdot}42(1-e^{-0{\cdot}1t})+0{\cdot}58(1-e^{-0{\cdot}002t})$. The number of observations for each data point is between nine and eleven pairs of muscles.

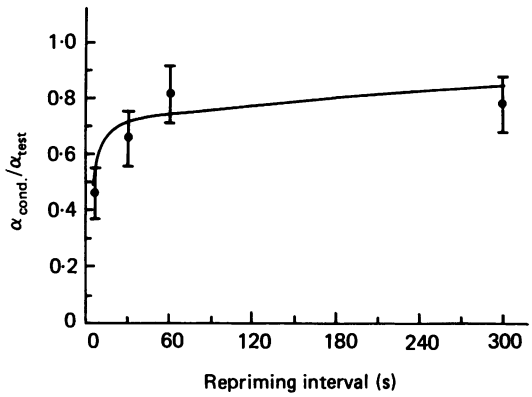

Fig. 3. A plot of the repriming of the time constant for the l.m.h. evolution as a function of the stimulus interval. Values given as the mean ± S.E. of mean. The continuing line is $\alpha_{cond.}/\alpha_{test} = 0{\cdot}72(1-e^{-0{\cdot}20t})+0{\cdot}28(1-e^{-0{\cdot}002t})$. The number of observations per data point is between nine and eleven.

Chemical changes

The high-energy phosphate usage of muscles undergoing conditioning and test tetani is shown in Table 2. Because the changes in AMP content were all less than 0·02 μmol/g and were insignificant, these values are not listed. The Table shows no significant change in ATP and an equivalence in the rise in Cr_f and P_i content. Thus PCr splitting is the only significant net reaction occurring. The PCr used in the test tetani is significantly less than that in the conditioning tetanus. For repriming

intervals of 30 and 300 s the difference from the control tetanus is highly significant ($P < 0.025$) while that at 6 s is not quite significant ($P < 0.01$, a consequence of the elevated P_i change). There are no significant differences in PCr utilization among the test tetani. To obtain a measure of the $\Delta \sim P$ utilization for 60 s repriming intervals, the test tetani data were pooled, yielding a value of 2.16 ± 0.08 μmol/g, or 0.43 ± 0.12

TABLE 2. Chemical changes in isometric tetani*

Parameter	Conditioning tetanus	Test tetani (s)		
		6	30	300
Observations	15	9	10	9
ΔCr_f (μmol/g)	+2·54	+2·14	+2·24	+2·12
(E–C)	±0·11	±0·11	±0·17	±0·12
ΔP_i (μmol/g)	+2·60	+2·40	+1·94	+2·23
(E–C)	±0·11	±0·17	±0·16	±0·11
ΔATP (μmol/g)	+0·07	+0·04	+0·11	+0·01
(E–C)	±0·04	±0·08	±0·06	±0·07
ΔADP (μmol/g)	+0·01	+0·02	+0·00	+0·02
(E–C)	±0·01	±0·04	±0·01	±0·02
Extent of PCr splitting (μmol/g)	+2·59	+2·28	+2·12	+2·16
(E–C)	±0·09	±0·14	±0·14	±0·11
Extent of ATP hydrolysis (μmol/g)	−0·03	0·00	−0·05	−0·02
(E–C)	±0·02	±0·04	±0·03	±0·03
Explained enthalpy (mJ/g)	86·5	77·1	69·5	75·2
	±3·2	±5·3	±5·2	±4·0
Unexplained enthalpy (mJ/g)	27·1	3·7	33·0	28·2
	±4·3	±6·8	±6·9	±7·2

*Values given as mean ± S.E. of mean. Extent of PCr splitting = $(\Delta Cr_f + \Delta P_i - [\{\Delta ADP - \Delta ATP\}/2])/2$. Extent of ATP splitting = $(\Delta ADP - \Delta ATP)/2$.

μmol/g less than that in the conditioning tetanus ($P < 0.03$). The difference (equivalent to 14·6 mJ heat/g muscle) is a consequence of either a decreased steady rate of ATP hydrolysis or a change in a non-linear component of ATP usage. If the former is true, the s.m.h. rate should be 2·5 mJ g^{-1} s^{-1} less than that in the conditioning tetanus. As this is not observed (Table 1), it is likely that the reduced rate of PCr utilization in the test tetani is a consequence of a fall in a non-linear usage. As shown earlier (Curtin & Woledge, 1977) enough PCr was split to account for the s.m.h.

Energy balance results

The amount of u.e. production in the conditioning and test tetani is tabulated in the last line of Table 2. The u.e. produced in the tetanus after a 6 s repriming interval is reduced to 18% of that in the conditioning tetanus ($P < 0.01$). However, at 30 and 300 s the amount of u.e. is not significantly different from that in the conditioning tetanus and is, if anything, slightly greater. Figure 4 is a bar graph in which the amount of u.e. produced in the conditioning and test tetani at 6, 30, 60 and 300 s are compared to the l.m.h. (The u.e. value at 60 s was computed from the average test tetanus $\sim$ P utilization.) In the conditioning and 6 s test tetani, the l.m.h. and u.e. are not significantly different. However, at 30 s, when the u.e. has fully reprimed (giving a time constant for repriming of 6–10 s), the l.m.h. is only about 50% of its

conditioning value. Thus, u.e. and l.m.h. are significantly different ($P < 0{\cdot}01$), and the results do not support the idea that the l.m.h. and u.e. are derived from the same chemical process.

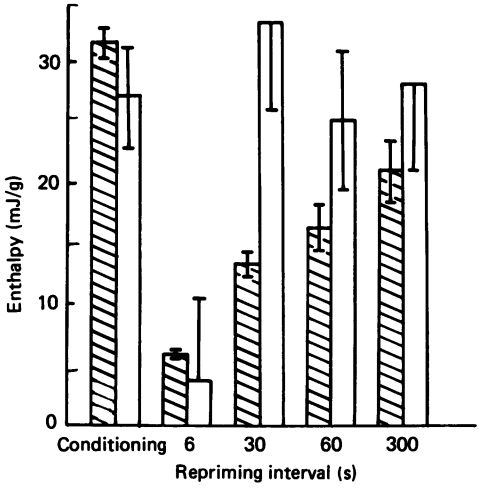

Fig. 4. A bar graph of the dependence of the l.m.h. (cross-hatched bars) and u.e. (open bars) production as a function of the repriming interval. The data on which this graph is based is in Tables 1 and 2.

The post-tetanic reversal of the energy imbalance

The foregoing results imply that reaction(s) producing the u.e. are reversed within 30 s of the last stimulus. Reversal of an exothermic reaction (that producing the u.e.) should be associated with an absorption of enthalpy (muscle cooling) as the reaction proceeds. Because the reversal is presumably driven by ~ P splitting (which produces heat), there need not be an actual cooling of the muscle. However, if an energy balance experiment were performed over the 30 s interval following a conditioning tetanus (assuming insignificant amounts of oxidative resynthesis), less heat should be produced than expected from the measured ~ P hydrolysis (i.e. there should be a negative energy balance). Further, the amount of enthalpy absorbed in this period should be equal in magnitude (though opposite in sign) to the u.e. produced during the 6 s tetanus.

To test this prediction, pairs of sartorius muscles were tetanized for 6 s. In the first series of experiments, the experimental muscles were frozen 6 s after the beginning of the stimulation, and their metabolic contents compared to unstimulated controls to measure the amount of u.e. produced during the tetanus. In a second series, both muscles of a pair were stimulated for 6 s and the control was immediately frozen while the experimental muscle relaxed and was then frozen 30 s after the last stimulus. In a third series of experiments, the amount of enthalpy produced by pairs of sartorius muscles during the 6 s of the tetanus and the 30 s following the last stimulus were measured. The results of these experiments are shown in Table 3. The amount of heat produced, high-energy phosphate used, and u.e. produced ($40{\cdot}6 \pm 8{\cdot}6$ mJ/g) *during* the tetanus are similar to the results shown in Tables 1 and 2. From the time of the last stimulus to 30 s later, there is a relatively small amount (9·7 mJ/g)

of heat produced and a significant amount ($P < 0.01$) of high-energy phosphate hydrolysed. The $\Delta \sim P$ would have produced the 22.0 ± 3.4 mJ/g of heat if no other concomitant reaction absorbed the heat. Thus there was a significant *negative* energy

TABLE 3. Energy balance in the 30 s following a 6 s isometric tetanus*

	Chemical changes				
Time interval (s)	ΔCr_f (μmol/g)	ΔP_i (μmol/g)	ΔATP (μmol/g)	ΔADP (μmol/g)	ΔPCr (μmol/g)
0–6	2·96	2·93	0·30	−0·03	−3·03
($n = 8$)	±0·17	±0·37	±0·07	±0·12	±0·17
6–36	0·78	0·66	0·09	−0·08	−0·77
($n = 16$)	±0·08	±0·18	±0·05	±0·04	±0·10

	Energy balance		
Time interval (s)	Observed enthalpy (mJ/g)	Explained enthalpy (mJ/g)	Unexplained enthalpy (mJ/g)
0–6	135·6	95·0	+40·6
	±6·4	±5·8	±8·6
	($n = 11$)	($n = 8$)	(d.f. = 17)
6–36	9·7	22·0	−12·3
	±1·9	±3·4	±3·9
	($n = 11$)	($n = 16$)	(d.f. = 25)

* Values given as mean ± S.E. of mean. d.f. = degrees of freedom. ΔPCr calculated as in Table 2.

balance (-12.3 ± 3.9 mJ/g, $P < 0.02$). The negative energy balance is significantly different in absolute magnitude from the u.e. produced during the tetanus. However, it is shown in the Discussion that most of this difference is a consequence of the oxidative recovery, so that the processes producing the u.e. are probably reversed in the first 30 s after the tetanus.

DISCUSSION

Comparison to previous results

The results reported here agree with published values of the amount of force developed in a tetanus, total heat, s.m.h. rate, l.m.h., α, amount of ATP utilization and u.e. production (Aubert, 1956, 1968; Hill & Woledge, 1962; Curtin & Woledge, 1977, 1979; Homsher *et al.* 1979; Peckham & Woledge, 1986). Further, like the one previous report on energy balance in consecutive tetani (Curtin & Woledge, 1977), we find that both the l.m.h. and u.e. are reduced to the same extent in a test tetanus immediately (< 6 s) following a conditioning tetanus and that the amount of ATP utilization in the second of two closely spaced tetani is reduced by about 15%. Finally our observation that the l.m.h. repriming follows a relatively slow time course agrees with reports by Aubert (1968) and Peckham & Woledge (1986) where they found that at least 15 min are required for complete repriming of the l.m.h.

Reversal of the u.e.

The repriming experiments show that the u.e. is reversed within a 30 s repriming interval. However, the measurement of the energy balance during the first 30 s after

stimulation *seems* to indicate that only 30–40% of the reversal has occurred (Table 3). This energy balance study underestimates the extent of u.e. reversal because during the 30 s interval, *both* u.e. reversal *and* oxidative resynthesis of high-energy phosphate (an exothermic set of reactions) occur. The energy balance for the reversal of u.e. *per se* can be estimated if account is taken of the heat produced and high-energy phosphate resynthesized by oxidative phosphorylation. Hill (1940) and Kushmerick & Paul (1976) have shown that in the 30 s following a tetanus, about 5% of the PCr used in the tetanus (about 0·20 μmol/g in the present case) will be resynthesized. This amount of oxidative resynthesis will generate 7·6 mJ heat/g of muscle (see Woledge, Curtin & Homsher, 1985, p. 219; 38 kJ/mol PCr $\times$ 0·2 μmol/g). Thus the heat produced by processes other than oxidative phosphorylation during the 30 s recovery period is 2·1 mJ/g (9·7 − 7·6 mJ/g). The explained heat production should be 33 mJ/g ((0·77 μmol/g measured $\Delta \sim$ P + 0·2 μmol/PCr formed/g) $\times$ 34 kJ/mol). Thus 30·9 mJ heat/g muscle must have been reabsorbed during this time interval, which is not significantly different from the absolute amount of u.e. produced during the tetanus. This calculation is consistent with the results of the repriming experiments.

Sources of u.e. and l.m.h.

The primary observations in this work are: (1) u.e. reprimes within 30 s; (2) unlike the s.m.h., l.m.h. is significantly reduced by previous activity and recovers with a biphasic time course; and (3) the amount of PCr split in the test tetani is about 0·4 μmol/g less than that in the conditioning tetanus. The return of the PCr splitting to conditioning values probably occurs with a time constant of *ca.* 500 s since 30–40 min is required for complete repriming (Kushmerick & Paul, 1976). These results show that u.e. and l.m.h. are not manifestations of the same process and raise questions about the source of l.m.h. and u.e. The above results can be quantitatively accounted for by relatively minor changes in existing (Woledge *et al.* 1985, pp. 257–260) hypotheses.

Three reactions produce significant amounts of enthalpy during an isometric contraction: PCr splitting, Ca^{2+} binding to regulatory sites on troponin, and Ca^{2+} binding to parvalbumin. Using data in the literature, one can compute the time course of PCr splitting and observed and unexplained enthalpy production during conditioning and test tetani.

PCr splitting. During contraction PCr is split in association with cross-bridge cycling and ionic transport, and 34 kJ of enthalpy is produced per mole of PCr split. The time course of PCr splitting (H_{PCr}) is biphasic (Kushmerick & Paul, 1976; Curtin & Woledge, 1979 (see Fig. 1); Homsher *et al.* 1979 (see Fig. 2)); i.e. there is a constant rate of utilization (equivalent to *ca.* 12–14 mJ g^{-1} s^{-1}) as well as an exponentially decaying rate of PCr usage occurring at the beginning of the tetanus (of duration t,) which decays to zero within 3–5 s (rate constant *ca.* 1 s^{-1}). The amount of extra energy associated with the exponentially decaying phase is about 15 mJ/g (see Fig. 2, Homsher, *et al.* 1979, or Fig. 1, Curtin & Woledge, 1979). Because the s.m.h. rate is not significantly depressed by previous activity, the *ca.* 15 mJ/g reduction in $\sim$ P hydrolysis in the test tetani, is probably caused by a reduction in the exponentially decaying phase of H_{PCr}. Since the muscle's ability to split PCr reprimes slowly over 30–40 min (τ = *ca.* 500 s; Kushmerick & Paul, 1976) the

amount of heat produced by PCr splitting (H_{PCr}) will depend on the time interval, Δt (in seconds), between the conditioning and test tetanus and will be given by:

$$H_{PCr} = (15[1-e^{-0.002\,\Delta t}]\,mJ/g)\,(1-e^{-1t}) + 12\,mJ\,g^{-1}\,s^{-1}(t). \quad (3)$$

The depression of PCr splitting and peak tension by previous activity may be related to the accumulation of intracellular inorganic phosphate which depresses isometric force (Herzig, Peterson, Ruegg & Solaro, 1981; Hibberd, Dantzig, Trentham & Goldman, 1985) and ATPase rate in muscle fibres (Kawai & Güth, 1986)).

Calcium binding to troponin. During activation calcium binds to calcium-specific sites on troponin C (2 mol calcium/mol troponin C) and produces enthalpy at 32 kJ/mol calcium bound (Potter, Hsu & Pownall, 1976). Since there is about 0·1 μmol troponin/g muscle (Yates & Greaser, 1983) and assuming a calcium-binding constant of 5×10^6 M^{-1} (Potter & Gergely, 1975), a resting level of calcium of 5×10^{-8} M (Weingart & Hess, 1984), and an activated level of calcium of 1×10^{-5} M^{-1} (Blinks, Wier, Hess & Prendergast, 1982), calcium binding to troponin C could produce 5 mJ of enthalpy per gram of muscle. As this enthalpy will be produced with a rate constant of $> 5\ s^{-1}$ (the rate of rise of force in a tetanus), the time course of this enthalpy production, H_{TR}, during the tetani in these studies is

$$H_{TR} = 5\,mJ/g\,(1-e^{-5t}). \quad (4)$$

Calcium binding to parvalbumin. There are 0·4 μmol of parvalbumin per gram of muscle (Gosselin-Rey & Gerday, 1977). Each mole of frog parvalbumin has two binding sites for calcium ($K = ca.1 \times 10^8$ M^{-1}) or magnesium ($K = 4 \times 10^4$ M^{-1}) (Haiech, Derancourt, Pechere & Demaille, 1979) so that in the resting muscle (assuming sarcoplasmic magnesium = 3 mM; Hess, Metzger & Weingart, 1982) these calcium–magnesium sites will have magnesium bound. When calcium displaces bound magnesium, *ca.* 31 kJ of enthalpy are produced per mole of calcium bound (Tanokura & Yamada, 1985). There are 0·2 μmol/g of similar calcium–magnesium sites on troponin (Robertson, Johnson & Potter, 1981; Potter *et al.* 1976). Thus there are about 1 μmol of calcium–magnesium binding sites per gram of muscle and calcium binding to these sites could provide as much as 31 mJ of enthalpy per gram of muscle during the tetanus. Using the affinity constants (K) above, the rise of free intracellular calcium concentration from 5×10^{-8} to 1×10^{-5} M will produce *ca.* 25 mJ of enthalpy per gram of muscle by calcium binding to the calcium–magnesium sites. The time course of this enthalpy production will depend on the rate of magnesium dissociation (calcium can bind only after magnesium dissociation) and the amount will depend on the rate of calcium dissociation from the sites and the time interval between tetani (magnesium cannot rebind after relaxation until calcium dissociates). The rate of magnesium dissociation is 2·7 s^{-1} and that of calcium is 0·5 s^{-1} at 20 °C for carp parvalbumin (Potter, Johnson & Mandel, 1978). Assuming a Q_{10} of 2–3 for these rate constants, at 0 °C in frog parvalbumin, magnesium may dissociate at 0·3 s^{-1} and calcium at 0·1 s^{-1}. Thus the time course of this heat production, H_{PA}, in a 6 s tetanus is given by:

$$H_{PA} = (4+21[1-e^{-0.1\,\Delta t}])\,(1-e^{-0.3t})\,mJ/g. \quad (5)$$

Here the 4 mJ/g term is the amount of heat *not* liberated in the conditioning tetanus because a 6 s tetanus is not of sufficient duration to totally fill the PA sites. Summation of eqns 3, 4 and 5 describes the time course of enthalpy production in

either a conditioning tetanus ($\Delta t > 2500$ s) or a test tetanus ($\Delta t = 6$, 30 or 300 s). The time course of the enthalpy production given by the summation of these equations was fitted to Aubert's equation (eqn 1) using the non-linear curve-fitting routine to obtain estimates of the l.m.h., s.m.h. and α for comparison to the experimental data. The value for H_{PCr} predicted by the model for the 6 s conditioning and test tetani can

TABLE 4. Comparison of Experimental results with model predictions*

Parameter	Conditioning tetanus	Test tetanus (s)		
		6	30	300
Total energy (mJ/g)	113±3	81±4	102±5	103±6
	(113)	(88)	(98)	(105)
Explained enthalpy (mJ/g)	87±3	77±5	70±5	75±4
	(87)	(72)	(73)	(79)
Unexplained enthalpy (mJ/g)	27±4	4±7	33±7	28±7
	(26)	(16)	(25)	(26)
l.m.h. (mJ/g)	32±1	6±1	13±1	21±3
	(29)	(9)	(14)	(21)
s.m.h. (mJ/g)	13±1	12±1	14±1	13±1
	(14)	(13)	(14)	(14)
α (s^{-1})	0·8±0·1	1·7±0·2	1·2±0·1	1·0±0·1
	(1·1)	(1·8)	(1·2)	(1·1)

* Observed values given as mean±S.E. of mean while the model predictions, enclosed in parentheses, are computed as described in the text.

be compared to the measured explained enthalpy in each case. Because the H_{TR} and H_{PA} describe an enthalpy production not associated with a ~ P hydrolysis during a tetanus, the sum of eqns 4 and 5 is the amount of u.e. production, and it can be compared to the result obtained in the repriming experiments above. Table 4 contains a comparison of the values observed in these experiments and those predicted by the equations. There is good agreement between the observed and predicted values. The predicted u.e. reprimes within 30 s while both l.m.h. and α follow a biphasic time courses similar to those data shown in Figs 2 and 3. According to this anaysis the u.e. production is solely associated with enthalpy changes involving calcium binding to troponin and parvalbumin. If so, the reason for the large variation of u.e. production by different batches of frogs may be related to variations in resting or activated levels of calcium, magnesium, or the amount of parvalbumin. The l.m.h. is largely produced by the exponentially decaying rate of PCr splitting in a tetanus, the enthalpy change with calcium binding to troponin, and a minor contribution from the initial phase of the calcium binding to parvalbumin.

The foregoing model makes three predictions that could be used to test the hypothesis. (1) The removal of calcium from the sarcoplasm after a tetanus at 0 °C will be slow, about 80% of it removed at a rate of 0·1 s^{-1}. Somlyo, McClellan, Gonzales-Serratos & Somlyo (1985) have shown using electron probe X-ray microanalysis that in frog muscles tetanized at 20 °C amounts of calcium substantially above resting values remain in the sarcoplasm even after the muscle has mechanically relaxed. Tormey & Homsher (1986), using the same technique in muscles tetanized at 0 °C, have found that the sarcoplasmic calcium content does not return to resting values until at least 30 s after the cessation of a tetanus. (2) The amount of un-

explained enthalpy produced by a muscle will be dependent on the amount of parvalbumin and troponin in the muscle. (3) The rate of calcium and magnesium release from frog parvalbumin at 0 °C will be 0·1 and 0·3 s^{-1} respectively.

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